ORIGINAL ARTICLE

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Zoonotic protozoa in diarrheic cats from the midwest of Brazil

Protozoários zoonóticos em gatos diarréicos no centro-oeste do Brasil

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ABSTRACT: Diarrhea, especially that of infectious origin, is a common problem in feline medicine. However, identifying a causative agent is difficult in routine clinical practice due to the many pathogens that lead to non-specific clinical signs. Among the various gastrointestinal parasites, protozoa, such as *Giardia duodenalis* (Lambl, 1859) and *Cryptosporidium* spp. (Tyzzer, 1907), pose a great threat because of their pathogenicity, prevalence in domestic animals, and zoonotic potential. This study aimed to report the occurrence of intestinal protozoa, *G. duodenalis,* and *Cryptosporidium* spp., in cats from Campo Grande, Mato Grosso do Sul, Brazil. Fecal samples and clinical and epidemiological data were obtained from 60 patients with diarrhea. Samples were subjected to DNA extraction and polymerase chain reaction (PCR) to identify the protozoa. Protozoa-positive samples were subjected to DNA sequencing and phylogenetic analysis. PCR assays revealed that one sample (1.67 %) was positive for *G. duodenalis,* and four samples (6.67 %) contained *Cryptosporidium* spp. *G. duodenalis* assemblage B and *Cryptosporidium felis* (Iseki, 1979) were identified through amplicon DNA sequencing and subsequent phylogenetic analysis. This is the first report of *C. felis* and *G. duodenalis* (assemblage B) in cats in Mato Grosso do Sul, central-western Brazil.

KEYWORDS: Zoonosis; Intestinal pathogens; Molecular analysis; Diarrhea; Felines

RESUMO: A diarreia, especialmente a de origem infecciosa, é um problema comum na medicina felina. No entanto, identificar um agente causador é difícil na prática clínica de rotina devido aos muitos patógenos que levam a sinais clínicos inespecíficos. Dentre os diversos parasitas gastrointestinais, protozoários, como *Giardia duodenalis* (Lambl, 1859) e *Cryptosporidium* spp. (Tyzzer, 1907), representam uma grande ameaça devido à sua patogenicidade, prevalência em animais domésticos e potencial zoonótico. Este estudo teve como objetivo relatar a ocorrência de protozoários intestinais, *G. duodenalis* e *Cryptosporidium* spp., em gatos de Campo Grande, Mato Grosso do Sul, Brasil. Amostras fecais e dados clínicos e epidemiológicos foram obtidos de 60 pacientes com diarreia. As amostras foram submetidas à extração de DNA e reação em cadeia da polimerase (PCR) para identificação dos protozoários. Amostras positivas para protozoários foram submetidas a sequenciamento de DNA e análise filogenética. Os ensaios de PCR revelaram que uma amostra (1,67%) foi positiva para *G. duodenalis* e quatro amostras (6,67%) continham *Cryptosporidium* spp. *G. duodenalis* assemblage B e *Cryptosporidium felis* (Iseki, 1979) foram identificados através de sequenciamento de DNA e subsequente análise filogenética. Este é o primeiro relato de *C. felis* e *G. duodenalis* (assemblage B) em gatos no Mato Grosso do Sul, Centro-Oeste do Brasil.

PALAVRAS-CHAVE: Zoonose; Patógenos intestinais; Análise molecular, Diarreia, Felinos

INTRODUCTION

Diarrhea is a common problem in feline medicine, especially from an infection (Bai *et al.*, 2023). Identifying gastrointestinal pathogens in cats is crucial because of the damage caused to the host's health and the zoonotic potential of certain species (VEYNA-SALAZAR et al., 2023). However, obtaining a definitive diagnosis, especially in chronic and intermittent diarrhea cases, is often difficult in clinical practice because of the wide variety of pathogens and the absence of specific clinical symptoms.

Among the various gastrointestinal parasites, protozoa play an important role, mainly due to their pathogenicity and high prevalence in domestic animals (Dall´agnol *et al.*, 2010). *Giardia duodenalis* and *Cryptosporidium* spp. are examples of intestinal protozoa distributed worldwide and frequently found in cat feces. In addition, certain species of

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Cryptosporidium spp. and strains of *G. duodenalis* can infect humans and cause diseases (Enbom *et al.*, 2023). Clinical signs of these infections are non-specific and have been associated with chronic diarrhea in many cases (Thompson; Palmer; O'handley, 2008).

According to the Instituto Pet Brazil (IPB) estimates, the cat population in Brazil is more than 27 million (IPB, 2023). Once cats are adopted as pets, their contact with humans increases, raising the possibility of pathogens such as *G. duodenalis* and *Cryptosporidium* spp. being transferred to humans.

