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Posted Date: 29 October 2025

doi: 10.20944/preprints202510.2306.v1

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Article

Emerging Risk of Flea-Borne *Bartonella* in Tropical Cities: Evidence from Stray Cats in the Klang Valley, Malaysia

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Simple Summary

Stray cats living in cities often carry fleas, which can spread pathogens that cause diseases to humans and animals. In this study, we examined fleas collected from stray cats in different sites within Klang Valley, Malaysia to find out if they carried any harmful pathogen. We first confirmed that all the fleas collected were cat fleas (*Ctenocephalides felis*), the most common flea that parasitize cats. We then tested the fleas for the presence of *Bartonella* bacteria, which can cause “Cat Scratch Disease (CSD)” in humans. Our results showed a very high number of infected fleas. These findings show that there may be a hidden health risk to communities living in close contact with stray cats, especially in crowded city areas. This study highlights the importance of monitoring fleas and the bacteria they carry as part of public health efforts. By understanding these risks early, health authorities and communities can take steps to manage stray animals and prevent the spread of diseases to humans.

Abstract

Urban stray cats are often associated with ectoparasites and zoonotic pathogens due to their unsanitary living conditions and lack of veterinary care. Fleas, especially *Ctenocephalides* spp., are competent vectors of *Bartonella* spp., a genus of emerging bacterial pathogens with both public health and veterinary relevance. This study investigated the presence of *Bartonella* DNA in ectoparasitic fleas infesting stray cats in various urban habitats within the Klang Valley, Malaysia. A total of 204 fleas were collected from 89 stray cats. Fleas were identified morphologically using established taxonomic keys under a light microscope and further validated through PCR amplification of the mitochondrial cytochrome *c* oxidase subunit I (*cox1*) gene. Detection of *Bartonella* spp. was conducted by targeting the citrate synthase (*gltA*) gene. All fleas were confirmed as *Ctenocephalides felis*, with an infestation prevalence of 39.33% among the cats sampled. Of 118 *C. felis* specimens tested, 86.44 % were positive for *Bartonella* DNA, one of the highest worldwide and significantly surpassing previous Malaysian reports. Sequencing of 12 positive samples showed identities with *B. claridgeiae* (58.3 %), *B. henselae* (25.0 %), an uncultured *Bartonella* species (8.3 %) and a *Bartonella* isolate from a dog in Chile (8.3 %). These results highlight the significant presence of *Bartonella*, causative agent of cat-scratch disease in stray cats, emphasizing their potential role as urban reservoirs and vectors. The findings underscore the need for ectoparasite surveillance and zoonotic pathogen control as integral components of stray animal management in Malaysia’s urban settings.

Keywords: vector; zoonosis; *Felis catus*; infectious disease; animal management

1. Introduction

Stray cats are ubiquitous in urban ecosystems, often living in close proximity to humans and other domestic animals. Many of these cats originate from abandoned pets or unplanned litters, forming unregulated populations that thrive in densely populated areas. Due to limited access to veterinary care and poor health management, stray cats are frequently infested with ectoparasites, which play a key role in maintaining and transmitting zoonotic pathogens of public health significance [1]. This human–animal interface, intensified by urbanisation, provides an ideal setting for the emergence and spillover of vector-borne infections under the One Health framework.

Among feline ectoparasites, fleas particularly the cat flea *Ctenocephalides felis* are the most common and globally widespread [2]. *C. felis* exhibits remarkable ecological adaptability, infesting a wide range of mammalian hosts across both temperate and tropical regions [3]. Beyond causing dermatological irritation and allergic reactions in animals, *C. felis* serves as a competent vector for several zoonotic agents, notably *Bartonella* species. Of these, *B. henselae* and *B. claridgeiae* are of major medical relevance, being the primary etiological agents of cat scratch disease (CSD) in humans [4]. Human infection typically results from scratches or bites contaminated with flea faeces [5], and while CSD is usually self-limiting in immunocompetent individuals, severe systemic manifestations such as neuroretinitis, endocarditis, and prolonged fever have been documented, particularly in immunocompromised patients.

Globally, urban stray cats are increasingly recognised as reservoirs for *Bartonella* spp. and their flea vectors, underscoring the need for integrated surveillance at the animal–vector–human interface. However, in Malaysia, studies on flea infestations and *Bartonella* infections in stray cats remain limited, often constrained by small sample sizes or narrow geographic coverage. The Klang Valley, encompassing Kuala Lumpur and its surrounding metropolitan districts, is one of the most densely populated and urbanised regions in the country, hosting a large and growing population of free-roaming cats. Despite the frequent human–cat interactions in this region, data on the prevalence of *C. felis* infestations and their role as vectors of *Bartonella* spp. are scarce [4,6,7]. This paucity of epidemiological evidence limits public health risk assessment and hinders the development of evidence-based vector control strategies.

The present study aims to address this knowledge gap by investigating the natural infection rates of *C. felis* and *Bartonella* spp. in urban stray cats within the Klang Valley, Peninsular Malaysia. By integrating morphological and molecular identification approaches, this research provides updated insights into flea-borne *Bartonella* circulation in urban environments. The findings are expected to contribute to the growing body of One Health evidence needed to inform zoonotic disease surveillance, risk mitigation, and integrated vector management strategies in tropical urban settings.

