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Rhodococcus equi pneumonia in foals: An assessment of the early diagnostic value of serum amyloid A and plasma fibrinogen concentrations in equine clinical practice

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ABSTRACT

Early diagnosis and prevention of *Rhodococcus equi* pneumonia in foals represent important goals for equine clinicians. Recent protocols for diagnosis and treatment of Rhodococcosis in foals typically rely on a multimodal approach based on sonographic evidence suggestive of pyogranulomas, sonographic abscess scores and laboratory findings including plasma fibrinogen concentrations, blood biochemistry testing and platelet and leukocyte counts. The aim of this study was to assess the utility of weekly testing of serum amyloid A (SAA) and plasma fibrinogen concentrations in foals to achieve early diagnosis of *R. equi* pneumonia prior to the onset of clinical signs. This testing was used to simulate a clinically practical screening procedure and compared with thoracic ultrasonography performed in parallel.

The present study suggests that SAA does not represent a reliable early marker of Rhodococcosis when plasma concentrations are tested weekly. However, when clinical signs of *R. equi* pneumonia are present, SAA concentrations may allow clinicians to obtain 'real-time' indications concerning both the progress of infection and the effectiveness of therapy. This study raises the possibility that plasma fibrinogen monitoring starting at 1 week of age and repeated on a weekly basis, could serve as a screening test allowing clinicians to identify foals as suspected of *R. equi* infection. Future investigations regarding both physiological plasma fibrinogen concentrations in foals as well as fibrinogen kinetics in foals affected with *R. equi* pneumonia, including the establishment of appropriate reference intervals for the test method employed in this study, will be necessary in order to clarify this possibility.

Introduction

Rhodococcosis, an important respiratory infection in foals, usually affects animals in their first few months of life (Chaffin et al., 2003). The disease typically manifests as a chronic pyogranulomatous inflammation in the form of broncopneumonia, enteritis and occasionally bacteremia and arthritis (Flaminio et al., 2009). Although many organisms can cause pneumonia in foals, infection with virulent *Rhodococcus equi* is considered to be the most common cause of severe pneumonia (Anon, 1978; Prescott, 1991; Takai et al., 1995).

R. equi, the etiologic agent of Rhodococcosis, is a Gram-positive, facultative intracellular, aerobic, coccobacillary bacteria. These bacteria are considered to be ubiquitous in equine housing environments where they can often be isolated from feces or soil. Exposure of foals is thought to occur during the early post partum period (i.e. within the first week of life) in endemic farms (Horowitz et al., 2001). *R. equi* strains isolated from horses affected by Rhodococcosis typically contain a plasmid encoding for a protein associated with virulence (VapA), which is thought to allow the bacteria to multiply within macrophages (Giguère et al., 1999).

Diagnosis of *R. equi* pneumonia can be challenging because the course of disease is often insidious and overt clinical signs may be subtle, particularly early in the course of the disease (Giguère and Prescott, 1997; Giguère, 2001). Early diagnosis is generally considered desirable as it enables both a timely implementation of treatment and is thought to be associated with improved outcomes including shorter duration of treatment and reduced case-fatality (Cohen et al., 2005).

At present, clinical suspicion of pneumonia in foals is primarily based on clinical signs and blood test results including, white blood cells (WBC) counts and plasma fibrinogen concentrations (Giguère et al., 2003; Takizawa and Hobo, 2006). In the case of *R. equi* pneumonia in foals, veterinary clinicians will typically rely on diagnostic

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imaging methods, bacterial culture, and cytological analysis of tracheobronchial aspirates as methods of empirical diagnosis (Bertone, 1998; Leclere et al., 2009).

Acute Phase Proteins (APPs) are endogenously synthesized alpha globulins produced in the liver in response to harmful stimuli associated with tissue injury and subsequently released into the blood stream. In horses, increases in plasma APP concentrations have been associated with a large variety of conditions including, aseptic and septic infections, inflammatory disease, neoplasia, exposure to endo- and exotoxins, trauma and surgery (Jacobsen and Andersen, 2007).

