EVALUATION OF MARES AS A SOURCE OF *Rhodococcus equi* FOR THEIR FOALS USING QUANTITATIVE CULTURE AND A COLONY IMMUNOBLOT ASSAY

A Thesis

by

MICHAEL BRADLEY GRIMM

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

MASTER OF SCIENCE

August 2006

Major Subject: Veterinary Medical Sciences

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Approved by:

Chair of Committee, Noah Cohen
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ABSTRACT

Evaluation of Mares as a Source of *Rhodococcus equi* for Their Foals Using Quantitative Culture and a Colony Immunoblot Assay. (August 2006)

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Chair of Advisory Committee: Dr. Noah Cohen

Fecal specimens from 130 different mares were collected from an endemic farm for 2 consecutive years at 4 different times pre- and post-foaling (41 mares contributed data in both years). A modified NANAT agar medium was used to quantitatively culture 1-g aliquots of the mare feces without inhibition of growth of *Rhodococcus equi*. Once the *R. equi* in the mare feces were quantified and the total concentrations of *R. equi* determined, a colony immunoblot procedure was performed to detect the presence of the virulence-associated protein antigen on the isolates. This allowed for the proportion and concentration of virulent *R. equi* to be determined. Foals that were found to have ultrasonographic evidence of peripheral pulmonary abscessation or consolidation underwent aseptic trans-cutaneous tracheobronchial aspiration. Positive results of TBA were used to categorize foals as affected with *R. equi* pneumonia. *R. equi* pneumonia developed in 31% of the foals. Shedding of virulent *R. equi* was observed in at least 1 sampling period for every mare examined, and >33% were culture-positive during all sampling periods. However, significant differences were not observed in either the fecal concentrations of total or virulent *R. equi* from dams of affected foals compared to dams of unaffected foals. No significant temporal changes in the fecal concentrations

of *R. equi* were observed. It was concluded that dams of affected foals do not shed more *R. equi* in feces than do dams of unaffected foals, indicating that heavier shedding by particular mares does not explain infection in their foals. However, the finding that virulent *R. equi* were excreted in the feces of all sampled mares indicates that mares are likely an important source of *R. equi* for their surrounding environment.

DEDICATION

I dedicate this manuscript to my parents, Larry and Kathy, and also to my sister, Megan, for their continual support throughout my life. Always inspiring me to accomplish more and never allowing me to give up have strengthened me to accomplish my goals.

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I am very thankful to have such wonderful advisors that helped guide me through my research. Drs. Noah Cohen, Ronald Martens, and Melissa Libal were all inspirational during the course of my graduate career.

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CHAPTER I

INTRODUCTION

Rhodococcus equi is a facultative-intracellular organism and an important cause of disease and death among foals, particularly at endemic farms.[1] *R. equi* is primarily a soil saprophyte with growth requirements that are met by herbivore manure and warm climates.[2] *R. equi* can be cultured from the feces of mature horses,[3] and foals as young as 3 days of age.[4] In 1923, it was first described from as the causative agent of pyogranulomatous pneumonia from young foals in Sweden.[5] Today, *R. equi* is considered the most common cause of severe pneumonia in foals and can be isolated from a number of different types of specimens including feces, soil, and tracheobronchial fluid. It has been suggested that *R. equi* is likely part of the normal flora of the equine lower gastrointestinal tract.[3]

Foal pneumonia caused by *R. equi* is endemic on some farms, intermittent on others, and absent on most farms.[6] Anecdotally, dams of some foals have been reported to have multiple affected foals, while foals of other mares from the same environment are consistently unaffected.[7] Recent epidemiological evidence indicates that foals with spontaneous *R. equi* pneumonia become infected early in life,[8] although the source of infection for foals remains unknown. Several previous studies have supported the notion of mare feces as a potential source of *R. equi* in the environment and possibly a direct

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source for their foals. For example, the prevalence of *R. equi* in feces of mares was similar for 2 farms in Kansas with recent cases of *R. equi* pneumonia (14%) and an unaffected control farm (31%).[9] In a report from Australia, the prevalence of fecal shedding of *R. equi* among 127 horses was 71%.[10] Furthermore, in a study of 43 foals from 2 farms in Japan, the prevalence of fecal shedding was high among foals from an *R. equi*-endemic farm (94%; 16/17) and a farm with no history of *R. equi* pneumonia (73%; 19/26).[11] The mean concentration of *R. equi* per gram of feces was similar among foals from the 2 farms (10^4/g feces). In 2 foals that developed disease, fecal concentrations of *R. equi* were observed to increase. The organism has been isolated from feces of foals <1 week of age and fecal shedding among foals has been shown to increase with age.[12-14] Evidence from Japan has revealed a seasonal variation in shedding of *R. equi* in feces of mares, with the highest concentrations of *R. equi* shedding during the spring (foaling season), although the average number of organisms shed by mares during the 5-week periods pre- and post-foaling was not significantly different.[12]

Expression of the virulence-associated protein antigen (VapA) by *R. equi* is strongly associated with disease in foals; the *vapA* gene encoding this protein is located on an 85-90 kb plasmid (VapA-P).[4,6,15] Because only virulent organisms are considered to cause disease, it is important to consider the virulence status of isolates in epidemiologic studies of *R. equi*. In a study of 2 breeding farms in Japan, the prevalence of virulent organisms was greater among foals at an *R. equi*-endemic farm than an unaffected farm, although differences were not observed in the prevalence of virulent isolates in the feces of their dams.[16]

Given the high prevalence of *R. equi* fecal shedding by foals, large volume of feces passed by adult horses, and an increased prevalence of *R. equi* shedding by dams during the foaling season, it is plausible that a dam may be an important source of virulent *R. equi* for her foal. The purpose of this study is to determine if dams of foals affected with *R. equi* pneumonia shed more *R. equi* in their feces than dams of foals that remain unaffected with *R. equi* pneumonia.

CHAPTER II

EVALUATION OF MARES AS A SOURCE OF *Rhodococcus equi* FOR THEIR FOALS USING QUANTITATIVE CULTURE AND A COLONY IMMUNOBLOT ASSAY

Introduction

Rhodococcus equi is a facultative-intracellular organism and an important cause of disease and death among foals, particularly at endemic farms.[1-4] *R. equi* is primarily a soil saprophyte with growth requirements that are met by herbivore manure and warm climates.[2] *R. equi* can be cultured from the feces of mature horses,[3] and foals as young as 3 days of age.[4] It has been suggested that *R. equi* is likely part of the normal flora in the equine lower gastrointestinal tract.[3]

Foal pneumonia caused by *R. equi* is endemic on some farms, intermittent on others, and absent on most farms.[2,6] Anecdotally, some mares have reportedly had multiple affected foals, while foals of other mares from the same environment are consistently unaffected.[7] Epidemiological evidence indicates that foals with spontaneous *R. equi* pneumonia become infected early in life.[8] The source of infection for foals remains unknown. Results of previous studies indicate that the feces of mares is a potential source of *R. equi* for the environment and possibly a direct source of infection for foals. *Rhodococcus equi* has been isolated from the feces of mares at 2 breeding farms in the United States.[9] In a report from Australia, the prevalence of fecal shedding of *R. equi* among 127 horses was 71%.[10] Evidence from Japan has revealed a seasonal variation in shedding of *R. equi* in feces of mares, with the highest levels of *R. equi*

shedding during the spring (foaling season), although the average number of organisms shed by mares during the 5-week-periods before and after foaling was not significantly different.[12]

Expression of the virulence-associated protein antigen (VapA) by *R. equi* is strongly associated with disease in foals; the *vapA* gene is located on an 85-90 kb plasmid (VapA-P).[4,6,15] Because only virulent organisms are considered to cause disease, it is important to consider the virulence status of isolates in epidemiologic studies of *R. equi*. In a study of 2 breeding farms in Japan, the prevalence of virulent organisms in feces was greater among foals at an *R. equi*-endemic farm than at an unaffected farm, although differences were not observed in the prevalence of virulent isolates in the feces of their dams.[16] To the authors' knowledge, no studies have been reported in which the concentration of virulent *R. equi* in fecal samples of mares or foals was determined and for which the association of these concentrations with disease in foals caused by this bacterium was examined.

Given the high prevalence of *R. equi* in feces of breeding mares, the large volume of feces passed by adult horses, and the reported increased prevalence of *R. equi* shedding by mares during the foaling season, it is plausible that a mare could be an important source of virulent *R. equi* for her foal. The primary objective of this study was to examine the association between *R. equi* pneumonia status of the foal and shedding of virulent *R. equi* by its dam. To address this objective, fecal specimens were collected for 2 years from mares at a single farm with a history of recurrent *R. equi* pneumonia at specified time-points pre- and post-foaling, and fecal concentration of total and virulent *R. equi* was quantified for each sample. Fecal concentrations of total and virulent *R. equi*

were compared between mares of foals that subsequently developed *R. equi* pneumonia and mares of foals that did not develop pneumonia. Comparisons were made for concentrations at each sampling time, and for the 6 differences among the 4 different sampling times.

Materials and Methods

Study population and fecal sample collection - Mares and their foals from a farm in central Kentucky with endemic *R. equi* pneumonia served as the study population. Only mares whose foals were born alive and resided at the farm from birth through weaning were included in the study. The rationale for this inclusion criterion was to ensure consistent monitoring for development of *R. equi* pneumonia by 2 of the authors (NMS and GDM). Fecal samples were collected from mares at the following time-points before and after foaling: approximately 2 weeks prior to foaling; approximately 1 week prior to foaling; the day of foaling; and 1 week after foaling.

Fecal samples were collected either from the top of a fresh fecal pile observed to have been voided in the stall or by manual evacuation of feces from the rectum. Following collection, fecal samples were refrigerated at 4ºC until shipped and transported within 48 hr of collection in insulated containers with icepacks. During 2004, fecal samples were frozen at -20ºC upon arrival and prior to culture. During 2005, quantitative culture of feces was performed on fresh samples either on the day of arrival or the following day for samples that arrived late in the day; specimens cultured the next day were maintained under refrigeration. The reason for the discrepancy in specimen handling was that sample collection was initiated in 2004 prior to funding for the project;

samples initially were saved frozen in the event that the study could not be conducted because of lack of funding. When funding was obtained, the investigators elected to freeze all fecal samples for 2004 so that they would be processed in a consistent manner.

Microbiologic culture of R. equi - A 1-g aliquot of each fecal specimen was mixed thoroughly with 5 ml phosphate-buffered saline[a] (PBS) in order to evenly distribute organisms present in the sample. Quantitative culture of the fecal suspensions was performed using a modified *R. equi* selective agar medium,[10] developed by a coauthor (ST). The modified NANAT agar medium consisted of the following: 300 ml boiled beef broth; 600 ml distilled water; 20 g peptone;[b] 5 g sodium chloride;[c] 5 g yeast extract;[d] 5 g glucose;[e] 0.2 g sodium dithionite;[f] 1.2 g sodium thiosulfate;[g] 2 g potassium phosphate;[h] 2 g sodium bicarbonate;[i] and 15 g technical agar.[j] The pH was adjusted to 7.2 and autoclaved at 121.6ºC for 30 min. The medium was allowed to cool to 55ºC before the following antibiotics were added: 25 µg/ml novobiocin;[k] 40 µg/ml cycloheximide;[l] 20 µg/ml nalidixic acid;[m] and 3.5% potassium tellurite.[n] Although either beef broth or horse meat broth can be used in this formulation, in this study beef broth was prepared using approximately 1.1 kg (2.5 lb) of lean ground beef mixed with 5 L of distilled water and incubated overnight at 4ºC. The mixture was then boiled for 2 hr, cooled at room temperature, and stored at 4ºC until completely cooled. The solidified fat was removed from the surface and broth was strained through gauze to remove residual fat and meat. The broth was then autoclaved at 121.6ºC for 30 min, and stored at 4ºC.

The use of the modified NANAT agar medium effectively minimized concomitant growth of bacterial and fungal contaminants. To examine the possibility of *R. equi*

growth inhibition on the modified NANAT agar, virulent and plasmid-cured (avirulent) *R. equi* bacterial strains were quantitatively cultured on the NANAT agar medium from minimal media.[18] Growth of each *R. equi* strain appeared uninhibited on the NANAT agar in both pure culture and after mixing with equine fecal material to mimic other potential growth-inhibiting sources.[o]

Ten-fold serial dilutions of the fecal/PBS suspensions were prepared and cultured in duplicate for all samples. A 100 µl volume of the fecal suspension was inoculated onto the surface of a NANAT medium agar plate and evenly dispersed using a sterile plate spreader. Both positive and negative controls were implemented to ensure validity of the results. The positive controls consisted of pure culture virulent *R. equi* (ATCC strain 33701) grown in *R. equi* minimal media[18] to approximately 10^8 colony forming units (cfu) per ml and 1 g of feces inoculated with approximately 10^8 cfu of virulent *R. equi*. Ten-fold serial dilutions were performed on both of the positive controls, and 100 ul of the dilution containing approximately 100 cfu/ml of virulent *R. equi* were plated onto separate agar plates. The negative controls consisted of 100 µl of *R. equi* minimal media (used to make the pure cultures) and 100 µl of PBS (used to resuspend feces for quantitative culture). All quantitative culture controls were plated on the modified NANAT agar medium used for all other samples in this study.

Fecal culture plates were allowed to incubate at 34°C for 48 hrs. The dilution containing between 15 and 150 cfu of *R. equi* per culture plate was counted, and these same plates were used for colony immunoblotting. The number of cfu on a plate were multiplied by the dilution factors (and divided by 1) to determine the concentration of *R.*

equi in feces. The average concentration of cfu/g of *R. equi* in feces of the 2 duplicates was determined and used for analysis.

Modified colony immunoblot assay - The concentration of virulent *R. equi* in feces was determined by modifying an immunoblotting technique for detecting VapA.[19] Colony immunoblotting, using a monoclonal antibody for VapA, has been previously used to determine the virulence status of 3 to 10 *R. equi* colonies subcultured from their original agar plates.[20,21] This approach was modified for this study to allow quantification of the concentration of virulent *R. equi* within a background of bacterial and fungal contamination. Nitrocellulose membranes[p] were placed onto previously incubated NANAT agar culture plates containing between 15 and 150 cfu of *R. equi*, and allowed to become completely saturated. Once saturated, membranes were removed, air-dried at room temperature for 30 min, and baked in an oven[q] at 100ºC for 1 min. The nitrocellulose membranes were incubated in 5% non-fat dry milk[r] for 60 min at 37ºC to block any unbound sites. The membranes were then washed 3 times with 0.05% Tween 20[s] in tris-buffered saline[t] (TBST). The membranes were incubated overnight at 4ºC on a rocker[u] with the monoclonal antibody (Mab)[v] diluted 1:10,000 in 5% non-fat dry milk. The membranes were washed for 10 min at 37°C with fresh TBST a total of 3 times. Horseradish peroxidase-conjugated goat immunoglobulin G fraction to mouse immunoglobulin G[w] was diluted 1:2,000 in 5% non-fat dry milk and added to the membranes, followed by a 1 hr incubation at 37ºC. The membranes were again washed for 10 min at 37°C with fresh TBST a total of 3 times. The membranes were then washed once with citrate-ethylenediaminetetraacetic acid (EDTA) buffer (10 mM sodium

citrate[x] and 10 mM EDTA[y]) (pH=5.0) for 5 min at 37°C, once with citrate-EDTA buffer + 1% dextran sulfate[z] for 10 min at 37°C, and 3 times with citrate-EDTA buffer for 5 min at 37°C. The substrates 3,3',5,5'-tetramethylbenzidine (TMB)[aa] and hydrogen peroxide were added to the membranes followed by a 30 min incubation at 37°C. The substrate was discarded and distilled water was added to stop development. Virulent *R. equi* colonies appeared blue, while the avirulent *R. equi* colonies and contaminates remained colorless. This colony immunoblotting method was validated by using both virulent and avirulent strains of *R. equi*. The immunoblotting method and a previously described colony picking method[16,22,23] of each strain of *R. equi*, grown on NANAT agar medium from minimal media,[18] were performed. In addition, plates of *R. equi*-negative feces experimentally inoculated with virulent or avirulent *R. equi* were also colony immunoblotted by directly placing a nitrocellulose membrane onto the agar plate to validate proper selectivity of the Mab to virulent *R. equi* isolates. Confirmation that only virulent *R. equi* colonies were immunoblot-postitive was made prior to implementation of this technique.[o] Positive and negative controls were also included along with each batch of colony immunoblots. The positive and negative controls were NANAT agar medium plates grown from pure culture of virulent and avirulent *R. equi* in minimal media,[18] respectively. Ten-fold serial dilutions were performed on the pure culture virulent and avirulent *R. equi* using PBS and 100 µl of the dilution containing approximately 10^4 cfu/ml were individually plated. All colony immunoblot control plates were incubated under the same conditions as the quantitative culture control plates and the fecal specimen plates. The proportion of virulent isolates in the fecal specimen plates were determined by dividing the total number of virulent colonies counted from both duplicate

plates by the total number of *R. equi* colonies that were present on both duplicate plates prior to the colony immunoblotting portion. Concentration of virulent isolates was determined by multiplying the proportion of virulent isolates by the concentration of *R. equi* present in the fecal sample.