2. Materials and Methods

2.1. Study Area and Flea Collection

All procedures involving animal handling and sample collection were conducted in strict accordance with institutional and international ethical standards for the humane treatment of animals. Approval was obtained from the Institutional Animal Care and Use Committee (IACUC), Universiti Malaya (Ethics Reference No: T/22052023/13032023-02/R). Flea sampling was conducted between October and December 2023 across ten urban sites within the Klang Valley, Peninsular Malaysia (Figure 1). This region represents one of the most densely populated and urbanised areas of the country, characterised by a large stray cat population living in close proximity to humans. Each site was visited only once to avoid resampling of individual cats.

Free-roaming cats were approached using food pellets as bait to encourage stationary behaviour. Only cats exhibiting no signs of illness or aggression were sampled. Fleas were collected using a fine-toothed stainless-steel comb, focusing on key anatomical regions such as the head, neck, torso, and tail. Immediately after combing, the flea-laden comb was sealed in a sterile zip-lock bag to prevent escape, and the bag was shaken to dislodge fleas. All animals were released unharmed upon completion of the procedure. Collected fleas were transported to the laboratory and preserved at -80°C until further processing. Morphological identification was performed under a stereomicroscope using standard taxonomic keys [8].

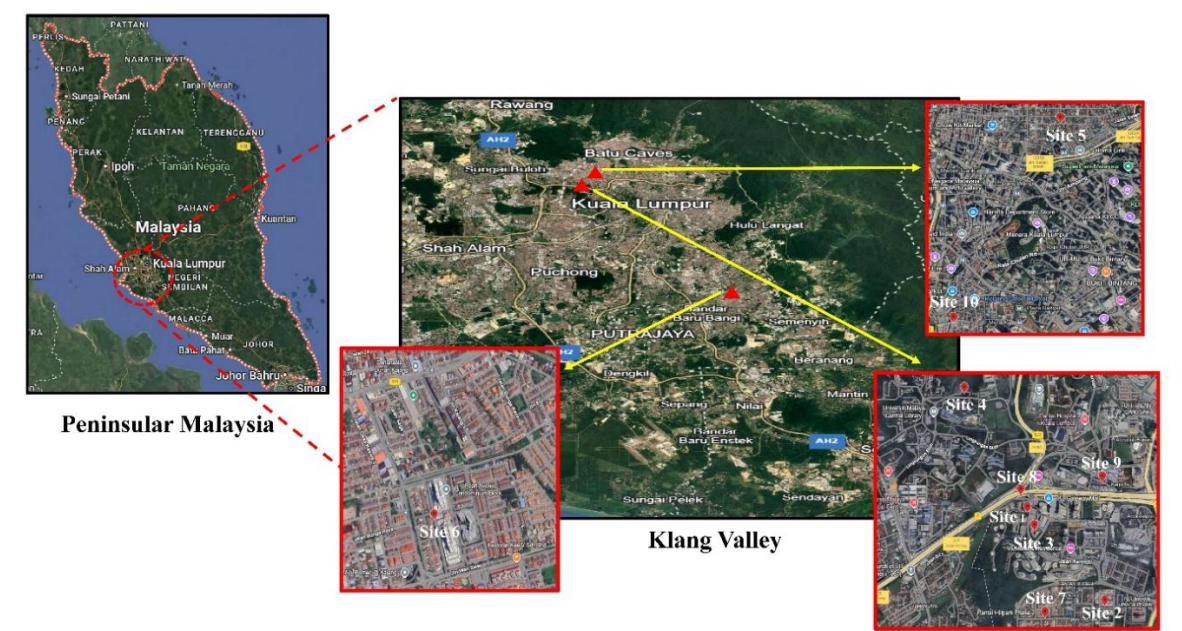


Figure 1. A map illustrating the distributions of the ten sampling sites for stray cat screening.

2.2. Study Area and Flea Collection

Genomic DNA was extracted from 118 individual fleas using the DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufacturer’s protocol, with minor modifications. Each flea was homogenized in 180 μL of ATL buffer using a sterile micropestle, followed by the addition of 20 μL of Proteinase K. The samples were incubated at 56°C with intermittent vortexing until complete tissue digestion was achieved. Subsequently, 200 μL of AL buffer and 200 μL of absolute ethanol were added to each lysate, which was then transferred to a spin column and centrifuged at $6000 \times g$ for 1 minute. The column was washed sequentially with 500 μL of AW1 and AW2 buffers. Finally, DNA was eluted in 50 μL of AE buffer and stored at -20°C until polymerase chain reaction (PCR) analysis.

2.3. Molecular Identification of Fleas

Molecular confirmation of flea species was performed via PCR amplification of the mitochondrial cytochrome *c* oxidase subunit I (*cox1*) gene using the primer pair LCO and Cff-R [9] (Table 1), yielding an expected amplicon size of approximately 550 bp. Each 25 μL PCR reaction contained 12.5 μL of DreamTaq Green PCR Master Mix (Thermo Scientific, Lithuania), 1 μL of each primer (10 μM), 2 μL of genomic DNA, and 8.5 μL of nuclease-free water. Amplification was carried out under the following thermocycling conditions: initial denaturation at 95°C for 1 minute, followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 15 seconds, and extension at 72°C for 10 seconds, with a final extension at 72°C for 5 minutes (Table 2). Positive controls (known *C. felis* DNA) and negative controls (nuclease-free water) were included in each PCR run. Amplicons were visualized by electrophoresis on 1.0 % agarose gels stained with GelRed and compared against

a 100 bp DNA ladder (GeneRuler™, 1st BASE, Malaysia). Positive PCR products were purified and sent for sequencing (Apical Scientific Sdn. Bhd., Malaysia) for species confirmation.

