Association of soil concentrations of *Rhodococcus equi* and incidence of pneumonia attributable to *Rhodococcus equi* in foals on farms in central Kentucky

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Objective—To determine whether soil concentrations of total or virulent *Rhodococcus equi* differed among breeding farms with and without foals with pneumonia caused by *R equi*.

Sample Population—37 farms in central Kentucky.

Procedures—During January, March, and July 2006, the total concentration of *R equi* and concentration of virulent *R equi* were determined by use of quantitative bacteriologic culture and a colony immunoblot technique, respectively, in soil specimens obtained from farms. Differences in concentrations and proportion of virulent isolates within and among time points were compared among farms.

Results—Soil concentrations of total or virulent *R equi* did not vary among farms at any time point. Virulent *R equi* were identified in soil samples from all farms. Greater density of mares and foals was significantly associated with farms having foals with pneumonia attributable to *R equi*. Among farms with affected foals, there was a significant association of increased incidence of pneumonia attributable to *R equi* with an increase in the proportion of virulent bacteria between samples collected in March and July.

Conclusions and Clinical Relevance—Results indicated that virulent *R equi* were commonly recovered from soil of horse breeding farms in central Kentucky, regardless of the status of foals with pneumonia attributable to *R equi* on each farm. The incidence of foals with pneumonia attributable to *R equi* can be expected to be higher at farms with a greater density of mares and foals. (*Am J Vet Res* 2008;69:385–395)

Rhodococcus equi is a facultative intracellular bacterium that causes pneumonia in foals.[1-4] Expression of VapA by *R equi* is strongly associated with disease in foals, and detection of this protein or the *vapA* gene, located on an 85- to 90-kilobase plasmid, is used to classify isolates of this bacterium as virulent.[4-6] Only virulent organisms are believed to cause disease; therefore, it is important to determine the virulence status of isolates in epidemiologic studies of *R equi*. The epidemiologic aspects of pneumonia in foals caused by this bacterium are not clearly characterized. Pneumonia in foals caused by *R equi* is endemic on some farms and develops intermittently on others but is not found on most farms.[2,5] There may be year-to-year variability in foals with pneumonia attributable to *R equi* at farms where the disease is endemic.[7] Generally, the proportion of foals with pneumonia attributable to *R equi* at endemic farms is approximately 10% to 20%, but proportions

Abbreviations

VapA	Virulence-associated protein A
NANAT	Nalidixic acid, novobiocin, cyclohexamide, and potassium telurite
ATCC	American Type Culture Collection
TBSS	Tris-buffered saline solution
ZINB	Zero-inflated negative binomial
IRR	Incidence-rate ratio
OR_{adj}	Adjusted odds ratio
CI	Confidence interval

Received May 29, 2007.
Accepted August 7, 2007.
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Supported by the Link Equine Research Endowment, Texas A&M University; and the University of Kentucky Equine Research Foundation.
The authors thank Drs. Chet Blackey, Terry Blanchard, Connie Brown, Doug Byars, Nancy Cole, Nathan Earl, William Fishback Jr, Diane Ganzer, Michelle LeBlanc, Carol McLeod, James Morehead, David O'Neal, John Park, Eric Peterson, Gary Priest, Thomas Riddle, Lynda Rhodes-Stewart, Linda Robbins, Trey Schott, Nathan Slovis, John Steiner, Jeremy Whitman, Perry Wornall, and Patricia Ziefle for assistance with collection of soil samples and Jessica Harrington, Michael O'Conor, and Lindsey Williams for technical assistance.
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> 20% are not unusual.[8,9] The reasons for this variation among and within farms remain unknown. Molecular epidemiologic data indicate that recurrence of the problem at a farm does not appear to be attributable to a particular genotypic strain of *R equi* at that farm, and various genotypes may be isolated from affected foals or their environment at a particular farm.[10,11] Although the disease appears to be most common at larger breeding farms that use practices deemed desirable for management of optimum foal health,[8,9] these criteria do not reliably differentiate farms with foals that have pneumonia attributable to *R equi* from farms with unaffected foals.

A possible explanation for the variation in prevalence of this disease among farms is that environmental exposure is greater at farms with affected foals than at farms with unaffected foals. *Rhodococcus equi* has been isolated from a wide range of environmental samples, including soil, air and airborne dust, chaff and animal feeds, troughs, and stall floors.[4,12-18] Results of studies quantifying virulent isolates of *R equi* in soil samples from breeding farms are exiguous. In Japan, the proportions of virulent isolates in soil samples and fecal samples obtained from foals were significantly greater at an affected farm than at an unaffected farm.[19] The proportions of virulent isolates from soil samples obtained from 3 farms with a history of foals with *R equi* were reportedly higher than historical data from unaffected farms in Japan.[20,21] In Australia, however, soil concentrations are not significantly associated with prevalence of pneumonia attributable to *R equi* among foals at horse breeding farms, whereas airborne concentrations of *R equi* are significantly associated with disease prevalence.[22] To our knowledge, data are lacking for North America regarding the association between disease status of farms with respect to pneumonia attributable to *R equi* and soil concentrations (or absolute and relative proportions) of virulent *R equi*. The purpose of the study reported here was to determine whether an association existed between the concentration or proportion of virulent *R equi* in soil samples and the incidence of foals with pneumonia attributable to *R equi* among horse breeding farms in central Kentucky. Selected characteristics of these farms also were recorded to account for potentially confounding effects in data analysis.

Materials and Methods

Study population—Listings of all members of the Kentucky Association of Equine Practitioners and members of the American Association of Equine Practitioners who resided in Kentucky were obtained, and duplicate entries were eliminated. All 337 members of the resulting list were contacted by mail to solicit participation in the study. Initially, 47 veterinarians responded to indicate their willingness and ability to participate. Participating veterinarians were requested to identify, on the basis of the medical history, 1 eligible horse breeding farm likely to have foals affected by pneumonia caused by *R equi* during 2006 and 1 eligible horse breeding farm unlikely to have foals affected by this disease; however, veterinarians were encouraged to identify 1 farm of either type when they could not contribute a farm of both types. To be eligible for inclusion, a farm had to have at least 10 foals born and residing on the farm through time of weaning during 2006.

For the purposes of the study, a farm was considered likely to be affected when there was a history of pneumonia attributable to *R equi* among foals born at and residing at the farm during at least 3 of the preceding 5 years; a farm was considered likely to be unaffected during 2006 when there was no history of foals affected with pneumonia attributable to *R equi* during the preceding 5 years.

