# Diagnostic evaluation of real-time PCR in the detection of *Rhodococcus equi* in faeces and nasopharyngeal swabs from foals with pneumonia

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*Rhodococcus equi* is a Gram-positive, facultative, intracellular bacterial pathogen and the most common cause of severe infectious pneumonia in foals of less than six months of age (Prescott 1991). The pathogen is ubiquitous in the environment, but becomes concentrated in breeding farm situations due to its ability to reproduce in the gastrointestinal tract of herbivores. A foal is suspected of having *R equi* pneumonia if it originates from an endemic farm, has signs of pneumonia, a severe inflammatory leukogram and a prominent alveolar and/or interstitial pattern on thoracic radiographs (Ainsworth 1999). Bacterial culture combined with cytological examination of a tracheobronchial aspirate is the only acceptable way of making a definitive diagnosis of *R equi* pneumonia. Although culture is the only means of identifying concurrent bacterial pathogens and testing in vitro antimicrobial susceptibility, it can yield false-negative results, possibly because of previous antimicrobial administration or overgrowth by multiple bacterial species (Sweeney and others 1987). Furthermore, culture does not allow differentiation between virulent and avirulent strains of *R equi*. In recent years the use of PCR amplification based on the *VapA* gene sequence has been shown to be a more sensitive means of identifying *R equi* in tracheal wash (TW) fluid samples than bacterial culture, especially if the foal sampled is being treated with antimicrobials at that time (Sellon and others 2001, Halbert and others 2005). Occasionally, the severity of clinical signs in foals suspected of having *R equi* pneumonia precludes the sampling of the lower airways.

This short communication describes a study in which, during the search for alternative diagnostic specimens, it was hypothesised that PCR analysis of nasopharyngeal swabs (NS) and faeces collected from foals diagnosed with *R equi* pneumonia would be a sensitive alternative to standard culture techniques.

Clinical samples were collected from 31 foals that had been referred to the Veterinary Medical Teaching Hospital, University of California, Davis, due to pneumonia. The foals ranged from four weeks to seven months of age (mean [sd] 3·3 [1·6] months). There were 17 female and 14 male foals, which included 15 thoroughbred, five quarter horse, five Arabian, two paint horse and one of each of Friesian, warmblood, standardbred and Percheron breeds. Before presentation, 19 of the 31 foals had been treated with a variety of antimicrobials (rifampin, azithromycin, erythromycin, doxycycline, penicillin procaine G, ampicillin, gentamycin, ceftiofur and trimethoprim-sulfamethoxazole) for one to 30 days (8·9 [7·7] days).

All foals displayed clinical signs (cough, nasal discharge, tachyponea, fever and adventitial lung sounds) consistent with bacterial pneumonia on presentation. A complete blood count (CBC) was available from all foals and showed a normal leukocyte count (range $5 \times 10^9$ to $11{\cdot}6 \times 10^9$ white blood cells/l) and fibrinogen (1 to 4 g/l) in four foals, leukocytosis in seven foals, hyperfibrinogenemia in five foals and leukocytosis with hyperfibrinogenemia in 14 foals. TW fluid was obtained via a standard aseptic technique in 29 foals and aliquoted into evacuated glass tubes containing no anticoagulant, for cytology, culture and real-time PCR analysis. Cytology showed marked to severe suppurative inflammation in all submitted samples. TW fluid samples were not collected from two of the foals because of severe respiratory distress. Chest radiographs performed on 27 foals showed a bronchointerstitial (11), alveolar (six) and/or interstitial (10) pattern. Pulmonary abscesses were suspected in seven foals.

For PCR analysis, NS and faeces were collected from all of the foals. Furthermore, faecal samples from 13 mares accompanying their sick foals (that is seven non-*R equi* and six *R equi* foals) were also collected for PCR analysis. Final classification of an *R equi* confirmed case was based on all available information, including history, clinical presentation, radiographic and laboratory (CBC, biochemical panel, TW fluid cytology) results, culture and PCR results, and postmortem results. The final classification was used as a reference standard for the calculation of diagnostic sensitivity and specificity for culture of TW fluid, and PCR of TW fluid, NS and faeces.

Genomic DNA (gDNA) extraction from TW fluid samples, NS and faeces was performed using a commercial DNA (TW fluid, NS – DNEasy Blood Kit; Qiagen) and stool (faeces – QIAamp DNA Stool; Qiagen) kit according to the manufacturer's instructions. A real-time TaqMan PCR assay for *R equi* was established and validated. The assay is based on the detection of a specific 75 base pair product of the *VapA* gene of *R equi* (GenBank accession number AF116907; oligonucleotides: forward primer CAGCAGTGCGATTCTCAATAGTG, reverse primer CGAAGTCGTCGAGCTGTCATAG, probe CAGAACCGACAATG CCACTGCCTG). Amplification efficiency was calculated from the slope of a standard curve generated from a 10-fold diluted *R equi*-positive DNA sample. High amplification efficiency for the target gene of 95 per cent indicated a high analytical sensitivity. Analytical specificity was verified by sequencing TaqMan PCR products for the target gene. PCR reactions contained 400nM of each primer, 80nM of the TaqMan probe and mastermix (TaqMan Universal PCR Mastermix; Applied Biosystems) and 1 µl of the gDNA sample, in a final volume of 12 µl. The samples were amplified in a combined thermocycler/fluorometer (ABI PRISM 7700 Sequence Detection System; Applied Biosystems) for two minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. DNA extraction and amplification efficiency was verified by quantifying the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase.