Table 1. Primers used for PCR assays in this study.

Gene	Primer Name	Sequence (5'-3')	Direction	Detection	Reference
cox1	LCO	GGT CAA CAA ATC ATA AAG ATA TTG G	Forward	Flea species	[9]
	Cff-R	GAA GGG TCA AAG AAT GAT GT	Reverse		
gltA	BhCS.781p	GGG GAC CAG CTC ATG GTG G	Forward	Bartonella	[10]
	BhCS.1137n	AAT GCA AAA AGA ACA GTA AAC A	Reverse		

Table 2. Cycling conditions used for PCR assays in this study.

Gene	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension	Cycle	Amplicon size (bp)	Reference
cox1	95 °C	95 °C	55 °C	72 °C	72 °C	35	550	[9]
	1 min	15 s	15 s	10 s	5 min			
gltA	94 °C	94 °C	51 °C	72 °C	72 °C	35	379	[10]
	10 min	30 s	45 s	30 s	7 min			

2.4. Detection of Bartonella spp. By PCR

Screening for *Bartonella* DNA was performed by amplifying a fragment of the citrate synthase (*gltA*) gene using the primer pair BhCS.781p (5'-GGGGACCAGCTCATGGTGG-3') and BhCS.1137n (5'-AATGCAAAAAGAACAGTAAACA-3') as described by Norman et al. [10] (Table 1), generating a 379 bp amplicon. Each PCR reaction (25 µL) contained 12.5 µL of DreamTaq Green PCR Master Mix (Thermo Scientific, Lithuania), 1 µL of each primer (10 µM), 2 µL of flea genomic DNA, and 8.5 µL of nuclease-free water. Thermocycling conditions consisted of an initial denaturation at 94°C for 10 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 51°C for 45 seconds, and extension at 72°C for 30 seconds, with a final extension at 72°C for 7 minutes (Table 2). Positive and negative controls were included in all PCR assays, using *Bartonella henselae* DNA as the positive control and sterile nuclease-free water as the negative control. PCR products were visualized via electrophoresis on 1.0 % agarose gels stained with GelRed and compared to a 100 bp molecular size marker. Positive amplicons were purified and submitted for bidirectional sequencing at Apical Scientific Sdn. Bhd. (Selangor, Malaysia).

2.5. Phylogenetic Analysis

Raw chromatograms were examined using SeqScanner v2 (Applied Biosystems) and manually edited for base-calling accuracy in BioEdit v7. Sequences were aligned using MEGA v11 [11] with default parameters, and consensus sequences were compared against the NCBI GenBank database using BLASTn for species identification. Phylogenetic trees were constructed using the Maximum Likelihood (ML) method in MEGA v11. The Tamura 3-parameter (T92) model was applied for *cox1* gene sequences of fleas, while the Kimura 2-parameter (K2) model was used for *Bartonella gltA* sequences. Node reliability was evaluated with 1,000 bootstrap replicates. *Pulex irritans* [12] and *Brucella melitensis* [13] were designated as outgroups for flea and *Bartonella* phylogenies, respectively. Reference sequences retrieved from GenBank were included to confirm genetic clustering and species assignment.

2.6. Statistical Analysis

Flea infestation parameters were quantified following the definitions proposed by [14] to provide a comprehensive assessment of parasite–host interactions. The prevalence of infestation was calculated as the proportion of cats harbouring at least one flea, expressed as a percentage of the total

number of cats examined. This parameter reflects the overall likelihood of flea presence within the sampled cat population.

Descriptive statistics were used to summarise flea infestation levels and *Bartonella* detection rates. Associations between categorical variables and flea infestation status were assessed using Fisher’s Exact Test. A p-value of < 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism v9 (GraphPad Software, USA).

3. Results

3.1. Flea Collection and Prevalence

A total of 204 fleas were recovered from 35 of the 89 stray cats sampled across various Klang Valley localities, yielding an overall infestation prevalence of 39.33 %. Fleas were detected in all surveyed locations except Kampung Baru. The highest flea burden originated from Pantai Dalam (n = 94; 45.19 %), followed by Vista Angkasa Apartment (n = 53; 25.98 %) and Kajang (n = 25; 12.25 %) (Table 3, Table S1). Female cats constituted the majority of the sampled population (n = 69; 77.53 %) compared to males (n = 20; 22.47 %). Of these, 27 females (39 %) and eight males (40 %) were infested (Table 4). Age distribution was similarly consistent, with infestation detected in 39.0 % of adults (33/84) and 40.0 % of juveniles (2/5) (Table 4). Fisher’s Exact Test revealed no significant association between flea infestation and either gender (P = 0.5711) or age group (P = 0.6571). Environmental assessment indicated that infestation prevalence was highest at public transportation areas (100 %), followed by housing (40 %) and eatery zones (34 %) (Table 4). Despite eatery areas contributing the most fleas (n = 136), their prevalence was comparatively low due to a higher number of sampled cats with few infested individuals.

Table 3. Number of fleas collected according to classification of area within Klang Valley.

