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Co-infection by *Tritrichomonas foetus* and *Pentatrichomonas hominis* in asymptomatic cats[1]

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ABSTRACT.- Dos Santos C.S., De Jesus V.L.T., McIntosh D., Berto B.P. & Lopes C.W.G. 2015. **Co-infection by *Tritrichomonas foetus* and *Pentatrichomonas hominis* in asymptomatic cats.** *Pesquisa Veterinária Brasileira 35(12):980-988*. Curso de Pós-Graduação em Ciências Veterinárias, Anexo 1, Instituto de Veterinária, Universidade Federal Rural do Rio de Janeiro, Campus de Seropédica, BR-465 Km 7, Seropédica, RJ 23897-970, Brazil. E-mail: carolinespitz@yahoo.com.br

Tritrichomonas foetus, a parasite well known for its significance as a venereally transmitted pathogen in cattle, has been identified as a cause of chronic large bowel diarrhea in domestic cats in many countries of the world. In Brazil, several studies on the diagnosis of bovine trichomoniasis have been performed, but until now, no study was made regarding feline trichomoniasis. Thus, this is the first study to report the occurrence of *T. foetus* and *Pentatrichomonas hominis* in cats using morphological and molecular analysis. Feces from 77 cats were examined, four of which (5.2%) were positive for the presence of parabasalids. Morphological analysis of stained smears revealed piriform trophozoites showing the three anterior flagella, elongated nucleus and axostyle ending abruptly in fillet, characteristic of *T*. *foetus*. In scanning and transmission electron microscopy, identification characters similar to those previously reported for *T. foetus* were observed. The cultures containing trophozoites were submitted for molecular analysis, which resulted positive for *T. foetus* DNA using specific primers (TFR3 and TFR4), and all samples were positive and subjected to sequencing in which they showed 99.7-100% similarity with another isolate sequencing of *T*. *foetus* (JX960422). Although no trophozoite with consistent morphology of *P. hominis* has been visualized in the samples, differential diagnosis was performed using specific primers for *P*. *hominis* (TH3 and TH5) amplicon. In three of the four samples (3.89%) sequencing revealed 100% similarity when compared with another sequence of *P. hominis* deposited in Genbank (KC623939). Therefore, the present study revealed through the diagnostic techniques employed the simultaneous infection by *T. foetus* and *P. hominis* in the feces of cats. However, it was necessary to use more than one technique for the diagnosis of the co-infection. These results demonstrate the importance of a correct diagnosis to allow an appropriate treatment by the veterinarian.

INDEX TERMS: Co-infection, *Tritrichomonas foetus, Pentatrichomonas hominis,* domestic cats.

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RESUMO.- [**Co-infecção por *Tritrichomonas foetus* e *Pentatrichomonas hominis* em gatos assintomáticos**.] *Tritrichomonas foetus*, um parasito bem conhecido por seu significado como um agente patogênico transmitido venereamente em bovinos, também foi identificado como causa de diarreia crónica do intestino grosso em gatos domésticos em muitos países. No Brasil, vários estudos sobre o diagnóstico de tricomonose bovina foram realizados, mas até agora, não há informação disponível em relação à trichomonose felina. Assim, este é o primeiro estudo a relatar a ocorrência

de *T. foetus* e *Pentatrichomonas hominis* em gatos por meio de análise morfológica e molecular. Fezes de 77 gatos foram examinadas, a partir da qual quatro (5,2%) foram positivas para a presença de parabasalídeos. A análise morfológica de esfregaços corados revelou trophozoitos piriformes com três flagelos anteriores, núcleo alongado e axóstilo cuja projeção termina abruptamente em formato de filete, características estas de identificação morfológica *T. foetus*. Além disso, microscopia eletrônica de varredura e transmissão, revelaram caracteres morfológicos semelhantes aos descritos na literatura para esta espécie. A análise molecular de culturas utilizando iniciadores específicos para trofozoítos de *T. foetus* (TFR3 e TFR4), mostrou que as quatro amostras foram positivas para este parasito e osequenciamento dos fragmentos amplificados demonstraram 99,7-100% de similaridade com seqüências depositadas no GeneBank de *T. foetus*. Nenhum trofozoíto com morfologia consistente com a descrição de *P. hominis* foi visualizado nas amostras. No entanto, a análise molecular, utilizando iniciadores específicos para esta espécie (TH3 e TH5) detectou que três das quatro amostras (75%) também foram positivas para *P. hominis* e o sequenciamento de nucleotideos revelou 100% de similaridade dos amplicons quando comparada com o mesmo fragmento de DNA de *P. hominis* depositado no GenBank. Como tal, o presente estudo relata a coinfecção de gatos com *T. foetus* e *P. hominis* e destacou a exigência de uma combinação de métodos para o diagnóstico de coinfecções. Estes resultados demonstram a importância do diagnóstico correto para a aplicação da terapia apropriada por parte dos veterinários.

TERMOS DE INDEXAÇÃO: Coinfecção, *Tritrichomonas foetus*, *Pentatrichomonas hominis*, gatos domésticos.

