UNIVERSITI PUTRA MALAYSIA

MOLECULAR DETECTION AND RISK FACTORS ASSOCIATED WITH POTENTIALLY ZOONOTIC ENTERIC PROTOZOA INFECTING SYNANTHROPIC MACAQUES (*Macaca fascicularis*) IN THE NORTHERN REGION OF PENINSULAR MALAYSIA

GOH KAI SHIN

lp
FPV 2023 107

MOLECULAR DETECTION AND RISK FACTORS ASSOCIATED WITH POTENTIALLY ZOONOTIC ENTERIC PROTOZOA INFECTING SYNANTHROPIC MACAQUES (*Macaca fascicularis*) IN THE NORTHERN REGION OF PENINSULAR MALAYSIA

GOH KAI SHIN

FACULTY OF VETERINARY MEDICINE
UNIVERSITI PUTRA MALAYSIA
SERDANG, SELANGOR
2024

GOH KAI SHIN

DEGREE OF DOCTOR OF VETERINARY MEDICINE

2023 / 2024

MOLECULAR DETECTION AND RISK FACTORS ASSOCIATED WITH POTENTIALLY ZOONOTIC ENTERIC PROTOZOA INFECTING SYNANTHROPIC MACAQUES (*Macaca fascicularis*) IN THE NORTHERN REGION OF PENINSULAR MALAYSIA

GOH KAI SHIN

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

November 2023

CERTIFICATION

It is hereby certified that I have read this project paper entitled "Molecular Detection and Risk Factors Associated with Potentially Zoonotic Enteric Protozoa Infecting Synanthropic Macaques *(Macaca fascicularis)* in the Northern Region of Peninsular Malaysia.", by Goh Kai Shin and in my opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4901 - Project.

DR. REUBEN SUNIL KUMAR SHARMA

DVM (UPM), MVSc (UPM), PhD (Cambridge)

Senior Lecturer,

Faculty of Veterinary Medicine

Universiti Putra Malaysia

(Supervisor)

ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude to my supervisor, Dr. Reuben Sunil Kumar Sharma for his invaluable guidance and feedback. He is always ready to assist from planning until the completion of my project, and generously provided his knowledge and expertise, besides funding my research. Additionally, this endeavour would not have been possible without the patience and support from Norhadila Binti Zulkefli, for guiding me step by step and taking all my questions patiently.

I am extremely grateful to my parents, for the endless support they gave me since the first day I was born. Thank you for believing me in anything I do, for providing all I need so that I can focus on my academics, for taking care of Kiko when I am away. I couldn't ask for a better mum & dad. Words can't express my gratitude to OwlOwl, PuiPui and Kiko, for always being a big mental support as well, thanks for coming into my life at the right time. How I wish to have your companion every day.

To my partner in crime, Avril Lim, thank you for putting up with my mood swings throughout the project, and prepping me for presentation. I will never forget those times where we stayed in the lab till late night, having deep conversations while carrying out the experiments. To be honest, sometimes I was annoyed by your lame jokes but sometimes your comforting words means a lot to me.

Besides, I could not have undertaken this journey without the support of by colleagues and friends, DVM24, The Six Musketeers, friends from high school, friends from UPMKB that we are still keeping in touch. Thanks for keeping me sane when this degree is trying to break me apart.

Last but not least, special thanks to my special one who took care of me when I was struggling in completing my project. Thanks for the words of advice and guidance based on your experiences, thanks for taking me to good food to make sure I have a good mood. Thank you for the love, even though you always laugh at my misery. You know who you are.

CONTENTS

2.0 LITERATURE REVIEW

2.1 Aetiological Agent of Zoonotic Enteric Protozoan

2.2 Non-human Primate Infection

2.3 Human Infection

2.4 Detection of Aetiological Agents

3.0 MATERIALS AND METHODS

4.0 RESULTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic acid
PCR	Polymerase Chain Reaction
18SrRNA	18S ribosomal ribonucleic acid
µm	Micrometer
pH	Potential of Hydrogen
ST	Subtype
AIDS	Acquired immune deficiency syndrome
WHO	World Health Organization
ELISA	Enzyme-linked immunosorbent assay
GPS	Global Positioning System
PERHILITAN	Jabatan Perlindungan Hidupan Liar dan Taman Negara Semenanjung Malaysia
UPM	Universiti Putra Malaysia
mg	Milligram
kg	Kilogram
°C	Degree Celsius
µL	Microlitre
mL	Millilitre
rpm	Revolutions per minute
USA	United States of America
bp	Base pairs

TAE	Tris-acetate EDTA
EDTA	Ethylene-diamine-tetraacetic acid
UV	Ultraviolet
NCBI	National Center for Biotechnology Information
BLAST	Basic Local Alignment Search Tool
ML	Maximum Likelihood
NJ	Neighbour Joining
N	Number of samples
CI	Confidence Interval
IBD	Inflammatory Bowel Disease
M. fascicularis	*Macaca fascicularis*
M. sylvanius	*Macaca sylvanius*
M. mulatta	*Macaca mulatta*
SSU-rRNA	Small subunit ribosomal ribonucleic acid
FAO	Food and Agriculture Organization
TAD	Transboundary animal disease

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4901 -Projek.

PENGESANAN MOLEKULAR DAN FAKTOR-FAKTOR RISIKO JANGKITAN PROTOZOA USUS YANG BERPOTENSI ZOONOTIK DI KALANGAN *Macaca fascicularis* DI WILAYAH UTARA SEMENANJUNG MALAYSIA

Oleh

Goh Kai Shin

2024

Penyelia: Reuben Sunil Kumar Sharma

Kera liar sering kali ternampak di pelbagai jenis habitat semula jadi dan kawasan peridomestik, dan menyebabkan peningkatan antara muka manusia-monyet. Kera ini merupakan takungan patogen zoonosis yang cekap. *Blastocystis, Cryptosporidium* dan *Giardia* ialah parasit protozoa usus yang yang menjangkiti pelbagai perumah termasuk primata bukan manusia dan manusia, dengan potensi kejangkitan zoonotic. Penerbitan mengenai prevalens dan factor-faktor risiko epidemiologi parasit-parasit tersebut di Malaysia masih terbatas walaupun kesedaran kesihatan awam sedunia telah berkembang. Kajian ini dijalankan bertujuan untuk menentukan prevalens protozoa usus dalam kalangan kera ekor panjang (*Macaca fascicularis*) yang menetap berdekatan dengan manusia, dan untuk mengkaji factor-faktor risiko yang mempengaruhi jangkitan. Sejumlah lapan puluh tujuh sampel najis yang diarkibkan telah terkumpul daripada kera ekor panjang yang mendiami wilayah Utara Semenanjung Malaysia dan tertakluk kepada pengekstrakan DNA dan amplifikasi PCR berdasarkan gen 18SrRNA. Amplicon daripada sampel positif telah diselaraskan dan tertakluk kepada analisis bioinformatik dan filogenetik menggunakan algoritma *Maximum Likelihood* dan *Neighbour Joining*. Ketiga-tiga protozoa usus telah dikesan dalam kera, di mana *Cryptosporidium* adalah yang paling lazim (16.09%), diikuti oleh *Blastocystis* (14.94%) dan *Giardia* (12.64%). Analisis statistik multivariate menunjukkan bahawa terdapat kaitan antara jangkitan *Blastocystis* dengan jenis habitat, manakala jangkitan *Cryptosporidium* dan *Giardia* tidak berkaitan dengan umur, jantina dan habitat kera. Analisis filogenetik mendedahkan bahawa *Blastocystis* dan *Giardia* yang menjangkiti *M. fascicularis* mempunyai pertalian genetik yang rapat dengan subjenis menjangkiti manusia, dan berkemungkinan zoonotik. Kajian ini telah berjaya mendedahkan bahawa kera ekor panjang di Malaysia yang mendiami kawasan bandar, luar bandar dan pertanian bertakung protozoa zoonosis dan berupaya untuk menjangkit manusia. Dengan ini penduduk tempatan dan pelancong dinasihatkan untuk menjauhkan diri daripada kera liar dan megamalkan penjagaan kebersihan untuk mengurangkan risiko penularan zoonotik dan antroponotik parasit-parasit tersebut.

Kata kunci: Kera ekor panjang, *Macaca fascicularis*, protozoa usus, penyakit zoonotik

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4901- Project.

MOLECULAR DETECTION AND RISK FACTORS ASSOCIATED WITH POTENTIALLY ZOONOTIC ENTERIC PROTOZOA INFECTING SYNANTHROPIC MACAQUES (*Macaca fascicularis*) IN THE NORTHERN REGION OF PENINSULAR MALAYSIA

by

Goh Kai Shin

2023

Supervisor: Reuben Sunil Kumar Sharma

Synanthropic macaques are known to inhabit a wide range of natural and peridomestic habitats, thus increasing human-macaque interface. These macaques are efficient reservoirs of zoonotic pathogens. *Blastocystis, Cryptosporidium* and *Giardia* are divergent enteric protozoal parasites infecting a wide host range including non-human primates and humans, with potential of zoonotic transmission. In spite of their public health importance, there is a dearth of published information on the prevalence and epidemiological risk factors of these parasites in Malaysia. The aim of this study was to determine the prevalence of enteric protozoans in local free-ranging Long-tailed macaques (*Macaca fascicularis*), and to elucidate the associated risk factors for infection. Eighty-seven archived faecal samples collected from *M. fascicularis* inhabiting Northern region of Peninsular Malaysia were subjected to DNA extraction and PCR amplification targeting the 18SrRNA gene of *Blastocystis, Cryptosporidium* and *Giardia*. Amplicons were sequenced and subjected to bioinformatics and phylogenetic analyses using the Maximum Likelihood and Neighbour Joining algorithms. All three enteric protozoa were detected in the macaques, whereby *Cryptosporidium* was the most prevalent (16.09%), followed by *Blastocystis* (14.94%) and *Giardia* (12.64%). Multivariate statistical analysis indicated that the infections with *Blastocystis* were significantly associated with habitat types, while infections with *Cryptosporidium* and *Giardia* were not significantly associated with the age, gender and habitat of the macaques. Phylogenetic analysis revealed that *Blastocystis* and *Giardia* infecting *M. fascicularis* have close genetic affinities with the human-infective subtypes, and may be potentially zoonotic. This study had successfully revealed that the local free-ranging *M. fascicularis* inhabiting the urban, rural and agricultural areas harbour zoonotic enteric protozoa and may be potential reservoirs for human infection. It is recommended that local residents and tourists limit their contact with these macaques and maintain hygienic precautions to minimize the risk of zoonotic transmission of these parasites.

Keywords: Long-tailed macaques, *Macaca fascicularis*, enteric protozoa, zoonotic diseases

INTRODUCTION

The increasing human-wildlife interactions around Peninsular Malaysia is raising a public health concern due to the narrowing disease transmission interface. Synanthropic mammals including long-tailed macaques (*Macaca fascicularis*) are known to inhabit a wide range of natural and peridomestic areas, including residential areas, recreational parks, tourist attractions spots, riparian zones, peripheral forests near villages and agriculture sites (Eudey, 2008). Besides deforestation, availability of resources such as food provision by humans directly or indirectly led to adaptations by the macaques. They adjusted their behaviour by living near anthropogenic influences, and are in close contact with humans.

Macaques in the urban environment are often perceived as pests and nuisance due to their ability to occupy human's space and utilize the same food resources as humans (Yong *et al*., 2021). The macaques may cause disturbances and possess risks to the local community by stealing food, rummaging trash bins and even attacking humans. These human-macaque interactions could potentially result in zoonotic disease transmission (Md-Zain *et al*., 2014).

Long-tailed macaques are known as efficient reservoirs of human pathogens by maintaining a sylvatic cycle of zoonotic diseases. (Wolfe *et al*., 1998). Despite the growing concern of zoonotic diseases globally, detection and identification of potentially zoonotic pathogens infecting synanthropic monkeys in Peninsular Malaysia remains scarce. One of the major disease is malaria caused by haemoparasite of the genus *Plasmodium*, which is a potentially fatal disease. In addition to *Plasmodium*, there are a few zoonotic enteric protozoans that are capable of interspecies transmission between human and macaques, which include *Blastocystis, Cryptosporidium*, and *Giardia* (John *et al*., 2017).

Interspecies transmission of zoonotic enteric protozoans can occur through ingestion of oocysts or cysts in contaminated food and water, often due to insufficient filtration or pre-treatment of water. Previous studies reported that a low infecting dose is sufficient to produce human infection. The clinical significance in humans consist of a wide range, usually self-limiting diarrhoea in mild cases, up to fatalities in immunosuppressed individuals due to dysentery, dehydration, malabsorption and liver abscess (Stark *et al*., 2009).

In 2016, Long-tailed macaques were the most reported species of human-wildlife conflict complaints, according to Department of Wildlife and National Parks (DWNP). Several approaches have been taken including culling and trapping of individual macaques (DWNP, 2016). To improve public awareness about macaque behaviour, campaigns and educational programs were launched. However, further assessments are required to manage the issues in the long run. Accurate and precise knowledge on the distribution range and abundance of zoonotic parasites in macaques is crucial for effective management of interactions (Dickman, 2010).

