UNIVERSITI PUTRA MALAYSIA

MOLECULAR DETECTION AND GENOTYPING OF HEPATOZOON (APICOMPLEXA: HEPATOZOIDAE) INFECTING PERI-DOMESTIC RODENTS IN SELANGOR, MALAYSIA

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CERTIFICATION

It is hereby certified that I have read this project entitled “Molecular Detection And Genotyping Of *Hepatozoon* (Apicomplexa : Hepatozoidae) Infecting Peri-Domestic Rodents In Selangor, Malaysia”, by Muhammad Syahir Najmi Bin Mohd Nasir and in my opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the final course VPD 4999- Final Year Project

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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan VPD 4999- Projek Akhir Tahun.

PENGESANAN MOLEKULAR DAN GENOTIP *HEPATOZOON* (APICOMPLEXA : HEPATOZOIDAE) YANG MENJANGKITI TIKUS PERI-DOMESTIK DI SELANGOR, MALAYSIA

oleh

Muhammad Syahir Najmi Bin Mohd Nasir

2020

Pegawai Pemantau: Dr Reuben Sharma

Kepelbagaian patogen yang dimiliki oleh tikus telah menjad ia sebagai tumpuan banyak penkaji kerana potensi mereka untuk menjadi vektor kepada penyakit zoonosis. Walaubagaimanapun, hanya sedikit maklumat mengenai pengesanan molekul hemoparasit di antara mamalia kecil ini di Malaysia. Kajian ini dilakukan untuk menentukan kelaziman molekul, kepelbagaian spesies dan kepelbagaian genetik *Hepatozoon* bawaan tikus di negara ini. Sebanyak 58 tikus peri-domestik diperoleh menggunakan perangkap dawai mesh dari 6 kawasan komersial dan perumahan di Selangor, Malaysia. Sebanyak 24% yg ditangkap adalah dari kawasan perumahan, sementara 76% berasal dari kawasan komersial. Darah diambil untuk pengesana

Hepatozoon melalui kaedah mikroskopi dan molekular. Setelah pengekstrakan DNA genom, fragmen separa dari gen *Hepatozoon* 18SSUrRNA diperkuat menggunakan primer khusus genus. Kaedah mikroskopi mengesan *Hepatozoon* pada 17% tikus, dan DNA *Hepatozoon* dikesan pada 25.9% dari tikus yang telah diperiksa. Kelaziman *Hepatozoon* antara tikus yang terperangkap dari kawasan komersial dan perumahan adalah sekitar 22.4% dan 3.5%. Analisis bioinformatik dan filogenetik lokus gen 18SSUrRNA sebanyak 15 amplikon positif menggunakan algoritma maximum parsimony (MP) dan neighbor-joining (NJ) mendedahkan pengelompokan isolasi Malaysia yang berbeza, dengan persamaan yang terdekat adalah *Hepatozoon* sp. isolasi yang dikesan di Chile (*Rattus norvegicus*), Thailand (Bandicote indica dan Amblyomma varanense). Kajian ini merupakan laporan pertama, pengesanan molekul dan genotip *Hepatozoon* di kalangan tikus di Malaysia.

ABSTRACT

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

MOLECULAR DETECTION AND GENOTYPING OF *HEPATOZOON* (APICOMPLEXA : HEPATOZOIDAE) INFECTING PERI-DOMESTIC RODENTS IN SELANGOR, MALAYSIA

by

Muhammad Syahir Najmi Bin Mohd Nasir

2020

Supervisor: Dr Reuben Sharma

The diversity of pathogens harbored by rodents have been the focus of many studies due to their potential as vectors of zoonotic diseases. However, there remains a paucity of information on the molecular detection of haemoparasites among these small mammals in Malaysia. The present study was undertaken to determine the molecular prevalence, species diversity and genetic variability of rodent-borne *Hepatozoon* in the country. A total of 58 peri-domestic rodents were obtained using mesh wire traps form five commercial and residential localities in Selangor, Malaysia. Of the rodents trapped, 24% were from residential areas, while 76% were from commercial areas. Blood was collected for microscopy and molecular detection of *Hepatozoon*. Following genomic DNA extraction, a partial fragment of the *Hepatozoon* 18SSUrRNA gene was amplified using genus-specific primers. Microscopy examination of thin blood films revealed a prevalence of 17%, while *Hepatozoon* DNA was detected in 25.9% of the rodents examined. The prevalence of

Hepatozoon among the rodents trapped from the commercial and residential areas were 22.4% and 3.5%, respectively. Bioinformatic and phylogenetic analyses of the 18SSUrRNA gene locus of 15 positive amplicons using Maximum Parsimony (MP) and Neighbor-joining (NJ) algorithms revealed distinct clustering of the Malaysian isolates, with closest affinities to *Hepatozoon* sp. isolates detected in Chile (*Rattus norvegicus*), Thailand (*Bandicote indica* and *Amblyomma varanense*). The present study constitutes the first report, molecular detection, and genotyping of *Hepatozoon* among rodents in Malaysia.

Keywords: *Hepatozoon*, molecular detection, rodents, Malaysia

INTRODUCTION

Rats of the family Muridae are one of the largest groups of rodents in Malaysia (Medway, 1983; Ow-Yang 1971). These small mammals inhabit a diverse array of habitats, and with their high adaptability, are able to thrive in urban environments worldwide. These rodents have a high reproductive rate and are often considered as pests in agricultural areas as well as in the peri-domestic environment (Siti Shafiyyah *et al*. 2012). The rapid destruction of forested habitats, the conversion of land use for urban development, and the lack of proper garbage disposal and sewerage, has escalated the population of urban rodents. This is one of the primary public health concerns as these rodents are known to be reservoirs of many zoonotic pathogens including viruses, bacteria and parasites (Paramasvaran *et al*, 2009; Zain *et al*., 2015).

