OCCURRENCE OF *BARTONELLA* SPP. IN STRAY CATS IN PENGKALAN CHEPA, KELANTAN

WONG SIN JIA
(D18A0043)

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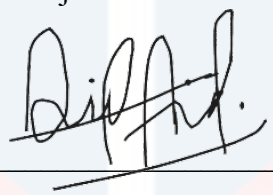
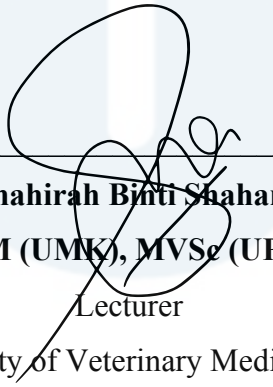
CERTIFICATION

This is to certify that we have read this research paper entitled 'Occurrence of *Bartonella* spp. in Stray Cats in Pengkalan Chepa, Kelantan' by Wong Sin Jia, and in our opinion, it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course DVT 55204 - Research Project.



Dr. Murshidah Binti Mohd Asri
DVM (UMK), MVSc (UPM)
Lecturer
Faculty of Veterinary Medicine
Universiti Malaysia Kelantan
(Supervisor)



Dr. Nurshahirah Binti Shaharulnizim
DVM (UMK), MVSc (UPM)
Lecturer
Faculty of Veterinary Medicine
Universiti Malaysia Kelantan
(Co-supervisor)

Assoc. Prof. Dr. Rumaizi Bin Shaari
DVM (UPM), MVM (UPM), PhD (University of Adelaide)
Associate Professor
Faculty of Veterinary Medicine
Universiti Malaysia Kelantan
(Co-supervisor)

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DEDICATIONS

This paper is dedicated to my parents, who gave me a simple yet incredible life, and always my strong pillar whenever I needed one. I also dedicate this research to many of my lecturers, especially Dr. Murshidah who have guided and supported me throughout the research process, with words of encouragement, lots of patience and time. Finally, I would like to dedicate this work to my dear friends, who have accompanied me through the ups and downs of my university years.

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ABSTRACT

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

Bartonella henselae is the main causative agent of cat-scratch disease, which is associated with a wide range of clinical signs in humans, mainly lymphadenopathy. The main reservoir of *B. henselae* is domestic cats, and it is transmitted to humans through cat scratches and bites. This possesses great zoonotic risk, especially towards immunocompromised individuals. Research has shown that it has a higher prevalence in warm and humid countries due to the suitable environment for the multiplication of the vector, cat fleas. In this study, the occurrence of *Bartonella* spp. in stray cats in Pengkalan Chepa, Kelantan was determined by analysing 15 blood samples. DNA was extracted from the blood samples using a commercial DNA extraction kit, and amplification of DNA was performed by polymerase chain reaction (PCR) using primers targeting the *gltA* gene (379bp) of *Bartonella* spp. Then, the amplified DNA fragments were separated using gel electrophoresis and viewed under Gel Doc Imager. From the results obtained, 3 out of 15 samples were suspected to carry *B. henselae* genes. This concluded that *Bartonella* spp. is present in stray cats, in Pengkalan Chepa, Kelantan, which contributes to the concern of being infected and the awareness among residents should be raised.

Keywords: *Bartonella henselae*, cat-scratch disease, domestic cats, zoonotic, stray cats, Pengkalan Chepa, blood, PCR

ABSTRAK

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

Bartonella henselae adalah agen penyebab utama penyakit cakar kucing, yang dikaitkan dengan pelbagai tanda klinikal pada manusia, terutamanya limfadenopati. Hos takungan utama *B. henselae* adalah kucing domestik, dan ia menjangkiti manusia melalui cakar dan gigitan kucing. Ini mempunyai risiko zoonosis yang besar, terutamanya terhadap individu yang mempunyai imuniti yang lemah. Pelbagai kajian yang telah dilaksanakan menunjuk bahawa ia mempunyai kelaziman yang lebih tinggi di negara-negara panas dan lembap disebabkan persekitaran yang sesuai untuk pertubuhan vector bagi *B. henselae*, iaitu kutu kucing. Dalam kajian ini, kehadiran *Bartonella* spp. dalam kucing liar di Pengkalan Chepa, Kelantan telah ditentukan dengan menganalisis 15 sampel darah dari kucing liar. DNA telah diekstrak daripada sampel darah menggunakan kit pengekstrakan DNA komersial, dan penguatan DNA dilakukan oleh tindak balas berantai polimeras (PCR) menggunakan primer menyasarkan gen *gltA* (379bp) *Bartonella* spp. Kemudian, serpihan DNA yang telah diperkuat, dipisahkan menggunakan elektroforesis gel dan dilihat bawah Gel Doc Imager. Daripada keputusan yang diperolehi, 3 daripada 15 sampel disyaki mengandungi gen *B. henselae.* Ini membuat kesimpulan bahawa *Bartonella* spp. terdapat dalam kucing liar di Pengkalan Chepa, Kelantan. Ini juga mampu menyumbang kepada kebarangkalian untuk dijangkiti dan disebabkan itu, kesedaran dalam kalangan penduduk harus dipupuk.

