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Identification of Intermediately Virulent *Rhodococcus equi* Isolates from Pigs

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We recently reported the existence of *Rhodococcus equi* isolates with at least three virulence levels, isolated from AIDS patients: virulent *R. equi* having 15- to 17-kDa antigens that kills mice with 10^6 cells, intermediately virulent *R. equi* having a 20-kDa antigen that kills mice with 10^7 cells, and avirulent *R. equi* that does not kill mice with 10^8 cells or more (S. Takai, Y. Imai, N. Fukumaga, Y. Uchida, K. Kamisawa, Y. Sasaki, S. Tsubaki, and T. Sekizaki, J. Infect. Dis. 172:1306–1311, 1995). Virulent *R. equi* having the 15- to 17-kDa antigens has been isolated frequently from horses and their environment, but the source of intermediately virulent *R. equi* having the 20-kDa antigen is poorly understood. There are many reports of the isolation of *R. equi* from the lymph nodes of pigs with and without lesions resembling those of tuberculosis. Therefore, we analyzed antigens of *R. equi* isolates from the submaxillary lymph nodes of pigs by immunoblotting with monoclonal antibodies against these virulence-associated antigens. Immunoblots of whole-cell antigen preparations of *R. equi* pig isolates revealed the presence of the 20-kDa antigen in almost all the pig isolates studied, and these isolates were intermediately virulent for mice. We also demonstrated that the expression of the 20-kDa antigen and its pathogenicity in mice were associated strongly with the presence of five large, distinct plasmids of 79 to 95 kb; two of the five plasmids from pig isolates were the same sizes as those from human isolates. These results suggest that *R. equi* having the 20-kDa antigen exists in the submaxillary lymph nodes of pigs and that the source of infection in some human cases might be associated with pigs and their environment.

Rhodococcus equi is a facultative, intracellular, gram-positive coccobacillus that causes chronic, suppurative bronchopneumonia and enteritis and is associated with a high mortality rate in 1- to 3-month-old foals (1, 13, 38). Recently, *R. equi* has emerged as an important pulmonary pathogen among immunosuppressed patients, especially those with human immunodeficiency virus infection (4, 6, 7, 11–13, 15, 27, 34, 35). We have recently reported that the 15- to 17-kDa antigens of *R. equi* are associated with virulence in foals and mice (10^6 bacteria needed for lethality) (23, 24, 28) and that the presence of large plasmids (85- and 90-kb virulence plasmids) is essential for virulence and for expression of the 15- to 17-kDa antigens (8, 16, 21, 24, 28, 31, 32). More recently, we reported that some isolates from AIDS patients were found by immunoblotting to contain a 20-kDa antigen and were intermediately virulent (10^7 bacteria needed for lethality) in mice (22). Moreover, these isolates contained one of four distinct large plasmids of 79 to 100 kb (22).

The route of infection in human cases remains obscure. Contact with farm animals and manure was reported in about one-third of the human cases (4, 7, 11). *R. equi* is a soil organism that is ingested into the guts of many herbivores and is widespread in their environment (1, 13, 30). Virulent *R. equi* with the 15- to 17-kDa antigens has been isolated frequently from horses and soil at horse-breeding farms (18, 25, 26), and transmission from soil or animals to human patients has been reported (4, 7, 11). However, *R. equi* with the 20-kDa antigen has not been isolated from horses and their environment so far (21, 33). *R. equi* has been isolated from the submaxillary lymph nodes of pigs with and without lesions resembling those of tuberculosis lesions (1, 3, 5, 9, 10, 14, 29, 30, 36), but the causative role of *R. equi* in granulomatous lymphadenitis in swine remains unclear.

The purpose of this study was to investigate the source of intermediately virulent *R. equi* with the 20-kDa antigen from domestic animals other than horses. We isolated *R. equi* having the 20-kDa antigen from the submaxillary lymph nodes of pigs and demonstrated that the expression of the antigen and its pathogenicity in mice are strongly associated with the presence of five large, distinct plasmids.

Strains ATCC 33701 and L1 (horse origin), strain ATCC 33704 (pig origin), and strains 2, 5, 11, and 39 (human origin) were used as reference strains because for some the protein profiles, plasmid characteristics, and virulence levels have already been described (22, 28, 31, 33).

