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Profiling *Bartonella* infection and its associated risk factors in shelter cats in Malaysia

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ABSTRACT

Background: Poor disease management and irregular vector control could predispose sheltered animals to disease such as feline *Bartonella* infection, a vector-borne zoonotic disease primarily caused by *Bartonella henselae.*
Objectives: This study investigated the status of *Bartonella* infection in cats from eight (n = 8) shelters by molecular and serological approaches, profiling the CD4:CD8 ratio and the risk factors associated with *Bartonella* infection in shelter cats.
Methods: *Bartonella* deoxyribonucleic acid (DNA) was detected through polymerase chain reaction (PCR) targeting 16S-23S rRNA internal transcribed spacer gene, followed by DNA sequencing. *Bartonella* IgM and IgG antibody titre, CD4 and CD8 profiles were detected using indirect immunofluorescence assay and flow cytometric analysis, respectively.
Results: *B. henselae* was detected through PCR and sequencing in 1.0% (1/101) oral swab and 2.0% (1/50) cat fleas, while another 3/50 cat fleas carried *B. clarridgeiae.* Only 18/101 cats were seronegative against *B. henselae*, whereas 30.7% (31/101) cats were positive for both IgM and IgG, 8% (18/101) cats had IgM, and 33.7% (34/101) cats had IgG antibody only. None of the eight shelters sampled had *Bartonella* antibody-free cats. Although abnormal CD4:CD8 ratio was observed in 48/83 seropositive cats, flea infestation was the only significant risk factor observed in this study.
Conclusions: The present study provides the first comparison on the *Bartonella* spp. antigen, antibody status and CD4:CD8 ratio among shelter cats. The high *B. henselae* seropositivity among shelter cats presumably due to significant flea infestation triggers an alarm of whether the infection could go undetectable and its potential transmission to humans.

Keywords: Bartonellosis; *Felis catus*; PCR; immunofluorescence; T cell; flea infestations

INTRODUCTION

Bartonella henselae is a gram-negative, haemotrophic, pleomorphic, and fastidious bacteria-causing cat-scratch disease, a zoonotic disease in humans [1]. *B. henselae*-infected individuals often experienced mild symptoms, such as fever and lymphadenopathy; however, in immunocompromised individuals, such as HIV/AIDS patient, pregnant women or children,

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Conflict of Interest

The authors declare no conflicts of interest.

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a more severe consequences, such as bacillary angiomatosis, endocarditis, convulsions, lymphadenitis, and neuroretinitis could occur.

Cats are considered reservoirs in the transmission of *B. henselae* to humans either directly via cat scratch and bite or indirectly by arthropod vectors, such as cat flea (*Ctenocephalides felis*) [2]. Although *B. henselae* often causes persistent bacteraemia, cats could remain asymptomatic for months to years.

Multiple reports evaluating molecular detection of *Bartonella* spp. within cat shelters throughout the world ranging from 39.9% in a tropical region, such as Brazil, to 27.8% in the low-climate region of Lithuania [3,4]. On the other hand, serosurveillance detected higher percentage of *Bartonella*-positive cats within the same study when compared to molecular detection by polymerase chain reaction (PCR), indicating the possibility of missed detection either due to limited window of bacteraemia episode or insensitive molecular assay [5,6]. There is a high possibility 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 the population of cats that are bacteremic in shelter homes [7]. Pet ownership has been associated with one's improved mental health and reduced loneliness, especially among those with immunosuppressive disease [8]. As cats are known to be a reservoir of *Bartonella* spp., and given the close relationship between humans and cats, it is important to determine *Bartonella* infection among shelter cats, as shelters remain the prime source where pathogens can be transmitted indefinitely from cat to cat.

