UNIVERSITI PUTRA MALAYSIA

INCIDENCE AND RISK FACTORS OF BARTONELLA HENSELAE INFECTION-ASSOCIATED MYOCARDITIS IN CATS

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INCIDENCE AND RISK FACTORS OF *BARTONELLA HENSELAE* INFECTION-ASSOCIATED MYOCARDITIS IN CATS

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Incidence and Risk Factors of *Bartonella henselae* infection-associated Myocarditis in Cats", by Khoo Kaiwen and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course VPD 4999 – Final Year Project.

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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999- Projek Ilmiah Tahun Akhir.

INSIDEN DAN PERHUBUNGAN ANTARA INFEKSI *BARTONELLA HENSELAE* DENGAN RADANG OTOT JANTUNG DALAM KUCING

Oleh

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2020

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Bartonella henselae merupakan zoonotik agen penyakit yang disebarkan oleh kutu *Ctenocephalides felis* dan menyebabkan kucing menghidapi Bartonellosis. Infeksi ini akan menyebar ke manusia melalui calar dan gigitan kucing dan menyebabkan penyakit calar kucing (CSD). Kes-kes laporan menunjukkan manusia yang menghidapi penyakit calar kucing akan menunjukkan tanda-tanda klinikal yang berhubung dengan radang otot jantung. Akan tetapi, informasi mengenai kejadian radang otot jantung dalam kucing yang menghidapi *Bartonella henselae* adalah

terhad. Penyelidikan ini mengenal pasti kejadian *Bartonella henselae* dan insiden radang otot jantung berhubung dengan infeksi tersebut dalam kucing. Populasi yang terlibat dalam penyelidikan ini terdiri daripada 64 kucing kemilikan pelanggan yang kelihatan sihat yang datang ke University Veterinary Hospital, Universiti Putra Malaysia. Sampel darah kesulurahan telah dikumpulkan melalui venipuncture. Pengekstrakan DNA dan pengesanan molekular dengan menggunakan tindak balas rantai polimerase (PCR) telah dijalankan untuk mengenal pasti kewujudan *Bartonella henselae* dalam sampel darah. Sampel serum telah dianalisis untuk mengenal pasti konsentrasi troponin I jantung (cTnI) yang merupakan suatu indikasi untuk penyakit jantung. Satu tinjauan telah dilakukan untuk mendapat maklumat pesakit termasuk umur, jantina, status neuter, pengurusan, status vaksin, pengetahuan pemilik dan sejarah berkutu, dan jenis produk pencegahan kutu yang digunakan. Keputusan menunjukkan semua kucing adalah negative untuk *Bartonella henselae* dan ada sesetengah kucing menunjukkan peninggian konsentrasi serum cTnI. Sehubuingan dengan itu, peninggian konsentrasi serum cTnI dalam kalangan kucing tersebut mungkin disebabkan oleh penyakit jantung, penyakit buah pinggang dan/atau penyakit respiratori. Kesimpulannya, maklumat yang diperoleh daripada penyelidikan ini tidak menunjukkan perhubungan antara infeksi *Bartonella henselae* dengan radang otot jantung dalam kucing. Untuk mendapat data yang lebih baik, penyelidikan ini boleh dijalani dalam kalangan kucing liar, kucing yang tinggal di luar rumah, kucing yang tidak diberi produk pencegahan kutu dan kucing yang sakit.

Kata Kunci: *kucing, Bartonellosis, tindak balas rantai polimerase, konsentrasi*

troponin I jantung (cTnI), radang otot jantung.

ABSTRACT

Abstract of the project paper presented to the Faculty of Veterinary Medicine in partial requirement for the course VPD 4999 – Final Year Project.

INCIDENCE AND RISK FACTORS OF *BARTONELLA HENSELAE* INFECTION-ASSOCIATED MYOCARDITIS IN CATS

By

KHOO KAIWEN

2020

Supervisor: Dr Khor Kuan Hua

Co-supervisors: Prof Dr Rasedee Abdullah and Dr Farina Mustaffa Kamal

Bartonella henselae is a zoonotic disease agent transmitted by the flea *Ctenocephalides felis* and causes Bartonellosis in cats. The infection is transmitted to humans through cat scratches and bites, which lead to cat scratch disease (CSD). It has been reported that humans infected with CSD show clinical signs associated with myocarditis. However, there is limited information on the occurrence of myocarditis in cats infected with *Bartonella henselae*. This study determined the occurrence of *Bartonella henselae* and the incidence of myocarditis associated with the infection in cats. The study population consisted of 64 client-owned overtly

healthy cats referred to the University Veterinary Hospital, Universiti Putra Malaysia. Whole blood samples were collected via venipuncture. DNA extraction and molecular detection using Polymerase Chain Reaction (PCR) were done to determine the presence of *Bartonella henselae* in blood samples. Serum samples were analysed to determine the cardiac troponin I (cTnI) concentration, an indicator of cardiac disease. A survey was also conducted to obtain patient information including age, sex, neutering status, management, vaccination status, owners' knowledge and history on flea infestation, and the type of flea prevention products used. The results showed that all cats tested negative for *Bartonella henselae* and some cats showed elevated serum cTnI concentrations. Therefore, elevated serum cTnI levels in these cats might be due to other causes such as heart, kidney, and/or respiratory diseases. In conclusion, the information obtained in the study did not show association between *Bartonella henselae* infection and myocarditis in cats. Perhaps, for better data, the study could also be expanded to stray, outdoor-maintained, non-flea-prevented, and unhealthy cats.

Keywords: *cats, Bartonellosis, polymerase chain reaction, cardiac troponin I, myocarditis.*

CHAPTER 1: INTRODUCTION

1.1 Study Background

Bartonella is a zoonotic agent of an infectious disease known as cat scratch disease (CSD) in humans. It is caused by the bacteria named *Bartonella henselae*, which can be found in the flea (*Ctenocephalides felis*) of infested cat. The prevalence of *Bartonella henselae* in fleas in healthy and asymptomatic cats was reported to be approximately 33% (Ishida et al., 2001). The bacteria have been reported found in flea faeces obtained from cats' claws and teeth. In humans, the infection can be transmitted through cats' scratches or bites. Humans infected with cat scratch diseases will show signs such as benign regional lymphadenopathy, low-grade fever, malaise, aching, headache, anorexia, Parinaud oculoglandular syndrome, encephalitis, endocarditis, haemolytic anaemia, hepatosplenomegaly, glomerulonephritis, pneumonia, relapsing bacteremia, and osteomyelitis (Chomel et al., 2006).

Almost 25% of the stray cat population were found seropositive for the bacterium (Brunetti et al., 2013). In 2016, the prevalence of *Bartonella hensalae* infection in pet cats presented to University Veterinary Hospital, Universiti Putra Malaysia was 16.9% (Hassan et al., 2016) based on molecular detection. The prevalence of *Bartonella hensalae* infection in pet cats in other countries were almost similar or higher in countries such as France was 16.5%, in Germany was 15%, in the Netherlands was 22%, in Austria was 33.3%, in United Kingdom was 40.6%, in Sydney, Australia was 35% and in Japan was 15.1% (Boulouis et al., 2004).

Cats infected with *Bartonella* often appear asymptomatic. However, in severe infections, clinical signs such as uveitis, lymphadenopathy, gingivitis, stomatitis,

urinary tract infection may become evident (Breitschwerdt and Kordick, 2000). Cats infected with *B. henselae* may manifest other clinical abnormalities, associated with endocarditis, myocarditis, lymphadenopathy, meningitis, radiculitis, and reproductive disorders (Gulbahar et al., 2019). Interestingly, myocarditis as a consequence of the Bartonellosis has not been vastly described in cats, but has been reported in humans (Magno and Spatar, 2009).

