Parasitol Res (2010) 106:457–461
DOI 10.1007/s00436-009-1686-4

ORIGINAL PAPER

Investigation of *Neospora caninum*, *Hammondia* sp., and *Toxoplasma gondii* in tissues from slaughtered beef cattle in Bahia, Brazil

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Received: 11 October 2009 / Accepted: 9 November 2009 / Published online: 27 November 2009

Abstract *Neospora caninum*, *Hammondia* sp., and *Toxoplasma gondii* are parasites with morphological and genetic similarities. *N. caninum* and *T. gondii* are important abortive agents of cattle and sheep, respectively, and may infect numerous animal species. *Hammondia* sp. is not known to induce disease in animals, but may cause confusion in the identification of closely related coccidia. The aim of this study was to investigate infection rates caused by *N. caninum*, *Hammondia* sp., and *T. gondii* in beef cattle using a nested PCR for Toxoplasmatinae rDNA, followed by sequencing of the PCR products. Antibodies to *N. caninum* and *T. gondii* were also investigated in the tested animals. Brains and hearts were obtained from 100 beef cattle in a slaughterhouse in Bahia. Seven samples from brain tested positive for Toxoplasmatinae DNA. No positive reactions were found in heart tissues. After sequencing of the PCR products from all positive tissues, five sequences matched with *N. caninum* and two matched with *T. gondii.* Antibodies to *N. caninum* and *T. gondii* were found in 20% and 26% of the animals, respectively. The confirmation of *N. caninum* and the absence of *Hammondia heydorni* in the tested animals is suggestive that cattle are not efficient intermediate hosts of *H. heydorni*; however further studies need to be performed using a greater variety of tissues and a higher sample size. The detection of *T. gondii* DNA in bovine tissues reinforces the potential risk of transmission of this parasite to humans and other animals through the consumption of bovine meat.

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Introduction

Neospora caninum, *Hammondia heydorni*, *Hammondia hammondi*, and *Toxoplasma gondii* are closely related cyst-forming coccidia. *N. caninum* is known to cause abortion and significant economic losses in cattle worldwide (Dubey 2003). *T. gondii* induces serious disease in humans and also causes abortion in sheep and other animal species (Tenter et al. 2000). *Hammondia* sp. is considered to be non-pathogenic for animals or humans (Dubey et al. 2002). Comparisons among these coccidia revealed that *N. caninum* and *H. heydorni* are phylogenetically more closely related than between *N. caninum* and *T. gondii* (Ellis et al. 1999; Mugridge et al. 1999; Siverajah et al. 2003). *N. caninum* and *H. heydorni* have canids as definitive hosts, while *T. gondii* and *H. hammondi* have felids as definitive hosts (Dubey et al. 2002). The oocysts of these parasites are morphologically similar (Schares et al. 2001; Slapeta et al. 2002). Studies about *H. heydorni* prior to the classification of *N. caninum* should be interpreted with caution, because no tests were available to differentiate these two parasites at that time (Schares et al. 2001; Slapeta et al. 2002).

Molecular techniques offer great possibilities to solve several questions regarding differentiation of Toxoplasmatinae organisms (Payne and Ellis 1996). The internal transcribed spacer 1 (ITS-1) of the rDNA has been a target for the development of primers that can differentiate sequences between these closely related coccidia. Variations of the ITS-1 sequences have allowed the classification of new parasite species as well as the presence of intra-species variations (Abel et al. 2006; Marsh et al. 1998; Schares et al. 2002; Slapeta et al. 2002; Sreekumar et al. 2003).

Although numerous serological studies have been done with *N. caninum* in a diversity of species, it is not clear whether *H. heydorni* induces cross-reaction in serological tests with *N. caninum* as observed between *T. gondii* and *H. hammondi* (Riahi et al. 1998). Epidemiologic studies involving *H. heydorni* are scarce because it could not be continuously maintained in cell culture, and serological tests have not been developed for this parasite (Mugridge et al. 1999; Riahi et al. 1995; Schares et al. 2003; Speer et al. 1988).

N. caninum frequently causes disease in cattle (Dubey 2003), while *T. gondii* is not commonly associated with disease in this animal species; however, the consumption of infected bovine meat may cause infection in humans and other animals (Tenter et al. 2000). To the authors' knowledge, there is no epidemiological study about *H. heydorni* infection in cattle. The aim of this study was to investigate the infection rates of Toxoplasmatinae parasites (*N. caninum*, *Hammondia* sp., and *T. gondii*) in beef cattle using a nested PCR followed by sequencing of the amplified fragments.

Materials and methods

Animals and sampling

Samples of blood, brain, and heart were collected from 100 beef cattle (mixed breed and Nelore) at a commercial slaughterhouse in Salvador metropolitan region, Bahia—Brazil, between March and November 2008. The animals originated from different counties (Itapetinga, Itaju do Colônia, Ipirá, Marcionílio Souza, Fátima, and Macajuba) which are located between 200 and 600 km from Salvador.

