

# Evaluation of a real-time quantitative polymerase chain reaction assay for detection and quantitation of virulent *Rhodococcus equi*

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**Objective**—To evaluate a real-time quantitative polymerase chain reaction (QPCR) assay in the detection and quantitation of virulent *Rhodococcus equi*.

**Sample Population**—1 virulent, 2 intermediately virulent, and 2 avirulent strains of *R equi* and 16 isolates of bacteria genetically related to *R equi*.

**Procedure**—The QPCR assay was evaluated for detection and quantitation of the virulence-associated gene (*vapA*) of *R equi* in pure culture and in samples of tracheobronchial fluid, which were inoculated with known numbers of virulent *R equi*. Results were compared with those derived via quantitative microbial culture and standard polymerase chain reaction methods.

**Results**—The QPCR assay detected the *vapA* gene in pure culture of *R equi* and in tracheobronchial fluid samples that contained as few as 20 CFUs of virulent *R equi*/mL and accurately quantitated virulent *R equi* to 10<sup>3</sup> CFUs/mL of fluid. The assay was highly specific for detection of the *vapA* gene of virulent *R equi* and was more sensitive than standard polymerase chain reaction for detection of *R equi* in tracheobronchial fluid.

**Conclusions and Clinical Relevance**—The QPCR assay appears to be a rapid and reliable method for detecting and quantitating virulent *R equi*. The accuracy of the QPCR assay is comparable to that of quantitative microbial culture. The increased sensitivity of the QPCR method in detection of virulent *R equi* should facilitate rapid and accurate diagnosis of *R equi* pneumonia in foals. (*Am J Vet Res* 2005;66:755–761)

*Rhodococcus equi* is a gram-positive, soil-saprophytic bacterium that shares characteristics with members of the genera *Mycobacterium* and *Corynebacterium*<sup>1</sup> and has worldwide distribution. *Rhodococcus equi* is a facultative intracellular pathogen of macrophages and causes severe pneumonia in foals.<sup>2</sup> The bacterium is also an emerging opportunistic pathogen in immunosuppressed humans.<sup>2,3</sup> In foals, virulence of *R equi* is associated with an 80- to 90-kbp plasmid containing the gene (*vapA*) that codes and mediates expression of a 15- to 17-kd virulence-associated protein (VapA) located on the cell wall surface.<sup>4–7</sup> Deletion of the *vapA*

gene attenuates virulence of the bacterium in mice.<sup>8</sup> The VapA surface protein is expressed by *R equi* isolates recovered from foals with pneumonia, but it is not uniformly expressed by isolates from other sources.<sup>2</sup> Although the exact function of VapA is not known, expression of the protein increases in response to iron deprivation, suggesting a role in the acquisition and utilization of iron by *R equi*.<sup>7,9</sup>

The quantitation of bacteria in biological specimens is typically performed by use of methods that are dependent on microbial culture (ie, determination of CFUs or the most probable number).<sup>10</sup> However, culture-dependent methods for quantitating intracellular bacteria, such as *R equi* and *Mycobacterium tuberculosis*, generally underestimate the number of viable organisms because of a tendency for the bacteria to clump.<sup>11,12</sup> Alternative methods such as immunofluorescence assays, flow cytometry, and radioisotope labeling have been successfully used to quantify *R equi*.<sup>11,13</sup> Unfortunately, these techniques are laborious and expensive and thus of marginal value for routine clinical and epidemiologic use.

The use of quantitative polymerase chain reaction (QPCR) assays in the study of infectious disease has greatly increased, and the method has rapidly gained acceptance as an effective and reliable diagnostic test.<sup>12,14,15</sup> Unlike standard polymerase chain reactions (PCRs), QPCRs can be monitored in real time by use of a fluorescent sequence detection system that allows the initial quantity of a gene to be determined. The initial copy number is deduced from the threshold cycle (Ct) value, in which the fluorescent signal generated by amplification of the target gene crosses a specified threshold.<sup>16</sup> Monitoring the reaction in real time eliminates the requirement for post-PCR analysis with agarose gel electrophoresis, thereby reducing the potential for contamination.

Diagnostic methods that use QPCR have been reported for pathogens that are slow growing and difficult to cultivate, such as *Rickettsia rickettsii* and *M tuberculosis*.<sup>12,14</sup> To the authors' knowledge, use of QPCR assays has not been reported for quantitation of *R equi*. The objective of this study was to evaluate a

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QPCR assay based on quantitation of the *vapA* gene for detection and quantitation of virulent *R equi*. Results of the assay were compared with results of quantitative microbial culture to evaluate the ability of the assay to determine the quantity of virulent *R equi*. The limit of detection of the assay was compared with that of standard PCR assay by use of serial dilutions of virulent *R equi* in phosphate-buffered saline solution and tracheo-bronchial fluid samples inoculated with known numbers of the bacterium.

