Rhodococcus equi-specific hyperimmune plasma administration decreases faecal shedding of pathogenic *R. equi* in foals

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Abstract

Rhodococcus equi is the most common cause of pneumonia in young foals. Pneumonic foals are an important source of environmental contamination as they shed higher amounts of *R. equi* in their faeces than unaffected foals. As *R. equi*-specific hyperimmune plasma (HIP) lessens clinical pneumonia, we hypothesise that its use would result in decreased faecal shedding of *R. equi* by foals. Neonatal foals were either given HIP (n=12) or nothing (n=9, control) shortly after birth and were then experimentally infected with *R. equi*. Faeces were collected before and on weeks 2, 3, 5 and 7 after infection. Presence of virulent *R. equi* was tested using qPCR. There was strong evidence of an association between HIP administration and a decrease in faecal shedding of virulent *R. equi* (P=0.031 by Pearson chi-squared test). Foals in the control shed significantly more *R. equi* (colony-forming units/ml) than foals that received HIP (P=0.008 by Mann-Whitney rank-sum test). While our study is the first to report this additional benefit of HIP administration, future studies are needed to evaluate the implications of its use under field conditions.

Introduction

Rhodococcus equi is a common cause of pneumonia in foals and has a major financial impact on the horse industry worldwide.[1] It is well established that foals become infected early in life[2] and that exposure of foals to airborne virulent *R. equi* is significantly (and likely causally) associated with the development of pneumonia.[3] Thus, minimising the concentration of pathogenic airborne *R. equi* is key to reducing the risk of development of rhodococcal pneumonia. While the sources of environmental contamination are multiple, foals that have rhodococcal pneumonia have been shown to shed higher amounts of *R. equi* in their faeces than unaffected foals.[4 5] This likely results from movement of pulmonary fluids from the pneumonic lung via the trachea to the oropharynx where it is subsequently swallowed.[6] Thus, a reduction in the number of pneumonic foals or the severity of pneumonia should result in a reduced pathogenic *R. equi* shedding in faeces by affected foals. *R. equi*-specific hyperimmune plasma (HIP) is widely used intravenously as prophylactic against *R. equi* pneumonia. Although the protective mechanism of HIP remains poorly understood, we recently reported that HIP administration decreased severity of pneumonia in newborn foals experimentally challenged.[7] While our results support those seen by others,[8–10] the effect of HIP administration on the foal's faecal shedding of *R. equi* has not been previously evaluated. We hypothesise that the decreased severity of pneumonia observed in our study after administration of HIP[7] would be accompanied by a decrease in faecal concentration of virulent *R. equi*. The study described here provides basic but essential information of an additional benefit of HIP use that has not been previously investigated.

Materials and methods

Faecal samples

All faecal samples used in this study were collected previously for a project evaluating the protective effect of HIP administration after experimental intratracheal

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challenge with *R. equi*.[7] Briefly, 12 neonatal foals received 1 litre of HIP intravenously 24–48 hours after birth, while nine foals were used as controls (no HIP administration). All foals were experimentally challenged with virulent *R. equi* (UKVDL206; total dose 10^3 colony-forming units [cfu]/foal) within the first week of life as described before[2] and were closely monitored for eight weeks. All foals were housed in the same pasture with their mares and were temporarily placed in stalls twice a week for physical and ultrasonographic evaluation as well as sample collection dictated by the original HIP study. Faeces were collected from the rectum by digital palpation or from the stall floor when available before and on weeks 2, 3, 5 and 7 after infection. Immediately after collection, faecal samples were stored at −80°C until processing by the Washington Animal Disease Diagnostic Laboratory (WADDL) in Pullman, WA, as described below.

DNA extraction from faeces

DNA extraction from faeces was performed at the Washington State University (WSU)-WADDL as previously described.[4 6] Briefly, faeces were thawed at room temperature and genomic DNA was extracted using a commercial kit (QIAamp DNA Stool, Qiagen, Germantown, MD) following manufacturer's instructions. The eluted DNA was appropriately labelled and stored at −80°C until its analysis. Similar to that described by Madrigal *et al*,[6] faeces spiked with an *R. equi* pure culture were used as an extraction control. Spiked faeces were processed as described above during each extraction.

