Rhodococcus equi Virulence Plasmids Recovered from Horses and Their Environment in Jeju, Korea: 90-kb Type II and a New Variant, 90-kb Type V

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ABSTRACT. *Rhodococcus equi* was isolated from fecal and soil samples from four native Jeju horse farms and six Thoroughbred farms in Jeju, Korea. The isolates were examined for the presence of virulence-associated 15–17-kDa antigens (VapA) by colony blotting, using the monoclonal antibody 10G5, and for the gene encoding VapA by PCR. *R. equi* was isolated from all 36 soil samples collected from the 10 farms with between 5.0×10^2 and 7.5×10^4 colony-forming units (cfu) per gram of soil, and from 37 of 40 fecal samples with between 5.0×10^1 and 1.1×10^5 cfu per gram of feces. Virulent *R. equi* was isolated from seven farms and appeared in 2.0% of isolates (10 of 508). Of the 10 virulent isolates, four contained a 90-kb type II plasmid, which has been found in isolates from the Kiso native horses of Japan, and the other six contained a new variant, which did not display the *Eco*RI and *Eco*T22I digestion patterns of the 10 representative plasmids already reported (85-kb types I, II, III, and IV; 87-kb types I and II; 90-kb types I, II, III, and IV). We designated the new variant as the "90-kb type V" plasmid, because its *Eco*RI digestion pattern is similar to that of the 90-kb type II plasmid. This is the first report of the prevalence of virulent *R. equi* in Jeju, Korea. The same virulence plasmid type is found in both Korean and Japanese isolates, providing insight into the origin, ancestry, and dispersal of native horses in Korea and Japan.
KEY WORDS: equine, Korea, plasmid, *Rhodococcus equi*, virulence.

Rhodococcus equi is a facultative, intracellular, Gram-positive coccobacillus that causes chronic, suppurative bronchopneumonia and enteritis, and is associated with high mortality in one- to three-month-old foals [1, 11, 25]. With a worldwide distribution, the disease generally occurs sporadically, becoming endemic on some farms [11, 14, 25]. Recent studies of its epidemiology and experimental infections in foals have shown that only virulent strains of *R. equi*, which express a 15–17-kDa virulence-associated protein antigen (VapA) and harbor a virulence plasmid of 85–90-kb, can cause the disease in foals, and that farms with endemic disease are more contaminated with virulent *R. equi* than those without the disease [15, 20, 24, 26, 29].

More recently, restriction-enzyme digestion patterns of virulence plasmids in foals and their environmental isolates from several foreign countries, including Japan, have been examined. These patterns have categorized the plasmids of virulent isolates into 10 closely related types [4, 8, 12, 22, 23, 27]. These studies revealed geographic differences in the distributions of the virulence plasmids, not only throughout the world, but also in Japan [31].

In Korea, the major horse-breeding region is Jeju Island. In Jeju, there are two horse breeds, Thoroughbreds and native Jeju horses. However, little is known about the prevalence of virulent *R. equi* in either the native Jeju horses or the Thoroughbreds. The purpose of this study was to identify the presence of virulent *R. equi* on Jeju Island and to compare the plasmid profiles of virulent isolates with those of Japanese isolates.

MATERIALS AND METHODS

Bacterial strains: The bacterial strains used in this study were the reference strains of *R. equi* 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), L1 (90-kb type I), S11–8 (90-kb type II), Kuma83–3 (90-kb type III), and Kuma83–10 (90-kb type IV) [16, 22, 23, 27].

Collection and isolation of R. equi from soil: Thirty-six soil samples and 40 fecal samples were collected from four native Jeju horse farms (Jaebangl, JaebanglI, Jeju, and Shin-jeju) and from six Thoroughbred horse farms (Hala, Keu-mak, Seongeub, Halim, Jungang, and OK) on Jeju Island in November 2002. Soil was scraped from the ground surface with a small spoon and poured into sterile tubes. Fecal samples were collected from the paddocks and poured into sterile bags. One gram of soil or feces was serially diluted 10-fold with sterile saline. Each dilution was inoculated onto two plates of nalidixic acid-novobiocin-actidione (cycloheximide)-potassium tellurite (NANAT) medium, as described by Woolcock *et al.* [30]. The plates were incubated at 30°C for two or three days. *Rhodococcus equi* colonies were counted, and the number of viable organisms per gram of soil was calculated. Three to 10 colonies of *R. equi* per specimen were subcultured and examined for VapA by colony blot enzyme-linked immunosorbent assay with the monoclonal antibody 10G5 [18]. For colony blot analysis, bacterial strains were injected onto brain-heart-infusion agar plates with an inoculation needle and incubated at 38°C for 24 hr. Nitrocellulose filters (pore size 0.45 mm, BAS 85;

Schleicher & Schuell, Dassel, Germany) were then placed over the cultures for a few minutes to wet them completely. The membrane was removed, air-dried, and treated by autoclaving at 105°C for 1 min. All buffers, antibodies and conjugate dilutions used for immunoblotting were as described previously [21].

