Genotypic Characterization of Virulent *Rhodococcus equi* Isolated from the Environment of Hokkaido Native Horses in Hakodate, Hokkaido

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Rhodococcus equi was isolated from soil samples obtained from seven Hokkaido native horse farms in Hakodate, Hokkaido. *R. equi* was prevalent in all 59 soil samples collected from the seven farms at concentrations of between 7.5×10^2 and 7.5×10^4 colony-forming units per gram of soil. The isolates were examined for presence of the gene that encodes the virulence-associated 15–17 kDa antigen protein, *VapA*, using PCR. *VapA*-positive *R. equi* was isolated from 1–6% of isolates from three of the farms. This represents 1.4% of isolates from all farms examined (8 out of 590). The eight virulent isolates were characterized by plasmid profiling, restriction fragment length polymorphism analysis, and pulsed-field gel electrophoresis. All of the virulent isolates contained an 87-kb type II plasmid, but there were eight distinct *VspI* profiles. These results suggest that, although the Hokkaido horses of Hakodate are exposed to a variety of distinct types of virulent *R. equi*, the incidence of exposure is low or zero. An 87-kb type II plasmid was strongly associated with Hokkaido native horses as has already been observed in virulent isolates from Hokkaido native horses at Bekkai, Hokkaido.

Key words: Hokkaido, horse, plasmid, *Rhodococcus equi*, virulence

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Rhodococcus equi is one of the most harmful bacterial pathogens for 1- to 3-month-old foals. Infections caused by this organism are characterized by chronic, suppurative bronchopneumonia, which is often accompanied by ulcerative enteritis and mesenteric lymphadenitis [14, 18]. Since the first report in 1949 by Harakawa and Morita [5] of *R. equi* infection in Japanese foals in Aomori, the occurrence of the disease in the northern part of Japan, especially in Aomori and Hokkaido, has been described [7, 11, 22]. The discovery of virulence-associated antigens and virulence plasmids has elucidated the virulence of *R. equi* [18, 24, 29, 32]. Virulent *R. equi*, which is characterized by *VapA* and a virulence plasmid of 85–90 kb, has been isolated from the lesions of infected foals and can reproduce the disease under experimental conditions

[30, 34]. However, avirulent *R. equi*, which has no virulence-associated antigens or plasmids, does not cause the disease and is widely distributed in horses and their environment [19, 27]. The disease generally occurs sporadically, becoming endemic on farms that are heavily contaminated with virulent *R. equi* [14, 18, 28].

Molecular epidemiological studies on the virulence plasmids of *R. equi* have been conducted in several countries, including Japan [2, 4, 8, 9, 12, 13, 15, 16, 20, 21, 25, 26, 30, 31, 36]. Restriction-enzyme digestion patterns of virulence plasmids from foals and their environmental isolates have been used to categorize them into 12 closely related types. These studies have revealed differences between the geographic distributions of various virulence plasmids. These differences exist on a global scale and within Japan [2, 4, 8, 9, 12, 13, 15, 16, 20, 21, 25, 26, 30, 31, 36].

Hokkaido horses are popularly known as *dosanko* in

Japan. In the fifteenth century, people from mainland Japan settled in Hokkaido, bringing horses with them [6]. These horses were mainly the offspring of Nanbu horses from the Tohoku breeding district [6]. Hokkaido is now the most important district for racehorse breeding in Japan. Molecular epidemiological studies on virulent *R. equi* in Thoroughbred foals and their environment have been conducted in Hidaka, Hokkaido [19, 22], but little is known about the prevalence of virulent *R. equi* in Hokkaido native horses in Hakodate, where the horses from the Tohoku region were originally brought ashore. The purpose of this study was to quantify the prevalence of virulent *R. equi* on Hokkaido horse stud farms and to characterize it using plasmid profiling, restriction fragment length polymorphism (RFLP) analysis, and pulsed-field gel electrophoresis (PFGE).