Peri-domestic rats can easily transmit zoonotic pathogens in the urban setting due to close contact with humans and the environment. The multitude of parasitic fauna that accompany the rat population presents many opportunities of transmission through direct contact and *via* arthropod vectors. In Malaysia, the first recorded prevalence of parasites in wild rat was reported by Adams (1993). Subsequently, a number of studies have demonstrated the presence of various haemoparasites infecting local rats that were captured from multiple sites in peninsular Malaysia (Siti Shafiyyah *et al*., 2012; Alias *et al*., 2014). To date, most studies on haemoparasites in rodents from Malaysia have relied on microscopy detection. There is a need for a systematic survey using molecular techniques like PCR which is more sensitive and specific in detecting parasitaemia. The present investigation aims at molecular detection and genotyping of *Hepatozoon* sp. infecting peri-domestic rats in Selangor, Malaysia.

Hepatozoon is an Apicomplexian alveolate which parasitized the erythrocytes of vertebrates including amphibians, reptiles, and mammals (Smith, 1996). In Malaysia, the genus is known to infect dogs (Rajmanickam *et al*., 1985), but there is a lack of information on the prevalence in rodents and other small mammals. It is envisaged that the data obtained will provide a clearer understanding on the diversity and prevalence of *Hepatozoon* sp. In Malaysia and the potential role of urban rats as reservoirs of the pathogen.

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The present study was undertaken with the following objectives:

1) To ascertain the molecular prevalence of *Hepatozoon* sp. infecting peri-domestic rodents in Selangor, Malaysia.
2) To determine the genotypes of *Hepatozoon* infecting these peri-domestic rodents.

LITERATURE REVIEW

Blood parasites in peri-domestic rodents in Peninsular Malaysia

In Malaysia, Adams (1933) pioneered research on the prevalence of parasites in wild rats, followed by Singh and Chee-Hock (1971), where out of 999 feral rats examined, 450 were positive with nematodes parasites. Lim *et al.* (1997) also recorded the presence of nematodes in wild rodent in various parts of the country in peninsular Malaysia. While focus has been mostly on nematode parasites of rodents in the past, there is a paucity on the species diversity, prevalence and epidemiology of blood parasites infecting peri domestic rodents.

Zainal and Noor (1999) recorded the presence of *Trypanosoma lewisi* among local rodents with a prevalence of 21.7%. A subsequent study (Paramasvaran *et. al.*, 2003) on rodents in Bukit Kemandol, an Orang Asli village, did not yield any blood parasites among the rodents. A further study by Paramasvaran *et al*., 2009) also did not demonstrate any haemoparasitaemia among rodents from five wet markets (Chow Kit, Dato Keramat, Setapak, Jinjang and Kepong) in Kuala Lumpur. In addition, Premaalatha *et. al.* (2010) did not detect any blood parasites among rodents trapped from the surrounding area of the Veterinary Research Instituted, Ipoh, Malaysia. A recent study, however, manage to record a low level of *Trypanosoma lewisi* infection (1.5%) among 137 wild rodents in Kuala Lumpur (Siti Shafiyyah *et al*., 2012). The use of microscopy detection only in the studies, may be the reason for the lack of detection or the low prevalence of haemoparasites among local rodents, thus confirming the need for molecular detection.

Hepatozoon

Members of the genus *Hepatozoon* are apicomplexan alveolates belonging to a group collective known as hemogregarines, all of which have a heteroxenous cycle involving an intermediate vertebrates host and a blood feeding definitive invertebrate host (Smith, 1996). It is an obligate intra-erythrocytic parasite that possesses a unique structure known as the apicoplast that is used to penetrate into the host cell. *Hepatozoon* tend to be host species specific. Smith (1996) has listed a few know vertebrates and invertebrates which had the capabilities to propagate this parasite. This wide array of vertebrate host include mammals (Wenyon, 1926), crocodilians (Hoare, 1932), lizards (Robin, 1936), snakes (Ball *et al*., 1967), turtles (Michel, 1973), and anurans (Desser *et al*., 1995). In addition, invertebrates such ticks, mites, reduviid bugs, sandflies, mosquitoes, and tsetse flies (Telford, 1984) are known vectors of this haemoparasite.

Hepatozoon employs two method for propagation; sexual reproduction which occur in the invertebrate host, and asexual reproduction which occurs in vertebrate host (Figure 1). The main routes for this infection to spread are by direct ingestion of invertebrates containing the mature oocyst, or by predation of infected paratenic host the secondary host. The transmission cycle occurs when the vertebrate host accidentally ingested the invertebrates which contain the mature oocyst or form paratenic host that contain high parasite burden of *Hepatozoon* sp.

