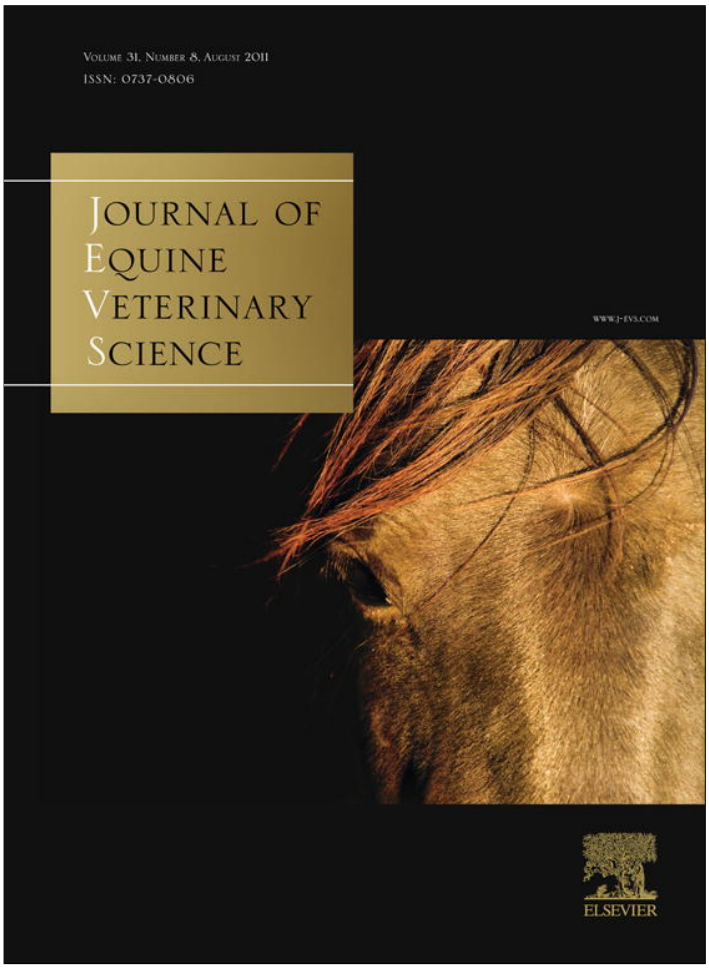

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Original Research

Associations between the Exposure to Airborne Virulent *Rhodococcus equi* and the Incidence of *R equi* Pneumonia among Individual Foals

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ABSTRACT

Rhodococcus equi is a significant cause of pneumonia, resulting in disease and sometimes death of foals. It is believed that infection occurs by inhalation of dust contaminated with virulent *R equi*. Although association between the airborne concentration of virulent *R equi* and the incidence of foal pneumonia at breeding farms has been documented, studies at the level of individual foals have not been reported. Thus, the objective of this study was to determine whether the magnitude of airborne virulent *R equi* was significantly associated with risk of *R equi* pneumonia for individual foals. The concentration of virulent *R equi* was significantly ($P < .001$) greater in stalls than paddocks among samples collected from 47 foals at a breeding farm in central Kentucky. The presence of airborne virulent *R equi* in stalls was significantly ($P = .045$) more likely at 7 days of age for foals subsequently found to be affected by rhodococcal pneumonia. Additionally, airborne concentrations of virulent *R equi* in stalls were significantly greater at 7 and 14 days of age than at birth. Presence of the mare and foal at the time of sampling was significantly ($P < .001$) associated with increased airborne concentrations of virulent *R equi* in stalls. These findings suggest that environments containing airborne virulent *R equi* during the first week of life may influence the risk of subsequent disease for a foal.

1. Introduction

The gram-positive, facultative intracellular, bacterium *Rhodococcus equi* is important for foal welfare and to the equine breeding industry because it causes severe pyogranulomatous pneumonia and lymphadenitis in foals [1-3]. Isolates capable of causing disease in foals possess an 85- to 90-kb plasmid with a pathogenicity island encoding at least one gene product, virulence-associated protein A, which is necessary for virulence [4].

It is commonly accepted that *R equi* is a soil saprophyte [5], but studies have shown that it is also possible to recover virulent and avirulent *R equi* from the feces of mares and foals, as well as from ambient air in paddocks and stalls at horse-breeding farms [6-9]. Current understanding of the pathogenesis of *R equi* pneumonia suggests that pulmonary infection results from inhaling soil-derived virulent organisms that have been aerosolized [8,10,11].

