



Molecular frequency and isolation of cyst-forming coccidia from free ranging chickens in Bahia State, Brazil

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ABSTRACT

The Toxoplasmatinae parasites *Toxoplasma gondii*, *Neospora caninum* and *Hammondia* spp. have carnivores as definitive hosts that shed the parasite oocysts in their feces. Birds that feed directly from the soil, such as chickens, are exposed to infection and may serve as indicators of the presence of the parasite in the environment and as a source of infection for other animals. The aims of this study were to determine the frequency of infection by these parasites in free ranging chickens, to test whether chickens are intermediate hosts of *Hammondia* spp., and to isolate *N. caninum* from chickens. One hundred chickens, which were raised in contact to cattle and dogs, were bought in five towns located in Bahia, Brazil. Blood and tissues (brain and heart) were used for serology, molecular tests and bioassay in mice for parasite isolation. *T. gondii* DNA was detected in 29 chickens, and *N. caninum* DNA was observed in six animals. *Hammondia* spp. DNA was not detected in tissues from any chicken. Tissues from eight *N. caninum* seropositive chickens were bioassayed in interferon-gamma gene knockout mice, but the mice did not become infected; *T. gondii* was isolated from six of 14 seropositive chickens after bioassay in outbred Swiss mice. The authors concluded that: chickens seem to be better hosts for *T. gondii* when compared to *N. caninum*, based on the molecular and bioassay results; *Hammondia* spp. probably does not infect chickens or is rarely found in this animal species.

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1. Introduction

Toxoplasma gondii, *Neospora caninum* and *Hammondia* spp. are cyst-forming coccidian parasites that belong to the subfamily Toxoplasmatinae (Frenkel and Smith, 2003). *T. gondii* is known to infect warm-blood animals, including birds, but most infected birds do not show sign of the disease (Dubey, 2002). Chickens are efficient intermediate

hosts of *T. gondii* and serve as good indicators of environmental contamination with oocysts (Dubey, 2010). *T. gondii* has been transmitted to mice and cats using tissues from naturally infected chickens (Dubey et al., 2005).

N. caninum infects several mammalian species and has been detected in chickens (Costa et al., 2008), sparrows (*Passer domesticus*) (Gondim et al., 2010), magpies (*Pica pica*) and buzzard (*Buteo buteo*) (Darwich et al., 2012), but the parasite has not been isolated from naturally infected birds so far. Experimental infections with *N. caninum* were performed in chickens and embryonated eggs; dogs that consumed *N. caninum*-infected embryonated eggs shed the

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parasite oocysts, suggesting that chickens may serve as intermediate hosts for the parasite (Costa et al., 2008; Gondim et al., 2010). Mansourian et al. (2009) have also demonstrated the susceptibility of embryonated eggs from broiler chickens to *N. caninum* infection. In both studies, embryo mortality and birth of sick chicks occurred after *N. caninum* infection. Recently, *N. caninum* antibodies were detected in 39.5% of 1324 chickens from several countries in America, showing a large exposure of chickens to the parasite in the studied regions (Martins et al., 2011). However, the role of chickens as intermediate hosts of *N. caninum* has not been demonstrated yet.

Hammondia hammondi and *H. heydorni* are closely related to *T. gondii* and *N. caninum*, respectively, and both have no clinical relevance in animals; however, infectious stages of *Hammondia* spp. are similar to *T. gondii* and *N. caninum* and should be differentiated from these parasites (Dubey et al., 2002b; Dubey and Sreekumar, 2003).

Chickens may be infected by *T. gondii* and *N. caninum* after ingestion of sporulated oocysts, mainly free ranging chickens which feed directly from the soil, being more exposed to oocysts (Dubey et al., 1993; Costa et al., 2008). Felines and canines are definitive hosts for *T. gondii* and *N. caninum* (Frenkel et al., 1970; McAllister et al., 1998), respectively, and may shed oocysts on the soil; these animals are also definitive hosts of *Hammondia* spp. (Frenkel and Dubey, 2000; Slapeta et al., 2002), so chickens are probably exposed to *Hammondia* spp. after ingestion of parasite oocysts from the soil. The aims of this study were to determine the molecular frequency of *N. caninum*, *T. gondii* and *Hammondia* spp. infection in free ranging chickens, to verify whether chickens serve as intermediate host of *Hammondia* spp., and to isolate *N. caninum* from naturally infected chickens.