In equine medicine, serum amyloid (SAA) is generally considered as the most sensitive reflector of the clinical condition of horses (Hulten and Demmers, 2002; Hulten et al., 2002; Petersen et al., 2004). Reference intervals for plasma SAA concentrations in horses, have been described to range from less than 0.5 to 20 mg/L (Hulten et al., 1999; Stoneham et al., 2001; Jacobsen et al., 2006) or as low or undetectable (Crisman et al., 2008). In response to infection and inflammation, concentrations of SAA increase markedly and values of above 100 mg/L and even 1000 mg/L are often observed (Crisman et al., 2008). Depending on the nature of the inflammatory stimulus, SAA values have been reported to start increasing by 4–12 h following acute inflammation, reach peak values within 48 h and return to baseline concentrations between 3 days and 3 weeks (Hulten and Demmers, 2002; Jacobsen and Andersen, 2007; Crisman et al., 2008; Prato et al., 2012). These characteristics render SAA monitoring in horses particularly suited for real-time evaluation of inflammatory activity. After having excluded the existence of coexisting noxious stimuli, plasma SAA concentrations may also serve as a good indicator of disease status, reflecting clinical improvement or deterioration as well as the quality of the response to treatment (Jacobsen and Andersen, 2007).

Notwithstanding the promising reports regarding the potential of SAA monitoring in the field of equine medicine, fibrinogen still represents the most commonly analyzed APP in routine practice (Giguère et al., 2003; Hobo et al., 2007). In clinically healthy adult horses, fibrinogen is normally present in concentrations ranging from 2 to 4 g/L (Crisman et al., 2008). In comparison with the SAA response, the fibrinogen response to noxious stimuli is more contained and slower to both develop and subside. This is why fibrinogen is often regarded as a less sensitive indicator of disease (Crisman et al., 2008). Increases in fibrinogen concentrations are reported to occur within 2 days in the course of acute inflammation, reaching maximum concentrations of two- to fourfold the normal values between 4 and 7 days following the introduction of harmful stimuli (Schalm, 1979; Andrews et al., 1994). In comparison, other studies report one- to 10 fold increases in plasma fibrinogen concentrations within the first 24–72 h following induction of the inflammatory response (Crisman et al., 2008). Increases in plasma fibrinogen concentrations have also been reported to occur early in the course of *R. equi* pneumonia in foals, following earlier increases in WBC counts. Both values have been described as markers for early diagnosis of Rhodococcosis (Giguère et al., 2003).

The aim of this study was to assess the utility of weekly testing of serum amyloid A (SAA) and plasma fibrinogen concentrations in foals to achieve early diagnosis of *R. equi* pneumonia prior to the onset of clinical signs. This testing was used to simulate a clinically practical screening procedure and compared with thoracic ultrasonography performed in parallel.

Materials and methods

Fifteen Thoroughbred foals, including 10 foals born and reared on a farm endemic for *R. equi* (see Discussion) and five foals from a farm in which *R. equi* infection had not been recorded for 10 years ('control'), were included in the study. All foals were subjected to a complete physical examination starting on the day of birth and repeated weekly for 2 months.

Blood samples

Blood samples were collected from all animals at 12 h from birth, followed by sampling weekly, for a total of 7 weeks (eight blood samples). Samples were obtained via jugular venipuncture into sterile tubes (Vacutainer) containing sodium citrate and EDTA for fibrinogen and SAA testing, respectively, in accordance with the manufacturer's (Equinostic EVA1) guidelines for APP testing. The collected blood was centrifuged at 2500 rpm for 10 min in order to separate plasma from corpuscular blood components.

