Isolation and biological and molecular characterization of *Neospora caninum* (NC-SP1) from a naturally infected adult asymptomatic cattle (*Bos taurus*) in the state of São Paulo, Brazil

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SUMMARY

The biological and genetic diversity of *Neospora caninum* is very limited because of availability of only a few viable isolates worldwide. This study describes the isolation and biological and molecular characterization of a new viable isolate of *N. caninum* (NC-SP1), from a cattle in Brazil. Approximately 400 g of brain from a naturally infected adult male cattle from an abattoir was fed to a 2-month-old dog. *Neospora*-like oocysts were observed on day 7 post-inoculation (PI) and the duration of oocyst shedding was 14 days. The DNA obtained from oocysts was characterized molecularly and the final sequence was 99% identical to homologous sequences of *N. caninum* available in GenBank®. For bioassay, gerbils (*Meriones unguiculatus*) were orally inoculated with 10 100 and 1000 oocysts; all gerbils remained clinically normal but developed *N. caninum* antibodies 14 days PI. Cell culture isolation was successful using the brain homogenate from one of the gerbils and tachyzoites were observed 24 days PI. Microsatellite genotyping revealed a unique genetic profile for this new reference isolate.

Key words: Neosporosis, cattle, bioassay, oocyst shedding, gerbils, microsatellite analysis.

INTRODUCTION

Neospora caninum is an important cause of abortion in cattle worldwide. It has a wide host range and can cause clinical disease in several species of animals, including dogs (Dubey *et al*. 2017). It is morphologically and ancestorily related to *Toxoplasma gondii*, but canids (dogs, coyote, dingo and wolf) and not felids are its definitive hosts.

Unlike *T. gondii, N. caninum* is very difficult to isolate from naturally infected animals. For this reason, little is known of the genetic variability among *N. caninum* isolates from different hosts and most of the isolates were obtained from clinical cases. This limitation is apparent when a comparison is made: for *T. gondii* 1457 isolates (Shwab *et al*. 2014) compared with 108 *N. caninum* isolates (Regidor-Cerrillo *et al*. 2013) were analysed.

The aims of this study were to isolate viable *N. caninum* from cattle, to perform biological and molecular characterization and to provide a new *N. caninum* isolate that may be used as a reference for further studies.

MATERIALS AND METHODS

Naturally infected cattle

Brain and serum samples were collected from nine cattle slaughtered in an abattoir in the municipality of Santa Rita do Passa Quatro, state of São Paulo, Brazil. Serum samples were tested by indirect fluorescent antibody test (IFAT) to detect antibodies against *N. caninum* using cut-off of 1:100. Tachyzoite cell cultures, derived from the NC-1 strain of *N. caninum*, were used as antigen (Dubey *et al*. 1988). Secondary fluorescein isothiocyanate (FITC)-labelled conjugated IgG anti-bovine antibodies were used (Sigma-Aldrich®, Saint Louis, MO, USA). One adult, male cattle had an IFAT titre of 200 for *N. caninum* antibodies and was used for the present study.

Approximately 400 g of brain from the seropositive cattle was cut into small pieces and pooled.

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Around 10 g was separated for trypsin digestion and four aliquots were collected for further PCR analysis. The remainder was used to isolate *N. caninum* by bioasssay in a dog.

Bioassay in a dog

The brain tissue was mixed with dry commercial dog food and fed over 3-days to a 2-month-old female mixed-breed *N. caninum* seronegative dog (IFAT titre <50). The dog was donated by the owner to the experiment. This dog had never eaten raw meat and was kept on a diet of dry commercial dog food during the experimental period. Blood samples from the dog were collected weekly via the cephalic vein [0–135 days post-inoculation (PI)] and IFAT tests were performed using FITC-labelled conjugated IgG anti-dog antibodies (KPL® Inc., Gaithersburg, MD, USA) with a cut-off point of 1:50.

Fecal samples were examined daily (0–40 days PI) for detection of *Neospora*-like oocysts by means of a standard sucrose flotation technique. Daily output of oocysts as described (Pena *et al*. 2007). When oocysts were observed, the fecal samples were mixed with 2% H_2SO_4 for sporulation at 25 °C and thereafter stored at 4 °C.