This study aims to identify the diversity and prevalence of zoonotic enteric protozoan species, and the factors that influenced the patterns of parasite transmission. Molecular diagnosis using PCR will be conducted to detect pathogens in the faecal samples among the Long-tailed macaques in Northern region of Malaysia. To date, however, there are no comprehensive studies on molecular detection of zoonotic enteric protozoans in these macaques in Malaysia. Therefore, the data from present study will enable us to understand the epidemiological risk factors that contribute to the dynamics of pathogen transmission, which in turn may be employed to drive public health policies and formulate control plans.

Objectives

The study was undertaken to understand the epidemiology of the zoonotic enteric protozoans, namely *Giardia, Cryptosporidium* and *Blastocystis* naturally infecting *M. fascicularis* in Northern region of Malaysia, with the following specific objectives:

1. To determine the diversity and prevalence of zoonotic enteric protozoan species among synanthropic macaques in Peninsular Malaysia using molecular detection methods.
2. To determine the risk factors for transmission in relation to intrinsic (gender, age group) and extrinsic (habitat) variables.

Hypothesis

Null Hypothesis 1:	Synanthropic macaques do not harbour potentially zoonotic enteric protozoa.
Alternative Hypothesis 1:	Synanthropic macaques harbour various species of potentially zoonotic enteric protozoa.
Null Hypothesis 2:	Zoonotic enteric protozoa are not significantly related to host intrinsic factors and habitat type.
Alternative Hypothesis 2:	Zoonotic enteric protozoa are significantly related to host intrinsic factors and habitat type.

LITERATURE REVIEW

2.1 Aetiological Agent of Zoonotic Enteric Protozoan

2.1.1 *Blastocystis*

Blastocystis is an enteric protozoan found in humans and wide range of animal hosts (Stenzel *et al*, 1996). They are taxonomically grouped with the stramenopiles, a complex and heterogeneous evolutionary assemblage of heterotrophic and photosynthetic protozoa (Wawrzyniak *et al*, 2013). The identification of *Blastocystis* is rising in trend, suggesting an emerging parasite with a worldwide distribution. To date, there were 38 different subtypes detected based on genetic variations, 14 of which are commonly found in human and animal hosts (Darwish *et al*, 2023).

Blastocystis exist in four major morphological forms which are vacuolar, granular, amoeboid and cyst forms. (Suresh *et al*., 2009). The cysts are the infectious stage in the life cycle; when ingested, excystation occurs in the large intestines and the parasite develops into vacuolar forms. The vacuolar forms divide by binary fission and may develop into amoeboid or granular forms. Encystation follows while pathogen enters colon, and cysts are excreted in the faeces.

2.1.2 *Cryptosporidium*

Cryptosporidium is one of the most common intestinal protozoan parasites of human and animals. It is classified under the phylum *Apicomplexa* that includes other pathogens such as *Plasmodium, Eimeria, Babesia,* and *Theileria* (Suarez *et al*, 2017) Cryptosporidiosis is a worldwide infection of various hosts such as birds, horses, cattle, sheep, rodents and dogs (Mousa *et al*, 2020). To date, there are more than 30 species of *Cryptosporidium*, however only two species infect human, namely *C. parvum* and *C. hominis* (Ryan, 2014).

Cryptosporidium is a monoxenic parasite, where they complete their life cycle in single host species, capable for both asexual and sexual reproduction (Tzipori, 2002). Infection occurs when oocysts were ingested and hatch in intestines, each releasing four infective sporozoites. After excystation, these parasites reside in the host extra-cytoplasmic region and undergo asexual reproduction known as schizogony, producing merozoites. These merozoites can invade the epithelial cells of intestines and propagate. Two distinct replicative cycles happen in this stage: asexual stage with multiplication of merozoites and/or sexual stage characterized by formation of microgametocyte and microgametocytes that fused to form a zygote.

2.1.3 *Giardia*

The enteric parasite, *Giardia* is a flagellate of the order *Retortomonadida*. Giardiasis occurs worldwide with wide range of mammalian species such as human, pet animals like dogs and cats, wildlife (non-human primates, cetaceans), farm animals like cattle, deer and pig (Martin, 2016). The life cycle of *Giardia* alternates between the trophozoite and cysts. The trophozoite appears pear-shaped and dorsally convex, measuring 9.5 to 21µm long by 5 to 15µm wide. It has a teardrop appearance when viewed dorsoventrally and spoon shaped when viewed laterally. The dorsal surface is the area for diffusion of nutrients (Jones, 1988) as sucking disk to the mucosal surface is thought to be from negative pressure, together with the beating of flagella (Nash *et al*., 1987). There are four pairs of flagella and two nuclei. The cyst form is oval shaped measuring 8 to 12µm long by 7 to 10µm wide. Mature cyst contains four nuclei usually at one end. Once the cysts of *Giardia* were ingested, they pass through the mouth, oesophagus, and stomach into the small intestine. In the small intestine, each cyst releases two trophozoites through excystation. The trophozoites then absorb nutrients through their dorsal surface. The trophozoites of *Giardia* divides by longitudinal binary fission, remaining in the intestines of the host, either freely in the lumen or attached to the intestinal microvilli surface. When moving towards the colon, *Giardia* trophozoites are able to transform into a cyst form called encystation. *Giardia* inhabits the duodenum and upper jejunum of the host, due to alkaline pH that favours their growth. They invade mucosa and submucosa of the intestinal tract.

2.2 Non-human Primate Infection

2.2.1 *Macaca fascicularis* as Natural Host for Zoonotic Parasites

The Long-tailed macaque (*Macaca fascicularis*) is one of the species of Old World Monkeys native to Malaysia. The national census outcome pointed out that the total macaque population in Peninsular Malaysia was 133,403 individuals in 2014, with a 5% per annum total population growth rate (Karuppannan *et al*, 2014). Human interventions had disturbed their natural habitats which is the natural forests, therefore the distribution of these macaques have expanded closer to human settlements. Karuppannan *et al* (2014) also suggested that agriculture plantation sites recorded the highest macaque populations in Peninsular Malaysia.

The prevalence of *Blastocystis* in free-ranging *M. fascicularis* in Thailand was reported to be 41.87%. Three subtypes comprising 19 different alleles were observed, namely ST 1(17.26%), ST2 (24.37%), and ST 3 (36.55). (Vaisusuk *et al*, 2017).

Another study carried out in China (Zanzani, 2016) indicated 87.63% prevalence of *Blastocystis* infection in *M. fascicularis*. *S*ubtype ST2 was the most predominant (77.5%), followed by ST1 (63.5%), ST7 (41.2%), ST3 (38.8%), and ST5 (1.2%). ST4 and ST6 were not isolated in the study. Co-infections of two or more subtypes (76.5%) were more frequent than single subtype infection (23.5%). The most common form of combinations in mixed infection are ST1 and ST2 (16.47%), followed by ST1, ST2, and ST3 (11.76%).

A study in Thailand employed the detection of the 18SrRNA gene revealed that 1.0% (2/200) of the macaques examined were positive for *Cryptosporidium* infection (Sricharern *et al*, 2016). Sequence analysis of the amplicons showed 99% identity to the *Cryptosporidium* spp. monkey genotype (GenBank accession no: AF112569). A low (0.5%) prevalence of *Cryptosporidium* among non-human primates was also reported among Cynomolgus monkeys in China (Ye *et al*, 2014). Similarly, Rhesus macaques, Cynomolgus monkeys, Slow lorries and Francois' leaf monkeys from China showed low (0.7%) infection rates (Karim *et al*, 2014).

A study conducted in 2014 located near San Phra Kan shrine and Phra Prang Sam Yod temple, Thailand by Sricharern *et al*. (2016) indicated that the prevalence of *Giardia duodenalis* in faecal samples tested was 7.0% (14/200). The prevalence of the infection in male macaques was 7.9% (9/114), while the female conspecifics had a 5.8 % (5/86) infection rate. This study found no significant associations between the gender and habitat of the macaques and the detection of *Giardia*. Analyses of the positive amplicons revealed that 85.7% (12/14) of the *Gardia* belonged to assemblage B, 7.1% (1/14) to assemblage A, and one unidentified assemblage (Sricharern *et al*, 2016).

2.3 Human Infection

2.3.1 Mode of Transmissions

Blastocystis, Cryptosporidium and *Giardia* are waterborne protozoa commonly found in the environment. The pathogen is spreading freely in the environment such as soil, food and water contaminated from faeces of infected persons or animals. Transmission occurs primarily through faecal-oral route, such as ingestion of viable oocysts contaminated food or water (Gerace, 2019). Humans with poor hygiene practices, close animal contact and consumption of contaminated food or water are at higher risk of infections. Immunocompromised individuals are commonly infected, such as infants, kindergarten and school-aged children (Adedolapo *et al*, 2021).

2.3.2 Clinical Signs

The intestinal protozoan species often results in gastrointestinal manifestations, however systemic signs are possible in severe infections. Clinical manifestations are influenced by several factors, including the virulence of the parasite strain, the number of cysts ingested, the age of the host, and the state of the immune system at the time of infection (Faubert, 2000).

Blastocystis induced intestinal symptoms are abdominal pain, diarrhoea and vomiting; as well as non-specific symptoms including nausea, fatigue and flatulence (Cristina *et al*, 2022). Studies showed ST4 isolates have higher prevalence in symptomatic patients, arguing for subtype as contributing factor of clinical manifestations (Dominguez-Marquez, 2009).

G. intestinalis infection results in various clinical manifestations ranging from acute to chronic, or asymptomatic carriers. The presenting clinical signs may be diarrhoea, nausea, weight loss, bloating and abdominal pain (Roxström-Lindquist, 2006; Cotton, 2011). Furthermore, *Giarida* infection has been associated with long-term post-infectious side effects, for example functional gastrointestinal disorders, chronic fatigue syndrome, arthritis, and cognitive impairment in children (Halliez, 2013).

Cryptosporidiosis often shows systemic signs rather than gastrointestinal illness, which is more difficult to treat and may cause mortality. The common signs are fever and malabsorption, and the parasite can cause biliary tree inflammation, leading to biliary tract obstruction, sclerosing cholangitis, papillary stenosis and pancreatitis (Wang *et al*, 2018). It is considered as one of the riskiest opportunistic infections in immunosuppressed individuals, especially in acquired immune deficiency syndrome (AIDS) patients (Gerace, 2019).

2.3.3 Public Health Significance

Inter-host species transmission is possible betwee*n Blastocystis, Cryptosporidium* and *Giardia*. They pose a serious public health threat, especially when they are harboured by wildlife species that occupy the peri-domestic environment. In developing countries, intestinal parasites affecting people caused most infections and death, where poor hygiene and unavailability of effective water treatment have facilitated their transmission (Sterling & Adam, 2006). While in developed countries they also cause significant illness (Ortega, 2008). However, less emphasis has been given on the impact in urban settings presumably due to better health standards. Therefore, they are often underdiagnosed and the disease burden is often complicated by lack of reliable data.

World Health Organisations (WHO, 2008) reported that diarrhoeal disease affected huge number of individuals compared to other illness, where some cases are associated with enteric protozoa. Some might cause severe debilitating illness especially in immunosuppressed individuals. Furthermore, several enteric protozoa infect other wildlife, livestock and domestic pets has featured in several zoonotic outbreaks and transmission to humans (Chalmers, 2011). This may potentially result in a greater economic burden through higher medical and treatment costs (Torgerson, 2011). The utility of One Health frameworks should be strengthened to characterize the risk factors and to construct comprehensive control strategies in the future.

2.4 Detection of Aetiological Agents

2.4.1 Molecular Detection

The diagnosis of pathogenic protozoan species and genotyping using molecular method has improved our understanding of their transmission and epidemiology. According to Verweij and Stensvold (2014), there are numerous advantages of nucleic acid-based methods such as increased sensitivity and specificity, and simpler standardization of diagnostic procedures. The extracted DNA samples can be stored and used for genetic characterization and molecular typing, which is useful for surveillance studies. However, molecular assays often require refrigeration for DNA samples and reagents, along with expensive consumables and equipment. Furthermore, molecular method only reveals the pathogens for which appropriate primers are used, hence, additional analysis is required to detect other suspected pathogens.

2.4.2 Microscopic Detection

Microscopic identification of cryptosporidiosis is generally performed in the faecal smears. Despite being simple to perform and relatively low cost, this method has a low sensitivity (<30%) (Gerace, 2019). Furthermore, an experienced microscopist is needed for accurate diagnosis, particularly when the availability of parasites is low. Gerace also pointed out that sensitivity is improved to 55% when it is viewed under microscope with modified acid-fast stain. However, it is impossible for interspecies identification with this method.

2.4.3 Serology Detection

The detection of parasites using serology methods are less defined compared to microscopy and molecular methods. Examples include Enzyme-linked Immunosorbent Assay (ELISA), Immunochromatography and Direct fluorescent antibody. The sensitivity of each test varies with assay types.

MATERIALS AND METHODS

3.1 Sampling Area

In the present study, Long-tail macaques (*Macaca fascicularis*) were sampled from 17 locations throughout the Northern region of Malaysia, encompassing four states in the country including Perlis, Kedah, Kelantan and Terengganu (Figure 3.1, Table 3.1). Macaques were captured based on convenience sampling method, whereas the most accessible individuals were captured. A total of 87 faecal swab were collected with sedation of the animals. All sampling sites were categorized into four habitat types (urban, rural, plantations, forest), and GPS coordinates of each sampling location was recorded. Institutional permits were obtained according to the sampling guidelines and ethical protocols of PERHILITAN. All samples were curated at -20°C at the Parasitology Laboratory, Faculty of Veterinary Medicine, UPM.

