UNIVERSITI PUTRA MALAYSIA

MOLECULAR DETECTION AND GENOTYPING OF *BARTONELLA* (PROTEOBACTERIA: BARTONELLACEAE) INFECTING PERI-DOMESTIC RODENTS IN SELANGOR, MALAYSIA

ROZIANA BINTI DAMING

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ROZIANA BINTI DAMING

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CERTIFICATION

It is hereby certified that I have read this project paper entitled "**Molecular Detection and Genotyping of *Bartonella* (Proteobacteria: Bartonellaceae) Infecting Peri-domestic Rodents in Selangor, Malaysia**" by Roziana Binti Daming and in my opinion it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course VPD 4999 - Final Year Project.

DR. REUBEN SHARMA

DVM, MVSc, PhD, MRSB, CBiol

Senior Lecturer,

Faculty of Veterinary Medicine

University of Putra Malaysia

(Supervisor)

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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 *BARTONELLA* (PROTEOBAKTERIA: BARTONELLACEAE*)* YANG MENJANGKITI TIKUS PERI-DOMESTIK DI SELANGOR, MALAYSIA

oleh

Roziana Binti Daming

Disember 2020

Pegawai Pemantau: Dr Reuben Sharma

Bartonella adalah proteobakteria yang pantas dan cekap juga dikenali mampu menyerang darah dan sel endotelial, menyebabkan jangkitan bakteria dalam darah pada manusia dan pelbagai jenis haiwan. Beberapa spesies *Bartonella* berkait rapat dengan tikus adalah zoonotik dan memerlukan kesedaran kesihatan awam. Walaupun penyakit ini boleh berjangkit kepada manusia, informasi dan data mengenai pembahagian, pertalian tuan rumah, kepelbagaian spesies dan epidemiologi penyakit ini masih terhad. Dengan itu, kajian ini dilaksanakan untuk mengesan kelaziman molekular, mengenalpasti spesies dan kepelbagaian genetik *Bartonella* yang menjangkiti tikus peri-domestik di Selangor, Malaysia. Sebanyak 58 ekor tikus telah ditangkap menggunakan perangkap besi berjaring di beberapa lokasi perumahan dan komersial sekitar kawasan Selangor. Tikus telah ditenangkan menggunakan dietil eter dan dibius menggunakan tiletamin/zolazepam. Darah diambil melalui tusukan jantung dan disimpan di tub EDTA dalam suhu sejuk. Selepas mengestrak genom DNA, separa bahagian *Bartonella* 18SSUrRNA gen disalin dan diperbanyakkan menggunakan genus-spesifik primer. DNA *Bartonella* telah dikesan sebanyak 6.9% daripada tikus yang dikaji daripada satu lokasi sahaja. Infeksi *Bartonella* ini dapat dikesan di tikus betina dewasa dengan kelaziman sebanyak 11.1%. Kadar infeksi pada dua spesis tikus (*Rattus norvegicus* and *R. rattus*) adalah sebanyak 6.5% dan 7.4%. Bioinformatik dan filogenetik analisis 18SSUrNA gen lokus dengan empat positif amplicon digunakan untuk melakar pokok filogenetik menggunakan dua kaedah iaitu Maximum-likelihood (ML) dan Neighbour-Joining (NJ) algoritma menunjukkan kluster berbeza dengan pertalian terdekat iaitu *B. tribocorum*, *B. elizabethae*, *B. rattimassiliensis* dan *Candidatus B. krasnovii*. Kehadiran *Bartonella* dikalangan tikus peri-domestik di Selangor perlu meraih kesedaran kesihatan awam oleh kerana kemampuan penularan bakteria ini secara zoonotik.

Kata kunci: *Bartonella*, pengesanan molekular, tikus, Selangor, 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 *BARTONELLA* (PROTEOBACTERIA: BARTONELLACEAE) INFECTING PERI-DOMESTIC RODENTS IN SELANGOR, MALAYSIA

by

Roziana Binti Daming

December 2020

Supervisor: Dr Reuben Sharma

Bartonella are fastidous proteobacterium that are known to infect blood and endothelial cells, causing persistent bacteraemia in humans and a variety of animals. Several rodent-associated *Bartonella* species are human infective and are of public health concern. However, in spite of their zoonotic potential, information on the global distribution, hosts affinities, species diversity and disease epidemiology are limited. This study was therefore undertaken to determine the molecular prevalence, species identification and genetic diversity of *Bartonella* infecting peri-domestic rodents in Selangor, Malaysia. A total of 58 rodents were collected using small mammal mesh wire traps, from several residential and commercial areas in Selangor. The rodents were sedated with diethyl ether and anaesthetized with tiletamine/zolazepam. Blood was collected by cardiac puncture and stored and chilled in EDTA tubes. Following genomic DNA extraction, a partial fragment of the *Bartonella* 18SSUrRNA gene was amplified using genus-specific primers. *Bartonella* DNA was detected in 6.9% of the rats examined, and all positive rats were obtained from only one location. Infection was only present in the adult female rats at a prevalence of 11.1%. Infection rates in the two rodent species examined (*Rattus norvegicus* and *R. rattus)* were 6.5% and 7.4%, respectively. Bioinformatic and phylogenetic analyses of the 18SSUrRNA gene locus of four positive amplicons using maximum parsimony (MP) and neighbor-joining (NJ) algorithms revealed distinct clustering of the Malaysian isolates, with closest affinities to *B. tribocorum*, *B. elizabethae*, *B. rattimassiliensis* and *Candidatus B. krasnovii*. The presence of *Bartonella* among peri-domestic rodents in Selangor is a public health concern due to the potential for zoonotic transmission.

Keywords: *Bartonella*, molecular detection, rodents, Selangor, Malaysia

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1.0 INTRODUCTION

Rodents are one of the largest groups of small mammals in Malaysia (Medway, 1983; Ow-Yang, 1971). Members of the family Muridae have high adaptability and are capable of inhabiting a wide array of habitats, including urban environments worldwide. Their high reproductive rate often leads to large populations in the peri-domestic setting, where they are often considered pests (Siti Shafiyyah *et al.*., 2012). In addition, these rodents are known to carry a wide range of microorganism that may cause zoonotic diseases, including many species of bacteria, virus and protozoa. They act as vectors or reservoirs for various kind of pathogens which may be transmitted by ectoparasites such as ticks, mites, fleas and lice (Paramasvaran *et al.*, 2009; Siti Nursheena *et al.*., 2015).

