UNIVERSITI PUTRA MALAYSIA

PREVALENCE OF ZOONOTIC ENTERIC PROTOZOA (*GIARDIA, BLASTOCYSTIS* AND *CRYPTOSPORIDIUM*) AMONG SHELTER CATS IN SELANGOR, PENINSULAR MALAYSIA

LIM MEI YI

PREVALENCE OF ZOONOTIC ENTERIC PROTOZOA (*GIARDIA*, *BLASTOCYSTIS* AND *CRYPTOSPORIDIUM*) AMONG SHELTER CATS IN SELANGOR, PENINSULAR MALAYSIA

LIM MEI YI

A project paper submitted to the
Faculty of Veterinary Medicine, Universiti Putra Malaysia
In partial fulfilment of the requirement for the
DEGREE OF DOCTOR OF VETERINARY MEDICINE
Universiti Putra Malaysia
Serdang, Selangor Darul Ehsan.

MARCH 2017

It is hereby certified that we have read this project paper entitled "Prevalence of zoonotic enteric protozoa (*Giardia, Blastocystis and Cryptosporidium*) among shelter cats in Selangor, Peninsular Malaysia", by Lim Mei Yi and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 – Final Year Project.

_____

DR. REUBEN SUNIL KUMAR SHARMA
DVM (UPM), MVSc (UPM), PHD (Cambridge), MRSB, CBiol.
Senior Lecturer,
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Supervisor)

_____

DR. GAYATHRI THEVI SELVARAJAH
DVM (UPM), PHD (Netherlands),
Senior Lecturer,
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Co-supervisor)

DEDICATIONS

This work is dedicated to my parents, who passed on a love of reading and respect for education, and to my friends, lecturers and coursemates of DVM 2017 who have made me who I am today.

ACKNOWLEDGEMENTS

Special thanks to those who have contributed time, efforts and aid to the completion of this project paper:

Dr. Reuben Sunil Kumar Sharma

Dr. Gayathri Thevi Selvarajah

Staff of Parasitology Lab, Faculty of Veterinary Medicine, UPM

PAWS Animal Shelter, Selangor

SPCA Animal Shelter, Selangor

Furry Friends Farm, Selangor

Mun Mun Furkid Haven, Selangor

Tan You Ken

Stephanie Magdalene

Ruviniyia Kalaiselvam

Daniel Mohan Jacob

Sujey Kumar

Foong Jia Ning

DVM Class of 2017

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

°C	Degree Celsius
× *g*	Relative centrifugal force
%	Percentage
µm	Micrometer
µL	Microliter
bp	Base pair
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
g	Gram
gp60	60 kDa glycoprotein
IACUC	Institutional Animal Care and Use Committee
kDa	Kilodalton
mg/kg	Milligram per kilogram
mL	Milliliter
PCR	Polymerase Chain Reaction
pH	Potential Hydrogen
SDS	Sodium dodecyl sulfate
SPSS	Statistical Package for the Social Sciences
SSU	Small Subunit Ribosomal RNA
tris	Trisaminomethane
USA	United States of America
V	Volt

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek Ilmiah Tahun Akhir

Prevalens protozoa enterik zoonotic (*Giardia, Blastocystis* dan *Cryptosporidium*) dalam kalangan kucing dari pusat perlindungan haiwan Selangor, Semenanjung Malaysia

oleh

Lim Mei Yi

2017

Penyelia: Dr. Dr. Reuben Sunil Kumar Sharma

Penyelia bersama: Dr. Gayathri Thevi Selvarajah

Species protozoa enterik seperti *Giardia, Cryptosporidium* dan *Blastocystis* dianggap mempunyai potensi zoonotik. Kucing biasanya dikaitkan dengan persekitaran yang ditinggal manusia. Namun, maklumat yang diterbit tentang epidemiologi protozoa enterik tersebut dalam kalangan kucing dibela dan terbiar di negara ini amat kurang sekali. Maka, kajian ini dijalankan untuk menentukan prevalens protozoa enterik zoonotik (*Giardia, Blastocystis* dan *Cryptosporidium*) dalam kalangan kucing dari pusat perlindungan haiwan

Selangor, Semenanjung Malaysia. Kapas kesat tinja telah dikumpul dari 105 kucing yang dipilih secara rawak dari pusat perlindungan haiwan terpilih. Saput tinja disediakan dan diwarnakan dengan "Giemsa" dan "Ziehl-Neelsen" ubah suai untuk pemeriksaan mikroskopi. Cerakin reaksi rantai polymerase (PCR) telah dijalankan dengan menggunakan primer genus tertentu untuk mengamplifikasi gen "Small Subunit Ribosomal RNA (SSU)" protozoa. Tiada protozoa enterik zoonotik yang dijumpai melalui pemeriksaan mikroskopi konvensional. Pengesanan melalui PCR menunjukkan bahawa 37.5% (15/40) dan 25% (10/40) daripada sampel adalah positif untuk *Giardia* dan *Blastocystis* masing-masing. *Cryptosporidium* tidak dikesan dalam semua sampel melalui dua teknik. Kehadiran protozoa enterik zoonotik ini tidak dikaitkan dengan umur atau ketekalan tinja. Prevalens *Giardia* dan *Blastocystis* yang tinggi dalam kalangan kucing dari pusat perlindungan haiwan dalam Malaysia wajar mendapat penyelidikan lanjutan dari segi epidemiologi dan faktor risiko jangkitan.

Kata kunci: *Giardia, Cryptosporidium, Blastocystis,PCR, zoonotik, kucing*

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4999 – Final Year Project

Prevalence of zoonotic enteric protozoa (*Giardia, Blastocystis* and *Cryptosporidium*) among shelter cats in Selangor, Peninsular Malaysia

by

LIM MEI YI

2017

Supervisor: Dr. Reuben Sunil Kumar Sharma

Co-supervisor: Dr. Gayathri Thevi Selvarajah

Enteric protozoan species like *Giardia, Cryptosporidium* and *Blastocystis* in cats are considered to have zoonotic potential. In Malaysia, cats are commonly associated with human living environments. However, there remains a paucity of published information about the epidemiology of these enteric protozoa among both owned and stray cats in the country. This study was carried out to determine the prevalence of zoonotic enteric protozoa among cats kept in animal shelters in Selangor, and to ascertain if the infection was related to age and fecal consistency. Fecal swabs of 105 cats were collected opportunistically from chosen animal shelters. Fecal smears were prepared and stained with Giemsa and Modified Ziehl-Neelsen for microscopy examination. Polymerase chain reaction (PCR) using genus-specific primers was carried out on 40 samples to amplify the

Small Subunit Ribosomal RNA (SSU) gene of the protozoa. No zoonotic enteric protozoa were detected in all fecal samples using conventional microscopy. PCR detection revealed that 37.5% (15/40) and 25% (10/40) of the samples were positive for *Giardia* and *Blastocystis*, respectively. *Cryptosporidium* was not detected in all the samples examined by both techniques. The presence of these zoonotic enteric protozoa was neither associated with age nor fecal consistency. The high prevalence of *Giardia* and *Blastocystis* among shelter cats in the country merits further investigation on the epidemiology and risk factors for infection.