Determining R. equi pneumonia status of foals – Beginning in the first week of life, foals were monitored daily by visual inspection for abnormalities (such as elevated respiratory rate or effort, joint swelling, etc.,) and assessment of rectal temperature; physical examination was performed immediately if fever (Temp >38.9ºC [>102^{o}F]) or physical abnormalities were detected by inspection. Thoracic ultrasonography began at approximately 2-weeks of age, to identify peripheral pulmonary abscessation, consolidation, or other abnormalities such as irregularities of the pleural surface, and was repeated at 2-week time-points until weaning (16-20 wk). Additionally, foals underwent thoracic ultrasonography within 48 hours if a fever or other physical abnormality was detected during daily screening. Each foal included in the study was assigned an ultrasound score based on sonographic findings, using a proposed scoring sytem.[bb] For purposes of this study, ultrasound scores for the thorax were categorized as no lesions, lesions of total scores of 1 to 3, or lesions with score >3. Foals that were found to have ultrasonographic evidence of peripheral pulmonary abscessation or consolidation underwent aseptic trans-cutaneous tracheobronchial aspiration (TBA). For purposes of this study, a case of *R. equi* pneumonia was defined as a foal >2 weeks of age with ultrasonographic evidence of pulmonary abscessation or consolidation and isolation of *R. equi* from TBA or gram-positive intracellular coccobacilli observed in cytology of TBA

fluid. Foals that had clinical signs of *R. equi* infection and from which either *R. equi* was isolated from a TBA or gram-positive intracellular coccobacilli were observed in cytology of TBA fluid were also included as cases. Clinical signs of early detection of *R. equi* pneumonia included: increased fever and respiratory rate, coughing, wheezing, and nasal discharge.[24-26]

Data analysis - The data were summarized using medians and interquartile (**IQ**) ranges (ie, the range from the 25th to the 75th percentiles of the data) for continuous data (such as concentrations or proportions of virulent organisms) and contingency tables for categorical data. Continuous variables were compared using the Wilcoxon rank-sum test (for comparison of 2 samples) or the Kruskal-Wallis test (for comparisons of >2 samples) because these data generally appeared non-Gaussian. Categorical variables were compared using either the χ^2 test or, when appropriate, Fisher's exact test. Because multiple comparisons were made, the method of Holm for adjustment was used to retain an overall significance level of $P < 0.05$ when any comparison was identified as being statistically significant.[27] Analyses were conducted using commercial software.[cc]

Results

There were 94 and 77 mares that had foals residing through weaning at the farm in 2004 and 2005, respectively; 41 mares contributed data in both years such that there were 130 different mares during the 2-year period of the study. During 2004, the 94 mares contributed 365 fecal samples (97% of a total of 376 [94 x 4] possible samples): there were 2, 4, 2, and 3 samples missed from mares at the sampling periods 2 weeks

prior to foaling, 1 week prior to foaling, day of foaling, and 1 week after foaling, respectively. During 2005, the 77 mares contributed 286 fecal samples (93% of a total of 308 [77 x 4] possible samples): there were 0, 4, 10, and 8 samples missed from mares at the same sampling periods, respectively as above. Because samples were processed differently in 2004 and 2005 (ie, frozen vs fresh cultures), the effects of year on shedding of *R. equi* and whether the mare's foal developed *R. equi* pneumonia were examined. Because there were significant differences observed between years for the concentration of *R. equi* and the proportion of virulent organisms in fecal samples (Table 6; Figures 3, 5, 7, 9, 11, 12), the data were analyzed separately for 2004 and 2005. There were, however, no significant differences between years in the distribution of the number of an individual mare's samples (up to 4 samples/mare) that were positive for virulent *R. equi*, the prevalence of mares with diseased foals (31% [29/94] in 2004 and 31% [24/77] in 2005), or the distribution of total scores from ultrasound examinations. All TBA isolates of *R. equi* obtained from affected foals were confirmed to be *R. equi* and determined to be positive for the *vapA* gene using a multiplex polymerase chain reaction method.[28]

Association of R. equi in feces with disease status of the foal for mares during 2004 - Of the 94 mares included in the study during 2004, 29 mares (31%) had foals that were ultimately diagnosed with *R. equi* pneumonia. An additional 11 foals had sonographic evidence of pulmonary abnormalities but *R. equi* was not isolated from their TBA fluid; β-hemolytic streptococcal organisms were isolated from 7 of these 11 foals and either no organisms (2 foals) or presumed contaminants (2 foals) were isolated from the remaining foals. The proportion of foals diagnosed with pneumonia (sonographic and clinical

findings indicating a TBA) after June 1, 2004 was significantly (P =0.0003) greater among the foals from which *R. equi* was not isolated from TBA fluid (82%; 9/11) than among the foals diagnosed with *R. equi* pneumonia (17%; 5/29). Overall, the age at diagnosis of pneumonia was significantly (P < 0.0001) greater for foals from which *R. equi* was not isolated (median, 13 weeks; IQ range, 6 to 15 weeks) than for foals from which the organism was isolated (median, 6 weeks; IQ range, 5 to 6 weeks).

There were no significant differences among mares that had foals affected with *R. equi* pneumonia (hereafter referred to as the affected group) and mares whose foals were unaffected with *R. equi* pneumonia (hereafter referred to as the unaffected group) in the concentrations of either *R. equi* or virulent *R. equi*, or the proportion of virulent *R. equi* (Table 7; Figures 4, 6, 8, 10). Virulent *R. equi* were detected in the feces of all mares during at least 1 sampling time: 1 (1%) mare was culture-positive for virulent *R. equi* only at 1 sampling time (a mare in the unaffected group), 16 (17%) mares were positive for 2 samples, 43 (46%) mares were positive for 3 samples, and 34 (36%) mares were positive for all 4 samples. There was no significant difference between the affected and unaffected groups in the distribution of the number of fecal samples in which virulent *R. equi* were detected: 26 mares (90%) in the affected group and 51 (78%) mares in the unaffected group were culture-positive in 3 or more samples.

There were no significant differences between groups for any of the fecal outcomes (concentrations of total or virulent *R. equi*, or proportion of virulent *R. equi*) among the 6 pairs of differences in sampling times (ie, difference 1 = [number for 2 weeks prior to foaling] – [number for 1 week prior to foaling], difference 2 = [number for 2 weeks prior to foaling] – [number for foaling date], etc.). Additionally, none of the

differences between pairs of sampling times were significantly different, irrespective of disease status. Irrespective of sample (ie, 2 weeks prior to, 1 week prior to, day of, or 1 week after foaling), there did not appear to be any seasonality in the pattern of shedding during 2004 (Figure 1). There was no significant difference between the concentration of either *R. equi* or virulent *R. equi*, or the proportion of virulent *R. equi* in the fecal samples of dams at any sampling time among the 3 categories of ultrasound scores.

Association of R. equi in feces with disease status of the foal for mares during 2005 - Of the 77 mares included in the study during 2005, 24 mares (31%) had foals that were ultimately diagnosed with *R. equi* pneumonia. An additional 11 foals had sonographic evidence of pulmonary abnormalities but *R. equi* was not isolated from their TBA fluid; β-hemolytic streptococcal organisms were isolated from 5 of these 11 foals and either no organisms (1 foal) or presumed contaminants (5 foals) were isolated from the remaining foals. Although the proportion of foals diagnosed with pneumonia (sonographic and clinical findings indicating a TBA) after June 1, 2005 was greater among the foals from which *R. equi* was not isolated from TBA fluid (40%; 4/10) than among the foals diagnosed with *R. equi* pneumonia (21%; 5/24), the difference was not significant. Although the age at diagnosis of pneumonia was greater for foals from which *R. equi* was not isolated (median, 8 weeks; IQ range, 5 to 9 weeks) than for foals from which the organism was isolated (median, 5 weeks; IQ range, 5 to 6 weeks), the difference was not significant ($P = 0.0649$).

There were no significant differences among mares in the affected group and mares in the unaffected group in the concentrations of either *R. equi* or virulent *R. equi*,

or the proportion of virulent *R. equi* (Table 8). Virulent *R. equi* were detected in the feces of all mares during at least 1 sampling time: 2 (3%) mares were culture-positive for virulent *R. equi* only at 1 sampling time (1 mare each in the affected and unaffected groups), 11 (14%) mares were positive for 2 samples, 36 (47%) mares were positive for 3 samples, and 28 (36%) mares were positive for all 4 samples. There was no significant difference between the affected and unaffected groups in the distribution of the number of fecal samples in which virulent *R. equi* were detected: 21 (91%) of the affected group and 43 (80%) of the unaffected group were culture-positive in 3 or more samples.

There were no significant differences between groups for any of the fecal outcomes (concentrations of total or virulent *R. equi*, or proportion virulent) among the 6 differences between pairs of sampling times. Too, none of the differences between sampling times were significantly different, irrespective of disease status. Irrespective of sample, there did not appear to be any seasonality in the pattern of shedding during 2005 (Figure 2). Significant differences were not detected between the concentration of either *R. equi* or virulent *R. equi*, or the proportion of virulent *R. equi* in the fecal samples of dams at any sampling time among the 3 categories of ultrasound scores.

Mares with foals born in both years of the study. There were 41 mares that had foals born during both 2004 and 2005. Only 3 of 41 mares had foals affected with *R. equi* pneumonia during both years, whereas there were 8 mares that had affected foals during 2004 but not 2005, 12 mares with foals affected during 2005 but not 2004, and 18 mares with foals that were unaffected in 2004 and 2005. Thus, there appeared to be no evidence

from this study that certain mares were more likely than other mares to have affected foals.

Discussion

Rhodococcus equi is a bacterial pathogen that causes disease in foals worldwide.[4] This bacterium can be isolated from many sources including feces, soil, and TBA fluid. Unfortunately, much is still unknown regarding the transmission of *R. equi*. The primary aim of this study was to determine if dams of affected foals were a potential source of *R. equi* for their foals. Although disease caused by *R. equi* is not commonly diagnosed in adult horses,[2] horses excrete varying fecal concentrations of virulent *R. equi* into their environment.[11-13,29,30] Foals exposed to this contaminated environment are thus likely to be exposed to virulent *R. equi* from birth, and epidemiologic evidence indicates that foals may be infected during the first few days of life.[8] The route of *R. equi* infection for foals is unknown, but is presumed to be by inhalation of organisms from the environment.[2] Because it is common for foals to ingest small quantities of fresh feces passed by their dam,[17] it is also possible that foals might become infected by coprophagia. Irrespective of the route of infection, results of this study indicate that foals at breeding farms may be exposed from the time of birth to virulent *R. equi* from their dams and other mares.

An important and novel finding in this study was that virulent isolates of *R. equi* were identified in the feces of all mares tested. This finding has not been previously reported. Although contamination of specimens either prior to or during collection, or after collection in the laboratory, could explain these findings, we do not believe this to be the case. Fecal samples were collected either from the top of a fresh fecal pile (ie, not

in contact with the stall) or by manual evacuation from the rectum. The presence of *R. equi* has been found to be greatest when recovered from the soil surface,[12,31] making dust during a windy day a potentially contaminating source of the organism. Therefore, contamination of the voided feces cannot be excluded. Samples were individually packaged and shipped in sealed sacks. In the laboratory, negative and positive 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 for the high prevalence of virulent *R. equi* in fecal specimens exist. Sequential sampling provided increased opportunity to identify *R. equi* in feces. Use of a modified selective NANAT media improved our ability to culture *R. equi* relative to other media, including standard NANAT media (data not shown). The high prevalence of *R. equi* in feces is consistent with at least 1 previous report from Australia.[10] To the authors' knowledge, this is the first report of a method for quantifying the concentration of virulent organisms in fecal samples. Previously, investigations have estimated the proportion of virulent isolates in fecal samples by selectively subculturing up to 10 colonies selected at random from plates and testing these specimens for expression of VapA using immunoblotting.[16,22,23] With the modified colony immunoblot protocol, placing nitrocellulose membranes directly on the culture plates allowed for evaluation of the virulence status of all *R. equi* isolates on a plate rather than just a portion. Conceivably, the finding of a higher prevalence of mares shedding virulent isolates in our study may be a function of improved culture techniques and the use of the complete plate colony immunoblot method used. Interestingly, the proportion of the concentrations of virulent isolates in this study was similar in magnitude to the

proportions of virulent isolates among isolates subcultured from fecal samples in previous reports.[16,22,30,32] Negative and positive control plates were also blotted along with the feces culture plates to ensure that the colony immunoblot method was properly controlled.

The concentrations of *R. equi* cultured from dam feces ranged from $>10^2$ to $>10^3$ cfu/g, which is consistent with past reports.[22,30,33] Isolation of *R. equi* from the air had previously been reported to increase on dry windy days.[31] Furthermore, it has been suggested that prevalence of disease appears to increase when climatic conditions are dry and dusty.[26] No efforts were made in this study to correlate climatic or weather conditions with fecal concentrations of *R. equi*. Although a previous report documented an increase in the isolation of *R. equi* in feces from breeding mares at the end of March,[12] no apparent seasonality of shedding or temporal variation of fecal concentrations of *R. equi* among breeding mares was observed during the months of the year in which this study was conducted (January through June).

There are a number of limitations of this study. Results of this study were obtained from 1 farm: it is unclear to what extent these results would be similar among other farms affected recurrently with *R. equi* foal pneumonia. Additionally, it would be interesting to know if the prevalence and concentration of fecal excretion of virulent *R. equi* varies among farms affected with *R. equi* foal pneumonia and unaffected farms. Results of such studies would help elucidate the association between *R. equi* shed in the feces of mares at breeding farms and the *R. equi* pneumonia status of foals at those farms.

Not all mares contributed 4 samples to the study. Because foaling dates were estimated on the basis of the last breeding date, some mares foaled earlier than expected

such that samples were not collected from all mares at each sampling time. In other instances, samples were not collected on the day of foaling or 1 week after because of absence of the technician responsible for collecting specimens. The impact of missed sampling was minimal in light of the fact that > 95% of potential samples were collected, and the finding that all mares were observed to shed virulent *R. equi* in feces.

Another limitation of this study was the possible misclassification of foals. Only foals from which *R. equi* was isolated from their TBA were classified as having *R. equi* pneumonia. Although the sensitivity of microbiologic culture is generally high (57-100%),[24,34-39] false-negative results may occur. Among foals confirmed by necropsy to have *R. equi* pneumonia, TBA has been reported to be 100% sensitive;[34,35] however, it is sometimes difficult to isolate *R. equi* from the TBA fluid of experimentally infected foals.[40] Conceivably, false-negative results might be more likely during the early stages of disease. Because foals included in the study were systematically screened for early detection of disease, pneumonia was detected during the early stages for foals in this study. In this study, approximately 28% of foals were culture-negative for *R. equi* from TBA fluid despite sonographic findings of pulmonary lesions. In some of these cases, *R. equi* was most likely not the cause of the associated lung lesions, particularly when β-hemolytic streptococcal organisms were isolated from the TBA fluid.[41] However, it is also possible that some foals with sonographic lesions but from which *R. equi* was not isolated were truly infected with *R. equi* but the culture results of TBA were falsely negative. To assess the potential impact on results of this misclassification, data were analyzed such that all foals with clinical and sonographic evidence of pneumonia (irrespective of results of microbiologic culture of TBA fluid) were considered as being

affected with *R. equi* pneumonia: results of these analyses yielded similar results to those reported here. Thus, misclassification of foals with false-negative TBA results did not appear to bias the results of this study. Moreover, the finding that foals undergoing TBA from which *R. equi* was not isolated were either significantly (for 2004) or more likely to be (for 2005) older and diagnosed later in the year is consistent with these foals being appropriately classified as different from those from which *R. equi* was isolated

Analyses were also conducted using the ultrasound scores as a possible indicator of severity (ie, were foals with more extensive sonographic lesions from dams with higher fecal concentrations or proportions of virulent *R. equi* than foals with less severe lesions?). No significant association was identified between the category of ultrasound severity score and the mare's group (affected versus unaffected).