Based on all the available information (clinical presentation and diagnostic test results) 12 of the 31 foals (39 per cent) were diagnosed with *R equi* pneumonia. TW fluid cultures yielded bacterial growth for 26 of 29 foals (90 per cent). The following bacterial species were isolated: *Streptococcus zooepidemicus* (16 foals), *R equi* (nine), *Actinobacillus* species (five), *Escherichia coli* (three), and *Bordetella bronchiseptica* (two). Multiple bacterial species were isolated from the TW fluid of nine of 29 foals (31 per cent). *R equi* was the only cultured bacteria in eight TW fluid samples, while one TW fluid sample grew *R equi* and *S zooepidemicus*. No bacteria were isolated from the TW fluid of three of the 29 foals (10 per cent). *R equi* was cultured postmortem from the lungs of one foal in which TW fluid sampling was not performed due to severe respiratory distress.

Real-time PCR analysis of TW fluid samples was positive in 11 of 29 foals (38 per cent). Agreement between culture

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**TABLE 1: Culture of tracheal wash (TW) fluid and PCR analysis of TW fluid, nasopharyngeal swabs (NS) and faeces from 12 foals diagnosed with *Rhodococcus equi* pneumonia**

| | TW fluid culture | TW fluid | *R equi* PCR NS | Faeces | Antimicrobial used before referral (days)† |
|---|---|---|---|---|---|
| 1 | *Escherichia coli*, *Streptococcus* species | + | + | + | None |
| 2 | *Streptococcus zooepidemicus* | + | – | – | Doxycycline (22) |
| 3 | *R equi* | + | – | + | TMS (1) |
| 4 | *R equi* | + | – | + | None |
| 5 | *R equi* | + | – | + (15)‡ | Ceftiofur (10) |
| 6* | ND | ND | – | + | Azithromycin (10) |
| 7 | *R equi* | + | – | – | Azithromycin (30) |
| 8 | *R equi* | + | – | + (4)‡ | Azithromycin (1) |
| 9 | *R equi* | + | – | + | None |
| 10 | *R equi* | + | – | – | Azithromycin (10) |
| 11 | *R equi* | + | – | + | Azithromycin (7) |
| 12 | *R equi*, *S zooepidemicus* | + | – | + (7)‡ | Amikacin, PPG (1) |
| Total | 9/11 (82 per cent) | 11/11 (100 per cent) | 1/12 (8 per cent) | 9/12 (75 per cent) | |

* Foal without TW fluid collection due to severe respiratory distress. *R equi* cultured postmortem from lungs
† Antimicrobial given before referral to VMTH
‡ Numbers in parentheses represent days of *R equi* PCR-positive faecal samples after initiation of antimicrobial treatment at VMTH
TMS Trimethoprim-sulfamethoxazole, ND Not done, PPG Penicillin procaine G
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**TABLE 2: Accuracy of tracheal wash (TW) fluid culture and TW fluid, nasopharyngeal swab (NS) and faecal PCR for *Rhodococcus equi* pneumonia**

| Diagnostic test* | Sensitivity (%) | Specificity (%) |
|---|---|---|
| TW fluid culture | 82 | 100 |
| TW fluid PCR | 100 | 100 |
| NS PCR | 8·3 | 100 |
| Faecal PCR | 75 | 100 |

* Diagnostic tests were compared to the reference standard cases (history, clinical presentation, radiographic and laboratory results, culture and PCR results and postmortem examination)

and PCR was 82 per cent (Table 1). Two foals (foal 1 and 2) were positive for *R equi* PCR in TW fluid, while *E coli* and/or *Streptococcus* species were cultured from their respective TW samples. PCR analysis of NS and faeces was positive in one of 31 foals (3 per cent) and nine of 31 foals (29 per cent), respectively. The only PCR-positive NS was collected from one foal (foal 1), which interestingly cultured negative for *R equi* in TW fluid, but yielded a positive PCR result from its TW sample. Agreement of faecal PCR results with culture and TW fluid PCR results was 64 per cent and 73 per cent, respectively. Disagreement of faecal PCR results with TW fluid PCR was recorded for three foals (foals 2, 7, 10) that had been treated with either doxycycline for 22 days or azithromycin for 10 and 30 days. Faecal PCR was positive in one foal (foal 1) that had a negative *R equi* culture of the TW fluid and in one additional foal (foal 6), for which TW sampling could not be performed due to the severity of the clinical signs. Culture from the lungs of this foal (foal 6) was *R equi* positive at postmortem. Sequential faecal PCR analysis was performed on three confirmed *R equi* pneumonia foals to document shedding time after initiation of a combination treatment of rifampin and azithromycin (two foals) or rifampin and clarithromycin (one). These three foals remained *R equi* PCR positive for four to 15 days (mean 8·6 days). The faecal samples collected from 13 mares accompanying their sick foals all tested negative for *R equi* by PCR. Using all available information to classify the foals as having *R equi* pneumonia, diagnostic accuracies (sensitivity and specificity) were calculated for the different diagnostic tests (Table 2).