Thus, this study aimed to report the occurrence of intestinal protozoa, *G. duodenalis,* and *Cryptosporidium* spp., in diarrheal cats from Campo Grande, Mato Grosso do Sul, Brazil.

MATERIALS AND METHODS

Sampling and molecular analysis

The convenience sample used in this study was initially collected for the investigation of *Enterocytozoon bieneusi* by Prado et al. (2019). This study was approved by the Animal Ethics Committee of Universidade Federal de Mato Grosso do Sul (protocol number: 787/2016).

Sixty fecal samples from diarrheal cats domiciled in the municipality of Campo Grande, Mato Grosso do Sul, Brazil, were analyzed in this study. The samples were collected from private residences and veterinary clinics between 2016 and 2017.

The cat's age ranged from 45 days to 17 years. All samples (approximately 8 g each) were placed in clean containers and immediately sent for processing to a molecular biology laboratory at a veterinary hospital. Aliquots of the samples (1 g) were transferred to 1.5 mL polypropylene tubes containing 500 µL of 0.9 % sterile saline solution and stored at −20 °C until DNA extraction.

DNA was extracted as described previously (PRADO et al., 2019). Briefly, 300 µL of the fecal suspensions stored in microtubes were centrifuged (10,000 x g for 10 min). After discarding the supernatant, the pellet was suspended in 500 µL of 20 % Sodium Dodecyl Sulfate (SDS), and 10 µL of Proteinase K (20 mg/mL) was added. The suspension was homogenized using a vortex mixer and incubated at 65 °C for 10 min. Then, 400 µL of chloroform was added, and the suspension was vortexed again, after which 300 µL of protein precipitation solution (5 M potassium acetate, 11 % glacial acetic acid) was added. The microtubes were centrifuged (10,000 × g for 10 min), and the supernatant was transferred to a new 1.5 mL microtube. One milliliter (1 mL) of ethanol was added to precipitate the DNA. After another centrifugation step (10,000 × g for 5 min), the supernatant was discarded, and the pellet was washed with 1 mL of 70 % ethanol. The samples were centrifuged for 2 min (10,000 × g), and the pellets were allowed to dry at room temperature. Then, 100 µL of nuclease-free water was added for DNA elution. Sample analysis using a BioPhotometer Plus spectrophotometer (Eppendorf; Hamburg, Germany) showed a DNA concentration ≥25 ng/µL and a ratio of 260/280 nm ≥1.75.

The epidemiological database used in the initial research (sex, age, outdoor access, presence of feline contactants, consistency of feces, frequency of diarrhea, and clinical signs of onset) was applied in this study.

Polymerase chain reaction and sequencing

Polymerase chain reaction (PCR) was performed using primers and parameters specific to each agent. Negative (ultrapure water) and positive controls (*G. duodenalis* and *C. parvum*) DNA previously characterized–Genbank accession numbers EF175936 and MZ044900, respectively) were used in each batch reaction.

For *Cryptosporidium* spp., a nested PCR was used to amplify 826–864 base pairs (*bp*) of the rRNA 18S subunit (XIAO et al. 1999). To detect *Giardia* spp., a semi-nested PCR method described by Read; Monis; Thompson (2004), which delimits a fragment of approximately 432 *bp* of the glutamate dehydrogenase gene (*gdh*), was used. The primer sets used are listed in Table 1.

Amplification products were visualized under ultraviolet (UV) light after agarose gel electrophoresis (2 %) and stained

Table 1. Primer sets used to amplify DNA fragments of *Cryptosporidium* spp. and *Giardia* spp. from cat feces from Campo Grande, Mato Grosso do Sul state, Brazil

Pathogen	Reaction	Primer set	Reference
Cryptosporidium spp.	First	F 5′-TTCTAGAGCTAATACATGCG-3′ R 5′-CCCTAATCCTTCGAAACAGGA-3′	Xiao et al. 1999
	Second	F 5′-GGAAGGGTTGTATTTATTAGATAAAG-3′ R 5′-AAGGAGTAAGGAACAACCTCCA-3′	
Giardia spp.	First	F 5′-TCAACGTYAAYCGYGGYTTCCGT-3′ R 5′-GTTRTCCTTGCACATCTCC-3′	Read; Monis; Thompson, 2004
	Second	F 5′-CAGTACAACTCYGCTCTCGG-3′ R 5′-GTTRTCCTTGCACATCTCC-3′	

with GelRed (Biotium; Fremont, CA, USA) according to the manufacturer's instructions.

For DNA sequencing, the amplicons were purified with a Clean Sweep PCR purification reagent (Thermo Fisher Scientific; Waltham, MA, USA) according to the manufacturer's instructions, and subsequently sequenced in both directions by the dideoxy method (SANGER; NICKLEN; COULSON, 1977) in an ABI 3130 automated sequencer (Applied Biosystems; Waltham, MA, USA). After processing the electropherograms, consensus sequences were obtained using BioEdit v.7.2.5. Species identification was performed with the aid of the BLASTn program (available at http://blast.ncbi.nlm.nih.gov/Blast.cgi) based on a search for homology with DNA sequences available in GenBank.

