MOLECULAR AND IMMUNOLOGICAL PROFILING OF *Bartonella* spp. IN SHELTER CAT POPULATION IN PENINSULAR MALAYSIA

By

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Chair : Farina Mustaffa Kamal, PhD
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Feline bartonellosis is a zoonotic disease caused by *Bartonella henselae* which may result in either mild or severe bacteremia in asymptomatic cats. Severe human infections such as cat scratch disease (CSD), endocarditis, and ocular bartonellosis have been associated with infections due to *Bartonella* spp. including *Bartonella henselae*. To date, no biosurveillance and immunological profiling in *Bartonella*-positive shelter cats have been conducted in Malaysia. A molecular prevalence study carried out in 2017 confirmed that 16.9 % (48/284) pet cats in Selangor, Malaysia were positive with *B. henselae*. However, this study was performed only on pet cats and represents a small cohort of cat population in Malaysia. Shelter environment remains as prime sources where emerging pathogens could arise as the result of mixing of animals from various origins; therefore, giving the possibility of inadvertent disease transmission between shelter cats to community cats and also human especially those with immunocompromised status. Therefore, this study was aim to determine the molecular, serological prevalence of *Bartonella* spp. in shelter cats, immunophenotyping of CD4 and CD8 as well as the association between each assay and also association with physical examination findings. Overall, blood was collected from 217 cats, flea from 100 cats and oral swabs from 176 cats were subjected to molecular analysis. A total of 122 serum collected were subjected to serological detection of IgM and IgG while 110 whole blood that were kept in -80°C freezer were subjected for immunophenotyping of CD4 and CD8. Molecular detection of *Bartonella* DNA was performed using conventional PCR detecting 16S-23S rRNA intergenic spacer sequence which yield a single band at 630 to 680 base pair. *Bartonella* DNA was detected from four cats (4/217;1.8%) in which three from cat's blood and one from oral swab. Meanwhile, 8.0% (8/100) *Bartonella* DNA were detected in flea from 100 cats. All the positive amplicon were sent for sequencing. Sequence analysis of the 16S-23S rRNA intergenic spacer gene fragments show the identification of *B. henselae* in blood (n=3), oral swab (n=1) and flea from one cat, *B. koehlerae* from one flea and *B. clarridgeiae* from six fleas. The sequence analysis reveals high sequence similarity of

98.7% with *B. henselae*, 97.9% *B. koehlerae* and 97.8%-100.0% with *B. clarridgeiae* respectively. Phylogenetic tree constructed based on *Bartonella* 16S-23S rRNA intergenic spacer gene sequences revealed a close genetic relationship between Malaysian *B. hensalae* strains from blood and oral swab samples with *B. henselae* of Korea and China strains while genetic relationship from flea samples were closely related with *B. henselae* from China strain, *B. koehlerae* from Palestine strain, and *B. clarridgeiae* from China and Australia strains. Of 122 cats with serum samples available for testing using IgM and IgG commercial indirect immunofluorescence assay (IFA) against *B. henselae*, 82.8% (101/122) cats demonstrated seroreactivity to *B. henselae* antigen. A total of 47.5% (58/122) cats were positive for IgM antibody alone, 65.6% (80/122) cats were positive for IgG antibody alone and 30.3% (37/122) were positive for both IgM and IgG. In addition, immunophenotyping of 110 frozen whole blood detecting CD4 and CD8 T cells were performed. Results showed that 60.0% (66/110) cats had abnormal ratio of CD4 to CD8. Only one cat was positive for both PCR and antibody assays against *Bartonella* spp. had abnormal CD4:CD8; however, it did not show any clinical signs. Statistical analyses suggested there were no association between PCR, serological test and CD4:CD8. Next, a survey on shelter management practices and their personnel awareness towards bartonellosis were also conducted by distributing a set of questionnaires to the shelter personnel. Surveys from management practices of shelter cats revealed that five out of ten shelters were practicing ectoparasite control and 93.3% (28/30) shelter's personnel were aware that cat and cat flea might contribute to zoonotic diseases. However, they were unaware about *Bartonella* can infect human and cat scratch disease (CSD). The high *B. henselae* seroreactivity among shelter cats with low molecular detection rate suggests previous infection or low bacteraemia level that was not detectable by conventional PCR analysis. In addition, this study observed the presence of more than one species in cat's fleas, while only *B. henselae* was detected in the cat hosts. As there were no clear symptoms and diagnostic markers that can be used to determine the status of *Bartonella* infection among cats, this may pose zoonotic risk to children, elderly and immunocompromised individual who are more susceptible to *Bartonella* infection.