Thoracic ultrasonography

Based on previous reports (Reef, 1998; Slovis et al., 2005; McCracken and Slovis, 2009), all foals were subjected to thoracic ultrasonographic screening using a portable ultrasound machine (Sonosite M-Turbo). Thoracic examinations were performed once every 2 weeks, starting at 2 weeks of age (for a total of four examinations), using a multi-frequency 7–10 MHz linear transducer. Sonographic findings were classified as follows: absence of irregularities, presence of 'comet tails', presence of abscesses <2 cm and presence of one or more abscesses >2 cm. On the basis of a recent study (Venner et al., 2012), animals exhibiting hypo or anechogenic alterations <2 cm were designated for future evaluation in order to establish whether the lesions were increasing or diminishing in size. Conversely, animals possessing one or more lesion ≥2 cm, were treated for 2 weeks using a combination of azithromycin (10 mg/kg BW PO once daily) and rifampin (10 mg/kg PO twice daily) and subsequently monitored for signs of lesion regression (Giguère et al., 2004; Venner, 2009). Scans and therapeutic cycles were repeated as described until the lesions completely cleared.

Trans-tracheal washing

Foals with sonographic findings suggestive of pulmonary lesions were further subjected to trans-tracheal washes (TTW). In order to perform this procedure, the animals were sedated using a combination of xylazine 0.6 mg/kg and butorphanol 0.02 mg/kg and a small area of the ventral neck, just proximal to the thoracic inlet, was clipped, scrubbed and anesthetized. The tracheal aspirates were obtained by advancing a 14G introduction catheter, coupled with a 16G × 70 cm flushing catheter (Mila), between two tracheal rings and into the tracheal lumen, for approximately 40–60 cm, depending on the length of the foal's neck and the position of the animal's head. Consequently, 30 mL of sterile saline solution were introduced into the trachea and then collected via aspiration.

Bacteriological examination

A bacteriological examination of the TTW samples was performed via culturing in 5% defibrinated sheep blood agar. Inoculated culture plates were incubated at 37 °C for 48 h under conditions of aerobiosis and, when present, mucoid, non-hemolytic, pink colonies suggestive of *R. equi* were analyzed using biochemical (RapID™ CB Plus System OXOID) and CAMP testing in order to obtain further confirmation. Colonies that were positively identified as *R. equi* were subjected to further polymerase chain reaction (PCR) and antibiogram testing.

APP monitoring

APP concentrations were measured using the Equinostic EVA1 diode array spectrophotometer within 5 h from sampling in all samples. SAA and fibrinogen concentrations were determined via a turbidimetric 'latex-enhanced' method and an immunoturbidometric method, respectively. The measurement of plasma SAA and fibrinogen concentrations as well as calibration of the equipment were all performed according to the manufacturer's guidelines. A recent study demonstrated how SAA concentrations could reliably be measured in heparinized plasma using a turbidimetric immunoassay previously validated for equine SAA concentrations in serum. No statistically significant differences were noted between SAA concentrations in serum and plasma using a paired *t* test ($P = 0.48$). The correlation between paired samples was 0.97 (Spearman's rank $P < 0.0001$; 95% confidence interval 0.95–0.99) (Howard and Graubner, 2014). Whether this correlation applies to EDTA plasma, as used in the present study, still remains to be demonstrated.

PCR testing

PCR testing was performed on both tracheal aspirates and pure culture isolates in order to attain a high level of certainty regarding the pathogenicity of the bacteria sampled from the upper airways of the test foals. DNA was extracted from the TTW aspirate using a commercial kit (QIAamp DNA Blood Mini Kit, Qiagen), in accordance with the manufacturer's instructions. In addition, in order to confirm that the colonies employed in antibiogram testing were VapA positive, DNA was also extracted from pure cultures, by dispersing a bacterial colony in 100 µl of PBS (phosphate buffered saline), followed by incubation (95 °C for 5 min) and centrifugation (4500 rpm for 5 min) of the resulting suspension. The supernatant was collected and assessed for DNA quality and quantity using both electrophoresis and spectrophotometry and subsequently subjected to PCR testing.