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Detection of natural occurrence of *Tritrichomonas foetus* in cats in Araçatuba, São Paulo, Brazil[1]

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ABSTRACT.- Duarte R.P., Rocha P.R.D.A., Nakamura A.A., Cipriano R.S., Viol M.A., Melo G.D., Meireles M.V. & Machado G.F. 2018. **Detection of natural occurrence of *Tritrichomonas foetus* in cats in Araçatuba, São Paulo, Brazil**. *Pesquisa Veterinária Brasileira 38(2):309-314*. Faculdade de Medicina Veterinária, Universidade Estadual Paulista "Julio de Mesquita Filho, Campus de Araçatuba, Rua Clóvis Pestana 793, Araçatuba, SP 16050-680, Brazil. E-mail: giselem@fmva.unesp.br

The aim of this study was to investigate the occurrence of *Tritrichomonas foetus* in cats in the area surrounding the city of Araçatuba municipality, State of São Paulo, Brazil. Fecal samples from 129 cats were collected by rectal flush technique. It was compared two diagnosis methods, direct examination of feces and PCR. The presence of *T. foetus* DNA was verified using PCR by amplification of 347-bp fragment from the primers TFR3 and TFR4 and amplicons of positive cases were sequenced. Statistical analyses were performed investigating the associations between *T. foetus* infection with gender, age, breed, presence of diarrhea and/or history of diarrhea, previous treatment, lifestyle, origin, environment, and co-infection. *T. foetus* was observed in one sample (n=129) by direct microscopic examination of feces while PCR was positive in five samples (3.9%). *Giardia* species and *Cryptosporidium* species co-infection was also observed. Statistical analyses showed no significant associations between *T. foetus* infection and all listed factors, although most positive cats were asymptomatic and lived in multi-cat household. The isolates of *T. foetus* showed 100% identical sequences with other *T. foetus* isolates from cats around the world. So, the occurrence of *T. foetus* was confirmed in cats in Araçatuba city (Brazil). This parasite must be considered as a differential diagnosis in cats with diarrhea and also in asymptomatic carriers as source of infection in multi-cat environments.

INDEX TERMS: *Tritrichomonas foetus*, cats, diarrhea, polymerase chain reaction, PCR, genetic sequencing, parasitoses.

RESUMO.- [**Detecção da ocorrência natural de *Tritrichomonas foetus* em gatos em Araçatuba, São Paulo.**] O objetivo deste estudo foi investigar a ocorrência de *Tritrichomonas foetus* em gatos na região do município de Araçatuba, SP, Brasil. Foram coletadas amostras fecais de 129 gatos através da técnica de lavado retal. Dois métodos diagnósticos foram comparados, o exame direto das fezes e a PCR. A presença de DNA de *T. foetus* foi verificada por meio da PCR através da amplificação de 347 pares de bases a partir dos iniciadores específicos TFR3 e TFR4. Posteriormente, os resultados amplificados das amostras positivas foram sequenciadas. Também foi feita análise estatística a fim de investigar a correlação entre infecção por *T. foetus* e sexo, idade, raça, presença e/ou histórico de diarreia, tratamento prévio, coinfecção, estilo de vida, origem e tipo de ambiente. O protozoário pôde ser observado em uma amostra através do exame direto das fezes e à PCR foram detectadas cinco amostras positivas (3.9%). Foram detectadas coinfecções por

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Giardia spp. e *Cryptosporidium* spp. Não foram observadas correlações entre infecção por *T. foetus* e todos os fatores listados anteriormente, embora a maioria dos felinos positivos fossem assintomáticos e vivessem em ambientes multigatos. O resultado do sequenciamento genético dos isolados das amostras positivas mostrou 100% de similaridade com outros isolados de felinos no mundo. Assim, a ocorrência de *T. foetus* foi confirmada em gatos em Araçatuba, São Paulo, Brasil. Sendo assim, o parasito deve considerado como diagnóstico diferencial em gatos com diarreia assim como em portadores assintomáticos como fontes de infecção em ambientes multigatos.

TERMOS DE INDEXAÇÃO: *Tritrichomonas foetus,* felinos, diarreia, reação em cadeia da polimerase, PCR, sequenciamento genético, parasitoses.

INTRODUCTION

Tritrichomonas foetus is a protozoan best known for causing sexually transmitted disease in cattle (BonDurant 1997). Likewise, *T. foetus* would be a synonym of *Tritrichomonas suis* (Doi et al. 2012). However, in cats *T. foetus* colonizes the colon causing chronic large bowel diarrhea and is transmitted by the fecal-oral route (Levy et al. 2003, Tolbert & Gookin 2009, Gruffydd-Jones et al. 2013). But not all infections by *T. foetus* in cats are associated with clinical signs, since diarrhea may resolve temporarily without treatment (Tolbert & Gookin 2009, Gruffydd-Jones et al. 2013).