MATERIALS AND METHODS

Animal and sample collection

A total of 101 cats were sampled from eight (n = 8) animal shelters across five different states within Peninsular Malaysia, which were Selangor, Kuala Lumpur, Johor, Pahang, and Penang, from August until November 2018. The animal ethics application was approved by the Universiti Putra Malaysia Institutional Animal Care and Use Committee (IACUC), with reference number UPM/IACUC/AUP-R029/2018. The profile of each cat, including age, sex, and clinical signs, were recorded. Approximately 2–3 mL of blood were collected from each cat and put into ethylenediaminetetraacetic acid (EDTA)-anticoagulant tube for PCR and flow cytometric assay, while serum was extracted from plain tube for serological evaluation. For flow cytometric assay, 10% dimethyl sulfoxide (DMSO; Himedia, India) were added into 1 mL of blood in a dropwise manner, mixed well, and stored frozen at −80°C for flow cytometric analysis. Meanwhile, the collected serum was stored at −20°C prior to serological testing. A minimum of two fleas per cat were collected when available. The site where fleas were spotted was sprayed using 70% ethanol to paralyse the fleas. These fleas were then removed using forceps and stored at −20°C in tightly enclosed tubes containing 80% ethanol prior to viewing under microscope and DNA extraction. Upon viewing, fleas were identified using morphological keys for the presence of genal and pronotal combs [9]. Saliva was obtained by rolling cotton swabs on the cat's cheek, and the swabs were air-dried for 10 min prior to storage in a sterile, 1.5-mL Eppendorf tube. The swabs were suspended in phosphate-buffered saline (PBS; Sigma, St. Louis, USA) prior to DNA extraction.

DNA extraction

The DNA was extracted from 200 μL of whole blood and oral swab using innuPREP DNA mini kit (Analytik Jena GmbH, Germany) according to the manufacturer's instructions. Then, the DNA was eluted in 40 μL of elution buffer and stored at −20°C before PCR screening. For DNA extraction from flea samples, the fleas were removed from storage tubes by using sterile forceps, as well as rinsed once in 80% ethanol and twice in sterile milli-Q water. A total of 20 μL PBS was added to each flea, followed by grounding with sterile mortar and pestle, of which the lysate from the same cat's flea was pooled in a tube. The lysate was then boiled with 0.7 M of ammonium hydroxide in a total volume of 110 μL for 20 min to extract the DNA from the body trunk [10]. The boiled lysate was left open to cool, incubated for another 10 min at 95°C to remove the excess ammonia, and stored at −20°C.

PCR and sequencing

PCR targeting the 16S-23S-rRNA gene internal transcribed spacer (ITS) DNA sequence of *Bartonella* spp. was performed in a 20 μL reaction mixture using MyTaq RedMix (Bioline, USA) containing DNA template, forward primer, reverse primer, MyTaq Mix, and sterile deionised water. The primers used were 321s forward primer: 5′-AGA TGA TGA TCC CAA GCC TTC TGG-3′ and 983as reverse primer: 5′-TGT TCT YAC AAC AAT GAT GAT G-3′, which amplify the ITS sequence of *Bartonella* species [11]. The genomic DNA of *B. elizabethae*, which was isolated from a rat was used as positive control [12], and sterile deionised water was used as negative control. DNA was amplified in a MyCycler thermal cycler (Bio-Rad, USA) using the following protocols: denaturation step at 95°C for 4 min, followed by 30 amplification cycles (94°C for 30 s, 54°C for 30 s, 72°C for 35 s) and final extension step at 72°C for 5 min 15 sec. Amplified PCR products were analysed on 1.5% agarose gel electrophoresis for 30 min at 90 volts and stained using RedSafe (INtRON Biotechnology, Korea) prior to visualisation under UV light. The PCR products were purified and later sequenced using the ABI PRISM 3730xl Genetic Analyser (Applied Biosystems, USA). Sequences obtained were checked for homologous sequences in National Centre for Biotechnology Information (NCBI, USA) GenBank database by using Basic Local Alignment Search Tool (BLAST) programme. Obtained sequences listed in the GenBank database were aligned and a phylogenetic tree was constructed using Maximum Likelihood method with bootstrap values calculated from 1000 replicates based on Tamura-Nei model via Molecular Evolutionary Genetics Analysis X (MEGA X) programme.

