

# Epidemiologic study of results of pulsed-field gel electrophoresis of isolates of *Rhodococcus equi* obtained from horses and horse farms

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**Objective**—To compare isolates of *Rhodococcus equi* on the basis of geographic source and virulence status by use of pulsed-field gel electrophoresis (PFGE).

**Sample Population**—290 isolates of *R equi* (218 virulent isolates from foals and 72 avirulent isolates from feces, soil, and respiratory tract samples) obtained between 1985 and 2000 from horses and horse farms from 4 countries.

**Procedure**—DNA from isolates was digested with the restriction enzyme *Asel* and tested by use of PFGE. Products were analyzed for similarities in banding patterns by use of dendrograms. A similarity matrix was constructed for isolates, and the matrix was tested for nonrandom distributions of similarity values with respect to groupings of interest.

**Results**—There was little grouping of isolates on the basis of country, virulence status, or region within Texas. Isolates of *R equi* were generally < 80% similar, as determined by use of PFGE. Isolates from the same farm generally were rarely of the same strain.

**Conclusions and Clinical Relevance**—Considerable chromosomal variability exists among isolates of *R equi* obtained from the same farm, sites within Texas, or among countries from various continents. Only rarely will it be possible to link infections to a given site or region on the basis of analysis of isolates by use of PFGE of chromosomal DNA. (*Am J Vet Res* 2003;64:153–161)

Pneumonia is a common and often severe disease among foals.<sup>1-6</sup> Pneumonia attributable to infection with *Rhodococcus equi* has a worldwide distribution and is considered the most common cause of severe pneumonia in foals.<sup>1,3-5</sup> The impact of this disease is

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large for several reasons, including the fact that prevalence and case-fatality rates are high. Prevalence of pneumonia attributable to infection with *R equi* can exceed 20%, and case-fatality rates as high as 80% have been reported.<sup>4,5</sup> Treatment is generally prolonged, expensive, associated with adverse effects, and often unsuccessful.<sup>4</sup> Pneumonia attributable to *R equi* may negatively impact future performance of affected foals.<sup>6</sup>

For most infectious diseases, methods for control and prevention have a greater impact on the burden of disease than does treatment of each affected animal. Methods for control and prevention of pneumonia attributable to *R equi* are limited. Currently, vaccines to prevent disease caused by *R equi* are lacking. Prevention by transfusion of hyperimmune plasma<sup>7</sup> is costly, labor intensive, and not uniformly effective.<sup>8,9</sup>

Understanding epidemiologic characteristics of the causal organism is essential to developing methods to control and prevent disease caused by infection with that organism. Reports of epidemiologic characteristics of *R equi* have generally been limited to studies of isolates obtained from foals, soil, and air (dust) from a small number of farms or studies monitoring *R equi*-specific antibodies in horses.<sup>3,5,10-15</sup> Isolates of *R equi* have been characterized serologically and on the basis of their plasmid patterns.<sup>5,16-18</sup> Although serotyping systems have been developed, they do not reflect the virulence or genetic relatedness of strains.<sup>5,16,17</sup> Restriction digestion of plasmid isolates has revealed that at least 5 subtypes of *R equi* exist, which manifest geographic differences.<sup>19</sup> Serologic groupings and examination of plasmid patterns, however, are not as discriminating as genetic fingerprinting (genotyping) techniques such as pulsed-field gel electrophoresis (PFGE) of restriction endonuclease-digested genomic DNA.<sup>20</sup>

In a few studies,<sup>21-23</sup> investigators have used PFGE to evaluate isolates of *R equi* obtained from horses. Differences in PFGE patterns among 21 European isolates of *R equi* from feces of healthy cattle and horses have been documented as well as variability within and among farms.<sup>21</sup> The PFGE analysis of *R equi* isolates obtained from Thoroughbred horse farms in Australia has been reported.<sup>22,23</sup> Analysis of those studies indicates that a large number of PFGE-defined strains of *R equi* exists, that individual farms tend to have particular strains, and that there is considerable heterogeneity in PFGE patterns among isolates. To the authors' knowledge, use of PFGE to compare a large ( $\geq 30$ ) number of isolates from various continents or from the

United States has not been reported. The purposes of the study reported here were to use PFGE to assess the genetic similarity of isolates obtained from affected foals or their environments with respect to country of origin and virulence status (ie, whether the isolate possessed a virulence-associated plasmid) and to assess the genetic similarity of isolates obtained from affected foals or their environment from farms and regions within Texas.

## Materials and Methods

**Sample population**—We obtained 290 isolates of *R equi* for use in the study. Investigators involved in the study submitted isolates to the Equine Infectious Disease Laboratory at Texas A&M University. All isolates of *R equi* used in this study were examined for virulence-associated protein antigen plasmids (VapA-P) by use of a polymerase chain reaction (PCR) technique, as described elsewhere.<sup>24</sup> Isolates that had positive results when tested by use of the PCR assay for VapA-P were classified as virulent, and those that had negative results were classified as avirulent.

Isolates were obtained by microbiologic culture of samples obtained from various sources, including tracheo-bronchial aspirates (TBAs) or pulmonary parenchyma of foals in which there was a diagnosis of pneumonia caused by *R equi* (203 isolates from 203 foals, including 198 virulent and 5 avirulent isolates); fecal (9 avirulent isolates) or soil (53 avirulent isolates) samples from horse farms that had foals affected by pneumonia attributable to *R equi* and horse farms that did not have foals affected by this disease; extrapulmonary sites of infection (17 isolates) such as joints and lymph nodes; and feces of foals affected with pneumonia or diarrhea (8 isolates [3 virulent and 5 avirulent]).

Samples were obtained from 4 countries (Argentina, Ireland, Japan, and the United States). Isolates from Argentina (28 virulent isolates) were obtained from samples from 28 pneumonic foals that had been submitted for microbiologic culture to the laboratory of 1 of the authors (TB). These isolates were from foals of 16 farms, with 10 farms each contributing only a single isolate and 2 farms contributing 2, 3, or 4 isolates, respectively. Exact year of isolation was not specified for the isolates from Argentina, but these isolates were collected between 1985 and 1999.