To measure the size of the oocysts, images of 100 unsporulated oocysts were captured at the magnification of 400× and analysed using the Image-Pro Plus® software, version 5.1 (Media Cybermetics, Inc., Silver Spring, MD, USA).

Brain inoculation in rodents and cell culture

Around 10 g of the cattle brain tissue were digested with 0·05% trypsin for 1 h at 37 °C (Dubey *et al*. 2013). After the digestion, the homogenized suspension was washed three times with HBSS buffer (Hank's Balanced Salt Solution, GIBCO®, Grand Island, NY, USA) by centrifuging it at 1500 × ***g*** for 10 min and the resultant pellet was suspended in RPMI 1640 medium (Roswell Park Memorial Institute medium). Two flasks containing a monolayer of VERO cells were seeded with the sediment obtained (500 μL per flask). Two gerbils (*Meriones unguiculatus*) were intraperitoneally inoculated (500 μL per animal).

Bioassay of oocysts in gerbils and isolation in cell culture

Eight groups of two gerbils each were orally inoculated with 200 oocysts per gerbil. Every week, blood samples were collected from all the animals for serological analysis and one group was randomly euthanized. Tissues were collected from each animal for PCR assay. For isolation in cell culture, nine gerbils, divided into three groups, were orally infected with 10^1, 10^2 and 10^3 oocysts. The brains of the gerbils were aseptically collected, homogenized, digested with 0·05% trypsin or acid–pepsin solution (pepsin, 1·3 g; NaCl, 2·5 g; HCl, 3·5 mL; and distilled water, 250 mL) as described in (Dubey, 1998), and inoculated into a monolayer of VERO cells.

The serum samples were tested by means of IFAT using anti-gerbil IgG FITC-labelled conjugate (Immunology Consultants Laboratory® Inc., Portland, OR, USA). The cut-off was 1:50; the positive samples were diluted until the endpoint and the titres were then attributed.

DNA extraction and PCR assay

DNA from animal tissues was obtained using DNeasy Blood and Tissue Kit® (Qiagen Inc., Hilden, Germany), in accordance with the manufacturer's instructions.

To extract DNA from the oocysts, the oocysts were ruptured by means of six cycles of freezing in liquid nitrogen (−192 °C for 2 min) and thawing at 37 °C. After this pre-treatment, DNA extraction was done using the QIAamp DNA Stool Mini Kit® (Qiagen).

The PCR assays were performed using a pair of primers based on the Nc-5 gene (Np6 plus and Np21 plus) (Müller *et al*. 1996) for both the animal tissues and oocysts; and based on the ITS-1 region [(JS4) (Šlapeta *et al*. 2002) and (CT2c, JS4b and CT2b) (Soares *et al*. 2011)] for the oocysts. The amplified DNA was viewed by electrophoresis on 2% agarose gels stained with SYBR® Safe DNA gel stain (Invitrogen™, Carlsbad, CA, USA).

Identity of Neospora-like oocysts

The DNA of the *Neospora*-like oocysts were tested with an ITS-1 nested PCR–RFLP, in order to confirm the identity of the *Neospora*-like oocysts and to rule out mixed infection with oocysts of *Hammondia heydorni*. The primers were designed based on 18S and 5·8SrRNA coding genes and flanked the ITS-1 region, as described by Soares *et al*. (2011). The amplicons were digested using the restriction endonuclease TaqαI. Also, the oocysts were tested with Np6 plus and Np21 plus primers. The amplified DNA was viewed as described above.

ITS-1 sequencing of the isolate NC-SP1

DNA from tachyzoites was obtained from the cell culture. The ITS1–PCR products based on sense and antisense primers (JS4 and CT2b) were sequenced using the BigDye® Terminator v3·1 cycle sequencing kit and the sequencing products were analysed in the ABI 3730 DNA analyser (Life Technologies; Applied Biosystems, Foster City,

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CA, USA). The PCR amplicons were sequenced four times in both directions. The sequences were assembled and the contig was formed with the phred base-calling and phrap assembly tools, which are available in the Codoncode aligner suite v.1·5·2. (Codoncode Corp., Dedham, Ma, USA).

