MOLECULAR DETECTION OF *BARTONELLA* SPP. IN BAT FLIES IN EAST-COAST MALAYSIA

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CERTIFICATION

It is hereby certified that we have read this research paper entitled "**Molecular Detection of *Bartonella* spp. in Bat Flies in East-Coast Malaysia**" by Azra Hafizah Binti Kamar and in our opinion it is satisfactory in terms of scope, quality, and presentation as part of the requirements for the course DVT 5436 - Research Project.

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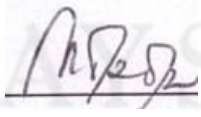

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Thank you

DEDICATIONS

I dedicate my dissertation work to

My supervisors

My family

Friends

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ABSTRACT

An abstract of the research paper presented to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, in partial requirement on the course DVT 5436 – Research Project

Bats are recognized as a major reservoir for numerous pathogens including *Bartonella* spp. and it is one of the emerging zoonotic bacterial diseases that can be transmitted to humans and may cause various unspecific clinical manifestations. Thus, Bartonellosis is rarely diagnosed and regarded as neglected vector borne disease (VBD). Bat flies have been hypothesized to be a vector in transmission of pathogens among bats. They are host-specific, which reduces the likelihood of pathogen transmission across bat species, however it is still likely to maintain the pathogen loads within its host species populations. To explore more on this neglected bacteria and its presence in bat flies, bat flies samples collected from East Coast Malaysia were used for molecular detection and sequences analysis of *Bartonella* spp.. A percentage of 38.71% bats were infested with bat flies from Terengganu and Kelantan and no bat fly was found in Pahang collection site. Both bat flies families, Nycteribiidae (79.59%) and Streblidae (20%) were identified. The bat flies were pooled into 37 samples and 70.27% were positive for *Bartonella* spp. by PCR and four positive samples were randomly selected for sequence analysis, all samples had 95% identity to *Bartonella* spp. from two different locations, which were LR9Brazil and B40400African strains. They were regarded as new species as the citrate synthase (*gltA*) sequence share less than 96% similarity to previously identified species and the obtained findings also suggest that the bat flies in East Coast Malaysia serve as a reservoir for a zoonotic potential *Bartonella* spp..

Keywords: bats, *Bartonella* spp., bat flies, PCR, sequence analysis

ABSTRAK

Abstrak daripada kertas penyelidikan dikemukakan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan untuk memenuhi sebahagian daripada keperluan kursus DVT 5436 – Projek Penyelidikan.

Kelawar diakui sebagai takungan utama bagi banyak patogen termasuk *Bartonella* spp. dan ini adalah salah satu penyakit bakteria zoonotik yang muncul yang dapat menular ke manusia dan boleh menyebabkan pelbagai manifestasi klinikal yang tidak spesifik. Oleh itu, Bartonellosis jarang didiagnosis dan dianggap sebagai penyakit bawaan vektor yang diabaikan (VBD). Lalat kelawar telah dihipotesiskan sebagai vektor penularan patogen di antara kelawar. Mereka khusus untuk kelawar spesies, yang mengurangi kemungkinan penularan patogen ke seluruh spesies kelawar, namun masih cenderung untuk mengekalkam beban patogen dalam populasi spesies perumahnya. Untuk mengetahui lebih lanjut mengenai bakteria yang diabaikan ini dan kehadirannya dalam lalat kelawar, sampel lalat kelelawar yang dikumpulkan dari Pantai Timur Malaysia digunakan untuk analisis pengesanan molekul dan urutan *Bartonella* spp.. Peratusan 38.71% kelawar dipenuhi dengan lalat kelawar dari Terengganu dan Kelantan dan tidak ada kelawar yang dijumpai di lokasi pengumpulan Pahang. Kedua-dua keluarga kelawar, Nycteribiidae (79.59 %) dan Streblidae (20 %) dikenal pasti. Lalat kelawar dikumpulkan menjadi 37 sampel dan 70.27 % positif untuk *Bartonella* spp. oleh PCR dan empat sampel positif dipilih secara rawak untuk analisis urutan, semua sampel mempunyai identiti 95% kepada *Bartonella* spp. dari dua lokasi yang berbeza, iaitu strain LR9Brazil dan B40400Africa.

Mereka dianggap sebagai spesies baru kerana urutan sitrat synthase (gltA) mempunyai persamaan kurang daripada 96% dengan spesies yang dikenal pasti sebelumnya dan penemuan yang diperoleh juga menunjukkan bahawa kelawar terbang di Pantai Timur Malaysia berfungsi sebagai takungan untuk potensi zoonotik *Bartonella* spp..

Kata kunci: kelawar, *Bartonella* spp., Kelawar kelelawar, PCR, analisis urutan

1.0 Introduction

Malaysia has the highest rate of deforestation compared to major countries that are reducing their forest cover (Butler, 2013). This is especially true in Peninsular Malaysia, where much of the old-growth forest is being or has already been logged and converted into agriculture, particularly for oil palm (Koh & Wilcove, 2008; Peh et al., 2006). These severe and rapid deforestation and degradation of tropical rainforest can lead to direct loss of wildlife habitat. Removal of trees and other types of vegetation reduces the availability of food, shelter and breeding habitat for the wildlife animals.

Bats are one of the wildlife animals that inhabit the tropical rainforests. Despite the benefits of bats, they are also recognized as a major reservoir for numerous pathogens that can be transmitted to humans through religious activities in forests or caves, bat consumption or contact with contaminated products. Deforestation and degradation of tropical rainforest may increase the widespread of bat populations in urban areas and come in close contact with both domestic animals and humans, contaminating the houses with urine and saliva as well as transmitting the infectious pathogens.

Bartonellosis is one of the emerging zoonotic bacterial diseases that is responsible for a wide variety of clinical syndromes in humans that can be transmitted by bat hosts. *Bartonella* spp. are fastidious, gram-negative and intracellular hemotropic bacteria, more than 20 *Bartonella* species have been identified and many of those being known as zoonotic pathogens (Breitschwerdt et al., 2010). It can be transmitted and spread among bats populations by hematophagous arthropods such as flies, fleas, lice and ticks.