For purposes of the study, a farm was considered affected when pneumonia attributable to *R equi* was diagnosed in at least 1 foal by use of the study definition for a farm-confirming case; this definition was applied to both farm history and year the study was conducted. A farm-confirming case of pneumonia attributable to *R equi* was defined as a 3-week-old to 6-month-old foal with clinical signs of pneumonia (tachypnea, fever, nasal discharge, and coughing) and from which *R equi* was isolated from a tracheobronchial aspirate or postmortem lung specimen or with clinical signs of pneumonia and at least 2 additional confirmatory results (multifocal pulmonary opacities on thoracic radiographs, ultrasonographically visible pulmonary abscesses, or cytologically visible gram-positive intracellular coccobacilli on tracheobronchial aspirates). Not all foals at participating farms underwent tracheobronchial aspiration or both radiography and ultrasonography of the thorax; therefore, not all foals with pneumonia attributable to *R equi* met the study criteria for a farm-confirming case. At farms with at least 1 farm-confirming case, pneumonia attributable to *R equi* was diagnosed in foals (other than those meeting farm-confirming criteria) on the basis of clinical signs of pneumonia (tachypnea, fever, nasal discharge, or coughing) and any 1 additional confirmatory result (multifocal pulmonary opacities on thoracic radiographs, ultrasonographically visible pulmonary abscesses, or cytologically visible gram-positive intracellular coccobacilli on tracheobronchial aspirates).

At each participating farm, veterinarians were instructed to collect soil samples from 3 areas used to house foals. Soil samples were collected on approximately January 15, March 15, and July 15 (9 samples/farm). Soil samples were to be collected from areas where foals were likely to gather, such as adjacent to watering troughs or creep feeders. Soil samples were to be collected from the same sites at each sample collection time. Soil samples were collected by scraping the surface soil with a metal teaspoon. For each location, a volume of 3 teaspoons (approx 15 g) of soil was collected and placed into a sterile plastic bag; samples were refrigerated until shipped chilled to the Equine Infectious Disease Laboratory at Texas A&M University within 24 hours after collection. The spoon used to collect soil samples was to be cleaned with alcohol between each subsequent sample to reduce contamination. All materials for specimen collection and shipping, including spoons, cold packs, and preaddressed shipping labels, were provided to participating veterinarians.

Bacteriologic culture of *R equi*—A 1-g aliquot of each soil sample was mixed thoroughly with 5 mL of PBS solution[a] to evenly distribute microorganisms.

Quantitative bacteriologic culture of soil suspensions was performed by use of a modified-NANAT, *R equi*–selective agar medium,[9] as described elsewhere.[23] Use of the modified NANAT agar medium effectively minimizes concomitant growth of bacterial and fungal contaminants without inhibiting growth of *R equi*.[23]

Ten-fold serial dilutions of the soil-PBSS suspensions were prepared and cultured in duplicate for all samples. A 100-µL volume of the soil suspension was inoculated on the surface of an NANAT agar medium plate and evenly dispersed by use of a sterile plate spreader. Positive and negative control specimens were cultured to ensure validity of the results. The 2 positive control specimens consisted of a pure culture of virulent *R equi* (ATCC strain 33701) grown in *R equi* minimal medium[3] to approximately 10^8 CFUs/mL or 1 g of soil that was inoculated with approximately 10^8 CFUs of virulent *R equi*. Ten-fold serial dilutions were used for both positive control specimens, and 100 µL of the dilution containing approximately 100 CFUs of virulent *R equi*/mL was plated onto separate agar plates. The 2 negative-control specimens consisted of 100 µL of *R equi* minimal medium (used to make the pure cultures) or 100 µL of PBSS (used to resuspend soil for quantitative bacteriologic culture). All quantitative culture control specimens were plated on modified NANAT agar medium used for all other samples in the study.

Culture plates for control and soil specimens were incubated at 34°C for 48 hours. For each dilution of each soil sample, the number of CFUs of *R equi* per culture plate was counted; the dilution containing 15 to 150 CFUs of *R equi*/culture plate was used for colony immunoblotting. In rare situations when 2 dilutions of a soil sample yielded 15 to 150 CFUs of *R equi*/culture plate (eg, 148 CFUs of *R equi* on a plate prepared from a given dilution and 21 CFUs on a plate prepared from the subsequent higher dilution), the plate with the lower number of CFUs was used. Number of CFUs on a plate was multiplied by the dilution factor to determine the concentration of *R equi* in soil. Mean concentration (number of CFUs/g of soil) of *R equi* in the 2 duplicates was determined and used for analysis.

Modified colony immunoblot assay—The concentration of virulent *R equi* in soil was determined by use of a modified immunoblotting technique for detection of VapA,[24] as described elsewhere.[23] This method allowed quantification of the concentration of virulent *R equi* within a background of bacterial and fungal contamination. Nitrocellulose membranes[b] were placed on previously incubated modified NANAT agar culture plates that contained 15 to 150 CFUs of *R equi* and were allowed to become completely saturated. Once saturated, membranes were removed, air-dried at 22°C for 30 minutes, and baked in an oven[c] at 100°C for 1 minute. Nitrocellulose membranes were incubated in 5% nonfat dry milk[d] (diluted in TBSS[e]) for 60 minutes at 37°C to block unbound sites. Membranes were then washed 3 times in TBSS with 0.05% Tween 20.[f] Membranes were incubated overnight at 4°C on a rocker[g] with a murine-derived IgG1 isotype monoclonal antibody directed against vapA diluted 1:10,000 in 5% nonfat dry milk. Membranes then were washed 3 times (10 min/wash) with fresh TBSS at 37°C. Horseradish peroxidase–conjugated goat IgG fraction against mouse IgG[h] was diluted 1:2,000 in 5% nonfat dry milk and added to the membranes, which were then incubated for 1 hour at 37°C. Membranes then were washed 3 times (10 min/wash) with fresh TBSS at 37°C, 1 wash with citrate-EDTA buffer (10mM sodium citrate[i] and 10mM EDTA[j] [pH, 5.0]) for 5 minutes at 37°C, 1 wash with citrate-EDTA buffer and 1% dextran sulfate[k] 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[l] and hydrogen peroxide were added to the membranes, which were then incubated for 30 minutes at 37°C. The substrate was discarded, and distilled water was added to stop development. Colonies of virulent *R equi* appeared blue, whereas colonies of avirulent *R equi* and contaminants remained colorless.