Upon arrival, fecal specimens collected in 2004 were immediately frozen at -20ºC. It was speculated that there would be a loss in the numbers of *R. equi* if the feces were frozen prior to being cultured, but the rate of that loss was unknown. Experimentally, we determined that freezing of fecal specimens at -20°C resulted in approximately a 0.5 $\log_{10}$ to 1 $\log_{10}$ reduction in growth of *R. equi*.[o] To avoid the reduction in numbers resulting from freezing, the 2005 samples were cultured upon arrival. A larger number of contaminants were observed during microbiologic culture of the 2005 samples relative to samples from 2004, which was considered likely to be an effect of samples having not been frozen prior to culture. Although the freezing reduced the growth of *R. equi*, it also reduced the growth of contaminants.

On the basis of a previous report, [31] the dilution containing between 15 and 150 *R. equi* colonies was used for quantitative culture and immunoblotting. Since fewer than 15

R. equi colonies were occasionally isolated from a specimen, continuous variables, such as the proportion of virulent isolates, included some outliers. This was more of an issue with the data from 2004, because they were frozen at -20°C and the resultant fecal concentrations of *R. equi* were lower. The Wilcoxon rank-sum and the Kruskal-Wallis tests were used for analyses because they are less sensitive to the effects of outliers and the non-Gaussian distribution of data. Analysis of the data following logarithmic or other transformations to render them more Gaussian in distribution did not alter results of this study. Nevertheless, the fact that the denominator for calculating the proportion of virulent organism was generally smaller in 2004 meant that these proportions were more sensitive to the presence or absence of a given colony (ie, the difference between 4 and 5 colonies being immunoblot-positive was more influential on the estimated proportion of virulent organisms when the denominator was 15 than when it was 100). It is probable that the significant difference between years in the proportion of virulent isolates was attributable to the fact that there were more plates with small total numbers of *R. equi* colonies enumerated in 2004 than in 2005. Alternatively, it is possible that virulent isolates were less affected by freezing at -20^{o}C than were avirulent isolates. To the authors' knowledge, experimental evidence to support this hypothesis is lacking.

The comparisons between years of the concentrations and proportions of *R. equi* assumed independence of samples and ignored the fact that some samples were correlated (ie, some mares contributed samples to both years). We believe the impact of this assumption is minimal for the following reasons. Nearly all samples from a given mare at a given time-point had lower concentrations for 2004 than for 2005 (data not shown). Ignoring the correlation in the data meant that we underestimated the variance of the data

used for significance testing. Although we may have overestimated the number of significant differences or the magnitude of significance of these differences, the fact that samples were processed differently between years and that exploratory data analysis between years supported our approach of analyzing data separately from each of the 2 years. Although it does not speak directly to this issue, the magnitudes of the P values for the between-year comparisons were all adjusted (inflated) for the 12 comparisons that were made.

The fact that all samples were not collected by manual evacuation from the rectum raises the possibility that samples included in the study were contaminated by the environment. Although some fecal samples were collected by manual rectal evacuation, the majority were collected from the stall. Although samples were collected fresh and from the top of piles to avoid contact with bedding, etc., it is possible that specimens were contaminated by isolates of *R. equi* that were either air-borne or in bedding.

The motivation for this study was to determine whether mares were a possible source of infection for their foals; however, exposure of foals to virulent *R. equi* was not directly assessed in this study. Serologic assessment of exposure to virulent *R. equi* in foals is problematic because of poor sensitivity and specificity.[42,43] Using the methods described in this study to evaluate fecal specimens might yield evidence of exposure to virulent *R. equi*. Such monitoring was beyond the scope of this study

Results of this study are important because they indicate that all mares at a breeding farm with recurrent *R. equi* foal pneumonia were shedding virulent *R. equi* in feces during the perinatal period: these virulent isolates are a potential source of infection for susceptible foals. It would be important to substantiate these findings at other farms,

and to compare findings of fecal concentrations of *R. equi* among mares from farms with recurrent *R. equi* pneumonia and farms without such history. The method of quantifying the concentration of virulent *R. equi* in fecal or other specimens (such as soil or tissue) may be useful to other investigators.

CHAPTER III

CONCLUSIONS AND DISCUSSION

In addition to the work described in Chapter II, a number of other experiments were conducted for this project. The principal objective of this chapter is to summarize that work.

Foal Fecal Specimens

Fecal specimens were collected from 171 foals on the day of foaling and 1 week post-foaling over a 2-year period. Originally, the foal feces were going to be analyzed for the study but because the majority of the fecal samples collected came in the form of swabs taken from the foals' rectums, the concentration of *R. equi* in feces could not be quantified. Since quantification was not possible, determining if the foal was shedding *R. equi* and virulent *R. equi* in its feces became the focus for these samples. A swab was cultured by directly plating it onto the surface of each of 2 NANAT agar plates (duplicate to maximize growth potential). Inoculated plates were allowed to incubate at 34ºC for 48 hr. Culturing rectal swabs was not an ideal method for determining if foals were shedding *R. equi* in their feces because of the small amount of fecal material and viable organisms present on the swabs. Swabs typically grew only a small amount of *R. equi*, which may or may not be representative of the number of *R. equi* excreted in feces by a particular foal. Enrichment of the fecal swabs using a modified NANAT medium broth for 24 hr at 34ºC was attempted, but was unsuccessful at minimizing other bacterial

growth. Since *R. equi* grows relatively slowly compared to other organisms, enrichment allowed for fecal contaminants to flourish and mask the *R. equi* growth.

Two methods were proposed to determine whether or not the foals that were shedding *R. equi* in their feces were shedding virulent *R. equi*: (1) Up to 10 colonies were picked (placing in same tube) from the duplicate culture plates, DNA was then extracted using the alkaline lysis procedure described below, and a real-time polymerase chain reaction (RT-PCR) assay[44] was performed for confirmation of the presence or absence of the *vapA* gene; and, (2) 2 NANAT agar plates were inoculated with swabs and the plates were tested for virulence by using the colony-blot technique. Since the proportion of fecal isolates of *R. equi* that were virulent was 14% in a previous report,[30] these methods were consistently unsuccessful, presumably because all swabs from 2004 were frozen at -20°C and freezing greatly reduced the viability of *R. equi*. There were low numbers of total *R. equi* grown from the swabs, and low proportions of virulent isolates determined, meaning that false-negatives were probable. In 2005, the swabs were cultured upon arrival and not frozen, but culture was unsuccessful because of the limited amount of fecal material present on the swabs. Varying amounts of data were accumulated from both of these methods, as summarized in Appendix A (Tables 13-17).

Swabs were cultured using NANAT agar medium during both years. The colony-blot technique was used in 2004 to determine virulence status of the limited numbers of *R. equi* grown. Both colony-blot and RT-PCR of DNA extracted using the alkaline lysis procedure of *R. equi* colonies were used to detect virulent isolates from the 2005 swabs. RT-PCR was not used on the 2004 swabs because of contamination of samples that occurred during extraction during that time.

In 2005, there was poor correlation between the 2 methods of determining virulence when performed on colonies from the same sample swab. Results of foal fecal data are summarized in Tables 9 through 17 (Appendix A); these data were not submitted for publication because of difficulties with their interpretation resulting from the aforementioned problems.

Morphology Assessment Experiments

Before mare and foal fecal samples were processed, studies were conducted to become familiar with the identification and growth characteristics of *R. equi*, and to confirm that the use of the NANAT agar medium did not inhibit its growth. These experiments involved using the virulent (ATCC 33701+) and avirulent (ATCC 33701-) strains of *R. equi* in minimal media,[18] referred to as pure culture. Serial dilutions of virulent and avirulent *R. equi* in pure culture were plated onto modified NANAT agar medium, counted, and cfu/ml determined in order to characterize accuracy of these procedures. Horse feces from Texas that were previously culture-negative for *R. equi* were also inoculated with these bacterial dilutions, plated, counted and the cfu/g calculated. These studies were important to perform because of the wide range of bacterial and fungal contaminants present in feces. Even using a modified NANAT media, these contaminants were not fully suppressed. Recognizing *R. equi* and understanding its morphology were vital for assessment during all projects. Colonies of *R. equi* appear to vary in size (2-4 mm), be irregularly round, smooth, semitransparent, glistening, and mucoid.[45]

Media Experimentation

Experimental analyses of several different types of agar media used to identify *R. equi* were performed. CAZ-NB containing ceftazidime[dd], novobiocin and cycloheximide is a medium that had been used by other researchers and its ability to reliably culture *R. equi* had been tested.[46] The original NANAT agar medium was described as the most accurate agar medium for isolating *R. equi*.[10] Both were prepared in the laboratory and virulent (ATCC 33701+) and plasmid-cured (ATCC 33701-, avirulent) *R. equi* in minimal media[18] were used to determine possible inhibitory effects on growth of *R. equi*. The virulent and avirulent strains of *R. equi* were simultaneously cultured onto trypticase soy agar with 5% sheep red blood cells (RBC),[ee] (BAM) as a base of comparison since BAM is not inhibitory to *R. equi*. Amphotericin B,[ff] a common antifungal,[47] was added to the CAZ-NB agar at the manufacturers recommendation of 5.6 mg/l to help reduce fungal growth. Approximately 10^8 cfu/ml of *R. equi* (both virulent and avirulent, independently) were used to perform serial dilutions (1:10). The dilution containing approximately 10^2 cfu/ml of *R. equi* was inoculated onto the various types of agar plates, in duplicate. Results after the inoculated agar plates were allowed to incubate at 34ºC for 48 hr are summarized in Table 1.

Table 1. BAM vs. CAZ-NB and NANAT agar media

Agar Medium	ATCC 33701+	ATCC 33701-
BAM	3.2×10^8	3.0×10^8
CAZ-NB	5.0×10^7	7.0×10^7
CAZ-NB + Amp B	4.2×10^7	3.4×10^7
NANAT	0	0

It was determined that both the CAZ-NB and NANAT agar media inhibited the growth of *R. equi*. It is likely that the failure to grow any *R. equi* on the initial NANAT agar medium was a result of a mistake made while making the medium or some kind of experimental error. Before further experimentation was performed, we became familiar with a proven modified version of the NANAT agar medium and decided to test it, rather than resuming efforts to succeed with the standard NANAT.

An experiment was performed upon receipt of the recipe for the modified NANAT agar medium, generously provided by Dr. Shinji Takai, for confirmation that it would not inhibit growth of *R. equi*. Using both virulent and avirulent *R. equi*, grown to approximately 10^8 cfu/ml in minimal media,[18] 10-fold serial dilutions were performed with PBS. The dilution containing approximately 10^2 cfu/ml of *R. equi* was plated, using 100 µl, onto the modified NANAT agar medium and BAM, in duplicate. Agar plates were placed at 34ºC for 48 hr. Following incubation, the plates were removed and counted; results from this experiment are summarized in Table 2. It was determined that the modified NANAT agar medium did not inhibit the growth of *R. equi* grown in minimal media.

Table 2. BAM vs. modified NANAT agar medium with *R. equi* in pure culture and inoculated feces

Agar Medium	ATCC 33701+	ATCC 33703-
BAM (PC)	2.5×10^8	3.0×10^8
Modified NANAT (PC)	3.3×10^8	2.7×10^8
Modified NANAT (feces)	2.0×10^8	2.5×10^8

Experimental analysis of the modified NANAT agar medium was also performed using equine feces that had been inoculated with similar amounts of virulent or avirulent *R. equi* grown in minimal media.[18] Since BAM is not selective and is beneficial for growth of all organisms that are present in a specimen, only the modified NANAT agar medium was used for this experiment. Fecal material (previously culture-negative for *R. equi*) was weighed into 3-g aliquots and approximately 10^8 cfu of virulent or avirulent *R. equi* in minimal media was added to the feces. The same virulent and avirulent *R. equi* in minimal media that were used for plating the pure culture portion of the experiment were used to inoculate the feces. Ten-fold serial dilutions were performed using PBS and 100 µl, which contained about 10^2 cfu of *R. equi*, and each dilution was plated in duplicate. Modified NANAT agar plates were allowed to incubate for 48 hr at 34ºC before being removed and counted. After the colonies were counted it was determined that the modified NANAT agar medium did not significantly inhibit the growth of *R. equi* in equine feces (Table 2). The feces were originally inoculated with 10^8 cfu of *R. equi*,

meaning that inhibition was not observed with the modified NANAT agar medium. The subtle difference in the cfu's counted on the modified NANAT agar plates between inoculated feces and pure culture (Table 2) could have been due to a pipetting error or to the feces being slightly inhibitory to the bacterium. Regardless, the modified NANAT agar medium was determined to be the best medium for culturing *R. equi*.

Effect of Duration on Freezing Fecal Specimens

Prior to acquiring funding for the feces project, fecal specimens from participating mares in the 2004 sampling period were collected and frozen at -20ºC upon arrival. It was speculated that freezing fecal specimens at -20ºC would have an effect on the amount of *R. equi* isolated. Experimentally, it was determined that between a 0.5 $\log_{10}$ to a 1 $\log_{10}$ reduction occurred due to freezing the specimens at -20ºC. No apparent difference between fresh and frozen samples was observed when fecal specimens were experimentally frozen at -80ºC, meaning that this would have been the better choice. Due to limitations of time and personnel, the experiment could not be performed prior to sample collection. Data from the relevant experiment are summarized in Table 3. Feces aliquoted in 1.5-g increments in multiple tubes were inoculated with approximately 10^8 cfu of virulent *R. equi*. One *R. equi* inoculated tube was immediately cultured onto modified NANAT agar medium, in duplicate, to serve as the control (baseline) value for comparison with frozen specimens; the control is described in Table 3 as the "fresh" sample. The remaining aliquots were then either frozen at -20ºC or -80ºC. Inoculated feces were then removed from the freezers in 1-week increments for 4 weeks to

determine if length of time frozen had any effect on the viable *R. equi*. The method for culturing was identical to the method used in Chapter II for the mare fecal specimens.

Table 3. Effects of freezing feces inoculated with virulent *R. equi* at different lengths of time and temperatures

Time Frozen (weeks)	-20ºC Freezer	-80ºC Freezer
1	1.0×10^8 cfu/g	1.8×10^8 cfu/g
2	2.8×10^7 cfu/g	1.5×10^8 cfu/g
3	2.6×10^7 cfu/g	1.4×10^8 cfu/g
4	2.7×10^7 cfu/g	1.8×10^8 cfu/g

"Fresh" inoculated fecal specimen contained 2.1×10^8 cfu/g

Effect of Thawing and Duration on Freezing Fecal Specimens

After the initial experimentation that was described above was completed, it was important to determine the probable detrimental effects of freezing samples and thawing them on multiple occasions. To examine the effects of thawing and duration, 1.5-g fecal samples were again aliquoted and inoculated with approximately 10^8 cfu of *R. equi*. For this experiment, both a virulent (ATCC 33701+) and an avirulent (ATCC 33701-) strain of *R. equi* were used. By using both virulent and avirulent *R. equi* it was possible to evaluate whether one biotype of *R. equi* was affected more than the other by thawing and duration of freezing at -20ºC and -80ºC. One virulent and one avirulent *R. equi*-inoculated tube were immediately cultured onto separate modified NANAT agar medium plates, in duplicate, to serve as the control values for comparison with frozen specimens;

these controls are described in Table 4 as the "fresh" samples. Each week, all sample tubes were removed from the freezer and allowed to thaw at room temperature for 45 min. After documenting that fecal specimens had thawed, the tubes that were not used for culture were placed back into the appropriate freezer. The number of weeks in Table 4 also represents the number of times that the sample was thawed.

