Case Report

Coinfection by avirulent *Rhodococcus equi* and *Klebsiella oxytoca* as a cause of atypical abortion in a thoroughbred mare

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Introduction: The majority of abortions in mares are associated with placental infections caused by opportunistic bacteria. *Rhodococcus equi* is widespread in the environment of stud farms and *Klebsiella oxytoca* is found on the mucosal surfaces of horses. Both can occasionally cause placental infection and abortion in mares. To the best of our knowledge, however, there have been no reports of *R. equi* and *K. oxytoca* coinfection in mares experiencing placentitis and abortion. The present report describes an uncommon case of abortion caused by *R. equi* and *K. oxytoca* coinfection in a thoroughbred mare in Brazil, in which the virulence profile of *R. equi* was investigated.

Case Presentation: A 6-year-old thoroughbred mare, in her first pregnancy, showed increased uteroplacental junction and suspected placentitis on transabdominal ultrasound. The mare aborted a 10-month-old female fetus. A field necropsy was performed and hydrallantois was diagnosed.

Conclusion: Although we isolated an avirulent strain of *R. equi*, coinfection of *R. equi* and *K. oxytoca* in the placenta and uterus probably potentiated the pathogenic effect of these micro-organisms, resulting in abortion.

Keywords: abortion; coinfection; hydrallantois; *Klebsiella oxytoca*; mare; placentitis *Rhodococcus equi*.

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Introduction

The majority of placental infections are caused by opportunistic bacteria migrating into the uterus from the caudal reproductive tract (Macpherson, 2005). The most commonly isolated bacteria in equine placentitis and/or abortion include *Streptococcus equi* subsp. *zooepidemicus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and nocardioform species (Giles *et al.*, 1993). Fungal and viral organisms can also infect the placentas of mares (Macpherson, 2005).

Rhodococcus equi is a Gram-positive facultative intracellular bacterium that is associated with pyogranulomatous infections in animals and humans (Prescott, 1991). Three virulence levels of *R. equi* are recognized, named virulent, intermediately virulent and avirulent, based on the presence or absence of virulence-associated antigens

and plasmids (Vap). Virulent isolates contain a large plasmid (80–90 kb) that contains a gene encoding a virulence-associated antigen (15–17 kDa) called VapA, which is subdivided into 11 classical types with different geographical distributions (Ribeiro *et al.*, 2005). Virulent *R. equi* strains (*vapA* positive) are the major cause of bronchopneumonia and enteritis in foals (Van Der Kolk *et al.*, 1999). Intermediately virulent (VapB) isolates present the *vapB* gene and are commonly found in the lymph nodes of swine and HIV-positive humans (Takai *et al.*, 2003). Avirulent isolates show no evidence of *vapA* or *vapB* genes and are found in HIV-positive patients, livestock environments and soil from parks (Takai, 1997).

R. equi is widespread in soil, farm manure and the faeces of healthy herbivores, especially foals. *R. equi* from soil samples is usually *vapA* negative. However, Makrai *et al.* (2006) reported *vapA* positivity in 20 % of isolates, highlighting that soil isolates are an important source of

Abbreviation: EHV-1, equine herpesvirus type 1.

virulent *R. equi* for horses. Nevertheless, *R. equi*-associated abortions in mares are unusual (Hines, 2007). The first description of placentitis and abortion as a result of *R. equi* infection was described with a virulent strain (Patterson-Kane *et al.*, 2002), although there is a paucity of data regarding the virulence-plasmid profile of *R. equi* isolates from mares that have experienced abortion. In contrast, Nakamura *et al.* (2007) reported an abortion in a thoroughbred mare caused by an avirulent *R. equi* strain.

Like *R. equi*, species of the *Klebsiella* genus are unusual causative agents of abortion in mares (Hines, 2007). The first case of *Klebsiella oxytoca* infection inducing the abortion of a 10-month-old female equine was described in Hungary (Szeredi *et al.*, 2008). In an aetiological study of 72 cases of abortion in mares from Brazilian farms, bacterial infections were observed in 36.1 % (Pereira *et al.*, 2012). From these, *K. pneumoniae* was diagnosed in only 5.6 % of the fetuses.