Isolates from Japan were from samples obtained from pneumonic foals (29 virulent isolates) of various farms that had been submitted for microbiologic culture to the laboratory of 1 of the authors (ST) or from microbiologic culture of fecal samples of individual foals (9 avirulent isolates) or soil samples (12 avirulent isolates) collected by that author from 3 horse farms in Japan. Isolates from the foals in Japan were collected during 1996 (n = 2), 1997 (5), 1998 (20), and 1999 (2); 3 of the virulent isolates from Japan were from intra-abdominal infections in foals with pneumonia. Fecal and soil samples from Japan were collected during 1997 (n = 9) and 1998 (12).

All isolates from Ireland (19 virulent isolates) were from samples obtained from pneumonic foals of various farms that had been submitted for microbiologic culture to the laboratory of 1 of the authors (DL). Isolates from Ireland were collected in 1997 (n = 4) or 2000 (15).

Isolates from the United States were from California (n = 6), Oklahoma (2), or Texas (185). Isolates from California were virulent isolates obtained in 1999 by microbiologic culture of specimens obtained from pneumonic foals of various farms (4 isolates) or extrapulmonary sources other than feces (2). Isolates from Oklahoma were virulent isolates obtained from TBAs of foals from 2 farms (1 in 1997 and 1 in 1998). Isolates from Texas were from samples obtained from clinical

specimens of foals (n = 144) or from the soil of horse farms (41). Samples were collected from foals during 1997 (n = 45), 1998 (43), 1999 (18), and 2000 (38); these foals were generally from separate farms, but there were 9 farms that contributed isolates from multiple foals. Isolates from foals in Texas included 119 virulent isolates obtained from the respiratory tract, 5 avirulent isolates obtained from the respiratory tract, 3 virulent isolates obtained from fecal samples, 5 avirulent isolates obtained from fecal samples, and 12 virulent isolates obtained from extrapulmonary sources other than feces. Soil samples were collected during 1997 (n = 32), 1998 (8), and 1999 (1) from 14 farms in Texas.

Four geographic regions were used to compare the isolates from Texas. These geographic regions were defined on the basis of the authors' knowledge of the state and distribution of the farms of origin of the isolates. The 4 regions were defined as east Texas (31 isolates), north-central Texas (57), southeast Texas (67), and west Texas (30).

**PFGE**—Bacteria were purified and grown on trypticase soy agar plates with 5% sheep blood.<sup>a</sup> Bacteria then were inoculated into 15-mL centrifuge tubes that contained 8 mL of modified brain-heart infusion broth (MBHIB). The MBHIB consisted of brain-heart infusion broth<sup>b</sup> supplemented with normal calf serum<sup>c</sup> (10%), glucose<sup>d</sup> (0.4%), and Tween 80<sup>e</sup> (0.2%). After incubation for 24 hours at 35°C with rotation achieved by use of a tissue culture rotator,<sup>f</sup> 100  $\mu$ L of each bacterial suspension was transferred into 8 mL of MBHIB in a 15-mL centrifuge tube and incubated for an additional 24 hours. The suspension was centrifuged at 1,500  $\times$  g for 5 minutes, and all but approximately 0.5 mL of supernatant was discarded. The bacterial pellet was resuspended in a 2-mL centrifuge tube and combined with 200  $\mu$ L of a 2% solution of lysozyme<sup>g</sup> and 200  $\mu$ L of a 0.1% buffered solution of lysostaphin.<sup>h</sup> Bacterial suspension (0.5 mL) and 1% agarose<sup>i</sup> (0.5 mL) was combined in a sterile 2-mL tube, then aliquots (100  $\mu$ L) of this mixture were placed in disposable plug molds<sup>j</sup> and allowed to harden. After they were polymerized, plugs were submerged in 200  $\mu$ L of a 2% solution of lysozyme and 200  $\mu$ L of 0.1% buffered lysostaphin in a sterile 2-mL tube and incubated for at least 1 hour at 37°C to cause breakdown of the cell wall and release of bacterial DNA. Plugs were then transferred to a new sterile 2-mL tube containing 1 mL of digestion buffer (2.934 g of N-laurylsarcosine,<sup>k</sup> 10 mL of distilled deionized water, and 90 mL of 0.5M EDTA) and 50  $\mu$ L of proteinase K.<sup>l</sup> Plugs were incubated in this solution for at least 3 hours in a water bath set at 50°C. Following this step, plugs were subjected to a series of 6 wash cycles in 1 mL of Tris-EDTA buffer (10 mL of 1M Tris-HCl,<sup>m</sup> 2 mL of 0.5M disodium EDTA,<sup>n</sup> 988 mL of distilled deionized water [pH, 8.0]) on ice. Wash solution was changed hourly, with the first 3 cycles incorporating 2  $\mu$ L of 0.1M phenylmethanesulfonyl fluoride<sup>o</sup> to neutralize the activity of proteinase K and preserve the restriction enzyme that was subsequently added. The final 3 wash cycles consisted only of fresh Tris-EDTA buffer. Following washing, the plugs were transferred to sterile 2-mL centrifuge tubes containing 200  $\mu$ L of 10 $\times$  restriction buffer B<sup>p</sup> and 16  $\mu$ L of the restriction enzyme, AseI.<sup>q</sup> Plugs then were incubated for at least 4 hours at 37°C.

