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SHORT COMMUNICATION

Evidence of congenital transmission of *Neospora caninum* in naturally infected water buffalo (*Bubalus bubalis*) fetus from Brazil

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Abstract The aim of this study was to determine the congenital infection by *Neospora caninum* in the water buffalo (*Bubalus bubalis*), a natural intermediate host. Nine pregnant water buffalos, raised under free-grazing condition, were slaughtered, and their fetuses were collected. Samples of brain and thoracic fluid were obtained from those fetuses, with gestational ages ranging from 2 to 5 months. The DNA of *N. caninum* was detected and identified in the brain of one of those fetuses, using two PCR assays, one directed to the *Nc5* gene and the other, to the common toxoplasmatiid ITS1 sequence. The DNA fragments produced on PCR were sequenced, and *N. caninum* was confirmed in the samples. No antibodies to *N. caninum* were detected on any sample of thoracic fluid by immunofluorescent antibody test (IFAT<25). This is the first confirmation of congenital transmission of *N. caninum* in water buffalos.

Introduction

The water buffalo (*Bubalus bubalis*) is an important livestock species raised in Asia, South America, Europe, Australia, and Africa, for dairy and meat production and even transport, living closely to several human and animal populations. They are natural intermediate hosts for *Neospora caninum* (Rodrigues et al. 2004). Seroprevalence studies were developed in experimentally and naturally infected animals in Brazil (Fujii et al. 2001; Rodrigues et al. 2005; Gennari et al. 2005; Gondim et al. 2007), Argentina (Campero et al. 2007), Egypt (Dubey et al. 1998), India (Meenakshi et al. 2007), Vietnam (Huong et al. 1998), and Italy (Guarino et al. 2000), with positivity ranging from 1.5% to 70.9%. In Italy, *Neospora*-like tissue cysts were found in two of four aborted fetuses from water buffalos (Guarino et al. 2000), and in Brazil, *N. caninum* was isolated from naturally infected water buffalos by bioassay in dogs (Rodrigues et al. 2004). Despite the high seropositivity to *N. caninum* found in some regions of Brazil, abortion caused by this etiologic agent has not been described. The objective of this study was to detect and identify *N. caninum* in the brains of fetuses obtained from pregnant water buffalos, slaughtered at a commercial abattoir, to verify congenital transmission of the parasite in this intermediate host.

Materials and methods

In a commercial abattoir located in the southern region of the state of São Paulo, Brazil, nine pregnant water buffalos, from properties which maintain their animals on free-grazing condition, were slaughtered. The fetuses had gestational ages ranging from 2 to 5 months. These fetuses were refrigerated at 4°C and immediately sent to the Parasitic Diseases Laboratory at the Department of Preventive Veterinary Medicine and Animal Health, Faculty of Veterinary Medicine, University of São Paulo. Samples of brain were collected for molecular investigation, and thoracic fluid was collected for serologic assay.

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Approximately 100 mg of fragments of brain from each fetus were macerated and placed in a lysis buffer containing 10 mM Tris–HCl, pH 8.0; 25 mM EDTA, pH 8.0; 100 mM NaCl; 1% SDS; and 20 μg/mL proteinase K for 12 h, incubated at 37°C. After phenol–chlorophorm extraction, the DNA solution was precipitated with ethanol. *N. caninum*-specific nested PCR protocol, Nc5-nPCR (Baszler et al. 1999), was applied on the total DNA obtained from each brain sample. To improve the molecular analysis, a second nested PCR protocol (ITS1-nPCR) was carried out, employing primers directed to the common toxoplasmatiid 18S and 5.8S coding genes, flanking the ITS1 region. The primary amplification was performed using primers JS4 (Slapeta et al. 2002) and CT2b (this study). The secondary amplification was performed using primers CT1 and CT2 (Sreekumar et al. 2003).

The amplicons from Nc5-nPCR and ITS1-nPCR were sequenced in both directions using the ABI chemistry. The sequencing was carried out at least four times, in order to increase the reliability of the results. The sequences were assembled, and the contig formed with the phred base-calling tool available in the site http://bioinformatica.ucb.br/. The sequences were recovered with each residue score equal to or greater than 20.

Fetal fluid from the nine fetuses was collected from the pleural cavity, and indirect fluorescent antibody test (IFAT) was used to determine the presence of antibodies anti-*N. caninum*, according to the methodology described by Dubey et al. (1998), with a cutoff value of 25 and *N. caninum* Nc-1 strain tachyzoites as antigen.

Results and discussion

All samples of fetal fluid from the nine sampled fetuses were negative for the presence of *N. caninum* antibodies. However, the presence of *N. caninum* DNA was detected in the brain of one fetus at approximately 3 months of gestation. Similar procedures were applied in water buffalos for the detection of other abortive agents (Perugini et al. 2009), as well as for the detection of *N. caninum* in other host species (Costa et al. 2008; Suteu et al. 2010).

In the *N. caninum*-positive sample, a fragment of 227 bp could be amplified by Nc5-nPCR. This sample was also successfully amplified by the ITS1-nPCR, which yielded, in this case, an amplicon 419 bp long. Both bands co-migrated with the positive control, and no band was amplified from the negative control. The Nc5 and ITS-1 gene PCR products from the positive brain tissue were sequenced, and the results were deposited in GenBank under accession numbers DQ059067 and DQ059068, respectively.

The absence of antibodies in the *Neospora*-positive fetus could be due to the lack of fetal immunocompetence, or a short interval between infection and sample collection. In bovine fetuses younger than 6 months, the lack of fetal immunocompetence may explain the low sensitivity of fetal serology for the detection of *N. caninum* infection (Wouda et al. 1997). Immunologic response of the fetus depends on the antigen as well as on the species and the age of the fetus. Ferritin elicits a response in the fetus from the fourth through the ninth month of gestation. The *Brucella* antigen induced an immune response in fetuses as early as the fourth month, but ovoalbumin or IBR viral antigens did not induce any response (Gibson and Zemjanis 1973). Nevertheless, it is noteworthy that fetal immunocompetence in water buffalo fetuses has never been studied.

The sequences obtained in this study were compared with the existing homologues in the GenBank. Except for a single nucleotide substitution (C→G), the fragment of the *Nc5* gene sequenced here is almost identical to the homologous sequences from the five Brazilian isolates from adult water buffalos NCBrBuf-1 (AY497041), NCBrBuf-2 (AY497042), NCBrBuf-3 (AY497043), NCBrBuf-4 (AY497044) and NCBrBuf-5 (AY497045). The same nucleotide substitution was encountered when the Nc5-nPCR sequence was compared to the NC-1 strain (AY665719).

The ITS sequence is identical to that from the NC-1 strain (AY665715), but differed at four nucleotides from that of NC-Bahia (AY259043), at three nucleotides from that of NC-Liverpool (AY259038), and at one nucleotide from those of the five Brazilian isolates from adult water buffalos (AY497041–AY497044).

There are many reports on isolation of viable *N. caninum* from fetuses from cattle, but there are none from water buffalos (Dubey 2003). *Neospora*-like tissues cysts and lesions were reported by Guarino et al. (2000) in two of four water buffalo fetuses examined in Italy, but no other test was done to confirm the presence of the agent in these fetuses.

Our finding is of most importance as relevant evidence of vertical transmission in this host species. This is the first molecular detection and identification of *N. caninum* in fetuses of naturally infected water buffalos, indicating that congenital transmission of *N. caninum* may occur in this species. However, the real importance of this protozoon as a causative agent of reproductive disorders in water buffalos remains to be elucidated.

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