Association of disease with isolation and virulence of *Rhodococcus equi* from farm soil and foals with pneumonia

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**Objective**—To determine whether isolation and virulence of *Rhodococcus equi* from soil and infected foals are associated with clinical disease.

**Design**—Cross-sectional and case-control study.

**Sample Population**—*R. equi* isolates from 50 foals with pneumonia and soil samples from 33 farms with and 33 farms without a history of *R. equi* infection (affected and control, respectively).

**Procedure**—*R. equi* was selectively isolated from soil samples. Soil and clinical isolates were evaluated for virulence-associated protein antigen plasmids (VapA-P) and resistance to the β-lactam antibiotics penicillin G and cephalothin. Microbiologic cultures and VapA-P assays were performed at 2 independent laboratories.

**Results**—VapA-P was detected in 49 of 50 (98%) clinical isolates; there was complete agreement between laboratories. *Rhodococcus equi* was isolated from soil on 28 of 33 (84.8%) affected farms and 24 of 33 (72.7%) control farms, but there was poor agreement between laboratories. Virulence-associated protein antigen plasmids were detected on 14 of 66 (21.2%) farms by either laboratory, but results agreed for only 1 of the 14 VapA-P-positive farms. We did not detect significant associations between disease status and isolation of *R. equi* from soil, detection of VapA-P in soil isolates, or resistance of soil isolates to β-lactam antibiotics. No association between β-lactam antibiotic resistance and presence of VapA-P was detected.

**Conclusions and Clinical Relevance**—On the basis of soil microbiologic culture and VapA-P assay results, it is not possible to determine whether foals on a given farm are at increased risk of developing disease caused by *R. equi*. *(J Am Vet Med Assoc 2000;217:220–225)*

Foal pneumonia is a major concern to the equine industry worldwide. A recent large-scale study indicated that respiratory disease was the most common cause of foal morbidity and mortality in Texas. Many different organisms may cause respiratory disease, but *Rhodococcus equi* is considered the most common cause of severe pneumonia in foals. *Rhodococcus equi* is a gram-positive, facultative-intracellular, soil saprophytic bacterium that characteristically infects foals <3 months of age. The disease caused by *R. equi* is endemic on some farms, sporadic on others, and does not develop on most farms. Environmental as well as individual foal health issues have been implicated in these differences in disease prevalence.1,2 Virulence factors of *R. equi* have not been identified; however, virulence-associated markers in the form of 15- to 17-kilodalton virulence-associated protein antigens (VapA) have been described.3,19 Ten different VapA plasmids (VapA-P), each of which possess a VapA gene (VapA-G) that encodes for the production of VapA, have been identified.3,19 The association of VapA-P and their associated genes and proteins with virulence has been established in foals and mice infected with *R. equi*.19,30 Only foals that possess VapA-P and VapA-G produce VapA. Virulence-associated protein antigen plasmids can be detected directly by plasmid profiling or indirectly by detection of VapA-G or VapA.3,19,21

*R. equi* is commonly cultured from feces of adult horses and foals, and foals with *R. equi* pneumonia are capable of shedding large numbers of the organism onto the soil.3,19,21 *Rhodococcus equi* can flourish and multiply in soil when environmental conditions are suitable. The greatest number of organisms are recovered from the soil surface.14,15 Early epidemiologic studies indicated that the number of *R. equi* organisms in soil on a given farm correlated with the incidence of *R. equi* pneumonia on that farm.1,4,16,17 Recent studies in Japan, however, indicate that the difference in disease incidence on a farm is not related to the number of *R. equi* organisms in soil but rather to the prevalence of *R. equi* isolates that contain VapA-P.19,30,31 Most of these observations and correlations have been made on farms on which *R. equi* infections are endemic.

Resistance to β-lactam antibiotics also has been correlated with virulence of *R. equi* obtained from humans and a limited number of other animals.20 This relationship has been refuted by investigators studying equine isolates of *R. equi*.21 If this correlation were valid, however, the routine use of β-lactam antibiotics for treatment of respiratory diseases in foals could inadvertently select for strains of *R. equi* that are resistant to β-lactam antibiotics.