Location type	Location Code	Location Name	Number of cats examined	Number of cats infested with fleas (Prevalence)	Total fleas collected
Housing Area	Site 1	Vista Angkasa Apartment	14	7 (50.0)	53
	Site 2	PPR Kerinchi	7	1 (14.2)	3
	Site 3	Pangsapuri 17 Tingkat Kerinchi	4	2 (50.0)	3
Eatery Area	Site 4	Universiti Malaya	26	6 (23.1)	17
	Site 5	Kampung Baru	7	0 (0.0)	0
	Site 6	Kajang	5	3 (60.0)	25
	Site 7	Pantai Dalam	21	11 (52.4)	94
Public Transportation Area	Site 8	LRT Universiti Station	2	2 (100.0)	2
	Site 9	LRT Kerinchi Station	1	1 (100.0)	3
	Site 10	LRT Pasar Seni Station	2	2 (100.0)	4
Total			89	35	204

Table 4. Prevalence of fleas (n = 204) on 89 sampled cats based on identified risk factors in the study areas.

Risk Factor	Variable	Flea-infested cat	Total fleas	Prevalence of Infestation (%)
Area	Housing area (n = 25)	10	59	40.0
	Eatery area (n = 59)	20	136	33.9
	Public transportation area (n = 5)	5	9	100.0
Gender	Male (n = 20)	8	35	40.0
	Female (n = 69)	27	169	39.0
Age	Adult (n = 84)	33	200	39.0
	Juvenile (n = 5)	2	4	40.0

Note: Prevalence of infestation was calculated as the proportion of cats harbouring at least one flea, expressed as a percentage of the total number of cats examined.

3.2. Flea Identification

All 204 fleas were morphologically identified as *Ctenocephalides felis* (the cat flea) using established taxonomic keys [8]. Diagnostic characteristics included a long head with an angular frons, genal comb spines 1 and 2 of nearly equal length, and a distinct narrow dorsal incrasation [15]. No other flea species were detected among the examined specimens. For molecular confirmation, DNA from 118 individual fleas was successfully amplified targeting the mitochondrial *cox1* gene (~ 550 bp). Ten representative sequences (479 bp) showed 97–100 % similarity with *C. felis* isolate Malay01 (GenBank accession no.: MT027230.1) in BLAST analyses, confirming morphological identification and demonstrating high sequence conservation among local populations (Table 5).

Table 5. BLASTn results for the obtained flea sequences based on the *cox1* gene region.

Flea in this study	Location	Closest GenBank match	Maximum Identity (%)	Query cover (%)	E-value	Origin
F02	University Malaya	<i>C. felis</i> isolate Malay01 (MT027230.1)	99.83	99	0.0	East and Southeast Asia
F04	University Malaya	<i>C. felis</i> isolate Malay01 (MT027230.1)	97.83	98	0.0	East and Southeast Asia
F21	Vista Angkasa Apartment	<i>C. felis</i> isolate Malay01 (MT027230.1)	100.00	98	0.0	East and Southeast Asia
F37	Vista Angkasa Apartment	<i>C. felis</i> isolate Malay01 (MT027230.1)	100.00	98	0.0	East and Southeast Asia
F50	PPR Kerinchi	<i>C. felis</i> isolate Malay01 (MT027230.1)	100.00	100	0.0	East and Southeast Asia
F60	Kajang	<i>C. felis</i> isolate Malay01 (MT027230.1)	100.00	100	0.0	East and Southeast Asia
F76	Kajang	<i>C. felis</i> isolate Malay01 (MT027230.1)	100.00	98	0.0	East and Southeast Asia
F90	Pantai Dalam	<i>C. felis</i> isolate Malay01 (MT027230.1)	100.00	100	0.0	East and Southeast Asia
F109	Pantai Dalam	<i>C. felis</i> isolate Malay01 (MT027230.1)	100.00	100	0.0	East and Southeast Asia
F118	Pantai Dalam	<i>C. felis</i> isolate Malay01 (MT027230.1)	99.83	98	0.0	East and Southeast Asia

3.3. Molecular Detection of *Bartonella* spp.

Of the 118 flea DNA samples, 102 (86.44 %) tested positive for *Bartonella* spp. via PCR targeting the *gltA* gene, producing amplicons of the expected 379 bp size (Table 1). Twelve representative positive samples from different locations were sequenced for species identification. BLAST analysis of the 266 bp partial *gltA* sequences revealed the presence of two recognized species, *B. henselae* and *B. claridgeiae*, along with two uncharacterized *Bartonella* strains (Table 6). Ten sequences exhibited ≥98 % similarity with known type strains, confirming species identity. One sample (F103) displayed 92.51 % similarity to *Bartonella* sp. isolate Dog_9 (GenBank accession no.: MN233800.1), suggesting a

potentially novel or divergent strain, though still above the 95.4 % similarity threshold for genus-level classification [16]. Specifically, three sequences (F01, F11, F33) matched *B. henselae* isolates from domestic cats in Brazil (MN107415.1), while seven (F23, F38, F44, F46, F83, F91, F109) were closely related to *B. claridgeiae* isolates PESET LAMADINUFF 23/25 (MH019300.1–MH019301.1). Another sample (F95) showed 98.48 % identity with an uncultured *Bartonella* sp. clone (MH279890.1) from a crab-eating fox (*Cerdocyon thous*) in southern Brazil.

Table 6. The BLASTn results for the obtained *Bartonella* sequences based on *gltA* gene region.

