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PREVALENCE OF *BARTONELLA spp. AND RICKETTSIA spp.* IN *CTENOPHALIDES FELIS* INFESTING STRAY CATS IN RURAL AREAS OF 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

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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.

One of the most popular pets owned by people are cats, which are also frequently found as stray animals that live without human care and must scavenge for food and shelter. *Ctenocephalides felis*, the cat flea that transmits zoonotic illnesses including Cat Scratch Disease by *Bartonella henselae* and Flea-borne Spotted Fever by *Rickettsia felis* (Lisa D. Brown a *et al*., 2022), mostly feeds on felines. The purpose of this study was to assess the prevalence of *Bartonella* and *Rickettsia* spp. in stray cats. Three locations of rural areas of Kota Bharu were chosen as sampling areas, including: Ketereh, Pulau Pisang, and Pantai Mek Mas. Total of six fleas were collected per cat which then proceeded in *Bartonella* and *Rickettsia* spp. detection by doing DNA extraction and Polymerase Chain Reaction (PCR) and gel electrophoresis. Samples that showed positive (clear and faint bands) were then chosen to be sent for gene sequencing to determine the exact DNA molecules of the samples. Samples number were 30 and results showed 100% prevalence for *Bartonella* spp., and 16.67% both for *Rickettsia* spp. and combination infections. Results of gene sequencing identified the presence of *Bartonella henselae* and *Bartonella clarridgeiae* for positive PCR samples of *Bartonella* spp., meanwhile *Rickettsia felis* was identified for positive PCR sample of *Rickettsia* spp.

This statistical study showed an important relationship between flea infestations and the age of the cat. Also, there were a lot more fleas on the head, neck, and ears than anywhere else on the animal that was studied. This was valid for the dorsal, ventral, forelimbs, hindlimbs, and tail. These results are useful for improving methods for getting rid of fleas on stray cats and lowering the chance that zoonotic diseases will spread to people.

Keywords: Ketereh, Pulau Pisang, Pantai Mek Mas, *Ctenophalides felis, Bartonella henselae, Bartonella clarridgeiae, Rickettsia felis*, Cat Scratch Disease, Flea-borne Spotted Fever, Polymerase Chain Reaction (PCR), Stray cats

ABSTRAK

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

Salah satu haiwan peliharaan yang paling popular dimiliki oleh orang ramai ialah kucing, yang juga sering dijumpai sebagai haiwan terbiar yang hidup tanpa penjagaan manusia, dan harus mencari makanan dan tempat tinggal sendiri. *Ctenocephalides felis*, kutu kucing yang menularkan penyakit zoonosis termasuk Penyakit Calar Kucing oleh *Bartonella henselae* dan Demam Bintik bawaan Kutu oleh *Rickettsia felis*, kebanyakannya hidup dengan memakan darah kucing. Tujuan kajian ini adalah untuk menilai prevalens *Bartonella* dan *Rickettsia* spp. pada kucing terbiar. Tiga lokasi kawasan luar bandar Kota Bharu telah dipilih sebagai kawasan persampelan, termasuk: Ketereh, Pulau Pisang, dan Pantai Mek Mas. Sebanyak enam ekor kutu telah dikumpul daripada setiap kucing yang kemudiannya diteruskan dengan melakukan pengekstrakan DNA dan Reaksi Rantaian Polimerase (PCR) dan elektroforesis gel untuk mengesahkan kehadiran *Bartonella* dan *Rickettsia* spp. di dalam kutu-kutu yang disampel. Sampel yang menunjukkan positif (jalur yang jelas dan samar) kemudiannya dipilih untuk dihantar untuk penjujukan gen bagi menentukan molekul DNA yang spesifik bagi sampel. Bilangan sampel adalah 30 dan keputusan menunjukkan 100% prevalens untuk *Bartonella* spp., dan 16.67% untuk *Rickettsia* spp. dan jangkitan gabungan kedua-dua bakteria. Keputusan penjujukan gen mengenal pasti kehadiran *Bartonella henselae* dan *Bartonella clarridgeiae* untuk sampel PCR positif *Bartonella* spp., manakala *Rickettsia felis* dikenal pasti untuk sampel PCR positif *Rickettsia* spp.

Kajian statistik ini menunjukkan hubungan penting antara serangan kutu dan umur kucing. Selain itu, terdapat lebih banyak kutu di kepala, leher, dan telinga berbanding tempat lain pada haiwan yang dikaji. Ini sah untuk dorsal, ventral, forelimbs, hindlimbs dan ekor. Keputusan ini berguna untuk menambah baik kaedah untuk menghilangkan kutu pada kucing liar dan mengurangkan kemungkinan penyakit zoonosis akan merebak kepada manusia.

Kata kunci: Ketereh, Pulau Pisang, Pantai Mek Mas, *Ctenophalides felis, Bartonella henselae, Bartonella clarridgeiae, Rickettsia felis*, Penyakit Calar Kucing, Demam Bintik Bawaan Kutu, 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 *Ctenophalides felis* Infesting Stray Cats in Rural areas of Kota Bharu, Kelantan'** by **Nik Ameera Syadiyah binti Azhar**, and in our opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirements for the course DVT 55204 – Research Project.