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Although studies have identified multiple environments as possible sources for exposure to virulent *R equi*, it remains unclear why some foals become affected, whereas others in the same environment remain unaffected [6-8,12]. Studies monitoring airborne virulent *R equi* have furthered understanding of the epidemiology of *R equi* pneumonia at horse-breeding farms: airborne concentrations of virulent *R equi* have been positively correlated with the cumulative incidence of *R equi* pneumonia at horse-breeding farms, and contaminated stables may pose a greater risk for infection [8,12]. Model-based [13] and observational epidemiological evidence [14] indicate that most foals become infected with *R equi* early in life. To the authors' knowledge, no studies have investigated the association between airborne concentrations of virulent *R equi* in stalls of individual foals early in life and the risk of developing *R equi* pneumonia. Thus, the primary purpose of this study was to determine whether the magnitude of exposure to virulent *R equi* in stalls and paddocks for individual foals at selected ages during early life was significantly associated with the risk of subsequently developing *R equi* pneumonia at a farm with recurrent history of this disease. Additionally, we examined the association of the airborne concentration of *R equi* with the following variables measured at the time of air sample collection: presence of the mare and foal in the stall, wind speed, ambient temperature, and humidity.

2. Materials and Methods

2.1. Farm and Sampling Criteria

In this study, samples were collected for 47 foals from a single horse-breeding farm in central Kentucky. This farm was selected on the basis of willingness to participate in the study, having a recurrent history of annual cumulative incidence of *R equi* pneumonia of >20% in at least 3 of the previous 5 years, and lack of the use of screening tests, such as thoracic ultrasonography, for early detection of *R equi* pneumonia. The rationale for excluding farms that used screening tests was to exclude foals diagnosed with *R equi* pneumonia that did not develop clinical signs of disease because the probability of disease recorded by positive screening tests is unknown [15].

2.2. Air Sampling Criteria and Procedures

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

Air samples were collected using a portable, commercially available air sampling device (MAS-100 Eco, Merck, Inc., Whitehouse Station, NJ). Petri dishes measuring 100 mm and containing a modified base medium supplemented by nalidixic acid, novobiocin, cycloheximide, and potassium tellurite (NANAT) were used for sampling. The NANAT medium is a selective growth media commonly used for epidemiological studies of *R equi* [7,17]. Samples were collected by placing the air sampler on the ground to collect air approximately 10 cm above the stall floor or paddock ground. In all, 500 L of air were aspirated onto a culture plate, at a rate of 100 L/min. Before each collection, the sieve of the air sampler was disinfected by swabbing with an isopropanol wipe [10]. Ambient temperature, relative humidity, and wind speed were also recorded at the time of collection of each air sample using another handheld device (Skywatch ATMOS, JDC Electronics, Yverdon-les-Bains, Switzerland). Whether mares and/or foals were present at the sampling location when a given air sample was collected was also recorded.

2.3. Modified Colony Immunoblot Assay

Culture plates were shipped to the Equine Infectious Disease Laboratory at the Texas A&M University in insulated containers chilled with icepacks. After being received at the Laboratory, plates were incubated at 37°C for 48 hours. After incubation, colonies of *R equi* were identified according to morphological characteristics. Plates positive for *R equi* growth were then analyzed for virulent *R equi* by using a modified colony immunoblotting assay for detection of virulence-associated protein A [6,7,16,18]. This method allows for the quantification of virulent *R equi* within a background of bacterial and fungal contamination. Airborne concentrations of virulent *R equi* were expressed as colony forming units (CFU) per cubic meter of air (CFU/m^3), calculated by the following equation:

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

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 sampling time, and F is the rate of airflow of the sampling device [19].

Each batch of colony immunoblots contained a positive and negative control specimen. The positive and negative controls consisted of pure cultures of virulent (American Type Culture Collection [ATCC] strain 33701) and avirulent (ATCC strain 33703) *R equi* grown on modified NANAT culture plates. The pure culture strains had been previously grown in *R equi* minimal media [9]. Colony immunoblot control plates were incubated under the same conditions and at the same time as the airborne sample plates.

