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VIABILITY OF SPORULATED OOCYSTS OF *NEOSPORA CANINUM* AFTER EXPOSURE TO DIFFERENT PHYSICAL AND CHEMICAL TREATMENTS

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ABSTRACT: The aim of the present study was to evaluate the viability of *Neospora caninum* sporulated oocysts after various chemical and physical treatments. Bioassays in gerbils and molecular techniques (PCR-RFLP) were used for identification of the oocysts shed by experimentally infected dogs. Sporulated oocysts were purified and divided into 11 treatment groups as follows: absolute ethanol for 1 hr; 20 C for 6 hr; 4 C for 6 hr; 60 C for 1 min; 100 C for 1 min; 10% formaldehyde for 1 hr; 10% ammonia for 1 hr; 2% iodine for 1 hr; 10% sodium hypochlorite for 1 hr; 70% ethanol for 1 hr; and one group was left untreated and kept as a positive control. All chemical treatments were performed at room temperature (37 C). A total of 33 gerbils, or 3 gerbils per treatment, were used for bioassays. After treatment, the oocysts were divided into aliquots of 1,000 oocysts and orally administered to gerbils. After 63 days, the gerbils were anesthetized and killed with 0.2 ml of T61; blood and tissue samples were collected for serological (IFAT and western blotting), molecular (real-time PCR), histopathology, and immunohistochemical tests. Treatments were considered effective only if all 5 detection techniques tested negative. High temperatures at 100 C for 1 min and 10% sodium hypochlorite for 1 hr were the only treatments that met this condition, effectively inactivating all oocysts.

Neospora caninum, a cyst-forming coccidian parasite, affects a wide range of host species and is one of the most important infectious causes of abortion in cattle. Dogs are the definitive hosts of the parasite and shed fecal oocysts; oocysts are approximately $11.7 \times 11.3 \mu\text{m}$ in size and become infectious after sporulation, at which time they acquire a spherical to sub-spherical shape. Oocysts have 2 sporocysts ($7.0\text{--}8.0 \times 2.0\text{--}3.0 \mu\text{m}$), each of which is, in turn, composed of 4 sporozoites (Lindsay et al., 1999).

Studies conducted mainly with *Eimeria* spp. show that, apart from lipids and carbohydrates, the cellular walls of coccidian oocysts possess a highly structured and resistant protein matrix (Mai et al., 2009). This tough cellular wall provides an effective barrier for sporozoites against mechanical and chemical threats (Belli et al., 2006). Resistance to proteolytic substances and impermeability to aqueous solutions, including various detergents and disinfectants, also creates real challenges for controlling coccidian infection in domestic animals (Mai et al., 2009). Lipid-soluble substances and small molecules are permeable to oocyst cell walls, as has been observed with ammonia and methylbromide (Kuticic and Wikerhauser, 1996).

Although several studies have dealt with the resistance and viability of other major coccidia such as *Toxoplasma gondii* (Frenkel et al., 1975; Dubey and Beattie, 1988; Dubey, 1998; Lindsay et al., 2003) and *Sarcocystis neurona* (Dubey et al., 2002), there is sparse information in the literature concerning the viability of *N. caninum* oocysts. The objective of the present study is to assess the resistance of sporulated *N. caninum* oocysts to different temperature conditions, treatments by disinfectants, and exposure times.

MATERIALS AND METHODS

Production of *N. caninum* oocysts

Oocysts used in the physical and chemical treatments were obtained from experimentally infected dogs through ingestion of naturally *N. caninum*-infected buffalo brains. Briefly, buffaloes were tested for anti-*N. caninum* antibodies using an indirect immunofluorescence antibody test (IFAT ≥ 100) as described by Dubey et al. (1988). Brains from the seropositive animals were collected and fed to 3, 2-mo-old domestic dogs (*Canis familiaris*). Each pup consumed 1 brain administered in equal portions throughout 2 consecutive days. Excepting a 24-hr fasting period prior to ingestion of the brains, the dogs received daily rations of appropriate commercial dog food and water ad libitum throughout the experimental period. They were maintained in individual stalls and vaccinated against parvovirus, parainfluenza, corona virus, leptospirosis, and canine distemper (Duramune® Max5CvK, Fort Dodge, Campinas, SP, Brazil) prior to *N. caninum* infection.