Keywords: *Bartonella*, cats, PCR, IFA, flow cytometric analysis

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PEMPROFILAN IMUNOLOGI DAN MOLEKUL *Bartonella* spp. DALAM POPULASI KUCING PERLINDUNGAN DI SEMENANJUNG MALAYSIA

Oleh

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Bartonelosis felin merupakan penyakit zoonotik yang disebabkan oleh *Bartonella henselae* yang sering menyebabkan bakteremia yang ringan atau teruk dalam kucing yang tidak bergejala. Jangkitan-jangkitan teruk kepada manusia seperti penyakit cakar kucing (CSD), endokarditis, dan bartonelosis okular telah dikaitkan dengan beberapa spesies *Bartonella* termasuk *Bartonella henselae*. Sehingga kini, tiada bio-pengawasan dan pemprofilan imunologi dalam kucing perlindungan yang positif dengan *Bartonella* yang telah dilakukan di Malaysia. Kajian prevalen molekular yang dilakukan pada tahun 2017 mengesahkan bahawa 16.9 % (48/284) daripada kucing peliharaan di Selangor, Malaysia telah positif dengan *B. henselae*. Walaubagaimanapun, kajian tersebut hanya dilakukan ke atas kucing peliharaan yang mewakili sebahagian kecil daripada populasi kucing di Malaysia. Persekitaran di tempat perlindungan tetap mejadi sumber utama di mana patogen boleh muncul daripada pergaulan antara haiwan-haiwan yang mempunyai pelbagai latarbelakang; justeru, meningkatkan kebarangkalian jangkitan penyakit yang tidak disengajakan antara kucing perlindungan dengan kucing komuniti dan juga dengan manusia, terutamanya yang terimunokompromi. Oleh itu, kajian ini bertujuan untuk mengenalpasti prevalen molekular dan serologi, immunofenotip CD4 dan CD8 terhadap *Bartonella* dalam kucing perlindungan, serta perkaitan antara setiap ujian dan kaitan dengan penemuan pemeriksaan fizikal. Secara keseluruhan, darah daripada 217 ekor kucing, pinjal daripada 100 ekor kucing, dan air liur daripada 176 ekor kucing telah diambil dan analisis molekular telah dijalankan. Sebanyak 122 serum yang dikumpul bertujuan untuk mengesan antibodi IgM dan IgG manakala 110 darah yang disimpan di penyejuk beku yang bersuhu -80°C bertujuan untuk ujian immunofetotip CD4 dan CD8. Pengesanan molekular DNA *Bartonella* telah dijalankan dengan menggunakan tindak balas berantai polimeras (PCR) konvensional yang mengesan intergenik peruang sekuen (ITS) rRNA 16S-23S dengan menghasilkan jalur berukuran 630 ke 680 pasangan bes. DNA *Bartonella* telah dikesan dalam empat ekor kucing (4/217; 1.8%) di mana tiga daripada darah kucing dan satu daripada air liur. Sementara itu, 8.0% (8/100) *Bartonella* DNA telah dikesan dalam sampel pinjal daripada 100 ekor kucing. amplikon positif telah dihantar untuk