Positive and negative control specimens were also included with each batch of colony immunoblots; positive and negative control specimens consisted of modified NANAT agar medium plates on which were grown pure cultures of virulent (ATCC strain 33701) and avirulent (ATCC strain 33703) *R equi* that had been grown from stock in *R equi* minimal media[3] prior to plating, respectively. Ten-fold serial dilutions were created from virulent and avirulent *R equi* by use of PBS solution, and 100 µL of the resulting dilution that contained approximately 10^4 CFUs of *R equi*/mL was individually plated. All colony immunoblot control plates were incubated under the same conditions as for the quantitative bacteriologic culture control plates and soil specimen plates. The proportion of virulent isolates in the soil specimen plates was determined by dividing the total number of virulent colonies from both duplicate plates by the total number of *R equi* colonies that were on both duplicate plates prior to colony immunoblotting. Concentration of virulent isolates was determined by multiplying the proportion of virulent isolates by the concentration of *R equi* in the soil sample.

Data collection for farms—In November 2006, participating veterinarians were sent a farm data questionnaire. Veterinarians were asked to record the following data about each farm included in the study: name of veterinarian; date form was completed; name of farm or farm owner; county where farm was located; whether the farm had a history of foals affected by pneumonia attributable to *R equi*, as previously defined; whether the farm had at least 1 affected foal during 2006; the predominate breed of horse at the farm (Thoroughbred vs other breeds); total area used to house mares and foals; number of years the farm had been used for raising horses; total number of adult horses at the farm during 2006; total number of foals residing at the farm during 2006; number of resident mares at the farm during 2006; number of transient (nonresident) mares at the farm during 2006; total number of foals affected with pneumonia attributable to *R equi* during 2006; number of affected foals that died or were euthanized because of infection with *R equi*; whether any of several methods (microbiologic culture of tracheobronchial aspirates, cytologic examination of tracheobronchial aspirates, ultrasonographic evidence of pulmonary abscesses or consolidation, radiographic evidence of multifocal pulmonary abscesses, clinical signs of pneumonia, find-

ings during gross necropsy, or microbiologic culture of lung samples obtained during necropsy) was used to diagnose pneumonia attributable to *R equi* in foals at the farm; type of flooring (dirt, sand, concrete, rubber mats, asphalt, or other) in foaling stalls; age at which foals were first turned out to pasture or paddock; age at which foals were no longer maintained in stalls; and whether hyperimmune plasma was administered to foals at the farm to prevent pneumonia attributable to *R equi* during 2006. Questionnaires were manually evaluated for errors, and apparent errors were resolved by telephone conversation with the relevant participating veterinarian.

Data analysis—For analysis, mean data for the 3 samples from a specific farm at a specific sample collection were used (ie, sum of the values for samples 1 to 3 for a specific farm/3). Total concentration of *R equi*, concentration of virulent *R equi*, and proportion of virulent *R equi* in soil samples among categories of farms at each sample collection point were compared. Furthermore, differences in soil outcomes between time points were calculated (ie, difference 1 = value for January sample – value for March sample; difference 2 = value for January sample – value for July sample; and difference 3 = value for March sample – value for July sample).

Previously affected versus currently affected and unaffected farms—Comparisons were made among the 3 calculated differences for the categories of farms. Data were summarized as mean and SD and as median values and interquartile (25th to 75th percentiles) ranges for continuous data (such as concentrations or proportions of virulent organisms). When data appeared to have a non-Gaussian distribution, they were transformed by use of the natural logarithm of the observed value to conform to assumptions of Gaussian distribution and homogeneity of variances necessary for valid application of ANOVA methods. Continuous variables were compared among farm categories by use of ANOVA; when a significant effect of farm category was detected, post hoc testing with the method of Sidak[25] was used to identify significant differences among the 3 pairwise contrasts of farm types (ie, affected vs unaffected, affected vs previously affected, and previously affected vs unaffected). After data were collected and analyzed, the association between the density of mares and foals and each of the various soil outcomes at each time point was examined by use of linear least-squares regression analysis. A significance value of $P < 0.05$ was used for all aforementioned analyses. Analyses were conducted by use of commercial software.[m]

Explaining differences of incidence rate among affected farms—To examine separately the potential for each of the *R equi* soil classifications (ie, total, total virulent, proportion virulent, and differences for each classification for sampling periods 1 minus 2, 1 minus 3, and 2 minus 3, respectively) to impact incidence on affected farms, a ZINB model[n] was used. First, variance was partitioned into factors associated with a farm being currently unaffected. Second, factors that influenced the incidence rate on farms that were currently affected were examined.[26]

Graphic approaches were used initially to examine the distribution of incidence rates of *R equi* by farm (by use of a histogram) and then to examine the relation between the incidence and each of the soil classifications stratified by affected versus unaffected farms (by use of a scatterplot). A distinctive bimodal distribution with a large number of zero-incidence farms would favor the ZINB model approach.

The ZINB model was constructed and evaluated as follows. First, factors with potential to explain currently unaffected farms (ie, zero-inflation) were each examined in bivariate models. The same variables listed previously were each assessed in this model. Then, a multivariable model was built forward, with zero-inflating terms deemed significant ($P < 0.10$) being forced into subsequent models to examine the incidence rates on farms. Coefficients from the zero-inflated component of the models were interpreted as the logarithmic odds for a farm being unaffected by *R equi* in the current foaling season. The overall significance of use of the ZINB model versus a simpler negative binomial model (ie, ignoring zero-inflation) was evaluated by use of the Vuong test[n] ($P < 0.05$).

To evaluate the impact of the 3 *R equi* soil classifications on incidence rate at each of 3 time points as well as the differences between time points, several strategies were used. First, each continuous variable representing total *R equi*, total virulent *R equi*, or proportion of virulent *R equi* at each of 3 time points was converted into a 3-level categoric variable at the 33.3 and 66.7 percentile cutpoints. Then, the 2 highest categories (66.7 and 100 percentiles) were examined relative to the referent category (33.3 percentile). The dependent variable was the number of *R equi*–affected foals in the current foaling season, offset by the number of foals at-risk during the current foaling season. Variables with a value of $P < 0.05$ for a likelihood-ratio test with 2 *df* were considered significant.

