UNIVERSITI PUTRA MALAYSIA

DETECTION OF GASTROINTESTINAL PROTOZOA IN PET CATS PRESENTED TO SELECTED VETERINARY CLINICS IN THE KLANG VALLEY AND RISK FACTORS ASSOCIATED WITH INFECTION

TAN LI PING

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DETECTION OF GASTROINTESTINAL PROTOZOA IN PET CATS PRESENTED TO SELECTED VETERINARY CLINICS IN THE KLANG VALLEY AND RISK FACTORS ASSOCIATED WITH INFECTION

TAN LI PING

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DEGREE OF DOCTOR OF VETERINARY MEDICINE

Universiti Putra Malaysia

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MARCH 2016

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CERTIFICATION

It is hereby certified that I have read this paper entitled, “Detection of gastrointestinal protozoa in pet cats presented to selected veterinary clinics in the Klang Valley and risk factors associated with infection” by Tan Li Ping, and in my opinion it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course VPD 4999 –Project.

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ABSTRACT

DETECTION OF GASTROINTESTINAL PROTOZOA IN PET CATS PRESENTED TO SELECTED VETERINARY CLINICS IN THE KLANG VALLEY AND RISK FACTORS ASSOCIATED WITH INFECTION

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2016

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The common gastrointestinal protozoa in cats that cause diarrhea are *Giardia spp.*, *Isospora spp.* and *Cryptosporidium spp.*, and recently *Tritrichomonas foetus* has been recognized as an emerging protozoa that causes chronic diarrhea in cats. *Tritrichomonas foetus* infection in cats has not yet been reported in Malaysia. *Entamoeba spp.* is found

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rarely but present in cats. This study aimed to investigate the prevalence of gastrointestinal protozoa in pet cats presented to selected veterinary clinics in Klang Valley as well as the risk factors associated with these protozoal infections. Rectal swabs were performed on 30 diarrheic cats presented to selected veterinary clinics in the Klang Valley to culture *Tritrichomonas foetus*. Another 30 fecal samples were collected randomly and subjected to staining for the detection of other gastrointestinal protozoa. Two out of 30 culture samples were positive for *Tritrichomonas foetus* with a prevalence of 6.7% and both positive samples were from young kittens. *Cryptosporidium spp.* was the only protozoa detected in 3 out of 30 samples through the staining method with a prevalence of 10%. This study detected *Tritrichomonas foetus* for the first time in the Malaysian cat population. The overall prevalence of gastrointestinal protozoa in pet cats in the Klang Valley was low.

Keywords : Gastrointestinal protozoa, *Tritrichomonas foetus*, Cat, Culture, Staining

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ABSTRAK

PENGESANAN PROTOZOA GASTRUSUS DALAM KUCING PERLIHARAAN DIBAWA KE KLINK VETERINAR TERPILIH DI KLANG VALLEY DAN FAKTOR-FAKTOR RISIKO BERKAITAN DENGAN INFEKSI.

Oleh

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Dr. Puteri Azaziah Megat Abdul Rani

Protozoa gastrousus sepunya dalam kucing yang menyebabkan cirit-birit termasuk *Giardia spp.*, *Isospora spp.* and *Cryptosporidium spp.*, dan kebelakangan ini *Tritrichomonas foetus* telah dikenali sebagai protozoa yang menyebabkan cirit-birit kronik dalam kucing. Infeksi *Tritrichomonas foetus* dalam kucing belum dilaporkan di

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Malaysia. *Entamoeba spp.* jarang ditemui tetapi kadang-kadang muncul dalam kucing. Kajian ini bertujuan untuk menyiasat prevalens protozoa gastrousus dalam kucing perliharaan dibawa klinik veterinar terpilih dan juga faktor-faktor risiko berkaitan dengan infeksi protozoa. Pengesatan rektum dilakukan pada 30 kucing cirit-birit yang dibawa ke klinik veterinar terpilih di Klang Valley untuk kultur *Tritrichomonas foetus*. 30 sampel najis dikumpul secara rambang dan diwarnakan dengan teknik pewarnaan untuk mengesan protozoa gastrousus lain. Dua daripada 30 sampel kultur positif untuk *Tritrichomonas foetus* dengan prevalens 6.7% dan dua sampel positif tersebut adalah daripada anak kucing. *Cryptosporidium spp.* adalah satu-satu protozoa yang ditemui dalam 3 daripada 30 sampel dengan pengunaan teknik pewarnaan dengan prevalens 10%. Kajian ini telah mengesan *Tritrichomonas foetus* untuk kali pertama di populasi kucing di Malaysia. Prevalens kesuluruhan untuk protozoa gastrousus di kucing perliharaan di Klang Valley adalah rendah.

Kata kunci : protozoa gastrousus, *Tritrichomonas foetus*, kucing, kultur, pewarnaan

1.0 INTRODUCTION

Protozoans are unicellular organisms that belong to the kingdom Protista which is further divided into several phyla (Hendrix and Robinson, 2006). The four different phyla of veterinary importance includes the flagellated protozoans, amoeboid protozoans, apicomplexans and ciliated protozoans (Hendrix and Robinson, 2006). Protozoa that infect domestic animals may reside in the gastrointestinal tract, circulatory system, urogenital system and also in the respiratory system (Hendrix and Robinson, 2006). Every phyla of unicellular protozoans have their own lifecycle, reproduction stage as well as their feeding behavior. They are transmitted from one host species to another via different means. Generally, protozoans are transmitted via four different routes, including direct contact, exposure to resistant stages in the environment, ingestion and through vectors such as blood feeding arthropods (Bowman et al, 2002).

