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PREVALENCE OF *BARTONELLA* SPP. AND *RICKETTSIA* SPP. IN *CTENOCEPHALIDES FELIS* INFESTING STRAY CATS IN URBAN AREAS IN KOTA BHARU, KELANTAN.

BY:
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A RESEARCH PAPER SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF VETERINARY MEDICINE

FACULTY OF VETERINARY MEDICINE
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PREVALENCE OF *BARTONELLA* SPP. AND *RICKETTSIA* SPP. IN *CTENOCEPHALIDES FELIS* INFESTING STRAY CATS IN URBAN AREAS IN KOTA BHARU, KELANTAN.

ABSTRACT

An abstract of the research paper presented to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, in partial requirement of the course DVT 55204 – Research Project.

Cats are not only among the most popular pets but also form a significant portion of the stray animal population, relying on foraging for food and shelter. Stray cats serve as primary hosts for the cat flea, *Ctenocephalides felis*, a vector responsible for transmitting zoonotic diseases, including Cat Scratch Disease (*Bartonella henselae*) and Flea-borne Spotted Fever (*Rickettsia felis*). This study aimed to assess the prevalence of *Bartonella* spp. and *Rickettsia* spp. in the stray cat population, identify factors contributing to flea infestations, and examine the correlation between specific body regions and infestation intensity. A total of 40 stray cats were sampled from three urban locations: Pantai Cahaya Bulan, Taman Tengku Anis, and Pasar Tok Guru, using random sampling. Fleas were collected from 30 of the 40 cats that were found infested, by combing various body regions (head, dorsal, ventral, forelimbs, hindlimbs, and tail/perineum) for two minutes. DNA from the flea samples was analysed using Polymerase Chain Reaction (PCR) to detect *Bartonella* spp. and *Rickettsia* spp., with positive results confirmed by gel electrophoresis. Selected PCR products were sequenced for species identification. All 30 samples tested positive for *Bartonella* spp. (100%), while 5 samples (16.67%) showed suspected positivity for *Rickettsia* spp., though insufficient PCR product concentration hindered further confirmation. Gene sequencing identified *Bartonella henselae* and *Bartonella clarridgeiae*. Statistical analysis revealed a significant association between flea infestations and the cat's age ($p < 0.017$). Furthermore, the flea counts on the head, neck, and ears were significantly higher ($p < 0.001$) compared to the other examined regions, including the dorsal, ventral, forelimbs, hindlimbs, and tail areas. These findings offer valuable insights for improving flea control strategies in stray cats and minimizing the risk of zoonotic disease transmission to humans.

Keywords: *Ctenocephalides felis, Bartonella henselae, Bartonella clarridgeiae, Rickettsia* spp., Polymerase Chain Reaction (PCR), Stray cats.

KELAZIMAN *BARTONELLA* SPP. DAN *RICKETTSIA* SPP. DALAM *CTENOCEPHALIDES FELIS* YANG MENJANGKITI KUCING TERBIAR DI KAWASAN BANDAR DI KOTA BHARU, KELANTAN.

ABSTRAK

Abstrak daripada kertas penyelidikan yang dibentangkan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan, sebagai keperluan sebahagian daripada kursus DVT 55204 – Projek Penyelidikan.

Kucing bukan sahaja antara haiwan peliharaan yang paling popular, tetapi juga membentuk sebahagian besar populasi haiwan terbiar yang bergantung kepada pencarian makanan dan tempat perlindungan. Kucing terbiar menjadi perumah utama bagi kutu kucing, *Ctenocephalides felis*, iaitu vektor yang bertanggungjawab menyebarkan penyakit zoonotik seperti Penyakit Cakaran Kucing (*Bartonella henselae*) dan *Flea Borne Spotted Fever* (*Rickettsia felis*). Kajian ini bertujuan untuk menilai kelaziman *Bartonella* spp. dan *Rickettsia* spp. dalam populasi kucing terbiar, mengenal pasti faktor yang menyumbang kepada infestasi kutu, serta mengkaji hubungan antara kawasan tubuh tertentu dengan intensiti infestasi. Sebanyak 40 ekor kucing terbiar telah diambil sampel dari tiga lokasi bandar: Pantai Cahaya Bulan, Taman Tengku Anis, dan Pasar Tok Guru, menggunakan pensampelan mudah. Kutu dikumpulkan daripada 30 daripada 40 kucing yang didapati mempunyai infestasi, dengan menyikat pelbagai kawasan tubuh (kepala, dorsal, ventral, anggota depan, anggota belakang, dan ekor/perineum) selama dua minit. DNA daripada sampel kutu dianalisis menggunakan Reaksi Rantai Polimerase (PCR) untuk mengesan *Bartonella* spp. dan *Rickettsia* spp., dengan keputusan positif disahkan melalui elektroforesis gel. Produk PCR terpilih telah diurut untuk mengenal pasti spesies. Kesemua 30 sampel diuji positif untuk *Bartonella* spp. (100%), manakala 5 sampel (16.67%) menunjukkan keputusan positif yang disyaki untuk *Rickettsia* spp., walaupun kepekatan produk PCR yang tidak mencukupi menghalang pengesahan lanjut. Pengurutan gen mengenalpasti *Bartonella henselae* dan *Bartonella clarridgeiae.* Analisis statistik menunjukkan hubungan signifikan antara infestasi kutu dengan umur kucing ($p < 0.017$). Selain itu, jumlah kutu pada bahagian kepala, leher, dan telinga adalah jauh lebih tinggi ($p < 0.001$) berbanding dengan kawasan lain yang diperiksa, termasuk bahagian dorsal, ventral, kaki depan, kaki belakang, dan ekor. Penemuan ini memberikan maklumat penting untuk meningkatkan strategi kawalan kutu dalam kalangan kucing terbiar dan mengurangkan risiko penularan penyakit zoonotik kepada manusia.

Kata Kunci: *Ctenocephalides felis, Bartonella henselae, Bartonella clarridgeiae, Rickettsia* spp., Reaksi Rantaian Polimerase (PCR), Kucing terbiar.

CERTIFICATION

This is to certify that we have read this research paper entitled 'Prevalence of *Bartonella* spp. and *Rickettsia* spp. in *Ctenocephalides Felis* Infesting Stray Cats in Urban Areas in Kota Bharu, Kelantan.' By Izzah Binti Yusof, and in our opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirements for the course DVT 55204 – Research Project.

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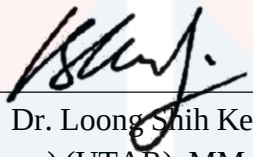

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TABLE OF CONTENT

LIST OF FIGURES

LIST OF SYMBOLS

%	Percentage
℃	Degree Celsius
µL	Microlitre
x g	Times Gravity
V	Volt

LIST OF TABLES

CHAPTER 1

INTRODUCTION

1.1 Research Background

Cats are among the most common pets worldwide. They are also one of the most populated stray animals. Stray cats are domesticated cats that have been lost, abandoned, or live without owners, relying on foraging for food and shelter. Cats serve as hosts for various pests, including the cat flea, *Ctenocephalides felis*, which is known to transmit several zoonotic diseases, such as Cat Scratch Disease (*Bartonella henselae*) and Flea-borne Spotted Fever (*Rickettsia felis*). Stray cats, in particular, often lack consistent veterinary care, leaving them vulnerable to diseases and infestations. They are therefore at a higher risk of flea infestations than domestic cats due to prolonged exposure to flea-infested environments (Gracia *et al*., 2013).

The cat flea acts as both a reservoir and a vector for various pathogens, including *Bartonella* spp. and *Rickettsia* spp., which can be transmitted to humans through bites and scratches, even when the cats themselves show no clinical symptoms. This asymptomatic nature of infections in cats can create a false sense of security, increasing the risk of human exposure (Šlapeta *et al*., 2018). Identifying the prevalence of flea-borne diseases, particularly *Bartonella* spp. and *Rickettsia* spp., in stray cats in urban areas of Malaysia is critical for public health management. Given the zoonotic potential of these diseases, understanding their prevalence in stray cat populations is essential for implementing effective control measures to protect both human and animal health. Besides, factors like age, breed, overall health, and environmental management play a role in the susceptibility of cats to flea-borne diseases (Azrizal-Wahid *et al*., 2019), aiding researchers and healthcare professionals in identifying potential disease agents.

