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MICROSCOPIC AND MOLECULAR DETECTION OF *GIARDIA* SPP. IN FECAL SAMPLES AMONG SHELTER DOG POPULATION IN SELANGOR

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Microscopic and Molecular Detection of *Giardia* spp. in Fecal Sample among Shelter Dog Population in Selangor", by Stephanie Magdalene and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999 - Project

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DEDICATIONS

This project paper is dedicated to Almighty God

To my family

Grandfather

Grandmother

Father

Mother

Brother, Sister

&

My furry family

And to all my lecturers who have committed themselves towards the noble cause of education.

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LIST OF ABBREVIATIONS

%	Percent
µL	Micro liter
nm	Nanometer
°C	Degree Celsius
µm	*Micrometer*
BID	Twice a day
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immunosorbent Assay
gDNA	Genomic Deoxyribonucleic Acid
IFA	*Immunofluorescence assay*
ME	Microscopic examination
PCR	Polymerase chain reaction
SDS	Sodium Dodecyl Sulphate
SID	Once a day
TAE	Mixture of Tris base, acetic acid and EDTA

ABSTRACT

MICROSCOPIC AND MOLECULAR DETECTION OF *GIARDIA* SPP. IN FECAL SAMPLE AMONG SHELTER DOGS POPULATION IN SELANGOR

By

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2017

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Giardia spp. is a protozoan that can be found in feces of human and animals and is considered to have zoonotic potential. In vertebrates, including mammals, birds, reptiles and fishes, clinical signs like diarrhea, vomiting, weight loss and lethargy are frequently observed while in humans, various clinical manifestations have been reported ranging from asymptomatic to acute, intermittent or chronic non-bloody diarrhea. This study aimed to determine the occurrence of *Giardia* spp. in fecal samples of dogs from various canine shelters in Selangor by using microscopic and polymerase chain reaction (PCR) detection methods. Secondly, the occurrence of *Giardia* spp. was investigated for

its association with occurrence of diarrhea. This project was approved by the UPM animal ethics committee and consent received from the five shelters. A total of 130 dogs were randomly selected based on convenience sampling. Fecal swabs were obtained and rolled onto glass slides, air-dried and stained with both Giemsa and Ziehl-Neelson and microscopically examined. Seventy fecal samples were sufficient for nested-PCR assay where primers specific for *Giardia* were used, and subsequent gel electrophoresis to determine the specific bands corresponding to the PCR product band size. Statistical analysis was done using SPSS where P-value<0.05 was considered significant. *Giardia* spp. was detected from four dogs out of 130 dogs (3.1%) on microscopic evaluation which was confirmed on PCR detection. Other parasites detected by microscopic examination include *Cryptosporidium* and coccidia. A total of 17.1% (n=12/70) of the samples were positive for *Giardia* spp. by nested-PCR detection method. There was four times higher positive detection of *Giardia* spp. in dogs with diarrhea and it was statistically significant through Pearson's chi-squared analysis (P-value=0.044). In conclusion, this study reports for the first time molecular detection of *Giardia* spp. in 17.1% of shelter dogs in Malaysia using molecular detection method. It is recommended that all shelter dogs should be periodically dewormed to prevent transmission of *Giardia* spp. among the shelter animals within the same enclosures and avoid potential zoonotic transmission to care takers of the shelter. Phylogenetic characterization of *Giardia* spp. in dogs in Malaysia merit further studies.

Keywords: *Giardia* spp., Zoonotic, Dog, Microscopy, PCR

ABSTRAK

PENGESANAN *GIARDIA* SPP. DARI SAMPEL TINJA DI KALANGAN ANJING PERLINDUNGAN DI SELANGOR DENGAN MENGGUNAKAN KAEDAH MIKROSKOPIK DAN MOLEKUL

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2017

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Giardia spp. adalah protozoa yang boleh didapati dalam tinja manusia dan haiwan dan dianggap mempunyai potensi zoonotik. Di kalangan vertebrata, termasuk mamalia, burung, reptilia dan ikan, tanda-tanda klinikal seperti cirit-birit, muntah, susut berat badan dan kelesuan sering dapat diperhatikan manakala pada manusia, pelbagai manifestasi klinikal dapat dilihat, dari asimptomatik kepada akut, berselangan atau kronik. Kajian ini bertujuan untuk mengesan *Giardia* spp. pada tinja anjing di pusat perlindungan haiwan di sekitar negeri Selangor dengan menggunakan kaedah mikroskopi dan polymerase chain reaction (PCR). Kedua, kaitan penemuan *Giardia* spp. telah dikaji

dengan jenis tinja dalam bentuk cirit-birit. Projek ini telah diluluskan oleh jawatankuasa UPM IACUC dengan persetujuan daripada lima pusat perlindungan haiwan tersebut. Sebanyak 130 ekor anjing telah dipilih secara rawak berdasarkan persampelan mudah. Calitan tinja diperolehi, digolek ke slaid kaca, dikeringkan dan diwarnakan menggunakan kedua-dua Giemsa dan Ziehl-Neelson dan diperiksa menggunakan kaedah mikroskopi. Tujuh puluh sampel tinja adalah memadai untuk digunakan bagi kaedah PCR dan seterusnya gel elektroforesis dilakukan untuk menentukan saiz produk PCR sepadan dengan *Giardia* spp. Analisa statistik dilakukan dengan menggunakan SPSS dengan P-value<0.05 dianggap signifikan. *Giardia* spp. dikesan daripada empat anjing (3.1%) pada penilaian mikroskopik yang disahkan dengan pengesanan PCR. Parasit lain yang dikesan melalui pemeriksaan mikroskopik termasuk *Cryptosporidium* dan coccidia. Sebanyak 17.1% (n=12/70) sampel adalah positif untuk *Giardia* spp. melalui kaedah pengesanan PCR bersarang. Terdapat empat kali pengesanan positif yang lebih tinggi untuk *Giardia* spp. pada anjing yang cirit-birit jika dibandingkan dengan anjing yang tidak mengalami cirit-birit dan ia adalah signifikan secara statistik melalui analisis Pearson chi-squared (P-value= 0.044). Ia adalah disyorkan bahawa semua anjing perlindungan harus diberi ubat cacing secara berkala untuk mencegah jangkitan *Giardia* spp. antara haiwan perlindungan dalam kurungan sama dan mengelakkan potensi jangkitan zoonotik kepada penjaga tempat perlindungan. Pencirian filogenetik untuk *Giardia* spp. pada anjing di Malaysia memerlukan kajian yang lebih.

1.0 INTRODUCTION

Giardia is one of the zoonotic gastrointestinal parasites known that can infect man and animals. The *Giardia* genus belongs to the type Sarcomastigophora, class Zoomastigophorea, order Diplomonadida, family Hexamitidae (Thompson, 2002). *Giardia* spp. is encountered in two forms which are trophozoite and cyst. The motile trophozoite is piriform to oval in shape with bilateral symmetry with a size of 9µm by 5µm to 21µm by 15µm. The extracellular trophozoite of the parasite attaches to upper small intestine enterocytes using a highly specialized ventral adhesive disc (House *et al*., 2011). Cysts on the other hand are oval-shaped with a thin hyaline wall with a size of 8-10µm by 7-10µm. The mature cyst has four nuclei, curved median bodies and longitudinal axonemes whereas immature cyst usually consists of only two nuclei (Ivanov, 2010).

Giardiasis is a chronic, intestinal protozoal infection seen worldwide. Infection is common in dogs, cats, ruminants and pigs. Most dogs and cats harbor subclinical infections; in some, acute watery diarrhea that contains mucus can be noticed. Besides that, steatorrhea can also be observed. Chronic or intermittent diarrhea and weight loss may be noted in animals with concurrent infections or immunocompromise (Lappin, 2014). Although the presence of *Giardia* in the gut may cause diarrhea, many hosts still remain asymptomatic in spite of shedding resistant cysts into the environment (Tysnes *et al*., 2014).

Giardia spp. have been reported in 0.44%-39% of fecal samples from pet and shelter dogs and cats, with higher rates of infection in younger animals (Kahn, 2010). Animal-

to-animal and animal-to-human transmission are major concerns (Olson, 2010). *Giardia* assemblages that infect dogs are assemblage C and D; whereas, cats are assemblage F. When animals share human environment like contaminated water, human assemblages A and B can also be found in feces of dogs and cats (Lappin, 2014). They are usually not associated with clinical disease in humans but they can be detected in a immunocompromised person's feces.

Risk factors for giardiasis in human are such as swimming in contaminated pools, in contact or travel to areas with low hygiene and certain sexual practices (Furness *et al*., 2000). Risk of people being infected from pets has been conflicting reports. An increased risk of infection in relation with exposure to pigs, dogs and cats was found from a case-control study done in England (Warburton *et al*., 1994).

Despite the large population of shelter dogs in Malaysia, not many studies have been carried out in Malaysia on *Giardia* spp. detection in dogs. The most recent study was done by Ngui *et al*., 2014 in Malaysia on the prevalence of various gastrointestinal protozoa including *Giardia* in rural stray dog population in Selangor and Pahang. The prevalence of *Giardia* spp. in rural stray dogs was 13%. Besides that, there is also another study done by Rahman, 1990 in Malaysia on the prevalence of *Giardia* spp. among pet dogs in Penang. An amount of 21.9% of the pet dogs in Penang was detected positive for *Giardia* spp. Most of the studies have shown that there is higher prevalence of *Giardia* spp. in younger animals compared to older animals, diarheatic animals and animals that were kept in high stocking density (Rambozzi, 2007). The insufficiency of current data

on the prevalence of *Giardia* spp. in dogs in Malaysia and the lack of prevalence of *Giardia* spp. in shelter dogs warranted this study.

