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OCCURRENCE OF *CRYPTOSPORIDIUM* SP. IN DOGS AND CATS FROM CURITIBA AND ITS METROPOLITAN AREA

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ABSTRACT: The present study was carried out with the aim of assess the occurrence of *Cryptosporidium* sp. infection in dogs and cats in Curitiba and its metropolitan area, state of Paraná, Brazil. Techniques employed to detect the protozoan in fecal samples were: staining by Ziehl-Neelsen for oocysts search and nested polymerase chain reaction (nPCR) targeting the 18SSU rDNA gene. To attempt the proposed aim, 91 feces samples of dogs and 25 of cats were collected and analyzed. Ziehl-Neelsen technique was unable to detect any oocyst in both groups analyzed, showing a very low sensitivity. Results of nPCR showed an infection rate of 13.2% (12/91) and 4% (1/25) in dogs and cats respectively. The implications of these epidemiological data are discussed in this work.
Key Words: *Cryptosporidium sp.; molecular diagnosis; nested PCR; 18SSU rDNA gene*

OCORRÊNCIA DE *CRYPTOSPORIDIUM* SP. EM CÃES E GATOS DA REGIÃO METROPOLITANA DE CURITIBA

RESUMO: O objetivo deste trabalho foi relatar a ocorrência de *Cryptosporidium* sp. em cães e em gatos de Curitiba e região metropolitana, no estado do Paraná, Brasil. As técnicas utilizadas foram: coloração pelo método de Ziehl-Neelsen para detecção de oocistos e *nested*-PCR para pesquisa do gene 18SSU rDNA do parasito. Noventa e uma amostras de fezes de cães e 25 amostras de fezes de gatos foram colhidas. A pesquisa de oocistos pela técnica parasitológica teve resultado negativo. Na pesquisa de DNA observou-se uma taxa de positividade de 13,2% (12/91) nas amostras fecais de cães e 4% (1/25) nas amostras fecais de gatos. As implicações epidemiológicas destes dados são discutidas no presente trabalho.
Palavras-chave: *Cryptosporidium***;** epidemiologia; diagnóstico molecular

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INTRODUCTION

Cryptosporidium sp. is a coccidian protozoan that belongs to the phylum Apicomplexa and has worldwide distribution, being found in 90 countries, over six continents, and infecting about 170 different species of wild and domestic animals. Some species of this protozoan have the ability to infect both man and animals, therefore *Cryptosporidium* can be considered a potential zoonotic parasite (O'Donoghue, 1995; Morgan *et al*., 2000, Cacciò, 2005; Jex *et al*., 2008a,b). Infection with *Cryptosporidium* sp. can occur by oral ingestion of infectious oocysts, through direct contact with infected people or animals, or also through ingestion in soil, food (mainly vegetables or fruit), or water contaminated with hosts feces (Amarante, 1992; O'Donoghue, 1995; Overgaauw *et al*., 2009; Bowman; Lucio-Forster *et al*., 2010; Xiao, 2010).

Cryptosporidium canis and *C. felis* are the main species that infect dogs and cats (Bowman; Lucio-Forster, 2010). However, others species were also detected in these animals, such as *C. parvum*, *C. meleagridis* e *C. muris* (Hamnes *et al.*, 2007; Huber *et al.*, 2007; Bowman; Lucio-Forster, 2010). Molecular analyses have revealed that *C. felis* and *C. canis* can infect humans (Cacciò, 2005).

A recent review reported *Cryptosporidium* infection rates varying from 0% to 44.8% in dogs, and 0.6% to 15.4% in cats worldwide (Bowman; Lucio-Forster, 2010). In Brazil, reported prevalence for the infection vary from 1.4% to 40% in dogs and 2.8% to 30% in cats (Figueiredo *et al*., 2004; Ederli *et al*., 2005; Lallo; Bondan, 2006; Mundim *et al*., 2007; Thomaz *et al*., 2007).

