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Prevalence of *Cryptosporidium* infection in domestic cats from an urban area in Brazil

Prevalência de infecção por *Cryptosporidium* em gatos domiciliados em uma área urbana no Brasil

Luiz da Silveira Neto[1*]; Giovanni Widmer[2]; Valéria Marçal Felix de Lima[3]; Marcelo Vasconcelos Meireles[3]; Sandra Valéria Inácio[4]; Marcel Gambin Marques[5]; Ana Elisa Gregui Watanabe Marques[6]; Lucas Vinícius Shigaki de Matos[7]; Mariele Fernanda da Cruz Panegossi[8]; Katia Denise Saraiva Bresciani[3]

Highlights:
Cryptosporidium infection in domestic cats is usual;
The DNA sequences were 99% identical to *Cryptosporidium felis* found in feline faeces;
Veterinary practitioners should guide cat owners to adopt preventive measures.

Abstract

We investigated the occurrence of *Cryptosporidium* oocysts shedding by domestic cats in an urban setting. The calculation of minimum sample size was based on an estimated prevalence of 10%, 5% absolute sampling error and a 5% significance level, resulting in 138 cats. A total of 612 owners of 2,290 cats had to be contacted for achieving the minimal sample size. In the end, only 55 owners accepted to participate in this investigation. Stool samples collected from 138 dogs were examined by microscopy using modified Kinyoun acid-fast staining, capture ELISA and nested-PCR followed by sequencing. Samples were considered positive when *Cryptosporidium* were detected by at least two diagnostic methods. Thirteen samples were positive (9.4%; 95% CI: 4.5 - 14.3). *Cryptosporidium* amplicons from seven out of the 13 samples were successfully sequenced and shared 99% genetic similarity to *Cryptosporidium felis*, GenBank access AF112575.1 was found. We concluded that *Cryptosporidium* infection is common in domestic cats from urban area and veterinary practitioners should guide cat owners to adopt preventive measures against the parasite to reduce the chance of infection in cats and householders.

Key words: Prevalence. Public Health. Epidemiology. Cryptosporidiosis. Feline.

[1] Prof. Dr., Curso de Graduação em Engenharia de Bioprocessos e Biotecnologia, Universidade Federal do Tocantins, UFT, Gurupi, TO, Brasil. E-mail: luiz.silveira@uft.edu.br
[2] Prof. PhD., Cummings School of Veterinary Medicine at Tufts University, North Grafton, Massachusetts, 01536, USA. E-mail: giovanni.widmer@tufts.edu
[3] Profs. Drs., Curso de Graduação em Medicina Veterinária, Universidade Estadual Paulista, UNESP, Araçatuba, SP, Brasil. E-mail: vmflima@fmva.unesp.br; m.meireles@unesp.br; katia.bresciani@unesp.br
[4] Pesquisadora, UNESP, Araçatuba, SP, Brasil. E-mail: sandra_byol@yahoo.com.br
[5] Prof. Dr., Curso de Graduação em Medicina Veterinária, Centro Universitário das Faculdades Integradas de Ourinhos, UNIFIO, Ourinhos, SP, Brasil. marcelvetcardio@hotmail.com
[6] Discente de Mestrado do Programa de Pós-Graduação em Ciência Animal, UNESP, Araçatuba, SP, Brasil. E-mail: ana_watanabe@hotmail.com
[7] Dr., Coordenador de Pesquisa e Desenvolvimento, Ipanema Indústria de Produtos Veterinários, Araçoiaba da Serra, SP, Brasil. E-mail: lucasvsm@hotmail.com
[8] Profª M.e, Curso de Medicina Veterinária, Centro Universitário Max Planck, Indaiatuba, SP, Brasil. E-mail: marielepanegossi@gmail.com
[*] Author for correspondence

