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Identification of Virulent *Rhodococcus equi* by Amplification of Gene Coding for 15- to 17-Kilodalton Antigens

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During a survey of the prevalence of virulent *Rhodococcus equi* at horse-breeding farms by plasmid and protein profiles, cryptic plasmids of various sizes were found in 66 (3.8%) of 1,725 isolates from feces of horses and 129 (5.9%) of 2,200 isolates from soil. Twenty-two isolates, which contained cryptic plasmids of different sizes, were found by plasmid profiles, and their protein profiles and mouse pathogenicities were examined. Of the 22 isolates, 7 were virulent *R. equi*, contained both virulence and cryptic plasmids, and expressed 15- to 17-kDa antigens. The remaining 15 isolates were avirulent and did not express the antigens: 6 strains contained cryptic plasmids of two different sizes and 9 strains contained cryptic plasmids of various sizes. A PCR assay was developed for the rapid identification of virulence plasmids of *R. equi*. Oligonucleotide primers, derived from the sequence of a gene coding for the 15- to 17-kDa virulence-associated antigens of *R. equi*, amplified a 564-bp product from all the tested isolates harboring a virulence plasmid. This PCR product hybridized with virulence plasmid DNA in the Southern hybridization assay. Virulence plasmid-cured derivatives and all of the tested isolates harboring cryptic plasmids only were negative. The PCR is a rapid, sensitive, and specific test for the identification of virulent *R. equi* from environmental isolates compared with standard techniques, such as plasmid and protein profiles and the mouse pathogenicity test, and is considered to be a useful tool for epidemiological studies.

Rhodococcus equi is one of the most important bacterial pathogens in 1- to 3-month-old foals (1, 5, 6, 22). Infections caused by this organism are characterized by chronic, suppurative bronchopneumonia with extensive abscesses and enteritis associated with suppurative lymphadenitis (1, 5, 6). Despite the importance of this highly pathological disease, the pathogenesis of *R. equi* infection remains unclear (5, 22).

Recent interest in the virulence mechanisms of *R. equi* has undoubtedly been stimulated by the discovery of virulence plasmids (19, 21). Virulent *R. equi* contains a large plasmid of 85 or 90 kb, which contains the gene responsible for the expression of 15- to 17-kDa antigens (3, 8, 15). Almost all the clinical isolates from lesions of infected foals contained one or the other virulence plasmid (9, 20). Curing of the plasmid coincided with loss of the antigens and a dramatic decrease in virulence in foals (10).

The virulence-associated antigens and plasmids have been used as epidemiological markers to identify virulent *R. equi* (11, 16, 17). The environment of stud farms having endemic *R. equi* infections demonstrated heavy contamination with the virulent strain of *R. equi*, but the farms without the problem did not (17). This characteristic inconsistent spreading of the disease on some farms, and its sporadic presence on others, suggests different levels of contamination with virulent *R. equi.*

During a survey of the prevalence of virulent *R. equi* at horse-breeding farms by plasmid and protein profiles, cryptic plasmids of various sizes were found in isolates from feces of horses and from soil (20). Some of the cryptic plasmids were very similar in size to the virulence plasmids of *R. equi*. In this communication, we present plasmid and protein profiles, evidence of virulence of the isolates harboring cryptic plasmids of various sizes, and application of the PCR by amplifying a region of the 15- to 17-kDa-antigen structural gene sequence to achieve rapid identification of virulent *R. equi.*

MATERIALS AND METHODS

Bacterial strains. The *R. equi* strains used in this study were from two sources: 1,725 isolates were from feces of foals and dams at horse-breeding farms in Aomori and Hokkaido, Japan, and 2,200 soil isolates were from paddocks and stables of 38 horse-breeding farms in Aomori and Hokkaido. Some of the results concerning the prevalence of virulence plasmids in these isolates have already been described (11). Twenty-two representative strains harboring cryptic plasmids were used (see Table 1). Strains ATCC 33701 and L1 and their plasmid-cured derivatives were used as reference strains because some of their plasmid characteristics and virulence levels have already been described (19, 20). The strains were stored frozen with 20% glycerol in small aliquots at −80°C.

