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First molecular detection of *Tritrichomonas foetus* in domestic cats in Klang Valley, Malaysia

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Abstract. *Tritrichomonas foetus* is known to cause chronic diarrhea in the feline species in many different regions of the world. However, there is a paucity of information on *T. foetus* among cats in Malaysia. This study was conducted to determine the prevalence of *Tritrichomonas foetus* in the pet and stray cat population in Klang Valley, Malaysia. A total of 201 pet and stray cats' fecal samples were collected in Klang Valley. 24 samples were cultured in the InPouch® TF Feline to observe for motile trophozoites. A nested PCR protocol was used to screen for *T. foetus* in the collected samples. The prevalence of *T. foetus* in the cat population in Klang Valley was 33%. There was no association between *Tritrichomonas* infection and age, sex, breed or management of the cats. However, statistical analysis revealed that stray cats were more likely to be infected with *T. foetus* compared to pet cats. This study confirmed for the first time the presence of *T. foetus* among the cat population in Klang Valley, Malaysia.

INTRODUCTION

Tritrichomonas foetus is the causative agent of venereal disease in cattle (Tolbert & Gookin, 2016). In recent years, *Tritrichomonas foetus* (*T. foetus*) emerged as a gastrointestinal protozoa that causes chronic large bowel diarrhea in the feline species (Levy *et al.*, 2003). Studies revealed only 1% genetic difference between the isolates of *T. foetus* in cats and cattle (Šlapeta *et al.*, 2012). A more recent study done using a more comprehensive molecular sequence analysis suggested that the cat *Trichomonad* spp. isolates may be distinct with the suggested name *Tritrichomonas blagburni* but this has not yet been widely accepted (Walden *et al.*, 2013; Yao & Köster, 2015). Trophozoites of *T. foetus* may be detected via direct fecal smear or a wet mount under light microscopy (Manning, 2010). However, the motile trophozoites may be mistaken easily with *Giardia* spp. and *Pentatrichomonas hominis* (*P. hominis*) as the size of the trophozoite form of these species is similar (Manning, 2010). In addition, InPouch® TF Feline which was previously known to be highly specific for the culturing of *T. foetus* has been recently found to culture *P. hominis* as well (Ceplecha *et al.*, 2013). Polymerase chain reaction (PCR) is currently the most sensitive and specific tool in the detection of *T. foetus* in cats' fecal samples (Gookin *et al.*, 2002; Manning, 2010). *T. foetus* has a wide geographical distribution and is found in Europe, United States, Canada and Australia (Gookin *et al.*, 2004; Gunn-Moore *et al.*, 2007; Holliday *et al.*, 2009; Kuehner *et al.*, 2011; Tysnes *et al.*, 2011; Hosein *et*

al., 2013; Arranz-Solís *et al.*, 2016). In Asia, only few countries including Japan, Korea and Hong Kong have reported the detection of *Tritrichomonas foetus* in cats (Doi *et al.*, 2012; Köster *et al.*, 2015; Lim *et al.*, 2010). The prevalence of *T. foetus* in the cat population in Malaysia is currently unknown. Our study aimed to determine the prevalence of *T. foetus* in the pet and stray cat population in the Klang Valley and to identify the risk factors related to the infection.

MATERIALS AND METHOD

Sample collection

Sample collection was conducted according to the Code of Practice for the Care and Use of Animals for Scientific Purposes of Universiti Putra Malaysia under the approval of the Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia. Fecal samples were collected from 101 pet cats that were presented for various conditions including healthy cats to University Veterinary Hospital, Universiti Putra Malaysia and 100 stray cats from Dewan Bandaraya Kuala Lumpur using fecal swabs during January 2017 until May 2017. All pet cat samples were collected upon receiving consent from owners. Fecal samples collected were then placed in 700ul lysis buffer and kept under -20°C until further use. Another 24 fecal swabs were performed on 24 stray cats and inoculated into the InPouch® TF Feline to culture for *T. foetus*. The age, sex, breed and management of the cats were noted upon collection. Stray cats were aged according to the eruption, wear and tear of the teeth (The Humane Society of United States, 1996). Cats that had an owner were categorized as pet cats, whereas cats without owners were considered as strays.