penentuan sekuen. Analisis sekuen daripada amplikasi fragmen dari sampel darah kucing yang positif (n=3), sampel air liur (n=1) dan sampel pinjal dari seekor kucing menunjukkan padanan dengan *B. henselae* manakala sampel pinjal daripada seekor kucing menunjukkan padanan dengan *B. koehlerae* dan pinjal daripada enam ekor kucing menunjukkan padanan dengan *B. clarridgeiae*. Analisis sekuen menunjukkan keserupaan yang tinggi terhadap *B. henselae, B. koehlerae* dan *B. clarridgeiae* iaitu masing- masing sebanyak 98.7%, 97.9% dan 97.8% sehingga 100%. Pokok filogenetik telah dibina dari sampel darah dan sampel air liur dan hasil analisa telah menunjukkan kaitan rapat dengan strain *B. henselae* dari Korea dan China manakala hasil analisa untuk sampel pinjal berkait rapat dengan strain *B. henselae* dari China, strain *B. koehlerae* dari Palestin, dan strain *B. clarridgeiae* dari China dan Australia. Daripada 122 ekor kucing yang mana sampel serum telah diambil untuk ujian IgM dan IgG menggunakan ujian imunofluoresens tidak langsung (IFA) terhadap *B. henselae*, 82.8% (101/122) kucing telah sero-reaktif terhadap antigen *B. henselae*. Sebanyak 47.5% (58/122) kucing positif untuk antibodi IgM sahaja, 65.6 % (80/122) kucing positif untuk antibodi IgG sahaja dan 30.3% (37/122) kucing positif untuk kedua-dua antibodi IgM and IgG. Tambahan pula, asai sitometri aliran untuk pengesanan CD4 dan CD8 telah dijalankan dimana 60.0% (66/ 110) ekor kucing mempunyai nisbah CD4 kepada CD8 yang abnormal. Hanya seekor kucing yang positif untuk kedua-dua ujian PCR dan antibodi untuk *Bartonella* spp. yang mempunyai CD4:CD8 yang abnormal; namun, ia tidak menunjukkan sebarang petanda klinikal. Analisa statistik mencadangkan bahawa tiada kaitan antara PCR, ujian serum, dan nisbah CD4 kepada CD8. Seterusnya, satu set soalan kaji selidik telah diedarkan kepada kakitangan pusat perlindungan haiwan kaji selidik bertujuan untuk mengenal pasti pengurusan pusat perlindingan haiwan dan kesedaran kakitangan pusat perlindungan haiwan terhadap penyakit bartonellosis. Analisa daripada pengurusan pusat perlindungan haiwan menunjukkan lima daripada sepuluh pusat perlindungan haiwan menggunakan racun ektoparasit ke atas kucing dan 93.3% (28/30) kakitangan pusat perlindungan haiwan menyedari bahawa kucing dan pinjal merupakan pembawa penyakit zonotik. Walaubagaimanapun, kebanyakan kakitangan tidak mengetahui apa itu *Bartonella* dan penyakit cakar kucing (CSD). Keputusan-keputusan menunjukkan terdapat kadar seroreaktif yang tinggi di dalam kalangan kucing perlindungan berserta peratusan yang rendah bagi pengesanan molekular, mencadangkan sama ada jangkitan lampau ataupun paras bakteremia yang tidak boleh dikesan menggunakan PCR konvensional. Tambahan lagi, beberapa spesies *Bartonella* telah dibawa oleh pinjal kucing, manakala hanya *Bartonella henselae* boleh dikesan dalam hos kucing. Tiada simptom dan penentu diagnosis yang jelas boleh digunapakai untuk menentukan status jangkitan *Bartonella* di dalam kalangan kucing. Hal ini boleh menyebabkan risiko zoonosis kepada kanak-kanak, orang tua, dan orang yang terimunokompromi yang lebih rentan terhadap jangkitan *Bartonella*.

Kata kunci: *Bartonella*, kucing, PCR, IFA, analisis sitometri aliran

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AIDS	Acquired Immunodeficiency Syndrome
BadA	*Bartonella* adhesion A
BAPGM	*Bartonella* Alpha-Proteobacteria Growth Medium
Beps	*Bartonella* effector proteins
BLAST	Basic Local Alignment Search Tool
CD11	Cluster of Differentiation 11
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
CI	Confidence Interval
CSD	Cat Scratch Disease
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EM	Electron Microscope
FBS	Fetal Bovine Serum
FeLV	Feline Leukemia Virus
FITC	Fluorescein Isothiocyanate
FIV	Feline Immunodeficiency Virus
FMO	Fluorescence Minus One
FPV	Feline Panleukopenia Virus
FSC-A	Forward Scatter Area
FSC-H	Forward Scatter Height
ftsZ	Cell Division Protein
gltA	Citrate Synthase Gene