Kata Kunci: *Bartonella henselae*, penyakit cakar kucing, kucing domestik, zoonosis, kucing liar, Pengkalan Chepa, darah, PCR

1.0 INTRODUCTION

Bartonella spp. are fastidious, hemotropic, gram–negative bacteria that are mainly transmitted by blood-sucking arthropod vectors (Chomel et al., 2006; Markey et al., 2013; Regier et al., 2016). They mainly infect mammalian hosts and most of them are zoonotic. Among the list of species that have been identified to cause disease in humans are *Bartonella quintana, Bartonella bacilliformis* and *Bartonella henselae* (Anderson & Neuman, 1997; Regier *et al.*, 2006; Mosbacher et al., 2011). *B. henselae* is the main agent for cat-scratch disease (CRD) and is commonly associated with a wide range of clinical signs from lymphadenopathy to ocular neuritis and encephalitis in humans (Chomel et al., 2006; Guptil, 2010; Stull & Stevenson, 2015). In cats, fleas play a major role in the transmission of feline *Bartonella*, but other potential vectors, such as ticks and biting flies have been recently identified to harbour *Bartonella* DNA (Chomel et al., 2006; Regier et al., 2016).

Open wound is the main portal entry for *Bartonella* infection or bartonellosis. Open wounds may happen when the cats bite or scratch, breaking the epidermis and dermis layer. Then, these portal entries are contaminated with flea dirt that contains *Bartonella* spp. Aside from that, horizontal transmission can also happen when infected cats bite or scratch the healthy cats (Chomel et al.; 2006, Markey et al., 2013; Regier et al., 2016). Cats of every age, breed and population may carry these bacteria, but it is more prevalent in young kittens, stray cats, as well as cats in warm and humid regions (Chomel et al., 2006; Mosbacher et al., 2011; Markey et al., 2013; Regier et al., 2016). Higher prevalence in young kittens (< 1 year old) may result from the slower immune response due to the first encounter of the bacteria (Heller et al., 1997). Meanwhile, higher prevalence in stray cats as compared to pet cats may be attributed to the higher exposure to flea infestation from the outdoor environment, as described by Chomel et al. (1995). The warm and humid regions also provide favourable conditions for the hatching of eggs and the development of larvae, rendering a higher ectoparasite burden in these areas and higher risk of infection among the cats (Mosbacher et al., 2011; Taylor et al., 2016). Most cats that are infected with *Bartonella* spp. are asymptomatic. However, in rare cases, it can cause inflammation of the heart, and develop infection in the mouth, eyes or urinary system (Regier et al., 2016).

In humans, *Bartonella* infections in immunocompetent individuals are normally self-limiting (Angelakis & Raoult, 2014; Regier et al., 2016). As the disease progresses, the lymph nodes near the site of infection will start to inflame and become painful upon palpation. Other signs may also be presented such as fever, anorexia and lethargy (Mosbacher et al., 2011; Regier et

al., 2016). Sporadic reports on *Bartonella* spp. infection in young children and immunocompromised people revealed to develop more serious complications such as endocarditis, optic neuritis and encephalomyelitis (Chomel et al., 2006; Regier et al., 2016).

Isolation of *Bartonella* spp. is difficult due to its fastidious nature, hence most of the time molecular detection of this bacteria through polymerase chain reaction (PCR) is done as a confirmative method (Regier et al., 2016; CDC, 2022)

1.1 Research Problem

Feline *Bartonella* spp., especially *B. henselae* possess great zoonotic potential and risk of infection to humans, as people are often in contact with cats, either keeping them as pets or merely just to socialize with stray cats. This special human-cat relationship contributes to a high incidence rate of humans getting bitten or scratched by an infected cat, thereby increasing the chance of contracting diseases caused by *Bartonella* spp. Bartonellosis is more prevalent in warmer and humid countries as it favours the life cycle of cat fleas, which is the primary vector of feline bartonellosis. However, there is scarce information about the occurrence of *Bartonella* spp. infection in cats in Malaysia, which is a well-known tropical country. Therefore, this study is conducted as preliminary data to determine the occurrence and prevalence of *Bartonella* spp. in stray cats, in Pengkalan Chepa, Kelantan.

1.2 Research Questions

What is the occurrence of *Bartonella* spp. infection in stray cats in Pengkalan Chepa, Kelantan?

1.3 Research Hypothesis

H_o: *Bartonella* spp. is not present in stray cats in Pengkalan Chepa, Kelantan.

H_A: *Bartonella* spp. is present in stray cats in Pengkalan Chepa, Kelantan.

1.4 Objectives

To investigate the occurrence of *Bartonella* spp. in stray cats in Pengkalan Chepa, Kelantan.