Table 4. Effects of freezing feces inoculated with virulent or avirulent *R. equi* at different lengths of time, temperatures, and number of times thawed

	ATCC 33701+		**ATCC 33701-**	
	-20ºC	**-80ºC**	**-20ºC**	**-80ºC**
1 week	1.33×10^7 cfu/g	1.93×10^8 cfu/g	2.60×10^6 cfu/g	1.83×10^8 cfu/g
2 weeks	1.85×10^6 cfu/g	1.20×10^8 cfu/g	1.02×10^6 cfu g	9.33×10^7 cfu/g
3 weeks	8.67×10^5 cfu/g	8.50×10^7 cfu/g	5.00×10^4 cfu/g	2.63×10^7cfu/g
4 weeks	1.22×10^5 cfu/g	8.33×10^7 cfu/g	2.42×10^2 cfu/g	9.50×10^6 cfu/g

"Fresh" inoculated fecal specimen with ATCC 33701+ contained 2.33×10^8 cfu/g

"Fresh" inoculated fecal specimen with ATCC 33701- contained 2.43×10^8 cfu/g.

From this study, it appeared that both the virulent and plasmid-cured (avirulent) *R. equi* could be dramatically affected by continuous thawing and freezing at -20ºC. The avirulent *R. equi* appeared to have greater loss when compared to the virulent *R. equi* at this temperature. These fecal specimens were experimentally inoculated with 10^8 cfu, but in the fecal study described in Chapter II, the average total concentrations of *R. equi* were much smaller (10^2-10^3 cfu/g). Multiple thawing in a farm-based study could be detrimental to the outcome. It also appears from the results that freezing at -80ºC,

regardless of the number of times thawed always yielded less loss than that of the -20ºC. This experiment needs to be repeated to ensure the validity and repeatability of the results. Also, it would be interesting to examine the effects of freezing and thawing on soil samples, because of the epidemiologic importance of studies of the concentration and proportion of virulent *R. equi* in soil of affected and unaffected farms.

Colony Immunoblotting Substrate and Membrane Experimentation

Trial experiments using the virulent and avirulent strains of *R. equi* (ATCC 33701+ and 33701-, respectively) blotted onto nitrocellulose membranes from modified NANAT agar plates were performed to ensure the validity of the colony-blot method. Problems arose when using several substates toward the end of the protocol. The substrate is designed to bind to the colonies previously selected by the secondary antibody, causing a color change in the virulent *R. equi*.

Three different substrates were tried along with 3 different types of membranes in order to obtain the best results from blotting fecal culture plates. The substrates tested were Western Blue[gg]-stabilized substrate for alkaline phosphatase, 3,5'-diaminobenzoic acid[hh] (DAB), and TMB. The Western Blue-stabilized substrate for alkaline phosphatase was unsuccessful for differentiating between virulent and avirulent *R. equi*. Alkaline phosphatase was present in both virulent and avirulent strains of *R. equi*. This finding was not surprising because alkaline phosphatase activity is also found in many other bacteria. The next substrate, DAB, was also tested by using the aforementioned virulent and avirulent strains of *R. equi*. This substrate never gave a positive reaction with either virulent ATCC 33701+ or avirulent ATCC 33701-. TMB, which was a substrate that had

previously been compared to DAB,[48] was successful for identifying the virulent strain of *R. equi*. It was tested thoroughly using both virulent and avirulent *R. equi* in minimal media to ensure the validity and repeatability of the results.

Nylon[ii], polyvinylidene difluoride[ij] (PVDF), and nitrocellulose membranes were tested to identify the best membrane for colony-blotting fecal culture plates. Nylon and nitrocellulose membranes both were considered efficient when blotting fecal culture plates inoculated with virulent and avirulent *R. equi*. The PVDF membranes did not work well for reasons unknown. All 3 of these types of membranes are used successfully for protein blotting.[49] Nylon membranes came in the form of sheets, while the nitrocellulose membranes were in a roll. Due to the packaging of the nylon membranes from the manufacturer, nitrocellulose membranes were selected because economically they were considered a better value and had been used during the substrate experiments.

Validation of the Colony Immunoblot Assay

The colony-blot method was modified from its original protocol[19] a number of times before achieving a working state. There were several experiments that were performed to validate the method. Colony-picking, which had been done numerous times in past reports,[16,22,23] was done using both virulent and avirulent strains of *R. equi* grown onto the modified NANAT agar medium from minimal media.[18] Plates were inoculated with approximately 10^2 cfu/ml of virulent and avirulent *R. equi*, separately. These plates were allowed to incubate at 34ºC for 48 hr. Using a toothpick, individual colonies (10 total) were then picked off of each type of plate and placed onto separate nitrocellulose membranes. Membranes were then subjected to the full colony-blot protocol. Using this

technique, the membrane that contained the virulent *R. equi* colonies had a color change and all 10 colonies picked from the modified NANAT agar plates were visible. The membrane which contained the avirulent strain of *R. equi* was blank, with no color change observed.

After having success with the colony-picking experiment, it was necessary to test the possibility of placing the nitrocellulose membrane onto the surface of the modified NANAT agar plate and blotting all the *R. equi* colonies from the plate's surface onto the membrane. This was again performed using separate plates containing either virulent or avirulent *R. equi* grown from minimal media.[18] Feces inoculated with approximately 10^2 cfu/g of both strains of *R. equi* were plated separately onto the modified NANAT for use in this experiment. Nitrocellulose membranes, cut to fit the circular plate, were carefully placed onto each plate's surface and allowed to become completely saturated before being removed. The colony-blot protocol was then carried out on these blotted membranes and the results were as expected: a positive color change was observed on the membrane which contained the virulent *R. equi* grown from minimal media and on the membrane that contained the virulent *R. equi* grown in feces; a color change was not observed on the membrane containing avirulent *R. equi* grown from minimal media or on the membrane that contained the avirulent *R. equi* grown in feces. On the plates that contained either strain of *R. equi* from feces, there was "background" observed. This background was not the blue color associated with virulent *R. equi*, but appeared to represent bacteria other than virulent *R. equi* that were grey in color and that yielded a non-specific reaction with the reagents (Figure 13). It might be possible that the background that is being observed could be due to other bacteria expressing VapA.

VapA is one of a cluster of genes encoded by a pathogenicity island. Pathogenicity islands are thought to be genes derived from other organisms. Many types of bacteria are present in the intestinal tract of horses. Because they share an environment with *R. equi*, it is possible that the pathogenicity island encoding VapA is possessed by other organisms in the intestinal environment.

The 2 experiments carried out above were used to validate the colony-blot method. One other experiment was carried out in an attempt to colony-blot NANAT agar plates containing known proportions of both virulent and avirulent *R. equi* in feces and minimal media and to verify the colony-blot results by amplifying the *vapA* gene using PCR.[28] Modified NANAT agar plates were inoculated with equal proportions (1:1) of pure culture of virulent ATCC 33701+ and avirulent ATCC 33701- (10^2 cfu/ml) of each. The same proportions of the virulent and avirulent *R. equi* were also added to 1-g of feces, which was plated. Plates were allowed to incubate at 34ºC for 48 hr before being removed and assessed. There were 14 *R. equi* colonies that were counted from the inoculated feces plate and 29 *R. equi* colonies from the pure culture plate. Diagrams were drawn of the plates and the locations of individual colonies were noted. Using disposable sterile loops, half of each colony was placed into 3 ml of modified NANAT broth and prepared for enrichment. The remaining half colonies were immunoblotted by placing a nitrocellulose membrane over the top of the agar plate and following the modified protocol. After enriching the half colonies for 24 hr at 34ºC, DNA was extracted from 1.5 ml of the enriched broth using the alkaline lysis method. Muliplex PCR[28] was performed on the extracted enrichment broth and the results are summarized in Table 5.

Table 5. Colony-blot and PCR results from equal proportion of virulent and avirulent *R. equi*

	PCR +	**PCR-**	**TOTAL**
Colony-blot +	7	12	19
Colony-blot -	5	19	24
TOTAL	12	31	43

The results from this experiment should have shown that the colonies that were PCR-positive were also colony-blot-positive and vice versa. This, however, was not the case. There are a number of possible explanations for this unexpected discrepancy of results between methods for identifying vapA. It is possible that there were PCR inhibitors in extracted DNA from the modified NANAT enrichment broth. Alternatively, removing half of each colony could have left too little residual antigen to detect with colony blotting. False-positive results of PCR might have occurred as a result of DNA contamination during the extraction or amplification phases.

Extraction of DNA from Horse Feces

Prior to the development of a modified colony immunoblot protocol, in an attempt to quantify virulent *R. equi* using RT-PCR, DNA extraction of equine feces was attempted using many variations to the original protocol[50,51] but without success. DNA recovered from the feces of mares collected at different sampling times both pre- and post-foaling was to be quantitatively assessed for virulent *R. equi* by extracting the DNA and using a RT-PCR assay[44] to detect VapA. Although success of extracting DNA from

feces of horses from Kentucky never occurred, there were some successes using a modified protocol when feces of horses from Texas were inoculated with the virulent and avirulent strains of *R. equi* and extracted using the identical protocol.

It was never determined what specifically was causing the difficulty in extracting DNA, but it was speculated that there were PCR inhibitors in the feces of mares from Kentucky that inhibited amplification. Polyphenolic substances present in plant tissues are considered highly inhibitory to PCR.[52] Bile salts, hemoglobin degradation products, and complex polysaccharides are commonly associated with human feces[53,54] and may to some extent extend to horse feces. It was speculated that differences in diet between Texas and Kentucky horses probably caused the RT-PCR inhibition of the extracted DNA (e.g., feed additives such as mycotoxin binders).

The protocol used in the attempt to accurately extract DNA from feces of both Texas and Kentucky horses was as follows: One g of fecal matter was incubated overnight at 4°C in 5 ml 1XTE (1 M tris-HCl[kk] pH=8.0, 0.5 M EDTA pH=8.0, ddH_2O) containing 0.5% Triton-X.[ll] The supernatant was transferred to a 2 ml microcentrifuge tube, making sure the fecal pellet was left undisturbed. The supernatant was centrifuged[mm] at 14,000 rpm (16,000 x g) for 20 min. The supernatant was removed and discarded while the bacterial pellet was mixed with the following: 45 µl 5 M NaCl; 200 µl lysing solution (50 mM EDTA, 50 mM tris HCl, 20% sucrose[nn] in ddH_2O); 36 µl 10% hexadecyltrimethylammonium bromide[oo] (CTAB)/0.7 M NaCl; 6.6 µl 10% β-mercaptoethanol.[pp] The tube containing the bacterial pellet was then vortexed in order to loosen it from the bottom. Using a transfer pipette, the entire contents of the microcentrifuge tube was transferred into a lysing matrix b tube[qq] (bead beater tube).

Using a specialized lysing matrix instrument called FastPrep®[rr], the tube was beat at speed 6 for 40 sec. After beating, the following was added to the bead beater tube: 166µl 5 M NaCl; 1 ml lysing buffer [100 mM NaCl, 25 mM EDTA, 10 mM tris HCl and 0.5% sodium dodecyl sulfate[ss] (SDS)]; 5 µl 20 mg/ml proteinase K[tt]; 134 µl 10% CTAB/0.7 M NaCl; and 40 µl 10% β-mercaptoethanol. The fluids that were added to the bead beater tube in this step aided in getting all the beated material out of the bead beater tube and into a new sterile 2 ml microcentrifuge tube. Without this addition, the fluid appeared very sticky and a severe loss of DNA was likely. The bead beater tube was quickly centrifuged so that material was not left on the side of the tube. The supernatant was then transferred back into a 2 ml microcentrifuge tube. The tube was vortexed and incubated at 60°C for 60 min. Upon completion the tube was vortexed and incubated at 65°C for 10 min. An equal volume of chloroform[uu] was added to the microcentrifuge tubes and shaken for 5 min. It was important to make sure that the tube was closed tightly to prevent DNA loss. The microcentrifuge tube was centrifuged at 13,000 rpm (14,000 x g) for 5 min before the aqueous layer was transferred to another sterile 2 ml microcentrifuge tube. One-tenth of the total volume of 10% CTAB/0.7 M NaCl was added into the new microcentrifuge tube. It was vortexed and incubated at 65°C for 10 min. An equal volume of chloroform was next added and shaken for 5 min. The tube was again centrifuged at 13,000 rpm (14,000 x g) for 5 min before the aqueous layer was transferred to new tube. An equal volume of phenyl/chloroform/isoamyl[vv] (PCI) was added and shaken for 5 min. For 5 min at 13,000 rpm (14,000 x g) the tube was centrifuged and the aqueous layer was transferred. The PCI extraction was repeated twice. An equal volume chloroform extraction was performed once more. One-fifth of the total volume of 10 M

Ammonium acetate[ww] was added to the previous aqueous layer and mixed by inverting the tube. An equal volume of isopropanol was added and again the tube was inverted. The extraction was allowed to centrifuge for 20 min at 13,000 rpm (14,000 x g) before the liquid was removed. An equal volume of 70% ethanol was added and the tube was vortexed until the pellet was dislodged from the bottom of the tube. The tube was centrifuged for 10 min at 13,000 rpm (14,000 x g) before the ethanol was removed. Using a SpeedVac,[xx] vaccum the DNA pellet was dried. The DNA pellet was resuspended using 25 µl 1XTE, pH 8.0. Resuspended DNA was placed in a 50°C water bath for 20 min in order to get the pellet to dissolve completely before freezing. The DNA was stored at -80°C.

A number of steps, after extracting the DNA from Kentucky feces, were also tried to attempt to purify the DNA pellet. Kits such as GenSpin[yy] and Puregene[zz] were used with no success. DNA Bind,[55] a solution made that had been previously used[50] was unsuccessful at removing the PCR inhibitors present in the fecal DNA. Magnetic beads were attempted by using a ChargeSwitch® gDNA kit[aaa] specialized for blood. The idea behind the magnetic bead method was to place the DNA in contact with the beads. The DNA automatically adheres to the beads in a low pH environment due to the positive charge of the bead and the negatively charged nucleic acid backbone. Washing the inhibitors from the DNA was possible after it became securely stuck to the beads. Proteins and other contaminants do not adhere and were washed away. All of these protocols were unsuccessful in purifying extracted DNA for detection by RT-PCR.

Lysozyme[bbb] was first used to lyse the gram-positive *R. equi* but proteinase K was thought to be more successful. Using lysozyme also added to the length of the protocol

because it required an extra incubation at 37ºC for 30 min. The proteinase K appeared to be sufficient when feces from Texas spiked with virulent ATCC 33701+ and avirulent ATCC 33701– were extracted.

Extraction of DNA from Pure Culture, TBA Fluid, and Isolated Colonies

The extraction of DNA from pure culture, TBA fluid, and isolated colonies was performed by the use of a modified alkaline lysis procedure[56] Depending on the type of sample from which DNA was extracted (isolated colonies, pure culture, or TBA fluid) the first 2 steps differed slightly. When extracting from isolated colonies, single colonies were picked from an agar plate with a sterile loop. Colonies were placed in 1 ml of PBS, and vortexed until the bacteria were thoroughly distributed with no clumps present. When extracting DNA from pure culture or TBA fluid, the protocol described below was used.

Samples were centrifuged at 10,000 rpm (8,500 x g) for 10 min in order to pellet the cells. The supernatant was decanted without disturbing the pellet, and then 300 µl of TENS (0.1 M NaOH,[ccc] 10 ml 10XTE, 25 ml 20% SDS) was added to the tube containing the pellet. The sample was vortexed briefly and incubated at room temperature for 10 min. After the sample has incubated for 10 min at room temperature, the sample was placed directly on ice. Then 150 µl of 3 N sodium acetate[ddd] pH 5.2 was added and vortexed briefly. Samples were centrifuged for 2 min at 14,000 rpm (16,000 x g) and 17°C. The supernatant was transferred to a sterile 1.5-ml microcentrifuge tube. To the supernatant, 10 µl poly acryl carrier[eee] (PAC) was added and this was inverted gently. One ml of room temperature 100% isopropanol was added and again inverted gently to

thoroughly mix. The sample was centrifuged for 20 min at 14,000 rpm (16,000 x g) and 17°C. Isopropanol was then removed without disturbing the DNA pellet. The DNA pellet was washed with 1 ml of ice cold ethanol, and vortexed until the pellet was dislodged from the bottom of the tube. Samples were centrifuged for 10 min at 14,000 rpm (16,000 x g) and 4°C. Ethanol was then removed without disturbing the DNA pellet, and the pellet was vacuum-dried and resuspended in 25 µl 1XTE, pH 8.0. DNA was stored at -80°C until use. It was sometimes useful to place resuspended DNA in a 50°C water bath for 20 min in order to get the pellet to dissolve completely before freezing.