Serological assay

A commercially available indirect immunofluorescent assay test kit (IFA) (Vircell, Spain) for human was used to detect the presence of IgM and IgG antibodies against *Bartonella henselae* in cats. IFA assays were conducted according to the manufacturer's protocol with some modifications for the detection of *Bartonella*-specific antibodies from feline serum [13]. Serum titration of 1:4 for IgM and 1:64 for IgG were used as determination for the presence of *Bartonella*-positive antibodies in the shelter cats. Briefly, the cat sera were diluted 1:4 for IgM and 1:64 in IgG using phosphate-buffered saline provided in the test kit as the diluent. Positive control of anti-human IgM and IgG fluorescein isothiocyanate (FITC) conjugate solution from the kit was added to positive and negative control wells and anti-feline IgM and IgG FITC conjugate solution (VMRD, USA) to the feline sample wells. The presence or absence of apple green fluorescent of bacillary morphology indicated positive or negative detection, respectively. The coated slides were visualised under a fluorescent microscope (Nikon, USA) at 200× magnification.

Sample processing for flow cytometric assay

A total volume of 1 mL frozen whole blood in EDTA tube was removed from the –80°C freezer and thawed briefly in a 95°C water bath [14]. The thawed whole blood was immediately washed twice with cold PBS supplemented with 2.0 mM EDTA (Sigma, USA) and 2% fetal bovine serum (FBS) (Gibco, USA) in a ratio of 1:5. The diluted blood was shaken gently and centrifuged at 300 × g at room temperature for 5 min. The supernatant was aspirated out and the pellet was resuspended in buffer containing PBS, 2.0 mM EDTA and 2% FBS, followed by cell counting. Cell numbers were adjusted to 1–2 × 10^5 cells/mL for each cat for immunophenotyping analysis by flow cytometry.

Flow cytometric staining, acquisition, and analyses

Immunophenotyping of T cells from each cat were performed by surface staining with 1 µL of FITC-conjugated anti-feline CD4 (clone 34F4; Southern Biotech, USA) and 1 µL of PE-conjugated anti-feline CD8 (clone vpg-9, Thermo Fisher Scientific, USA). Two types of controls were used, which were the unstained controls and fluorescence minus one controls, whereby one of each fluorescent-conjugated antibodies were excluded from a tube. All tubes were incubated with the anti-CD4 and CD8 antibodies at room temperature in the dark for 30 min. Then, the samples were centrifuged and washed twice with PBS, 2.0 mM EDTA and 2% FBS. The supernatant was discarded and aspirated out, with the pellets resuspended in 1% paraformaldehyde and stored at 4°C for up to 24 h before acquisition. All samples were acquired using BD FACSCanto (BD, USA) with 50,000 events set for lymphocyte samples. The lymphocyte population was first gated, followed by filtering out the doublet using the forward scatter area (FSC-A) vs. height (FSC-H) plot (**Supplementary Fig. 1**). Unstained control for each stained dye act as a control for gating stained CD4 and CD8 plot. Data analyses were performed using NovoExpress software (ACEA Biosciences, USA). The percentages of CD4 and CD8 were obtained from the final gating, whereas the ratios of CD4:CD8 were calculated and value of less than 1.10 or more than 3.05 was regarded as an abnormal ratio [15].

Statistical analyses

Statistical analyses were performed using Statistical Package for the Social Sciences software programme (version 25; SPSS, USA). The association between positive results from PCR and serology, coupled with physical examination findings were analysed. Potential associations were compared in univariate analysis using chi-square Fisher's exact test. All risk factors were regarded as significant at $p \leq 0.05$. Univariate odds ratio (OR) and 95% confidence interval (CI) were calculated.