GroEl	60 kDa Heat-Shock Protein
HIV	Human Immunodeficiency Virus
IFA	Indirect Immunofluorescence Assay
IFN-γ	Interferon Gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHC	Immunohistochemistry
IL	Interleukin
IL-10	Interleukin 10
IL-4	Interleukin 4
ITS	Intergenic Transcribed Spacer
LPS	Lipopolysaccharides
MEGA X	Molecular Evolutionary Genetics Analysis X
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear Factor Kappa B
OR	Odd Ratio
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
qPCR	Real-Time Polymerase Chain Reaction
RBC	Red Blood Cell
ribC	Ribflavin Synthase Gene
rRNA	Ribosomal Ribonucleic Acid
spp.	Species
SSC-A	Side Scatter Area

T4SS	Type IV Secretion Systems
TAA	Trimeric Autotransporter Adhesins
Th-1	T Helper 1
Th-2	T Helper 2
TLR	Toll-Like Receptor
TNF-α	Tumor Necrosis Factor Alpha
VEGF	Vascular Endothelial Growth factor
VirB4/D4	Virulence Factor B4/D4
Vomp	Variably Expressed Outer-Membrane

CHAPTER 1

INTRODUCTION

1.1.1 Overview

Bartonella spp. are gram-negative, hemotrophic, pleomorphic and fastidious bacteria classified under the family *Bartonellaceae*, order Rhizobiales and class Alpha-proteobacteria. Within the last few years, 45 *Bartonella* species have been discovered and 13 species have been recognized as being pathogenic for humans (Okaro et al., 2017). These pathogenic species include *Bartonella henselae*, *Bartonella elizabethae*, *Bartonella koehlerae*, *Bartonella clarridgeiae*, *Bartonella vinsonii* subsp. *berkhoffii* and *Bartonella rochalimae*.

Among these *Bartonella* species, *B. henselae* is the zoonotic species causing cat scratch disease (CSD) in human (Eremeeva et al., 2007; Avidor et al., 2004; Chomel et al., 2004) . *B. henselae* has two genotypes which are *B. henselae* type I and II and they are also known as *B. henselae* Houston I strain and *B. henselae* Marseille strain, respectively. Cats are considered as reservoirs in transmission of *B. henselae* to humans (Álvarez-Fernández et al., 2018). *Bartonella* infections are spread directly via cat scratch and bite or indirectly by flea vector. Although *B. henselae* often causes persistent bacteremia but cats could remain asymptomatic for months to years. Consequently, human can get infected by the bacteria through cat scratch, causing CSD whereby immunocompetent individuals often develop swollen lymph nodes, fever or chronic disease such as bacillary angiomatosis, endocarditis, convulsions, lymphadenitis, and neuroretinitis which are usually seen in immunocompromised individuals especially, HIV/AIDS patient, pregnant women or children (Amer and Tugal-Tutkun, 2017). In human, several *Bartonella* spp. detection were recorded which were 48% (24/55) in Japan and 15 clinical reports in China from 1980 to 2010 that describe similar CSD cases (Liu et al., 2012; Yanagihara et al., 2010). This also include Malaysia as there is a case reported in 2017 where 26.3% (5/19) patients that in contact with cats were positive *Bartonella* spp. and had neuroretinits (Tan et al., 2017). The isolation of *Bartonella* spp. from human is difficult as compared from cats, hence mostly human cases were diagnosed with clinical manifestations, serological or PCR-based assays (Arvand et al., 2007; Dillon et al., 2002). The detection of *Bartonella* spp. either using molecular or serological varies according to geographic regions, groups of examined people and method used for diagnostic (Oteo et al., 2017).

The prevalence of feline bartonellosis throughout the world varies widely from 0% in low climate region of Antarctica to 59% in very high warm and humid region such as South America (Levy et al., 2008; Pressler, 2006). The variation of prevalence depends on environments with condition that favored flea infestation.

