Veterinary Parasitology 239 (2017) 37–41

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Short communication

Neospora caninum abortion in a Malayan tapir (*Tapirus indicus*)

M. Peters[a,*], C. Osmann[b], P. Wohlsein[c], G. Schares[d]

[a] Chemisches und Veterinäruntersuchungsamt Westfalen (CVUA) Westfalen, Zur Taubeneiche 10-12, D-59821 Arnsberg, Germany
[b] Zoo Dortmund, Mergelteichstraße 80, D-44225 Dortmund, Germany
[c] Department of Pathology, University of Veterinary Medicine Hannover, Bünteweg 17, D-30559 Hannover, Germany
[d] Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Epidemiology, Südufer 10, D-17493 Greifswald, Insel Riems, Germany

ARTICLE INFO

Keywords:
Neospora caninum
Tapir
Tapirus indicus
Abortion
Microsatellite typing

ABSTRACT

A captive 17-year old female Malayan tapir (*Tapirus indicus)* aborted a fetus with a crown rump length of 19 cm in early pregnancy. The fetus showed an early state of mummification. Histologically, a multifocal mononuclear encephalitis, myocarditis and periportal hepatitis was present indicating a possible protozoal cause of abortion. Although immunohistologically, *Neospora (N.) caninum* antigen could not be demonstrated, *N. caninum* DNA was detected by Polymerase Chain Reaction (PCR) in brain, heart, liver and lung of the fetus. *N. caninum* DNA was extracted from the aborted fetus and the microsatellite marker MS10 was amplified by PCR and sequenced. The obtained MS10 microsatellite pattern has not been described in Germany yet. Nevertheless, the MS10 pattern was very similar to those reported for *N. caninum* isolated from dogs and cattle in Germany. Because of the histological pattern and extent of the lesions, neosporosis was suspected as the cause of fetal death and abortion. This case report describes for the first time transplacental transmission of *N. caninum* and abortion due to neosporosis in a tapir.

1. Introduction

The Malayan tapir (*Tapirus indicus*) is the largest of the four extant tapir species (Baird's tapir, Lowland tapir, Mountain tapir und Malayan tapir) and the only extant old world species living in the rainforests of Southeast Asia. Phylogenetically, tapirs are an ancient group of herbivorous mammals. Due to habitat destruction and poaching the Malayan tapir is categorized as "endangered" on the IUCN red list of threatened animal species (Naveda et al., 2011). Together with horses and rhinoceroses, tapirs belong to the order of odd toed ungulates (Perissodactyla).

N. caninum is a globally distributed cyst-forming apicomplexan protozoan parasite closely related to *Toxoplasma gondii*. Definitive hosts are dogs (McAllister et al., 1998), coyotes (Gondim et al., 2004), dingoes (King et al., 2010) and gray wolves (Dubey et al., 2011). They excrete *N. caninum* oocysts within their feces. Natural intermediate hosts are several species of mammals, including ruminants, canines and horses (Dubey et al., 2007). They become infected either by ingestion of contaminated water or food or vertically by transplacental infection. In cattle, the transplacental route of infection is highly efficient resulting in fetal transmission rates as high as 95% (Donahoe et al., 2015). The parasite has emerged as a major pathogen for cattle, but also affects sheep and goats causing abortions, stillbirth and perinatal death (Dubey et al., 2007) *N. caninum* associated abortion and stillbirths are also reported in some wild and zoo ruminants (Donahoe et al., 2015) and transplacental transmission of *N. caninum* was very recently confirmed for the first time in moose (*Alces alces*) (Schlieben et al., 2017). In odd toed ungulates, *N. caninum* infections have hitherto only been reported in rhinoceroses (Sangster et al., 2010; Sommanustweechai et al., 2010; Williams et al., 2002) and horses. In a southern white rhinoceros it was identified as the cause of an abortion (Sangster et al., 2010). Horses can be infected by *N. caninum* and by another *Neospora* species, *N. hughesi*, which differs from *N. caninum* ultrastructurally, immunologically and on molecular base (Marsh et al., 1998). Whereas *N. caninum* in horses is associated with reproductive disorders like abortion and neonatal death (Dubey and Porterfield, 1990; Pitel et al., 2003), *N. hughesi* is the second most important cause of equine protozoal myeloencephalitis (EPM) next to *Sarcocystis neurona* (Reed et al., 2016).