Some gastrointestinal protozoa are known to cause chronic diarrhea in the feline species. The gastrointestinal protozoa of pathogenic importance in cats include *Giardia spp.*, *Tritrichomonas foetus* and *Cryptosporidium spp.* which cause problems and shed more in younger cats or kittens (Zajac and Conboy,2012 ; Gookin et al,2001 ; Craven, 2010; Rambozzi, 2007). *Tritrichomans foetus* has recently garnered attention as an important aetiological agent of chronic diarrhea in cats in the United States and Eurrope. *Isospora spp.* however usually causes infections without obvious clinical signs, even though occasional mild diarrhea might be observed in very young and immunosuppressed cat (Craven, 2010).

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Toxoplasma gondii is a common coccidian protozoa in cats that may or may not cause clinical signs (DeFeo et al, 2002).

The host of these protozoa, cats, are primarily kept as companion animals. As more and more people keep these domesticated cats as pets which share the same household as humans, the frequency of contact between them increases. This leads to an increased risk of zoonotic disease transmission between the feline species and humans. These gastrointestinal protozoa are not just clinically important for cats but also carry a zoonotic risk to humans and subsequently pose a one health problem.

Despite the large population of stray and pet cats in Malaysia, not many studies have been carried out in Malaysia on gastrointestinal protozoa of cats. The most recent study was done by Ngui et al, 2014 in Malaysia on the gastrointestinal protozoa of cats in the stray population. Furthermore, there has been as of yet no studies on the detection and prevalence of *Tritrichomonas foetus* in cats in Malaysia. A lot of studies have shown that the prevalence of gastrointestinal protozoa is higher in younger animals, diarrheic cats as well as cats kept in multi-cat households (Barutzki and Schaper,2013 ; Labarthe, et al, 2008 ; Zajac and Conboy, 2012 ; Rambozzi, 2007). The paucity of available and current data on the gastrointestinal protozoa of pet cats in Malaysia and the lack of any studies on Tritrichomonas foetus in cats in Malaysia warranted this study.

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The objectives of this study were to:-

1. detect the gastrointestinal protozoa in pet cats presented to selected veterinary clinics in Klang Valley.
2. determine the prevalence of gastrointestinal protozoa in pet cats presented to selected veterinary clinics in Klang Valley.
3. investigate risk factors associated with infection with these protozoa.

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2.0 LITERATURE REVIEW

2.1 Common gastrointestinal protozoa in cats

Gastrointestinal protozoa that sit in the gastrointestinal tract of the feline species include *Giardia* sp., *Tritrichomonas sp.* (Scorza, et al., 2006), *Cryptosporidium spp., Toxoplasma gondii*, *Isospora felis* and *Isospora rivolta ,*and *Entamoeba histolytica.* (Zajac and Conboy, 2012). These feline protozoa are clinically important in cats and some of these protozoa are of zoonotic importance.

Crypstosporidium spp.

The primary species detected in the feline species is *Cryptosporidium felis* which resides in the small intestine (Zajac and Conboy, 2012). *Cryptosporidium parvum* hads also been detected in cats by Tyzzer during1912 (Tzipori and Widmer, 2008).These protozoans' lifecycle begins when the host ingests the sporulated oocyst (Scorza, et al., 2006). The minute oocysts sized around 4.0-4.5µm, consist of four sporozoites (Urquhart, et al., 1996). *Cryptosporidium spp*. usually cause clinical disease in immunosuppressed cats (Zajac and Conboy, 2012). When cats are infected with *Cryptosporidium parvum*, it may lead to diarrhea in some. *Cryptosporidium* has been known to be associated with an increase in severity of diarrhea and shedding of *Tritrichomonas foetus* trophozoites in cats with a coinfection (Gookin, et al., 2001). It has also been reported that *Cryptosporidium*

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spp. is zoonotic and can transmit to humans causing health issues especially in those that are immunosuppressed (Lucio-Forster, et al., 2010).

Giardia spp.

Giardia spp. are commonly found in the feces of dogs, cats, cattle, sheep, goats and other domestic and wild mammals. Cross transmission with humans is possible which makes it a zoonotic protozoan (Bowman, 2003). Giardia infections mostly cause inapparent disease but may cause severe enteritis in some humans (Bowman, 2003). In cases of the feline species, *Giardia spp.* infections are usually asymptomatic, however in some cases, it may cause acute, chronic or intermittent diarrhea, particularly in young cats (Zajac and Conboy, 2012). Transmission of this protozoa is through the fecal-oral route by ingestion of feces or contaminated water and food (Barr, 2002). *Giardia spp.* shed in the faeces in two morphologic forms, the rarely observed trophozoite form and the frequently observed form in the resistant cyst form (Hendrix and Robinson, 2006). The motile trophozoites are pear shaped with four pairs of flagella with prominent adhesive disc and two nuclei. The trophozoite stage size ranges from 9µm by 5-µm to 21µm by 15µm. The mature cysts of *Giardia spp.* are oval and are 8-10µm by 7-10µm. The cyst have a refractile wall and four nuclei. Immature cysts contain only two nuclei (Hendrix and Robinson, 2006).

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Tritrichomonas spp.

The agent for trichomoniasis in cats was previously known to be *Pentatrichomonas hominis*. However, it has recently been demonstrated by Levy et al. (2003) that the trichomonads detected from cats has a 99.7% - 100% sequence identity with *Tritrichomonas foetus* using rRNA gene sequencing; restrict enzyme digestion, and microscopy. It has also been proven by Stockdale et al, 2007 that the *T. foetus* feline isolate and the bovine isolate produce similar but not identical lesions in cats and heifers. Now, *Tritrichomonas foetus* is considered one of the protozoans that are infective to cats causing large bowel chronic diarrhea (Gookin, et al. , 2001) especially in young cats <1 year of age (Gunn-Moore, et al., 2007). Very little is known about the transmission of *T. foetus* (Zajac and Conboy, 2012). *T. foetus* is a pear shaped approximately 8 to 22µm long flagellate with one nucleus. This organism has three free anterior flagella and a recurrent one, forming a well-developed undulating membrane (Payne and Artzer, 2009). Trichomonads also do not produce a cyst stage (Payne and Artzer, 2009). There is a case of meningioencephalitis in an immunosuppressed human caused by *Trichomonas foetus* (Okamoto, et al. ,1998) and therefore the zoonotic potential of *Tritrichomonas foetus* cannot be diregarded.