Sample ID	Location	Closest GenBank match	Maximum Identity (%)	Query cover (%)	E-value	Origin
F01	University Malaya	<i>B. henselae</i> isolate 15 (MN107415.1)	99.70	100	5e-172	Brazil
F11	University Malaya	<i>B. henselae</i> isolate 15 (MN107415.1)	99.70	100	5e-172	Brazil
F23	Vista Angkasa Apartment	<i>B. claridgeiae</i> isolate PESET LAMADINUFF 23 (MH019300.1)	99.41	100	2e-170	Brazil
F33	Vista Angkasa Apartment	<i>B. henselae</i> isolate 15 (MN107415.1)	98.53	100	2e-166	Brazil
F38	Vista Angkasa Apartment	<i>B. claridgeiae</i> isolate PESET LAMADINUFF 23 (MH019300.1)	99.41	100	2e-170	Brazil
F44	LRT Pasar Seni	<i>B. claridgeiae</i> isolate PESET LAMADINUFF 25 (MH019301.1)	99.69	96	6e-166	Brazil
F46	LRT Pasar Seni	<i>B. claridgeiae</i> isolate PESET LAMADINUFF 23 (MH019300.1)	99.70	100	2e-171	Brazil
F83	Pantai Dalam	<i>B. claridgeiae</i> isolate PESET LAMADINUFF 25 (MH019301.1)	99.11	100	1e-167	Brazil
F91	Pantai Dalam	<i>B. claridgeiae</i> isolate PESET LAMADINUFF 25 (MH019301.1)	98.24	100	1e-163	Brazil
F95	Pantai Dalam	Uncultured <i>Bartonella</i> sp. clone IPVDF_18 (MH279890.1)	98.48	98	2e-160	Brazil
F103	Pantai Dalam	<i>Bartonella</i> sp. isolate Dog_9 (MN233800.1)	92.51	99	4e-128	Chile
F109	Pantai Dalam	<i>B. claridgeiae</i> isolate PESET LAMADINUFF 23 (MH019300.1)	99.41	100	8e-170	Brazil

3.4. Phylogenetic Analysis

Phylogenetic reconstruction of the *cox1* gene sequences confirmed that all flea samples obtained in this study belonged to *Ctenocephalides felis* (Figure 2). All sequences formed a strongly supported monophyletic cluster (bootstrap = 96 %) together with *C. felis* isolate Malay01 (GenBank accession no.: MT027230.1), previously reported from cats in Southeast Asia. This cluster was clearly distinct from *Ctenocephalides canis* and *Ctenocephalides orientis*, which grouped into a separate, well-supported clade (bootstrap = 94 %). The topology thus corroborates both the morphological and molecular identification of the fleas as *C. felis*. Phylogenetic analysis of the *Bartonella gltA* gene sequences revealed the presence of two well-defined clades, corresponding to *B. henselae* and *B. claridgeiae* (Figure 3). Sequences F01, F11, and F33 clustered tightly with *B. henselae* isolates previously reported

from domestic cats in Brazil and the United States, supported by a bootstrap value of 99 %. This *B. henselae* clade formed a sister relationship with *B. koehlerae* (GenBank accession no.: AF176091.1), indicating close evolutionary relatedness (bootstrap = 81 %). The seven *B. claridgeiae* sequences (F23, F38, F44, F46, F83, F91, F109) formed a robust monophyletic clade (bootstrap = 98 %) alongside reference strains from Brazil, the USA, and Switzerland. Notably, sequence F103, although related to this cluster, branched independently with relatively lower sequence similarity, suggesting possible intraspecific divergence or geographic variation. Sequence F95 was genetically distinct from both major clades but clustered closely with an uncultured *Bartonella* sp. clone (GenBank accession no.: MH279890.1) previously detected in a crab-eating fox (*Cerdocyon thous*) from Brazil (bootstrap = 99 %). This unique positioning may reflect lineage diversification within *Bartonella* species infecting *C. felis* in Malaysia.