In human, cardiac troponin I (cTnI) is a serum marker for cardiac myocyte injury and is often used in combination with other test for diagnosis of myocarditis (Smith et al., 1997). The types of myocarditis are differentiated into subclinical acute myocarditis, probable acute myocarditis, definite myocarditis, myocarditis resembling a heart attack and myocarditis resembling acute or chronic heart failure (Willis, 2020). In a group of 53 patients with myocarditis, 18 (34%) patients had elevated cTnI concentration. It was found that the measurement of cTnI concentration was more accurate in identifying patients with histological evidence of myocarditis as compared to creatinine kinase isoenzyme MB (CK-MB) levels (Smith et al., 1997).

In cats, cTnI was shown to be a specific biomarker for myocardial injury (Langhorn and Willesen, 2016). In this study, cTnI will be used as a serum parameter in the screening for myocarditis in overtly healthy cats and to determine if these affected cats were infected with Bartonellosis.

1.2 Justification

Previous studies on Bartonellosis and myocarditis associated with the infection were documented in humans. To date, there are limited information to our knowledge

on the occurrence of myocarditis in cats infected with *Bartonella henselae*. As a screening test of myocarditis, serum cardiac troponin I concentration can be used as a biomarker of cardiac disease to facilitate the diagnosis heart disease (myocarditis) in *B. hensalae*-infected cats confirmed using molecular investigation.

1.3 Objectives

The objectives of the study were to determine

1. the occurrence of *B. hensalae* infections in cats.
2. the incidence of myocarditis in *B. hensalae*-infected cats using serum cardiac troponin I concentration as the biomarker.
3. the association between risk factors and incidence of myocarditis in *B. hensalae*-infected cats.

1.4 Hypothesis

Null hypothesis: There was no occurrence of *Bartonella henselae* infections in cats.

Alternative hypothesis: There was occurrence of *Bartonella henselae* infections in cats.

Null hypothesis: Cats infected with *B. hensalae* did not developed myocarditis.

Alternative hypothesis: Cats infected with *B. hensalae* developed myocarditis.

Null hypothesis: There was no identifiable risk factor for myocarditis in *B. hensalae*-infected cats.

Alternative hypothesis: There were identifiable risk factors of myocarditis in *B. hensalae*-infected cats.

CHAPTER 2: LITERATURE REVIEW

2.1 *Bartonella* spp.

Bartonella spp. are fastidious, haemotropic, gram negative bacteria mainly transmitted by vectors such as fleas, ticks and biting flies. Pets such as dogs and cats act as a large reservoir of *Bartonella* spp. and Bartonellosis poses zoonotic risk to pet owners (Chomel et al., 2006). Eight *Bartonella* spp. or subspecies have been recognised, including *B. henselae, B. elizabethae, B. grahamii, B. vinsonii* subsp. *arupensis, B. vinsonii* subsp. *berkhoffii, B. grahamii, B.* v*washoensis* (Kosoy et al., 2002) and more recently *B. koehlerae* (Aidor et al., 2004) has been identified. Cats are host for *Bartonella henselae, B. clarridgeiae*, and *B. koehlerae* (Breitschwerdt and Kordick, 2000). In dogs, *B. vinsonii subsp. Berkhoffii* has been reported to cause of canine endocarditis (Breitschwerdt et al, 1994). Other *Bartonella* sp. detrimental to humans are *Bartonella bacilliformis* which causes Carrión's disease or Oroya fever and *Bartonella quintana* which causes trench fever. *Bartonella henselae*, formerly known as *Rochalimaea henselae* is the main agent of CSD. The bacteria were isolated from flea faeces present in cats' claws and teeth. Therefore, the infection can be transmitted to humans through cats' scratches or bites. It has also been speculated that *Bartonella henselae* can be transmitted to cat owners via direct contact with the cat flea (*Ctenocephalides felis*) (Chomel et al., 1996).

2.2 Clinical manifestation of *Bartonella henselae*

Naturally infected cats are usually asymptomatic but in symptomatic cats, clinical signs observed may differs between infected cats. Clinical signs such as anterior uveitis and predisposed to neurological diseases (Girma et al., 2019) has been reported. Some research groups had found that experimentally infected cats were asymptomatic whereas some were reported with fever and lymphadenopathy (Chomel, 2000). Lymphadenopathy were often presented in infected cat compared to cats with signs of stomatitis and gingivitis followed by cats with anaemia, chest disorders and abdominal disorders (Ueno et al., 1996). Besides that, clinical signs such as anorexia, lethargy, redness and swelling at the site of bite (exposure), myalgia and behavioural changes were reported (O'Reilly et al., 1999).

Persistent *Bartonella henselae* infection in cats may lead to pathological changes in heart such as myocarditis. *Bartonella henselae* DNA are more often amplified from the hearts of cats with feline endomyocarditis–left ventricular endocardial fibrosis (FEMC–LVEF) complex compared with cats with hypertrophic cardiomyopathy (HCM) or cats with grossly and microscopically unremarkable hearts (designated non-cardiac disease controls) (Donovan et al., 2018). Granulomatous inflammation involving the heart, liver, lymph nodes and other tissues, endocarditis, bacillary angiomatosis, peliosis hepatitis, uveitis and vasoproliferative tumours have been reported in cats, dogs and humans infected with *Bartonella henselae* (Alejandra et al, 2018). Cats infected with *Bartonella henselae* had moderately enlarged hearts with myocardium of both left and right ventricles and to a lesser extent the atria and interventricular septum contained numerous multifocal spherical, 1-3 mm discrete,

uniform, slightly firm, white-creamy elevated nodules (Berkowitz et al., 2020) and pyogranuloma with larger granulomas with haemorrhage and central necrosis with loss of cardiocytes (Varanat et al., 2012). *Bartonella henselae* were consistently found in cats with pyogranulomatous myocarditis and diaphragmatic myositis through molecular and immunohistochemical studies (Varanat et al, 2012).

2.3 Detection of *Bartonella henselae*

Since *Bartonella* spp. are fastidious, slow-growing bacteria, therefore the most frequently used techniques for the detection of acute and chronic infections were specialised microbiological culture techniques, polymerase chain reaction (PCR), immunohistochemistry (IHC) and serology test (Clarridge et al., 1995). Specialised culture techniques including lysis centrifugation, cell culture isolation and growth enrichment in insect biochemical composition growth media are the "gold standard" for confirmation of Bartonella infection (Alejandra et al., 2018).

(i) Bacteria culture and isolation

Bacteria culture and isolation provides the definitive diagnosis for detection of Bartonellosis in cats using blood and tissue samples. *Bartonella* spp. are fastidious and slow growing bacteria that can be cultured successfully using agar plates containing 5% defibrinated rabbit or sheep blood maintained at 35 °C in a high humidity chamber with 5% CO2 concentration. Prolonged incubation times are required for isolation as bacterial colonies that may be visible 10–56 days after inoculation. *Bartonella* spp. are

small, curved, gram-negative rods that stain positively with silver stains such as the Warthin–Starry stain (Breitschwerdt, 2008).

The organisms have been found much easier to culture from hosts such as cats with *Bartonella henselae* as compared to infected humans and dogs. *Bartonella henselae* cultured from blood collected using sterile equipment and aseptic technique was much more likely to be successful in cats with severe bacteraemia (Breitschwerdt, 2008).