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Resumo

Investigamos a ocorrência de eliminação de oocistos de *Cryptosporidium* em fezes de gatos domésticos em ambiente urbano. O cálculo do tamanho mínimo amostral baseou-se em uma prevalência estimada de 10%, erro amostral absoluto de 5% e nível de significância de 5%, resultando em 138 gatos. Um total de 612 proprietários de 2.290 gatos precisou ser contatado para atingir o tamanho mínimo amostral. No final, apenas 55 proprietários aceitaram participar dessa investigação. As amostras de fezes coletadas de 138 gatos foram examinadas por microscopia, usando coloração de Kinyoun modificada, ELISA de captura e nested-PCR, seguida de sequenciamento. As amostras foram consideradas positivas quando *Cryptosporidium* foi detectado por pelo menos duas técnicas de diagnóstico. Treze amostras foram positivas (9,4%; IC95%: 4,5 - 14,3). Os *amplicons* de *Cryptosporidium* de sete das 13 amostras foram sequenciados com sucesso e compartilharam 99% de similaridade genética com *Cryptosporidium felis* (acesso ao GenBank: AF112575.1). Concluímos que a infecção por *Cryptosporidium* é comum em gatos domésticos em área urbana e os médicos veterinários devem orientar os proprietários de gatos a adotarem medidas preventivas contra o parasita para reduzir a chance de infecção em gatos e humanos.

Palavras-chave: Prevalência. Saúde Pública. Epidemiologia. Criptosporidiose. Felinos.

Introduction

Cryptosporidium oocysts are transmitted by the faecal-oral route through water and contaminated food, and the infective dose can be very low (DuPont et al., 1995; Chappell, Okhuysen, Sterling, & DuPont, 1996; Chappell et al., 2006). Transmission person-to-person can occur (Alpert et al., 1986). Currently, 38 species are recognized in the genus *Cryptosporidium* (Feng, Ryan, & Xiao, 2018).

Microscopy and immunodiagnostic assays are genus-specific because of the high morphological similarity and the presence of conserved antigens among *Cryptosporidium* species, respectively (Chalmers & Katzer, 2013; Xiao, Fayer, Ryan, & Upton, 2004). Thus, the use of molecular characterization in epidemiological surveys has provided new insights into the diversity of *Cryptosporidium* spp. species and genotypes and its zoonotic potential (Bajer, 2008)

Humans are mainly infected by *Cryptosporidium hominis* and *Cryptosporidium parvum*, but can also be infected by other species, including *Cryptosporidium felis* (Feng, Li, Duan, & Xiao, 2009; Smith & Nichols, 2010) typically found in cats. Significantly, *C. felis* was the second most common *Cryptosporidium* species in HIV-positive patients living in urban areas in Brazil (Lucca et al., 2009).

Our objective was to investigate the occurrence and the species of *Cryptosporidium* in fecal samples of domestic cats living in a defined urban area using a random sampling method. Further, we discussed the potential relevance of this host in the epidemiology of human cryptosporidiosis.

Material and Methods

Area of study

The study was conducted in the municipality of Araçatuba located at longitude -50.43° and latitude -21.2º, in northwestern São Paulo State, Brazil. The city has an estimated population of 182,204 inhabitants in an area of 1,167 km^2, divided into seven urban areas.

Sampling and study population

A cat population census was performed in Araçatuba between August 2010 and January 2011. A total of 5,744 cats were living in the urbanized area, which is divided into seven areas: area 1 (N = 1,061 cats), area 2 (N = 1,033 cats), area 3 (N =

1,104 cats), area 4 (N = 416 cats), area 5 (N = 544 cats), area 6 (N = 774 cats) and area 7 (N = 812 cats).

For the calculation of minimum sample size the followed parameters were considered: 10% of *Cryptosporidium* estimated prevalence, 5% absolute sampling error and a 5% significance level (Hajian-Tilaki, 2011), resulting in 138 cats. The estimated prevalence was based on studies conducted previously in Brazil (Coelho, Amarante, Soutello, Meireles, & Bresciani, 2009; Funada, Pena, Soares, Amaku, & Gennari, 2007; Gennari, Kasai, Pena, & Cortez, 1999; Huber, Bomfim, & Gomes, 2002; Ragozo et al., 2002).