PCR assay

Real-time quantitative PCR (qPCR) has been used to accurately quantify the number of virulent *R* in samples.[6] Real-time qPCR was performed at WSU-WADDL as previously described.[4] This real-time qPCR evaluates the presence of the *vapA* gene, which is present only in pathogenic strains of *R. equi*. In order to obtain an absolute quantification, a standard dilution curve was created as described before.[6] Briefly, the purified plasmid (pGEX-2T; 4948 base pair) was obtained from an *Escherichia coli* clone containing an *R. equi* strain 103^+ plasmid vector with the *vapA* gene (570 base pair) that was generously provided by Dr. Steeve Giguère, University of Georgia. Plasmid DNA was extracted using a commercially available kit (QIAamp DNA Stool, Qiagen) and the concentration was determined spectrophotometrically. Serial dilutions of the plasmid DNA were made in phosphate buffered saline and samples were processed using real-time qPCR. A standard curve was constructed using linear regression analysis of the $\log_{10}$ quantity of the pGEX-2T copies per sample and the corresponding Ct values.[6]

Statistical analysis

A commercial software (SigmaPlot, SPSS, Chicago, IL, USA) was used for data analysis. Descriptive statistics were used to characterise the number of weeks that faeces were positive for virulent *R. equi*. Comparison of qPCR faecal sample results was performed using Pearson chi-squared test. The amount of *R. equi* colonies found in faeces (cfu) was compared between groups using Mann-Whitney rank-sum test. Significance level was set at $P<0.05$.

Results

A total of 101 faecal samples were tested; 58 belonged to the 12 foals that received HIP shortly after birth prior to experimental infection with *R. equi* and 43 were from the nine foals included in the control group (no HIP prior to infection). Faecal samples from 20/21 foals were collected before experimental infection; faeces were not present and could not be collected in one foal. The remainder 81 samples were collected after infection. Of these, four faecal samples could not be obtained from the foal's rectum and were not available in the stall floor. One of the samples belonged to a foal in the HIP group (second week after infection). The other three samples belonged to foals in the control group (before, and five and seven weeks after experimental infection).

Only one faecal sample was positive before infection. It belonged to a foal in the control group that was challenged at six days of age. The amount of virulent *R. equi* was low in this sample (2 cfu/100 µl).

Eleven (10.8 per cent) postinfection faecal samples were qPCR positive for the *vapA* gene (table 1). Two samples from different foals in the HIP were positive on weeks 3 and 5 after experimental infection. The additional nine positive samples belonged to seven foals in the control group; two foals shed for two weeks. Shedding in the control group occurred two (n=2) and three (n=7) weeks after experimental infection. The amount of *R. equi* (cfu/100 µl) was calculated from the standard curve using Ct values as shown in figure 1. Strong evidence of an association between HIP treatment the first week of life and a decrease in faecal shedding of virulent *R. equi* (P=0.031) was found. In addition, foals in the control group shed a statistically significant larger amount of virulent *R. equi* (cfu/ml) than foals that received HIP the first week of life (P=0.008).

As dictated by the original HIP study, foals that developed clinical signs of pneumonia were treated with antimicrobials until resolution of disease.[7] All 4/9 foals that were treated for pneumonia in the control

Table 1 Results of qPCR for *vapA* gene in faeces of foals experimentally infected with virulent *Rhodococcus equi* the first week of life after intravenous hyperimmune plasma (HIP) administration or not (control)