Rodents are also involve in the transmission cycle of *Bartonella* infection when it is accompanied with the presence of vectors such as fleas and ticks. This microorganism infects the erythrocytes of many species of mammalian hosts and may cause chronic bacteraemia, fever, and cardiac diseases in humans. Several species of rodents-borne *Bartonella* including *B. elizabethae* and *B. washoensis* are known to be able to infect humans and have been isolated from patients with endocarditis (Daly *et al.*., 1993; Kosoy *et al.*., 2003). In addition, case of neuroretinitis or bilateral retinal artery branch occlusions have been observed in patients infected with *B. grahamii* (Kerkhoff *et al.*, 1999; Serratrice *et al.*, 2003). These findings direct attention to the importance of small mammals especially rodents, as major reservoirs for various zoonotic *Bartonella* species.

The study of *Bartonella* and its genotypes infecting rodents in Malaysia is limited to a few of studies conducted in the peninsula (Tay *et al.*, 2014, 2016) and in

Sarawak, Malaysian Borneo (Blasdell *et al.*, 2019). The present study was therefore undertaken to expand the knowledge on rodent-borne *Bartonella* in Peninsular Malaysia with the following objectives:

1) To determine the molecular prevalence of *Bartonella* sp. infecting peri-domestic rodents from Selangor, Malaysia.
2) To characterize the *Bartonella* genotypes present among these per-domestic rodents.

2.0 LITERATURE REVIEW

2.1 Classification of *Bartonella*

Members of the genus *Bartonella* are fastidious, facultative intracellular and rod-shaped, Gram-negative bacteria. They are taxonomically classified in the phylum Proteobacteria, class Alphaproteobacteria, order Rhizobiales and family Bartonellaceae. (Peters and Wigland, 1955; Relman *et al.*, 1992; Brenner *et al.*, 1993). In the early 1990's, *Bartonella* were classified under order Rickettsiales which consisted of three families, including Rickettsiaceae, Anaplasmataceae and Bartonellaceae. However subsequent work (Relman *et al*., 1992) proposed that there organisms were more closely related to the Alphaproteobacteria, especially the Rhizobiaceae, and the genus *Rochalimaea* and *Bartonella* were unified under the family Bartonellaceae (Brenner *et al.*, 1993). The genus *Bartonella* represents a diverse group of vector-borne bacteria that are usually found in the blood of various species of mammalian hosts, and arthropod vectors (Birtles, 2005).

2.2 Rodent host of *Bartonella*

Rodents are known to be one of important mammalian reservoirs of *Bartonella*, which cause high infection rates worldwide (Bai *et al*., 2007). Rodents of the family muridae are widely distributed and comprise one of the largest groups of mammals in Malaysia (Medway, 1978; Ow-Yang, 1971). These rodents have high adaptability traits and are able to thrive in various ecological niches including natural and human-altered environments (Siti Shafiyyah *et al*., 2012). These rodents also reproduce rapidly due to the availability of food resources and the lack of proper garbage disposal, making them amongst the common domiciliary pests in their country (Siti

Shafiyyah *et al.*, 2012). The rapid destruction of their natural habitats and land-use conversion for human activities has escalated the population of urban rodents, closing the gap between the human-rodent interface. This may escalate the transmission of rodent-borne zoonotic pathogens including viruses, bacteria and parasites (Paramasvaran *et al.*, 2009; Siti Nursheena *et al.*, 2015). Rodents of the genus *Rattus* in Malaysia consist of nearly 200 sub-species that can be grouped into 20 species (Paramasvaran *et al*., 2009). The two most successful species that can be found widespread in the world due to its ability in adapting to all kinds of environments are *R. tanezumi* and *R. norvegicus* (Blanchard *et al*., 1977).

Rattus tanezumi / Rattus rattus

Rattus tanezumi is a medium size rodent with tail of similar length or longer than the head and body (Miller *et al.*, 2008). The adults weigh from 65 to 300g, with a body length of 114-224mm, a hind foot length of 30-43mm, a tail length of 110-231mm, and with ear length of 16-25mm. The dorsal fur is coated with a shade of brown, but may vary in colour from dark-brown to greyish-brown or reddish-brown. (Aplin *et al.*, 2003). The fur at abdomen is usually white and often seen with some pale yellow or orange tipping, and grey-based fur flexible with grooved and spine-like hairs on the dorsal pelage may be seen. 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. They also have thinly furred, rounded and relatively large ears which are uniformly dark/black throughout and covered with numerous short stiff black hairs. It also occasionally has a short white tip on the ears. Females have five or six pairs of mammary teats; one pair at the pectoral region, one or two pairs post-axillary, and three pairs at the inguinal region. It has a wide range distribution in which Musser and Cartelon (2005) recorded the presence of *R. tanezumi* in Myanmar, Thailand, Malaysia and Singapore. They also reported that *R. tanezumi* is common in Afghanistan, Bangladesh, Bhutan, Cambodia, Korea and Thailand, Indonesia, Malaysia, Philippines, Fiji and Papua New Guinea.

Rattus norvegicus

Rattus norvegicus or Brown rat is a large rat species (Medway, 1983), with brown fur on the back with pale grey fur on the abdomen part. Adults normally weigh from 150-300g, and are up to 390mm in length. They have small ears and has short fur which is slightly stiff without bristles and also has guard hairs. The dorsal fur ranges 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 abdomen area. It has large front and hind feet. The female has six pairs of mammary teats; three pairs at the pectoral region, and the other three pairs are at the inguinal region. *Rattus norvegicus* is believed to have originated from northeast China and is now distributed worldwide, especially in cooler climates. In warmer climates of the tropics, they are usually found in habitats that are in close proximity to humans. Pimsai *et al*., (2014) mentioned that *R. norvegicus* may be found in Myanmar, Thailand, Malaysia and Singapore. This species is highly adaptable and is common in urban areas where food resources are abundant.