INTRODUCTION

Historically, the observation of parabasalids in the feces of cats with diarrhea and/or dysentery owned low importance and attributed to an overgrowth of gut microflora that favoured the proliferation of protozoa (Dimski 1989, Barr 1998). An association between dysentery in the large intestine in cats and the presence of parabasalids as *Pentatrichomonas hominis* and *Tritrichomonas foetus* was recognized by Romatowski (1996, 2000), Gookin et al. (1999), Levy et al. (2003). There are practically one or more reports of the occurrence of feline trichomoniasis or by *T. foetus* or *P. hominis* in one or more countries in the five continents. The mode of transmission is the direct type, for oral-fecal route (Brugerolle 1973, Farmer 1993) having pseudocysts important role in the transmission cycles of these protozoa. The pseudocyst, which can reverse the process and turn again a mobile trophozoite (Granger et al. 2000), is able to multiply and maintain their form infecting new hosts (Pereira-Neves et al. 2003).

P. hominis and *T. foetus* have been found in cats (DA Cunha & Muniz 1922, Levy et al. 2003). The difference between the two species requires more refined knowledge of the veterinary practitioner, because both species have the same type of motility and similar form. Therefore, the differentiation can not be made through examination of fresh stool, and not through specific culture media, because they are equally able to grow (Ceplecha et al. 2013). The importance of the correct parasitological diagnosis between both species is directly related to the therapeutic employed in cats with clinical signs of trichomoniasis, which differ according to the species involved (Gookin 2006, Ceplecha et al. 2013).

The laboratory methods used for the diagnosis of feline trichomoniasis include traditional and recent techniques. Among these techniques, the most common are fresh examination and culture, but for both techniques stool samples should be fresh, because dead organisms due to cooling of samples or failures in the processing may generate false negative results (Gookin et al. 2003). They are considered of low sensitivity and specificity, because they require a considerable number of motile protozoa for diagnosis, and do not differentiate between species. However, the culture of feces allows the confection of stained smears for the morphological diagnosis (Lun & Ghadanger 1999).

Currently, molecular biology in fecal samples is being used with greater frequency. But as it is an expensive method that requires adequate facilities and qualified staff, its use is still limited for routine experimental research laboratories in Brazil. Nevertheless, it has higher sensitivity and specificity than previous methods (Gookin et al. 2004). Although highly desirable to employ a unique technique that can diagnose accurately and quickly an etiologic agent, the use of molecular biology alone can lead to false negative results (Gunn-Moore et al. 2007); to improve the diagnostic sensitivity more than one technique must be employed (Hale et al. 2009).

Due to the absence of studies on feline trichomoniasis in Brazil, the present study aimed to investigate the frequency of cats positive for trichomoniasis through standard techniques: fresh examination of feces, culture, morphological and molecular analysis of isolated samples.

MATERIALS AND METHODS

Ethical considerations

The studies concerning animals were processed in strict accordance with the recommendations approved by the Ethic Commission (CEUA/IV/UFRRJ # 006/2014). We declare that the cats were not harmed in any way during the procedure.

Sample collection and processing

Fecal samples were collected from 77 domestic cats with or without diarrhea, at the Hospital Veterinário de Pequenos Animais, Instituto de Veterinária, UFRRJ, located in the municipality of Seropédica in the State of Rio de Janeiro, Brazil. Some animal data were required to evaluate possible risk factors associated with the infection, as age, sex, feces form (classified as consistent (normal) or not consistent (pasty, poorly formed or liquid), diarrhea history and contact with other cats. Samples were collected immediately after defecation or directly from the rectum by infusion of warm saline and were immediately transported to the Laboratório de Patologias da Reprodução, Anexo 1, UFRRJ. Thereafter, the feces were diluted in Hanks solution supplemented with 10% inactivated horse serum, incubated at 28-33°C, and examined by microscopy at 48 hour intervals. Fecal samples considered as positive upon direct examination were inoculated into specific medium (Diamond 1957) incubated at 35°C, and subcultured at 72h intervals.

Morphology analysis

Observation, measurement and illustration of trophozoites. Microscope slides were prepared using smears of culture fixed in methanol and staining by Panotico® protocol (Lun & Gadhajar 1999). Morphological observations, line drawings, photomicrographs and measurements were made using an optic microscope (Primo Star Zeiss® trinocular microscope coupled to a digital camera. The morphometric analysis of trophozoites was done using the computer program (AxionVision Release 4.8.2, 2010). Line drawings were edited using two software applications from CorelDRAW® (Corel Draw Graphics Suite, Version 11.0, Corel Corporation, Canada), specifically Corel DRAW and Corel PHOTO--PAINT. All measurements are in micrometres and are given as the range followed by the mean in parentheses.

Electron microscopy

The Electron Microscopy Center of the Federal University of Minas Gerais (CEM/UFMG), Pampulha, Belo Horizonte, MG, performed analyses of electron microscopy.