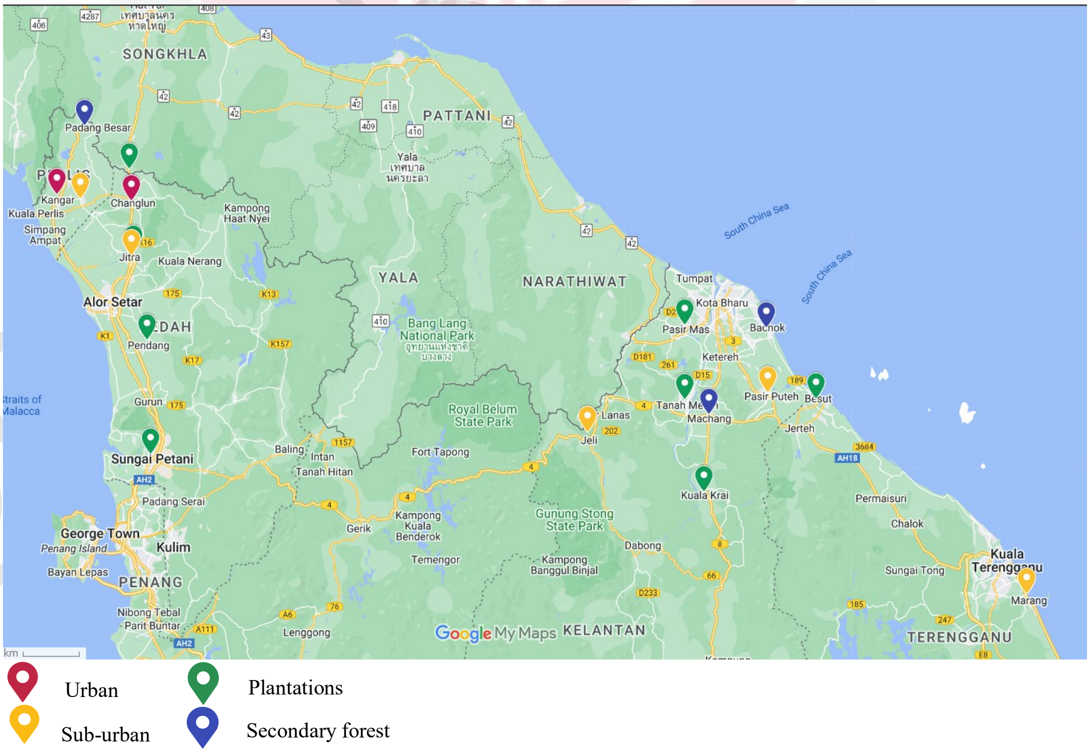

Figure 3.1: Sampling locations of Long-tailed Macaques (*Macaca fascicularis*) in the Northern region of Peninsular Malaysia.

Table 3.1: Location of sampling sites with and number of samples in each location.

States	District	Location	No. of samples
Perlis	Arau	Bukit Keriang, Ulu Pauh	2
	Arau	Kubang Paya	5
	Kangar	Kg. Nesam	1
	Kangar	Kg. Padang Melangit	1
	Kangar	Sekolah Batu Bertangkup	1
	Kangar	Taman Bukit Kaya	7
	Padang Besar	Kg. Titi Tinggi	1
Terengganu	Besut	Kg Tembila	5
	Marang	Pasir Puteh Merchang	5
	Marang	Pusat Latihan Tm, Rusila	7
Kelantan	Bachok	Kg. Cabang Empat, Gunong	1
	Jeli	Kg. Batu Melintang	1
	Kuala Krai	Taman Batu Lada	5
	Machang	Wisma Madu	6
	Pasir Mas	Alor Pasir	4
	Pasir Puteh	Kg. Temila	2
	Tanah Merah	Kg. Kelewek, Batu Gajah	1
Kedah	Bukit Kayu Hitam	Kg. Padang Satu	2
	Changlun	Kg. Mak Pap	4
	Changlun	Taman Beringin Changlun	2
	Changlun	Taman Teja	6
	Kubang Pasu	Kg. Lahar	4
	Pendang	Kg. Lubuk Ular	7
	Sg. Petani	Kg Jurong Atas	4
	Sg. Petani	Kg. Merbok	1
	Sg. Petani	Ldg. Sungai Puntar	2
Total			87

3.2 Faecal Sample Collection

The macaques were captured using baited traps and information such as age, gender and habitat were recorded. The macaques were anesthetized with 10mg/kg body weight of ketamine hydrochloride. Age of macaques were estimated by comparing dental eruption together with physical characteristics (Seethamchai *et al.*, 2008), whereby macaques less than two years of age were classified as juveniles, those between two and four as subadults and those more than four as adults. Faecal swab was collected through the rectum of the macaques. Each swab sample was transported in a centrifuge tube filled with lysis buffer. The samples were kept at -20°C.

3.3 DNA Extraction from Faecal Sample

Genomic DNA from each faecal sample was extracted using a conventional DNA extraction kit (QIAamp® Fast DNA Stool Mini Kit, Qiagen, Germany) according to the manufacturer's protocol. 200µL of faecal sample in preserved in lysis buffer was pipetted into 2mL microcentrifuge tubes. One millilitre of InhibitEX Buffer was added to each faecal sample and homogenized thoroughly through vortexing. The suspension is then heated for five minutes in a 70°C water bath. The sample is then centrifuged at room temperature for one minute at 14,000rpm. Subsequently, 600µL of the supernatant was mixed with 25µL of proteinase. Next, 600µL of Buffer AL was added and vortex for 15 seconds. The mixture was incubated in a 70°C water bath for 10 minutes. Next, 200µL of 96% ethanol was added to the lysate and mixed by vortexing, and 600µL of the lysate was carefully pipetted into the QIAamp spin column and centrifuge at 14,000rpm for one minute. The QIAamp spin column was placed in a new 2mL collection tube and 500µL of Buffer AW1 was added. The mixture was then centrifuged at 14000 rpm for 1 minute. The collection tube containing the filtrate was discarded and replaced with a new 2mL collection tube and 500µL of Buffer AW 2 was added and centrifuged at 14,000 rpm for three minutes. The collection tube was discarded, and the spin column was transferred into a new 1.5mL microcentrifuge tube. Next, 200µL of Buffer ATE was pipetted into the centre of spin column membrane followed by a one-minute incubation at room temperature and centrifugation at 14,000rpm for one minute to harvest the eluted DNA.

3.4 Polymerase Chain Reaction (PCR) Amplification of the 18SrRNA Gene

All PCR reactions were performed using a thermocycler (C1000 Touch™ thermal cycler, Bio-Rad Laboratories, USA). Amplification was done into a final volume of 25µL, made up of 4µL extracted DNA template, 8.4µL distilled water, 1µL forward primer (Primer 1) and 1µL of reverse primer (Primer 2), 10.6µL of GoTaq® G2 Hot Start.

Blastocystis

The extracted DNA was used to perform PCR to amplify a partial fragment (600 bp) of the Small Subunit ribosomal RNA genes (SSU-rRNA) gene. Amplification of *Blastocystis* was done using the primers RD5 (5′-ATCTGGTTGATCCTGCCAGT-3′) and BhRDr (5′-GAGCTTTTTAACTGCAACAACG-3′) (Scicluna *et al*., 2006). Thermal cycling conditions were set up at 94°C for 5 minutes; 30 cycles of 94°C for 1 minute, annealing step at 56°C for 1 minute, and extension of 72°C for 1 minute; final extension at 72°C for 4 minutes.

Cryptosporidium

For primary PCR analysis, a fragment (826-864bp) of the 18SrRNA gene from *Cryptosporidium* was amplified with the primers XF2 (5'-GGAAGGGTTGTATTTATTAGATAAAG-3') and XR2 (5' AAGGAGTAAGGAACAACCTCCA 3'). The thermal cycling protocol were as follows: Initial denaturation at 94°C for 5 minutes; 30 cycles of 94°C for 45 seconds for denaturation, annealing at 45°C for 20 minutes, extension at 72°C for 1.5 minutes, and final extension at 72°C for 10 minutes. Nested PCR of a 240bp fragment was performed with the primers; pSSUf (5'-AAAGCTCGTAGTTGGATTTCTGTT-3') and pSSUr (5'- ACCTCTGACTGTTAAATACRAATGC-3'). The nested PCR was carried under the following conditions: Initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension step of 10 min at 72°C.

Giardia

Primary PCR amplification of *Giardia* was carried out on a 292bp fragment of the 18SrRNA gene. The genus-specific primers used in this study were RH11 (5′-CATCCGGTCGATCCTGCC-3′) and RH4 (5′-AGTCGAACCCTGATTCTCCGCCAGG-3′) (Hopkins *et al*., 1997). The thermocyclic conditions for the PCR amplification are as follows: Initial Denaturation at 96°C for 2 minutes; 35 cycles at 96°C for 20 seconds, annealing at 59°C for 20 seconds, extension at 72°C for 20 seconds, and a final extension at 72°C for 7 minutes. The nested PCR amplification for *Giardia* was done using the primers GiarF (5′-GACGCTCTCCCCAAGGAC-3′) and GiarR (5′-CTGCGTCACGCTGCTCG-3′) (Read *et al*., 2002) to produce an amplicon of 130-200bp in size, using 4μL from the primary PCR amplification product. The amplification reaction mixture and cycling conditions for the nested PCR was similar to the primary amplification.

Table 3.2: Summary of primers and Thermocyclic Conditions used to Amplify targeted Gene of protozoan species

Protozoan Species	Gene	Primers (Amplicon size)	Sequence (5’ – 3’)	Thermocyclic conditions	References
Blastocystis	18S rRNA	RD5 BhRDr (600bp)	5’- ATCTGGTTGATCCTGCCAGT- 3’ 5’ - GAGCTTTTTAACTGCAACAACG- 3’	ID: 94°C (5 mins) No of cycles (D+A+E) : 30 D: 94°C (1 min) A: 56°C (1 min) E: 72°C (1 mins) FE: 72°C (4 mins)	Scicluna et al., (2006)
Cryptosporidium	18S rRNA	*1st nest* XF2 XR2 (826-864bp)	5’- GGAAGGGTTGTATTTATTAGATAAAG 3’ 5’ - AAGGAGTAAGGAACAACCTCCA - 3’	ID: 94°C (5mins) No of cycles (D+A+E) : 30 D: 94°C (45 secs) A: 45°C (2 mins) E: 72°C (1.5 mins) FE: 72°C (10 mins)	Xiao et al., (1999) Nolan et al., (2010)
		2nd nest pSSUf pSSUr (240bp)	5’- AAAGCTCGTAGTTGGATTTCTGTT- 3’ 5’ - ACCTCTGACTGTTAAATACRAATGC- 3	ID: 94°C (5mins) No of cycles (D+A+E) : 35 D: 94°C (30 secs) A: 55°C (30 secs) E: 72°C (30 secs) FE: 72°C (10 mins)	
Giardia	18S rRNA	*1st nest* RH11 RH4 (292bp)	5′-CATCCGGTCGATCCTGCC-3′ 5′-AGTCGAACCCTGATTCTCCGCCAGG-3′	ID: 96°C (2 mins) No of cycles (D+A+E) : 35 D: 96°C (20 secs) A: 59°C (20 secs) E: 72°C (30 secs) FE: 72°C (7 mins)	Hopkins et al., (1997)
		2nd nest GiarF GiarR (130-200bp)	5′-GACGCTCTCCCCAAGGAC-3′ 5′-CTGCGTCACGCTGCTCG-3′	ID: 96°C (2 mins) No of cycles (D+A+E) : 35 D: 96°C (20 secs) A: 59°C (20 secs) E: 72°C (20 secs) FE: 72°C (7 mins)	Read et al., (2002)

3.5 Agarose Gel Electrophoresis of PCR Amplicons

The amplicons of the PCR were electrophoresed at 80V on a 1.5% agarose gel (HyAgarose™, USA) with TAE (Tris-acetic acid-EDTA) buffer, stained with RedSafe™ Nucleic Acid Staining Solution. The solidified gel was placed into an electrophoresis tank immersed with TAE buffer. The power source was set at 80V and allowed to run at 50 min. The resulting gel is viewed under a UV transilluminator (Gel Doc XR ™, Bio-Rad Laboratories) with 100bp DNA ladders as standard size markers (Vivantis, USA). Image captured were processed using a computer software (Quantity One 1-D Analysis Software, Bio-Rad Laboratories, USA).

3.6 Sequencing of Positive Amplicons

The positive amplicons were sequenced bi-directionally and the chromatograms were edited, aligned and assembled using a bioinformatics software (Bioedit Sequence Alignment Editor). To facilitate identification of the enteric protozoans, the sequences obtained were compared to known gene fragments curated by the National Center for Biotechnology Information (NCBI) GenBank using the Basic Local Alignment Search Tool (BLAST).

3.7 Phylogenetic Analysis

Sequence alignment was done using the ClustalW program as implemented in MEGA 11.0 phylogenomics analytical software. (MEGA 11.0, Pennsylvania State University, USA).