For selective isolation of *R. equi*, NANAT medium was used with the following modifications (37). The medium, consisting of nutrient agar supplemented with yeast extract and Bacto Peptone, was modified by the addition of nalidixic acid (20 μg/ml), novobiocin (2.5 μg/ml), and potassium tellurite (5 μg/ml) and was termed modified NANT agar.

Submaxillary lymph nodes were removed from freshly slaughtered pigs at the slaughterhouse in Aomori Prefecture and placed in a sterile dish for transport to the laboratory. The lymph nodes were immersed in boiling water for 3 s prior to being cut up finely with sterile scissors, and the pieces were placed onto a modified-NANT agar plate and incubated for 2 to 3 days at 30°C. All suspected colonies of *R. equi* were counted, and 2 to 10 colonies per specimen were subcultured and then identified in our laboratory.

Whole-cell antigens, which were prepared by harvesting bac-

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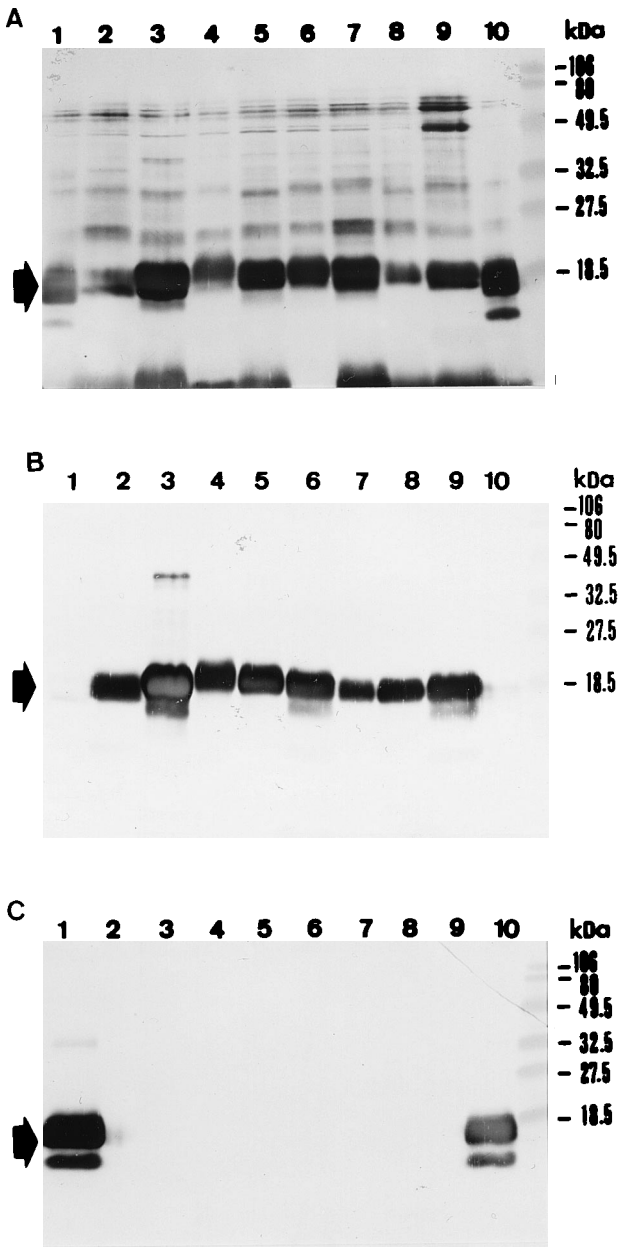

FIG. 1. Immunoblot profiles of pig isolates. Whole-cell antigens were analyzed by immunoblotting with serum from a naturally infected foal (A), a monoclonal antibody against the 20-kDa antigen (B), and a monoclonal antibody against the 15- to 17-kDa antigens (C). Lanes (plasmid sizes in parentheses): 1, isolate PL308 (90 kb); 2, isolate PL57 (95 kb); 3, isolate PL1011 (89 kb); 4, isolate PL28 (88.5 kb); 5, isolate PL887 (88 kb); 6, isolate PL459 (79 kb); 7, strain 5 (isolate from an AIDS patient, 95 kb); 8, strain 2 (isolate from an AIDS patient, 79 kb); 9, strain ATCC 33704 (79 kb); 10, strain ATCC 33701 (85 kb). The arrows on the left indicate the virulence-associated antigens. Molecular mass markers are indicated by bars on the right.

teria grown at 38°C for 48 h in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) and solubilized in sodium dodecyl sulfate (SDS) reducing buffer, were analyzed by SDS-polyacrylamide gel electrophoresis, electrotransfer of proteins to nitrocellulose sheets, and Western blot (immunoblot) analysis, as described previously (23, 28). Serum from a foal naturally infected with *R. equi* and monoclonal antibodies against the 20-kDa antigen and the 15- to 17-kDa antigens were used for the immunoblotting procedures (20, 22).