1.2 Research Problem

Bartonella spp. and *Rickettsia* spp. are among the most common vector-borne pathogens transmitted by *C. felis*, both of which have zoonotic potential. These diseases pose a risk to humans, particularly in urban areas where people frequently come into contact with stray cats, either as pets or through direct interaction. Infected cats often do not show clinical symptoms, which may lead to a false sense of security among cat owners and the general public. This increases the risk of human exposure to infected fleas. There is a lack of awareness regarding the potential risks associated with *Bartonella* spp. and *Rickettsia* spp. infection, which may result in delayed diagnosis and treatment. Stray cats, common in urban environments, act as reservoirs for these pathogens, thereby heightening the risk of human exposure. Factors such as overcrowding, poor sanitation, and limited access to veterinary care contribute to the spread of these pathogens. However, the extent of the risk and the prevalence of these pathogens in flea populations infesting stray cats remain underexplored in Kelantan. This study aims to assess the occurrence of *Bartonella* spp. and *Rickettsia* spp. among stray cats infested with *C. felis* in the urban areas of Kota Bharu, Kelantan to better understand the public health risks.

1.3 Research Questions

- What is the intensity of flea infestations in stray cats in Kota Bharu, Kelantan?
- What is the prevalence of *Bartonella* spp. and *Rickettsia* spp. in *C. felis* infesting stray cats in urban communities in Kota Bharu, Kelantan?
- What are the risk factors contributing to flea infestations among stray cats in urban communities in Kota Bharu, Kelantan?

1.4 Research Hypothesis

- Stray cats in Kota Bharu, Kelantan, exhibit a high intensity of flea infestations.
- It is hypothesized that the prevalence of *Bartonella* spp. among stray cats infested with cat fleas will be high, while the prevalence of *Rickettsia* spp. is expected to be low.
- The risk factors contributing to flea infestations in stray cats include age, body condition, hair length, and weight.

1.5 Objectives

- To assess the intensity of flea infestations in stray cats in Kota Bharu, Kelantan.
- To determine the prevalence of *Bartonella* spp. and *Rickettsia* spp. in *C. felis* fleas collected from stray cats in urban communities in Kota Bharu, Kelantan.
- To identify the risk factors (age, body condition, hair length, and weight) that contribute to flea infestations among stray cats in urban communities in Kota Bharu, Kelantan.

CHAPTER 2

LITERATURE REVIEW

2.1 Cat Population in Urban Area

Cats have received a reputation of being one of the world's invasive species due to their relations with the human population and this is believed due to human's preference for pets to be cats (Duffy & Capece, 2012). Therefore, the population of cats either stray or pets are linked with human populations (Ferreira *et al*., 2011). Stray cats, needing to scavenge for food, are frequently found around restaurants, alleyways, streets, and even residential areas. Areas with high food availability can lead to an increase in the stray cat population (Legge *et al*., 2017). The worldwide feral cat population is estimated at least 100 million, including about 60 million in the United States (Roebling *et al*., 2014). In Malaysia, cats are one of the most popular terrestrial animals chosen as a pet and the ownership of cats was document at 1,000,000 in 2022 (Mordor Intelligence, 2022). However, pets that are not adequately kept can easily become part of the stray animal population. Factors such as owners being unable to manage large numbers of pets, ineffective housing management leading to escapes, irresponsible breeding, and poor pet care all contribute to the rapid increase in feral and stray animal populations.

2.2 Common Ectoparasites in Cats

Based on a study conducted by Mohd Zain *et al*., 2013, the 5 common parasites that were recovered in stray cat populations from urban cities in Peninsular Malaysia included *C. felis, Felicola subrostratus, Haemaphysalis bispinosa, Heterodoxus spiniger* and *Lynxacarus radovskyi.*

In addition, a study on the prevalence of ectoparasites on a stray cat population from Kota Samarahan, Sarawak, Malaysia by Kamaruddin *et al*., 2020 examined a total of 150 cats for ectoparasites. Among these, 113 individuals (75.3%) of the stray cats were infested by at least one species of ectoparasites. There were nine species of ectoparasites belonging to four groups (louse, flea, mite, and tick). Louse known as *F. subrostratus*, (44.7%) was the most frequent species of ectoparasite infesting the hosts in this area, followed by flea species, namely, *C. felis* (18.7%) and *Ctenocephalides felis orientis* (16.0%). *Lynxacarus radovskyi* (24.0%), *Otodectes cynotis* (0.7%), *Haemaphysalis* sp. 1 (0.7%) and *Haemaphysalis* sp. 2 (0.7%).

2.3 Fleas

2.3.1 Host Range, Prevalence and Risk Factors of Fleas Infestation

The cat flea, scientifically known as *C. felis*, is a blood-sucking ectoparasite that causes significant discomfort for pets and stray cats globally (Rust, 2005). It is the most common flea species found on both domestic cats and dogs. While each flea species has a preferred host, they are not exclusive to one type of animal and can infest a variety of mammals. (Clark *et al*., 2018).

The cat flea is a wingless, laterally compressed insect measuring around 2 mm in length. These fleas are reddish-brown to black, with females typically being slightly larger and differently coloured than males. Aside from size and colour, males are distinguished by their complex, snail-shaped genitalia. Cat fleas can be identified by their distinctive combs, or ctenidia; they possess both a pronotal ctenidium and a genal ctenidium with more than five teeth. Cat fleas have a characteristic sloping forehead and lack an outer apical tooth on the hind tibia, setting them apart from other flea species (Rothschild & Traub, 2024).

2.3.2 Risk Factors Associated with Flea Infestation on Cat

According to a few studies, there are various factors that influence flea infestation in stray cats, including age, sex, health status, body condition, and hair length. Cats that are very young or old are particularly vulnerable due to their weaker immune systems. Male cats, known for their aggressive and territorial behaviour, may have a higher risk of flea infestation. Poor health or compromised immune systems also increase the likelihood of flea problems. Furthermore, cats with poor body condition, often due to insufficient nutrition or chronic illnesses, are less susceptible to fleas. Hair length plays a crucial role as well; long-haired cats offer a more suitable environment for fleas to hide and reproduce. Recognizing these risk factors is important for effectively managing and tackling flea infestations in stray cat populations (Azrizal-Wahid, *et al*., 2019).

2.4 Common Flea Borne Pathogens

Cat fleas are recognized for hosting a range of bacteria, including *Rickettsia* spp., *Bartonella* spp., and *Mycoplasma* spp. (Abdullah, *et al*., 2019). Moreover, illnesses such as rickettsioses, attributed to *Rickettsia* spp., and Bartonellosis, caused by *Bartonella* spp., are vector-borne diseases with extensive global distribution. This widespread dissemination is facilitated by the fleas, which possess a cosmopolitan presence and exhibit a broad spectrum of host preferences (Lawrence, *et al*., 2019).

2.4.1 Background of *Bartonella* spp.

Bartonella sp. is a fastidious, facultative intracellular, hemotropic gram-negative bacterium that has a distribution with the highest prevalence in areas where condition is most favourable

for arthropod vectors (Breitschwerdt, *et al*., 2010). Infected cats are thought to very rarely exhibit clinical signs, and at least thirteen *Bartonella* species or subspecies have been recognized as agents of human disease, three species are reportedly responsible for the majority of clinical illness: *Bartonella bacilliformis*, *Bartonella quintana* and *Bartonella henselae* (Lamas, *et al*., 2008). Infected immunocompromised humans will exhibit clinical indications such as pustules on the skin and lymphadenopathy at the site of inoculation, whereby the affected lymph nodes become swollen and painful, muscular aches, low-grade fever (Cunningham, *et al*., 2000).

2.4.2 Background of *Rickettsia* spp.

Rickettsia felis is the agent of flea-borne spotted fever, an obligate intracellular gram-negative bacterium (Blanton, *et al*., 2015). The main arthropod reservoir and vector is the cat flea, *C. felis*. The bacterium can be spread when the flea feeds on the host's blood or when the flea bite wound is contaminated with flea faeces left on the skin (Reif, *et al*., 2011). Infected cats tend to be asymptomatic however it causes manifestation of clinical signs in the infected human, such as fever, fatigue, headache, maculopapular rash, and eschar (Schriefer, *et al*., 1994, Richter, *et al*., 2002).