2.0 LITERATURE REVIEW

2.1 Background of *Bartonella* spp.

There are at least 20 species identified under the genus *Bartonella*, present in a wide range of animals and human (Regier et al., 2016). The principal pathogen in the genus is *Bartonella henselae*, which has domestic cats as the primary reservoir and is the main causative agent of cat-scratch disease (CSD) in humans (Markey et al., 2013). This bacterium is a pleomorphic, gram-negative, and slightly curved bacilli in shape. It is intracellular, typically found in red blood cells and vascular endothelium due to its ability to invade hematopoietic progenitor cells in humans after infection. (Markey et al., 2013)

2.2 Transmission of *Bartonella* spp.

Studies have proven that cat flea (*Ctenocephalides felis*) is the main transmission vector for *B. henselae* among cats, and occasionally in humans (Chomel et al., 2006; Regier et al., 2016). Meanwhile, other blood-sucking arthropods, such as ticks, lice and sand fly have been proven to be capable of transmitting other *Bartonella* spp. (Chomel et al., 2006; Mosbacher et al., 2011; Regier et al., 2016). Besides that, when the cats scratch or groom themselves, flea dirt containing *Bartonella* spp. will get trapped in the claws and teeth. This leads to the introduction of *Bartonella* spp. into tissue and bloodstream, when cats bite or scratch hard enough to cause deep wounds, or when they lick an open wound. (Regier et al., 2016; CDC, 2022). Humans usually acquire *Bartonella* infection through scratches and bites of an infected cat (Chomel et al., 2006; Mosbacher et al., 2011; Regier et al., 2016). Ultimately, people in frequent contact with cats are at higher risk of developing bartonellosis. Veterinarians, cat shelter volunteers and pet owners are examples of individuals at a high risk of infection due to their occupational hazards and way of living (Regier et al., 2016). Cats of every age, breed and population may harbour these bacteria, but it is more prevalent in young kittens, stray cats, and cats in warmer regions due to the higher ectoparasite burden (Chomel et al., 2006; Markey et al., 2013; Mosbacher et al., 2011; Regier et al., 2016).

2.3 Health Effects on Domestic Cats

As domestic cats are the main reservoir of *B. henselae*, most of them are clinically normal being infected by the bacterium (Regier et al., 2016; Nelson and Couto, 2020). Infected cats

sometimes will show fever that lasts for two to three days at the beginning of the bacteraemia state. Other clinical manifestations like fever, lethargy, lymphadenopathy, uveitis, gingivitis, endocarditis, myocarditis, hyperglobulinemia, osteomyelitis, cutaneous vasculitis, and neurologic diseases have also been reported (Chomel et al., 2006; Regier et al., 2016; Nelson and Couto, 2020). The exact reason why some cats exhibit more severe clinical signs is not known. It has been discussed that the lack of immunocompetency, such as through feline immunodeficiency virus (FIV) or feline leukaemia virus (FeLV) infection, could lead to the enhanced pathogenicity of Bartonellosis (Bergmann et al., 2017).

2.4 Health Effects on Humans

To date, a wide range of clinicopathological effects are reported to be associated with *Bartonella* infection in humans (Chomel et al., 2006; Mosbacher et al., 2011; Mosepele et al., 2012; Angelakis & Raoult, 2014; Regier et al., 2016). The three most significant human pathogenic *Bartonella* spp. have been identified, namely *B. bacilliformis*, which causes a biphasic disease characterized by a primary haemolytic fever and high mortality; *B. quintana*, which causes debilitating cyclic fever; and *B. henselae*, which causes CSD (Regier et al., 2016).

Among these, *B. henselae* is the most common agent that causes symptomatic infection and their severity was in relation to the immune status of patients (Angelakis & Raoult, 2014; Regier et al., 2016). In healthy patients, *Bartonella* infections are normally self-limiting where patients may only experience nonspecific symptoms such as fever, headache and inappetence (Chomel et al., 2006; Mosbacher et al., 2011; Angelakis & Raoult, 2014; Regier et al., 2016). Regional lymphadenitis may be observed within two to three weeks if the disease progresses (Regier et al., 2016). The most commonly affected lymph nodes are located at the axilla, neck and the groin region, as they drain the area of cat scratches or bites. It typically lasts 2 to 3 months, and occasionally longer. (Rolain et al., 2004). Aside from that, *B. henselae infection* has also been regarded as a common cause of fever of unknown origin (Chung et al., 2005; Zenone, 2011)

In immunocompromised hosts, such as patients undergoing chemotherapy or steroid treatments, as well as HIV/AIDS patients, Bartonellosis may cause rare but severe, life-threatening clinical signs. This includes but is not limited to bacillary angiomatosis, encephalopathy, neuroretinitis, endocarditis, granulomatous hepatitis, splenitis as well as osteomyelitis (Chomel et al., 2006; Mosepele *et al.*, 2012; Regier et al., 2016). Whereas in

Malaysia, neuroretinitis was commonly reported as the presenting symptom for ocular bartonellosis (Tan et al., 2017; Shariffudin et al., 2021).

