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List of abbreviations: ***A. fulica*** - *Achatina fulica*, **BLAST** - Basic Local Alignment Search Tool, **CAPES** - Coordenação de Aperfeiçoamento do Pessoal do Ensino Superior, **CNPq** - Conselho Nacional de Pesquisa e Desenvolvimento Tecnológico, **DNA** - desoxirribonucleic acid, **nPCR** - Nested-Polymerase Chain Reaction, **PCR** - Polymerase Chain Reaction, **RFLP** - Restriction Fragment Length Polymorphism, **RNA** - ribonucleic acid, **SISBIO** - Sistema de Autorização e Informação em Biodiversidade, **SISGEN** - Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado, **UEFS** - Universidade Estadual de Feira de Santana.

First detection of *Toxoplasma gondii* DNA in African Giant Snails, Achatina fulica (Bowdich, 1822)

Primeira detecção de DNA de Toxoplasma gondii em caramujos gigantes africanos, Achatina fulica (Bowdich, 1822)

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ABSTRACT

Introduction: Environmental contamination by *Toxoplasma gondii* oocysts poses a risk to human and animal health, especially in urban areas with poor sanitation. The giant African snail (*Achatina fulica*), due to its direct contact with soil and consumption of vegetables, may act as a mechanical vector of the parasite's oocysts. **Objectives:** To investigate the presence of Sarcocystidae DNA in fecal samples of *A. fulica* collected in Feira de Santana, Bahia, Brazil. **Methods:** Epidemiological observations were conducted at the collection sites, and climate variables were provided by the National Institute of Meteorology. In the laboratory, the animals were washed in running water, measured, placed in individual plastic containers, and fed daily with water and cabbage until fecal samples were processed. The cabbage leaves were subjected to parasitological testing to detect the presence of protozoa and helminths. Fecal samples were resuspended in an equal volume of absolute ethanol and frozen until DNA extraction, which was performed using a commercial kit. Nested PCR (nPCR) was conducted to detect ribosomal DNA fragments of protozoa from the Sarcocystidae family. **Results:** Of the 220 samples subjected to nPCR, 58 (26.36%; 95% CI: 20.99–32.57) amplified the expected 290-base pair fragment and were then subjected to RFLP. Of these, 41 (70.69%; 95% CI: 57.92–80.81) were cleaved by the restriction enzymes used in this study, showing a profile compatible with T. gondii. **Conclusion:** This is the first report on the presence of DNA compatible with *T. gondii* in *A. fulica*.

Keywords: Toxoplasma. Feces. Molluscum Contagiosum. DNA.

RESUMO

Introdução: A contaminação ambiental por oocistos de *Toxoplasma gondii* representa risco à saúde humana e animal, especialmente em áreas urbanas com saneamento precário. O caramujo africano gigante (*Achatina fulica*), por viver em contato direto com o solo e consumir vegetais, pode atuar como vetor mecânico de oocistos do parasito. **Objetivos:** Investigar a presença de DNA de Sarcocystidae em fezes de *A. fulica* coletados em Feira de Santana, Bahia, Brasil. **Métodos:** Observações epidemiológicas foram realizadas nos locais de coleta, e o Instituto Nacional de Meteorologia forneceu as variáveis climáticas. No laboratório os animais foram lavados em água corrente e mensurados, colocados em frascos plásticos individuais e alimentados com água e repolho diariamente até que as amostras de fezes fossem processadas. As folhas de repolho foram submetidas a um teste parasitológico para detectar a presença de protozoários e helmintos. As amostras de fezes foram ressuspensas em igual volume de etanol PA e congeladas até a extração de DNA, realizada com um kit comercial. Nested PCR (nPCRs) foi realizada para detectar fragmentos de DNA ribossomal de protozoários da família Sarcocystidae. **Resultados:** Das 220 amostras submetidas à nPCR, 58 (26,36%; IC95%: 20,99-32,57) amostras amplificaram o fragmento esperado de 290 pares de bases, sendo em seguida submetidas à RFLP. Destas, 41 (70,69%; IC95%: 57,92-80,81) amostras foram clivadas pelas enzimas de restrição utilizadas neste estudo, com perfil compatível com *T. gondii*. **Conclusão:** Este é o primeiro relato da presença de DNA compatível com *T. gondii* em *A. fulica*.