The objectives of this study were to:-

1. Determine the prevalence of *Giardia* spp. among shelter dogs in Selangor using microscopy and molecular detection techniques.
2. Ascertain the occurrence of infection with *Giardia* spp. in relation to age, gender and fecal consistency among shelter dogs.

The hypothesis of this study is:-

1. There is positive detection of *Giardia* spp. in dogs from shelter population.
2. Dogs with diarrhea have higher positive detection for *Giardia* spp. compared to dogs without diarrhea.

2.0 LITERATURE REVIEW

2.1 Giardiasis in dogs

Giardia spp. is a protozoan in the flagellate group that has been recognized in the feces of animals and man for many years (Lappin, 2014). *Giardia* spp. shed in the feces of animal and human in two morphologic forms which is the rarely observed trophozoite form and the frequently observed resistant cyst form (Hendrix and Robinson, 2006). Trophozoites have two nuclei positioned anteriorly and they are both transcriptionally active. The cytoskeleton involves a ventral adhesive disc, a median body and four pairs of flagella that behave differently during motility (Ankarklev *et al*., 2010). The size of the parasite in the trophozoite stage ranges from 9µm by 5µm to 21µm by 15µm. The mature cysts of *Giardia* spp. are oval in shape and are 8-10µm by 7-10µm in size. The mature cysts have a refractile wall and four nuclei while the immature cysts contain only two nuclei (Hendrix and Robinson, 2006). The trophozoite is motile and is found in the intestinal lumen, whereas the cyst is the transmissible form and is capable of prolonged survival in the environment (Barr, 2006).

Giardia spp. are usually transmitted through the faecal–oral route and can cause gastroenteritis in vertebrates, including mammals, birds, reptiles and fishes (Adam, 2013). This disease is usually transmitted from human-to-human (anthroponotic) or animal-to-human (zoonotic) (Xiao and Fayer, 2008 and Feng and Xiao, 2011). In dogs and cats, they can be either asymptomatic or can have diarrhea due to maldigestion, malabsorption and increased motility (Jarvinen, 2007). Giardiasis is seen commonly in

younger animals and in shelter populations compared to owned animals (Itoh, 2009). On the other hand, in humans, symptoms include abdominal cramping, nausea, vomiting, steatorrhea, anorexia and weight loss (Ortega and Adam, 1997).

2.2 Prevalence of giardiasis in Malaysia

In Malaysia, a recent study done on the *Giardia* prevalence in rural stray dogs and cats in Selangor and Pahang state showed a prevalence of 13% in rural stray dogs and 10.7% in rural stray cats. *Giardia* spp. was found as the most prevalent protozoan detected in the rural stray cats population (Ngui *et al*., 2014). Besides that, another study done in pet dog population in Penang showed 21.9% of the pet dogs were infected with *Giardia*.

Giardiasis in human population in Malaysia is considered an endemic infection. The prevalence varies according to the population studied and the infection is predominantly seen in children, especially in the underprivileged community (Norhayati *et al*., 1998, Al-Mekhlafi *et al*., 2005 and Mohammed *et al*., 2008). It was reported in a study that the highest prevalence were in the Proto-Malays (33.3%) followed by Negritos (20.1%) and Senois (10.4%) and the positive cases showed a decrease with increasing age. Most of the positive cases were observed in individuals less than 24 years old. Male is also reported to have significantly higher prevalence than females (Anuar *et al*., 2012). Besides that, a recent study done in Malaysia also identified that drinking tap water and consuming raw vegetables as risk factors for giardiasis (Mohammed *et al*., 2008). This probably caused by the contamination of animal feces such as dogs and cats. Besides that,

there is also a possibility of contaminated manure used as fertilizer during vegetables planting.

2.2 Prevalence of giardiasis in other countries

One of the most common parasites of dogs and cats around the world is *Giardia duodenalis.* A recent survey in Australia found *Giardia* cysts in 9.3% of 1400 dogs studied (Palmer *et al*., 2008). On the other hand, a survey of dogs and cats in the United States using a commercial ELISA-based test revealed a positive antigen result in the feces of 15.6% of 16,114 dogs that were considered symptomatic by the testing clinic (Carlin *et al*., 2006). *Giardia* spp. of dogs in Brazil reported a prevalence of 16.9% (Katagiri and Oliveira-Sequeira, 2007). In Europe, the prevalence of some 0.3–36% of dogs and cats have patent infections with up to 70% of dogs being infected during the first year of life (Tenter and Deplazes, 2006).

In other species like cats, a study done in New Zealand recorded a high prevalence of 32% of giardiasis (Kingsbury *et al*., 2011); whereas, in Germany, the prevalence was as high as 46% (Pallant *et al*., 2015). Another study done in Florida showed that the prevalence of *Giardia* spp. was 20% in cats with diarrhea and 8% in cats without diarrhea (Sabshin *et al*., 2012).

In domestic livestock, *Giardia duodenalis* infections are highly prevalent in domestic ruminants throughout the world (reviewed by O'Handley and Olson, 2006). Many reports indicate *Giardia* infections occur in lambs and calves with a prevalence of up to 100%

(Buret *et al*., 1990a, Taylor *et al*., 1993, Xiao and Herd, 1994, Olson *et al*., 1997a, Olson *et al*., 1997 and O'Handley *et al*., 1999). Calves as young as 4 days old can become infected with *Giardia* and this infection is reported to be able to persist for more than 4 months (Xiao and Herd, 1994 and O'Handley *et al*., 1999). *G. duodenalis* infections are also associated with the occurrence of diarrhea and ill thrift in calves although subclinical infections are also frequently reported (O'Handley *et al*., 1999, Geurden *et al*., 2006).

In humans, *G. duodenalis* has a global distribution. It is the most common intestinal parasite of humans in developed countries. In Asia, Africa and Latin America, about 200 million people have symptomatic giardiasis with some 500 000 new cases reported each year (WHO, 2006). In Latin America, recent studies from Venezuela (2008) have found giardiasis prevalence in human ranges from 7.41% to 7.69% (Quintero, Duran and Duri, 2012). In Cuba, the last national survey carried out in 2009 estimated an overall prevalence of *Giardia* infection of 6.02% (Rojas *et al*., 2012). Besides that, a published study in Colombia reported a point-prevalence of 11.17% in children (1–5 years old) from day care centers in Ibagué Tolima (Rodriguez, 2014). In Poland, according to the National Institute of Public Health–National Institute of Hygiene (NIPH-NIH) there is about 2000 symptomatic cases of giardiasis annually registered and it is most frequent in children and adolescents.

2.3 Detection method of *Giardia* spp.

In previous studies, test evaluation for the diagnosis of *G. duodenalis* in dogs used either microscopic examination (Hopkins *et al*., 1993), ELISA (Cirak and Bauer,

2004 and Dryden *et al*., 2006) or PCR as gold standard reference test. Microscopic examination (ME) has never been properly evaluated, although it is traditionally considered the gold standard reference test for the diagnosis of *G. duodenalis* (Hopkins *et al*., 1993). Recent studies have shown that the sensitivity of ME is lower compared to more recently developed diagnostic techniques such as PCR and ELISA (Cirak and Bauer, 2004, Geurden *et al*., 2004, Gundlach *et al*., 2005 and Dryden *et al*., 2006). Due to the sensitivity of the test, IFA was found to be the most preferred test for the diagnosis of *G. duodenalis* in dogs, both in epidemiological studies and for clinical diagnosis. The high IFA sensitivity may be due to the initial concentration of the feces in the IFA protocol which is not done in the SNAP® protocol. With the development of new diagnostic techniques, such as the polymerase chain reaction (PCR) and the enzyme-linked immunosorbent assay (ELISA) for detecting gastrointestinal parasites, the sensitivity of tests in detecting protozoas, particularly protozoa, has increased remarkably (Jenkins, 2000).

2.4 Treatment and control of *Giardia* spp.

As for the treatment of *Giardia* infection in dogs, fenbendazole, at the nematocidal label dosage, is an effective drug (Zajac, 1998). Treatment of *Giardia*-infected dogs and cats should be given, whether or not they are clinically ill, because of the potential for zoonotic transmission. If *Giardia* is found on faecal examination, it should be treated, regardless of whether the animal is ill or asymptomatic. Treatment is necessary, given the zoonotic potential of this parasite. According to study done by Barr *et al*., 1993b, it is shown that

albendazole was effective in treating dogs with *Giardia* infection. Oral metronidazole has also been used often to treat giardiasis in dogs and cats. However, detrimental side effects such as the acute development of anorexia and vomiting with progression to signs of central nervous system toxicity have been associated with this drug (Dow *et al.*, 1989). On the other hand, according to Zimmer and Burrington, 1986, metronidazole has shown to be only 67% effective in removing *Giardia* from infected dogs and its regular usage may cause resistance to the drug. Besides that, fenbendazole given at a dosage of 50 mg/kg, orally, once a day for 3 days is effective at ceasing the shedding of *Giardia* cysts (Barr and Bowmann, 1994). Fenbendazole is also shown to be successful in eliminating hookworms, whipworm and roundworms, and it is safe to be administered in pregnant dogs and puppies (Barr and Bowmann, 1994).

