Neosporosis in an Aborted Southern White Rhinoceros (*Ceratotherium simum simum*) Fetus

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NEOSPOROSIS IN AN ABORTED SOUTHERN WHITE RHINOCEROS (*CERATOTHERIUM SIMUM SIMUM*) FETUS

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Abstract: In December 2008, a southern white rhinoceros (*Ceratotherium simum simum*) aborted a 7-mo gestation male fetus. On hematoxylin and eosin-stained sections of fetal tissues, foci of necrosis were noted in the hepatic parenchyma and were associated with low numbers of lymphocytes, plasma cells, and neutrophils. Protozoal zoites were identified within the hepatic lesions and within the cerebellum. Evaluations utilizing immunohistochemistry, polymerase chain reaction, and DNA sequencing identified the protozoan as *Neospora caninum*. A microsatellite analysis using MS10 marker showed a unique trinucletoide repeat pattern $(ACT)_6$ $(AGA)_{19}$ $(TGA)_8$ distinct from all studied *N. caninum* to date. This is the first report of *N. caninum*-related abortion of a rhinoceros fetus of any species and the first report of polymerase chain reaction-confirmed *N. caninum* infection in any rhinoceros.

Key words: Protozoal abortion, *Neospora caninum*, pathology, Rhinocerotidae, genotyping, internal transcribed spacer 1.

BRIEF COMMUNICATION

The family Rhinocerotidae is represented by five species in four genera, the white rhinoceros (*Ceratotherium simum*), black rhinoceros (*Diceros bicornis*), Sumatran rhinoceros (*Dicerorhinus sumatrensis*), Javan rhinoceros (*Rhinoceros sondaicus*), and Indian rhinoceros (*Rhinoceros unicornis*).[5] All five species are listed on the International Union for Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Species, ranging from near threatened to critically endangered.[6] In an effort to produce insurance populations of members of this family, captive breeding programs have been established in facilities globally. Any mechanism, disease or otherwise, which negatively impacts these breeding programs could have significant repercussions on the future of these animals.

Although only first recognized as a disease entity in the 1980s, neosporosis is now considered a major cause of abortion in cattle worldwide.[3] Abortion or stillbirth has been associated with natural infection of *Neospora* spp. in a number of other species including goats, sheep, horses, a deer (*Cervus eldi siamensis*), and two twin antelope (*Tragelaphus imberberis*).[3] Death of a 16-day-old white rhinoceros (*Ceratotherium simum*) calf as a result of *Neospora* sp. infection has also been reported.[13] This is the first report of *Neospora*-related abortion of a rhinoceros fetus, of any species, and the first report of polymerase chain reaction (PCR)-confirmed *Neospora caninum* infection in any rhinoceros.

In December 2008, at Taronga Western Plains Zoo in Australia, a southern white rhinoceros (*Ceratotherium simum simum*) spontaneously aborted a 7-mo gestation male fetus. Expulsion of fetal membranes was facilitated by administration of two doses of 30IU oxytocin (Ilium Syntocin, Troy Laboratories Australia Pty Limited, Smithfield, New South Wales 2164, Australia) given intramuscularly via dart approximately 4 hr and 6 hr after discovery of the fetus. The placenta was passed within 24 hr and had a normal gross appearance. The dam, estimated to be between 13 and 15 yr of age, was in good general health. This individual had been wild caught in South Africa in 2002 and was transferred to Taronga Western Plains Zoo in 2003. Her reproductive history at the zoo featured an uneventful pregnancy in 2004–2005 resulting in birth and successful rearing of a healthy female calf. The dam's medical history featured transient, discreet episodes of muscle fasciculations, ataxia, hypermetria, seizures, and collapse, which occurred from 3 to 11 mo following the 2004–2005 pregnancy. Diagnostic evaluation including hematology, serum biochemistry, viral serology, blood culture, toxicology, and vitamin status failed to identify a

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causative etiology. The episodes resolved spontaneously by late 2005 and her general health in proceeding years was considered good.

Necropsy of the aborted fetus was performed on site. Gross findings included enlargement of the liver, with rounded edges, and herniation of a small segment of intestine through the umbilical opening. Kidney, spleen, adrenal gland, bladder, liver, lung, brain, placenta, and multiple sections of intestine were collected, fixed in 10% neutral buffered formalin, and submitted to the staff diagnostic veterinary pathologist. Additional sections of liver were frozen at −80°C.

On hematoxylin and eosin (H&E)-stained sections, multiple random foci of necrosis were noted in the hepatic parenchyma and were associated with low numbers of lymphocytes, plasma cells, and neutrophils. Protozoal tachyzoites were present as clusters within the cytoplasm of hepatocytes and were free amongst necrotic debris (Fig. 1). These tachyzoites were lightly basophilic, round to oval, 2–3 μm × 5–6 μm, and contained a single 1-μm diameter, deeply basophilic nucleus. Clusters of similar tachyzoites were seen on H&E-stained sections of the cerebellum but were associated with little inflammatory reaction.