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Toxoplasma gondii, Isospora felis, Isospora rivolta, Entamoeba histolytica

Toxoplasma gondii are shed in cat feces in the oocyst form. It causes ocular, respiratory and nervous signs in cats especially young or immunosuppressed animals (Zajac and Conboy, 2012*). Isospora felis* and *Isospora rivolta* are also shed in the oocyst form in the feces (Zajac and Conboy 2012). *Isospora spp,* usually affect kittens with clinical signs of diarrhea, abdominal pain, anorexia and weight loss. *Entamoeba histolytica* occurs in two morphologic forms which is the trophozoite and the cyst form (Hendrix and Robinson, 2006) *Entamoeba histolytica* are rarely seen in cats, however, it is believed to cross transmit to humans.

2.2 Prevalence and epidemiology of gastrointestinal protozoa in cats

The prevalence of *Cryptosporidium spp.* varies in different countries and in different studies. This wide range might be due to the different diagnostic methods used, ranging from direct microscopy to PCR. However, generally the prevalence of *Cryptosporidium spp* in cats ranges from 0% - 29.4%. In the United Kingdom, the prevalence was seen to be slightly low at 1% (Tzannes, 2007), and in another study done by Gow, et al (2009) *Cryptosporidium spp*. was not detected in cats. South Africa had a high prevalence rate of 32% (Samie, 2013) as well as Turin, Italy with a prevalence rate of 24.5%. The prevalence in Florida, USA was 12%, and slightly lower prevalence of 2.2% was reported in Australia (Rambozzi, 2007; Palmer, et al., 2008). In a recent study in Malaysia the prevalence of this protozoan

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infection in cats was around 7.1% (Ngui, et al., 2014). *Cryptosporidium spp.* most commonly infects younger cats and shedding is more in these cats as well (Rambozzi, 2007). The epidemiology of *Cryptosporidium spp.* depends on the location and the management of the cats. Rural areas have a higher prevalence rate compared to urban parts in South Africa, *Cryptosporidium spp.* was detected more in stray cats and also from places that are less hygienic (Samie, 2013). Cats managed outdoors were more likely to be infected with *Cryptosporidium spp.* (Rambozzi, et al, 2007).

The prevalence of giardiasis in cats depends on the population, area of study and also on the method of diagnosis, the prevalence was recorded in an average range of 5 - 15%. Even so a study in New Zealand recorded a high prevalence of 32% of giardiasis in cats (Kingsbury, et al., 2011). In Germany, the prevalence was 46% (Pallant, et al., 2015). Prevalence of *Giardia* spp. in cats in a recent study in Malaysia showed a prevalence of 10.7% and it was the most prevalent protozoan detected in cats (Ngui, et al, 2014) *Giardia* was able to be identified predominantly in cats with loose or watery diarrhea (Olson, et al, 2010). This is also supported by a study done in Florida which showed that the prevalence of *Giardia spp.* was 20% in cats having diarrhea and only 8% in cats without diarrhea (Sabshin, et al., 2012). Younger cats are known to be more susceptible to giardiasis supported by a few studies (Barutzki and Schaper,2013 ; Labarthe, et al, 2008).

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Tritrichomonas foetus is a protozoan recently found to be the common cause of chronic large bowel diarrhea in cats. There are now reports on *Tritrichomonas foetus* associated with chronic large bowel diarrhea in cats from the United States, United Kingdom, Europe, Canada, Australia but so far only two countries in Asia have reported *T. foetus* which are Japan and Korea. The prevalence rate ranged from 10 to 31%. The prevalence was seen to be higher in cattery populations compared to pet cats, as shown in studies in the United States (Stockdale et al, 2008) which revealed a 10% prevalence rate among pet cats compared to a prevalence of 31% of cats owned by 89 different breeders at an international cat show (Gookin et al, 2004). Studies in the United Kingdom also agreed where *T. foetus* infection was more prevalent in pedigree cats (Gunn-Moore et al, 2007). Cats that are less than 1 year old seem to be more susceptible to *T. foetus* (Gunn-Moore et al, 2007). *T. foetus* is believed to be shed more in cats with chronic diarrhea, in Australia, 77% of the cats detected with *T. foetus* had diarrhea (Bell et al, 2010). *T. foetus* trophozoites only survive in the environment for a short while 30 minute in water, 180 min in urine and do not survive in cat litter (Rosypal et al, 2011)

Isospora spp., Toxoplasma gondii and *Entamoeba spp*. are among the gastrointestinal protozoa in cats detected with slightly lower prevalence rates. *Isospora spp.* prevalence rates were 6.97%, 11.3%, 3% and 3.4% in Iraq, China, United Kingdom and Malaysia respectively (Al. Aredhi, 2015 ; Yang and Liang, 2015 ; Tzannes et al, 2007, ; Ngui et al, 2014). Seroprevalence of *Toxoplasma*

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gondii in cats in Malaysia was around 14.55% (Chandrawathani et al, 2008) and is considered to be quite high in comparison to other countries such as Italy with a prevalence rate of 7.5% and China with a prevalence rate of 2.78% (Mancianti et al, 2014 ; Yang and Liang, 2015). *Entamoeba spp.* is a protozoan not commonly detected in cats, however a recent study in Malaysia reported a prevalence of 10.7% in stray cats which is among the highest prevalence for protozoa in that study (Ngui et al, 2014).