In the state of Paraná, the only previous published study with *Cryptosporidium* infection in dogs was conducted by Navarro *et al*. (1997). They detected *Cryptosporidium* sp. in fecal samples of dogs from the city of Londrina (north region of the state). These authors reported 2.25% of positive dogs in 133 samples that were analyzed by parasitological tests. As the prevalence data vary from one geographical region to another, the objective of this study were to survey the occurrence of *Cryptosporidium* sp. in dogs and cats living in the metropolitan area of the city of Curitiba, state of Paraná, Brazil using direct parasitological methods, without prior purification and concentration of the fecal samples and molecular methods in finding *Cryptosporidium* sp. oocysts or the parasite DNA, respectively.

MATERIAL AND METHODS

1. Fecal samples and studied population

Fresh fecal samples from 91 dogs and 25 cats from different shelters and veterinary clinics were collected and analyzed. Of the fecal samples from dogs, 62 were collected in Curitiba, 10 were from São José dos Pinhais and 19 from Araucária (cities in the metropolitan area of Curitiba). All samples from cats were collected in Curitiba. All cats and dogs were asymptomatic at the time of sample collection. Ages varied from 2 months to 10 years. Fecal samples were collected immediately after defecation, placed in sterile plastic bags, and kept under refrigeration (4ºC) until further examination, which occurred after a maximum of 48 hours.

2. Parasitological methods

Detection of fecal *Cryptosporidium* sp. oocysts was performed using acid-fast staining (Ziehl-Neelsen) (Rigo; Franco, 2002). To perform this method, thin fecal smears were directly analyzed on glass slides, without any concentration technique, and stained. Smears were analyzed under light microscopy (Olympus BX41)

at a magnification of 40x. When any structure similar to *Cryptosporidium* sp. oocysts were observed, it was re-analyzed at a magnification of 100x to confirm the diagnosis.

3. Molecular methods

3.1. Purification of oocysts with sucrose gradient and DNA extraction

All samples were submitted to the technique of Hoffman, Pons and Janer, as a first purification method. The supernatant was collected and then subjected to the sucrose gradient method for purification and isolation of oocysts, according to Paulino (2005). For DNA extraction, lysis buffer (500 µL) was added to the purified material, which was then submitted to 15 cycles of freezing and thawing (-70ºC and 56ºC) and then submitted to DNA extraction with magnetic beads using ChargeSwitch® gDNA Mini Tissue Kit (Invitrogen™, Carlsbad, CA) following the manufacturer's protocol.

3.2. Polymerase chain reaction (PCR) and Nested-PCR (nPCR)

PCR analysis was performed in two different steps. The first used the pair of primes Xiao (F) 5' TTCTAGAGCTAATACATGCG 3' and Xiao (R) 5'CCCATTTCCTTCGAAACAGGA3' for the *Cryptosporidium* sp. region of rDNA gene 18SSU. This set of primers amplifies a segment of 1325 base pairs. The second step (nPCR) was performed using as template the specific amplified products from the previous PCR with a second pair of primers Xiao 1 (R) 5'AAGGAGTAAGGAACAACCTCCA 3' and Xiao 2 (F) 5'GGAAGGGTTGTATTTA TTAGATAAAG 3' (Xiao *et al*., 1999, 2000). This set of primers amplifies a segment of 826 to 864 base pairs.

The reactions of PCR and nPCR were standardized previously in our laboratory (Osaki, 2009). In this assay, PCR was able to amplify five oocysts, while nPCR was able to detect two oocysts (data no showed). Samples containing *Cryptosporidium parvum* DNA and samples without DNA (i.e., sterile water as template) were included in all the PCR reactions (as controls).

The amplification reactions were performed in a thermal cycler with a heated lid GeneAmp (Hybaid-US, Franklin, USA). Amplification products were electrophoresed in a 1.6% (w/v) agarose gel in TBE buffer at 60 V for 2 h., stained with ethidium bromide (0.5 µg/mL) for 20 min, illuminated under UV, and photographed with a UV transilluminator documentation system (Gibco BRL, Life Technologies, Grand Island, NY, USA).