The recent vaccine development against *Giardia* able to reduce the duration of shedding of cysts and this can provide an effective method for reducing carrier rates in animals and can prevent future environmental contamination. This is particularly important to immunocompromised individuals who are dealing with infected animals (Olson *et al*., 2000). On the other hand, in humans, nitroimidazole drugs such as metronidazole, tinidazole, and ornidazole are highly effective in eliminating *Giardia* infection. Metronidazole can be given at a 5 to 7 day course and it is expected to cure over 90% of individuals, and a single dose of tinidazole or ornidazole will cure a similar number. Quinacrine, which is no longer produced in the United States, has extremely good

efficiency but may be poorly tolerated, especially in young children (Gardner and Hill, 2001).

Good husbandry, including the frequent removal of feces from animal's environment is likely to minimize the chances of re-infection and transmission of *Giardia* in all species. In order to eliminate *Giardia* in the environment effectively, quaternary ammonium compound products (QATS) which are found in some household cleaning products can be used. Besides that, bleach mixed with water (3/4 cup of bleach to 1 gallon of water) is also sufficient to remove *Giardia* from the environment (Jenkins *et al*., 2001).

Great impact had been made by molecular epidemiology on the taxonomy of *Giardia* at both the species and intraspecific levels. With this, we are able to analyze the risk factors for public health from parasitic infections in companion animals and livestock (Thompson *et al*., 2008). Owners, on the other hand, should be given advice on the risks of *Giardia* infection from their dogs, particularly in young children, immunocompromised individuals or pregnant women (Mircean *et al*., 2012).

3.0 MATERIALS AND METHOD

3.1 Sample population and ethics approval

A total of 130 dogs were sampled from five different animal shelters in Selangor. This study was approved by the Institutional Animal Care and Use Committee of Universiti Putra Malaysia. The approval code is UPM/IACUC/FYP.2016/FPV(14,47).

3.2 Sample collection

Fresh fecal samples were collected from dogs randomly selected in the shelters. Sterile swabs were used to collect the fresh fecal sample. Fecal samples were smeared onto two clean glass slides each and the glass slides were air-dried and fixed with methanol for 5 minutes before staining it with 5% Giemsa or Ziehl-Neelson stain. The tip of the sterile swabs were then cut and soaked in a 1.5mL Eppendorf Tube® filled with 700µL lysis buffer solution and was transported on ice and later stored in 20°C freezer. The lysis buffer is made by adding 121.4g of Tris HCL pH8 into 500mL distilled water. Then the mixture was stirred and the pH was adjusted by using HCL until it reaches pH8. Then, distilled water was topped up to 950mL and the mixture was autoclaved. Lastly, 50mL of Sodium Dodecyl Sulphate (SDS) was added before the buffer was being kept in the fridge. The mixture was then diluted (1x) with distilled water before using.

3.3 Microscopic Examination

For 5% Giemsa staining method, the slide was immersed in a freshly prepared 5% Giemsa stain solution for 30 minutes, then flushed with tap water and left to dry at room

temperature. On the other hand, for the acid-fast staining method, the slides were flooded with Carbol Fuchsin for 5 minutes, and rinsed off in tap water until all traces of red are gone from thin part of smear. Then, the slides were flooded with 1% acid alcohol for 3 seconds to remove the Carbol Fuchsin, thus removing the stain from cells that are unprotected by a waxy lipid layer. Lastly, the cells were stained in Loeffer's methylene blue solution for 30 seconds. Fecal specimens were then examined under light microscope to detect for the presence of *Giardia* cyst and trophozoite.

3.4 Polymerase Chain Reaction (PCR)

3.4.1 Extraction of genomic DNA

Total gDNA was extracted using a commercially available kit called QIAamp® Fast DNA stool Mini Kit (QIAGEN®, Germany) by following the instructions provided by the manufacturer. An amount of 250µL of stool together with the buffer solution was placed in a 2mL microcentrifuge tube and was placed on an ice. One mL of QIAGEN® InhibitEX Buffer (QIAGEN®, Germany) was added to each stool sample. The stool samples were vortex for 1 minute until the stool samples were thoroughly homogenized. Then, the suspension was heated for 5 minutes at 70°C. Samples were then centrifuged for 1 minute to pellet stool particles. A total of 15µL of QIAGEN® Proteinase K (QIAGEN®, Germany) was pipetted into a new 1.5mL microcentrifuge tube. Then, two hundred µL of supernatant was then pipetted from step 4 into the 1.5mL microcentrifuge tube containing QIAGEN® Proteinase K (QIAGEN®, Germany). Next, 200 µL

QIAGEN® Buffer AL (QIAGEN®, Germany) was added and vortex to homogenize the sample. Then, the sample was incubated at 70°C for 10 minutes. Later, 200µL of ethanol was added to the lysate and the lysate was vortex before a total volume of 600µL lysate was carefully added into the QIAamp® spin column (QIAGEN®, Germany) and centrifuged for 1 minute. Then, the QIAamp® spin column (QIAGEN®, Germany) was placed in a new 2mL collection tube and the tube containing the filtrate was discarded. The QIAamp® spin column (QIAGEN®, Germany) was carefully opened and 500µL QIAGEN® Buffer AW1 (QIAGEN®, Germany) was added in to the QIAamp® spin column and was centrifuged for another 1 minute. Then the QIAamp® spin column was placed in a new 2mL collection tube and the tube containing the filtrate was discarded. The QIAamp® spin column was carefully opened and 500µL QIAGEN® Buffer AW2 (QIAGEN®, Germany) was added and was centrifuged for 3 minutes. The centrifuge containing the filtrate was discarded. Then the QIAamp® spin column (QIAGEN®, Germany) was placed in a new 2mL collection tube and the old collection tube with the filtrate was discarded and was centrifuged for 3 minutes. Then, the QIAamp® spin column (QIAGEN®, Germany) was transferred into a new, labeled 1.5mL microcentrifuge tube and 200µL of QIAGEN® Buffer ATE (QIAGEN®, Germany) was pipette directly onto the QIAamp® membrane (QIAGEN®, Germany) and was incubated for 1 minute at room temperature before centrifuging it for 1 minute to elute DNA.

3.4.2 Quantification of gDNA

Quantification of gDNA was performed using spectrophotometer via ultraviolet absorbance. Blank containing the ultrapure water was prepared separately for calibration purposes. Nuclei acids were diluted in ultrapure water at 1:25 dilution and measured in ng/µL at 260 and 280nm wavelengths.

3.4.3 Nested Polymerase Chain Reaction (PCR)

PCR is done to amplify the region of interest in this study. A total of 4µL of sample DNA, 5µL each of 5X green GoTaq® Flexi Buffer (Promega, US), 5µL each $MgCl_2$ 25mM solution, 0.3µL of each deoxynucleoside triphosphate (dNTP), 1µL each of forward primer P1 (5'-CATCCGGTCGATCCTGCC-3') and reverse primer P2 (5'-AGTCGAACCCTGATTCTCCGCCAGG-3') (Lim *et al*., 2013), 0.3µL each of GoTaq® DNA polymerase 5u/uL and 8.4µL of nuclease-free water (ddH20) were mixed and subjected to PCR. The reaction included 95°C for 2 minutes for initial denaturation, followed by 35 cycles at 95°C, 59°C and 72°C for 20 seconds each and by one cycle at 72°C for 7 minutes for final extension. PCR reactions were done in a thermal cycler (BioRad, US).

Nested PCR amplification was next carried out in 25µL containing 4µL of the PCR products from the gDNA templates generated in the first round of PCR, 5µL each of 5X green GoTaq® Flexi Buffer (Promega, US), 2.5µL each $MgCl_2$ 25mM solution, 0.3µL of each deoxynucleoside triphosphate (dNTP), 1µL each of forward primer P1 (5'-

GACGCTCTCCCCAAGGAC-3') and reverse primer P2 (5'-CTGCGTCACGCTGCTC-3'), (Lim *et al*., 2013), 0.3µL each of GoTaq® DNA polymerase 5u/uL and 10.9µL of nuclease-free water (ddH20) were reacted in a thermal cycler at 95°C for 2 minutes for initial denaturation, which was followed by 35 cycles at 95°C, 59°C and 72°C for 20 seconds each and by one cycle at 72°C for 7 minutes for final extension. The PCR product was stored at -20°C for further analysis.

3.4.5 Gel electrophoresis

The products were then visualized by electrophoresis on a 1.5% agarose gel following 15minutes Ethidium Bromide staining on Molecular Imager® Gel Doc™ XR System (Bio-Rad®, US) with UV illuminator. The 1.5% agarose gel was made by mixing 1.5g Hyagarose™ (HydraGene) powder with 100 mL 1xTAE in a microwavable flask. Then, the mixture was microwaved for 1-3 min until the Hyagarose™ powder was completely dissolved. Then, the solution was let to cool down to about 50°C before pouring the solution into a gel tray with the well comb in place. The newly poured placed at room temperature for 20-30 minutes to let it completely solidified. An amount of 0.5µL of Vivantis™ VC100bp Plus DNA ladder (Vivantis™, Malaysia) was loaded into the first well, followed by 0.5µL of positive control and negative control on the second and third well. 0.7µL samples were then loaded into the additional wells of the gel. The gel was run at 110 V for 45minutes until the dye line is around 75-80% of the way down the gel.