For each gel, 10 plugs containing isolates were loaded onto a 15-well comb, with lanes 1, 8, and 14 containing *Saccharomyces cerevisiae* chromosomal DNA molecular weight standards<sup>r</sup> and lanes 2 and 15 containing  $\lambda$  DNA size standards.<sup>s</sup> Agarose<sup>t</sup> (concentration of 1.5%) with 0.5 $\times$  trizma-boric acid-EDTA (TBE) buffer (5.4 g of trizma base,<sup>u</sup> 2.75 g of boric acid,<sup>v</sup> 0.465 g of disodium EDTA,<sup>w</sup> 1 L of distilled deionized H<sub>2</sub>O) was then poured into the gel mold and allowed to harden and form a gel with plugs in place. This gel was then submerged in 2 L of 0.5 $\times$  TBE and processed by

electrophoresis, using a PFGE apparatus<sup>w</sup> with the following conditions: 33.66 hours, 6 V/cm, included angle of 120°, initial switch time of 9.14 seconds, final switch time of 154.76 seconds, and temperature of 11°C. Following electrophoresis, the gel was stained by submersion in 500 mL of a 5% solution of ethidium bromide,<sup>x</sup> destained with tap water, and photographed. Digital photographic images of the gels were stored for subsequent analysis.

**Data analysis**—Digitized gel images were processed and analyzed for similarities in PFGE banding patterns by use of commercial software.<sup>y</sup> The Dice coefficient was used to calculate similarities by pairwise comparison of PFGE banding patterns, and the unweighted pair group method, using arithmetic averages, was used to generate dendrograms.<sup>25,26</sup> Isolates were considered to be within a cluster when the index of similarity was > 80%.

Only bands  $\geq 150$  kilobases (kb) were included in the analysis. The rationale for this inclusion criterion included 2 factors. First, evaluation of our gels indicated that there was greatest diversity among bands > 150 kb. Second, we wanted to exclude differences among isolates explained on the basis of DNA of the VapA-P; because the VapA-P is < 90 kb, differences observed among isolates in our study were not attributable to plasmid DNA.

Inferential statistical methods were used to determine whether there were significant differences for comparisons among countries for virulent isolates obtained from foals; between virulent and avirulent isolates for the US isolates, the Japanese isolates, or both; and among the 4 regions of Texas for virulent isolates from foals. A similarity matrix was constructed for all isolates by use of the Dice similarity coefficient. The matrix was tested for nonrandom distributions of similarity values with respect to the 3 aforementioned groupings. Average within- and between-group similarity values were calculated for each subset. Each test consisted of a bootstrap simulation in which 1,000 randomized similarity matrices were generated by sampling the original matrix with replacement. Average within- and between-group similarity values were calculated for each group, and the results were compared to the original values for each group.<sup>27</sup> A *P* value was determined for each group by calculating the percentage of simulated matrices that contained a greater difference between average within- and between-group similarity values than the original dataset. Values of *P* < 0.05 indicated a member of that group was significantly more likely to be more similar to a member of its own group than to a member of any other group. The bootstrap method was selected to avoid assuming a particular distribution of similarity values across the matrix.

Virulent and avirulent isolates from Texas that were obtained from the same farm were compared to determine the frequency with which isolates were of identical or closely related strains, using strain definitions for PFGE described elsewhere.<sup>28</sup> Briefly, isolates with PFGE-derived banding patterns that had the same number of bands and in which the corresponding bands were of identical size were defined as indistinguishable. Isolates that were not identical but that had banding patterns with 3 or fewer different bands were considered closely related.

## Results

**Comparison of isolates among countries**—Agarose gels of isolates of *R equi* were used for the analysis (Fig 1). A dendrogram was constructed on the basis of Dice coefficients of similarity for 198 virulent isolates of pulmonary origin (ie, obtained by microbiologic culture of samples obtained from TBAs or lung tissue) from foals in the 4 countries (Table 1). Analysis of the dendrogram did not reveal distinct grouping of iso-

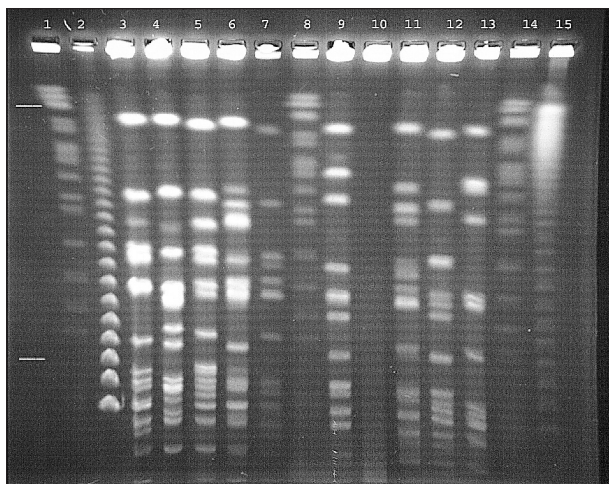


Figure 1—Ethidium bromide-stained 1% agarose gel containing isolates of *Rhodococcus equi* analyzed by use of pulsed-field gel electrophoresis, using the restriction enzyme *Asel*. Lanes 1, 8, and 14 contain  $\lambda$  DNA ladder standards that range from 225 to 2,200 kilobases (kb). Lanes 2 and 15 contain  $\lambda$  DNA ladder standards that range from 48.5 to 1,018.5 kb. The thin white horizontal bars to the left of lane 1 indicate 150 kb (lower bar) and 1,125 kb (upper bar). Analysis was restricted to bands within this range. Lanes 3, 5, 6, 7, 9, 11, 12, and 13 contain DNA from virulent isolates of *R equi* obtained from foals in the United States. Lanes 4 and 10 contain avirulent isolates of *R equi* obtained from soil samples collected in the United States.

Table 1—Country of origin and virulence status\* of 290 isolates of *Rhodococcus equi* examined by use of pulsed-field gel electrophoresis

Country	Virulent pulmonary	Virulent extrapulmonary	Avirulent	Total
Argentina	28	0	0	28
Ireland	19	0	0	19
Japan	26	3	21	50
United States	125	17	51	193
<b>Total</b>	<b>198</b>	<b>20</b>	<b>72</b>	<b>290</b>

\*Virulent organisms were those that had positive results when tested by use of a polymerase chain reaction (PCR) assay for the plasmid-encoded gene for virulence-associated protein A; avirulent organisms were those that had negative results when tested by use of the PCR assay for this gene.

lates among countries (Fig 2). Clusters (ie, index of similarity > 80%) of > 2 isolates for a given country were rare. Of the 19 virulent pulmonary isolates from Ireland, there was 1 cluster of 4 isolates, 1 cluster of 3 isolates, and 3 clusters of 2 isolates. Of the 125 virulent pulmonary isolates from the United States, there was 1 cluster of 4 isolates, 2 clusters of 3 isolates, and 19 clusters of 2 isolates. There was 1 cluster of 4 isolates and 6 clusters of 2 isolates among samples from Argentina. Isolates from Japan did not have clusters of > 2 isolates; there were 3 clusters of 2 isolates from Japan.