2. Materials and methods

2.1. Animals

One hundred adult chickens were bought from small farmers in towns located 50–300 km far from Salvador, Bahia. This sample size is able to detect at least one infected chicken with 99% probability assuming a constant prevalence of 5% and infinite population. In all farms where the chickens were obtained, there were bovines and at least one dog. The animals were acquired in five towns: Serinha (48 chickens), São Gonçalo (11), Irará (13), Itaparica Island (08), and Conceição de Feira (20). The chickens were transported to the Veterinary Hospital at the Universidade Federal da Bahia and were kept in individual metallic cages with water and commercial food until blood collection and euthanasia. After euthanasia the whole brain and heart tissues were collected for molecular analysis and mice bioassay.

Fourteen female Swiss mice and eight female interferon-gamma gene knockout (KO) mice, with ages between eight and 12 weeks, were obtained from Laboratório Central (LACEN/Bahia) and the Instituto Oswaldo Cruz (Fiocruz, Bahia), respectively. The Swiss mice were used in the bioassay for *T. gondii* and the KO mice in the bioassay for *N. caninum*.

All the animal procedures were performed in compliance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation, and approved by the Institutional Bioethics Committee (Protocol: 001/2009).

2.2. Nested PCR

Heart and brain tissues from 100 chickens were used for DNA extraction. Half from each sample was individually homogenized with liquid nitrogen and the DNA was extracted using a commercial DNA extraction kit (Easy-DNA™, Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. After the extraction, DNA samples were stored at -20°C until the execution of the PCR reactions.

A nested PCR was performed to detect the internal transcribe spacer 1 (ITS1) of Toxoplasmatinae (*T. gondii*, *N. caninum*, and *Hammondia* spp.) rDNA, as previously described (Silva et al., 2009). Two pairs of primers complementary to portions of the ITS1 sequences conserved among *Hammondia* spp., *N. caninum* and *T. gondii* were employed. The first reaction used the external primer JS4 (5'-CGA AAT GGG AAG TTT TGT GAA C-3') (Slapeta et al., 2002), that anneals to the 3' conserved region of the 18S rRNA gene and CT2b (5'-TTG CGC GAG CCA AGA CAT C-3') (Monteiro et al., 2007), that anneals to the 5' conserved region of the 5.8S rRNA gene. The second reaction used primers CT1 (5'-TGA ATC CCA AGC AAA ACA-3') and CT2 (5'-G CGC GAG CCA AGA CAT CCA T-3') (Sreekumar et al., 2003) that anneal to the 3' region of the ITS1 and to the 5' conserved region of the 5.8S rRNA gene, respectively.

Each amplification reaction was conducted in 25 μl reaction mixtures containing 0.5 μl of each of the respective primer, 12.5 μl of PCR commercial mix (Master Mix, Promega, Madison, WI, USA), 10.5 μl of ultra-pure water and 1.0 μl of template DNA. Reactions were run in GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) thermal cyclor.

The first PCR reaction (JS4/CT2b) was done under the following conditions: 40 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. The second reaction (CT1/CT2) consisted of 40 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min; both preceded by an initial denaturation step of 94°C for 5 min and followed by a final extension at 72°C for 7 min. The expected size of the amplicon was about 500 bp for the first reaction and 400 bp for the second one. Positive control DNA was from *H. heydorni* oocysts from a naturally infected dog (Monteiro et al., 2007) and negative control consisted of ultra-pure water.