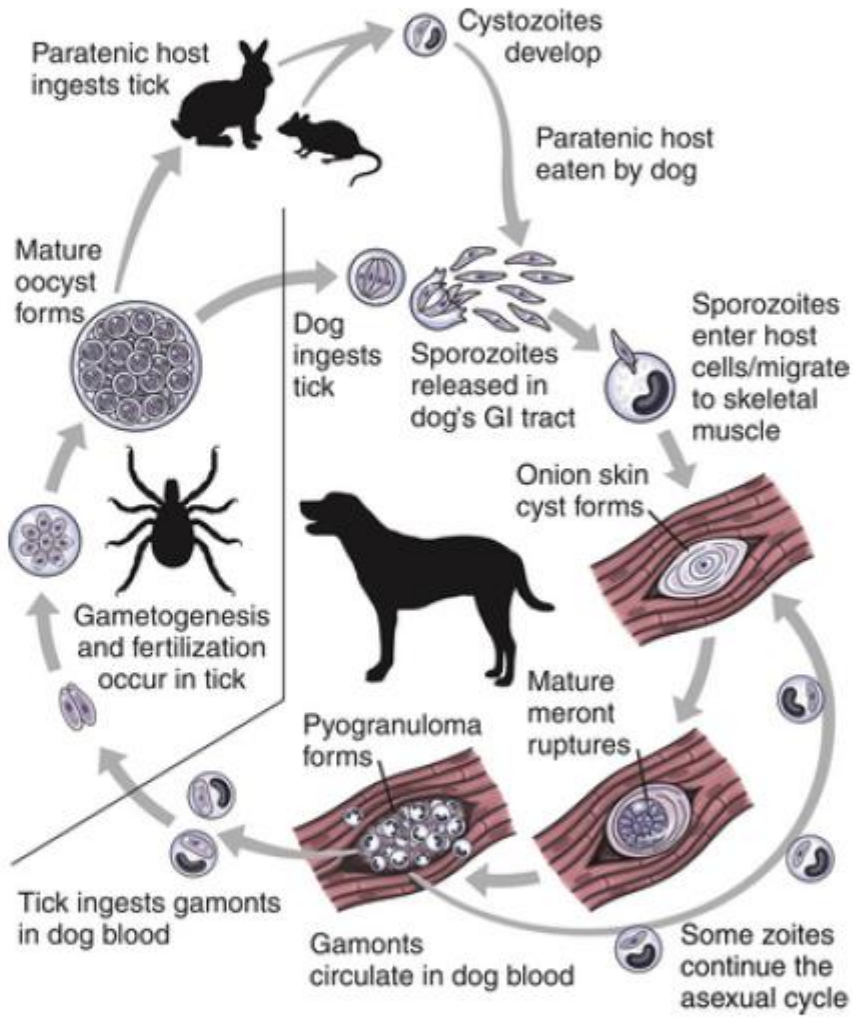

Figure 1. The Life cycle of *Hepatozoon* in the primary vertebrate host, the paratenic host and invertebrate intermediate host. Adapted from: Vincent-Johnson (2013). Canine and Feline Infectious Diseases, 747–759.

The life cycle of *Hepatozoon* in rodents has not been well studied, thus, the life cycle of *H. americanum* in the canine host will be used as a reference. The life cycle starts from the primary host (vertebrae) which accidentally ingested the intermediate host (ticks) which contain mature oocyst of *Hepatozoon*. Each mature oocyst contains hundreds of sporocysts, where each of the sporocyst contains 10 to 26 infective sporozoites. The sporozoites will be released once in contact with the digestion juice (bile's juice for *H. americanum*), which enables the sporozoites to easily penetrated the intestinal epithelium wall. The sporozoites will then be transport to the targeted organ using mononuclear cell. *Hepatozoon americanum* has a predilection for the skeletal muscle cells where the sporozoites will lodge between the myocytes (Vincent, 2013). Concentric layers of mucopolysaccharide will act as protection from the immune response. Because of the appearance which is onion-like, the formation is termed as "onion-skin cyst".

The sporozoites will further develop into mature meront *via* merogony and upon maturation, the cysts will rupture and release the merozoites. Upon the rupture of the cyst, inflammatory response will be initiated by the body and signal for neutrophils and monocytes to gather. Many of the inflammatory cells will be infected and be transported throughout the body. Some of the infected cells will enter the blood circulation *via* angiogenesis and travel to other target sites. The infected cell can either continue the asexual reproduction cycle or develop into gamonts. Gamonts will be ingested by the tick vector, thus completing the life cycle. The life cycle could also include other infected paratenic host which may be eaten by the primary hosts, thus completing the life cycle.

Peri-domestic rodents in Peninsular Malaysia

Rodents are one of the most well-known mammals in the world. They consist of 42% of the whole mammals in the world. The Rodent family (order Rodentia) can be further divided into five distinct orders, namely the Sciuromorpha (squirrel-like), Castorimorpha, Myomorpha (mouse-like), Anomaluromorpha, and Hystricomorpha (porcupine-like) (Carleton and Musser, 2005). Collective they include 33 families, 481 genera and 2277 species. Among all of them, one the family (the Muridae) has been extremely successful in propagating and dominating most of the regions and habitats in the world. Their ability to adapt and exploit new environments and habitats is one of the major reasons why this species can strive in rural or even the urban area where most species have a hard time to cope and adjust efficiently. Based on a previous literature (Paramasvaran *et al*., 2009; Paramasvaran and Sani, 2012; Zain *et al*., 2012; Siti Shafiyyah *et al*., 2012; Alias *et al*., 2014), some of the common species that have been recorded in the peri-domestic setting in peninsular Malaysia include *R. novergicus*, *R. rattus* and *R. argentiventer*.

Rattus rattus / Rattus tanezumi

This species has a range that extends from Afghanistan through north India, Nepal, Bhutan to southern China, and Korea to Tai-wan and Japan (Musser and Carleton, 2005). Based on the study conducted by Pimsai *et al*. (2014), the species was also recorded in Myanmar, Thailand, Malaysia and Singapore. It is listed by IUCN as 'Least Concern' "due to its wide distribution and tolerance of a wide variety of habitat types.

It is a medium to large size rodent with thin, flexible, grooved, spine-like hairs on the dorsal pelage (Pimsai *et al.*, 2014). They also have a pale basal area with black tips which are more evident in older individuals. The spines vary between populations. It has long guard hairs that reach beyond the over hairs and not that evident over the lower part of the back and rump which are mostly black, although some are also paler. Generally, the upperparts are grizzled olive brown in colour, or sometimes with greyish or reddish-orange tones, or there is darkening on the midline of the back and the crown on the head which some individuals are paler, and the others are a little bit darker. Their feet are brown, but the hairs on the toes are white. They also have thinly furred, rounded and relatively large ears which uniformly dark/black throughout and covered with numerous short stiff black hairs. It also occasionally presence of a short white tip on the ears. There are around five or six pairs of mammae which is one pair of them are at the one pectoral, one or two pair are at post-axillary and three pairs ate the inguinal region.

Rattus norvegicus

This species is distributed worlwide but are more easily found in colder latitudes of the northen and southen hemisphere. In the warmer climate of the tropics, they are usually found in habitats which near to human habitation/civilization. Pimsai *et al*. (2014) recorded this species in Myanmar, Thailand, Malaysia and Singapore, and considered it to be widely distribution in urban areas. Based on the ICUN, they are considered as “Least Concern” as it “is a common species with no major threat” (Ruedas, 2008).

R. norvegicus is considered a large rat with mass of between 150–400 g (Pimsai *et al.,* 2014). It has short fur which is slightly stiff and without bristles; it has long. It also has guard hairs. The dorsal surface is range from grey-brown to brown in colour, and pale-brown or grey on the ventral surface. There is often a white patch on the chest but no clear line of demarcation between the flanks and the belly area. Black (melanistic) individuals are common in certain areas. It has long and broad nose meanwhile the eyes and ears are relatively small. The tail is almost always shorter when comparing with the head and body. It is usually weakly bicoloured, dark above and slightly paler below, and sometimes mottled. It has large front and hind feet with poorly developed foot pads and inconspicuous ridges. It also have six pairs of mammae which three pairs on the pectoral region and the other three pairs are on the inguinal region.