2.3 Diagnostic method for gastrointestinal protozoa in cats

Direct Wet Mount and Fecal Floatation

This is the simplest technique for the laboratory diagnosis of intestinal protozoa. This method can detect the motile trophozoite stage of the protozoan species (Neimeister et al 1989), though it usually requires an experienced laboratory technician to be able to detect the protozoa (Koontz and Weinstock, 1996), especially when the protozoa might appear transparent. This is an economical and rapid screening method however due to the small volume of faeces utilized in this method it yields a low sensitivity. The differentiation of trophozoites using this method can be difficult as well (Dryden and Payne, 2010).

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Concentration technique

The concentration technique is used to increase the probability of detecting the protozoa trophozoites or cyst, to increase the sensitivity. In general there are two common concentration techniques that is zinc sulphate flotation technique and formol ether sedimentation technique (Truant et al, 1981). Fecal floatation is also used to recover cysts for observation in certain laboratories, however the sensitivity of this technique for detecting protozoa is generally lower than other diagnostic methods. The sensitivity to detect *Giardia* cysts is about 38.5% with a negative predictive value of 93.6% (Hoopes et al, 2013). The sensitivitiy of flotation technique to detect oocysts is around 50% with a negative predictive value of 98.8% (Hoopes et al, 2013). The formalin-ether technique has the advantage of less distortion and the ability to recover protozoa (Truant et al, 1981). Fecal floatation still remains a useful method to detect *Giardia spp.* together with other protozoa, however, trained technicians are required to be able to diagnose the infection together with good laboratory procedures (Mekaru et al, 2007). A study indicated that 3 consecutive zinc sulphate fecal flotations may be necessary to achieve 94% accuracy in a positive *Giardia* spp. diagnosis (Dryden et al, 2006). Cysts and oocysts such as *Cryptosporidium* spp and *Giardia* spp shed periodically which causes the limited reliability of a single fecal specimen (Mekaru et al, 2007). However, formalin-ether sedimentation confirmed an increase of detection efficiency of parasites in stool compared to other concentration methods used in various studies (Mergani, 2014) which makes the

concentration technique increase the sensitivity before staining (Kellogg and Elder, 1999).

Staining methods

Common staining methods used are Giemsa staining, Ziehl-Neelsen and Trichrome staining. Permanently stained smears of faecal samples, by trichrome provide a better means for the detection and diagnosis of parasitic infections as compared to the conventional wet mounts. Studies have proven that trichrome-stained smears are a very effective method for detecting parasitic infections (Shoaib et al, 2002; Agrawal et al,,2006). A study stained *Cryptosporidium spp*. and *Isospora spp*. using acid fast trichrome stain and were able to distinguish the protozoa (Ignatius et al, 1997). However, there are some studies on the sensitivity of Ziehl-Neelsen and Trichrome stain to stain *Cryptosporidium spp*. whereby acid-fast Trichrome stain method was only able to detect 42% of the *Cryptosporidium* cysts identified by Ziehl-Neelsen stain, further supporting that Ziehl-Neelsen has a greater sensitivity compared to other staining methods (Rigo and Franco, 2002) Studies also have compared the sensitivity of Ziehl-Neelsen to other diagnostic methods such as enzyme immunoassays and ELISA and showed no significant differences (Kehl et al, 1995 ; Ignatius, et al, 1997 ; Weitzel et al., 2006 ; Pacheco et al, 2013).

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InPouch® *Tritrichomonas* Feline culture

InPouch® TF Feline is one of the most sensitive diagnostic methods for *Tritrichomonas fetus* infection (BIOMED® Diagnostic method) Inpouch® are made of clear plastic containing a proprietary culture media and the contents in the pouch can be viewed under the microscope (Gookin et al, 2003). The culture media is selective for the growth of *Tritrichomonas foetus* while inhibiting the growth of yeast, mold and bacteria (BIOMED® Dianostic). InPouch® contains trypticase, protease, peptone, yeast extract, maltose, amino acids, salts, antifungal and antimicrobial agents in a normal saline phosphate buffer (BIOMED® Dianostic). Hale et al, 2009 stated the InPouch® had a sensitivity of 83% in culturing *Tritrichomonas foetus*. This InPouch® claims to inhibit the growth of *Pentatrichomonas hominis* and *Giardia spp.* (Gookin et al, 2003). Though there was one study that detected *Pentatrichomonas hominis* using InPouch (Ceplecha et al, 2013), it has been shown that *Pentatrichomonas hominis* infection does not lead to a misdiagnosis of *T. foetus* infection because *P. hominis* is always accompanied by *T. foetus* but not vice versa in cats (Gookin, 2007).

Enzyme Immunoassay

A SNAP® test kit for *Giardia spp* was produced. According to the product insert, the sensitivity and specificity of the SNAP test was 95% and 97% when compared to similar immunoassay tests produced by other manufacturers. In a study, Enzyme Immunoassay (ProSpecT Giardia Microplate Assay) was said to have the

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highest sensitivity of 91.2% and specificity of 99.4% compared to other enzyme immunoassay tests for detection of *Giardia spp*.(Mekaru et al, 2007). However, the disadvantage of this method is that it is cost and labor intensive (Mekaru et al, 2007) Also, false-negative results can occur as the antigen might not be evenly distributed in the fecal mass (Dryden and Payne,2010).