(ii) Serology

Serology can be performed using indirect immunofluorescence assay (IFA), Enzyme-linked Immunosorbent assay (ELISA) or Western Immunoblot. Serological tests appeared to have good specificity and can be used to confirm exposure or ongoing infection, but due to poor sensitivity, serology has limitation in its used for predicting bacteraemia in potentially sick cats (Alejandra et al., 2018). It was reported that *Bartonella henselae* specific antibodies were not detectable in some bacteraemia cats and the culture of *Bartonella henselae* from some cats with detectable antibodies were not successful (Breitschwerdt, 2008). Using serologic testing as an only diagnostic tool may provide inaccurate results as false-positive test results are common regardless of the types of assay used. The serological results should be interpreted in conjunction with blood culture or molecular test such as PCR. Serum IgG antibodies will persist in experimentally infected animals for prolonged periods and the duration of antibodies persist following clearance of an infection is unknown. The clearance of Bartonella infection is difficult to be documented due to the relapsing nature of feline

bacteraemia and the insensitivity of culture and molecular methods in detecting low levels of bacteraemia (Guptill, 2010).

(iii) Molecular detection

PCR technique is a highly specific and rapid method for definitive species identification in the diagnosis of Bartonella species infections (Dauga et al., 1996). Using PCR, samples such as blood and tissue samples (i.e. lymph node, cardiac valve, liver, spleen and cutaneous skin lesions) can be used for diagnosis of *Bartonella* spp. Besides that, PCR enables rapid detection of cultured and uncultured bacteria. Detection of *Bartonella henselae* are based on the recognition of sequences within the 16S rRNA gene. The products of PCR reactions are then sequenced and species and/or strain of *Bartonella* can be identified. If compare with bacteria culture, the pathogenic *Bartonella* spp. can be differentiated rapidly (Fenollar and Raoult, 2004).

Real time PCR has advantages such as speed, simplicity, reproducibility, quantitative capacity and low risk of contamination as compared to conventional PCR (Fenollar and Raoult, 2004). Recently, a newer tool, LightCycler apparatus (LCN-PCR) using real- time nested PCR assay had allowed the diagnosis of endocarditis due to *Bartonella* in a shorter time frame. The test has allowed clinical detection of patients with blood culture-negative endocarditis and patients with both unexplained fevers directly associated with elevated titres *Bartonella* spp. antibodies. Besides, LCN-PCR can also be used in detection of systemic *Bartonella* infections such as chronic bacteraemia, bacillary angiomatosis, peliosis hepatis and cat scratch disease with visceral involvement (Zeaiter et al., 2003).

Immunohistochemistry is superior than other antigen detection techniques and able to identify the *Bartonella* spp. directly in the tissue samples such as cardiac valves or lymphoid organs. When combine with histology, immunohistochemistry can establish correlations between antigen localisation and histopathological lesions more effectively (Webster et al., 2010).

2.4 Zoonotic implications of *Bartonella henselae*

Bartonella spp. are zoonotic and humans especially cat owners are at risk of the disease. CSD caused by *Bartonella henselae*, Carrion's disease caused by *Bartonella bacilliformis*, trench fever caused by *Bartonella quintana*, endocarditis caused by *Bartonella quintana* and *Bartonella henselae*, bacillary angiomatosis caused by *Bartonella quintana* and *Bartonella henselae* and hepatic peliosis caused by *Bartonella henselae* (Vayssier-Taussat et al., 2016). In humans infected with Bartonellosis, *Bartonella henselae* had colonised endothelial progenitor cells (EPCs) which then reduced the repair and regeneration of endothelial cells and lead to vascular inflammation and degeneration (Salvatore et al., 2015).

However, *Bartonella henselae* remains to be the most commonly diagnosed in humans. The bacteria are transmitted from cats to humans via a scratch or bite through a wound on the skin (Magno and Spatar, 2009). It can also be transmitted via a bite from an infected flea (Magno and Spatar, 2009). Clinical sign reported in human patients diagnosed with CSD was benign regional lymphadenopathy especially in immunocompetent patients (Chomel et al., 2016). A papule followed by a pustule may developed within 7 to 12 days at site of scratch and regional lymphadenopathy may

developed which usually involved a single lymph node 1 to 3 weeks later. The lesion will persist for few weeks to months (Chomel et al., 2016). The affected lymph node is often warm, tender and erythematous (Girma et al., 2019). Approximately 50% to 85% of patients have only single node involvement and the most commonly affected node is the axillary and epitrochlear, head and neck and inguinal nodes (Lieberman, 2017). *Bartonella henselae* causes vascular proliferative disease of the skin with multiple, blood filled and cystic nodules. Abscessed lymph nodes occur occasionally. Besides skin lesion, low-grade fever, malaise, headache, anorexia, aching and splenomegaly were often reported. In some cases, atypical manifestations may develop in 5% to 9% of CSD patients, such as Parinaud oculoglandular syndrome, encephalitis, endocarditis, hemolytic anaemia, hepatosplenomegaly, glomerulonephritis, pneumonia, relapsing bacteraemia, and osteomyelitis (Chomel et al., 2016).

In immunocompromised patients, *Bartonella henselae* infection can cause prolonged fever with or without bacteraemia. Bacillary angiomatosis and bacillary peliosis has been reported in this group of immunocompromised individuals (Koehler et al., 1993). Study has shown that affected individuals with chronic vascular proliferative lesions that were clinically and histologically similar to verruga peruana were caused by *Bartonella bacilliformis*. HIV patients with CD4+ cell counts of < $50/mm^3$ had been associated with greater risk to develop bacillary angiomatosis. Cutaneous bacillary angiomatosis is a tumour-like growth pattern with capillaries proliferation that have protuberant epithelioid endothelial cells histologically. These

lesions were always mistaken as pyogenic granuloma, Kaposi sarcoma and angiosarcoma (Chomel et al., 2004).

2.5 Risk Factors of Bartonellosis in Cats

Stray cats, shelter cats, cats living in multi-cat household, free roamers and cats living in tropical countries with hot and humid conditions and predisposed to flea infestation were found at greater risk of *Bartonella henselae* infection (Girma et al., 2019). Bites, scratches (in the absence of fleas), grooming, sharing of litter boxes and food dishes among cats cannot transmit *Bartonella henselae*. The transmission of *Bartonella henselae* will not occur during mating between bacteraemia female cats and uninfected males or during gestation period of infected female or in the neonatal period, provided that the cats are in flea-free environments (Pennisi et al., 2013).

Cats were reported to have higher chances to be infected with *Bartonella henselae* during the first year of life as bacteraemia appears to be more common in cats less than 1 year old compared to the adult cats. Chomel (1995) reported that more than 90% of cats less than one year old had *Bartonella henselae* antibodies and this indicates infection at early age. For bacteraemia cats, flea infestation were more frequent observed as compared to non-bacteraemia cats and this association was higher in pet cats (Chomel et al., 1995). Bacteraemia usually lasts from a few weeks to a few months (Chomel, 2000). Stray cats or cats acquired from shelter were more bacteraemic than cats bred at home or acquired (adopted) from friends or breeders. Cats that hunt and managed outdoor might be predisposed to flea exposure. However,

there was no significant association between indoor or outdoor status of cats and either bacteraemia or positive serologic status (Guptill et al., 2003).

Cats living in multi cat households were more likely to be bacteraemia and seropositive than cats living in single cat household (Gurfield et al., 2001). Non-neutered and especially male cats were twice more likely to be bacteraemia than neutered cats (Zangwill et al., 1993). In contrast, Sander (1996) stated that female cats were more at risk than the male cats. However, another study found that the sex of the cats, bacteraemia and bartonellosis were not associated (Gurfield et al., 2001), and therefore sex of the cat was not a risk factor. Cats infested with fleas during the preceding 6 months were more likely to be seropositive than cats without fleas and this risk factor were found associated with CSD (Gurfield et al., 2001).