2.5 Clinical Diagnosis and Treatment of Cat-Scratch Disease in Humans

CSD is usually suspected in the presence of enlarged lymph nodes, small vesicles or granuloma at the site of inoculation, in conjunction with a history of scratched or bitten by a cat. As it is usually self-limiting, it does not require treatment (Anderson & Neuman, 1997). However, it can go severe in some cases and requires antibiotic therapy (Rolain et al., 2004; Boulouis et al., 2005). There is no specific treatment approach for *Bartonella*-related diseases but rather depends on the clinical situation as they can be very different.

In a few studies, antibiotic treatment is regarded as ineffective in treating CSD (Anderson & Neuman, 1997; Boulouis et al., 2005; Angelakis & Raoult, 2014). It also increased the risk of adverse drug reactions and emerging of resistant flora, hence the current recommendation for mild to moderately ill, immunocompetent patients with CSD is no antibiotic treatment. Nevertheless, analgesics are still given to manage pain, as well as needle aspiration for suppurative lymph nodes. For patients with severely enlarged and painful lymphadenitis, azithromycin is recommended. As for complicated CSD involving systemic illnesses such as retinitis and encephalopathy, a combination of doxycycline and rifampin has been proven to be successful in treatment. However, there is no definite optimum duration for the therapy. It has also been found that there is a dramatic clinical response to antibiotics observed in immunocompromised patients, in contrast with the minimal clinical response observed in immunocompetent patients (Rolain et al., 2004).

2.6 Vaccination

There have been attempts to develop vaccines against feline Bartonellosis, to reduce the spread of infection among cats, and the risk of infection in humans. Unfortunately, the attempt has been deemed unsuccessful due to the wide diversity of *Bartonella* isolates even within the same genotype (Boulouis et al., 2005).

2.7 Diagnostic Test

The best diagnostic test for bartonellosis can be very subjective. Bacterial culture, PCR assay, and serologic tests have all been used to assess for *Bartonella* infection (Boulouis et al., 2005; Chomel et al., 2006; Regier et al., 2016).

Serologic tests including immunofluorescence and enzyme-linked immunosorbent assay (ELISA) are useful, non-invasive diagnostic methods but have varied specificities and sensitivities due to the cross-reactivity between *B. henselae* and other species (Rolain et al., 2004; Chung et al., 2005). Besides that, it is of limited diagnostic value in cats as many of them are likely to be seropositive against *B. henselae* (Boulouis et al., 2005).

According to Stepanić et al. (2019), the gold standard for diagnosing is to culture *Bartonella* spp. from blood or tissue samples, followed by molecular sequencing. Despite its fastidious nature, they managed to isolate *Bartonella* colonies as early as 6 days after inoculation. On the other hand, Gutiérrez et al. (2017) and Agan & Dolan (2002) suggested that conventional or real–time PCR is more practical and sensitive than culture. To date, most of the published studies have described the usage of PCR as a method of diagnosing Bartonellosis (Ohno et al., 2021; Saengsawang et al., 2021; Köseoğlu et al., 2022). Among these publications, the target genes used to identify presence of *Bartonella* spp. include the partial *gltA, rpoB, ftsZ, ribC, and groEL* (Saengsawang et al., 2021) and 16S–23S rRNA internal transcribed spacer region (Köseoğlu et al., 2022).

3.0 MATERIALS AND METHODS

3.1 Sample Collection and Physical Examination

A total of 15 stray cats, regardless of age, sex, breed, and health status in Pengkalan Chepa were recruited. Physical examination was performed, with all the signalments and abnormal findings recorded. Whole blood samples were collected by jugular venipuncture, and saphenous or cephalic venipuncture if cats are fractious and not cooperative. About 1 – 3 ml of collected blood were filled into ethylenediaminetetraacetic acid (EDTA) tubes and stored at -12°C.

3.2 Sample Preparation

Blood samples were thawed at 25°C for 30 minutes before the DNA extraction process.

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

Total genomic DNA was extracted by using commercial DNA extraction kits (Genomic DNA Mini Kit, *Geneaid*, Taiwan) following the instructed procedures by the manufacturer. The basic procedure starts from mixing the blood samples with RBC lysis buffer, to lyse the cells and release the DNA into the solution. After removing the supernatant, GB buffer was added and incubated at 60°C, to ensure the sample lysate is clear of cellular debris. After that, RNase A was added to completely clear the lysate by vigorous shaking. Following that, absolute ethanol was added to the lysate to precipitate the DNA and bind onto purification matrix in a GD Column. Next, wash buffers will be added to remove any contaminants from the sample. Lastly, elution buffer is added to release DNA from the matrix, while eluate is collected and discarded.

The primers used for amplification of the *gltA* gene (379bp) of *Bartonella* spp. were 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).