Paraffin-embedded, formalin-fixed liver was submitted to the Tasmanian Department of Primary Industries, Parks, Water and Environment (DPIPWE) Animal Health Laboratory (Kings Meadows, Tasmania 7249, Australia) for immunohistochemistry (IHC). Polyclonal antibodies against *N. caninum* antigen (generously provided to Tasmanian DPIPWE by J. P. Dubey) at 1:2,000 were applied to the tissue, positively staining the tachyzoites. Antibodies to *Toxoplasma gondii* faintly stained some protozoal structures, but as the antibodies to *N. caninum* failed to stain a *T. gondii* control tissue, this finding was considered insignificant.

Total DNA was extracted from the liver of the rhinoceros fetus using a PureLink™ Genomic DNA Kit (Invitrogen Australia Pty Limited, Mulgrave, Victoria 3170, Australia) following manufacturer's instructions. For PCR, 2× Econo-*Taq* PLUS GREEN MasterMix (Lucigen Corporation, Middleton, Wisconsin 53562, USA) containing *Taq* DNA polymerase was used according to manufacturer's instructions. *Neospora caninum* was identified using positive PCR with Np6+/21+ targeting the Nc5 polymorphic region (Fig. 2).[9] This result was validated by sequencing four markers in order to unequivocally characterize *N. caninum*; JV1/JV2 (small subunit ribosomal DNA, 'SSU rDNA'), CR1/CR2 (hypervariable D1/D2 domain of large subunit ribosomal DNA, 'LSU rDNA'), JS4/TIM11 (internal transcribed spacer 1, 'ITS1'), and JS4/T13R (internal transcribed spacer one–5.8S ribosomal RNA–internal transcribed spacer 2, 'ITS1-5.8S-ITS2 rDNA').[4,9,12]

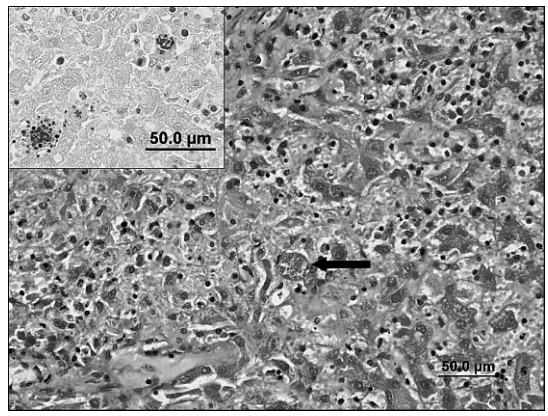

Figure 1. Liver from aborted rhinoceros fetus. Intracellular protozoal tachyzoites (arrow) are present within multifocal areas of necrosis. H&E, ×400. Inset: IHC of same tissue using antibodies against *N. caninum*. ×400.

Positive bands of expected sizes for all four marker genes were bidirectionally sequenced, directly, using both amplification primers at the Sydney University and Prince Alfred Molecular Analysis Centre. The sequences were assembled, aligned with related sequences, analyzed using CLC Main Workbench 5.5 (CLC bio, Katrinebjerg 8200, Aarhus N, Denmark), and deposited in GenBank (National Center for Biotechnology Information) under the accession numbers GQ899204–GQ899207, GU128955–GU128956. In each case, obtained sequences of SSU rDNA, ITS1-5.8S-ITS2 rDNA, and LSU rDNA revealed 100% identity with *N. caninum*.

Furthermore, a microsatellite analysis using MS10 marker revealed a unique pattern of repeats in the fetal liver DNA isolate when compared to other studies.[1,10] The three variable trinucletoide repeat units $(ACT)_6$ $(AGA)_{19}$ $(TGA)_8$ were distinct from all other isolates studied to date, including two Australian isolates—Nc Nowra featuring a $(ACT)_6$ $(AGA)_{22}$ $(TGA)_8$ pattern and Nc WA-K9 (FJ824923).[1] Nc Nowra is the first Australian strain in culture isolated from the brain of a congenitally infected calf, and Nc WA-K9 is a strain that was isolated from a skin lesion of a naturally infected dog.[7,8]