The number of randomly sampled cats was proportional to the feline population of each urbanized area (Table 1). After sampling, the complete address of each cat owner was recorded and used to search for telephone number. The presence of a residential landline was required for inclusion in this study. During the initial telephone contact, the owners were briefed on the research objectives. One stool collector for each cat was provided to owners who agreed to participate in the survey. The owners were contacted daily to verify if a fecal sample had been collected.

Table 1
Number of cats, contacted and participant owners and their cats in seven urbanized area of the municipality of Araçatuba in northwestern São Paulo State, Brazil

Area	Cat population	Contacted		Participants	
		Cats	Owners	Cats	Owners
1	1061	530	134	26	12
2	1033	295	68	25	7
3	1104	471	157	25	10
4	416	154	28	11	4
5	544	348	119	13	7
6	774	245	52	19	4
7	812	247	54	19	11
Total	**5744**	**2290**	**612**	**138**	**55**

To achieve the minimum sample size (n= 138 cats), it was necessary to register 612 owners of 2,290 cats. In the end, only 55 out of 612 owners contacted accepted to participate in this investigation (Table 1). On average, samples from three cats were collected per household, ranging from two to five cats. The refusal rate to participate in the survey was 91%.

Samples were taken from cats of both sexes (male = 57, female = 81) and categorized according to age: younger (n = 72) or older (n = 66) than 12 months, as estimated by the owners and confirmed by a veterinarian. Cats were living in indoor/outdoor (n = 123) or indoor (n = 15) conditions. All the animals were mixed breed.

The survey was approved by the Ethics Committee on Animal Experimentation of the Universidade Estadual Paulista, Faculdade de Medicina Veterinária de Araçatuba, under Protocol 2008-002166. All owners signed an informed consent form before enrolling their cats in the study.

Sample processing

The 138 fecal samples were separated as follows: 2 g were suspended in 2.5 % (mass/volume) potassium dichromate for microscopy; 1 g was stored in 10% formalin for capture ELISA, and two portions of 200 µg were frozen at -20 ºC in 1.5 mL DNAse and RNAse-free microcentrifuge tubes for nested PCR (n-PCR) analysis. All samples were processed within 24 h of harvest.

Microscopy

Oocysts were concentrated by water-ether centrifugation (Meloni & Thompson, 1996) and stained with modified Kinyoun acid-fast staining (Ma & Soave, 1983) before visualizing by microscopy at 40x magnification.

Enzyme-linked immunosorbent assay (ELISA)

An ELISA (*Cryptosporidium* Stool Antigen Detection Assay kit; MEDIVAX, Brazil) was used to detect oocyst antigens. The optical density (OD) was read with a spectrophotometer (Packard Bio Science Company) at a wavelength of 490 nm. One sample was used per well. Since the antigen capture ELISA was developed to detect *C. parvum* antigens in human feces and cats are infected mainly by *C. felis*, the cut-off value was recalculated using the mean optical density plus three standard deviation obtained from 124 samples found to be negative by microscopy. Based on this analysis the cut-off calculated was set at 0.080, which is the same recommended by the manufacturer.

DNA extraction and nested-PCR (n-PCR)

DNA was extracted from fecal samples using a QIAamp Gel Extraction kit (Qiage, USA) in accordance with the manufacturer's recommendations. Subsequently, for amplification of a fragment of the ribosomal RNA 18S gene, n-PCR was performed with primers 5 ´ TTC TAG AGC TAA TAC ATG CG 3 ' and 5 ' CCC ATT TCC TTC ACA GAA GGA 3 ' for the primary reaction (amplicon size 1325 bp), and primers 5 ' GGA AGG GTT GTA TTT ATT AGA TAA AG 3' and 5' AAG GAG TAA GGA ACA ACC TCC to 3 ´ for the secondary reaction (amplicon size 826-840 bp) (Xiao et al., 1999a).

