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Short communication

Shedding of *Neospora caninum* oocysts by dogs fed different tissues from naturally infected cattle

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ABSTRACT

Neospora caninum is one of the most important causes of abortion in dairy cattle worldwide. The distribution of *N. caninum* in tissues of adult cattle is unknown and the parasite has not been demonstrated histologically in tissues of cows. In the present study the distribution of *N. caninum* in different tissues of adult cattle was evaluated by bioassays in dogs. Seventeen dogs (2–3 month-old) were fed different tissues of 4 naturally exposed adult cattle (indirect fluorescent antibody test *N. caninum* titer ≥400): 5 were fed with masseter; 5 with heart, 3 with liver, 4 with brain, and 3 pups were used as non-infected control. Two dogs fed masseter, 2 fed heart, 1 fed liver, and 3 fed brain shed oocysts, and all dogs presented no seroconvertion to *N. caninum* during the observation period of 4 weeks. The oocysts were confirmed as *N. caninum* based on the detection of *N. caninum*-specific DNA by PCR and sequencing. The results indicate that dogs can be infected by *N. caninum* with different tissues of infected cattle.

1. Introduction

Neospora caninum is a protozoan parasite of domestic and wild animals. Coyotes and dogs are the definitive hosts of the parasite (McAllister et al., 1998; Lindsay et al., 1999; Gondim et al., 2004) and it was firstly demonstrated they shed oocysts after consuming infected mice (McAllister et al., 1998; Lindsay et al., 1999). Despite the importance of dogs in the epidemiology of the disease, few are the reports of *N. caninum* oocysts shedding by naturally infected dogs (Basso et al., 2001; Gondim et al., 2002; Slapeta et al., 2002; McGarry et al., 2003; Schares et al., 2005; McInnes. et al., 2006).

Gondim et al. (2002) fed dogs with a pool of different tissues (brain, vertebral column, heart, liver, kidney, tongue, diaphragm and other muscles) of experimentally infected calves and these dogs shed oocysts, however no information is available about which bovine tissue was the source of cysts to the dogs.

Data from shedding of oocysts in puppies and adult dogs suggest that young dogs present oocysts production significantly higher than adult dogs, indicating that the age of the dogs can influence oocysts production, and it may be related to protective immunity resulting from a prior exposure (Lindsay et al., 1999; Gondim et al., 2002; Gondim et al., 2005).

In the present experiment the distribution of *N. caninum* in tissues of naturally exposed cattle was studied by bioassays in young dogs.

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Table 1
Identification of cattle used as inoculums and dogs (2–3 month old) infected by different bovine tissues and *Neospora caninum* or *Hammondia heydorni* oocysts shedding.

Inocula	Bovine no.	Dog no.	Shedding (d.a.i.)	Total number of oocysts	PCR and sequencing analysis
Brain	B1	2	N	0	ND
	B2	6	8	218	*N. caninum*
	B3	11	10, 11	1495	*N. caninum*
	B4	18	7, 8, 9	1165	*N. caninum*
Masseter	B1	1	N	0	ND
	B2 + B3	7	8, 9, 10, 12	ND	*H. heydorni*
	B2 + B3	12	6, 9, 10, 11, 12	ND	*H. heydorni*
	B4	16	9, 10, 14	993	*N. caninum*
	B4	17	8, 9, 10	1311	*N. caninum*
Liver	B1	3	N	0	ND
	B4	21	N	0	ND
	B4	22	14	317	*N. caninum*
Heart	B1	5	12, 13, 15, 16, 17	5100	*N. caninum*
	B2 + B3	9	6, 7, 8, 10, 11, 12, 13, 14	ND	*H. heydorni*
	B2 + B3	14	N	0	ND
	B4	19	8, 9, 12, 13, 14	ND	*H. heydorni*
	B4	20	14	309	*N. caninum*
Control		4	N	0	ND
		10	N	0	ND
		15	N	0	ND

N = negative; ND = not done; d.a.i. = day after infection.

2. Materials and methods

2.1. Dogs

Twenty mixed-breed puppies were raised since birth in captivity with their mothers. A week before the onset of the experiment serum samples of pups and dams were tested for the absence of *N. caninum* antibodies by indirect fluorescent antibody test (IFAT) using a 1:50 serum dilution as cut off (Dubey et al., 1988). After weaning, the puppies were vaccinated against Canine Distemper Virus, Parvovirus, Adenovirus type 2, Parainfluenza, Hepatitis, Coronavirus and Leptospirosis (Duramune® Max5CvK – Fort Dodge, Brazil). Pups and dams were fed only processed commercial dry dog food and water *ad libitum*. The dogs had never been fed with meat products.