For differences between time points, a strategy for classifying variables was used. For total *R equi* and virulent *R equi*, differences in logarithm of the number of CFUs between 2 time points were evaluated by dividing data into tertiles; however, in this case, the referent category (ie, omitted from the model) was the category that spanned a difference of 0 (typically, but not always, the middle category). This permitted comparison of farms with positive differences between 2 time points (ie, a decrease in total logarithms of *R equi* bacteria) with farms that had little or no change. Similarly, farms with a negative difference (ie, an increase in total logarithms of *R equi* bacteria) could be compared with farms that had little or no change. Finally, for the difference in proportion (percentage) of *R equi* that was virulent, when comparing 2 time points, the cutpoints for the 3-level categoric variable were established for all 3 time-point differences. Category 1 was farms for which the difference was < –2%. Category 2 (referent) was farms for which the difference was ≥ –2% and < 2%. Category 3 was farms for which the difference was ≥ 2%. Large positive differences indicated that the proportion of virulent *R equi* (relative to total *R equi*) had decreased between 2 time points. Conversely, a negative difference indicated the proportion of virulent *R equi* had increased. Values of $P < 0.05$ were deemed significant

for a likelihood-ratio test with 2 *df*. The antilog of each model coefficient represented the IRR of pneumonia attributable to *R equi* relative to the referent category for all 3-level categoric soil classifications.

Results

Participating farms—Of the 47 veterinarians who initially indicated willingness to participate, 24 (51%) ultimately submitted soil samples and data for 37 horse breeding farms. These farms were from 6 counties in the region of Lexington, Ky, including Bourbon (7 farms), Clark (3), Fayette (12), Scott (4), Versailles (3), and Woodford (9). There were 16 farms that had foals affected by pneumonia attributable to *R equi* during 2006 and 21 farms that did not have affected foals during that year. Median number of foals with pneumonia attributable to *R equi* at affected farms was 5 (interquartile range, 3 to 10 foals; range, 1 to 17 foals), and the median cumulative incidence of affected foals at farms was 18% (interquartile range, 10% to 26%; range, 1% to 54%). Analysis of the histogram of incidence rates for the 37 farms revealed a bimodal distribution with marked inflation (ie, excess) of zero-incidence farms (**Figure 1**). Of the 16 affected farms, 15 had a history of foals with pneumonia attributable to *R equi*. Of the 21 farms without affected foals during 2006, 9 had a history of foals with pneumonia attributable to *R equi*. Consequently, farms were categorized into 3 groups (affected farms [n = 16], farms with foals affected during 2006; previously affected farms [9], farms with a previous history of foals with pneumonia attributable to *R equi* but no affected foals during 2006; and unaffected farms [12], farms with no history of affected foals and no affected foals during 2006). These farms housed 3,749 mares and foals (1,984 at affected farms, 847 at previously affected farms, and 918 at unaffected farms), of which 1,380 (37%) were foals residing at a farm through weaning (671 foals at affected farms, 330 foals at previously affected farms, and 379 foals at

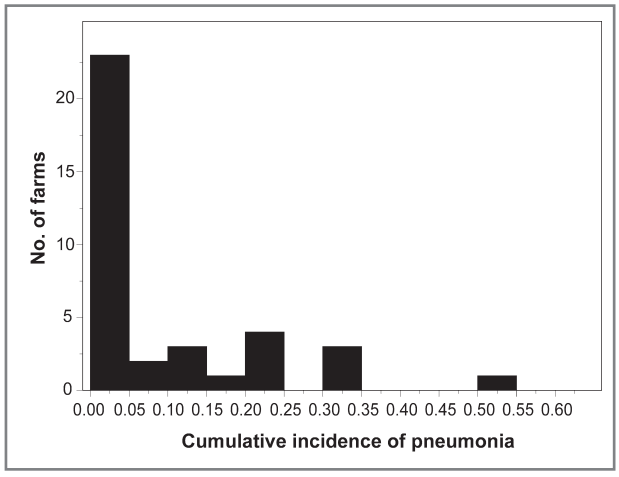

Figure 1—Frequency distribution of the cumulative incidence of foals with pneumonia attributable to *Rhodococcus equi* at 37 horse breeding farms in central Kentucky during the 2006 foaling season (March to July 2006).

Table 1—Characteristics of 37 horse breeding farms in central Kentucky used to evaluate pneumonia in foals attributable to *Rhodococcus equi* and *R equi* in soil samples.

Variable	Value	Affected	Previous	Unaffected	*P**
Total hectares for horses	Median (range)†	68 (24–126)	81 (61–101)	61 (30–118)	—
	Mean ± SD	96 ± 86	95 ± 70	127 ± 173	—
Log (total hectares)	Mean ± SD	4.1 ± 0.4	4.3 ± 0.3	4.2 ± 0.4	0.870
Horses and foals at farm	Median (range)†	100 (50–152)	45 (40–140)	45 (30–87)	—
	Mean ± SD	124 ± 103	94 ± 93	76 ± 71	—
Log (horses and foals)	Mean ± SD	4.5 ± 0.8	4.1 ± 1.0	3.8 ± 0.8	0.288
Foals at farm	Median (range)†	30 (20–55)	20 (15–60)	17 (11–28)	—
	Mean ± SD	42 ± 30	37 ± 32	32 ± 36	—
Log (foals)	Mean ± SD	3.5 ± 0.8	3.2 ± 0.9	3.0 ± 0.9	0.364
Resident mares	Median (range)†	31 (15–62)	22 (14–85)	23 (14–34)	—
	Mean ± SD	44 ± 35	52 ± 49	41 ± 50	—
Log (resident mares)	Mean ± SD	3.4 ± 0.8	3.5 ± 1.1	3.2 ± 1.0	0.735
Transient mares	Median (range)†	15 (4–34)	10 (5–10)	2 (0–12)	—
	Mean ± SD	40 ± 74	8 ± 5	9 ± 12	—
Log (transient mares)	Mean ± SD	2.5 ± 1.8	1.9 ± 0.9	1.3 ± 1.5	0.154
Density of horses and foals (equids/hectare)	Mean ± SD	1.63 ± 0.57[a]	0.94 ± 0.47[b]	0.89 ± 0.47[b]	0.008
Density of foals (foals/hectare)	Mean ± SD	0.59 ± 0.32[a]	0.37 ± 0.20[a,b]	0.32 ± 0.17[b]	0.015
Years the farm was used for horses	Median (range)	20 (18–26)	20 (20–30)	30 (18–42)	—
	Mean ± SD	23 ± 16	36 ± 37	34 ± 26	—
Log (years)	Mean ± SD	2.9 ± 0.7	3.0 ± 1.3	3.2 ± 0.9	0.756

Data that did not appear to have a Gaussian distribution were transformed by use of the natural logarithm to meet assumptions for an ANOVA. Farms were defined as follows: Affected = farm with foals affected by pneumonia attributable to *R equi* during 2006; Previous = farm with a previous history of affected foals but none affected during 2006; and Unaffected = farm with no history of affected foals and no affected foals during 2006.

*Values reported are *P* values for the *F* statistic obtained by use of an ANOVA; values were considered significant at $P < 0.05$. †Range represents the interquartile (25th to 75th percentiles) range.