Microsatellite (MS) genotyping and eBURST analysis

Neospora caninum multilocus genotyping (MLG) based on nine MS markers (MS4, MS5, MS6A, MS6B, MS7, MS8, MS10, MS12 and MS21) was performed for the NC-SP1 isolate (DNA was derived from tachyzoites) under PCR conditions that had previously been described (Regidor-Cerrillo *et al*. 2013). These multiplex PCRs were performed on approximately 200 ng of DNA. *N. caninum* (Nc-Spain7) DNA was included in each batch of amplifications as a reference isolate. MS allele assignment was performed in accordance with the sizes determined by capillary electrophoresis and sequencing of the MS5, MS10 and MS7 markers was done as previously described (Regidor-Cerrillo *et al*. 2006, 2013). The size of the 6-FAM-labelled PCR products for all of the MSs was determined using a 48-capillary 3730 DNA analyser (Applied Biosystems) with Gene Scan-500 (LIZ) size standards (Applied Biosystems) and MS sequencing with the Big Dye Terminator v3·1 cycle sequencing kit (Applied Biosystems) and a 3730 DNA analyser (Applied Biosystems) at the Genomic Unit of the Madrid Science Park, Spain. The sizes of the PCR products and sequences were analysed using the GeneMapper1 v3·5 software (Applied Biosystems) and BioEdit Sequence Alignment Editor v.7·0·1 (Copyright_ 1997–2004 Tom Hall, Ibis Therapeutics, Carlsbad, CA, USA), respectively, as described previously (Regidor-Cerrillo *et al*. 2013).

The eBURST software was used to explore the closest genetically related genotypes of NC-SP1 in a dataset involving 81 *N. caninum* 9-MLGs from Argentine, Spanish, German and Scottish populations (Regidor-Cerrillo *et al*. 2013). The eBURST software generates networks composed of MLGs represented as dots, linked to their single-locus variants (SLV: 8 shared loci out of 9) and double-loci variants (DVL: 7 shared loci out of 9), line by line (Feil *et al*. 2004).

Animal ethics

All the animals were handled in accordance with protocols that had been approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine, University of São Paulo, São Paulo, Brazil (CEUA no. 8634270114), following the National Research Council Guide for the Care and Use of Laboratory Animals.

RESULTS

Bioassay in a dog

DNA of *N. caninum* was detected in one out of four aliquots from the cattle brain.

Neospora-like oocysts were detected in dog feces on day 7 PI. The duration of oocyst shedding was 14 days and the number of oocysts produced during the patent period was 144 500 oocysts. The average number of oocysts per gram of feces was 102 oocysts/g (a range of 2–946) (Supplementary Table S1). The unsporulated oocysts measured 9·85–12·20 (11·19 ± 0·45) μm × 10·28–12·42 (11·46 ± 0·47) μm.

No clinical signs were observed and antibodies against *N. caninum* (IFAT <50) were not detected in the dog serum during the period analysed (19 weeks).

Inoculation of the cattle brain in rodents and cell culture

The cell culture flask inoculated with the cattle brain tissue was examined for 2 months and no parasites were observed. Also, none of the gerbils seroconverted.

Infection of gerbils with oocysts and isolation in cell culture

All gerbils fed 200 oocysts seroconverted 14 days PI. The range of titres was from 100 to 25 600 (Supplementary Fig. S1). The gerbils remained clinically healthy. The PCR analysis based on the Nc-5 gene showed that the parasite was distributed in the tissues of the 16 gerbils (Supplementary Table S2).

Successful isolation in cell culture occurred with the brain homogenate from a gerbil inoculated with 10^2 oocysts that was euthanized 78 days PI. The acid–pepsin digestion method had been used for this sample. Tachyzoites were observed 24 days PI in the cell culture, but the isolate had slow growth. Six tissue cysts from fresh brain homogenate of the same gerbil were viewed under a microscope and were found to measure 23·84 ± 4·14 μm × 25·69 ± 5·30 μm.

Identity of Neospora-like oocysts

DNA of *N. caninum* was detected on 6 of the 14 days of oocyst shedding (days 7, 8, 9, 10, 11 and 13 PI) using nested ITS-1 PCR. In the same fecal samples, using Np6 plus and Np21 plus primers, it was also possible to detect DNA of *N. caninum*. The results based on ITS-1 nested PCR-RFLP indicated that only oocysts of *N. caninum* were present.