Purification of PCR amplicons and molecular characterization

Amplicons were purified with a QIAamp® DNA Mini Kit (Qiagen, USA), according to the manufacturer's recommendations and sequenced by ET Dye Terminator Cycle Sequencing Kit (MegaBACE, USA). Sequencing reactions were performed in duplicate and in both directions, i.e. four reactions total per amplicon.

Program CodonCode Aligner v. 1.5.2. (CodonCode Corp. Dedham) was used to infer a consensus sequence. Only nucleotides with sequence quality values ≥20 were considered. Consensus sequences were aligned with CLUSTAL_X (Thompson, Gibson, Pletwniak, Jeanmougin, & Higgins, 1997) and curated using the BioEdit Sequence Alignment Editor (Hall, 1999), using as reference homologous 18SrRNA sequences downloaded from GenBank AF112575.1 (Xiao et al., 1999b).

Statistical analysis

To estimate the number of *Cryptosporidium* oocyst positive cats in the population in the survey area. We used the following formula: $CI95\% = P \pm z\sqrt{[P(1-P)/n]}$, in which P is the sample prevalence expected based on previous surveys, n is the sample size, *z* refers to the Gaussian distribution to estimate the 95% confidence interval. Only samples positive with at least two diagnostic methods were considered.

All data and laboratory results were entered into an Excel spreadsheet, and the GraphPad Prism

version 5.0 (GraphPad™) was used for statistical analysis.

Results and Discussion

Cryptosporidium was detected by at least two of three diagnostic methods in 13 of 138 stool samples (9.4%; 95% CI: 4.5- 14.3) of cats. Among the 55 households surveyed, nine (16.4%) had at least one cat excreting *Cryptosporidium* oocysts. Two or three cats were excreting *Cryptosporidium* oocysts in 80% of positive households.

Six samples were positive by capture ELISA and microscopy, two samples were positive by capture ELISA and n-PCR, and five samples were positive by three methods. *Cryptosporidium* DNA from seven of the 13 positive cats was successfully sequenced and shared 99% genetic similarity to *C. felis* found in feline fecal samples GenBank accession AF112575.1 (Xiao et al., 1999), and in human fecal samples AF323563-323566 (Pedraza-Diaz, Amar, Iversen, Stanley, & McLauchlin, 2001, AF356786 (Tiangtip & Jongwutiwes, 2002) AY282720 (Hajdušek, Ditrich, & Šlapeta, 2004, FJ233037-FJ233040 (Lucca et al., 2009), and FJ707310-FJ707311 (Wang et al., 2011) from England, Thailand, the Czech Republic, Brazil and China, respectively.

To the best of our knowledge, this is the first time a probabilistic random sampling method has been applied to attempt to estimate the occurrence of *Cryptosporidium* in a population of domestic cats from an entire urban area. Considering a 95% confidence interval and a feline population of 5,744, up to 821 cats were estimated to shed *Cryptosporidium felis* oocysts in the urban area of Araçatuba during the period of this investigation.

The rate of positive samples is in agreement with other studies conducted in Brazil by only parasitological techniques (Coelho et al., 2009; Funada et al., 2007; Gennari et al., 1999; Huber, Silva, Bomfim, Teixeira, & Bello, 2007; Moreira et al., 2018; Pivoto, Lopes, Vogel, Botton, & Sangioni, 2013; Ragozo et al., 2002). The molecular characterization was performed in just a few studies in Brazil (Alves et al., 2018; Huber et al., 2007; Thomaz, Meireles, Soares, Pena, & Gennari, 2007).

To achieve the minimum sample size of 138 cats, it was necessary to contact 612 owners of 2,290 cats, representing a refusal rate of 91% (Table 1). The main reasons cited by non-participants were not knowing where their cats defecated, adversion to handling feces, lack of time or lack of interest in collaborating. Moreover, some owners were concerned that *Cryptosporidium* positive cats might be euthanized. Unfortunately, this perception is common in the survey area, because canine visceral leishmaniasis is endemic in Araçatuba, and in Brazil euthanasia is recommended for dogs infected with *Leishmania*. Another possible explanation for the high rate of refusal might be the fact that owners were contacted by telephone, a means of communication that is less reliable than face-to-face contact. The real reasons of high refusal rate of cat owners to participate in an epidemiological survey of an enteric parasite with zoonotic potential should be investigated for establishing a better relationship between the academic and local communities.

