Clinical application of a polymerase chain reaction assay in the diagnosis of pneumonia caused by *Rhodococcus equi* in a horse

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EQUINE

- Diagnosis of pneumonia caused by *Rhodococcus equi* can be made more rapidly by use of a polymerase chain reaction (PCR) assay than by use of conventional bacteriologic culture techniques.
- Use of a PCR assay aids in the differentiation between virulent and avirulent strains of *R equi,* and the assay may be used to identify *R equi* in feces and soil of breeding farms.

A 6-week-old Quarter Horse filly was examined by the Equine Field Service of the North Carolina State University College of Veterinary Medicine for signs of lethargy, cough, and fever (105 F [40.5 C]) of 1 day's duration. The filly had been born at the owner's farm and was transported to the current location, a breeding farm, at 2 weeks of age. There was no history of *Rhodococcus equi* pneumonia at either farm.

At the initial examination, rectal temperature was 103.8 F (39.8 C), respiratory rate was 40 breaths/min, and heart rate was 92 beats/min and regular. Mucous membranes were severely hyperemic, and capillary refill time was 2 seconds. There was moderate scleral injection and hyperemia of the optic disc in both eyes. Severe inspiratory and expiratory wheezes and crackles were heard bilaterally on auscultation of the thorax.

Radiographs of the thorax were obtained in the field by use of a portable X-ray machine. A fluid specimen obtained by transtracheal aspiration appeared tan and had a fetid odor. Blood was obtained for CBC and biochemical and electrolyte analyses. A presumptive diagnosis of bacterial pneumonia was made and, pending results of radiography and other diagnostic tests, treatment with potassium penicillin (20,000 U/kg [9,091 U/lb] of body weight, IV, q 6 h) and gentamicin (6 mg/kg [2.73 mg/lb], IV, q 24 h) was initiated.

Radiographic findings of the thorax included a generalized increase in interstitial opacity throughout the caudodorsal lung fields and a considerable increase in opacity in the caudoventral aspect of the thorax, with multiple foci of gas opacities superimposed over this region. The radiographic findings were consistent with pneumonia, possibly attributable to infection with *R equi*.[1,2] Considerable septic-suppurative inflammation with intracellular coccoid or coccobacillus-shaped bacteria were found on cytologic examination of the tracheal aspirate specimen. The WBC count was 10,200 cells/µl, platelet count was 249,000 thrombocytes/µl, and fibrinogen concentration was 900 mg/dl. No abnormalities were detected on serum biochemical and electrolyte analyses.

The initial antibiotic regimen for the foal was chosen to address pneumonia caused by pathogens such as *Escherichia coli, Klebsiella pneumoniae,* and *Streptococcus* spp, although the differential diagnoses also included pneumonia caused by *R equi*. Because of the severity of clinical signs, radiographic and laboratory findings, and the length of time required to identify *R equi* by routine bacteriologic culture methods (often 1 week), identification of *R equi* by use of **polymerase chain reaction (PCR)** was attempted to obtain a diagnosis more rapidly. The method of using PCR to detect the presence of *R equi* in transtracheal aspirates has been described.[3-5] Using amplification of the *R equi* 16S **ribosomal RNA (rRNA)** gene and 85 to 90 kilobase virulence plasmid, PCR can be used to detect *R equi* and differentiate between virulent and avirulent strains. Polymerase chain reaction was performed on the tracheal wash sample as described,[3,4] and it was revealed that results of the fluid analysis were positive for the virulent strain of *R equi*.

With the establishment of a definitive diagnosis of pneumonia caused by *R equi* by use of PCR, the antibiotic regimen was changed to erythromycin estolate (25 mg/kg [11.36 mg/lb], PO, q 8 h) and rifampin (5 mg/kg [2.27 mg/lb], PO, q 12 h) within 24 hours of the initial examination. The presence of *R equi* in the fluid obtained by transtracheal aspiration was confirmed by bacteriologic culture 7 days after the specimen was obtained and was identified as the sole pathogen. This treatment regimen was continued for 2 months on the basis of physical examination findings, auscultation of the thorax, and increased plasma fibrinogen concentrations. When the foal appeared clinically normal, a fluid sample was obtained by transtracheal aspiration, which yielded negative results for *R equi* by use of PCR and bacteriologic culture. Treatment with antibiotics was discontinued at that time, and the foal remained clinically normal.

The owners of the foal were extremely concerned that pneumonia caused by *R equi* had been diagnosed in their foal. They had operated a horse farm at their current location for several years and never had a diagnosis of pneumonia caused by *R equi*. Despite counseling that *R equi* is a ubiquitous pathogen found in soil, they were convinced that the breeding farm to which the foal was sent at 2 weeks of age was the source of the infection.