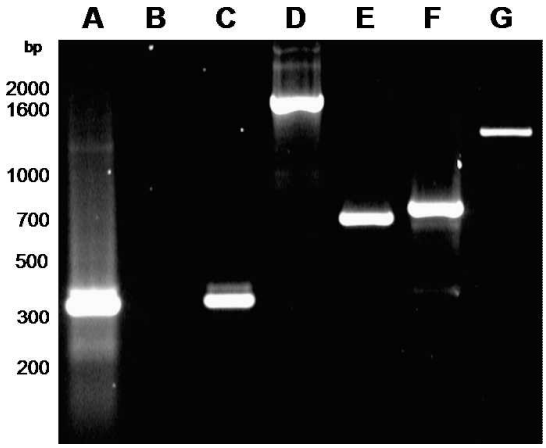

Figure 2. Molecular identification of *N. caninum* in white rhinoceros fetus using five different PCR amplifications. **A.–C.** Amplification of Nc5 region using Np6+/21+ yielding an approximately 320 bp product (A), negative control (B), and positive control (DNA from *N. caninum* NC-Liverpool) (C). **D.** Amplification of small subunit ribosomal DNA using JV1/JV2 primers yielding an approximately 1,700 bp product. **E.** Amplification of hypervariable D1/D2 region of large subunit ribosomal DNA with primers CR1/CR2 yielding an approximately 600 bp product. **F.** Amplification of a partial internal transcribed spacer of the ribosomal DNA unit using primers JS4/TIM11 yielding an approximately 620 bp product. **G.** Amplification of a complete internal transcribed spacer of the ribosomal DNA unit using JS4/T13R yielding an approximately 1,300 bp product. All PCR sets of reactions contained positive and negative controls (water instead of the DNA template). Negative and positive controls for D–G are not shown. A size marker is shown on the left.

Based on the presence of *Neospora* sp. zoites identified by IHC within necrotic hepatic lesions, and on molecular identification of *N. caninum* in the same tissue, abortion of this rhinoceros fetus was attributed to the protozoal infection. It is unclear when and how the dam was exposed to *N. caninum*. Although this female rhinoceros had produced a seemingly healthy calf since being brought into captivity, it is possible she carried this infection with her from South Africa. In domestic cattle, neosporosis can be acquired by a cow through exposure to oocysts shed into the environment by canine definitive hosts or as a result of transplacental vertical transmission from her own dam.[3] Abortion of this rhinoceros calf confirms that vertical transmission occurs in this rhinoceros species. Given that horizontal transmission must have occurred originally for infection to have become established in the species, both routes are deemed possible. However, it is not yet determined whether rhinoceroses can produce subclinically affected female calves that would pass the infection vertically to their own offspring.

Alternatively, this dam may have been exposed to the pathogen once arriving in Australia. The open range zoo where the animal is housed is within an agricultural landscape in which beef farming is a main occupation. Although not as prevalent as within dairy herds in the same state, *Neospora*-positive serology has been documented in beef cattle in this area (B. Moloney, Industry and Investment, New South Wales, pers. comm.). To date, domestic dogs and coyotes (*Canis latrans*) are the only identified definitive hosts of *N. caninum*, although *N. caninum*-like oocysts have been identified by PCR in red fox (*Vulpes vulpes*) feces, albeit in low numbers.[3] Domestic dogs are abundant in the farming landscape around the zoo and red foxes are a common pest species, both amongst farms and on zoo grounds. It is feasible that rhinoceros paddocks could be contaminated with *N. caninum*-infected canine feces, providing exposure.

Neospora caninum bradyzoites have a known predilection for central nervous system tissue in both canine definitive hosts and a wide range of intermediate hosts.[3] Relevance of this organism to the history of neurologic disease in the dam of the aborted fetus is only speculative at the time of publication. It is plausible that a latent infection in this animal developed into clinical disease during her first pregnancy and lactational period. Although very little is known regarding the epidemiology of neosporosis in domestic perissodactylids, evidence to date suggests that horses do encounter the organism under natural conditions and that they only succumb to disease during immune system compromise.[2] Retrospective serologic evaluation of the dam and her surviving female offspring is planned. This may aid in determining whether this animal was exposed to *N. caninum* during her most recent pregnancy or, alternatively, had a persistent quiescent infection which resulted in abortion due to recrudescence.

Southern white rhinoceros are listed by the IUCN Red List of Threatened Species as near threatened, making the impact of neosporosis on the reproductive success of these animals of utmost importance.[6] Captive breeding programs have been established for four extant species of rhinoceros. The sustainability of such programs is already undermined by subfertility and, in some taxa, offspring sex-ratio bias.[11] Efforts of those working

in this field will be further challenged if neosporosis proves to be an emerging disease of rhinoceroses that is characterized by vertical and horizontal transmission and by recurrent abortion.

To date, no cases of *Neospora* sp. related abortion or neonatal death in any other Rhinocerotidae species have been reported, but an occurrence is possible, given that these events have occurred in the white rhinoceros. All efforts to exclude canine species from rhinoceros enclosures and feed stuffs are recommended.

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