

Prevalence of Virulent *Rhodococcus equi* in Isolates from Soil and Feces of Horses from Horse-Breeding Farms with and without Endemic Infections

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The prevalence of virulent *Rhodococcus equi* in isolates from soil and feces of foals on a farm with endemic *R. equi* infections was significantly higher than that of a farm with no history of the disease. Foals bred on a farm with the endemic disease might be constantly exposed to virulent *R. equi* in their environment.

Rhodococcus equi is a pathogen causing pneumonia or enteritis or both, characterized by severe infections and high mortality rate, in foals 1 to 3 months old (1). The natural route of *R. equi* transmission is generally regarded as inhalation and ingestion. The primary source of infection is believed to be via soil (1, 6). It is characteristic of the disease that it occurs endemically on some farms and sporadically on others and is not recognized on most (5). Attempts to analyze this aspect of the disease have been made (4, 10, 12), but a clear explanation has not been determined.

We have recently shown by immunoblotting the association of 15- to 17-kDa antigens in clinical and environmental isolates of *R. equi* with virulence in a mouse model and suggested that those antigens may be useful virulence markers for *R. equi* isolated from horses and their environment (7). The purposes of this study were twofold: (i) to compare the prevalence of virulent *R. equi* among environmental isolates collected from a farm in which *R. equi* infection is endemic as well as from a farm in which *R. equi* infection has not occurred and (ii) to verify the assumption that farms with a potential for endemic infection can be distinguished on the basis of whether virulent *R. equi* has contaminated their environment.

Two horse-breeding farms in Hokkaido and Aomori prefecture, Japan, were used in the study. Farm S was a horse-breeding farm where the disease had been a problem for several years, clinically affecting at least 10 to 15% of the foals born and one or two foals diagnosed by postmortem examination every year. Farm M had no history of the disease. Soil samples were collected from 26 sites at farm S and 9 sites at farm M from June to July 1990. The soil was collected from small paddocks used for a mare with a foal at foot, from paddocks for mares and older foals near the stables, and from the stables. The number of samples collected corresponded to the size of the farms (the number of paddocks). The soil was scraped from the surface of the ground with a small spoon and poured into sterile tubes. Fecal samples were collected from 27 foals and 25 mares on farm S and from 6 foals and 12 mares on farm M. Fecal samples were taken from freshly passed materials in the stables.

For the selective isolation of *R. equi*, nalidixic acid-novobiocin-actidione (cycloheximide)-potassium tellurite (NANAT) medium, previously described by Woolcock et al. (10), was used. One gram of feces or soil was diluted serially with a 10-fold volume of sterile saline. Each dilution was inoculated onto two plates of NANAT medium. The plates were incubated at 37°C for 2 or 3 days. All suspect colonies of *R. equi* were counted, and the number of viable organisms per gram of feces or soil was calculated (8). Two to ten colonies per specimen were subcultured and identified in our laboratory and were tested for the presence of 15- to 17-kDa antigens by immunoblotting, as described previously (7). The antigens were examined by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4.5% stacking gel and 12.5% separating gel) by the method of Laemmli (2). Whole-cell preparations contained approximately 10^7 cells per 10 μ l of sample buffer per lane. The proteins were transferred to nitrocellulose membranes (Toyo Roshi, Tokyo, Japan) at 200 mA for 1.5 h. The nitrocellulose blots were incubated with 100-fold dilutions of a naturally infected foal serum in Block Ace (a blocking agent made from milk; Yukijirushi Nyugyoku Ltd., Tokyo, Japan) at 37°C for 2 h. Blots were rinsed three times with 0.05% Tween 20 in Tris-buffered saline (pH 7.4), incubated at 37°C for 1 h with horseradish peroxidase-conjugated goat anti-horse immunoglobulin G (Cappel Laboratories, Cochranville, Pa.), and reacted with substrates.

The mean numbers of *R. equi* and the isolation rate of *R. equi* from the soil were $3.8 \times 10^3 \pm 0.9 \times 10^3$ /g and 100% (26 of 26) on farm S and $1.2 \times 10^4 \pm 0.5 \times 10^4$ /g and 100% (9 of 9) on farm M, respectively. The mean numbers of *R. equi* and the isolation rate of *R. equi* from the feces of the mares were $5.7 \times 10^2 \pm 1.5 \times 10^2$ /g and 72% (18 of 25) on farm S and $8.3 \times 10^2 \pm 3.2 \times 10^2$ /g and 92% (11 of 12) on farm M, respectively. Quantitative culture of *R. equi* from the feces of 26 foals on farm S and 6 foals on farm M was done individually from June to July 1990. The mean numbers of *R. equi* found in the feces of foals on the farms increased to 10^5 /g of feces for 4-week-old foals, held steady for 2 weeks, and gradually decreased to 10^2 /g for >12-week-old foals. There were no significant differences in the mean numbers of *R. equi* in the soil and feces of the mares and foals between the farms with and without a history of *R. equi* infection. Several researchers also reported that *R. equi* was wide-