Keywords: *Giardia, Cryptosporidium, Blastocystis, PCR, zoonotic, cats*

INTRODUCTION

Gastrointestinal protozoa in cats including *Giardia, Cryptosporidium* and *Blastocystis* are zoonotic and major causes of diarrhoeal diseases worldwide through foodborne or waterborne transmission (Thompson & Smith, 2011). *Giardia* is a flagellate diplomonad from family Hexamitidae which cause chronic intestinal infection characterized by continual or intermittent diarrhoea or steatorrhoea. The most representative species of this genus is *G. duodenalis* (also known as *G. intestinalis* and *G. lamblia*), a species complex infecting a wide range of domestic animals and humans (Paoletti *et al*., 2011). *Cryptosporidium* is a genus of apicomplexan protozoans with *C. parvum* and *C. felis* usually found in cats (Li *et al*., 2015). There is also increasing evidence that *Blastocystis*, a stramenophile, is a significant cause of intestinal disorders due to its association with individuals suffering from diarrhoea or irritable bowel syndrome (Tan, 2004). In cats, these infections are usually subclinical, although kittens and immunocompromised individuals may develop watery, sometimes foul-smelling diarrhoea, frequently accompanied with abdominal pain, vomiting, hyperthermia and weight loss (Thompson & Smith, 2011).

In Malaysia, these zoonotic protozoa may be highly prevalent in humans, animals and the environment. The prevalence of cryptosporidiosis and giardiasis in human is between 0.9 – 23% and 0.21 – 25% respectively, with children and immunosuppressed patients as the most susceptible population (Lim *et al*., 2008). The prevalance of *Giardia* in pet dogs in Malaysia is reported to be 21.9% (Lim

et al., 2008). They also found that among 174 river water samples in Malaysia, 39% were positive for *Giardia* cysts and 11.5% of *Cryptosporidium* oocysts. There remains little published information on the epidemiology of *Blastocystis*, particularly in animals in Malaysia. A study showed that 20.4% of Proto-Malay, Negrito and Senoi tribes of Orang Asli in Malaysia were infected with *Blastocystis* (Anuar, 2013).

In spite of their important zoonotic potential, diseases caused by these protozoa among animals have not been studied in much detail, and basic epidemiological information is still lacking in the country. As such, the present study was undertaken to determine the prevalence and basic epidemiology of these infections among shelter cats in Selangor, Peninsular Malaysia.

The objectives of this study were:

1. To determine the prevalence of *Giardia*, *Cryptosporidium* and *Blastocystis* among shelter cats in Selangor using microscopy and molecular detection techniques.
2. To ascertain the prevalence of infection with these protozoa in relation to the animals age and fecal consistency.

LITERATURE REVIEW

Parasites of domestic cats are important not only for cats but also for human health due to the zoonotic potential of certain parasite species. Common zoonotic enteric protozoa which reside in the gastrointestinal tract of the feline species include the flagellate *Giardia*, the coccidian *Cryptosporidium* and the stramenophile *Blastocystis* (Thompson & Smith, 2011).

Giardia

Giardia was first discovered by Antony van Leeuwenhoek in 1681. The species is a diplomonad, a complex nucleus-associated karyomastigonts possessing two nuclei and bilateral symmetry. It has two developmental stages: the trophozoite and the cyst. The trophozoite is characterized by its pyriform shape (10-30 μm long), two intracytoplasmic granular axonemes, bilateral symmetry with two equal nuclei lying adjacent to each other and six to eight flagella. The cyst is ovoid to ellipsoid (11-14 μm in width), membrane bound, and possess 4 nuclei, axonemes and median bodies. These characteristics can be identified by using the light microscope. Genetic characterization of *Giardia* isolates from different mammalian hosts around the world has identified the existence of species which are host specific and two species which have broad host ranges and are zoonotic (Monis *et al*., 2009) (Table 1).

Table 1. Genotypic groupings (assemblages) and hosts repertoire of *Giardia.*

Species (= assemblage)	**Host**
G. duodenalis (assemblage A)	Humans and other primates, dogs, cats, livestock, rodents and other wild mammals
G. enterica (assemblage B)	Humans and other primates, dogs, some species of wild mammals
G. agilis	Amphibians
G. muris	Rodents
G. psittaci	Birds
G. ardeae	Birds
G. microti	Rodents
G. canis (=assemblage C/D)	Dogs, other canids
G. cati (assemblage F)	Cats
G. bovis (=assemblage E)	Cattle and other hoofed livestock
G. simondi (= assemblage G)	Rats

Further molecular epidemiological studies of *Giardia* revealed that zoonotic and host-specific species can be transmitted through four main cycles. Transmission of *G. duodenalis* and *G. enterica* can be maintained through direct transmission between humans (e.g.: between infants in a day-care centre), *G. bovis* between livestock (e.g.: dairy cattle in enclosed environment of farm), *G. canis* between dogs (e.g.: puppies in a breeding kennel) and novel wildlife

genotypes between various wildlife species. However, *G. duodenalis* and to lesser extent *G. enterica* can infect other host populations (Monis *et al*., 2009) (Table 1). A number of studies have revealed that zoonotic species of *Giardia* can be found in individual pet dogs residing in urban areas, thus emphasizing the potential public health risk from domestics dogs, cats and livestock as well as the potential risk of wildlife to become reservoir of human infection (Leonhard *et al*., 2007; Inpankaew *et al*., 2007; Salb *et al.*, 2017; Traub *et al.*, 2004).

The life cycle of *Giardia* (Monis *et al*., 2009) is simple whereby non-invasive trophozoites will multiply rapidly on the mucosal surface of the small intestine through binary fission, which then leads to the formation of environmentally resistant cysts. The cysts are passed in the faeces and can be transmitted directly or indirectly to another susceptible host (Monis *et al*., 2009). There is also increasing evidence from molecular genetic and epidemiological studies that this species is capable of sexual reproduction other than binary fission, but the recombination frequency and its effect on the epidemiology is still unknown. This genetic diversity undoubtedly contributed many years of controversy and confusion about the taxonomy of *Giardia* (Poxleitner *et al*., 2008).

Cryptosporidium

Cryptosporidium is an obligate intracellular protistan parasite that can infect a wide range of animals and human hosts worldwide. The oocysts shed in

faeces are ellipsoid with a size of 4 by 6 µm (Scorza and Lappin, 2006). *Cryptosporidium parvum* and *C. hominis* cause the majority of human infections among the species of *Cryptosporidium* thus far described. Humans can also be infected by other species of *Cryptosporidium* like *C. meleagridis, C. canis, C. felis, C. andersoni*, and *C. muris* (Leoni, 2006; Morse *et al.*, 2007; Robinson *et al.*, 2008). *Cryptosporidium parvum* (bovine genotype) is found to be infectious to many mammalian hosts worldwide, but *C. parvum* has several distinct genotypes in which their relationships and host specificities are still largely unknown (Amidou *et al*., 2013).