MATERIALS AND METHODS

Sampling sites

This study was conducted at six different sites with a minimum of four rodents captured from each site. The sites chosen were characterize as commercial (shop lots and eateries) or residential areas, and either less or more than 250m from forest. The sites sampled were Bukit Gita Bayu, Taman Kajang Utama. Taman Kajang Prima, Taman Putra Permai, and Taman Perindustrian Bukit Serdang.

Trapping method and identification

Conventional wire traps (18x28x13 cm) was used to trap the rats. The traps were baited with breads, peanut butter, bananas and dried fish and the surrounding of the traps was sprinkled with water which had been fermented with dried fish to further attract the rodents. Trapping was done from August till September and a total of 58 rodents were caught at the end of the trapping session. The traps that contain the rodents then placed into a cloth bag and transported to the lab for further processing. Morphometric measurements (weight, tail length, head and body length, ear length, and hind limb length) were recorded and the gender and age group (adult, juvenile) were determined. The rodents were identified to species using taxonomic references (Pimsai *et al*., 2014).

Blood Sampling

The rodents were sedated with diethyl ether in a closed plastic container and subsequently anaesthetized with zolazepam/tiletamine intra-muscularly. Blood was then drawn *via* cardiac puncture using 3mL syringes and 23-gauge needles. The blood was placed in EDTA tubes and chilled. The rodents were then euthanized with pentobarbital sodium via the intracardiac route.

Microscopy examination

Thin blood films were prepared from the EDTA blood, and allowed to air-dry. The slides then were fixed in methanol solution for two minutes and air-dried. The fixed blood films were then stained for 30mins with PBS-Giemsa at pH 7.2. The slides were then rinsed with water and air-dried. Each stained blood film was examined unter the compound microscope at 400x and 1000x magnification. A total of at least 1000 erythrocytes were examined per slide.

DNA Extraction

DNA extraction was done using a conventional extraction kit (DNeasy Blood & Tissue Kit, Qiagen) according to the manufacturer’s protocol. The kit contained DNeasy Mini spin columns, 2mL collection tubes, Buffer AL, Buffer AW1, Buffer AW2, Buffer AE, and proteinase K. Personal Protective Equipment (PPE) were used including lab coats, gloves and masks. This is to reduce chances of sample contamination and exposing oneself to unknown pathogens. For each sample, 20µL proteinase K and 200 mL anticoagulant-treated blood was mixed in a 2mL centrifuge tube. 200µL Buffer AL was added into each stool sample. The samples were mix thoroughly by vortexing and incubated in 56°C water bath for 10 minutes. The samples were then added with 200µL ethanol (100%) and mixed thoroughly by vortexing. The samples were transferred into a DNeasy mini spin column which was attached with 2mL collection tube and centrifuged for 1 minute at 8000 rpm. The flow through and the collection tube were discarded and replace with new a collection tube. 500µL of

Buffer AW1 was added to each sample and centrifuged for 1 minutes at 8000 rpm. The flow through and the collection tube were discarded and replace with a microcentrifuge tube. 200µL of Buffer AE was added by targeting at the center of the spin column membrane, incubated for 1 minute at room temperature and centrifuged for 1 minutes at 8000 rpm. The spin column was then removed and the extracted DNA was stored at -30°C.

Polymerase Chain Reaction (PCR) amplification

Molecular detection of *Hepatozoon* DNA was performed by amplification of a partial fragment of the 18SrRNA (*Ujvari et al.* (2004). The PCR Master Mix included nuclease-free water (dH_2O), $MgCl_2$**,** *Taq* DNA Polymerase, deoxyribonucleotide triphosphate (dNTP) solution and buffer solution. The primer pairs (5'-GTT TGT GAC CTA TCA GCT TTC GAC G -3 and 5'C AAA TCT AAG AAT TTC ACC TCT GAC - 3 (Ujvari *et al.*, 2004) were used to amplify a 600bp fragment of the *Hepatozoon* 18SrRNA gene. The PCR reaction was run on all 58 samples, in separate batches, with positive (*Hepatozoon* DNA) and negative controls (dH_2O). All the solutions, except *Taq* DNA Polymerase were mixed in a microcentrifuge, starting with Nuclease-free water. The PCR was run in a 25µL reaction with the following thermocyclic profiles: denaturation at 94°C for 30s, annealing at 60°C for 30s, and elongation at 72°C for 1 min for a total of 35 cycles (Maia *et al*., 2014).

The PCR amplicons were electrophoresed on a 1.5% agarose gel (Vivantis, USA) at 80V for 60 minutes with TAE (Tris-acetic acid-EDTA) buffer, stained with Red Safe loading dye and viewed under a UV transilluminator with 100bp DNA ladders as size markers. Images were captured using a digital camera and computer software (GeneSnap^{TM}, Bio-Rad Laboratories). To prevent cross contamination, work areas were designated solely for DNA extraction, PCR reagent preparation and PCR amplification. Reagent preparation was done in a biosafety cabinet class II which was UV illuminated before and at the end of each session.

Sequence analysis and phylogenetics

Positive amplicons were sequenced bi-directionally using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) and the resulting electropherogram was checked and edited manually. In order to facilitate identification of *Hepatozoon*, 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) (Altschul *et al*., 1990). Phylogenetic analysis involved 19 nucleotide sequences of the 18SrRNA region of *Hepatozoon* curated at the NCBI GenBank and 15 isolates obtained from the rodents in the present study. Sequence alignment was done using the ClustalW program as implemented in MEGA6 (Tamura *et al*. 2013). The best fit model with the lowest BIC (Bayesian Information Criterion) score was computed after trimming and removal of gaps and ambiguous residues to a total of 436 positions in the final dataset. Phylogenetic tree construction was done following the Maximum Likelihood (ML) parameter estimation using the Tamura 3-parameter (T92) model with evolutionary rate differences inferred by discrete Gamma (+G) distribution. The confidence level of the phylogenetic tree was assessed using 100 (ML) and 1000 (NJ) bootstrap replicates.