## Materials and Methods

**Preparation of *R equi* cultures**—Five strains of *R equi* were used: a *vapA*<sup>+</sup> reference strain (American Tissue Culture Collection [ATCC] 33701p+), a *vapA*<sup>-</sup> reference strain (ATCC 33703), an isogenic plasmid-cured strain (ATCC 33701p-),<sup>17</sup> and 2 *vapB*<sup>+</sup> strains (S1 and S3). Bacteria were inoculated into *R equi*-minimal medium<sup>7,18</sup> and rotated<sup>b</sup> overnight (10 revolutions/min) at 37°C.

Ten-fold serial dilutions of *R equi* strain 33701p+ (range, 10<sup>1</sup> to 10<sup>8</sup> CFUs/mL) were prepared in PBS<sup>c</sup> solution, and CFU numbers were determined by use of plate counts on trypticase soy blood agar.<sup>d</sup> To permit visualization of the bacterial pellet and standardize the concentration of *R equi* from which DNA was extracted, dilutions were inoculated with approximately 10<sup>8</sup> CFUs of *R equi* 33701p-. Six sets of serial dilutions (approx range, 10<sup>1</sup> to 10<sup>8</sup> CFUs/mL) were prepared for quantitative microbial culture and QPCR analysis.

**Preparation of *R equi* DNA**—The DNA was extracted from each of the 6 sets of dilutions by use of a modified alkaline lysis procedure.<sup>19</sup> After centrifugation for 20 minutes at 10,000 × g at 4°C, bacteria were incubated for 10 minutes at 21°C with 300 μL of lysing solution, which contained Tris-HCl EDTA (TE) buffer (10mM Tris-HCl and 1mM EDTA [pH, 8.0]), 0.1N NaOH, and 0.5% SDS. Cellular debris was precipitated by addition of 150 μL of sodium acetate (3.0N; pH, 5.2) and centrifugation for 2 minutes at 14,000 × g. The supernatant was decanted into a fresh microcentrifuge tube, and 10 μg of yeast tRNA<sup>e</sup> was added to each dilution as a source of carrier nucleic acid. The DNA was precipitated with cold ethanol and centrifugation for 20 minutes at 14,000 × g at 4°C. After the pellets were dried, 50 μL of TE buffer (pH, 7.4) was added to reconstitute the DNA, which was stored at -20°C until analyzed.

**Plasmid standard for absolute quantitation of DNA copy number**—The *vapA* coding sequence was amplified from strain 33701 by use of published<sup>20</sup> primer sequences modified by insertion of a *PacI* restriction site at the 5' end. The plasmid (pVAPA01) was constructed by inserting the 564-bp product into a cloning vector.<sup>f</sup> Plasmid DNA was purified by use of a commercially available kit,<sup>g</sup> and the concentration was determined via spectrophotometer.<sup>h</sup> Stock solutions of known concentration of plasmid DNA were stored at -86°C until analyzed.

**Primer selection**—Primer sequences were designed by use of a commercial software program<sup>i</sup> and were based on the 564-bp coding sequence of *vapA* for *R equi* strain 33701. The primers were selected based on their lack of sequence homology with other known organisms in a database search.<sup>j</sup> The following sequences were chosen for use in the assay on the basis of their specificity for *vapA*: VPF5, 5' AACGGTCGAGCAAGCGATAC 3'; and VPB6, 5'GGCCC-GAATACGTGAAACCT 3'.

**QPCR conditions**—Each reaction contained the following: 2.5 μL of plasmid or template DNA (approx 4.8 μg of template DNA); 0.25 μL of each primer (12 and

17 pmol of VPF5 and VPB6, respectively); 12.5 μL of a commercially prepared PCR reagent mixture<sup>k</sup>; 2.5 μL of a commercial PCR enhancer<sup>l</sup> designed to reduce primer dimer formation; and molecular-grade water<sup>m</sup> to a final reaction volume of 25 μL. Amplification and data analyses were carried out on a real-time thermocycler.<sup>n</sup> The thermal profile consisted of an initial denaturation at 95°C for 10 minutes, followed by 40 cycles (94°C for 15 seconds, 62°C for 30 seconds, and 72°C for 20 seconds). A final extension was carried out for 7 minutes. To confirm the specificity of each QPCR assay, a dissociation protocol was performed, generating a melting curve profile. The dissociation protocol consisted of a 15-second hold at 95°C and a 20-second hold at 60°C, followed by a 20-minute slow ramp from 60° to 95°C during which the dissociation data were recorded.