Scanning electron microscopy. The culture in logaritmic phase was centrifugated and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. Afterwards, the samples were washed in PBS, post-fixed in 1% OsO_4, dehydrated in ethanol, critical point dried with CO_2, and sputter-coated with gold--palladium.The samples were examined in a JEOL 5800 scanning electron microscope.

Transmission electron microscopy. The culture of in logaritmic phase was centrifugated and fixed at room temperature with 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2. Afterwards, the trofozoites were washed with PBS. Post-fixation was performed for 30 min with 1% OsO_4 in cacodylate buffer containing 5mM $CaCl_2$ and 0.8% potassium ferricyanide. Parasites were washed with PBS, dehydrated in acetone and embedded in Epon. Ultrathin sections were stained with 10% uranyl acetate and 5% lead citrate and observed with a JEOL 1210 transmission electron microscope.

Molecular analysis

DNA was extracted from about $1x10^6$ trophozoites (enumerated microscopically), as follows: culture were harvested by centrifugation (16,000xg for 5 min) and supernatants were discarded, and the cell pellets were washed once with 1mL of phosphate buffered saline (PBS: pH7.2). Washed cells were resuspended in 100µL of chelex matrix (InstageneTMmatrix BIO RAD) with incubation at 56ºC for 30 minutes, and then boiled for 10 minutes. The tubes were centrifuged (16,000xg for 5 min), to sediment cell debris together with the chelex matrix, and 60µL of each supernatant were transferred to new tubes, with storage at -20ºC. A Polymerase chain reaction (PCR) assay design for *Tritrichomonas foetus* was performed, using the primers TFR3 and TFR4 according to Felleisen et al. (1998). For the differential diagnosis, specific primers Th3 and Th5 for *Pentatrichomonas hominis* were employed (Crucitti et al. 2004). The K strain of *T. foetus* isolated by H. Guida (Embrapa, Seropédica, RJ, Brazil) from the urogenital tract of a bull was used as a positive amplification PCR control in the first assays. PCR products reaction was examined by 2% agarose gel electrophoresis stained by ethidium bromide to confirm the presence of amplicons with the predicted molecular weight.

PCR products (339 base pairs), from the *P. hominis* specific assay were digested using 10 units of the restriction endonucleases H*ae* III (Promega) and H*inf* I (Invitrogen) for 2h at 37ºC. Digestion products were separated using 12% polyacrylamide gels electrophoresis followed by staining with ethidium bromide. The resulting banding patterns were compared with those predicted by in silico digestion using the computer program (Program NEB-cutter V 2.0; New England Biolabs) of the amplified region present in the 18S rDNA of a feline isolate of *P. hominis*, GenBank accession number (KC594038.1).

Sequencing of *T. foetus* 5.8s rDNA, ITS 1 e 2 region and *P. hominis* 18S rDNA amplicons was performed as follows: 10µL of PCR products were treated with Exo-Sap-IT (GE Healthcare), according to the manufacturer's protocol and sequenced in both directions, employing the amplification primers, by use of the BigDye Ready Reaction mix (ABI Corp); reaction products were analyzed on a 3500 automated genetic analyzer (ABI Corp). Sequence alignments were performed using Sequencher (Version 5.2, Genecodes Corporation, CA). Aligned sequences were entered into the BLAST search algorithm and the NCBI nucleotide database to determine gene identity.

Statistical analyses

Statistical analysis was based on BioEstat (AYRES et al. 2007) and Sampaio (2007) to analyze the variables found in this study.

RESULTS

Tritrichomonas foetus diagnosis in cats

Seventy-seven feces samples were sent by the Veterinary Hospital for protozoan fecal research. Only four samples (5.2%) were positive by standard tests. Two samples were positive on fresh examination and two were positive only after culture in Diamond´s media, all were positive in cultures. The average age of the positive cats was 3 and a half years, as to gender, three were females and only one male. There was no significant difference in any of the variables evaluated (Table 1), even the conviviality with other cats did not show significance, although the positive cats coexisted with other cats.

Table1. Risk factors evaluated for the presence of Parabasalids in cats attended at the Veterinary Hospital of UFRRJ

Variables	Animals		*P* value	Risk fator	95% IC*
	Positive	Negative			
Diarrhea					
Yes	7 (17)	8 (19)†			
No	4 (10)	22 (54)	0,03	1,5865	0,9614-2,6183
Total	11 (27)	30 (73)			
Consistency of stools					
Normal	5 (12)	13 (31,6)	0,5894	1,0234	0,7030-1,4899
Abnormal	6 (15)	17 (41,4)			
Total	11 (27)	30 (73)			
Fecal diagnosis					
Fresh exam	10 (24,4)	30 (73)			
Culture	11 (26,8)	30 (73)	≤ 0,000	11,0000	1,6974-71,2847
Total	11 (27)	30 (73)			

* Using the approximation of Katz; †Percentual values.

Morphologic diagnosis

The trophozoites of *Tritrichomonas foetus* showed almost always piriform form sometimes more elongated, but in older cultures became more rounded. The size of trophozoites (n=100) of T. foetus isolates of cats (excluding the projecting part of the axostyle) was 10.4±1.0 (8.5-13.0) µm in length and 6.1±0.8 (4.2-8.0) µm in width. The undulating membrane, extending throughout the body of the protozoan, ended with the free portion of the posterior flagellum (Fig.1A).