RESULTS

Cats' signalment and identification of fleas

A total of 101 cat's blood and oral swab samples were obtained, where 68 (67.3%) of these cats were females and 33 (32.7%) were males (**Table 1**). Cats' age varied from six months to six years old, with four (4.0%) cats were less than two years old and categorised as junior, and 97 (96.0%) were in the range of two to six years were grouped as adults. A total of 40/101 (39.6%) cat had enlargement of lymph nodes, 17/101 (16.8%) cats had ocular discharge, 45/101 (44.6%) cats with respiratory-associated problems, 35/101 (34.7%) cats with gingivitis and 13/101 (12.9%) cats with diarrhoea. Flea infestation was observed in 50/101 cats, where the collected fleas were subsequently identified as cat fleas (*Ctenocephalides felis*) based on

Table 1. Shelter cats' signalment, physical, clinical finding and χ^2 analysis to determine the association between physical examination findings, CD4:CD8 ratio and antibody detection

Characteristics	Categories (n = 101)	Seropositive (n = 83)	Seronegative (n = 18)	OR	95% CI	Univariate *p* value
Sex	Female (n = 68)	53 (63.9)	15 (83.3)	0.4	0.1–1.3	0.165
	Male (n = 33)	30 (36.1)	3 (16.7)			
Age	Adult (n = 97)	79 (95.2)	18 (100.0)	N/A	N/A	1.000
	Junior (n = 4)	4 (4.8)	0 (0)			
Flea infestation	Yes (n = 50)	47 (56.6)	3 (16.7)	6.5	1.8–24.3	0.003*
	No (n = 51)	36 (43.4)	15 (83.3)			
Enlarged lymph nodes	Normal (n = 71)	48 (57.8)	13 (72.2)	0.5	0.2–1.5	0.291
	Abnormal (n = 40)	35 (42.2)	5 (27.8)			
Ocular discharge	Presence (n = 17)	14 (16.9)	3 (16.7)	1.0	0.3–4.0	1.000
	Absence (n = 84)	69 (83.1)	15 (83.3)			
Respiratory-associated problem	Presence (n = 45)	39 (47.0)	6 (33.3)	1.8	0.6–5.2	0.433
	Absence (n = 56)	44 (53.0)	12 (66.7)			
Gingivitis	Presence (n = 35)	27 (32.5)	8 (44.4)	1.7	0.6–4.7	0.414
	Absence (n = 66)	56 (67.5)	10 (55.6)			
Diarrhoea	Yes (n = 13)	8 (9.6)	5 (27.8)	0.3	0.1–1.0	0.052
	No (n = 88)	75 (90.4)	13 (72.2)			
CD4:CD8 ratio	Normal (n = 40)	35 (42.2)	5 (27.8)	1.9	0.6–5.1	0.299
	Abnormal (n = 61)	48 (57.8)	13 (72.2)			

The percentage in the bracket represent the number for each subcategory over their antibody status.
OR, odds ratio; CI, confidence interval.
*The result is statistically significant $p \leq 0.05$.

the presence of genal and pronotal comb described by the presence of comb above the mouth and comb-like bristles on the tail-edge of the first thoracic segment behind the head, respectively (data not shown). Sixty-one out of 101 (60.4%) cats were reported to show abnormal CD4:CD8 ratio in the present study (normal CD4 to CD8 ratio: 1.10 to 3.05).

Serological and molecular detection of *Bartonella* DNA from shelter cat's blood, oral swab, and fleas

Molecular detection of *Bartonella* infection using 16S-23S rRNA ITS-specific primers was performed on blood and oral swab samples from 101 shelter cats and pooled flea samples collected from 50/101 of the shelter cats. ITS amplification reveals that one of 101 (1.0%) oral swabs samples and four flea samples out of the 50 cats (8.0%) were positive for *Bartonella* DNA (**Table 2**). However, no *Bartonella* DNA was detected from blood samples. DNA sequencing revealed the presence of *B. henselae* (BUPM115) in the only oral sample and one (BUPM55) out of four cat flea samples, while the other three cat flea samples (BUPM199, BUPM206, BUPM207) harboured *B. clarridgeiae*.