On the third and fourth day post-ingestion (dpi), 2 dogs were immunosuppressed with an intramuscular injection of 80 mg of methylprednisolone acetate (Depo-Medrol®, Pfizer, São Paulo, SP, Brazil). After 5 dpi and beyond, feces of each dog were collected daily and sampled for oocysts resembling the morphology of *N. caninum* or *Hammondia heydorni* (in the following referred to *Neospora-Hammondia*-like oocysts) using a sugar flotation technique (Ogassawara and Benassi, 1980).

Positive samples were mixed in 2% potassium dichromate solution and stored in bottles at 27 C for 14 days to induce sporulation. After this, sporulated oocysts were maintained at 4 C. Prior to their use, oocysts were purified and potassium dichromate was removed with 3 successive washes of distilled water and centrifugation.

Purified material was homogenized and the number of oocysts determined by triplicate measurements using a Neubauer counting chamber. The number of oocysts for inocula was determined by the average number of oocysts obtained at the triplicates. The oocysts were maintained at 4 C for no longer than 87 days from the time they were eliminated in feces until gerbil inoculation.

Identification of oocysts

Prior to the chemical and physical treatments, it was confirmed that the oocysts were *N. caninum* using molecular (PCR-RFLP) and biological (gerbil bioassay) methods. PCR-RFLP was performed as described by Monteiro et al. (2008) using primers targeting the Hsp70 coding gene. The species-specific enzymes *StuI* and *KpnI* were used to digest target regions of *N. caninum* and *H. heydorni*, respectively. The PCR amplification and digestion pattern were visualized after electrophoresis in a 2.5% agarose gel stained with ethidium bromide. Positive controls were extracted from known samples of *N. caninum* (Nc-1) and oocysts of *H. heydorni* (stock HheBR) that were previously characterized by molecular methods (Monteiro et al., 2008) and compared to sampled material.

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Oocysts treatments

Eleven tubes with approximately 3,000 sporulated *N. caninum* oocysts each were submitted to 1 of the following chemical or physical treatment groups: (G1) absolute ethanol at 24 C for 1 hr; (G2) -20 C in saline solution for 6 hr; (G3) 4 C in saline solution for 6 hr; (G4) 60 C in saline solution for 1 min; (G5) 100 C in saline solution for 1 min; (G6) 10% formalin at 24 C for 1 hr; (G7) 10% ammonia at 24 C for 1 hr; (G8) 2% iodine at 24 C for 1 hr; (G9) 10% sodium hypochlorite at 24 C for 1 hr; (G10) 70% ethanol at 24 C for 1 hr; and (G11, control) saline solution at ambient temperature, 24 C.

For the chemical inactivation tests (G1, G6, G7, G9, and G10), 475 µl of the oocyst suspensions were mixed in 10 ml of the disinfectant solution and incubated for 1 hr at 24 C. Following treatment, oocysts were twice washed with saline solution and centrifuged to remove chemical residues. For thermal resistance tests (G2–G5, G11) oocysts were suspended in saline solution and microtubes were kept at -20 C, 4 C, 60 C, and 100 C according to the period of incubation mentioned above.

Gerbil bioassay

Thirty-three gerbils were divided into 11 groups of 3. After the treatments, inocula containing approximately 1,000 oocysts were orally administered to each gerbil with the aid of a feeding tube. Gerbil groups G1 to G10 received oocysts from the corresponding oocyst treatments, while G11 received the untreated control oocysts. Each group was maintained in a separate cage that was sanitized weekly. Gerbils were observed daily throughout the entire experimental period and received adequate daily rations and water ad libitum.