The first report of *Cryptosporidium* infection among the companion animals was in 1979 in Japan occurring in cats (Iseki, 1979). Since then, this parasite has been implicated in persistent diarrhoea, anorexia and weight loss in symptomatic and asymptomatic cats (Iseki, 1979). A report has shown that the prevalence of *Cryptosporidium* among animal shelter dogs in Florida, USA was 12% (Tupler *et al*., 2012). Another report revealed that 10.7% of the 28 dogs tested in Brazil were infected with *Cryptosporidium* (Dado *et al*., 2011).

The life cycle of *Cryptosporidium* (Leitch & He, 2012) starts with a host ingesting sporulated oocysts. The oocysts then excyst in the gastrointestinal tract releasing infective sporozoites which become enclosed within parasitophorous vacuoles of the microvilli surface of enterocytes as trophozoites. The trophozoites can produce two types of meronts. Type I meronts will leave the parasitophorous vacuoles to invade other epithelial cells within 24 hours, where

they develop into more type I or type II meronts. Type I meronts can be formed indefinitely through asexual reproduction. The type II meronts produce gamonts, which can reproduce sexually. The fusion of macrogamete and microgametes produce either thick-walled or thin-walled oocyst that contains four sporozoites. Around 20% of the oocysts in the gastrointestinal tract are thin-walled. The thick-walled oocysts are passed through the faeces into the environment. Oocysts of *Cryptosporidium* are extremely resistant and infective upon excretion, thus allowing direct fecal-oral transmission.

Blastocystis

Blastocystis is another emerging parasite associated with enteric disease with zoonotic potential. Studies in defined endemic areas like zoos have proved that there is risk of zoonotic transmission (Parker *et al*., 2010). It is a polymorphic organism with four main forms: vacuolar, granular, amoeboid and cyst forms. The vacuolar form, namely central vacuole form, has an average range of 4-15 μm. It is characterized by large vacuole and a thin rim of peripheral cytoplasm. The exact function of the central vacuole is still unclear. The granular form is morphologically similar to vacuolar forms except that granules are observed in the cytoplasm, or in central vacuoles. In the amoeboid form, one or more pseudopods will be present that are involved in locomotion. It is still unclear about the factors that cause the transition to the amoeboid form (Tan, 2004). An environmentally resistant cyst form is a relatively recent discovery which contributed to our understanding of its transmission mode (Zaman, 1998).

It is usually smaller in size (2-5 μm) than other forms and more environmentally resistant. It also has a thick, multi-layered cyst wall with internal contents including one to four nuclei, multiple vacuoles and glycogen and lipid deposits replacing the large central vacuole. The cysts form is resistant to hydrolysis and in room temperature up to 19 days, but is unable to survive under extreme heat and cold as well as common disinfectants. However, the vacuolar and granular forms are sensitive to temperature changes, exposure to air, as well as hypertonic and hypotonic environments (Zaman *et al*., 1997).

The life cycle of *Blastocystis* (Tan, 2004) starts with the cyst ingested by the host develops into vacuolar form. The vacuolar form may become amoeboid or granular, or even undergo binary fission to produce more vacuolar forms. The vacuolar and granular forms can differentiate into cysts, which is then shed through the faeces to infect other susceptible animals through ingestion (Tan, 2004). A prevalence study of *Blastocystis* among domestic dogs and cats in Brisbane, Australia has shown that 70.8% of the dogs and 67.3% of the cats tested were infected (Duda *et al*., 1998). However, there is a still lack of epidemiological information of *Blastocystis* infection among companion animals in Malaysia.

MATERIALS AND METHODS

Animals and Sample Collection

A total of 105 local Domestic Short-haired cats were conveniently selected from four animal shelters in Selangor. The animals comprised adults more than a year old (67/105) and kittens (38/105). Their fecal condition was observed or a brief history about their bowel condition was also noted. Sterile swabs were used to collect per-rectal fecal sample from each animal. Two fresh faecal smears were made for each faecal sample by smearing the faecal swabs on glass slides with a drop of normal saline solution. Faecal swabs were also placed in empty microcentrifuge tubes and microcentrifuge tubes containing 700 µL of lysis buffer (Appendix 1). The faecal swabs were then stored at -20°C for subsequent DNA extraction. This research project was approved by the Institutional Animal Care and Use Committee (IACUC) with reference number: UPM/IACUC/FYP.2016/FPV (14, 47) dated 30 December 2016.

Faecal Smear Staining and Microscopy Detection

The faecal smears were fixed with methanol for five minutes and air dried. One faecal smear from each faecal sample was stained with 10% Giemsa solution, and another faecal smear was stained with cold modified Ziehl-Neelsen's acid-fast. For Giemsa staining, the fixed faecal smears were flooded with 10% Giemsa solution for 30 minutes, rinsed with tap water and air-dried.

For cold modified Ziehl-Neelson's acid fast staining, the faecal smears were flooded with carbol fuchsin for 10 minutes, rinsed with tap water, and decolourised with 1% acid alcohol until all traces of red were gone from the thin part of the smear. The acid alcohol was rinsed with tap water and Loeffler's methylene blue was used to flood the smears for 30 seconds, rinsed with tap water and air-dried. The stained fecal smears were examined microscopically using 20 times magnification for screening with a Nikon Eclipse 50i microscope. Upon detection of any enteric protozoa, a magnification of 100 times oil-immersion was used to further analyze the morphology of the protozoa as well as to obtain morphometric data using a Nikon Eclipse 50i microscope fitted with a camera and NIS-Elements D Documentation Software.

DNA Extraction

DNA extraction was carried out on 40 fecal samples using a commercial DNA extraction kit according to the manufacturer's instruction (QIAmp® Fast DNA Stool Mini Kit). The faecal swabs stored in lysis buffer were vortexed briefly before 250 µL was pipetted out into a new microcentrifuge tube and 1 mL InhibitEX Buffer was added. The solution was then briefly vortexed, heated for five minutes at 70°C, and centrifuging at 20,000 × *g* for 1 minute to pellet the stool particles. 200 µL of the centrifuged supernatant was placed into the new microcentrifuge tube and 15 µL of Proteinase K and 200 µL of Buffer AL was added. The mixture was vortexed for 15 seconds and incubated at 70°C for 10 minutes. 200 µL of 99% ethanol was then added to the lysate and mixed by

vortexing. 600 µL of the lysate was transferred into the QIAmp® spin column and centrifuged for one minute. The filtrate in the collection tube was discarded, and 500 µL of Buffer AW1 was added into the spin column. The spin column was centrifuged at 20,000 × *g* for one minute. The filtrate in the collection tube was discarded and 500 µL of Buffer AW2 was added into the spin column. The spin column was then centrifuged at 20,000 × *g* for three minutes, the filtrate discarded, followed by centrifugation at 20,000 × *g* for three minutes. The spin column was transferred into a new, labelled microcentrifuged tube and 200 µL of Buffer ATE was added directly onto the column membrane. The column was incubated for one minute at room temperature and centrifuged at 20,000 × *g* for one minute to elute the DNA. The final DNA concentration was determined by diluting the eluted DNA sample in deionized water (1:49 dilution) and absorbance was measured in a spectrophotometer (BiophotometerPlus, Eppendorf, Germany).