2.4. Disease Status Data Collection

In September 2009, the farm veterinarian was provided with a follow-up questionnaire for each foal, which included information about the following variables:

whether the foal developed clinical signs of *R equi* pneumonia, age at and date of diagnosis of *R equi* pneumonia, outcome of disease (lived or died), methods used for determining the diagnosis of *R equi* pneumonia in the foal, and whether the foal had other forms of *R equi* infection (abdominal lymphadenitis, osteomyelitis, enterocolitis). Four of the foals enrolled in the study were omitted from the final data analysis because one foal was euthanized (because of a scrotal hernia) and three were transferred off the farm before all airborne samples were obtained. For the purposes of this study, at least one foal at the farm had to demonstrate clinical signs of pneumonia and have microbiologic culture of *R equi* from a tracheobronchial aspirate (TBA) along with cytologic evidence indicating septic pneumonia, to substantiate that pneumonia caused by *R equi* occurred at the farm. Foals were defined as having *R equi* pneumonia if they had clinical signs of pneumonia and any one of the following diagnostic findings: (1) multifocal pulmonary opacities on thoracic radiographs, (2) ultrasonographically visible pulmonary consolidation or abscessation, (3) positive results of microbiologic culture of *R equi* from TBA fluid, and (4) cytologically visible gram-positive intracellular coccobacilli in the TBA fluid. These diagnostic criteria were determined a priori and have been used previously by the investigators [6,7,16,20].

2.5. Data Analysis

Because most air samples yielded no isolates of virulent *R equi*, the data set contained a large proportion of zeros. Consequently, a zero-inflated Poisson (ZIP) model was used to analyze the data [21]. The ZIP model is a mixture of two components: a Poisson distribution for the unbounded counts, and a point-mass at zero to account for the inflated number of zeros. The zero versus one data were modeled by a logistic regression model, whereas the count data were modeled such that the mean of the Poisson distribution was a linear combination of the variables under investigation. The analyses were performed using the expectation-maximization (EM) algorithm for mixture modeling, as described as a general methodology in McLachlan and Peel [22]. Nested ZIP models were compared using a likelihood ratio test [23]. Non-nested models (eg, a Poisson model vs. a ZIP model) were compared using a Vuong test [24]. All coding was done using the R (R, http://cran.r-project.org/) computing platform. A value of $P < .05$ was used for significance. Proportions of air samples positive for virulent *R equi* between stalls and paddocks were compared using χ^2 analysis, and the proportion of samples collected with mares and foals present at the time of sampling were compared between foals that went on to develop *R equi* pneumonia and foals that remained free of *R equi* pneumonia using Fisher's exact test.

3. Results

3.1. Location

Using ZIP modeling, the concentrations of virulent *R equi* were significantly greater in samples collected in stalls than those collected in paddocks ($P < .001$), even after adjusting for effects of age of the foal, disease status of the foal, and presence of the mare and foal at the time of sampling. Comparison of these proportions using χ^2 analysis also identified a significant ($P < .001$) difference between these two locations. Of the 81 samples collected in paddocks, only six (7.4%) yielded positive results for virulent *R equi*, whereas positive results were obtained in 40 (31.5%) of 127 samples from stalls. The median concentration of virulent *R equi* was 0 CFU/m^3 from both paddocks and stalls (range: 0 to 2 CFU/m^3 and 0 to 32 CFU/m^3, respectively). Among air samples yielding positive results for virulent *R equi*, the concentration of virulent *R equi* in each of the six air samples collected from paddocks was 2 CFU/m^3, whereas the median for positive samples from stalls was 4 CFU/m^3 (range: 2 to 32 CFU/m^3).

3.2. Association of Age and Disease Status with the Concentration of Virulent R equi in Air Samples Collected in Stalls

Seven of the 47 foals enrolled in the study were subsequently diagnosed with pneumonia attributed to *R equi*. The median age at the time of diagnosis was 3 months, and all of the foals had clinical signs of pneumonia and sonographic evidence of pneumonia; one foal had *R equi* recovered by microbiologic culture from joint fluid.