InPouch® TF Feline (Biomed Diagnostics, Oregon, USA)

24 fecal samples that were inoculated into the pouch were incubated at 37°C for 24 hours. After a day of incubation, the InPouch® were observed under the light microscope at 100x magnification to identify motile trophozoites. If no motile trophozoites were observed, the InPouch® was kept in the dark at 18–25°C. The pouches were then checked for motile trophozoites every day until the fourth day of inoculation. Subsequently every other day until the 12th day to confirm a negative result.

Molecular analysis

DNA was extracted from the 201 fecal samples using QIAamp stool mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Extracted DNA was subjected to nested PCR to amplify *T. foetus* at a conserved portion of the internal transcribed spacer (ITS) region and the 5.8S rRNA gene. The first PCR included two primers TFR3 and TFR4 (Felleisen *et al.*, 1998), then followed by second PCR with the primers TFITS-F and TFITS-R (Gookin *et al.*, 2002). These primers were used to amplify a product size of 208bp. First amplification reaction mix consisted of 5µl of Promega 5x Green GoTaq®Flexi Buffer (Promega,Wisconsin, USA), 2mM $MgCl_2$, 0.16mM dNTP mix, 1.5U of Taq polymerase with 0.16pmol of both first set of primers and 5µl of DNA samples with a total volume of 25µl. 1µl of the first PCR product was then used in the second PCR amplification mix which included 5µl of Promega 5x Green GoTaq®Flexi Buffer, 2mM of $MgCl_2$, 0.16mM of dNTP mix, 2U of Taq polymerase and 0.5pmol of second set of primers with a total volume of 25µl. The amplification conditions were as shown in Table 1. PCR amplified products were then loaded on 2% agarose gel for gel electrophoresis. The gel was post-stained with ethidium bromide and viewed under Biorad Gel Doc™ XR (Biorad, Hercules, CA, United States). Positive samples were purified using Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany) and sent for sequencing for confirmation.

Statistical analysis

Samples were grouped into different risk factors which included age, sex, breed and management of the cats. Comparative analysis of each group was performed using *Chi*-square test and Fisher exact test. Statistical analysis was performed using

Table 1. Amplification conditions

1ST PCR			
Initial denaturation	:	94°C 5min	
Denaturation	:	94°C 30s	40×
Annealing	:	67°C 30s	
Extension	:	72°C 1min 30s	
Final extension	:	72°C 15min	
2ND PCR			
Initial denaturation	:	95°C 5min	
Denaturation	:	95°C 30s	55×
Annealing	:	62°C 30s	
Extension	:	72°C 30s	
Final extension	:	72°C 5min	

IBM SPSS Statistics 22. The differences were considered statistically significant when $P<0.05$.

RESULT

InPouch® TF Feline

Motile trophozoites were observed in 6 pouches out of the 24 samples cultured. All positive pouches were confirmed on the second day of inoculation. The remaining 18 InPouch® were confirmed to be negative after the 12th day of inoculation.

Prevalence of *Tritrichomonas foetus*

201 fecal samples (100 stray cats and 101 pet cats) were collected to screen for the presence of *T. foetus*. A total prevalence of 33% (67/201) was obtained for both pet and stray cats. A large number of stray cats sampled were positive for *T. foetus* with 53 out of the total 100 positive. A lower number of positive samples (14/101) were obtained from the pet cat population. 34 amplified products of approximately 208bp as shown in Figure 1 were extracted using QIAquick Gel Extraction Kit and were sent for sequencing. All sequenced data were compared with those in the GenBank database using BLAST (Basic Local Alignment Search Tool) and were identified to be *T. foetus*. Based on the PCR result, four out of the six samples that were cultured positive using the InPouch were confirmed *T. foetus*.