The number of anterior flagella was constant in all four positive samples being visualized three anterior flagella and one posterior flagellum (Fig.1B). The first anterior flagella measured 14.0±1.3 (10.9-16.9), the second was 13.8±1.2 (10.3-15.7) and the third has 13.4±1.1 (11.6-15.7) µm length. The pelta was visualized like a comma shaped structure positioned in anterior portion of the trophozoite in the emergence of the anterior flagella.

The nucleus lays in the anterior portion o the body, with oval/elongated shape located dorsally over the capitulum of axostyle, and measuring 3.5±0.8 (2.2-7.0) µm in length and 2.5±0.6 (1.6-5.9) µm in width. The parabasal body

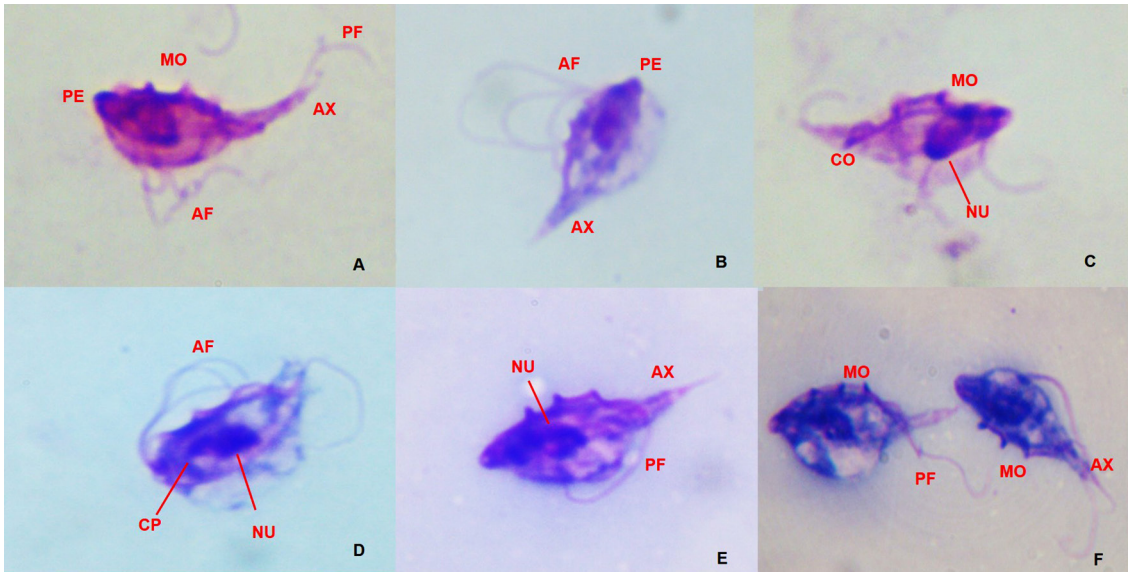

Fig.1. Cellular structures present in *Tritrichomonas fetus* isolated from cats. (**A**) Presence of clearly Pelta (PE) comma-shaped on the front of the parasite body and the Capitulum of axostyle (CA). (B) Trophozoite presented three anterior flagella (FA). (**C**) Trophozoite with clearly visible coast beneath the undulating membrane and following up its entire length. (**D**) *T. foetus* exhibiting three anterior flagella (FA), elongated oval shape nucleous (NU) and visible parabasal body (CP) located dorsally to the nucleous. (**E**) Typical axóstilo format (AX), posterior flagellum (FP) marking the end of the undulating membrane. (**F**) Trophozoites formats of *Tritrichomonas foetus* isolated from cats. One with characteristic piriformis shape of elongated appearance and another with oval/round shape. It is possible to view structures as axostyle, undulating membrane and posterior flagellum. Fast Panoptic®, obj.100x.