The variety of organisms cultured from TW fluid samples from foals with clinical signs of pneumonia were in agreement with previous studies (Hoffman and others 1993). In the present study, mixed infections were reported in nine of 29 (31 per cent) TW fluid samples, although mixed infection involving *R equi* was only reported in one of nine samples (11 per cent). A similar study reported mixed infection with *R equi* in one of five cases (20 per cent), which highlights the importance of culture in comparison to the selective PCR detection (Hoffman and others 1993). When compared to the reference standard cases, the sensitivity and specificity of TW culture was 82 per cent and 100 per cent respectively. Mixed infection from the reference cases may have been the reason why *R equi* did not grow from two of the 11 available TW fluid samples. Interestingly, *R equi* grew from the TW fluid collected from three foals on short-term antimicrobial treatment (one day) and from four foals on long-term (seven to 30 days) antimicrobial treatment.

PCR detection of *R equi* from TW fluid samples showed 100 per cent sensitivity and specificity when compared to the reference standard cases. High accuracy of *R equi* PCR (*VapA* gene) on TW fluid collected from foals with pneumonia has been previously reported with a sensitivity and specificity of 100 per cent and 90·6 per cent, respectively (Sellon and others 2001). As with any diagnostic technique, PCR results should always be interpreted in light of the individual history, clinical signs and other diagnostics from the foal and should only be used in conjunction with standard culture.

Foals with *R equi* pneumonia actively cough up *R equi*-containing secretions that will contaminate their nasopharyngeal mucosa. The collection of non-invasive specimens, such as NS, would help assess the infection status of a severely affected foal, whose condition does not allow TW fluid sampling. A previous study showed that culture of NS collected from foals with pneumonia showed poor accuracy in detection of *R equi*-infected patients (Hashikura and others 2000). Although PCR is able to differentiate between virulent and avirulent *R equi*, its use with NS to detect *R equi* infection had low accuracy in the present study, which is in agreement with a previous study showing that six of 33 foals with *R equi* pneumonia had a positive NS by PCR (Sellon and others 2001).

The quantitative culture of the faeces of foals at weekly intervals has been advocated as an aid in early diagnosis of *R equi* infection in foals because the bacterial count per gram of faeces increased at the same time as clinical signs appeared (Takai and others 1986). However, a single faecal sample from a foal has no diagnostic value because of individual as well as farm-to-farm variation in the number of *R equi* in the faeces (Nakazawa and others 1983). However, the diagnostic value of faeces using PCR has, to the authors' knowledge, not been reported in the veterinary literature. Furthermore, this alternative specimen can easily be collected in situations in which TW fluid collection is not possible because of the severity of the clinical signs. Faecal PCR for *R equi* detected nine of the 12 (75 per cent) reference cases and showed 64 per cent and 73 per cent agreement with TW fluid culture and TW fluid PCR, respectively. Faecal shedding is thought to occur either passively via swallowing of *R equi*-containing pulmonary secretions or due to the colonisation of the gastrointestinal tract, as shown to occur in 50 per cent of cases of *R equi* pneumonia (Zink and others 1986). The use of faecal material for molecular diagnostics has been associated with false negative results due to the presence of inhibitory substances in the faeces that can interfere with nucleic acid extraction or amplification (Machiels and others 2000). However, the development and use of specific extraction kits, such as the QIAamp DNA Stool, has improved the yield of nucleic acid from faeces and removed inhibitory substances (Li and others 2003). Disagreement of faecal PCR results with the reference cases was recorded for three foals that had been treated

with *R equi* susceptible antimicrobials (azithromycin, doxycycline) for 10 to 30 days. Although the long-term use of antimicrobials may affect the *R equi* load in faeces, molecular detection time of *R equi* in faeces may also be influenced by host, organism and environmental factors. Based on the small number of reference cases, it seems that antimicrobial use for up to 10 days before faecal sampling has a minimal effect on PCR results. In an attempt to follow molecular detection of *R equi* in faeces the authors performed sequential analysis in three foals treated with azithromycin/clarithromycin and rifampin. These foals remained *R equi* PCR positive for a mean of 8·6 days. The ideal situation for which faecal PCR would be diagnostically relevant was encountered in one foal (foal 6) for which TW sampling could not be performed due to the severity of the clinical signs. Faecal PCR results also highlight the contamination risk associated with the shedding of *R equi* in the environment and the role of affected foals as bacterial amplifiers. Based on a small number of tested mares, the authors believe that adult horses do not represent a high risk for *R equi* shedding via the faeces. Future studies are necessary to determine whether molecular detection of *R equi* in faeces from foals with pneumonia can be used as an adjunct to monitor the response to antimicrobial treatment.

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