Young cats with one year old or less seen to be more susceptible to *T. foetus* infection, whereas older cats are generally asymptomatic (Levy et al. 2003, Tolbert & Gookin 2009). Other risk factor, also the most important, is multi-cat household (Gruffydd-Jones et al. 2013, Yao & Köster 2015), since the trophozoites do not persist for a long time out in the environment and cats become infected probably through close contact, specially by mutual grooming (Tolbert & Gookin 2009, Gruffydd-Jones et al. 2013).

Comparative analysis of molecular markers suggests the differentiation of "*cat genotype"* and "*cattle genotype"* of *T. foetus* (Slapeta et al. 2010). Cats that were experimentally infected with *T. foetus* isolates from heifers and heifers that were infected with *T. foetus* isolates from cats showed no similarities on the infectivity and pathogenicity (Stockdale et al. 2007, Stockdale et al. 2008). A recent study suggested that *T. foetus* isolated from feces of different cats may belong to new specie, which would justify the use of a new name, *Tritrichomonas blagburni* (Walden et al. 2013).

The occurrence of *T. foetus* in cats has been cited as worldwide distribution (Yao & Köster 2015). It has been reported in several countries such as United Kingdom (Gunn-Moore et al. 2007), The United States of America (Stockdale et al. 2009), Switzerland (Frey et al. 2009), Italy (Holliday et al. 2009), Australia (Bell et al. 2010), South Korea (Lim et al. 2010), New Zealand (Kingsbury et al. 2010), Greece (Xenoulis et al. 2010), Germany (Kuehner et al. 2011), Spain (Miró et al. 2011), Norway (Tysnes et al. 2011), Japan (Doi et al. 2012), Canada (Hosein et al. 2013) and France (Profizi et al. 2013). Due to the increasing interest in this protozoan in feline medicine, the current study was designed to investigate the occurrence of *T. foetus* in Brazilian cats using direct examination of feces, PCR, and further genetic sequencing. Additionally, to investigate the associations between *T. foetus* infection and some risk factors such as gender, age, breed, presence and/or history of diarrhea, previous treatment, co-infection, lifestyle, origin and multi-cat household.

MATERIALS AND METHODS

Data collection, fecal samples and direct feces examination. Data about the cats were obtained by asking the owners or caretakers and approval was ensured by a written consent before samples collection. Fecal samples were collected from 129 cats chosen by convenience living in the area surrounding the city of Araçatuba, São Paulo, Brazil. Kittens under two months of age were excluded from the study. The domestic shorthairs were classified as mixed breed by the absence of pedigree. Fecal samples were collected by rectal flush technique consisting in infusion of 10mL of 0.9% saline solution with the usage of a syringe attached to a urethral probe nº8 (https://cvm.ncsu.edu/research/labs/clinical-sciences/tfoetus). Within six hours (Hale et al. 2009), the aspirated fluid was placed in a conical tube and stored at room temperature for 15 minutes for precipitation of its content. A precipitate drop of each sample was examined under a coverslip at 100x and 400x magnification by a light microscopy direct examination (Tolbert & Gookin 2009, Gruffydd-Jones et al. 2013). Subsequently, DNA was extracted using the ZR Fecal DNA Kit®, Zymo Research, Orange, CA, USA, and so the extracted DNA was stored at -20°C for PCR.

PCR for *Tritrichomonas foetus*. A total volume of 25µL for each reaction mixture was prepared with 500nM of primers TFR3 and TFR4 (Felleisen et al. 1998); 0.2mM deoxynucleotide (A,C,T,G) solution,, Deoxinucleotide (dNTP) Mix, Sigma®, Saint Louis, Missouri 63103, USA; 0.5U Taq DNA polimerase, Platinum®, Invitrogen™, by Life technologies™; 2mM $MgCl_2$; 2.5µL buffer 10x; 2µL of extracted DNA sample; and the remaining volume was completed with ultrapure water, Sigma®, SIGMA-ALDRICH, UK. DNA amplification followed the conditions established by Gookin et al. (2002) with minor modifications in a thermal cycler, Mastercycler eppendorf, Realplex^2: initial denaturation at 94°C for 5 minutes, denaturation at 95°C for 30 seconds, annealing at 67°C for 30 seconds, extension at 72°C for 45 seconds, repeated for 50 cycles, followed by final extension at 72°C for 5 minutes. The positive control was commercially purchased, VetMAX™ *T. foetus Control*, Invitrogen™, by Life technologies™, and the negative control was ultrapure water. The presence of *T. foetus* DNA was verified by amplification of 347-bp fragment after electrophoresis on a 1.5% agarose gel and visualized by UV illumination.