Among the bat ectoparasites, bat flies (Diptera: Nycteribiidae and Streblidae) are one of the common potential vectors in transmitting and maintaining *Bartonella* spp. in bat populations. They are hematophagous ectoparasites that can carry diverse infectious agents including viruses, bacteria, fungi and blood parasites (Szentiványi, Christe, et al., 2019). *Bartonella* spp. can be transmitted vertically and horizontally. The vertical transmission of pathogens occurs during the milk feeding from the female to its larva. On the other hand, horizontal transmission from bat to bat may occur by direct contact with contaminated bat flies saliva during feeding or parasitoids of bat flies larvae on bat hosts.

Bat flies are highly host-specific ectoparasites that are found living in the fur and on wing membranes of the bat host (Dick et al., 2006). Female flies will immediately return to their host after depositing their larva on roosting substrate. For the offspring, depending on several factors, such as host presence and temperature, they will eventually emerge and actively find their bat host to continue their life cycles. These offspring may become the vectors of the infectious pathogens and spread to another bat host.

Since bats are recognized as a major reservoir for numerous zoonotic pathogens, the obligation of bat flies on bats may play an important role in maintaining the *Bartonella* spp. among bat populations. In this project, sampling of bat ectoparasites particularly on bat flies in East-Coast Malaysia is conducted to detect the presence of *Bartonella* spp. by using Polymerase Chain Reaction (PCR) analysis and sequence analysis.

2.0 Problem Statement

There is no data on the occurrences of *Bartonella* spp. in bat flies collected from bat populations in East Coast Malaysia.

3.0 Research Questions

- What are the common bat flies family that can be found on bat populations in the East Coast Malaysia?
- Can *Bartonella* spp. be detected in bat flies collected from bat populations located in East Coast Malaysia?

4.0 Research Hypothesis

- Nycteribiidae and Streblidae are both the common bat fly families that can be found in bat populations in East Coast Malaysia.
- *Bartonella* spp. is present in bat flies collected from the bat populations in East-Coast Malaysia.

5.0 Objectives

- To determine the bat flies family collected from the bat population from East Coast Malaysia.
- To detect the presence of *Bartonella* spp. in bat flies collected from bat populations located at the East-Coast of Malaysia through Polymerase Chain Reaction (PCR).

6.0 Literature Review

6.1 Bats population and their public health concerns

Bats (Order:Chiroptera) are the second most varied order of mammalian orders, with a wide range of physiological and ecological characteristics (Hutson *et al*.,2001). Bat species have developed a huge range of roosting and eating behaviors. They roost in foliage, caves, rock crevices, tree hollows, beneath exfoliating bark, and various man-made structures during the day. Bats have a pair of unique wing structures that enable them to fly long distances during migrations. They also have capabilities to live in diverse colonial populations. These characteristics play a major role for their widespread distribution and diversity (Kasso & Balakrishnan, 2013). Bat serves an important ecological role in prey and predators, consumption of pathogen-carrying arthropods, pollination, seed dispersal, material and nutrient distribution (Kunz et al., 2011). Despite the multiple benefits attributed to them, they are also recognized as a major reservoir for numbers of pathogens, including viruses, protozoans, bacteria, and also fungi (Mühldorfer, 2013). In recent years, one of the important emerging zoonotic pathogens, bat-associated *Bartonella* genotypes have been detected in humans, indicating the public health importance of this bacteria (Bai et al., 2018; Urushadze et al., 2017). The potential for zoonotic transmission increases as the bat populations overlap with both domestic animals and human populations. Bats come in urban areas, contaminating houses with their urine and guano. Bat to human disease transmission also may occur because humans occasionally encroach their natural habitat for religious activities in caves, as well as consuming infected bats' meat (Bai et al., 2018).

6.2 Bat flies and pathogens associated

Bats host numerous ectoparasites, such as bat flies, louse, mites and ticks. Bat flies (Diptera: Nycteribiidae and Streblidae) are the most common bat ectoparasite. Among the ectoparasites that inhabit the bat hosts, bat flies have a high degree of host specificity to bat hosts compared to the others (Azhar et al., 2015; Dick et al., 2006). This condition is believed to reduce the likelihood of pathogen transmission across species, but the bat flies are still likely to carry and maintain the pathogen loads within the host populations (Dick et al., 2006).

Based on literature data, five main groups of pathogens in bat flies have been identified. The most common reported pathogens in bat flies are bacteria, followed by fungi, blood parasites and viruses. Within bacteria, the three most frequently detected were *Bartonella* spp., *Arsenophonus* spp. and *Wolbachia* spp.. While all observed fungi were *Laboulbeniaceae* (Ascomycota: Laboulbeniales) and belong to three genera, *Arthrorhynchus*, *Gloeandromyces*, and *Nycteromyces*. For blood parasites, *Polychromophilus* spp. (Haemosporida: Plasmodiidae) was mostly detected in bat flies. Virus represents a much smaller proportion of all pathogen observations in bat flies (Szentiványi, Christe, et al., 2019).

As one of the most frequently detected pathogens in bat flies, *Bartonella* spp. can be regarded as important bacterial pathogens. *Bartonella* spp. have been discovered in over 60 bat species from Central and South America, Africa, Europe and Southeast Asia, representing over 40 genera and 11 families (McKee et al., 2017). *Bartonella* spp. diversification appears to have followed diversification of bats, where specific taxonomy

of *Bartonella* spp. confined to specific bat families, superfamilies and suborders (Lei & Olival, 2014).

In Malaysia, bats and their ectoparasites have been laboratory-confirmed to harbor *Bartonella* spp. (Morse et al., 2012). A recent research study that had been done on Malaysia small flying foxes (*Pteropus hypomelanus*) and the associated bat flies, two strains of *Bartonella* spp. (*Bartonella* sp. KS013a and KS013b) were isolated from PCR-positive for both blood samples and the bat flies. Both strains were regarded as potentially novel *Bartonella* spp. as their citrate synthase (*gltA*) sequence only exhibits less than 96% similarities compared to previously identified *Bartonella* spp. (Hou et al., 2018).