[a,b]Within a row, values with different superscripts differ significantly ($P < 0.05$; post hoc testing).

— = Not applicable.

unaffected farms). Of the 671 foals residing at the 16 affected farms, 123 (18%) developed pneumonia attributable to *R equi*. Of the 123 affected foals, 2 (2%) died from the disease.

Diagnostic methods most commonly used to identify foals with pneumonia attributable to *R equi* at the 16 affected farms were ultrasonography (15 farms) and clinical signs (15), followed by microbiologic culture of tracheobronchial aspirates (6) and cytologic evaluation of tracheobronchial aspirates (5). All affected farms whose veterinarian used ultrasonography also concurrently used clinical signs for diagnosis, except 1 farm at which the veterinarian used ultrasonographic findings in combination with results of microbiologic culture and cytologic evaluation of tracheobronchial aspirates collected from foals; this farm had at least 1 clinically affected foal, as determined in accordance with the study definition for a farm-confirming case.

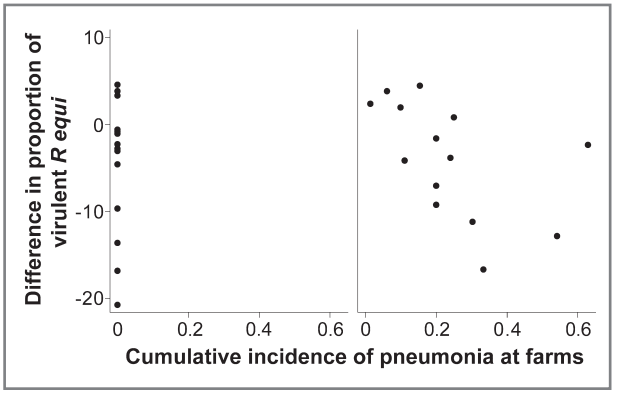

Figure 2—Scatterplot of the absolute difference of the proportion of virulent *R equi* in soil samples collected in March and July 2006. Data were stratified on the basis of farm status for the 2006 foaling season, with the panel on the left representing data from farms at which no foals had pneumonia attributable to *R equi* during 2006 (ie, cumulative incidence = 0) and the panel on the right representing data from farms at which foals had pneumonia attributable to *R equi* during 2006.

The predominant breed was Thoroughbred at affected farms (15/16), previously affected farms (8/9), and unaffected farms (9/12); these proportions did not differ significantly. Total area used for horses did not differ significantly among farms (**Table 1**). Although the total numbers of horses, foals, resident mares, and transient mares at affected farms typically were greater than those at other types of farms, there was no significant difference in the distributions. However, distribution of densities of horses, foals, and the sum of horses plus foals differed significantly among categories of farm, with density of horses and foals being significantly greater at affected farms.

Number of years the farm had been used to house horses did not differ significantly between affected farms (median, 20 years; interquartile range, 18 to 28 years) and unaffected farms (median, 28 years; interquartile range, 19 to 39 years). Similarly, there was no

Table 2—Concentrations of total *R equi* and virulent *R equi* and proportion of virulent isolates in soil samples collected from 37 farms in central Kentucky during 2006.

Collection period	Variable	Value	Affected	Previous	Unaffected	*P**
January			n = 15	n = 5	n = 11	
	Total *R equi*	Median (range)†	35,778 (15,360–61,083)	50,678 (38,667–76,888)	41,778 (15,200–69,305)	—
	(CFUs/g)	Mean ± SD	44,323 ± 36,422	62,880 ± 33,000	43,608 ± 34,587	—
	Log (total *R equi*)	Mean ± SD	10.3 ± 1.0	10.9 ± 0.5	10.3 ± 1.0	0.395
	Virulent *R equi*	Median (range)†	2,266 (1,397–6,673)	4,459 (3,004–7,040)	1,380 (1,093–5,926)	—
	(CFUs/g)	Mean ± SD	5,373 ± 6,905	8,140 ± 9,007	4,271 ± 5,036	—
	Log (virulent *R equi*)	Mean ± SD	8.0 ± 1.1	8.6 ± 0.9	7.7 ± 1.3	0.335
	Proportion virulent	Median (range)†	8.2 (6.2–13.1)	8.9 (8.5–15.8)	8.6 (6.2–11.9)	—
	(%)	Mean ± SD	10.2 ± 5.5	12.4 ± 7.4	9.1 ± 4.0	—
	Log (proportion virulent)	Mean ± SD	2.2 ± 0.5	2.4 ± 0.6	2.1 ± 0.5	0.651
March			n = 14	n = 5	n = 11	
	Total *R equi*	Median (range)†	16,750 (12,417–30,611)	24,461 (17,210–30,639)	28,278 (10,139–69,157)	—
	(CFUs/g)	Mean ± SD	23,258 ± 18,568	23,209 ± 10,272	43,337 ± 41,247	—
	Log (total *R equi*)	Mean ± SD	9.8 ± 0.8	10.0 ± 0.5	10.2 ± 1.0	0.484
	Virulent *R equi*	Median (range)†	1,638 (793–2,874)	3,581 (2,713–3,728)	1,742 (694–3,219)	—
	(CFUs/g)	Mean ± SD	2,146 ± 2,372	2,860 ± 1,579	2,177 ± 1,838	—
	Log (virulent *R equi*)	Mean ± SD	7.1 ± 1.3	7.7 ± 1.0	7.2 ± 1.1	0.641
	Proportion virulent	Median (range)†	9.6 (5.2–11.4)	10.6 (7.5–13.9)	5.1 (3.6–7.7)	—
	(%)	Mean ± SD	8.9 ± 4.6	10.9 ± 5.6	6.1 ± 3.1	—
	Log (proportion virulent)	Mean ± SD	2.0 ± 0.6	2.3 ± 0.6	1.7 ± 0.5	0.158
July			n = 14	n = 5	n = 11	
	Total *R equi*	Median (range)†	44,555 (27,820–69,516)	84,389 (78,333–88,889)	54,11 (34,278–83,667)	—
	(CFUs/g)	Mean ± SD	48,723 ± 30,686	82,988 ± 11,313	62,616 ± 33,785	—
	Log (total *R equi*)	Mean ± SD	10.5 ± 0.8	11.3 ± 0.2	10.9 ± 0.6	0.092
	Virulent *R equi*	Median (range)†	6,090 (2,074–9,274)	6,673 (4,880–7,396)	6,541 (4,121–6,935)	—
	(CFUs/g)	Mean ± SD	8,010 ± 9,185	6,907 ± 2,643	5,978 ± 3,542	—
	Log (virulent *R equi*)	Mean ± SD	8.5 ± 1.1	8.8 ± 0.4	8.4 ± 0.9	0.769
	Proportion virulent	Median (range)†	10.3 (8.1–14.3)	9.6 (8.8–11.6)	9.2 (6.9–15.3)	—
	(%)	Mean ± SD	12.9 ± 8.4	9.7 ± 3.0	12.3 ± 7.7	—
	Log (proportion virulent)	Mean ± SD	2.4 ± 0.6	2.2 ± 0.4	2.3 ± 0.6	0.861

n = No. of farms.
See Table 1 for remainder of key.