Palavras-chave: Toxoplasma. Fezes. Molusco Contagioso. DNA.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan, occurring in various homeothermic species, including humans. This parasite is responsible for toxoplasmosis, which is usually asymptomatic, but may cause severe manifestations in infants infected *in utero* and encephalitis in immunocompromised patients[1]. Felidae act as definitive hosts and, therefore, the only ones that can contaminate the environment with oocysts[2]. The transmission of this zoonotic disease occurs via ingestion of raw meat containing tissue cysts, via transplacental transmission when a primary infection occurs during pregnancy, or by ingesting sporulated oocysts present in the water, soil, or food contaminated[3-4].

Toxoplasma gondii oocysts in the soil become infective by sporulation within 21 days[5] and can remain viable for up to 18 months[6]. They are highly resistant to environmental conditions and various chemical and physical agents[7]. In the environment, these oocysts are distributed through wind, water, fertilizer, and land invertebrates, contaminating water, soil, and

plants[8]. Thus, sporulated oocysts in the environment can be a source of infection for humans, domestic and wild animals.

The mollusk *Achatina fulica*, known as the giant African snail, has become a public health problem, being responsible for transmitting a variety of diseases[9], including eosinophilic meningoencephalitis and abdominal angiostrongyliasis caused by nematodes *Angiostrongylus cantonensis* and *A. costaricensis*, respectively[10-11]. In addition, the fact that the species *A. fulica* lives in direct contact with the soil, preferably in humid and polluted places[10], and the habit of feeding on moist soils and vegetables[11] make these snails mechanical vectors of parasites that can cause disease in humans and animals.

Aiming to investigate the possibility of terrestrial snails of the invasive species *Achatina fulica* mechanically carrying *T. gondii* oocysts and acting as bioindicators of environmental contamination by this parasite's oocysts, this study aims to investigate the presence of Sarcocistidae protozoa in *A. fulica* stool samples in Feira de Santana, Bahia, checking the possible role of this gastropod in the epidemiological chain of toxoplasmosis.

METHODS

Ethical aspects

Snail collections are submitted and approved by Brazilian Ministry of Environment Biodiversity Authorization and Information System – SISBIO (authorization #35442-1) and SISGEN (authorization ACEEFE5).

Area of study and sampling

Between May 2012 to June 2013, 223 *A. fulica* were collected in eight areas (Figure 1) of the urban perimeter of the city of Feira de Santana, Bahia (12°16'S, 38°58'W): Mangabeira (12°15'50.85"S, 38°57'14.70"W; 12°13'57.50''S, 038°57'41.10''W), Queimadinha (12°16'1.26"S, 38°56'59.70"W), Campo Limpo (12°16'55.18"S, 38°57'27.14"W; 12°12'00.30''S, 038°58'23.60''W; 12°12'28.00"S, 038°58'18.60"W), Novo Horizonte (12°14'28.78"S e 38°56'45.98"W) and Caseb (12°13'0.50''S e 038°56'42.90''W).

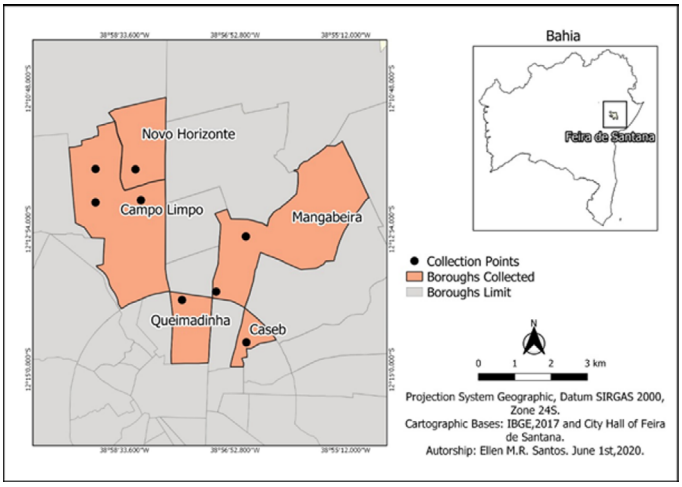

Figure 1. Collection points of *Achatina fulica* in Feira de Santana, Bahia. 2012-2013[12].