Association between serum antibody titre and shelter cats' signalment, physical examination findings, and CD4:CD8 ratio

As the number of shelter cats positive for *Bartonella* DNA were too low for statistical analysis, only serological data were used to determine the association between cats' signalment, physical examination findings, CD4:CD8 ratio and serological status. Univariate analysis showed that

Table 2. Polymerase chain reaction detection of *Bartonella* spp. and BLAST analysis of the amplified DNA sequence

Cat ID	Blood	Oral swab	Flea	BLAST analysis
BUPM-55	–	–	+	*B. henselae* (Accession number: MW603780)
BUPM-115	–	+	–	*B. henselae* (Accession number: MN123554)
BUPM-199	–	–	+	*B. clarridgeiae* (Accession number: MN123544)
BUPM-206	–	–	+	*B. clarridgeiae* (Accession number: MN123545)
BUPM-207	–	–	+	*B. clarridgeiae* (Accession number: MN123546)

BLAST, Basic Local Alignment Search Tool.

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the presence of antibodies to *B. henselae* detected by the IFA test was strongly associated with flea infestation (**Table 1**). Cats with flea infestation were 6.5 times (95% CI, 1.8–24.3; $p = 0.003$) more likely to be infected with *Bartonella* spp. than cats that were free from fleas. No association were recorded in this study between sex, age, enlarged lymph nodes, ocular discharge, respiratory-associated problem, gingivitis, diarrhoea, CD4:CD8 ratio, and seroreactive cats.

Distribution of positive molecular and serological cases among sampled shelters

Positive cases by molecular detection were observed in 3/8 sampled shelters (two in an urban area, one in a rural location); however, all eight shelters housed *Bartonella*-seropositive cats (**Table 3**). Highest detection rates of seropositive cats (100%) were observed in two shelters that also had positive molecular detection of *Bartonella* spp. of flea samples. In addition, 2/3 of these shelters with positive *Bartonella* DNA detection did not practice routine ectoparasite control. Although ectoparasite controls were practiced routinely in 4/8 shelters, only one shelter had no flea infestation among their cats upon observation.

Serological detection of *B. henselae*-specific IgM and IgG in shelter cats and its correlation with the detection of *Bartonella* DNA

From the 101 shelter cats, 83/101 (82.2%) cats had antibodies to *B. henselae* (**Table 4**). Eighteen (17.8%) cats were positive for IgM antibody only and 34/101 (33.7%) cats were positive for IgG antibody only, while 31/101 cats (30.7%) were positive for both IgM and IgG. The one cat that had detectable *Bartonella* DNA in its oral swab, was also seronegative (**Tables 2** and **4**). The other four cats that had detectable *Bartonella* DNA in their flea samples had either IgG+ (2/5; 40%) or both IgM+ and IgG+ antibodies (2/5; 40%). Meanwhile, 29/96 (30.2%), 18/96 (18.8%) and 32/96 (33.3%) cats that were PCR negative for *Bartonella* DNA were IgM+/IgG+, IgM+/IgG-, and IgM-/IgG+ antibodies, respectively. Only 17/96 (17.7%) PCR-negative cats were also negative for antibodies. There was no significant association between the status of PCR and detection of IgM and/or IgG antibodies among the shelter cats ($p = 0.299$).

Table 3. Molecular and serological findings of *Bartonella* spp. based on shelter homes, their ectoparasite control, observation of flea infestation and location

Shelter	Molecular detection of *Bartonella* spp.	Seroprevalence of IgM/IgG antibodies against *Bartonella henselae*	Routine of ectoparasite control	Observation of flea infestation	Location (rural/urban)
1	+[F]	12/12 (100%)	No	Yes	Urban
2	-	14/16 (87.5%)	No	Yes	Urban
3	+[O]	6/15 (40%)	Yes	Yes	Urban
4	-	1/10 (10%)	Yes	Yes	Urban
5	-	5/8 (62.5%)	Yes	Yes	Urban
6	-	12/18 (67%)	Yes	No	Rural
7	+++[F]	12/12 (100%)	No	Yes	Rural
8	-	3/10 (30%)	No	Yes	Urban

+ = positive *Bartonella* spp. detection by polymerase chain reaction with the number of + signs indicates the no. of positive samples found.
F, flea sample; O, oral sample.