Isolation of plasmid DNA: Plasmid DNA was isolated from *R. equi* by the alkaline lysis method [3], with some modifications, as described previously [28]. Plasmid DNAs were analyzed by digestion with restriction endonucleases *Bam*HI, *Eco*RI, *Eco*T22I, and *Hin*dIII for detailed comparison and estimation of plasmid sizes. Samples of the plasmid preparations were separated on 0.7% or 1.0% agarose gels at approximately 5 V/cm for 2 hr.

PCR: The target DNA for PCR amplification was the published sequence of the VapA gene of the virulence plasmid (GeneBank accession number D21236l) from *R. equi*, ATCC 33701 [13]. Primer 1 (5'-GACTCTTCACAA-GACGGT-3') corresponded to the sense strand at nucleotides 6 to 23, and primer 2 (5'-TAGGCGTTGTGCCAGC TA-3') corresponded to the antisense strand at nucleotides 569 to 552 in the sequence of the VapA gene [19].

PCR amplification was performed with 10 μl of DNA preparation in a 50-μl reaction volume containing 10 mM Tris-HCl (pH 8.3 at 25°C), 50 mM KCl, 1.5 mM MgCl$_2$, 0.2 mM (each) deoxynucleotide triphosphates, 1 mM each primer, and 2.5 U *Taq* DNA polymerase (Takara, Tokyo, Japan). The samples were subjected to 30 cycles of amplification in a GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT, U.S.A.). The cycling conditions were as follows: denaturation, 90 s at 94°C; primer annealing, 60 s at 55°C; and extension, 2 min at 72°C [19].

Southern hybridization: The VapA gene was detected in fragments of plasmid DNA by Southern hybridization [19]. Digested fragments of plasmid DNA were transferred from gels to a sheet of nylon membrane (Hybond N; Amersham

Japan Corp., Tokyo, Japan) using a vacuum transfer method with VacuGene (Pharmacia-LKB Biotechnology AB, Uppsala, Sweden), without depurination, according to the manufacturer's instructions. Hybridization was carried out at 68°C for at least 6 hr in $5 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl, 0.015 M sodium citrate [pH 7.0]) containing 0.1% sodium N-lauroylsarcosine, 0.02% SDS, and 1% blocking reagent. After hybridization, the membranes were washed twice at room temperature for 5 min in $2 \times$ SSC containing 0.1% SDS, and then twice again for 15 min at 68°C in $0.1 \times$ SSC containing 0.1% SDS. Immunological detection of hybridized digoxigenin-labeled probe was performed with a commercial kit (Boehringer-Mannheim-Yamanouchi, Tokyo, Japan). For Southern analysis, a digoxigenin-11-dUTP-labeled probe was amplified with the primers by PCR, as described above [19].

RESULTS

Isolation of R. equi from soil and fecal samples from native Jeju horse farms and Thoroughbred horse farms: *Rhodococcus equi* was cultured quantitatively from the soil and fecal samples collected from four native Jeju horse farms and six Thoroughbred horse farms on Jeju Island, Korea (Table 1). Soil samples and all but two fecal samples from the 10 farms were positive for *R. equi*. The numbers of *R. equi* recovered ranged from 5.0×10^2 to 7.5×10^4 colony-forming units (cfu) per gram of soil, and from 5.0×10^1 to 1.1×10^5 cfu per gram of feces. One to 10 colonies per sample were subcultured; 508 colonies were identified and tested for the presence of VapA antigens by colony blotting and for the gene encoding VapA by PCR. Positive colonies were then tested for the presence of virulence plasmids. As shown in Table 2, virulent *R. equi* was isolated from seven farms and appeared in 2.0% of samples (10 of 508).

Characterization of plasmid types in isolates of virulent

Table 1. Isolation of *Rhodococcus equi* from feces of native Jeju horses and Thoroughbred horses and from soil collected from their farms

Breed	Specimen	No. of specimens	No. of positive culture (%)	Number[a] of *R. equi*/g of soil (range)
Native Jeju	Feces	20	20 (100)	$1.0 \times 10^3 - 7.2 \times 10^4$
	Soil	18	18 (100)	$1.0 \times 10^3 - 4.3 \times 10^4$
Thoroughbred	Feces	20	17 (85)	$5.0 \times 10^1 - 1.1 \times 10^5$
	Soil	18	18 (100)	$5.0 \times 10^2 - 7.5 \times 10^4$

a) Colony-forming units.