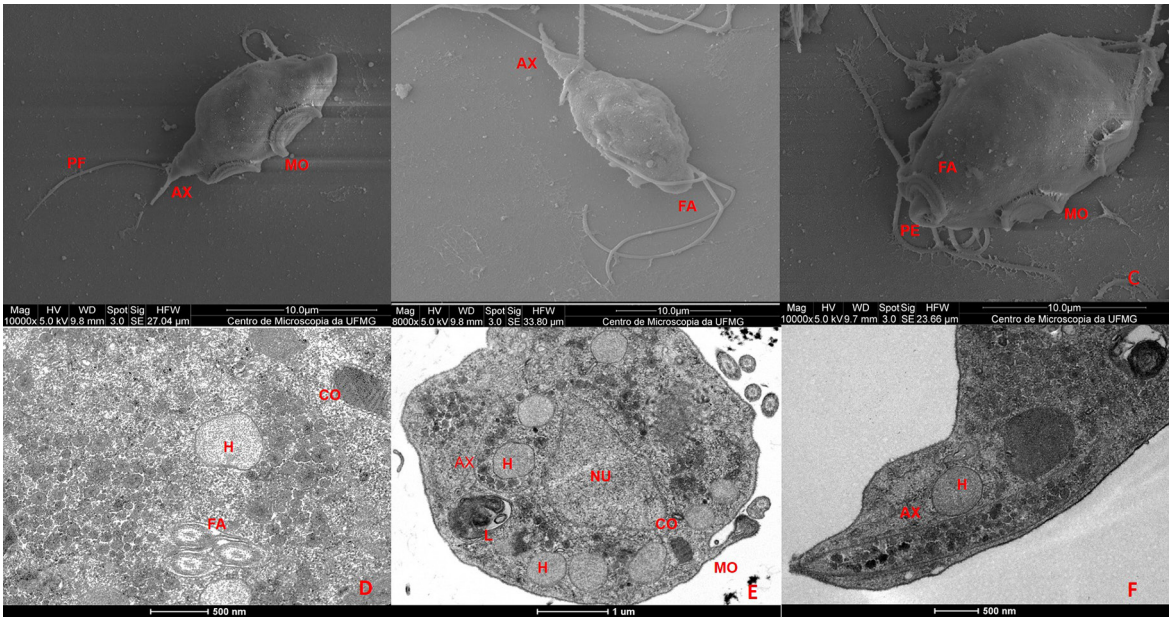

Fig.2. *Tritrichomonas foetus* isolated from cat. (**A**) In featured, structures as axostyle (AX) undulating membrane (UM), posterior flagellum (FP) and one of the anterior flagella (AF). (**B**) Trofozoite presented three anterior flagela and axostyle. (**C**) *T. foetus* presented visible pelta (PE), flagelar channel; undulating membrane associated to posterior flagella connected to parasite the body by a thin layer. SEM. (**D**) Ultrastructural details of the group of anterior flagella, Costa (CO), and hydrogenossomes. (**E**) *T. foetus* demonstrating ultra structural details axostyle (axostyle trunk), oval-shaped nucleus (NU); Costa, undulating membrane and posterior flagellum; Glycogen granules near the axóstilo; several hydrogenosomes of varying size. (**F**) Axostyle details, presented glicogen granules (rosettes fomation), hydrogenossomes arrayed along the axostyle. TEM.

found itself over the nucleus and had elongated shape. The costa was identified as a slender filament located just below the undulating membrane and going through its entire length. The length of the posterior flagellum was 11.3±2.6 (6.0-18.0) μm in length (Fig.1C and D).

The axostyle showed itself clear in its entire length, with a cylindrical shape, larger in diameter at the beginning of the body, known as the Capitulum, and was tapering until partly extended (Fig.1E). The extended part of axostyle presented itself after its emergence with a larger diameter and tapered abruptly in a small fillet (Fig.1G). Their average length was 5.4±1.5 (2.16-8.39) μm.

Scanning eletronic microscopy

Trophozoites were piriform displaying typical morphology of the Tritrichomonadidae family with three anterior flagella, undulating membrane stepping through the entire body of the parasite until the end portion, and a recurring flagella continuing beyond the undulating membrane in a free portion (Fig.2A and B). The pelta was visible forming a kind of wall that supports the anterior flagella (Fig.2C). The trophozoites showed an undulating membrane with 3 to 4 undulations, the extended part of axostyle presented its thickest projection straight in the emergence of body and was becoming thinner in the final portion, similar to a thread and with diameter like the posterior flagellum (Fig.2A). It was possible to visualize the undulating membrane as a single structure formed by the posterior flagellum connected to the body by a thin plasma membrane layer.

Transmission electron microscopy

Piriform trophozoites had three anterior flagella and in its interior numerous hydrogenosomes and lysosomes. The hydrogenosomes had rounded shape and double membrane of varied size and granule content, most of which was largely associated with axostyle (Fig.2D and E). The axostyle appeared as a ribbon delimited by two filaments, one dorsal and the other ventral, containing inside several granules including granules of glycogen that were darker points in the form of rosettes and tapering in the final portion (Fig.2F). The axostyle trunk was visualized as partly involve the nucleus, comma-shaped which later became straight (Fig.2D). Glycogen granules were seen as darker points (rosette formation) present and distributed in the cytoplasm, but mostly located along the axostyle. The nucleus had an irregular shape, but ovaler and located between the costa and axostyle. The posterior flagellum was seen always associated with the bodie's undulating membrane. Another structure which was present in the cytoskeleton was visualized, the costa, in form of a tape with light and dark bands. Based on the main morphological characters, a schematic drawing was made (Fig.3) highlighting all the features for morphological identification of *T. foetus*, differentiating it from *P. hominis* (Dos Santos 2015).