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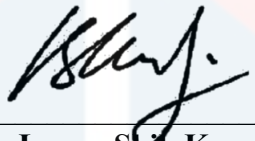

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LIST OF ABBREVIATIONS

C. felis - *Ctenocephalides felis*

R.felis - *Rickettsia felis*

B.clarridgeiae - *Bartonella clarridgeiae*

PCR - Polymerase Chain Reaction

CHAPTER 1

1.0 INTRODUCTION

Humans and companion animals share a unique and intimate bond that sets them apart from other types of domesticated animals (such farm animals or lab animals). Cats, namely *Felis catus*, are one of the most popular companion species for humans. Meanwhile, stray cats are the offspring of domestic cats, *Felis catus*, that roam freely without owners and have to find their own shelters and foods. *Bartonella henselae*, which is the primary culprit behind cat scratch disease in humans, is present in the feces of *Ctenophalides felis* on infected cats' skin, with fleas serving as the agents for transmitting *B. henselae* between cats (Lappin, 2011).

The cat flea serves as both a reservoir and a vector for numerous pathogens, such as Bartonella spp. and Rickettsia spp. These pathogens can be transmitted to humans via bites and scratches, even in the absence of clinical symptoms in the cats themselves. The asymptomatic nature of infections in cats may lead to a misleading sense of safety, thereby heightening the risk of human exposure (Slapeta *et al*., 2018). Determining the occurrence of flea-borne diseases, especially Bartonella spp. and Rickettsia spp., in stray cats within urban regions of Malaysia is essential for effective public health management. Considering the zoonotic potential of these diseases, it is crucial to learn about their prevalence in stray cat populations and start effective control measures that protect both human and animal health. Additionally, elements such as age, breed, overall health, and environmental management contribute to the vulnerability of cats to flea-borne diseases (Azrizal-Wahid *et al*., 2019), assisting experts in pinpointing possible disease agents.

1.1 RESEARCH PROBLEM STATEMENT

Bartonella spp. and *Rickettsia* spp. are common vector-borne diseases transmitted by fleas (*Ctenocephalides felis*), which both have zoonotic potential. The absence of routine detection and control of flea-borne diseases in felines creates a considerable public health issue. Infected felines frequently exhibit no clinical signs, which promotes a misleading sense of security among cat owners and the general population. In rural areas, there is a higher prevalence of stray cats following the factors of lack of spaying and neutering programs, abundant food sources in agricultural settings, limited access to veterinary care, and cultural factors which increase the risk of human exposure to infected fleas. Stray cats may serve as reservoirs for these pathogens, hence increasing the risk of human exposure. The scale of the risk and the incidence of these infections in flea populations infesting stray cats remain minimally investigated. This study is done to improve understanding of public health issues of these cat fleas towards humans.

1.2 RESEARCH QUESTIONS

- What is the prevalence of *Bartonella* spp. and *Rickettsia* spp. in *Ctenophalides felis* fleas collected from stray cats in rural areas of Kota Bharu, Kelantan?
- What is the intensity of flea infestations in stray cats in Kota Bharu, Kelantan?
- Does age as risk factor contributing to flea infestations among stray cats in rural area of Kota Bharu, Kelantan?

1.3 RESEARCH HYPOTHESIS

- There are significant differences in the prevalence of *Bartonella* spp. and *Rickettsia* spp. among different rural areas within Kota Bharu, Kelantan which is significantly higher at Ketereh, than at Pulau Pisang and Pantai Mek Mas.
- Stray cats in Kota Bharu, Kelantan, exhibit a high intensity of flea infestations.
- Age does contribute as risk factor to flea infestations in stray cats in rural areas of Kota Bharu, Kelantan.

1.4 RESEARCH OBJECTIVES

- To determine the prevalence of *Bartonella* spp. and *Rickettsia* spp. in *Ctenophalides felis* fleas collected from stray cats in rural areas of Kota Bharu, Kelantan.
- To assess the intensity of flea infestations in stray cats of Kota Bharu, Kelantan.
- To identify age as risk factor that contribute to flea infestations among stray cats in rural areas of Kota Bharu, Kelantan.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Background of *Bartonella* spp.

Bartonella spp. are Gram-negative bacteria that are fastidious and can infect a wide range of mammalian hosts, including humans. These pathogens are primarily transmitted by fleas, lice, and ticks (Chomel *et al.*, 2006). Breitschwerdt *et al*. (2010) have stated that *Bartonella henselae* and *Bartonella quintana* are the most clinically significant species in humans, causing diseases such as trench fever, bacillary angiomatosis, and Cat Scratch Disease (CSD).

The reservoir hosts are usually asymptomatic and the arthropod vectors that facilitate transmission shows the genus' complicated biological cycle (Harms & Dehio, 2012). Their capacity to infiltrate endothelial cells and endure intracellularly is a factor in both immune evasion and persistent infections, as mentioned by Seubert *et al*. (2010). The increased interest in Bartonella is due to its emerging status as an underdiagnosed pathogen, which has been associated with a variety of acute and chronic illnesses (Maggi *et al*., 2013).

2.2 Background of *Rickettsia* spp.

Rickettsia felis is an obligate intracellular Gram-negative bacterium responsible for flea-borne spotted fever (FBSF). *Rickettsia felis* needs both vertebrate and invertebrate hosts for its survival and reproduction. The cat flea (*Ctenocephalides felis*) is considered as the principal vector and reservoir host of this infection. *Rickettsia felis* has also been detected in various flea species, with increasing evidence of its identification in other arthropods, including ticks, mites, lice, and mosquitoes (Tsokana *et al*., 2022). Typically, *R.felis* infection results in mild to moderate illness, marked by fever, cutaneous rash which is sometimes accompanied by an inoculation eschar, as well as neurological and gastrointestinal symptoms (Socolovschi *et al*., 2010).

