

Molecular prevalence of trichomonad species from pet shop puppies and kittens in Japan

Molecular prevalência de espécies trichomonas em filhotes de cães e gatos de pet shop no Japão

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Abstract

Pentatrachomonas hominis and *Tritrichomonas foetus* (cat genotype) have been commonly identified as intestinal trichomonads in both dogs and cats. Although *P. hominis* is considered as non-pathogenic protozoa in many kinds of mammals, it has the potential for zoonotic transmission. *T. foetus* has been recognized as the emerging causative agent of diarrhea in cats without the risk of zoonotic transmission. As pet shops are the major source of young companion animals, the present study discusses the molecular prevalence of *P. hominis* and *T. foetus* from 544 pet shop puppies and 409 kittens. The results suggest that the prevalence of *P. hominis* (puppies: 7.0%; kittens: 0.5%) and *T. foetus* (puppies: 0%; kittens: 2.4%) in pet shop young animals are low. In addition, the infections of *P. hominis* and *T. foetus* are not always associated with the clinical signs (soft or diarrhea feces).

Keywords: *Pentatrachomonas hominis*, *Tritrichomonas foetus*, puppy, kitten, pet shop.

Resumo

Pentatrachomonas hominis e *Tritrichomonas foetus* (genótipo de gato) têm sido comumente identificados como trichomonas intestinais em cães e gatos. Apesar de *P. hominis* ser considerado como protozoário não patogênico em muitos tipos de mamíferos, tem potencial para transmissão zoonótica. Enquanto o *T. foetus* foi reconhecido como o agente causador emergente de diarréia em gatos sem o risco de transmissão zoonótica. Devido às lojas de animais serem as principais fontes de filhotes de animais domésticos, o presente estudo discute a prevalência molecular e/ou o potencial zoonótico de *P. hominis* e *T. foetus* em 544 filhotes de cachorro e 409 gatos de “pet shop”. Os resultados sugerem que a prevalência de *P. hominis* (cães: 7,0%; gatos: 0,5%) e *T. foetus* (cães: 0%; gatos: 2,4%) em animais jovens de “pet shop” é baixa. Além disso, as infecções de *P. hominis* e *T. foetus* nem sempre estão associadas aos sinais clínicos (fezes moles ou diarréia).

Palavras-chave: *Pentatrachomonas hominis*, *Tritrichomonas foetus*, filhote de cachorro, filhote de gato, pet shop.

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Introduction

In both dogs and cats, two species of intestinal trichomonads, *Pentatrichomonas hominis* and *Tritrichomonas foetus* (cat genotype), have been identified (Gookin et al., 2007; Li et al., 2016a). It remains still controversial whether these parasites are pathogenic or not. In general, *P. hominis* is considered as non-pathogenic opportunistic protozoa in many kinds of mammals including dog, cats, and humans (Maritz et al., 2014). However, some previous reports suggest an association of *P. hominis* infection with the occurrence of diarrhea (Bastos et al., 2018; Kim et al., 2010; Meloni et al., 2011; Zhang et al., 2019). In addition, the dogs and cats infected with *P. hominis* have the potential as reservoir for zoonotic transmission. *T. foetus* has been recognized as the emerging causative agent of chronic diarrhea in cats (Bastos et al., 2019; Hora et al., 2017; Yao & Köster, 2015). Hence, intestinal trichomonads in dogs and cats are obviously significant as potential pathogen and/or as zoonotic agent. As pet shops are the major source of young companion animals for owners, the present study discusses the molecular prevalence of *P. hominis* and *T. foetus* from pet shop puppies and kittens in Japan.

Materials and Methods

Between October 2017 and September 2018, a total of 544 and 409 fecal samples were collected randomly from pet shop puppies and kittens in Japan, respectively. All of the animals were aged 1-3 months. After natural defecation, the fresh feces were visually evaluated by veterinarians as follows: Normal (hard or moderate soft formed stool), Soft (evidently moist formed stool), and Diarrhea (watery liquid stool). The stool specimens were immediately submitted to the laboratory on ice from three pet shops located in two different prefectures (Shop A-C) for puppies and from two pet shops (Shop A, B) for kittens, and were stored at 4 °C prior to DNA extraction (within three days). The outlines of pet shops were as follows: Shop A (223 puppies; 234 kittens, Koto-ku in Tokyo), Shop B (282 puppies; 175 kittens, Hirosaki-city in Aomori), and Shop C (39 puppies, Hachinohe-city in Aomori). The trichomonad genomic DNA extraction from fecal samples was performed using QIAamp Fast DNA Stool Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA samples were stored at -20 °C until polymerase chain reaction (PCR) analysis.