3.5 Statistical analysis

Statistical test was done using Statistical Package for the Social Sciences (SPSS). Pearson's chi-squared test was done by analyzing a 2×2 contingency tables at 95% confidence level. The occurrence of *Giardia* was evaluated with respect to age, gender, fecal consistency and *Giardia* detection on polymerase chain reaction (PCR) assay.

4.0 RESULTS

4.1 Demographics

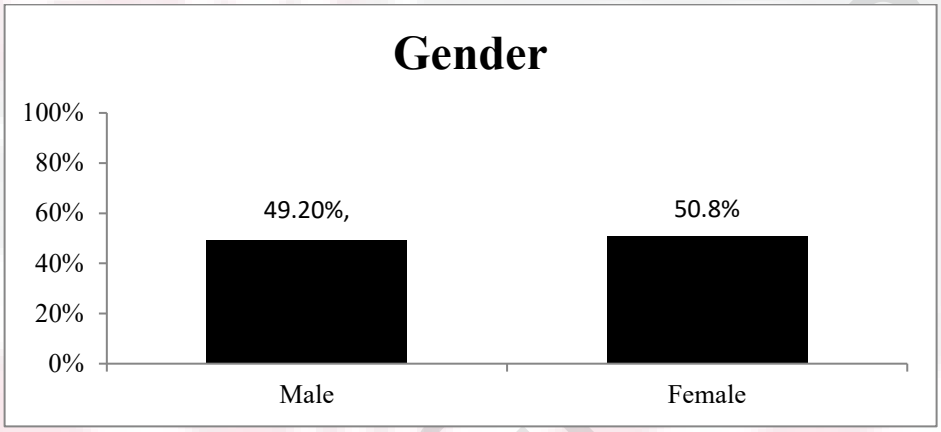

Figure 4.1: Gender of the dogs in 5 different shelters in Selangor

Out of 130 fecal samples collected, 64 fecal samples were collected from male dogs; whereas, 66 samples were collected from female dogs.

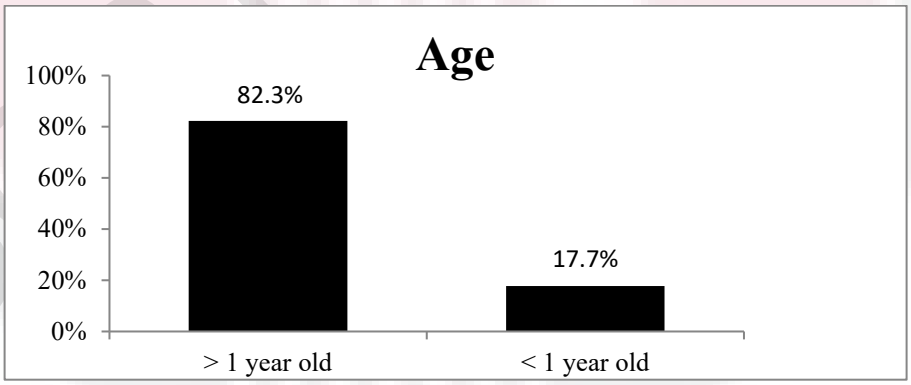

Figure 4.2: Age of the dogs in 5 different shelters in Selangor

Out of 130 fecal samples collected, 107 samples were collected from dogs aged more than 1 year old; whereas, 23 samples were collected from dogs less than 1 year old.

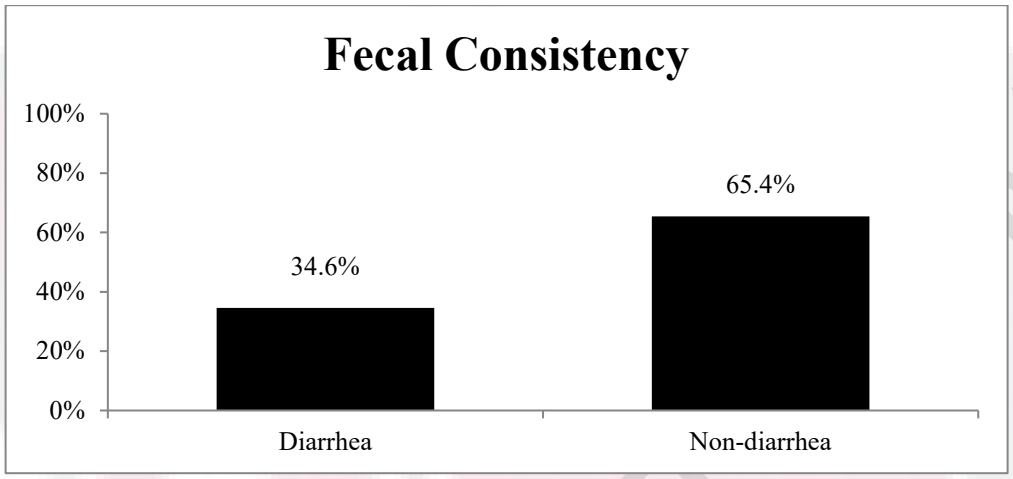

Figure 4.3: Fecal consistency of the dogs in 5 different shelters in Selangor

For the fecal consistency of dogs, 45 fecal samples were taken from dog with diarrhea; whereas, 85 samples were taken from dogs without diarrhea.

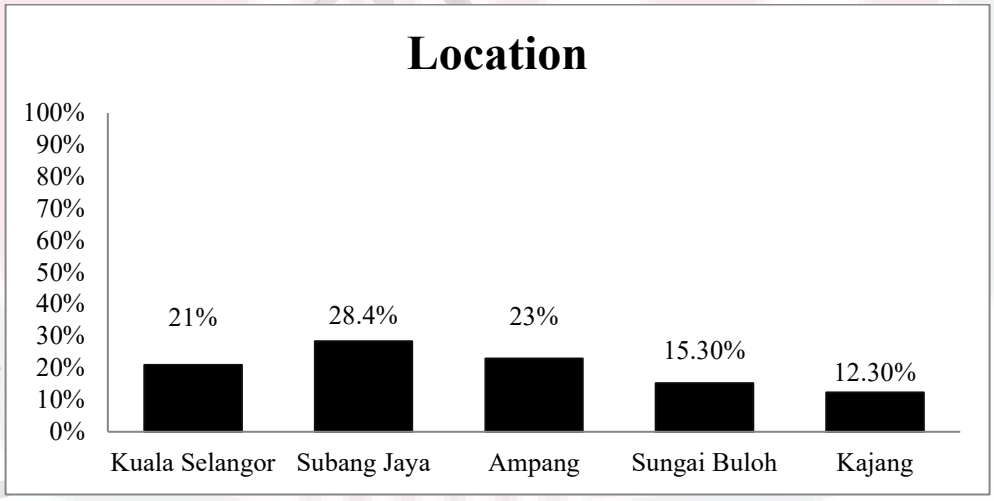

Figure 4.4: Number of dogs from each shelter in Selangor

Out of 130 fecal samples collected, 27 samples were from Kuala Selangor, 37 samples from Subang Jaya, 30 samples from Ampang, 20 samples from Sungai Buloh and 16 samples from Kajang.

4.2 Microscopic Examination

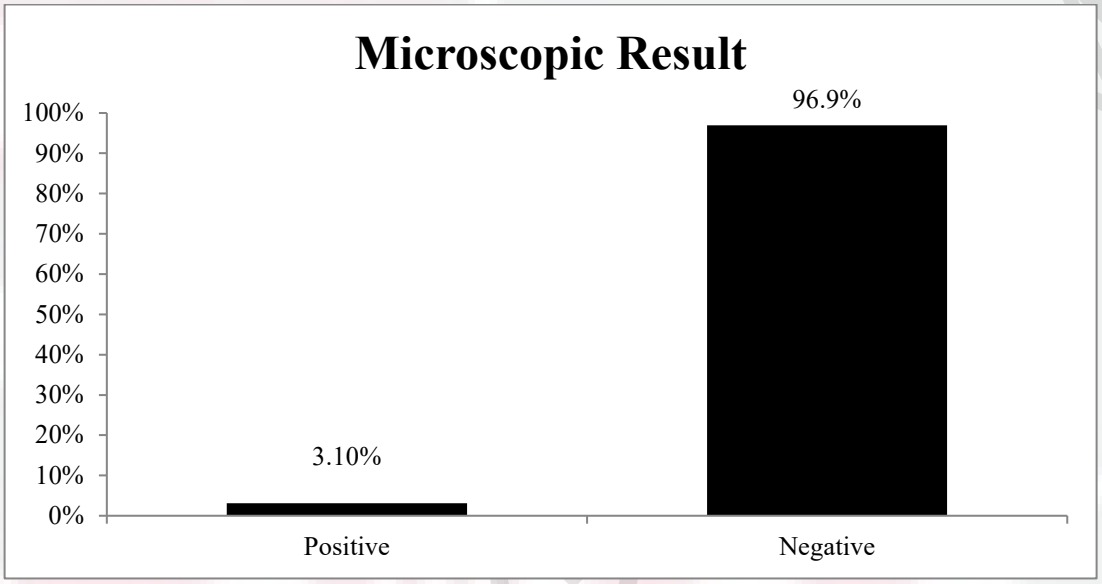

Figure 4.5: Microscopic results from 130 dogs that were examined

For the microscopic result from 130 dogs examined, 4 dogs were positive for *Giardia* spp.