Isolates from Ireland were significantly (*P* < 0.001) more similar to each other than to isolates from any of the other countries (ie, within-group similarity was significantly greater than between-group similarity). Isolates from the United States were also significantly (*P* < 0.001) more similar to each other than to isolates from other countries. Within- and between-group similarities did not differ significantly for isolates from Japan or Argentina.

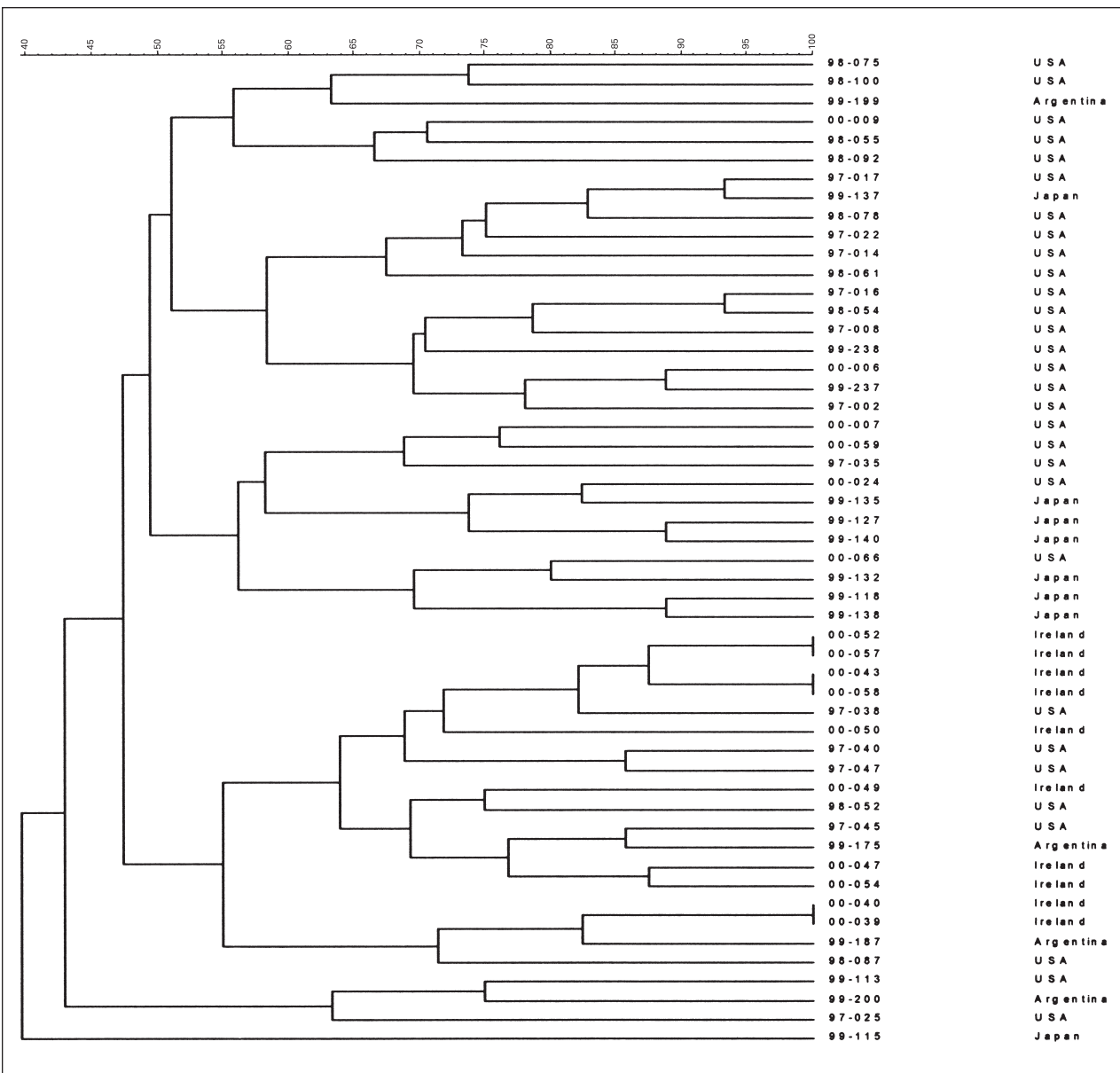


Figure 2—Representative portion of a dendrogram of 198 virulent isolates of *R equi* obtained from foals from 4 countries (Argentina, Ireland, Japan, and the United States of America [USA]). Notice the genetic heterogeneity among isolates within and among countries. Scale at the top of the figure is the index of similarity among isolates.

#### Comparison of virulent and avirulent isolates—

The 29 virulent isolates from Japan were significantly ( $P < 0.001$ ) more likely to be similar to other virulent isolates than to any of the 21 avirulent isolates (ie, within-group similarity was significantly greater than between-group similarity). However, the within-group similarity for the 21 avirulent isolates was not significantly ( $P = 0.304$ ) different from the between-group similarity. Virulent isolates from the United States ( $n = 142$ ) were significantly ( $P < 0.001$ ) more likely to be similar to other virulent isolates than to any of the 51 avirulent US isolates. The within-group similarity was greater, but not significantly so ( $P = 0.074$ ), than the between-group similarity among avirulent US isolates.