Phylogenetic trees [Maximum Likelihood (ML) and Neighbour Joining (NJ)] were constructed following computed best fit models with confidence levels set at 100 (ML) and 1000 (NJ) bootstrap replicates. Fifty sequences of similar organism were undertaken to construct the phylogenetic tree including sequences isolated from positive samples and reference sequences from GenBank. Reference sequences were gathered from isolates from other hosts such as human, non-human primate, livestock, companion animals and environmental water bodies, originated from local and foreign countries.

3.8 Statistical Analysis

All data obtained were statistically analysed using SPSS version 21 (SPSS Inc. Chicago, Illinois). Putative epidemiological risk factors associated with the infection were categorized into host intrinsic factors (age, gender, age x gender), agent-based factors (co-infection with number of species, co-infection with specific species), and extrinsic factors (geographical location, habitat). Chi-square test was applied to evaluate the significance of associations between epidemiological risk factors and infection rate. Univariate and multivariate binary logistic regression models were constructed. Odds ratios for the relative risk of enteric protozoa infection were calculated with statistical significance set at $p<0.05$.

RESULTS

4.1 PCR Amplification and Agarose Gel Electrophoresis

A total of 87 archived faecal samples of *M. fascicularis* were screened for presence of *Blastocystis, Cryptosporidium* and *Giardia.* The PCR assay was performed using specific primers on the selected genes. Positive results were defined as a band expression at the targeted base pair (bp) after gel electrophoresis.

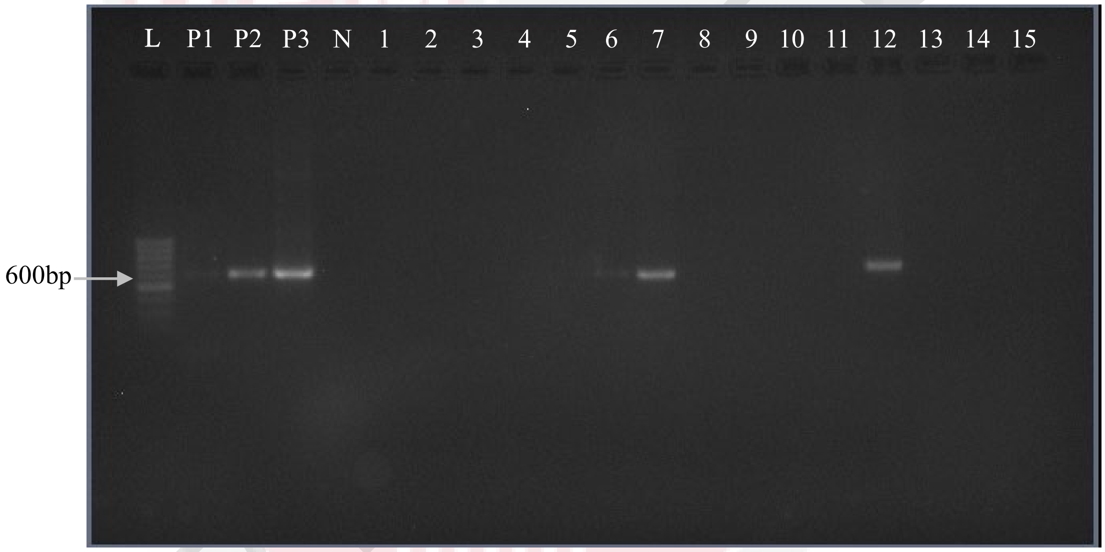

Figure 4.1. PCR amplification products of *Blastocystis.* Lane L: 100bp DNA ladder, Lane P1 – P3: Positive Controls, Lane N – Negative Control. Lane 1-15: PCR amplicons of study samples. The target bands were observed on lane 6,7 and 12.

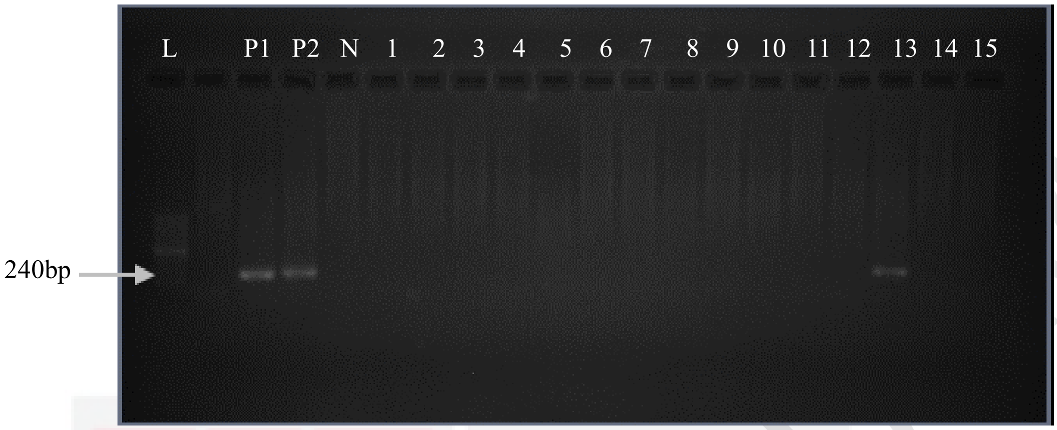

Figure 4.2: PCR amplification products of *Cryptosporidium*. Lane 1: 100bp DNA ladder, Lane P1-P2: Positive Controls, Lane N – Negative Control. Lane 1-15: PCR amplicons of study samples. Positive bands were observed on lane 13.

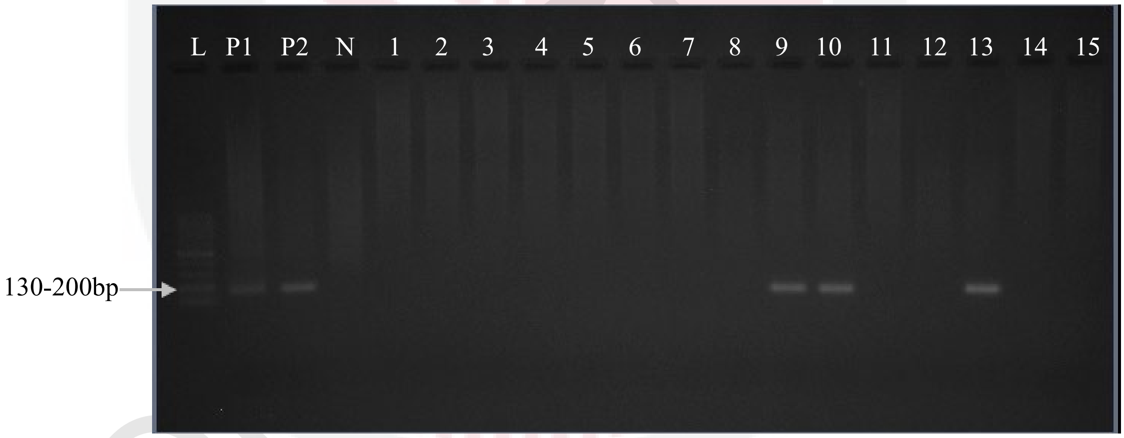

Figure 4.3: PCR amplification products of *Giardia*. Lane L: 100bp DNA ladder, Lane P1-P2: Positive Control, Lane N: Negative Control. Late 1-14 – PCR amplicons of study samples. The target bands were observed on lane 8, 9 and 12.

4.2 Spatial Distribution of *Blastocystis, Cryptosporidium* and *Giardia* in Northern Region of Peninsular Malaysia

The spatial distribution and prevalence of each protozoa infection in each sampling area and geographical zone are presented in Table 4.1 and illustrated in Figure 4.4, 4.5 and 4.6. For the spatial analysis of *Blastocystis*, highest infection was recorded at Marang, Terengganu with the prevalence of 10/11 (90.9%). Similar to *Cryptosporidium*, as highest prevalence was recorded in Marang, Terengganu with the prevalence of 4/11 (36.36%). Whilst for *Giardia*, they were found most in Kangar, Perlis with the prevalence of 3/13 (23.08%).

Table 4.1: Summary of the Spatial distribution and prevalence for *Blastocystis, Cryptosporidium* and *Giardia* infection among Long-tailed Macaques (*Macaca fascicularis*) in various sampling zones throughout Northern Peninsular Malaysia.

States	**District**	**No. of samples**	**No. of positive samples (prevalence)**		
			Blastocystis	***Cryptosporidium***	***Giardia***
Kedah	Sg. Petani	7	0	1 (14.3%)	1 (14.3%)
	Kubang Pasu	4	0	0	0
	Pendang	7	0	1 (14.3%)	1 (14.3%)
	Changlun	13	0	1 (7.69%)	1 (7.69%)
	Bukit Kayu Hitam	2	0	0	0
	Jitra	2	0	0	0
Kelantan	Pasir Mas	4	1 (25%)	1 (25%)	0
	Jeli	1	0	0	0
	Bachok	1	0	0	0
	Tanah Merah	1	0	0	0
	Pasir Puteh	2	0	0	0
	Kuala Krai	3	0	1 (33.33%)	1 (33.33%)
	Machang	6	0	0	2 (33.33%)
Perlis	Arau	7	1 (14.3%)	0	0
	Kangar	13	0	3 (23.08%)	**3 (23.08%)**
	Padang Besar	1	0	0	0
Terengganu	Besut	2	1 (50%)	2 (100%)	0
	Marang	11	**10 (90.9%)**	**4 (36.36%)**	1 (9.09%)
TOTAL		**87**	13	14	11

Blastocystis

The screening for *Blastocystis* infection among the macaques revealed that 13/87 (14.94%) were positive. The positive samples showed a band in 600bp. (Figure 4.1). The parasites were discovered in 4 out of 17 sampling sites as shown in the map (Figure 4.4).

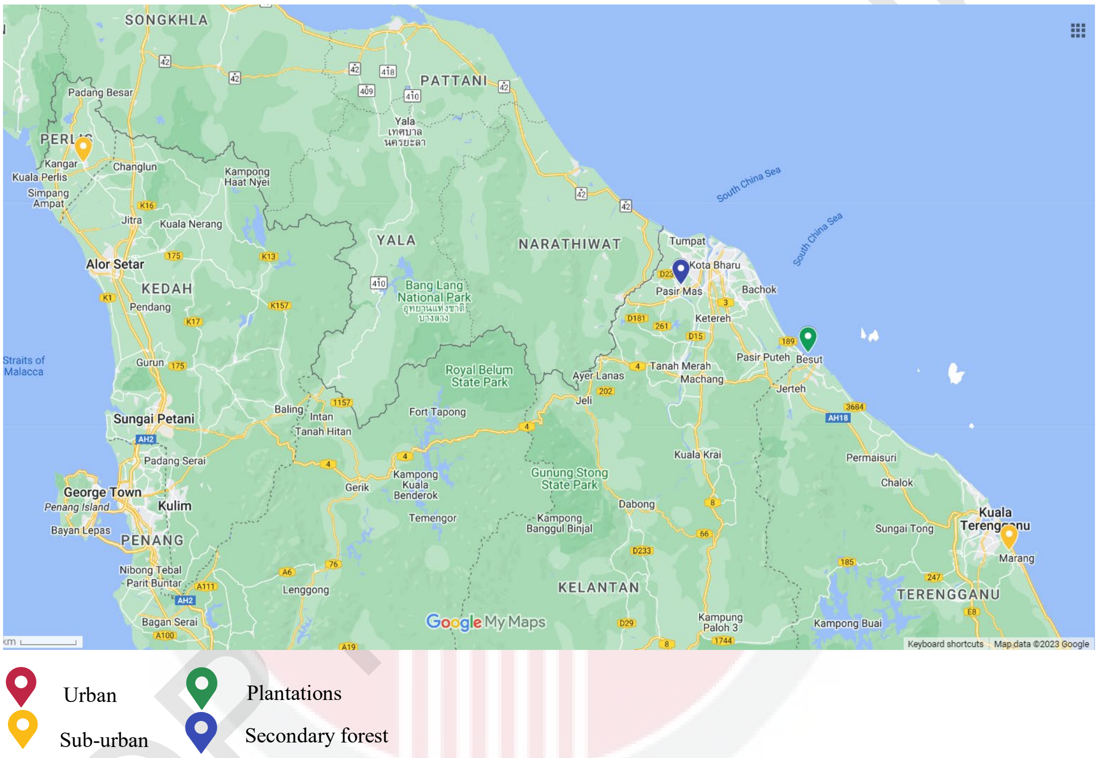

Figure 4.4: Spatial distribution of *Blastocystis* among *Macaca fascicularis* in the Northern region of Peninsular Malaysia.

Cryptosporidium

Amplicons of 200bp consistent with *Cryptosporidium* were observed from 16.09% (14/87) of the macaque faecal samples tested. The parasites were discovered in 8 out of 17 sampling sites as shown in the map (Figure 4.5).