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

The scratching of cats, whether domestic or wild, can infect humans with *Bartonella henselae*, especially kittens. The bacteria *B. henselae* can be carried by fleas in cats. The bacteria can be transferred from cats to humans through scratches that are infected with flea feces. Another way that infected cats might transfer bacteria is by biting or licking someone who has an open wound. The transmission of these bacteria to humans through the bite of infected cat fleas has been suggested but not proved.

Most rickettsial pathogens are spread to people directly by sick arthropods that feed on people, like fleas, lice, mites, and ticks. Rickettsia can also be spread when someone accidentally introduces rickettsial pathogens into an arthropod bite wound or other skin break. This can happen by scratching skin that has been contaminated with an arthropod's infectious fluids or feces, or by crushing the arthropod vector at the bite site. Some rickettsial pathogens can also cause infections by breathing in pathogens or putting infectious material in the conjunctiva (Nicholson & Paddock, 2023).

2.4 Geographical variations of rural areas associated with *Ctenophalides felis* infestation

In rural places, *Ctenocephalides felis* infestations are affected by the environment, the number of hosts that are available, and interactions between the pest and animals and people. In contrast to urban areas, rural areas often have a bigger range of hosts, such as roaming pets, livestock, and wildlife, that fleas can attach to and feed on (Bitam *et al*., 2010).

In rural tropical and subtropical areas, *C. felis* can reproduce and live all year thanks to good weather conditions like high temperatures and humidity (Rust & Dryden, 1997). For example, cats and dogs that walk freely in these areas are often important hosts for fleas because they are close to wildlife reservoirs and don't get much veterinary care (Blanton & Morales, 2006).

2.5 Prevalence of Bartonellosis and *Rickettsia felis* infection

Bartonellosis are very common in some places but not in others, and they can be hard to spot in others. *Bartonella henselae* is mostly spread by cats, and the infection rate can be anywhere from 15% to 40% in moderate areas and up to 70% in warm, humid areas (Chomel *et al*., 2006; Breitschwerdt *et al.,* 2010). Different parts of the world have different levels of *Rickettsia felis* in fleas. Higher rates have been found in tropical and subtropical places (Bitam *et al*., 2010; Parola *et al.*, 2005).

2.6 Public health concern

Bartonellosis, a new zoonotic disease, threatens global public health. Infected cats or fleas spread *Bartonella* spp., especially *Bartonella henselae,* which causes cat scratch disease (CSD). Infections can cause fever, lymphadenopathy, endocarditis, neuroretinitis, and hepatosplenic abscesses in humans (Chomel *et al*., 2006; Breitschwerdt, 2010). *Bartonella*

infections can cause life-threatening bacillary angiomatosis and peliosis hepatis in immunocompromised people like HIV/AIDS patients (Koehler *et al*., 1994). Bartonellosis' zoonotic origin and wide clinical range make it relevant in human and veterinary medicine.

The flea-borne bacterium *Rickettsia felis* is increasingly linked to human fevers. The pathogen is spread by *Ctenocephalides felis* and is found in flea-infested areas. Underdiagnosis of *R. felis* infections is common due to their nonspecific febrile symptoms (Bitam *et al*., 2010; Mediannikov & Parola, 2013). *R. felis* has spread across Africa, Asia, and South America, especially in low-resource areas with minimal diagnostic infrastructure. Its potential as a neglected tropical disease is concerning (Parola *et al.*, 2005). *R. felis*' extensive host range and febrile epidemics make it a growing threat to public health systems globally.

2.7 Diagnostic test of Bartonellosis and Rickettsiosis

Bartonella henselae may be detected by ELISA, immunofluorescence antibody assay (IFA), polymerase chain reaction (PCR), Culture, and Western Blot. Each test has benefits and downsides, and none stands out. The ELISA, IFA, and Western Blot assays identify antibodies against *Bartonella*, assuming *Bartonella* is present. PCR is a sensitive DNA test for *Bartonella*, however as the bacterium only sporadically circulates, false negatives might occur. Bartonella species may be identified quickly using PCR. Blood cultures are the most accurate test, although many are needed since the organism circulates sporadically. A positive culture indicates infection. A delayed hypersensitivity skin test is used to diagnose cat scratch illness in humans, but not in cats. Similar to the TB test, a skin scratch is produced, and a response to the injected antigens may occur immediately or in 48 hours. Not good delayed hypersensitivity responders, cats (Brooks, 2003).

The diagnosis of rickettsial illnesses can be accomplished using a variety of laboratory approaches. The methods vary in terms of what is available, how and when specimens are collected, and how the results are interpreted. Common testing methods for rickettsial disorders include molecular methods like PCR assays and serologic techniques like the indirect IFA.

CHAPTER 3

3.0 MATERIALS AND METHODOLOGY

3.1 Ethical Considerations

This research project got approval from the Institutional Animal Care and Use Committee (IACUC) to conduct research involving animals with approval code: UMK/FPV/ACUE/FYP/011/2024

3.2 Study Area

The study area where samples were collected are the selected rural areas of Kota Bharu: Pantai Mek Mas, Kampung Kadok, Pulau Pisang.
These 3 locations were chosen as they are inhabited by an abundance of stray cats which can be the carrier for *Bartonella* spp. and *Rickettsia* spp. for the humans in the area.