According to the previous study (Kamaruddin et al., 2014), a nested PCR targeting the partial small subunit ribosomal RNA gene was employed for the detection of trichomonad species. The sequences of each primer and the cycling conditions are shown in Table 1. In the primary reaction, the PCR mixture comprised 1× buffer containing 3.0 mM of MgCl₂, 200 μM of each dNTP, 0.5 μM of each primer, 1.25 units of GoTaq DNA polymerase (Promega Corporation, Madison, WI, USA), and 3.0 μl of template DNA (1-5 ng DNA) in a total reaction volume of 25 μl. In the secondary reaction, the PCR mixture was the same as that for the primary reaction, with the exception of the primary PCR amplicons, which were used as a template (3.0 μl).

Table 1. Polymerase chain reaction primers, annealing temperatures, and expected sizes of amplicons.

PCR	Primers names	Primers sequence (5' to 3')	Annealing temperature (°C)	Amplicon size (bp)	Reference
Primary	MK1	GTAGGCTATCACGGGTAACG	66	10,80	Kamaruddin et al. (2014)
	MK6	GTTGACACACATTTACAAGGGATTCC			
Secondary	MK5	GCAGCAGGCGCAAACCTTAC	64	850	
	MK4	GGACATCACGGACCTGTTATTGCTAC			

The cycling conditions of PCRs were as follows. In primary reaction, after an initial denaturation of 3 min at 94 °C, 35 cycles were performed, each consisting of 30 sec 94 °C for denaturation, 30 sec at 66 °C for annealing, and 60 sec at 72 °C for extension, with a final extension of 5 min at 72 °C. In secondary reaction, the cycling conditions were the same as primary reaction, except that the annealing temperature was 64 °C.

All secondary PCR amplicons were identified via electrophoresis on 1.5% agarose gels. The specific DNA fragments were confirmed after staining with AtlasSight DNA Stain (Bioatlas, Tartu, Estonia) under UV light using a transilluminator. After the amplicons of predicted size were purified using a QIAquick Gel Extraction Kit (QIAGEN), those were sequenced in a commercial laboratory (FASMAC Co., Ltd., Atsugi, Kanagawa, Japan). Sequence alignment and compilation were carried out using the MEGA 7.0.26 program (MEGA, 2020). To determine the species of trichomonad, the obtained DNA sequences were compared to GenBank references by BLAST searches (NCBI, 2020).

The data were stratified according to pet shop and fecal condition (normal vs. soft vs. diarrhea) and were analyzed statistically with pairwise comparisons using Fisher's exact probability test. The *P* values were adjusted for multiple test procedure by Holm's method and significant level was set to $P < 0.05$. Statistical analysis was performed using the software of R version 4.0.2 (The R Foundation for Statistical Computing, Vienna).

Results

The results of the present study are summarized in Table 2. Overall prevalence of trichomonad species in pet shop puppies and kittens were 7.0% (38/544) and 2.9% (12/409), respectively. According to the sequencing analysis, all of 38 isolates from puppies were corresponding to *P. hominis* with the similarity of 99.0-100% (accession number KC594038). From kittens, 10 isolates (2.4%; 10/409) were corresponding to *T. foetus* with the similarity of 99.8-100% (accession number AF466749), and the remainder two isolates (0.5%; 2/409) were *P. hominis* with the similarity of 99.4% (accession number KC594038).

Table 2. Molecular prevalence of trichomonad species from pet shop puppies and kittens in Japan.