The sera were separated and stored at −20°C until the execution of the serological tests. The entire brains and hearts were individually packaged, refrigerated, and transported to the Universidade Federal da Bahia. One third of brain and 200–250 g of different segments of heart were stored at −20°C until DNA extraction. All materials used in between each tissue collection were decontaminated with sodium hypochlorite solution (2.5%) followed by dH_2O to prevent DNA cross-contamination.

DNA extraction and nested PCR

A total of 200 samples (100 from brains and 100 from hearts) were individually homogenized with pestle and mortar in liquid nitrogen, and in each procedure, the same DNA decontamination protocol described before was used in the implements. DNA extraction was performed using an Easy-DNA™ kit (Invitrogen, São Paulo, SP, Brazil).

A nested PCR was performed for detection of the ITS-1 of Toxoplasmatinae rDNA, as previously described (Silva et al. 2009). The primers JS4 that anneals to the 3′ conserved region of the SSU rRNA gene (Slapeta et al. 2002) and CT2b that anneals the 5.8S rRNA (Monteiro et al. 2007) were selected as external primers (~500 bp of band size), while CT1/CT2 (Sreekumar et al. 2003) were employed as internal primers for the ITS-1 sequences (~400 bp of band size) of *N. caninum*, *Hammondia* sp., and *T. gondii*.

Each amplification was performed in 12.5 μL volumes containing 0.25 μL of each primer, 6.5 μL of PCR Master Mix (50 U/mL TaqDNA polymerase in a proprietary reaction buffer, pH 8.5, 400 lM each deoxynucleoside triphosphate and 3 mM $MgCl_2$; Promega, Madison, WI, USA), 5.25 μL of ultra-pure water and 0.5 μL of DNA template. Reactions were run in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) thermal cycler.

The first PCR reaction (JS4/CT2b) was done under the following conditions: 1 cycle at 94°C for 5 min; 40 cycles of 94°C for 1 min, 60°C for 1 min; 72°C for 1 min followed by 72°C for 7 min. The conditions of the second PCR reaction (CT1/CT2) were the same as the first, except for the annealing temperature that was 55°C. PCR products were analyzed after the second reaction by electrophoresis on 2% agarose gel stained with blue green and visualized under ultraviolet light. DNA from *H. heydorni* oocysts obtained from a naturally infected dog (Monteiro et al. 2007) was used as a positive control and ultra-pure distilled water as a negative control.

Sequencing procedures

When amplicons were visualized, reactions were repeated using the original genomic DNA for a total volume of 50 μL, and 30 μL of each positive sample was again subjected to electrophoresis in 1% agarose for amplicon isolation. The amplicons were extracted from the gel using the Wizard SV Gel and PCR Clean-Up System kit (Promega) according to the manufacturer's instructions.

The purified PCR products were directly sequenced in the forward and reverse directions using the Big Dye terminator system, version 3.1 (Applied Biosystems) and an ABI 3130XL sequencer (Applied Biosystems). The sequence chromatograms were edited using BioEdit Sequence Align-

ment Editor version 7.0.9.0 (Ibis Biosciences, Carlsbad, CA, USA). BLAST searches were performed in order to compare the sequences with those in the public database (http://www.ncbi.nlm.nih.gov).

Immunofluorescent antibody tests

The serum samples were diluted at 1:200 and 1:50 for *N. caninum* and *T. gondii* immunofluorescent antibody tests (IFAT), respectively. Tachyzoites of *N. caninum* (NC-Bahia strain; Gondim et al. 2001) and *T. gondii* (RH strain) were used as antigens in each test. A fluorescein isothiocyanate-labeled anti-mouse IgG was used as a secondary antibody (Sigma, St. Louis, MO, USA). Positive sera were two-fold diluted until the end point.

Results

Brain and heart tissues from 100 beef cattle were tested for *N. caninum*, *Hammondia* sp., and *T. gondii*. PCR-positive results were observed in 7% of the tested animals. Positive reactions were only found in brain tissues. ITS-1 sequences of the amplicons were obtained for the seven samples and subjected to a BLAST search to obtain the best score. Five sequences matched with *N. caninum* (GenBank TM accession numbers FJ966043, FJ966044, FJ966045, FJ966046, and FJ966047), and two matched with *T. gondii* (GenBank TM accession numbers FJ966048 and FJ966049). The sequences of each identified species shared 97–100% identity with sequences from other strains of these coccidia present in GenBank (AF432123, AY582110, U16161, AY665718.1, and AF432123.1).