RESULTS AND DISCUSSION

1. Parasitological detection

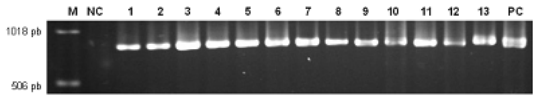
By direct parasitological method we could not detect any positive case for infection with *Cryptosporidium* sp. This negative result could be explained by the small amount of oocysts that was probably shed in the feces of the animals evaluated. According to Pereira *et al.* (2008), to detect *Cryptosporidium* sp. by Ziehl-Neelsen staining 50,000 to 500,000 oocysts per gram of feces are needed. We hypothesized that, since any cat or dog in this study had no clinical signs of disease, the amount of oocysts shed in the feces was probably too small to detect by direct parasitological methods.

2. DNA detection (nPCR)

Out of the 91 dogs analyzed, 12 (13.2%) presented specific bands to *Cryptosporidium* sp. in nested PCR (Figure 1). Only one cat (4%) showed a positive result (Table 1). The infection rates of *Cryptosporidium* reported in the literature varied from 0% to 44.8% in dogs and 0.6% to 15.4% in cats worldwide (Abe *et al.,* 2002; Giangaspero *et al.,* 2006; Funada *et al*.,

2007; Hamnes *et al.,* 2007; Thomaz *et al.,* 2007; Palmer *et al.,* 2008; Katagiri and Oliveira-Siqueira, 2008; Meireles, 2010).

Figure 1. Products amplified by nested-PCR containing 826 to 864 base pairs of *Cryptosporidium* sp. from fecal samples of dogs and cats.



(reference strain of *C. parvum*).
M: Standard molecular marker (1kb)
NC:Negative control; samples 1 to 12: *Cryptosporidium* sp. in dogs; sample 13: *Cryptosporidium* sp. in cat;
PC: Positive control

Table 1. Nested-PCR results by counties and specie of animal studied

City	*Samples number*	Positives samples	%
	Dogs/cats	Dogs/cats	Dogs/cats
Curitiba	62/25	7/1	11.3/4
São Jose dos Pinhais	10/0	2/0	20/0
Araucária	19/0	3/0	15.8/0
Total	91/25	12/1	13.2/4

The variation of *Cryptosporidium* prevalence rates among dogs and cats might be due to geographic differences (temperature, season), use of different diagnostic methods, studied population, age groups and living conditions (Hamnes *et al.,* 2007). According to Hamnes *et al*. (2007) other factors can affect the prevalence in animals, such as purebred/mixed breed, feeding, urban/rural living conditions, single or multiple household dogs, previous treatments, and immune status.

There is much contradiction in the literature regarding the pathogenicity of *Cryptosporidium* sp. in animals. In dogs, for example, some authors affirm that most infections with *C. canis* are usually asymptomatic, while others maintain that infections with *C. canis* are usually symptomatic (Hamnes *et al*., 2007; Smith *et al*., 2009). The same opposition occurs when we try to define the pathogenicity of *C. felis* in cats, since infections with *C. felis* may be asymptomatic or cause diarrhea (Smith *et al.*, 2009). Kittens seem to be more susceptible to *C. felis* infection probably due to the immaturity of their immune system (Huber *et al.*, 2007).

Very possibly for this reason, shedding of a larger number of oocysts is more common in young animals. Adult animals, in turn, are usually resistant to *Cryptosporidium* infection. However, stress conditions can favor infection with the parasite and induce shed of oocysts in older animals (Palmer *et al.,* 2008).

Molecular approaches suggest that subclinical infections may be more frequent, as shown in our study, as 13.2% of the dogs and 4% of the cats were infected with *Cryptosporidium* sp., but none of them showed clinical signs of the disease.

CONCLUSION

Our initial approach in this survey was to demonstrate that the parasite is definitively present in our region and is endemic. To analyze the role of dogs or cats in the transmission of *Cryptosporidium* to human beings it is necessary to further identify the parasite species in order to study the phylogenetic relationship in *Cryptosporidium* from dogs, cats and human.

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