	Examined no.	<i>Pentatrichomonas hominis</i>		<i>Tritrichomonas foetus</i>		Total	
		Positive no.	Prevalence	Positive no.	Prevalence	Positive no.	Prevalence
Puppies							
Overall	544	38	7.0%	0	0%	38	7.0%
Pet shops							
Shop A	223	7	3.1%	0	0%	7	3.1% *
Shop B	282	28	9.9%	0	0%	28	9.9% #
Shop C	39	3	7.7%	0	0%	3	7.7%
Fecal condition							
Normal	519	36	6.9%	0	0%	36	6.9%
Soft	13	1	7.7%	0	0%	1	7.7%
Diarrhea	12	1	8.3%	0	0%	1	8.3%
Kittens							
Overall	409	2	0.5%	10	2.4%	12	2.9%
Pet shops							
Shop A	234	0	0%	7	3.0%	7	3.0%
Shop B	175	2	1.1%	3	1.7%	5	2.9%
Fecal condition							
Normal	384	2	0.5%	10	2.6%	12	3.1%
Soft	18	0	0%	0	0%	0	0%
Diarrhea	7	0	0%	0	0%	0	0%

*vs #; $P < 0.01$.

The prevalence of *P. hominis* in each pet shop puppies were as follows: 3.1% (7/223) in Shop A, 9.9% (28/282) in Shop B, and 7.7% (3/39) in Shop C. The ratio in Shop B was significantly ($P < 0.01$) higher than that in Shop A.

The frequencies of trichomonads in two pet shop kittens were 3.0% (7/234) in Shop A and 2.9% (5/175) in Shop B, with no significant difference. The trichomonad species from kittens in Shop A demonstrated that all seven isolates were *T. foetus*. While in Shop B, the identified species were three isolates of *T. foetus* and two isolates of *P. hominis*.

According to the fecal condition, in puppies, the prevalence was 6.9% (36/519) in normal, 7.7% (1/13) in soft, and 8.3% (1/12) in diarrhea. Hence, there was no significant differences among fecal conditions. In kittens, the frequency of normal vs. soft vs. diarrhea was 3.1% (12/384) vs. 0% (0/18) vs. 0% (0/7), without significant differences.

Discussion

The present study is the first report that evaluated the prevalence of intestinal trichomonad species from pet shop puppies and kittens in Japan. In puppies, only *P. hominis* infection was determined. In contrast, *T. foetus* infection was identified in kittens, with a few cases of *P. hominis* infections, but the case of coinfection with two organisms was none. Corresponding with the present results, the previous reports demonstrated that *P. hominis* and *T. foetus* infections were dominant for dogs and cats, respectively (Bastos et al., 2019; Grellet et al., 2013; Li et al., 2016a, 2019; Maritz et al., 2014). In addition, *P. hominis* was sometimes isolated from cats, but *T. foetus* infection was determined in cats, and it was markedly rare in dogs (Bastos et al., 2018; Gookin et al., 2007; Li et al., 2016a, 2019;). To our knowledge, in the last decade, there were no available investigations for trichomonad infections, targeting the pet shop puppies and kittens. However, there are several reports that represent the status in pet shop animals. The recent molecular prevalence of *P. hominis* from young dogs has been recorded as follows: 17.4% (19/109; breeding kennel dogs aged <8 weeks) in France (Grellet et al., 2013), 41.5% (49/118; police dogs and rural district dogs aged ≤6 months) in China (Li et al., 2016b), 12.5% (4/32; breeding kennel dogs aged ≤6 months) in Poland (Michalczyk et al., 2015) and 53.3% (24/45; household dogs aged ≤12 months) in China (Li et al., 2016a). In young cats, the following molecular prevalence of *T. foetus* was lately described: 26.3% (5/19; show cats aged <12 months) in Norway (Tysnes et al., 2011), 11.7% (7/60; household cats aged ≤12 months) in China (Li et al., 2019) and 33.3% (12/36; household cats aged ≤12 months) in Poland (Dąbrowska et al., 2020). Authors started the present investigation with a hypothesis that pet shop puppies and kittens showed a high-level prevalence for intestinal trichomonads. Because the higher prevalence of *P. hominis* and *T. foetus* was suggested in young dogs and cats, based on the high-density animals in limited space, suspected stressful situation, and their immature immune system (Bastos et al., 2019; Dąbrowska et al., 2020; Grellet et al., 2013; Li et al., 2016a, b; Tysnes et al., 2011; Yao & Köster, 2015). Additionally, the behavior of puppy, particularly the habit of biting and licking objects which has the potential of contamination with trichomonads is also likely a significant contribution factor for transmission. Similarly, the behavior of grooming, characteristic in cats, seems to associate with transmission through the physical contamination. The present study was carried out in puppies and kittens aged ≤3 months. Nevertheless, the overall prevalence was unexpectedly low. It is difficult to determine the exact reasons for the above contradicting results. However, several factors are suspected. Considering the investigated populations, a part of the results in previous studies were obtained from smaller size of examined numbers, which included subconsciously a higher risk for misunderstanding. The fecal condition is also probably one of the factors that influenced the results. The higher prevalence of trichomonad infections was recorded from the fecal samples of soft/diarrhea in dogs and cats (Dąbrowska et al., 2020; Li et al., 2016a). Although we could not obtain the detailed data from referring studies here, the fecal conditions of soft/diarrhea were of high percentage such as 13.8-41.1% in overall (Dąbrowska et al., 2020; Li et al., 2016a, 2019; Tysnes et al., 2011). In contrast, the percentages of the feces with soft and diarrhea in the present study were low such as 2.4% (13/544) and 2.2% (12/544) in puppies, and 4.4% (18/409) and 1.7% (7/409) in kittens, respectively. Another suspected factor is surely the environmental contamination. The infectious route of *P. hominis* and *T. foetus* is limited only via fecal-oral transmission (Bastos et al., 2019; Maritz et al., 2014; Yao & Köster, 2015). The intestinal trichomonads are shed into the environment at trophozoite stage and the several days' survival of *P. hominis* and *T. foetus* in feces, which means environmental contamination, has been suggested (Grellet et al., 2013; Meloni et al., 2011; Yao & Köster, 2015). Thus, it is supposed that the lower prevalence in the present study is due to satisfactory sanitary control in all pet shops. Of course, there was a difference among facilities, and a significant difference of *P. hominis* infection was observed between pet shops.