Polymerase Chain Reaction (PCR)

This technique has proven to have good specificity and good sensitivity to detect the specific protozoa up to the species level (Kuzehkanan et al, 2011 ; Gookin et al, 2002 ; Fotedar et al, 2007). PCR sensitivity can go up to as high as 100% in certain studies to detect protozoa as shown in Iran by Kuzehkanan et al. Nested PCR was able to detect all cases of *Cryptosporidium spp.* in the study (Kuzehkanan et al, 2011). This is also supported by a recent study that stated that by using multiplex PCR to detect *Giardia intestinalis* and *Cryptosporidium spp.* the sensitivity was 98% and 100% respectively (Laude et al, 2015). However, it has also been stated that single-tube nested PCR is less superior to diagnose feline *T. foetus* infection in comparison to fecal culture (Gookin et al, 2002). Fecal samples are considered to be one of the complex specimens for direct PCR sampling due to presence of heme, bilirubin, bile salts and others PCR inhibitors (Fotedar et al, 2007). Due to presence of these PCR inhibitors, immunomagnetic separation (IMS) technique and culture enrichment was used for *Cryptosporidium* and *Giardia spp.* prior to DNA extraction to remove inhibitory substances

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(Skotarczak ,2009) However, certain protozoal organisms have no IMS procedures or cannot be cultured (Skotarczak, 2009). Even with the superior specificity and sensitivity of PCR one of the major disadvantages of PCR is that it is expensive.

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3.0 MATERIALS AND METHOD

3.1 Sample population

Two different groups of cats were sampled during the 5 week sampling between 11/1/2016- 21/2/2016. The first group (Group 1) consisted of 30 cats with diarrhea presented to selected veterinary clinics in the Klang Valley.

The second group of samples (Group 2) consisted of 30 fresh fecal samples from random cats from selected clinics in the Klang Valley.

Consent was obtained from all the cat owners prior to sampling.

3.2 Sample collection

Rectal swabs were performed on the presented diarrheic cats using a sterile swab. The sterile swab was placed gently into the cat's anus and into the rectum and the swab was rotated gently to obtain a fecal sample.

The fecal samples for group 2 were collected when they were still fresh. Samples had to be processed as soon as possible within one day and were kept between 4-10°C if they were not processed immediately.

3.3 Sample processing

The first group of samples were inoculated into TF-InPouch® culture within 15 minutes after the rectal swabs were performed. Then, the culture pouch was incubated at 37°C for 24hours.

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The second group of fecal samples were collected and direct wet mount was performed to observe for any motile protozoa under the light microscope. One gram of fresh feces was then concentrated using the formol-ether sedimentation technique (Appendix 1). After performing the concentration technique, the sediments were smeared on glass slides and the glass slides were fixed with methanol for 5 minutes. The slides were then stained using permanent staining methods to increase the sensitivity to detect gastrointestinal protozoa. Three staining techniques were performed for each sample, trichrome staining for *Entamoeba spp., Giardia spp., Isospora spp.* and *Cryptosporidium spp.,* Ziehl-Neelsen stain for *Cryptosporidium spp.* and Giemsa stain to detect oocyst of *Toxoplasma spp.*, *Giardia spp., Isospora spp*., and *Entamoeba spp*.

3.4 Interpretation of results

The first group of diarrhea samples that had been cultured in the TF-InPouch® were removed from the 37°C incubator after 24 hours and were observed under the light microscope at 100x magnification. The result was considered positive when motile trophozoites of *Tritrichomonas foetus* were visible. 400x magnification was used to further confirm the motile trophozoites. If no motile trichromonads were observed, the InPouch® were then placed in room temperature between 18-25°C in the dark. The pouches were observed every day until the fourth day of inoculation and then were observed every other day until the 12th day to confirm a negative result.

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For the second group of samples, after staining, the slides were observed under light microscope 1000x magnification with emulsion oil to detect for the cyst of *Entamoeba spp.,* cyst or the trophozoites of *Giardia spp.,* oocyst of *Isospora spp,* oocyst of *Cryptosporidium spp.* and *Toxoplasma spp.* Detection of these gastrointestinal protozoa were considered positive when at least one cyst, trophozoite or oocyst was found.

3.5 Data analysis

The data collected from both sampling groups were recorded and tabulated into Microsoft Excel spreadsheets. The percentage of positive samples among the total sample size for both groups were computed. The prevalence of the different protozoa among pet cats presented in selected veterinary clinics was determined according the following formula :

$$Prevalence : \frac{Number\ of\ positive\ for\ specific\ protozoa}{Total\ number\ of\ samples\ collected} \times 100$$

The two sample groups' information were analyzed by grouping them into age, breed, management and sex.

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4.0 RESULTS

4.1 Study population

For the first sampling group, among the 30 diarrheic cats presented to selected veterinary clinics in Klang Valley, 22/30 (73%) cats were from multi-cat households. Five cats were the only cat at home and 3 more cats only had information that the cats were kept entirely indoor as shown in Table 1. The number of cats per household was reported for 19 cases; the median number of cats per household was 5 (range from 1-43). Seventeen cats (56%) were male and 13 cats (44%) were female. The median age for the sample group was 5.5 months ranging from 1 month old to 16 years old. Twenty one (70%) of the cats were Domestic Short Hair , 4 (13%) were Persian and the remaining were Persian cross, Maine coon, American Short Hair, E. Burnexx and Domestic Long Hair (one each).

For the second sampling group, not much information was available on the management of the 30 cats as shown in Table 2. Information was only available for 12 cats; that included 4 cats from multi-cat households, 1 was the only cat, 6 cats were exclusively indoor, 1 semi-roamer and 1 was a stray cat found that was brought to one of the clinics. Six (20%) cats were less than one year old and the remaining were all adult cats. Twelve (40%) of the sampled cats were male and 18 (60%) were female. Twenty four out of 30 cats were Domestic Short Hair, 2 Domestic Long Hair, 2 Persian and 2 Persian cross. 22 (73%) of the cats had

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normal fecal consistency, 5 (17%) had soft and pasty stool consistency and 3 (10%) had watery diarrhea.

4.2 *Tritrichomonas foetus* culture

Among the 30 samples from Group 1 for which InPouch® was used, only 2 were positive where motile trichomonads were clearly visible under light microscopic examination (100X magnification)(Figure 1). The prevalence of *Tritrichomonas foetus* from the selected veterinary clinics was 6%. Both positive samples were able to be confirmed after 24 hours incubation at 37°C. The positive cultures were smeared on slides and stained with Giemsa and revealed that each trichomonad possessed 3 anterior flagella and 1 posterior flagellum and an axostyle(Figure 2). The trichomonads had sizes ranging from 7µm to 13µm in length and from 3µm to 7µm in width.