RESULTS

Two species of rodents were captured at fairly equal rates, namely 31 (53%) *R. norvegicus* and 27 (47%) *R. rattus*. There was a higher number of females (64%) captured compared to their male conspecifics. In relation to the habitat, a majority (62%) of the rodents were obtained from commercial areas and >250m from forest. Microscopy detection reveal presence of *Hepatozoon* in multiple samples (Figures 1a and 1b). Out of 58 rodent that were caught, 10 (17%) were microscopy positive for *Hepatozoon*. Molecular detection targeting the 18SSUrRNA gene of *Hepatozoon* revealed higher prevalence, where 15 (26%) of the rodents were positive, with an amplicon size of 600bp (Figure 2). The prevalence rate of *Hepatozoon* infection varied based on habitat type. Rodents captured from the residential areas and <250m to forest had a prevalence of 59.0% (Figure 3). Rodents sampled in the commercial areas and >250m from forest had a low prevalence rate of 5.5%.

Phylogenetic analysis carried out for the 16 isolates obtained in this study and 35 isolates curated at the NCBI Genbank (Table 3) based on maximum likelihood and neighbour-joining algorithms revealed Based on the results of both Neighbour Joining (Figure 4) and Maximum Likelihood (Figure 5) phylogenetic trees, the data that has the highest similarities when comparing the DNA arrangement of the *Hepatozoon* are only *Hepatozoon sp.* which identify inside *R.norvegicus* caught in Chille as it is inside the same clades. The sister clades are also *Hepatozoon sp* but identify in different host species which is *B.indica*, caught in Thailand.

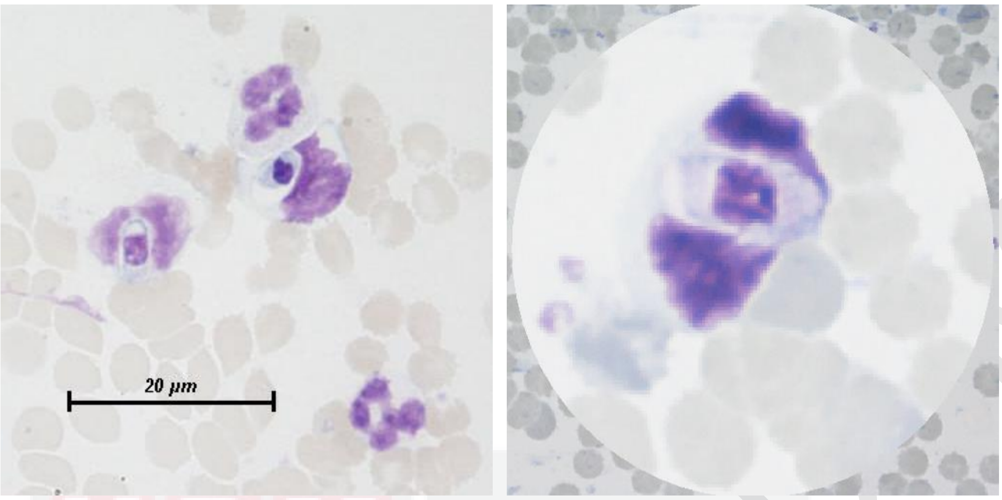

Figure 2a and 2b. *Hepatozoon* sp. detected from rodents (*Rattus*) from Selangor, Malaysia. Primary magnification 1000x.

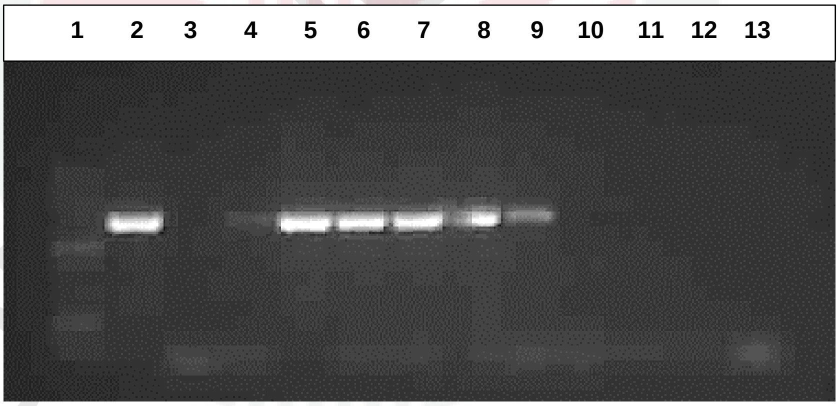

Figure 3. Gel electrophoresis showing the positive amplicons of *Hepatozoon* sp. amplified from blood of rodents (*Rattus*) from Selangor Malaysia. Lane 1: 100bp size marker, Lane 2; positive control, Lane 3: negative control, Lanes 4-9: positive samples, Lanes 10-13: negative samples.