In conclusion, stray cats or cats living in a shelter, cats with flea infestations and cats that hunt were at higher risk for *Bartonella henselae* infection. Indoor cats free of fleas and cats that were seronegative for *Bartonella henselae* were the least likely to be bacteraemic (Guptill et al., 2003).

2.6 Myocarditis in Cats

Myocarditis is the inflammation of the heart's muscular wall, also known as myocardium in the absence of ischemia that results in injury to the cardiac myocytes and cardiac dysfunction (Schober, 2016). Primary myocarditis can be caused by an acute viral infection such as feline parvovirus (FPV) or a post viral autoimmune response whereas secondary myocarditis caused by specific pathogens including

bacteria such as *Bartonella henselae*, protozoa such as *Trypanosoma cruzi* and *Toxoplasma gondii* and fungi and by drugs, chemicals, physical agents and systemic inflammatory diseases (Schober, 2016).

Myocarditis may be focal or diffused throughout the myocardium. Cats with myocarditis will show clinical signs such as arrhythmias, cough, exercise intolerance, difficulty in breathing, weakness, collapse, fever and other symptoms related to infections (German, 2010). On gross pathological examination, patchy myocardial discoloration and minute bleeding may be seen. Histopathologic lesions include inflammatory cell invasion, interstitial oedema, myocyte degeneration and necrosis will be seen and has been extensive and diffuse (Schober, 2016).

Cats with focal myocardial inflammation may be asymptomatic. Endomyocarditis were commonly reported to cause acute death with or without signs of pulmonary oedema lasting for 1 to 2 days in young cats. Histopathological changes showed focal or diffuse lymphocytic or plasmocytic and histiocytic infiltrates with few neutrophils. In adjacent myocytes, degenerative and lytic changes were observed. In chronic endomyocarditis, it was associated with a minimal inflammatory response with myocardial degeneration and fibrosis (Ware, 2011).

Subclinical lymphoplasmacytic myocarditis has been found in cats as a result from sepsis, bacterial endocarditis or pericarditis (Ware, 2011). *Bartonella henselae* causes pyogranulomatous myocarditis and diaphragmatic myositis in cats. The coalescing granulomas were found distributed throughout the myocardium and macrophages with a rare multinucleated giant cell are observed histologically in cats (Varanat et al., 2012).

2.7 Cardiac Biomarkers

Cardiac biomarkers are used in cats with cardiac and systemic diseases, and the two cardiac biomarkers generally used in practice are N-terminal pro-B type natriuretic peptide and cardiac troponin I. The cardiac biomarkers offered a straight forward and accessible adjunct test for veterinarians in diagnosing feline cardiac diseases (Borgeat et al., 2015).

2.8 Cardiac Troponin I (cTnI)

Serum proteomics which includes the analysis of circulating biomarkers of myocardial injury such as cardiac troponin (cTn) concentration is used to diagnose acute myocardial injury (Schober, 2016). cTn is recognised as an objective measure of cardiomyocyte status in both cardiac and noncardiac disease, acting as additional information to echocardiography and electrocardiogram (Langhorn and Willesen, 2016). It is a structurally bound proteins which regulate the calcium-mediated interaction between actin and myosin in skeletal and heart muscle (Burgener et al., 2006). Injury and damage to the cardiomyocytes and sarcolemmal membrane dissociates troponin from the actin. This causes the leakage of troponin into the extracellular space and troponin will then enters the circulation (Oyama, 2013).

cTnI is an excellent marker for myocardial injury because the increase in level and duration are proportional to the severity of injury (DeFrancesco, 2011). The normal cTnI concentration range established by Lean et al., (2019) was 0.00 – 0.05 ng/mL and any value above 0.05 was considered abnormal. cTn may be helpful in the

diagnosis of myocardial contusion, myocarditis, congestive heart failure, doxorubicin-induced cardiotoxicity and in distinguishing between idiopathic pericardial effusion and pericardial effusion caused by hemangiosarcoma (Burgener et al., 2006).

In the veterinary clinical settings, the cTnI concentration were found highly elevated in animals with suspected or confirmed myocarditis (DeFrancesco, 2011). Infectious diseases such as Babesiosis or Ehrlichiosis will lead to an increase in cTnI concentration due to myocarditis (Burgener et al., 2006). Acute myocardial infarction is rare in dogs and cats. The common heart disease in cats and dogs are chronic heart diseases such as myxomatous mitral valve degeneration (MMVD) and dilated cardiomyopathy (DCM) in dogs and hypertrophic cardiomyopathy (HCM) in cats. Thus, cTn may provide both diagnostic and prognostic information (Oyama, 2013) as a guide for clinician to monitor disease progression.

2.9 The use of cardiac biomarkers

cTnI is normally use to detect myocarditis and feline cardiomyopathy whereas N-terminal pro-B type natriuretic peptide (NT-proBNP) is used to detect occult cardiomyopathy and differentiate cardiac versus noncardiac causes of respiratory signs (Oyama, 2013).

The cardiac biomarkers NT-pro BNP and cTnI are increased in hyperthyroid cats, but the levels returned within the normal range 3 months after treatment with Iodine-131. Thus, it was suggested that these cardiac biomarkers cannot efficiently differentiate between cats with primary HCM and cats with thyrotoxic

cardiomyopathy (Sangster et al., 2013). Both NT-pro BNP and cTnI were higher in cats with cardiomyopathy than hyperthyroidism, but there were significant overlaps between groups and this suggests that neither biomarker can effectively differentiate between cats with hyperthyroidism and primary cardiomyopathy (Sangster et al., 2013).

CHAPTER 3: MATERIALS AND METHODS

3.1 Sample collection

Prior to the conduct of this study, an approval from Institutional Animal Care and Use Committee (IACUC) was obtained (UPM/IACUC/AUP-U105/2020). Overtly healthy cats presented to University Veterinary Hospital (UVH) were recruited upon the cat owners' approval to participate in this study.

A total of 64 client-owned overtly healthy cats were recruited among patients of the University Veterinary Hospital, Universiti Putra Malaysia and subjected to physical and oral examination. Cats with oral diseases or gingivitis were noted. The cats were gently restrained and 3 ml of blood was collected via jugular venipuncture. Blood samples was stored in plain tube and ethylenediaminetetraacetic acid (EDTA) tubes. Blood in plain tube were centrifuged at 300 × g for 5 minutes and serum collected. Both serum and whole blood samples were stored at -20°C freezer until analysis.

3.2 Survey

Information of the cats including age, sex, neutering status, management, vaccination status, owners' knowledge on flea infestation, history of flea infestation, types of flea prevention products used and application of flea prevention products were collected. For age, each cat was further categorised in grouping based on AAFP-AAHA Feline Life Stage Guidelines 2010 (Vogt et. al., 2010). For management, the cats were categorised into free roamer, indoor, outdoor and semi roamer. After

physical and oral examination, the cats were categorised into group with evidence of oral diseases such as gingivitis, stomatitis, halitosis and oral ulcer and also group without any oral disease. For the history of fleas, the cats were categorised into yes, no and uncertain. As for the owners that did applied flea prevention products on their cats, the brand of the flea prevention products used were recorded. The frequency of application of flea prevention products on the cats were categorised into every month, every 3 months, every 6 months and only when there are fleas.