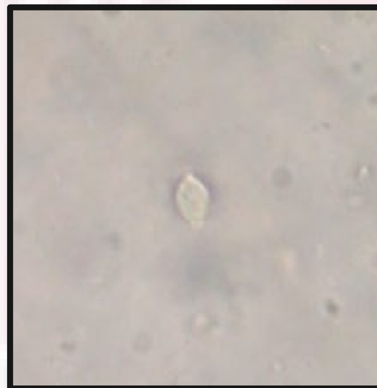

FIGURE 1 : *Tritrichomonas foetus* observed under the light microscope in the InPouch (400x)

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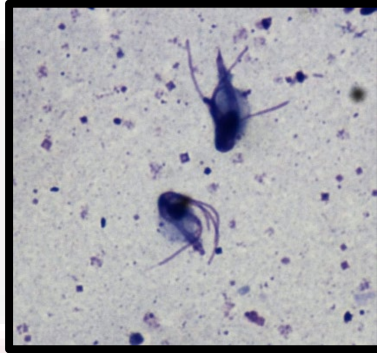

FIGURE 2 : *Tritrichomonas foetus* stained with Giemsa stain

4.3 Overall prevalence of gastrointestinal protozoal infections in pet cats

For the 30 samples that were collected from pet cats (Group 2) presented to selected veterinary clinics, *Giardia spp.* ,*Isospora spp.*, and *Entamoeba spp.* were not detected in any of the cat fecal samples through the staining methods used.

However, *Cryptosporidium spp*. was found in 3 out of 30 cats using Trichrome stain with the prevalence of 10% (Figure 3).

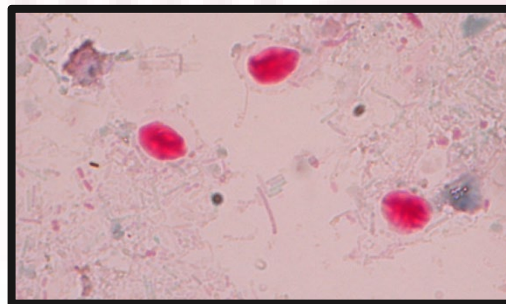

FIGURE 3 : *Cryptospodirium spp.* stained with Trichrome stain

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NO	AGE	SEX	BREED	MANAGEMENT	RESULT
1	1month	M	DSH	Multi cat household	Negative
2	1 month	F	DSH	Multi cat household	Negative
3	1 month	M	DSH	Multi cat household	Negative
4	7 years	F	Persian	Multi cat household	Negative
5	7 years	M	Persian	Multi cat household	Negative
6	8 years	M	DSH	Only cat	Negative
7	4 months	F	DSH	Indoor	Negative
8	4 months	M	DSH	Only cat	Negative
9	7 years	CM	Persian cross	Indoor	Negative
10	6 months	M	DSH	Multi cat household	Positive
11	2 months	F	DSH	Multi cat household	Negative
12	7 months	F	Maine coon	Multi cat household	Negative
13	Adult	F	DSH	Only cat	Negative
14	3 months	M	DSH	Multi cat household	Negative
15	Adult	F	DSH	Multi cat household	Negative
16	6 months	M	DSH	Multi cat household	Negative
17	4 months	M	DSH	Multi cat household	Negative
18	5 months	M	E. burnexx	Multi cat household	Positive
19	16 years	M	DSH	Multi cat household	Negative
20	3 months	F	DSH	Multi cat household	Negative
21	1 year old	M	DSH	Multi cat household	Negative
22	6 months	F	DSH	Multi cat household	Negative
23	2years	F	Persian	Indoor	Negative
24	4 months	M	DSH	Multi cat household	Negative
25	4 months	F	DSH	Multi cat household	Negative
26	4 months	M	American Short Hair	Multi cat household	Negative
27	6 months	M	Persian	Only cat	Negative
28	6years	M	DLH	Only cat	Negative
29	2 months	F	DSH	Multi cat household	Negative
30	2month	F	DSH	Multi cat household	Negative

TABLE 1 : Tabulated information of the 30 cats (Group 1) sampled for *Tritrichomonas foetus* culture

NO	AGE	SEX	BREED	MANAGEMENT	STOOL CONSISTENCY	RESULT
1	4 months	F	DSH	Indoor	Watery diarrhea	Negative
2	4 months	M	DSH	Indoor	Watery diarrhea	Negative
3	3 years	F	DSH	-	Normal	Negative
4	1 year	M	DSH	Caged indoor	Normal	Negative
5	3-4months	F	DSH	-	Normal	Negative
6	3 years	SF	DSH	-	Normal	Negative
7	Adult	M	DSH	Only cat	Soft stool	Negative
8	6 months	M	DSH	Multi cat household	Soft stool	Negative
9	16yo	M	DSH	Multi cat household	Watery diarrhea	Negative
10	>1yr old	M	DSH	Indoor cat	Normal	Negative
11	1 year	CM	DSH	Semi-roamer	Normal	Negative
12	10year	SF	DSH	-	Normal	Negative
13	>3years	F	DSH	Multi cat household	Normal	Negative
14	2years	F	DSH	Multi cat household	Normal	Negative
15	6 months	F	Persian	Caged Indoor	Soft pasty stool	Positive
16	2 months	F	DLH	Indoor	Soft pasty stool	Negative
17	Adult	F	DSH	-	Normal	Negative
18	Adult	F	DSH	-	Normal	Negative
19	Adult	M	DSH	-	Normal	Negative
20	1 year	F	DSH	-	Normal	Negative
21	Adult	M	DSH	-	Normal	Positive
22	4 years	F	Persian cross	-	Normal	Negative
23	10years	F	DSH	-	Normal	Negative
24	Adult	M	DSH	-	Normal	Negative
25	Adult	F	DSH	-	Normal	Positive
26	Adult	M	DSH	-	Normal	Negative
27	Adult	F	Persian	-	Normal	Negative
28	Adult	F	Persian cross	-	Normal	Negative
29	3years	M	DSH	-	Normal	Negative
30	6 years	SF	DLH	-	Normal	Negative