3.3 Study Design

In this study, a cross-sectional study is used as the flea samples are collected from random stray cats at a single point in time from three different locations. These samples were processed and diagnosed for the presence of *Bartonella* spp. and *Rickettsia* spp.

3.4 Study Population

Based on Ariffin's (2024) sample size calculate and the assumption of proportion, a study by Azrizal-Wahid *et al.* (2019) in four different regions of Peninsular Malaysia found that 71.83% of stray cats have fleas. With a 95% confidence level, a 50% chance of prevalence, and a 10% chance of dropping out, the sample number (n) needed for this study is 317. This lowers the chance of bias and raises the study's external validity. However, the sample size, n, was determined to be a total of 35 stray cats that were randomly selected from three different rural areas in Kota Bharu due to the limitation of time and costs.

3.5 Selection Criteria

3.5.1 Inclusion Criteria

In selecting stray cats for taking flea samples, cats were selected with no bias such as age, sex, breed, and body condition score.

3.5.2 Exclusion Criteria

Aggressive and fierce were not selected for sampling. Cats that have skin lesions also were excluded to reduce the risk of zoonotic disease transmission.

3.6 Sample Collection and Physical Examination

Thirty-five stray cats were randomly selected from three rural areas in Kota Bharu (Pantai Mek Mas, Kampung Kadok, Pulau Pisang) regardless of age, sex, breed, and health status. A general examination was conducted to see if the animals possessed any of the exclusion criteria.

In order to take flea samples from the cats, a flea comb was required. Cats were approached slowly and gentle restraint was applied, by putting the palm of the hand on the cat's dorsal part which will make the cat to be on sternal. Then, using the comb, begin combing the cat's fur, focusing on areas where fleas commonly gather, such as the head, neck, and tail base. Use gentle strokes to remove fleas and flea dirt, being careful not to cause discomfort to the cat.

3.7 Flea Collection and Identification

For flea collection from cats, the cat was securely restrained while combing its fur in a downward motion from head to tail. Begin at the top of the head and proceed gradually and consistently, ensuring thorough coverage of all body areas. Focus on areas where fleas commonly hide, such as around the ears, under the chin, and near the tail. Use gentle pressure while combing to avoid causing discomfort or pain. For cats with long fur, consider sectioning it off with clips to facilitate access to all coat areas and effectively capture fleas, if present. 3-6 fleas were taken from each cat and were kept in a vial container filled with 90% Ethanol (C_2H_6O). Flea samples were then brought to the Parasitology Laboratory, Faculty of Veterinary Medicine, UMK and observed under a stereo microscope for further identification of the species.

Table 1. Preparation of 90% Ethanol dilution

From 100% Ethanol to 90% Ethanol dilution:
Desired volume of 90% Ethanol dilution, V2 = 200 ml

$$M1V1 = M2V2$$
$$(100\%)(V1) = (90\%)(200\ ml)$$
$$V1 = 180\ ml$$

180 ml of 100% Ethanol is diluted with 20 ml Normal Saline to make 200 ml of 90% Ethanol dilution.

3.8 DNA extraction, Polymerase Chain Reaction (PCR), Gel electrophoresis, and species identification

Total genomic DNA was extracted by using the NucleoSpin® Tissue DNA isolation kit (Macherey-Nagel, Düren, Germany) following the instructed procedures by the manufacturer. Firstly, the fleas were transferred to a microcentrifuge tube, then homogenized the fleas using homogenizer. Then, pre-lysed the sample by adding 180µL Buffer T1 and 25µL Proteinase K solution. Vortex to mix. It must be sure that the samples were completely covered with lysis solution then incubate at 56°C overnight.

Next, the samples were lysed by vortexing the samples and 200µL Buffer B3 was added and incubated at 70°C for 10 minutes. DNA binding condition was adjusted by adding 96-100% ethanol to the sample then vortex vigorously. To bind the DNA, for each sample, place one NucleoSpin® Tissue Column into a Collection Tube. Sample was applied to the column and centrifuge for 1 min at 11,000 x g. The flow-through was discarded and placed back into the Collection Tube. After that, the silica membrane is washed by adding Buffer BW and is centrifuged for 1 min at 11,000 x g and the flow-through is discarded. Second wash was done by adding Buffer B5 and again centrifuged for 1 min at 11,000 x g and the flow-through is discarded again. Residual ethanol was removed during drying the silica membrane by centrifuging the column for 1 min at 11,000 x g. The highly pure DNA was eluted by placing the NucleoSpin® Tissue Column into a 1.5 mL microcentrifuge tube and 50µL prewarmed Buffer BE (70 °C) is added, and then incubated at room temperature for 1 min. Centrifuge 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-GGG GAC CAG CTC ATG GTG G-3) and BhCS. 1137n (5-AAT GCA AAA AGA ACA GTA AAC A-3) as forward and reverse primers,

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

A Polymerase Chain Reaction (PCR) assay was conducted to detect *Bartonella* spp. DNA in the extracted flea DNA samples. The PCR cycling conditions were as follows: an initial denaturation at 95°C for 5 minutes, followed by 34 cycles of denaturation at 95°C for 20 seconds, annealing at 51°C for 30 seconds, extension at 72°C for 2 minutes, and a final extension at 72°C for 5 minutes.