2.2. Tissues from cattle used as inoculum

Adult male and female mixed breed cattle from three abattoirs on the country-side of São Paulo State were selected for experimental infection of dogs. They had an IFAT titer of 400 or higher. Masseter, heart, liver and brain were collected and kept refrigerated (4 °C) for up to 48 h before being administered to the dogs.

2.3. Experimental infection of dogs

Seventeen 2–3 month-old puppies were fed different tissues from four bovine cattle over a period of 1–3 days. Five puppies were fed with masseter, 5 with heart, 3 with liver, and 4 with brain; each puppy consumed approximately 350 g, 400 g, 300 g and 300 g of tissue. Three puppies were used as non-infected control and received only processed commercial dry dog food and water *ad libitum* (Table 1).

During the observation period (4 weeks), dogs were housed individually in metal cages and sera were collected at day 0 (the day which the animals were orally challenged with bovine tissues), day 15 and day 30 post infection (p.i.), for the presence of *N. caninum* antibodies (IFAT $\geq$50).

2.4. Fecal examination and processing of oocysts

Feces were collected individually from day 5 p.i. and examined by the sucrose flotation technique for the detection of oocysts. Each day, the total volume of feces collected in 24 h was individually weighed, homogenized, and 5 g were analyzed as described by Gondim et al. (2002). When *Neospora*-like oocysts were observed, they were counted and the total number of oocysts produced was estimated. Fecal examinations were carried out until 10 days after the last shedding day, and the dogs that presented no oocysts shedding had feces examined until 30 days p.i.

2.5. Molecular identification of oocysts

After microscopic examinations of fecal samples in which *Neospora*-like oocysts were detected, slides were rinsed with 1 ml of TE buffer (10 mM Tris–HCL, pH 8.0; 1 mM EDTA, pH 8.0) to recover the oocysts. Each oocyst suspension was cleared by centrifugation (1500 × *g* for 10 min) and the sediment was resuspended in 1 ml of TE. This process was repeated once. The supernatant was discarded and the pellet was resuspended in 500 μl of lyses buffer (10 mM Tris–HCL, pH 8.0; 25 mM EDTA, pH 8.0; 100 mM NaCl, 1% SDS). This suspension was submitted to three freeze–thaw cycles and proteinase K was added to a concentration of 10 μg/ml. The suspension was incubated at 37 °C. After overnight digestion the DNA was extracted using a mixture of phenol–chlorophorm, isoamyl-alcohol and ethanol

(25:24:1) precipitated as described by Sambrook et al. (1989).

DNA of oocysts was tested by PCR based on primers directed to 18S and 5.8SrRNA coding genes common to all toxoplasmatinae (PCR-ITS-1). The forward primer anneal to 3′ end of 18S locus whereas the reverse primer are directed to the 5′ end of 5.8S locus. The primers flank the complete internal transcribed spacer 1 (ITS-1). The primers are depicted as follows: JS4 CGA AAT GGG AAG TTT TGT GAA C (Slapeta et al., 2002) and CT2b TTG CGC GAG CCA AGA CAT C (Monteiro et al., 2007). DNA of oocysts was also tested by a PCR based on primers directed to the *Neospora* spp. exclusive locus Nc-5, using primers Np6 and Np7 (PCR-Nc5) (Bazler et al., 1999).

The PCR-ITS-1 products were sequenced using the original primers and the Big Dye® chemistry (Applied Biosystems, Foster City, California). Sequencing products were analyzed on an ABI377 automated sequencer. Both strands of each PCR products were sequenced at least four times in both directions to increase the confidence of sequencing. The sequences were assembled and the contig formed with the phred-base calling and the phrap-assembly tool available in the suite Codoncode aligner v.1.5.2. (Codoncode Corp. Dedham, MA, USA). The PCR-ITS1 derived sequences were submitted to the BLAST search (blastn, www.ncbi.nlm.nig.gov/BLAST) in order to identify the species of the parasite.

All experimental procedures were in accordance with the ethical principles in animal research and approved by the Committee for the care and use of laboratory animals of the Faculty of Veterinary Medicine at the University of São Paulo, Brazil.

3. Results

Eight of 17 puppies fed naturally infected bovine tissues shed *N. caninum*-like oocysts in their feces (Table 1). All the oocysts shed by these puppies yielded a specific fragment the expected size for *N. caninum* after PCR-Nc5 and PCR-ITS-1 amplification. The PCR-ITS-1 sequences amplified from all the oocysts shed by these dogs were 100% identical to other *N. caninum* homologous sequences available in GenBank. Two ITS-1 haplotypes were detected and each of them corresponds to either one of the two haplotypes existing in *N. caninum* genome. These sequences were deposited in GenBank under accession numbers HQ542298 and HQ542299 and they were 100% similar to GQ899205 and GQ899204, respectively.