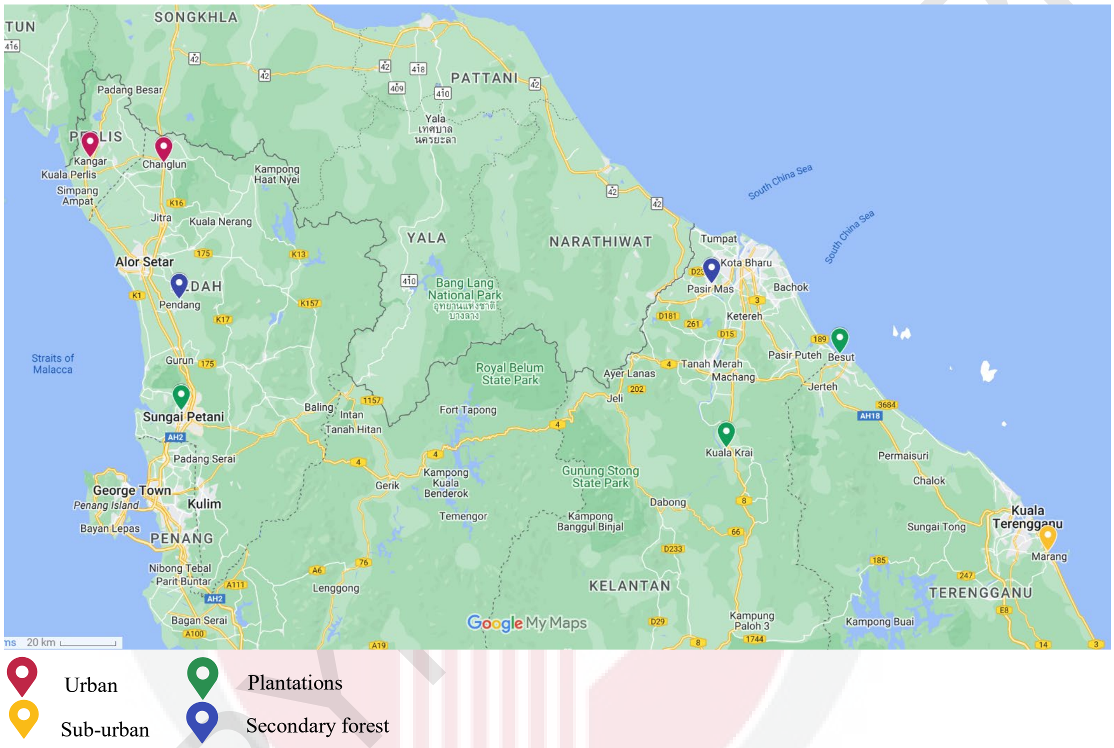

Figure 4.5: Spatial distribution of *Cryptosporidium* among *Macaca fascicularis* in the Northern region of Peninsular Malaysia.

Giardia

Nested PCR amplification of the 18SrRNA gene revealed the prevalence of *Giardia* among the macaques to be 12.64% (11/87). The parasites were discovered in 7 out of 17 sampling sites and the locations were illustrated in Figure 4.6.

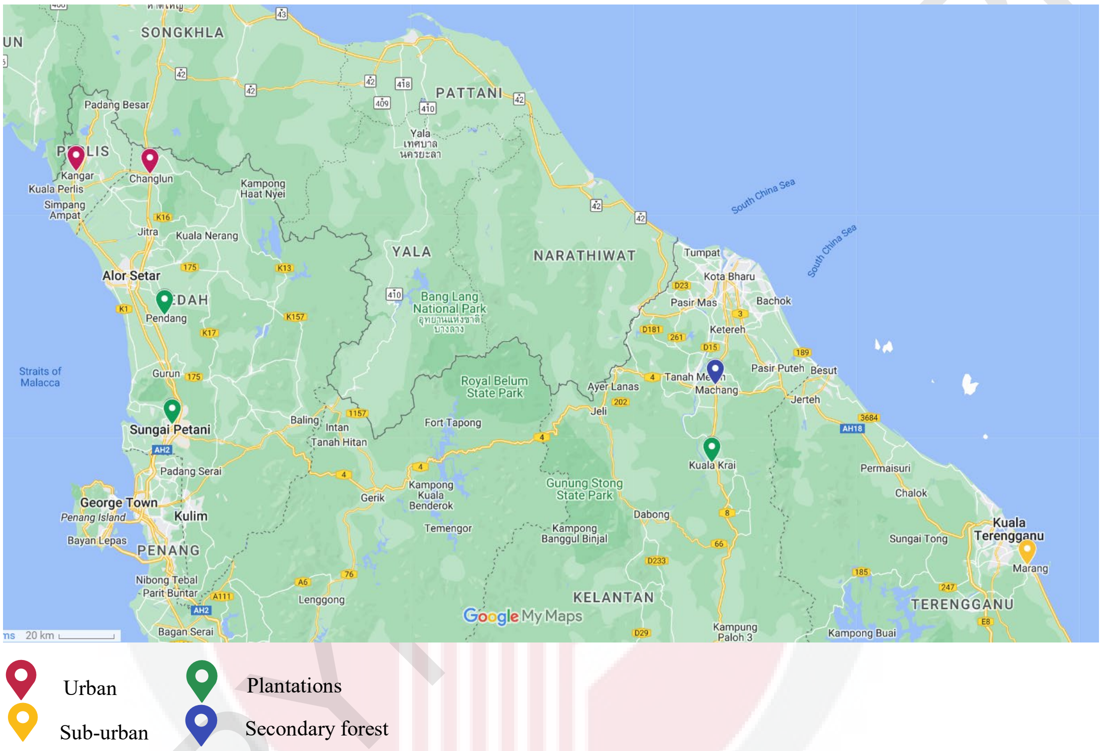

Figure 4.6: Spatial distribution of *Cryptosporidium* among *Macaca fascicularis* in the Northern region Peninsular Malaysia.

4.3 Molecular Prevalence and Co-infection Status of *Blastocystis, Cryptosporidium* and *Giardia* among *Macaca fascicularis* in Northern region of Peninsular Malaysia.

The PCR assay result produced from 87 archived faecal samples were analysed to investigate the presence of *Blastocystis, Cryptosporidium* and *Giardia. Cryptosporidium* infection recorded the highest prevalence of 14/87 (16.09%), followed by *Blastocystis* 13/87 (14.94%) and *Giardia* 11/87 (12.64%). Positive samples were submitted for DNA sequence analysis to identify the species of each protozoa. Single infections were more commonly in *M. fascicularis* compared to double infections. The prevalence of *Blastocystis* + *Cryptosporidium* was 6.9% (6/87), which is similar to *Cryptosporidium* + *Giardia.* The prevalence of *Blastocystis* + *Giardia* recorded the lowest combination with the prevalence of 1.15% (1/87). None of the macaques were co-infected with all three types of enteric protozoa. The prevalence and co-infection status were summarized in Table 4.1.

Table 4.2: Prevalence of zoonotic enteric protozoans and their co-infection status among Long-tailed Macaques *(Macaca fascicularis)* in the Northern Region of Peninsular Malaysia. [Prevalence (%) = No. infected / 87 x 100].

Species co-infection	No. infected	Prevalence (%)
Single		
Blastocystis	13	14.94
Cryptosporidium	14	16.09
Giardia	11	12.64
Double		
Blastocystis + Cryptosporidium	6	6.9
Blastocystis + Giardia	1	1.15
Cryptosporidium + Giardia	6	6.9

Blastocystis

The occurrence of *Blastocystis* infection according to the host factors (Table 4.3) revealed that the highest prevalence was among the sub-adult macaques (23.33%) followed by adults (16.67%), while juvenile macaques had the lowest prevalence of infection (3.7%). For the gender factors, female macaques had a slightly higher rate of infection (17.07%) than males (13.04%). The macaques sampled at the rural areas had a distinct increase in trends of infection with *Blastocystis* (45.83%). Macaques inhabiting plantation sites possessed a prevalence of 4.54%, which is faintly higher than secondary forests (4.17%). The infection with *Blastocystis* was not detected among macaques sampled from the urban areas.

Table 4.3: Prevalence (%) of *Blastocystis* among Long-tailed Macaques (*Macaca fascicularis*) in Northern Region of Peninsular Malaysia in relation to the host intrinsic factors (Age, Gender, Age x Gender) and extrinsic factors (Habitat). [Prevalence (%) = Positive cases / N x 100].

Host Variables	No of Samples (N)	Positive Cases	Prevalence (%)
Age			
Juvenile	27	1	3.7
Sub-adult	30	7	23.33
Adult	30	5	16.67
Gender			
Male	46	6	13.04
Female	41	7	17.07
Age x Gender			
Juvenile Male	16	1	6.25
Juvenile Female	11	0	0
Sub-adult Male	19	3	15.79
Sub-adult Female	11	4	36.26
Adult Male	10	2	20
Adult Female	20	3	15
Habitat			
Urban	17	0	0
Rural	24	11	45.83
Plantation	22	1	4.54
Secondary Forest	24	1	4.17
Total samples	87	13	14.94

The prevalence of *Cryptosporidium* infection among *M. fascicularis* in relation to host variables (age, gender, age x gender) and habitat were presented in Table 4.4. Adult macaques carry the highest prevalence 26.67% (8/30), followed by juveniles 11.11% (3/27), and sub-adults 10% (3/30). The infection rate among female macaques was 21.95% (9/41), which was higher than the rate among male macaques 10.87% (5/46). With regards to the habitat of the sampling areas, the macaques sampled from the rural areas harboured the highest rate of infection with *Cryptosporidium* 20.83% (5/24). The second highest infection were observed in plantation sites 18.18% (4/22).

It is followed closely by macaques inhabiting urban areas with a prevalence of 17.65% (3/17). Macaques inhabiting secondary forest were the least infected 8.33% (2/24) with *Cryptosporidium*.

Table 4.4: Prevalence (%) of *Cryptosporidium* among Long-tailed Macaques (*Macaca fascicularis*) in Northern Region of Peninsular Malaysia in Relation to the host intrinsic factors (Age, Gender, Age x Gender) and extrinsic factors (Habitat). [Prevalence (%) = Positive cases / N x 100].

Host Variables	No of Samples (N)	Positive Cases	Prevalence (%)
Age			
Juvenile	27	3	11.11
Sub-adult	30	3	10
Adult	30	8	26.67
Gender			
Male	46	5	10.87
Female	41	9	21.95
Age x Gender			
Juvenile Male	16	1	6.25
Juvenile Female	11	2	18.18
Sub-adult Male	19	2	10.53
Sub-adult Female	11	1	9.09
Adult Male	10	2	20
Adult Female	20	6	30
Habitat			
Urban	17	3	17.65
Rural	24	5	20.83
Plantation	22	4	18.18
Secondary Forest	24	2	8.33
Total	87	14	16.09

The occurrence of infection in relation to the host variables were represented in Table 4.5. The juvenile macaques recorded a higher prevalence of 18.52% (5/27). The sub-adults had a similar rate of infection as adult macaques of 10% (3/30). Gender wise, the prevalence of infection among the females was 17.07% (7/41), approximately twice that of the males (8.7%, 4/46).

The habitat analysis showed the highest occurrence in the urban region, with 23.53% (4/17) prevalence, followed by secondary forest 16.67% (4/24), and plantation 9.09% (2/22), with the rural-inhabiting macaques recording the least prevalence of 4.17% (1/24).

Table 4.5: Prevalence of *Giardia* among Long-tailed Macaques (*Macaca fascicularis*) in Northern Region of Peninsular Malaysia in Relation to the host intrinsic factors (Age, Gender, Age x Gender) and extrinsic factors (Habitat). [Prevalence (%) = Positive cases / N x 100].

Host Variables	No of Samples (N)	Positive Cases	Prevalence (%)
Age			
Juvenile	27	5	18.52
Sub-adult	30	3	10
Adult	30	3	10
Gender			
Male	46	4	8.7
Female	41	7	17.07
Age x Gender			
Juvenile Male	16	2	12.5
Juvenile Female	11	1	9.09
Sub-adult Male	19	1	5.26
Sub-adult Female	11	3	27.27
Adult Male	10	2	20
Adult Female	20	2	10
Habitat			
Urban	17	4	23.53
Rural	24	1	4.17
Plantation	22	2	9.09
Secondary Forest	24	4	16.67
Total	87	11	12.64

4.4 Epidemiological Risk Factors Associated with *Blastocystis, Cryptosporidium* and *Giardia* Infection among *Macaca fascicularis* in the Northern Region of Peninsular Malaysia

These putative risk factors were then subjected to univariate and multivariate regression analysis to determine the association between the epidemiological factors (age, gender & habitat) and infection of *Blastocystis, Cryptosporidium* and *Giardia* Infection among the Long-tailed macaques. The statistical analysis results are summarised in Tables 4.6 to 4.11. The reference category was labelled with R. Univariate analysis revealed that there is a significant association with *Blastocystis* infection and habitat type of the macaques. Only one environmental factor (habitat) shows significance towards infection with *Blastocystis* with a P-value of <0.05. The odds of Long-tailed macaques inhabiting the rural region to be infected with *Blastocystis* were 22 times higher than those living in secondary forest. There was no significant association between *Blastocystis* infection in age and gender variables. There was no significance in the odds for infection of *Cryptosporidium* and *Giardia* and any of the putative risk factors. All macaques regardless of age, gender and habitat has the same chance of getting infection with *Cryptosporidium* and *Giardia.*

Table 4.6: Univariate Logistic Regression Analysis for *Blastocystis* infection in Long-tailed Macaques (*Macaca fascicularis*) in the Northern region of Peninsular Malaysia in relation to the host intrinsic variables.