3.3 DNA extraction and molecular detection of *Bartonella henselae*

Frozen whole blood samples were thawed at 20 to 30 minutes at room temperature prior to DNA optimisation. The buffer for DNA optimisation is prepared. The buffers are RBC lysis Buffer (RLB): 0.155 mol/L NH_4Cl, 10 mmol/L $KHCO_3$ and 0.1 mol/L EDTA (Na_2) in 1000 mL of distilled H2O. The pH was adjusted to 7.6. The extraction buffer used is 1.5 mol/L Tris pH 7.6, 0.4 mol/L disodium salt of ethylenediaminetetra acetic acid (Na_2EDTA), 2.5 mol/L NaCl, 2% Cetyl trimethyl ammonium bromide (CTAB) 850 mL H_2O. The pH is adjusted to 8.0 and the final volume is made to 1 L. 10% SDS (Sodium dodecyl sulfate), β-Mercaptoethanol, Chloroform: Isoamyl alcohol (24:1), Isopropanol, 70% and 90% ethanol are prepared.

DNA were extracted using the conventional method (Guha et al., 2017). Two hundred and fifty microlitre of blood sample in EDTA tube was transferred to an Eppendorf tube. The samples were centrifuged at 2700 RCF for 7 minutes at 4°C and the plasma was aspirated out and discarded. One millilitre of RLB was added to the precipitate, mixed gently and was placed at room temperature for 2 minutes. The

mixture was then centrifuged at 2700 RCF (4900 rpm) for 6 minutes at room temperature. The supernatant was discarded and this step was repeated 1 to 2 times until a white coloured pellet was obtained. 500 µL of prewarmed DNA extraction buffer was added to the pellet followed by 30 µL of 10% SDS and 2 µL of B-Mercaptoethanol respectively and the mixture was mixed gently. The mixture was then incubated at 60°C for 1 hour. Five hundred microlitre of Pheno-Chloroform: Isoamylalcohol (25:24:1) was added to the mixture and shaken well. The mixture was then centrifuged at 11000 RCF for 12 minutes at 4°C. Four hundred microlitre of supernatant was pipetted out in another fresh sterilized centrifuge tube containing 400 µL chilled absolute ethanol. The tube was shaken for a while until fine white threads appeared in the solution. The sample was then centrifuged at 11000 RCF for 12 minutes at 4°C. The supernatant was discarded without disturbing the pellet and 400 µL of 70% ethanol was added to it. The sample was then centrifuged at 11000 RCF for 12 minutes at 4°C. These two steps were repeated again with 70% ethanol. The supernatant was discarded and the pellet was allowed to dry at 37°C. The pellet was then dissolved in 100 µL of TE buffer overnight. The DNA solution was then stored at −20°C for future use (Guha et al., 2017).

PCR was performed in a 25.0 µL reaction mixture containing 12.5 µL My Taq RedMix (Bioline, USA), 3.5 µL deionized distilled water (ddH_2O), 7.0 µL of DNA template , 1.0 µL of forward primer (321s forward: 5'- AGA TGA TGA TCC CAA GCC TTC TGG- 3') and 1.0 µL of reverse primer (983as reverse: 5'-TGT TCT YAC AAC AAT GAT GAT G- 3') targeting the internal transcribed spacer (ITS) region of Bartonella species (Maggi and Breitschwerdt, 2005). Positive control comprising of 3 µL of *Bartonella henselae* and 4 µL of deionized distilled water (ddH_2O) and negative

control of 7 µL deionized distilled water (ddH_2O) were included in the reaction. DNA were amplified in Mycycler thermal cycler (Biorad, USA) with denaturation step at 95°C for 20 seconds, annealing at 51°C at 30 seconds and extension at 72°C for 2 minutes. Amplified DNA fragments were separated via agarose gel electrophoresis.

In gel electrophoresis, 1.5% agarose gel were made by adding 1.5g agarose powder in 100 mL 1x TAE. The mixture was pre-heated and allowed to cool for 1 minute before mixing with 4 µL Red Safe staining. The gel was casted and it was left for harden for 30 minutes. The gel was then placed into gel tank and 1x TAE was added. 10.0 µL PCR product and 4.0 µL ladder were loaded into gel wells. Electrophoresis was set to 90V, 350 mA and run for 30 minutes.

3.4 Cardiac Troponin I (cTnI)

The concentration of serum cTnI were determined using the Siemens Dimension ® Xpand Plus analyser. Eighty microliters of serum were required for the "sandwich" one-step immunoassay. cTnI concentration were measured based on colour change of the samples quantitatively. The normal range used is 0.00 – 0.05 ng/ml (Lean et al., 2019).

3.5 Statistical Analysis

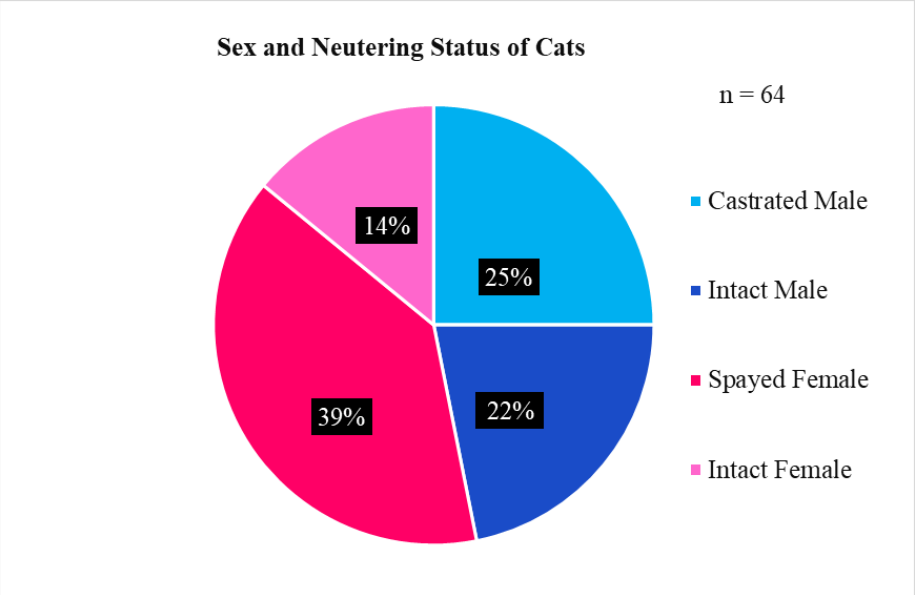
The data were analysed descriptively using Microsoft Excel 2016. The distribution of data and serum cTnI concentration were analysed using descriptive analysis and the data were presented using pie charts. Risk factors analysis will be

evaluated by Chi Square Test using IBM SPSS software version 25.0. Significance among means was determined at p=0.05.

CHAPTER 4: RESULTS

In this study, a total of 64 overtly healthy cats were recruited. The majority of the cats (53%, n= 34/64) were female, in which 39% (n= 25/64) were spayed female and 14% (n= 9/64) were intact female. The remaining 47% (n= 30/64) of the cats were male cats, in which 25% (n= 16/64) were castrated and 22% (n=14/64) were intact males (Figure 1).

Figure 1: The distribution of sex and neutering status (%) among cats (n = 64) recruited in this study



These cats were in age groups as follows; junior (n= 25) and prime (n = 20) categories, followed by kitten (n= 9) and mature (n= 9) and lastly senior (n= 1) (Figure 2). The mean age of the cats was 3.25 ± 3.03 years ranging from 3-month to 14-year-old. Majority were managed indoor (n= 44), followed by as semi roamer cats (n= 13), outdoor cats (n= 6) and lastly free roamer cats (n= 1) (Figure 3).