Table 2. Detection of *vapA* by PCR in *Rhodococcus equi* isolates and their plasmid profiles

Breed	Specimen	No. of isolates	No. of *vapA* positive (%)	Plasmid type	
				90-kb type II	90-kb type V[a]
Native Jeju	Feces	170	0	–	–
	Soil	138	1 (0.7)	–	1
Thoroughbred	Feces	97	3 (3.1)	1	2
	Soil	103	6 (5.8)	3	3

a) New type.

Table 3. Comparison of the virulence plasmid ratios in clinical and environmental *vapA*-positive *Rhodococcus equi* isolates from Jeju, Korea, and Hokkaido, Kiso, Misaki, and Kagoshima, Japan

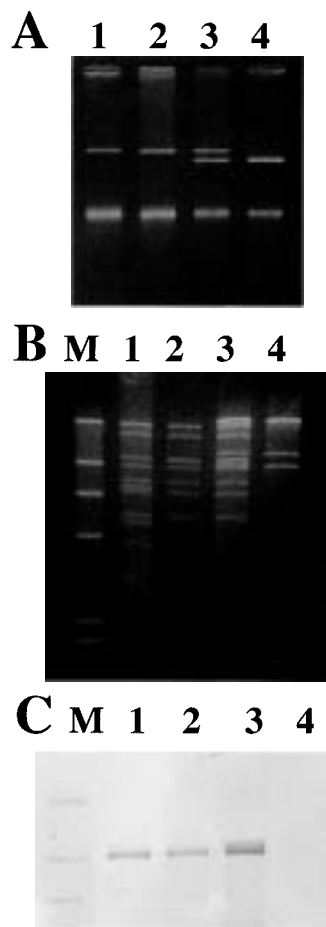| Native horse	Location	87-kb type II	90-kb type I	90-kb type II	90-kb type III	90-kb type IV	New type 90-kb type V	Reference
Jeju	Jeju, Korea	–	–	+	–	–	+	This study
Hokkaido	Hokkaido, Japan	+	–	–	–	–	–	Takai *et al.* [23]
Kiso	Nagano, Japan	+	–	+	–	–	–	Takai *et al.* [23]
Misaki	Miyazaki, Japan	–	+	–	–	–	–	Takai *et al.* [23]
Thoroughbred	Kagoshima, Japan	–	+	–	+	+	–	Yuyama *et al.* [31]

Fig. 1. Plasmid profiles of *Rhodococcus equi* isolates from Jeju, Korea (A). Plasmid DNA was extracted, separated on a 0.7% agarose gel, and stained with ethidium bromide. Lanes: 1, strain S11-8 (90-kb type II); 2, isolate J21–2 (90-kb type V); 3, isolate J10–2 (90-kb type V, and cryptic plasmid); 4, a plasmid-cured derivative of isolate J10–2 (cryptic plasmid). (B) *Eco*RI restriction fragments of the plasmids of *R. equi*. The digestion patterns of the plasmids correspond to the gel stained with ethidium bromide in panel A. (C) Southern hybridization of digoxigenin-labeled PCR *vapA* product used to probe the *Eco*RI restriction fragments of the plasmids. The Southern blot of the gel Marker

R. equi from Jeju: Plasmid DNAs were extracted from the 10 isolates, and four of the 10 isolates contained two different plasmids: a virulence plasmid and a cryptic plasmid. The other six contained one virulence plasmid (Fig. 1A). The plasmid DNAs from these six isolates were digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Eco*T22I, and *Hin*dIII. The digestion patterns that resulted grouped the plasmids into two closely related types (which included four and two isolates; some results are shown in Fig. 1B). The digestion patterns were then compared with 10 representative virulence plasmid types (85-kb types I, II, III, and IV; 87-kb types I and II; and 90-kb types I, II, III, and IV), which have already been reported [16, 22, 27, 31]. As shown in Fig. 1B and Table 2, the *Eco*RI digestion pattern of a 90-kb type II plasmid matched one of the two types. None of the *Eco*RI or *Eco*T22I digestion patterns of the 10 representative plasmid types matched the other plasmid type found in the two isolates (Fig. 1B). Plasmid DNA of the new plasmid type and the 10 representative types digested with *Eco*RI were examined by Southern analysis with PCR probes (Fig. 1C). The PCR products labeled with digoxigenin-11-dUTP hybridized with one of the fragments of each plasmid DNA. From these results, we designated the new plasmid the "90-kb type V" plasmid, because its *Eco*RI digestion pattern was very similar to that of the 90-kb type II plasmid (Fig. 1B).