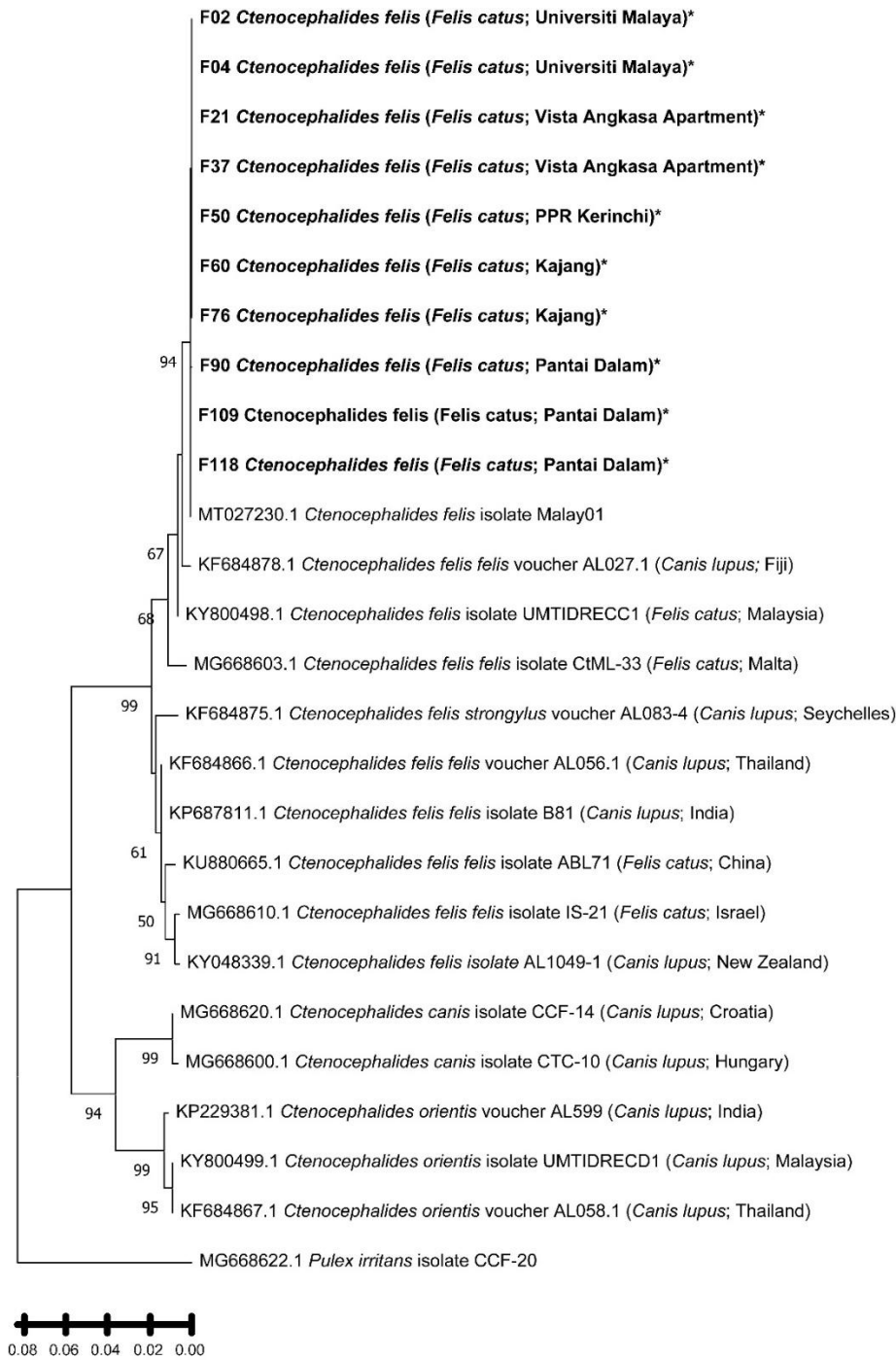


Figure 2. Phylogenetic tree based on 479 bp of *cox1* gene of flea sample from Klang Valley and constructed by using MEGA11 software. The tree was obtained by using the Maximum Likelihood method and Tamura 3-

parameter model. Bootstrap support was calculated by using 1000 replicates and >60 % bootstrap values are shown. *Pulex irritans* isolate CCCF-20 was used as an outgroup for this study. Bold and asterisk (*) indicate the samples in this study.