Analysis of dendrograms revealed that there was considerable heterogeneity in the PFGE patterns of vir-

ulent and avirulent isolates and that there was not a distinct grouping of virulent and avirulent isolates (ie, clusters generally did not include  $> 2$  isolates [Fig 3 and 4]). Among isolates from Japan, there were 5 clusters of 2 virulent isolates and 2 clusters of 2 avirulent isolates. Among US isolates, there was 1 cluster of 5 virulent isolates, 2 clusters of 4 virulent isolates, 2 clusters of 3 virulent isolates, 24 clusters of 2 virulent isolates, and 4 clusters of 2 avirulent isolates. Moreover, we did not detect specific bands that consistently differentiated virulent from avirulent isolates.

Isolates from Japan and the United States were combined for analysis, and comparisons were made on the basis of country and virulence status (ie, comparison of within- and between-group similarities for virulent US isolates, avirulent US isolates, virulent isolates from

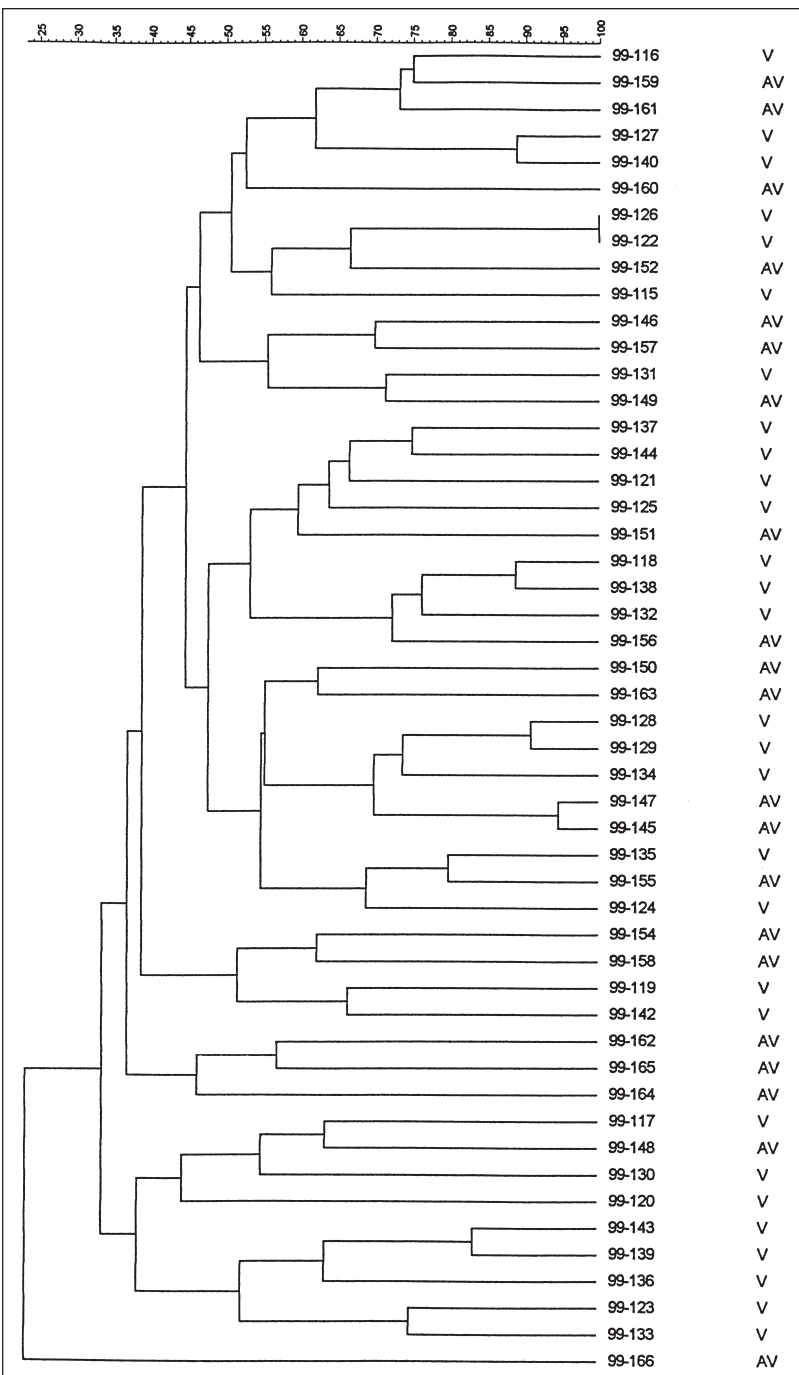


Figure 3—Dendrogram of 29 virulent (V) and 21 avirulent (AV) isolates of *R. equi* obtained from samples from Japan. Notice the genetic heterogeneity between and within V and AV groupings of isolates. Scale at the top of the figure is the index of similarity among isolates.

Japan, and avirulent isolates from Japan). Evaluation of dendrograms again revealed principally clusters of 2 isolates (30 clusters of 2 isolates [24 from virulent isolates and 6 from avirulent isolates], 6 clusters of 3 virulent isolates, and 1 cluster of 4 virulent isolates). A few clusters consisted of 2 virulent isolates from different countries or an avirulent and a virulent isolate from the same country. The within-group similarity was significantly greater than the between-group similarity for virulent

US isolates ( $P < 0.001$ ), avirulent US isolates ( $P < 0.001$ ), virulent isolates from Japan ( $P = 0.017$ ), and avirulent isolates from Japan ( $P = 0.032$ ).

**Comparison of isolates by region in Texas**—We did not detect a significant difference among regions in Texas with respect to within- and between-group similarity. Evaluation of a dendrogram did not reveal any apparent grouping of isolates on the basis of the 4 defined regions.