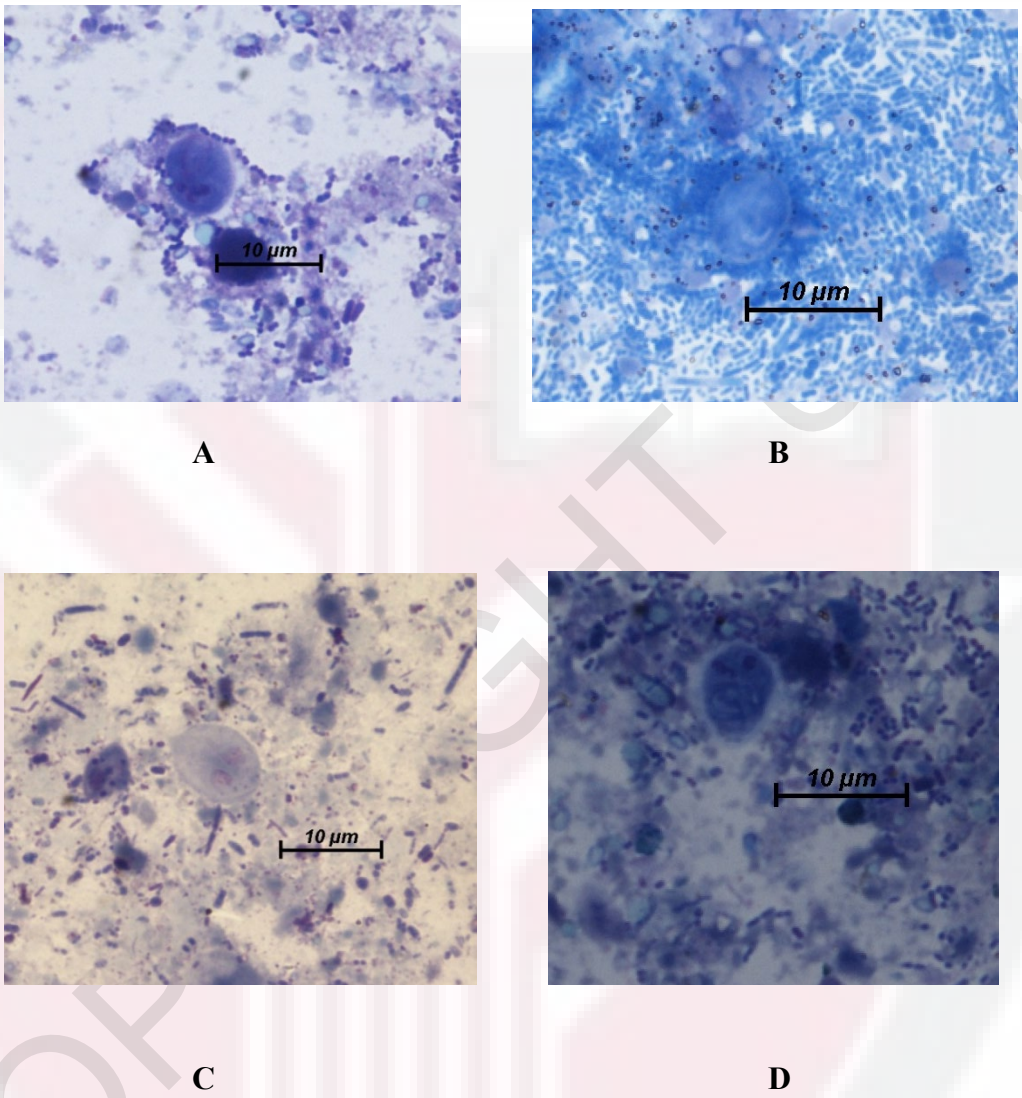

Figure 4.6: (A) *Giardia* spp. cyst found in a dog from a shelter in Kuala Selangor. **(B)** *Giardia* spp. cyst found in a dog from shelter in Subang Jaya. **(C)** *Giardia* spp. trophozoite found in a dog from shelter in Subang Jaya. **(D)** *Giardia* spp. cyst found in a dog from shelter in Sungai Buloh.

4.3 Polymerase Chain Reaction (PCR)

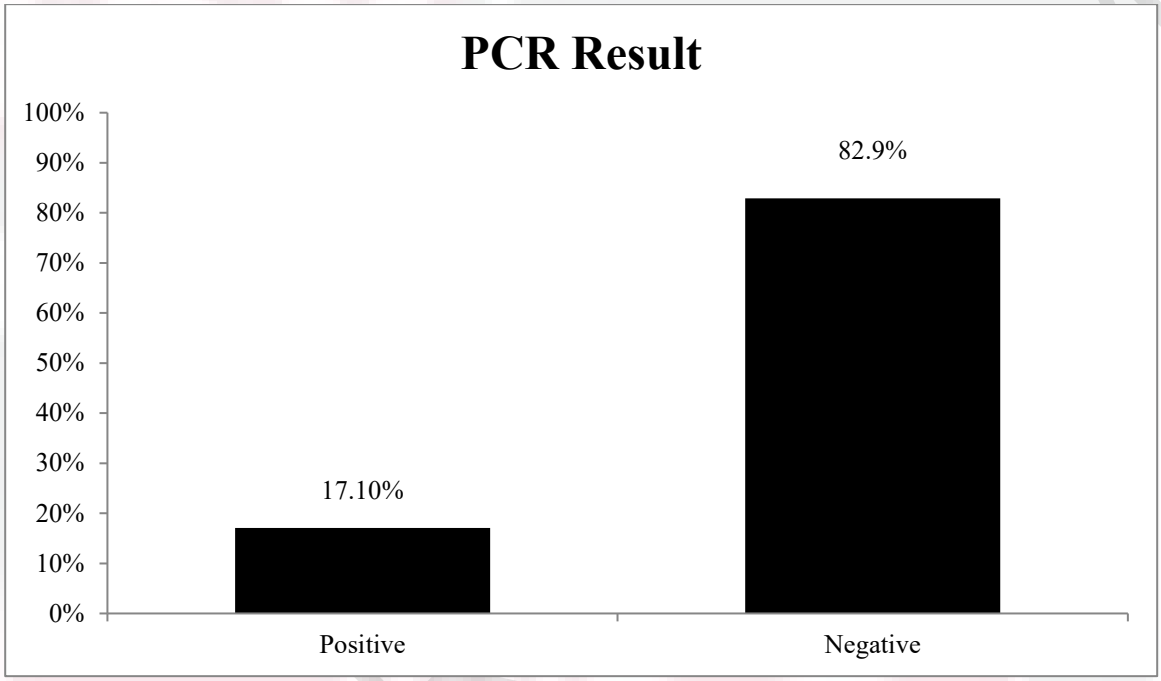

Figure 4.7: PCR results from 70 dogs that were examined

For the Polymerase Chain Reaction (PCR) result from 70 dogs examined, 12 dogs were tested positive for *Giardia* spp.