TABLE 2 : Tabulated information of the 30 cats (Group 2) sampled for gastrointestinal protozoa through staining techniques

4.4 Demographics of the positive cats

By grouping according to age developed by the Feline Advisory Bureau, both cats positive for *Tritrichomonas foetus* were from the age group less than 6 months old (Table 3). The three positive cats for *Cryptosporidium spp.* included 2 adult cats and 1 kitten less than 6 months old (Table 4).

Age	Positive Results *(Tritrichomonas foetus)*
<6 months	2/19
7months – 2 years	0/4
3 – 6 years	0/2
7 – 10 years	0/4
11 -14 years	0/0
15 years	0/1

Table 3 : Group 1 Sample by Age

Age	Positive Results *(Cryptosporidium spp.)*
<6 months	1/6
7months – 2 years	0/5
3 – 6 years	0/6
7 – 10 years	0/2
11 -14 years	0/0
15 years	0/1
Adult	2/10

Table 4 : Group 2 Sample by Age

By grouping according to management, *Tritrichomonas foetus* positive cats were both from multi-cat households and for the *Crypotosporidium spp*. positive cat the management was unknown (Table 5 and Table 6).

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Management	Positive Results *(Tritrichomonas foetus)*
Multi-cat household	2/22
Only cat	0/5
Unknown	0/3

Table 5 : Group 1 Sample by Management

Management	Positive Results *(Cryptosporidium spp.)*
Multi-cat household	0/4
Only cat	0/1
Unknown	3/25

Table 6 : Group 2 Sample by Management

When the samples were grouped according to breed and of the Group 1 samples, one Domestic Short Hair cat and one Pure bred cat were positive for *Tritrichomonas foetus* (Table 7). The *Cryptosporidium spp.* positive cats included one Domestic Short Hair, one Domestic Long Hair and a pure breed cat. (Table 8)

Breed	Positive Results *(Tritrichomonas foetus)*
DSH (Domestic Short Hair)	1/23
Pure breed	1/7

Table 7 : Group 1 Sample by Breed

Breed	Positive Results *(Cryptosporidium spp.)*
DSH (Domestic Short Hair)	1/24
DLH (Domestic Long Hair)	1/2
Pure breed	1/4

Table 8 : Group 2 Sample by Breed

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The two *Tritrichomonas foetus* positive cats were male cats and for *Cryptosporidium spp*. two were females and one was a male cat. (Table 9 and Table 10)

Gender	Positive Results *(Tritrichomonas foetus)*
Male	2/17
Female	0/13

Table 9 : Group 1 Sample by Gender

Gender	Positive Results *(Cryptosporidium spp.)*
Male	1/12
Female	2/18

Table 10: Group 2 Sample by Gender

When the group 2 samples were grouped according to fecal consistency it revealed that both of the *Cryptosporidium spp*. positive cats had a normal fecal consistency and one had soft and pasty feces as shown in Table 11.

Fecal Consistency	Positive Results *(Cryptosporidium spp.)*
Normal	2/22
Soft & Pasty	1/5
Watery Diarrhea	0/3

Table 11: Group 2 Sample by Fecal Consistency

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5.0 DISCUSSION

This study was able to detect and demonstrate *Tritrichomonas foetus* infection in 2 out of 30 cats presented to selected veterinary clinics in Klang Valley with the complaint of diarrhea. To the best of our knowledge this is the first confirmed case of *Tritrichomonas foetus* infection in the feline species in Malaysia and also Southeast Asia. Previously, only Japan and South Korea reported the occurrence of *Tritrichomonas foetus* in Asia (DOI et al, 2011; Lim et al, 2010). The prevalence of *Tritrichomonas foetus* in this study was 6.7%. The prevalence is relatively lower compared to that reported in the United States and in some European countries which ranged from 10 to 31% (Gookin et al, 2004; Frey et al, 2008; Kuehner et al, 2011; DOI et al, 2011; Gunn-Moore et al, 2007; Stockdale et al,2008). However, a study in Japan demonstrated a prevalence of 8.8% of *Tritrichomonas foetus* infection, also lower than other reported studies (DOI et al, 2011). The low prevalence rate in this study in comparison to studies in other countries may be due to the smaller sample size. This study sampled only 30 cats with diarrhea which may be the reason for yielding lower positive results. The prevalence of *Tritrichomonas foetus* was lower in pet cats compared to cats from catteries showed in the United States and Germany (Gookin et al, 2004; Stockdale et al, 2008; Kuehner et al, 2010) and this study only focused on pet cats which may have also contributed to the low prevalence. Two cats that tested positive for *Tritrichomonas foetus* in this study were aged 5 months and 6 months old respectively. This finding was consistent with several studies that cats aged

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less than 1 year of age were more susceptible to *Tritrichomonas foetus* infection (Yao and Koster, 2015). An association between age of cats and development of clinical signs has also been found whereby younger cats infected with *Tritrichomonas foetus* will show clinical signs of diarrhea (Tysnes et al, 2011) which may relate to the positive culture from young cats in this study.