Because the number of paddock air samples that yielded positive results was small, and because paddock samples were not collected at birth, only data collected from stalls were evaluated to determine the association of airborne concentration of virulent *R equi* with subsequent development of *R equi* pneumonia and age at the time of sampling. ZIP regression analysis revealed that the zero-inflation parameter (the parameter which captures extra zeros in the airborne concentration of virulent CFU) was significantly affected by age. Also, the interaction between the presence of *R equi* pneumonia and age had a statistically significant ($P = .045$) effect on the zero-inflation parameter (Table 1). In the count data model, airborne concentrations of virulent *R equi* were significantly greater at 1 and

Table 1
Results of zero-inflated Poisson mixture modeling of airborne concentration of virulent *Rhodococcus equi* as a function of age and *R equi* pneumonia status, obtained from stalls that housed 47 foals and their dams at a horse-breeding farm in central Kentucky during 2009; 7 of 47 foals developed clinical signs of pneumonia attributed to infection with *R equi*

Variable	Coefficient	Standard Error of Coefficient	*P* Value
Count data model			
Intercept	0.6898	0.2205	.002
Age 1 week	1.4999	0.2368	<.001
Age 2 weeks	0.9466	0.2555	<.001
Zero values data model (model for the zero-inflation parameter)			
Intercept	0.4645	0.4001	.2456
R equi pneumonia	1.1541	1.1742	.3257
Age 1 week	0.4516	0.5478	.4097
Age 2 weeks	0.4435	0.5484	.4187
R equi pneumonia × age 1 week	−2.9870	1.4897	.0450
R equi pneumonia × age 2 weeks	−1.1541	1.4909	.4389

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

Table 2
Distribution of the results of colony immunoblots for foals that did not later develop *R equi* pneumonia (no *R equi* pneumonia, N = 35) versus foals that did later develop *R equi* pneumonia (*R equi* pneumonia, N = 7) by day

Day of Life and Disease Status	0 CFU/m^3	>0 CFU/m^3
Day 1—No *R equi* pneumonia	23 (65.7%)	12 (34.3%)
Day 1—*R equi pneumonia*	6 (85.7%)	1 (14.3%)
Day 7—No *R equi pneumonia*	25 (71.4%)	10 (28.6%)
Day 7—*R equi* pneumonia	2 (28.6%)	5 (71.4%)
Day 14—No *R equi pneumonia*	25 (71.4%)	10 (28.6%)
Day 14—*R equi* pneumonia	5 (71.4%)	2 (28.6%)

2 weeks of age than at birth. Neither effects of subsequent *R equi* pneumonia nor the interaction term for subsequent pneumonia and age were significantly associated with the mean airborne concentration of virulent *R equi* in the count model (Table 1, count data model). The crude odds of subsequent development of *R equi* pneumonia were approximately 2.5-fold greater among foals that had positive values of airborne concentrations of virulent *R equi* (ie, *R equi* detected) at 7 days of age than for foals that had zero values in stalls at the same age (Tables 1 and 2). Including a term for *R equi* pneumonia in the count model of the ZIP regression did not alter the magnitude or significance of findings (Table 3). A likelihood ratio test indicated that the model including the term for *R equi* pneumonia in the count data model (Table 3) was not significantly ($P = .371$) better than the model without this term (Table 1). However, the ZIP model was significantly ($P < .001$) better than a standard Poisson regression model (data not shown) representing the same terms.

3.3. Association of Other Variables with Airborne Concentrations of Virulent R equi

A secondary aim of the study was to determine whether airborne concentration of virulent *R equi* was associated with presence of mares and foals in the stall, humidity, temperature, and wind speed (all determined at the time of sampling). ZIP modeling indicated that presence of the mare and foal was significantly associated with the concentration of airborne virulent *R equi* at the time of sampling (Table 4), both for the count data model and the zero values data model (model for the zero-inflation parameter). In this modeling, age was significantly associated with the airborne concentration of virulent *R equi*, similar to what was observed in modeling to examine effects of disease (Table 1). A ZIP model identical to that seen in Table 4, but which also included age in the model for the zero-inflation parameter, did not alter significance or magnitude of associations and did not significantly improve fit of the data ($P = .655$). Wind speed, humidity, and ambient temperature were not significantly associated with airborne concentration of virulent *R equi*, either individually or after adjusting for effects of age and presence of mares and foals at the time of sample collection.

