

determine whether the management practices influenced the RFLP type.

Fifteen different RFLP types of PRRSV have been reported previously.^{4,5} This study identified all of these except type 1-8-2. As well, 17 additional RFLP types of the virus were identified in this study. Two strains may have a natural deletion in ORF 5, ORF 4, or ORF 6. This study indicates that many RFLP types of PRRSV exist on Ontario farms. The great variety of PRRSV in Ontario suggests that the virus undergoes frequent mutation under field conditions.

Sources and manufacturers

- a. IKA Ultra-Turrax T25, IKA Laboratory Technology, Staufen, Germany.
- b. Fisher Scientific, Nepean, ON, Canada.
- c. RNeasy® Mini Kit, Qiagen, Mississauga, ON, Canada.
- d. Qiaamp® Viral RNA Mini Kit, Qiagen, Mississauga, ON, Canada.
- e. PE Applied Biosystems, Mississauga, ON, Canada.
- f. Molecular Supercenter, University of Guelph, Guelph, ON, Canada.
- g. GIBCOBRL, Burlington, ON, Canada.
- h. SIGMA, Oakville, ON, Canada.
- i. AmpliWax® PCR Gem, PE Applied Biosystems, Mississauga, ON, Canada.
- j. Amersham Pharmacia Biotech, Baie d'Urfé, PQ, Canada.
- k. NuSieve agarose, BioWhittaker Molecular Applications, Rockland, ME.

- l. Ingelvac® RespPRRS vaccine, Boehringer Ingelheim Ltd., Burlington, ON, Canada.
- m. PRIME PAC® PRRS vaccine, Schering-Plough Animal Health, Omaha, NE.

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Detection of *Rhodococcus equi* by polymerase chain reaction using species-specific nonproprietary primers

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Abstract. Species-specific primers for the polymerase chain reaction (PCR) for the detection of *Rhodococcus equi* were developed. These primers were based on unique DNA fragments produced from *R. equi* reference strains and field isolates. Following random amplification of polymorphic DNA from *R. equi* and *R. rhodochrous* with a set of 40 arbitrary 10–base pair (bp) primers, a pair of species-specific primers was designed to detect a unique 700-bp fragment of *R. equi* chromosomal DNA. This PCR product was limited to *R. equi* and was not detectable in other *Rhodococcus* species or in a panel of additional gram-positive and gram-negative bacteria.

Rhodococcus equi is an aerobic gram-positive pleomorphic bacterium with worldwide distribution.^{24,33,37} Although this facultative intracellular pathogen can infect a wide range of animals, it is primarily a pathogen of foals.²⁴ Nearly all isolates of *R. equi* from affected foals contain an 85–90-kilobase (kb) plasmid that possesses a gene that encodes a 15–17-kD protein antigen, commonly referred to as the vir-

ulence-associated protein antigen (VapA).³⁹ *Rhodococcus equi* is being more frequently recognized as a pathogen of immunocompromised humans, particularly patients with AIDS.^{13,19,27,28}

The primary clinical manifestation of *R. equi* infection in foals is severe suppurative bronchopneumonia.^{3,24} Pneumonia is an important cause of morbidity and mortality for foals. Approximately 9% of all foals in the United States are affected by pneumonia, and about 12% of these foals die.⁹ In Texas, respiratory disease is the most common cause of disease and death in foals.⁶ Although many different organisms have been associated causally with pneumonia in foals, *R. equi* is considered the most common cause of severe

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Table 1. *Rhodococcus equi* isolates tested by polymerase chain reaction using primers specific for *R. equi*.

| Source | No. isolates | Comment* |
|-----------------|--------------|---------------------------|
| Lung | 4† | Virulent/clinical disease |
| TBA‡ | 15 | Virulent/clinical disease |
| Soil | 12 | Avirulent |
| Intestinal swab | 2 | Virulent/clinical disease |
| Pig lymph node | 1§ | Avirulent |

* Virulent = isolate determined to contain virulence-associated protein antigen (VapA) by immunoblot²² or VapA gene by PCR.²²

† One of these 4 isolates was the reference strain ATCC 33701.

‡ Isolate obtained by tracheobronchial aspiration of an affected foal.