The PCR products were analyzed by electrophoresis in a 2% agarose gel stained with "Blue Green Loading Dye I" (LGC, Biotecnologia, Cotia, SP, Brazil) and visualized under ultraviolet light. A 100 bp DNA ladder (Invitrogen, Carlsbad, CA, USA) was used as a marker. When amplicons were visualized, reactions were repeated using the original genomic DNA and each positive sample was again subjected to electrophoresis in 1% agarose gel for amplicon isolation. The amplicons were excised with a fine scalpel blade and extracted from the gel using the Wizard SV Gel

and PCR Clean-Up System kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

2.3. Species-specific PCR for *T. gondii* and *N. caninum*

To confirm the identity of the tachyzoites isolated in cell culture, conventional species-specific PCRs were conducted for *T. gondii* and *N. caninum* using the primers TgB1.1/TgB1.4 (Burg et al., 1989) and Np21/Np6 (Yamaga et al., 1996), respectively, according to protocols described by the mentioned authors.

2.4. Sequencing procedures

The PCR products were directly sequenced in the forward and reverse directions using the primers CT1/CT2 and the Big Dye Terminator 3.1 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The thermocycling conditions consisted of 96 °C for 1 min, 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. These steps were all performed in slow ramp (1 °C/s). The purification was conducted by ethanol/EDTA method. The basecalling was done using the software Sequence Analysis (Applied Biosystems, Foster City, CA, USA) and the quality of the sequences was evaluated using the software Sequence Scanner (Applied Biosystems, Foster City, CA, USA). The sequencing results were edited using the programs Pre-gap 4, version 1.5, and Gap, version 4.10 (Staden Package, <http://staden.sourceforge.net/>). The sequences were submitted to homology search by BLASTN at the NCBI server (<http://www.ncbi.nlm.nih.gov/BLAST/>).

2.5. Immunofluorescent antibody tests

Chicken and mouse sera were tested for *N. caninum* and for *T. gondii* using immunofluorescent antibody tests (IFAT) according to methods previously described (Costa et al., 2008). IFATs for *N. caninum* and *T. gondii* were performed using tachyzoites of the strains NC-Bahia (Gondim et al., 2001) and RH as antigens, respectively. Commercial fluorescein-labeled anti-chicken IgG (Zymed, Invitrogen, Camarillo, CA, USA) or anti-mouse IgG (Sigma, St. Louis, MO, USA) were used as the secondary antibodies. The samples were screened for *T. gondii* at a 1:50 dilution (mouse sera) and at 1:16 dilution (chicken sera). The sera for *N. caninum* were screened at 1:50 for both chicken and mouse (Costa et al., 2008). A reaction was considered positive if fluorescence occurred around the complete periphery of tachyzoites. Positive sera were diluted 2-fold until the end point. Negative and positive controls sera were included on each slide.

2.6. Bioassay and in vitro isolation

Brain and heart tissues (1/4 of each organ) from chickens that tested positive for *T. gondii* or *N. caninum* antibodies were collected and kept for 1–48 h in PBS with 2% of antibiotic/antimycotic solution (Invitrogen, Auckland, New Zealand) for parasite isolation attempts. The tissues were processed as previously described with slight modifications (Gondim et al., 2001). Briefly, the tissues

were macerated in an equal volume of PBS with antibiotic/antimycotic. The suspension was filtered in sterile gauze, centrifuged at 1000 × g for 10 min, and the sediment was treated with 0.25% trypsin solution for 30 min at 37 °C. After incubation the mixture was washed with PBS by centrifugation three times at 1000 × g for 5 min. The sediment was separated and used for animal inoculations.

The KO mice and Swiss mice were employed for *N. caninum* and *T. gondii* isolation, respectively. Each mouse was inoculated intraperitoneally with 0.5 ml of processed brain and heart from each seropositive chicken. Animals which presented any clinical sign were euthanized in a chamber with enflurane (Farmasa, SP, Brazil). The remaining mice were maintained until 50 days after inoculation, and then euthanized as described above. Blood samples from mice were collected after euthanasia for serology. Only the seropositive mice were used for cell culture isolation attempts, according to previously described methods (Gondim et al., 2001). Tachyzoites observed in cell culture were submitted to species-specific PCR for *N. caninum* and *T. gondii* (described above).