Preparation of DNA. Plasmid DNA was isolated from *R. equi* by the alkaline lysis method (2) with the following modifications. *R. equi* isolates were grown for 2 days at 30°C in 5 ml of brain heart infusion broth (Difco). One milliliter of bacterial culture was centrifuged, and bacteria were resuspended in 200 μl of a freshly prepared solution containing 0.05 M Tris-HCl (pH 8.0), 0.01 M EDTA (pH 8.0), 0.5 M NaCl, and 20% (wt/vol) sucrose plus 5 mg of lysosome per ml. The bacteria were incubated at 37°C for 2 h. Cells were then lysed by adding 400 μl of a solution containing 2.0% (wt/vol) sodium dodecyl sulfate (SDS) and 0.3 M NaOH. Chromosomal DNA was precipitated with 5 M potassium acetate-acetic acid buffer (pH 4.8) and centrifuged at 10,000 × *g* for 15 min. Samples of plasmid preparations were separated along with the plasmids of *Escherichia coli* V517 in 0.7% agarose gels at approximately 5 V/cm for 2 h. Molecular weights were estimated by observing migration in the gels as described by Rochelle et al. (7).

Primers. The selection of two primers was based on the gene sequence published by Sekizaki et al. (8). Two oligonucleotide primers were custom synthesized (Nisseiken Co., Ltd., Tokyo, Japan): 5′-GACTCTTCACAAGACGGT-3′ (primer 1) and 5′-TAGGCGTTGTGCCAGCTA-3′ (primer 2). Primer 1 corresponds to the sense strand at positions 6 to 23 of the gene; primer 2 corresponds to the antisense strand at positions 569 to 552.

PCR amplification. PCR amplification was performed with 10 μl of the DNA preparation in a 50-μl reaction mixture containing 10 mM Tris-HCl (pH 8.3 at 25°C), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphos-

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phates, 1 μM each primer, and 2.5 U of *Taq* DNA polymerase (Takara, Tokyo, Japan).

The samples were subjected to 30 cycles of amplification in a PC-700 thermal cycler (ASTEC, Tokyo, Japan). The cycling conditions consisted of denaturation for 90 s at 94°C, primer annealing for 1 min at 55°C, and extension for 2 min at 72°C. After amplification, 10 μl of the reaction mixture was electrophoresed in a 0.7% agarose gel, and the DNA fragment was visualized by UV fluorescence after staining with ethidium bromide.

Preparation of probe DNA. A nonradioactive DNA labeling and detection kit (Boehringer Mannheim-Yamanouchi, Tokyo, Japan) was used for the hybridization procedures. For Southern analysis, a digoxigenin-11-dUTP-labeled probe was amplified with the primers by PCR as described by the supplier.

Southern hybridization analysis of plasmid DNA. Plasmid DNA was transferred to a nylon membrane sheet (Hybond N; Amersham Japan Corp., Tokyo, Japan) by using the vacuum transfer method with VacuGene (Pharmacia-LKB Biotechnology AB, Uppsala, Sweden) without depurination, as instructed by the manufacturer. Hybridization was carried out at 68°C for at least 6 h in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]) containing 0.1% sodium *N*-lauroyl sarcosine, 0.02% SDS, and 1% blocking reagent. After hybridization, the sheets were washed twice at room temperature for 5 min in 2× SSC containing 0.1% SDS and then twice for 15 min at 68°C in 0.1× SSC containing 0.1% SDS. Immunological detection of the hybridized digoxigenin-labeled probe was performed with the commercial kit.

Western blot analysis. Whole-cell antigens, which were prepared by harvesting bacteria grown at 38°C for 48 h from brain heart infusion broth and solubilized in SDS reducing buffer, were analyzed by SDS-polyacrylamide gel electrophoresis, electrotransfer of proteins to nitrocellulose sheets, and Western blot (immunoblot) analysis as described previously (15). Serum from a foal naturally infected with *R. equi* and monoclonal antibody against 15- to 17-kDa antigens were used for the immunoblotting procedures (12).