The primers used for PCR amplification for the gItA 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 (expected 830 bp for forward primer and CS-1069 5'CAGGGTCTTCGTGCATTTCTT'3 with the reading frame of 1069 to 1049 as reverse primer (Labruna *et al*., 2004). The positive control 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 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.

Lastly, the PCR products then were separated by gel electrophoresis in a 1.5% agarose gel, at 100 V for 40 minutes. Separated DNA fragments were visualized using Gel Doc EZ Imager.

3.9 Data Analysis

To estimate prevalence of *Bartonella* spp. and *Rickettsia* spp. in *Ctenophalides felis* fleas collected for each location, number of fleas detected positive of *Bartonella* spp. or *Rickettsia* spp., or both, divided by the total cats sampled from each location.

Considering a p-value of 0.05 as statistically significant, the chi-square test was carried out to determine the correlation between risk factor (age) and flea infestation. Furthermore, used was the Chi-square test to find the relationship between flea infestation intensity and body parts. Moreover, a one-way ANOVA was carried out to investigate for variations in flea infection intensity among the five separate body parts of the stray cats.

CHAPTER 4

4.0 RESULTS

4.1 Fleas collection and identification

Upon sampling in three different areas: Ketereh, Pulau Pisang, and Pantai Mek Mas, the number of cats sampled was 35 stray cats and 180 fleas were collected from 30 cats, making it 6 fleas per cat. The vials were labelled as K1-K10 for cats sampled at Ketereh, P1-P10 for cats sampled at Pulau Pisang, and M1-M10 for cats sampled at Pantai Mek Mas.

Table 2. Number of cats sampled, and fleas collected per cat

Location	**Number of cats sampled**	**Fleas collected per cat (n)**
Ketereh (K)	10 (K1-K10)	6
Pulau Pisang (P)	10 (P1-P10)	6
Pantai Mek Mas (M)	10 (M1-M10)	6

Fleas collected were then brought to Parasitology Laboratory, FPV UMK to identify its species using stereo microscope. *Ctenophalides felis* can be identified by characteristic ctenidia, or combs; it has a pronotal ctenidium and a genal ctenidium, and sloping forehead. Female fleas also are bigger in size compared to male fleas.

Figure 1.0. Female *Ctenophalides felis*

Figure 2.0. Male *Ctenophalides felis*

4.2 Correlation of Risk Factor (Age) and Flea Infestation

Table 3. The Chi-square test On Risk factor (Age) and Flea Infestation

Risk Factor	Variable	Fleas Infestation (Num. of Cats)		Chi-Square	*p*-value	Significance
		Yes	No			
Age	Juvenile (<6 months old)	21	1	4. 589	0.032	Yes
	Adult (>6 months old)	9	4			

A *p*-value of 0.032 showed that there was a significant correlation between age and flea infection. This means that fleas are more likely to infest juvenile cats (less than 6 months old) than older cats (more than 6 months old) in the stray cat population.

4.3 Correlation of Flea Infestation Intensity on Five Different Body Parts

Table 4. Chi-square test of Flea Intensity on Different Body Parts

Body Parts	Fleas Infestation (Num. of Cats)		Chi-Square	*p*-value	Significance
	Yes	No			
Head, ear, and neck	29	1	39.249	<0.001	Yes
Dorsal region	23	7			
Ventral region	11	19			
Forelimb & Hindlimb	9	21			
Tail & Perianal Region	20	10			

As stated in table 4, a Chi-square test was used to determine the association between the body section and flea infestation. With a p-value of <0.001, the Chi-square test of independence demonstrated a significant relationship among all five body areas. Therefore, there is a strong correlation between the severity of flea infestation and various cat body parts.

In order to evaluate the extent of flea infestation across various body sections of stray cats, a one-way ANOVA was conducted. The analysis revealed a statistically significant difference in flea infestation levels among the body sections, as demonstrated by a p-value of <0.001. This suggests that the location on the body is an important variable in determining the intensity of flea infestation. In the body sections that were examined, the head, ear, and neck region were the most substantially affected, suggesting that the flea infestation is concentrated in this area.

Flea infestations are most prevalent in the head, ear, and neck region, as indicated by the post-hoc results. The flea intensities in the dorsal, ventral, forelimb, and hindlimb regions, as well as the tail and perineum regions, are comparable to one another, and there are no notable variances between them.