significant difference in the number of years for which the farm was used to house horses between farms with a history of pneumonia attributable to *R equi* (median, 20 years; interquartile range, 18 to 28 years) and farms that had no such history (median, 30 years; interquartile range, 19 to 39 years). All foals at all farms were born in specifically designated foaling stalls. There was no significant difference among the categories of farms in the distribution of the types of flooring in foaling stalls (rubber mats [15 farms], dirt [11], and asphalt [9] were the flooring types most commonly reported, with 2 farms reporting other types of flooring). All farms indicated that foals were exposed to the outdoors at < 1 week after birth. The distributions of age at which foals were no longer housed in a stall did not differ among categories of farm (mean and median age for this factor was 4 weeks).

Soil data—Veterinarians were generally compliant with respect to submission of samples. Median dates of specimen collection in 2006 for the January, March, and July samples were January 30, March 21, and July 18. Soil samples for each sample collection period were not obtained from all 37 farms that provided samples for at least 1 collection point (31 samples were obtained in January [15 affected, 5 previously affected, and 11 unaffected farms], 28 samples were obtained in March [14 affected, 4 previously affected, and 10 unaffected farms], and 30 samples were obtained in July [14 affected, 5 previously affected, and 11 unaffected farms]). Soil samples from all farms yielded both virulent and avirulent *R equi* for all sample collection points. There were no significant differences at any sample collection point among affected, previously affected, and unaffected farms in soil concentrations of total *R equi* or virulent *R equi* or in the proportion of virulent organisms in soil samples (**Table 2**).

Three soil samples were collected at each time point such that 267 individual soil samples were submitted. Of these, 265 (99%) yielded *R equi*, and virulent isolates were detected in 253 of 265 (95%) samples yielding *R equi*. No farm had > 1 soil sample that had negative results for *R equi* or virulent *R equi* at a given time point, and only 1 farm (with foals affected with pneumonia attributable to *R equi*) had soil samples from the same location that yielded no growth of virulent *R equi* at > 1 time point; soil from this location at this farm yielded negative results for virulent *R equi* on 2 of 3 time points, whereas samples from the 2 other locations yielded virulent *R equi*.

Differences (and logarithms of these differences) in soil concentrations of total and virulent *R equi* and the proportion of virulent *R equi* in soil samples between each pair of sample collection times were calculated. Samples were obtained for 26 farms for both the first and second sample collection points, for 27 farms for the first and third sample collection points, and for 28 farms for the second and third sample collection points. There were no significant differences among farm types in any paired differences of soil concentrations for concentrations of total or virulent *R equi* or for paired differences in the proportion of virulent isolates. Use of linear regression analysis revealed that there were no significant associations at any sample collection time between the density of horses, foals, or the sum of horses and foals and soil concentrations of total *R equi* or virulent *R equi* or the proportion of virulent isolates in the soil.

Among those variables assessed as to their potential impact on inflating the zero-incidence, prior history of *R equi* ($P = 0.019$; OR_{adj}, 0.04; 95% CI, 0.002 to 0.58) and increasing foal density (for each additional foal/0.4 hectares [ie, 1 acre]; $P = 0.064$; OR_{adj}, < 0.0001; 95% CI, 6.1×10^{-10} to 1.80) greatly reduced the relative odds of being an unaffected farm. The Vuong test indicated that the ZINB model was a significant ($P < 0.001$) improvement over a standard negative binomial model. With these 2 zero-inflation terms forced into subsequent models to examine the relative incidence rates for each of the 3-level categoric variables of total *R equi* (logarithms), virulent *R equi* (logarithms), proportion of virulent *R equi*, and differences between time points, only 1 soil classification had a significant association with incidence on affected farms. When comparing time points 2 (March) and 3 (July), farms with a decrease in the proportion of virulent *R equi* between the 2 time points had a lower incidence (IRR, 0.53; 95% CI, 0.21 to 1.34) when compared with farms with little or no difference in the proportion between the 2 time points. Conversely, farms with an increase in the proportion of virulent *R equi* between the 2 time points had an increased incidence (IRR, 2.18; 95% CI, 0.96 to 4.94) when compared with farms with little or no difference in the proportion between the 2 time points. Overall, the importance of this 3-level variable was significant ($P = 0.002$) with a likelihood-ratio χ^2 test of 13.06 with 2 *df*. In other words, among affected farms, those with a decrease in the proportion of virulent *R equi* in soil from March to July had a significantly lower incidence of foals with pneumonia attributable to *R equi* when compared with those farms for which the proportion increased between the same time points (**Figure 2**). However, this association cannot be interpreted to extend to unaffected farms.

Discussion

Analysis of results of the study reported here indicated that concentrations of total *R equi* (ie, virulent and avirulent isolates), virulent *R equi*, and the proportion of isolates that were virulent in soil samples did not differ significantly among farms with foals affected by pneumonia attributable to *R equi*, farms with a previous history of the disease but no foals affected during the year of the study, and farms without affected foals and with no history of disease. Thus, differences in soil concentration of virulent *R equi* or the proportion of virulent isolates in the soil do not appear to predict or correlate with the incidence of foals with pneumonia attributable to *R equi* at breeding farms in central Kentucky. These results are consistent with a report[22] in which soil concentrations of virulent *R equi* were not significantly correlated with prevalence of pneumonia caused by *R equi* among foals at Thoroughbred breeding farms in Australia. However, in that study, airborne concentrations of virulent *R equi* were correlated with prevalence of pneumonia in foals caused by this bacterium. Unfortunately, we did not collect air samples in

our study. Additional investigations are indicated to assess the association between air concentrations of virulent *R equi* and the incidence of pneumonia attributable to *R equi* among foals at horse breeding farms in North America.