Animals were collected in the early morning, around five o'clock. Mollusks were manually collected with hands protected by rubber gloves. Snails that were found were placed in coolers with the fitting lid to allow aeration. After each collection, the specimens were transported to the Laboratory of the Zoonoses and Public Health Research Team (LABZOO) at the *Universidade Estadual de Feira de Santana* (UEFS).

During the collection period, some observations were made about garbage, domestic animals, and sewage in the places where the snails were captured. In addition, climatic data (average temperature, daily precipitation, and relative humidity) were obtained from the National Institute of Meteorology[13].

At the laboratory, the animals were washed in running water. Then, they were measured by the total length of the shell with a caliper (accuracy of 0.1 mm) and the total mass of the snail on a digital balance (accuracy of 0,01 g). Age development stage was used according to Tomiyama[14], which has considered juveniles the snails with shells from 10 to 40 mm; young individuals with shells measuring 41 to 90 mm, and adults those with shells above 90 mm in length. Animals were individually kept in plastic bottles with a perforated lid until the processing of stool samples.

The cabbage leaves (*Brassica oleracea var. capitata*) used to feed the gastropods were initially examined for the presence of protozoa and helminths[15], but no parasites were found in these samples. The gastropods were then fed daily with water and these cabbage leaves until approximately 2 g of feces were released. The fecal samples were previously analyzed for the presence of *Giardia* sp. and oocysts using the zinc flotation centrifuge technique[16]. Subsequently, the feces were resuspended in an equal volume of PA ethanol and frozen for future DNA extraction.

Molecular analysis

Extraction and purification of stool samples DNA were initiated with three steps of freezing at -80°C in liquid nitrogen and thawing at 70ºC to break oocysts, followed by the use AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen®), according to the manufacturer's instruction.

For Sarcocystidae, DNA detection was used oligonucleotides directed to a fragment of the 18S rRNA gene by nested-PCR, using primers and conditions of reaction standardized by da Silva et al.[17]. The first reactions were performed in a volume of 25.0 µl using the *Top Taq Master Mix* (Qiagen®); for the second reaction was used 5,0 µl of the first amplification product, using the same conditions and a new pair of primers. Each amplification batch included a positive control (DNA extracted from *T. gondii*) and two negative controls (ultrapure water).

Analysis of length polymorphism of DNA fragments generated by restriction enzymes on products amplified (PCR-RFLP) was performed using *DdeI* restriction enzymes, *Hpy188III* and *MspI* on the amplification product with 290 DNA base pairs of Sarcocystidae, to differentiate *Toxoplasma gondii, Hammondia hammondi, Neospora caninum,* and *Sarcocystis neurona*, respectively. Digestions were performed in a total volume of 6.0 µl, containing 3.0 µl of the product from *nested*-PCR mixed with 1.0 µl of buffer NEB, 1.0 µl of bovine serum albumin (1 mg/ml), 0.4 µl of ultra-pure water, 0,4 µl (4U) of *DdeI*, 0,1 µl (1U) of *Hpy188III* and 0,8 µl (8U) of *MspI*. The samples were incubated at 37°C for 60 minutes, followed by enzyme inactivation at 65°C for 10 minutes. In each batch of enzymatic cleavage were added positive controls of DNA extracted from *Toxoplasma gondii, Hammondia hammondi, Neospora caninum* and *Sarcocystis neurona* from *Universidade Federal da Bahia* Animal Parasitosis Diagnostic Laboratory.