**Construction of a standard curve**—Ten-fold serial dilutions of plasmid DNA (approx range, 10<sup>1</sup> to 10<sup>8</sup> copies of pVAPA01/μL) were prepared in TE buffer (pH, 8.0). Plasmid DNA standards were processed in triplicate via QPCR assay. A standard curve was constructed by use of linear regression analysis of the log<sub>10</sub> quantity of pVAPA01 copies per sample and the corresponding Ct values.

**Assay validation**—Assay validation was performed to determine intra- and interassay variability and to demonstrate repeatability of the method used for sample preparation. Experiments were evaluated in terms of the coefficient of variation (CV), which was calculated by dividing the mean SD value by the respective Ct. Sets of DNA (each extracted from 8 dilutions of virulent *R equi*) were chosen at random for use in the validation experiments.

To validate the method of sample preparation, all 6 sets of DNA (ie, 48 samples) were analyzed concurrently by use of QPCR. Coefficients of variation were calculated for corresponding *R equi* concentrations.

To determine the interassay variability, 1 set (8 samples) of DNA was analyzed in 3 separate QPCR assays. Coefficients of variation were calculated across reactions for each concentration of *R equi*.

Intra-assay variability was assessed with 10 replicates of 1 set of DNA extracted from dilutions containing approximately 10<sup>4</sup>, 10<sup>6</sup>, and 10<sup>8</sup> CFUs of *R equi*/mL of PBS solution. The dilutions were chosen because they represented the range over which virulent *R equi* organisms are commonly detected in clinical and biological specimens.<sup>21,22</sup> Coefficients of variation were calculated for each concentration of *R equi*.

**Standard PCR**—By use of the thermal profile described, a thermocycle unit<sup>o</sup> was used to perform standard PCR assays, with the exception that the initial denaturation was conducted at 94°C for 5 minutes and followed by 30 amplification cycles. Each reaction contained the following: 2 μL of template DNA; 0.2 μL of *Taq* DNA polymerase<sup>p</sup>; 2 μL of MgCl<sub>2</sub> (2.5mM final concentration); 2 μL of Mg<sup>2+</sup>-free buffer<sup>q</sup>; 2.5 μL of a commercial PCR enhancer<sup>r</sup>; 0.4 μL of dNTPs (0.1mM each dNTP); 0.25 μL of each primer (12 and 17 pmol VPF5 and VPB6, respectively); and molecular-grade water to a total reaction volume of 20 μL. After sequence amplification, 15 μL of the PCR mixtures was electrophoresed on 2.0% agarose gel stained with ethidium bromide. Amplicons were visualized by use of UV fluorescence.

**Specificity of the QPCR assay**—To determine the specificity of the assay for *vapA*, 1 *vapA*<sup>+</sup> and 2 *vapB*<sup>+</sup> *R equi* strains and 16 genetically related non-*R equi* isolates were analyzed via QPCR assay (Appendix). By use of the alkaline lysis technique, the DNA was extracted from single colonies incubat-

ed on blood agar plates for 48 hours at 37°C. Results were considered negative for a sample if there was no melting peak at approximately 83°C and if the Ct value on the amplification plot was  $\geq 35$  cycles.

**Detection and quantitation of virulent *R equi* in tracheobronchial fluid**—Tracheobronchial fluid was obtained from a foal with pneumonia by use of a standard tracheobronchial aspirate procedure. The fluid specimen was submitted to the clinical microbiology laboratory for bacterial species identification. Results of microbial culture of the fluid were positive for growth of *Streptococcus zooepidemicus* and *Bacillus* sp. *Rhodococcus equi* bacteria were not isolated from the sample.

Aliquots of broth incubated overnight for approximately 16 hours with *R equi* strain 33701 were centrifuged for 10 minutes at  $1,600 \times g$ , the supernatant was discarded, and the bacteria were resuspended in sterile PBS solution. A 100- $\mu$ L aliquot of the bacterial suspension was added to 900  $\mu$ L of tracheobronchial fluid and subjected to 10-fold serial dilutions (approx range,  $10^1$  to  $10^7$  CFUs of virulent *R equi*/mL). Quantitative microbial culture was performed to confirm the concentration of *R equi* in each tracheobronchial fluid sample. The concentration of *R equi* inoculated into the tracheobronchial fluid was determined via quantitative microbial culture to adjust for any growth inhibition that may have resulted from bacteriostatic agents in the tracheobronchial fluid.

The alkaline lysis method was used to extract DNA from each fluid sample. The QPCR analysis of each dilution was performed in duplicate, and results were compared with those obtained via quantitative microbial culture. The DNA extracts were analyzed by use of standard PCR assay and agarose gel electrophoresis, and a dichotomous outcome of detection or failure was used for comparison with results of QPCR assays.