Here we report for the first time a *N. caninum* associated abortion in another odd toed ungulate animal species, the Malayan tapir (*Tapirus indicus*).

* Corresponding author.
E-mail address: martin.peters@cvua-westfalen.de (M. Peters).

http://dx.doi.org/10.1016/j.vetpar.2017.04.018
Received 27 January 2017; Received in revised form 6 April 2017; Accepted 12 April 2017

2. Material and methods

2.1. History

In Dortmund zoo, a 17-year-old Malayan tapir aborted a male fetus of 19 cm crown rump length (CRL) in November 2013. The female tapir was born in captivity in Oklahoma Zoo, kept at Munich Zoo before she came to Dortmund in 2000. She had three healthy calves previously in 2003, 2007, and 2009 before the abortion occurred. The cow and the bull are the only Malayan tapirs in Dortmund zoo and are living together as a breeding pair since the year 2000. The bull was aged 14 years when the abortion occurred. Both were accommodated in a rainforest house together with a group of Sumatran orangutans.

2.2. Necropsy and sampling

The fetus was submitted without placenta for necropsy to the Chemisches und Veterinäruntersuchungsamt Westfalen (CVUA), a North Rhine-Westfalian State Veterinary laboratory in Arnsberg, Germany in November 2013. A complete post mortem examination was performed and samples of brain, lung, heart, liver were fixed in 4% neutral buffered formalin for histological and immunohistological examination. Native samples of stomach content, lung and liver were taken for routine cultural bacteriological examination. Additionally, lung samples were used for PCRs to exclude infection with *Chlamydia* spp. (Kaltenböck et al., 1997) and *Coxiella burnettii* (Schrader et al., 2000). Samples of spleen, lung and brain were used to rule out infection with equine herpesvirus by direct immunofluorescence staining technique on frozen tissue sections. Brain, lung, heart and liver samples were taken to examine for DNA of *Toxoplasma (T.) gondii* and *N. caninum* by PCR.

Serum samples of the tapir cow and bull were provided by the zoo veterinarian in March and September 2014, respectively.

2.3. Histology and immunohistology

Formalin-fixed tissue samples were routinely processed for histology and embedded in paraffin wax. Sections were cut at 4 µm and stained with hematoxylin and eosin (H & E) for microscopic examination. The immunohistochemical examination was performed as described previously (Peters et al., 2000). Briefly, the tissue sections were deparaffinized. After incubation in isopropanol and 96% [v/v] ethanol, endogenous peroxidase was quenched with 0.5% [v/v] hydrogen peroxide in methanol for 30 min. After rehydration in 75% [v/v] and 50% [v/v] ethanol the slides were rinsed in phosphate buffered saline (PBS) and incubated with 0.05% [v/v] pronase E (Merck, Darmstadt, Germany) for 20 min at 37 °C. After another rinse in TRIS-PBS, sections were blocked in TRIS-PBS containing 20% [v/v] normal goat serum and were incubated (45 min, 37 °C) with a rabbit antiserum (1:500 in PBS) developed against *N. caninum* (NC-1) tachyzoites (Schares et al., 1997). Antibodies were detected using the avidin-biotin-peroxidase complex method with a commercial kit (Vectastain ELITE ABC Kit, Vector Laboratories, USA) as described by the manufacturer. 3,3-diaminobenzidine tetrahydrochloride (0.05 mg/ml in 0.05 M TRIS PBS, pH 7,6) and 0.03% [v/v] hydroxide peroxide were used for color development. The enzyme reaction was stopped after 5 min by thorough rinsing in tap water. The sections were counterstained with hematoxylin, dehydrated and mounted. Substitution of the primary antibody by normal rabbit antiserum was used as negative control.