Materials and Methods

Bacterial strains

The following bacterial reference strains of *R. equi* were used in this study: ATCC 33701 (85-kb type I), 96E35 (85-kb type II), T47-2 (85-kb type III), T43 (85-kb type IV), 222 (87-kb type I), 96B6 (87-kb type II), Brazil (87-kb type III), L1 (90-kb type I), S11-8 (90-kb type II), Kuma83-3 (90-kb type III), Kuma83-10 (90-kb type IV) and J21-2 (90-kb type V) [16, 20, 25, 26, 30, 31].

Collection and isolation of *R. equi* from soil

Fifty-nine soil samples were collected from seven farms in Hakodate, Hokkaido, as shown in Table 1. A small spoon was used to scrape soil samples from the surface of the ground and each sample was poured into

a sterile tube. One gram of the soil was serially diluted with a 10-fold volume of sterile saline. Each dilution was inoculated onto two plates of nalidixic acid-novobiocin-actidione (cycloheximide)-potassium tellurite (NANAT) medium as previously described [35]. The plates were incubated at 30°C for two or three days. The *R. equi* colonies were counted and the numbers of viable organisms per gram of soil were estimated.

Polymerase chain reaction

Suspected colonies of *R. equi* were subcultured and examined for the presence of the 15–17 kDa antigen (VapA) gene using PCR. Published sequences of the VapA gene (GenBank database accession number D212361) from the *R. equi* strain, ATCC 33701, were used as target DNA for polymerase chain reaction (PCR) amplification [17]. Primer 1 (5'-GACTCTTCACAAGACGGT-3') corresponded to the sense strand at positions 6–23, and primer 2 (5'-TAGGCGTTGTGCCAGCTA-3') corresponded to the antisense strand at positions 569–552 in the sequence of the 15–17 kDa antigen gene [17]. PCR amplification was performed as described previously [23].

Isolation of plasmid DNA

Plasmid DNA was isolated from *R. equi* by the alkaline lysis method [3] with modifications as described previously [32]. Plasmid DNA was digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Eco*T22I, and *Hind*III for estimation of plasmid size. Samples of plasmid preparations were separated on 0.7% or 1.0% agarose gels at approximately 5 V/cm for 2 hr.

Analysis of chromosomal DNA restriction pattern using pulsed field gel electrophoresis

The isolates of *R. equi* were transferred to a BHI

Table 1. Isolation of virulent *R. equi* from soil samples on Hokkaido stud farms

Farm	No. of soil samples	No. of positive cultures	Range of the number of <i>R. equi</i> per gram of soil	No. of isolates	No. of virulent isolates	Virulence plasmid type
Su	10	10	$4.4 \times 10^3 - 4.7 \times 10^4$	100	1	87 kb type II
Oo	10	10	$1.5 \times 10^3 - 1.7 \times 10^4$	100	4	87 kb type II
Do	10	10	$1.1 \times 10^3 - 2.8 \times 10^4$	100	0	–
Se	10	10	$7.5 \times 10^2 - 6.1 \times 10^3$	100	0	–
Ts1	5	5	$3.3 \times 10^3 - 6.4 \times 10^3$	50	3	87 kb type II
Ts2	6	6	$3.3 \times 10^3 - 1.1 \times 10^4$	60	0	–
Sa	8	8	$5.7 \times 10^3 - 7.5 \times 10^4$	80	0	–
Total	59	59	–	590	8	–