## Results

**Selection of primers**—Four primer pairs were evaluated for use in the QPCR assay. The primer sequences were designed to amplify inside the 564-bp coding region of the *vapA* gene, the region from which the plasmid standards were constructed. The QPCR assay and melting curve analyses were performed on 10-fold dilutions of the quantitation standards. A single sharp peak at approximately 83°C on the melting curve indicated amplification of *vapA*. Additional peaks at lower temperatures indicated nonspecific amplification.

The formation of primer dimers occurred frequently with amplification involving the first 2 primer pairs, especially at the lower end of the range of target concentrations. The addition of a PCR enhancer to the master mix reduced the formation of secondary structures, but not enough to abolish interference with the quantitative ability of the assay. The second set of primers yielded nonspecific amplification of *R equi* isolates positive for the *vapB* gene. These results rendered the first and second primer pairs unsuitable for use in the assay.

The third and fourth primer pairs differed from each other only in the sequence of the reverse primer. Although both primer pairs amplified *vapA*, the reverse primer (VPB6) was chosen for use in the assay because it contained a greater number of mismatches for *vapB*, thus decreasing the possibility of nonspecific amplification.

Occasionally, the melting curve profiles of the nontemplate and negative control samples contained a peak at approximately 75°C, a discrepancy of approximately 10°C from the predicted melting temperature of the primers. Primer dimer formation is typically evident by a peak at or  $< 65^\circ\text{C}$ .<sup>23</sup> This anomaly suggested formation of a cross dimer between the forward and reverse primers. Subsequent analysis of the primer pair by use of a commercial software program<sup>†</sup> predicted the formation of a 3' cross dimer. However, amplification of the cross dimer occurred only in *vapA*<sup>-</sup> samples and therefore had no adverse effects on the quantitative function of the assay.

**Construction of a standard curve**—Results of regression analysis of amplification of pVAPA01 revealed a strong linear correlation ( $R^2 = -0.996$ ; slope =  $-3.10$ ) between the initial copy number of the plasmid and the corresponding Ct value. A single peak was observed at approximately 83°C in the melting curve profile of the entire dilutional range of pVAPA01 (Figure 1).

**Assay validation**—To determine the efficiency and reproducibility of the method used to prepare the samples, 6 sets of DNA were analyzed concurrently via QPCR assay. Mean CV across all sets was 3.54% (range, 2.3% to 4.9%).

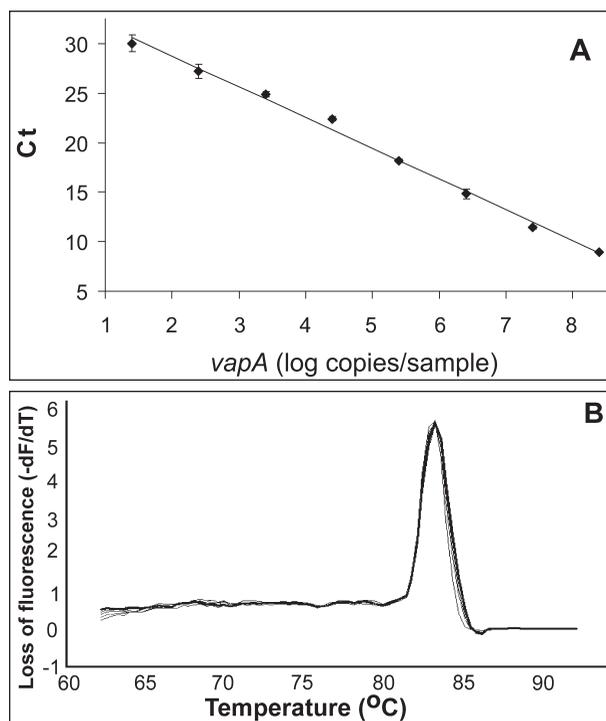


Figure 1—Results from quantitative polymerase chain reaction (QPCR) analysis of a series of 10-fold dilutions of a plasmid (pVAPA01) containing the virulence-associated gene (*vapA*; range,  $2.5 \times 10$  to  $2.5 \times 10^9$  copies/reaction). A—Standard curve of the log<sub>10</sub> quantities of pVAPA01 plotted against threshold cycle (Ct) values. The points about the regression line represent the mean Ct value of triplicate QPCR amplification of the pVAPA01 sequence. Error bars represent 1 SD from the mean. The slope and correlation coefficient of the data points were  $-3.10$  and  $R^2 = -0.996$ , respectively. B—Melting curve profile of pVAPA01. Notice the single peak at approximately 83°C.