2.7. Statistical analysis

Data from cross tabulation between serological tests of *N. caninum* and *T. gondii*, and nested PCR performed in the heart and brain, were used to test the correlation by Kappa statistics. *P* values ≤0.05 were considered as statistically significant.

3. Results

Nested PCR-positive reactions for the subfamily Toxoplasmatinae were observed in 42 of 100 tested chickens (42%). DNA fragments detected in the tested tissues had approximately 400 bp. DNA from the studied species were identified in 35 of the 42 PCR-positive chickens. The poor amount or quality of DNA obtained from seven chickens impaired the nucleotide sequencing of these bird samples.

After sequencing of the PCR products from the second amplification, *T. gondii* DNA was observed in 29 chickens (GenBank™ accession nos. JF810931–JF810959), and *N. caninum* DNA was detected in six birds (GenBank™ accession nos. JF810960–JF810965). The sequences identified as *T. gondii* matched 99–100% with each other and with other sequences deposited in GenBank™ (accession nos. EU025025, AY582110, AF252408, and U16161), except the sequence from one chicken (accession no. JF810938), which shared 92% of similarity with the sequences mentioned above. The sequences identified as *N. caninum* matched 99–100% with each other and with other sequences deposited in GenBank™ (accession nos. GQ899204, GU194959, AF432123, and AF029702). No chickens presented simultaneous infection with both parasites.

Positive PCR reactions for Toxoplasmatinae DNA were observed in 30 brains and in 25 hearts; 13% of the samples had simultaneous infections in the brain and heart for a unique parasite species. There was a significant agreement between the PCR results in both organs (Kappa = 0.27; *P* = 0.006). *T. gondii* DNA was detected in 22 brains and in 15

Table 1

Nested PCR for Toxoplasmatinae, tissue containing parasite DNA, and species identification by nucleotide sequencing in 100 free ranging chickens from Bahia, Brazil.

Parasite DNA	Brain	Heart	In both brain and heart	Total of infected chickens
Toxoplasmatinae	30	25	13	42
<i>Toxoplasma gondii</i>	22	15	8	29
<i>Neospora caninum</i>	3	3	0	6

hearts; eight chickens presented the parasite DNA in both brain and heart. *N. caninum* DNA was observed in three brains and in three hearts (Table 1). *Hammondia* spp. DNA was not detected in any chicken.

Antibodies to *T. gondii* were found in 25% of the serum samples, while antibodies to *N. caninum* were detected in 17%. A total of 9% of the chickens were seropositive for both parasites (Table 2). A significant agreement was observed in the serology for both *T. gondii* and *N. caninum* (Kappa = 0.28; $P = 0.03$). Twenty-two out of the 42 PCR-positive chickens for Toxoplasmatinae DNA were also positive by IFAT for at least one of the tested parasites (*T. gondii* and *N. caninum*) (Kappa = 0.34; $P < 0.001$). *T. gondii* DNA was detected in 15 seropositive chickens and in 14 seronegative chickens. The six PCR-positive chickens for *N. caninum* were seronegative for the parasite by IFAT. The antibody titers ranged from 16 to 4096 for *T. gondii*, and from 50 to 400 for *N. caninum*.

Tissues from 14 of 25 *T. gondii*-seropositive chickens were processed for bioassay in mice. Eight mice seroconverted and were euthanized for *in vitro* isolation attempts. Six isolates were obtained from the eight seropositive mice. Tachyzoites were grown in cell culture and confirmed to be *T. gondii* by a species-specific PCR. The isolates were obtained from chickens which had antibody titers for *T. gondii* between 64 and 4096.

Isolation attempts of *N. caninum* using the KO mice inoculated with tissues from eight *N. caninum*-seropositive chickens were negative. No seroconversion occurred in the eight inoculated mice. The chickens which were seropositive simultaneously for both parasites (nine birds) were not used in the bioassay for *N. caninum*.

