Isolation of Virulent and Intermediately Virulent *Rhodococcus equi* from Soil and Sand on Parks and Yards in Japan

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**ABSTRACT.** *Rhodococcus equi* is an emerging opportunistic pathogen of human immunodeficiency virus-infected patients. However, little is known about the distribution of virulent and intermediately virulent *R. equi* in human environment. In the present study, *R. equi* was isolated from 173 of 234 (73.9%) samples collected from soil and sand on 115 parks and 49 yards in Japan. The numbers of *R. equi* from soil and sand ranged from 2.5 × 10^2 to 1.2 × 10^5 per gram of sample. None of the isolates from these samples showed virulence-associated 15- to 17-kDa antigens and a 20-kDa antigen. These results suggest that avirulent *R. equi* is widespread in parks and yards, but the human environment has not been contaminated with virulent and intermediately virulent *R. equi* strains yet. — KEY WORDS: *Rhodococcus equi*, soil.


*Rhodococcus equi* is a facultative, intracellular, Gram-positive cocccobacillus that causes chronic, suppurative bronchopneumonia and enteritis and is associated with high mortality in 1- to 3-month-old foals [1, 9]. Recently, *R. equi* has emerged as an important pulmonary pathogen among immunosuppressed patients, especially those with HIV infection [3–5, 6, 7, 10–12, 26, 27]. We have recently revealed that there are at least two different virulence levels in *R. equi*: virulent *R. equi* needs 10^6 bacteria for lethality in mice and intermediately virulent *R. equi* needs 10^3 bacteria for lethality in mice [16]. Virulent *R. equi* showing 15- to 17-kDa antigens contains either an 85- or a 90-kb plasmid [24], and intermediately virulent *R. equi* contains one of four distinct large plasmids of 79 to 100 kb in size [16]. The majority of the isolates from patients with AIDS were virulent and of intermediate virulence [16].

Infection through contact with farm animals and manure was reported in about one-third of the human cases [3, 5, 7]. *R. equi* is a soil organism that is ingested into the gut of many herbivores and is widespread in their environment [1, 9, 23]. Virulent *R. equi* showing 15- to 17-kDa antigens has been isolated frequently from horses and soil at horse-breeding farms [13, 18, 19], and transmission from soil to animals to human patients has been reported [3, 5, 7]. However, the route of infection in the two-thirds of the human cases is unclear [4, 8–10, 26, 27], and the source of intermediately virulent *R. equi* showing the 20-kDa antigen has not been revealed yet.

The purpose of this study was to investigate the source of virulent *R. equi* from the human environment as possible sources of human infection. In the present study, we isolated *R. equi* from soil and sand from parks and yards in Japan, and investigated the presence of virulence-associated antigens and plasmids.

One hundred and forty one soil samples and 93 sand samples were collected from one to two sites from 115 parks and 49 yards at 41 prefectures in Japan from January to August, 1993. The soil or sand was scraped from the surface of the ground or sand box with a small spoon and poured into sterile bags. For the selective isolation of *R. equi*, nalidixic acid-novobiocin-actidione (cycloheximide)-potassium tellurite (NANAT) medium, previously described by Woolcock et al. [28], was used. One gram of soil or sand 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 30°C for 2 or 3 days. All *R. equi* suspected colonies were counted and the number of viable organisms per gram of soil or sand was calculated. One to ten colonies per specimen were subcultured and identified in our laboratory as described previously [17] and then tested for the presence of 15- to 17-kDa antigens or 20-kDa antigen by colony blotting and Western blotting with a infected foal serum and monoclonal antibodies [14, 16, 18], and for the presence of virulence plasmids by agarose gel electrophoresis. Strains ATCC 33701, L1 and strain 5 were used as reference strains because some of their plasmid and protein profiles have already been described [16, 24].

Plasmid DNA was isolated from *R. equi* by an alkaline lysis method [2], with some modifications, as described previously [22]. Samples of the plasmid preparations were separated along with the plasmids of *R. equi* ATCC 33701 (pREAT701) and L1 (pREATL1) in 0.7% agarose gels at approximately 5 V/cm for 2 hr. The oligonucleotide primers used were selected from the sequence of the 15- to 17-kDa virulence-associated antigens of *R. equi* as described before [6, 11]. A 564-bp fragment was amplified from the plasmid DNA preparations with primers 1 and 2 as described previously [15].