2.3 Vectors of *Bartonella*

Ectoparasites have been described to be a major contribution to *Bartonella* infection as they are efficient vectors. Ectoparasites such as ticks, sand flies, fleas, and lice are among the key vectors of *Bartonella* (Angelakis *et al.*, 2009), of which fleas are considered major vectors and reservoirs of rodent-associated *Bartonella* (Birtles, 2005; Deng *et al.*, 2018). This is because they harbour a wide diversity of *Bartonella* species and strains and demonstrate great efficiency in the transmission of these bacteria among rodents (Brinkerhoff *et al*., 2010).

2.4 Epidemiology

The genus *Bartonella* are well adapted to a wide range of vertebrate animals including small mammals and humans. In general, *Bartonella* can establish long-term infections in mammalian reservoirs with a silent strategy and are able to hinder clearance from the host's immune system (Harms and Dehio, 2012). This is parallel with a previous study which demonstrated that most of the rodents with bacteraemia had low antibody level against various *Bartonella* antigens (Kosoy *et al.*, 1997), suggesting that these animals may serve as competent reservoirs. Furthermore, the transmission of *Bartonella* is associated with the presence of arthropod vectors (Kosoy *et al*., 2012) and these could lead to spreading the bacteria within and between natural reservoir species. The transmission routes of *Bartonella* among animals and their vector is crucial in maintaining the infection and transmission cycle in nature (Figure 1). The bacteria are transmitted *via* vertical and/or horizontal pathways to spread within rodents. Various studies (listed in Figure 1) have demonstrated the capability of fleas to acquire and transmit *Bartonella* strains under experimental conditions, and pioneering work has shown that species like *Xenopsylla cheopis* are competent vectors for *Bartonella* in voles (*Myodes glareolus*) pioneer study (Krampitz, 1962). Further studies including Birtles (2005) showed that this transmission route from arthropod to mammal is done through the gastrointestinal content, mainly faeces.

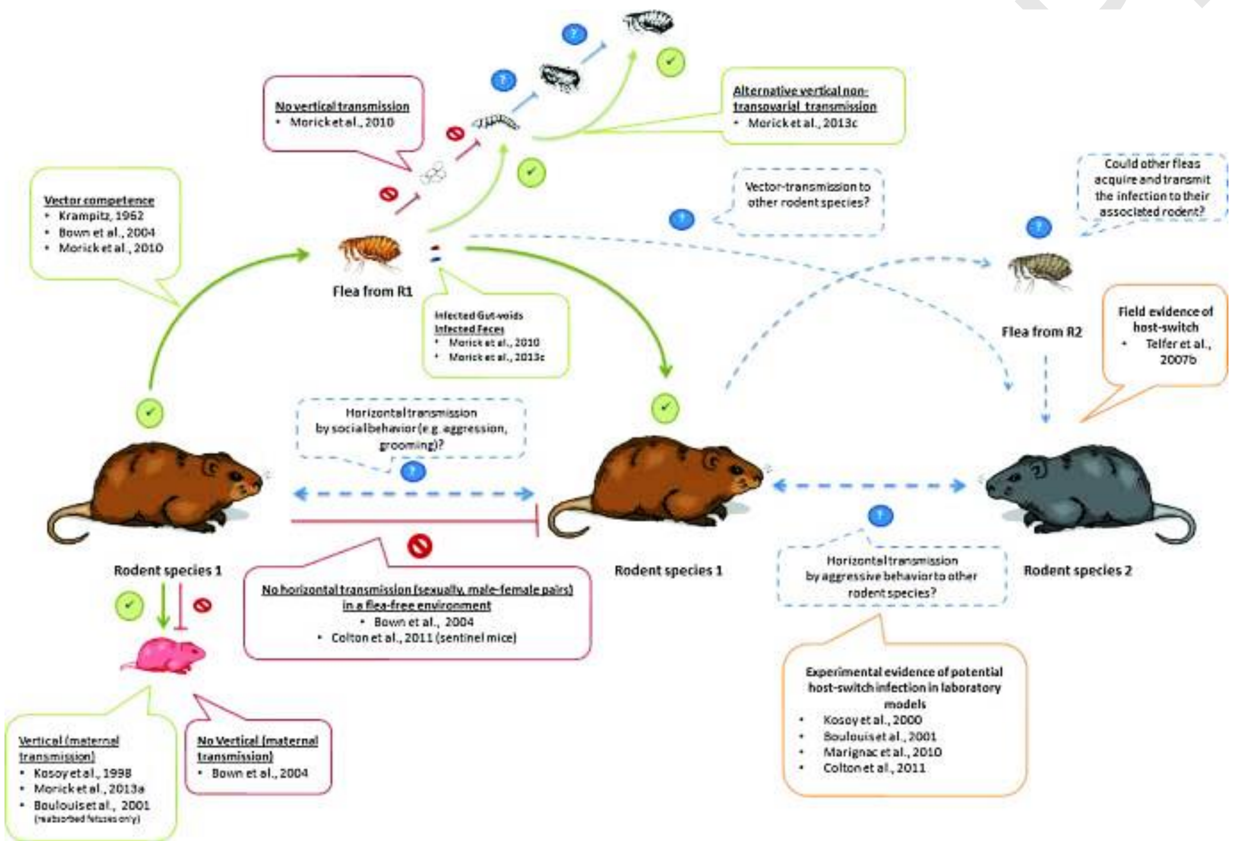

Figure 1. Pathways and potential transmission routes of *Bartonella* sp. in wild rodents and their fleas. Adapted from Gutiérrez *et al.* (2015).