From the *nested*-PCR products or the enzyme digestions; 5.0 µl were homogenized with 5.0 µl of bromophenol blue solution and submitted to 2% agarose gel horizontal electrophoresis (*n*PCR) or 3% (RFLP) agarose gel, containing 0.3 µg ethidium bromide, in Tris-Borate-EDTA buffer (TBE) 1X, pH 8.0, for 45 (*n*PCR) or 55 minutes (RFLP) at 120V. Amplified and digested products were visualized and photographed with L-Pix Image System (Loccus Biotechnology).

Nested-PCR amplicons were submitted to sequencing using Bigdye (Applied Biosystems, USA) according to the manufacturer. It was used 5 pmol from the internal primers used in nested-PCR and 10ng of

purified DNA product. The sequencing reactions were performed in a sequencer, Genetic Analyzer 3130XL (Applied Biosystems, USA). The 18S rRNA genes sequenced were edited and submitted to BLASTn (http://www.ncbi.nlm.nih.gov/BLAST) and compared with the sequences deposited in databases.

Data analysis

Association between dependent and independent variables was checked on contingency tables by Pearson χ^2, Fisher's Exact Test or Williams G test, using EpiInfo[18]. The biometric variables were compared between positive and negative animals for the presence of DNA compatible with the parasites by the Mann-Whitney test. In all analyses were considered significant P values lower than 0.05[19].

RESULTS

Of the 220 samples submitted to the nPCR for Sarcocystidae detection, 58 (26.36%; 95% IC: 20.99-32.57) samples amplified the 290 base pair fragment expected, then subjected to RFLP. Of these, 41 (70.69%; 95% CI: 57.92-80.81) samples were cleaved by restriction enzymes used in this study, producing a profile compatible with *T. gondii*. (Figure 2). Other 17 samples revealed cleavage profiles not compatible with the Sarcocystidae described by Da Silva et al.[17]. In 11 of 58 samples, sequencing was successful, and one was compatible with the GenBank sequence EF472967.1 of *T. gondii*, while the other 10 showed similarities with EF472967.1 and AY663792.1 sequences. However, the remaining samples showed low-quality sequences, not allowing the establishment of a reliable consensus sequence.

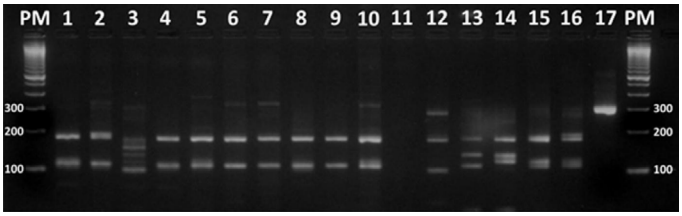

Figure 2. Differentiation of Apicomplexa parasites by RFLP technique of 18S rRNA gene fragment. The nested-PCR product was digested with the restriction enzymes DdeI, MspI and Hpy188III.
Caption: PM: molecular weight marker of 100 pb; 1-12: stool samples of *A. fulica*; 1, 2, 4, 5, 6, 7, 8, 9, 10: samples compatible with *T. gondii* DNA; 13: product of *H. hammondi* digestion; 14: product of *N. caninum* digestion; 15: product of *T. gondii* digestion; 16: product of *S. neurona* digestion; 17: no-digest *T. gondii*.

It was not found an association between climatic and epidemiological variables (data not shown) with the DNA of *T. gondii* in stools. However, there was a significant difference between the total length of the shell and body mass with the *T. gondii* DNA presence (Table 1), indicating a more frequent occurrence of *T. gondii* DNA in large (older) mollusks, compatible with and hypothesis that older animals are more frequently exposed and become infected.

Table 1. Descriptive statistics of body weight and total length of 220 shell *Achatina fulica* specimens collected, according to the PCR-RFLP result. Feira de Santana, Bahia, Brazil. 2012-2013.