Mouse pathogenicity test. Virulence of the parent strains and derivatives was examined by the mouse pathogenicity test, as described previously (15, 19). These strains were grown for 48 h in brain heart infusion broth at 37°C. Cultures were diluted 10-fold with sterile saline. Six mice were used for each environmental isolate. Two groups of three mice each for each strain were tested at two inoculum levels (approximately 10^7 and 10^6 cells). ddY mice (20 to 23 g each) were each given 0.2 ml of the bacterial suspension intravenously. The mice were observed for 10 days, and deaths were recorded. Strains that killed two or more mice at a concentration of 10^6 cells were considered to be virulent. Inocula of two different sizes were used to ensure that the avirulent strain did not kill mice even at a concentration of 10^7 cells.

RESULTS

Prevalence of the cryptic plasmid in *R. equi* isolates from feces of horses and soil at horse-breeding farms. Virulent *R. equi* contains either an 85-kb or a 90-kb virulence plasmid and expresses 15- to 17-kDa virulence-associated antigens (20). Plasmid and protein profiles are useful markers in epidemiological studies of *R. equi* infection (11, 16, 17). During a survey of the prevalence of virulent *R. equi* isolates from feces of horses and soil at horse-breeding farms with endemic infection by the two methods, all of the isolates were screened for the presence of plasmids by agarose gel electrophoresis, and then the plasmid-positive isolates were tested for the presence of virulence-associated antigens by immunoblotting. Cryptic plasmids of various sizes were found in these isolates. Some of the results concerning the prevalence of virulence plasmids have been described elsewhere (11). Cryptic plasmids of various sizes were found in 66 (3.8%) of 1,725 isolates from feces of foals and dams and in 129 (5.9%) of 2,200 isolates from soil. These isolates lacked 15- to 17-kDa antigens by protein profiles. Virulence plasmids were detected in 224 (10.2%) of 2,200 soil isolates and in 366 (21.2%) of 1,725 fecal isolates. As shown in Fig. 1, some of the cryptic plasmids were very similar in size to the virulence plasmids of *R. equi*. Therefore, during these epidemiological studies, plasmid profiles were used as a screening test and definitive identification of virulent *R. equi* depended on the identification of virulence-associated antigens by Western blotting.

Diversity of *R. equi* plasmids. Twenty-two representative strains were chosen from plasmid-positive isolates on the basis of the sizes and numbers of plasmids. Plasmid and protein profiles of these strains and control strains ATCC 33701 and L1 are shown in Fig. 1. Of the 22 strains, 7 strains were virulent in the mouse pathogenicity test, and they contained either 85- or 90-kb virulence plasmids and cryptic plasmids of various sizes (Table 1). The remaining strains were avirulent: six strains contained cryptic plasmids of two different sizes, and nine strains contained cryptic plasmids of various sizes. Twenty cryptic plasmids of different sizes were found in these strains. The environmental strains varied little in plasmid size, since 224 soil isolates and 366 fecal isolates contained either 85-kb or 90-kb virulence plasmids.

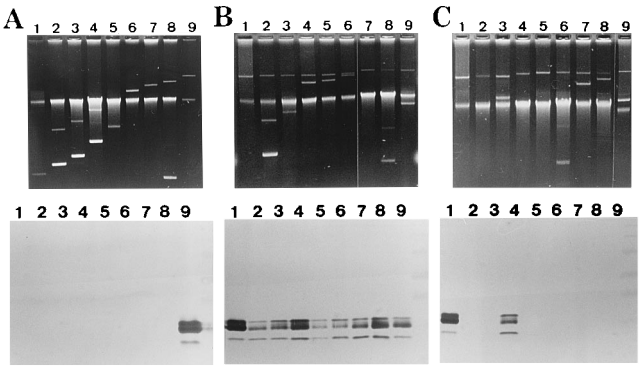

FIG. 1. Plasmid profiles and immunoblot profiles of *R. equi* isolates from feces of horses and soil at horse-breeding farms. Plasmid DNA was extracted, resolved in a 0.7% (wt/vol) agarose gel, and stained with ethidium bromide. Whole-cell preparations were analyzed by immunoblotting with monoclonal antibody 10G5 against 15- to 17-kDa antigens. (A) Lanes: 1, K101; 2, K56; 3, NB16; 4, K71; 5, K93; 6, K14; 7, K98; 8, Y804; 9, ATCC 33701. (B) Lanes: 1, ATCC 33701; 2, NB19; 3, KT3435; 4, K92; 5, K513; 6, K34; 7, L1; 8, KT29414; 9, KT2910. (C) Lanes: 1, ATCC 33701; 2, KT223; 3, KT672; 4, L1; 5, KT507; 6, KT3647; 7, KT658; 8, Y310; 9, KT210.