Table 4. *Bartonella* DNA and antibody detection results among shelter cats

B. henselae-specific antibody detection by IFA	*Bartonella* DNA detection by PCR			
	Positive (n = 5)	%	Negative (n = 96)	%
IgM+/IgG−	0	0.0	18	18.8
IgM−/IgG+	2	40.0	32	33.3
IgM+/IgG+	2	40.0	29	30.2
IgM−/IgG−	1	20.0	17	17.7

Odd ratio, 0.527; 95% confidence interval, 0.172–1.616; $p = 0.299$.
IFA, immunofluorescence assay; PCR, polymerase chain reaction.

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Phylogenetic tree analysis

BLAST analysis of amplified fragments from positive oral swab (n = 1) samples (BUPM-115) demonstrated a high sequence similarity (97.8% to 99.3%) to *Bartonella henselae* strain M1BJ-CW from China (Genbank accession number JQ316963), *B. henselae* strain BNC07 from France (Genbank accession number JN646684), *B. henselae* strain Q5BJ-CW from Korea (Genbank accession number JQ009430), *B. henselae* isolate 55-1 from New Zealand (Genbank accession number MF196158), *B. henselae* strain 36 from Malaysia (Genbank accession number KT318619), *B. henselae* strain 112 from Malaysia (Genbank accession number KT318618) (**Fig. 1**).

Sequence analysis of the ITS region obtained from the fleas demonstrated that flea samples from one cat contained *B. henselae* (BUPM-55), and the remaining three had *B. clarridgeiae*-like sequences (BUPM-199, BUPM-206, BUPM-207) (**Fig. 2**). BLAST result for sequence analysis showed high sequence similarity (98.7%) with *B. henselae* strain Brazil-1 (Genbank accession number DQ346666) and *B. henselae* isolate 77 from Austria (Genbank accession number MF374385); sequence similarity (97.8%–100.0%) with *B. clarridgeiae* strain from USA (Genbank accession number DQ683194), *Bartonella clarridgeiae* isolate C49 from France (Genbank accession number AF312501), *B. clarridgeiae* isolate ML0094 from Greece (Genbank accession number MN170543), *Bartonella clarridgeiae* strain M9HN-SHQ from China (Genbank accession number EU589237), Uncultured *Bartonella clarridgeiae* clone MU9/KCF21from Australia (Genbank accession number HM990962). *Rickettsia typhi* was used as an outgroup (**Fig. 1**) for positive cat samples, while *Agrobacterium* and *Neorickettsia* strains were used as outgroup (**Fig. 2**) for positive flea samples.

DISCUSSION

Animal shelters often placed mix population of animals that came in from multiple unknown sources with minimal or no prior health care. As animal shelters could play a role as the central source of pathogens mixing, good shelter management with routine disease