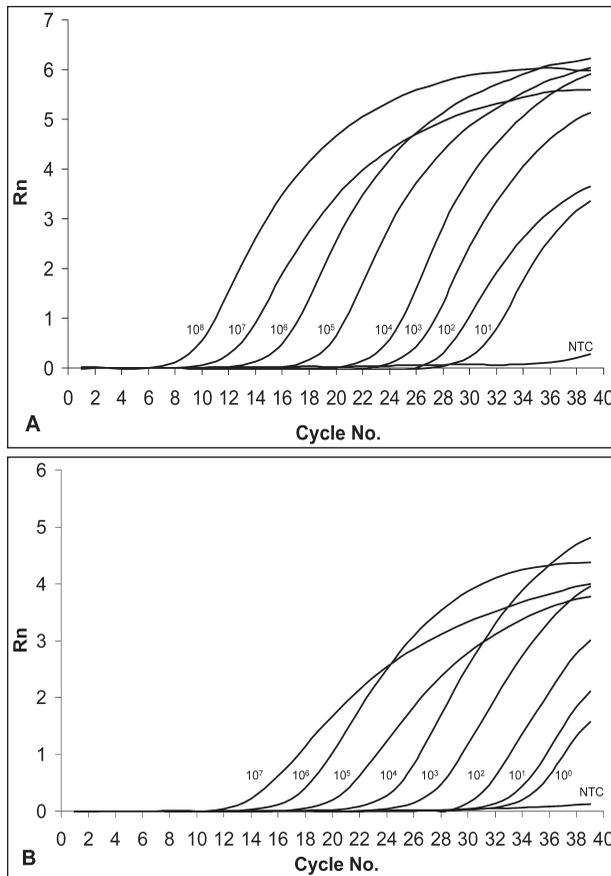


Figure 2—Relationship between the QPCR assay and quantitative microbial culture for quantitation of virulent *Rhodococcus equi* and linear regression analysis of a series of dilutions of virulent *R equi* American Tissue Culture Collection (ATCC 33701) present in pure culture at concentrations of  $10^1$  to  $10^8$  CFUs/mL (A) and tracheobronchial fluid at concentrations of  $10^1$  to  $10^7$  CFUs/mL (B). For each dilution, datum point represents mean Ct value and error bars indicate 1 SD from the mean. NTC = Nontemplate control. Rn = Fluorescence.

Intra-assay variation was evaluated by use of 10 replicates each of DNA extracted from dilutions that contained approximately  $10^4$ ,  $10^6$ , and  $10^8$  CFUs of virulent *R equi*/mL of PBS solution. Mean CVs were 3.58%, 2.0%, and 1.31% for the  $10^4$ ,  $10^6$ , and  $10^8$  dilution factors, respectively.

Interassay variation was determined by use of 3 separate QPCR assays involving dilutions from another set of DNA. Mean CVs for each dilution factor across reactions ranged from 0.6% to 1.69%. Mean overall CV was 0.90%.

No correlation was observed between the CV and concentration of bacteria in the sample for any of the validation experiments. The range of CVs for all validation assays was 0.6% to 4.9%, with most being < 2.5%. Amplification plots of pure *R equi* DNA had general linearity that was comparable to that of the amplification plots of pVAPA01 (Figure 2). The melting curve profile of virulent *R equi* was consistent with that of pVAPA01.

**Detection and quantitation of virulent *R equi* from pure culture and from tracheobronchial fluid—**Via QPCR analysis of DNA extracted from samples of virulent *R equi* that had been diluted to concentrations