2.5 Zoonotic potential of *Bartonella*

Rodent-borne *Bartonella* are associated with public health importance, and *B. vinsonii arupensis, B. elizabethae, B. grahamii,* and *B. washoensis* have been isolated from humans (Welch *et al*., 1999). Studies have shown that *Rattus* sp. are potential reservoirs for *B. elizabethae*, and endocarditis and neuroretinitis have been reported in humans in Indonesia (Winoto *et al.*, 2005). These findings parallel a previous study conducted by (Daly *et al.*, 1993) which stated that *B. elizabethae* is a zoonotic species and has been reported in a case of endocarditis in human patients. Myocarditis has also been associated with *Bartonella* infection in humans (Kosoy *et al.*, 2003). In addition, fever and neurologic disorders (Welch *et al.*, 1999), intraocular neuroretinitis (Kerkhoff *et al.*, 1999), meningitis (Probert *et al*., 2009), splenomegaly (Eremeeva *et al.*, 2007), and lymphadenopathy (Oksi *et al.*, 2013) have also been reported in humans infected with *Bartonella*.

2.6 Prevalence of *Bartonella* among rodents in Southeast Asia

The presence of *Bartonella* in rodents in several countries in Asia have been documented, including Bangladesh, China, Japan, Indonesia, Laos, Cambodia, Taiwan, Thailand, and Nepal (Ying *et al.*, 2002; Winoto *et al*., 2005; Bai *et al*., 2007; Inoue *et al.*, 2008; Kosoy *et al.*, 2010; Jiyipong *et al.*, 2012). A study by Jiyipong *et al.* (2015) revealed an overall 10.7% prevalence of *Bartonella* among rodents in the region with specific infection rates of 11.9% in Lao PDR, 11.0% in Thailand, and 9.6% in Cambodia. In Malaysia, a study by Tay *et al*. (2014) revealed that 13.5% of *R. rattus* (n = 58) and 13.8% of *R. norvegicus* (n =37) were positive for *Bartonella*, with five species (*B. rattimassiliensis, B. coopersplainsensis, B. tribocorum, B. elizabethae,* and *B. queenslandensis*) detected. In Sarawak, Malaysian Borneo, the overall prevalence of *Bartonella* was 57.3%, with a specific prevalence of 47.1% and 87.0% in *Rattus* sp. and *Sundamys muelleri*, respectively (Blasdell *et al.*, 2018).

2.7 Control and prevention

Rodent population control programs are vital for the control and prevention of *Bartonella* infection among humans, and involve collaboration between public health professionals and local communities. The control program may include direct disinfections by using poisonous bait or traps. Poisonous bait such as mixing rodenticide with bait for rodent consumption. Traps such as cage or snap traps can be used. As for fundamental control, communities shall improve the sanitary condition of the environment by reducing food spillage and waste food availability to reduce rodent population from harbouring the waste area. Proper waste management should be practiced in the communities to prevent the presence of rodents and their flea vectors. Controlling the geographical and/or habitat spread of rodents and their fleas may restrict the diversity of *Bartonella* and therefore afford better control of the disease (Jardine *et al.*, 2006)

3.0 MATERIAL AND METHODS

3.1 Study sites and sampling

A total of 58 rats (31 *R. norvegicus* and 27 *R. rattus*) were captured at six different urban localities in Selangor, Malaysia, namely Bukit Gita Bayu, Taman Kajang Utama, Taman Kajang Prima, Taman Putra Permai and Taman Perindustrian Bukit Serdang, and Subang Jaya (Figure 2). The rats were trapped from several sites with two different habitat characteristics; residential and commercial areas. These sites were further classified as being more or less than 250m from forest. Trapping was performed using mesh wire traps (18x28x13cm) baited with banana and salted fish. The rats collected were processed at the Faculty of Veterinary Medicine, UPM. The rats were sedated using an ether-charged chamber and anesthetized with tiletamine and zolazepam (Zoletil®, Virbac). Blood was drawn *via* cardiac puncture using a 23G needle and 1mL syringe. The blood was collected in EDTA tubes and stored at -80°C for further molecular identification of *Bartonella* species. The rodents were sexed, aged, and identified using published taxonomic references (Medway, 1983; Payne *et al.*, 1985; Pimsai *et al.*, 2014). Each rat was humanely euthanized with pentobarbital sodium and morphometric measurements (total body length, tail length, hind foot length and ear length) and body weight were recorded.

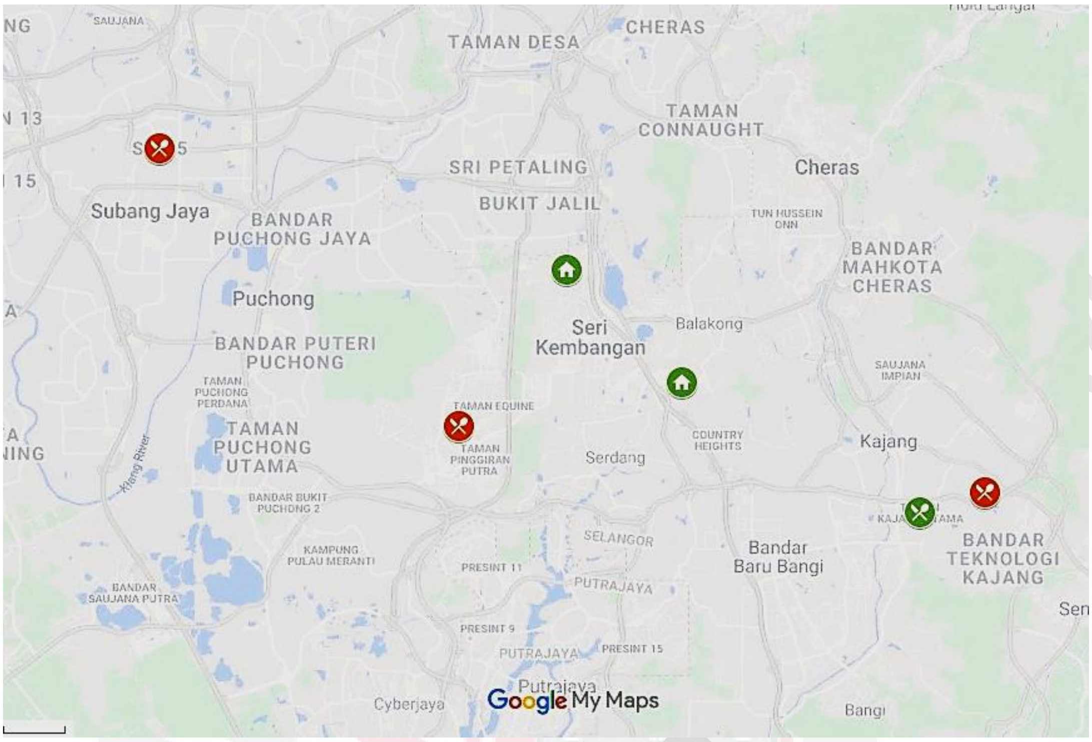

Figure 2: Map showing the rodent trapping sites in Selangor, Malaysia indicated with green and red icon

3.2 Microscopy examination

Thin blood films were prepared from the EDTA blood, and allowed to air-dry. The slides then were fixed in methanol 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, air-dried and examined under the compound microscope at 400x and 1000x magnification. A total of at least 1000 erythrocytes were examined per slide.