Gene amplification was carried out in a total of 25 µL reaction volumes, containing:

- 5.0 µL of extracted DNA sample
- 1.0 µL of forward and reverse primer each

- 12.5 µL Thermo Scientific™ DreamTaq™ Master Mix (2X) solution
- 5.5 µL of nuclease-free water

DNA amplification was performed using the following cycling conditions: initial denaturation at 95°C for 5 minutes, followed by 35 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 PCR products were then separated by gel electrophoresis in a 1.5% agarose gel, at 100 V for 40 minutes. Separated DNA fragments were then visualized using Gel Doc EZ Imager.

4.0 RESULTS

PCR amplification of the extracted DNA using the described protocol has produced non-specific bands in gel electrophoresis. It was initially assumed that the annealing temperature was too low, causing primers to bind non-specifically to the template.

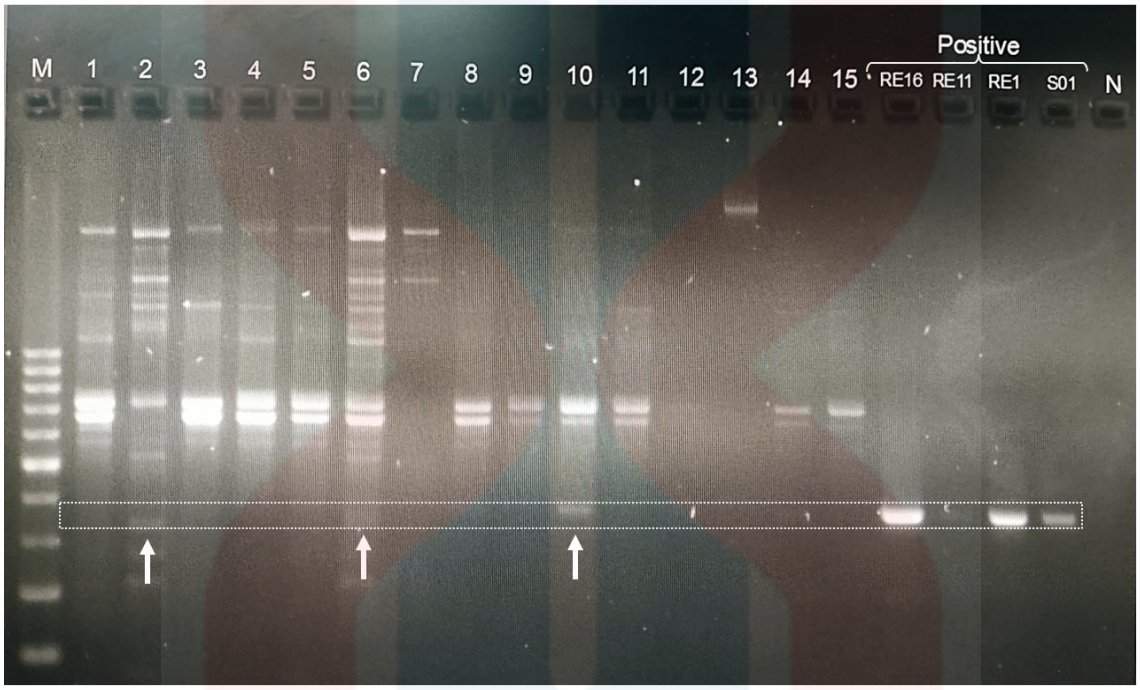

Figure 4.1: Agarose gel electrophoresis of the PCR products. M: DNA ladder marker; 1–15: Isolated samples; RE16, RE11, RE1, S01: Positive controls; N: Negative control; Arrows showing the 3 suspected samples containing Bartonella spp. genome, which are samples 2, 6, and 10.

Figure 4.1 shows most of the samples (12 out of 15) have multiple bands in between 600 and 800 bp. However, in reference to the positive controls, *Bartonella* spp. (379 bp) was only in cats number 2, 6 and 10, which makes the positive result to be 20% (3 out of 15) of the blood samples.

Due to the presence of non-specific bands, annealing temperature was optimized stepwise in 1-2°C increments by using a thermal gradient, ranging from 52°C to 62°C. In this procedure, only 4 samples were used, namely 2, 4, 11 and 13. The temperatures selected were 52.0°C, 52.9°C, 55.1°C, 56.4°C, 57.9°C, 60.2°C, 61.1°C and 62°C.