PCR amplification of 15- to 17-kDa-antigen genes. A PCR method was developed by using two primers derived from the

TABLE 1. Expression of 15- to 17-kDa antigens, mouse pathogenicity, and PCR results of *R. equi* isolates

Strain (source)	Plasmid size(s) (kb)	Expression of 15- to 17-kDa antigens	Mouse pathogenicity	PCR result
K101 (soil)	5.2	−	−	−
K56 (soil)	6.3	−	−	−
NB16 (feces of a foal)	7.3	−	−	−
K71 (soil)	10	−	−	−
K93 (soil)	15	−	−	−
K14 (soil)	47	−	−	−
K98 (soil)	56	−	−	−
Y804 (feces of a foal)	4.6, 64	−	−	−
ATCC 33701 (lung abscess)	85	+	+	+
NB19 (feces of a foal)	7.3, 85	+	+	+
KT3435 (soil)	21, 85	+	+	+
K92 (soil)	62, 85	+	+	+
K513 (soil)	64, 85	+	+	+
K34 (soil)	80, 85	+	+	+
KT223 (soil)	88	−	−	−
KT672 (soil)	40, 88	−	−	−
L1 (lung abscess)	90	+	+	+
KT2914 (soil)	5.5, 90	+	+	+
KT2910 (soil)	23, 90	+	+	+
KT507 (soil)	105	−	−	−
KT3647 (soil)	7.3, 105	−	−	−
KT658 (soil)	62, 105	−	−	−
Y310 (feces of a dam)	70, 105	−	−	−
KT210 (soil)	22, 120	−	−	−

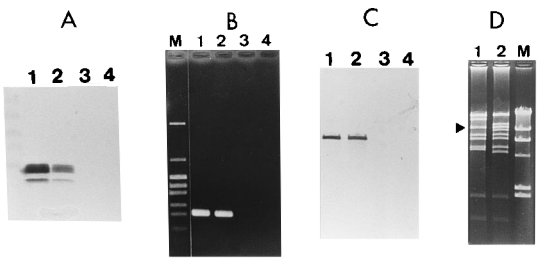

FIG. 2. (A) Immunoblot profiles of *R. equi* ATCC 33701 and L1 and their derivatives; (B) PCR amplification patterns of these strains analyzed by 1% agarose gel electrophoresis; (C) Southern hybridization of digoxigenin-labeled PCR product probes to the *Eco*RI restriction fragments of the plasmids; (D) *Eco*RI restriction fragments of the plasmids. Arrowhead, fragment to which the probe was hybridized. Whole-cell antigens were analyzed by immunoblotting with monoclonal antibody 10G5. Lanes: 1, ATCC 33701; 2, L1; 3, plasmid-cured derivative of strain ATCC 33701; 4, plasmid-cured derivative of strain L1. The markers (lanes M) in panels B and D are *Hin*dIII and *Hae*III digestion products of pHY300PLK DNA and *Hin*dIII digestion products of bacteriophage lambda DNA, respectively.

sequence of the gene coding for the virulence-associated 15- to 17-kDa antigens of *R. equi* (8) to identify virulence plasmids. Strains ATCC 33701 and L1 and their plasmid-cured derivatives were used to develop and evaluate the specificity of the PCR. Virulent strains showing 15- to 17-kDa antigens in immunoblots gave a 564-bp product of the expected size in the PCR amplification, but the plasmid-cured derivatives did not (Fig. 2A and B). PCR products labeled with digoxigenin-11-dUTP hybridized with the 10.5-kb *Eco*RI fragment of pREAT701 and pREL1 (Fig. 2C and D). No hybridization was observed with the plasmid-cured derivatives.