2.5 Transmission of *Bartonella* spp. and *Rickettsia* spp.

Fleas are important ectoparasites vectoring several pathogens which have been considered veterinary problems (Hamzaoui *et al*., 2020; Lappin, 2018; Oguntomole *et al*., 2018). The domestic cat is the major reservoir for three zoonotic *Bartonella* spp., namely *B. henselae, Bartonella clarridgeiae and Bartonella koehlerae* and *C. felis* are the primary vectors of feline *Bartonella* spp. *C. felis* has been proven to serve as both the principal vector and the reservoir of *R. felis* in the environment. *R. felis* has been identified in both male and female cat flea reproductive tissue, including the ovaries and testicular epithelial sheath, suggesting strong evidence for this process. *R. felis* was first identified to transmit transovarially, with *R. felis* found in freshly deposited cat flea eggs, followed by *R. felis* in newly emerging unfed adult cat fleas, suggesting transstadial transmission. Cats can become infected with *Bartonella* spp. and *Rickettsia* spp. by the bites of infected fleas or contact with contaminated blood, such as during cat fights.

2.6 Prevalence of Bartonellosis and Rickettsiosis

A cross-sectional study was conducted to investigate the prevalence of Bartonellosis in cats at the University Veterinary Hospital, Selangor, Malaysia. *Bartonella* spp. was discovered in 48 (16.9%) of the 284 cats examined using Polymerase Chain Reaction (PCR) (Hassan, *et al*., 2017). Based on a study by Tay, *et al*. (2014), out of 177 *C. felis* fleas collected, 56 (32.2%) were found to be positive for *R. felis.* Sequence analysis of the gltA amplicons revealed two genotypes of *R. felis* (Rf31 and RF2125) in the fleas.

2.7 Public Health Concern

The most frequent pathogenic *Bartonella* spp. for humans include the *B. bacilliformis*, *B. henselae* and *B. quintana*. Infected animals are frequently asymptomatic and may act as a reservoir for infection in a more general population, resulting in vector transmission and zoonotic diseases with these bacteria (Gomes & Ruiz, 2018). Infected individuals frequently have milder clinical indications such as swollen lymph nodes, fever, and papules at the inoculation site, which are treatable if diagnosed early (Zangwill, 2021). Unfortunately, for immunocompromised persons due to HIV/AIDS or transplant patients, this infection could be fatal since it allows opportunistic diseases to cause further immune system damage and severe clinical symptoms such as in a more disseminated form with hepatosplenomegaly or meningoencephalitis, or with bacillary angiomatosis (Lamas, *et al*., 2010).

Rickettsial diseases are considered as neglected and overlooked by physicians due to the clinical presentation resembling any other tropical diseases therefore the clinical diagnosis is difficult which leads to known prevalence being underreported (Newton & Guerin, 2020). Furthermore, considering fleas feed often and are mobile, flea-borne *R. felis* can spread quickly to human populations. Close contact with domesticated companion animals, such as dogs and cats, and their ectoparasites, can increase the risk of contracting rickettsial illnesses (Colella, *et al*., 2020).

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Study Area

The study area involved random collection in three locations in the urban area of Kota Bharu, namely: residential area, wet market area and recreational area. First location is community park Taman Tengku Anis (6° 8' 35.88" N, 102° 15' 5.04" E) followed by wet market at Pasar Tok Guru Cabang Tiga Pengkalan Chepa (6° 9' 37.44" N, 102° 16' 37.92" E) and lastly hawker spot in Pantai Cahaya Bulan (6° 12' 0.36" N, 102° 16' 1.92" E). These three locations are chosen as they are surrounded by a high number of stray cats which possess the risk of zoonotic disease to the public.

3.2 Study Design

This study used a cross-sectional study design in which collected samples will be processed and analysed for the presence or absence of *Bartonella* spp. and *Rickettsia* spp. from three separate places at one point in time.

3.3 Study Population

Based on the sample size calculation by Ariffin (2024), with assumption of proportion 71.83% of stray cats are infested with cat fleas, as reported in a study by Azrizal-Wahid *et al*. (2019) conducted across four distinct regions in Peninsular Malaysia. Considering an expected prevalence (P_{exp}) of 50%, a 95% confidence level, and a desired precision of 5%, a minimum of 317 cats were required for the study. The minimum sample size helps ensure that the sample is representative of the population being studied, reducing bias and increasing the external validity of the study. However, due to the constraint of time, the number is reduced to a total of 40 stray cats will be chosen at random from three different areas throughout Kota Bharu.

3.4 Selection Criteria

3.4.1 Inclusion Criteria

The inclusion criteria for this study are stray cats which will be randomly selected with no bias concerning age, breed, coat length, body condition and health status.

3.4.2 Exclusion Criteria

Pets and aggressive stray cats are excluded from this study.

3.5 Sample Collection and Physical Examination

The fleas were collected from stray cats in Kota Bharu, Kelantan to detect the presence of flea-borne disease. A random sampling method was used based on manageable handling. Information regarding the ages, body condition, hair length and weight was recorded. With respect to age, the stray cats were categorised into juvenile (less than 6 months) and adults (more than 6 months old) based on their dentition. Physical examination was performed and abnormal findings were recorded.

At first, each of the stray cat was handled with gloved hands and appropriately restrained by either in lateral or sternal position. To restrain a stray cat in a lateral position, the cat was placed in a sternal or standing position, then the cat was grabbed at the scruff of the neck firmly and the other hand was slid under the cat's rear legs, gripping above the hocks and maintaining fingers between the cat's legs. The cat was gently stretched out and held against the ground. To restrain the stray cat in a sternal position, one hand was placed on the cat's shoulder and the other on the hindquarters to provide moderate control without applying too much pressure. Once the cat is in a sternal position, support its body to ensure its stability and comfort. The cat's breathing and overall demeanour while restrained must be observed attentively. The restraint must be released immediately and allow the cat to walk freely once it showed signs of distress or difficulty in breathing.

3.6 Flea Collection and Identification

Fleas were collected using a dampened flea comb, particularly on the areas of the body most likely to harbour fleas, which were divided into five regions based on Figure 1,

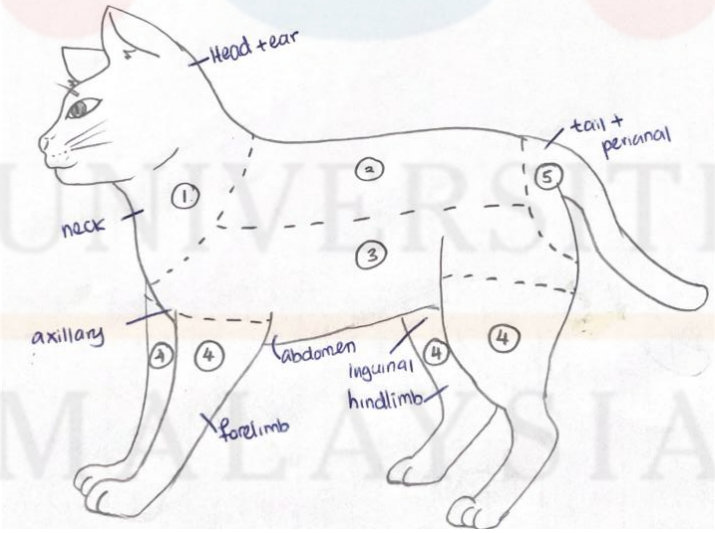


Figure 1: Collection of fleas from specific body areas on a stray cat. Region 1 – head, neck & ear, region 2 – dorsal, region 3 – ventral, region 4 – forelimb and hindlimb, region 5 – tail & perineum.

The procedure of collecting fleas on the stray cat would take roughly 1 minute, with each part being timed and checked for 15 seconds. At the end of the grooming process, the fleas were placed inside the hard glass container filled with 90% ethanol and closed with a tightly fitting

lid. The flea samples were stored in a freezer at -20°C. The flea was brought to the Parasitology Laboratory, Faculty of Veterinary Medicine, UMK to be examined under a compound microscope to determine the species and sex of each flea collected.