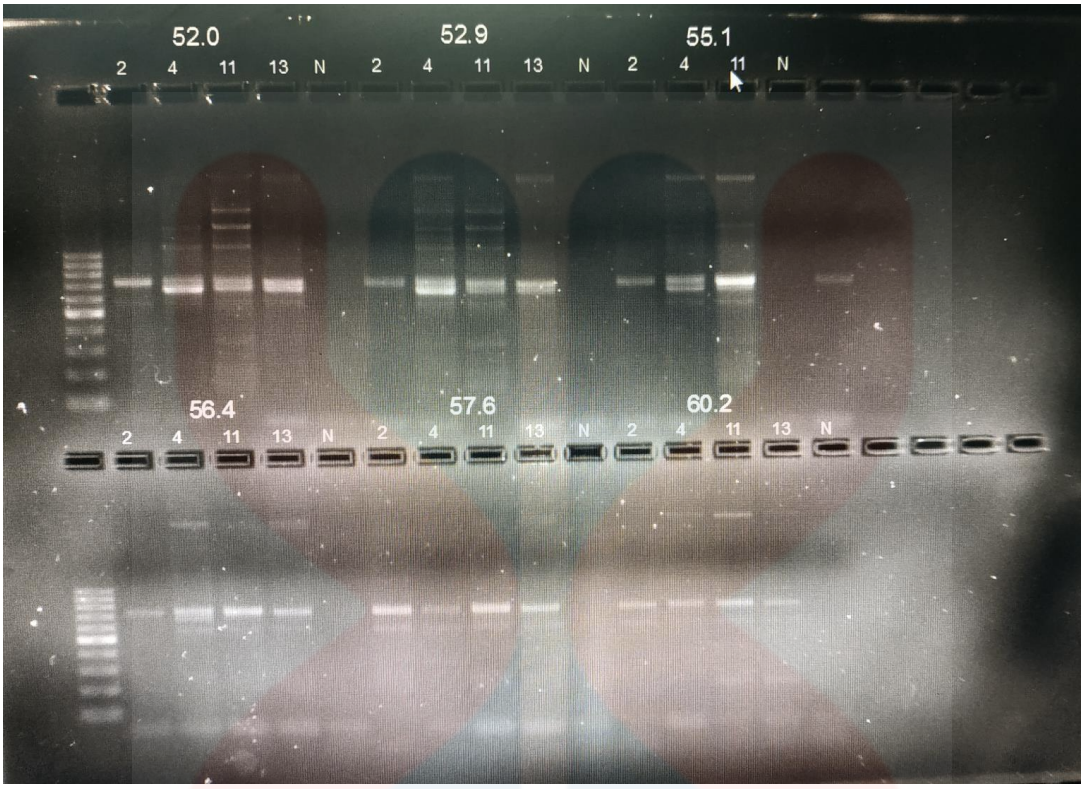

Figure 4.2: Agarose gel electrophoresis of the PCR products, using thermal gradient

From the result, both annealing temperatures 61.1°C and 62°C produced no significant bands, indicating poor annealing efficiency and hence were not included in the final result. Nevertheless, a higher temperature from a range of 56°C to 60°C seems to have reduced the appearance of nonspecific bands, but the bands are not of the desired size (379 bp). Instead, all of them have clear bands between 600 and 800 bp.

Table 4.1 shows physical examination records of the cats which were positive for PCR detection.

Table 4.1: Characteristics of cats suspected to be infected by *Bartonella henselae.*

Cat No.	Sex	Age Group	Physical Examination Findings
2	M	<1 year old	• Flea dirt on hair coat • Ear wax in both ears
6	F	1 – 5 years old	• Polydactyl; No other abnormal findings
10	M		• Mild gingivitis • Indolent ulcer • Bilateral crusty lesion and alopecia at ear pinna

From the findings listed, there seems to have no specific correlation between the positive result and health status of stray cats. There is also no predisposed sex. This indicates that *B. henselae* can be found in stray cats regardless of health status, age, and sex.

5.0 DISCUSSION

The result that we obtained concludes that 3 out of 15 (20%) of the samples contain *Bartonella* spp., based on the presence of positive bands in alignment with the positive controls. It is highly suspected to be *Bartonella henselae* as it is the most commonly found species in domestic cats (Sykes & Chomel, 2014). However, the bands appeared to be quite faint and further confirmation is very much needed. Hence, the amplified products are planned to be sent for sequence analysis, for sequence similarity verification with *B. henselae* strain Houston-I chromosome with GenBank accession number L35101.

According to health records of the positive-cats, cat No. 2 was presented with flea dirt on the hair coat, indicating presence of flea infestation. It is also very young (<1 year old), which may contribute to the establishment of infection due to the low immunity level. However, the kitten showed no other symptoms, suggesting that the immune system is tackling the infection well. Cat No. 6 showed more abnormal signs, which includes mild gingivitis, indolent ulcer, as well as alopecic and crusty skin lesions on both ear pinna. Most studies have included gingivitis as one of the common clinical signs of feline bartonellosis, but some stated that it is more prevalent in cats that are co-infected with diseases that cause immunosuppression, such as FIV and FeLV infections. (Ueno et al., 1996; Boulouis et al., 2005; Bergmann et al., 2017).