The virulence of pig isolate was examined by the mouse pathogenicity test as described previously (23, 28).

Plasmid DNA was isolated from *R. equi* by the alkaline lysis method (2), with the following previously described modifications (21). Samples of plasmid preparations were separated in 0.7% agarose gels at approximately 5 V/cm for 2 h. Plasmid DNAs were analyzed by digestion with the restriction endonucleases *Bam*HI, *Eco*RI, *Eco*T22I, *Hin*dIII, and *Pvu*II for comparison in detail and estimation of the plasmid size.

R. equi was isolated from 56 (3.1%) of 1,832 submaxillary lymph nodes from apparently healthy pigs. The majority (43 of 56 lymph nodes) of the lymph nodes showed fewer than 20 colonies on the NANT agar plate. Two to 10 colonies per positive specimen were subcultured, and all of the isolates (392 isolates) were identified by biochemical characteristics and production of factors associated with *R. equi* (13). There was no difference in macroscopic observation between *R. equi*-positive and -negative lymph nodes.

All of the isolates were tested for the presence of virulence-associated antigens by immunoblotting with serum from a naturally infected foal and monoclonal antibodies against the 15- to 17-kDa antigens and the 20-kDa antigen (some of the results are shown in Fig. 1). Three hundred sixty-eight (93.9%) of the 392 isolates showed the 20-kDa antigen, 2 (0.5%) of the isolates showed the 15- to 17-kDa antigens, and the remaining 22 isolates did not show any virulence-associated antigens. Forty-three (76.8%) of the 56 lymph nodes contained *R. equi* with only the 20-kDa antigen, and 11 (19.6%) of the lymph nodes contained *R. equi* with and without the 20-kDa antigen. However, the majority of the isolates from the 11 lymph nodes were positive for the 20-kDa antigen. One (1.8%) of the lymph nodes contained *R. equi* with the 15- to 17-kDa antigens, and the remaining one (1.8%) contained *R. equi* without any virulence-associated antigens.

Fifty-six representative isolates were chosen from the 56 culture-positive lymph nodes. The plasmid profiles of the representative isolates and reference strains ATCC 33701, ATCC 33704, and L1 were examined. The 56 isolates could be classified according to plasmid profile as follows: 1 isolate had the 15- to 17-kDa antigens and contained a 90-kb virulence plasmid, 54 isolates had the 20-kDa antigen and contained large plasmids of various sizes, and the remaining 1 isolate had no virulence-associated antigens and contained no plasmids. As these similar plasmids were not easily distinguished by migration on the gel, all of the plasmids were analyzed by digestion with restriction endonucleases *Bam*HI, *Eco*RI, *Eco*T22I, *Hin*dIII, and *Pvu*II for comparison in detail and estimation of plasmid size. On the basis of the restriction cleavage patterns of the plasmid DNA, the 54 isolates were shown to have plasmids of into five sizes: 79, 88, 88.5, 89, and 95 kb (Table 1).

TABLE 1. Prevalence of plasmids and virulence-associated antigens in *R. equi* isolates from pig submaxillary lymph nodes

Plasmid size (kb)	Size (kDa) of virulence-associated antigen(s)	No. (%) of isolates
79	20	24 (42.8)
88	20	3 (5.4)
88.5	20	11 (19.6)
89	20	13 (23.2)
95	20	3 (5.4)
90	15–17	1 (1.8)
		1 (1.8)

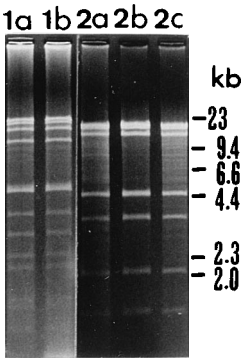

FIG. 2. Comparison of restriction endonuclease *Eco*RI digestion profiles of plasmid DNA from pig and human isolates. Lanes (plasmid sizes in parentheses): 1a, isolate PL57 (95 kb); 1b, strain 5 (isolate from an AIDS patient, 95 kb); 2a, isolate PL459 (79 kb); 2b, strain 2 (isolate from an AIDS patient, 79 kb); 2c, strain ATCC 33704 (79 kb). Molecular size markers are indicated by bars on the right.