C. felis is identified by its distinct morphological features. Adults are small, measuring 1-3 mm in length, with a laterally compressed, wingless body adapted for movement through the host's fur. They exhibit a reddish-brown to dark brown coloration. Key identifying characteristics include a rounded head with an elongated first segment of the antenna, comb-like structures (ctenidia) on the head and thorax, and strong, well-developed hind legs for jumping. The presence of a genal comb with evenly spaced spines and a pronotal comb on the thorax further differentiates *C. felis* from other flea species (Lawrence, *et al*., 2015).

Male and female *C. felis* can be differentiated through distinct morphological traits. Males are typically smaller, while females are slightly larger, although both share a similar reddish-brown to black coloration. The abdomen shape varies between the sexes, with males having a narrower, more tapered abdomen and females displaying a larger, rounded abdomen suited for egg production. Additionally, their reproductive structures differ: males are equipped with claspers at the tip of the abdomen, whereas females have visible spermatheca.

Table 1: Comparison in differentiating male and female *C. felis*.

	Male	Female
Size	Generally smaller	Slightly larger
Colour	Reddish brown to black	
Abdomen shape	Narrower, more tapered abdomen.	Larger, more rounded abdomen which accommodates egg production.
Reproductive organs	Presence of claspers at the tip of the abdomen	Visible spermatheca

Source: Sanches, *et al*., 2024.

3.7 Flea Occurrence, Risk Factors and Flea Infestation Intensity

The occurrence of flea infestation was assessed based on the number of flea-infested stray cats, with age, sex, health state, body condition and hair length being considered risk factors. The average level of flea infestation intensity in the study were calculated according to the formula of Krasnov *et al*. (2002), to determine the flea intensity and flea prevalence: flea intensity = (total number of fleas / total number of infested cats); flea prevalence = (number of infested cats / total number of sampled cats) ×100.

The severity of flea infestation was categorized based on the number of fleas present on the cat's body and the estimated blood consumption per day. Based on a study conducted by Blakely *et al*. (2023), the blood consumption was calculated using the average volume of blood consumed by a single flea, approximately 5.7 µL every 48 hours (equivalent to 2.85 µL/day). Female cat fleas consumed an average of 13.6 µL of blood per day amounting to approximately

15.15 times their body weight (Dryden *et al.*, 1991). The total blood loss was calculated by multiplying the number of fleas by their daily blood consumption, which enabled the classification into three severity levels: mild, moderate, and severe.

Table 2: Categorization of cat flea infestation severity based on the number of fleas on the cat's body and the estimated blood consumption per day (µL).

Number of fleas	Severity	Estimation blood consumption per day (µL)
1-100	Mild	2.85-285.0
101-219	Moderate	287.85-624.15
>220	Severe	>627.0

Source: Greenfieldvets, 2015.

3.8 DNA Extraction, Polymerase Chain Reaction (PCR), Gel Electrophoresis and Genus Identification for *Bartonella* spp. and *Rickettsia* spp.

DNA extraction was performed by using the NucleoSpin® Tissue DNA isolation kit (Macherey-Nagel, Düren, Germany) following the instructed procedures by the manufacturer. Firstly, six fleas were transferred into microcentrifuge tube and homogenise using homogenizer followed by adding 50-75µL phosphate buffered saline (PBS) and homogenise. In the pre-lyse sample step, 180 µL Buffer T1 and 25 µL Proteinase K solution will be added and a vortex to mix and the sample was incubated at 56 °C overnight. Next, the samples were mixed by a vortex and 200 µL Buffer B3 was added, mixed by the vortex vigorously and incubate at 70 °C for 10 min followed by a brief vortex. For the adjusted DNA binding conditions, 210 µL ethanol (96–100 %) was then added into the sample and vortex vigorously. For each sample, one NucleoSpin® Tissue Column was placed into a Collection Tube. Centrifuge for 1 min at 11,000 x g.

The flow through was discarded and placed the column back into the Collection Tube. Next was washing the silica membrane in 2 washes. For the first wash, 500 µL Buffer BW was added. Centrifuged for 1 min at 11,000 x g. The flow-through was discarded and placed back into the Collection Tube.

This was followed by the second wash which is 600 µL Buffer B5 that was added into the column and centrifuge for 1 min at 11,000 x g. The flow-through was discarded and placed back into the Collection Tube. Next step was the silica membrane was dried by centrifuged the column for 1 min at 11,000 x g and residual ethanol is removed during this step. Lastly eluted the highly pure DNA by NucleoSpin® Tissue column placed into a 1.5 mL microcentrifuge tube and 50 µL prewarmed Buffer BE (70°C) was added. Incubated at room temperature for 1 min followed by centrifuged 1 min at 11,000 x g.

The primers were used for amplification of the *gltA* gene (379 bp) of *Bartonella* spp. DNA detection is BhCS.781p (5-GGGGACCAGCTCATGGTGG-3) and BhCS.1137n (5-AATGCA AAAAGAACAGTAAACA-3) as forward and reverse primers, respectively, as described by

(Pangjai *et al*., 2022). The positive control that was used for *Bartonella* spp. was *Bartonella henselae* strain Houston-1 (ATCC 49882).

A Polymerase Chain Reaction (PCR) assay was performed to detect the presence of *Bartonella* spp. DNA in the extracted flea DNA samples using the following PCR cycling conditions: initial denaturation at 95°C for 2 minutes, followed by 45 cycles of denaturation at 95°C for 1 minute, annealing at 60°C for 1 minute, extension at 72°C for 30 seconds and a final extension at 72°C for 5 minutes (Jensen, *et al*., 2000).

The primers used for PCR amplification for the *gltA* gene (834 bp) *Rickettsia* spp. DNA detection was, CS-239 5'GCTCTTCTCATCCTATGGCTATTAT'3 with position on gene relative to the open reading frame 239 to 263 for forward primer and CS-1069 5'CAGGGTCTTCGTGCATTTCTT'3 with the reading frame of 1069 to 1049 as reverse primer (Labruna, M.B. *et al*., 2007). The positive control that will be used was cultured *Rickettsia raoultii.*

A Polymerase Chain Reaction (PCR) assay was performed to detect the presence of *Rickettsia* spp. DNA in the extracted flea DNA samples using the following PCR cycling conditions: initial denaturation at 93°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 48°C for 30 seconds, extension at 72°C for 30 seconds and a final extension at 72°C for 7 minutes (Eremeeva, M., *et al*., 2003).

The PCR products were separated by gel electrophoresis in a 1.5% agarose gel at 100 V for 40 minutes. Separated DNA fragments were visualised using Gel Doc EZ Imager.

The identification of the species *Bartonella* spp and *Rickettsia* spp. was determined through molecular sequencing techniques. Specifically, DNA was extracted from *C. felis*, and a targeted gene region, such as *gltA* gene, was amplified using polymerase chain reaction (PCR). The resulting sequences were compared against reference databases, such as NCBI BLAST® to confirm the species identity with high accuracy.

3.9 Data Analysis

Chi-Square test was conducted to determine the relationship between various risk factors and flea infestation, considering a *p*-value <0.05 as statistically significant. In addition, Chi-square test was to determine the association between body sections and fleas' infestation intensity. Several risk factors were assessed for their potential link to flea infestations in stray cats. A total of 40 individual cats from three different locations were studied, and the table below presents the results of the association between these risk factors (gender, age, hair length, and body hygiene) and flea infestation, using the Chi-square test. Furthermore, a one-way ANOVA was conducted to compare for differentiation of flea infestation intensity across the five different body sections of the stray cats.

3.10 Ethical Consideration

Research project received approval code from the FPV Animal Ethics Committee: UMK/FPV/ACUE/FYP/019/2024.

CHAPTER 4

RESULTS

4.1 Occurrence of *Bartonella* spp. and *Rickettsia* pp., Flea Infestation Intensity and Affected Body Sections of Stray Cats

A total of 202 fleas were collected from 30 out of 40 stray cats that were found infested with fleas, sampled from three distinct areas: Pantai Cahaya Bulan, Pasar Tok Guru, and Taman Tengku Anis. The overall mean intensity of flea infestation was 6.73 fleas per cat. The flea species *C. felis* was found to be overwhelmingly predominant, identified on the infested cat (202/202; 100%).