Analysis of results of the study reported here indicated that exposure to virulent *R equi* in soil is widespread at horse breeding farms. Virulent *R equi* were identified in soil from all farms at all sample collection times included in the study. This result differs from that in other reports[4,5,19-22] in which the proportions of farms or soil samples with virulent isolates were < 50%. Although the reason for this discrepancy is unknown, it may be attributable to differences among studies in methods of specimen collection, specimen handling, microbiologic culture, and detection of virulent isolates. Although contamination of soil specimens before or during collection at a farm or in the laboratory after collection could explain this result, we do not believe this to be the case for several reasons. Farm was the unit of analysis, and samples from a specific farm were collected and submitted individually, with soil samples placed in separate sterile bags. Nevertheless, contamination of soil sample by isolates of *R equi* that were in the soil or the air from the same farm cannot be excluded. With regard to contamination of soil samples at the laboratory, samples were individually packaged and shipped in sealed bags. At the laboratory, negative control plates were used during each culture procedure such that contamination of reagents and instruments with virulent *R equi* would have been detected.

Other explanations exist for the high prevalence of virulent *R equi* in soil specimens. Collection and pooling of results of 3 samples from a farm provided increased opportunities to identify *R equi* in soil samples, relative to a single sample. The impact of this pooling was small because 265 of 267 (99%) samples yielded *R equi* and 253 of 265 (95%) *R equi*–positive samples yielded virulent *R equi*. Use of a modified NANAT medium improved our ability to culture *R equi*, relative to that achieved by use of other media, including standard NANAT medium (data not shown). Interestingly, the proportion of the concentrations of virulent isolates in this study was similar in magnitude to the proportions of virulent isolates among isolates from soil samples in other studies.[4,5,19-21]

The rationale for collecting soil samples at 3 time points was 2-fold. First, we were interested in determining whether the soil variables evaluated in the study differed among farms in soil samples collected early in the year (January) prior to development of pneumonia attributable to *R equi* among foals because this would have indicated whether differences in these variables preceded (and therefore predicted) pneumonia among foals. Second, we were interested in determining whether there was evidence of a temporal pattern for the samples collected during the study. Analysis of results of the study indicated that the concentration or proportion of virulent *R equi* in soil samples early in the year did not predict the disease status of participating farms and that there were no significant differences in these values during the study among the categories of farms. Soil samples from the 3 locations were pooled for analysis. This pooling could have resulted in loss of information relative to analysis on individual samples. We do not believe that pooling samples meaningfully altered our results. First, 99% of samples submitted yielded *R equi*, and 95% of the samples yielding *R equi* had virulent isolates; no farm ever had > 1 sample at a particular time point that yielded no *R equi* or no virulent *R equi*. Moreover, no farm had multiple samples that failed to yield growth of *R equi*, and only 1 affected farm had soil samples from the same location that failed to yield growth of virulent *R equi* at 2 time points (a sample from this same location yielded virulent *R equi* on 1/3 samples). Second, results of analysis were similar when samples were considered individually by location (results not shown).

Of the farm characteristics recorded, only density of mares and foals (or foals alone) was significantly associated with farms having foals affected with pneumonia attributable to *R equi*. This result is consistent with that in another report,[27] which indicates that reducing the density of horses may help reduce the burden of pneumonia attributable to *R equi* among foals at horse breeding farms. One explanation for this association is that a greater density of horses results in a greater environmental burden of virulent *R equi*, either directly from excretion of virulent *R equi* by mares and foals or indirectly from a relatively greater concentration of feces in which the organism could proliferate. In the study reported here, we found no significant association between the concentration or proportion of virulent *R equi* and the density of horses and foals (or foals alone). Thus, other explanations for this association must be considered. Conceivably, a significant correlation may exist between airborne concentrations of virulent *R equi* and density of horses; however, some other aspect or aspects of intensive management also may explain the association of increased density of horses or foals with an increased likelihood of pneumonia attributable to *R equi* among foals.

Our finding that a farm history of pneumonia attributable to *R equi* as well as an increase in foal density reduced the odds that a farm was unaffected (as determined by use of the ZINB model) was not unexpected and remains consistent with the aforementioned results of the ANOVA analyses. Analysis by use of the ZINB model permitted us to account for factors that caused a farm to be unaffected or affected, and then to attempt to distill those factors that further contributed to an increased (or decreased) incidence of pneumonia at affected farms. In our study, none of the soil classifications at a single time point (ie, total concentration, virulent concentration, or proportion virulent) helped to explain differences in incidence rate determined by use of the ZINB or ANOVA. Among differences between sample collection time points, only those differences of the proportion of virulent *R equi* between March and July were significant ($P = 0.002$) by use of the ZINB model. The impacts of differences in the proportion of virulent *R equi* between January and March were not significant ($P = 0.797$), whereas the differences between January and July were also not significant ($P = 0.204$); these latter differences were differentially more important for farms with an increase in virulence (IRR, 2.27;

95% CI, 0.72 to 7.10), as opposed to those that had a decrease (data not shown). However, models for January to March and January to July became extremely unstable, probably because of our relatively sparse data, with up to 11 missing values/period comparison.

The clinical and epidemiologic importance of the temporal change in proportion of virulent isolates among affected farms was unclear. The purpose of collecting sequential samples during the foaling season was to attempt to discern whether the association of the concentration or proportion of virulent *R equi* in soil samples was a predictor (cause) or a result (effect) of foals developing pneumonia attributable to *R equi* at the farm. Finding that the concentration or proportion of virulent isolates was higher in January (before the birth of most foals) would have been evidence consistent with a causal relationship between virulent *R equi* in soil and pneumonia caused by this bacterium; alternatively, differences between affected and unaffected farms in July (after all foals had been born and when most would have developed signs of pneumonia) would have been as or more likely to have been attributable to an increase in environmental burden resulting from shedding by affected foals.

Pneumonia caused by *R equi* is a slowly progressive disease.[1] Epidemiologic evidence suggests that foals become infected early after birth and that clinical signs develop some number of weeks after the likely time of infection.[28] Thus, the authors' opinion is that the finding that the proportion of virulent isolates typically increased later during the foaling season was most probably an effect of disease incidence on the soil at affected farms, rather than the soil being a cause of an increase in incidence, because for the increase to have been a cause of pneumonia attributable to *R equi*, the increase would have had to precede the time of infection (ie, earlier in the season when foals were extremely young). Although the July sample collection was after the birth of all foals in the study, the age of infection of foals was unknown, and the age at onset of pneumonia was not recorded for each foal. Consequently, it was not possible to establish the temporal relationship between age of infection or age at which pneumonia attributable to *R equi* was diagnosed and the interval from March to July when the increase in the proportion of virulent isolates was detected. Thus, the possibility of a causal relationship between soil and disease cannot be excluded on the basis of these results. For example, virulent organisms may preferentially replicate as ambient temperatures become warmer,[4,20] and this increase in the proportion of virulent organisms may increase the risk of disease in foals as ambient temperatures increase. Additional studies are needed to determine the temporal relationship of disease incidence with soil and airborne bacteria.