§ Reference strain ATCC 33703.

pneumonia.^{9,24,33,37} Prevalence and fatality rates for *R. equi* pneumonia are high,^{24,37} and pneumonia caused by *R. equi* may negatively impact future performance.¹

Because an effective vaccine for prevention of infection by *R. equi* is not available, treatment of affected foals remains the principal approach for managing disease caused by this organism. Erythromycin and rifampin are considered the standard antibiotics for treatment.^{1,10,14,32,37,46} This protocol must be prolonged, is expensive, and has associated risks, including such adverse reactions as diarrhea and hyperthermia in treated foals and severe acute colitis in the dams of treated foals (Phelps MS, et al.: 1998, Proc Annu Conv Am Coll Vet Intern Med 16:708).^{12,24}

Because of the insidious onset and severity of clinical signs, prompt and accurate diagnosis of pneumonia caused by *R. equi* is of considerable importance to enable improved clinical outcomes of affected foals by earlier medical intervention. Efforts to facilitate early diagnosis using serologic methods have been proposed, but these methods have limitations, which include questionable specificity and, when paired titers are required, a considerable time lag for diagnosis.^{2,10} Traditionally, microbiologic culture of a tracheobronchial aspirate (TBA) has been used for definitive clinical diagnosis of pneumonia caused by *R. equi*.^{32,45} This approach has a number of limitations. Tracheobronchial aspiration can be technically difficult for veterinarians, and in some foals the invasiveness of the procedure can induce or exacerbate life-threatening respiratory distress. There is a lag of several days from the time of submission until results are obtained from microbiologic culture of TBA fluid; this lag may delay implementation of appropriate treatment. Sensitivity of microbiologic culture is imperfect, and false-negative results of microbiologic culture of fluid obtained by TBA are possible.³² A more sensitive, specific, and noninvasive diagnostic test for earlier detection of *R. equi* infection would be of considerable value to equine clinicians, human physicians, and laboratory diagnosticians.

The polymerase chain reaction (PCR) assay has been used to detect *R. equi* in clinical specimens from foals.^{38,42} The PCR assay is more rapid and sensitive than microbiologic culture and is highly specific.²⁹ To date, 2 methods for detecting *R. equi* have been described: 1) PCR using primers that amplify a region of the 16S ribosomal RNA (rRNA) gene specific for *R. equi*^{24,30} and 2) PCR using primers that

Table 2. *Rhodococcus* species other than *R. equi* and bacteria closely related to *Rhodococcus* tested by polymerase chain reaction using primers specific for *R. equi*.

| Isolate | Origin |
|---|--|
| <i>R. coprophilus</i> (ATCC # 29080) | Pond mud (England) |
| <i>R. erythropolis</i> (ATCC # 4277) | Soil |
| <i>R. opacus</i> (ATCC # 51882) | Soil |
| <i>R. percolatus</i> (DSM # 44240) | Sludge and sediments |
| <i>R. rhodnii</i> (ATCC # 35071) | Reduvid bug |
| <i>R. rhodochrous</i> (ATCC # 271) | Soil |
| <i>Gordona aichensis</i> (ATCC # 33611) | Sputum of human patient with pulmonary disease |
| | Sputum of human patient with pulmonary disease |
| <i>G. bronchialis</i> (ATCC # 25592) | Soil |
| <i>G. rubropertincta</i> (ATCC # 14352) | Soil |
| <i>G. sputi</i> (ATCC # 29627) | Sputum of human patient with pulmonary disease |
| <i>G. terrae</i> (ATCC # 25594) | Soil |
| <i>Dietzia maris</i> (ATCC # 35013) | Soil (Ukraine) |
| Unidentified <i>Gordona/Rhodococcus</i> W6867 | Unknown |
| Unidentified <i>Gordona/Rhodococcus</i> W6875 | Unknown |
| Unidentified <i>Gordona/Rhodococcus</i> W6876 | Unknown |

amplify a region of the 85–90-kb plasmid found in virulent strains of *R. equi*.^{29,34} Because the 16S rRNA primers are based on proprietary sequences of DNA (patent no. 06037122), their commercial use is limited by associated costs. Although the association of the 85–90-kb VapA plasmid with virulence is strong in foals, it is not absolute.^{22,34} Furthermore, occasional negative results occur when PCR primers are used to detect the virulence plasmid in samples obtained from affected foals.²⁹ Because of these limitations of available PCR methods, species-specific PCR primers directed toward a nonproprietary segment of *R. equi* chromosomal DNA have been developed.