To the best of our knowledge, there have been no reports of *R. equi* and *K. oxytoca* coinfection in mares experiencing placentitis and abortion. The present report describes a rare case of abortion caused by *R. equi* and *K. oxytoca* coinfection in a thoroughbred mare in Brazil, in which the plasmid-virulence profile of *R. equi* was investigated.

Case report

A 6-year-old thoroughbred mare, in her first pregnancy, showed increased uteroplacental junction and suspected placentitis on transabdominal ultrasound. The mare was treated with trimethoprim/sulfamethoxazole and anti-inflammatories, and given a daily progesterone supplement. However, the prognosis was guarded and the mare aborted a 10-month-old female fetus. After the abortion, a field necropsy was performed by the attending veterinarian and hydrallantois was diagnosed. This case occurred on a stud farm in the State of Rio Grande do Sul, Brazil.

Investigations

Necropsy revealed that the whole placenta was thickened, and oedema and nodular areas (2 cm in diameter) without villi was seen in the pregnant horn, which had a ruptured area of about 5 cm in diameter. Amnion showed vascular congestion, which is suggestive of placentitis. Fragments of the heart, lung, liver, spleen, kidney, adrenal gland, small and large intestine, mesenteric lymph nodes and brain were collected for histopathological examination. Simultaneously, swab samples from the peritoneal and abdominal cavities, trachea, stomach and aspirate of stomach contents were subjected to microbiological culture for fungi and bacteria. Virological analysis was carried out using samples of the stomach contents.

The tissues fragments were fixed in 10 % buffered formalin and embedded in paraffin to perform haematoxylin and

eosin staining. In addition, Gomori's methenamine silver stains for fungi were performed using liver fragments. The samples collected for bacterial cultures were inoculated on defibrinated sheep blood agar (5 %) and MacConkey agar, and incubated under aerobic conditions at 35 °C for 48 h. Bacteria were identified on the basis of conventional phenotypical properties (Quinn *et al.*, 2011). The presence of *R. equi* colonies was confirmed by species-specific multiplex PCR, and a virulence profile analysis according to the presence of the *vapA* gene was performed as described previously (Krewer *et al.*, 2008). Virological tests were performed on RK13 (rabbit kidney cell line) to identify equine herpesvirus type 1 (EHV-1) and equine arteritis virus. Samples were grown in minimal essential medium containing penicillin (1.6 mg l⁻¹), streptomycin (0.4 mg l⁻¹) and nystatin (0.02 mg l⁻¹) supplemented with 5 % fetal bovine serum for virus isolation. The supernatant of homogenized and centrifuged fetal stomach content was inoculated in RK13 cell monolayers and the cultures were incubated at 37 °C and 5 % CO₂, and monitored for four consecutive passages of 96 h each to check for a cytopathic effect.

Diagnosis

Microscopically, significant lesions were observed only in the lungs and liver. In the lungs, alveolar spaces were filled with large numbers of foamy macrophages, epithelioid cells, neutrophils and multinucleated Langhans giant cells (Fig. 1). Bronchioles were obliterated by large numbers of neutrophils. In the liver, there were multifocal areas of necrosis, neutrophil infiltration and haemorrhage (Fig. 2). Both Gram-negative intracytoplasmic bacilli and Gram-positive intracytoplasmic coccobacilli were observed. The

Fig. 1. Lung showing alveolar space filled with large numbers of foamy macrophages, epithelioid cells, neutrophils and multinucleated Langhans giant cells (haematoxylin and eosin staining; bar, 50 µm).

Fig. 2. Liver showing multifocal areas of necrosis, neutrophil infiltration and haemorrhage (haematoxylin and eosin staining; bar, 50 µm).

fungal stain was negative, excluding this possible cause of abortion. The histological changes observed in this fetus were suggestive of bacterial intracellular infection.

K. oxytoca was isolated from all of the samples except aspirate of stomach content, while *R. equi* was isolated only from the stomach contents. Species-specific multiplex PCR for *R. equi* confirmation amplified a single 458 bp fragment, confirming the presence of *R. equi*, although samples were negative for the *vapA* gene. Virological tests did not demonstrate virus isolation from the fetal samples.