Soil Study

Quantitative culturing of soil from affected and unaffected farms in Kentucky was performed to determine the total concentrations of *R. equi*, and colony-blotting of the culture plates was conducted to determine the proportion of virulent *R. equi* present in a given sample. The primary aim of this particular study was to determine if an association existed between the number of virulent organisms cultured from the soil and the current *R. equi* pneumonia status of different affected and unaffected farms.

Soil was weighed upon arrival and cultured onto the modified NANAT agar medium. The plates were allowed to incubate at 34°C for 48 hr. Using the morphologic description previously described, plates were counted and the number of cfu/g present were calculated. Nitrocellulose membranes were placed directly on the cultures and the colony immunoblotting technique previously described was used to assess the number of virulent *R. equi* isolates present on the plates.

This study is important because the results of the work described in this thesis showed no association between individual mares shedding *R. equi* in their feces and the disease status of their specific foals. Thus, revisiting the role of the soil in the environment that the foals are placed in after birth as a potential source of infection seemed logical. Results of the work described here indicate that virulent *R. equi* is in the soil of both affected and unaffected farms at varying levels. There may be an association between proportion of virulent *R. equi* and time of year that the soil sample is collected. More sampling times are planned to aid us in evaluating this relationship.

ENDNOTES

[a] Phosphate-buffered saline, pH 7.2, Invitrogen Co, Carlsbad, CA.

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

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

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

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

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

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

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

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

[j] Technical agar, Difco by BD Biosciences Co, San Jose, CA.

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

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

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

[n] Potassium tellurite powder, Sigma Chemical Co, St Louis, MO.

[o] Grimm MB. Evaluation of mares as a source of *Rhodococcus equi* for their foals using quantitative culture and a colony immunoblot assay. MS thesis, Department of Large Animals Clinical Sciences, Texas A&M University, College Station, TX, 2006.

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

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

[r] Non-fat dry milk, Bio-Rad Laboratories, Hercules, CA.

[s] Tween 20 Solution, Bio-Rad Laboratories, Hercules, CA.

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

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

[v] Provided by Dr. Shinji Takai, Department of Animal Hygiene, School of Veterinary Medicine and Animal Science, Kitasato University, Towada, Aomori, Japan.

[w] Horseradish peroxidase-conjugated goat immunoglobulin G fraction to mouse immunoglobulin G, MP Biomedicals Inc, Aurora OH.

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

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

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

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

[bb] Slovis NM, McCracken JL, Mundy G. How to use thoracic ultrasound to screen foals for *Rhodococcus equi* at affected farms (abstr), in *Proceedings*. Annu Meet Am Assoc Equine Pract 2005;274-278.

[cc] S-PLUS, Version 7.0, Insightful, Inc., Seattle, Wash.

[dd] Ceftazidime, Sigma Chemical Co, St Louis, MO.

[ee] Trypticase Soy Agar with 5% sheeps RBC, BBL Prepared Media, Beckton-Dickinson Microbiology Sysytems, Cockeysville, MD.

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

[gg] Western Blue, Promega, Madison, WI.

[hh] 3,5'-diaminobenzoic acid, Sigma Chemical Co, St Louis, MO.

[ii] Nylon membranes, Bio-Rad Laboratories, Hercules, CA.

[jj] Polyvinylidene difluoride membranes, Bio-Rad Laboratories, Hercules, CA.

[kk] Tris-HCl, Bio-Rad Laboratories, Hercules, CA.

[ll] Triton-X, Sigma Chemical Co, St Louis, MO.

[mm] Microcenrifuge Eppendorf 5417 R, VWR International, West Chester, PA.

[nn] Sucrose, Sigma Chemical Co, St Louis, MO.

[oo] Hexadecyltrimethylammonium bromide, Sigma Chemical Co, St Louis, MO.

[pp] β-mercaptoethanol, Sigma Chemical Co, St Louis, MO.

[qq] Lysing Matrix B tubes, Qbiogene, Carlsbad, CA.

[rr] FastPrep® Instrument, Qbiogene, Carlsbad, CA.

[ss] Sodium dodecyl sulfate, Sigma Chemical Co, St Louis, MO.

[tt] Proteinase k, Sigma Chemical Co, St Louis, MO.

[uu] Chloroform, EMD Pharmaceuticals, Durham, NC.

[vv] Phenyl/Chloroform/Isoamyl, Fisher Scientific International Inc, Hampton, NH.

[ww] Ammonium acetate, Sigma Chemical Co, St Louis, MO.

[xx] Savant SpeedVac Instrument, Global Medical Instrumentation Inc, Ramsey, MN.

[yy] Whatman GenSpin™ Genomic DNA Purification Kit, Lablink Scientific Supply Inc, Mobile AL.

[zz] Puregene DNA Purification Kit, Gentra Systems Inc, Minneapolis, MN.

[aaa] ChargeSwitch® gDNA kit, Invitrogen Co, Carlsbad, CA.

[bbb] Lysozyme, Sigma Chemical Co, St Louis, MO.

[ccc] Sodium hydroxide, Sigma Chemical Co, St Louis, MO.

[ddd] Sodium acetate, Sigma Chemical Co, St Louis, MO.

[eee] Poly acryl carrier, Molecular Research Center, Cincinnati, OH.

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APPENDIX A

Table 6. Median (interquartile range) values for the concentrations of total *R. equi* and virulent *R. equi*, and the proportion of virulent *R. equi* in 171 fecal samples from 135 mares collected during 2004 and 2005 from a farm in central Kentucky.

Sample	**Concentration of *R. equi*** (cfu/g)			**Concentration of virulent *R. equi*** (cfu/g)			**Proportion of virulent *R. equi*** (%)		
	2004	**2005**	**P***	**2004**	**2005**	**P***	**2004**	**2005**	**P***
2 weeks prior to foaling	1,525 (1,100-2,206)	2,450 (1,225-7,500)	0.0040	160 (74-252)	125 (50-504)	1.000	11 (6-15)	6 (3-10)	0.0216
1 week prior to foaling	1,225 (950-1,819)	2,450 (1,225-3,800)	<0.0001	115 (52-211)	125 (51-276)	0.7700	9. (5-16)	7 (3-10)	0.0385
Foaling date	1,262 (750-1,969)	2,450 (1,038-9,625)	<0.0001	120 (54-226)	100 (25-290)	1.000	10 (3-16)	5 (3-8)	0.0045
1 week after foaling	1,475 (762-3,000)	2,500 (800-10,500)	0.0492	70 (0-198)	98 (25-502)	0.7700	5 (0-12)	4 (1-8)	0.7700

* P values adjusted for multiple comparison; results of significance testing were similar without adjustment (ie, classification of any comparison as significant was not altered by adjustment, only the magnitude of the associated P value was changed)

Table 7. Median (interquartile range) values for the concentrations of total *R. equi* and virulent *R. equi*, and the proportion of virulent *R. equi* in fecal samples collected from 94 mares from a breeding farm in Kentucky during 2004 by disease status of their foals. (Affected group = dam's foal was affected by *R. equi* pneumonia [N=29]; Unaffected group = dam's foal was not affected by *R. equi* pneumonia [N=65])

Sample	**Concentration of *R. equi*** (cfu/g)			**Concentration of virulent *R. equi*** (cfu/g)			**Proportion of virulent *R. equi*** (%)		
	Affected	**Unaffected**	**P**	**Affected**	**Unaffected**	**P**	**Affected**	**Unaffected**	**P**
2 weeks prior to foaling	1,525 (1,150-2,144)	1,525 (1,019-2,225)	0.7859	170 (90-215)	158 (69-260)	0.8751	11 (6-15)	11 (6-15)	0.9932
1 week prior to foaling	1,175 (775-1,450)	1,300 (975-1,825)	0.3185	100 (60-180)	125 (50-240)	0.5254	11. (5-18)	9 (5-15)	0.4733
Foaling date	1,225 (794-1,556)	1,350 (750-2,156)	0.3616	110 (55-176)	135 (49-264)	0.5538	10 (4-16)	10 (3-16)	0.9999
1 week after foaling	1,912 (988-3,106)	1,450 (562-2,800)	0.4288	48 (0-141)	125 (0-208)	0.4195	5 (1-10)	5 (0-12)	0.9895

Table 8. Median (interquartile range) values for the concentrations of total *R. equi* and virulent *R. equi*, and the proportion of virulent *R. equi* in fecal samples collected from 77 mares from a breeding farm in Kentucky during 2005 by disease status of their foals. (Affected group = dam's foal was affected by *R. equi* pneumonia {N=24]; Unaffected group = dam's foal was not affected by *R. equi* pneumonia [N=53])

Sample	**Concentration of *R. equi*** (cfu/g)			**Concentration of virulent *R. equi*** (cfu/g)			**Proportion of virulent *R. equi*** (%)		
	Affected	**Unaffected**	**P**	**Affected**	**Unaffected**	**P**	**Affected**	**Unaffected**	**P**
2 weeks prior to foaling	1,812 (1,025-6,375)	2,675 (1,475-7,500)	0.1887	75 (50-312)	150 (73-748)	0.2391	6 (4-9)	6 (3-12)	0.9255
1 week prior to foaling	2,050 (988-3,212)	2,525 (1,512-3,975)	0.3699	125 (50-275)	150 (75-367)	0.5521	6. (4-9)	7 (3-10)	0.7771
Foaling date	2,250 (1,588-4,088)	2,962 (994-10,312)	0.5006	101 (25-239)	100 (25-565)	0.7558	5 (2-7)	5 (3-9)	0.7709
1 week after foaling	1,825 (788-14,125)	2,600 (1,481-10,188)	0.6744	125 (0-627)	87 (25-438)	0.8726	4 (0-8)	4 (1-7)	0.4190

Table 9. *R. equi* pneumonia status vs. *R. equi* growth in foal 2004 foaling date (FD) samples

		Developed *R. equi* pneumonia		
		Yes	*No*	*Total*
Feces grew *R. equi*	Yes	1	8	9
	No	28	57	85
	Total	29	65	94

Table 10. *R. equi* pneumonia status vs. *R. equi* growth in foal 2004 1 week after foaling (1WAF) samples

		Developed *R. equi* pneumonia		
		Yes	*No*	*Total*
Feces grew *R. equi*	Yes	13	23	36
	No	16	42	58
	Total	29	65	94

*The one foal that grew *R. equi* in the 2004 foaling date (FD) sample and developed disease, failed to grow *R. equi* in its 2004 1WAF specimen.

Table 11. *R. equi* pneumonia status vs. *R. equi* growth in foal 2005 foaling date (FD) samples

		Developed *R. equi* pneumonia		
		Yes	*No*	*Total*
Feces grew *R. equi*	Yes	3	4	7
	No	20	50	70
	Total	23	54	77

Table 12. *R. equi* pneumonia status vs. *R. equi* growth in foal 2005 1 week after foaling (1WAF) samples

		Developed *R. equi* pneumonia		
		Yes	*No*	*Total*
Feces grew *R. equi*	Yes	13	32	45
	No	10	22	32
	Total	23	54	77

*Two foals grew *R. equi* in their 2005 foaling date (FD) sample and developed disease but failed to grow *R. equi* in their 1WAF samples.
*One foal grew *R. equi* in both its FD and 1WAF samples and developed disease.

Table 13. *R. equi* pneumonia status vs. detection of virulent *R. equi* growth (colony immunoblot) in foal 2004 1 week after foaling (1WAF) samples

		Developed *R. equi* pneumonia		
		Yes	*No*	*Total*
Feces contained virulent *R. equi*	Yes	5	12	17
	No	8	11	19
	Total	13	23	36

*Table includes only those foals that grew *R. equi* to begin with.

Table 14. *R. equi* pneumonia status vs. detection of virulent *R. equi* growth (colony immunoblot) in foal 2005 1 week after foaling (1WAF) samples

		Developed *R. equi* pneumonia		
		Yes	*No*	*Total*
Feces contained virulent *R. equi*	Yes	3	4	7
	No	10	28	38
	Total	13	32	45

*Table includes only those foals that grew *R. equi* to begin with.

Table 15. *R. equi* pneumonia status vs. detection of virulent *R. equi* growth as determined using RT-PCR in foal 2005 foaling date (FD) and 1 week after foaling (1WAF) samples

		Developed *R. equi* pneumonia		
		Yes	*No*	*Total*
Feces contained virulent *R. equi*	Yes	4	6	10
	No	12	30	42
	Total	16	36	52

Table 16. Foal 2004 samples that contained virulent *R. equi* detected using colony immunoblot method

		Collection time		
		foaling date (FD)	*1 week after foaling (1WAF)*	*Total*
Grew virulent *R. equi*	Did	1	17	18
	Didn't	93	77	170
	Total	94	94	188

* The foal that had the 1 foaling date (FD) sample that did grow virulent *R. equi*, was negative for disease.

Table 17. Foal 2005 samples that contained virulent *R. equi* detected using either the colony immunoblot or RT-PCR method

		Collection time		
		foaling date (FD)	*1 week after foaling (1WAF)*	*Total*
Grew virulent *R. equi*	Did	0	13	13
	Didn't	77	64	141
	Total	77	77	154

*5/13 foals that grew virulent *R. equi* in their 1WAF sample, developed disease
*A sample was considered to contain virulent *R. equi* if either or both detection methods detected virulent *R. equi*.
*A sample was considered not to contain virulent *R. equi* only if both methods failed to detect virulent *R. equi*

APPENDIX B

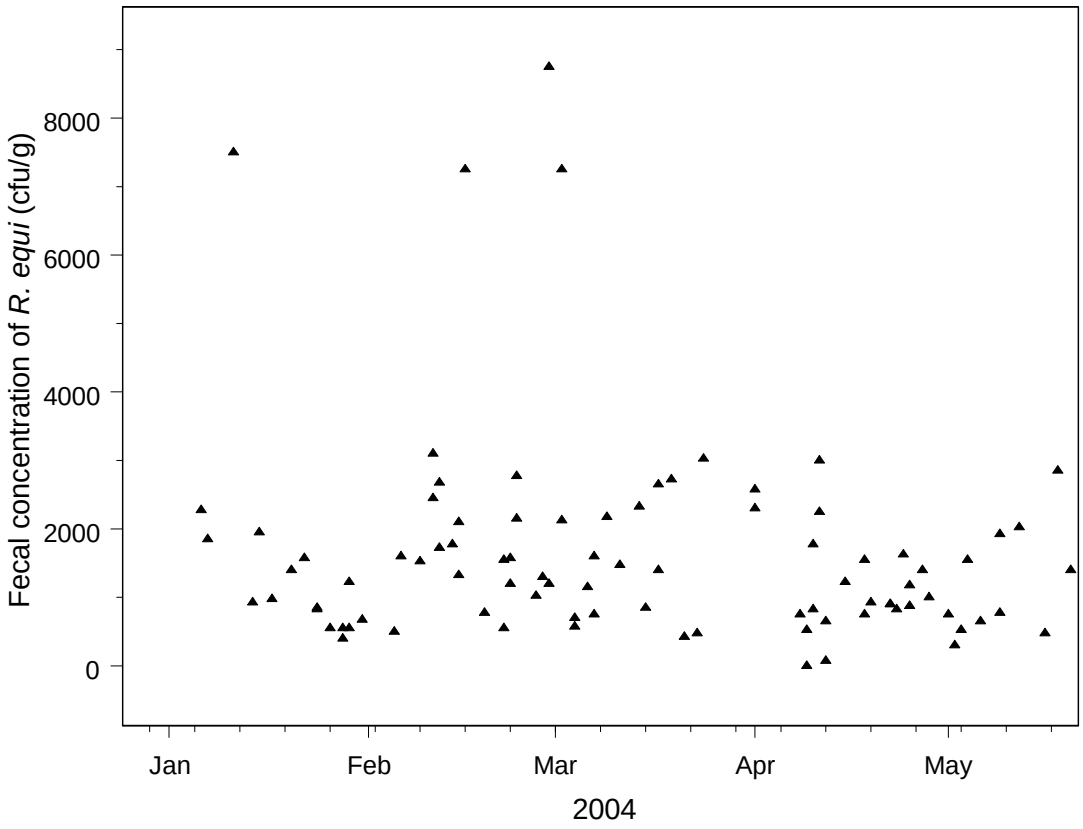

FIG. 1. Distribution of the total fecal concentrations of *R. equi* from 94 mares during the foaling day sampling time-point in 2004. No seasonal pattern was observed for temporal shedding. All other sampling time-points from 2004 appeared similar in distribution to this figure.