6.3 *Bartonella*, the neglected vector-borne pathogens

Bartonella spp. are small (approximately 0.3 μm × 1 μm), gram negative, pleomorphic coccobacilli. The bacteria are facultative intracellular pathogens and many of which are haemin dependent and employ haemotrophy as their parasitic strategy (Minnick et.al. 2006; Wong et al., 1995) where they infect erythrocytes and cause persistent bacteremia. In vitro, all members of this genus are notoriously fastidious and grow slowly.

Bartonellosis is an emerging zoonotic disease and commonly known diseases caused by *Bartonella* spp.. Infection in humans are Carrion's disease, Cat-scratch disease, and Trench Fever (Angelakis et al., 2014). The clinical manifestations of these diseases can range from benign and self-limited to severe and life threatening.

Several *Bartonella* spp. have been isolated and identified with a wide range of animal species including cats, dogs, rodents and livestock as well as wild animals such as coyotes, deer and wildcats (Breitschwerdt & Kordick, 2000). At least three of *Bartonella* spp.

identified are common human pathogens which are *B. bacilliformis, B. henselae, and B. quintana* (Minnick et al., 2006). *Bartonella* spp. can be transmitted interspecies from animals to humans through a variety of insect vectors including fleas, louse and flies (Breitschwerdt, 2017). There is also new evidence suggesting that ticks, red ants and spiders also can transmit this bacterium (Mosbacher et al., 2011).

The presence *Bartonella* spp. in diverse ecological animal reservoir hosts and a wide variety of potential arthropod vectors capable of transmitting this bacteria species from animal to human are major causes for public health concerns. However, in European countries, Bartonellosis is rarely diagnosed and regarded as neglected vector borne diseases (VBD) (Krügel et al., 2020). Since *Bartonella* spp. causes different diseases with various symptoms e.g. vasoproliferative lesions of the skin and abdominal organ, regional lymphadenomegaly and endocarditis in human, the infection caused by this bacteria is usually remain undetected and inadequate therapy may be life threatening (Prutsky et al., 2013).

7.0 Materials and Methods

7.1 Study sites and sample collection

Fieldworks were carried out by postgraduate students in 3 different states of East-Coast Malaysia (Terengganu, Pahang and Kelantan). The study sites are particularly in Sekayu Recreational Forest (Terengganu), Gunung Reng (Kelantan) and Pahang National Park, Merapoh (Pahang).

Mist nets and harp traps were set up before the emergence of bats. Each of the captured bats were placed in different cloth bags for further species identification and ectoparasite collection. Flea combs and tweezers were used for the ectoparasites collection at the fur area of the body. The samples were stored in 80% diluted ethanol and kept in -80°C in the laboratory.

All procedures were reviewed and approved by the Animal Care and Use Committee of Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, Malaysia (UMK/FPV/ACUC/PG/6/2021).

The archived ectoparasites, particularly on bat fly samples collected by the postgraduate students, were used for further detection and identification of *Bartonella* spp. by Polymerase Chain Reaction (PCR) and sequence analysis in this project.

7.2 Identification of bat flies

All collected bat flies were identified to the family using a dissecting microscope. The bat flies of the same family from the same bat species and the same collection site were placed

in separate microcentrifuge tubes that contain 80% ethanol and stored in -80°C. The ID of each of the flies was recorded.

7.3. DNA Extraction, Polymerase Chain Reaction (PCR) and Gel Electrophoresis

Prior to DNA extraction, the bat flies were removed from the ethanol vials and rinsed in sterile phosphate-buffered saline (PBS). Two to four bat flies of the same family from the same bat species from the same collection site will be treated as a pool. The flies were triturated and manually homogenized using a plastic pestle in the microcentrifuge tube.

DNA was extracted by using commercial DNA extraction kits and the procedures recommended by the manufacturer were to be followed.

Primers used for amplification of *glt*A (379 bp) are BhCS.781p (5-GGG GAC C AG CTC ATG GTG G-3) and BhCS.1137n (5-AAT GCA AAA AGA ACA GTA AAC A-3) as forward and reverse primers respectively.

Mastermix was prepared for 25 µL reaction volume containing 5.0 µL of extracted DNA, 1.0 µL of 10 uM of each primer, 12.5µL 2X GoTaq Green Buffer (Promega), 5.5µL Nuclease free water (Promega) for each reaction. The mastermix was centrifuged for 5s.

The PCR condition was including an initial denaturation step at 95°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 20s, annealing at 51°C for 30s, extension at 72°C for 2 minutes and a final extension step at 72°C for 5 mins in a thermal cycler.

The products of PCR were separated by gel electrophoresis in 1.5% agarose gel at 100 V and 400 mA for 40 minutes n 1 × TBE (89 mM Tris, 89 mM Boric Acid and 2 mM EDTA)

containing 1 µL Midori Green loading dye and was observed under ultraviolet (UV) light for the size of amplified fragments by comparison to 100-bp DNA molecular weight marker.

7.4 Sequence analysis and alignment

Four PCR products with positive detection of *Bartonella* spp. were sent to the Apical Scientific SDN. BHD. for sequencing services.

The sequences were edited and analyzed using BioEdit software (Informer Technologies) and aligned using the ClustalW (NCBI) and ClustalW program (EMBL-EBI) and blasted using the NCBI BLAST program.

8.0 Results

8.1 Bat flies infestation

A total 186 bats were captured, with 53 bats from Sekayu Recreational Forest, Terengganu, 103 bats from Gunung Reng, Kelantan and 30 bats from Pahang National Park, Merapoh, Pahang.

As documented in Table 1, 38.71% (72/186) of various bat species were infested with bat flies.

In Sekayu Recreational Forest, bat species with the highest bat flies infestation was *Hipposideros kunzi* 50%(1/2), and followed by *Rhinolophus affinis* 47.37%(9/19). Bat species of *Balionycteris seimundi (1/3), Hipposideros dyacorum (1/3)* and *Hipposideros larvatus (3/9)* had 33.33% of bat flies infestation and then followed by *Cynopterus horsfieldii* 28.57% (2/7) and *Cynopterus brachyotis* 14.29% (1/7). *Kerivoula minuta* and *Nycteris tragata* were found negative for bat flies infestation.