Statistical analysis of the risk factors

A total of 100 stray cats were sampled, 38 cats were 2 years old and below and 59 were 3 years old and above with 3 cats' age unknown. Half of the sampled stray cats were male cats and the other half were female. Whereas the majority (64/101) of the pet cats sampled were 2 years and below, 33 of them were 3 years old and above the age of the four remaining cats was unknown. 51 of the pet cats sampled were male and 49 of them were female with one of unknown sex. 86% (83/96) of the sampled pet cats

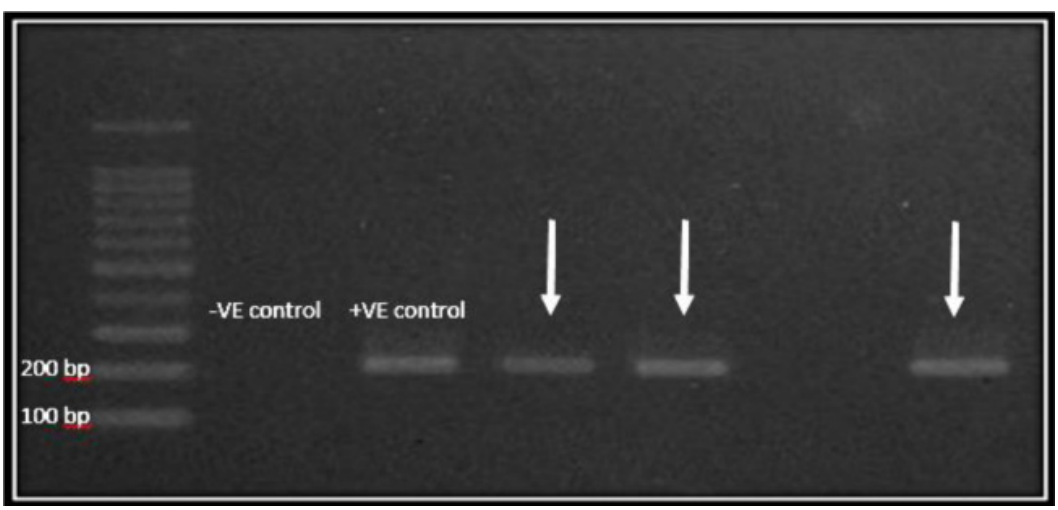

Figure 1. Result of nested PCR amplification products on 2% agarose gel. White arrows indicate the positive samples.

Table 2. Statistical analysis of risk factors using *Chi*-square test and fisher exact test

	Risk Factors	Odd Ratio (OR)	95% Confidence Interval	P value
Stray cats	Age (<2 years old / ≥ 3 years)	0.499	Lower : 0.218 Upper : 1.141	0.097
	Sex (Male/Female)	1.496	Lower : 0.679 Upper : 3.294	0.316
Pet cats	Age (<2 years old / ≥ 3 years)	1.852	Lower : 0.473 Upper : 7.523	0.533
	Sex (Male/Female)	0.683	Lower : 0.219 Upper : 2.137	0.511
	Breed (Pure/Domestic short hair)	2.500	Lower : 0.675 Upper : 9.265	0.160
	Multi/Single household	1.964	Lower : 0.466 Upper : 8.270	0.397
	Indoor/Outdoor	1.755	Lower : 0.507 Upper : 6.078	0.370

were from multi-cat households and 14% (13/96) of them were from single-cat households and for 5 the information was unavailable. 61% of the pet cats sampled were completely indoor cats and the other 39% had access to outdoors with 7 no information available. Only 16 pure breed cats were sampled and 5 of them were positive for *T. foetus*, the other 62 positive cats were all domestic short hair. Statistical analysis using *Chi*-square test and Fisher exact test showed no significant difference between age, sex, breed, single/multi cat household and management indoor/outdoor with infection status as listed in Table 2. Stray cats had demonstrated significantly higher prevalence compared to the pet cats (OR=7.008; 95% CI=3.523, 13.937; P=0.00), using *Chi*-square analysis.