Regarding the fecal condition, there was no evidence that *P. hominis* and *T. foetus* infections always induced digestive tract irritation, because no significant differences were demonstrated here between normal feces and soft or diarrhea ones. In agreement with the present results, *P. hominis* infection was commonly presumed to be asymptomatic in dogs and cats (Maritz et al., 2014). However, it has been recently suggested that this organism has the potential for diarrhea in dogs, cats, and humans (Bastos et al., 2018; Kim et al., 2010; Meloni et al., 2011; Zhang et al., 2019). Especially, in humans under the suppressive immune system (e.g., children and adult patients with cancer), *P. hominis* has been implicated as a causative agent of diarrhea (Meloni et al., 2011; Zhang et al., 2019). In dogs and cats, the coinfection of other protozoa with *P. hominis* is also presumed to increase the incidence of

diarrhea (Grellet et al., 2013; Michalczyk et al., 2015). As to protozoan *T. foetus*, the pathogenicity has not been demonstrated in dogs (Li et al., 2016a). In contrast, it is well known that *T. foetus* is a complete pathogen and can cause persistent large-bowel diarrhea in cats (Bastos et al., 2019; Hora et al., 2017; Yao & Köster, 2015). However, the present study suggests that *T. foetus* is not always associated with the symptomatic infection in cats. Some previous studies have also reported asymptomatic feline cases of *T. foetus* infection (Li et al., 2019; Tysnes, et al., 2011). Similar to in dogs, other factors, such as coinfection, presumably induce the diarrhea in cats (Bastos et al., 2019; Yao & Köster, 2015).

In conclusion, the results of the present study suggest that the trichomonads parasitized in the intestinal tract are mainly *P. hominis* and *T. foetus* in pet shop puppies and kittens in Japan, respectively, with the low-level invasion. *P. hominis* is determined not only from puppies but also from kittens with a few cases. In contrast, the isolation of *T. foetus* is limited from kittens. The infections of *P. hominis* and *T. foetus* are not always associated with the clinical signs (soft or diarrhea feces). However, as the present results are obtained from limited regions and only a few pet shops, further investigations are necessary for credible evaluation.

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