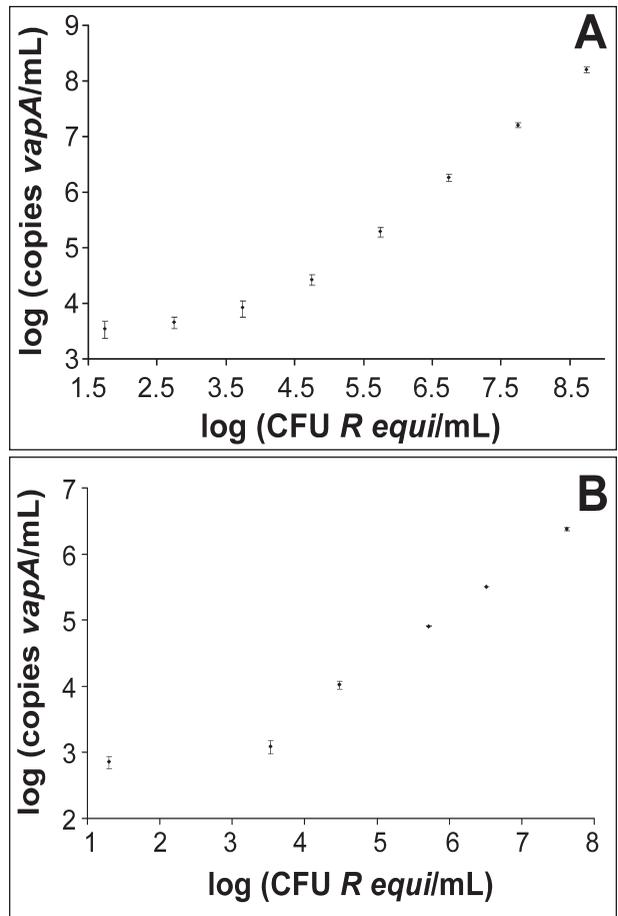


Figure 3—Amplification plots for pVAPA01 in concentrations of  $2.5 \times 10^1$  to  $2.5 \times 10^8$  copies/reaction (A) and for DNA extracted from pure culture dilutions of virulent *R equi* ATCC 33701 in final concentrations of  $10^1$  to  $10^7$  CFUs/reaction (B).

in the range of approximately  $10^1$  to  $10^8$  CFUs/mL of pure culture and  $10^0$  to  $10^7$  CFUs/mL of tracheobronchial fluid, *vapA* was detected in all samples. When results were expressed as a dichotomous outcome (ie, detected or not detected), the QPCR assay detected *vapA* in pure culture and in tracheobronchial fluid samples inoculated with as few as 20 CFUs of virulent *R equi*/mL. Standard PCR assays were performed on the fluid to compare the minimum limit of detection with that of the QPCR assay. Agarose gel electrophoresis of the standard PCR products yielded positive results for *vapA* in tracheobronchial fluid samples that contained approximately  $10^5$  to  $10^7$  CFUs of virulent *R equi*/mL; however, *vapA* was not detected via standard PCR assay in samples containing <  $10^4$  CFUs of virulent *R equi*/mL of fluid.

The ability of the assay to quantify organisms in pure culture and tracheobronchial fluid was evaluated by comparing the  $\log_{10}$  quantity of *R equi* (determined by quantitative microbial culture) with the value determined by QPCR assay (Figure 3). For concentrations  $\geq 10^4$  CFUs/mL of PBS solution and  $\geq 10^3$  CFUs/mL of tracheobronchial fluid, the QPCR assay underestimated the  $\log_{10}$  quantity of *R equi* by approximately 7% and 11%, respectively. For concentrations  $\leq 10^4$  CFUs/mL of PBS solution and  $\leq 10^3$  CFUs/mL of tra-

cheobronchial fluid, the QPCR assay overestimated the  $\log_{10}$  quantity of *R equi*.

**Specificity of the assay**—All bacteria used to assess the specificity of the assay for *vapA* were *vapA*<sup>-</sup> (ie, absence of a melting peak at 83°C and Ct value  $\geq 35$  cycles; Appendix).

## Discussion

*Rhodococcus equi* is an important cause of disease and death in foals and is a recognized opportunistic pathogen in humans.<sup>2,21</sup> The insidious onset of *R equi* pneumonia in foals makes detection difficult early in the course of disease. Although foals appear to be infected early in life,<sup>24</sup> clinical signs do not usually become apparent before 2 to 3 months of age, by which time pyogranulomatous lesions in the lungs are well established.<sup>2,25</sup> Early diagnosis of the disease is important in achieving a favorable outcome in affected foals.

A diagnosis of *R equi* pneumonia is presently made on the basis of radiographic evidence of characteristic pulmonary opacities, high plasma fibrinogen concentrations, and detection of the bacterium via microbial culture of tracheobronchial fluid specimens. Although microbial culture of tracheobronchial fluid is valuable in the diagnosis of rhodococcal pneumonia, some disadvantages are associated with its use. The time required for *R equi* to grow in culture may delay appropriate treatment of the disease,<sup>25</sup> and false-negative results may occur because of bacteriostatic agents and contaminating bacteria. Despite the disadvantages of invasiveness and a level of restraint needed to perform the tracheobronchial procedure that is potentially dangerous in foals with respiratory distress, a tracheobronchial fluid specimen is necessary to confirm *R equi* infection.<sup>26-28</sup>