In bats that were captured in Gunung Reng, Kelantan, bats with highest bat flies infestation was *Eonycteris spelaea* 66.67% (38/57), followed by *Rhinolophus pusillus* 50% (1/2), *Rhinolophus affinis* 43.75% (14/32) and *Rhinolophus stheno* 33.33% (1/4). *Hipposideros dyacorum, Hipposideros larvatus, Taphozous melanopogon, Hipposideros kunzi* and *Myositis muricola* were found to be negative for bat flies infestation.

In the capture site of Pahang National Park, Merapoh, Pahang, all of the 30 captured bats were found negative for bat flies infestation.

Table 1: Bat flies infestation in bat captured in Sekayu Recreational Forest, Terengganu and Gunung Reng, Kelantan.

Capture site	Bat Species (n)	Number of bat(s) were infested with bat flies	
		Positive	Negative
Sekayu Recreational Forest, Terengganu	*Balionycteris seimundi (3)*	1	2
	Cynopterus brachyotis (7)	1	6
	Cynopterus horsfieldii (7)	2	5
	Hipposideros dyacorum (3)	1	2
	Hipposideros kunzi (2)	1	1
	Hipposideros larvatus (9)	3	6
	Kerivoula minuta (2)	0	2
	Nycteris tragata (1)	0	1
	Rhinolophus affinis (19)	9	10
Gunung Reng, Kelantan	*Eonycteris spelaea (57)*	38	19
	Hipposideros dyacorum (2)	0	2
	Hipposideros kunzi (1)	0	1
	Hipposideros larvatus (2)	0	2
	Myositis muricola (1)	0	1
	Rhinolophus affinis (32)	14	18
	Rhinolophus pusillus (2)	1	1
	Rhinolophus stheno (4)	1	3
	Taphozous melanopogon (2)	0	2
	Hipposideros armiger (1)	0	1
	Hipposideros kunzi (12)	0	12

Pahang National Park, Merapoh, Pahang	*Hipposideros larvatus (16)*	0	16
	Rhinolophus stheno (1)	0	1

n: total number of bats

8.2 Bat flies family identification

A total of 98 bat flies were found inhabiting the bats in Sekayu Recreational Forest, Terengganu and Gunung Reng, Kelantan. The total number of bat flies collected from Sekayu and Gunung Reng were 27 and 71 respectively. The percentage of 20.41% (20/98) were found to be in Streblidae and 79.59% (78/98) were in Nycteribiidae families. Both families can be distinguished by the absence of wings (Nycteribiidae) and presence of wings (Streblidae) as shown in Figure 1 and Figure 2 respectively.

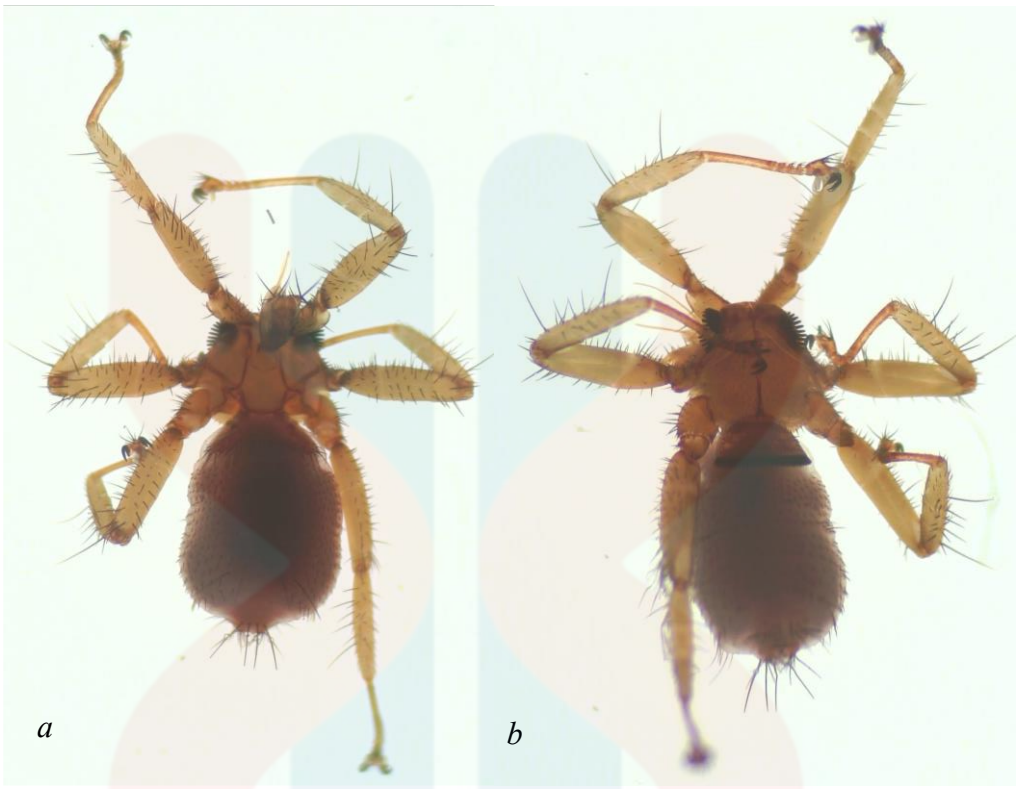


Figure 1: *(a) Dorsal view, (b) Ventral View. Bat flies under the Nycteribiidae family that was collected from Eonycteris spelaea (R39) bat species in Gunung Reng, Kelantan.*

Figure 2: *(a) Dorsal view, (b) Ventral view. Bat flies under the Streblidae family that was collected from Hipposideros dyacorum (S24) bat species in Sekayu Recreational Forest, Terengganu.*

8.3 *Bartonella* spp. detection by Polymerase Chain Reaction (PCR)

A total of 37 samples were tested for *Bartonella* spp. detection by Polymerase Chain Reaction (PCR). Two to four bat flies from the same bat flies family found on the same species of bat host in each state were pooled together to form a sample except for *Rhinolophus stheno* and *Rhinolophus pusillus* bat species, their bat flies were pooled together with *Rhinolophus affinis* bat flies according to their bat flies family (Table 2).

The expected band size for *Bartonella* spp. is 379bp. Among the 37 samples tested, 70.27% (26/37) were found positive for *Bartonella* spp.. The results were then divided into their collection site with 73.33% (11/15) from Sekayu Recreational Forest, Terengganu and 68.18% (15/22) from Gunung Reng, Kelantan were found positive for *Bartonella* spp. (Table 2).