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In an attempt to identify the possible source of *R equi* infection, a small epidemiologic study was performed. Fecal samples were obtained from the foal's dam, 4 adult horses at the owner's farm, and 5 horses at the breeding farm and examined for the virulent strain of *R equi* by use of PCR. Briefly, DNA was extracted from fecal samples, using a modification of the technique used on the samples obtained by transtracheal aspiration.[3,5] Fecal samples (1 g) were suspended in 10 ml of a solution containing 25 m*M* EDTA, 50 m*M* Tris-HCl, and 0.5% nonidet P-40. The solution was gently mixed for 1 hour at room temperature (20 to 22 C). Each sample was centrifuged for 5 minutes at 100 × g to settle the debris. The supernatant was removed and centrifuged for 12 minutes; the resulting pellet was resuspended in 300 µl of sucrose solution (25% sucrose, 50 m*M* Tris [pH 8.0], and 1 m*M* EDTA [pH 8.0]). After adding 30 µl of lysozyme (40 mg/ml), the solution was incubated for 2 hours at 37 C. The mixture was then centrifuged for 10 minutes; the supernatant was discarded and the pellet was resuspended in 600 µl buffer with 0.5% sodium dodectyl sulfate and 100 µg of proteinase K/ml. After incubation for 1 hour at 37 C, 100 µl of 5*M* NaCl was added, followed by 80 µl of cetrimonium bromide solution. After incubation for 10 minutes at 65 C, the sample was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1); DNA was precipitated with isopropanol and washed with ethanol. After drying, the samples were resuspended in buffer. Amplification of DNA and identification of *R equi* and virulence plasmid by use of PCR was performed as described.[3,5] For all PCR reactions (tracheal wash and feces), positive controls included DNA extracted from a pure broth culture of *R equi* strains 238 (contains the virulence plasmid) and American Type Culture Collection 6939 (contains no virulence plasmid). Negative controls included a sample to which no DNA had been added. For fecal samples, DNA extracts were simultaneously amplified with primers that recognized a conserved sequence of the 16S rRNA gene of all bacteria. This control should be positive for any DNA isolated from feces and was used to confirm the lack of nonspecific polymerase inhibitors in the reaction. Positive control reactions for fecal samples included reactions in which primers that recognize a highly conserved sequence of all bacterial 16S rRNA genes were used. The *R equi* 16S rRNA gene and the 85 to 90 kilobase virulence plasmid of *R equi* were detected in the feces from 1 of the adult horses from the owner's farm. The virulence plasmid of *R equi* was not detected in feces from the dam of the foal or the horses from the breeding farm. With this information, the owners accepted that their foal may have become infected with *R equi* at their farm.

The insidious course of infection with *R equi* often makes early diagnosis of disease difficult.[6] The signalment, history of *R equi* infections on the farm, and severity of clinical signs are indicative of *R equi* infection in foals with pneumonia. The diagnosis is further supported by laboratory evidence of chronic inflammation, including increased plasma fibrinogen concentration, WBC and platelet count, and radiographic or ultrasonographic evidence of pulmonary abscesses. Serologic confirmation of the diagnosis is often unreliable.[6,7] Bacteriologic culture of fluid obtained by transtracheal aspiration is the most definitive method of diagnosis[6,8]; however, it can be difficult to reliably grow *R equi* from a single tracheal aspirate sample,[9] possibly because the organism is a facultative intracellular pathogen.[10] The presence of multiple pathogenic bacterial species in a tracheal aspirate sample can further delay positive identification of *R equi*.[3] Standard bacteriologic culture techniques do not discriminate between virulent and avirulent strains of *R equi* and may take up to 1 week for final results to be available. In contrast, performing PCR on the tracheal fluid from the foal of this report provided results in < 24 hours, and the presence of the virulence plasmid was immediately confirmed. A rapid, specific diagnosis allowed for immediate implementation of appropriate antimicrobial treatment against *R equi*. Considering the severity of the clinical signs, the rapid implementation of treatment with erythromycin and rifampin may have been an important factor in this foal's ultimate recovery. Although use of PCR was extremely helpful in providing a rapid diagnosis for this foal, the assay would be inappropriate as the sole diagnostic test. Concurrent bacteriologic culture and antimicrobial susceptibility is necessary to identify bacteria other than *R equi* that may be present and determine antimicrobial susceptibility patterns of those organisms.

In this instance, use of PCR on fecal samples was helpful in resolving a difficult public relations problem between the foal's owners and the breeding farm. The PCR analysis did not provide definitive evidence that the foal was initially infected on the owner's farm, but it did offer convincing evidence that this was a possibility. Although results of PCR analysis obtained from the horses on the breeding farm were negative, the limited number of samples analyzed and the possibility of intermittent shedding precluded any definitive statements regarding the presence of the virulent strain of *R equi* on the breeding farm. Indeed, previous epidemiologic studies suggest that virulent and avirulent strains of *R equi* are present in the soil of almost all horse farms.[11-13] The negative results obtained from the manure specimens from the horses on the breeding farm may be a reflection of the sensitivity of the assay. The PCR analysis described here was not quantitative, and the sensitivity of the assay has not been determined. Submission of samples in which *R equi* is not detected does not rule out *R equi* as a cause of pneumonia in all cases. Use of PCR for identification of *R equi* is available for veterinarians through North Carolina State University[a] and Washington State University.[b]

Polymerase chain reaction may be a useful epidemiologic tool that may be used to identify virulent strains of *R equi* in the manure or soil of breeding farms. Bacteriologic culture techniques can be used to identify *R equi*, but they do not discriminate between virulent and avirulent strains of the organism without additional labor and time-intensive plasmid purification and identification steps.[11,14] The quantity of organisms per gram of soil may be the most important factor

in *R equi* infections in foals. By adapting methods for more quantitative results, PCR may prove useful for identification of horses shedding high quantities of the virulent strain of *R equi* and paddocks and pastures that are heavily contaminated. This would facilitate the implementation of control measures to decrease the risk of infection, including immunoprophylaxis,[15] minimizing overcrowding, pasture and paddock management, and control of dust. In addition, PCR may be useful in epidemiologic studies to determine factors that influence shedding of the organism in feces and persistence of the virulent strain of *R equi* in the soil of horse farms.

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