Antibodies to *N. caninum* were found in 20% of serum samples with titers of 1:200 (nine animals), 1:400 (six), and 1:800 (five), while antibodies to *T. gondii* were found in 26% of animals with titers of 1:50 (one animal), 1:100 (nine), and 1:200 (16). In a total of 20 serum samples positive for *N. caninum*, 14 sera reacted solely with *N. caninum*, and six sera reacted with both *N. caninum* and *T. gondii*. Most of PCR-positive animals were seronegative for *N. caninum* or *T. gondii* (Table 1).

Discussion

Infections by *N. caninum* and *T. gondii* have been confirmed in beef cattle using a nested PCR for Toxoplasmatinae DNA followed by sequencing of the amplified fragments. *H. heydorni* has not been detected in the tested animals. In a recent study using 102 slaughtered goats in Bahia, a higher number of *H. heydorni*-infected animals was detected when compared to *N. caninum*-infected ones, and *H. heydorni* DNA was mostly found in the heart (Silva et al. 2009). In the present report, the detection of *N. caninum* in 5% of the animals and the absence of *H. heydorni* is suggestive that cattle are less susceptible to *H. heydorni* infection when compared with *N. caninum*.

In a previous review article (Heydorn and Mehlhorn 2002), the authors mentioned a study from 1972 reporting that dogs fed with cattle tissues shed *H. heydorni* oocysts. However, at the time that study was done, *N. caninum* had not been described. It is not possible to be certain about the identity of the oocysts without molecular differentiation (Ellis et al. 1999).

It is important to note that in different experiments using cattle or buffaloes to induce excretion of *N. caninum* oocysts in dogs (Dijkstra et al. 2002; Gondim et al. 2002; Rodrigues et al. 2004), *H. heydorni* has not been detected. Shedding of *H. heydorni* oocysts has been reported in dogs fed with other non-bovine ruminants (Schares et al. 2001; Mohammed et al. 2003; Dubey et al. 2004) or in dogs with spontaneous oocyst shedding without a clear history of consuming any specific meal (Slapeta et al. 2002). Despite the phylogenetical and morphological similarities between *H. heydorni* and *N. caninum*, it is possible that the susceptibility of cattle for these parasites is different.

In a recent study (Soares et al. 2009), two foxes (*Cerdocydon thous*) shed *H. heydorni* oocysts after consuming a pool of masseter muscle and brain from two adult cattle, which had IFAT titers of 100 for *N. caninum*. The shedding of *H. heydorni* oocysts by the two foxes after consumption of bovine tissues (Soares et al. 2009) seems to be the unique confirmation of cattle as intermediate host of the parasite.

Table 1 Results of a nested PCR followed by sequencing for detection of Toxoplasmatinae DNA in bovine tissues and IFAT for *Neospora caninum* and *Toxoplasma gondii* using the cattle sera

Sample ID	Tissue	Anti-*N. caninum* antibodies	Anti-*T. gondii* antibodies	Sequencing result
04	Brain	Negative	Negative	*Neospora caninum*
09	Brain	Positive (1:400)	Positive (1:200)	*Neospora caninum*
46	Brain	Negative	Negative	*Toxoplasma gondii*
63	Brain	Negative	Negative	*Toxoplasma gondii*
67	Brain	Negative	Positive (1:50)	*Neospora caninum*
75	Brain	Negative	Negative	*Neospora caninum*
80	Brain	Negative	Negative	*Neospora caninum*

The finding of *T. gondii* DNA in 2% of beef cattle enhances the potential risk of transmission of this parasite through bovine meat for humans and other animal species. The cattle used in this study were raised extensively and are supposed to be less exposed to parasite oocysts than dairy cattle or intensively cared beef cattle are.

The frequencies of antibodies to *N. caninum* and *T. gondii* detected by IFAT were 20% and 26%, respectively. The frequency of *N. caninum*-seropositive animals was similar to a previous report in Bahia (Gondim et al. 1999a); however, the seropositivity observed for *T. gondii* was higher than previously reported in the same region (Gondim et al. 1999b), suggesting that cattle in the current study had a higher exposure to *T. gondii*. The nested PCR resulted in a lower number of infected animals than the serology, which may be explained by the reduced number of tissues used for the PCR and because only a portion of brain and heart (not the entire organs) was used for DNA extraction.

In a similar study performed with 102 goats, the use of heart and brain allowed the detection of *H. heydorni*, *T. gondii*, and *N. caninum* (Silva et al. 2009). Therefore, more studies on *H. heydorni* infection need to be performed using a greater variety of bovine tissues and a higher number of animals.

Acknowledgments This work has been supported by a research grant from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) of Brazil. Sara L. Santos was recipient of a fellowship from FAPESB (Fundação de Amparo à Pesquisa do Estado da Bahia). Luís F.P. Gondim was recipient of a productivity fellowship from CNPq, Brazil.

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