Figure 2: The distribution of age (%) among cats (n = 64) recruited in this study

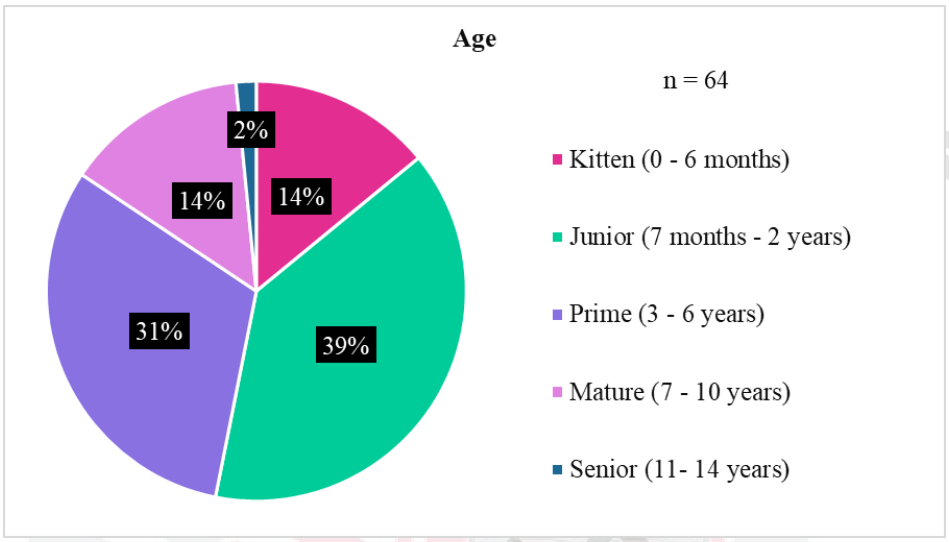
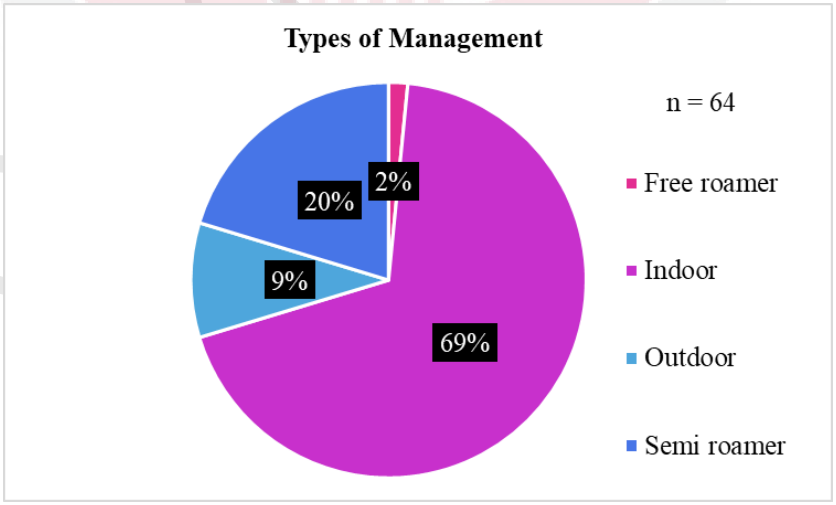


Figure 3: The distribution of types of management (%) among cats (n = 64) recruited in this study



As for health screening and preventive medicine, it was found that 64% (n= 41/64) of the cats received yearly medical check-up and 72% (n= 46/64) of the cats were vaccinated (Figure 4 and 5).

Figure 4: The distribution of vaccination status (%) among cats (n = 64) recruited in this study

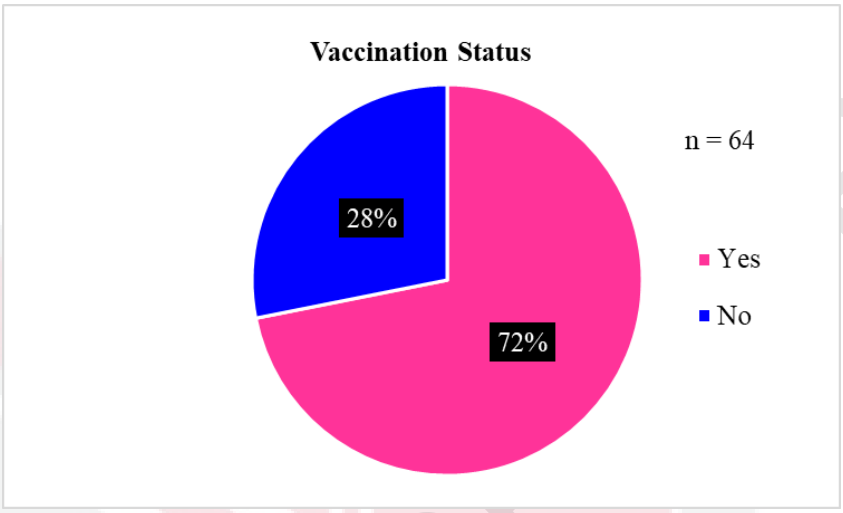
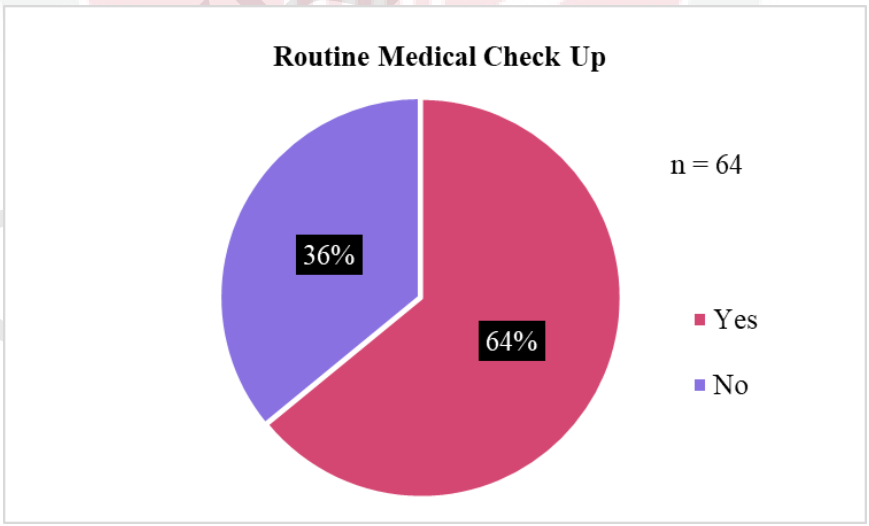


Figure 5: The distribution of routine medical check-up (%) among cats (n = 64) recruited in this study



A routine physical examination for all the cats found that 20.3% (n= 13/64) of the cats had gingivitis or oral diseases, in which 15.6% (n= 10/64) had gingivitis, 1.6% (n= 1/64) had stomatitis, 1.6% (n= 1/64) had halitosis and 1.6% (n= 1/64) had oral

ulcer. The remaining 79.7% (n= 51/64) of cats were free from gingivitis or oral diseases (Table 6).

Table 6: The distribution of evidence of oral disease (%) among cats (n = 64) recruited in this study

	Evidence of Oral Disease				
	Gingivitis	Stomatitis	Halitosis	Oral ulcer	No oral disease
n	10	1	1	1	51
%	15.6	1.56	1.56	1.56	79.7

All the cats recruited were found free of fleas upon recruitment. Based on the history obtained from their owners, 39 out of 64 (61%) cats claimed that their cats were flea infestated whereas 14 (22%) never had flea infestations and lastly, 11 (17%) cat owners were uncertain if their cats had fleas or not.