After a 63-day period, the gerbils were anesthetized via an intraperitoneal injection of a mixture of 0.015 ml of 2% xylazine (Kensol®, König, São Paulo, SP, Brazil) and 0.03 ml of 10% ketamine (Vetaset®, Fort Dodge). After anesthesia, 1.0 ml of blood was collected by intracardiac puncture. Serum samples were divided into aliquots and kept at -4 C until later use. After blood collection, the gerbils were killed with 0.2 ml of T61® (Intervet, São Paulo, SP, Brazil) and necropsy was performed to remove the brain, heart, liver, spleen, and thigh muscle. Half of each tissue was placed in a vial with buffered formalin (100 ml of 40% formaldehyde, 900 ml of distilled H₂O, 4 g of NaH₂PO₄, and 6.5 g of Na₂HPO₄) for histopathology and immunohistochemical (IHC) diagnosis. An additional aliquot of each tissue was placed in microtubes and frozen at -20 C for real-time PCR analysis.

Fresh brain smears were also examined, for detection of *N. caninum* cysts, using a 100-fold magnification in light microscopy.

Animal management and veterinarian procedures with dogs and gerbils were approved by, and in accordance to, the Animal Use Ethics Committee of the Faculty of Veterinary Medicine of the University of São Paulo (protocol number 1187/2007).

IFAT of gerbil serum

Gerbil sera were tested for anti-*N. caninum* antibodies by indirect immunofluorescence antibody tests (Dubey et al., 1988) using secondary fluorescein isothiocyanate-labeled anti-gerbil antibodies (Immunology Consultants Laboratory, Newberg, Oregon) and intact tachyzoites of *N. caninum* (strain NC-1) as antigen. The cutoff was set at 1:50 (Dubey et al., 1988) and the samples were tested at dilutions of 1:50 and 1:100.

Western blotting (WB) of gerbil serum

Nc-1 strains of *N. caninum* (Dubey et al., 1988) were maintained in Vero cell cultures and purified following the protocol of Schares et al. (1998, 1999). Tachyzoites obtained from the culture were frozen at -80 C until needed for WB.

Affinity chromatography employing monoclonal antibodies mAb 4.15.15, as described by Schares et al. (2000), was used to purify *N. caninum* surface antigens p38 (NcSRS2).

Whole tachyzoite antigens of *N. caninum* and purified surfaced antigens (Schares et al., 1998) were used for the WB test. Pellets containing 4×10^7 *N. caninum* tachyzoites and purified p38 proteins (0.05 µg) were incubated in buffer (2% [w/v] SDS, 10% [v/v] glycerol, 62 mM Tris-HCl, pH 6.8) for 1 min at 94 C and separated in polyacrylamide gel (SDS) at 12% (w/v; 60 × 70 × 1 mm in size). The antigens were subsequently transferred to PVDF membranes (Immobilon-P, Millipore, Billerica, Massachusetts)

which, after the transfer, were blocked with PBS-TG (PBS with 0.05% [v/v]), Tween 20 (Sigma, St. Louis, Missouri), and 2% liquid fish gelatin (v/v) (Serva, Heidelberg, Germany). The membranes were cut into strips for later examination.

When using total antigen of *N. caninum*, the reactivity of sera with immunodominant antigens of *N. caninum* tachyzoites (Nc-IDA) of 29, 30, 33, and 37 kDa was recorded (Schares et al., 2000). For the WB employing the purified p38 protein (NcSRS2), reactivity of the sera with a single band at 38 kDa was recorded.

To detect anti-*N. caninum* antibodies, incubations of the WB strips followed the methodology described by Schares et al. (1988), with modifications. Sera were diluted at 1:100 in PBS-TG and serum from an experimentally infected gerbil was used as a positive control (Schares et al., 2005).

Histopathology and immunohistochemistry (IHC)

Tissue sections stained with hematoxylin and eosin (H&E) were employed for the histological examination. *Neospora caninum* detection was performed by an IHC test using anti-*N. caninum* primary antibody (VMRD, Pullman, Washington).

The tissues were initially incubated in 3% hydrogen peroxide solution for 10 min to block endogenous peroxidase. Antigen retrieval was performed using 0.1% trypsin for 10 min at room temperature (37 C), followed by heat (microwave on full power 2 min) with the slides immersed in citrate buffer, pH 6.0 (1 L of distilled water, 2.1 g C₆H₈O₇; adjusted to pH 6.0 with 0.5% NaOH). Nonspecific labeling was reduced by applying 5% reduced-fat milk for 15 min. The primary antibody was applied for 1 hr at 37 C, followed by 20 min of the secondary biotinylated antibody and avidin-peroxidase conjugate solution (Dako do Brasil, São Paulo, Brazil), also at 37 C. Labeling was performed using the chromogen 3, 5-diaminobenzidine tetra-hydrochloride as a substrate (DAB, Dako do Brasil) for 5 to 10 min. Hematoxylin applied for 1 min was used as a counter stain (Mills, 1992).