3.3 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.

3.4 PCR Assay

Specific primer sets (Forward: 5′-GCTATGGTAATAAATGGACAATGAAATAA-3′ and Reverse: 5′-GACGTGCTTCCGCATAGTTGTC-3′) were used to amplify a 209-base pair partial fragment of the *Bartonella* SSUrRNA gene following protocols described by Diaz *et.al*., (2012) and Mylonakis *et al.,* (2018). The PCR reactions were performed using GoTaq® G2 Green Master Mix Kit (Promega). Each PCR reaction was carried out in PCR tubes by adding 5µL of buffer, 5µL of $MgCI_2$, 0.3µL of deoxynucleotide triphosphates (dNTP) and 1µL of each forward and reverse primer, 7.4µL of nuclease water and 0.3µL of DNA Taq Polymerase. 5 µL of DNA template was added to make the final volume of 25µL. The PCR amplification was performed using a thermal cycler (T100™ Thermal Cycler, Bio-rad, USA). Thermal cycling conditions consisted of an initial denaturation step at 98°C for 3 min followed by 40 amplification cycles (98°C for 15s, 62°C for 15s, and 72°C for 15s) and final extension at 72°C for 3 min.

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™, 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.

3.5 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 *Bartonella*, 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 27 nucleotide sequences of the *Bartonella* SSUrRNA region of curated at the NCBI GenBank and four 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 131 positions in the final dataset. Phylogenetic tree construction was done following the Maximum Likelihood (ML) parameter estimation using the Kimura 2-parameter (K2) model with evolutionary rate differences inferred by discrete Gamma (+G) distribution. The confidence level of the phylogenetic tree was assessed using 1000 (NJ) and 100 (ML) bootstrap replicates.

4.0 RESULTS

The rodents captured and sampled comprise 31 (53%) *R. norvegicus* and 27 (47%) *R. rattus.* There was a higher number of female 36 (62%) as compared to the male 22 (35%) rodents. The highest number of rats captured from a single location was from Taman Putra Permai 36% (21/58), followed by Bukit Gita Bayu and Taman Kajang Utama 17% (10/58). Nine (16%) of the rats were obtained from Taman Kajang Prima, whereas SS15 Subang Jaya and Taman Perindustrian Bukit Serdang each recorded a catch rate of 7% (4/58). The majority of the rodents (62%) were caught in commercial areas >250m from forest, while the rest (38%) were caught from residential areas <250m from forest.

Microscopy examination of Giemas-stained thin blood films revealed that *Bartonella* infection was present in 5.2% (3/58) of rodents. The bacilli form was readily observed in the erythrocytes at 400x magnification (Figures 3 and 4). PCR amplification revealed an overall *Bartonella* prevalence of 6.9% (4/58), where amplicons of 209bp were detected on the agarose gel electrophoresis (Figure 5). *Bartonella* was only detected among rodents captured from Bukit Gita Bayu with a prevalence rate of 40% (4/10). No *Bartonella* infections were apparent in the other locations. The prevalence of *Bartonella* was 7.4% in *Rattus rattus* and 6.5% in *Rattus norvegicus*. All of positive *Bartonella* were found in adult and female rodents (Table 1).

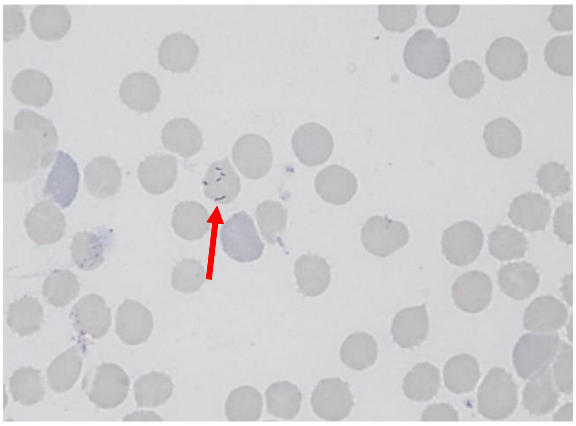

Figure 3: *Bartonella* sp. seen within red blood cell under 40X magnification (red arrow)

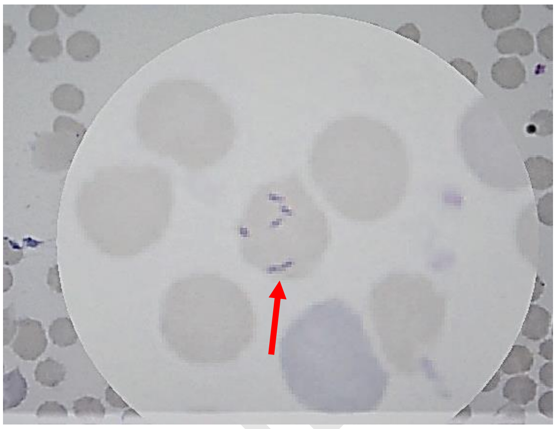

Figure 4: *Bartonella* sp. seen within red blood cell under 400X magnification (red arrow)