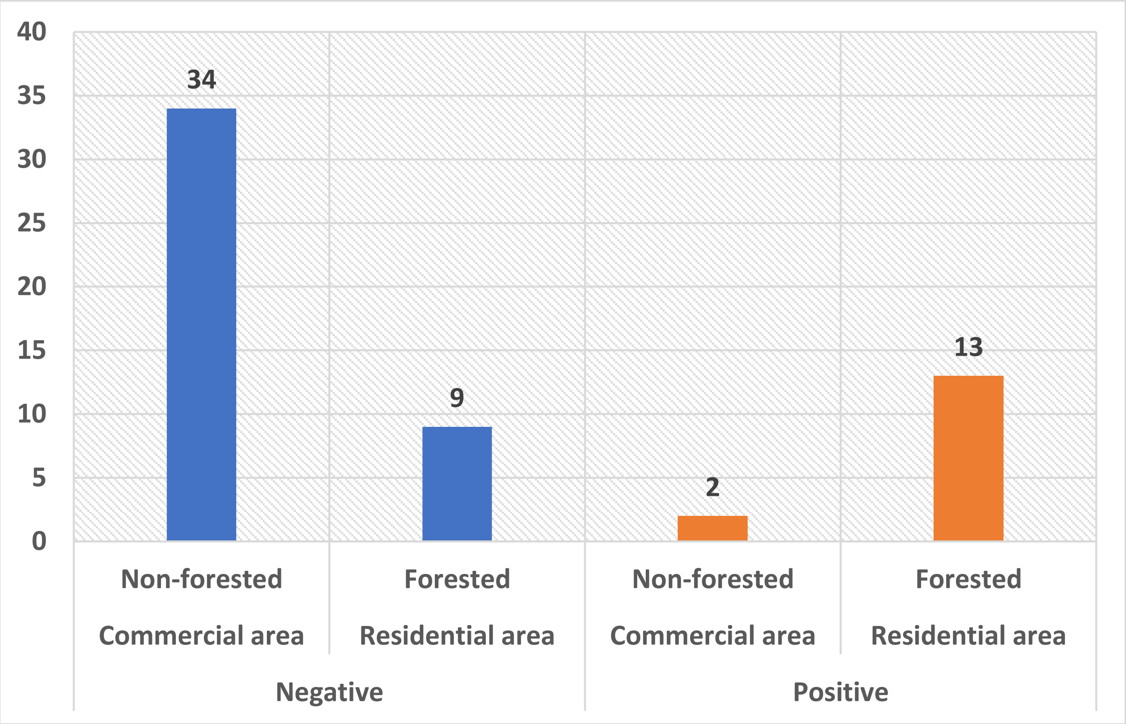

Figure 4. Prevalence of *Hepatozoon* among rodents (*Rattus*) captured from various habitat types in Selangor, Malaysia.

Table 1. *Hepatozoon* 18SSUrRNA sequences from this study and those curated at the NCBI Genbank that were used for phylogenetic analysis.

Accession No.	**Species**	**Host**	**Locality**	**Reference**
HRMH001	*Hepatozoon* sp.	*Rattus norvegicus*	Selangor, Malaysia	This study
HRMH002	*Hepatozoon* sp.	*Rattus norvegicus*	Selangor, Malaysia	This study
HRMH003	*Hepatozoon* sp.	*Rattus sp.*	Selangor, Malaysia	This study
HRMH004	*Hepatozoon* sp.	*Rattus sp.*	Selangor, Malaysia	This study
HRMH005	*Hepatozoon* sp.	*Rattus sp.*	Selangor, Malaysia	This study
HRMH006	*Hepatozoon* sp.	*Rattus sp.*	Selangor, Malaysia	This study
HRMH007	*Hepatozoon* sp.	*Rattus norvegicus*	Selangor, Malaysia	This study
HRMH008	*Hepatozoon* sp.	*Rattus norvegicus*	Selangor, Malaysia	This study
HRMH009	*Hepatozoon* sp.	*Rattus norvegicus*	Selangor, Malaysia	This study
HRMH010	*Hepatozoon* sp.	*Rattus norvegicus*	Selangor, Malaysia	This study
HRMH011	*Hepatozoon* sp.	*Rattus sp.*	Selangor, Malaysia	This study
HRMH012	*Hepatozoon* sp.	*Rattus norvegicus*	Selangor, Malaysia	This study
HRMH013	*Hepatozoon* sp.	*Rattus norvegicus*	Selangor, Malaysia	This study
HRMH014	*Hepatozoon* sp.	*Rattus norvegicus*	Selangor, Malaysia	This study
HRMH015	*Hepatozoon* sp.	*Rattus norvegicus*	Selangor, Malaysia	This study
MK454898	*Hepatozoon* sp.	*Rattus norvegicus*	Chile	Cordova *et al*. (2019) unpublished
AB181504	*Hepatozoon* sp.	*Bandicota indica*	Thailand	Dantrakool *et al*. (2004) unpublished
JQ670909	*Hepatozoon* sp.	*Aponomma varanense*	Thailand	Sumrandee *et al*. (2015)
MH615006	*H. canis*	*Canis lupus familiaris*	Israel	Leveille *et al*. (2019)

Table 1 continued

Accession No.	**Species**	**Host**	**Locality**	**Reference**
KF322145	*H. canis*	*Canis aureus*	Hungary	Farkas *et al*. (2014) unpublished
KF322144	*H. canis*	*Vulpes vulpes*	Hungary	Farkas *et al*. (2014) unpublished
MT107098	*H. canis*	*Canis* sp.	Hanzhong, China	Guo *et al*. (2020)
MT107097	*H. canis*	*Haemaphysalis longicornis*	Hanzhong, China	Guo *et al*. (2020)
MK910143	*H. canis*	*Canis lupus familiaris*	Colombia	Thomas (2019)
MK836092	*H. felis*	*Felis* sp.	Maio Island	Pereira *et al*. (2019)
KY056823	*H. felis*	*Rhipicephalus sanguineus*	Thailand	Bhusri & Changbunjong (2016)
KX017290	*H. felis*	*Panthera leo leo*	India	Rafiqi *et al*. (2016) unpublished
MN150506	*H. ursi*	*Ursus arctos*	Turkey	Guven *et al*. (2019) unpublished
HQ829437	*H. ursi*	*Melursus ursinus*	India	Pawar (2011)
EU041718	*H. ursi*	*Ursus thibetanus japonicus*	Japan	Kubo *et al*. (2007)
MN905023	*H. ursi*	*Ixodes ricinus*	Turkey	Orkun & Emir (2020)
MN463021	*H. ursi*	*Hyalomma marginatum*	Turkey	Orkun & Emir (2020)
MK645858	*Hepatozoon* sp.	*Ailuropoda melanoleuca*	USA	Yu *et al*. (2019)
MK424119	*Hepatozoon sp.*	*Chrysocyon brachyurus*	Brazil	Barranco *et al*. (2019) unpublished