Real-time PCR

Brain and heart samples of each gerbil were fragmented and macerated in a mortar and pestle with liquid nitrogen. The tissues were then homogenized with DNazol® (DNazol Reagent, GIBCO, Auckland, New Zealand) until cellular lysis was complete. Subsequently, the material was centrifuged at 10,000 g for 10 min to remove cellular debris. Absolute ethanol was used to precipitate nuclear material and subsequently extracted from the supernatant. After several washes with 70% ethanol, the DNA was solubilized in 8 mM NaOH solution. DNA concentration and quality were estimated by a spectrophotometer reading at 260 nm and 280 nm (BioPhotometer, Eppendorf, Hamburg, Germany).

Fifty nanograms of DNA were used in each amplification reaction using universal real-time PCR buffer (TaqMan Universal PCR Master Mix, Applied Biosystems, Carlsbad, California), with a specific set of primers and probe for *N. caninum* (gene NC5) and an endogenous control 18S (Eukariotic 18S rRNA Endogenous Control, Applied Biosystems). Oligonucleotide sequences (5'-3') for gene NC5, based on Genbank Accession number AY665719, were: Sense GCG GAC GTG TCG TTG TTG; Anti-sense GTT CAC ACA CTA TAG CCA ACA ACA A; and Probe (VIC-TAMRA) CCT GCG GCA GCA AGG CTC CTT.

DNA from tachyzoites of *N. caninum* (strain Nc-1) and Milli-Q water served as positive and negative controls, respectively. Amplifications were performed using Applied Biosystems 7500 Real-Time PCR System equipment and subjected to 50 C for 2 min, 95 C for 10 min, 40 cycles at 95 C for 15 sec, and 60 C for 1 min. Amplification curves were analyzed by Sequence Detection Software (SDS, v1.3, Applied Biosystems).

Reactions were considered positive if the fluorescent signal increased during amplification and negative if the fluorescent signal remained at the same basal level observed during the beginning of the amplification cycles.

Each gerbil tissue was analyzed in duplicate.

RESULTS

One of the immunosuppressed dogs died on 5 dpi and the other shed *Neospora-Hammondia*-like oocysts from 8 to 17 dpi. The dog that had not received an immunosuppressive treatment failed to shed oocysts. The oocysts present in dog feces were analyzed