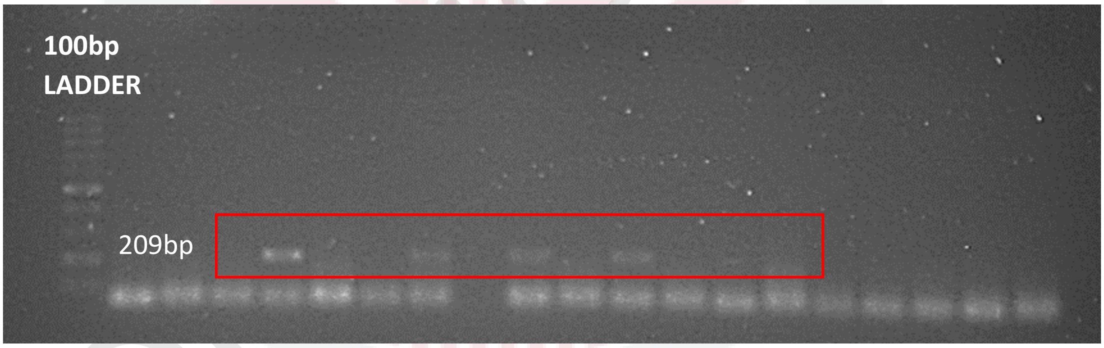

Figure 5. Gel electrophoresis showing the positive amplicons running at a size of 209 bp for *Bartonella* among rats from Selangor, Malaysia.

Table 1. Molecular prevalence of *Bartonella* among rats in Selangor, Malaysia according to the various host variables.

Host variables	Molecular prevalence
Species	
Rattus rattus	2/27 (7.4%)
Rattus norvegicus	2/31 (6.5%)
Age	
Adult	4/50 (8.0%)
Juvenile	0/8 (0.0%)
Gender	
Male	0/22 (0.0%)
Female	4/36 (11.1%)

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All four sequence obtained from the four positive amplicons were aligned and compared to known *Bartonella* sequences curated at the NCBI Genbank using the BLAST tool. The result obtained indicate close identities with isolates of B. *tribocorum*, uncultured *Bartonella* and *B. doshiae* with percentage identity of (97.6 – 100.0) (Table 2). Comparative phylogenetic analysis of the SSUrRNA gene of *Bartonella* isolated from this study with those retrieved from the NCBI Genbank was carried out using both Maximum-likelihood (Figure 6) and Neighbour-joining (Figure 7) algorithms. A total of 31 *Bartonella* SSUrRNA gene sequence were used for the phylogenetic tree construction (Table 3). The isolates in this study all grouped together indicating that they could be the same species or have a very close evolutionary relationship. These isolates were closest to *B. tribocorum* from *Rattus* sp. in Rwanda, *B. elizabethae* from a rodent in China, *Candidatus B. krasnovii* from cleopatrae in Israel, and *Bartonella* sp. from *Rattus rattus* in Tanzania.

Table 2. Results of BLAST search in the NCBI Genbank database with *Bartonella* isolated detected among rodents in Selangor Malaysia.

Bartonella isolate	Closest BLAST hit	Strain	Accession number	% Identity
BRM001	*B. tribocorum*	RWARO124-1	MH142454.1	97.60
BRM002	Uncultured *Bartonella*	R0024	MN256486	100.00
BRM003	*B. tribocorum*	GDHL73	MF65681.1	99.18
BRM004	B. *doshiae*	R18	JN0297568	99.19

Table 3. *Bartonella* SSUrRNA sequences from this study and those curated at the NCBI Genbank that were used for phylogenetic analysis.

Accession No.	Species	Host	Locality	Reference
BRM001	*Bartonella* sp.	*Rattus rattus*	Selangor, Malaysia	This study
BRM002	*Bartonella* sp.	*Rattus rattus*	Selangor, Malaysia	This study
BRM003	*Bartonella* sp.	*Rattus norvegicus*	Selangor, Malaysia	This study
BRM004	*Bartonella* sp.	*Rattus norvegicus*	Selangor, Malaysia	This study
MH618854	*Can. Bartonella krasnovii*	*Synosternus cleopatrae*	Israel	Gutierrez *et al*. (2018)
KF003133	*Bartonella naantaliensis*	*Myotis daubentonii*	Finland	Veikkolainen *et al.* (2014)
KM215711	*Bartonella chomelii*	Bovine	France	Antequera-Gomez *et al.* (2015)
KM215712	*Bartonella chomelii*	Bovine	Spain	Antequera-Gomez *et al.* (2015)
MT394903	Uncultured *Bartonella* sp.	*Pulex irritans*	Laos	Calvani *et al*. (2020)
MT394902	Uncultured *Bartonella* sp.	*Ctenocephalides felis*	Laos	Calvani *et al*. (2020)
MH486970	*Bartonella* sp.	Nycteribiid bat	Yunnan, China	Sui and Han (2018) Unpublished
KT327039	*Bartonella* sp.	*Meriones libycus*	Georgia	Kandelaki et al. (2016)
KF218229	*Bartonella bovis*	*Bubalus bubalis*	Thailand	Bai *et al.* (2013)
KF218227	*Bartonella bovis*	Bovine	Georgia	Bai *et al.* (2013)
LC460850	*Bartonella* sp.	*Rousettus aegyptiacus*	Zambia	Qiu *et al.*(2019)
MN529315	*Bartonella* sp.	*Artibeus jamaicensis*	Guatemala	Mckee (2019) Unpublished
MN529303	*Bartonella* sp.	*Desmodus rotundus*	Guatemala	Mckee (2019) Unpublished
MN654368	*Bartonella alsatica*	Fleas from rabbit	Colarado,USA	Sato *et al.* (2020)
MN654367	*Bartonella vinsonii*	Fleas from rabbit	Colarado,USA	Sato *et al.* (2020)