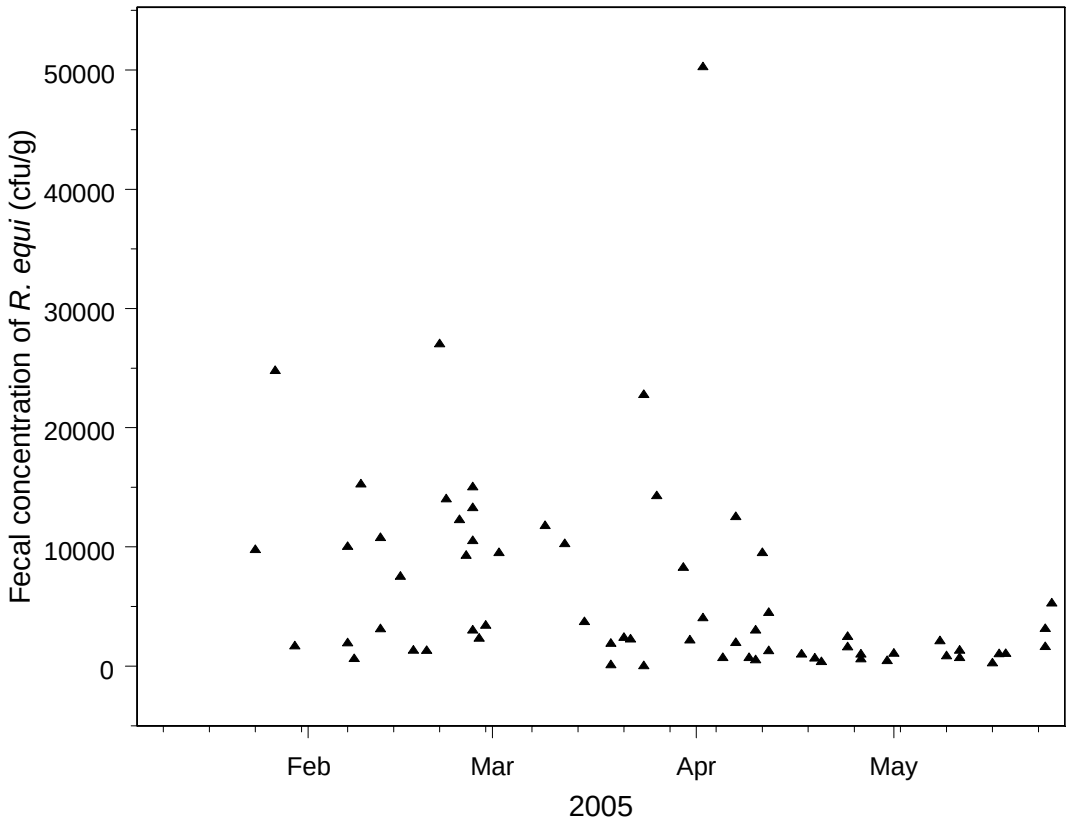

FIG. 2. Distribution of the total fecal concentrations of *R. equi* from 77 mares during the foaling day sampling time-point in 2005. No seasonal pattern was observed for temporal shedding. All other sampling time-points from 2005 appeared similar in distribution to this figure.

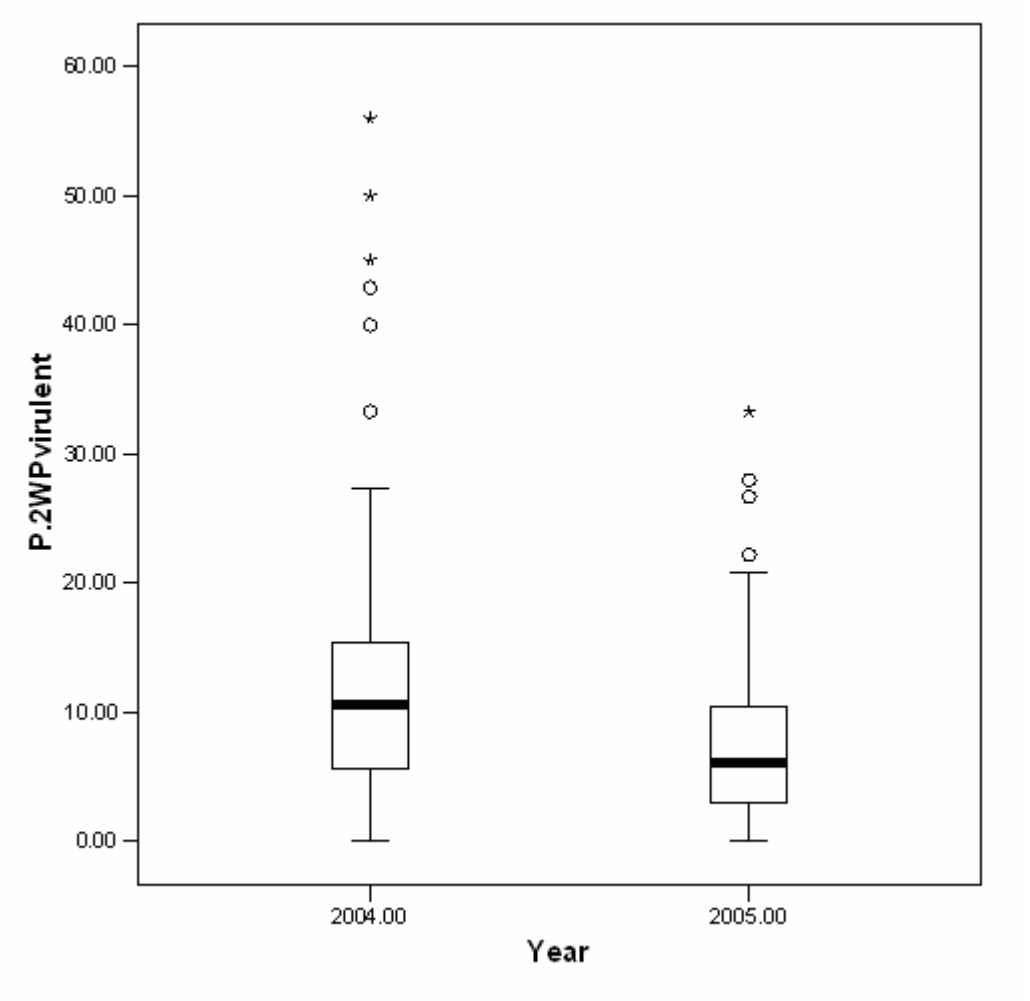

FIG. 3. Boxplots of the distribution of the proportion of virulent *R. equi* in mare fecal specimens 2 weeks prior to foaling. The difference in the distribution of the proportions of virulent isolates between the two sampling years was significantly different (P = 0.0216). Due to the difference in how the specimens were treated before culturing (i.e., frozen in 2004 and processed fresh on arrival in 2005), samples from each year were analyzed separately. P.2WPvirulent is the percentage (proportion X 100) of virulent *R. equi* isolates in mare fecal specimens. Looking at the boxplots, the thick bar is the median, ends of the boxes are the 25th and 75th percentiles, any outliers are marked with a circle and extreme outliers are marked with an asterisk. The outliers are cases with the values between 1.5 and 3 box-lengths from the 75th percentile or 25th percentile. The extreme outliers are cases with the values more than 3 box-lengths from the 75th percentile or 25th percentile.

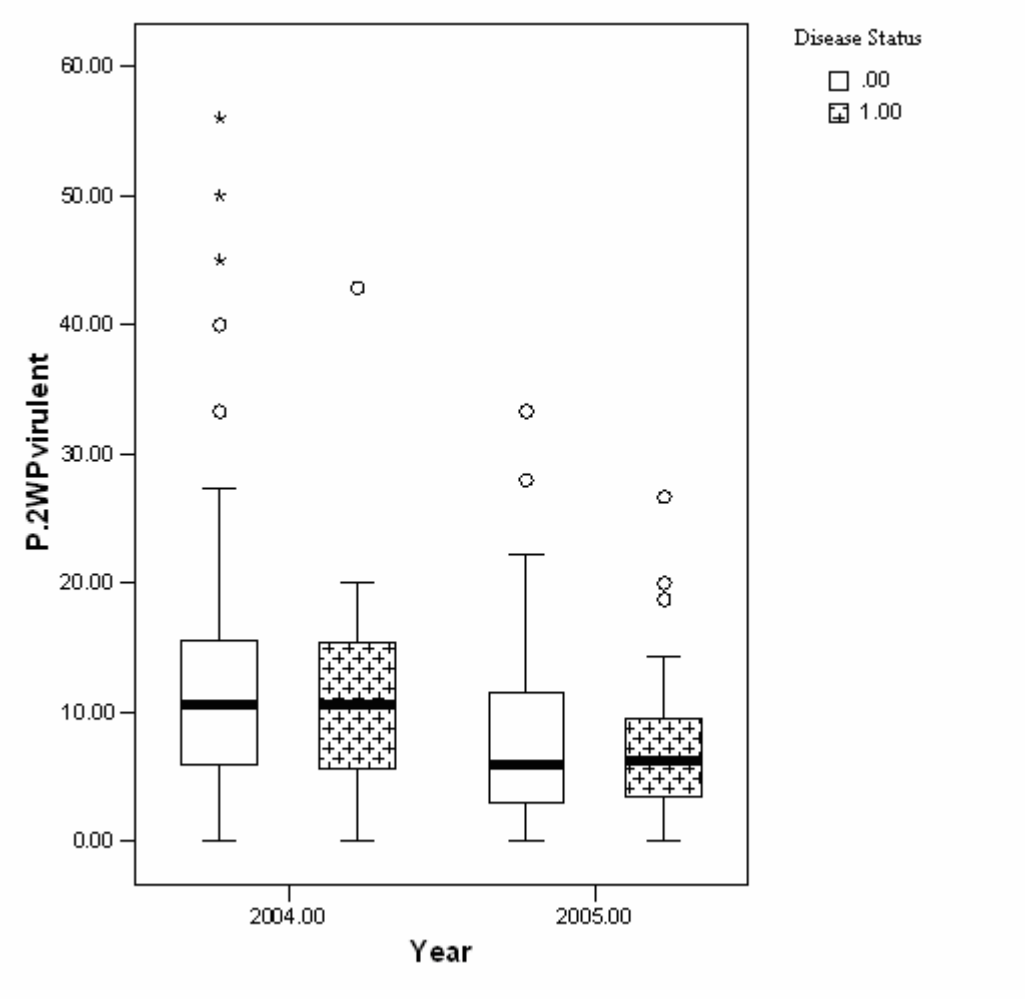

FIG. 4. Boxplots describing the distribution of the fecal proportions of virulent *R. equi* in mare fecal specimens collected 2 weeks prior to foaling, according to year and disease status of the foal. "0" indicates that the foal did not develop *R. equi* pneumonia and "1" indicates that it did develop *R. equi* pneumonia. When comparing within a year, the proportion virulent are similar for mares with affected or unaffected foals. When looking between years, it is evident that mares in 2004 had higher proportions of virulent *R. equi* in their feces. This was most likely due to smaller total concentrations of *R. equi* grown because of freezing the specimens upon arrival in 2004, leading to some unstable point estimates of these proportion data. No significant difference between disease status was detected in either 2004 (P = 0.9932) or 2005 (P = 0.9255). P.2WPvirulent is the percentage (proportion X 100) of virulent *R. equi* isolates in mare fecal specimens. Looking at the boxplots, the thick bar is the median, ends of the boxes are the 25th and 75th percentiles, any outliers are marked with a circle and extreme outliers are marked with an asterisk. The outliers are cases with the values between 1.5 and 3 box-lengths from the 75th percentile or 25th percentile. The extreme outliers are cases with the values more than 3 box-lengths from the 75th percentile or 25th percentile.

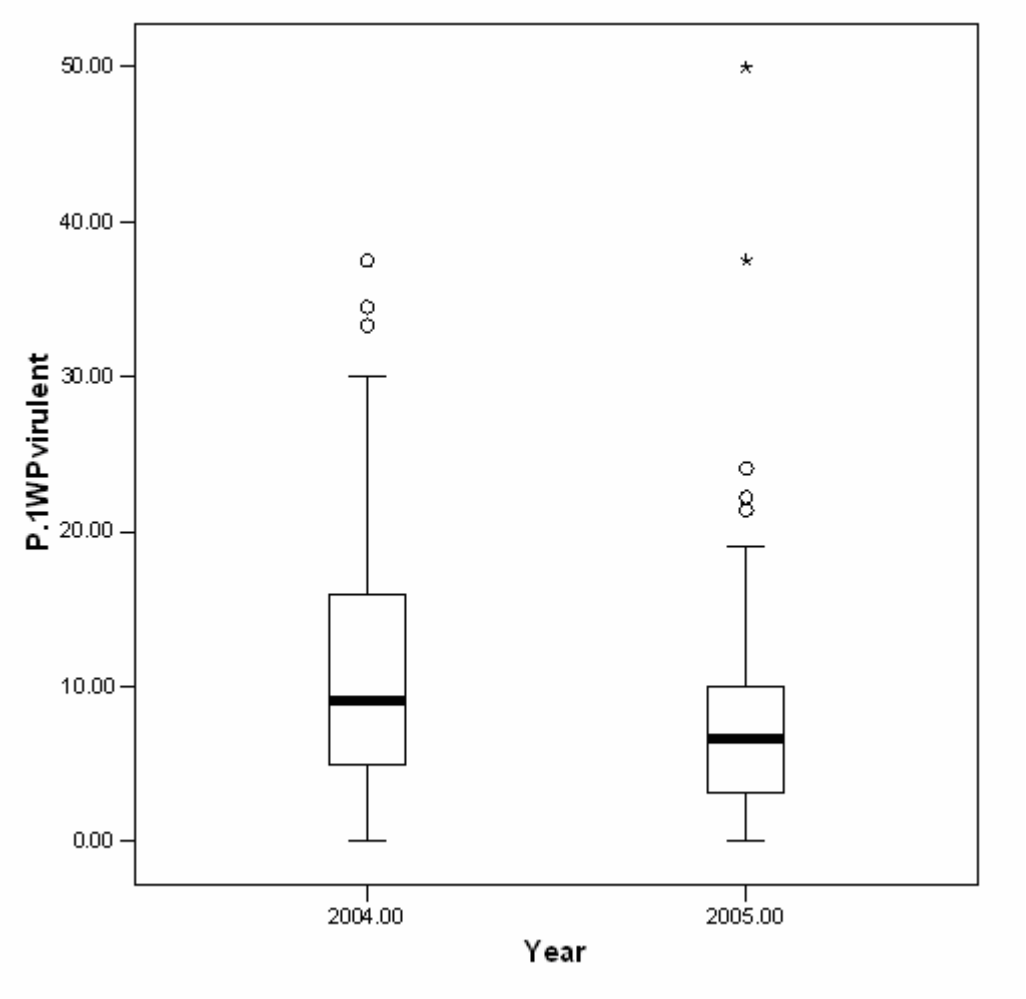

FIG. 5. Boxplots of the distribution of the proportion of virulent *R. equi* in mare fecal specimens 1 week prior to foaling. The difference in the distribution of the proportion of virulent isolates between the two sampling years was significantly different (P = 0.0385). Due to the difference in how the specimens were treated before culturing (i.e., frozen in 2004 and processed fresh on arrival in 2005), samples from each year were analyzed separately. P.1WPvirulent is the percentage (proportion X 100) of virulent *R. equi* isolates in mare fecal specimens. Looking at the boxplots, the thick bar is the median, ends of the boxes are the 25th and 75th percentiles, any outliers are marked with a circle and extreme outliers are marked with an asterisk. The outliers are cases with the values between 1.5 and 3 box-lengths from the 75th percentile or 25th percentile. The extreme outliers are cases with the values more than 3 box-lengths from the 75th percentile or 25th percentile.