PCR for *Giardia* species. A total volume of 25µL for each reaction mixture was prepared with 200nM of primers GDH1 and GDH4 (Homan et al. 1998); 0.2mM deoxynucleotide (A,C,T,G) solution; 0.5U Taq DNA polymerase; 2mM $MgCl_2$; 2.5µL buffer 10x; 0.5pmol/µL bovine serum albumin (BSA); 2.5µL of extracted DNA sample; and the remaining volume was completed with ultrapure water. DNA amplification followed the conditions established by Homan et al. (1998). The presence of *Giardia* DNA was verified by amplification of approximately 768-bp fragment.

Nested-PCR for *Cryptosporidium* species. Both primary and secondary reactions were prepared with 200nM of primers stablished by Xiao et al. (1999a, 2000); 0.2mM deoxynucleotide

(A,C,T,G) solution; 0.5U Taq DNA polymerase; 2mM $MgCl_2$; 2.5µL buffer 10x and 2.5µL of extracted DNA sample. For primary reaction 0.5µg/µL BSA was added. Remaining volume was completed with ultrapure water for a total volume of 25µL for each reaction mixture. DNA amplifications were done according to the following conditions: initial denaturation at 95°C for 3 minutes, denaturation at 95°C for 45 seconds, annealing at 55°C for 45 seconds, extension at 72°C for 1 minute, repeated for 39 cycles, followed by final extension at 72°C for 7 minutes. The presence of *Cryptosporidium* DNA was verified by final amplification of approximately 840-bp fragment.

Genetic sequencing. DNAs of positive cases were purified using the Gel Extraction Kit QIAquick®,Quiagen®, ICI Americas Inc. After DNAs quality and quantity were monitored, they were sequenced in both directions. The identities of the sequencings were checked using the BLAST® platform (Basic Local Alignment Search Tool). *T. foetus* sequences of positive cases were compared with the *T. foetus* bovine sequence, GenBank accession no. U85967 (Felleisen et al. 1998) and *T. blagburni* sequences, GenBank accession nos. EU569301 - EU569312 (Walden et al. 2013).

Statistical analysis. The correlation between both the diagnostic methods was determined using the *kappa* test (Conraths & Schares 2006) and the x^2 test which was applied to evaluate the relationship between *T. foetus* infection and data about the cats, like gender, age, breed, presence and/or history of diarrhea, previous treatment, lifestyle, origin, environment, and co-infection.

RESULTS

Samples. Most cats were females (n=83), adults, older than one year (n=80), and mixed breed (n=126). 19 cats presented with diarrhea at the moment of collecting samples. Clinical history was available from 14 cats and 3 of them had already been treated for *Giardia* species. 77 cats lived in environments with more than one cat, only 7 alone, and of 45 information was not available. About the origin of the animals, 20 cats were from a rescue colony of an animal hoarder, 35 were from rescue colonies of non-governmental organizations, 17 were from a city shelter, 55 were from homes and 2 cats were from a Persian cattery. And about lifestyle, 64 cats lived exclusively indoor, 36 outdoor, both in urban areas, nine were from farms, and of 20 information was not available.

Diagnosis. By direct microscopic examination *Tritrichomonas foetus* was observed in one sample of feces and *Giardia* species was observed in another one. *Toxocara* species, *Ancylostoma* species, coccidia, *Aelurostrongylus abstrusus* and *Dipylidium caninum* infections were present in 19 cats and three of these cats had co-infection with two parasites, one with *Ancylostoma* species and coccidia, and two with *Ancylostoma* species and *Dipylidium caninum*.

PCR was positive for *T. foetus* in five cats (3.9%), including positive sample by direct feces examination. Data about positive cats is available on Table 1. It was possible retrieve clinical history of just one positive cat. This cat lived exclusively indoors but had outdoor access in the past. His playmates were tested, and they were negative for *T. foetus* by PCR. One positive cat lived at a rescue colony of a hoarder and it was not possible to test all cats because new kittens arrived in the colony every day. One of the females was a stray cat and was caught by non-governmental organization for neutering. (Table 2)

Table 1. Comparison categorical data between PCR-positive and negative cats for *Tritrichomonas foetus*