Table 3 continued

Accession No.	**Species**	**Host**	**Locality**	**Reference**
KT355813	*Bartonella coopersplainsensis*	*Rattus* sp.	Thailand	Klangthong *et al.* (2016) Unpublished
KT355817	*Bartonella rattimassiliensis*	Rodent	Thailand	Klangthong *et al.* (2016) Unpublished
MH142454	*Bartonella tribocorum*	*Xenopsylla brasiliensis - Rattus rattus*	Rwanda	Nziza *et al.* (2018)
MF765676	*Bartonella elizabethae*	Rodent	Guandong	Li *et al.* (2018)
MF765638	*Bartonella japonica*	Rodent	Guandong	Li *et al.* (2018)
MF765682	*Bartonella henselae*	Rodent	Guandong	Li *et al.* (2018)
MN256484	Uncultured *Bartonella*	*Rattus rattus*	Tanzania	Theonest *et al.* (2018)
MK780191	*Bartonella rochalimae*	*Canis* sp.	Iran	Greco *et al.* (2019)
KR733196	*Bartonella bovis*	Bovine	Malaysia	Kho *et al.* (2015)
KM382247	*Bartonella* sp.	*Rousettus aegyptiacus*	Kenya	Kosoy *et al*. (2010)
JN394654	*Bartonella vinsonii*	Human blood	Thailand	Bai *et al.* (2012)
MK298177	*Bartonella grahamii*	Flea	UK	Abdullah *et al.* (2019)

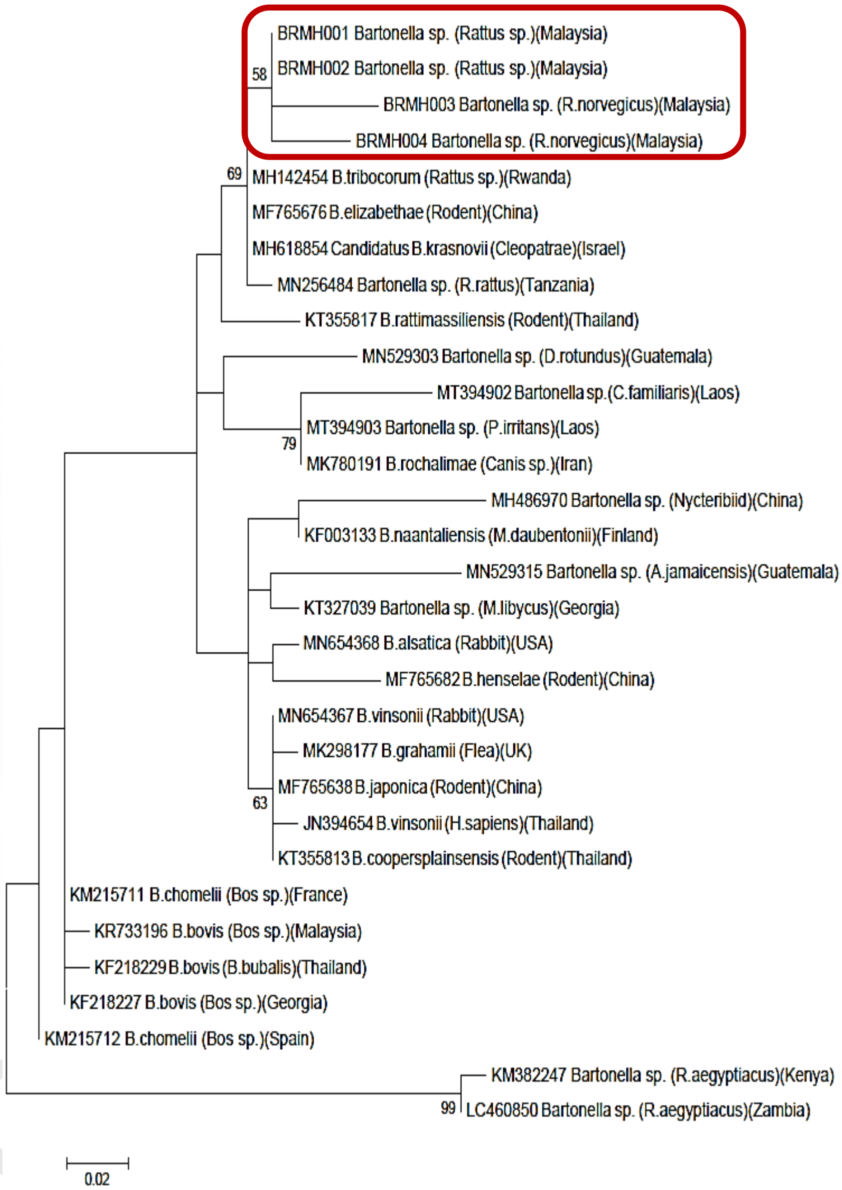

Figure 6. Maximum-likelihood phylogenetic tree of the *Bartonella* SSUrRNA gene with 100 bootstrap replicates indicating the evolutionary relationship of isolates from Selangor, Malaysia (in the red box) and those isolated from other hosts and regions.