For the second sampling group, *Giardia spp.*, *Isospora spp.*, and *Entamoeba spp.* were not detected in any fecal samples collected through staining techniques. Only *Cryptosporidium spp.* was detected in three fecal samples collected. Three of the samples were identified using Trichome stain, however it was not identified using Ziehl-Neelsen stain possibly due to the difference in staining technique. This finding contrasts with the statement by Rigo and Franco, 2002 that Ziehl-Neelsen had a greater sensitivity compared to other staining methods for the detection of gastrointestinal protozoa. The prevalence of *Cryptosporidium spp.* in the selected veterinary clinics was 10% (3/30) which is higher than the previously reported prevalence by Ngui et al, 2014 of 7.1%. *Cryptosporidium spp.* oocyst are usually shed in cats with normal stool consistency, however it may cause diarrhea in younger cats (Scorza and Tangtrongsup, 2010). This statement could be correlated to our findings where only the younger cat with cryptosporidiosis had pasty stool, however the adult cats with cryptosporidiosis had normal stool.

Based on previous studies, *Giardia spp.* were the most common gastrointestinal protozoa detected in cats with the highest prevalence among other protozoa

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species (Tzannes et al, 2008; Barutzki and Schaper, 2011; Ngui et al, 2014). This was not the case in our study and may be because of the small sample size and due to the age of the cats in our study. *Giardia spp.* shed more in younger cats whereby in our study group only 20% of the cats were less than one year of age. In the studies in other countries including United Kingdom, Florida and Iran, *Isospora spp.* were found more frequently in cats than *Cryptosporidium spp.* in pet cats (Tzannes et al, 2008; Sabshin et al,2013; Khademvatan et al,2014), however in this study *Isospora spp.* was not identified in any of the cats, but three cats were positive for *Cryptosporidium spp.*. Similar to the study done by Ngui et al, 2014, *Cryptosporidium spp.* were detected more frequently in comparison to *Isospora spp.* in rural areas in Selangor and Pahang states. This could lead us to suggest that possibly there is an actual higher prevalence of *Cryptosporidium spp.* compared to *Isospora spp.* among the cat population in Malaysia.

Even though *Giardia spp.* are usually the most prevalent protozoa in cats, *Giardia spp.* was not detected in any of the samples in this study possibly due to the small sample size and also the age of the cats. Once again among the 30 cats sampled, only 20% were cats younger than 1 year. This protozoa is shed more in younger cats from 3 to 6 months of age (Barutzki and Schaper, 2011) which could possibly explain the inability to detect *Giardia spp.* in this study. Moreover, Trichrome staining and Giemsa staining methods have lower sensitivity in detection of *Giardia spp.* compared to other diagnostic methods such as enzyme immunoassay and PCR (McGlade et al, 2002; Mekaru et al, 2007).

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Entamoeba histolytica was not detected in any of the cats consistent with previous studies where *Entamoeba spp.* was rarely observed and only sporadically with a low prevalence in cats as humans are the host for this protozoa (Shaw and Ihle, 2006; Gracenea et al., 2009 ; Merck's Veterinary Manual,2011). In 2014, Ngui et al found *Entamoeba spp.* with a prevalence of 10.7% which makes it the highest prevalence amongst the other protozoa in stray cats. As this protozoa originates from the human host, the pet cats sampled in this study may have a lower prevalence compared to the stray population due to improved hygienic conditions of the environment the cats live in.

Toxoplasma gondii oocyst was not detected in this study, as these oocyst only shed for a short period of time when the cat first gets infected (Hartmann et al,2013), thus *Toxoplasma gondii* oocyst are usually difficult to be detected through fecal samples. In 2007 in California, Dabritz et al. collected fecal samples to detect the prevalence of *Toxoplasma gondii* and only 0.9% were detected. In the same year, Dabritz et al. performed serologic testing and 26% of cats were positive for IgM and IgG for *Toxoplasma gondii.* Previous studies in Malaysia showed a prevalence of 14.5% *Toxoplasma gondii* in pet cats in Malaysia (Chandrawathani et al, 2008), Chandrawathani et al used indirect fluorescent antibody test (IFAT) to detect the seroprevalence of this protozoa in cats. Serological detection techniques would naturally reveal higher prevalence rates as it detects past infection or exposure as well as active infections. In our study

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we only performed fecal staining to detect *Toxoplasma gondii* and were unable to detect any *Toxoplasma gondii* oocyst.

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6.0 CONCLUSION

Tritrichomonas foetus was detected in pet cats in Malaysia for the first time. Clinicians should be aware of this protozoa as a possible differential diagnosis for cats with chronic diarrhea. The prevalence of *Tritrichomonas foetus* was 6.7% and *Cryptosporidium spp.* was 10% in pet cats presented to selected veterinary clinics in Klang Valley. Having detected these potentially zoonotic protozoa, it warrants further investigation with the ultimate aim to control and prevent these infections.

7.0 RECOMMENDATIONS

For future studies:-

1. Increasing the sample size would be preferable for more reliable data.
2. Expanding the location of sample collection for a better picture of prevalence rates of gastrointestinal protozoa in the whole of Malaysia.
3. Sampling at catteries as well as the pet cat population to compare the prevalence of *Tritrichomonas foetus* for both populations in Malaysia.

   Performing Polymerase Chain Reaction (PCR) to detect gastrointestinal protozoa due to its higher sensitivity and the ability to detect up to the species level.

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APPENDICES

APPENDIX 1

Formol-ether sedimentation technique

1. Place 1 gram of faeces into the white tube.
2. Add 10ml of 4% formol saline, then add 2ml of diethyl ether.
3. Mix the solution thoroughly until the fecal sample has broken down properly.
4. Replace green cap of the tube with an emulsification cap with the long straw facing outwards.
5. Cap the 15ml falcon tube onto the other end.
6. Turn the tube upside down.
7. Once all the feces filters into the falcon tube, centrifuge the falcon tube at 2000rpm for 5 minutes.
8. Remove the top layer until 0.5ml is left in the falcon tube.
9. Emulsify with pipette and smear a few drops onto slides.

(As per method used at Laboratory of Veterinary Parasitology, Faculty of Veterinary Medicine, UPM)

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