PCR Amplification

PCR for the detection of *Giardia*, *Cryptosporidium* and *Blastocystis* was carried on 40 fecal samples out using published genus-specific primers targeting the Small Subunit Ribosomal RNA (SSU) gene of the protozoa (Table 2). The PCR reaction was performed using the GoTaq® Flexi DNA Polymerase (Promega, USA). The primary amplification was carried out in a 25 µL volume comprising 8.4 µL sterile deionized water, 5 µL 5X Green GoTaq® Flexi Buffer, 5 µL 25mM magnesium chloride ($MgCl_2$) solution, 0.3 µL 10mM dNTP Mix, 0.3

µL GoTaq® DNA Polymerase, 1 µL of forward primer, 1 µL of reverse primer, and 4 µL of DNA template. The nested PCR reaction (25 µL) comprised 10.9 µL sterile deionized water, 5 µL 5X Green GoTaq® Flexi Buffer, 2.5 µL 25mM magnesium chloride ($MgCl_2$) solution, 0.3 µL 10mM dNTP Mix, 0.3 µL GoTaq® DNA Polymerase, 1 µL of forward primer, 1 µL of reverse primer, and 4 µL of DNA template. The PCR amplification was performed using a thermocycler (MyCycler™ Thermal Cycler System, Bio-Rad, USA).

Table 2. Primers used for PCR detection of the various enteric protozoa from faecal samples of cats in this study.

Protozoa	Primers	Fragment	Size (bp)	Reference
Giardia	**Primary PCR** RH11 (5'-CAT CCG GTC GAT CCT GCC-3') RH4 (5'-AGTCGAACCCTGATTCTCCGCCAGG-3') **Nested PCR** Giar-F (5'-GAC GCT CTC CCC AAG GAC-3') Giar-R (5'-CTG CGT CAC GCT GCTC-3')	Small Subunit Nuclear Ribosomal RNA (SSU)	200	Lim *et al.*, 2013
Cryptosporidium	**Primary PCR** XF2 (5'-GGAAGGGTTGTATTTATTAGATAAAG-3') XR2 (5'-AAGGAGTAAGGAACAACCTCCA-3') **Nested PCR** pSSUf (5'-AAAGCTCGTAGTTG- GATTTCTGTT-3') pSUr (5'-ACCTCTGACTGTTAAATACRAATGC-3')	Small Subunit Nuclear Ribosomal RNA (pSSU) 60 kDa glycoprotein (p*gp60*)	250-380	Yap *et al.*, 2016
Blastocystis	BhRDr (5'- GAGCTTTTTAACTGCAACAACG -3') RD5 (5'-ATCTGGTTGATCCTGCCAGT-3')	Small Subunit Ribosomal RNA (SUU-rDNA)	600	Scicluna *et al.*, 2005

Agarose Gel Electrophoresis and Imaging

HyAgarose™ (HydraGene Co. Ltd, USA) was used to prepare 1.5% agarose gel for electrophoresis. 100 ml of tris-acetate-ethylenediaminetetraacetic acid buffer (TAE buffer) and 1.5 g of agarose powder was mixed thoroughly and dissolved in a microwave (Panasonic, Malaysia). The agarose solution was cooled to 60°C and one microliter nucleic acid staining solution (Redsafe™, Intron, Korea) was added and mixed thoroughly by swirling and allowed to set in a tray. Electrophoresis was carried out in TAE buffer at 110 V for approximately 45 minutes using the PowerPacTM Basic unit (Bio-Rad, USA). Amplicons were visualized under ultraviolet illumination using the in a GelDoc XR + UV transilluminator (Bio-rad, USA) and photographed using Quantity One Basic Software (Bio-rad, USA).

Statistical Analysis

Statistical analysis was done using the Statistical Package for the Social Sciences (SPSS) version 22 (SPSS, IBM Inc, USA) software. The prevalence rates of *Giardia, Cryptosporidium* and *Blastocystis* was and Pearson Chi-Square was used to ascertain the prevalence of infection in relation to age and fecal consistency among shelter cats in Selangor. All significance values were determined at a 95% confidence and P values of less than 0.05 were considered significant.

RESULTS

Demographics

Out of the 105 cats examined, 38 (36.2%) were less than a year old and 67 (63.8%) were adults. Only 10 (9.5%) out of 105 fecal samples were classified as soft faeces or diarrhoea according to the fecal scoring system for cats by Royal Canin® (Canin, 2015) (Appendix 3).

Microscopy Detection

None of the Giemsa or Ziehl-Neelsen's acid-fast stained fecal smears were found to be positive for *Giardia, Cryptosporidium* or *Blastocystis* by microscopy examination. However, *Tritrichomonas* was detected in two of the fecal smears using microscopy (Figure 1).

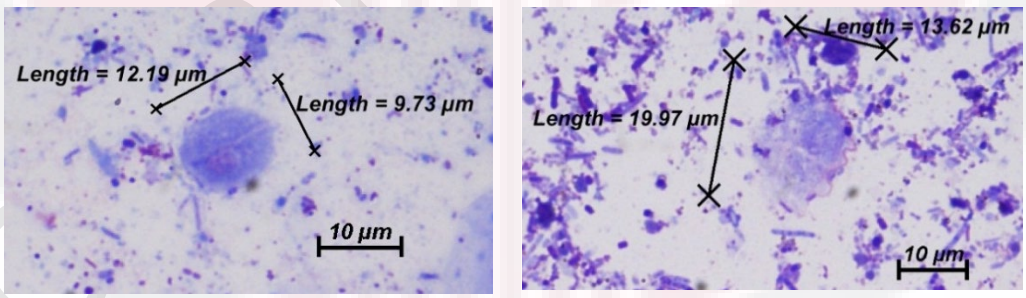

Figure 1. *Tritrichomonas* was detected in two of the fecal smears from the cats.