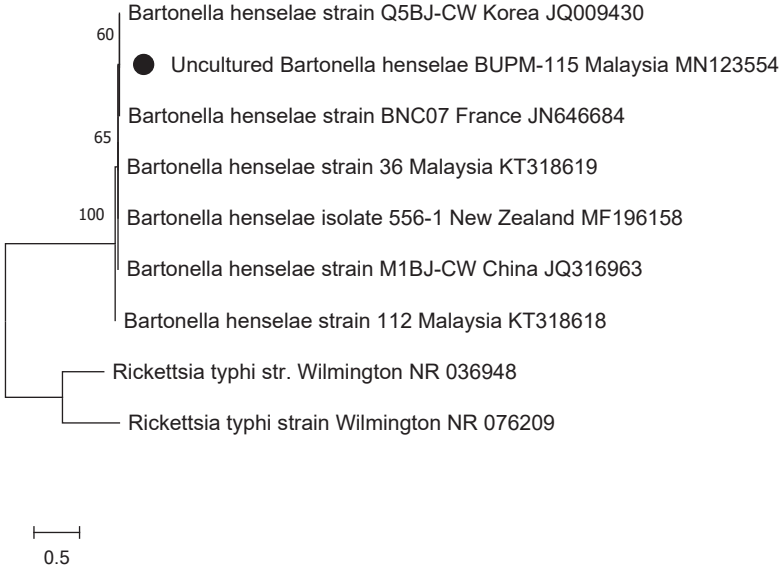

Fig. 1. Maximum-likelihood phylogenetic tree analysis for the partial internal transcribed spacer gene of *Bartonella* spp. by MEGA X for positive cat samples. The phylogenetic tree was constructed using Tamura-Nei model and bootstrap analysis of 1000 replicates. Oral swab sample sequenced in present study are marked as ●.

Fig. 2. Maximum-likelihood phylogenetic tree analysis for the partial internal transcribed spacer gene of *Bartonella* spp. by MEGA X for positive cat flea samples. The phylogenetic tree was constructed using Tamura-Nei model and bootstrap analysis of 1000 replicates. Different marking indicates different species of *Bartonella*. (e.g.: ♦ indicates *B. clarridgeiae*, and ● indicates *B. henselae*).

prevention is crucial to prevent occurring of disease outbreak especially in overcrowding situation. The study reported here attempted to determine the status of *Bartonella* infection in eight different cat shelters both at molecular and serological level, risk factors associated with *Bartonella* DNA detection and characterisation of positive *Bartonella* samples. Positive detection of *Bartonella* DNA were obtained from oral swabs (1/101 cats; 1%), and fleas (4/50 cats; 4%) of shelter cats; however, none of the shelter cats sampled in this study were bacteremic. In addition, no cats had simultaneous detection in blood, oral swabs and their flea samples, which could probably due to a number of reasons: 1) sampled fleas harbouring *Bartonella* spp. were feeding on different cats within the same household at the time of collection [16]; 2) different swabbing protocols used and different method in detecting *Bartonella* could result in the absence of detection from oral samples [17,18]; (3) the different composition of food fed to the cats and the health status of cats may also influence the variety of normal flora in the cat mouth, thus, it might affect the detection of *Bartonella* [19,20]; and 4) transient bacteremic state, where the bacterial load could not be detected by conventional PCR [21].

Subsequent species confirmation through DNA sequencing of both oral and flea samples confirmed the presence of *B. henselae* and *B. clarridgeiae*. It was well known that several *Bartonella* spp. could be harboured by *Ctenocephalides felis* (*C. felis*) such as *B. henselae*, *B. clarridgeiae* and *B. koehlerae* [22,23]. *B. henselae* and *B. clarridgeiae* DNA were detected in the fleas sampled in these studies; however, none of the other *Bartonella* spp. reported in flea was detected. Other reports have detected as high as 90% of flea-positive samples harbouring *B. henselae*, while other studies have reported the presence of co-infection of *B. henselae* and *B. clarridgeiae*, which indicates multiple *Bartonella* spp. can be harboured by fleas [22,24].

When compared to pet cats within the same geographical region, the molecular prevalence of *Bartonella* in shelter cats were lower [25]. In this study, four out of eight shelters practiced routine ectoparasite control. Out of these four shelters, positive PCR detection was found in

only one shelter indicating routine ectoparasite control and health management of cats could prevent the occurrence of *Bartonella* spp. transmission.