As a result of the small number of foals that developed pneumonia attributed to *R equi*, it was not possible to include a term for presence of mares and foals at the time of sampling in models examining the association of airborne concentrations of virulent *R equi* with the development of pneumonia because of the problem of complete separation (ie, some cells of stratified tabulations having no observations). Exploratory data, however, indicated that the effect of presence of mares and foals at the time of sampling would not have confounded the association between airborne concentration of virulent *R equi* and disease. For example, there were 35 foals for whom data regarding presence or absence of mares and foals at the time of sample collection was recorded at 7 days of age (30 foals that did not go on to develop *R equi* pneumonia and five that did). At 7 days of age, the proportion of samples collected with the mare and/or foal present at the time of sampling did not differ significantly between the five foals that ultimately developed *R equi* pneumonia (40%, 2/5) and among the 30 (60%, 18/30) that did not go on to develop *R equi* pneumonia ($P = .6313$, Fisher's exact test; Table 5).

4. Discussion

In our study, there was a significant association between airborne concentration of virulent *R equi* and the risk of subsequent development of *R equi* pneumonia that

Table 3
Results of zero-inflated Poisson mixture modeling of airborne concentration of virulent *R equi* as a function of age and *R equi* pneumonia status, obtained from stalls that housed 47 foals and their dams at a horse-breeding farm in central Kentucky during 2009; 7 of 47 foals developed clinical signs of pneumonia attributed to infection with *R equi*: same model as Table 1, but with disease status included in the count data model

Variable	Coefficient	Standard Error of Coefficient	*P* Value
Count data model			
Intercept	0.6996	0.2208	.002
R equi pneumonia	−0.1386	0.1644	.399
Age 1 week	1.5341	0.2400	<.001
Age 2 weeks	0.9584	0.2558	<.001
Zero values data model (model for the zero-inflation parameter)			
Intercept	0.4695	0.3989	.2392
R equi pneumonia	1.0963	1.1854	.3550
Age 1 week	0.4467	0.5470	.4142
Age 2 weeks	0.4394	0.5475	.4222
R equi pneumonia × age 1 week	−2.9298	1.4986	.0500
R equi pneumonia × age 2 weeks	−1.1035	1.500	.4619

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

Table 4
Results of zero-inflated Poisson mixture modeling of airborne concentration of virulent *R equi* as a function of age and presence of mares and foals at the time of sample collection; air samples were obtained from stalls that housed 47 foals and their dams at a horse-breeding farm in central Kentucky during 2009

Variable	Coefficient	Standard Error of Coefficient	*P* Value
Count data model			
Intercept	0.1660	0.2655	.5317
Age 1 week	1.6795	0.2350	<.001
Age 2 weeks	1.1524	0.2606	<.001
Presence of mares and foals	0.5687	0.1698	<.001
Zero values data model (model for the zero-inflation parameter)			
Intercept	1.0972	0.3399	0.001
Presence of mares and foals	−0.8717	0.4363	0.046

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

Table 5
Presence of the mare and/or foal in the stall at the time of day 7 sample collection and the subsequent development of *R equi* pneumonia for 35 foals

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

Percentages are of column totals.

depended on age. The observed effects of age were difficult to interpret. At 7 days of age, airborne concentrations of virulent *R equi* in stalls were significantly ($P = .045$) greater in the zero values data component of the ZIP mixture model for the seven foals that later developed *R equi* pneumonia (median: 4 CFU/m^3, range: 0 to 26 CFU/m^3) than those that did not later develop *R equi* pneumonia (median: 0 CFU/m^3, range: 0 to 32 CFU/m^3); however, this difference was not significant for the count data component of the ZIP mixture model. There was no significant association between the presence of airborne *R equi* and subsequent development of rhodococcal pneumonia at 2 weeks of age. The importance and interpretation of these findings remain unclear. It is possible that the observed difference in the presence of airborne *R equi* at approximately 1 week of age in the stalls of foals that subsequently developed pneumonia attributed to this bacterium may have occurred simply by chance alone. Alternatively, it is possible that exposure to airborne virulent *R equi* in stalls during early life contributes to increased risk of *R equi* pneumonia. Inhalation of virulent *R equi* is considered to be the route of infection, and one study found that concentrations of virulent *R equi* in the air are positively associated with the risk of disease [8]. Clinical signs of *R equi* pneumonia are generally observed when foals are between 30 and 90 days of life [2], but the age of infection and the incubation period remain unknown. Recent evidence suggests the hypothesis that foals become infected early in life [13,14], rather than during the time when maternal antibodies are decreasing to their nadir [25,26]. If the observed association between airborne virulent *R equi* at approximately 1 week of age and the subsequent development of *R equi* pneumonia reported in this article is true, it is further evidence that infection may occur early in life. Although our results do not indicate any association between the magnitude of airborne concentration of virulent *R equi* (ie, no significant association in the count model) and the risk of disease, exposure to virulent *R equi* was significantly associated with the risk of disease. Exploratory data analysis (Fig. 1) suggests that the distribution of absolute concentrations of airborne virulent *R equi* on day 7 (as well as the presence or absence of the bacterium) differed between affected and unaffected foals, but the small number of affected foals may have limited statistical power to detect a true difference. A larger study is warranted to confirm or refute these results.