Phylogenetic analysis to identify the giardia assemblage

Multiple DNA alignments were performed to determine the *G. duodenalis* genotype, and a phylogenetic tree was constructed based on the neighbor-joining method using the MEGA 6 program (TAMURA et al., 2013). Bootstrap resampling (1000 replicates) was performed to statistically support the reliability of the nodes on the trees (Felsenstein, 1985). *Trypanosoma grayi* was used as an outgroup.

Statistical analysis

The association between clinical and epidemiological variables and the presence of infection was evaluated using Fisher's exact test with a significance level of 5 %.

RESULTS

Of the 60 fecal samples analyzed, 1.67 % (1/60) were positive in the PCR assay for *G. duodenalis*. In contrast, 6.67 % (4/60) of samples tested positive for *Cryptosporidium* spp. No co-infections were observed between the protozoa investigated in the study.

DNA sequencing of the amplicons and subsequent analysis identified the species *G. duodenalis* and *C. felis*. In the phylogenetic analysis using the *Giardia* DNA sequence identified in this study and the sequences of different assemblages, clustering with assemblage B segregated from the others was observed, which was supported by a high bootstrap value (Figure 1). The DNA sequences were deposited in GenBank under accession numbers MH155187 (*G. duodenalis*) and MH137200, MH137201, MH118292, and MH118293 (*C. felis*).

Due to the low infection frequency observed for *Giardia* spp., the significance of the association between clinical and epidemiological factors and infection was evaluated only for *C. felis*. In this analysis, a significant association was observed only for the sex variable (Table 2).

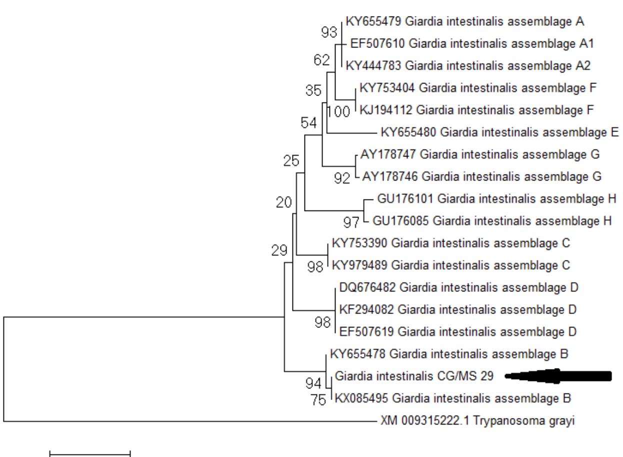

Figure 1. Phylogenetic tree constructed with sequences of the glutamate dehydrogenase gene of *Giardia duodenalis* available in Genbank and the sequence obtained in this study (arrow); the number at each knot corresponds to the bootstrap percentage; the scale represents the number of replacements per site of sequence

Table 2. Evaluation of the association between *Cryptosporidium felis* infection and clinical/epidemiological factors in diarrheal cats in the municipality of Campo Grande, MS, Brazil

Variable	Category	*Cryptosporidium felis*		*p-value*
		Positive	Negative	
Sex	Male Female	0 4	35 21	0.0259*
Age	≤1 year >1 year	3 1	40 16	0,5556
Outdoor access	Yes No	2 2	12 44	0.2245
Feces consistency	Pasty Liquids	3 1	41 15	0.7129
Clinical signs onset	Acute Chronic	1 3	26 30	0.6199
Frequency of diarrhea	Normal High	3 1	25 31	0.2570

* Significant association by Fisher's exact test.

DISCUSSION

Zoonotic protozoa in cats have received increased attention in recent years because of the increasing number of cats kept as pets and their consequent proximity to humans. This favors the exchange of infectious agents between species, which is important from a zoonotic perspective, especially in immunocompromised individuals (Llorente *et al.*, 2006; Muthusamy *et al.*, 2006; Guadano Procesi *et al.*, 2022; Enbom *et al.*, 2023).

Giardiasis is an intestinal disease affecting millions of people and animals worldwide. The disease is caused by protozoa of the *G. duodenalis* complex, whose member's present limited morphological and remarkable genetic variability. This species is divided into eight distinct genetic assemblages (A–H); however, only assemblages A and B are epidemiologically

important to humans. The remaining assemblages are considered to be host-specific, such as assemblages F, G, and H in cats, rats, and marine mammals, respectively (Monis *et al.*, 1999; Lasek-Nesselquest; Welch; Sogin, 2010; Fantinatti *et al.*, 2020), although assemblage exchange between hosts is not uncommon (Heyworth, 2016).