Our estimate should be considered with caution due some experimental design limitations. We collected only a single sample per cat. This strategy could underestimate the prevalence rates because cats can shed *Cryptosporidium* oocysts intermittently (Fayer, Santín, Trout, & Dubey, 2006). Collecting samples on two consecutive days could have increased the estimate (Marks, Hanson, & Melli, 2004). Furthermore, we sampled only 55 households, due the high refusal rate, and it is not a representative number of cat owners in Araçatuba.

Clinical signs of cryptosporidiosis in cats are not common (Sargent, Morgan, Elliot, & Thompson, 1998; Keith, Radecki, & Lappin, 2003; Marks et al., 2004; Fayer et al., 2006; Ballweber et al., 2009; Coelho et al., 2009; Neira, Muñoz, & Rosales, 2010;

Beser, Toresson, Eitrem, Krusnell, & Lebbad, 2015; Samie, Tsipa, & Bessong, 2013; Ito, Itoh, Kimura, & Kanai, 2016). Unfortunately, Brazilian owners tend to take their cats to a veterinarian primarily when the animals are clinically sick or need to be vaccinated. Furthermore, coprological exams are rarely requested during veterinary check-ups. These factors could increase the risk of zoonotic transmission to immunodeficient individuals, because if owners knew their cats were shedding *Cryptosporidium* oocysts, they may manage potentially infected animals and their feces with more care.

Humans are infected mainly by *C. parvum* and *C. hominis* (Xiao et al., 2004; Xiao & Fayer, 2008), but some authors claim that there is a slight risk of *C. felis* infection in humans (Lucio-Forster, Griffiths, Cama, Xiao, & Bowman, 2010; Elwin, Hadfield, Robinson, & Chalmers, 2012; Pereira & Ferreira, 2012). In addition, *C. felis* transmission from cats to their owners has already been suggested (Beser et al., 2015). These observations raise the question whether *C. felis* is relevant in the epidemiology of human cryptosporidiosis. We believe the answer depends on the management of the animals and the owners' habits of hygiene. *C. parvum* and *C. hominis* are often waterborne and foodborne pathogens (Tzipori & Widmer, 2008; Ryan, Hijjawi, & Xiao, 2018), but *C. felis* is probably mainly found in soil, gardens and sandboxes. In a study conducted in Monterey Bay, California, *Cryptosporidium* oocysts were found in 8 out of 74 samples of cat feces collected from the ground. *Cryptosporidium*-positive cats were shedding in average 75,000 oocysts per defecation (Oates et al., 2012). Moreover, *Cryptosporidium* oocyst resistance contributes to environmental contamination with infectious oocysts. *Cryptosporidium* oocyst can remain viable in feces for months (Robertson, Campbell, & Smith, 1992) and is resistant to common disinfectants as quaternary ammonium, hypochlorite and alcohol (Barbee, Weber, Sobsey, & Rutala, 1999). *Cryptosporidium* oocysts are sensitive to solar irradiation and desiccation (Rochelle, Upton, Montelone, & Woods, 2005; Méndez-Hermida et al., 2007) and the habit of cats burying their own feces might allow the pathogen to be viable for longer in the environment. On the other hand, handling cats seems to be safe, because in a study conducted in The Netherlands, *Cryptosporidium* oocysts were found in cat faeces, but not in their fur (Overgaauw et al., 2009)ELISA, and PCR. From 159 households, 152 dogs (D, probably because cats normally bury their excrements and frequently lick their fur.

Conclusions

Cryptosporidium felis infection is common in domestic cats from urban area and veterinary practitioners should guide cat owners to adopt preventive measures against the parasite to reduce the chance of infection in cats and householders.

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