On the basis of the strong association between...
prevalence of virulent \textit{R equi} in farm soil and incidence of \textit{R equi} pneumonia on farms for which \textit{R equi} is endemic, it has been postulated that the prevalence of virulent \textit{R equi} in soil may be useful to determine the relative risk of infection for foals on those farms.\textsuperscript{10} As part of a disease management strategy, veterinary practitioners may consider submitting soil samples for \textit{R equi} culture and assessing prevalence of virulence markers. The purposes of the study reported here were to determine, under conditions that may be available to practicing veterinarians through diagnostic laboratories, the frequency distribution of virulence marker-positive isolates of \textit{R equi} obtained from soils with pneumonia, the association between soil-borne \textit{R equi} and \textit{R equi}-induced disease status on the farm, the association between virulence marker-positive, soil-borne \textit{R equi} and \textit{R equi}-induced disease status on the farm, and the association between virulence of \textit{R equi} and resistance of \textit{R equi} to β-lactam antibiotics.

**Materials and Methods**

Clinical samples—Fifty isolates of \textit{R equi} were obtained during 1997 from soils with pneumonia in Texas. These bacteria were harvested from respiratory tract specimens submitted by veterinary practitioners throughout Texas in 1 of 3 major diagnostic laboratories. Following isolation and identification of \textit{R equi} by the respective laboratories, the organisms were transported to 2 independent study laboratories in Texas and Japan for confirmation of identity and assessment of virulence markers.

Soil samples—Soil samples were obtained as part of a prospective matched case-control study of horse-breeding farms. Veterinarians in Texas who were members of the American Association of Equine Practitioners were invited to participate in the study by submitting soil samples from a pair of actively operating horse-breeding farms in their practice. Each pair consisted of a farm that had at least 1 foal with \textit{R equi} pneumonia in 1997 (affected farm) and 1 farm that had no affected foals in 1997 and no known history of \textit{R equi} infections (control farm). A foal with \textit{R equi} pneumonia was defined as a 3-week- to 6-month-old foal with either clinical signs of pneumonia for which \textit{R equi} was isolated from tracheobronchial samples or lung specimens obtained at necropsy or clinical signs of pneumonia and at least 1 of the following: multifocal lung opacities apparent on thoracic radiographs, ultrasonographic evidence of pulmonary abscesses, gram-positive intracellular cocobacilli in transmural wash specimens, or a positive response to treatment aimed at \textit{R equi} infection.

Soil samples were submitted from 33 pairs of farms throughout Texas. Two to 3 teaspoons of surface soil were collected from 2 sites (no less than 10 yds apart) most frequently by foals on each farm. The samples were combined and submitted in sterile plastic bags. Each sample was homogenized by mixing and divided equally for the 2 participating laboratories. Samples for Texas were processed within 1 week, and those for Japan were stored at –80°C, delivered by private courier, and processed within 6 months of collection.

**Bacterial culture**—\textit{Rhodococcus equi} isolates obtained from clinical cases were transported to the study laboratories on trypticase soy agar (TSA)\textsuperscript{11} slants and propagated at 30°C on TSA plates with sheep blood\textsuperscript{12} and in brain heart infusion broth (BHI)\textsuperscript{13} with 10% newborn calf serum (NCS).\textsuperscript{14} All isolates were confirmed to be \textit{R equi} on the basis of described morphologic and biochemical characteristics, including synergistic hemolysis with \textit{Corynebacterium pseudotuberculosis} or \textit{Staphylococcus aureus}.\textsuperscript{15}

For the selective isolation of \textit{R equi} from soil samples, the study laboratory in Texas used a medium containing ef-tazidine and novobiocin (CAZ-NB),\textsuperscript{16} and the laboratory in Japan used a medium containing nalidixic acid, novobiocin, cycloheximide, and potassium tellurite (NANAT).\textsuperscript{17} Each composite soil sample was mixed, a 10% saline (0.9% NaCl) suspension prepared, and then 10-fold serial dilutions were inoculated onto selective agar plates. The plates were incubated at 30°C for 2 or 3 days. When present, 1 to 10 \textit{R equi}-suspect colonies per soil specimen were subcultured and identified on the basis of morphologic and biochemical characteristics.