The final sequence deposited in GenBank®, under accession number KT581980, was 99% identical to

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Table 1. Microsatellite (MS) genotyping of the *Neospora caninum* isolate NC-SP1 and comparison with the genetically closest isolates analysed and with other Brazilian isolates

			MS markers								
Isolates	Host	Location	MS4	MS5	MS6A	MS6B	MS7	MS8	MS10	MS12	MS21
NC-SP1 (BRA-15)	Cattle	BRA	13	19	15	12	9·1	13	6·14·10	16	6
NC-Bahia	Dog	BRA	13	13	15	12	9·1	14	5·14·9	16	6
NC-Goiás	Cattle	BRA	13	18	14	12	9·1	12	6·16·9	16	6
SP02-GAL-S-26	Cattle	SPA	13	19	15	12	14	13	6·14·9	16	6
SP05-GAL-W-40	Cattle	SPA	13	15	15	12	9·1	13	6·14·8	16	6
SP06-GAL-W-48	Cattle	SPA	13	14	15	12	9·1	13	6·14·8	16	6
ARG-04-E-1	Cattle	ARG	13	17	15	12	9·1	13	6·15·8	16	6

BRA, Brazil; SPA, Spain; ARG, Argentina.

homologous sequences of many *N. caninum* isolates available in the same database.

MS genotyping and genetic relationship

Alleles for each of the nine MS markers analysed were amplified and identified from a DNA sample obtained from NC-SP1 tachyzoites. No new alleles were detected, although comparison of the NC-SP1 MLG with the database of 81 MLGs showed that it has a unique and different genetic profile. In addition, the eBURST analysis detected the absence of SLVs with NC-SP1. Only four DLVs (with variation in two MS markers) were found associated with the NC-SP1 MLG. These genetically closest MLGs involved three MLGs of Spanish and one MLG of Argentine origin (Supplementary Table S3, Figs. S2 and S3). In addition, comparison of NC-SP1 MLG with other MLGs of *N. caninum* isolates obtained in Brazil, the NC-Bahia (Gondim *et al*. 2001) and NC-Goiás (García-Melo *et al*. 2009) isolates, showed variation in three and four out of nine markers, respectively (Table 1).

DISCUSSION

Successful isolation of the NC-SP1 isolate of *N. caninum* from a naturally infected cattle was possible due to the bioassay in a dog. Attempts to isolate the parasite from the primary sample (cattle brain) in cell cultures and rodents were not successful in this study. Dogs are efficient experimental hosts for isolation of *N. caninum*, because they can ingest large amounts of animal tissues (Gondim *et al*. 2002). In our study, the dog ate approximately 390 g of brain, compared with 10 g used for cell cultures and rodents. However, not all laboratories have the proper facilities to house and use dogs as experimental models, and therefore standardized protocols to isolate *N. caninum* need to be improved. Dubey and Schares (2011) emphasized in their review that isolation of viable parasites is difficult and that finding DNA is not as valuable as isolating viable parasites.

In this report, the results related to the *N. caninum* bioassay in a dog corroborate previous findings. The number of oocysts excreted by the dog can be considered to be a medium quantity of oocysts for *N. caninum*, in comparison with other reports (Lindsay *et al*. 1999; Rodrigues *et al*. 2004). Gondim *et al*. (2002) reported variance in the quantity of oocysts shed by the dogs and they pointed out that the dogs shed more oocysts when they were fed with infected cattle tissues than when fed with infected mouse tissues. Furthermore, the dog remained clinically healthy and did not develop antibodies against *N. caninum*. These facts were also consistent with the findings from other studies (Basso *et al*. 2001; Gondim *et al*. 2005; Pena *et al*. 2007).