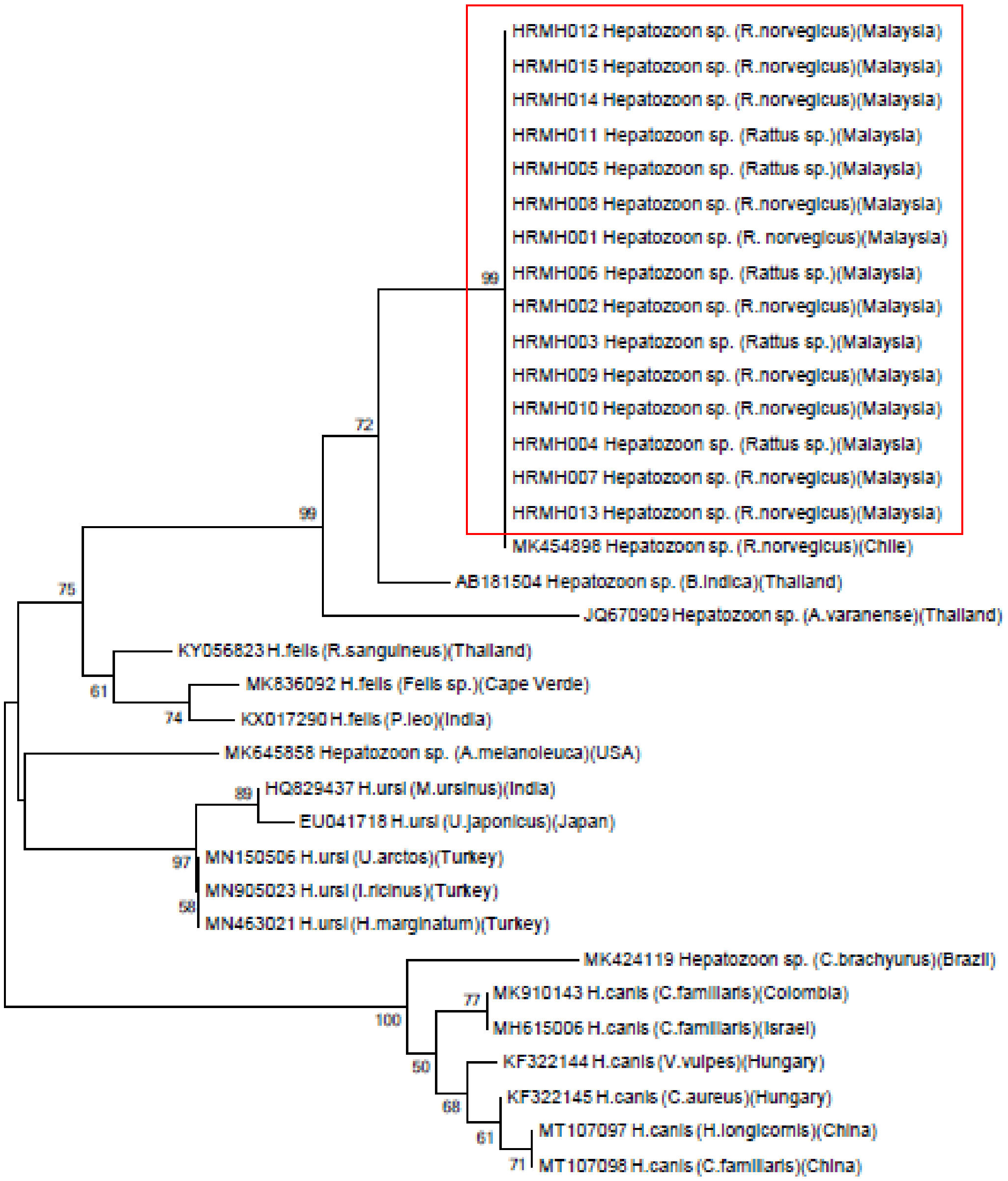

Figure 5. Neighbour Joining phylogenetic tree showing the evolutionary relationship of *Hepatozoon* sp. isolates from *Rattus* in Selangor, Malaysia (red box) and other geographical locations and hosts, based on a partial fragment of the 18SrRNA gene. Branch confidence was assessed with 1000 bootstrap replicates.