Risk factors	PCR status		*P* value
	Negative	Positive	
Gender			
Male	44	2	0.8362
Female	80	3	
Age			
< 1 year old	48	1	0.3980
≥ 1 year old	76	4	
Breed			
Mixed breed	121	5	0.7249
Purebred	3	0	
Diarrhea			
Yes	18	1	0.7344
No	106	4	
History of diarrhea			
Yes	13	1	0.6919
No	23	1	
No information	88	3	
Previous treatment			
Yes	3	0	0.7368
No	53	2	
No information	68	3	
Lifestyle			
Indoor	60	4	-
Outdoor	35	1	
Rural	9	0	
No information	20	0	
Origin			
Rescue colony of animal hoader	19	1	
Rescue colony of NGOs	34	1	-
Shelter	17	0	
Home	52	3	
Cattery	2	0	
Multi-cat household			
Yes	73	4	0.5366
No	7	0	
No information	44	1	
Co-infection (other parasites)			
Yes	19	0	-
No	110	0	
Giardia spp. co-infection			
Yes	60	4	0.1657
No	64	1	
Cryptosporidium spp. co-infection			
Yes	19	1	0.7769
No	105	4	

- Impossibility of chi-square analysis.

Genetic sequencing. Sequencing of *T. foetus* isolated in the present study GenBank accession no. KX267765 showed 100% identity to homologous sequences of *T. foetus* isolated from other domestic cats in USA, AF466749 (Levy et al. 2003) and EU569309 (Stockdale et al. 2007, Walden et al. 2013); Switzerland, JN006994 (Reinmann et al. 2012); French,

Table 2. Data about *Tritrichomonas foetus* PCR-positive cats

Gender	Age	Breed	Diarrhea	History of Diarrhea	Treated	Environment	Multi-cat	Other positives	Origin	Direct examination of feces	PCR *Cryptosporidium* species	PCR *Giardia* species
Male	Adult	Mixed	No	No	No	Indoor	Yes	No	Hoarder	Negative	Negative	Negative
Male	Adult	Mixed	No	Yes	No	Indoor	Yes	No	Home	Negative	Negative	Positive
Female	Adult	Mixed	Yes	*	*	Indoor	Yes	*	Home	Negative	Negative	Positive
Female	Adult	Mixed	No	*	*	Indoor	Yes	*	Home	T. foetus	Negative	Positive
Female	Young	Mixed	No	*	*	Outdoor	*	*	NGO	Negative	Positive	Positive

* Information not avaliable.

JX960422 (Profizi et al. 2013); Norway, HM856630 (Tysnes et al. 2011) and EF165538 (Dahlgren et al. 2007); and Australia, GU170216 (Slapeta et al. 2010) and JX187000 (Slapeta et al. 2012). Furthermore, revealed a single nucleotide polymorphism (T>C) when compared with *T. foetus* isolated from cattle, similar to the findings previously described (Slapeta et al. 2010).

Four of *T. foetus* positive cats had co-infection with *Giardia* species and one with *Cryptosporidium* species. Sequencing of *Giardia* showed 99% similarity with *Giardia duodenalis Assemblage F*, accession no. AB569373 (Suzuki et al. 2011) and sequencing of *Cryptosporidium* showed 100% similarity with *C. felis*, accession no. AF112575 (XIAO et al. 1999b).

Statistical analysis. Statistical analyses showed no significant associations between *T. foetus* infection, with gender, age, breed, presence of clinical signs, history of diarrhea, previous treatment, lifestyle, origin, multi-cat housing, and/or co-infection. According to the *kappa* test result, a fair agreement (0,324) between diagnosis by direct examination of feces and PCR was found.

DISCUSSION

This is the first study about the occurrence of *Tritrichomonas foetus* in Brazilian cats and the occurrence was 3.9%. Worldwide, the occurrence of this parasite has varied among countries. This variation may be due to two factors: 1) the cat population chosen to be studied; and 2) the diagnostic method (Gruffydd-Jones et al. 2013). It has been reported occurrence of 6.2% - 15/241 - in a study comparing the occurrence of *T. foetus* from cats sampled at cat shows, humane society and clinic (Hosein et al 2013), and also 82% - 18/22 – in another study only from purebred cats sampled at cat shows (Kingsbury et al. 2010).