**Virulence assays**—Virulence-associated protein antigen plasmids in \textit{R equi} obtained from soils with pneumonia and isolated from soil were identified at the laboratory in Texas by use of described polymerase chain reaction (PCR) techniques with minor modifications. Plasmid DNA was extracted from \textit{R equi} by the respective laboratories, the isolates were transported to 2 independent study laboratories in Texas and Japan by private courier, and processed within 1 week, and those for Japan were stored at –80°C, delivered by private courier, and processed within 6 months of collection. One milliliter of the bacterial culture was centrifuged and the pellet processed, using a commercially available plasmid preparation kit. Primers\textsuperscript{18} for PCR were synthesized. Primers 1 (5’-T[GCTT]CCAAGACCGGTT-3’) corresponded to positions 6 to 23 of the sense strand of \textit{VapA}-G, and primer 2 (5’-[TAGC]TTTGGCAGCTA-3’) corresponded to the antisense strand at positions 569 to 552.\textsuperscript{19} Amplification was performed with 1.0 µl of the DNA preparation in a 20-µl reaction containing 2.4 mM MgCl\textsubscript{2}, 0.25 mM of each deoxynucleoside triphosphate, 2.5 µM of each primer, 1.25 U Taq DNA polymerase, 0.5 µl DMSO, and 2.0 µl 10X PCR buffer without MgCl\textsubscript{2}. After an initial heating phase (95°C for 2 minutes), the samples were subjected to 35 cycles of amplification, consisting of a denaturation step (95°C for 45 seconds), a primer annealing step (57°C for 1 minute), and an extension step (57°C for 30 seconds). After amplification, 10 µl of the reaction mixture was electrohoresed on a 1% agarose gel, and DNA fragments were identified by ultraviolet fluorescence after staining with ethidium bromide.

Virulence-associated protein antigen plasmids in \textit{R equi} obtained from soils with pneumonia and isolated from soil were identified at the laboratory in Japan by use of a colony-blot ELISA with a monoclonal antibody against \textit{VapA}.\textsuperscript{20} For colony-blot analysis, bacterial strains were injected onto brain heart infusion agar plates, and incubated at 30°C for 24 hours. A sheet of nitrocellose filter (pore size, 0.45 µm) was placed on colonies for a few minutes until completely wet. The filter was removed, air-dried, and autoclaved at 105°C for 1 minute. All buffers, antibodies, and conjugate dilutions were as described.\textsuperscript{21}

Resistance of \textit{R equi} obtained from soils with pneumonia and those isolated from soil to the β-lactam antibiotics penicillin G and cephalothin was determined at the laboratory in Texas by use of a commercially available antibiotic susceptibility assay.\textsuperscript{22} Briefly, bacteria were grown for 24 hours at 30°C on TSA plates. Colonies were picked, and an 8 µl suspension of each colony was prepared to achieve a turbidity equivalent to a 0.5 McFarland standard. Sterile cotton swabs were used to inoculate Mueller-Hinton agar plates to achieve a confluent lawn of growth. Separate plates were used for each antibiotic, and tests were conducted in triplicate. The Mueller-Hinton agar plates were incubated for 24 hours at 30°C, and the mean inhibitory concentration (MIC) values were read at the point of intersection between the growth zone edge and the antibiotic impregnated test strip. The range of sensitivity of the penicillin G test strip was 0.002 to 32 µg/ml, and the range for cephalothin was 0.016 to 256 µg/ml.
with at least 1 foal with R equi pneumonia and identified as R equi by 1 of 3 submitting diagnostic laboratories, were transmitted to the study laboratories for confirmation of identity and characterization of virulence. All cultures contained pure growths of R equi.

Identity of soil isolates—Fifty bacterial isolates, obtained from respiratory tracts of foals with pneumonia and identified as R equi by 1 of 3 submitting diagnostic laboratories, were transmitted to the study laboratories for confirmation of identity and characterization of virulence. All cultures contained pure growths of R equi.