2.4. Toxoplasma gondii and Neospora caninum PCR

To detect *T. gondii* and *N. caninum* DNA, conventional end-point PCRs were performed as described (Legnani et al., 2016; Schares et al., 2011). PCR primers (Toxoplasma: Tox5/Tox-8 (Homan et al., 2000; Reischl et al., 2003); Neospora: Np6 + /Np21 + (Müller et al., 1996)) were used at a final concentration of 0.5 µM and dNTPs at 250 µM each (Amersham Biosciences, Piscataway, USA). DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland) was added at 1 U/25 µl with the provided buffer. The reaction mix was supplemented with bovine serum albumin at a concentration of 20 µg/ml. Water PCR Reagent (Sigma-Aldrich, Taufkirchen, Germany) served as a negative control. The reactions were performed in a thermal cycler (Eppendorf Mastercycler, Personal Thermal Cycler, Hannover, Germany). Amplification products were visualized after electrophoresis in 1.5% agarose gels stained with ethidium bromide. A 100 bp DNA ladder (Invitrogen GmbH, Karlsruhe, Germany) served as a size standard.

2.5. Immunoblot (IB) analysis and Indirect Fluorescent Antibody Tests (IFAT) analysis

The NC-1 strain of *N. caninum* (Dubey et al., 1988) and RH strain of *T. gondii* (Sabin, 1941) were maintained in cell cultures and purified as previously described (Schares et al., 1998, 1999). Cell-culture-derived tachyzoites were frozen as a pellet at −80 °C until used for immunoblot. Pellets of 8×10^7 tachyzoites of *N. caninum* or 2×10^8 *T. gondii* were incubated in non-reducing sample buffer (2% [w/v] sodium dodecyl sulfate [SDS], 10% [v/v] glycerol, 62 mM TrisHCl, pH 6.8) for 1 min (94 °C), separated in 12% [w/v] SDS polyacrylamide minigels of 60 × 70 × 1 mm size, and transferred to PVDF membranes (Immobilon-P, Merck-Millipore GmbH, Germany). After the transfer, membranes were blocked using PBS-TG (PBS with 0.05% [v/v] Tween 20 (Sigma, Germany) and 2% [v/v] liquid fish gelatine [Serva, Germany]) and cut into 50 stripes and examined as described below. To detect antibodies against *N. caninum* or *T. gondii* tachyzoite antigens, the incubation of stripes was performed as previously described (Schares et al., 1998), with a few modifications. Sera were diluted 1:100 in PBS-TG. Serum of an experimentally *N. caninum*-infected cow (Schares et al., 1999) was used as a *N. caninum* positive control. A serum of an experimentally *T. gondii*-infected pig (Azevedo et al., 2010) was used as a *T. gondii* positive control. As negative controls preinfection sera of cattle and pigs were applied. Stripes incubated with tapir-serum were examined using an anti-horse IgG H/L peroxidase conjugate (Dianova, Germany), 1:500 diluted in PBS-TG, while stripes with bovine or pig sera were analysed with an anti-bovine IgG H/L or anti-pig IgG H/L peroxidase conjugate (Dianova, Germany), both 1:500 diluted in PBS-TG.

For IFAT ten µl of a suspension of cell culture-derived *N. caninum* or *T. gondii* tachyzoites (5 × 106 ml − 1) in PBS were used to sensitize IFAT slide wells. Slides were air-dried and stored frozen at −20 °C until use. The slides were fixed with ice-cold acetone for 10 min and then incubated in PBS for 10 min. Moose serum was titrated in PBS in 2-fold steps starting at a dilution of 1:25. The test was performed as described (Schares et al., 1998) and rabbit anti-horse IgG (whole molecule) coupled to FITC (Dianova, Hamburg, Germany) diluted 1:50 in PBS with 0.2% Evans Blue was used to detect the primary antibodies. The slides were examined using a Nikon Eclipse Ti microscope (Nikon GmbH, Düsseldorf, Germany). Only complete peripheral fluorescence of the tachyzoite was considered specific. A titre of 1:100 was defined as the positive cut-off titre. The same sera as described for IB were used as positive or negative controls in IFAT and detected with rabbit anti-bovine or anti-pig IgG (whole molecule) coupled to FITC (Dianova, Hamburg, Germany) diluted 1:50 in PBS with 0.2% Evans Blue, respectively.

2.6. Microsatellite analysis

To further characterize the *N. caninum* DNA, positive samples were subjected to microsatellite analysis. For nested-PCR amplification of the *N. caninum* microsatellites (MS) MS1B, 2, 3, 5, 6A, 6B, 10, 12 and 21 and amplicon sequencing previously described methods were used (Basso et al., 2009a,b, 2010).