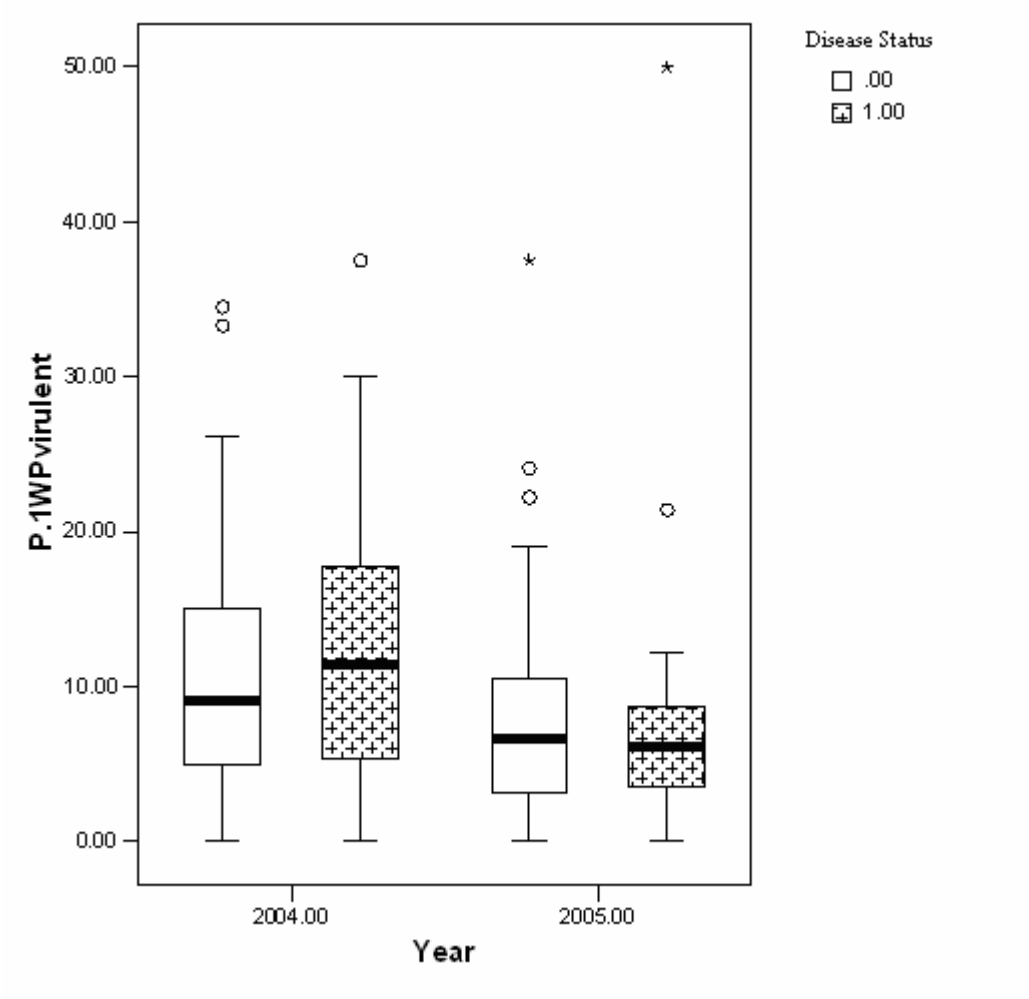

FIG. 6. Boxplots describing the distribution of the proportions of virulent *R. equi* in mare fecal specimens collected 1 week prior to foaling, according to year and disease status of the foal. "0" indicates that the foal did not develop *R. equi* pneumonia and "1" indicates that it did develop *R. equi* pneumonia. When comparing within a year, the proportion virulent are similar for mares with affected or unaffected foals. When looking between years, it is evident that mares in 2004 had higher proportions of virulent *R. equi* in their feces. This was most likely due to smaller total concentrations of *R. equi* grown because of freezing the specimens upon arrival in 2004, leading to some unstable point estimates of these proportion data. No significant difference between disease status was detected in either 2004 (P = 0.4733) or 2005 (P = 0.7771). P.1WPvirulent is the percentage (proportion X 100) of virulent *R. equi* isolates in mare fecal specimens. Looking at the boxplots, the thick bar is the median, ends of the boxes are the 25th and 75th percentiles, any outliers are marked with a circle and extreme outliers are marked with an asterisk. The outliers are cases with the values between 1.5 and 3 box-lengths from the 75th percentile or 25th percentile. The extreme outliers are cases with the values more than 3 box-lengths from the 75th percentile or 25th percentile.

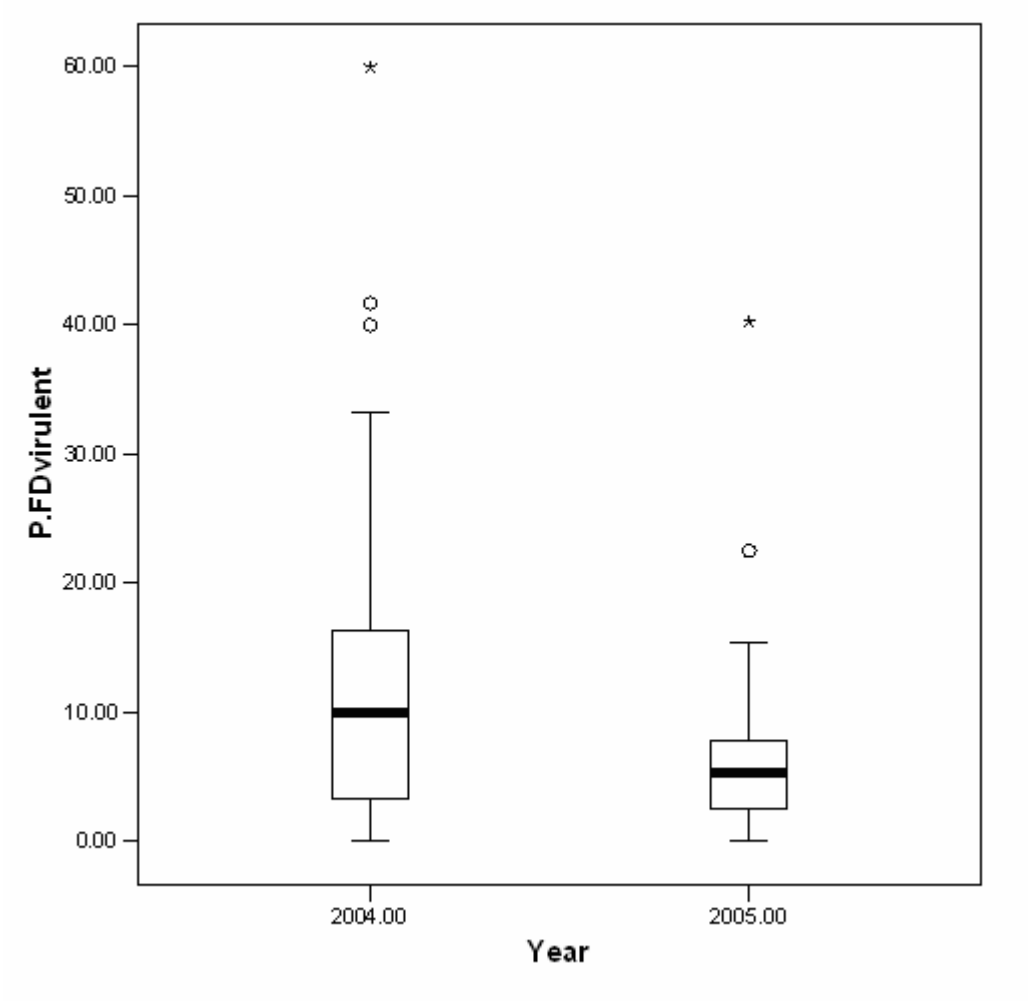

FIG. 7. Boxplots of the distribution of the proportion of virulent *R. equi* in mare fecal specimens collected on the foaling day. The difference in the distribution of the proportion of virulent isolates between the two sampling years was significant (P = 0.0045). Due to the difference in how the specimens were treated before culturing (i.e., frozen in 2004 and processed fresh on arrival in 2005), samples from each year were analyzed separately. P.FDvirulent is the percentage (proportion X 100) of virulent *R. equi* isolates in mare fecal specimens. Looking at the boxplots, the thick bar is the median, ends of the boxes are the 25th and 75th percentiles, any outliers are marked with a circle and extreme outliers are marked with an asterisk. The outliers are cases with the values between 1.5 and 3 box-lengths from the 75th percentile or 25th percentile. The extreme outliers are cases with the values more than 3 box-lengths from the 75th percentile or 25th percentile.

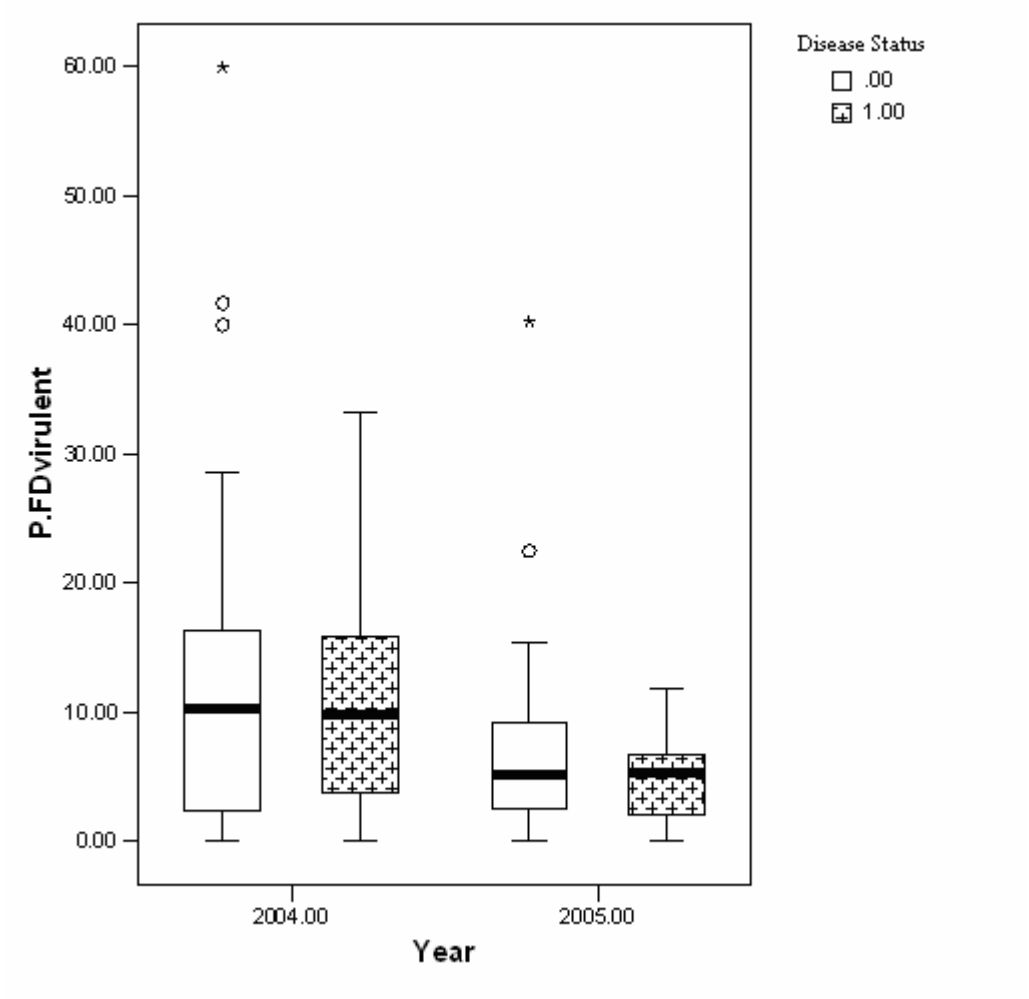

FIG. 8. Boxplots describing the distribution of the proportions of virulent *R. equi* in mare fecal specimens collected on the day of foaling, according to year and disease status of the foal. "0" indicates that the foal did not develop *R. equi* pneumonia and "1" indicates that it did develop *R. equi* pneumonia. When comparing within a year, the proportion virulent were similar for mares with affected or unaffected foals. When looking between years, it was evident that mares in 2004 had higher proportions of virulent *R. equi* in their feces. This was most likely due to smaller total concentrations of *R. equi* grown because of freezing the specimens upon arrival in 2004, leading to some unstable point estimates of these proportion data. No significant difference between disease status was detected in either 2004 (P = 0.9999) or 2005 (P = 0.7709). P.FDvirulent is the percentage (proportion X 100) of virulent *R. equi* isolates in mare fecal specimens. Looking at the boxplots, the thick bar is the median, ends of the boxes are the 25th and 75th percentiles, any outliers are marked with a circle and extreme outliers are marked with an asterisk. The outliers are cases with the values between 1.5 and 3 box-lengths from the 75th percentile or 25th percentile. The extreme outliers are cases with the values more than 3 box-lengths from the 75th percentile or 25th percentile.

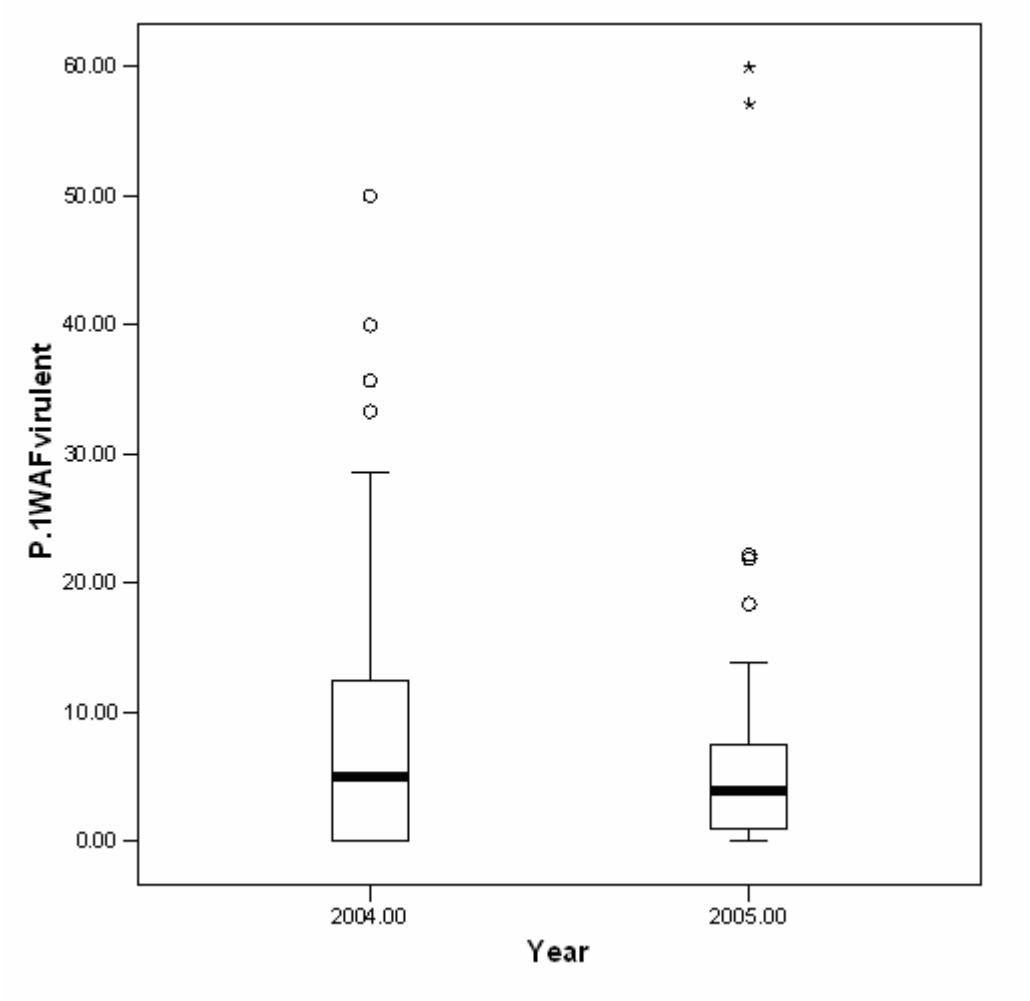

FIG. 9. Boxplots of the distribution of the proportion of virulent *R. equi* in mare fecal specimens collected 1 week after foaling. The difference in the distribution of the proportion of virulent *R. equi* between the two sampling years was not significant (P = 0.7700). Due to the difference in how the specimens were treated before culturing (i.e., frozen in 2004 and processed fresh on arrival in 2005), samples from each year were analyzed separately. P.1WAFvirulent is the percentage (proportion X 100) of virulent *R. equi* isolates in mare fecal specimens. Looking at the boxplots, the thick bar is the median, ends of the boxes are the 25th and 75th percentiles, any outliers are marked with a circle and extreme outliers are marked with an asterisk. The outliers are cases with the values between 1.5 and 3 box-lengths from the 75th percentile or 25th percentile. The extreme outliers are cases with the values more than 3 box-lengths from the 75th percentile or 25th percentile.