4. Discussion

In the present study, Toxoplasmatinae DNA was investigated in tissues from 100 chickens that were raised close to cattle and dogs; since dogs and cattle are important hosts for *N. caninum* and *H. heydorni* (McAllister et al., 1998; Soares et al., 2009), the authors intended to select

chickens exposed to both parasites and to reach two of the three proposed objectives in the study, which were to *in vitro* isolate *N. caninum* from chickens and to detect *H. heydorni* DNA in chicken tissues.

A nested PCR was used in this work because it successfully detected DNA of Toxoplasmatinae parasites (*N. caninum*, *T. gondii* and *Hammondia heydorni*) in previous studies (Silva et al., 2009; Gondim et al., 2010; Santos et al., 2010). Brain and heart tissues were used in this study because Silva et al. (2009) were able to detect *N. caninum*, *T. gondii* and *H. heydorni* in Bahia using brain and heart tissues from goats. Toxoplasmatinae DNA was observed in 42% of the chickens. After sequencing the amplicons, we observed that the number of chickens infected with *T. gondii* was higher than the number infected with *N. caninum*. The higher proportion of *T. gondii*-infected chickens may be due to the fact that the amount of *T. gondii* oocysts shed in cat feces is significantly greater than the amount of *N. caninum* oocysts shed by dogs. One single cat may shed over 20 million oocysts (Dubey, 1995), while the maximum numbers of oocysts shed by dogs is usually inferior to one million (Schares et al., 2001; Gondim et al., 2002). In addition, it is possible that chickens are less susceptible to *N. caninum* infection when compared to *T. gondii*.

All chickens were tested for *T. gondii* and *N. caninum* antibodies by IFAT. Antibodies were not tested to *H. heydorni* or *H. hammondi* because there is no serology available for these parasites. *T. gondii* DNA was detected from seropositive and seronegative chickens. Some chickens may harbor the parasite in their tissues without presenting detectable specific antibodies or with low antibody titers, as previously observed (Dubey et al., 2002a), what may explain the detection of the parasite DNA in seronegative birds. Nine chickens that were seropositive for *T. gondii* or *N. caninum* did not show positive reactions in the nested PCR for Toxoplasmatinae DNA; however, the specific antibody response might have been induced by parasites present in other tissues not included in the study.

Table 2

Immunofluorescent antibody tests (IFAT) for IgG to *Toxoplasma gondii* and *Neospora caninum* versus parasite DNA in tissues (brain and heart) from free ranging chickens in Bahia, Brazil.

Towns	Number of chickens	Positive IFAT			DNA		
		<i>T. gondii</i>	<i>N. caninum</i>	<i>T. gondii</i> and <i>N. caninum</i>	Toxoplasmatinae	<i>T. gondii</i>	<i>N. caninum</i>
Conceição de Feira	20	0	1	0	2	1	0
Ilha de Itaparica	8	2	6	2	1	1	0
Irará	13	4	2	2	10	7	2
São Gonçalo	11	5	5	4	8	7	0
Serrinha	48	14	3	1	21	13	4
Total	100	25	17	9	42 ^a	29	6

^aParasite identification could not be determined in seven of the 42 PCR-positive chickens for Toxoplasmatinae DNA.

Hammondia spp. DNA was not detected in tissues from any chicken. *Hammondia* spp. oocysts are shed in feces of cats, dogs and foxes (Dubey and Sreekumar, 2003; Schares et al., 2005; Soares et al., 2009), so chickens are probably exposed to ingestion of these parasites from the ground. A new *Hammondia* sp. of foxes has recently been described and named as *Hammondia truffittae* (Gjerde and Dahlgren, 2011), however their definitive hosts, the red fox (*Vulpes vulpes*) and the arctic fox (*Vulpes lagopus*), are not natural habitants of South America. In a previous study also conducted in Bahia state, goats were shown to be infected with *H. heydorni* (Silva et al., 2009), what confirms the presence of the parasite in the studied region. Both *N. caninum* and *H. heydorni* have dogs as definitive hosts; the detection of *N. caninum* DNA in chicken tissues is indicative of the exposure of chickens to dog feces. The lack of *H. heydorni* DNA detection in chicken tissues is suggestive that chickens are not intermediate hosts for this parasite or *H. heydorni* infection in chickens is rare (at least less than 5% prevalence). More studies need to be conducted to prove whether *Hammondia* spp. infection occurs in chickens.