Three different groups of bacteria were used in these experiments. The first group consisted of 2 reference strains obtained from a commercial repository^a and 32 isolates of *R. equi* consisting of 20 virulent and 12 avirulent strains. The 32 isolates were obtained by microbiologic culture of intestinal swabs, lung abscesses, and TBA from foals with respiratory tract disease and of soil collected from horse breeding farms, as previously described^{b,c} (Table 1).²² The second group included 15 isolates of *Rhodococcus* other than *R. equi* that were obtained from a diagnostic laboratory^d (Table 2). The third group consisted of other bacterial isolates obtained from various sources^{a–d} (Table 3).

Bacterial species identification schemes based on polymorphic DNA fragments have been developed using random amplification of polymorphic DNA techniques.¹⁷ Single arbitrary primers have been used to generate different fragment patterns identifiable by gel electrophoresis and used as species-specific genetic markers among species and strains of various organisms.^{17,43} The random primers detect polymorphisms without any specific information about the nucleotide sequence of the organism.⁴⁴ In the experiments reported here, a total of 40 arbitrary primers 10 base pairs (bp)

Table 3. Non-*Rhodococcus* isolates tested by polymerase chain reaction using primers specific for *R. equi*.

| Isolate |
|---|
| <i>Mannheimia haemolytica</i> |
| <i>Nocardia asteroides</i> |
| <i>Mycobacterium smegmatis</i> |
| <i>Bordetella</i> sp. |
| <i>Salmonella typhimurium</i> |
| <i>Actinobacillus equuli</i> |
| <i>Pasteurella multocida</i> |
| <i>Streptococcus equi</i> |
| <i>Streptococcus zooepidemicus</i> |
| <i>Escherichia coli</i> |
| <i>Klebsiella pneumoniae</i> |
| <i>Corynebacterium pseudotuberculosis</i> |
| <i>Staphylococcus aureus</i> |
| <i>Pseudomonas aeruginosa</i> |

long were initially used to amplify chromosomal DNA from *R. equi* and *R. rhodochrous*. Bacterial genomic DNA from these strains was extracted using a commercial kit.^f Amplification reactions were performed in a 50- μ l volume containing 25 pmoles of a decamer primer, 50 mM KCl, 10 mM Tris pH 8.3, 0.2 mM dNTPs, 2.5 mM MgCl₂, 50–100 ng of template DNA, and 2.5 U of *Taq* DNA polymerase.^g The reactions were run in a thermocycler.^h Following an incubation period of 12 minutes at 95 C, the PCRs were run for 45 cycles of 30 seconds at 94 C, 30 seconds at 36 C, and 30 seconds at 72 C, followed by a period of extension at 72 C for 10 minutes. The reaction products were analyzed by gel electrophoresis on a 1.5% agarose gel containing 0.5 μ g/ml of ethidium bromide. Eighteen of 40 random primers generated amplification patterns that differed between these 2 species of *Rhodococcus*. The fragments amplified for *R. equi* but not for *R. rhodochrous* were cut from the gels and cleaned using a commercially available column.ⁱ Ten of these PCR fragments were ligated into a plasmid cloning vector and transformed into competent cells using a commercially available cloning kit.^j Approximately 10–15 positive clones were picked from each ligation and grown overnight in 3 ml of Luria-Bertani medium containing 50 μ g/ml ampicillin. Plasmid DNA was recovered by an alkali-lysis miniprep procedure²⁶ and analyzed by restriction mapping to verify the presence of the insert. Inserts were sequenced from plasmid DNA using a dye terminator cycle sequencing kit and an automated DNA sequencer.^k Ten primer pairs for sequences internal to random primers were designed using a computer software program^l with the following criteria: 18–25 bp in length, 55–80 C, 45–55% G+C content, and a product size between 400 and 900 bp. Primers were synthesized in the DNA core technologies laboratory at the College of Veterinary Medicine, Texas A&M University. These 10 primer pairs were tested for specificity for *R. equi*.