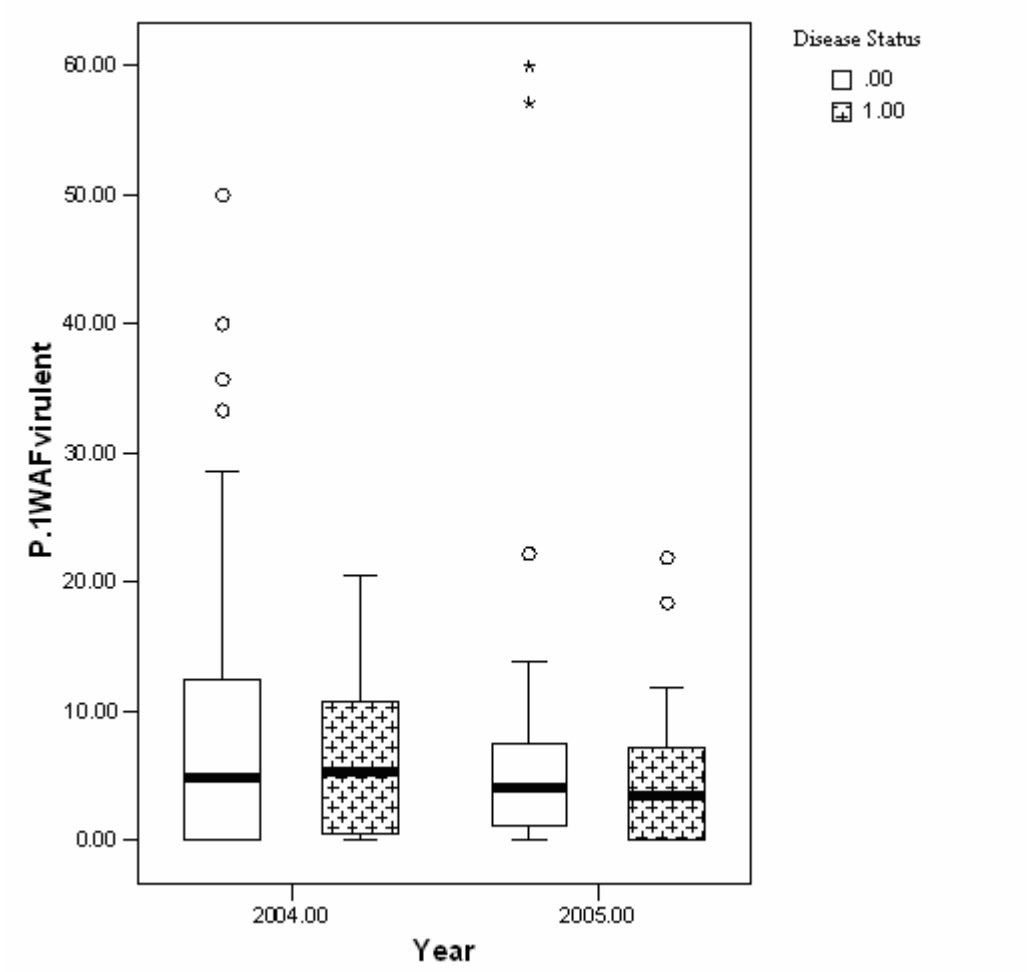

FIG. 10. Boxplots describing the distribution of the fecal proportions of virulent *R. equi* in mare fecal specimens collected 1 week after foaling, according to year and disease status of the foal. "0" indicates that the foal did not develop *R. equi* pneumonia and "1" indicates that it did develop *R. equi* pneumonia. When comparing within a year, the proportion virulent are similar for mares with affected or unaffected foals. When looking between years, it is evident that mares in 2004 had higher proportions of virulent *R. equi* in their feces. This was most likely due to smaller total concentrations of *R. equi* grown because of freezing the specimens upon arrival in 2004, leading to some unstable point estimates of the proportion data. No significant difference between disease status was detected in either 2004 (P = 0.9895) or 2005 (P = 0.4190). P.1WAFvirulent is the percentage (proportion X 100) of virulent *R. equi* isolates in mare fecal specimens. Looking at the boxplots, the thick bar is the median, ends of the boxes are the 25th and 75th percentiles, any outliers are marked with a circle and extreme outliers are marked with an asterisk. The outliers are cases with the values between 1.5 and 3 box-lengths from the 75th percentile or 25th percentile. The extreme outliers are cases with the values more than 3 box-lengths from the 75th percentile or 25th percentile.

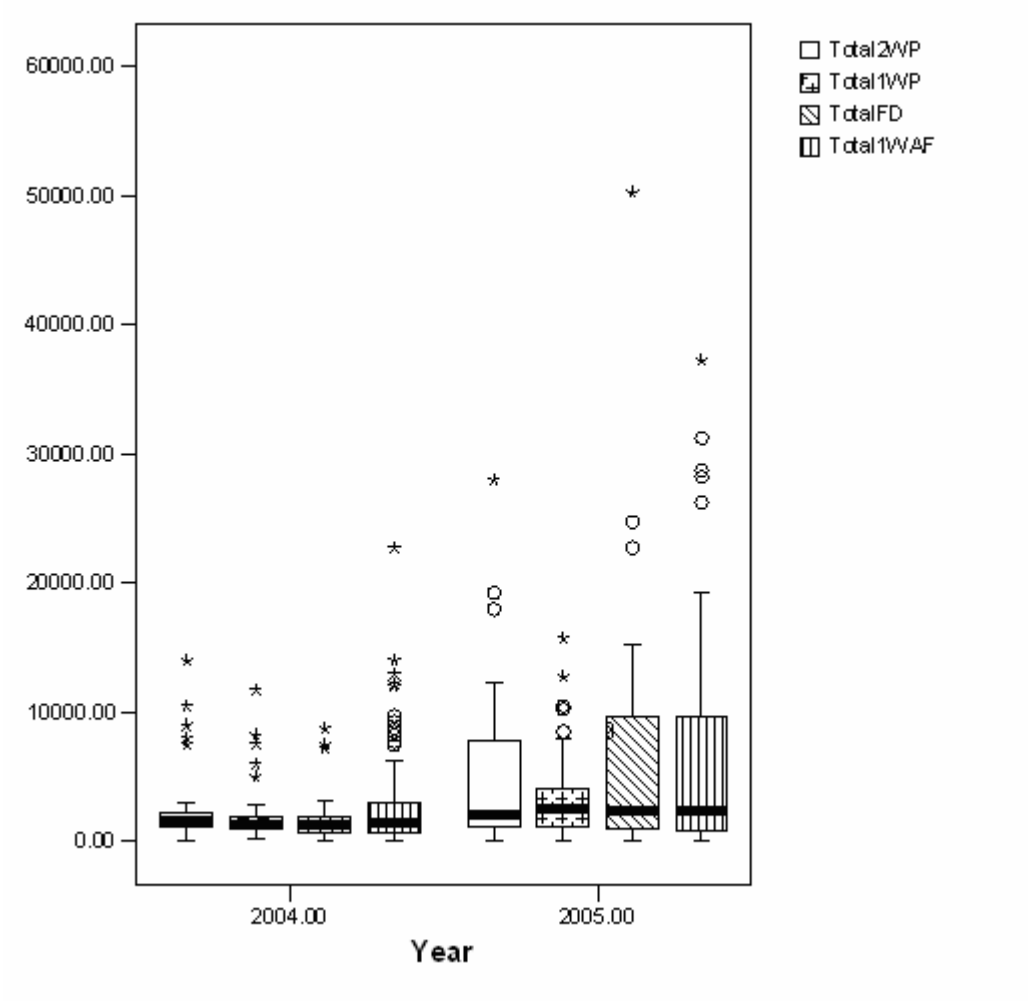

FIG. 11. Boxplots describing the distributions of total concentrations (cfu/g) of *R. equi* in all mare fecal specimens collected (2-weeks prior to foaling, 1-week prior to foaling, day of foaling, and 1-week after foaling) over both years (2004 vs. 2005). Overall, concentrations were much lower in 2004 (due to freezing), than in 2005. Ranges and medians were all higher for the 2005 data. The lower total concentrations of *R. equi* in 2004 could help explain why the proportion virulent for 2004 was so much higher. Less growth of *R. equi* by culture means that an extra virulent colony, determined by the immunoblotting assay, would yield a greater proportion virulent in the sample. Looking at the boxplots, the thick bar is the median, ends of the boxes are the 25th and 75th percentiles, any outliers are marked with a circle and extreme outliers are marked with an asterisk. The outliers are cases with the values between 1.5 and 3 box-lengths from the 75th percentile or 25th percentile. The extreme outliers are cases with the values more than 3 box-lengths from the 75th percentile or 25th percentile.

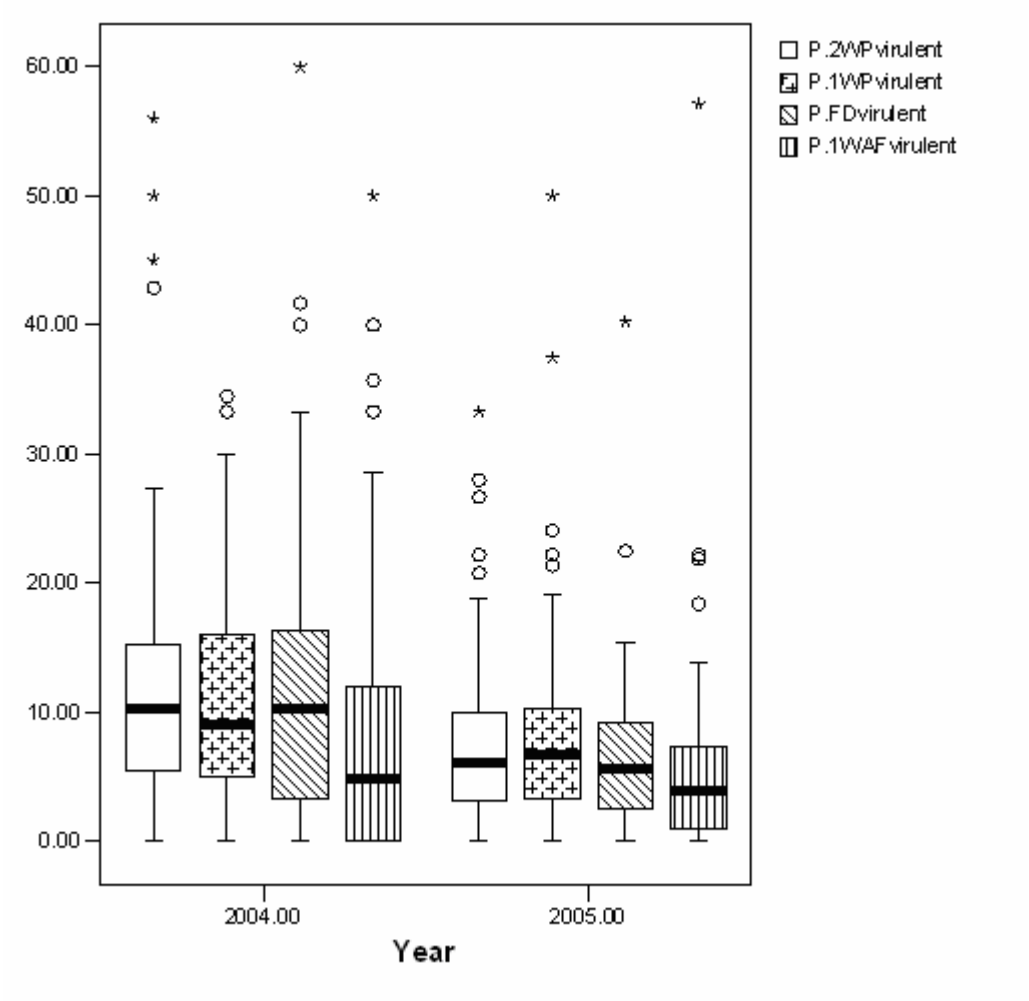

FIG. 12. Boxplots describing the distributions of the proportion of virulent *R. equi* by sample and by year. Larger ranges and slightly higher medians were observed for the 2004 specimens. This could be attributed to the freezing of the fecal specimens in 2004, leading to less *R. equi* growth and having an effect on the proportion virulent. No significant difference in proportions was determined. Looking at the boxplots, the thick bar is the median, ends of the boxes are the 25th and 75th percentiles, any outliers are marked with a circle and extreme outliers are marked with an asterisk. The outliers are cases with the values between 1.5 and 3 box-lengths from the 75th percentile or 25th percentile. The extreme outliers are cases with the values more than 3 box-lengths from the 75th percentile or 25th percentile.

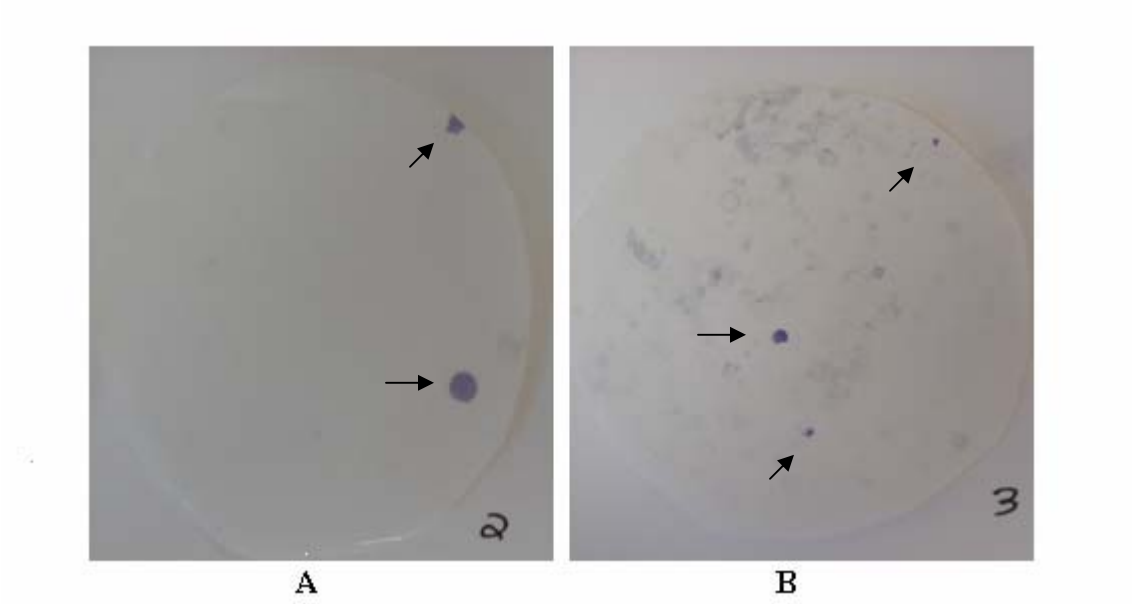

FIG. 13. Colony-blot results showing intensity of virulent *R. equi* colonies cultured from mare feces. Since there are many types organisms shed in equine feces, there is often growth of organisms other than *R. equi* on plates that can vary in density. The modified NANAT agar medium is effective at keeping this growth minimal, which makes the immunoblotted membranes easier to analyze (Figure A). The foci of background that can occasionally be observed with the cultured feces are grey in color and thus can be easily distinguished from virulent *R. equi* colonies appearing blue (Figure B). The numbers that appear in the lower right corner of each picture are the number of virulent colonies that were counted on the membrane.

VITA

Michael Bradley Grimm attended Texas A&M University from 2000 - 2006 and received a BS in Biomedical Science and an MS in Veterinary Medical Sciences. During his undergraduate time, he first worked as a student worker and then as a Technician I in the Large Animal Intensive Care Unit at Texas A&M University. In August of 2004 he entered the graduate program in the Department of Large Animal Clinical Sciences where he completed his MS in August of 2006.

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