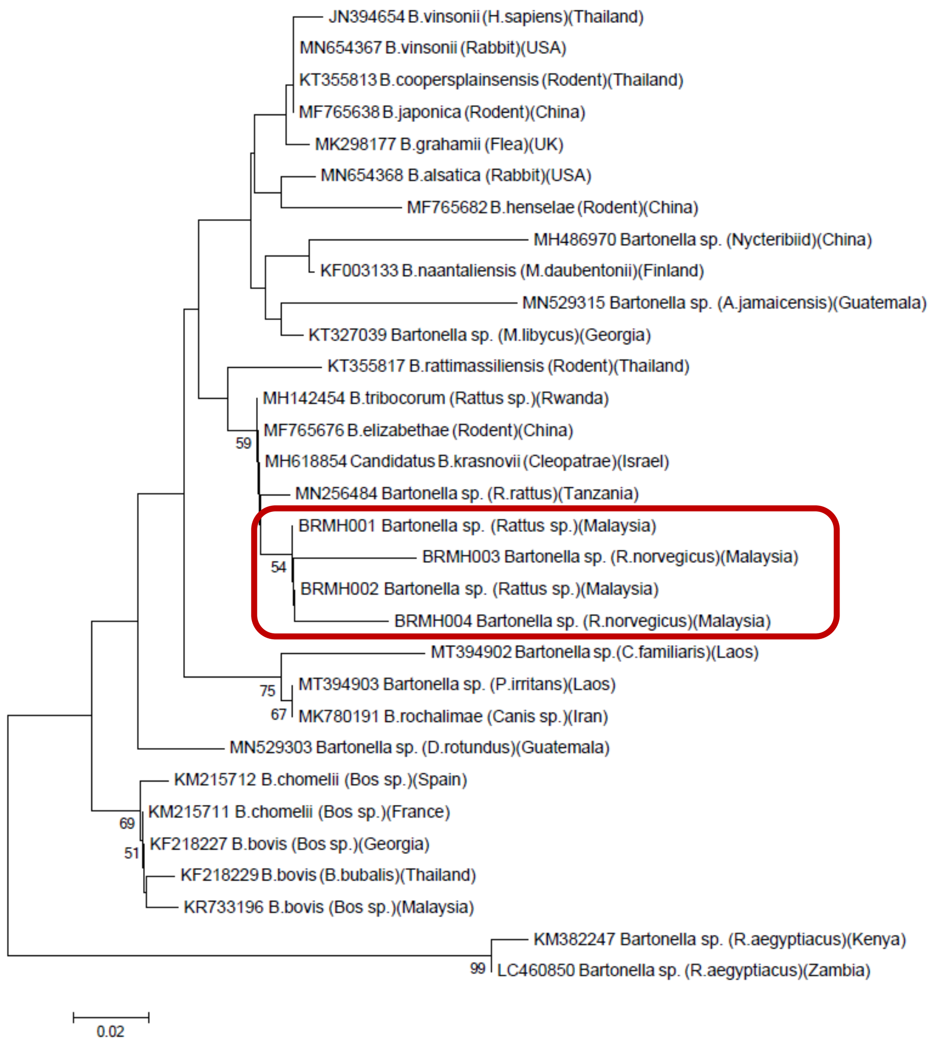

Figure 7. Neighbour-joining phylogenetic tree of the *Bartonella* SSUrRNA gene with 1000 bootstrap replicates indicating the evolutionary relationship of isolates from Selangor, Malaysia (in the red box) and those isolated from other hosts and regions.

5.0 DISCUSSION

The overall molecular prevalence of *Bartonella* sp. detected in this study (6.9%) was lower than that reported previously (Tay *et al.*, 2014), who reported an infection rate of 13.7% among rodents in Kuala Lumpur and Penang, Malaysia. The data however is comparable with the prevalence reported in other Asian countries namely Cambodia (9.6%), Taiwan (10.3%) (Jiyipong et al.., 2012). Whereas, the highest prevalence of *Bartonella* sp. found in Japan (25.7%) and Nepal (23.7%) with the highest prevalence noted across Asia (Tay *et al*., 2014). The prevalence of *Bartonella* by location showed that it appeared to be site specific depending on location characteristics. Bukit Gita Bayu has the highest prevalence among other locations. This site is characterized by presence of a residential area and forest <250meter radius. It is established that the prevalence of *Bartonella* is affected by the abundance and distribution of reservoir host or arthropod vectors in that area (Gutierrez *et al.*, 2015). The Gita Bayu area was observed to have a high population of rodents and accompanied with the presence of residential garbage and leftover food. In addition, its close proximity to the forest may provide close contact among the peri-domestic rodents with the forest rodents that may be sylvatic reservoirs for both the vectors and haemoparasites. The reasons for the low prevalence of *Bartonella* sp. found in the other sampling sites are not fully understand, but it may include few potential factors such as low levels of exposure and transmission, better host immunity and greater ecologic separation of sylvatic potential rodent species.

Rattus norvegicus and *R. rattus* had similar infection rates with *Bartonella*, a finding comparable to thet reported previously by Tay *et al.* (2014) which showed no host specificity to the parasite. Molecular detection using PCR yielded a higher prevalence of *Bartonella* infection compared to microscopy. This is expected as the PCR technique is more sensitive and specific, and able to detect small amounts of parasite DNA within the sample. In addition, microscopy detection is laden with challenges in parasite identification especially when parasitaemia is low.

Phylogenetic analysis revealed close association among the isolates in this study with sequences for a number of Bartonella species including *B. tribocorum, B. elizabethae*, and *Candidatus B. krasnovii*. This finding is consistent with a previous study by Tay *et al.* (2014), where they identifies *B. tribocorum, B. rattimassiliensis, B. coopersplainsensis* and *B. elizabethae* in the rodents sampled from penang and Kuala Lumpur, Malaysia. Both *B. tribocorum* and *B. elizabethae* are known to be zoonotic, with the latter implicated in a case of endocarditis in humans (Daly *et al.*, 1993). In addition, both these *Bartonella* speciesare of public health significance in Southeast Asia, having been isolated from febrile patients in Thailand (Kosoy *et al.*, 2010). The limited data on the pathogenicity, epidemiology and transmission of *Candidatus B. krasnovii* precludes its potential as a zoonotic pathogen, and therefore must await further investigation. The high prevalence of *Bartonella* sp. in Bukit Gita Bayu warrants an effective rat control program. It is associated with residential areas where people live in close proximity to rodents and this could bring potential risk of transmission of zoonotic *Bartonella* into the local communitiey.

6.0 CONCLUSION

The current study provides information on the distribution and genetic diversity of *Bartonella* found on rodents in Selangor. The finding compliments previous studies conducted in Malaysia and Asia. The prevalence of *Bartonella* in peri domestic rodents appears to be site-specific depending on its location characteristic. Bukit Gita Bayu has the highest prevalence among all other locations. Since this parasite is zoonotic, an effective rat control program should be initiated. The communities living in this area should be aware of its potential risk of zoonosis. With regards to this, the local community should practice good hygiene and better waste management to reduce the population of rodents in those areas. Vectors play a big role in transmission of *Bartonella* sp., and active vector control should also be implemented. Further studies are warranted on the transmission dynamics of *Bartonella* within the rodent population and also its potential to spill-over to humans in the area. In addition, a study of longer duration may yield other rodent host species and a wider diversity of *Bartonella* genotypes in the area specifically, and also in the country. The pathogenic and zoonotic potential of other rodent-borne *Bartonella* should also be investigated in order to fully appreciate the role of these peri-domestic mammals in the transmission and spread of emerging human diseases.

7.0 REFERENCE

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