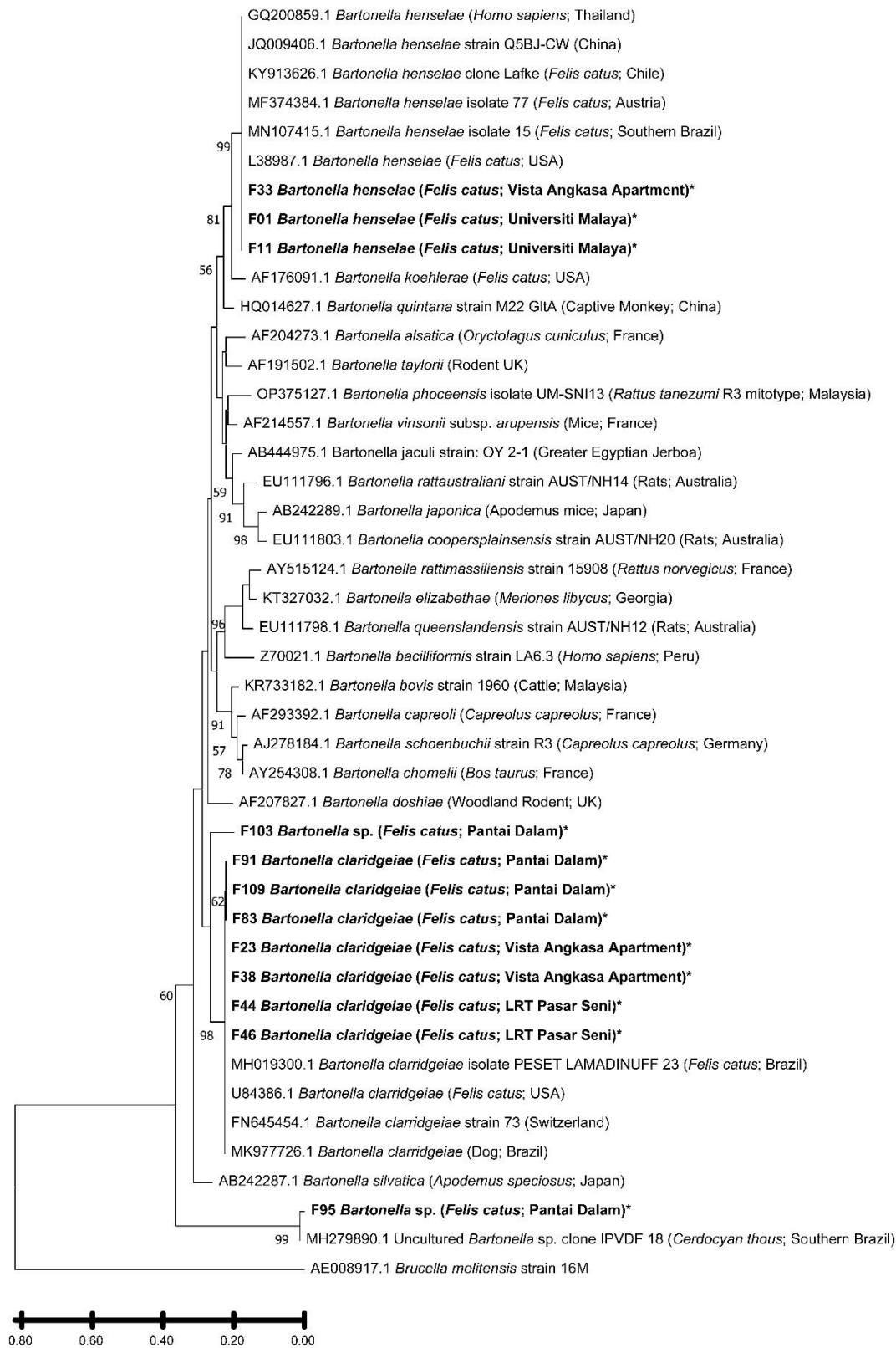


Figure 3. Phylogenetic tree based on 266 bp citrate synthase (*gltA*) gene of *Bartonella* sample from Klang Valley and constructed by using MEGA11 software. The tree was obtained by using the Maximum Likelihood method and Kimura 2-parameter model. Bootstrap support was calculated by using 1000 replicates and >55 % bootstrap

values are shown. *Brucella melitensis* strain 16M was used as an outgroup for this study. Bold and asterisk (*) indicate the samples in this study.

4. Discussion

This study offers the latest insight into flea infestation patterns among urban stray cats in the Klang Valley, a region marked by close human–animal interactions and limited vector surveillance. The overall flea infestation rate of 39.33 % observed here, although lower than the national average of 71.83 % reported by [17], remains epidemiologically important and aligns with thresholds that raise public health concerns [18]. Similar levels of infestation have been reported in other urbanised tropical region such as Mexico (53.0 %) [19], though they are substantially lower than reports from Thailand (95.8 %) [20], Egypt (85.7 %) [21], and Iran (92.3 %) [22]. Variations in prevalence likely stem from differences in climate, host density, sampling methods, and local veterinary practices.

Urban Malaysia's tropical humidity and steady temperatures create ideal conditions for continuous flea reproduction [23]. Stray cats frequenting busy areas, housing complexes, eateries, and transport hubs act as sentinels for urban flea persistence. The widespread infestation across these human-related zones highlights the need for integrated ectoparasite surveillance within a One Health approach to reduce zoonotic exposure risks among city populations.

No significant link was found between flea infestation and host sex or age, aligning with previous studies in Asia and the Middle East [20–22]. Although juvenile cats showed marginally higher infestation rates, these differences were not statistically significant, supporting findings that grooming behaviour, social structure, and environmental contact have a greater influence than intrinsic host factors [24,25].

The predominance of female cats in the sampling pool aligned with previous studies conducted by [26] and [17]. This situation may be explained behavioural accessibility, as males often avoid human contact due to territorial aggression. Such sex-biased sampling should be considered in future designs to better estimate true infestation demographics.

Morphological and molecular analysis confirmed *Ctenocephalides felis* as the only flea species parasitising stray cats in the Klang Valley. This finding aligns with global data recognising *C. felis* as the most widespread flea species associated with both companion and stray animals [27–29]. The use of *cox1* barcoding improved taxonomic accuracy, confirming species identity and showing close genetic ties to the *C. felis* isolate Malay01 previously reported from Southeast Asia.

Given its broad host range and vector competence, *C. felis* acts as a crucial epidemiological link between animals and humans, especially in environments lacking regular veterinary care. The predominance of *C. felis* supports the hypothesis that this species is the main vector of *Bartonella* and other flea-borne pathogens in Malaysia's urban ecosystems [4,8].

The detection of *Bartonella* DNA in 86.44 % of fleas is remarkably high, one of the highest recorded worldwide, and significantly surpasses previous Malaysian reports (28.0 %) [30] and figures from France (26.2 %) [31], the USA (22.8 %) [32], and Palestine (50.4 %) [33]. Such an elevated prevalence indicates a well-established enzootic cycle of *Bartonella* within stray cat populations and their flea vectors in the Klang Valley.

Sequencing of the *gltA* gene identified two pathogenic species, *B. henselae* and *B. claridgeiae*, both recognized as causative agents of cat scratch disease (CSD) in humans [5]. *B. claridgeiae* predominated (58.3 %), mirroring emerging trends in Asia and Latin America where it is increasingly implicated in human bartonellosis [34,35]. The co-circulation of *B. henselae* and *B. claridgeiae* reinforces the dual-vector reservoir role of stray cats and their fleas in maintaining zoonotic transmission cycles.