Among them, two plasmids of 79 and 95 kb from the five representative isolates of pigs were revealed to be the same plasmids as those from two of the four human strains (Fig. 2). These results suggest that there are at least seven plasmids from the isolates showing the 20-kDa antigen.

The pathogenicities of the 56 representative isolates were tested in mice. The 54 isolates showing the 20-kDa antigen were found to be intermediately virulent (10^7 bacteria needed for lethality), and the 1 isolate with the 15- to 17-kDa antigens was found to be virulent (10^6 bacteria needed for lethality). The one isolate without any virulence-associated antigens was avirulent. These results confirm the previous observations for human isolates of *R. equi* (22).

The present study revealed that almost all the isolates of *R. equi* from swine submaxillary lymph nodes contained the 20-kDa antigen and that these isolates were intermediately virulent for mice and contained one of five large plasmids of 79 to 95 kb. This is the first report concerning the isolation of *R. equi* containing the 20-kDa virulence-associated antigen from domestic animals. Recently, we demonstrated that of human isolates, those with the 20-kDa antigen contained one of four large, distinct plasmids which have DNA homology (22) and that two of them, 79- and 95-kb plasmids, were the same sizes as plasmids found in the pig isolates. Therefore, there were at least seven distinct plasmids of 79 to 100 kb found in human and pig isolates that were associated with the expression of the 20-kDa antigen.

The virulence-associated antigens and plasmids have been used as epidemiological markers to identify virulent *R. equi* from horses and their environment (18, 25, 26). Virulent *R. equi* has been isolated from almost all pulmonary abscesses of infected foals (17, 23). The environment of stud farms having endemic *R. equi* infections demonstrated heavy contamination with the virulent strain of *R. equi*, but farms without the problem did not (25, 26). During the survey of the prevalence of virulent *R. equi* at horse-breeding farms, we found 20 different cryptic plasmids of 5.2 to 120 kb in isolates from horses and their environment (21). These environmental isolates containing cryptic plasmids were screened by immunoblotting; however, we have never found the 20-kDa antigen in those horse isolates (21). At the beginning of this study to find out the source of intermediately virulent *R. equi*, we tried to isolate *R. equi* from soil collected from public parks and gardens as a possible source of human infection, but no virulent *R. equi* was isolated from these specimens (19). Then, we focused on pigs, since *R. equi* has frequently been isolated from swine submaxillary lymph nodes with and without lesions at a high prevalence (3, 5, 9, 10, 13, 14, 29, 30, 36) and since Tkachuk-Saad and Prescott (33) have already reported *R. equi* plasmids of different sizes from pig lymph nodes. Unfortunately, they did not determine the relationship between the plasmids and the virulence of *R. equi*. In the present study, *R. equi* having the 20-kDa antigen was isolated from swine lymph nodes; however, we cannot explain whether or not pig *R. equi* is the source of infection in AIDS patients. It is very important to reveal the route of infection in AIDS patients with *R. equi* from the standpoint of zoonotic potential. We are now investigating soil isolates from pig environments.

The pathogenesis of *R. equi* in pigs remains unclear, and the pathogenicities of *R. equi* isolates for pigs are controversial (9, 36, 39): *R. equi* has been isolated from lymph nodes with and without lesions which strikingly resemble those of tuberculosis (5, 10, 14, 30), and efforts to reproduce the lesions in swine submaxillary lymph nodes by feeding pigs cultures of *R. equi* have been unsuccessful (3, 9). However, it was amazing that almost all the pig isolates contained the 20-kDa antigen and were intermediately virulent in mice, since almost all isolates from clinical specimens of horses are virulent (33). The relationship between the lesions in pigs and the virulence of isolates is now being investigated by pathological and bacteriological means. The discovery of virulence-associated antigens and plasmids will help to understand the pathogenesis of *R. equi* infection in pigs as well as in AIDS patients.

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