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

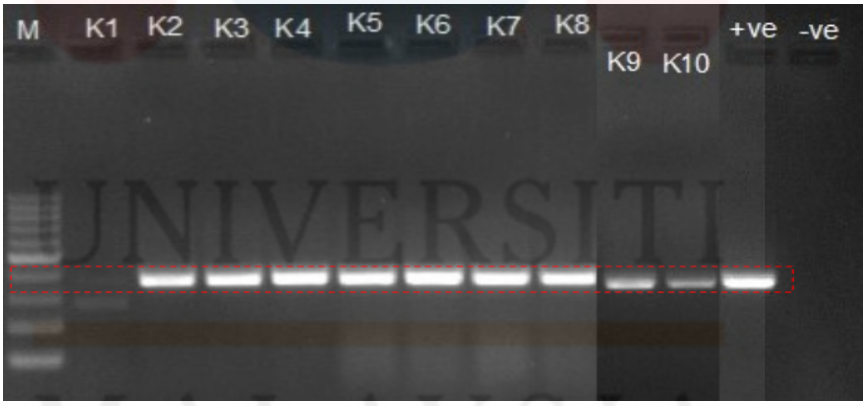

Figure 3.0 Agarose gel electrophoresis of the PCR products of K1-K10

Figure 4.0. Agarose gel electrophoresis of the PCR products of M1-M10

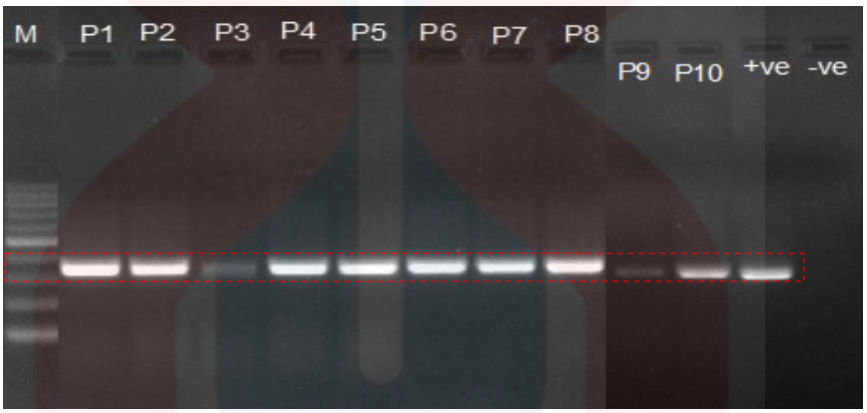

Figure 5.0. Agarose gel electrophoresis of the PCR products of P1-P10

M: DNA ladder marker
+ve: positive controls
-ve: negative control

The gel electrophoresis results from the 30 samples tested for *Bartonella* spp. showed clear bands in many samples, indicating the presence of the target DNA. Several samples had a clear DNA fragment at the expected size of 379 base pairs (bp), confirming *Bartonella* spp. DNA. The positive samples had strong and consistent bands matching the expected size for the primers used. However, four samples—K1, M2, M8, and P9—showed faint bands, possibly due to lower DNA amounts or partial amplification.

4.5 *Rickettsia* spp. detection by Polymerase Chain Reaction (PCR)

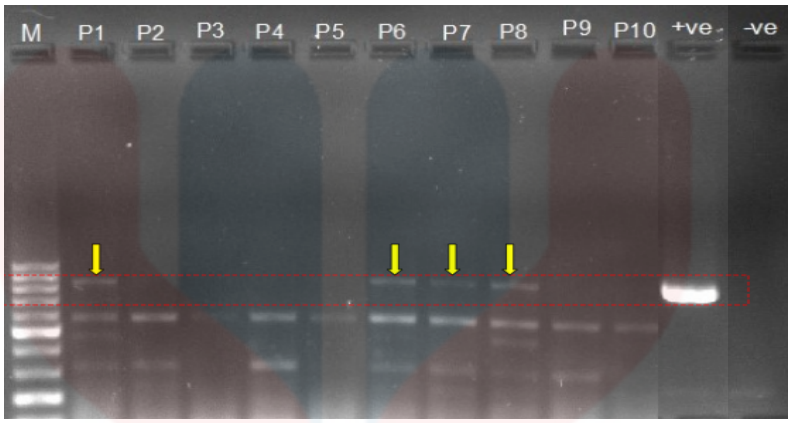

Figure 6.0. Agarose gel electrophoresis of the PCR products of P1-P10

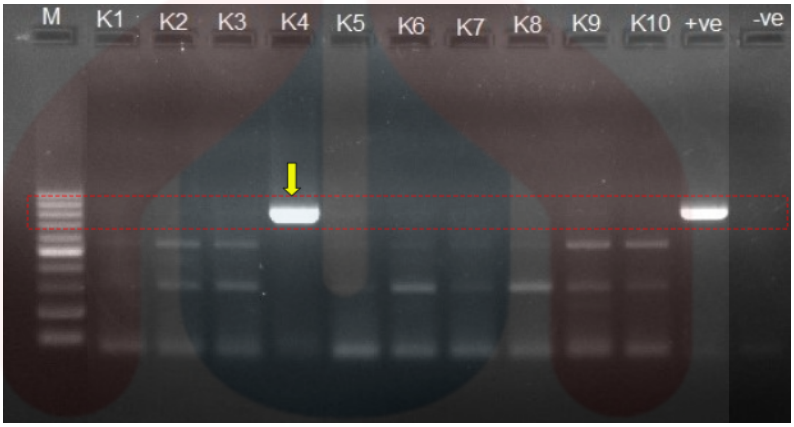

Figure 7.0. Agarose gel electrophoresis of the PCR products of K1-K10

The gel electrophoresis results from the 30 samples tested for *Rickettsia* spp. showed clear bands only in K4, meanwhile P1, P6, P7, and P8 at the expected size of 834 base pairs (bp), confirming *Rickettsia* spp. DNA. Gene sequencing was performed on six selected samples: K2, K3, K9, P10, M9, and M10, to confirm the results.

4.6 Prevalence of *Bartonella* spp. and *Rickettsia* spp. of positive samples

Table 5.0. Prevalence of *Bartonella* spp. and *Rickettsia* spp. and co-infections

	Positive samples (n)	**Prevalence (%)**
***Bartonella* spp.**	30	100.00
***Rickettsia* spp.**	5	16.67
Combination infections	5	16.67

Bartonella spp. had a 100% prevalence in 30 samples, with all samples testing positive. *Rickettsia* spp. has a prevalence of 16.67%, which means there are five positive samples (P1, P6, P7, P8, and K4). Therefore, the prevalence of combination infections is also 16.67%.