Giardia spp. are thought to be waterborne microorganisms closely related to low-income areas and poor sanitation infrastructure (Hotez; Gurwith, 2011). Thus, their distribution, including cats, varies greatly between regions and hosts (Faria *et al.*, 2016; Coelho *et al.*, 2017). In Brazil, the reported prevalence ranges between 4.2–34.5 % (Funada *et al.*, 2007; Coelho *et al.*, 2009; Dall'agnol *et al.*, 2010; Pivoto *et al.*, 2013).

In Mato Grosso do Sul, *G. duodenalis* has mainly been studied in humans (Higa-Junior *et al.*, 2017; Curval *et al.*, 2017; Rodrigues *et al.*, 2019), dogs (Marques; Borges, 2014), and capybaras *(Hydrochoerus hydrochaeris)* (Marta *et al.*, 2023). However, little is known about the frequency of infection in cats or assemblages of this species. In this study, the frequency of *G. duodenalis* infections was determined using molecular tools in diarrheal and domiciled cats in Campo Grande, MS. The frequency of infection (1.67 %) may be considered low when compared to the frequencies observed in other regions of Brazil, such as the metropolitan region of São Paulo (5.2 %) (Gennari *et al.*, 2016), Santos (13.3 %) (Lima *et al.*, 2021), and the state of Rio de Janeiro (15.6 %) (Labarthe *et al.*, 2008). This is the first study to identify the assemblage B of *G. duodenalis* in cats in Mato Grosso do Sul, Brazil.

Assemblages A and B of *G. duodenalis* mainly infect humans worldwide, with assemblage B being the most widespread (Fantinatti *et al.*, 2020). The importance of dogs and cats as sources of human infection remains controversial. While in a few studies there was no significant association between infection in humans and contact with pets (Hoque *et al.*, 2003; Stuart *et al.*, 2003; Tapia-Veloz *et al.*, 2023), in others, human giardiasis was observed to be positively associated with the number of household cats, increasing the odds for infection by approximately 25 % for each additional cat in the household (Pereira; Atwill; Barbosa, 2007). In Pakistan, the risk of giardiasis was 5.72 times higher among children with companion animals compared to those without (Khattak *et al.*, 2023).

The protozoan *C. felis* was detected in 6.67 % (4/60) of the samples analyzed by nested PCR and subsequent DNA sequencing. In Brazil, the prevalence of *C. felis* in cats ranges between 1.45–11.3 % (Funada *et al.*, 2007; Coelho *et al.*, 2009; Pivoto *et al.*, 2013; Oliveira *et al.*, 2021). Despite this, the protozoan has not been given importance as a cause of disease in cats since Dall'Agnol *et al.* (2010) reported that the presence of infection in healthy and asymptomatic cats was relatively common.

In this study, excluding the fact that all animals had diarrhea, there was no significant association between infection and any clinical/epidemiological factors analyzed, except for the sex variable. Female cats had a significantly higher frequency of infection (p-value = 0.0259) than in male cats (Table 2).

Although the zoonotic potential of *C. parvum* is well known (Chalmers *et al.*, 2011; Guo *et al.*, 2022), other species of *Cryptosporidium* have been associated with zoonotic cryptosporidiosis cases, among them *C. felis* (Cacciò *et al.*, 2002; Llorente *et al.*, 2006; Muthusamy *et al.*, 2006). In most reported cases, infected humans had diarrhea and were immunocompromised due to HIV infection. The cryptosporidiosis transmission route remains unclear. In a study conducted in Portugal, only one of four immunocompromised patients with *C. felis* was in close contact with cats at home (Matos *et al.*, 2004).

Assessing the risk of zoonotic transmission of *Giardia* and *Cryptosporidium* spp. from pet animals (mainly dogs) is challenging (De Lucio *et al.*, 2017). Genotyping analyses of parasite isolates are useful for ascertaining the genetic diversity of these pathogens and generating baseline molecular epidemiological information. However, these data alone should not be used to infer the occurrence of zoonotic transmission (Ballweber *et al.,* 2010). The following three factors may result in partial or unproven conclusions: lack of molecular data at the sub-genotype level, possible existence of reverse zoonosis, and unrecognized potential of companion animals to act as passive carriers of parasitic oocysts of anthroponotic origin (Gil *et al.,* 2017).

CONCLUSION

Cats can participate in transmitting *G. duodenalis* and *C. felis* to humans; the possibility of them being transmitters cannot be ruled out, nor should we underestimate their importance as an indirect source of infection. This is the first report of *C. felis* and *G. duodenalis* (assemblage B) in cats in Mato Grosso do Sul, central-western Brazil.

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