In this group of cats, 53 of the cat owners applied flea prevention products for their cats, whereby a majority of them used Frontline (n= 21), followed by Advocate (n= 19), Advantage (n= 12) and Revolution (n= 5). Some owners used more than one types of flea prevention products on their cats as they interchanged between brand despite being compliant with flea prevention. Only 5 cat owners could not recall what brand of flea prevention products used (Figure 7). However, majority of the owners were not compliant with the recommended frequency of the flea prevention products as they only applied on their cats every 6 months (n= 17) and every 3 months (n=7),

and some applied only when there are fleas (n= 14). There were 15 cat owners that routinely adhere to the advice and recommendation of the flea prevention regime. (Figure 8). followed by every month (n= 15)

Figure 7: The distribution of application of flea prevention products (%) among cats (n = 64) recruited in this study

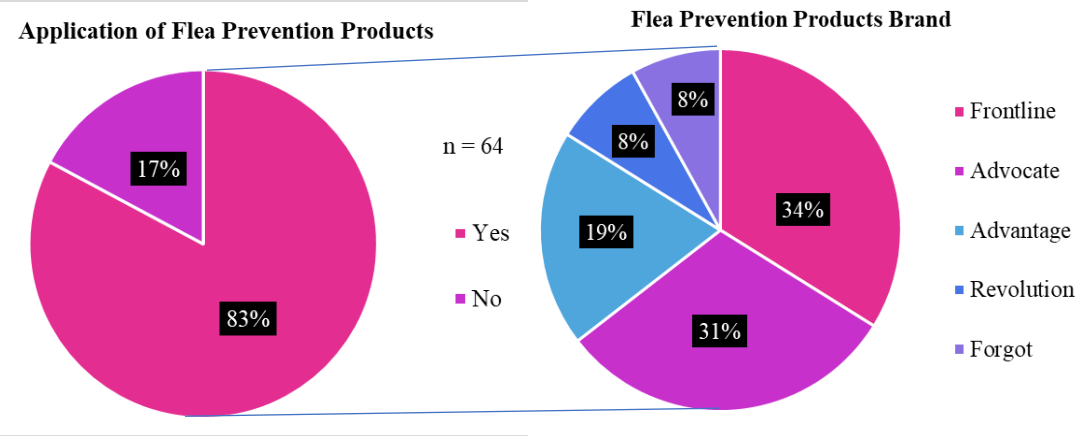
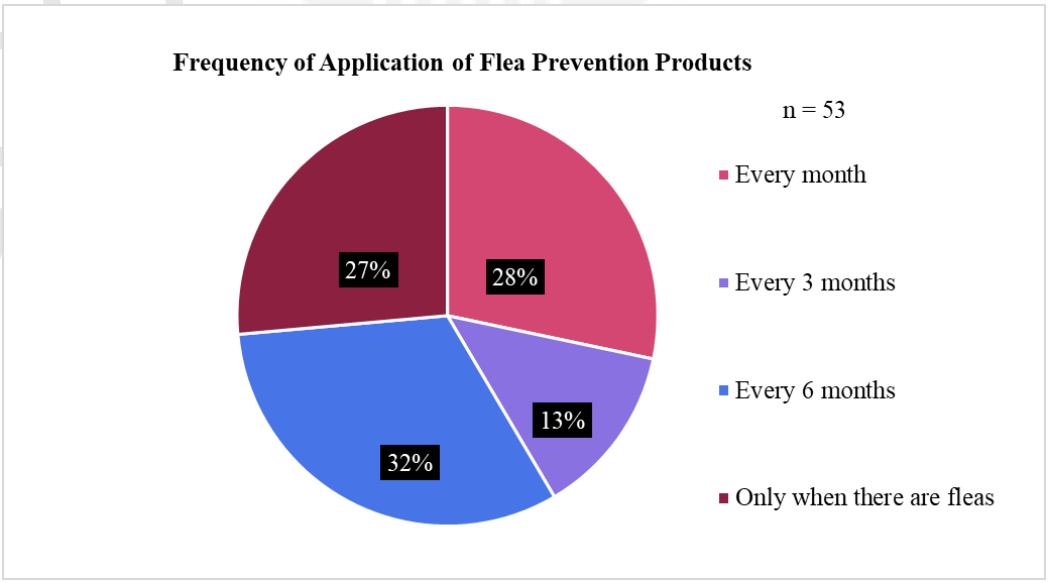
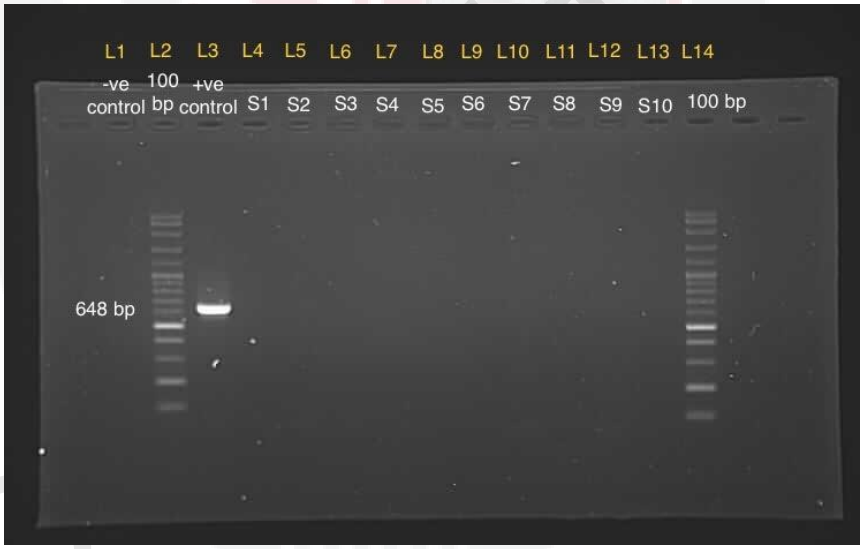


Figure 8: The distribution of frequency of application of flea prevention products (%) among cats (n = 64) recruited in this study



By using the extracted DNA from the blood samples of the 64 cats, Polymerase Chain Reaction (PCR) followed by gel electrophoresis were performed. In this study, all the cats were negative for *Bartonella henselae* (Figure 9).

Figure 9: A representative of gel image of negative *Bartonella henselae* DNA from blood samples using specific primer targeting ITS sequence gene of *Bartonella henselae* to produce 600 – 700 PCR products. Lane 1 were negative control. Lane 2 and Lane 14 were 100 bp DNA marker. Lane 3 were positive control. Lane 4 to 13 were extracted DNA from samples 1 to 10.



From the 64 overtly healthy cats, 51 cats had normal serum cTnI concentration with mean value of 0.029 ± 0.014 ng/mL and 13 cats had high serum cTnI level with mean value of 0.352 ± 0.868 ng/mL (Table 10). The clinical presentation and disease diagnosed in cats were as listed in Table 11.