Based on the bat flies family, Nycteribiidae bat flies pools were found to have higher positive results of *Bartonella* spp. with 75.86% (22/29) compared to Streblidae bat flies pools of 50% (4/8).

Table 2: *Bartonella* spp. detection in bat flies by Polymerase Chain Reaction (PCR) collected from Sekayu Recreational Forest, Terengganu and Gunung Reng, Kelantan.

Capture site	Bat species	Number of bat flies pool (n)*	PCR results (379bp)	
			Positive	Negative
Sekayu Recreational Forest, Terengganu	*Balionycteris seimundi*	Nycteribiidae (1)	0	1
	Cynopterus brachyotis	Nycteribiidae (1)	1	0
	Cynopterus horsfieldii	Nycteribiidae (2)	2	0
	Hipposideros dyacorum	Streblidae (1)	1	0
	Hipposideros kunzi	Streblidae (1)	0	1
	Hipposideros larvatus	Nycteribiidae (1)	1	0
	Rhinolophus affinis	Nycteribiidae (3)	3	0
		Streblidae (5)	3	2
Gunung Reng, Kelantan	*Eonycteris spelaea*	Nycteribiidae (17)	13	4
	Rhinolophus affinis	Nycteribiidae (3)	2	1
	Rhinolophus affinis + *Rhinolophus pusillus*	Streblidae (1)	0	1
	Rhinolophus affinis + *Rhinolophus stheno*	Nycteribiidae (1)	0	1

* *Two to four bat flies from the same family were pooled together for each sample.*

8.4 Sequence Analysis

Four positive samples were randomly selected and sent for sequencing.

Sequencing Analysis using Blast Program:

Below were the sequence comparisons obtained from NCBI BLAST. This software was useful for checking the similarity of *Bartonella* spp. isolates with published *Bartonella* spp. sequences from National Center for Biotechnology Information (NCBI) Genbank.

All four samples had 95% identity with *Bartonella* spp. from two different locations. Sample from Sekayu Recreational Forest, Terengganu (S7) had the similarity with LR9 Brazil and samples collected from Gunung Reng Kelantan (RE9, RE10 and RE15) had the similarities with B40400 Africa as shown in Table 4.

Table 3: BLAST result of *Bartonella* spp. isolates from Sekayu Recreational Forest, Terengganu and Gunung Reng, Kelantan.

Site	Isolates	Strain	Identities (%)	Accession Number
Sekayu Recreational Forest, Terengganu	S7 (*Rhinolophus affinis*, Nycteribiidae)	LR9 Brazil	95	MZ388461.1
Gunung Reng, Kelantan	RE10 (*Eonycteris spelaea,* Nycteribiidae)	B40400 Africa	95	KM030523.1
	RE9 (*Eonycteris spelaea,* Nycteribiidae)	B40400 Africa	95	KM030523.1

	RE15 (*Eonycteris spelaea,* Nycteribiidae)	B40400 Africa	95	KM030523.1

ClustalW sequences alignment demonstrated that scores were higher when the samples from Gung Reng were paired to each other, RE9: RE10 (99.6644%), RE10:RE15 (98.6577%), and RE9: RE15 (98.3221%). Whereas the scores were lower when the samples from Gunung Reng were paired with the sample from Sekayu Recreational Forest, RE9: S7 (85.2349%), S7:RE10 (85.2349%), and S7:RE15 (85.906%) as documented in Table 4.

Table 4: ClustalW sequences alignment

Sequences Aligned	Score (%)
RE9: S7	85.2349
RE9: RE10	99.6644
RE9: RE15	98.3221
S7:RE10	85.2349
S7:RE15	85.906
RE10:RE15	98.6577

9.0 Discussions

In the total of 186 bats that were captured in the East coast Malaysia particularly at Sekayu Recreational Forest, Terengganu, Gunung Reng, Kelantan and Pahang National Park, Merapoh, Pahang, only bats from Pahang collection site were found to be completely negative for bat flies infestation. Based on the results, the bats species captured in Pahang National Park were 96.67% (29/30) were from *Hipposideros* spp.. Comparing it to other capture sites, the captured bat genus from *Hipposideros* spp. were also having low infestation rates. This can be supported by the previous study that was conducted in Gabon (Obame-Nkoghe et al., 2016), which the bats species from *Hipposideros* spp. has a lower bat flies infestation rate compared to *Miniopterus* spp. and *Rousettu*s spp. that were captured in three different caves. This may be due to the large body size of *Hipposideros* spp., and presumably lower basal metabolic rate, they may generally maintain individual distance during roosting, instead of clustering together that contribute low bat flies transmission across hosts (Ho & Lee, 2003).

In both collection sites that were found positive for bat flies infestation, *Eonycteris spelaea* was the most captured bat species and has the highest rate of bat flies infestation (66.67%, 38/57) compared to other bat species. This is especially true, when this bat species was reported to have roosting behavior in large single-species colonies and it increases contact between bats leading to higher ectoparasite infestation rates (Francis, 2019). Comparatively to *Cynopterus brachyotis* bat species as reported in the same study, they roost in small groups of four to 12 individuals and regularly switch between roosts which decreases probability of bat flies transmission between bats (Francis, 2019), that consistent with our result of this bat species were having lowest infestation rate of bat

flies with only 14.286% (1/7). However, the total number of bats according to bat species were varied, thus leading to differences in percentage of infestation even with one positive result, for example of *Hipposideros kunzi* and *Rhinolophus pusillus* that have 50% (1/2) of bat flies infestation that were captured in Sekayu Recreational Forest and Gunung Reng respectively compared to *Rhinolophus affinis* that was captured in Gunung Reng that had only 43.75% of infestation despite had high number of bats infested (14/32).

Among the 98 bat flies that were parasitizing the bats in both Sekayu Recreational Forest, Terengganu and Gunung Reng, Kelantan, 20.41% (20) bat flies were identified as Streblids and 79.59% (78) were identified as Nycteribiids. These results are relatable as Nycteribiids that comprise of 275 species are more speciose in the Eastern Hemisphere, including Europe, Africa, Asia and Australia, whereas the Streblids which comprise of 227 species are mainly to be found in the Western Hemisphere (Dick et al., 2006; Szentiványi, Haelewaters, et al., 2019).