Mean *N. caninum* oocysts elimination was 2705, 1152, 960 and 317 for dogs that ingested heart, masseter, brain and liver, respectively. The dogs started shedding *N. caninum* oocysts at day 7 p.i. and oocysts were shed for 1–5 days (Table 1).

Four puppies (dogs 7, 9, 12 and 19) shed *N. caninum*-like oocysts, but none of these oocysts yielded fragments after PCR-Nc5 amplification. On the other hand, DNA extracted from these oocysts was successfully amplified by PCR-ITS-1. The PCR-ITS-1 sequences amplified from all the oocysts shed by these dogs were 100% identical to each other and 100% identical to other *Hammondia heydorni* homologous sequences available in GenBank. One of these sequences was deposited in GenBank under accession number HQ542300.

Dogs that were fed brain and liver shed only *N. caninum* oocysts. Two of the dogs that shedding *H. heydorni* oocysts received heart tissue, whereas the other two dogs were fed masseter. Although heart and masseter also caused *N. caninum* infection in 4 dogs, the molecular analysis of oocysts shed by each dog revealed that dogs that shed *N. caninum* did not shed *H. heydorni* and vice versa.

The total amount of *H. heydorni* oocysts shed by each dog was not determined and these dogs started the shedding at day 6 p.i. and oocysts were shed for 4–8 days (Table 1).

All dogs were negative for the presence of antibodies anti-*N. caninum* during all the experimental period (IFAT <50) and displayed no clinical signs of *N. caninum* infection after consuming cattle tissues.

4. Discussion

The results of the present study show, for the first time, that dogs fed other tissue than neural or placenta can shed *N. caninum* oocysts.

Tissue cysts have been demonstrated by electron microscopy in muscular and neural tissues of fetuses or neonatal cattle and dogs (Dubey et al., 1988). Nothing is known regarding the presence of tissue cysts of *N. caninum* in visceral tissues of animals however, in this study, one of the three dogs fed with liver of naturally infected bovine shed *N. caninum* oocysts.

All dogs, despite shed *N. caninum* oocysts, remained clinically healthy and did not seroconvert (IFAT <50) during the observation period, as previously described (McAllister et al., 1998; Lindsay et al., 1999; Bergeron et al., 2001; Dijkstra et al., 2001; Schares et al., 2001; Gondim et al., 2002; Cedillo et al., 2008). Many studies report low *N. caninum* oocysts shed by dogs experimentally or naturally infected by cattle tissue (McAllister et al., 1998; Lindsay et al., 1999; Basso et al., 2001; Dijkstra et al., 2001). Despite the low age of the dogs and the amount of tissue they received, the amount of oocysts were relatively low with a maximum number of oocysts shedding by dog number 5 that received heart, with a total of 5100 oocysts in 5 days of elimination and a minimum of 218, shedding by dog number 6 that received a brain tissue and shed only during one day.

It is noteworthy mentioning that tissues from the naturally infected cattle might contain insufficient number of organisms to induce optimal infection of dogs and this may be a possible cause of the differences in oocysts production by dogs fed different source of tissue cysts and failure to induce seroconversion in the dogs.

The presence of *N. caninum* was confirmed by a specie-specific PCR (PCR-Nc5) in 8 out of 12 cases in which dogs shed *Neospora*-like oocysts. The sequencing analysis of PCR-ITS-1 fragments derived from these oocysts revealed homogeneous chromatograms, i.e. the samples appear to not contain other *Neospora*-like oocysts than *N. caninum*. Analogous situation was observed with the 4 dogs that shed *Neospora*-like oocysts which were negative by PCR-Nc5. In this case, the sequencing anal-

ysis of chromatograms derived from PCR-ITS-1 revealed sequences similar to *H. heydorni* and only homogeneous chromatograms were observed. The oocysts identified as *H. heydorni* were eliminated by 4 dogs, and all of them had ingested muscular tissues (masseter or heart), which confirms the importance of muscles in the *H. heydorni* infection for dogs (Schares et al., 2005). Soares et al. (2009) also infected crab-eating foxes (*Cerdocyon thous*) with *H. heydorni* after they were fed with masseter and brain tissue of naturally infected bovines.

In the present study bovines that infected dogs with *H. heydorni* (B2, B3 and B4) were also able to infected dogs with *N. caninum*, however all the infected dogs shed or *H. heydorni* or *N. caninum*, never both coccidia were detected in the same dog. Rodrigues et al. (2004), using brain of buffaloes (*Bubalus bubalis*) *N. caninum* seropositives to infected dogs, found dogs shedding oocysts of both coccidia simultaneously, fact that was not observed in the present study.

In summary, this study confirms for the first time that other cattle tissues, such as heart, liver, and masseter, than brain or placenta are possible sources of *N. caninum* infection in dogs.

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