Genotypic identification of trophozoites

Polymerase chain reaction (PCR) and sequencing. In the present study two different pairs of specific species--primers were used, TFR3 TFR4 (Felleisen et al. 1998) for

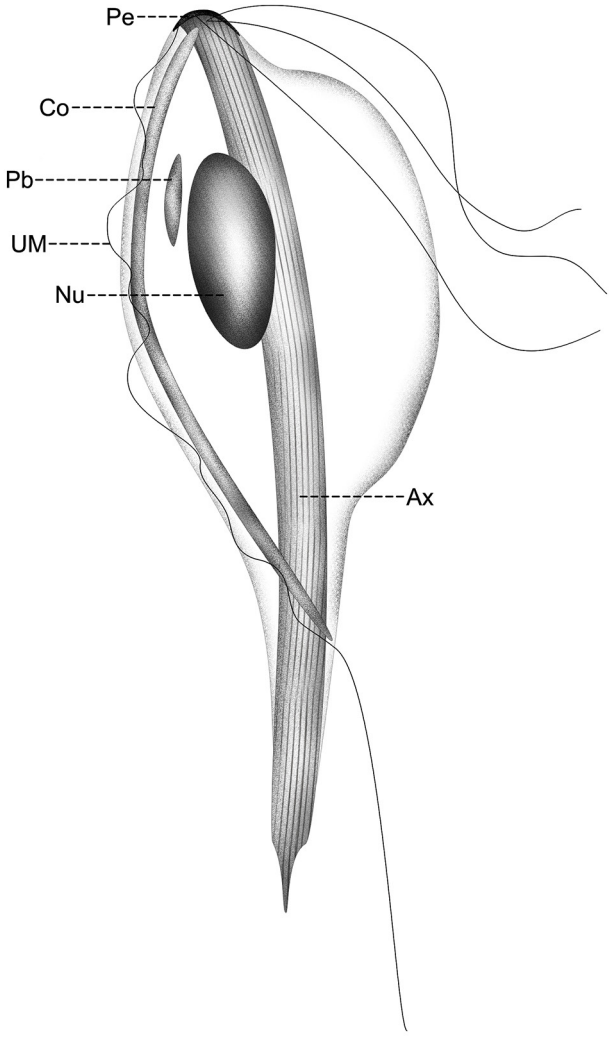

Fig.3. Schematic drawing of the *Tritrichomonas foetus* trophozoite isolated from domestic cats (*Felis catus*). Ax = axostyle; Co = costa; Nu = nucleus; PB = parabasal body; PE = pelta; UM = undulating membrane; FA = anterior flagella; FP = posterior flagellum.

diagnosis of *T. foetus* and TH3, and TH5 (Crucitti et al. 2004) for differential diagnosis of *P. hominis*. Using the primers for *T. foetus* four isolates in culture were positive, showing an amplification product of about 347 bp size, as well as the positive control strain K of *T. foetus*. The PCR products with the TFR3 and TFR4 primer three samples were sequenced and showed 99.7-100% similarity with other sequences deposited in Genbank of feline *T. foetus* isolated (accession number JX960422).

The alignments of gene amplification products of the 18S, 5.8S and ITS1 and ITS2 demonstrated that the feline *T. foetus* isolates had almost identical sequences (99.7-100%) with *T. foetus.* The comparison with *P. hominis* exhibited a low similarity sequence (56.6-82.6%) demonstrating a high degree of separation between the two species.

For differential diagnosis specific primers for *Pentatrichomonas hominis* (TH3 and TH5) were used and surprisingly, three of the four samples were positive for the spe-

cies; although none trophozoite has been identified in the morphological analysis. With the positive results also for *P. hominis* DNA, the samples were subjected to RFLP-PCR using the restriction enzymes. The amplified products with TH3 and TH5 primers were subjected to digestion with H*ae*III restriction enzymes and H*inf* I resulting in DNA sections, and the generation of two bands in the gel with an approximate size of 251 bp and 99 bp with H*ae* III, and the generation of three bands of 163bp, 133bp and 62 bp with H*inf* I. The banding patterns observed were corresponding to that specified in digestion silica. Thus, the DNA from *P. hominis* samples was considered identical. The amplified products with the TH3 and TH5 primers were subjected to sequencing and showed 100% similarity between them, also when compared with other sequence of *P. hominis* deposited in Genbank (KC623939).

DISCUSSION

In Brazil, several studies have been conducted with *Tritrichomonas foetus* of bovine origin; these studies primarily targeted at the parasite-host interaction, biochemical mechanisms and ultrastructural analysis (Pereira-Neves et al. 2009, Vilela & Benchimol 2011, Midlej et al. 2011). However, the study of *T. foetus* and *Pentatrichomonas hominis* in small animals is almost non existent were it not for the account given by Da Cunha & Muniz (1922) on *P. hominis* in cats. The present paper appears to be the first morphological and molecular study in Brazil describing the diagnosis of *T. foetus* in the feline population, and also the first one on co-infection with *P. hominis*. In the literature, infection rates vary widely among each study, two (Mancianti et al. 2014) to 31% (Gookin et al. 2004). In the present study, only 5.2% of the culture samples tested were positive. Correct identification of these two species is of extreme clinical relevance, because each species has a different treatment. Currently, *T. foetus* infection is treated with ronidazole, a neurotoxic drug for cats and potentially carcinogenic to humans who handle the drug (Gookin et al. 2006, Papich et al. 2013); but there are already reports of parasite resistance to this compound (Gookin et al. 2010). Treatment of trichomoniasis caused by *P. hominis* can vary from a simple change in diet (Ceplecha et al. 2013) to the use of drugs such as metronidazole (Gookin et al. 2005), and has no effect on *T. foetus* isolates of cats (Gookin 2006).