Among 37 bat flies pool samples tested for *Bartonella* spp., a total of 26 samples (70.27%) were found positive. Based on the bat flies family, Nycteribiidae bat flies pools were found to have higher positive results of *Bartonella* spp. with 75.86% (22/29) compared to Streblidae bat flies pools of 50% (4/8). This could be due to differences in the total number of collected bat flies families that influence the percentage of positive results of both bat flies families. The reason of *Bartonella* spp., favors to harbor specific bat flies family has yet to be found. Comparing the overall percentage positive results to a previously reported study that was conducted in Borneo, particularly in Lahad Datu Sabah (Low et al., 2022), 31.11% (14/45) were found to be positive of *Bartonella* spp., our positive results percentage was relatively higher. However, the study was conducted particularly on

Nycteribiids collected from *Cynopterus brachyotis* bat species, the results were believed to increase if the bats and bat flies of varying species were captured and collected for detection.

For sequence analysis, the nucleotides BLAST demonstrated that a positive sample from Sekayu (S7) of Nycteribiid bat flies from *Rhinolophus affinis* bat species had 95% identity to LR9 Brazil. Meanwhile, samples collected from Gunung Reng (RE10, RE9 and RE15) of Nycteribids bat flies from *Eonycteris spelaea* had 95% identity to B40400 Africa. The sequencing results that we obtained were aligned with the results from reported studies that were also conducted on bat flies and bat species in Malaysia for *Bartonella* spp. sequence analysis in different states which were Sabah (Low et al., 2022) and Pahang (Hou et al., 2018). These identified strains were related to previously reported *Bartonella* spp.. However, the strains may be regarded as potentially new *Bartonella* species as their citrate synthase (*gltA*) sequences exhibit less than 96% identity to previously identified *Bartonella* spp. based on the species definition (La Scola et al., 2003).

Based on ClustalW sequence alignment, the results suggested that *Bartonella* spp. strain in the particular samples are carried and spread from different countries during bat migrations. But these results cannot conclude that all bat flies from Sekayu Recreational Forest carry the particular strain-related to LR9 Brazil and bat flies samples from Gunung Reng carry the B40400 African-related-strain because the positive samples were selected randomly and they appeared to be in the same bat flies family from the same bat species for Gunung Reng samples.

10.0 Conclusion

In this study, 70.27% of bat flies pool samples collected in East Coast Malaysia were found positive with *Bartonella* spp. by Polymerase Chain Reaction (PCR) and sequence analysis served as an important tool to recognize the strain origin of the *Bartonella* spp. The *Bartonella* spp. identified from these samples were regarded as a new species as the citrate synthase sequences (*gltA*) exhibit less than 96% identity to previously identified *Bartonella* spp. These findings also suggest that the bat flies in East Coast Malaysia serve as a reservoir for a zoonotic potential *Bartonella* spp. infection.

11.0 Recommendations

For future work, it is recommended to capture bats in multiple study sites for a particular state to increase sample size for strong evidence for us to get into conclusion of the bat flies infestation status. For example, in Pahang, Tioman Island Wildlife Reserve can be added as a sample collection site for Pahang state that was previously reported to have bat flies infestation on the captured bats. Meanwhile in Kelantan, Gua Musang district also can be one of the collection sites.

Moreover, the collected bat flies may be further identified into species based on identification key of bat flies, to study and compare the host-specificity of our findings with previous reported studies.

Besides, more samples for sequence analysis may be sent including different type bat species and bat flies family to obtain more inclusive results of strain carried by the bat flies collected in East Coast Malaysia.

Last but not least, detection and sequence analysis of *Bartonella* spp. also is recommended to be conducted in the captured bats by using bat blood samples to compare the strain carried by bat flies and the bat itself because there was a reported study that showed *Bartonella* spp. strain detected in bat flies were not overlapped with those detected from the bat host, suggesting the bat flies themselves serve as reservoirs.