Discussion

Hydrallantois is a rare clinical manifestation in horses (Vandeplasseche *et al.*, 1976). Affected mares are usually multiparous, and the condition generally becomes apparent at 7–10.5 months' gestation (Koterba *et al.*, 1983) in mares aged 5–21 years (Blanchard *et al.*, 1987). Different pathogeneses have been suggested to explain this pathological condition, including excessive umbilical cord torsions, immunological incompatibility (Rüsse & Grunert, 1993) and hereditary factors (Waelchli & Ehrensperger, 1988). Although it is generally accepted that placental dysfunction is the reason for this disorder in mares, only a few reports have described inflammatory changes in the placenta (Koterba *et al.*, 1983; Stich & Blanchard, 2003; Shanahan & Slovis, 2011). In the case reported, any dysfunction cited above was observed in the mare or fetus, then hydrallantois was probably a result of ascending placentitis.

Inhalation and consumption of contaminated water and pastures appears to be the major source of transmission of *R. equi* in horses, particularly foals (Prescott, 1991). However, routes of *R. equi* transmission to mares as an

agent of abortion remain controversial, although transmission probably occurs by septicaemia or genital contamination from the farm environment (Hines, 2007). The common presence of avirulent *R. equi* strains in the soil and faeces of stud farms (Bell *et al.*, 1998), associated with the immunosuppression that occurs during pregnancy, may have contributed to the opportunistic infection by *R. equi* in the mare described in the current case. *R. equi* survives and replicates within macrophages through an ability to prevent the phagosome–lysosome fusion, resisting clearance by the organism's defences (Kanaly *et al.*, 1993). The lesions observed in the abortion described here are similar to those in the first case of abortion as the result of an avirulent *R. equi* isolate described in a thoroughbred mare (Nakamura *et al.*, 2007).

In contrast to the common presence of *R. equi* in the environment, *K. oxytoca* is found on the mucosal surfaces of horses (Podschun & Ullmann, 1998), and can occasionally cause placental infection and abortion. Although *K. oxytoca* is considered to be an extracellular pathogen, Szeredi *et al.* (2008) reported numerous intra-alveolar neutrophils, macrophages and multinucleated Langhans giant cells containing Gram-negative intracytoplasmic bacilli in a mare with *K. oxytoca*-associated abortion. In the current case, both Gram-negative intracytoplasmic bacilli and Gram-positive intracytoplasmic coccobacilli, suggestive of enterobacteria and actinomycetes, were observed; these were later diagnosed as *K. oxytoca* and *R. equi* coinfection, respectively.

Several aetiological agents can be involved in cases of placentitis and abortion in mares. Hong *et al.* (1993) noted a high prevalence of *Leptospira* species and nocardioform actinomycetes in a 2-year study. Abortions related to *Leptospira* species and nocardioform actinomycetes in mares are associated with diffuse placentitis, jaundice and chronic placentitis at the junction of the body and horns (Hong *et al.*, 1993; Pereira *et al.*, 2012). In the present report, the macroscopic lesions observed were not suggestive of infection by *Leptospira* species or nocardioform actinomycetes. Moreover, there was no history of *Leptospira* species infection from serological tests performed on the stud farm.

EHV-1 is the major viral cause of abortion in mares (Leon *et al.*, 2012). In late-term abortions, gross lesions found in fetuses consist of multiple foci of acute necrosis, randomly distributed on the affected organs, especially the liver. In the lung, pneumonia with fibrin has been observed in the tracheas of fetuses (Foster, 2009). In the present case, although some areas of liver necrosis were observed (Fig. 2), there were no lesions characteristic of EHV-1 in other organs and no organisms were grown in cell culture. In addition, this virus typically causes abortion earlier in gestation (Marenzoni *et al.*, 2012).

This is the first report of *K. oxytoca* and *R. equi* coinfection causing an uncommon abortion in a mare. Although the role of *R. equi* virulence in the pathogenicity of abortion in

mares remains unclear, the combined infection of *R. equi* and *K. oxytoca* in the placenta and uterus probably potentiated the pathogenic effect of these micro-organisms, resulting in abortion.

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