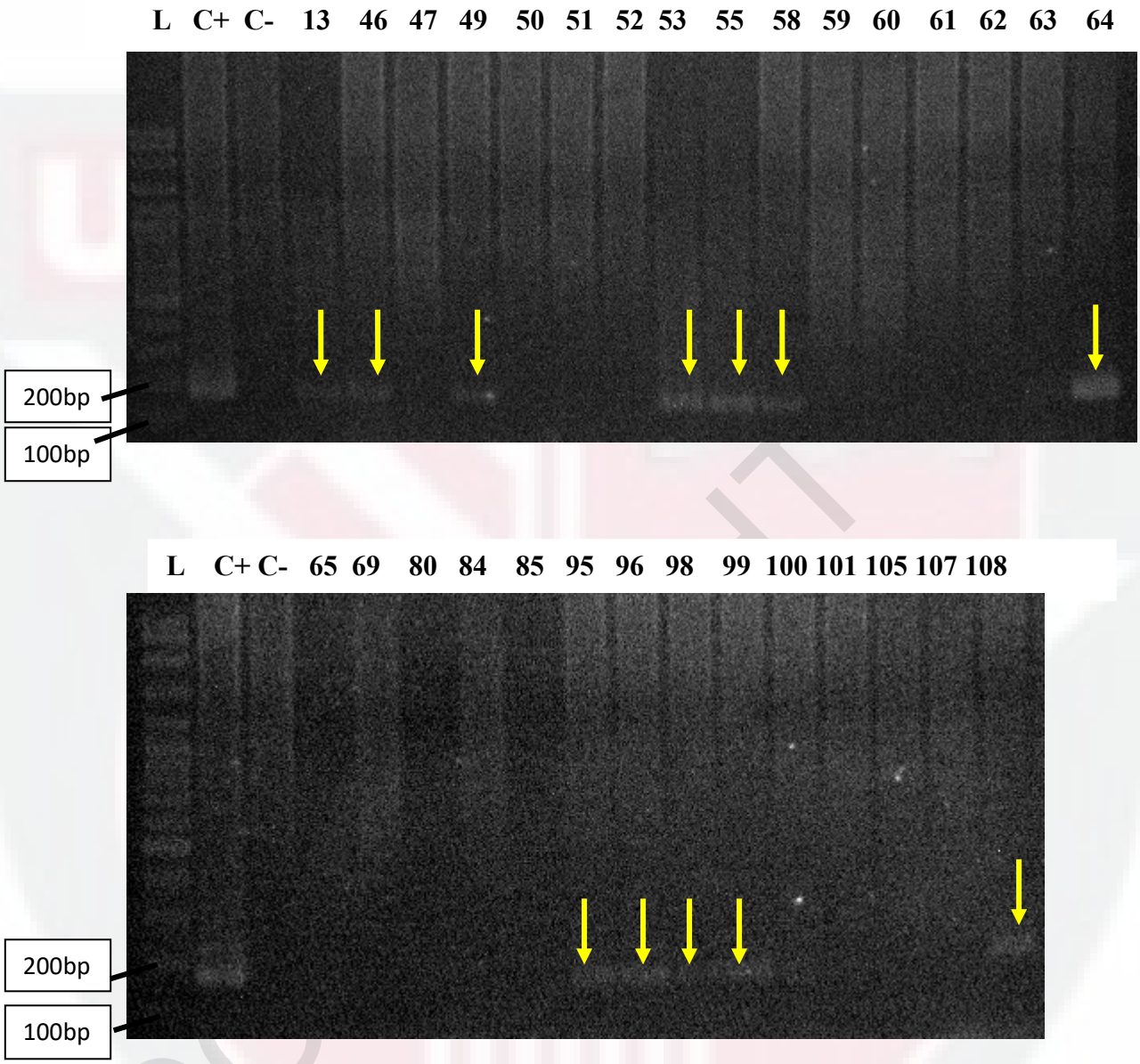

Figure 4.8: *Giardia* spp. positive samples detected on PCR assay (as shown in arrow). *Giardia* spp. generic DNA was detected in a 1.5% agarose gel electrophoresis showing band size of *Giardia* spp. which is 200bp. Lane L; VC 100bp plus DNA ladder

(Vivantis™, Malaysia), Lane C+; Positive control, Lane C-; Sterile distilled water (Negative control).

4.4 Statistical Analysis

Statistical data analysis was done using Pearson's chi-squared analysis to determine the association between animal variables such as gender, age and fecal consistency with presence of *Giardia* spp. Pearson's chi-squared test was done at 95% confidence level.

Table 4.9: The association of animal variables (gender, age and occurrence of diarrhea) with the presence of *Giardia* spp.

		***Giardia* PCR**		**P-value (2-sided)**
		Positive	**Negative**	
Gender	**Male**	9/70 (12.9%)	30/70 (42.9%)	P= 0.14
	Female	3/70 (4.3%)	28/70 (40%)	
Age	**< 1 year old**	2/70 (2.3%)	7/70 (10%)	P=0.665
	> 1 year old	10/70 (14.3%)	51/70 (72.9%)	
Fecal consistency	**Diarrhea**	9/70 (12.3%)	25/70 (35.8%)	P= 0.044
	Non-diarrhea	3/70 (4.3%)	33/70 (47.1%)	
Microscopic result	**Positive**	4/70 (5.8%)	0/70	P<0.001
	Negative	8/70 (11.4%)	58/70 (82.9%)	

Dogs with diarrhea have higher positive detection for *Giardia* spp. by using PCR compared to dogs without diarrhea. This was statistically significant at P-value= 0.044. All 4 dogs that were positive in microscopic result were also positive in *Giardia* detection through PCR, the rest were only positive on PCR. This was also statistically significant at P-value<0.001.

4.5 Other Gastrointestinal Parasites detected on Microscopic Examination

Other gastrointestinal parasites detected were such as *Cryptosporidium* and coccidia. Out of 130 slides that were examined, 27 samples were found to be infected with coccidia with a percentage of 20.8%. On the other hand, out of 130 slides that were examined, only 2 samples were found to be infected with *Cryptosporidium*, with a percentage of 1.5%.

5.0 DISCUSSION

Detection of *Giardia* spp. was done through microscopic and polymerase chain reaction (PCR) methods among shelter dogs in Selangor. Both domestic and stray dogs may be a potential source of infection for humans. Recent surveys of gastrointestinal parasites in dogs have demonstrated high levels of *Giardia* in stray as well as domestic dogs with a prevalence of 7.2 –22.1% in both developing and developed countries (Bugg *et al*., 1999, Itoh *et al*., 2001, Jacobs, Forrester & Yang, 2001, Sequeira *et al*., 2002).

In this study, a total of 130 fecal samples from dogs kept in 5 different animal shelters were collected. Fewer samples were collected from dogs less than one year of age compared to adult dogs. The reason is because puppies are more frequently being adopted by the public compared to adult dogs. Besides that, younger puppies are also more susceptible to diseases and could die easily from diseases. The puppies that are kept for adoption purposes at the shelters would have been dewormed in the recent months hence reducing the chances of detection. This result corroborates previous studies which have shown a negative linear relationship was discovered between dogs' age and proneness for adoption (Lepper, 2002). This is similar to Nemcova & Novak's, 2003, finding that the majority of dogs adopted from the shelters were usually two years old or younger. This was further supported by Wells, 2000, stating that, puppies are the highest to be purchased from the animal shelter because puppies seldom exhibit fearfulness, sexual problems and straying tendencies than juvenile or adult dogs. However, according to Hamnes *et al*., 2007, the individual prevalence for *Giardia* infection in dogs was between 6.0% and

11.4%, with the highest level in dogs more than 6 months old, but the differences between age groups were not statistically significant. In this study, it was found that there was no association between dogs' age and PCR detection on *Giardia* spp. although it is biased to make such an assumption due to too small sample size.

According to the result in this study, dogs with diarrhea have higher positive detection for *Giardia* spp. by using PCR technique compared to dogs without diarrhea. This was statistically significant at P-value equals to 0.044. According to Carlin *et al*., 2006, based on the IDEXX SNAP® Test (IDEXX, US), *Giardia* is common in dogs presenting with gastrointestinal disease such as vomiting and/or diarrhea. The same study also revealed that 15.6% of 16,114 dogs were considered symptomatic by the tests done by the clinic using a commercial ELISA-based test to test for positive antigen result in the fecal samples. According to Barr, 2006, acute diarrhea, when seen, tends to occur in very young dogs and cats; in older animals, diarrhea may be acute, intermittent, or chronic.

In this study, only 33.3% of the PCR positive samples were positively detected by microscopy. This was also statistically significant at P-value<0.001. This may be compounded by the fecal smear quality and the skill and experience of the observer. According to Verveij *et al*., 2003, microscopy is considered to be the gold standard for diagnosis of *G. lamblia* infection. However, microscopy is time-consuming and not sensitive. The real-time PCR is more specific and sensitive than microscopy. Besides that, the sensitivity of the microscope depends highly on the number of samples examined and finally on the skills and experience of the technician. Another study by Gotfred-

Rasmussen *et al*., 2016, also stated that qPCR and immunofluorescence assay (IFA) were significantly more sensitive than microscopy of iodine-stained concentrates. It is seen that the major predictor for prevalence rate of *Giardia* spp. is the diagnostic method used, with standard microscopy rated very poor against ELISA (226% higher), IFA (248%) and PCR (242%) in dogs (Bouzid *et al*., 2015). In contrast, Schuurman *et al*., 2007, found qPCR, rapid immunoassay, and microscopy to be equally sensitive in a study that compared samples initially detected positive for *G. duodenalis* by microscopy.

Giardia is a microscopic parasite that causes the diarrheal illness known as giardiasis. *Giardia duodenalis* is also known as *Giardia intestinalis* or *Giardia lamblia*. It is found on surfaces or in soil, food, or water that has been contaminated with feces from infected humans or animals. For further study, gene sequencing of the positive samples can be done. *Giardia duodenalis* (assemblage A) usually affects humans and other primates, dogs, cats, livestock, rodents and other wild animals whereas *Giardia canis* (assemblages C and D) affects dogs and other canids. According to Ballweber *et al*., 2010, the potential zoonotic risk from dogs arises from the detection of assemblage A and B in dogs. The scale of this risk may differ in locations, especially in those locations that frequently contaminated with human feces. Assemblage C, D, E, F, G, and H on the other hand are defined as host restricted (Li *et al*., 2016; Thompson, 2000). Some reports showed that the prevalence of *Giardia* is roughly around 10% in owned dogs, 36 to 50% in puppies and up to 100% in shelters and kennels (Simonato *et al*., 2015). Assemblages C and D seem to be predominant in dogs with many studies indicating high prevalence of these assemblages in owned and stray dogs (Berrilli *et al*., 2004, Claerebout *et al*.,

2009, Lebbad *et al*., 2010 and Upjohn *et al*., 2010). However, the identification of assemblages A and B in the dogs examined in the present study is not extremely surprising as studies in Brazil, Thailand and Mexico only identified zoonotic assemblages in dogs (Lalle *et al*., 2005, Traub *et al*., 2004 and Volotão *et al*., 2007). In a study in Germany, a high prevalence of assemblage A also was found in owned dogs (Leonhard *et al*., 2007) while in studies conducted in overcrowded communities in India and Thailand, assemblage B was identified in dogs (Traub *et al*., 2004 and Inpankaew *et al*., 2007). It has been shown that two transmission cycles exist in domestic urban environments, with the transmission of dog-specific assemblages among dogs and the transmission of assemblage A between pets and humans. The transmission of dog-specific genotypes may be cause by close contact among large numbers of dogs living together and may outdo the transmission of other assemblages. In household dogs, the frequency of transmission between dogs may be lower and infections with potentially zoonotic assemblages in dogs are likely to continue to exist (Thompson and Monis, 2012 and Claerebout *et al*., 2009)

For the treatment of *Giardia* infection, metronidazole is the most commonly used extra-label therapy. Dogs should be treated with metronidazole (25 mg/kg, BID) for 5 days. Besides that, fenbendazole (50mg/kg, SID) for 3 to 5 days are also effective in eliminating *Giardia* infection in dogs. Animal shelters should screen all new animals coming into the shelter to prevent the transmission of disease from the incoming dogs to the other dogs in the shelter. Lastly, volunteers in the shelter should wear gloves when

handling with *Giardia* infected dogs and wash their hands after handling dogs with *Giardia* infection to prevent zoonotic transmission. Quaternary ammonium compound products (QATS) which are found in household cleaning products are able to remove *Giardia* from the environment. Lastly, ¾ cup of bleach mixed with 1 gallon water can also be used to eliminate *Giardia* (Jenkins *et al*., 2001).