To date, there are only several reports describing the prevalence of *Bartonella* in Malaysia in various hosts (Hassan et al., 2017; Kho et al., 2015; Kernif et al., 2012; Mokhtar & Tay, 2011). The only study conducted in cats in Malaysia reported 16.9% (48/284) were positive for *B. henselae* (Hassan et al., 2017). In addition, *Bartonella* was studied in 209 cat fleas where 11.5% (24/209) sampled fleas were positive for *B. henselae* and 19.1% (40/209) were positive for *B. clarridgeiae* (Mokhtar & Tay, 2011). This was followed by *Bartonella* detection in wild rats whereby 13.7% (13/95) rats were positive with *B. queenslandesis* (6/95), *B. elizabethae* (4/95), *B. tribocorum* (2/95), *B. rattimassiliensis* (1/95) and *B. coopersplainsensis* (1/95) (Tay et al., 2014). *B. bovis* (4.5%; 10/224) was also reported in *Rhipicepalus microplus* tick in cattle (Kho et al., 2015). All these studies indicate that *Bartonella* persists in vectors and animal reservoirs surrounding us, and thus, may have a high probability of transmission to humans.

The prevalence of shelter cats was reported in Taiwan (19.4%;20/103) and Denmark (26.5%;13/49) (Tsai et al., 2011; Chomel et al., 2002). Therefore, there is a high chance for shelter cats to acquire *Bartonella* infection due to their living condition in a multi-cat environment that promotes flea-infestation which eventually lead to population of cats that are bacteremic in shelter homes (Fleischman et al., 2015). Besides, there is no study on molecular investigation of shelter cats in Malaysia and associated risk factors with *Bartonella* infection. As cats are known to be reservoir of *Bartonella* spp., and given the close relationship between humans and cats, it is important to determine the prevalence of infection among shelter cats as shelters remain the prime source where pathogens can be transmitted indefinitely from cat to cat. Thus, this study aims to assess the molecular and seroprevalance of *Bartonella* spp. among shelter cats in Peninsular Malaysia.

The diagnosis to detect *Bartonella* is commonly by culturing the blood samples but the culturing method is difficult which usually takes about two weeks to a month for the bacteria to growth, and only suitable for some cases with clear manifestations of *Bartonella* infection such as endocarditis and neuroretinitis in human and lymphadenopathy in cats (Okaro et al., 2017; Mosbacher et al., 2011). The other methods to diagnose *Bartonella* also by PCR or serological detection. PCR is the gold standard method in detecting *Bartonella* but there is possibility of false positive result if only depend on PCR result. Therefore, serological assay needs to conducted along with the molecular assay (Mito et al., 2016)). Nevertheless, the information on antibody profiles of cats infected with *Bartonella* spp. and its relationship with PCR status are lacking. Hence, it is important to detect the molecular and antibody profile against these bacteria.

The CD4 to CD8 ratio is a reflection of immune response whereby an abnormal ratio indicates compromised adaptive immune system. This can also occur in cats as a result of pathogen infection such as feline immunodefieiency virus (FIV) infection, and feline leukemia virus (FeLV) infection (Leung et al., 2013). There are limited information in T cells phenotype that are predominant in *Bartonella* infection in cats. Therefore, this study aims to provide information on potential immune markers to aid in diagnosis of bartonellosis.

1.1.2 Research Objectives

In this study, the objectives were;

1. To identify the presence of *Bartonella* in shelter cats using PCR targeting 16S-23S rRNA ITS gene.
2. To detect IgM and IgG antibodies against *Bartonella henselae* in shelter cats using indirect immunofluorescence assay (IFA).
3. To determine the frequency of CD4 and CD8 T cells among shelter cats using immunophenotyping by flow cytometry.
4. To determine association between molecular, serological status, the frequency of CD4 and CD8 with their other clinical findings.
5. To determine the shelter management practices and their personnel awareness towards bartonellosis.

1.1.3 Hypothesis

The hypothesis of this study were;

1. There is presence of *Bartonella* spp. in shelter cats in Peninsular Malaysia.
2. There is detection of IgM and IgG antibodies against *Bartonella henselae* in shelter cats using indirect immunofluorescence assay (IFA).
3. There is no difference in frequency of CD4 and CD8 T cells among shelter cats using immunophenotyping by flow cytometry.
4. There is no association between molecular, serological status, the frequency of CD4 and CD8 with their other clinical findings.

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