	PCR-RFLP (pattern of restriction compatible with *Toxoplasma gondii*)							
	Positives				Negatives			
	Mean ± pattern deviation	P25	Median	P75	Mean ± pattern deviation	P25	Median	P75
Body mass (g)	27.71 ± 11.84	18,73	28.42[b]	34,54	23.57 ± 11.59	13,55	24.60[a]	30,08
The total length of the shell (cm)	58.98[b] ± 10.35	52,75	59,00	64,50	54.53[a] ± 9.86	47,75	55,00	62,00

Statistics: average or median values followed by different letters indicate significant differences between the groups by Student t and Kruskal-Wallis test, respectively.

DISCUSSION

In this study, the presence of DNA of *T. gondii* in the stool of *A. fulica* was verified by nested PCR and identified by restriction polymorphism and sequencing, consisting of the first *T. gondii* register in the stool of terrestrial mollusks. This could be related to *A. fulica* in urban and peri-urban areas, associated with the narrow interaction between cats and their stools. Felines are the only source of oocyst elimination and are responsible for environmental contamination[6].

In soil, *T. gondii* sporulated oocysts survive for long periods in favorable conditions of temperature and humidity. They are found on the soil's surface or at a depth of up to four inches and remain viable for 18 months under various temperatures[6]. The actual load of oocysts of *T. gondii* in the soil is not well known. Studies held in California and France were able to estimate the oocysts burden through production's estimates of oocysts by cats; the number of oocysts of *T. gondii* ranged between 9-3 and 434-335 per square meter, respectively[20].

The domestic cats tend to bury their stools in soil layers, therefore a variety of terrestrial invertebrates, including beetles, isopods, mollusks and fly larvae, can ingest infective oocysts of *T. gondii* by direct contact with the cat's stools[6]. Experimental studies have already shown that earthworms and cockroaches[5] could act as mechanical vectors of *T. gondii* oocysts. Thus, the results of this study indicate the possibility of *A. fulica* acting as a mechanical carrier of *T. gondii* oocysts in the environment.

The soil plays an essential role for the mollusk *Achatina fulica*, besides being used as a source of food, it is also used as a defense strategy. Therefore, these snails are susceptible to contamination with *T. gondii* due to contact and ingestion of contaminated soil with oocysts of this parasite, which pass through the intestinal tract of these invertebrates intact and are re-eliminated into the environment. In addition, vegetables contaminated by direct exposure to the stools of cats or irrigation with contaminated water[21] can be a source of contamination for *A. fulica* because they use plants as a food source. *Toxoplasma gondii* DNA has also already been found contaminating the surface of raw vegetables[22], indicating probable contamination by the parasite's oocysts. Thus, the molecular detection of *T. gondii* in these invertebrates can be an important tool for assessing environmental contamination by the protozoan's oocysts, making these mollusks important bioindicators.

It is difficult to assess the implications of this discovery for human health, but it can be a risk for toxoplasmosis, as these snails can defecate in vegetables or other food that is barely sanitized can be a source of infection for humans. Concerning animal health, the mollusk *A. fulica* can be a source of infection with *T. gondii* and other parasites to various mollusk predators since these are members of a food chain and a food source for invertebrates and vertebrates.

In this study, it was observed that the largest snails were contaminated, and this may be related to the snails' dispersal behavior, because the animals of greater body size are equipped with sharp dispersibility to travel in search of new territories and sexual partners. Hence, they had exposure to the parasite because more time of exposure increases the chance of infection. Thus, there is still the possibility of dispersing these oocysts to new areas through mechanical transmission.

Although this study did not observe a significant association between epidemiological variables and the occurrence of DNA with *T. gondii* in stools of *A. fulica*, it is known that in places with poor

health infrastructure, the presence of cats and their droppings can be determining factors for the presence of the parasite on mollusks.

Then, these snails can serve as mechanical vectors of *T. gondii*, constituting a potential risk for humans and animals. Therefore, epidemiological studies are necessary to understand the real implications of this discovery in the scientific area of human and animal health.

CONCLUSION

In this study, nested PCR and sequencing of the 18S rRNA gene fragment were used to detect *Toxoplasma gondii* DNA in Achatina *fulica* stool samples. This is the first report, both in Brazil and globally, of the detection of *T. gondii* in A. *fulica'*s stools.

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