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Short Communication

Fecal shedding of *Rhodococcus equi* in mares and foals after experimental infection of foals and effect of composting on concentrations of *R. equi* in contaminated bedding

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ABSTRACT

Rhodococcus equi, a soil saprophyte, is a common cause of pneumonia in foals and a frequent opportunistic pathogen in immunosuppressed people. Because it is widespread in the environment, *R. equi* can be detected in the feces of most horses. However, the exact timing and rate of shedding relative to infection is unknown. The objectives of this study were to quantify shedding of *R. equi* in mares and foals after experimental infection of foals with 2 different inocula and to determine the effect of composting on concentrations of *R. equi* in contaminated bedding. Foals were infected intratracheally with virulent *R. equi* using inocula of 1×10^7 CFU/mL (n = 16) or 1×10^6 CFU/mL (n = 12) at 23 ± 2 days (range 21 to 27 days) of age. Fecal samples were collected from mares and foals prior to infection and on days 3, 7, and 14 post-infection for quantitative culture of total and virulent *R. equi*. Waste from the horses was composted for 7 days. Concentrations of total and virulent *R. equi* in foal feces were significantly higher on day 14 post-infection compared to day 0, regardless of inoculum size. Concentration of total *R. equi* in mare feces was significantly higher on days 3, 7 and 14 compared to day 0 regardless of inoculum size, whereas shedding of virulent *R. equi* only increased on day 14 post-infection. Composting for 7 days significantly decreased concentrations of total *R. equi* and virulent *R. equi* by an average of 1.08 ± 0.21 and 0.59 ± 0.26 $\log_{10}$ CFU/g, respectively.

1. Introduction

Rhodococcus equi, a Gram-positive facultative intracellular pathogen that preferentially infects macrophages, is one of the most common causes of disease in foals. *R. equi* is also a common opportunistic pathogen in immunosuppressed people, particularly in individuals receiving chemotherapy or infected with the human immunodeficiency virus (Arlotti et al., 1996; Donisi et al., 1996; Yamshchikov et al., 2010). The most common clinical manifestation of infection caused by *R. equi* in both species is pyogranulomatous bronchopneumonia with abscessation although infections at other sites have been described (Reuss et al., 2009; Yamshchikov et al., 2010). *R. equi* is an environmental saprotroph that can be found in the soil of horse farms as well as areas not inhabited by horses, such as city parks and house yards (Cohen et al., 2008; Takai et al., 1996). Because it is widespread in the environment, *R. equi* can be detected in the feces of most horses (Grimm et al., 2007). Unlike most environmental *R. equi*, isolates from pneumonic foals contain an 80–90 kb plasmid (designated pVAPA) encoding the highly immunogenic virulence-associated protein A (VapA) (Haites et al., 1997; Takai et al., 2000). Plasmid-cured derivatives of virulent *R. equi* do not induce pneumonia and are rapidly cleared from the lungs of foals (Giguère et al., 1999). In contrast, only approximately 15% of *R. equi* isolates from people contain pVAPA with the majority of isolates cultured not containing known virulence plasmids (Bryan et al., 2018).

Studies have shown that foals with pneumonia caused by *R. equi* shed higher concentrations of total or virulent *R. equi* in their feces compared to healthy foals (Shaw et al., 2015; Takai et al., 1986). However, the exact timing and rate of shedding relative to infection is unknown. Similarly, it is unknown if infection of foals with virulent *R. equi* affects fecal shedding of *R. equi* in healthy mares stalled with their foals. Experimental infection of foals with *R. equi* to study the pathogenesis of the disease and to test the efficacy of candidate vaccines creates waste which contains the bacteria. Wastes from animals infected with potentially pathogenic infectious agents is considered biomedical waste in many countries. In the United States, medical waste disposal is mainly controlled by state law and associated regulations

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vary considerably between states. Because a single adult horse produces approximately 15–30 kg of feces per day (Wehrman et al., 2017), commonly used decontamination procedures such as incineration or autoclaving are impractical for horse manure and bedding and alternative methods are needed. Composting is a commonly used method to reduce bacterial pathogen in the manure of herbivores (Manyi-Loh et al., 2016). The mechanisms leading to pathogen inactivation during composting are complex but temperature is known to play a key role (Turner, 2002). Exposure of *R. equi* to temperatures between 55 and 45 °C in broth under laboratory conditions resulted in eradication of the pathogen after 1 to 6 days, suggesting that short term composting might be an effective method to eradicate or at least decrease concentrations of *R. equi* in feces and bedding (Hebert et al., 2010). However, the laboratory conditions used in the aforementioned study did not mimic all the variables involved in composting and it is unknown if large scale composting is an effective means of decreasing concentrations of *R. equi* in horse waste. The objectives of this study were to quantify shedding of *R. equi* in mares and foals after experimental infection of foals with different inocula and determine the effect of short term large scale composting on concentrations of *R. equi* in contaminated bedding.