Isolation of genomic DNA was performed using a modified version of a phenol–chloroform extraction.²⁶ Bacterial isolates were grown in brain-heart infusion broth for 36 hours at 35 C. The bacterial suspensions were centrifuged for 10 minutes at 5,000 \times g. After removing the supernatant the pellets were resuspended in 700 μ l of deionized distilled

water and parboiled at 96 C for 6 minutes. The parboiled bacteria were transferred to 2-ml microcentrifuge tubes, and an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1) was added to each sample. The samples were shaken for 5 minutes and centrifuged for 8 minutes at 13,800 \times g. The top layer of each sample was transferred to a new 2-ml microcentrifuge tube, and an equal volume of chloroform was added to each sample. The samples were shaken for 5 minutes and centrifuged for 5 minutes at 13,800 \times g. The top layer of each sample was transferred to a new 1.5-ml microcentrifuge tube and 1/5 volume of 10 M ammonium acetate and 1 volume of 100% isoamyl alcohol were added to each tube. Samples were allowed to sit for 10 minutes at room temperature and then centrifuged for 30 minutes at 16,800 \times g. Then, 500 μ l of 70% ethanol was added to each sample, and samples were centrifuged for 15 minutes at 16,800 \times g and dried for 30 minutes at room temperature. The pellets were resuspended with 50 μ l of deionized distilled water. Absorbencies were read at 260 nm to quantify DNA concentrations. Samples then were diluted with water to a concentration of 100 ng/ μ l for the PCR. DNA extracted from bacterial isolates was subjected to amplification by PCR to test the designed primers. A PCR mixture was prepared consisting of 4.3 pmoles of forward and reverse primers, and the reaction was run using a thermocycler using the following reagents: 10 mM Tris HCl, 50 mM KCl, and 0.1% Triton X-100, 2.5 mM MgCl₂, 0.5% dimethyl sulfoxide, 0.2 mM dNTPs, and 2.5 U *Taq* polymerase.^m A 2.0 μ l volume of a 100-ng/ μ l solution of DNA was added to 18 μ l of the PCR cocktail buffer. The reaction started with an incubation period of 2 minutes at 95 C and was followed by 25 cycles of 20 seconds at 94 C and 40 seconds at 62 C, with a final extension period of 10 minutes at 72 C. Products of the PCR were analyzed by gel electrophoresis on a 1.5% agarose gel containing ethidium bromide, visualized by use of a ultraviolet transilluminator, and photographed. All PCR were sized via a molecular mass standard.ⁿ The product of these primers was sequenced, and a computer database^o search was performed to identify sequences with homology to the product.

Ten internal primer pair sequences to random primers were generated, but only forward primer 5'-TCCAGAAGCGGGATGAGGATTC-3' and reverse primer 5'-TGGTGTGATGGC-GGAAGATC-3' were specific for *R. equi*. Use of these primers for the PCR with DNA from 2 reference strains and 32 other isolates of *R. equi* consistently resulted in amplification of a 700-bp region of chromosomal DNA identifiable as a band in a 1.5% agarose gel (Fig. 1; Table 1). The 700-bp band was absent (Fig. 2) when the PCR contained DNA from 15 bacteria that were either *Rhodococcus* or closely related to *Rhodococcus* (Table 2) and 14 species of bacteria other than *R. equi* (Table 3). A database analysis of the 700-bp sequence showed there was no homology to either the 16S rRNA gene or to the VapA plasmid found in *R. equi*. The sequence of the species-specific segment also did not show homology to any coded gene found in GenBank.

The PCR primers developed in this experiment were species specific for detection of *R. equi*. They consistently amplified a 700-bp region of chromosomal DNA from 2 reference strains of *R. equi* and 20 virulent and 13 avirulent isolates of *R. equi*. They did not amplify any fragment of