6.0 CONCLUSION AND RECOMMENDATION

In this study, the prevalence of *Giardia* spp. among shelter dogs in Selangor was found to be 5.6% by using the microscopic method and 17.1% by using the Polymerase Chain Reaction (PCR) technique. PCR is found to be more sensitive and reliable in detecting *Giardia* spp. in dogs compared to the demonstration of *Giardia* spp. in dogs using a Giemsa stain fecal smear. Besides that, it was also shown that dogs with diarrhea have high positive detection for *Giardia* spp. in PCR.

Further studies should be done to detect the prevalence of *Giardia* spp. by expanding the location of sample collection which comprises not only shelters in Selangor but also shelters in other states of Malaysia. Through this study conducted, treatment can be given to the affected dogs and control and preventive measures can be taken to prevent the transmission of zoonotic diseases from dogs to human.

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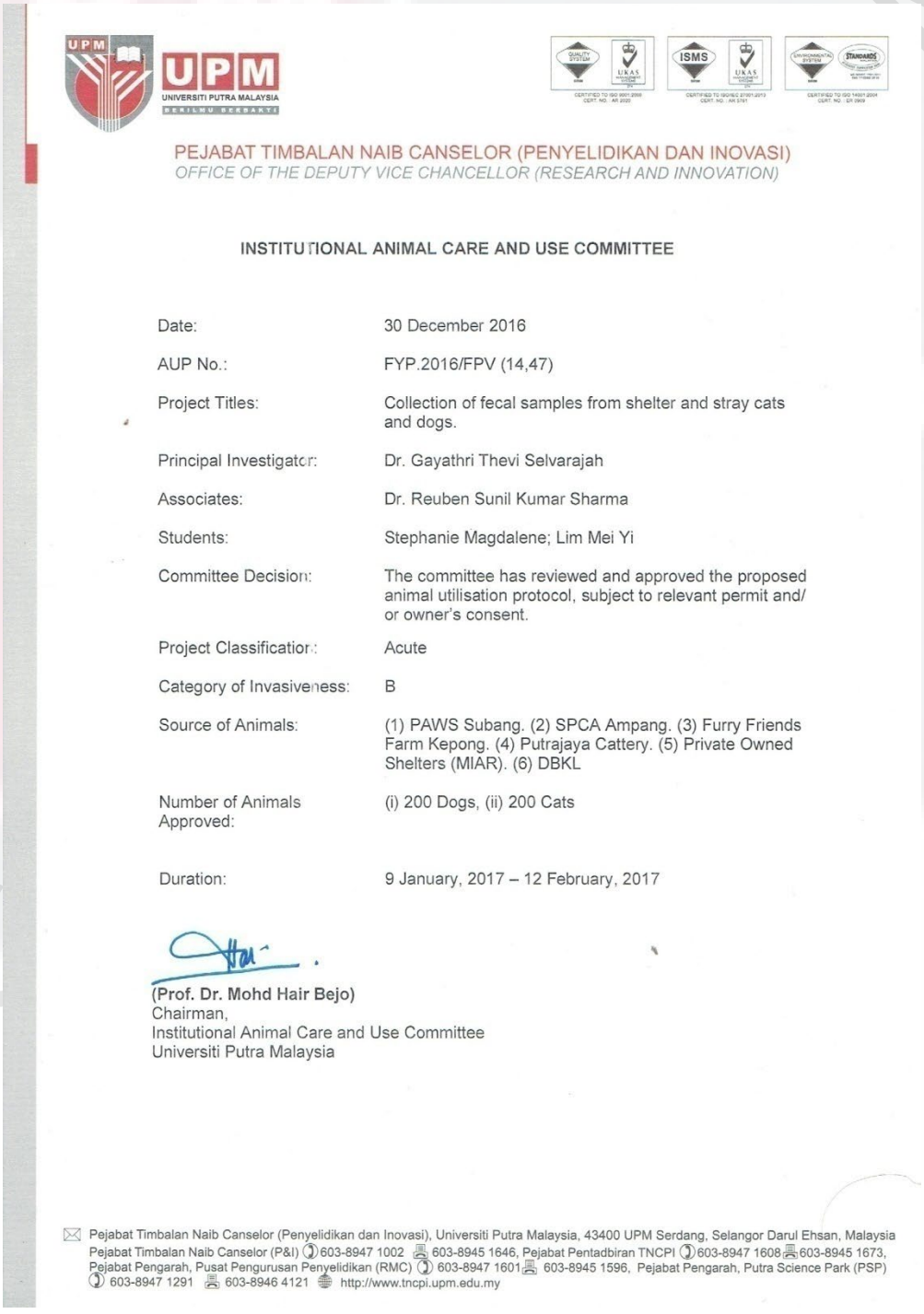
APPENDICES

APPENDIX A: Institutional Animal Care and Use Committee (IACUC) approval letter



UPM
UNIVERSITI PUTRA MALAYSIA
BERILMU BERBAKTI

PEJABAT TIMBALAN NAIB CANSELOR (PENYELIDIKAN DAN INOVASI)
OFFICE OF THE DEPUTY VICE CHANCELLOR (RESEARCH AND INNOVATION)

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

Date:	30 December 2016
AUP No.:	FYP.2016/FPV (14,47)
Project Titles:	Collection of fecal samples from shelter and stray cats and dogs.
Principal Investigator:	Dr. Gayathri Thevi Selvarajah
Associates:	Dr. Reuben Sunil Kumar Sharma
Students:	Stephanie Magdalene; Lim Mei Yi
Committee Decision:	The committee has reviewed and approved the proposed animal utilisation protocol, subject to relevant permit and/ or owner's consent.
Project Classification:	Acute
Category of Invasiveness:	B
Source of Animals:	(1) PAWS Subang. (2) SPCA Ampang. (3) Furry Friends Farm Kepong. (4) Putrajaya Cattery. (5) Private Owned Shelters (MIAR). (6) DBKL
Number of Animals Approved:	(i) 200 Dogs, (ii) 200 Cats
Duration:	9 January, 2017 – 12 February, 2017

(Prof. Dr. Mohd Hair Bejo)
Chairman,
Institutional Animal Care and Use Committee
Universiti Putra Malaysia

Pejabat Timbalan Naib Canselor (Penyelidikan dan Inovasi), Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia
Pejabat Timbalan Naib Canselor (P&I) 603-8947 1002 603-8945 1646, Pejabat Pentadbiran TNCPI 603-8947 1608 603-8945 1673,
Pejabat Pengarah, Pusat Pengurusan Penyelidikan (RMC) 603-8947 1601 603-8945 1596, Pejabat Pengarah, Putra Science Park (PSP)
603-8947 1291 603-8946 4121 http://www.tncpi.upm.edu.my

APPENDIX B: Tabulated information of the 130 dogs sampled for *Giardia* spp.

ID	Gender	Age	Fecal Consistency	PCR Result	Microscopy Result
1	Male	>1 year old	Diarrhea	-	Negative
2	Male	>1 year old	Diarrhea	Negative	Negative
3	Female	>1 year old	Diarrhea	-	Negative
4	Female	>1 year old	Non diarrhea	-	Negative
5	Female	>1 year old	Non diarrhea	-	Negative
6	Female	>1 year old	Diarrhea	Negative	Negative
7	Female	>1 year old	Non diarrhea	Negative	Negative
8	Male	>1 year old	Non diarrhea	-	Negative
9	Male	>1 year old	Non diarrhea	-	Negative
10	Male	>1 year old	Non diarrhea	Negative	Negative
11	Male	>1 year old	Non diarrhea	Negative	Negative
12	Female	>1 year old	Non diarrhea	Negative	Negative
13	Male	>1 year old	Non diarrhea	Positive	Positive
14	Male	>1 year old	Diarrhea	Negative	Negative
15	Female	>1 year old	Non diarrhea	-	Negative
16	Female	>1 year old	Diarrhea	-	Negative
17	Female	>1 year old	Diarrhea	-	Negative
18	Female	>1 year old	Non diarrhea	-	Negative
19	Female	>1 year old	Non diarrhea	-	Negative
20	Male	>1 year old	Non diarrhea	-	Negative
21	Male	>1 year old	Non diarrhea	-	Negative
22	Male	>1 year old	Diarrhea	Negative	Negative
23	Male	>1 year old	Non diarrhea	-	Negative
24	Female	>1 year old	Non diarrhea	-	Negative
25	Female	>1 year old	Diarrhea	Negative	Negative
26	Female	>1 year old	Non diarrhea	Negative	Negative
27	Female	>1 year old	Diarrhea	Negative	Negative
28	Female	>1 year old	Non diarrhea	Negative	Negative
29	Male	>1 year old	Non diarrhea	Negative	Negative
30	Male	>1 year old	Non diarrhea	Negative	Negative
31	Male	>1 year old	Non diarrhea	Negative	Negative
32	Female	>1 year old	Non diarrhea	Negative	Negative
33	Female	>1 year old	Non diarrhea	Negative	Negative
34	Male	>1 year old	Non diarrhea	Negative	Negative
35	Female	>1 year old	Diarrhea	Negative	Negative
36	Female	>1 year old	Non diarrhea	Negative	Negative
37	Female	>1 year old	Diarrhea	Negative	Negative
38	Male	>1 year old	Non diarrhea	Negative	Negative