Gerbils are considered to be susceptible hosts for clinical neosporosis, but results are inconsistent (see Dubey *et al*. 2007). However, the stage of the parasite needs to be taken into consideration. In our study, none of the gerbils orally fed 1000 oocysts developed clinical signs, thus suggesting that the new isolate has low pathogenicity in gerbils. The capacity of isolates of *N. caninum* to induce clinical disease or death in the experimental models and the efficiency of *in vitro* proliferation are important features to be evaluated in virulence studies (Regidor-Cerrillo *et al*. 2011; Dellarupe *et al*. 2014). Nevertheless, the results from virulence studies should be evaluated with caution because the interpretation can change according to the hosts, laboratory conditions and biological characteristics. The NC-SP1 isolate grew slowly in the cell culture and did not have the capacity to destroy the monolayer of VERO cells. More efforts will be made to adapt the new isolate to cell culture.

The sequencing of the *N. caninum* ITS-1 region confirmed that the new isolate is a member of the species *N. caninum*. MS genotyping has the power to distinguish between individual organisms and this genetic analysis showed that NC-SP1 has a unique genetic profile, in comparison with the database of 81 *N. caninum* MLGs. In addition, the four genetically closest MLGs were from isolates that originated

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from aborted bovine fetuses in Spain and Argentina (Regidor-Cerrillo *et al*. 2013). Unfortunately, there is only a limited number of Brazilian isolates, and therefore NC-SP1 MLG could only be compared with two other isolates: NC-Bahia (Gondim *et al*. 2001) and NC-Goiás (García-Melo *et al*. 2009), isolates from a dog and a bovine, respectively.

In summary, our study presented the biological and genetic characterization of a new isolate of *N. caninum*, named NC-SP1, from an adult naturally infected cattle. New attempts to isolate *N. caninum* from domestic and wild animals would be important for improving the understanding of the biological and genetic diversity among *N. caninum* isolates in Brazil.

SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at https://doi.org/10.1017/S0031182016002481

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REFERENCES

Basso, W., Venturini, L., Venturini, M. C., Hill, D. E., Kwok, O. C., Shen, S. K. and Dubey, J. P. (2001). First isolation of *Neospora caninum* from the feces of a naturally infected dog. *Journal of Parasitology* **87**, 612–618.

Dellarupe, A., Regidor-Cerrillo, J., Jiménez-Ruiz, E., Schares, G., Unzaga, J. M., Venturini, M. C. and Ortega-Mora, L. M. (2014). Comparison of host cell invasion and proliferation among *Neospora caninum* isolates obtained from oocysts and from clinical cases of naturally infected dogs. *Experimental Parasitology* **145**, 22–28.

Dubey, J. P. (1998). Refinement of pepsin digestion method for isolation of *Toxoplasma gondii* from infected tissues. *Veterinary Parasitology* **74**, 75–77.

Dubey, J. P. and Schares, G. (2011). Neosporosis in animals – the last five years. *Veterinary Parasitology* **180**, 90–108.

Dubey, J. P., Carpenter, J. L., Speer, C. A., Topper, M. J. and Uggla, A. (1988). Newly recognized fatal protozoan disease of dogs. *Journal American Veterinary Medicine Association* **192**, 1269–1285.

Dubey, J. P., Schares, G. and Ortega-Mora, L. M. (2007). Epidemiology and control of neosporosis and *Neospora caninum*. *Clinical Microbiology Reviews* **20**, 323–367.

Dubey, J. P., Choudhary, S., Ferreira, L. R., Kwok, O. C. H., Butler, E., Carstensen, M., Yu, L. and Su, C. (2013). Isolation and RFLP genotyping of *Toxoplasma gondii* from the gray wolf (*Canis lupus*). *Veterinary Parasitology* **197**, 685–690.

Dubey, J. P., Hemphill, A., Calero-Bernal, R. and Schares, G. (2017). *Neosporosis of Animals*. CRC Press, Boca Raton, Florida, USA. In press.

Feil, E. J., Li, B. C., Aanensen, D. M., Hanage, W. P. and Spratt, B. G. (2004). eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *Journal of Bacteriology* **186**, 1518–1530.

García-Melo, D. P., Regidor-Cerrillo, J., Ortega-Mora, L. M., Collantes-Fernández, E., de Oliveira, V. S. F., de Oliveira, M. A. P. and da Silva, A. C. (2009). Isolation and biological characterisation of a new isolate of *Neospora caninum* from an asymptomatic calf in Brazil. *Acta Parasitologica* **54**, 180–185.