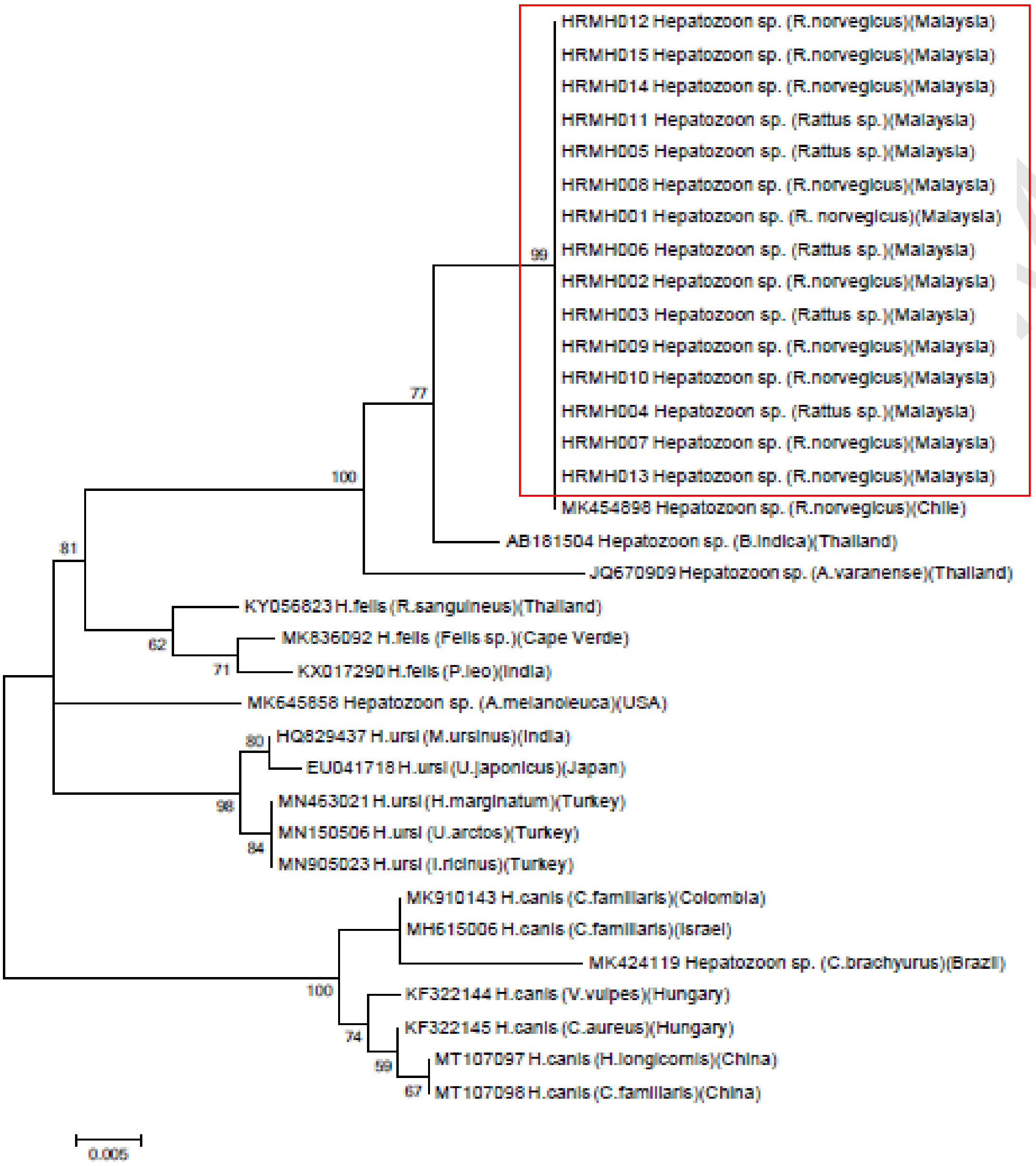

Figure 6. Maximum Likelihood phylogenetic tree showing the evolutionary relationship of *Hepatozoon* sp. isolates from *Rattus* in Selangor, Malaysia (red box) and other geographical locations and hosts, based on a partial fragment of the 18SrRNA gene. Branch confidence was assessed with 100 bootstrap replicates.

DISCUSSION

The present study constitutes the first report on the molecular detection, prevalence and genotyping of rodent *Hepatozoon* in Peninsular Malaysia. The relatively high prevalence detected by PCR targeting the parasite 18SSUrRNa gene, is a clear indication that molecular techniques should be employed for screening of rodent-borne blood parasites in the future. Microscopy revealed a lower prevalence rate, and this is expected due to the relatively low levels of parasitaemia.

Phylogenetic analysis revealed a close association among the isolates in this study with those previously reported to infect *R. norvegicus* in Chile (Cardova, 2019). This may indicate a broad geographical distribution of *Hepatozoon* genotypes, which are not restricted to location. *Hepatozoon* genotypes from Thailand that were the next closes in terms of phylogenetic origin, were isolated from the blood of *Bandicota indica* (Dantrakool, 2004) and ticks from reptiles (Sunramdee, 2015). The phylogenetic tree also showed that that was a clear host specificity in terms of the *Hepatozoon* genotypes. These isolates were unique to the hosts where there was a clear distinction among the *Hepatozoon* found in bears, canids and felids. Thus, the results indicate that *Hepatozoon* sp. are host specific in natural infections, where *Hepatozoon* species such as *H. ursi, H. canis* and *H. felis* have not been thus far isolated from rodents.

The phenomenon of a host specificity among *Hepatozoon*, however, may be challenged by an experiment conducted by Johnson *et al.* (2008), where canine *Hepatozooon* (*H. americanum*) was successfully inoculated and into mice, producing parasitaemia. This however, is not a naturally acquired infection. Host specificity may also depend on the specific arthropod vectors that infect mammals. However it does open the possibility of the cross infection of *Hepatozoon* between mammal species. As such, rodents could possibly transmit the parasite to domestic animals and pets, and vice versa. At the moment the present body of evidence is insufficient to provide any clear conclusion on the role of rodents in transmitting *Hepatozoon* and allied haemoparasites to other mammals. In addition the zoonotic potential of this pathogen has not been fully investigated. To date, there is only a single case of *Hepatozoon* infection in a person. The infected patient showed clinical symptoms of anemia and icterus, and microscopy examinations revealed gamonts of *Hepatozoon* sp. in circulating leukocytes. Therefore the potential for zoonotic transmission of *Hepatozoon* is apparently very low since the transmission involves the ingestion of infected haematophagous arthropods, especially ticks (Greene, 2012).

Based on the results, residential areas near a forest area had higher prevalence of *Hepatozoon* compared to other places. This could be due to the presence of abunndant sylvatic rodent reservoirs that may be naturally harboring *Hepatozoon*. Pathogen spill-over from sylvatic reservoirs is not uncommon, and may be seen in various diseases of peri-domestic and domestic animals. In addition, these residential area and forested area do provide a huge array of habitats that can support many rodent species. Furthermore, these areas do provide a surplus amount of food source either from human spoilage or from nature. Thus, these important elements for survival including a diverse habitat and surplus food may encourage the reproduction, propagation and interaction of rodents. There will also be close approximation and therefore efficient transmission of arthropod vectors that carry pathogens.

CONCLUSION

The current study constitutes the first report on rodent-borne *Hepatozoon* in Peninsular Malaysia. The parasite was visible in microscopy examination of blood films, and PCR amplification revealed a moderately high prevalence among peri-domestic rodents. The parasite is genetically similar to that isolated from rats (*R. norvegicus*) in Chile, indicating a wide geographical distribution of genotypes. The higher prevalence in residential areas close to forests indicates the possibility of a sylvatic cycle of the parasite, where wild rodents may act as reservoirs. In addition, the close proximity of urban rodents due to the abundance of food from human waste, and the availability of suitable habitats supports the effective transmission of this arthropod-borne blood parasite. Since the zoonotic potential of *Hepatozoon* is considered to be low, there is a minimal risk in handling the blood samples and even the hosts. However, rodents are known to harbor many pathogens, including zoonotic species. Thus, it is always prudent to practice appropriate personal protective and biosafety measures when handling rodents or their blood.

Future studies may want to look into the potential role of peri-domestic rodents in the transmission and epidemiology of *Hepatozoon* infection among domestic animals and pets. It is suggested to that rodents living in areas for endemic *Hepatozoon* infection among cats and dogs be investigated further to determine the species diversity and prevalence of *Hepatozoon*. In addition the possible occurrence of shared *Hepatozoon* genotypes among these rodents and domestic animals in the vicinity will be of interest, and will indicate the transmission of the pathogen among the sympatric mammals. The successful cross infection of a canine Hepatozoon (*H.* americanum) to laboratory rodents, opens the possibility for natural transmission among mammal species, which warrants further investigation.

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