broth and incubated for 36 hr at 30°C in a shaking incubator. Bacterial cells were washed and resuspended in SE buffer (25 mM EDTA, 75 mM NaCl; pH 7.5). Agarose plugs were made from a 1:1 mixture of 2% low-melting-point agarose and the cell suspension. The plugs were lysed in a resuspension buffer, which contained lysozyme (20 mg/ml), for 18 hr at 37°C. The cells were then treated for 24 hr at 50°C with the same volume of a solution (50 mM Tris-HCl, 50 mM EDTA, 1% vol/vol lauroyl sarcosine; pH 9.0) containing proteinase K (300 µg/ml) and were washed three times with Tris-EDTA buffer. *VspI* (MBI Fermentas) was used for restriction endonuclease digestion in accordance with the instructions of the manufacturer. The fragments were resolved by PFGE in electrophoresis-grade agarose (1%; Pharmacia Biotech) using a CHEF-MAPPER system (Bio-Rad). The following conditions were applied: running time, 22 hr; temperature, 14°C; voltage gradient, 200 V; included angle, 120°. During the first run (7 hr), an initial pulse time of 6 s and a final pulse time of 15 s was used, and during the second run (15 hr), an initial pulse time of 23 s and a final pulse time of 40 s was used. The gels were stained with ethidium bromide (0.5 µg/ml) for 15 min, destained in distilled water, and photographed under UV light. Mid Range PFG Maker II (New England Bio Labs) was used for estimation of molecular size.

Results

Isolation of R. equi from soil samples from Hokkaido horse farms:

R. equi was cultured from soil samples collected in

Hakodate, Hokkaido, from seven farms at which the only horse breed present was the Hokkaido horse (Table 1). All 59 of the soil samples from the seven farms were positive for *R. equi*. The number of *R. equi* ranged from 7.5×10^2 to 7.5×10^4 colony-forming units per gram of soil. Ten colonies per sample were subcultured; 590 colonies were identified and tested by PCR for the gene that encodes VapA. Positive colonies were then tested for the presence of virulence plasmids. As shown in Table 1, virulent *R. equi* was present in 1–6% of isolates taken from three of the farms, representing 1.4% of all isolates (8 out of 590).

Genotyping of virulent R. equi from Hokkaido native horses

Plasmid DNA from the eight isolates was digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Eco*T22I, and *Hind*III. All of the digestion patterns were those of an 87-kb type II plasmid (Table 2). PFGE patterns of the eight isolates were compared to assess the genetic diversity of VapA-positive *R. equi* isolates containing an 87-kb type II plasmid in Hokkaido horses. PFGE yielded eight distinct *VspI* profiles (Fig. 1).

Discussion

Our study demonstrates that Hokkaido native horses in Hakodate are exposed to virulent *R. equi* with a variety of distinct PFGE types, but the presence of these is low. An 87-kb type II plasmid was strongly associated with Hokkaido native horses.

In recent studies, we showed differences in the geographic distribution of virulence-associated plasmids found in the Americas, Europe, Australia, Africa, and Japan [2, 4, 8, 9, 12, 13, 15, 16, 20, 21, 25, 26,

Table 2. Comparison of the virulence plasmid ratios of *vapA*-positive *Rhodococcus equi* isolates from Hokkaido, Aomori, Kiso, Misaki, Kagoshima, Kumamoto, and Jeju, Korea

Breed	Location	87-kb type II	90-kb type I	90-kb type II	90-kb type III	90-kb type IV	90-kb type V	Reference
Hokkaido	Hakodate	⊙	—	—	—	—	—	This study
Hokkaido	Bekkai	⊙	—	—	—	—	—	Takai <i>et al.</i> [26]
Kiso	Nagano	○	—	⊙	—	—	—	Takai <i>et al.</i> [26]
Misaki	Miyazaki	—	⊙	—	—	—	—	Takai <i>et al.</i> [26]
Thoroughbred	Hidaka,	⊙	○	—	—	—	—	Takai <i>et al.</i> [22]
Thoroughbred	Aomori	⊙	○	—	—	—	—	Takai <i>et al.</i> [22]
Thoroughbred	Kagoshima	—	⊙	—	○	○	—	Yuyama <i>et al.</i> [36]
Crossbred	Kumamoto	—	—	—	○	○	—	Takai <i>et al.</i> [25]
Cheju	Korea	—	—	○	—	—	⊙	Takai <i>et al.</i> [31]

⊙, major isolates (more than 50% of isolates); ○, minor isolates; —, not found.