Chickens are known to be efficient hosts of *T. gondii* and are good markers of environmental contamination with oocysts; several studies have reported *T. gondii* isolation from chicken tissues in Brazil using bioassay in mice (da Silva et al., 2003; de Oliveira et al., 2009; Dubey et al., 2010). *N. caninum* is known to infect chickens (Costa et al., 2008), but no cysts of the parasite were detected in naturally infected chickens neither viable isolates have been obtained from this animal species.

The *in vitro* isolation of *T. gondii* in this study was performed using Swiss outbreed mice, which are known to be susceptible to infection by the parasite (Dubey, 1967; Dubey et al., 1995). *T. gondii* was isolated from six of 14 mice which had been inoculated with tissues from seropositive chickens; mouse tissues were used for isolation of the parasite in cell culture. The attempts to isolate *N. caninum* from seropositive chickens were performed by inoculation of chicken tissues into interferon-gamma KO mice, because heterozygous immunocompetent mice are resistant to *N. caninum* infection (Lindsay and Dubey, 1989). Tissues from eight *N. caninum*-seropositive chickens were processed and inoculated into eight interferon-gamma KO mice, but no mouse presented clinical signs or seroconverted to *N. caninum*. Isolation of *N. caninum* from mammalian tissues has been successfully conducted using interferon-gamma KO mice (Dubey et al., 1998; Basso et al., 2001; Canada et al., 2004). Due to ethical concerns, only one mouse was used for each seropositive chicken in the attempts to isolate *T. gondii* or *N. caninum*. The authors speculate that a higher number of parasite isolates would be obtained if more than one mouse per infected chicken were used in the bioassay.

The lack of isolation of *N. caninum* from eight seropositive chickens using a well-established animal model is suggestive that infected chickens, in contrast to *T. gondii*, have parasite in very low numbers with irregular distribution in tissues or do not possess mature parasite stages in their tissues capable to infect mice and possibly in other animals. The authors suspect that the temperature range required by *N. caninum* to develop into a mature tissue cyst is different from that required by *T. gondii*. The

body temperature of chickens is around 42 °C (Yalcin et al., 2008), what is quite higher than the body temperature of some efficient hosts of the parasite, such as cattle and dogs (Angle and Gillette, 2011; Wenz et al., 2011). In a previous study, dogs were induced to shed oocysts after ingestion of chicken embryonated eggs infected with *N. caninum* (Furuta et al., 2007). However, the temperature used to incubate chicken eggs is between 37.5 °C (Shim and Pesti, 2011), what is lower than the body temperature of adult chickens that were used in the current work. Infective stages of *T. gondii* may develop in a wider temperature range, as demonstrated by bioassays using bird tissues (Dubey, 2002).

The authors concluded that chickens seem to be better hosts for *T. gondii* when compared to *N. caninum*, based on the molecular detection of the parasites and on the results of the bioassays in mice. So far, neither tissue cysts of *N. caninum* nor viable isolates of the parasite have been reported using chicken tissues. Recently, tissue cysts positively immunostained to *N. caninum* were reported in two wild birds (Mineo et al., 2011); however, the authors could not confirm the identity of the cysts because no DNA was available.

Due to the global distribution of chickens and their use for human and animal nutrition, more studies are necessary to confirm whether *N. caninum* can be transmitted to animals through the consumption of infected tissues from chickens. It is also important to conduct more studies about *Hammondia* spp. infection in birds and to test their potential role as intermediate hosts of these parasites.

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