The study reported here has a number of limitations. A relatively small proportion of the veterinarians contacted provided soil samples and data, such that the participating veterinarians may represent a biased sample. Data and resources were not available with which to compare respondents and nonrespondents to assess this potential bias. Regardless, it is impossible to know the extent to which the results of this study may be extrapolated to other horse breeding farms in Kentucky or other regions of the United States. It is the authors' opinion that the farms studied could be expected to reasonably represent breeding farms in central Kentucky.

Another limitation of the study was the possible misclassification of farms as affected or unaffected. Diagnostic criteria used for diagnosis of pneumonia attributable to *R equi* varied among participating veterinarians but most commonly involved use of ultrasonographic evidence of lesions consistent with pneumonia attributable to *R equi* and clinical signs of pneumonia. Because microbiologic confirmation of diagnosis was often lacking, some foals deemed to have been affected with pneumonia attributable to *R equi* may have had pneumonia caused by other agents. We believe the impact of this potential misclassification was likely to be small. First, each of the veterinarians participating in the study had extensive experience with health management at breeding farms, including recognition and treatment of foals with pneumonia caused by *R equi*. Second, sensitivity and specificity of microbiologic culture of tracheobronchial aspirates for diagnosis of *R equi* are imperfect (sensitivity ranges from 57% to 100%[29-35]), and it is sometimes difficult to isolate *R equi* from tracheobronchial aspirates of experimentally infected foals.[36] False-positive results may result among healthy foals that aspirate *R equi* from their environment. Third, results were unchanged when data were analyzed to restrict the definition of affected farms to only those farms for which positive results of microbiologic culture of tracheobronchial aspirates was used for diagnosis of pneumonia attributable to *R equi* (data not shown). Fourth, misclassification may have been more likely at the level of the foal than at the level of farm for 2 reasons. First, participating farms had multiple affected foals such that there was greater opportunity for correct classification. Second, only 1 foal had to meet the case definition for a confirmed case of pneumonia attributable to *R equi* for a farm to be classified as affected by the disease, whereas additional foals were classified as having pneumonia attributable to *R equi* when they had clinical signs of disease and at least 1 other factor (isolation of *R equi* from microbiologic culture of tracheobronchial aspirates, cytologically visible gram-positive intracellular coccobacilli in tracheobronchial aspirates, multifocal pulmonary opacities on thoracic radiographs, or ultrasonographically visible pulmonary abscesses).

The issue of misclassification of foals as affected by pneumonia attributable to *R equi* in farm-based studies is problematic. It would be desirable to have all foals defined as affected by *R equi* meet the diagnostic criteria of having clinical signs of pneumonia, findings on thoracic imaging consistent with multifocal pyogranuloma formation, and cytologic and microbiologic findings consistent with *R equi* infection. Unfortunately, in the authors' experience, most experienced equine practitioners forego use of all of these procedures to confirm a diagnosis once the disease has been confirmed in some number of foals at a farm because of time, costs of procedures, and risks of some of these diagnostic procedures to foals. As mentioned previously, none of these diagnostic procedures has a sensitivity or specificity

of 100%, so some degree of misclassification of foals is to be expected, regardless of the case definition selected. Nevertheless, results of farm-based studies must be interpreted cautiously in light of the potential for misclassification resulting from variability in diagnostic criteria.

For all but 1 farm included in the study, clinical signs of pneumonia were used as a criterion for diagnosis of pneumonia attributable to *R equi* among foals. The farm representing the exception did have at least 1 foal with clinical signs, but pneumonia attributable to *R equi* was diagnosed in most foals on the basis of ultrasonographic evidence of pulmonary abscesses identified during sequential screening evaluations and results consistent with *R equi* infection from microbiologic culture and cytologic evaluation of tracheobronchial aspirates collected from foals with ultrasonographic evidence of pulmonary abscesses. Thus, some of the 17 foals with pneumonia attributable to *R equi* (of the 110 total foals) at that farm may have represented subclinically affected foals. Because ultrasonographic screening was not used at all farms, the extent to which there was subclinical pulmonary infection with *R equi* in foals at other farms is unclear. To assess the impact of including this farm, analyses were repeated to exclude data from this farm; neither the effects nor the significance of any results was altered.

As mentioned relative to the ZINB modeling, the number of farms included in the study was modest. This limited the statistical power of the study and may have precluded the identification of important associations.

Results of the study reported here are important because they indicated that neither the concentration nor proportion of virulent isolates of *R equi* in soil explained the reason that some farms have foals affected with pneumonia attributable to *R equi* during a given year, whereas others are not affected. Moreover, exposure to virulent *R equi* in soil samples was widespread and common at breeding farms, irrespective of their status with respect to incidence of pneumonia attributable to *R equi* among foals. Thus, although virulent *R equi* is necessary to cause pneumonia attributable to *R equi*, it is clearly not a sufficient cause, at least with regard to its concentration in the soil. Farm-to-farm differences in the incidence of pneumonia attributable to *R equi* likely arise through contributions and interactions of a variety of environmental, host, and farm management factors. Additional studies are warranted to explain the epidemiologic aspects of pneumonia attributable to *R equi* at horse breeding farms in the United States, including assessment of airborne concentrations of *R equi* in barn areas and paddocks or pastures.

a. Phosphate-buffered saline solution, pH 7.2, Invitrogen Co, Carlsbad, Calif.
b. Nitrocellulose membranes, pore size 0.45 µm, Bio-Rad Laboratories, Hercules, Calif.
c. Hybridization oven, VWR International, West Chester, Pa.
d. Nonfat dry milk, Bio-Rad Laboratories, Hercules, Calif.
e. Tris-buffered saline solution, Bio-Rad Laboratories, Hercules, Calif.
f. Tween 20 solution, 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. Sodium citrate, Sigma Chemical Co, St Louis, Mo.
j. Ethylenediaminetetraacetic acid, Sigma Chemical Co, St Louis, Mo.
k. Dextran sulfate, Sigma Chemical Co, St Louis, Mo.
l. 3,3′,5,5′-tetramethylbenzidine, Sigma Chemical Co, St Louis, Mo.
m. S-PLUS, version 7.0, Insightful Inc, Seattle, Wash.
n. Stata, version 9.2, Stata Corp, College Station, Tex.

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