Results of the present study indicate that the QPCR assay has high sensitivity for detection of the *vapA* gene. As few as 20 CFUs of virulent *R equi*/mL of fluid, or approximately 0.7 CFUs/QPCR, were detected. Because this value is  $< 1$ , some (approx 3/10) reactions should have yielded negative results (ie, failed to detect *R equi*). A possible explanation for the greater than predicted sensitivity of the assay is that there was contamination of the QPCR assay, which resulted in false-positive findings; however, stringent precautions were taken in handling samples and reagents (eg, handling of DNA and QPCR reagents in separate rooms and use of barrier pipette tips) to minimize the possibility of contamination. Furthermore, the controls in each QPCR experiment yielded no amplification plots or melting peaks, indicating that false-positive results from contamination were unlikely. Another explanation is that the QPCR assay may have been amplifying nucleic acids from dead (nonviable) virulent *R equi*, as has been reported<sup>29,30</sup> in PCR amplification of nucleic acids from other bacteria, including species related to *R equi*. The authors believe that the latter possibility likely explains the greater than expected sensitivity of the assay. In the present study, QPCR assay was 4  $\log_{10}$  units greater in sensitivity than the standard PCR assay. The sensitivity of standard PCR assay in detecting

*R equi* in tracheobronchial fluid was consistent with that reported<sup>26</sup> previously.

Because the QPCR assay was more sensitive for the detection of *R equi*, compared with the standard PCR assay, it is anticipated that use of the QPCR assay will enable detection of minute quantities of virulent *R equi* in clinical specimens. There is evidence that quantitation of *R equi* in such specimens may be of diagnostic value in clinically affected foals; exhaled breath samples of clinically affected foals were found to yield significantly more virulent *R equi* than unaffected foals from the same environment.<sup>31</sup> The high specificity of the assay makes it potentially useful for detecting and quantitating virulent *R equi* in heavily contaminated specimens such as feces and soil, samples that may be studied for epidemiologic purposes.<sup>21</sup> For example, the assay could be used to determine whether dams of affected foals have greater fecal shedding of virulent *R equi* prior to onset of disease in their foals, compared with dams of nonaffected foals. A recent report<sup>31</sup> described the use of selective media and colony-blotting DNA hybridization to quantify and detect virulent and avirulent isolates of *R equi* from environmental specimens, but the method is laborious and requires use of radioisotopes, which limits its usefulness for diagnostic and epidemiologic purposes.

Advantages of the QPCR assay over traditional methods used to quantitate *R equi* in a specimen include rapidly available results, the ability to detect and quantify virulent organisms with high sensitivity and specificity, elimination of post-PCR analyses and the problem posed by clumping of bacteria.<sup>10-12</sup> The assay was conducted with SYBR Green, making it less expensive than QPCR methods that use sequence-specific probes, an important advantage for clinical and research laboratories.

The QPCR assay detected the *vapA* gene in virulent *R equi*. A problem encountered in choosing appropriate primers for the assay was nonspecific amplification of *R equi* isolates that contained the *vapB* gene. That gene, an isogene of *vapA*, was originally identified in isolates of *R equi* from human infections and later from a pig.<sup>22,32</sup> On the basis of murine challenge studies, *R equi* isolates with the *vapB* genotype are classified as intermediate in virulence.<sup>3,22</sup> The clinical relevance of *vapB* in equine isolates is unknown; the genotype has not been reported in equine infections, and foals challenged with *vapB*<sup>+</sup> *R equi* do not develop disease.<sup>32</sup> A previous study<sup>33</sup> revealed that *vapA* and *vapB* genes share approximately 85% sequence homology, and this similarity is likely responsible for the observed amplification of *vapB*<sup>+</sup> *R equi* in the samples in our study. Therefore, to avoid nonspecific amplification, we chose the set of primers that had the greatest number of mismatches with the nucleotide sequence of *vapB*.

Another problem was apparent loss of target DNA during the extraction process. Loss of DNA can be minimized by reducing the number of steps used to process the specimens. We used a simple alkaline lysis method to extract DNA from pure culture dilutions of virulent *R equi*, thereby reducing loss of DNA and eliminating use of organic compounds, such as phenol and

chloroform, that are toxic and require special handling. Alkaline lysis DNA extraction procedures are typically used for purification of plasmid DNA. However, the method used in the present study was effective in purifying chromosomal DNA as well as plasmid DNA, justifying its use in the extraction of DNA from all bacteria. The alkaline lysis method was also used to extract DNA from tracheobronchial fluid, which commonly contains potent inhibitors of PCRs. Surprisingly, the alkaline lysis extraction resulted in high-quality DNA. To the authors' knowledge, this is the first report of successful extraction of *R equi* DNA from tracheobronchial fluid by use of this method.