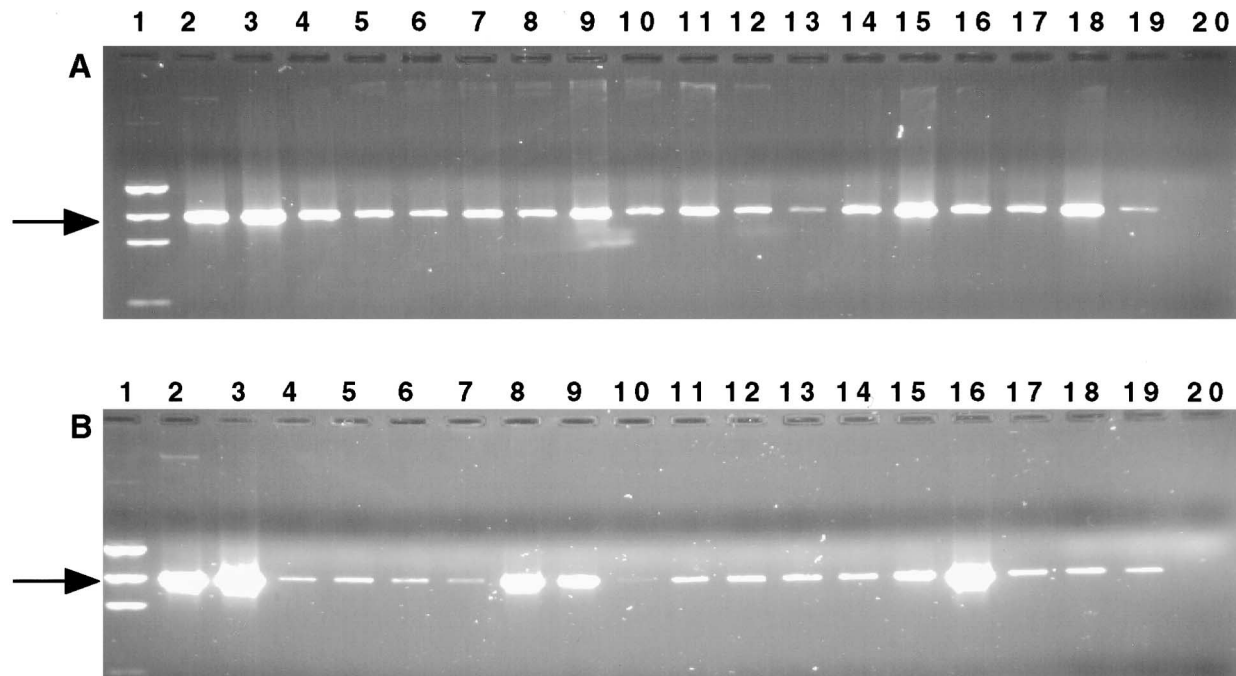


Figure 1. Images of 2 ethidium bromide-stained 1.5% agarose gels indicating the results of PCR amplification using *R. equi*-specific primers. Reference strains ATCC 33701 and ATCC 33703 (used as positive controls) showed amplification of a 700-bp fragment seen as a band in lanes 2 and 3; a band of the same size visible in lanes 4–19, which represent genomic DNA of *R. equi* isolates obtained by microbiologic culture of intestinal swabs, lung abscesses, and transtracheal aspirates from foals and from soil collected from horse-breeding farms (Table 1). Negative control (distilled water) showed no amplification of the band (lane 20). Lane 1 contains a molecular mass standard. **A.** Lanes, samples: 4, 97.01; 5, 97.02; 6, 97.03; 7, 97.05; 8, 97.07; 9, 97.09; 10, 97.10; 11, 97.11; 12, 97.12; 13, 97.13; 14, 97.14; 15, 97.15; 16, 97.16; 17, 97.17; 18, 97.18; 19, 97.19; 20, distilled water. **B.** Lanes, samples: 4, 97.20; 5, 97.21; 6, 97.22; 7, 97.23; 8, 6N2; 9, 9N1; 10, 11N1; 11, 12P1; 12, 11N2; 13, 11N3; 14, 12P2; 15, 13N1; 16, 13P3; 17, 16N1; 18, 16N2; 19, 16N3. Arrow indicates 700 bp.

chromosomal DNA from a wide range of other bacteria, including other *Rhodococcus* species and other bacteria commonly isolated from foals with respiratory disease (e.g., *Streptococcus* spp.).