Molecular Detection

15 (37.5%) out of 40 samples examined by PCR were positive for *Giardia* and 10 (25%) were positive for *Blastocystis* (Figure 2). *Cryptosporidium* was not detected by PCR in any of the cat fecal samples examined. Of the 15 *Giardia* positive samples, 9 (60%) were single *Giardia* infections while 6 (40%) were also positive for *Blastocystis*. Of the 10 *Blastocystis* positive samples, 4 (40%) were single *Blastocystis* infections while 6 (60%) harbored *Giardia* as well. The overall prevalence for single protozoal infection was 85% while that of mixed infections was 15%. Pearson's Chi-square test (Tables 3 and 4) revealed that fecal consistency and age were not significantly correlated ($p>0.05$) with either *Giardia* or *Blastocystis* infections.

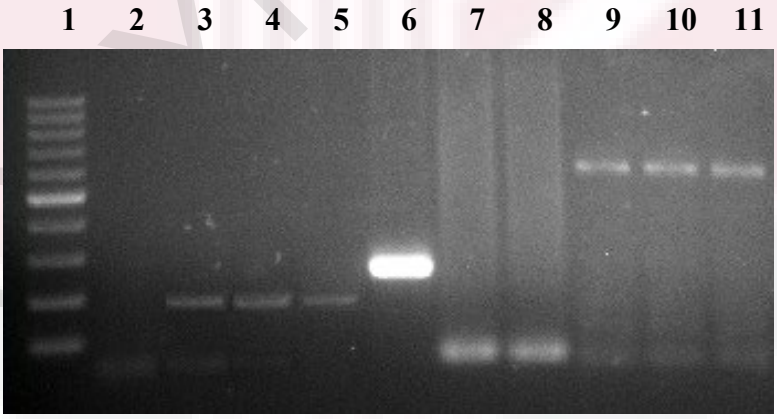

Figure 2. Detection of *Giardia, Cryptosporidium* and *Blastocystis* in faecal samples of shelter cats in Selangor. Lane 1 - 100bp DNA ladder marker, Lane 2 - negative control, Lane 3, 6 and 9 - positive controls of *Giardia* (200bp), *Cryptosporidium* (300bp) and *Blastocystis* (600bp), respectively. Lanes 4 and 5 positive for *Giardia*, Lanes 7 and 8 negative for *Cryptosporidium*, Lanes 10 and 11 positive for *Blastocystis*.

Table 3. Pearson Chi-square analysis of positive detection for *Giardia* using PCR in relation to age, fecal consistency and positive detection for *Giardia* using microscopy detection at 95% confidence level.

Parameters		**PCR Positive for *Giardia***		**P value (2-sided)**
		Positive	Negative	
Age	< 1 year old	4	6	0.850
	> 1 year old	11	19	
Fecal Consistency	Diarrhoea	4	2	0.109
	Normal	11	23	

Table 4. Pearson Chi-square analysis of positive detection for *Blastocystis* using PCR in relation to age and fecal consistency at 95% confidence level.

Parameters		**PCR Positive for *Blastocystis***		**P value (2-sided)**
		Positive	Negative	
Age	< 1 year old	1	9	0.206
	> 1 year old	9	21	
Fecal Consistency	Diarrhoea	3	3	0.125
	Normal	11	27	

DISCUSSION

The present study is the first to employ molecular techniques to detect zoonotic enteric protozoa infection among cats in Malaysia. The prevalence among shelter cats in Selangor using PCR assay in this study was 37.5% for Giardia, 0% for *Cryptosporidium* and 25% for *Blastocystis*. A previous study (Tan, 2016) using microscopy detection showed that 3/30 (10%) random cats from selected clinics in Klang Valley were positive for *Cryptosporidium*. Microscopy evaluation on fecal samples from captive wild cats in a local zoo revealed that 4/28 (14.3%) of the animals were positive for *Cryptosporidium* (Lim *et al.*, 2008). A recent study done on rural cats from Selangor and Pahang showed that the prevalence of *Giardia duodenalis* and *Cryptosporidium* were 12% and 8%, respectively (Ngui *et al*., 2014). All these studies done on cats in Malaysia used microscopy detection. The present study reports for the first time the presence of *Blastocystis* among cats in the country. The prevalence of zoonotic enteric protozoa among shelter cats in Selangor in this study was higher compared to other previous reports. This is expected as PCR detection is considered to be more sensitive in detecting the presence of the pathogen compared to conventional microscopy.

The introduction of stray animals without prior screening for enteric protozoa is one of the possible causes of the high prevalence of zoonotic enteric protozoa among shelter cats in Selangor. Stray or surrendered animals are usually exposed to pathogens present in the environment outside of shelter such as water

or soil. Oocysts and cysts of zoonotic enteric protozoa can be easily transmitted to the animals directly through fecal-oral route or indirectly through water-borne or food-borne route. The infected animals are reservoirs of zoonotic enteric protozoa when they are transferred into shelters. Oocysts or cysts will be shed into the faeces of infected animals and become a source of infection to other susceptible animals in the shelter.

Some shelters keep high number of animals although they have limited space, which contributes to the high population density of resident animals. This will increase the possibility of cross-infection if there is an infected animal among them. Poor hygiene and management of shelters is also another factor contributing to the high prevalence in shelter cats. Dirty cattery or infrequent change of litter boxes and disinfection of facilities will cause higher risk of transmission of zoonotic enteric protozoa among cats. Sharing of equipment between quarantine area and clean area will also increase the risk of transmission of pathogens from sick animals to healthy animals. It is also possible that wildlife and domestic animals like wild birds, rats and even resident dogs can be reservoirs that transmit these protozoa to shelter cats. When there is no proper barrier to separate the resident animals from the wild animals, strains or subtypes of zoonotic enteric protozoa can be transmitted to resident animals. A cat infected with any of the zoonotic enteric protozoa can transmit to susceptible host like naïve cats, dogs in the shelter. The cysts or oocysts shed by the cat can also remain resistant in the environment, waiting to infect another animal some other time. Infected cats which are potential candidates for adoption can become

a source of infection to humans. Zoonotic enteric protozoa can have serious implications to the immunocompromised human like old people, young children and HIV patients.

In this study, the overall prevalence for single protozoan infection was 85% while that of mixed infections was 15%. Infection with multiple species of enteric protozoa is common in the developing countries where poor hygiene, insufficient sanitation and close vicinity to zoonotic reservoirs like livestock and small animals (Thompson & Smith, 2011). However, the effect of mixed infections has not been well-studied (Ouattara *et al.*, 2008). Enteric protozoa are part of a complex ecological system which is shared with other helminths and bacteria. Therefore, they must be studied together in terms of their impact of disease dynamics (Rohani *et al.*, 2003).