**Comparison of isolates from the same farm in Texas**—Multiple isolates were obtained from 9 farms (range, 3 to 10 isolates/farm; median, 5 isolates/farm). For 1 farm that contributed 5 isolates, 2 isolates appeared indistinguishable. These isolates had been obtained during 1998 from 2 affected foals by use of TBAs collected approximately 6 weeks apart. The other 3 isolates from that farm (virulent isolates from affected foals) differed by  $\geq 6$  bands. Of the isolates from any of the other farms that contributed multiple isolates, no 2 were found to be identical. Three farms had isolates that appeared to be from closely related strains within each farm. One farm that contributed 10 virulent isolates from foals had 2 isolates that were obtained by using TBAs and differed by 4 bands. One farm that contributed 6 virulent isolates from foals had a pair of isolates that differed by 4 bands. One farm that contributed 6 avirulent isolates obtained from soil samples during 1997 had 2 isolates, which were from samples collected from soils in different regions of the farm, that differed by 2 bands. Another farm that contributed 6 avirulent isolates from soil samples obtained during 1997 had 2 isolates, which were from samples collected from soils in different regions of the farm, that differed by 4 bands. In general, there was considerable heterogeneity in banding patterns among isolates from the same farm (differences of  $< 6$  bands; indices of similarity,  $< 60\%$ ). There were 2 farms (1 farm that contributed 10 samples and 1 farm that contributed 6 samples) that had samples from multiple years (ie, 1997, 1998, and 2000 for the farm that contributed 10 samples and 1997 and 2000 for the farm that contributed 6 samples). Analysis of dendrograms revealed grouping of the isolates collected during the year 2000, compared with other isolates obtained at these farms.

**Comparison of isolates from pulmonary and extrapulmonary samples from Texas**—Of the 134 vir-

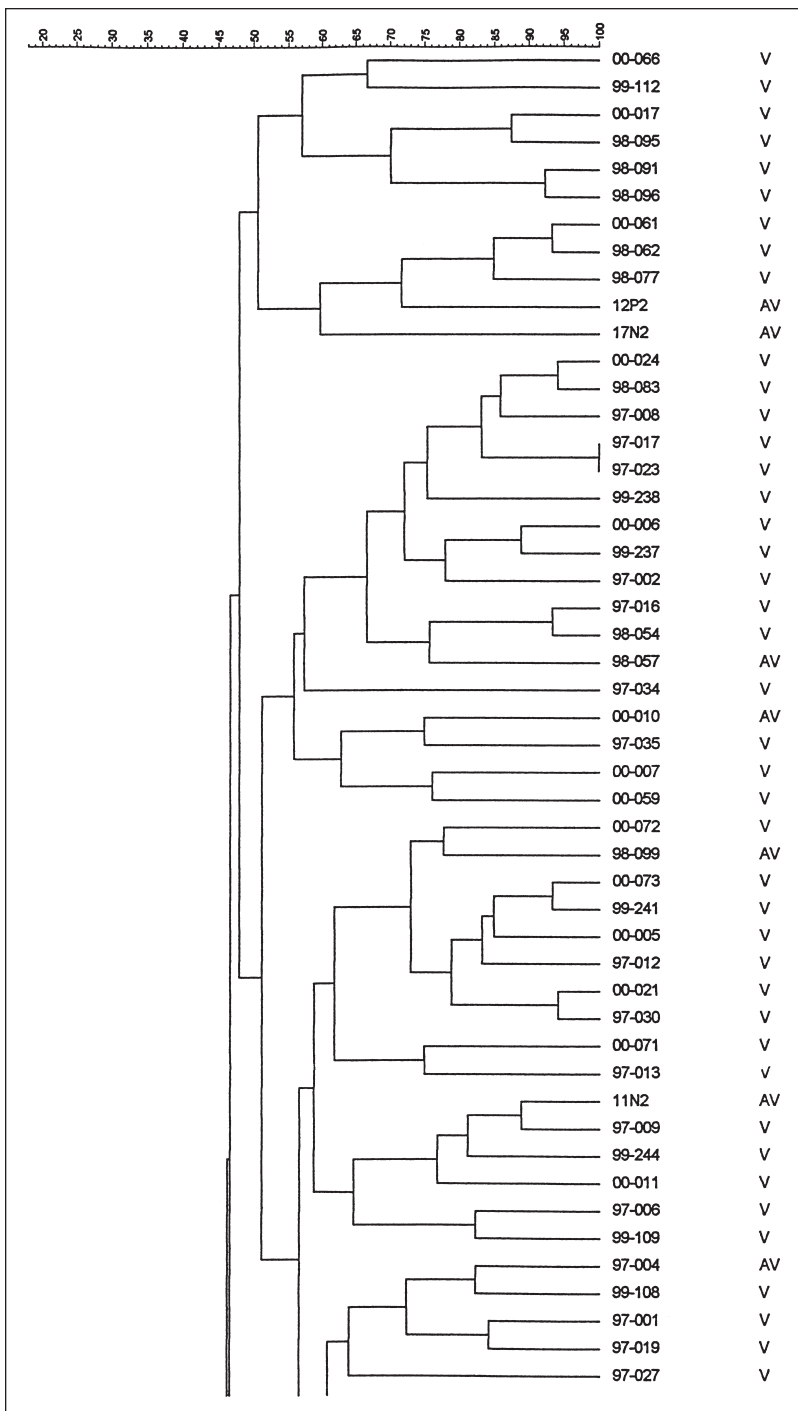


Figure 4—Representative portion of dendrogram of 142 V and 51 AV isolates of *R. equi* obtained from samples from the United States. Notice the genetic heterogeneity between and within V and AV groupings of isolates. Scale at the top of the figure is the index of similarity among isolates.

ulent isolates from Texas, 12 were of extrapulmonary origin other than feces. Sources included mesenteric lymph nodes, a vertebra with osteomyelitis, and cutaneous abscesses. Analysis of a dendrogram did not reveal apparent clustering on the basis of pulmonary or extrapulmonary sources, and the within-group similarity was not significantly different from the between-group similarity for these isolates.

geographic differences in the distribution of virulence plasmids of *R. equi* isolates from 5 countries<sup>19</sup>; that method may be useful for molecular epidemiologic studies of virulent *R. equi*.