Identification of *Rhodococcus* has been difficult because of its tortuous taxonomic history and the instability of its nomenclature.¹¹ To date, biochemical and culture methods have been used as standard tests to identify *R. equi* strains.^{2,11,15} Identification of *R. equi* by these conventional methods is difficult, problematic, labor intensive, and time consuming.¹⁸ Biochemical tests are laborious and insufficiently specific and only give a presumptive identification of *R. equi*.¹¹ A large number of biochemical tests must be performed to distinguish *R. equi* from other *Rhodococcus* species.^{11,18,40} Investigators have reported that conventional biochemical tests cluster different species into groups based on identical reaction patterns.¹⁸ Microbiologic culture properties of *R. equi*, including hemolytic properties and pigmentation, only allow presumptive identification of the species.³¹ Thus, these methods are not satisfactory for *R. equi* identification,¹⁸ and misclassification of *Rhodococcus* spp. and related bacteria can occur.¹¹ For example, 4 isolates were provided for this study that were described as *Rhodococcus* species other than *R. equi*, but amplification by these *R. equi*-specific primers and further genomic analysis (including analysis of the 16S rDNA sequence) identified them as *R. equi* (unpublished data).

Genomic methods for identification of bacteria are based on examination of chromosomal or plasmid DNA. The presence of conserved chromosomal and plasmid regions allows for differentiation among related species of bacteria.⁴¹ Genotypic methods have higher discriminatory power and provide reproducible results for identification of related species of bacteria.⁸ Because composition of DNA is not affected by conditions of microbiologic culture of the bacteria, genomic methods are more reliable.⁸ Genomic methods such as plasmid typing, ribotyping, and PCR-based typing have been used for identification of *R. equi*. All of these methods have higher discriminatory power, typeability, and reproducibility than do conventional biochemical and culture methods.^{4,30,31,34,42}

Clinically, identification of *R. equi* as a cause of pneumonia is most commonly based on microbiologic culture of TBA.¹⁰ One of the major limitations of this method is the delay in positive identification of *R. equi*, which may be due to the presence of multiple pathogenic bacteria in a TBA sample, prior antibiotic administration to a foal, or the fact that *R. equi* is a facultative intracellular pathogen.^{7,16,20,32} Also, false-positive results can occur for samples obtained from healthy foals that have inhaled *R. equi* present in dust.

Recently, PCR has been used as a simple and rapid diagnostic test to identify bacteria.^{23,25} The PCR may be used to detect microorganisms below levels that can be detected by microbiologic culture.^{5,23,25} Results can be obtained in a