Table 10: The distribution of cTnI concentration (ng/ml) among cats (n = 64) recruited in this study

	cTnI (ng/ml)	
	Normal (n= 51 cats)	High (n=13 cats)
Range	0.00 - 0.05	0.051 - 3.45
Mean	0.029	0.352

Table 11: Serum cTnI concentration, body system involved, differential diagnosis in cats

Cat Number	**serum cTnI concentration (ng/ml)**	**Body systems**	**Differential Diagnosis**
Cat 50	0.09	Cardiovascular System	Hypertrophic cardiomyopathy
Cat 11	3.45	Cardiovascular System	Restrictive cardiomyopathy
Cat 40	0.09	Urinary System	Suspected kidney disease
Cat 62	0.19	Urinary System	Kidney and liver disease
Cat 60	0.09	Urinary System	Feline Lower Urinary Tract Disease (FLUTD)

Cat 63	0.08	Respiratory System	Feline Upper Respiratory Disease (FURD)
Cat 1	0.07	Respiratory System	Feline asthma
Cat 28	0.06	Respiratory System	Feline asthma
Cat 57	0.09	Visual System	Corneal ulcer
Cat 2	0.06	Digestive System	Gastrointestinal issues (Foreign body obstruction)
Cat 36	0.11	Skeletal System	Pelvic fracture
Cat 58	0.11	Skeletal System	Locked jaw
Cat 48	0.07	Reproductive System	Castration

CHAPTER 5: DISCUSSION

In this study, all the pet cats recruited were tested negative for *Bartonella henselae* and therefore were not at risk of *Bartonella henselae* infections as compared to the study done by Hassan et al., (2016) with high risk at a prevalence of 16.9%. From that study, it was noted that 30 out of 284 cats were infested with fleas. Another study locally (Kam et al., 2020) shown a low detection rate of *Bartonella henselae*-infected client-owned cats in Klang Valley at 2% in the blood samples of 50 cats. Kam et al., (2020) noted that only the cat with *Bartonella henselae* positive was infested with fleas at the time of study. Therefore, it could be speculated that flea was an important risk factor for Bartonellosis but the cats recruited in this study were free of flea infestation upon recruitment.

Flea-infested cats possessed a higher risk to be infected with *Bartonella henselae* (Guptill et al., 2003). However, none of the cats recruited had fleas but only 3 cats were found to have mite and 2 cats had lice infestation. Since all the cats were free from flea infestations during recruitment, that may directly contribute to low or no risk towards detection of *Bartonella* spp. Besides that, most cats appeared overtly healthy and if infected, it could be suspected that these cats might be having transient bacteraemia at the time when the blood were collected.

The risk factors such as sex and the neutering status could not be determined in this current study but it has been reported as not an important risk of *Bartonella henselae* infections in cats (Gurfield et al., 2001). However, younger cats which were less than one year old have higher chances to be infected with *Bartonella henselae* as bacteraemia appeared more common in younger cats (Chomel et al., 1995). Cats with

gingivitis or any other oral diseases may be associated with *Bartonella henselae* infections or other causes such as dental diseases (Ueno et al., 1996). It was observed that a total of 13 cats had mild oral lesion but it was uncertain if it was associated to *Bartonella henselae* infection in this study. Using clinical sample such as blood, molecular detection using PCR was suitable if the cats were at bacteraemic phase but the viable *Bartonella henselae* circulating in the blood was low after onset of bacteraemia due to host defence mechanisms (Heininger et al., 1999), thus detection may be negative.

The better method for detection of transient bacteraemia cats is using Indirect Immunofluorescence Assay (IFA) but this was not carried out at the time of the study. Therefore, it could not be confirmed as to whether the cats were really free from *Bartonella henselae* or that these cats were asymptomatic due to low bacteraemia. Serological testing using IFA allows detection of IgG antibodies against *Bartonella henselae* antigen. IgG antibodies was reported detected in the cats' bodies that had past infection as the antibodies will remain in the body for more than 2 years (Metzkor et al., 2003). This current study could not confirm that the pet cats were free from the infection against *Bartonella henselae* by doing PCR alone as they might have transient bacteraemia. The detection rate of *Bartonella henselae* will be higher if IFA was used together with PCR as the detection sensitivity increases (Yanagihara et al., 2018).

It could be speculated that no detection of feline Bartonellosis in this study could be related to the owners' compliance in the usage of flea prevention products on their pet cats. The application of flea prevention products routinely (i.e. every month (28% of the cats) may had prevented their cats from being infected with *Bartonella*

henselae, despite having a majority of these cat owners that did not adhere to the monthly application practice as has been advised.

Most of the cats were maintained indoor opposed to free roamers and outdoor cats that have been reported to have higher risk to be infected with *Bartonella henselae* as their chances of flea exposure would be higher (Guptill et al., 2003). Besides that, cats living in multi cat households had higher potential to be infected as there might be co-infection of *Bartonella henselae* among cats and strict flea prevention protocol is hard to accomplish due to the large number of cats (Gurfield et al., 2001). Despite having different types of management, the cats that possess higher risk were still not infected with *Bartonella henselae*, thus, we can suggest that the flea prevention practices by among these cat owners were good.

The association between *Bartonella henselae*-infected cats and the occurrence of myocarditis could not be proven as *Bartonella henselae* were not detectable in the blood samples obtained. These cats were then not at risk of the disease and thus, risk factors were not determined. Despite that all cat appear apparently healthy, it was found that 13 cats had an elevated serum cTnI concentration and it could be speculated that these cats may had some underlying disease such as heart and kidney diseases. Elevated cTnI have been reported in heart diseases such as HCM, DCM, myocarditis, myocardial infarction (MI) and heart damage secondary to infectious disease. In cats with heart diseases such as HCM, the abnormal intramural coronary artery will lead to prolonged ischemia followed by myocardial fibrosis had shown increased in cTnI concentration (Maron et al., 1986). In a study done by Ghiassi et al., (2020), cats with respiratory diseases will become hypoxia which subsequently causes decreased in

myocardial oxygen supply. The death of the myocardial cells due to lack of oxygen causes sub-lethal injury results in the release of serum cardiac troponin I into the bloodstream and thus contributes to the elevation of cTnI concentration.

Besides that, other causes such as traumatic injury, immune mediated haemolytic anaemia (IMHA), snake bite envenomation, Adder bite poisoning, heat stroke and myotoxic injury due to ingestion of monensin which lead to cTnI elevation have been reported. Non-cardiac diseases, especially renal disease have been reported to indirectly contribute to an elevation in cTnI due to reduced renal clearance as troponins are too large for renal elimination (Langhorn and Willesen, 2016).

Therefore, apparently healthy cats with elevated cTnI may then directly alert their pet owners about possible underlying early stage or the risk of subclinical cardiovascular disease or renal diseases in their cats. This result obtained would prompt the cat owners to have their cats to carry out health screening. Early intervention and treatment of these disease at early stage may prevent serious and life-threatening condition and prolong life span in these cats.

The low detection rate of bartonellosis in cats might be due to the limitations present in this study in which all cats were not flea infested at the point of recruitment, owners carry out good flea prevention practices, most of the cats are kept indoor, vaccinated and did receive routine medical check-up. Perhaps the study could also be expanded to other groups of cats being stray, maintained outdoor, not on flea prevention and unhealthy cats. Another limitation is that cats with transient bacteraemia were not detected with the usage of PCR alone.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

This study shown that all the pet cats were free from *Bartonella henselae* infection, thus, it can be concluded these client-owned overtly healthy cats were not at risk of bartonellosis. However, the cat owners should still be made be aware of the zoonotic potential of *Bartonella henselae* infection and as they are at high risk for CSD especially in immunocompromised individuals. Routine ectoparasite control was practiced among the cat owners diligently in order to prevent flea infestations in their cats therefore might have reduced the risk of exposure to *Bartonella henselae*.

Serum cTnI concentration is a good biomarker to detect myocarditis and/or any heart diseases in cats. Although the association between *Bartonella henselae* and the development of myocarditis cannot be proven, however, the elevation of serum cTnI concentration some cats have indicated that these cats may have a heart diseases or non-cardiac diseases such as renal diseases, hyperthyroidism and many more. These cats are recommended for further health check.

Our recommendations for future study would be to increase the sample size to increase the possibility of getting cats that are infected with *Bartonella henselae*. By increasing the sample size, the results obtained will be more accurate and significant. Heavily flea infested cats, free roamers and outdoor cats should be recruited to increase the chances of detection of *Bartonella henselae* in cats. We also recommend to carry out serological testing such as Indirect Immunofluorescence Assay (IFA) to increase the sensitivity and thus increase the detection rate of *Bartonella henselae*.

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