Appendix A

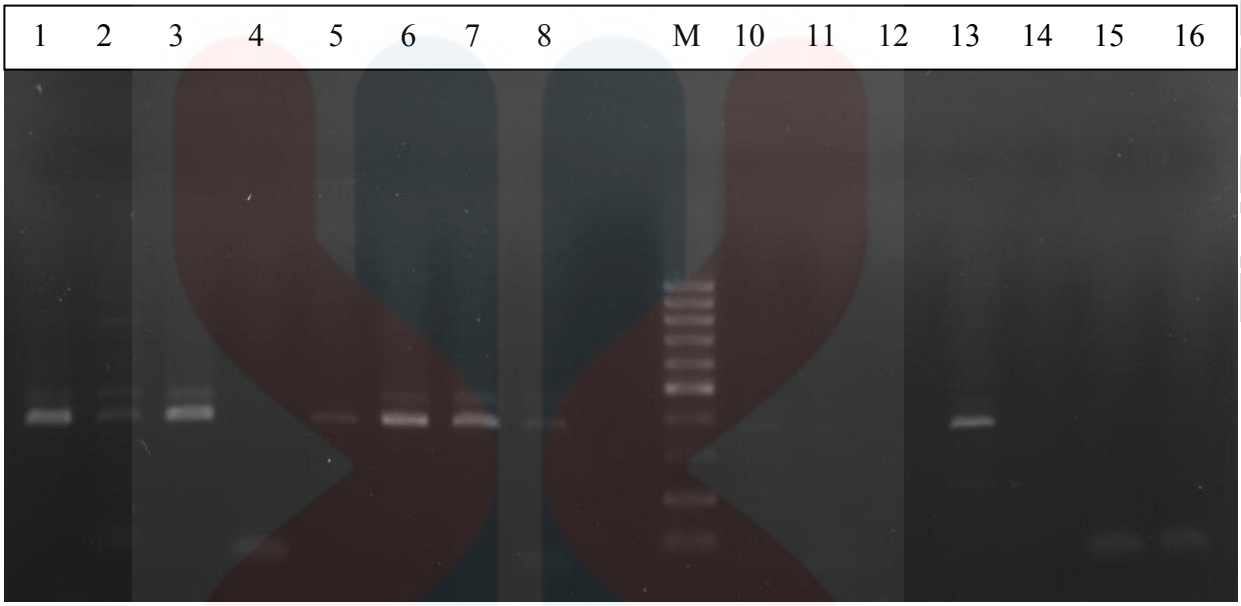
Appendix A 1: Bat flies' pool

No. of bat flies pool sample	Sample code*	Bat species	Bat flies family
1	RE1	*Eonycteris spelaea*	Nycteribiidae
2	RE2	*Eonycteris spelaea*	Nycteribiidae
3	RE3	*Eonycteris spelaea*	Nycteribiidae
4	RE4	*Eonycteris spelaea*	Nycteribiidae
5	RE5	*Eonycteris spelaea*	Nycteribiidae
6	RE6	*Eonycteris spelaea*	Nycteribiidae
7	RE7	*Eonycteris spelaea*	Nycteribiidae
8	RE8	*Eonycteris spelaea*	Nycteribiidae
9	RE9	*Eonycteris spelaea*	Nycteribiidae
10	RE10	*Eonycteris spelaea*	Nycteribiidae
11	RE11	*Eonycteris spelaea*	Nycteribiidae
12	RE12	*Eonycteris spelaea*	Nycteribiidae
13	RE13	*Eonycteris spelaea*	Nycteribiidae
14	RE14	*Eonycteris spelaea*	Nycteribiidae
15	RE15	*Eonycteris spelaea*	Nycteribiidae
16	RE16	*Eonycteris spelaea*	Nycteribiidae
17	RE17	*Eonycteris spelaea*	Nycteribiidae
18	RA1	*Rhinolophus affinis*	Nycteribiidae
19	RA2	*Rhinolophus affinis*	Nycteribiidae
20	RA3	*Rhinolophus affinis* + *Rhinolophus stheno*	Nycteribiidae
21	RA4	*Rhinolophus affinis*	Nycteribiidae

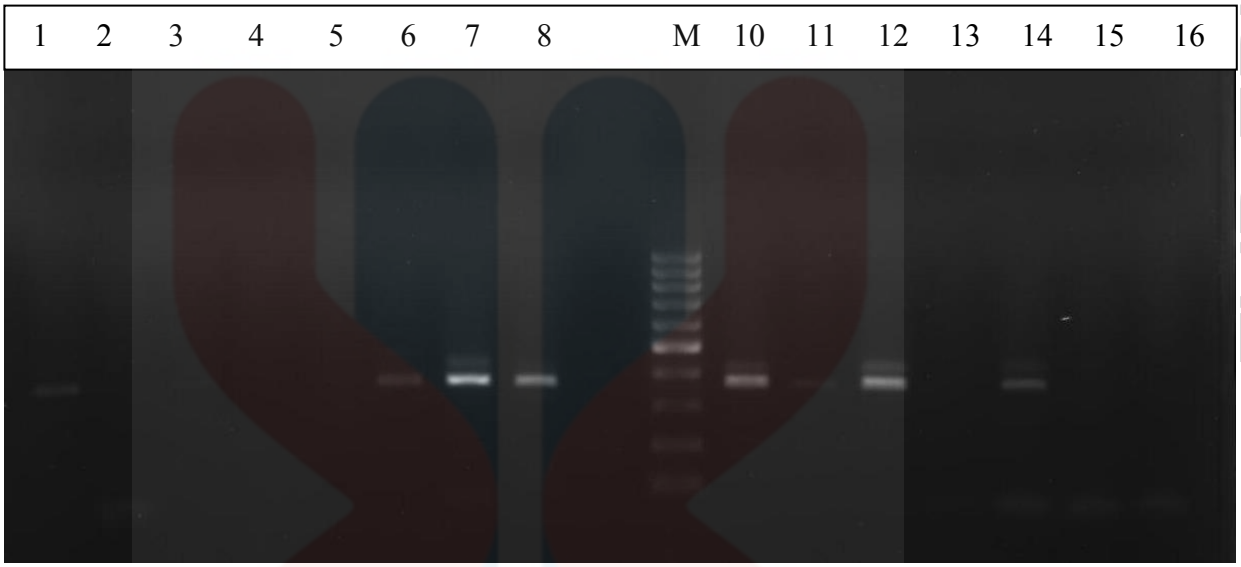
22	RR1	*Rhinolophus affinis + Rhinolophus pusillus*	Streblidae
23	S37	*Cynopterus horsfieldii*	Nycteribiidae
24	S7	*Rhinolophus affinis*	Nycteribiidae
25	S33	*Rhinolophus affinis*	Streblidae
26	SB1	*Balionycteris seimundi*	Nycteribiidae
27	SL1	*Hipposideros larvatus*	Nycteribiidae
28	SA1	*Rhinolophus affinis*	Nycteribiidae
29	SR3	*Rhinolophus affinis*	Streblidae
30	SK1	*Hipposideros kunzi*	Streblidae
31	S02	*Cynopterus horsfieldii*	Nycteribiidae
32	S01	*Rhinolophus affinis*	Nycteribiidae
33	SR2 (1)	*Rhinolophus affinis*	Streblidae
34	SD1	*Hipposideros dyacorum*	Streblidae
35	SR2 (2)	*Rhinolophus affinis*	Streblidae
36	SR1	*Rhinolophus affinis*	Streblidae
37	SC1	*Cynopterus brachyotis*	Nycteribiidae

** 'R' indicated samples were collected from Gunung Reng, Kelantan and 'S' indicated samples were collected from Sekayu Recreational Forest, Terengganu.*