2. Material and methods

2.1. Animals

Twenty-eight mixed breed foals were used in this study in conjunction with a separate study aimed at documenting the occurrence of septic polysynovitis and uveitis in foals experimentally infected with *R. equi* (Huber et al., 2018). The study was approved by the Institutional Animal Care and Use Committee of the University of Georgia. Adequate transfer of passive immunity was confirmed in foals at approximately 24 h of age by measurement of plasma IgG concentration using glutaraldehyde coagulation or a commercial immunoassay (Snap Foal IgG Test, Idexx Laboratories Inc., Westbrook, ME, USA). Foals were kept on pasture with their dams at a farm never used for breeding horses previously. Mares and foals were moved to individual concrete stalls bedded with pine wood shavings in an isolation facility 3–4 days prior to infection.

2.2. Intratracheal challenge with virulent R. equi

A virulent strain of *R. equi*, confirmed to contain the virulence plasmid by amplification of *vapA* by PCR was used for inoculation (Huber et al., 2018). The inocula were kept as frozen stabilates in individual 10 mL aliquots and a concentration of 1×10^7 colony forming units (CFU)/mL was confirmed after thawing. Prior to infection, foals were determined to be healthy based on a complete physical examination. The mean (± SD) age of the foals at time of infection was 23 ± 2 days (range 21–27 days). The foals were infected transtracheally with a low (10^7 CFU; n = 12) or high (10^8 CFU; n = 16) inoculum of virulent *R. equi* as described previously (Huber et al., 2018). The day of infection was designated as day 0 of the study.

2.3. Sample collection and composting

Animals were assessed throughout the study based on twice daily complete physical examination by experienced veterinarians. Fecal samples were collected from mares and foals prior to infection (day 0) and on days 3, 7 and 14 post-infection. Fecal samples from the foals were collected directly from the rectal ampulla through digital palpation. Fecal samples from mares were collected either from the top of a fresh fecal pile that the mare was known to have voided or by manual evacuation of feces from the rectum. When collected from a freshly voided pile, only the center of a fecal ball was collected to avoid the possibility of contamination from surrounding bedding. The samples were collected in sterile bags and kept frozen at −80 °C until processing. Foals were euthanized 14 days post-infection to assess the severity of pulmonary disease (Huber et al., 2018).

Composting material consisted of the mixed waste (bedding, urine, and manure from several different stalls) produced only by the animals used in this study. Stalls were cleaned daily and waste was placed in 208 L plastic barrels. Once full, the barrels were sealed and stored in the isolation facility until being transported to the composting facility every 3 to 4 days. The composting material was placed in 2.5 × 1.8 m open-top wooden bins with concrete floors to a depth of 1.2 m (volume of 5.4 m^3). The bins were under a roof to protect contents from rainfall. Composting material was kept in the bins for 10–14 days after which the content was spread on a pasture at a University-owned farm. Composting samples were collected from 5 separate bins on the day the composting process started (day 0) and again 7 days later. At each sampling time, 10 samples consisting of approximately 3 g (g) of composting material were collected from each bin; 5 samples were collected from different areas at the surface and 5 samples were collected from the same areas but approximately 50 cm below the surface. The samples were placed in sterile bags and kept frozen at −80 °C until processing. Temperature of the compost was taken prior to each sample collection as well as on day 2,4 and day 7, using two thermometers per bin designed for measuring composting temperature for up to 32 in. of depth. The compost was turned when reached 5–7 days of the process to provide oxygenation.

2.4. Sample processing

Fecal and compost samples were processed according to the same protocol. A 1 g aliquot of each sample was placed in a sterile conical tube and mixed thoroughly with 5 mL of phosphate-buffered saline (PBS) solution. Serial dilutions were plated on modified NANAT *R. equi* selective medium, as described previously (Grimm et al., 2007), and incubated aerobically at 37 °C for 48 h. Bacterial colonies characteristic of *R. equi* were counted and total colony forming unit (CFU) per gram of sample was calculated. *R. equi* was identified by colony morphology and confirmed by PCR amplification of the *choE* gene from 30 colonies per sample using previously published primers (Ladron et al., 2003). Presence of pVAPA was assessed in the same colonies by PCR amplification of *vapA* using previously published primers (Giguère et al., 2010). The number of virulent (containing pVAPA) *R. equi* was estimated by multiplying the proportion of virulent *R. equi* by the total *R. equi* CFU.

2.5. Data analysis

Normality of the data was assessed based on examination of histograms and normal quantile plots of residuals and using the Shapiro-Wilk test. Variance of the data was assessed by plotting residuals against predicted values. The CFU data was subjected to $\log_{10}$ transformation to meet the assumptions for testing using parametric statistical methods. Comparison of baseline fecal concentration and proportion of virulent *R. equi* between mares and foals was done using the paired t-test. Fecal concentration and proportion of virulent *R. equi* over time data were analyzed using linear mixed-effects modeling with foal or mare modeled as a random effect to account for repeated measurements and inoculum (high vs. low), day of the study, and interaction between inoculum and day modeled as fixed nominal effects. Composting data were also analyzed using linear mixed-effects modeling with bin modeled as a random effect to account for the correlated structure of the data (sample nested within bin) and day of study modeled as fixed nominal effect. Temperature of the compost and sample depth were also evaluated in the initial model but they were not statistically significant and did not improve model fit; hence they were not retained in the final model. Model fit was assessed using Akaike's information criterion values. When appropriate, multiple pairwise comparisons were performed using the method of Sidak to control for

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