Results of quantitative culture of *R. equi* from soil and sand samples collected in 164 parks and yards are shown in Table 1. The numbers of *R. equi* from 102 soil samples and 71 sand samples ranged from 2.5 × 10^2 to 1.2 × 10^5 per gram of soil and 5.0 × 10^1 to 4.3 × 10^5 per gram of sand, respectively. The mean numbers of *R. equi* from the soil and sand on the parks and yards examined were almost the same as those from horse-breeding farms [13, 18]. However, the isolation rates of *R. equi* from the soil and sand were slightly lower than those from horse-breeding farms [13, 18].
The 1,294 \textit{R. equi} isolates (723 isolates from the soil samples and 571 isolates from the sand samples) were then tested for the presence of 15- to 17-kDa antigens and 20-kDa antigen by colony blot enzyme-linked immunosorbent assay and Western immunoblotting with monoclonal antibodies against these antigens (some of the results are shown in Fig. 1). Neither of the 723 isolates from soil nor the 571 isolates from sand showed positive signals (Table 2).

To investigate the prevalence of cryptic plasmids in these environmental isolates, all isolates were then analyzed for the presence of plasmid DNA by agarose gel electrophoresis. Cryptic plasmids of various sizes were found in 239 (33.1\%) of the 723 isolates from soil and 168 (29.4\%) of the 571 isolates from sand (Table 2 and some of the results are shown in Fig. 2). Although some of the cryptic plasmids were very similar in size to the virulence plasmids of \textit{R. equi}, these isolates gave the same negative results by PCR as shown by immunoblotting (data not shown).

The present study revealed that \textit{R. equi} is widespread in human environment such as soil and sand in parks and yards. However, none of the isolates from the soil and sand samples examined in this study showed virulence-associated 15- to 17-kDa and 20-kDa antigens. These results suggest that human environment such as parks and yards has not been contaminated with virulent \textit{R. equi} strains yet.

Virulence markers, such as virulence-associated antigens and virulence plasmids, have led to advance epidemiological and ecological studies on \textit{R. equi} infection in foals [13, 17, 18, 20, 22, 24]. \textit{R. equi} is well-known to be widespread in the environment of domestic animals, especially in the horse-breeding farms [1, 9, 22, 23]. Natural infections in foals are caused principally by virulent \textit{R. equi} but not by avirulent organisms [12, 24]. The virulent \textit{R. equi} showing 15- to 17-kDa antigens was frequently isolated from feces of horses and soil at horse-breeding farms with the problem of \textit{R. equi} infection in foals [13, 19, 22]. The incidence of infection depends on the magnitude of contamination of horses and their environment with virulent \textit{R. equi} [13, 19, 22]. Infection through contact with horses was reported in some of the human cases [3, 5, 7]. We are now investigating the source of intermittently virulent \textit{R. equi} with the 20-kDa antigen from domestic animals except horses as possible source of human infection.

Cryptic plasmids of various sizes were previously detected in isolates from horses and their environment with low frequency (<5\%) [15]. This frequency was found to be much lower than that of the present study. Therefore some of the cryptic plasmids might have phenotypic characteristics of clinical importance [25]. Further studies will be done characterize these plasmids by physical and genetic means.

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REFERENCES

Fig. 1. Immunoblot profiles of R. equi isolates from soil and sand at parks and yards. Whole-cell preparations were analyzed by immunoblotting with a infected foal serum (A), monoclonal antibodies against 15- to 17-kDa antigens (B) and a 20-kDa antigen (C). The arrows on the right indicate 15- to 17-kDa antigens and a 20-kDa antigen. Lanes: 1, isolate 11114; 2, isolate 12111; 3, isolate 12115; 4, isolate 12419; 5, isolate 12628; 6, isolate 12712; 7, isolate 20264; 8, isolate 30041; 9, ATCC 33701; 10, strain 5. The markers (lanes M) in the panels A to C are phosphorylase b (Mr, 106,000), bovine serum albumin (Mr, 80,000), ovalbumin (Mr, 49,000), carbonic anhydrase (Mr, 32,500), soybean trypsin inhibitor (Mr, 27,500), and lysozyme (Mr, 18,500).