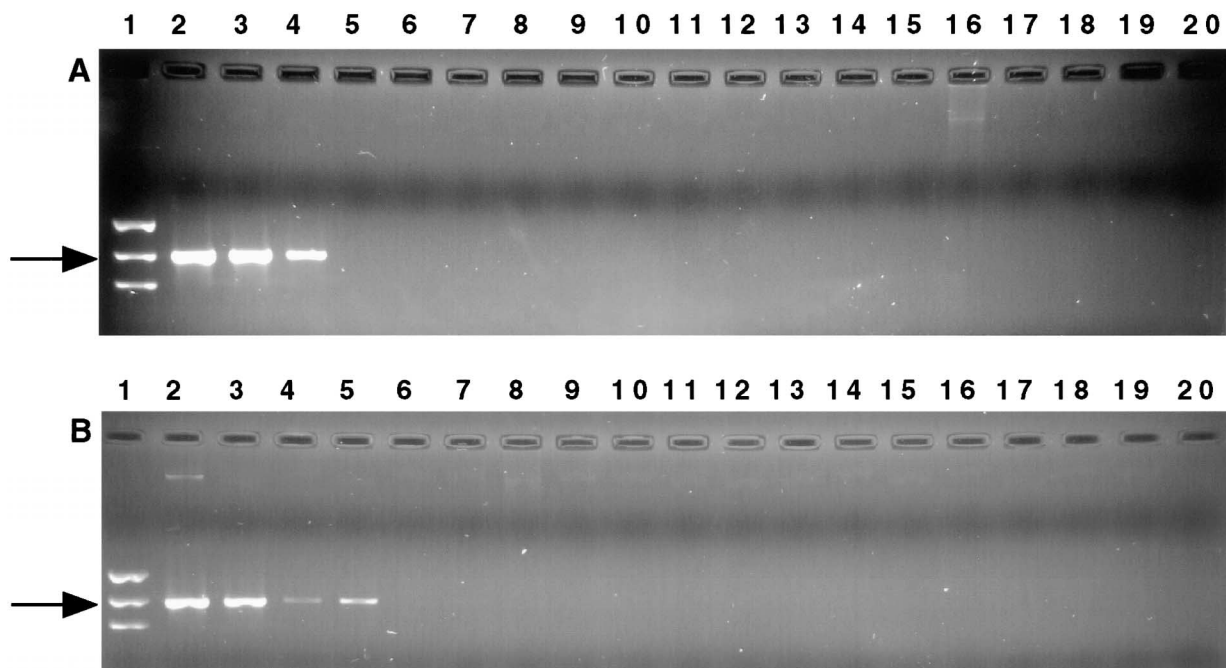


Figure 2. Images of 2 ethidium bromide-stained 1.5% agarose gels indicating the results of PCR amplification using *R. equi*-specific primers. Reference strains ATCC 33701 and ATCC 33703 and *R. equi* isolates from transtracheal aspirates from a foal were used as positive controls and showed amplification of a 700-bp fragment seen as a band. The 700-bp band was absent when the PCR contained DNA from 15 bacteria that were either *Rhodococcus* species or closely related bacteria (Table 2) and 14 species of bacteria other than *R. equi* (Table 3). Negative control (distilled water) showed no amplification of the band (lane 20). Lane 1 contains a molecular mass standard. **A.** Lanes, samples: 2, ATCC 33701; 3, ATCC 33703; 4, 97.11; 5, *R. coprophilus*; 6, *R. erythropolis*; 7, *R. opacus*; 8, *R. percolatus*; 9, *R. rhodnii*; 10, *R. rhodochrous*; 11, *Gordona aichensis*; 12, *G. bronchialis*; 13, *G. rubropertincta*; 14, *G. sputi*; 15, *G. terrae*; 16, *Dietzia maris*; 17, unidentified *Gordona/Rhodococcus* W6867; 18, unidentified *Gordona/Rhodococcus* W6875; 19, unidentified *Gordona/Rhodococcus* W6876. **B.** Lanes, samples: 2, ATCC 33701; 3, ATCC 33703; 4, 97.11; 5, 97.18; 6, *Pasteurella haemolytica*; 7, *Nocardia asteroides*; 8, *Mycobacterium smegmatis*; 9, *Bordetella* sp.; 10, *Salmonella typhimurium*; 11, *Actinobacillus equuli*; 12, *Pseudomonas aeruginosa*; 13, *Pasteurella multocida*; 14, *Streptococcus equi*; 15, *S. zooepidemicus*; 16, *Escherichia coli*; 17, *Klebsiella pneumoniae*; 18, *Corynebacterium pseudotuberculosis*; 19, *Staphylococcus aureus*. Arrow indicates 700 bp.

few hours and are more sensitive and specific.³⁰ The PCR technique is applicable to samples of blood and tracheal wash fluid.³⁰ Administration of antibiotics does not interfere with test accuracy because results do not depend on bacterial growth.³⁰ *Rhodococcus equi* residing in macrophages can be detected by PCR, whereas they may not be detected by routine culture.

Two methods for using PCR to detect *R. equi* have been described. The first incorporated use of PCR primers amplifying a region of the 16S rRNA gene, and the second incorporated primers amplifying a region of the VapA plasmid found in some *R. equi* isolates.^{30,34} There are limitations to these 2 PCR methods for detecting *R. equi*. The PCR primers that amplify a region of the 16S rRNA gene specific for *R. equi* are based on propriety sequences of DNA (patent no. 06037122); therefore, their commercial use is limited by associated costs. Plasmids are autonomous, self-replicating DNA elements not essential for bacterial growth that can be mobilized and exchanged between bacteria by conjugation. Also, they are subject to DNA rearrangement by transposition or integration into the host chromosome.²¹ Because of the biologic properties of plasmids, the PCR using plasmid-specific primers could give false-positive results with other species⁴ and only allows detection of bacteria that contain

the plasmid. Moreover, plasmid DNA is less stable than chromosomal DNA,⁸ and this lack of stability could yield false-negative results for the PCR.