Identity of soil isolates—Data were obtained from 33 pairs of farms. Selective R equi culture from soil samples by the 2 study laboratories yielded significantly different results. Rhodococcus equi was cultured from soil samples significantly (P = 0.006) more often at the laboratory in Japan than the laboratory in Texas (Table 1). Moreover, the Japanese laboratory identified isolates from significantly more affected (P = 0.05) and control farms (P = 0.05; Tables 2 and 3) than the Texas laboratory. In addition, there was poor agreement between the laboratories regarding detection of R equi from individual soil samples. Culture results were in agreement on 12 of 33 (36.4%) affected farms, 18 of 33 (54.5%) control farms, and 32 of 66 (48.5%) farms overall. For purposes of this study, a farm was considered culture-positive if R equi was isolated from soil by either laboratory. Thus, 28 of 33 (84.8%) affected farms and 24 of 33 (72.7%) control farms were culture-positive. There was no significant or apparent association of disease status with isolation of R equi from soil.

Virulence of clinical isolates—Virulence-associated protein antigen plasmids in R equi isolates obtained from respiratory tracts of 50 foals with pneumonia were identified indirectly by detection of VapA-G (Texas laboratory) and VapA (Japanese laboratory). Plasmids were identified in 49 of 50 (98%) isolates, and there was complete agreement between laboratories.

Virulence of soil isolates—Virulence-associated protein antigen plasmids in representative R equi isolates independently obtained from the same soil samples by each laboratory were identified in Texas and Japan by use of PCR and colony-blot ELISA, respectively. Although the difference was not significant, VapA-P was identified in isolates from more affected and control farms at the Japanese laboratory (6/33 [18.2%] and 4/33 [12.1%], respectively) than at the Texas laboratory (4/33 [12.1%] and 1/33 [3%], respectively). In addition, there was poor agreement between laboratories regarding detection of VapA-P on individual farms (Table 4). Although there was 80% agreement regarding detection of VapA-P on all 66 farms, this result was skewed, because VapA-P was not detected on 52 of 66 (78.8%) farms by either laboratory. Of the 14 VapA-P-positive farms, the laboratories were only in agreement on 1 farm. For purposes of this study, a farm was considered VapA-P-positive if VapA-P was detected in R equi by either laboratory. Thus, 9 of 28 (32.1%) affected farms and 5 of 24 (20.8%) control farms were VapA-P positive.

For 10 pairs of farms, R equi was cultured from the soil of only 1 farm (either affected or control), so only 23 pairs of farms were used to examine the association of disease status with isolation of VapA-P. Rhodococcus equi isolates from 7 of 23 (30.4%) affected farms were identified indirectly by detection of VapA-G (Texas laboratory) and VapA (Japanese laboratory). Plasmids were identified in 49 of 50 (98%) isolates, and there was complete agreement between laboratories.

Table 1—Results of microbiologic culture of Rhodococcus equi obtained at 2 study laboratories from soil samples collected from 66 actively operating horse-breeding farms

<table>
<thead>
<tr>
<th>Texas laboratory</th>
<th>Japanese laboratory</th>
<th>Total (%)</th>
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<tbody>
<tr>
<td>Negative</td>
<td>Negative</td>
<td>14 (40)</td>
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<tr>
<td>Positive</td>
<td>Positive</td>
<td>25 (60)</td>
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<tr>
<td>Total</td>
<td></td>
<td>39 (100)</td>
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</table>

<table>
<thead>
<tr>
<th>Texas laboratory</th>
<th>Japanese laboratory</th>
<th>Total (%)</th>
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</thead>
<tbody>
<tr>
<td>Negative</td>
<td>Negative</td>
<td>9 (27)</td>
</tr>
<tr>
<td>Positive</td>
<td>Positive</td>
<td>18 (54)</td>
</tr>
<tr>
<td>Total</td>
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<td>27 (100)</td>
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*Results determined by use of a colony-blot ELISA. #Results determined by use of a polymerase chain reaction-based assay.
VapA-P positive, whereas isolates from 5 of 23 (21.7%) control farms were VapA-P positive. We did not detect a significant association between disease status of a farm and VapA-P in R equi isolated from soil on that farm.

Virulence-associated protein antigen plasmids were detected significantly (P = 0.001) more often in R equi isolated from foals with pneumonia (49/50, 98%) than in R equi isolated from soil samples (6/57, 10.5%).