In the present study, it was included samples from cats of different populations, young and adult, with and without diarrhea, from various origins, and with diverse lifestyle. Although we did not find any correlation between *T. foetus* infection and all risk factors listed before, some of these relationships should be considered with caution, mainly due to the small number of representative samples of each population and lack of detailed information about some cats. For instance, *T. foetus* infection was detected in five cats and four of them were asymptomatic. Also, four cats have been living with more than one cat. Generally, *T. foetus* infection is associated as a result of multi-cat household, previously described as the main risk factor (Holliday et al. 2009, Bell et al. 2010, Kuehner et al. 2011). Simultaneously, the stress generated by the pressure of overcrowding, like in shelters and rescue colonies (Holliday et al. 2009), contributes to trigger episodes of diarrhea even in asymptomatic cats, which facilitates the dissemination of the parasite (Kuehner et al. 2011). In this way, screening of asymptomatic carriers, which can be a source of infection, should be considered in multi-cats environments (Gookin et al. 2001, Xenoulis et al. 2010, Miró et al. 2011).

Despite the high prevalence of this parasite in susceptible cat populations and although we did not find *T. foetus* positive cats from farms, this is the first study that also included a cat population from rural areas. The transmission route, if the cattle for the cat or the opposite, is an issue discussed by some authors (Stockdale et al. 2007, Stockdale et al. 2008, Slapeta et al. 2010, Slapeta et al. 2012, Reinmann et al. 2012) and molecular studies still disagree about the genetic identity of *T. foetus* (Yao & Köster 2015). Until now, the genetic sequence isolated in the present study may be classified as "cat genotype" like suggested by Slapeta et al. (2010), because this sequence showed the same single nucleotide polymorphism (T> C) in the ITS2 region. We believe that further studies, with more cats which live exclusively in rural areas may contribute to clarify the epidemiological relationship between *T. foetus* infection in cats and the contact with farm animals.

Co-infection with another parasite was not detected by direct examination of feces in *T. foetus* positive cats, however, it was found *Giardia duodenalis* (n=4) and *C. felis* (n=1) co-infection by PCR. Co-infection with other intestinal parasites, such as *Giardia* species (Gookin et al. 2004, Bissett et al. 2008, Stockdale et al. 2009, Kingsbury et al. 2010, Köster et al. 2015), *Cryptosporidim* species (Stockdale et al. 2009), coccidia like *Isospora* species (Stockdale et al. 2009), *Sarcocystes* species (Kingsbury et al. 2010), and *Toxoplasma gondii* (Miró et al. 2011), has been already reported. The most common co-infections are *Giardia* species and coccidia (Tolbert & Gookin, 2009). In cats with co-infection by *Cryptosporidium* species there is an increased severity of diarrhea, and concomitantly increase in the spread of *T. foetus* (Gookin et al. 2001).

As mentioned before, the diagnosis method is the second point that may interfere with the *T. foetus* occurrence. The sensitivity of direct examination of feces showed a much lower *T. foetus* diagnosis than the PCR, as highlighted in previous studies (Gookin et al. 2004, Holliday et al. 2009, Miró et al. 2011, Tysnes et al. 2011). Molecular diagnosis is a good choice mainly in the absence of viable organisms in fecal samples, reducing the false negative diagnosis (Holliday et al. 2009, Doi et al. 2012, Tysnes et al. 2011) and even the false positive diagnosis using other methods such as direct examination of feces and cultivation (Miró et al. 2011). According to the literature, it is difficult to differentiate *T. foetus* from the *Pentatrichomonas*

hominis, a commensal trichomonad of cats, especially by direct examination of feces (Gookin et al. 2007, Yao & Köster 2015). However, even the PCR may have limitations for diagnosis (Hale et al. 2009). The sensitivity of PCR may be impaired by the degradation of DNA and the presence of inhibitors, similar to what happens with the diagnosis of *T. foetus* infection in cattle. Fecal samples would be considered a challenge to PCR rightly by the presence of large amounts of inhibitors, such as bilirubin, bile salts, complex carbohydrates, which are often extracted concurrently with the DNA of the pathogen (Gookin et al. 2007). We tried to overcome these limitations through the extraction kit (Stauffer et al. 2008).

Besides, a single fecal sample often results in a false negative diagnosis due to intermittent elimination of the parasite (Thurmond & Johnson 2004, Hosein et al. 2013). However, repeated sampling was not possible in the present study due to practical limitations, therefore the occurrence of *T. foetus* might be higher.

CONCLUSIONS

The occurrence of *Tritrichomonas foetus* was confirmed in cats living in Araçatuba city (Brazil) and also by the genetic sequencing of the parasite.

Although co-infection with other intestinal parasites was observed, *T. foetus* must be considered as differential diagnosis in cats with diarrhea and also in asymptomatic carriers as source of infection in multi-cat environments.

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Ethical approval.- All procedures performed in this study were in accordance with ethical standards of the institution, CEUA, FOA/Unesp, process no. 2014-00898.

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