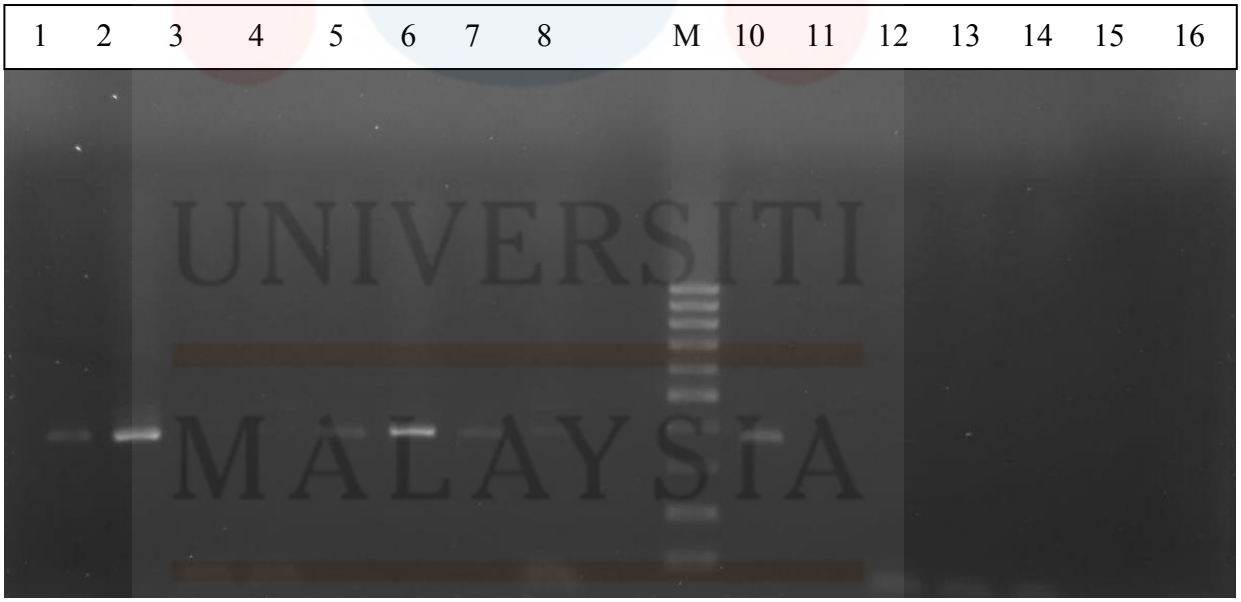
Appendix B



Appendix B.1: Agarose Gel Electrophoresis of PCR of Bartonella spp. at 379bp. Lane 1: RE15, lane 2: S37, lane 3: S7, lane 4: SK1, lane 5: SL1, lane 6: RE9, lane 7: RE10, lane 8: RE14, lane M: 100 bp DNA ladder, lane 10: RE7, lane 11: RE3, lane 12: SR1, lane 13 RE8, lane 14: RE4, lane 15: Positive control, lane 16: Negative control (No Template Control).



Appendix B.2: Agarose Gel Electrophoresis of PCR of Bartonella spp. at 379bp. Lane 1: SR2 (1), lane 2: RA3, lane 3: RE17, lane 4: RE6, lane 5: S33, lane 6: S01, lane 7: RE16, lane 8: RE1, lane M: 100bp DNA ladder, lane 10: RE11, lane 11: RE2, lane 12: RE12, lane 13: RA1, lane 14: RE5, lane 15: Positive control, lane 16: Negative control (No Template Control).



Appendix B.3: Agarose Gel Electrophoresis of PCR of Bartonella spp. at 379bp. Lane 1: SR2 (2), lane 2: SC1, lane 3: SB1, lane 4: RR1, lane 5: SD1, lane 6: SR3, lane 7: RA2, lane 8: SA1, lane M: 100bp DNA ladder, lane 10: RA4, lane 11: RE13, lane 12: S02, lane 13 Positive control, lane 14: Negative control (No Template Control).

Appendix C

Appendix C.1: Sequence analysis result obtained from Apical Scientific SDN. BHD..

Site	Samples	Sequences
Sekayu Recreational Forest, Terengganu	S7	CCTGAATTTATTGCACGAGCAAAAGATAAAAAAG ATCCTTTTCGTCTTATGGGTTTTGGTCATAGAGTTT ATAAAAATTATGATCCACGTGCAAAAATTATGCA GCAAACTTGCTATGAAGTTTTAAAAGAACTAGATA TTCAGAATGATCCACTCCTTGATATCGCTATGGAG CTTGAAAAAATTGCTTTGAATGATGAATATTTTAT TGAAAAAAAGCTTTACCCTAATGTCGATTTCTATT CTGGTATTACATTAAAAGCTTTAGGCTTTCCTACC GAAATGTTTACTGTTCTTT
Gunung Reng, Kelantan	RE9	CCTGAATTCATTGCGCGTGCAAAAGATAAAAATG ACCCTTTCCGTCTTATGGGATTTGGTCATAGAGTC TACAAAAATTATGATCCACGTGCAAAAATTATGCA GCAAACTTGTCACGAAGTTCTAAAGGAACTCAAT ATTAAAGATGACCCACTTCTTGATATCGCTGTCGA ACTTGAAAAAATCGCTCTACACGATGATTACTTTA TTGAAAAGAAGCTCTATCCTAATGTCGATTTTTAT TCTGGAATTACATTAAAAGCTTTAGGTTTTCCGAC TGAAATGTTTACTGTTCTTT
	RE10	CCTGAATTCATTGCGCGTGCAAAAGATAAAAATG ACCCTTTCCGTCTTATGGGATTTGGTCATAGAGTC TACAAAAATTATGATCCACGTGCAAAAATTATGCA GCAAACTTGTCACGAAGTTCTAAAGGAACTCAAT ATTAAAGATGACCCACTTCTTGATATCGCTGTTGA ACTTGAAAAAATCGCTCTACACGATGATTACTTTA TTGAAAAGAAGCTCTATCCTAATGTCGATTTTTAT TCTGGAATTACATTAAAAGCTTTAGGTTTTCCGAC TGAAATGTTTACTGTTCTTT
	RE15	CCTGAATTCATTGCGCGTGCAAAAGATAAAAATG ATCCTTTCCGTCTTATGGGATTTGGTCATAGAGTCT ACAAAAATTATGATCCACGTGCAAAAATTATGCA GCAAACTTGTCACGAAGTTTTAAAAGAACTCAATA TTAAAGATGACCCACTTCTTGATATCGCTGTTGAA CTTGAAAAAATCGCTCTACACGATGATTACTTTAT TGAAAAGAAGCTCTATCCTAATGTCGATTTTTATT

CTGGAATTACATTAAAAGCTTTAGGTTTTCCGACT
CAAATGTTTACTGTTCTTT

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