Although infections with *Giardia*, and to a lesser extent *Cryptosporidium*, are common in dogs and cats, infections are usually asymptomatic (Thompson & Smith, 2011). No drug has been proved to be effective to remove *Cryptosporidium* from the gastrointestinal tract, hence only symptomatic treatment for diarrhoea is instituted (Lappin, 2015). *Giardia* infection is commonly found in dogs and cats having small-bowel diarrhoea. Although healthy pets are unlikely to be reservoirs of *Giardia* infection to human, clinical manifestations of *Giardia* are usually intermittent and some species of *Giardia* are known to be zoonotic. Therefore, it is wise to consider treating healthy dogs and cats, particularly animals from shelters which are highly exposed to infected

oocysts or cysts (Lappin, 2015). The increased recognition of *Blastocytis* as being pathogenic, at least in humans (Boorom *et al*., 2008), also warrants more studies of *Blastocystis* among animals as they are commonly associated to human living environments. However, the pathogenesis of *Blastocystis* is still not well-understood due to the absence of suitable animal model which can be exploited to confirm whether *Blastocystis* can fulfil Koch's postulates (Tan, 2004). *Blastocystis* infections commonly cause non-specific clinical sings such as diarrhoea, abdominal cramps and nausea, and the infections are usually self-limiting.

CONCLUSION AND RECOMMENDATIONS

Genetic and phylogenetic analysis is recommended to determine the presence of known human-infective strains among the fecal samples. Further study of zoonotic enteric protozoa in other areas in Malaysia should also be explored. For the stakeholders in this study, which are animal shelters, it is recommended that the shelters quarantine and screen new animals coming in shelters before releasing them to mingle around with resident animals. Besides, it is also strongly suggested to treat infected animals with antiprotozoal drugs like metronidazole. The dose for cats is 25 mg/kg BID for 5-7 days. For shelter management, regular disinfection of cattery and changing of cat litter is important to improve the hygiene of the shelter. Strict prohibition of equipment sharing between quarantine area and clean area like broom, brush, scrubs, food or water bowls can also help to reduce the risk of transmission of pathogens from sick animals to healthy animals. Finally, proper deworming and vaccination protocols must be practiced in shelters to avoid immunosuppression from concurrent infections. In conclusion, *Giardia* and *Blastocystis* were successfully detected through PCR among shelter cats in Selangor in this study. There is a high prevalence of *Giardia* (37.5%) and *Blastocystis* (25.0%) among the animals, and this warrants further investigation into strain typing to identify the human-infective genotypes. Host age and fecal consistency are not related to the prevalence of these zoonotic enteric protozoa.

REFERENCES

Amidou, S., Machuene, A. T., & Pascal, B. (2013). The epidemiology of C*ryptosporidium* in cats and dogs in the Thohoyandou region, South Africa. African Journal of Microbiology Research, 7(21), 2510-2518. doi:10.5897/ajmr12.1391

Anuar, T. S., Ghani, M. K., Azreen, S. N., Salleh, F. M., & Moktar, N. (2013). *Blastocystis* infection in Malaysia: Evidence of waterborne and human-to-human transmissions among the Proto-Malay, Negrito and Senoi tribes of Orang Asli. Parasites & Vectors, 6(40), 1-12. Retrieved October 18, 2016, from http://www.parasitesandvectors.com/content/6/1/40

Boorom, K. F., Smith, H., Nimri, L., Viscogliosi, E., Spanakos, G., Parkar, U., . . . Jones, M. S. (2008). Oh my aching gut: irritable bowel syndrome, *Blastocystis*, and asymptomatic infection. Parasites & Vectors, 1(1), 40. doi:10.1186/1756-3305-1-40

Canin, R. (2015). Science Happens. Retrieved March 1, 2017, from http://sciencehappens.royalcanin.com/

Dado, D., Izquierdo, F., Vera, O., Montoya, A., Mateo, M., Fenoy, S., . . . Miró, G. (2011). Detection of Zoonotic Intestinal Parasites in Public Parks of Spain. Potential Epidemiological Role of *Microsporidia*. Zoonoses and Public Health, 59(1), 23-28. doi:10.1111/j.1863-2378.2011.01411.x

Duda, A., Stenzel, D., & Boreham, P. (1998). Detection of *Blastocystis* sp. in domestic dogs and cats. Veterinary Parasitology, 76(1-2), 9-17. doi:10.1016/s0304-4017(97)00224-0

Inpankaew, T., Traub, R., Thompson, R. C., & Sukthana, Y. (2007). Canine parasitic zoonoses in Bangkok temples. . Asian Journal Tropical Medicine Public Health, 38, 247-255. Retrieved March 1, 2017.

Iseki, M. (1979). *Cryptosporidium felis* sp. n. (Protozoa: Eimeriorina) from the domestic cat [Abstract]. Japanese Journal of Parasitology, 28, 285-307. Retrieved March 1, 2017.

Lappin, M. R. (2015). Feline intestinal protozoa - zoonotic importance or not? European Journal of Companion Animal Practice, 25(1), 4-7. Retrieved March 1, 2017.

Leitch, G. J., & He, Q. (2012). Cryptosporidiosis-an overview. Journal of Biomedical Research, 25(1), 1-16. doi:10.1016/s1674-8301(11)60001-8

Leonhard, S., Pfister, K., Beelitz, P., Wielinga, C., & Thompson, R. (2007). The molecular characterisation of *Giardia* from dogs in southern Germany. Veterinary Parasitology, 150(1-2), 33-38. doi:10.1016/j.vetpar.2007.08.034

Leoni, F. (2006). Genetic analysis of *Cryptosporidium* from 2414 humans with diarrhoea in England between 1985 and 2000. Journal of Medical Microbiology, 55(6), 703-707. doi:10.1099/jmm.0.46251-0

Li, W., Li, Y., Song, M., Lu, Y., Yang, J., Tao, W., . . . Xiao, L. (2015). Prevalence and genetic characteristics of *Cryptosporidium*, *Enterocytozoon bieneusi* and *Giardia duodenalis* in cats and dogs in Heilongjiang province, China. Veterinary Parasitology, 208(3-4), 125-134. doi:10.1016/j.vetpar.2015.01.014

Lim, Y. A., Ahmad, R. A., & Smith, H. V. (2008, January). Current status and future trends in *Cryptosporidium* and *Giardia* epidemiology in Malaysia. Journal of Water and Health, 6(2), 239-254. Retrieved October 17, 2016, from jwh.iwaponline.com/content/ppiwajwh/6/2/239.full.pdf.

Monis, P. T., Caccio, S. M., & Thompson, R. A. (2009). Variation in *Giardia*: towards a taxonomic revision of the genus. Trends in Parasitology, 25(2), 93-100. doi:10.1016/j.pt.2008.11.006

Morse, T., Nichols, R., Grimason, A., Campbell, B., Tembo, K., & Smith, H. (2007). Incidence of cryptosporidiosis species in paediatric patients in Malawi. Epidemiology and Infection, 135(08). doi:10.1017/s0950268806007758

Outtara, M., Silue, K. D., Yapi, A., N'Guessan, A. N., Barbara, M., Raso, G., . . . N'Goran, J. (2008). Prevalence and Polyparasitism of intestinal protozoa and spatial distribution of *Entamoeba histolytica E. dispar* and *Giardia intestinalis* from pupils in the rural zone in Cote d'Ivoire. Sante, 18, 215-222. Retrieved March 1, 2017.