In general, domestic cats are the main host that transmit *B. henselae* (Regier et al., 2016; Nelson and Couto, 2020). However, certain studies have also reported that dogs are the occasional host and cause *Bartonella* transmission. These studies reported that *B. henselae* has been isolated from many samples obtained which include the saliva, gingival swab, nails, blood samples and lymphoid tissue of dogs (Tsukahara et al., 1998; Oskouizadeh et al., 2010; Nelson and Couto, 2020). Like cats, *B. henselae* is transmitted by fleas and bacteremia in dogs rarely show signs of illness, unless the host is immunocompromised. In the later situation, dogs will exhibit nonspecific signs such as uveitis, gingivostomatitis, chylothorax, polyarthritis, chronic rhinitis, idiopathic lower urinary tract disease, lymphadenopathy, reproductive disease, or neurologic disorders (Sykes & Chomel, 2014). On top of that, Nelson and Couto (2020) also stated that *Bartonella* infection in dogs is commonly associated with a co-infection, particularly with *Anaplasma* spp. or *Erlichia* spp., which was believed to contribute to the pathogenesis of the disease. Signs of CSD such as lymphadenitis and fever had been shown in human beings after exposure to dogs, and *B. henselae* detected in the dogs' saliva. These suggest that dogs may also cause transmission of the disease to humans (Tsukahara et al., 1998; Nelson and Couto, 2020). However, the mode of transmission is not fully understood.

Although stray cats have a higher prevalence of harbouring *Bartonella* species, Oskouizadeh et al. (2010) suggested that pet cats are more likely to infect humans. This is due to the cautious nature of stray cats that do not allow physical touch from humans, thereby reducing the contact and risk of being scratched and bitten. However, from the experience of luring and capturing stray cats in this study, the cats are mostly friendly and approachable, unless they are agitated and scared from the blood withdrawal procedure. This confirms the truth of the occupational hazards in certain risk groups, such as veterinarians and veterinary technicians.

In this study, there were nonspecific PCR products, as viewed in the gel electrophoresis. According to Bio-Rad (n.d.), the factors of production of nonspecific bands can be related to cycling times and temperatures, which includes excessive cycling, excessive extension time, excessive annealing time, annealing temperature too low, thermal cycler ramping speed too slow, inaccurately calculated primer concentration and annealing temperature. Among these, the annealing temperature, as well as the inaccurate primer concentration are the main suspects of cause, as other factors had adhered to the recommended protocols. If the annealing temperature is too low, the primers may bind non-specifically to the template, causing amplification of undesired gene sequence. Besides that, incorrect calculation of primer concentration can also result in incorrect calculation of annealing temperature, leading to the same consequence.

Besides that, Bio-Rad (n.d.) also suggested that it can also be related to PCR components, such as impurities in the primer, too much primer was used, primers were designed or synthesized incorrectly by the user or manufacturer, impure dNTPs, too much Mg^{2+} or impure water was used. From the listed factors, impure dNTPs and excess amount of Mg^{2+} were not possible. This is because a ready-made master mix solution containing the accurate amount of dNTPs and Mg^{2+} were used, with the recommended volume from protocol. Besides that, nuclease-free water was also used throughout the DNA extraction steps, which ruled out the presence of impure water. Since the amount of primer used was also standardized, too much primer used is also deemed impossible. Hence, it is left with the error within the primer itself. However, the lack of nonspecific bands in the positive controls proved that primers containing impurities or incorrectly designed are unlikely.

Since other factors are believed to be ruled out as the recommended protocol was followed, the annealing temperature was the main suspect of causing the nonspecific band on the template. Hence, annealing temperature was optimized stepwise in 1-2°C increments by using a thermal

gradient, as described in the results. The outcome of the result has led to the question of whether the primer is specifically used to isolate *Bartonella* spp. in domestic cats, as it has been done in several studies. Hence, the primer sequence was analysed using the Basic Local Alignment Search Tool (BLAST) from National Library of Medicine (NCBI), and was found to be not specific to *Bartonella* spp. Besides that, this set of primers have been widely used to detect *Bartonella* spp. in other host species, including ticks, rats, deer, and bats, indicating that this primer is also not host-specific.

In PCR, selection of an appropriate primer is the principal factor to help identify a unique part of the genome. It must be exceptionally specific that it only binds to a certain genomic sequence and does not have matches to other targets in certain orientations or within certain distances that allow undesired amplification. Primers that are not specific enough for a particular DNA template will result in mispriming or failure in amplifying the target gene. For future studies, a more specific primer design for *Bartonella* spp. isolation should be selected, and preferably for cat blood samples. For example, a study from Hassan et al. (2017) had used a primer sequence which targets the internal transcribed spacer region of *B. henselae*, to detect the presence of the bacteria from 284 domestic cat blood samples. The primer is found to be highly specific to *Bartonella* species, and is able to recognize *B. henselae* from domestic cats at 648 bp.

Nevertheless, several recent papers that used the same primer sequence for isolation of *Bartonella* spp. have used a range of annealing temperature between 53°C to 56°C (Boonmar et al., 2021; Azimi et al., 2021; Böge et al., 2021), supporting our findings that a higher temperature serves a more optimal annealing temperature.