Antibiotic resistance of clinical isolates—Clinical isolates of R equi were evaluated for their susceptibility to penicillin G and cephalothin. Forty-nine of 50 (98%) were resistant to penicillin G, 49 of 50 (98%) were resistant to cephalothin, and 49 of 50 (98%) were VapA-P positive on the basis of results of PCR analysis. Overall agreement between resistance to either penicillin G or cephalothin with VapA-P results was good (96 and 100%, respectively), largely because of the number of samples that yielded positive results by use of both assays. Although there were 2 discordant observations for resistance to penicillin G versus VapA-P, all observations were concordant for resistance to cephalothin versus VapA-P.

Antibiotic resistance of soil isolates—Resistance to β-lactam antibiotics was only evaluated at the study laboratory in Texas. For 25 pairs of farms, R equi was cultured at the Texas laboratory from the soil of only 1 farm (either affected or control), so only 8 pairs of farms were used to examine the association of disease status with β-lactam antibiotic resistance.

For the 8 pairs of farms (16 farms), 35 R equi isolates were obtained (median, 2.5 isolates/farm; range, 1 to 3 isolates/farm). For antibiotic resistance data, the median MIC value for all isolates from a given farm was used to characterize that farm. Eight of 8 (100%) affected and 7 of 8 (87.5%) control farms yielded isolates resistant to penicillin G and cephalothin. One control farm had isolates that were susceptible to penicillin G and cephalothin. The median MIC of penicillin G was 28 µg/ml (range, 20 to >32 µg/ml) for isolates from affected farms and 32 µg/ml (range, 1.5 to >32 µg/ml) for isolates from control farms. The median MIC of cephalothin was 128 µg/ml (range, 48 to >256 µg/ml) for isolates from affected farms and 112 µg/ml (range, 3.5 to >256 µg/ml) for isolates from control farms. For the 8 pairs of farms from which R equi was isolated from both farms, there was no apparent association of disease status with resistance of isolates to penicillin G or cephalothin.

The isolates from the 8 pairs of farms were also considered without accounting for matching. Again, there was no significant association of disease status with resistance to β-lactam antibiotics. Although the proportions of isolates resistant to penicillin G or cephalothin were greater on affected farms (18/19 [94.7%] and 18/19 [94.7%, respectively) than on control farms (13/16 [81.2%] and 13/16 [81.2%, respectively), these differences were not significant. Only 4 of the 35 isolates from these 16 farms were susceptible to either penicillin G or cephalothin.

Antibiotic resistance of clinical isolates versus that of soil isolates—The proportion of R equi isolates resistant to penicillin G did not differ between clinical isolates (49/50 [98%]) and soil isolates from affected farms (18/19 [94.7%]); however, the proportion of resistant R equi isolates was significantly (P = 0.041) less from soil isolates from control farms (13/16 [81.2%]) than from clinical isolates. Similarly, there was no significant difference between the proportion of isolates resistant to cephalothin among isolates from foals with pneumonia and soil from affected farms; however, the proportion of cephalothin-resistant isolates was significantly (P = 0.041) greater among clinical isolates than among soil isolates from control farms. These significant differences were not considered clinically meaningful.

When considering all R equi isolated from clinical cases and soil, the median MIC of penicillin G for all β-lactam-resistant isolates was 32 µg/ml (range, 8 to 32 µg/ml), and median MIC for cephalothin was 192 µg/ml (range, 16 to 256 µg/ml). Median MIC of penicillin G for susceptible isolates was 1.5 µg/ml (range, 1.5 to 2 µg/ml), and median MIC for cephalothin was 3.5 µg/ml (range, 3.5 to 8 µg/ml).

Association of VapA-P with antibiotic resistance—The association of resistance of R equi to penicillin G or cephalothin with detection of VapA-P was evaluated in 124 isolates (50 clinical isolates, 29 soil isolates from affected farms, and 45 soil isolates from control farms). Of these isolates, 56 (45.1%) were VapA-P-positive and 68 (54.8%) were VapA-P-negative, as determined by use of PCR.

No significant correlation was found between resistance to either penicillin G or cephalothin and presence of VapA-P. Of the 124 isolates, 116 (93.5%) were resistant to penicillin. Overall agreement for penicillin resistance and presence of VapA-P was low (62/124 [50%]); 55 of the 56 (98.2%) VapA-P-positive and 61 of the 68 (89.7%) VapA-P-negative isolates were resistant to penicillin. This difference was not significant. The distribution of data and results were identical for the association of resistance to cephalothin with presence of Vap-P.