Paoletti, B., Otranto, D., Weigl, S., Giangaspero, A., Cesare, A. D., & Traversa, D. (2011). Prevalence and genetic characterization of *Giardia* and *Cryptosporidium* in cats from Italy. Research in Veterinary Science, 91(3), 397-399. doi:10.1016/j.rvsc.2010.09.011

Parkar, U., Traub, R. J., Vitali, S., Elliot, A., Levecke, B., Robertson, I., . . . Thompson, R. A. (2010). Molecular characterization of *Blastocystis* isolates from zoo animals and their animal-keepers. Veterinary Parasitology, 169(1-2), 8-17. doi:10.1016/j.vetpar.2009.12.032

Patton, S. (n.d.). Overview of Giardiasis - Digestive System. Retrieved January 27, 2017, from http://www.merckvetmanual.com/digestive-system/giardiasis/overview-of-giardiasis

Poxleitner, M. K., Carpenter, M. L., Mancuso, J. L., Wang, C. R., Dawson, S. C., & Cande, W. Z. (2008). Evidence for karyogamy and exchange of genetic material in the binucleate intestinal parasite *Giardia intestinalis*. Science, 319, 1530-1533. doi:10.1126/science.1153752

Robinson, G., Elwin, K., & Chalmers, R. M. (2008). Unusual *Cryptosporidium* Genotypes in Human Cases of Diarrhea. Emerging Infectious Diseases, 14(11), 1800-1802. doi:10.3201/eid1411.080239

Rohani, P., Green, C. J., Mantilla-Beniers, N. B., & Grenfell, B. T. (2003). Ecological interference between fatal diseases. Nature, 422(6934), 885-888. doi:10.1038/nature01542

Salb, A. L., Barkeman, W. B., Elkin, B. T., Thompson, R. A., Whiteside, R. A., Black, S. R., . . . Kutz, S. J. (2008). Parasites in dogs in two northern Canadian communities: implications for human, dog, and wildlife health. . Emergency Infectious Disease, 14, 60-63. Retrieved March 1, 2017.

Scorza A.V., Lappin M. R. (2014, October 27). An update on three important protozoan parasitic infections in cats: cryptosporidiosis, giardiasis, and tritrichomoniasis. Retrieved January 25, 2017, from http://veterinarymedicine.dvm360.com/update-three-important-protozoan-parasitic-infections-cats-cryptosporidiosis-giardiasis-and-tritrich?id=&sk=&date=&pageID=2

Tan S. K. W. (2004). *Blastocystis* in humans and animals: new insights using modern methodologies. Veterinary Parasitology, 126 (2004), 121-144. doi:10.1016/j.vetpar.2004.09.017

Thompson, R., & Smith, A. (2011). Zoonotic enteric protozoa. Veterinary Parasitology, 182(1), 70-78. doi:10.1016/j.vetpar.2011.07.016

Traub, R. J., Monis, P. T., Robertson, I., Irwin, P., Mencke, N., & Thompson, R. C. (2004). Epidemiological and molecular evidence supports the zoonotic transmission of *Giardia* among humans and dogs living in the same community. Parasitology, 128(3), 253-262. doi:10.1017/s0031182003004505

Tupler, T., Levy, J. K., Sabshin, S. J., Tucker, S. J., Greiner, E. C., & Leutenegger, C. M. (2012). Enteropathogens identified in dogs entering a Florida animal shelter with normal feces or diarrhea. Journal of the American Veterinary Medical Association, 241(3), 338-343. doi:10.2460/javma.241.3.338

Zaman, V. (1998). The differential identification of *Blastocystis hominis* cysts. Annals of Tropical Medicine And Parasitology, 92(2), 233-235. doi:10.1080/00034989860094

Zaman, V., Howe, J., Ng, M.. (1997). Variation in the cyst morphology of *Blastocystis hominis*. Parasitology. Research, 83, 306–308.

APPENDICES

Appendix 1

Recipe of Lysis Buffer

In order to make 1L of 10 times lysis buffer, 121.4 g of trisaminomethane (tris) was mixed into 500 mL of distilled water. Hydrogen chloride was slowly mixed into the diluted tris and stirred until the solution reaches pH 8. 292.44g of ethylenediaminetetraacetic acid (EDTA) and 5.844 g of sodium chloride were added into the mixture and stirred well. 950 mL of distilled water was added into the mixture to make it into 1 L before it was autoclaved. 50 mL of sodium dodecyl sulfate (SDS) was then added into the autoclaved mixture before it was kept in the fridge. The 10 times lysis buffer stock was diluted to 1 times with distilled water before use.

Appendix 2

Results

No	Shelter	Age	Fecal Consistency	Microscope Detection	PCR *Giardia*	PCR *Cryptos-poridium*	PCR *Blasto-cystis*
1	A	< 1 y/o	Not diarrhea	Negative	Negative	Negative	Negative
2	A	> 1 y/o	Not diarrhea	Negative	Negative	Negative	Negative
3	A	> 1 y/o	Not diarrhea	Negative	-	-	-
4	A	> 1 y/o	Not diarrhea	Negative	-	-	-
5	A	> 1 y/o	Not diarrhea	Negative	Negative	Negative	Negative
6	A	> 1 y/o	Not diarrhea	Negative	Negative	Negative	Negative
7	A	> 1 y/o	Not diarrhea	Negative			
8	A	> 1 y/o	Not diarrhea	Negative	Negative	Negative	Negative
9	A	> 1 y/o	Not diarrhea	Negative			
10	A	> 1 y/o	Not diarrhea	Negative	Negative	Negative	Negative
11	A	> 1 y/o	Not diarrhea	Negative			
12	A	> 1 y/o	Not diarrhea	Negative	Negative	Negative	Negative
13	A	< 1 y/o	Not diarrhea	Negative	Negative	Negative	Negative
14	A	> 1 y/o	Not diarrhea	Negative	Negative	Negative	Negative
15	A	< 1 y/o	Not diarrhea	Negative	-	-	-
16	A	> 1 y/o	Not diarrhea	Negative	Negative	Negative	Negative
17	A	> 1 y/o	Not diarrhea	Negative	-	-	-
18	A	> 1 y/o	Not diarrhea	Negative	-	-	-
19	A	> 1 y/o	Not diarrhea	Negative	-	-	-
20	A	> 1 y/o	Not diarrhea	Negative	Positive	Negative	Negative
21	A	> 1 y/o	Diarrhea	Negative	Negative	Negative	Negative
22	A	> 1 y/o	Not diarrhea	Negative	Positive	Negative	Negative
23	A	> 1 y/o	Not diarrhea	Negative	Negative	Negative	Negative
24	B	< 1 y/o	Not diarrhea	Negative	-	-	-
25	B	> 1 y/o	Diarrhea	Positive	Positive	Negative	Negative
26	B	> 1 y/o	Not diarrhea	Negative	Positive	Negative	Negative
27	B	< 1 y/o	Not diarrhea	Positive	Positive	Negative	Negative
28	B	> 1 y/o	Not diarrhea	Negative	-	-	-
29	B	< 1 y/o	Not diarrhea	Negative	-	-	-
30	B	> 1 y/o	Not diarrhea	Negative	-	-	-
31	B	> 1 y/o	Not diarrhea	Negative	-	-	-
32	B	> 1 y/o	Not diarrhea	Negative	-	-	-
33	B	> 1 y/o	Not diarrhea	Negative	-	-	-