In the present study, none of the parameters described as risk factors for the disease were significant, possibly due to the low rate of positivity found. Though the infected cats had a history of diarrhea in the past six months and all divided the same space with other cats, they were at the time of sample collection asymptomatic. Foster et al. (2004) presented similar results among cats with chronic *T. foetus* infection without clinical signs after resolution of diarrhea.

The main factors of risk associated with infection/transmission of the parasite are related to high population density and could exert higher pressure for infection resulting in an increased risk of oro-fecal contact with the protozoan. This could be attributed to increased stress or reinfection of cats under dense housing conditions (Foster et al. 2004). The occurrence of co-infection with other enteropathogens, especially *Giardia* spp. and *Cryptosporidium* spp., would be directly related to clinical signs displayed by animals positive for *T. foetus*. Feces consistency and history of diarrhea and/or dysentery are factors to be considered when collecting samples for the diagnosis of trichomoniasis, since there is a strong association between infection with *T. foetus*, the diarrhea history (Gookin et al. 2004) and abnormal fecal score (Kuehner et al. 2011). While clinical impressions indicate that, the infection is most common in young animals (Gookin et al. 1999), cats of any age can acquire the infection (Gookin et al. 2004). Apparently, older cats that acquire the infection may be asymptomatic and young cats may be more vulnerable to infection due to their immune system under developement (Gookin 2001).

The importance of positive diagnosis for trichomoniasis in asymptomatic cats is related to environmental contamination with feces containing protozoa that can infect other cats, mainly in environments where there is a high density of cats or even animals of other species that may acquire the infection of this parasite. Foster et al. (2004) observed that cats usually continue to eliminate trophozoites of *T. foetus* for up to 39 months after resolution of diarrhea; thus, each cat, with or without diarrhea, in a closed population should be evaluated.

The morphological details described in this study aim to give veterinary clinicians tools for easier and faster diagnosis, so that they could be able to differentiate the two species which can parasitize cats. According to the results obtained on the morphology, small differences in the size and shape of the analyzed *T. foetus* trophozoites and to other morphological studies may be related with the medium used, cultivation time or fixing methods, or even with bacteria present in the medium (Kirby 1951, Taquezy et al. 2002, Clothier et al. 2015). The trophozoites of *T. foetus* show consistently three anterior flagella, unlike *P. hominis* which presents five flagella (Li et al. 2014). This is one of the features which can be used to differentiate among species (Kirby 1951). In several studies with a variety of strains of *T. foetus*, the trophozoites show three anterior flagella, even between different hosts such as cattle, pigs and cats (Wenrich & Emmerson 1933, Hibler et al. 1960, Levy et al. 2003). Other characters can also be observed as the form of the axostyle which gradually narrows in *P. hominis,* and in *T. foetus* is continuous until the point that is tapered abruptly forming a small fillet (Wenrich & Emmerson 1933, Wenrich 1944).

By morphological examination was seen that parabasalids observed in the feces of infected cats at HVPA/UFRRJ were diagnosed as *T. foetus*. This was based on the number of flagella, shape and location of the nucleus and axostile format. Due to these morphological details, it was possible to differentiate the trophozoites of *T. fetus* and *P. hominis*. Contradicting these results, Levy et al. (2003) stated that the two species are not easily to be distinguished by examining the organisms stained with standard histological dyes. Then the anterior flagella are difficult to number and morphology axostilemorphology, which is an important criterion to distinguish *T. foetus* from *P. hominis* requires specific and expensive staining techniques, as the silver impregnation, for their correct identification.

In the past, *T. foetus* in cats was mistakenly classified as *P. hominis*, which because *P. hominis* may have varying numbers of 3 to 5 anterior flagella (Romatosky 1996, 2000, Gookin et al. 2001). However, it is known that *T. foetus* possesses a constant number of flagella, with three anterior and one posterior, in old and recent records on that constancy (Riedmüller 1928, Wenrich & Emmerson 1933, Kirby 1951, Benchimol 2004, Pereira-Neves Benchimol 2009). The confusion with the number of previous flagella of *P. hominis*, is still subject of inquiry, but more recent literature (Honinberg et al. 1978, Li et al. 2014) on this species may present four to five anterior flagella as has been shown by several studies including ultrastructural analysis. Thus, the confusion in the morphologic diagnosis between the two species which occur in cat feces is something that should not be taken into account, since several studies have demonstrated the possibility of differentiation of the two species.