Gondim, L. F. P., Pinheiro, A. M., Santos, P. O. M., Jesus, E. E. V., Ribeiro, M. B., Fernandes, H. S., Almeida, M. A. O., Freire, S. M., Meyer, R. and McAllister, M. M. (2001). Isolation of *Neospora caninum* from the brain of a naturally infected dog, and production of encysted bradyzoites in gerbils. *Veterinary Parasitology* **101**, 1–7.

Gondim, L. F. P., Gao, L. and McAllister, M. M. (2002). Improved production of *Neospora caninum* oocysts, cyclical oral transmission between dogs and cattle, and *in vitro* isolation from oocysts. *Journal of Parasitology* **88**, 1159–1163.

Gondim, L. F. P., McAllister, M. M. and Gao, L. (2005). Effects of host maturity and prior exposure history on the production of *Neospora caninum* oocysts by dogs. *Veterinary Parasitology* **134**, 33–39.

Lindsay, D. S., Dubey, J. P. and Duncan, R. B. (1999). Confirmation that the dog is a definitive host for *Neospora caninum*. *Veterinary Parasitology* **82**, 327–333.

Müller, N., Zimmermann, V., Hentrich, B. and Gottstein, B. (1996). Diagnosis of *Neospora caninum* and *Toxoplasma gondii* infection by PCR and DNA hybridization immunoassay. *Journal of Clinical Microbiology* **34**, 2850–2852.

Pena, H. F. J., Soares, R. M., Ragozo, A. M. A., Monteiro, R. M., Yai, L. E. O., Nishi, S. M. and Gennari, S. M. (2007). Isolation and molecular detection of *Neospora caninum* from naturally infected sheep from Brazil. *Veterinary Parasitology* **147**, 61–66.

Regidor-Cerrillo, J., Pedraza-Díaz, S., Gómez-Bautista, M. and Ortega-Mora, L. M. (2006). Multilocus microsatellite analysis reveals extensive genetic diversity in *Neospora caninum*. *Journal of Parasitology* **92**, 517–524.

Regidor-Cerrillo, J., Gómez-Bautista, M., Sodupe, I., Aduriz, G., Álvarez-García, G., Del Pozo, I. and Ortega-Mora, L. M. (2011). *In vitro* invasion efficiency and intracellular proliferation rate comprise virulence-related phenotypic traits of *Neospora caninum*. *Veterinary Research* **42**, 41.

Regidor-Cerrillo, J., Díez-Fuertes, F., García-Culebras, A., Moore, D. P., González-Warleta, M., Cuevas, C., Schares, G., Katzer, F., Pedraza-Díaz, S., Mezo, M. and Ortega-Mora, L. M. (2013). Genetic diversity and geographic population structure of bovine *Neospora caninum* determined by microsatellite genotyping analysis. *PLoS ONE* **8**, 1–12.

Rodrigues, A. A. R., Gennari, S. M., Aguiar, D. M., Sreekumar, C., Hill, D. E., Miska, K. B., Vianna, M. C. B. and Dubey, J. P. (2004). Shedding of *Neospora caninum* oocysts by dogs fed tissues from naturally infected water buffaloes (*Bubalus bubalis*) from Brazil. *Veterinary Parasitology* **124**, 139–150.

Shwab, E. K., Zhu, X. Q., Majumdar, D., Pena, H. F. J., Gennari, S. M., Dubey, J. P. and Su, C. (2014). Geographical patterns of *Toxoplasma gondii* genetic diversity revealed by multilocus PCR-RFLP genotyping. *Parasitology* **141**, 453–461.

Šlapeta, J. R., Koudela, B., Votýpka, J., Modrý, D., Hořejš, R. and Lukeš, J. (2002). Coprodiagnosis of *Hammondia heydorni* in dogs by PCR based amplification of ITS 1 rRNA: differentiation from morphologically indistinguishable oocysts of *Neospora caninum*. *Veterinary Journal* **163**, 147–154.

Soares, R. M., Lopes, E. G., Keid, L. B., Sercundes, M. K., Martins, J. and Richtzenhain, L. J. (2011). Identification of *Hammondia heydorni* oocysts by a heminested-PCR (hnPCR-AP10) based on the *H. heydorni* RAPD fragment AP10. *Veterinary Parasitology* **175**, 168–172.