The concentrations of virulent *R equi* isolated from stalls were significantly ($P < .001$) greater in stalls than paddocks, and results indicated that the proportion of air samples yielding positive results for virulent *R equi* in stalls (31.5%) was significantly ($P < .001$) greater than in paddocks (7.4%). This finding is consistent with results from a previously

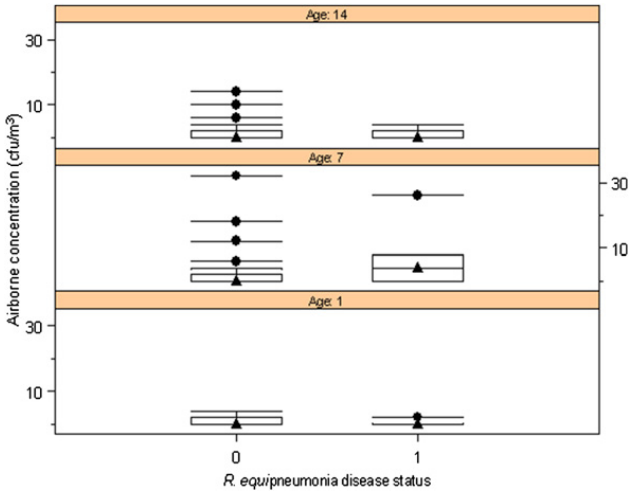

Fig. 1. Box and whisker plots of the airborne concentrations of virulent *Rhodococcus equi* at age of sampling. Group 0 foals included 35 foals that did not later develop *R equi* pneumonia, whereas group 1 foals included seven foals that did later develop *R equi* pneumonia. Boxes represent the interquartile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

reported study involving three horse-breeding farms in Ireland [8], but is in contrast to the results of a previous study conducted in Kentucky by our laboratories [16]. The reason for the differences of results among studies is unclear, but could be attributable to variations among farms in factors that influence airborne concentrations in barns (eg, barn ventilation), or variation among studies with regard to the cumulative incidence of disease and criteria for defining *R equi* pneumonia on affected and unaffected farms [6,7,10].

In our previous study [16], data analysis revealed that horses being present at the *general* location of sampling (ie, whether horses were predominately in the barn or in paddocks at the time of sampling) significantly increased the chances of recovering virulent *R equi* from the ambient air. This finding was a serendipitous and unexpected finding because that study had not been designed a priori to determine whether presence of mares and foals influenced the results of airborne concentrations, in fact, one of the authors (J.L.S.) astutely recorded whether horses were predominately in the barns or in paddocks at the time of sampling. A major limitation of that observation was that it was not known whether mares and foals were present *specifically* at the site of sampling (ie, we did not ascertain whether specific stalls were occupied by a mare or foal at the time of sampling in that study). In this study, however, we attempted to determine whether a given mare–foal pair was present in the stall at the time of sampling to better assess the association between presence of mares and foals at the time of sampling. The presence of mares and foals in the stalls was positively and significantly associated both with the absolute concentrations of virulent *R equi* in airborne samples and whether virulent *R equi* was isolated from the air ($P < .001$ and $= .046$ for the counts and zero values components of the ZIP model, respectively [Table 4]). This finding is important for at least two reasons. First, it indicates that studies of airborne concentrations of