Avirulent isolates were available from Japan and the United States. Comparisons between avirulent and virulent organisms were performed for isolates from each country and all isolates from Japan and the United

## Discussion

In the study reported here, evidence of distinct geographic clustering was not apparent from analysis of PFGE of restriction-digested genomic DNA from *R. equi*. Virulent pulmonary isolates obtained from foals from various countries were not grouped distinctly on the basis of country, as determined by analysis of a dendrogram, size of cluster (ie, isolates with coefficients of similarity  $\geq 80\%$ ) rarely exceeded 2 for isolates from the same country, and there were a number of clusters consisting of 2 isolates from different countries. Using bootstrap methods, the within-group similarity was significantly greater than the between-group similarity for isolates from Ireland and the United States; however, isolates from these 2 countries were also similar in banding patterns to isolates from other countries and were not clearly grouped separately in the dendrogram (Fig 1). These data indicated that there is considerable diversity among PFGE patterns of isolates from various continents. Evidence that transmission and spread of *R. equi* is associated with dissemination of isolates with similar PFGE patterns within a country was lacking. Although a smaller number of international isolates was examined in another study,<sup>22</sup> similar findings were reported by use of PFGE for examination of isolates from infected foals from Australia (212 isolates), Argentina (3), Brazil (1), Canada (1), Germany (6), and Japan (10). It has been suggested that the apparent genetic heterogeneity among isolates from various continents could be attributable to spread resulting from international movement of horses.<sup>22</sup> Alternatively, differences among countries may be of minimal importance relative to other factors that predispose foals to development of *R. equi* infections such as status with regard to the virulence-associated plasmid, susceptibility status of the host, and characteristics of the farm. Disease transmission and spread of *R. equi* may be most closely related to dissemination of the plasmid encoding virulence-associated protein A. Restriction fragment-length polymorphisms have been used to identify geo-

States. For both countries, some virulent isolates were more similar to each other than to avirulent isolates, as determined on the basis that within-group similarity was significantly greater than between-group similarity for virulent isolates. Virulent isolates, however, were not distinctly grouped separately from avirulent organisms by use of dendrograms (Fig 2), and there were no particular bands that differentiated virulent from avirulent isolates. Because only bands  $\geq 150$  kb were considered in this study, the differences between virulent and avirulent isolates were not attributable to VapA-P, which is  $< 90$  kb. These findings are consistent with the concept that virulence of *R equi* is principally associated with VapA-P and that virulence is largely dependent on dissemination of this plasmid. The virulent isolates (ie, those with VapA-P) were almost exclusively from respiratory tract samples obtained from foals with clinical signs of pneumonia, whereas the avirulent isolates that lacked VapA-P were obtained principally from microbiologic culture of soil samples, samples from adult horses, or samples from atypical sites of infection such as synovial fluid. The finding that there was some degree of nonrandom distribution between virulent and avirulent isolates may indicate that at least some of the plasmid-mediated effects are modulated by chromosomal genes. Because virulence is principally associated with VapA-P, it is possible that molecular epidemiologic study of this plasmid could be useful. Indeed, evidence exists that there are differences among countries from various continents in the patterns of restriction digestion fragments of VapA-P.<sup>19</sup>

We did not detect evidence that isolates were clustered in geographic regions within Texas. There was considerable heterogeneity among isolates from various regions of this large state, and isolates from the various regions were equally likely to resemble isolates from other regions as isolates from their own region. Moreover, there were numerous clusters, but cluster size was usually limited to 2 isolates. These findings indicate that there is considerable chromosomal variability among isolates from the same geographic region within the United States. These findings are similar to the genotypic diversity of *R equi* isolates from Australia.<sup>22,23</sup> Guidelines for molecular epidemiologic characterization of strains by use of PFGE indicate that strain characterization should only be applied to small sets of isolates related to potential outbreaks.<sup>28</sup> In the study reported here, isolates from the same farm met these criteria for strain characterization. Among these isolates, there did not appear to be evidence of strains of *R equi* within a specific farm or region of Texas.

Pneumonia caused by *R equi* may be endemic at some farms.<sup>10-14</sup> Theoretically, endemicity may be explained on the basis of persistence of farm-specific strains. Evidence supporting this theory was limited in the study reported here. When considering isolates from the same farm, there was only 1 instance in which isolates (a pair of isolates) were deemed indistinguishable, and only 1 other pair of isolates were considered closely related; 3 isolates were considered possibly related. The closely related isolates were obtained from soil samples collected from different areas on the farm during the same year. At another farm, 2 slightly relat-

ed isolates were temporally separated by approximately 3 years. For 2 farms for which there were isolates obtained from foals in various years, use of dendrograms revealed that isolates obtained in 2000 at each farm were grouped separately from isolates obtained in other years. These data indicate that isolates from affected farms are quite variable within and among farms, particular strains may occasionally be isolated from foals or soil at affected farms, a given strain may persist at a farm over time, and strains may change over time at a given farm. Thus, it rarely will be possible to link infections to a given site or region on the basis of analysis of isolates by use of PFGE of chromosomal DNA. *Rhodococcus equi* is ubiquitous in the environment, and there is no evidence that the organism is more likely to be isolated from soil of farms affected by pneumonia caused by this organism than from the soil of unaffected farms.<sup>24</sup> Consequently, the epidemiologic characteristics of disease caused by *R equi* may be better defined in terms of dissemination of the virulence-associated plasmid and horse- or farm-level factors that predispose horses to development of disease.

A number of limitations were evident for our study. Isolates were obtained from collaborators (convenience sampling) rather than by probability (random) sampling. Thus, these isolates cannot be considered representative of any population of isolates or foals. One important potential bias of the use of a convenience sample would be that isolates may have been more homogeneous than for a probability sample as a result of nonrandom selection. Assuming this to be true, this bias would have resulted in overestimating the relatedness of isolates. Because we observed considerable heterogeneity (ie, little genetic relatedness) of isolates, the impact of this bias on our results appears to have been limited.