In this study, a pair of species-specific primers for *R. equi* not based on a proprietary chromosomal sequence of *R. equi* was identified. These primers, however, are not specific for virulent organisms because they amplify a 700-bp region of chromosomal DNA present in both virulent and avirulent *R. equi* isolates. The majority of *R. equi* isolates from immunocompromised human patients and patients with AIDS do not contain the VapA plasmid.^{35,36} Because this pair of *R. equi*-specific primers is based on a chromosomal sequence, it can also be used for the diagnosis of *R. equi* infections in humans. For example, 2 of 11 *R. equi* isolates obtained from human patients⁹ were identified as virulent *R. equi* strains by PCR amplification using VapA primers, but all 11 isolates were identified as *R. equi* using the present *R. equi*-specific primers (unpublished data). Amplification of DNA using PCR can be accomplished rapidly and is of particular value when the concentration of bacteria is low, organisms are not viable, or isolation of bacteria is difficult. These species-specific primers can be used in a PCR-based diagnostic test to identify clinical and environmental isolates of *R. equi*. Additional studies of this diagnostic test with clinical sam-

ples from *R. equi*-infected and noninfected equine and human patients are needed to evaluate its clinical usefulness.

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Sources and manufacturers

- a. American Type Culture Collection, Manassas, VA.
- b. Texas Veterinary Medical Diagnostic Laboratory, College Station, TX.
- c. Veterinary Clinical Microbiology Laboratory, Texas A&M University, College Station, TX.
- d. Dr. June Brown, Centers for Disease Control, Atlanta, GA.
- e. Operon Technologies Inc., Alameda, CA.
- f. DNeasy Tissue Kit, Qiagen, Valencia, CA.
- g. AmpliTaq Gold, Perkin-Elmer Applied Biosystems, Foster City, CA.
- h. GeneAmp PCR system 9700, Perkin-Elmer Applied Biosystems, Foster City, CA.
- i. QIAquick PCR purification kit, Qiagen, Valencia, CA.
- j. Original TA cloning Kit, Invitrogen, Carlsbad, CA.
- k. ABI PRISM 377 DNA sequencer, Perkin-Elmer Applied Biosystems, Foster City, CA.
- l. MacVector[®] 6.5, Genetics Computer Group, Madison, WI.
- m. Promega Corp., Madison, WI.
- n. EZ Load Precision Molecular Mass Standard, Bio-Rad Laboratories, Hercules, CA.
- o. National Center for Biotechnology Information, Bethesda, MD.
- p. Ms. Marianne P. Garcia, Texas Department of Health, Austin, Tx.

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Development of a polymerase chain reaction and restriction typing assay for the diagnosis of bovine herpesvirus 1, bovine herpesvirus 2, and bovine herpesvirus 4 infections

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Abstract. A multiplex polymerase chain reaction (PCR) method coupled with a restriction analysis of PCR products (PCR with restriction fragment length polymorphism) was developed for the simultaneous detection of bovine herpesvirus 1, bovine herpesvirus 2, and bovine herpesvirus 4 infections. The specificity, sensitivity, and practical diagnostic applicability of this method were evaluated. This assay may be also adapted to the diagnosis of suid herpesvirus 1 and equine herpesviruses 1 and 3 and could become a powerful diagnostic tool.

The family Herpesviridae is divided into 3 subfamilies known as alpha, beta, and gamma on the basis of the genome organization. The recognized bovine herpesviruses are assigned to two subfamilies:¹⁴ bovine herpesvirus 1 (BHV1), bovine herpesvirus 2 (BHV2), and bovine herpesvirus 5 (BHV5) to the alpha-Herpesvirinae and bovine herpesvirus 4 (BHV4) and alcelaphine herpesvirus 1 to the gamma-Herpesvirinae.

Herpesviruses are agents of a wide range of disease syndromes in cattle and are the object of control and eradication

plans worldwide, because infections are associated with serious economic losses. A definitive diagnosis of herpesvirus infections in cattle is difficult on the basis of the clinical signs alone; in particular, BHV4 infections are often associated with nonspecific signs such as a febrile response, occasional mild respiratory distress, and a postpartum metritis.^{5,9,19} In BHV1 infections, although typical clinical signs may be recognized in overt outbreaks of disease, no pathognomonic signs are observed and a variety of atypical manifestations have also been described. BHV2 infections can be difficult to diagnose especially when secondary bacterial infections alter the appearance of the lesions. In areas where the disease rarely occurs, it may be confused with other viral infections of the bovine teat.¹⁸ Related herpesviruses such as BHV1 and BHV5 may cross-react in neutralization assays

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