	vapA Positive	*vapA* Negative	Total
HIP	2	56	58
Control	9	34	43

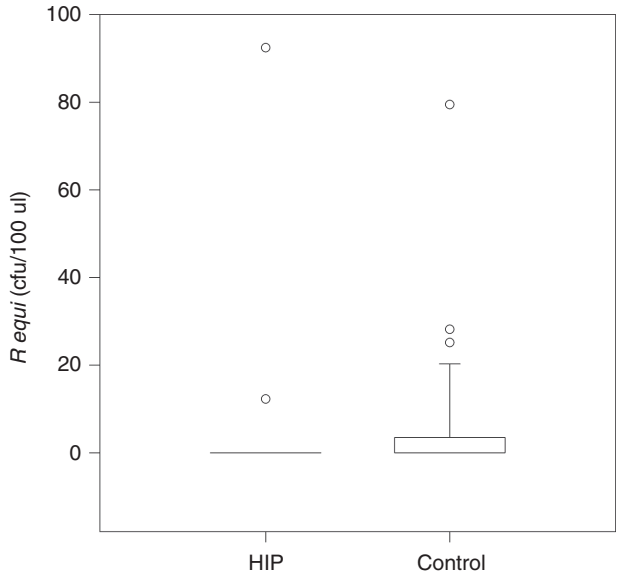

Figure 1 Over the study period, foals that received HIP shed significantly less (P=0.008) virulent *Rhodococcus equi* (cfu/100 µl) in their faeces than foals that did not receive HIP (control) prior to experimental infection with virulent *R. equi*. Faeces were collected before and on weeks 2, 3, 5 and 7 after infection, the cumulative results are shown. Colony-forming unit values were calculated using the Ct value and a standard curve. cfu, colony-forming units; HIP, hyperimmune plasma.

group shed virulent *R. equi* in their faeces at least one week during the study period. In contrast, none of the two foals treated with antimicrobials in the HIP group shed virulent *R. equi* in their faeces.

Discussion

It is well established that exposure of newborn foals to airborne virulent *R. equi* is associated with the development of pneumonia.[3] Moreover, pneumonic foals shed higher amounts of virulent *R. equi* in their faeces than unaffected foals and as a result, they become the major source of environmental contamination.[4 5 11 12] As HIP administered intravenously shortly after birth has been shown to decrease the severity of rhodococcal pneumonia,[7–10] it is valid to speculate that its use would be also associated with a reduction in virulent *R. equi* faecal shedding. Thus, the purpose of our study was to evaluate the effect that HIP administration had in faecal shedding of virulent *R. equi* over time after experimental infection of neonatal foals.

One foal shed virulent *R. equi* in the faecal sample collected before experimental challenge. This finding is not unexpected as foals are exposed to environmental *R. equi* shortly after birth,[13 14] and the foal was challenged at six days of age. The amount of virulent *R. equi* present in the faeces before infection was minimum (Ct 41, cfu 2/100 µl) and the subsequent sample collected two weeks after infection was negative for this foal, supporting an initial low exposure. Environmental contamination of the faecal sample is unlikely as collection was performed from the rectum by digital palpation.

As expected with this experimental model of *R. equi* infection in neonatal foals,[2] all the foals developed pulmonary lesions consistent with *R. equi* infection two to three weeks after experimental infection that were visible using thoracic ultrasonography. Six out of the 21 infected foals developed clinical pneumonia four to five weeks after infection and required antimicrobial treatment (oral azithromycin and rifampin).[7] Two of those foals had received HIP and did not shed any *R. equi* in their faeces. In contrast, all four foals that developed pneumonia in the control group shed *R. equi* for one or two weeks after infection. The fact that all faecal samples were negative seven weeks after infection was expected as foals that developed clinical pneumonia were treated with antimicrobials until resolution of the disease. In addition, all foals were deemed clear of the disease based on physical examination, blood work, thoracic ultrasonography and transtracheal wash culture and cytology eight weeks after infection.[7] Individual cultures of each faecal sample were not performed as conventional microbiological methods have low accuracy and can lead to misidentification.[15] In addition, culture does not differentiate between pathogenic and non-pathogenic *R. equi* strains.

Thus, our study shows that HIP administration to neonatal foals significantly decreases virulent *R. equi* faecal shedding after experimental challenge. While this finding was expected due to the fact that foals that received HIP developed significantly less severe pneumonia in our study,[7] to the author's knowledge, there are no previous reports of this additional effect of HIP administration. Although lower faecal shedding would decrease environmental contamination, this study was not designed to evaluate the effects of the environmental virulent *R. equi* reduction on subsequent foal crops. Thus, future studies are needed to see if the observed faecal *R. equi* reduction turns into a decrease risk of infection in farms with endemic or severe rhodococcal pneumonia.

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Competing interests None declared.

Ethics approval All work was approved by the University of Kentucky's Institutional Animal Care and Use Committee and the Institutional Biosafety Committee.

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