R equi at horse farms must account for whether horses are present at the sampling site. Second, it may have implications for control and prevention of *R equi* pneumonia. Epidemiological studies have determined that soil and feces from the mare are important sources of exposure of foals to *R equi*, but were unable to show an association between the concentration of *R equi* in these environmental sources and the cumulative incidence of *R equi* pneumonia [6,7,20]. It has been suggested previously that the stabling of mares and foals for extensive periods could increase the prevalence of disease at a farm because of a more concentrated and constant exposure to dust contaminated with virulent *R equi* from the feces of mares and foals [8,24]. Our results regarding presence of mares and foals are consistent with this suggestion, and indicate the need to evaluate the effect on the incidence of foal pneumonia of methods to reduce either airborne *R equi* in stalls where mares and foals are housed or extent to which foals are housed in stalls where higher airborne concentrations of *R equi* may occur. Similarly, studies on the effect of increased density of horses at a farm and how the activities of horses and farm personnel contribute to increased airborne concentrations of *R equi* are also warranted.

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

Third, the moderate sample size of only 47 total foals, including only seven that developed *R equi* pneumonia, limited our statistical power. This precluded us from being able to simultaneously examine the association of airborne concentrations of virulent *R equi* with age, subsequent development of *R equi* pneumonia, and presence of mares or foals when samples were collected because of the problem of complete or near complete separation (ie, zero values in some strata of multivariate analysis). Although subsequent studies using a larger sample size will help address this limitation, we found no significant association between disease and whether mares and foals were present at the time of sampling, indicating that confounding was improbable (Table 5). Another limitation of this study is that not all samples were collected in a uniform manner with respect to presence of mares or foals at the time of sample collection. Ideally, we would have collected all samples with mares or foals present (or absent) to avoid potential confounding by this factor. However, at the time the study was designed and implemented, the association between the presence of mares or foals and airborne concentrations of virulent *R equi* was not known to us and was not specified in previous studies. As a consequence, we only recorded whether mares and foals were present at the time of sampling. Moreover, we only recorded these data for 35 of 47 foals. Still, an important result of this study is that it is first to document that presence of mares or foals at the time of sample collection significantly increases airborne concentrations of virulent *R equi* in stalls. As with any farm-based study of *R equi*, the potential for misclassification of disease status exists. In this study, all foals diagnosed with *R equi* pneumonia had clinical signs of pneumonia (fever and either cough, nasal discharge, or elevated respiratory rate) and ultrasonographic evidence of pulmonary consolidation or abscessation concurrent with clinical signs; however, isolation of *R equi* by microbiologic culture of fluid recovered by tracheobronchial aspiration was only confirmed in one foal. Three of the seven foals diagnosed with *R equi* pneumonia that had clinical signs of pneumonia and ultrasonographic evidence also had elevated white blood cell concentrations, and another foal also had radiographic evidence consistent with *R equi* pneumonia. One foal was diagnosed with pneumonia other than *R equi* on the basis of findings of clinical examination, thoracic ultrasonography, microbiologic culture, and response to treatment; virulent *R equi* were not identified in air samples from the stall of this foal at any age. However, the effect of any disease misclassification cannot be predicted, but the expected effect would be to bias the association toward the null (ie, toward observing no association of disease with exposure) when the misclassification is non-differential (ie, when the proportion of foals misclassified as diseased does not depend on exposure status) [27]. Selection of an appropriate model for the data was another difficult task. A Poisson model, which excluded a zero-inflation model, yielded similar results to the count model used in this study. In the Poisson model, the effects at birth and subsequent development of *R equi* pneumonia was less and not significant ($P = .412$, data not shown). Comparison of the models with a Vuong test indicated that a ZIP model was significantly ($P < .001$) better than the Poisson model. Using the zero-inflated term including both age and *R equi* pneumonia provided the best fit.

5. Conclusions

In summary, airborne concentrations of virulent *R equi* were significantly higher in stalls than in paddocks at this farm, and virulent *R equi* were more frequently isolated from air samples obtained from stalls than from paddocks. Additionally, the concentration of virulent airborne *R equi* was significantly higher in stalls when mares and foals were present at the time of sample collection. Among the foals that later developed *R equi* pneumonia, it was more likely that virulent airborne *R equi* was present in the stall at approximately 1 week of age than for foals that remained free of this disease. Further evaluation of the potential causal relationship between airborne concentrations of virulent *R equi* in stalls of foals and

subsequent risk of *R equi* pneumonia are needed to substantiate the results of this study.

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