No	Shelter	Age	Fecal Consistency	Microscope Detection	PCR *Giardia*	PCR *Cryptos-poridium*	PCR *Blasto-cystis*
34	B	> 1 y/o	Not diarrhea	Negative	Negative	Negative	Negative
35	B	> 1 y/o	Not diarrhea	Negative	Negative	Negative	Negative
36	B	> 1 y/o	Not diarrhea	Negative	Negative	Negative	Negative
37	B	< 1 y/o	Not diarrhea	Negative	Positive	Negative	Negative
38	B	> 1 y/o	Not diarrhea	Negative	-	-	-
39	B	> 1 y/o	Not diarrhea	Negative	-	-	-
40	B	> 1 y/o	Not diarrhea	Negative	-	-	-
41	B	> 1 y/o	Not diarrhea	Negative	-	-	-
42	B	> 1 y/o	Not diarrhea	Negative	-	-	-
43	B	> 1 y/o	Not diarrhea	Negative	Positive	Negative	Negative
44	B	> 1 y/o	Not diarrhea	Negative	-	-	-
45	B	> 1 y/o	Not diarrhea	Negative	-	-	-
46	B	> 1 y/o	Not diarrhea	Negative	-	-	-
47	B	> 1 y/o	Not diarrhea	Negative	-	-	-
48	B	> 1 y/o	Not diarrhea	Negative	-	-	-
49	B	> 1 y/o	Diarrhea	Negative	-	-	-
50	B	> 1 y/o	Diarrhea	Negative	Positive	Negative	Negative
51	B	< 1 y/o	Not diarrhea	Negative	-	-	-
52	B	< 1 y/o	Not diarrhea	Negative	Negative	Negative	Negative
53	B	< 1 y/o	Not diarrhea	Negative	-	-	-
54	B	< 1 y/o	Not diarrhea	Negative	-	-	-
55	B	< 1 y/o	Not diarrhea	Negative	Negative	Negative	Negative
56	B	> 1 y/o	Diarrhea	Negative	-	-	-
57	B	> 1 y/o	Diarrhea	Negative	-	-	-
58	B	> 1 y/o	Diarrhea	Negative	-	-	-
59	C	> 1 y/o	Not diarrhea	Negative	-	-	-
60	C	< 1 y/o	Not diarrhea	Negative	-	-	-
61	C	< 1 y/o	Not diarrhea	Negative	-	-	-
62	C	> 1 y/o	Not diarrhea	Negative	-	-	-
63	C	< 1 y/o	Not diarrhea	Negative	-	-	-
64	C	> 1 y/o	Not diarrhea	Negative	-	-	-
65	C	> 1 y/o	Not diarrhea	Negative	-	-	-
66	C	< 1 y/o	Not diarrhea	Negative	-	-	-
67	C	< 1 y/o	Not diarrhea	Negative	-	-	-
68	C	< 1 y/o	Not diarrhea	Negative	-	-	-
69	C	< 1 y/o	Not diarrhea	Negative	-	-	-
70	C	< 1 y/o	Not diarrhea	Negative	-	-	-

No	Shelter	Age	Fecal Consistency	Microscope Detection	PCR *Giardia*	PCR *Cryptos-poridium*	PCR *Blasto-cystis*
71	C	< 1 y/o	Not diarrhea	Negative	Negative	Negative	Negative
72	C	< 1 y/o	Not diarrhea	Negative	-	-	-
73	C	< 1 y/o	Not diarrhea	Negative	-	-	-
74	C	< 1 y/o	Not diarrhea	Negative	-	-	-
75	C	< 1 y/o	Not diarrhea	Negative	-	-	-
76	C	< 1 y/o	Not diarrhea	Negative	-	-	-
77	C	< 1 y/o	Not diarrhea	Negative	-	-	-
78	C	< 1 y/o	Not diarrhea	Negative	-	-	-
79	C	< 1 y/o	Not diarrhea	Negative	-	-	-
80	C	< 1 y/o	Not diarrhea	Negative	-	-	-
81	C	< 1 y/o	Not diarrhea	Negative	-	-	-
82	C	< 1 y/o	Not diarrhea	Negative	-	-	-
83	C	< 1 y/o	Not diarrhea	Negative	-	-	-
84	C	< 1 y/o	Not diarrhea	Negative	-	-	-
85	C	< 1 y/o	Not diarrhea	Negative	-	-	-
86	C	> 1 y/o	Not diarrhea	Negative	-	-	-
87	C	< 1 y/o	Not diarrhea	Negative	Positive	Negative	Negative
88	D	> 1 y/o	Not diarrhea	Negative	Negative	Negative	Negative
89	D	> 1 y/o	Not diarrhea	Negative	-	-	-
90	D	> 1 y/o	Not diarrhea	Negative	-	-	-
91	D	> 1 y/o	Not diarrhea	Negative	-	-	-
92	D	> 1 y/o	Not diarrhea	Negative	-	-	-
93	D	> 1 y/o	Not diarrhea	Negative	Negative	Negative	Negative
94	D	> 1 y/o	Not diarrhea	Negative	-	-	-
95	D	< 1 y/o	Not diarrhea	Negative	Negative	Negative	Negative
96	B	> 1 y/o	Not diarrhea	Negative	Negative	Negative	Positive
97	B	> 1 y/o	Not diarrhea	Negative	Negative	Negative	Positive
98	B	> 1 y/o	Diarrhea	Negative	Negative	Negative	Positive
99	B	> 1 y/o	Not diarrhea	Negative	Positive	Negative	Positive
100	B	> 1 y/o	Not diarrhea	Negative	Positive	Negative	Positive
101	B	> 1 y/o	Not diarrhea	Negative	Positive	Negative	Positive
102	B	> 1 y/o	Not diarrhea	Negative	Negative	Negative	Positive
103	B	> 1 y/o	Diarrhea	Negative	Positive	Negative	Positive
104	B	> 1 y/o	Not diarrhea	Negative	Positive	Negative	Positive
105	B	< 1 y/o	Diarrhea	Negative	Positive	Negative	Positive

Appendix 3

Royal Canin® Fecal Scoring System for Cats

Appendix 4

Institutional Animal Care and Use Committee Approval Letter