In terms of flea developmental stages, adults accounted for the majority (202/202; 100%) highlighting a predominantly mature flea population and potentially indicating recent infestations. Additionally, two of the 30 stray cats were co-infested with the louse species *F. subrostratus*.

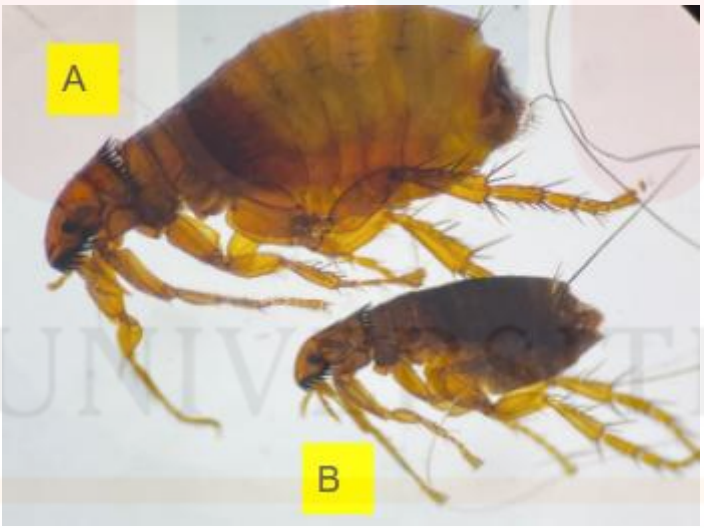


Figure 2: (A) Female and (B) male *C. felis* was collected from the stray cats

Figure 3: *F. subratus* was found from two of the stray cats

The study found that *Bartonella* spp. was significantly more prevalent in fleas infested stray cats, with an occurrence rate of 100% (30/30 cats positive for *Bartonella* spp.). Flea samples from all three locations tested positive for *Bartonella* spp., suggesting a widespread presence of this pathogen across the areas. In contrast, *Rickettsia* spp. was found in a much smaller proportion of fleas, with an occurrence rate of 23.33% (7/30 cats). Pantai Cahaya Bulan had four cases of *Rickettsia* spp., while Taman Tengku Anis had three cases. Co-infection was also detected amongst seven sampled cats which were positive for both *Bartonella* spp. and *Rickettsia* spp. These findings suggest that *Bartonella* spp. was more commonly associated with flea infestations in stray cats, while *Rickettsia* spp. occurs less frequently.

Table 3: Occurrence of *Bartonella* spp and *Rickettsia* spp. in fleas' infestation on stray cats in urban areas in Kelantan

Location	Prevalence of flea infestation (%)	Average intensity of cat flea	Number of fleas collected (n)	Number of cats sampled	Detection of		Occurrence rate (%)	
					Bartonella spp.	*Rickettsia* spp.	*Bartonella* spp.	*Rickettsia* spp.
Pantai Cahaya Bulan (A)	55.55	4.05	73	18	10/10	4/10	33.33	13.33

Taman Tengku Anis (B)	100.0	5.9	59	10	10/10	3/10	33.33	10.0
Pasar Tok Guru (C)	83.33	5.83	70	12	10/10	0	33.33	0
Total	238.88	15.78	202	40	30	7	75.0	17.5

The analysis revealed a significant association between age and flea infestation, with a *p*-value of 0.017. This suggests that flea infestations are more prevalent in juvenile cats (under 6 months old) compared to adult cats (over 6 months old) in the stray cat population. Furthermore, the Chi-square test showed no significant relationship between flea infestation and gender (*p*-value = 0.090), hair length (*p*-value = 0.126), or body hygiene status (*p*-value = 0.830).

Table 4: The Chi-square test to determine the association of risk factors with flea infestation in this study

Risk factor	Variable	Cats with fleas	Cats without fleas	Chi-Square	*p*-value	Significance
Gender	Male	9	6	2.880	.090	No
	Female	21	4			
Age	Juvenile (less than 6 months old)	12	0	5.714	.017	Yes
	Adult (more than 6 months old)	18	10			
Hair length	Short, <10mm	25	6	2.342	.126	
	Medium, 10-20 mm	5	4			

Body hygiene state	Clean	22	7	0.0417	.830	No
	Dirty	8	3			

A Chi-square test was done to determine the association of the body section with fleas' infestation as shown in table 3. The Chi-square test of independence showed that there was a significant association between all five body regions with a *p*-value of <0.001. Thus, different body parts of the cats have a significant association with the flea infestation intensity.

Table 5: Association between flea intensity and body parts in cats

Body section	With fleas	Without fleas	Chi-Square	*p*-value	Significance
1 (Head, ear and neck)	26	4	88.174	<.001	Yes
2 (Dorsal region)	21	9			
3 (Ventral region)	14	16			
4 (Forelimb and hindlimb)	7	23			
5 (Tail and perianal region)	13	17			

One-way ANOVA was carried out to assess the variation in flea infestation intensity across different body sections of stray cats. The analysis indicated a statistically significant difference in flea infestation levels among the body sections, as evidenced by a *p*-value of <0.001, suggesting that the location on the body plays a crucial role in determining flea intensity. Among the examined body sections, the head, ear and neck region stood out as the most significantly affected area, indicating that flea's infestation is concentrated in this region.

Based on the post hoc results, the head, ear, and neck region have the highest flea infestation. The dorsal, ventral, forelimb and hindlimb and tail and perineum regions show similar flea intensities, with no significant differences among them.

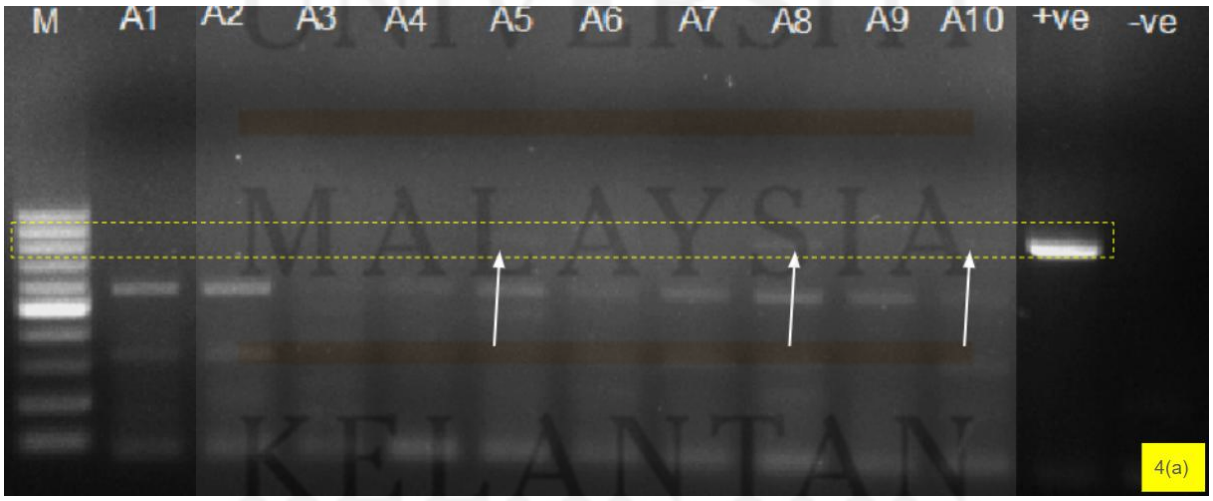
Table 6: ANOVA test to determine the association of body sections of stray cats with flea infestation

Body Sections	Post Hoc (Mean ± SE)
1 (Head, Ear and Neck)	$3.90 \pm 2.36_a$
2 (Dorsal)	$1.20 \pm 1.62_b$
3 (Ventral)	$0.60 \pm 0.93_b$
4 (Forelimb and hindlimbs)	$0.37 \pm 0.80_b$
5 (Tail and perineum)	$0.67 \pm 1.97_b$

Means in the Post Hoc column sharing the same letters are not significantly different at a significance level of $p < 0.05$.