Host Intrinsic Variables	**N**	**Parasites +ve**	**Parasites -ve**	**Odds Ratio (95% CI)**	**P-value**
Age					
Juvenile	27	0	27	0.192 (0.021, 1.764)	0.145
Sub-adult	30	8	22	1.522 (0.423,5.472)	0.520
^{R}Adult	30	5	25	1.00	-
Gender					
^{R}Male	46	7	39	1.00	
Female	41	6	35	1.300 (0.399, 4.239)	0.664
Age x Gender					
Juvenile Male	16	0	16	0.356 (0.33, 3.805)	0.393
Juvenile	11	0	11	-	-
Female	19	5	14	1.000	
^{R}Sub-adult	11	3	8	3.048 (0.535, 17.374)	0.210
Male	10	2	8	1.333 (0.184, 9.660)	0.776
Sub-adult	20	3	17	0.941 (0.165, 5.361)	0.946
Female					
Adult Male					
Adult Female					
Total	87	13	74		

^{R}Reference category

Table 4.7: Univariate Logistic Regression Analysis for *Cryptosporidium* infection in Long-tailed Macaques (*Macaca fascicularis*) in the Northern region of Peninsular Malaysia in relation to the host intrinsic factors.

Host Intrinsic Variables	**N**	**Parasites +ve**	**Parasites -ve**	**Odds Ratio (95% CI)**	**P-value**
Age					
^{R}Juvenile	27	2	25	1.000	
Sub-adult	30	4	26	0.889 (0.164, 4.828)	0.891
Adult	30	8	22	3.273 (0.774, 13.832)	0.107
Gender					
^{R}Male	46	5	41	1.000	
Female	41	9	32	2.439 (0.744, 7.997)	0.141
Age x Gender					
Juvenile Male	16	0	16	-	
Juvenile Female	11	2	9	1.185 (0.166, 6.471)	0.866
^{R}Sub-adult Male	19	3	16	1.000	
Sub-adult Female	11	1	10	0.533 (0.049, 5.862)	0.607
Adult Male	10	2	8	1.333 (0.184, 9.660)	0.776
Adult Female	20	6	14	2.286 (0.480, 10.883)	0.299
Total	87	14	73		

^{R}Reference category

Table 4.8: Univariate Logistic Regression Analysis for *Giardia* infection in Long-tailed Macaques (*Macaca fascicularis*) in the Northern region of Peninsular Malaysia in relation to the host intrinsic factors.

Host Intrinsic Variables	**N**	**Parasites +ve**	**Parasites -ve**	**Odds Ratio (95% CI)**	**P-value**
Age					
^{R}Juvenile	27	5	22	1.000	
Sub-adult	30	3	27	0.489 (0.105, 2.276)	0.362
Adult	30	3	27	0.489(0.105, 2.276)	0.362
Gender					
Male	46	4	42	1.000	
Female	41	7	34	2.162 (0.584, 8.004)	0.248
Age x Gender					
Juvenile Male	16	2	14	2.571 (0.211, 31.326)	0.459
Juvenile Female	11	3	8	6.750 (0.605, 75.270)	0.121
^{R}Sub-adult Male	19	1	18	1.000	
Sub-adult Female	11	2	9	4.000 (0.319, 50.229)	0.283
Adult Male	10	1	9	2.000 (0.112, 35.807)	0.638
Adult Female	20	2	18	2.000 (0.166, 24.069)	0.585
Total	87	11	76		

^{R}Reference category

Table 4.9. Univariate Logistic Regression Analysis for *Blastocystis* infection in Long-tailed Macaques (*Macaca fascicularis*) in the Northern region of Peninsular Malaysia in relation to the environmental factors.

Habitat	**N**	**Parasites +ve**	**Parasites -ve**	**Odds Ratio (95% CI)**	**P-value**
Urban	17	0	17	1.428E-7 (1.428E-7)	0.000*
Rural	24	11	13	22.000 (2.534, 190.998)	0.005*
Plantations	22	1	21	1.143 (0.067, 19.424)	0.926
^{R}Secondary forest	24	1	23	1.000	

^{R}Reference category *Significant ($p<0.05$)

Table 4.10: Univariate Logistic Regression Analysis for *Cryptosporidium* infection in Long-tailed Macaques (*Macaca fascicularis*) in the Northern region of Peninsular Malaysia in relation to the environmental factors.

Habitat	**N**	**Parasites +ve**	**Parasites -ve**	**Odds Ratio (95% CI)**	**P-value**
^{R}Urban	17	3	14	1.000	
Rural	24	5	19	1.228 (0.251, 6.017)	0.800
Plantations	22	4	18	1.037 (0.199, 5.410)	0.966
Secondary forest	24	2	22	0.424 (0.063, 2.867)	0.379
Total	87	14	73		0.153

^{R}Reference category

Table 4.11: Univariate Logistic Regression Analysis for *Giardia* infection in Long-tailed Macaques (*Macaca fascicularis*) in the Northern region of Peninsular Malaysia in relation to the environmental factors.

Habitat	**N**	**Parasites +ve**	**Parasites -ve**	**Odds Ratio (95% CI)**	**P-value**
Urban	17	4	13	1.000	
Rural	24	1	23	0.148 (0.015, 1.468)	0.103
Plantations	22	2	20	0.325 (0.052, 2.037)	0.230
Secondary forest	24	4	20	0.619 (0.132, 2.913)	0.544
Total					0.297

^{R}Reference category

4.5 Nucleotide Sequencing and Phylogenetic Analysis of Malaysian *Blastocystis* and *Giardia* sequences

Blastocystis

Seven out of thirteen sequences for *Blastocystis* were subjected to phylogenetic analyses. Sequences obtained from this study and those retrieved from Genbank revealed close phylogenetic clustering into four main clades. Pylogenetic trees (Figures 4.7) showed local *Blastocystis* sample sequences were dispersed into two out of the four main clusters. The homology is indicated by the distance of each sample in the tree. The *Blastocystis* samples attained in this study were labelled with “MF” initials, while the reference samples were labelled according to the host type and country of origin. Percentage bootstrap supports (100 replicates) are shown by the numbers at the respective nodes. The resulting phylogenetic tree was divided into four main clusters as shown in each box. The sequences obtained from this study (labelled with MF) showed a high homology to sequences recovered from Orangutan from Malaysia, human from Thailand and Malaysia, Chicken from Malaysia, Long-tailed macaques from Phillipines, and *Macaca silenus* from Spain.

Giardia

Six out of eleven *Giardia* sequences were subjected to phylogenetic analyses together with 44 sequences from Genbank. Phylogenetic trees showed that all six of the local *Giardia* sample sequences belongs to the same clusters (Figure 4.8). The homology is indicated by the distance between each sample in the tree. The *Giardia* attained in this study were labelled with “MF” initials, while the reference samples were labelled according to the host type and country of origin. Percentage bootstrap supports (100 replicates) are shown by the numbers at the respective nodes. The sequences of study samples (labelled with MF) showed a high homology to sequences recovered from human from Malaysia, Goat from Malaysia*, Macaca mulatta* from China, and environmental water from Malaysia.

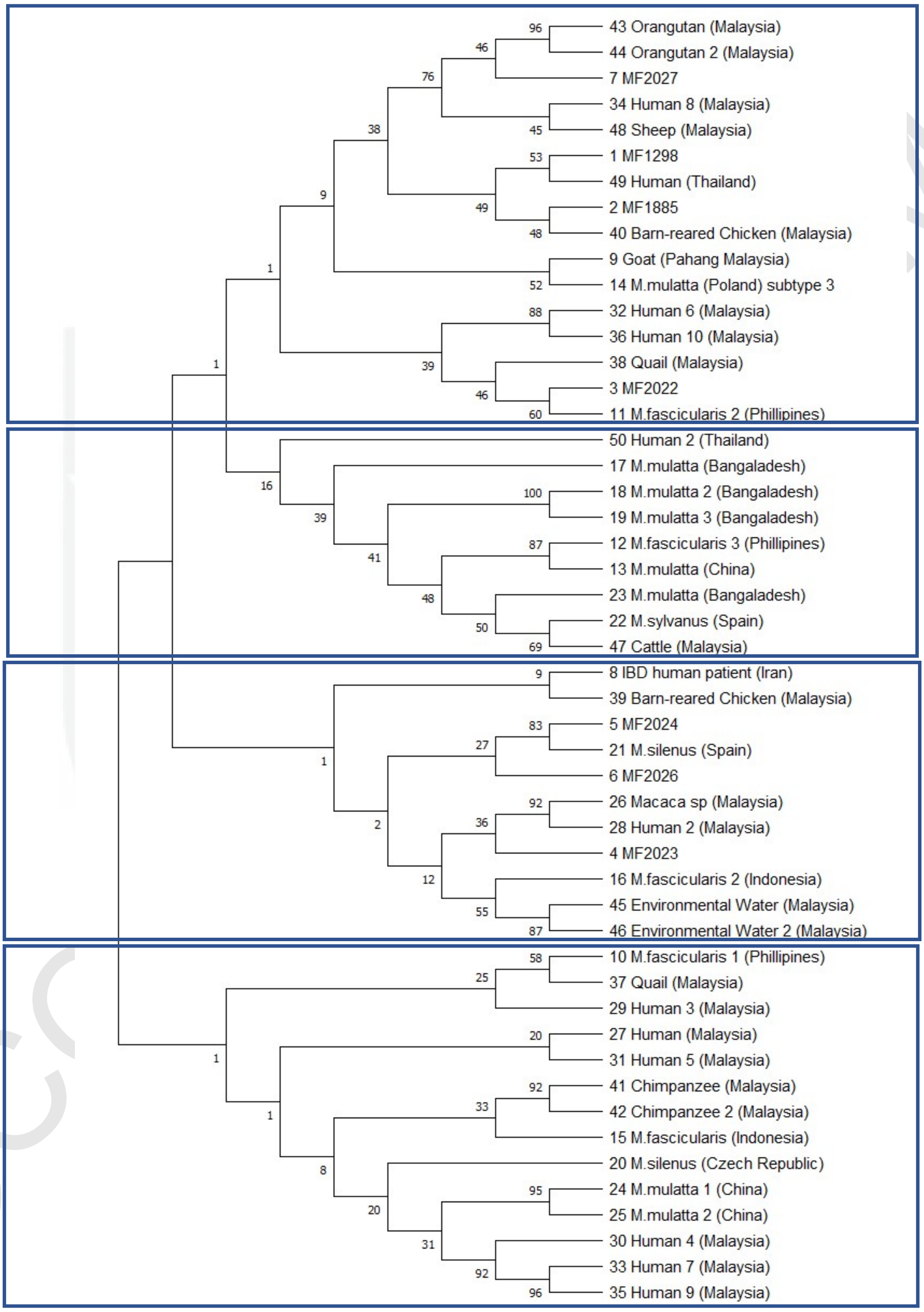

Figure 4.7: Phylogenetic tree of *Blastocystis* by the Maximum Likelihood algorithm using MEGA11.0 program that made up of 50 sequences.

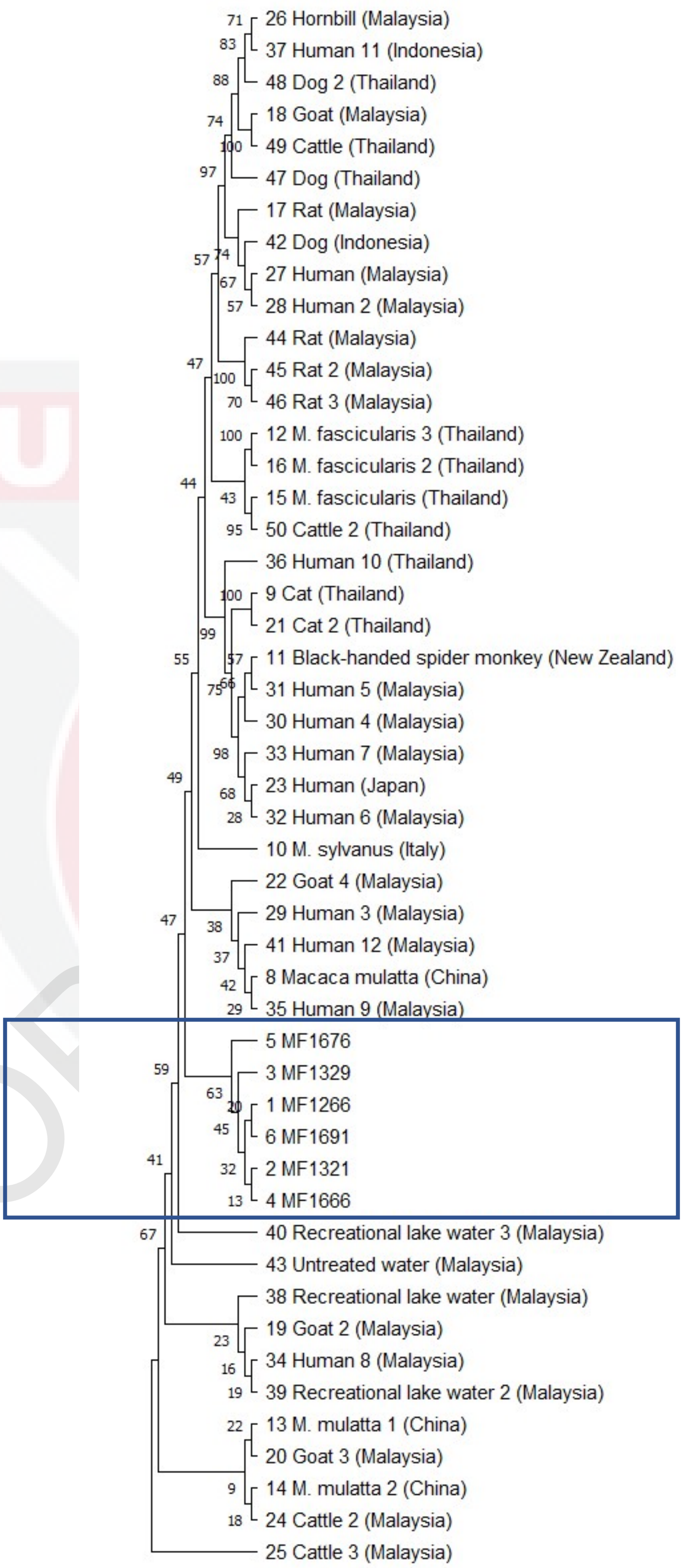

Figure 4.8: Phylogenetic tree of *Giardia* using Maximum Likelihood algorithm generated by MEGA11.0 program using 50 nucleotide sequences.