DISCUSSION

This was the first molecular study on *T. foetus* in Malaysia and a prevalence of 33% in cats in the Klang Valley was obtained. The finding of this study agrees with many reported prevalence rates ranging between 30-39% in the United States, Spain and Italy (Holliday *et al.*, 2009; Polak *et al.*, 2014; Arranz-Solís *et al.*, 2016). Even though, most of the studies showed prevalence of above 10% for *T. foetus* in cats, a lower prevalence of 8.8% was reported in cats in Japan (Gunn-Moore *et al.*, 2007; Xenoulis *et al.*, 2010; Tysnes *et al.*, 2011; Doi *et al.*, 2012). Moreover, in a study in Canada, there were no *T. foetus* identified in feral and shelter cats sampled (Raab *et al.*, 2016). These findings suggested that *T. foetus* has a wide range of prevalence that differs with the geographical location. In our study, we expressed agreement where InPouch® TF Feline does not specifically culture *T. foetus*, as there were two pouches that were confirmed to have motile trophozoites under the microscope but were negative for *T. foetus* on nested-PCR. However, PCR was not performed to confirm if the cultured trophozoites were *P. hominis*.

When comparing the stray and pet cat populations separately, a glaring difference in *T. foetus* prevalence was noticed. More than half of the samples collected from the stray cats were positive for *T. foetus*. This could be due to higher risk for stray cats to be exposed to the *T. foetus* trophozoites from the environment. *T. foetus* trophozoites have been shown to survive in water, cats' urine and in the feces (Hale *et al.*, 2009; Rosypal *et al.*, 2012). The trophozoites can survive in the feces for up to 7 days which can act as source of transmission (Hale *et al.*, 2009). Statistical analysis in our study

showed that stray cats had a significantly higher risk for *T. foetus* infection than the pet cat population ($P < 0.05$).

Age of the cats showed no significant association with the positive status of *T. foetus* in cats for both stray and pet cats. Despite our finding in this study, younger age has been consistently reported by several studies to be a risk factor for *T. foetus* infection (Manning, 2010; Kuehner *et al.*, 2011; Köster *et al.*, 2015; Arranz-Solís *et al.*, 2016). However, older cats were also reported in a few studies to have higher risk for *T. foetus* infection (Holliday *et al.*, 2009; Xenoulis *et al.*, 2010). In our study, younger cats in the pet cat population demonstrated more risk for feline trichomoniasis (OR: 1.852), despite *Chi*-square test that showed no significant association ($P > 0.05$). In contrast, younger cats in the stray cat population were two times less likely to have *T. foetus* infection compared to the older cats (OR: 0.499). The contradiction of this finding between these two populations could be due to the living conditions of the sampled cats. Recurrent shedding of *T. foetus* has been reported in cats that are from a dense household and stressful environment (Foster *et al.*, 2004; Yao & Köster, 2015). All pet cats that were 3 years and older that were shedding *T. foetus* came from a multi-cat household. Due to the roaming lifestyles of the stray cats, the prevalence of *T. foetus* was probably higher compared to the pet cats. Similar to other studies, the sex of the cat does not show any significant association with feline trichomoniasis (Arranz-Solís *et al.*, 2016). Several studies had reported that multi-cat households have a higher risk for *T. foetus* infection (Gookin *et al.*, 2004; Hosein *et al.*, 2013; Arranz-Solís *et al.*, 2016). Even though in the current study most of the positive pet cats were from multi-cat households, however statistical analysis showed no significant difference between single-cat and multi-cat households. Cats from single-cat households that tested positive for *T. foetus* could have acquired the infection before they were brought into the household at an earlier point in their lives (Xenoulis *et al.*, 2010; Yao & Köster, 2015). In our study, breed of the cat also showed no association with the shedding of *T. foetus* trophozoite as in other studies (Manning, 2010; Doi *et al.*, 2012; Arranz-Solís *et al.*, 2016). This study was the first to detect *Tritrichomonas foetus* in cats in Malaysia. The prevalence of *T. foetus* in the cat population in Klang Valley was 33%. The high prevalence of *T. foetus* necessitates the inclusion of this protozoal infection as a differential diagnosis for chronic diarrhea in cats in Malaysia. Stray cats were significantly more at risk of *T. foetus* infection compared to the pet cats.

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