4.2 Identification of *Rickettsia* spp.

Figure 4(a) and 4 (b) showed samples from A and B group for detection of *Rickettsia* spp.,



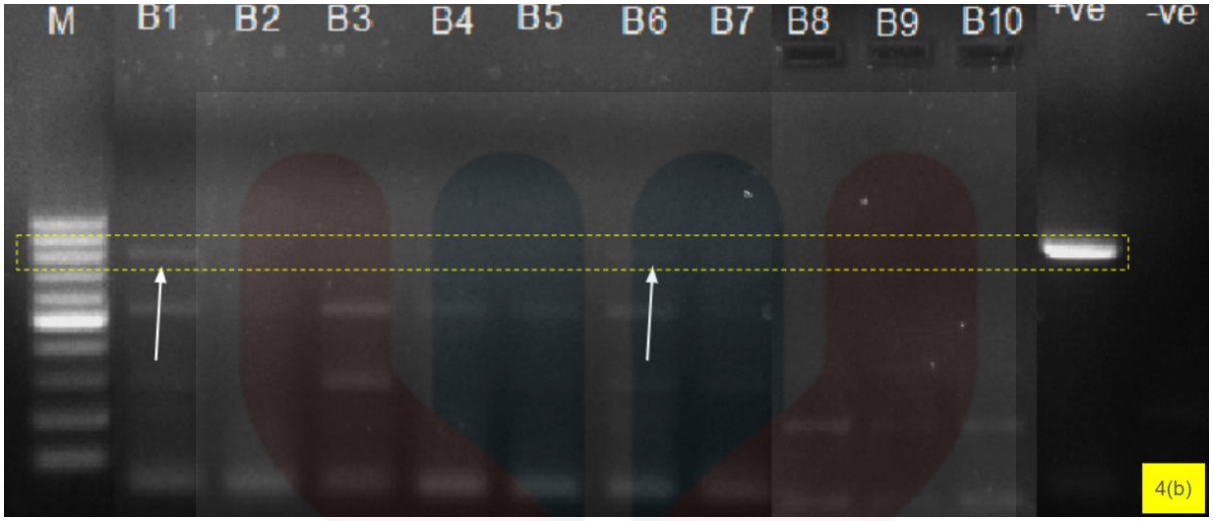
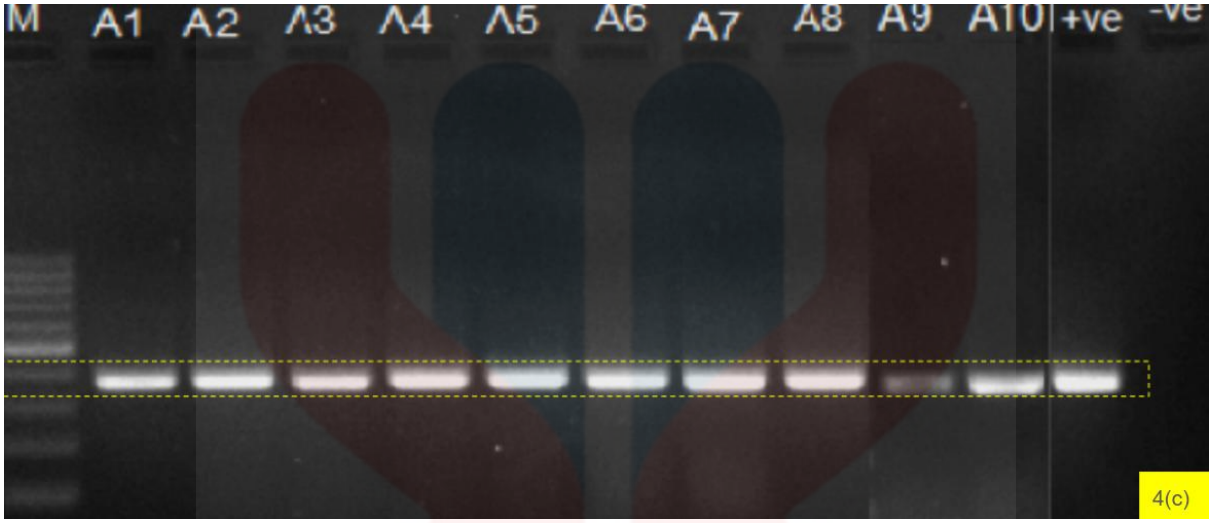


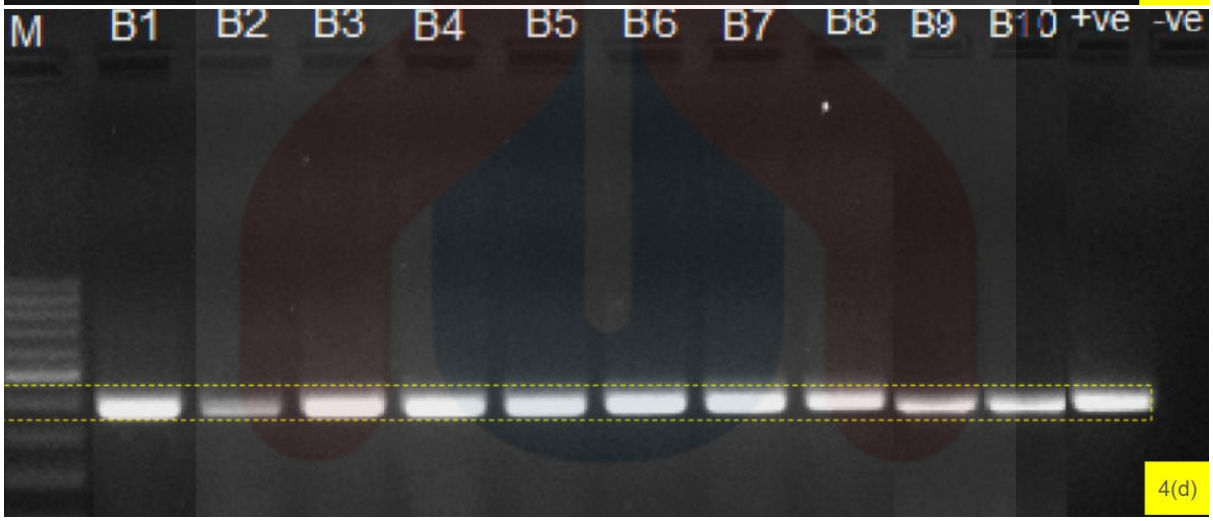
Figure 4(a) & 4(b): Agarose gel electrophoresis of the PCR products. M: DNA ladder marker, A1-A10 samples from Pantai Cahaya Bulan, B1-B10 from Taman Tengku Anis; +ve: positive controls; -ve: negative control; arrows showing the suspected samples containing *Rickettsia* spp. genome, which are A5, A8, A10, B1, B6

The figure shows that all samples exhibited multiple smearing bands between 100 and 600 bp. However, in comparison to the positive controls, *Rickettsia* spp. (834 bp) was only detected in the samples from cats A5, A8, A10, B1, and B6, resulting in a positive detection rate of 17% (5 out of 30) of the flea samples. Notably, all samples from the C group tested negative for *Rickettsia* spp.

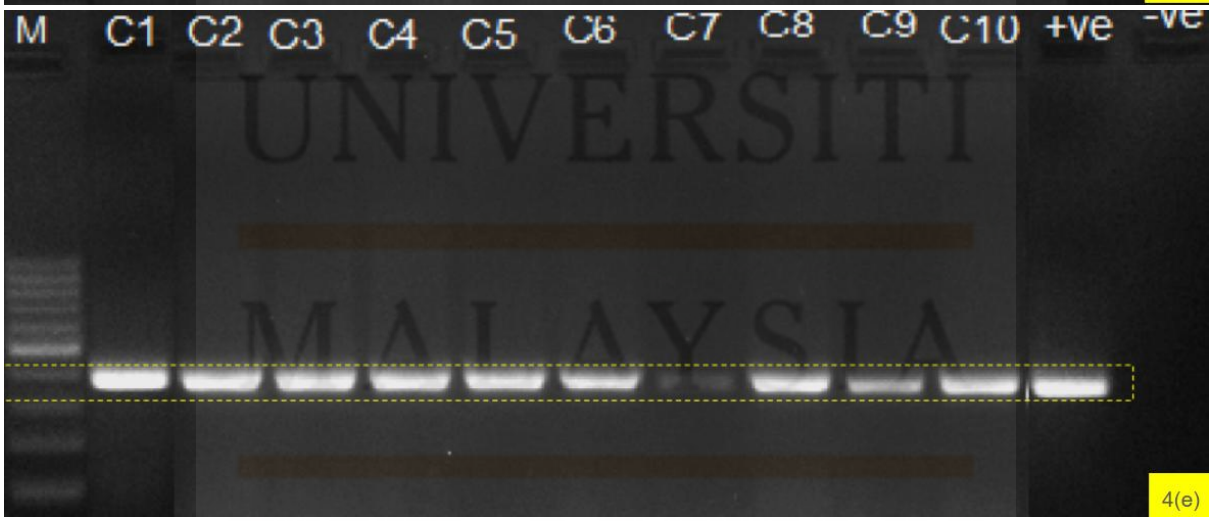
4.3 Identification of *Bartonella* spp.