DISCUSSION

This cross-sectional study is the first in the country to compare the prevalence, internal risk factors (age, gender), and risk factors (habitat) for infection of three enteric parasites, namely *Blastocystis, Cryptosporidium* and *Giardia* of Long-tailed macaques in Northern region of Malaysia. This study also constitutes the first attempt to identify the infection status of zoonotic enteric parasites among *M. fascicularis* in Malaysia. Long-tailed macaques (*Macaca fascicularis*) have a wide range distribution across Southeast Asia (Eudey, 2008; Groves, 2011; Gumert, 2011). According to the IUCN Red list, the status of *M. fascicularis* is endangered (Assessed in March 7, 2022) primarily as consequences of multiple threats. The IUCN primate specialist proposed that their population declined due to rapid development on Southeast Asia coupled with heavy demand in international trading (Eudey, 2008). Other than that, *M. fascicularis* are constantly under pressures from hunting and trapping (Weisman, 2007), persecution from human-macaque conflict (PERHILITAN, 2018), subsistence food (San & Hamada, 2011). PERHILITAN, 2018 reported that 493,823 individual *M. fascicularis* were culled from 2011-2018 in Malaysia pertaining to complaint of macaque intrusion. These macaques are generalist and opportunist species, adapted to wide range of habitats such as forests, coasts, hills, and mountains (Fooden, 1995). They are commonly found in mangrove swamps, particularly riverine habitats. However, they have a significant synanthropic population as they are often seen in human altered habitats, e.g., temples, plantation sites, rural and urban settlements (Gumert, 2011).

Blastocystis, Cryptosporidium and *Giardia* are intestinal protozoa capable of infecting a range of host species and give rise to many waterborne epidemics around the world (Debenham *et al*, 2016). With the rise of global concern in public health and zoonotic diseases, it is important to understand the epidemiology of these parasites. The data gathered in this study is a direct evidence of the presence of zoonotic pathogens among macaques which are commonly seen in anthropogenic areas in the country.

The prevalence of *Blastocystis* (14.94%) is relatively low compared with several studies in Old World and New World monkeys from Thailand, where colonization rates up to 41.9% have been observed (Vaisusuk *et al*, 2018). The infection of *Cryptosporidium* among *M. fascicularis* in this study indicated a relatively high rate (16.09%) compared to previous studies in Thailand (1%) (Sricharern, 2016). Same goes by the higher prevalence of *Giardia* (12.64%) compared to that reported in Thailand (7%), in free range rhesus macaques in public parks in China (8.5%), and among laboratory long-tailed macaques in China (2.4%) (Ye et al, 2014). Nonetheless, there is a possibility of underdiagnosed infection due to low availability of oocysts and cysts in faecal samples (Karim et al, 2014). It cannot be excluded that macaques showing negative results were intermittently shedding oocysts and cysts. Mixed infection analysis reveals that 13 out of 87 (14.94%) individuals were co-infected with two species of protozoa, where none of the macaques had triple co-infection. It is difficult to draw conclusion of the possibility of triple infections due to low number of samples. To date, there is a limited study regarding the co-infection dynamics between these parasites in non-human primates.

For spatial analysis, highest distribution of infections was recorded in Marang, Terengganu for both *Blastocystis* and *Cryptosporidium*. The location with the most *Giardia* infection in *M. fascicularis* was in Kangar, Perlis. It is apparent that Marang, Terengganu which is located near to the coast is the hotspot location for enteric protozoans among the macaques in Peninsular Malaysia. While the exact reason for the high prevalence in this region remains uncertain, it is known that the waterborne nature of these protozoa plays a part in their transmission (Marshall, 1997). As macaques in the coastal region mainly feed on crustaceans, they spend more time in the water hunting for food.

The only environmental risk factor that showed significant relationship with *Blastocystis* infection was the habitat type. In addition, the macaques colonizing the rural areas had a markedly higher odds of infection. This high prevalence could be attributed to the fact that in the rural areas, there is a narrowed human-macaque interface, whereby zoonotic and anthroponotic transmissions may occur (Beisner et al, 2016). The assumption is made based on the similar subtypes infecting human and macaque, indicating the possibility of zoonotic and anthroponotic transmission (Boonslip, 2009). Long-tailed macaques in secondary forests of South-east Asia have a wide range of human interactions (Adhikari, 2017), such as tourists or local residents. Low awareness regarding disease transmission between human and anthropogenic macaques for people residing close proximity to the forest could be a driving force of zoo-anthroponotic transmission of these parasites. Macaques in the secondary forest can acquire these parasites from other wildlife that share the same habitat, such as wild boar and jungle fowl. These animals may shed the organism in their faeces and contaminate the food and water of macaques (Mul *et al*, 2007).

Interestingly, none of the epidemiological risk factors shows significant effect on infection of *Cyrptosporidium* and *Giardia* among these macaques. Multivariate analysis revealed no significant relationship between the infection with all three enteric parasites and all risk factors examined. Macaques inhabiting the urban regions had a generally lower prevalence of the enteric protozoa compared to other habitat types. This finding could be related with urbanization, public investment in basic sanitation and improvement of general living conditions (Boonslip, 2009). Hence, there will be lower parasite harbouring in the environment of urban region.

Although statistical analysis shows no significant association between gender and infection of *Blastocystis, Cryptosporidium* and *Giardia*, the female macaques more often than not had a higher prevalence than their male conspecifics in all three protozoa group. The phenomenon of gender-related susceptibility is also seen in orangutan (*Pongo abelii*). A study in Indonesia by Mul *et al.* (2007) stated that female orangutans had a significantly high prevalence of intestinal parasites than males, and it is related to the degree of physical contact with other individuals. There are several hypotheses made based on the studies; first, it is believed that female macaques travel relatively often than males to search for food and feed their young. Second, females are accompanied by their offspring for most of the time. While the females are feeding, juveniles often socialize together and contact with parasites, passing to the females (Mul *et al*, 2007). Adult males only engage in physically contact during mating and fights, which are relatively rare occurrences (Singleton & van Schaik, 2001). Statistical analysis revealed that there was no association between infection of *Blastocystis, Cryptosporidium* and *Giardia* with the age group of macaques. The age-infection pattern is commonly related with their immunity status. However, the findings with regards to epidemiological risk factors may need future verification using a more comprehensive study of the possible host variables that may affect the prevalence of the parasites.

Based on the phylogenetic analysis, the *Blastocystis* isolated from the macaques were situated diversely in the four different clades, indicating high genetic variability of this parasite in Northern Region of Peninsular Malaysia. Vaisusuk *et al*. (2018) pointed out that *Blastocystis* exhibits extensive genetic diversity and comprises not less than 17 subtypes (ST) based on analysis of SSU-rRNA genes. The phylogenetic tree of Maximum Likelihood algorithm also indicates that the local *Blastocystis* sequences were found to be evolutionary close related to *Blastocystis* infecting Orangutan from Malaysia, human from Malaysia and Thailand, poultry from Malaysia, *Macaca* sp. from the Philippines and Spain. Some homology was identified with local *Blastocystis* and those isolates from environmental water in Malaysia.

The phylogenetic tree of *Giardia* was successfully constructed using a total of 50 sequences including 4 DNA fragments of the *Giardia* positive study samples and 44 reference sequences obtained from Gene bank. The sequences showed high homology to the *Giardia* recovered from other macaque species, human and goat in Malaysia. Humans might acquire infection by *Giardia* from these peri-domestic macaques. However, it is difficult to verify the zoonotic potential of these isolates as multi-locus typing based on several gene fragments is warranted. A close genetic proximity was observed in the isolates from study sample and *Giardia* in environmental water bodies, suggesting that the macaques are able to shed *Giardia* in their faeces that will contaminate environmental waters. According to Gallas-Lindemann (2012), the robustness of the cysts allows *Giardia* to persist in the environment, and water plays an important role transmission cycle. To corroborate the findings, further genotype grouping and investigation regarding the relationship with epidemiology, symptoms, virulence and pathogenesis is recommended. The phylogenetic tree of *Cryptosporidium* was not generated due to insufficient local sample sequences.

The study focused on *M. fascicularis* from the Northern region of peninsular Malaysia, which is near the border of neighbouring country, Thailand. This will further facilitate studies on potential trans-boundary spread of zoonotic pathogens. Food and Agriculture Organisation (FAO) of the United Nations defines trans-boundary animal disease (TAD) as those disease that are of significant socioeconomic, trade and public health importance, which can be spread easily to other countries. They require cooperation between several countries. Wildlife plays a major role in the transmission of many important TAD, which many of them are zoonotic. Wildlife is the original source and maintenance host of pathogens, they continue to harbour and spread the pathogens without expressing clinical symptoms.

Human activities such as intensification of farming, altering landscapes and demographics, regional conflict, illegal animal trade, animal translocations are one of the most important mechanisms of establishing new interfaces between humans and wildlife, driving disease emergence (Siembieda *et al*, 2011). Due to increased proximity between animal populations, interspecies transmission occurs whereby novel diseases are introduced to new hosts. Pathogen evolutions are possible with interspecies progression through a variety of mechanisms, including genetic reassortment and recombination (VandeWoude & Apetrei, 2006). Besides enhancing interspecies disease transmission, these activities result in changing the spatial distribution of hosts.

In developing countries including Malaysia, enteric protozoan infections in humans are recognized as an important cause of morbidity, with children being the most vulnerable population (Harhay et al, 2010). The protozoa are mainly transmitted through faecal-oral route, followed by ingestion of infective stages through contaminated water and food. Understanding the epidemiology of this group of parasites is crucial to public health, as their wide range of hosts increases the likelihood of emergence of novel infections in humans and domestic animals. Therefore, the study of the factors affecting disease ecology and pathogen cycle is essential to minimize pathogen from establishing infection in potential host and induce clinical disease, which eventually cause death.

Despite the worldwide prevalence and the unfavourable consequences of zoonotic enteric protozoa, especially in immunocompromised patients, children and elderlies, current control program is still inadequate. Public health precautions are necessary to reduce the transmission of pathogens. First of all, improvements should be done on detection of pathogens in wildlife and humans. Therefore, professional training and capacity building need to be initiated to enhance regional surveillance and early detection of disease. It is important for One Health forces especially veterinarians, wildlife professionals, human medical personals, and ecologists to work across disciplines and sharing knowledge regarding monitoring and control of disease outbreak. The regions with higher rates of infection needs to be prioritized.

Furthermore, local authorities, stakeholders and communities are responsible for the sustainable urban development to meet the needs of a community without compromising wildlife resources. It is the combination of strategies to reduce pollution and preserve natural resources. Rapid urbanization has caused drastic environmental changes such as landscape and topography, due to deforestation and landscape conversion. Therefore, wild macaques are forced to scavenging for food and water at peridomestic area due to the loss of habitat. In Malaysia, it is suggested that Department of Wildlife and National Parks (PERHILITAN) should work alongside with Ministry of Local Government Development to improve living conditions of both human and macaque populations. Hence, the human-wildlife interface will be overcome alongside with human-macaque conflict

In addition, there are some zoonotic diseases where the mechanism of transmission is still open to question. With the increasing morbidity and mortality due to these pathogens, it is critical to learn more about the complex ecology, identify the reservoirs and changing epidemiological patterns to limit disease transmission. Understanding wildlife and their role is important especially in transboundary pathogen transmission. Further studies should include identification of these zoonotic protozoans in other host species, such as other primates including humans, as well as companion animals living in the same environment. The location of study should be expanded to cover more regions in Malaysia.

The limitations of this study are the small sample size and the nature of single sample collected from each animal; opportunistic sampling may have led to selection bias and underestimation of the prevalence rates. A larger sample size or a longer duration of research period are needed for further studies. In addition, only age, sex, and habitat of the macaques were studied; however, other important risk factors, e.g., season, diet, or water source could be suggested for future study to help in prevention and control of intestinal parasitic infection in this population.