Only data from bands  $\geq 150$  kb were considered in our analyses. The rationale for this criterion was that we wanted to exclude DNA from the VapA-P, we observed greater diversity in banding patterns for bands  $\geq 150$  kb, and we sometimes found it difficult to accurately identify the size of bands near the bottom of gels. Because there may have been additional useful information from bands at the bottom of gels, we repeated the analysis to consider bands between approximately 48 to 149 kb alone or in combination with data for bands  $\geq 150$  kb. Generally, the number of bands in the lower kilobase region was small. Results were not changed by including these smaller bands (data not shown). Moreover, our results are consistent with those in a study<sup>22</sup> of a large number of isolates of *R equi* from foals at farms in Australia tested by use of PFGE in which bands  $< 150$  kb were considered for analysis.

The relatively large sample size and bootstrap methods used in the study reported here provided considerable statistical power. Consequently, several distributions were found to be significantly nonrandom despite the fact that there did not appear to be a clear or distinct pattern of grouping when dendrograms were visually evaluated. The statistical methods used ignored the fact that some isolates were derived from the same farm. Failure to account for this correlation likely contributed to significance of comparisons.

The number of isolates available for comparison within farms was small, thus we had limited ability to assess within-farm similarity of isolates. Among the small number of isolates considered, however, there was little similarity in the PFGE patterns among isolates from a given farm. The number of extrapulmonary isolates also was quite small, limiting our statistical power for comparisons; however, the extrapulmonary isolates did not appear to be genetically closely related.

Year of isolation was determined for all isolates except those from Argentina. Isolates from countries other than Argentina were collected during a relatively brief period (1996 to 2000). The 28 isolates from Argentina were collected between 1985 and 1999, but year of collection for specific isolates was unknown. Because those samples were collected over a wider time frame, compared to the time frame for isolates from the 3 other countries, it is possible that the diversity among isolates from Argentina was partly attributable to temporal changes in chromosomal composition over time. Diversity of isolates from Argentina, however, did not appear to be greater than that among isolates from other countries.

Use of PFGE may not have been the best method for assessing the relatedness of the diverse isolates of *R equi* with respect to the epidemiologic goals of the study. In some circumstances, PFGE may be more discriminating and lead to less clear grouping than other methods. For example, a study<sup>29</sup> of the host specificity of vancomycin-resistant *Enterococcus faecium* strains that used PFGE and amplified fragment-length polymorphisms (AFLP) found that AFLP more clearly resolved isolates into groups on the basis of host. Although PFGE is considered the standard method for genotyping strains in outbreaks of disease in hospitals, it may be less suited for analysis of the genetic relatedness of samples in which the epidemiologic links among isolates are less clear or unknown.<sup>29</sup> Analysis of AFLP patterns may establish genetic relatedness among strains that is not observed by use of PFGE. To our knowledge, molecular epidemiologic studies of *R equi* by the use of AFLP have not been reported.

Analysis of results of the study reported here indicated that it often is not possible to use PFGE to provide a strong genotypic link among isolates on the basis of source, time point, or location. Isolates from the same farm are rarely identical. Based on results obtained by use of PFGE, it appears that a diverse array of isolates of *R equi* capable of causing disease are widely distributed.

<sup>†</sup>Trypticase soy agar with 5% sheep blood, Becton, Dickinson & Co, Franklin Lakes, NJ.

<sup>‡</sup>Brain heart infusion, Becton, Dickinson & Co (Difco/BBL), Annapolis, Md.

<sup>§</sup>Newborn calf serum, Sigma Chemical Co, St Louis, Mo.

<sup>¶</sup>D-(+)-glucose anhydrous, Sigma Chemical Co, St Louis, Mo.

<sup>¶¶</sup>Tween 80, Sigma Chemical Co, St Louis, Mo.

<sup>¶¶¶</sup>Laboratory rotator, Glas-Col Apparatus Co, Terre Haute, Ind.

<sup>¶¶¶¶</sup>Lysozyme, Sigma Chemical Co, St Louis, Mo.

<sup>¶¶¶¶¶</sup>Lysostaphin, Sigma Chemical Co, St Louis, Mo.

<sup>¶¶¶¶¶¶</sup>InCert agarose, Bio-Molecular Applications, Rockland, Me.

<sup>¶¶¶¶¶¶¶</sup>CHEF Mapper disposable plug mold, Bio-Rad Laboratories, Hercules, Calif.

<sup>¶¶¶¶¶¶¶¶</sup>N-Laurylsarcosine, Sigma Chemical Co, St Louis, Mo.

<sup>¶¶¶¶¶¶¶¶¶</sup>Proteinase K, Gibco BRL, Rockville, Md.

<sup>¶¶¶¶¶¶¶¶¶¶</sup>Trizma hydrochloride, Sigma Chemical Co, St Louis, Mo.

<sup>¶¶¶¶¶¶¶¶¶¶¶</sup>Ethylenediaminetetraacetic acid disodium salt dihydrate, Sigma Chemical Co, St Louis, Mo.

<sup>¶¶¶¶¶¶¶¶¶¶¶¶</sup>Phenylmethanesulfonyl fluoride, Sigma Chemical Co, St Louis, Mo.

<sup>¶¶¶¶¶¶¶¶¶¶¶¶¶</sup>NEBuffer 3, New England Biolabs, Beverly, Mass.

<sup>¶¶¶¶¶¶¶¶¶¶¶¶¶¶</sup>Ase I, New England Biolabs, Beverly, Mass.

<sup>¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶</sup>CHEF DNA size standard (*Saccharomyces cerevisiae*), Bio-Rad Laboratories, Hercules, Calif.

<sup>¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶</sup>CHEF DNA size standard: lambda ladder, Bio-Rad Laboratories, Hercules, Calif.

<sup>¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶</sup>Pulsed-field certified agarose, Bio-Rad Laboratories, Hercules, Calif.

<sup>¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶</sup>Trizma base, Sigma Chemical Co, St Louis, Mo.

<sup>¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶</sup>Boric acid, Sigma Chemical Co, St Louis, Mo.

<sup>¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶</sup>CHEF Mapper, Bio-Rad Laboratories, Hercules, Calif.

<sup>¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶</sup>Ethidium bromide, Sigma Chemical Co, St Louis, Mo.

<sup>¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶¶</sup>BioNumerics version 2.5, Applied Maths, St-Martens-Latem, Belgium.

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