6.0 CONCLUSION AND RECOMMENDATION

Bartonella species are important zoonotic agents and are responsible for a wide range of diseases, ranging from benign and self-limited to severe and life-threatening. This study has shown that *Bartonella henselae* is present in stray cats in Pengkalan Chepa. The presence of *Bartonella* species in stray cats in Pengkalan Chepa, Kelantan is signifying the risk of developing cat-scratch disease among the residents. With the findings, the level of awareness among people should be raised and more safety precautions should be taken, such as washing and disinfecting hands after handling cats, performing routine flea prevention, and keeping pet cats indoor to reduce exposure to *Bartonella* species.

In future studies, it is recommended to have a larger sample size collected from a variety of locations. These could ensure a more reliable result and a better representative of the cat population. As the primer used in this study is not specific to the DNA sequence of *B. henselae* in domestic cats, a better primer design should be used in future study to have a more accurate diagnosis. Last but not least, sequence analysis is also highly suggested to determine the origin of the genome shown by the clear, nonspecific bands.

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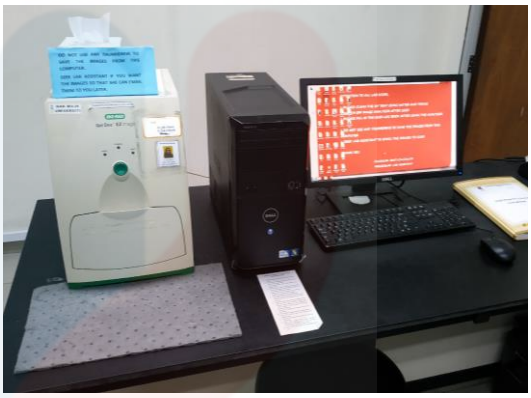
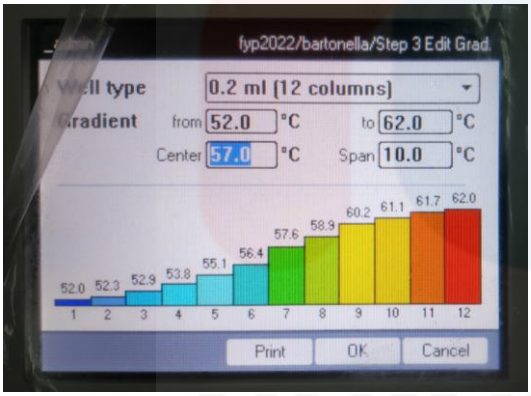
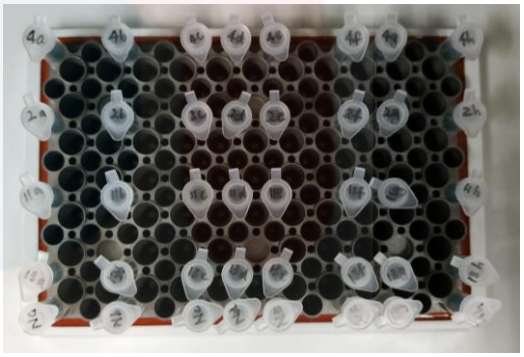
APPENDIX A

Appendix A1: All 15 stray cats, their signalments and physical examination findings

Age Group	Cat Number	Gender	Physical Examination Findings
<1 Year Old	02	M	Presence of flea dirt on hair coat; Presence of ear wax in both ears
	12	M	Pale pink and tacky mucous membrane; Presence of both fleas on skin; Both ears are dirty and filled with earwax
	14	F	Presence of flea and lice on haircoat
	15	M	Presence of flea dirt on haircoat; Gingivitis on both upper and lower arcades; Crusty skin lesion on both ear pinna
1 – 5 Year Old	01	F	Pregnant
	03	M	Skin tenting 3 seconds; Presence of circular, wet, not well-demarcated wound at the head area; Bilateral submandibular, right prescapular, bilateral popliteal lymph node enlargement;
	04	F	No abnormal findings
	05	M	Rough hair coat; Presence of lacerated wound caudal to the left ear base; Left submandibular lymph node enlargement; Presence of tear stain bilaterally; Presence of ear wax in left ear

	06	F	No abnormal findings; Polydactyl
	07	M	Presence of lice on haircoat; Mild left popliteal lymph node enlargement; Presence of soft mucoid faeces on perineal area
	08	M	Presence of lice on haircoat; Mild bilateral submandibular lymph node enlargement; Tachycardic
	09	F	Presence of fleas on skin; Tear stain present on both eyes; Mild crusty skin lesion on both ear pinna; Dirty ear canals filled with earwax
	10	M	Crusty skin lesion and alopecia on both ear pinna tips; Gingivitis on upper arcades; Presence of indolent ulcer
	11	F	Presence of lice on haircoat; Bilateral submandibular and popliteal lymph node enlargement
	13	F	Bilateral submandibular lymph node enlargement

APPENDIX B

	
Appendix B1: Stray cat kept in cage, with water, food and litter provided	Appendix B2: Gel Doc Imager used to analyse PCR products
	
Appendix B3: Gradient temperature settings from Bio-Rad thermal cycler	Appendix B4: Arrangement of PCR tubes for amplification of DNA using gradient temperature