4(c)



4(d)



4(e)

Figure 4(c), 4(d) & 4(e): Agarose gel electrophoresis of the PCR products. M: DNA ladder marker, A1-A10 samples from Pantai Cahaya Bulan, B1-B10 from Taman Tengku Anis; C1-C10 samples from Pasar Tok guru; +ve: positive controls; -ve: negative control; all well showing the suspected samples positive of *Bartonella* spp. genome

The gel electrophoresis results from the 30 samples for the detection of *Bartonella* spp. using universal primers showed distinct bands indicating the presence of the target DNA. Out of the 30 samples, several displayed a clear amplicon at the expected size (379 bp), confirming the presence of *Bartonella* spp. DNA. The positive samples exhibited bands of consistent intensity, corresponding to the expected molecular weight, which aligns with the universal primer's binding region for *Bartonella* spp. In contrast, there are two samples that showed slight faint bands which are A9 and C7. Gene sequencing was conducted on samples A1, A5, A8, A10, B1, B6 and C10.

Gene sequencing revealed that there are 3 samples namely A1, A10 and C10 were positive for *B. henselae* and A5, A8, B1 and B6 were positive for *B. clarridgeiae.*

Table 7: Gene sequencing of samples A1, A5, A8, A10, B1, B6 and C10.

Samples ID	**Gene sequencing result**
A1	*Bartonella henselae*
A5	*Bartonella clarridgeiae*
A8	*Bartonella clarridgeiae*
A10	*Bartonella henselae*
B1	*Bartonella clarridgeiae*
B6	*Bartonella clarridgeiae*
C10	*Bartonella henselae*

4.4 Discussion

The study revealed the occurrence and severity of flea infestations on stray cats in urban areas of Kota Bharu, Kelantan. A total of 40 stray cats were carefully inspected for flea infestations. The findings showed that 75% of the stray cats were affected by fleas. Fleas are widely distributed and serve as vectors for several significant zoonotic diseases, including *Bartonella* spp. and *Rickettsia* spp. (Mokhtar & Tay, 2011). Azrizal Wahid *et al*. (2019) reported a prevalence rate of 72.71% for cats in Peninsular Malaysia, with variations potentially influenced by factors such as geographic location and urban density. For instance, our study

was conducted in Kota Bharu, a densely populated urban area with a high number of stray cats, which may explain the observed prevalence.

Moreover, the presence of co-infection of *Bartonella* spp. and *Rickettsia* spp. is suggesting a mixed ectoparasite burden in a subset of the population. Since fleas are blood-feeding parasites, they can acquire various bacterial strains by intermittently feeding on multiple infected hosts. This process may lead to co-infections with different bacteria types, highlighting the need for further research into the health and living environments of stray cat populations to understand these dynamics more thoroughly (Azad, *et al* 1997).

Furthermore, flea infestation rates tend to be influenced by the climate conditions, with tropical regions providing an ideal environment for flea survival and reproduction (Wong *et al*., 2009). These factors may explain why flea prevalence in Southeast Asia tends to be higher than in temperate regions and this is evident by international studies, such as those that were conducted in Thailand and Indonesia, report similar flea infestation rates, suggesting a broader trend in Southeast Asia (El-Seify *et al*., 2016).

The average flea infestation intensity was 6.73 fleas per cat, indicating a mild level of infestation within the stray cat population. Compared to other studies, this intensity falls within the mild range, as infestations with fewer than 100 fleas per cat are typically considered low, while those exceeding 220 fleas are classified as high (Greenfieldvets, 2015). In cats, persistent flea exposure may lead to serious conditions such as anaemia and skin problems, as repeated infestations can cause an allergic reaction to flea saliva, resulting in flea allergic dermatitis (Newbury & Moriello, 2006). Additionally, healthy cats may act as subclinical carriers of the bacterial infections (Álvarez-Fernández, *et al*., 2018). These infections are transmitted through bites from infected fleas or exposure to contaminated blood, with cats that engage in fights or receive blood transfusions being particularly vulnerable to infection.

This study found a higher prevalence of fleas in female cats compared to male cats; however, the difference was not statistically significant, suggesting that gender may not be a critical factor in flea infestation. This observed trend may be influenced by the characteristics of the population sampled, particularly the gender distribution. Supporting this context, a study by Mohd Zain *et al*. (2013) also reported a predominance of female cats in their sample population from selected regions in Peninsular Malaysia. The higher number of females in such studies might reflect sampling biases or local demographic trends within cat populations, which could inadvertently influence the observed prevalence of flea infestations.

Out of 40 stray cats, a higher proportion of adult cats (18/40; 45%) were infested with fleas compared to younger cats (12/40; 30%). The age of the cats had a notable impact on flea infestation with all juvenile cats being infested (12/12; 100%), whereas some adult cats showed no signs of fleas (10/28; 35.71%). Despite this, there was a significant association between age and flea infestation in this study which align with studies conducted in Thailand (Jittapalapong *et al*., 2008).

The higher infestation rate in young cats could be due to several factors such as limited grooming ability. Juvenile cats, especially those that are still growing, often lack the fine motor coordination and strength required for effective self-grooming, which is essential in reducing flea infestation (Salant *et al*., 2013). Without the ability to groom thoroughly, young cats are more likely to harbour fleas compared to adults who are generally better at reducing their infestations through regular grooming. Additionally, young cats may have immature immune systems, making them more susceptible to flea infestations as their bodies may not respond as effectively to flea bites or the pathogens they carry (Moriello, 2006). In addition, maternal transmission of fleas could also play a role, as young kittens are often in close contact with their mothers, who may be carrying fleas and this close proximity increases the likelihood of flea transfer, especially during the early stages of life (Jittapalapong *et al*., 2008). Another contributing factor is environmental exposure as kittens often have more limited mobility and are more likely to remain in high-risk areas, where flea populations are dense thus increasing their chances of flea exposure (Beugnet & Marie, 2009).

In terms of body hygiene as a risk factor for flea infestation, no significant association was found in this study. Moreover, cats categorized as "clean" showed a higher number of flea infestations compared to "dirty" cats. However, this difference was not significant (*p*-value = 0.830), suggesting that the cleanliness of a cat's body does not influence the likelihood of flea infestation. This finding indicates that other factors, such as environmental conditions or exposure to infested animals, may play a more significant role in determining flea prevalence. This also applied to the risk factor of hair length in which there was no significant association (*p*-value= 0.126) and it suggests that hair length may not be a determining factor for flea infestation, and other variables, such as environmental exposure or grooming habits, may have a greater impact on flea prevalence.

A significant association was found among the five body sections, with the head, ear, and neck identified as the most favoured sites for flea infestation on stray cats. In a study by Meng *et al*. (2002), the mean number of fleas was significantly higher on the head and neck region compared to other body sections. The head and neck are particularly challenging to groom through oral grooming, leading to more flea-related problems in these areas. While scratching with the hind claws may help remove fleas from the head and neck, it is less effective due to the significantly shorter duration (only 1/50th of the time spent on oral grooming), making it insufficient for thorough grooming (Eckstein & Hart, 2000). As for the dorsal, ventral, forelimb and hindlimbs, tail and perineum are similar in flea infestation intensity, with no significant differences among them, but they differ significantly from the head.