Twenty-four strains harboring plasmids of various sizes and numbers were analyzed by PCR (Fig. 3). All 9 strains showing the virulence-associated antigens gave positive results, but the remaining 15 strains without the antigens gave negative results. PCR results were consistent with protein profiles and the mouse pathogenicity test results (Table 1). Plasmid DNAs from the 22 strains were examined by Southern analysis with the PCR probes (data not shown). Virulence plasmid DNAs from the 7 strains were hybridized with the probes, but no hybridization was observed with cryptic plasmid DNAs from the 22 strains.

DISCUSSION

In agreement with the results of Tkachuk-Saad and Prescott (21), we observed the presence of various cryptic plasmids in *R. equi* from environmental isolates. *R. equi* plasmids of six different sizes from clinical materials were reported by Tkachuk-Saad and Prescott (21); we also found 20 different cryptic plasmids of various sizes in isolates from horses and their environment. Of the 22 representative isolates, 7 had an 85- or a 90-kb virulence plasmid and cryptic plasmids of various sizes and the others had one or two cryptic plasmids of various sizes. In contrast to these environmental isolates, almost all the clinical isolates from infected foals harbored either an 85- or a 90-kb virulence plasmid (9, 20), and no isolates harboring cryptic plasmids only were found. Tkachuk-Saad and Prescott (21) found plasmids of six different sizes in isolates from specimens obtained from horses, pigs, and cats with high frequency (49 of 54 isolates). More recently, we observed cryptic plasmids in isolates from patients with and without AIDS (4, 18). On the other hand, the prevalence of cryptic plasmids in our environmental isolates was low: 3.8% in fecal isolates and 5.9% in soil isolates. These results suggest that cryptic plasmids among clinical isolates from pigs and cats might have phenotypic characteristics of clinical importance, such as antibiotic resistance and virulence. Further studies are needed to clarify the function of these cryptic plasmids.

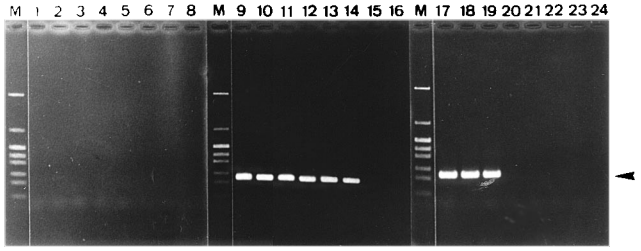

FIG. 3. Agarose gel electrophoresis of PCR products obtained after amplification of DNAs from 24 isolates of *R. equi* with the primers. Lanes: 1, K101; 2, K56; 3, NB16; 4, K71; 5, K93; 6, K14; 7, K98; 8, Y804; 9, ATCC 33701; 10, NB19; 11, KT3435; 12, K92; 13, K513; 14, K34; 15, KT223; 16, KT672; 17, L1; 18, KT29414; 19, KT2910; 20, KT507; 21, KT3647; 22, KT658; 23, Y310; 24, KT210. The markers (lanes M) are *Hin*dIII and *Hae*III digestion products of pHY300PLK DNA. Arrowhead, 564-bp amplified fragment.

Although standard techniques, such as plasmid and protein profiles, for identifying virulent *R. equi* from environmental samples are reliable, they require several days or weeks to complete, depending on the quantity of samples. The monoclonal antibody-based colony blot test has been developed to identify rapidly and accurately virulent *R. equi* (16). However, the colony blot test is not completely accurate because the expression of the 15- to 17-kDa antigens is regulated by growth conditions such as temperature and pH (13, 14). The gene coding for the 15- to 17-kDa antigens proved to be a promising target for PCR for detection of virulent *R. equi*. The 564-bp segment was amplified from the strains containing either an 85- or a 90-kb virulence plasmid but not from the virulence plasmid-cured derivatives or the representative strains harboring cryptic plasmids of various sizes.

In conclusion, the PCR has the potential for identifying virulent *R. equi* rapidly by amplification of gene sequences unique to virulence plasmids, and it will be useful not only in the epidemiological investigations but also in early diagnosis of infection. The application of PCR for detection of virulent *R. equi* in bronchial lavage fluid from foals with a suspected infection is in progress.

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