Fig. 1. PFGE patterns of *Rhodococcus equi* isolates from Hakodate. Lanes: 1, Mid Range PFG Maker II; 2, isolate 11-4 (87-kb type II); 3, isolate 19-2 (87-kb type II); 4, isolate 21-3 (87-kb type II); 5, isolate 21-10 (87-kb type II); 6, isolate 22-2 (87-kb type II); 7, isolate 45-1 (87-kb type II); 8, isolate 45-5 (87-kb type II); 9, isolate 46-3 (87-kb type II).

30, 31, 36]. Most clinical isolates from the Americas, Australia, and Europe contain 85-kb type I or 87-kb type I plasmids. The 85-kb type II, III, IV, and V plasmids are unique to France, Texas, Tennessee, and Brazil, respectively [15, 20, 30, 33]. On the other hand, the 87-kb type II and 90-kb type I, II, III and IV plasmids have only been found in clinical or environmental isolates from Japan, and 90-kb type II and V plasmids have only been found in those from Korea [25, 30, 31, 36].

Surveillance studies of virulent *R. equi* have been conducted in Thoroughbred horses and native horses in Japan (Table 2). Virulent isolates from Thoroughbred horses in Hokkaido and Aomori contained an 87-kb type II or a 90-kb type I plasmid. The ratio of 87-kb type II plasmids to 90-kb type I plasmids in the clinical and environmental isolates in Hidaka was about 3 to 1. Interestingly, virulent isolates from Thoroughbred horses in Kagoshima contained a 90-kb type I, III, or IV plasmid, and most of the isolates contained a 90-kb type I plasmid. In our previous study, Japanese native horse breeds (Hokkaido, Kiso, Noma, Misaki, Tokara, Miyako, and Yonaguni) were also examined, but virulent isolates were isolated only from the Hokkaido, Kiso and Misaki breeds [26]. Hokkaido

isolates in Bekkai contained an 87-kb type II plasmid, and Kiso isolates contained an 87-kb type II plasmid and a 90-kb type II plasmid. Misaki isolates contained a 90-kb type I plasmid. These results suggest that there are differences in the geographic distribution of virulence plasmids within Japan, *viz.* that the 90-kb plasmids are dominant in the western part of Japan, and an 87-kb type II plasmid is dominant in the northern part of Japan.

The Hokkaido breed is thought to be descended from the Nanbu native breed, but the Nanbu breed is now extinct in the Tohoku region today [6]. However, as the 87-kb type II plasmid was predominant in isolates from Aomori (Table 2), it may have been introduced to the island of Hokkaido by Nanbu horses imported from the Tohoku region during the past 300 years.

There has been a report of an outbreak of *R. equi* infection in Hokkaido native horses [26]. According to a field survey conducted by local veterinarians in Hakodate, the disease was not recognized, as it had not occurred there before. The prevalence of virulent *R. equi* at the seven farms surveyed was comparatively low or absent compared with that at Thoroughbred horse-breeding farms in Hokkaido [18, 19]. Furthermore, eight isolates showed distinct PFGE patterns. An infected foal may shed a large amount of virulent *R. equi* in the feces, in which it may multiply. Contamination of the environment with monoclonal organisms of virulent *R. equi* may also occur via the lesions. Supporting this concept, PFGE analysis of clinical isolates collected in Australia demonstrated that there are different strains of *R. equi* that carry the VapA gene. These strains tended to be widespread on the farms that were examined in this study, but particular strains were associated with individual farms [10]. The present study detected eight distinct genotypic isolates of virulent *R. equi* in samples taken from three farms. This suggests that *R. equi* infection will not become a problem in Hokkaido native foals in the future. In conclusion, virulent *R. equi* organisms were introduced by native horses in the past and are presently being transferred between horse-breeding regions in Japan by Thoroughbred horses.

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