On the basis of the size of the virulence plasmid (85 to 90 kbp), it was assumed that virulent *R equi* and the *vapA* copy number occurred in a 1:1 ratio. With that assumption, the log<sub>10</sub> quantity of *R equi* in pure culture and in tracheobronchial fluid was underestimated by approximately 7% and 11%, respectively. Correcting for this discrepancy allowed the quantity of virulent *R equi* (in concentrations ranging from 10<sup>3</sup> to 10<sup>8</sup> CFUs/mL) to be estimated with reasonable accuracy. Plots of the *vapA* copy number against the Ct value became nonlinear at concentrations ≤ 10<sup>3</sup> CFUs/mL, indicating that *R equi* was not quantified accurately when present in low numbers. The observed deviation from linearity was expected because the relative difference among dilutions containing low concentrations of *R equi* is much greater in magnitude than in dilutions containing high concentrations of *R equi*. Use of a nonlinear regression model for calibration may enable the QPCR assay to be used to quantify virulent *R equi* at concentrations ≤ 10<sup>3</sup> CFUs/mL, but will require additional experiments. The sensitivity with which the QPCR assay detected *vapA* permitted detection of < 10<sup>3</sup> CFUs of virulent *R equi*/mL, demonstrating that the assay was semiquantitative with regard to detection of virulent *R equi* across an 8 log range (ie, 10<sup>1</sup> to 10<sup>8</sup>) and accurately quantitative above 10<sup>3</sup> CFUs/mL. Furthermore, the high sensitivity of the QPCR assay in detecting virulent *R equi* in tracheobronchial fluid provided compelling evidence of its potential diagnostic applications.

- a. Provided by Dr. Shinji Takai, Department of Animal Hygiene, School of Veterinary Medicine and Animal Science, Kitasato University, Towada, Aomori, Japan.
- b. Laboratory rotator, Glas-Col Apparatus Co, Terre Haute, Ind.
- c. Phosphate-buffered saline, Gibco BRL, Frederick, Md.
- d. Trypticase soy agar with 5% sheep RBC, BBL Prepared Media, Becton-Dickinson Microbiology Systems, Cockeysville, Md.
- e. Yeast tRNA, Sigma Chemical Co, St Louis, Mo.
- f. pENTR/D-TOPO, Invitrogen Corp, San Diego, Calif.
- g. Qiagen Plasmid Midi, Qiagen, Germantown, Md.
- h. Smartspec 3000, Bio-Rad Laboratories, Hercules, Calif.
- i. Primer Express, Applied Biosystems, Foster City, Calif.
- j. Basic Local Alignment Search Tool (BLAST), National Center for Biotechnology Information, Bethesda, Md. Available at: [www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/). Accessed May 24, 2004.
- k. SYBR Green PCR Master Mix, Applied Biosystems, Foster City, Calif.
- l. MasterAmp, Epicentre, Madison, Wis.
- m. Distilled water, DNase RNase free, Gibco BRL, Frederick, Md.
- n. GeneAmp 5700 Sequence Detection System, Applied Biosystems, Foster City, Calif.
- o. GeneAmp PCR System 9700, Applied Biosystems, Foster City, Calif.
- p. Taq DNA polymerase in storage buffer B, Promega, Madison, Wis.
- q. Mg<sup>2+</sup>-free buffer, Promega, Madison, Wis.
- r. Net Primer, PREMIER Biosoft International, Palo Alto, Calif.

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Appendix—Bacteria morphologically similar or genetically related to *Rhodococcus equi* that were used to assess specificity of a quantitative polymerase chain reaction assay for detection of the virulence-associated gene (*vapA*) in *R equi*.

Species	Source or identification
<i>Gordonia aichiensis</i>	ATCC 33611
<i>Gordonia rubripertincta</i>	ATCC 14352
<i>Gordonia bronchialis</i>	ATCC 25592
<i>Gordonia sputi</i>	ATCC 29627
<i>Gordonia terrae</i>	ATCC 25594
<i>Rhodococcus equi</i>	ATCC 33703*
<i>R equi</i>	S1† <sup>a</sup>
<i>R equi</i>	S3b† <sup>a</sup>
<i>Rhodococcus coprophilus</i>	ATCC 29081
<i>Rhodococcus fascians</i>	ATCC 12794
<i>Rhodococcus erythropolis</i>	ATCC 4277
<i>Rhodococcus opacus</i>	ATCC 51882
<i>Rhodococcus percolatus</i>	DSM 44240
<i>Rhodococcus rhodnii</i>	ATCC 35071
<i>Rhodococcus rhodochrous</i>	ATCC 271
<i>Rhodococcus ruber</i>	Mayo Clinic
<i>Nocardia brasiliensis</i>	Mayo Clinic
<i>Nocardia nova</i>	Mayo Clinic
<i>Tsukamurella tyrosinosolvans</i>	Mayo Clinic

\**vapA*. †*vapB*.  
 ATCC = American Tissue Culture Collection. DSM = Deutsche Sammlung von Mikroorganismen und Zeeellkulturen (German Collection of Microorganisms and Cell Cultures).