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TABLE 1. Prevalence of virulent *R. equi* in various isolates

Farm	Soil		Feces from mares		Feces from foals	
	No. of total isolates	No. (%) of virulent isolates ^a	No. of total isolates	No. (%) of virulent isolates	No. of total isolates	No. (%) of virulent isolates
S	100	28 (28) ^b	35	5 (14) ^c	54	13 (24) ^b
M	100	5 (5)	43	7 (16)	77	3 (4)

^a Virulent *R. equi* showing 15- to 17-kDa antigens by immunoblotting.

^b $P < 0.05$, compared with results for farm M (in the same column) by the chi-square test.

^c $P > 0.05$, compared with results for farm M (in the same column) by the chi-square test.

spread in horses and their environment (1, 8, 9, 11). Therefore, it might be difficult to explain the characteristics of the disease only by the number of *R. equi* present in the horse-breeding farm environment.

We have recently shown that the 15- to 17-kDa proteins are virulence-associated antigens with immunogenicity in foals and that they may be useful in marking virulent *R. equi* contamination in the environment of a horse-breeding farm (8). The isolates were tested for the presence of the virulence marker antigens by immunoblotting. Virulent *R. equi* showing 15- to 17-kDa antigens from soil was found in 28% of the isolates from farm S and 5% of the isolates from farm M (Table 1). Virulent *R. equi* from feces of mares and foals appeared in 14 and of 24% of the isolates from farm S, and 16 and 4% of the isolates from farm M, respectively. There was a significant difference in the prevalence of virulent *R. equi* isolates from the soil and the feces of foals between farms S and M (Table 1). Mouse pathogenicity tests were done with the isolates (7). All 61 isolates showing the 15- to 17-kDa antigens (Table 1) killed mice at concentrations of 10^6 cells and were confirmed to be virulent.

To study how foals are exposed to virulent *R. equi*, three foals from farm S were used to collect fecal specimens at weekly intervals for 6 weeks. As shown in Table 2, the number of *R. equi* in the feces of foals ranged from 10^3 to 10^5 /g of feces. Ten colonies per specimen were subcultured, and the isolates were tested for the presence of the marker antigens. Virulent *R. equi* appeared in 27% of the isolates from foal 1, 50% of the isolates from foal 2, and 63% of the isolates from foal 3. During the observation period, the three foals appeared to be healthy.

The present study revealed that the environment of the horse-breeding farms with endemic *R. equi* infections was heavily contaminated with virulent *R. equi*, whereas the environment of the farm with no history of *R. equi* infection was only slightly contaminated with the virulent organism. Furthermore, foals bred on a farm with the endemic disease were exposed more frequently to virulent *R. equi* in their intestine and environment than those of a farm without the problem. The main routes of infection are inhalation and

ingestion. Susceptible foals, those whose maternal immunity wanes before development of their own immune response, readily develop disease if exposed to sufficient numbers of virulent *R. equi* (12). Susceptible foals on a farm with the endemic disease may therefore be at higher risk for the many dangers of infection than such foals located on a farm without a history of endemic disease.

Since *R. equi* was first described by Magnusson (3), soil has been suspected as a potential source of infection. Magnusson (3) observed that outbreaks of *R. equi* infection were averted sometimes by removing in-foal mares to farms on which the disease had not occurred. Prescott et al. (4) pointed out the danger of progressive development of infection in affected soil on horse farms with prolonged use, because conditions favorable for proliferation of *R. equi* are provided in the environment of horse-breeding farms. These empirical observations suggest that management and environmental circumstances have a major role to play in determining the magnitude of an infection and therefore in the prevalence of the disease. The present study suggests one more important fact to explain the characteristic of *R. equi* infection in foals: the difference in the prevalence of the disease on the farms is related not only to the number of *R. equi* in the environment but also to the prevalence of virulent *R. equi* in the environment.

Further studies are necessary to confirm this assumption and to prevent progressive contamination of virulent *R. equi* in horse-breeding farms with endemic infections.

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TABLE 2. Prevalence of virulent *R. equi* in the isolates from the feces of three healthy foals on farm S

Foal no.	CFU/g of feces	Age (wks)	No. of specimens	No. of total isolates	No. (%) of virulent isolates ^a
1	2×10^3 – 3×10^5	4–10	6	59	16 (27)
2	8×10^3 – 3×10^5	4–10	6	60	30 (50)
3	4×10^3 – 2×10^5	4–8	4	40	25 (63)

^a Virulent *R. equi* showing the 15- to 17-kDa antigens by immunoblotting.

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