Furthermore, archived samples were kept in the refrigerator for up to 6 years, DNA degradation might lead to underdiagnosing of results. The nucleotides tend to break into smaller fragments when degrades. DNA degradation may be results from few factors including tissue preservation methods, exposure to UV radiation, temperature, pH and salt concentration of the environment (Dean & Ballard, 2001). Repetitive freezing and thawing of DNA samples during the procedures, leaving DNA samples at room temperature for too long, exposing DNA samples to heat might damage the nucleotides.

To minimize error in results, PCR optimization is important, and it is achieved by optimizing the reaction conditions which can influence the specificity and sensitivity of the reactions. The process includes determining the appropriate concentration of primers, annealing temperature, DNA template concentrations and quality, primer concentrations and several other factors (Grunenwald, 2013). Various problems may occur if PCR optimization is not performed correctly such as insufficient amplification of the target template or absence of PCR products, and presence of non-specific bands or fuzzy background.

CONCLUSION

In summary, *Macaca fascicularis* in the Northern Region of Peninsular Malaysia harbours *Blastocystis, Cryptosporidium* and *Giardia* which are potentially zoonotic. Macaques inhabiting the rural areas had a significantly higher prevalence of *Blastocystis* than those living in other habitat types. These intestinal protozoa are mainly transmitted through faecal-oral route, *via* contaminated food and water. It is also proven that these parasites persist in the environment such as water bodies, facilitating their transmission. Thus, public health measures are needed to minimize the risk of zoonotic and anthroponotic transmission of these parasites, for example sustainable urban development, sustainable disease identification, resolving human-macaque conflict and public awareness programme. However, more in-depth investigation is warranted to understand the epidemiological risk factor of transmissions of these parasites.

REFERENCES

Belkessa, S., Ait-Salem, E., Laatamna, A., Houali, K., Sönksen, U. W., Hakem, A., Bouchene, Z., Ghalmi, F., and Stensvold, C. R. (2021). Prevalence and Clinical Manifestations of *Giardia intestinalis* and Other Intestinal Parasites in Children and Adults in Algeria. ASTMH. 104(3): 910-916. https://doi.org/10.4269/ajtmh.20-0187

Bouzid, M., Hunter, P. R., Chalmers, R. M., and Tyler, K. M. (2013). *Cryptosporidium* pathogenicity and virulence. Clin. Microbiol. Rev. 26(1): 115–134. https://doi.org/10.1128/cmr.00076-12

Conners, E. E., Miller, A. D., Balachandran, N., Robinson, B. M., and Benedict, K. M. (2021). Giardiasis outbreaks — United States, 2012–2017. MMWR. 70(9): 304–307. https://doi.org/10.15585/mmwr.mm7009a2

Darwish, B., Aboualchamat, G., and Nahhas, S. A. (2023). Molecular characterization of *Blastocystis* subtypes in symptomatic patients from the southern region of Syria. PloS One, 18(3), e0283291.

Gerace, E., Di Marco Lo Presti, V., and Biondo, C. (2019). *Cryptosporidium* infection: epidemiology, pathogenesis, and differential diagnosis. Eur. J. Microbiol. Immunol. 9(4): 119–123. https://doi.org/10.1556/1886.2019.00019

Grunenwald, H. (2003). Optimization of polymerase chain reactions. In Humana Press eBooks. pp. 89–100. https://doi.org/10.1385/1-59259-384-4:89

Heyworth, M. F. (2016). *Giardia duodenalis* genetic assemblages and hosts. Parasite. 23:13. https://doi.org/10.1051/parasite/2016013

John, J. D., Kristoffer, T., Sandhya, K. and Lucy, J. R. (2017): Occurrence of *Giardia*, *Cryptosporidium*, and *Entamoeba* in wild rhesus macaques (*Macaca mulatta*) living in urban and semi-rural North-West India. IJP:PAW. 6: 29-34.

Haghi, M.M., Khorshidvand, Z., Khazaei, S., Foroughi-Parvar, F., Sarmadian, H., Barati, N., Etemadifar, F. and Ghasemikhah, R. (2020): *Cryptosporidium* animal species in Iran: a systematic review and meta-analysis. Trop Med Health. 48:97. https://doi.org/10.1186/s41182-020-00278-9

Hopkins, R..M., Meloni, B.P., Groth, D.M., Wetherall, J.D., Reynoldson, J.A., Thompson, R.C.A . (2012): Ribosomal RNA Sequencing Reveals Differences between the Genotypes of *Giardia* Isolates Recovered from Humans and Dogs Living in the Same Locality. JSTOR. 83(1): 44-51.

Jian, L., Yijing, R., Haiying, C., Weiyi, H. and Xinyu, F. (2021): Risk Evaluation of Pathogenic Intestinal Protozoa Infection Among LaboratoryMacaques, Animal

Facility Workers, and Nearby Villagers From One Health Perspective. Front Vet Sci. 8 (696568). https://doi.org/10.3389%2Ffvets.2021.696568

Jones-Engel, L. (2022). *IUCN Red List of Threatened Species: Macaca fascicularis*. IUCN Red List of Threatened Species. https://www.iucnredlist.org/fr/species/12551/221666136

Karuppannan, K., Saaban, S., Mustapa, A. R., Zainal Abidin, F. A., Azimat, N. A. and Keliang, C. (2014): Population Status of Long-Tailed Macaque (*Macaca fascicularis*) in Peninsular Malaysia. J Primatol. 3(1): 118. https://doi.org/10.4172/2167-6801.1000118

Koehler, A. V., Korhonen, P. K., Hall, R. S., Young, N. D., Wang, T., Haydon, S. R., and Gasser, R. B. (2017). Use of a bioinformatic-assisted primer design strategy to establish a new nested PCR-based method for *Cryptosporidium*. Parasit Vectors. *10*(1). https://doi.org/10.1186/s13071-017-2462-4

Luis, E. J. P., Fidel, A. N-F., Jorge, F. N., Iraís, A. M. and Iredys, C. R. (2020): Diagnosis of intestinal protozoan infections in patients in Cuba by microscopy and molecular methods: advantages and disadvantages. J. Micmeth. 179 (106102).

Martin, S. W. (1992): Giardiasis. Clin. Microbiol. Rev. 5(1): 93-100.

Md-Zain, B.M., Ruslin, F. and Idris, W.M.R. (2014): Human-macaque conflict at the main campus of Universiti Kebangsaan Malaysia. Pertanika J. Trop. Agric. Sci. 37 (1): 73 – 85.

Nantavisai, K., Mungthin, M., Tan-ariya, P., Rangsin, R., Naaglor, T. and Leelayoova, S. (2007). Evaluation of the Sensitivities of DNA Extraction and PCR Methods for Detection of *Giardia duodenalis* in Stool Specimens. J. Clin. Microbiol. *45*(2): 581–583. https://doi.org/10.1128/jcm.01823-06

Newell, D. G., Koopmans, M., Verhoef, L., Duizer, E., Aïdara-Kane, A., Sprong, H., Opsteegh, M., Langelaar, M., Threfall, J., Scheutz, F., Van Der Giessen, J., and

Kruse, H. (2010). Food-borne diseases — The challenges of 20years ago still persist while new ones continue to emerge. Int. J. Food Microbiol. 139: S3–S15. https://doi.org/10.1016/j.ijfoodmicro.2010.01.021

Ortega, Y. R. and Eberhard, M. L. (2008). Protozoan diseases: cryptosporidiosis, giardiasis and other intestinal protozoan diseases. In *Elsevier eBooks* (pp. 354–366). https://doi.org/10.1016/b978-012373960-5.00485-8

Peter F.M. T, Arie H. H. (2002). Risk assessment for protozoan parasites. Int. Biodeterior. Biodegradation. 50 (2002): 185 – 193.

Ramírez, J. D., Heredia, R. D., Hernández, C., León, C., Moncada, L. I., Reyes, P., Pinilla, A. E. and López, M. C. (2015). Molecular diagnosis and genotype analysis of *Giardia duodenalis* in asymptomatic children from a rural area in central Colombia. Infect. Genet. Evol. 32: 208–213. https://doi.org/10.1016/j.meegid.2015.03.015

Rauff-Adedotun, A. A., Zain, S. N. M. and Termizi, F. H. M. (2020). Current status of *Blastocystis sp*. in animals from Southeast Asia: a review. Parasitol. Res. 119(11): 3559–3570. https://doi.org/10.1007/s00436-020-06828-8

Rauff-Adedotun, A. A., Termizi, F. H. M., Shaari, N. and Lee, I. L. (2021). The Coexistence of *Blastocystis spp*. in Humans, Animals and Environmental Sources from 2010–2021 in Asia. Biol. 10(10): 990. https://doi.org/10.3390/biology10100990

Ryan, U., Fayer, R., Xiao, L. (2014). Cryptosporidium species in humans and animals: current understanding and research needs. Parasitology. 141(13):1667–85.

Scicluna, S. M., Tawari, B. and Clark, C. G. (2006). DNA barcoding of *Blastocystis*. Protist. 157(1): 77–85. https://doi.org/10.1016/j.protis.2005.12.001

Siembieda, J. L., Kock, R., McCracken, T. A. and Newman, S. H. (2011). The role of wildlife in transboundary animal diseases. Anim. Health Res. Rev. 12(1): 95–111. https://doi.org/10.1017/s1466252311000041

Sricharern, W., Inpankaew, T., Keawmongkol, S., Supanam, J., Stich, R. W. and Jittapalapong, S. (2016). Molecular detection and prevalence of *Giardia duodenalis* and *Cryptosporidium spp.* among long-tailed macaques (*Macaca fascicularis*) in Thailand. Infect. Genet. Evol. 40: 310–314. https://doi.org/10.1016/j.meegid.2016.02.004

Sricharern, W., Inpankaew, T., Kaewmongkol, S., Jarudecha, T. and Inthong, N. (2021). Molecular identification of *Trichuris trichiura* and *Hymenolepis diminuta* in long-tailed macaques (*Macaca fascicularis*) in Lopburi, Thailand. Vet. World, 14(4): 884–888. https://doi.org/10.14202/vetworld.2021.884-888

Sotiriadou, I., Pantchev, N., Gassmann, D. and Karanis, P. (2013). Molecular identification of *Giardia* and *Cryptosporidium* from dogs and cats. Parasite. 20: 8. https://doi.org/10.1051/parasite/2013008

Stark, D., Barratt, J.L.N., Hal, S.V., Marriott, D., Harkness, J. and Ellis, J.T. (2009). Clinical Significance of Enteric Protozoa in the Immunosuppressed Human Population. Clin. Microbiol. Rev. 22(4): 634–650.

Tangtrongsup, S., Sripakdee, D., Malaivijitnond, S., Angkuratipakorn, R. and Lappin, M. R. (2019). Intestinal Parasites and the Occurrence of Zoonotic *Giardia* *duodenalis* Genotype in Captive Gibbons at Krabokkoo Wildlife Breeding Center, Thailand. Front. vet. sci. https://doi.org/10.3389/fvets.2019.00110

Vaisusuk, K., Saijuntha, W., Sedlak, S., Thanchomnang, T., Pilap, W., Suksavate, W., Stensvold, C. R. and Tantrawatpan, C. (2018). *Blastocystis* subtypes detected in long-tailed macaques in Thailand—Further evidence of cryptic host specificity. Acta Tropica. 184: 78–82. https://doi.org/10.1016/j.actatropica.2017.09.002

Verweij, J. J. and Stensvold, C. R. (2014). Molecular testing for clinical diagnosis and epidemiological investigations of intestinal parasitic infections. Clin. Microbiol. Rev. 27(2): 371–418. https://doi.org/10.1128/cmr.00122-13

Wawrzyniak, I., Poirier, P., Viscogliosi, E., Meloni, D., Texier, C., Delbac, F. and Alaoui, H. E. (2013). *Blastocystis*, an unrecognized parasite: an overview of pathogenesis and diagnosis. TAID. 1(5): 167–178. https://doi.org/10.1177/2049936113504754

Ye, J., Xiao, L., Ma, J., Guo, M., Liu, H. and Feng, Y. (2012). Anthroponotic enteric parasites in monkeys in public Park, China. Emerg. Infect. Dis. 18(10): 1640–1643. https://doi.org/10.3201/eid1810.120653

Ye, J., Xiao, L., Li, J., Huang, W., Amer, S., Guo, Y., Roellig, D. M. and Feng, Y. (2014). Occurrence of human-pathogenic Enterocytozoon bieneusi, *Giardia* duodenalis and Cryptosporidium genotypes in laboratory macaques in Guangxi, China. Parasitol. Int. 63(1): 132–137. https://doi.org/10.1016/j.parint.2013.10.007

Yong, Y. Z., Ikki, M., Anna,W. and Henry, B. (2021): The Distribution and Abundance of Long-Tailed Macaques in the Main Campus of Universiti Malaysia Sabah and its Vicinity. Trop. Bio. and Conserv. 18: 185–202.

Zanzani, S. A., Gazzonis, A. L., Epis, S. and Manfredi, M. T. (2015). Study of the gastrointestinal parasitic fauna of captive non-human primates (*Macaca fascicularis*). Parasitol. Res. 115(1): 307–312. https://doi.org/10.1007/s00436-015-4748-9

Zhang, K., Zheng, S., Wang, Y. et al. (2021) Occurrence and molecular characterization of *Cryptosporidium spp*., *Giardia duodenalis, Enterocytozoon bieneusi*, and *Blastocystis sp.* in captive wild animals in zoos in Henan, China. BMC Vet Res 17, 332.