A low annealing temperature in PCR can promote the formation of primer dimers, leading to non-specific bands in the gel electrophoresis. The annealing step in PCR is crucial for primers to bind to their complementary sequences on the template DNA. If the annealing temperature is too low, the primers may not bind specifically to the target DNA, and instead, anneal to each other. According to Nucleics (n.d.), to minimize primer dimer formation, the annealing temperature should be optimized to ensure that the primers bind specifically to the target DNA and not to each other and this can be done using gradient PCR. In gradient PCR it

functions by varying the temperature across the PCR block, generating a temperature gradient. This enables testing of multiple annealing temperatures in a single run, typically within a 5°C to 10°C range around the estimated optimal temperature for the primers (Padmakumar, *et al*., 2003). This approach allows for the evaluation of different annealing temperatures to influence PCR performance, eliminating the need for separate reactions at each temperature. If primer dimers are observed at lower temperatures, increasing the annealing temperature in the gradient PCR can help prevent primer dimer formation (Prezioso & Jahns, 2000).

The samples were sent for sequencing, which identified them as *B. henselae* and *B. clarridgeiae*. According to a study by Kamrani, *et al*. (2008), domestic cats are the primary reservoirs for three species of Bartonella—*B. henselae, B. clarridgeiae,* and *B. koehlerae*. In addition, based on Raimundo, *et al*. (2022), *B. henselae* and *B. clarridgeiae* are prevalent in shelter cats in South Brazil, representing 10.63% and 6.38% of the infectious cases which is similar with the result of this study, representing 10% and 13.33%. The higher occurrence of *B. clarridgeiae* in our study (13.33%) compared to the study by Raimundo, *et al*. (2010) (6.38%) may be attributed to several factors. One possible explanation is the regional variation in flea populations, as the local flea species in our study area may be more efficient vectors for *B. clarridgeiae*. In the midst of this, *B.henselae* is the primary pathogen responsible for Cat Scratch Disease but also by *B. clarridgeiae* (Iannino *et al*., 2017; Lamas *et al*., 2008), which can be life-threatening for individuals with weakened immune systems. Infected cats usually do not display any clinical signs of the infection. Throughout the time, the bacterium and cats have developed a symbiotic relationship allowing it to persist in the host while minimizing its pathogenic impact (Guptill, 2010).

The detection rate of *Rickettsia* spp. in this study was notably lower than that of *Bartonella* spp., likely influenced by factors such as sample size and geographic distribution. Pathogens like *Rickettsia* spp. often exhibit irregular prevalence, with their distribution tied to environmental factors such as flea density and host availability. Sampling from a limited number of locations can overlook areas with high infection prevalence. Bitam *et al*. (2010) highlighted that small sample sizes increase the risk of missing pathogens that are rare or unevenly distributed. This unevenness often results from factors like variations in flea populations, host availability, and environmental conditions, which create localized infection hotspots. Without broader and more diverse sampling efforts, such concentrated areas of infection might go undetected, leading to underrepresentation of the true pathogen prevalence.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Ultimately, there was a prevalence number of flea infections (75%) among stray cats in Kota Bharu, Kelantan. Furthermore, *Bartonella* spp. were discovered to be more frequently occurring than *Rickettsia* spp. Age of the cat was found to be the most significant risk factor for flea infestation. The head, neck, and ears were discovered to be the most common body regions for flea infestation, most likely caused by the cats' difficulties grooming these areas. The findings provide useful information that can be used to improve flea control techniques and reduce the spread of zoonotic diseases.

5.2 Recommendation and Future Work

This study faced several limitations such as the small sample size poses significant challenges, examples are increasing the likelihood of random variability, which can affect the precision and reliability of the data. As a result, the conclusions drawn may not fully represent the entire population of stray cats in the area. Additionally, certain body parts of the cats could not be thoroughly examined due to the animals becoming agitated or aggressive during the examination process. This may have led to missed flea samples, potentially unable to fully visualise the flea infestation. The inability to fully inspect these areas introduces a level of bias that may affect the overall accuracy of the results. Moreover, optimization of the annealing temperature can eliminate the formation of primer dimer which are the unspecified bands in the gel electrophoresis. Finally, the study can further improve by using species-specific primers, which are known for their higher sensitivity and specificity in detecting the desired amplicon. This would have likely improved the precision of the molecular analysis, ensuring more reliable identification of *Bartonella* spp. and *Rickettsia* spp. in the samples.

Overall, addressing these limitations in future studies would enhance the accuracy and reliability of the results.

APPENDIX

Figure 5: Abundance of flea dirt during sampling.

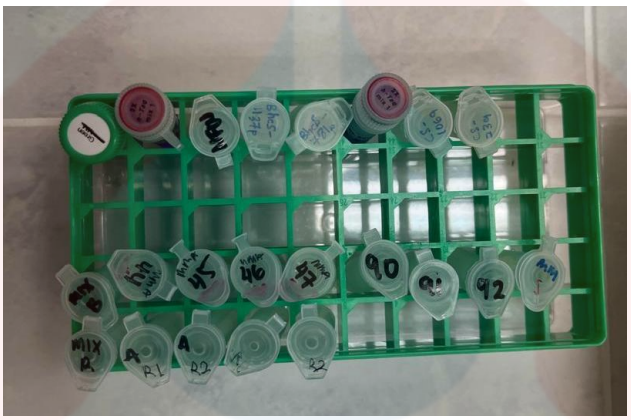


Figure 6: Primers and positive controls for *Bartonella* spp. and *Rickettsia* spp., reagents for PCR.

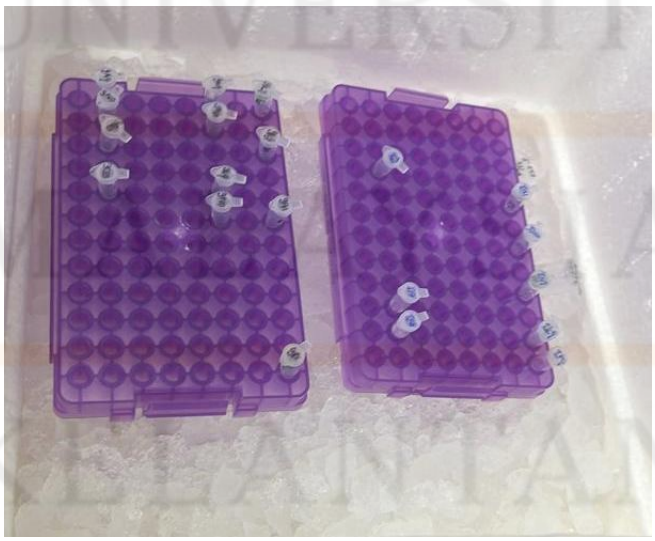


Figure 7: Samples preparation.

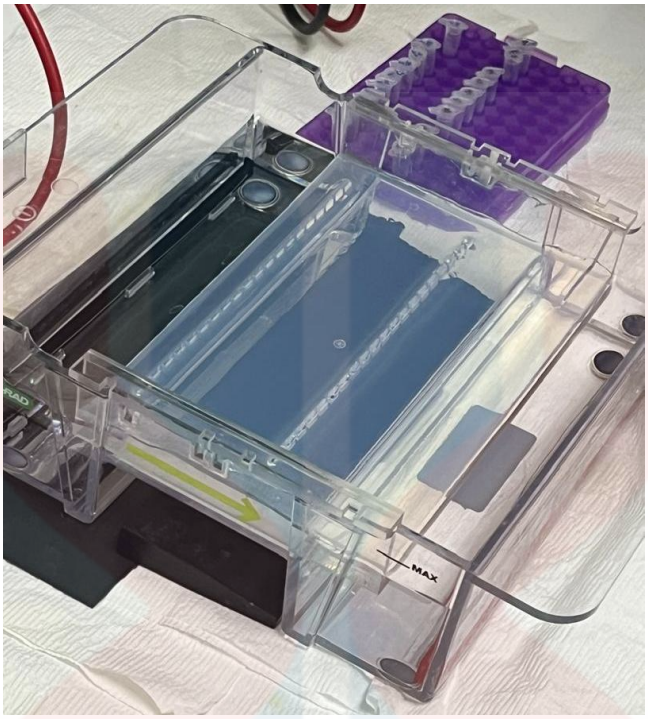


Figure 8: Gel placed in the electrophoresis machine ready for loading of ladder, samples and positive and negative controls.

Figure 9: Samples wrapped with film to send for gene sequencing.

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