In electron microscopy results obtained were similar to those of the optical microscopy for *T*. *foetus* isolates from cats and were similar to those reported in the literature for bovine and feline strains described in other studies (Benchimol et al. 2000, Frey et al. 2009, Midlej et al. 2011, Walden et al. 2013). The ultrastructural analysis has been used for a long time to better understand the structure of different organisms and potential drug targets. It has been well established that the ultrastructure of the Trichomonadidae family is specific to gender (Brugerolle 1986) and is of little help to the differentiation of species. Parabasalids from the same genus can be quite similar such as the case of *Tritrichomonas mobil*ensis and *T. foetus*. However, Midlej et al. (2011) that despite *T. mobiliensis* possesses morphology very similar to *T. foetus*, the projection of axostile differentiates them. The authors noted that there was no formation of a fine tip at the end of axostile projection, as in *T. foetus*.

Few studies have been concerned in making the differential diagnosis between these two species in cats with diarrhea/dysentery. This because it is part of the premise that *P. hominis* is merely commensal. Molecular analysis used in this survey found the infection of cats by *T. foetus* and also the co-infection of the same animals by *P. hominis*. No trophozoite analyzed showed morphological characteristics of *P. hominis*; however, by PCR was found the presence of DNA compatible with the species. The reduced number of trophozoites of *P. hominis* culture in relation to *T. foetus* may be related to the metabolic rate of each species, causing survival variability by competition for nutrients. Frey et al. (2009) affirmed that the survival of the parasites is extremely variable among isolates. When performing subcultures in Diamond media, just some of them are recovered as stable cultures.

Co-infection with these two species has been described by Gookin et al. (2007), and despite the morphology of trophozoites has not been examined, the authors affirmed that the detection of *P. hominis* in feces would not contribute to a wrong diagnosis of infection by *T. foetus*. This**,** because *P. hominis* was found in 1.9 to 2.1% of samples, and all cats in which *P. hominis* was identified, were also infected with *T. foetus*.

Similar results were obtained in the present study, but we disagree with the fact that the identification of *P. hominis* does not interfere with the diagnosis of infection by *T. foetus* when using other tools as not only DNA analysis. For example, fresh examination of feces or specific culture media that does not differentiate between the two species and, once the PCR directly from feces alone is not the method more reliable, it could generate false negative samples, according to Stokdale et al. (2009).

The results obtained with molecular analysis of the isolates only corroborate the results of the morphological diagnosis made in this study. Therefore, its use with the analyzed samples was made in a complementary manner for the morphologic diagnosis of *T. foetus*. The combination of the three techniques allowed a more accurate diagnosis of *T. foetus* isolates from fecal samples, apart from identifying the presence of *P. hominis* three of the four samples examined.

Currently, there is a general tendency to opt for direct molecular analysis of feces for the diagnosis of trichomoniasis (Gookin et al. 2002, Paris et al. 2014). According to these authors it is not possible to differentiate *T. foetus* from other protozoa with similar appearance and size as *Giardia* spp. and *P. hominis* in optical microscopy or for sample conditions of handling, such as exposure to low temperatures, which can lead to a false negative result of culture, besides the time spent in culture and morphological analysis (Oyhenart et al. 2013). Due to the presence of a range of PCR inhibitors in feces the extraction method is one of the key points for using the technique (Stauffer et al. 2008). The presence of these inhibitors may result in false-negative samples as was demonstrated by Stockdale et al. (2009). Therefore, to improve the sensitivity of diagnosis more than one technique should be employed (Hale et al. 2009). False-negative results can occur when using only one technique and a single sample. The ideal would the use of a routine for the diagnosis of feline similar trichomoniasis which is made in the bovine, as the realization of more than a collection of material for analysis to rule out the infection thereby increasing the diagnostic sensitivity (Da Silva et al. 2011).

The morphological and molecular findings presented here indicate the presence of carrier cats both *T. foetus* as *P. hominis* and the absence of clinical signs of disease in these animals, making them asymptomatic carriers, and therefore disseminators of these protozoa. Not only between animals, but also for humans. Several reports in the literature suggest respiratory and intestinal infections in humans by these two etiologic agents (Jongwutiwes et al. 2000, Kutisova et al. 2005, Duboucher et al. 2006, Mantini et al. 2009). Considering these findings, these species should be carefully monitored as a question of public health in humans and domestic animals, related potential from the viewpoint of zoonotic transmission and poorly studying pathogenicity. In contrast to the previous image to consider them supposedly as commensal, because there is a close relationship between humans and companion animals, mainly dogs and cats that are considered in some cases as part of the family.

Due to lack of information in Brazil about these paraba-

salids, there are possibly a greater number of clinical cases of these infections in humans and animals than is currently recognized. Thus influencing not only the health of the animals but also humans through direct pathologies, but also indirectly by dysbiosis of the microflora of the intestinal mucosa and local inflammation, thus favouring transmission of other pathogens.

CONCLUSION

The present study detected with the diagnostic techniques employed *Tritrichomonas foetus* and *Pentatrichomonas hominis* in the feces of cats. However, it was necessary to use more than one technique for the diagnosis of the co-infection. These results demonstrate the importance of correct diagnosis for the application of appropriate therapeutic measures in veterinary medicine.

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