4.5 Sequence Analysis

Gene sequencing was also done in order to further confirm the samples that showed positive results on the Agarose gel electrophoresis using the PCR products. For *Bartonella* spp., six samples were selected: K2, K3, K9, P1, P6, P7, P8, and M9, for gene sequencing to further confirm the results.

Table 6.0 Gene sequencing result for few of *Bartonella* spp. positive samples

Sample Name	**Gene Sequence Result**
K2	*Bartonella henselae*
K3	*Bartonella clarridgeiae*
K9	*Bartonella henselae*
P1	*Bartonella clarridgeiae*
P6	*Bartonella clarridgeiae*
P7	*Bartonella clarridgeiae*
P8	*Bartonella clarridgeiae*
M9	*Bartonella clarridgeiae*

The results show presence of *Bartonella clarridgeiae* and *Bartonella henselae* with *B.clarridgeiae* being more common.

For *Rickettsia* spp., one sample, K4, was sent for gene sequencing to further confirm the results.

Table 7.0 Gene sequencing result for *Rickettsia* spp. positive samples

Sample Name	**Gene Sequence Result**
K4	*Rickettsia felis*

The result shows the presence of *Rickettsia felis.*

CHAPTER 5

5.0 DISCUSSION

Out of 180 fleas collected it was revealed a significant prevalence of *Bartonella* spp. (100%) and *Rickettsia* spp. (16.67%) in *Ctenocephalides felis* collected from stray cats in rural areas of Kota Bharu, Kelantan. These findings show the zoonotic potential of these pathogens and highlight the role of *C.felis* as a key vector for their transmission. The results have important implications for public health, especially in regions where stray cats are abundant, and flea control measures are limited.

Out of 35 stray cats, a higher proportion of juvenile cats (21/35; 60%) were infested with fleas compared to adult cats (9/35; 25%). This implies that flea infections are more likely to affect young, less than six-month-old cats than adult or older cats. There are several reasons why younger cats may have more fleas than older cats. These include having a weaker immune system, spending more time with their mother and littermates, and not being able to clean themselves as well (Beugnet & Franc, 2010).

Young cats often stay in places where fleas like to nest, which makes them even more likely to get an infestation (Rust, 2017). Additionally, adult cats may have got some sort of resistance to flea infestations as a result of their increased mobility, improved grooming habits, or prior exposure, which enables them to avoid environments that are severely infested (Hsu *et al*., 2020). Also, fleas are more common on younger animals, especially in places where flea control methods have not been implemented (Blagburn & Dryden, 2009).

Stray cats' flea infestations were most common in the head, ear, and neck. The one-way ANOVA showed considerable variation in flea infestation levels among body areas (p-value <0.001). The post-hoc study confirmed that the head, ear, and neck had the most fleas. This trend matches previous research that found more fleas in the head and neck. According to Meng *et al*. (2002), this area had a considerably higher mean flea count than other body regions. One possible explanation is that the head and neck are particularly difficult for cats to groom using oral grooming, which is the primary method of flea removal. Lack of grooming increases flea buildup in the head and neck.

As for the dorsal, ventral, forelimb, hindlimb, tail, and perianal regions, their flea infestation intensities were found to be similar, with no significant differences among them. However, they differed significantly from the head, ear, and neck, confirming that fleas favour certain cat body parts. Fleas are concentrated in these locations, suggesting they are primary feeding sites due to greater vascularization and warmth, which aid flea survival and reproduction (Rust, 2017).

The detection of *Bartonella* spp. in 100% of flea samples represents a remarkably high prevalence, consistent with studies from tropical and subtropical regions where environmental conditions such as high temperature and humidity favor flea survival and pathogen transmission (Bitam *et al*., 2010). *C.felis* is a well-documented vector for *Bartonella* spp., particularly *B.henselae* and *B.clarridgeiae,* which are commonly linked with cats and fleas over the world (Chomel *et al*., 2006; Lappin *et al*., 2020).

Bartonella clarridgeiae, was isolated from a cat and linked to cat scratch disease (CSD) in a human. The person developed symptoms like fever, enlarged lymph nodes, and a skin bump at the infection site. This means that cats may harbor multiple *Bartonella* species, including *B.clarridgeiae* and *B.henselae,* and can transmit either through scratches, bites, or blood transfusion (Kordick *et al.,* 1997).

In this study, *Bartonella clarridgeiae* was identified more often than *Bartonella henselae.* Similar studies have shown this pattern, which might have numerous causes: First, *B.clarridgeiae* is well-known to be strongly associated with *C. felis,* where it can remain in the gut of the flea and be passed on to new hosts during feeding (Foil *et al.,* 1998). Second, the biology of *B.clarridgeiae* might enable it to adapt more successfully to the flea habitat than *B.henselae,* thereby maybe increasing its frequency in flea populations. Third, ecological elements in rural settings, including the existence of untreated flea infestations and poor veterinary care, could promote the development of *B.clarridgeiae* over *B.henselae* (Kelly *et al.,* 2015).

The fact that 16.67% of flea samples show *Rickettsia* spp. suggests that *C.felis* is also a major vector for the infection linked with fever diseases in people (Parola *et al*., 2005). Although the frequency of *Rickettsia* spp. was less than *Bartonella* spp., its presence in the same fleas emphasizes the possibility of combination infections, which could complicate identification and treatment of zoonotic diseases even if their frequency was smaller.