Other than PCR, infection with *Bartonella* spp. can be detected using several other methods such as culture and serological assay; however, culture may require a longer time to get the result [26]. Therefore, in this study, serum antibody titres against *Bartonella henselae* among the shelter cats were evaluated. Although the detection of *Bartonella* DNA was less than 4% among the sampled shelter cats, surprisingly, 82.1% shelter cats were found to be positive for *B. henselae* antibodies. However, analyses of several recent studies highlighted that the discrepancy of molecular and seroprevalence detection either within pet cats or stray population were not that uncommon [5,27]. The combined result of IgM and IgG could be used as a diagnostic approach for *Bartonella* infection because current infection is more likely if both IgM and IgG are positive, since IgM result is indicative for recent infection, while IgG result is indicative for persistent infection [28]. Further evaluation of antibody profiles against *B. henselae* indicates that 18.8% of shelter cats were IgM+ alone, 33.3% were IgG+ alone and 30.2% were positive for both IgM and IgG. In contrast, the one cat that was tested positive for *Bartonella* DNA in oral sample had no detectable antibodies. IgM detection has been observed as early as four days versus detection of IgG at one week post-inoculation in an experimental study, while *B. henselae* detection was observed by culture within two weeks of inoculation of high dose *B. henselae* [29]. Therefore, it is plausible cats that had IgM+ detection in this study were actually harbouring *Bartonella* spp.; however, the number of bacterial load was too low to be detected by conventional PCR. In the absence of reinfection, IgM level has been detected in *Bartonella*-inoculated cats up to 16 weeks post-infection, whereas IgG level persisted. It is therefore reasonable to assume that the cats that had IgG+ alone were most likely had been able to clear off the bacteria. Therefore, serologic testing is best used along with PCR testing to avoid false-negative. The false-negative result was reported less common and increase the diagnosis accuracy when a combination of serological and molecular assays was used [30].

There were contradicting observation related to age with the risk of getting *Bartonella* infection in cats [23,25]. Additionally, in regards to sex, male cats had higher risk of becoming seropositive due to *B. henselae* infection compared to female [31]. Nevertheless, in the present study, no association was observed between sex and seropositivity status. Bartonellosis has been proposed as a cause of enlarged lymph nodes, gingivitis, as well as ocular disease, but true associations could not be defined [32,33]. However, in this study, the presence of enlarged lymph nodes, ocular disease, respiratory-associated problem, gingivitis, and diarrhoea showed no association with seropositivity to *B. henselae*. Flea infestation was the only significant risk factor observed in this study ($p = 0.003$). The high number of seropositive cats along with flea infestation was expected and in agreement with a study by Guptill et al. [26]. Another study stated that flea infestation was associated with seropositivity of *B. henselae* but not with bacteraemia in cats [34]. The climate factors such as an increase in temperature and humidity might contribute to flea infestations since their biological cycle is more favourable in this condition, which may lead to an increase of seropositivity due to *Bartonella* infection [35,36].

As cell-mediated immunity has been shown to play a role in warding off *Bartonella* spp. colonisation, CD4 to CD8 ratio was included as part of the analysis in this study. Although the results in this study pointed that *Bartonella*-seropositive cats had an abnormal CD4 to CD8 ratio where the percentage of CD4 T cells was lower than the percentage of CD8

T cells; however, it was not significant when compared with seropositivity status. The possible explanation for the high percentages of shelter cats having abnormal CD4 to CD8 ratio in this study might be due to the infection and/or co-infection with other feline pathogens that can induce immunosuppression, such as feline immunodeficiency virus (FIV) and feline leukaemia virus (FeLV), which are common in shelter cat population. There was still contrasting evidence on FIV and/or FeLV co-infections in *Bartonella*-infected cats; however, co-infections with these feline viruses were not investigated in this study [37]. In addition, previous supporting evidence showed that the presence of *Bartonella* does not cause immunosuppression in cats [38,39]. Indeed, one bacteraemia cat that was seronegative in this study also showed an abnormal CD4:CD8; however, the animal appeared healthy. Therefore, further studies are warranted to investigate the kinetics of *Bartonella* infection in cats associated with humoral and cell-mediated immune responses. To the best of our knowledge, this study provides a novel overview on the different result parameters using either PCR, serology or/and immunophenotyping assays in assessing the presence of *Bartonella* spp. among shelter cats. Overall, 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. The CD4:CD8 ratio is not that useful especially in shelter environment in which cats are exposed to other potential pathogens that could trigger immune response. 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.

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SUPPLEMENTARY MATERIAL

Supplementary Fig. 1

The gating strategies for frozen whole blood.

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