To obtain a cryptic-plasmid-cured derivative, one of the four isolates (isolate J10–2) that contained two plasmids was cultured and passaged 20 times at 38°C in broth. Every five passages, 10 individual colonies of each culture were isolated and screened for the presence of plasmid DNA by agarose gel electrophoresis. A mutant lacking the virulence plasmid was isolated, but at this time, no mutant lacking the cryptic plasmid has been isolated (Fig. 1A). The *Eco*RI digestion patterns of the plasmid DNA from isolate J10–2 and its virulence-plasmid-cured derivative were compared with that of the 90-kb type V plasmid (Fig. 1B). The *Eco*RI pattern of the plasmid DNA from isolate 10–2 was found to

corresponds to the gel stained with ethidium bromide in panel A. "Markers" indicates *Hin*dIII digestion products of bacteriophage lambda DNA.

be a mixture of the 90-kb type II plasmid and the cryptic plasmid from a mutant of isolate J10–2. Therefore, the four isolates containing the two plasmids were shown to harbor the 90-kb type V plasmid.

DISCUSSION

The present study demonstrates that the environments of the native Jeju horse farms and the Thoroughbred horse farms on Jeju Island, Korea, are contaminated with virulent *R. equi.* Two distinct plasmid types were found in their isolates: one was a 90-kb type II plasmid, which has been found in isolates from the native Kiso horses of Japan [23], and a new variant, the 90-kb type V plasmid. Therefore, at this time there are at least 11 distinct though closely related plasmids present in isolates from horses throughout the world. This is the first report of the prevalence of virulent *R. equi* in Korean horses. We also report that the same plasmid type occurs in both Korean and Japanese isolates, suggesting a common origin, ancestry, and dispersal of the native horses of Korea and Japan.

In our recent studies, we have demonstrated geographic differences in the distributions of the virulence-associated plasmids found in the Americas, Europe, Australia, Africa, and Japan [2, 6, 7, 10, 15–17, 22, 23, 27, 31]. 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 plasmid is found only in French isolates and the 85-kb type III and type IV plasmids only in isolates from Texas [12, 16, 27]. These five types of virulence plasmids have never been isolated from Japanese samples. On the other hand, the 87-kb type II and 90-kb type I, II, III, and IV plasmids have been found only in clinical and environmental isolates from Japan [22, 27, 31]. More recently, a surveillance study of virulent *R. equi* was conducted in seven Japanese native horse breeds (Hokkaido, Kiso, Noma, Misaki, Tokara, Miyako, and Yonaguni), and virulent *R. equi* was isolated from the fecal samples from Hokkaido, Kiso, and Misaki horses [23]. Virulent isolates from the Hokkaido horses contained an 87-kb type II plasmid. Virulent isolates from the Kiso horses, which are bred in Nagano, central Honshu, contained an 87-kb type II plasmid or a 90-kb type II plasmid. The 90-kb type II plasmid was not found in isolates from the other native horses or from Thoroughbred horses [23]. Isolates from the Misaki horses, which are bred in Miyazaki on Kyushu Island, contained a 90-kb type I plasmid [23, 31]. Isolates from crossbred and Thoroughbred foals in Kumamoto and Kagoshima contained either a 90-kb type III plasmid or a 90-kb type IV plasmid. These results suggest that these virulent *R. equi*, which contain five distinct plasmid types, have been established differently in Japan in accordance with the dispersal of native horses, over a long period.

The native horses raised in northeast Asia—that is, in Korea and Japan—are considered to have originated predominantly from the Mongolian horse [9]. The breeding history of the native Jeju horses of Korea supports this. It is reasonable to consider that the Mongolian-type horses were imported for the first time into Japan in the Kofun period (in the fifth to sixth century) through the Korean peninsula. Recently, using genetic characterization, Jung *et al.* [5] demonstrated that horses inhabited Jeju Island before the Mongolian invasion of the 13th century. In the present study, the same plasmid type was found in isolates from both Jeju Island, Korea, and Kiso, Japan. This finding also supports the above hypothesis of a common origin and ancestry of the native horses of northeast Asia. At present, a molecular epidemiological study of *R. equi* in China and Mongolia is in progress.

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