Notably, the detection of two divergent genotypes, *Bartonella* sp. clone IPVDF_18 and isolate Dog_9, highlights potential novel or regionally adapted *Bartonella* lineages. The close phylogenetic relationship of these Malaysian sequences to South American isolates [36,37] may suggest global dissemination through companion animal movement or vector migration, an increasingly recognised phenomenon in molecular epidemiology.

Phylogenetic analyses of *cox1* and *gltA* sequences provided molecular confirmation of both flea and bacterial species identity. All *C. felis* sequences clustered monophyletically with *C. felis* isolate Malay01, forming a lineage distinct from *C. canis* and *C. orientis*, thus validating morphological diagnosis. Similarly, *Bartonella* sequences formed two well-supported clades representing *B. henselae* and *B. claridgeiae*. The branching pattern of sequences F95 and F103, which diverged from these main clades, suggests possible host adaptation or local genetic diversification within Malaysian *Bartonella* populations. These evolutionary insights strengthen the understanding of regional *Bartonella* ecology and its zoonotic potential.

The coexistence of high *C. felis* infestation and elevated *Bartonella* infection underscores the One Health importance of flea-borne zoonoses in urban Malaysia. Stray cats serve as both reservoirs and amplifiers of vector populations capable of transmitting CSD to humans, particularly in densely populated communities, open eateries, and waste accumulation zones.

Inadequate vector control and the absence of animal health monitoring facilitate ongoing pathogen circulation. Integrating flea surveillance with urban health strategies, particularly in collaboration with municipal authorities and animal welfare organizations, could help mitigate zoonotic risks. This aligns with WHO's and OIE's (WOAH) global call to strengthen vector-borne disease surveillance through cross-sectoral approaches [38,39].

While the study provides critical baseline data, limitations include restricted sampling coverage and reliance on a single gene marker (*gltA*). Expanding future research to include multiple loci (e.g., *rpoB*, *ftsZ*, *ssrA*) and additional pathogens such as *Rickettsia felis* and *Mycoplasma haemofelis* would enhance diagnostic resolution [40,41]. Broader spatial sampling across peri-urban and rural gradients, coupled with next-generation sequencing and vector-host interaction modelling, could further elucidate the ecological and evolutionary dynamics of flea-borne *Bartonella* in Southeast Asia.

Future studies should broaden sampling to include peri-urban and rural areas across Peninsular Malaysia, increase the sample size, and incorporate multi-host analysis involving cats, dogs, and rodents. They should also screen for additional flea-borne pathogens such as *Rickettsia*, *Mycoplasma*, and *Brucella*, and utilise whole-genome sequencing to investigate *Bartonella* evolution and virulence factors. An integrated surveillance approach that combines molecular diagnostics, vector ecology, and community education is crucial for reducing zoonotic risks. These findings emphasise the urgent need for coordinated One Health interventions that target the urban stray cat population as both a sentinel and reservoir of flea-borne zoonoses in Malaysia.

5. Conclusions

This study presents updated molecular and phylogenetic evidence of *Ctenocephalides felis*-borne *Bartonella* species circulating among stray cats in urban areas of the Klang Valley, Malaysia. The high prevalence of *C. felis* infestations and the notable detection rate of *Bartonella* DNA highlight a potentially underestimated zoonotic risk within densely populated urban environments. Phylogenetic analyses based on *cox1* and *gltA* genes confirmed that all flea specimens were *C. felis* and identified *B. henselae* and *B. claridgeiae*, with several genetically diverse lineages suggesting possible novel or geographically adapted strains. These findings emphasise the importance of ongoing molecular surveillance of flea-borne pathogens in stray and domestic animal populations, especially in tropical cities where close human-animal contact facilitates transmission. Integrated control strategies combining vector management, stray animal health programmes, and public awareness are crucial to reducing the risk of *Bartonella* and other flea-borne pathogens spilling over to humans. Future research employing multi-locus or whole-genome approaches will further clarify the evolutionary dynamics and public health implications of these emerging pathogens in Southeast Asia.

Supplementary Materials: Table S1: The number of fleas collected by hosts within Klang Valley area.

Author Contributions: Conceptualization, Z.Y.; Methodology, J.T.A.A., Z.Y., M.A.A., and M.R.A.H.; Validation, M.R.A.H., A.A.A.H, M.A.A. and Z.Y.; Formal Analysis, J.T.A.A, M.R.A.H and A.A.A.H; Investigation, J.T.A.A.,

Z.Y., M.A.A., A.A.A.H. and M.R.A.H.; Resources, Z.Y., M.A.A., M.R.A.H. and S.A.B.; Writing – Original Draft Preparation, J.T.A.A., Z.Y., M.A.A., and A.A.A.H.; Writing – Review and Editing, J.T.A.A., Z.Y. and A.A.A.H.; Visualization, J.T.A.A., Z.Y., and M.A.A.; Supervision, Z.Y., M.A.A., M.R.A.H. and S.A.B.; Project Administration, Z.Y., and M.A.A.; Funding Acquisition, Z.Y., S.A.B. and M.A.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Universiti Malaya Research Grant (RU Grant) (Code: RU006-2025A).

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Acknowledgments: The authors would like to extend their gratitude for the assistance of TIDREC surveillance team during the field sampling. We are grateful for the laboratory accessibility from Department of Microbiology, Universiti Malaya and their staff for the kind assistance.

Conflicts of Interest: The authors declare no conflicts of interest.

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