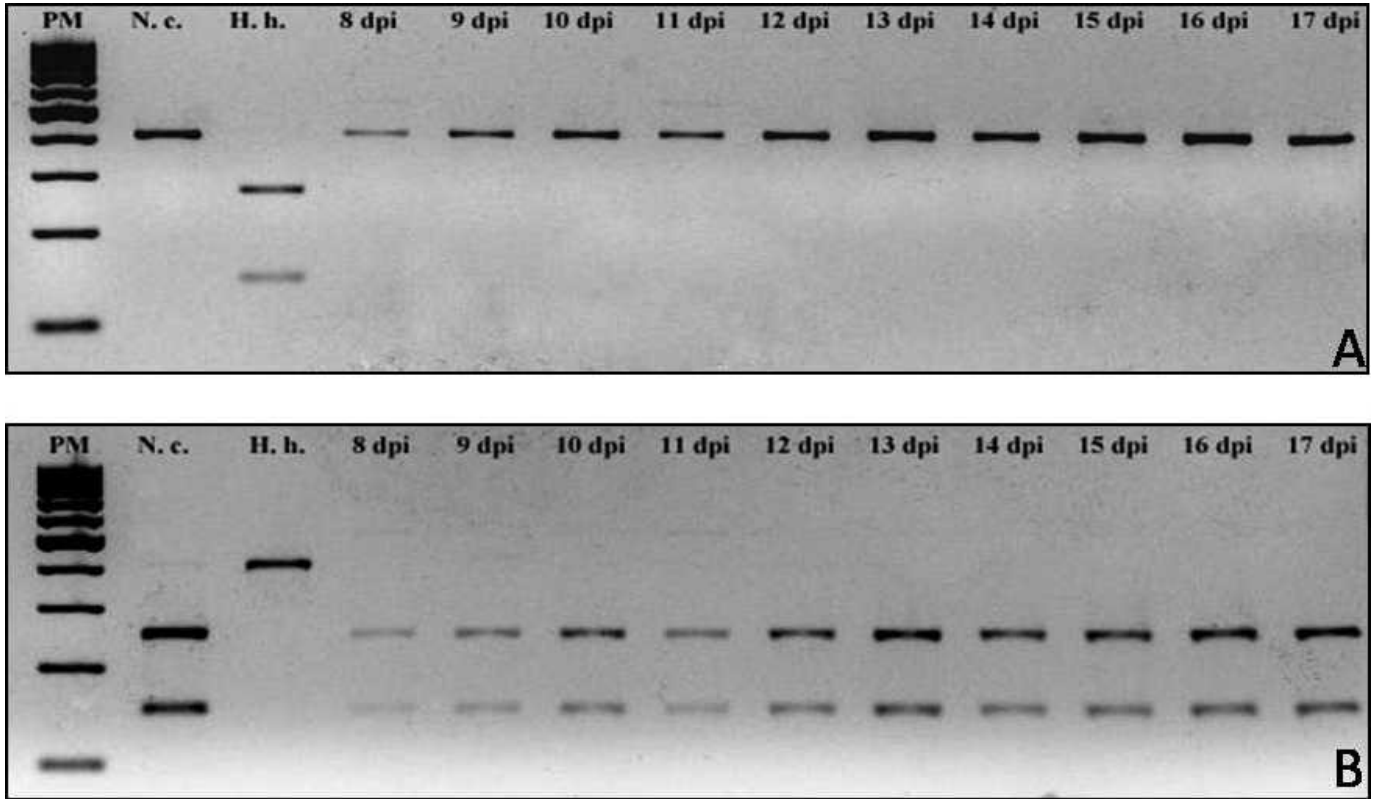


FIGURE 1. Agarose gel electrophoresis of the PCR-RFLP products amplified from *Hammondia*-like oocysts using primers directed to the HSP 70 coding sequences and the restriction enzymes *KpnI* and *StulI* from 8 to 17 dpi. (A) *KpnI* enzyme – N.c.: positive control for *N. caninum* (400 bp); H.h.: positive control for *H. heydorni* (136 + 264 bp). (B) *StulI* enzyme – N.c.: positive control for *N. caninum* (150 + 250 bp); H.h.: positive control for *H. heydorni* (400 bp). PM: molecular weight; weight marker (100 bp).

separately each day and confirmed to be *N. caninum* by PCR-RFLP (Fig. 1).

Table I shows the results of the gerbil bioassay evaluating the viability of *N. caninum* oocysts after the physical and chemical treatments. Two treatments, 100 C for 1 min (G5) and 10% hypochlorite at ambient temperatures for 1 hr (G9), were the only ones to successfully inactivate oocysts, as confirmed by the 5 tests performed.

Via histologic examination, only gerbil 12 from G4 presented moderate suppurative meningitis in the brain, with deposition of fibrin and bacterial clots and discrete focal mononuclear encephalitis. In the same animal, *N. caninum* were observed in the interior of inflammatory cells of the brain by IHC.

Tissue cysts were not detected in the brain of the gerbils by light microscope examination.

By the real-time PCR, tissue samples from 7 (3 controls, 1 gerbil from G3, 2 from G4, and 1 from G7) of the 33 gerbils were positive to *N. caninum* DNA. All positive samples were from brain material.

The serological techniques (WB and IFAT) presented similar results, with all gerbils from G5 and G9, and 2 from G8, negative. The remaining gerbils were all positive.

DISCUSSION

Oocysts used in the assay were confirmed to be *N. caninum* by molecular and biological methods. Rather than pooling samples,

oocysts shed in feces were analyzed daily, with each analysis conferring similar results. After identification was firmly established through repeated analysis, the oocysts were pooled from different samples in order to prepare the numerous inoculations.