Discussion

Since the discovery and characterization of R equi VapA-P and their associated genes and protein antigens, several detection assays have been developed. These assays have been used to assess the association of virulence markers with clinical disease and investigate the importance of virulence markers as risk factors for infection. Although results of standard techniques, such as plasmid and protein profiles for identifying virulent R equi are reliable, they take several days or weeks to generate. The monoclonal antibody-based colony-blot ELISA for VapA and the PCR-based VapA-G assay were developed to more rapidly identify virulent R equi. The colony-blot ELISA, which detects VapA, could yield false-negative results, because expression of VapA is regulated by growth conditions such as temperature and pH. The PCR-based assay for VapA-G does not have these limitations.

Fifty isolates of R equi from foals with pneumonia...
were examined for VapA-P by both study laboratories; each laboratory used a different test. There was complete agreement between laboratories regarding detection of VapA-P. The association of VapA-P with clinical disease was strong and corroborated previous findings.3,4,8

Prior to the use of selective media, it was nearly impossible to culture R equi from soil because of overgrowth by other bacteria and fungi. Several types of selective media have been developed.12,13,17 In addition to suppressing the growth of other microorganisms, selective media may also inhibit growth of some strains of R equi.21,23,27 In this study, 2 types of selective media, CAZ-NB23 and NANAT,23 were used to culture R equi from soil. The use of NANAT agar by the laboratory in Japan resulted in isolation of R equi from a significantly greater number of farms than use of CAZ-NB agar. In addition, there was poor agreement between laboratories regarding isolation of R equi from individual soil samples. For the 66 soil samples, there was 49% agreement in culture results, but for the 52 culture-positive samples, there was only 33% agreement between results. Consequently, both laboratories recovered R equi from samples that yielded negative culture results at the other laboratory. Because the microbiologists who performed the cultures in this study were experienced in the propagation and identification of R equi, the disparity in results may be attributable to different inhibitory effects of the 2 types of selective media. There is also the possibility that the soil samples were not adequately homogenized, thereby yielding different results. Because of the disparity in culture results between these 2 types of selective media, it may be advantageous to use more than 1 type of selective media when attempting to culture R equi from soil.

Previous epidemiologic studies that evaluated the association of soil-borne R equi and VapA-P with clinical disease compared far fewer farms than we did or selected specifically for farms on which R equi disease compared far fewer farms than we did or

Because there was excellent correlation between laboratories regarding detection of VapA-P in R equi isolates from infected foals, the most probable explanation for divergent results from soil isolates is that each laboratory was testing different R equi isolates from the same soil sample. This may have been attributable to inadequate homogenization of the soil samples or the presence of various R equi strains within a single sample.

Despite the disparity in culture and VapA-P assay results for soil-borne R equi between the study laboratories, whether individual or combined laboratory results were analyzed, the associations between R equi and VapA-P in soil isolates with that of disease status remained the same. We did not detect an apparent association of disease status with isolation of R equi from soil or detection of VapA-P.

Although there was a significant correlation between resistance to β-lactam antibiotics and VapA-P in R equi from infected foals, this was skewed by the fact that 98% of the clinical R equi isolates were Vap-P-positive. When we compared 124 R equi isolates from infected foals and soil samples, of which 33% were VapA-P-negative, we did not detect a significant correlation between β-lactam resistance and VapA-P. In addition, there was no significant correlation between β-lactam resistance and disease status. These results refute the positive association between β-lactam antibiotic resistance and virulence of R equi, as proposed by Nordman et al,21 and substantiate results from other investigators.23

The results of the present study suggest that the optimal conditions for soil sample collection and microbiologic culture have not been established to determine whether a farm or site on a farm harbors R equi or VapA-P-positive R equi. These results also suggest that the association between presence of R equi or VapA-P-positive R equi in farm soil and prevalence of R equi pneumonia on that farm have not been adequately established. Instead, we believe that it is not possible to determine whether foals on a given farm are at increased risk of developing disease caused by R equi on the basis of culture results or results of assays to detect VapA-P.


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