39	Female	>1 year old	Diarrhea	Negative	Negative
40	Female	>1 year old	Diarrhea	Negative	Negative
41	Female	>1 year old	Non diarrhea	-	Negative
42	Female	>1 year old	Non diarrhea	Negative	Negative
43	Female	>1 year old	Non diarrhea	Negative	Negative
44	Female	>1 year old	Non diarrhea	Negative	Negative
45	Female	>1 year old	Non diarrhea	Negative	Negative
46	Female	< 1 year old	Diarrhea	Positive	Negative
47	Female	< 1 year old	Diarrhea	Negative	Negative
48	Male	< 1 year old	Non diarrhea	-	Negative
49	Male	< 1 year old	Diarrhea	Positive	Negative
50	Male	< 1 year old	Non diarrhea	Negative	Negative
51	Male	< 1 year old	Diarrhea	Negative	Negative
52	Male	< 1 year old	Non diarrhea	Negative	Negative
53	Male	>1 year old	Diarrhea	Positive	Positive
54	Male	>1 year old	Non diarrhea	-	Negative
55	Male	>1 year old	Diarrhea	Positive	Negative
56	Male	>1 year old	Non diarrhea	-	Negative
57	Male	>1 year old	Non diarrhea	-	Negative
58	Male	>1 year old	Non diarrhea	Positive	Positive
59	Male	>1 year old	Diarrhea	Negative	Negative
60	Male	>1 year old	Non diarrhea	Negative	Negative
61	Male	>1 year old	Diarrhea	Negative	Negative
62	Male	>1 year old	Diarrhea	Negative	Negative
63	Male	>1 year old	Diarrhea	Negative	Negative
64	Male	>1 year old	Diarrhea	Positive	Negative
65	Female	< 1 year old	Non diarrhea	Negative	Negative
66	Female	< 1 year old	Non diarrhea	-	Negative
67	Female	< 1 year old	Non diarrhea	-	Negative
68	Female	< 1 year old	Non diarrhea	-	Negative
69	Female	< 1 year old	Non diarrhea	Negative	Negative
70	Female	< 1 year old	Non diarrhea	-	Negative
71	Male	< 1 year old	Non diarrhea	-	Negative
72	Female	< 1 year old	Non diarrhea	-	Negative
73	Female	< 1 year old	Non diarrhea	-	Negative
74	Female	< 1 year old	Non diarrhea	-	Negative
75	Female	< 1 year old	Non diarrhea	-	Negative
76	Female	< 1 year old	Non diarrhea	-	Negative
77	Male	< 1 year old	Non diarrhea	-	Negative
78	Female	< 1 year old	Non diarrhea	-	Negative
79	Female	< 1 year old	Non diarrhea	-	Negative
80	Female	>1 year old	Non diarrhea	Negative	Negative

81	Male	>1 year old	Non diarrhea	-	Negative
82	Male	>1 year old	Non diarrhea	-	Negative
83	Male	>1 year old	Non diarrhea	-	Negative
84	Male	>1 year old	Non diarrhea	Negative	Negative
85	Male	>1 year old	Non diarrhea	Negative	Negative
86	Female	>1 year old	Non diarrhea	-	Negative
87	Male	>1 year old	Non diarrhea	-	Negative
88	Male	>1 year old	Non diarrhea	-	Negative
89	Female	>1 year old	Non diarrhea	-	Negative
90	Female	>1 year old	Non diarrhea	-	Negative
91	Female	>1 year old	Non diarrhea	-	Negative
92	Male	>1 year old	Non diarrhea	-	Negative
93	Female	>1 year old	Non diarrhea	-	Negative
94	Female	>1 year old	Non diarrhea	-	Negative
95	Male	>1 year old	Non diarrhea	Positive	Negative
96	Male	>1 year old	Diarrhea	Positive	Positive
97	Female	>1 year old	Diarrhea	Negative	Negative
98	Female	>1 year old	Diarrhea	Positive	Negative
99	Female	>1 year old	Diarrhea	Positive	Negative
100	Female	>1 year old	Non diarrhea	Negative	Negative
101	Male	>1 year old	Non diarrhea	Negative	Negative
102	Female	>1 year old	Non diarrhea	-	Negative
103	Female	>1 year old	Diarrhea	-	Negative
104	Male	>1 year old	Diarrhea	-	Negative
105	Female	>1 year old	Non diarrhea	Negative	Negative
106	Male	>1 year old	Diarrhea	-	Negative
107	Female	>1 year old	Diarrhea	Negative	Negative
108	Male	>1 year old	Diarrhea	Positive	Negative
109	Female	>1 year old	Non diarrhea	-	Negative
110	Male	>1 year old	Non diarrhea	-	Negative
111	Female	>1 year old	Non diarrhea	-	Negative
112	Female	>1 year old	Non diarrhea	-	Negative
113	Female	>1 year old	Diarrhea	Negative	Negative
114	Male	>1 year old	Non diarrhea	Negative	Negative
115	Male	>1 year old	Diarrhea	Negative	Negative
116	Male	>1 year old	Non diarrhea	Negative	Negative
117	Male	>1 year old	Non diarrhea	-	Negative
118	Female	>1 year old	Non diarrhea	-	Negative
119	Male	>1 year old	Non diarrhea	-	Negative
120	Male	>1 year old	Diarrhea	Negative	Negative
121	Male	>1 year old	Diarrhea	Negative	Negative
122	Female	>1 year old	Diarrhea	-	Negative

123	Male	>1 year old	Non diarrhea	-	Negative
124	Male	>1 year old	Non diarrhea	Negative	Negative
125	Male	>1 year old	Diarrhea	Negative	Negative
126	Male	>1 year old	Diarrhea	-	Negative
127	Female	< 1 year old	Non diarrhea	Negative	Negative
128	Male	>1 year old	Diarrhea	Negative	Negative
129	Male	>1 year old	Diarrhea	Negative	Negative
130	Female	>1 year old	Diarrhea	-	Negative

APPENDIX C: Other gastrointestinal parasites detected on ME

Sample ID	Types of GIT Parasites	
	Cryptosporidium spp.	Coccidia
1.	-	-
2.	-	-
3.	-	-
4.	-	-
5.	-	-
6.	-	-
7.	-	+
8.	-	-
9.	-	-
10.	-	-
11.	-	+
12.	-	-
13.	-	+
14.	-	-
15.	+	+
16.	-	+
17.	-	-
18.	-	-
19.	-	-
20.	-	-
21.	-	-
22.	-	-
23.	-	-
24.	-	-
25.	-	-
26.	-	+
27.	-	-
28.	-	-
29.	-	-
30.	-	-
31.	-	-
32.	-	-
33.	-	-
34.	-	-
35.	-	-
36.	-	-
37.	-	-
38.	-	-
39.	-	-
40.	-	-
41.	-	-
42.	-	-
43.	-	+
44.	-	-
45.	-	-
46.	-	+
47.	-	+
48.	-	-
49.	-	-
50.	-	-
51.	+	-
52.	-	-
53.	-	+
54.	-	-
55.	-	+
56.	-	-
57.	-	+
58.	-	-
59.	-	-
60.	-	-
61.	-	+
62.	-	-
63.	-	-
64.	-	+
65.	-	-
66.	-	-
67.	-	-
68.	-	-
69.	-	-
70.	-	-
71.	-	+
72.	-	-
73.	-	-
74.	-	-
75.	-	+
76.	-	-
77.	-	-
78.	-	-
79.	-	-
80.	-	-
81.	-	-
82.	-	-
83.	-	-
84.	-	-

85.	-	-
86.	-	-
87.	-	-
88.	-	-
89.	-	-
90.	-	-
91.	-	-
92.	-	-
93.	-	-
94.	-	-
95.	-	-
96.	-	-
97.	-	+
98.	-	+
99.	-	-
100.	-	+
101.	-	-
102.	-	+
103.	-	-
104.	-	-
105.	-	-
106.	-	+
107.	-	-
108.	-	-
109.	-	-
110.	-	-
111.	-	-
112.	-	-
113.	-	-
114.	-	-
115.	-	-
116.	-	+
117.	-	-
118.	-	-
119.	-	-
120.	-	+
121.	-	-
122.	-	-
123.	-	-
124.	-	-
125.	-	+
126.	-	+
127.	-	-

128.	-	-
129.	-	+
130.	-	+

APPENDIX D: Carbol fuchsin stain preparation

- Basic fuchsin 0.3g
- Ethyl alcohol 95% 10.0mL
- Phenol, melted crystals 5.0mL
- Distilled water 95.0mL

1. Dissolve 0.3g basic fuchsin in 10ml 95% ethyl alcohol (Solution 1).
2. Dissolve 5mL phenol in 95mL distilled water (Solution 2).
3. Mix the 2 solution (1 & 2).
4. Let stand for several days