In this study, the prevalence of combination infections is 16.67% which was detected in 5 samples. Combination infections of *Bartonella* spp. and *Rickettsia* spp. in *C.felis* are concerning as this affects human as well as veterinary medicine since similar symptoms of Bartonellosis and Rickettsiosis can delay proper therapy and accurate diagnosis (Fournier & Raoult, 2009).

The outcomes of this study are particularly significant for rural areas of Kota Bharu, where stray cats are abundant, and veterinarian interventions are limited. Stray cats serve as reservoirs for flea populations, which in turn act as vectors for zoonotic infections. The high incidence of *Bartonella clarridgeiae* in rural areas may indicate a increased exposure risk for humans in close contact with stray cats or their fleas, particularly those involved in handling or caring for these animals.

Rural communities generally lack the infrastructure and resources for efficient flea management, increasing the risk of flea-borne diseases. Public health interventions should focus on boosting

knowledge of zoonotic infections, advocating routine flea control methods for stray and domestic animals, and improving environmental hygiene to eliminate flea habitats.

CHAPTER 6

6.0 CONCLUSION

This study shows a high prevalence of *Bartonella* spp. (100%) and a significant presence of *Rickettsia* spp. and combination infections (16.67%) in *Ctenocephalides felis* collected from stray cats in rural areas of Kota Bharu, Kelantan. The majority of *Bartonella clarridgeiae* over *Bartonella henselae* highlights its strong relationship with fleas and recommends different ecological or biological factors favoring its proliferation in this region. Age of the stray cats also can be a significant risk factor for flea infestation. The most common places for flea infestation in cats were found to be the head, neck, and ears. This is probably because it is hard for them to groom these parts of their bodies. The finding of combination infections further points to the significance of *Ctenophalides felis* as a responsible vector for numerous zoonotic bacteria, increasing concerns about the potential risks to public health.

There were some limitations with this study. For example, sampling a cat was a bit of a challenge as not all cats can cooperate with all the handlings as they might get irritated and reluctant to stay for sampling. Other than that, optimizing the annealing temperature also needed to be done for a few times as the primer dimers or unidentified band keep appearing on the gel electrophoresis.

For recommendations, I recommend doing a study that includes the whole rural areas of Kota Bharu, Kelantan to get a more conclusive data regarding prevalence of *Bartonella* spp., *Rickettsia* spp., and combination infections in *Ctenocephalides felis* collected from stray cats in rural areas of Kota Bharu, Kelantan. Other than that, it is also recommended to take a bigger sample size to reduce margin of error compared to smaller sample size. Also, larger sample sizes are more likely to show how varied the population really is, including differences in age, sex, health condition, and other factors. Sample sizes that are smaller are more likely to not show the true diversity of the population, which can cause figures of prevalence to be wrong.

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Lisa D. Brown a, a, b, c, Highlights•A total of 3643 cat fleas were collected from 283 free-roaming domestic cats.•Flea intensity was highest in the month of June and in juvenile cats (, AbstractThe cat flea (Ctenocephalides felis) is a competent vector of numerous bacterial pathogens in the genera Bartonella and Rickettsia. In the United States, Akucewich, L. H., Breitschwerdt, E. B., Durden, L. A., Eckstein, R. A., Luria, B. J., Matos, M., Shanks, D., Šlapeta, Thomas, J. E., Wallace, J. L., Azrizal-Wahid, N., Boulouis, H. J., Cdc, … Curtis, T. M. (2022, May 30). *Detection of bartonella spp. and Rickettsia spp. in cat fleas (ctenocephalides felis) collected from free-roaming domestic cats in southeastern Georgia, USA*. Veterinary Parasitology: Regional Studies and Reports. https://www.sciencedirect.com/science/article/abs/pii/S2405939022000594?via%3Dihub

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APPENDIX

Figure 8.0 One of the cats sampled at Ketereh

Figure 9.0 Water bath for DNA extraction

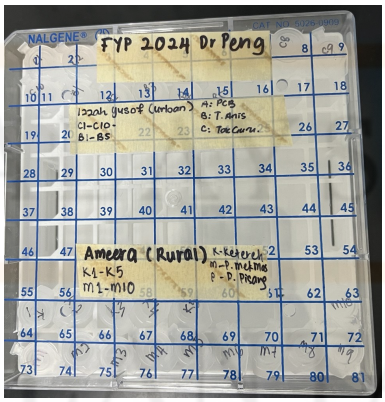

Figure 10.0 Samples storage

Figure 11.0 PCR Products of our samples

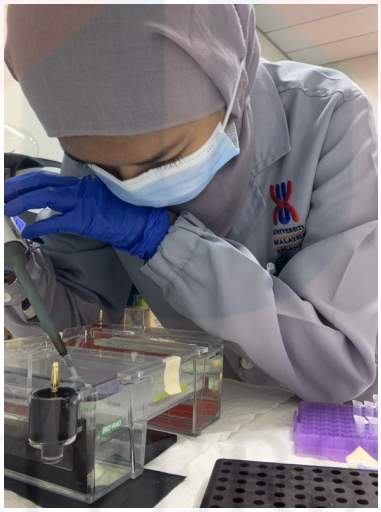

Figure 12.0 Loading wells for gel electrophoresis