Not all tests were equally effective in detecting *N. caninum*. Although 5 brain smears were performed per gerbil, none of the 30 experimentally infected gerbils was found to have brain tissue cysts. In a study on *T. gondii* infections, Dubey (1988) predicted a frequency of 1 cyst per 50 g of tissue in large-bodied mammals. Similar data for *N. caninum* infection do not exist. However, given that *N. caninum* cysts are rarely observed in organs other than the brain, expected frequencies may be much less than in *T. gondii* infection. Furthermore, the number of *N. caninum* cysts in tissues may vary depending on the isolate (Gondim et al., 2001; Pena et al., 2007).

The detection of *N. caninum* stages by IHC also showed low sensitivity, with only 1 gerbil from G4 (–60 C for 1 min) testing positive. However, molecular (real-time PCR) and serological (IFAT and WB) techniques proved to be more sensitive, suggesting their effectiveness in trials utilizing gerbils as experimental models. All control gerbils receiving oocysts without treatment resulted in positives for *N. caninum* for all 3 tests. Real-time PCR tests were positive only for brain tissues of 7 gerbils. In the heart samples, *N. caninum* was not found, reinforcing the importance of examining brain samples.

TABLE I. Results of direct examination for tissue cysts of *N. caninum* in the brain (cysts), immunohistochemistry tests (IHC), indirect immunofluorescence antibody tests (IFAT ≥ 100), western blotting (WB), and real-time PCR (PCR) in gerbils that had received different treatments (G1–G11) and were inoculated with 1,000 oocysts (P = Positive, N = Negative).

Group	Treatment	Gerbil					
		No.	Cysts	IHC	IFAT	WB	PCR
1	Absolute	1	N	N	P	P	N
	Alcohol	2	N	N	P	P	N
	24 C/1 hr	3	N	N	P	P	N
2	–20 C/6 hr	4	N	N	P	P	N
		5	N	N	P	P	N
		6	N	N	P	P	N
3	4 C/6 hr	7	N	N	P	P	N
		8	N	N	P	P	N
		9	N	N	P	P	P
4	60 C/1 min	10	N	N	P	P	P
		11	N	N	P	P	P
		12	N	P	P	P	N
5	100 C/1 min	13	N	N	N	N	N
		14	N	N	N	N	N
		15	N	N	N	N	N
6	10% Formaldehyde	16	N	N	P	P	N
		17	N	N	P	P	N
		18	N	N	P	P	N
7	10% Ammonia	19	N	N	P	P	N
		20	N	N	P	P	N
		21	N	N	P	P	P
8	2% Iodine	22	N	N	P	P	N
		23	N	N	N	N	N
		24	N	N	N	N	N

Both WB and IFAT produced identical results. In each case, 25 of the 33 gerbils tested positive for anti-*N. caninum* antibodies.

A given treatment was considered effective only if all 5 diagnostic tests proved negative, a condition only observed in 2 groups, G5 (100 C for 1 min) and G9 (10% sodium hypochlorite for 1 hr). In other treatments, at least 1 positive outcome after the inoculations of 3 gerbils was observed.

While 10% sodium hypochlorite for 1 hr at room temperature proved effective, shorter time periods should be tested to avoid prolonged exposure times in closed environments, especially if animals are present in the same area. The diversity of dilutions that are commercially available makes it difficult to establish specific guidelines for proper use in controlling *N. caninum*. Nonetheless, knowledge of the various dilutions, and their application times, should be further studied before being discarded as a practical solution. In contrast, high temperatures, either from flame, steam, or water sources, for at least 1 min may be the most practical and effective form of control in a closed environment.

Dubey et al. (2002) reported that a treatment of absolute (29.9%) ammonia hydroxide at temperatures ranging between 55 C and 70 C effectively inactivated *S. neurona*. In the present study, however, 10% ammonia hydroxide at room temperature was not effective. These results confirm the resistance of sporulated *N. caninum* oocysts to most chemical treatments, suggesting the use of high temperatures to inactivate sporulated oocysts.

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