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RESEARCH ARTICLE

Detection of *Mycoplasma haemocanis* and *Candidatus Mycoplasma haematoparvum* in shelter and pet dogs in Malaysia

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ABSTRACT

Canine haemotrophic mycoplasmosis is caused by mycoplasma haemopathogens, which includes *Mycoplasma haemocanis* (Mhc) and *Candidatus Mycoplasma haematoparvum* (CMhp). The Mhc and CMhp pose a health risk to dogs, particularly in immunocompromised and splenectomised dogs, as they lead to haemolytic anaemia. There is scarce information on the detection of *Mycoplasma* in dogs in Malaysia. Therefore, this study aims to detect the presence of *Mycoplasma* in the blood of shelter and pet dogs and identify associated risk factors in Malaysian dog populations. Blood samples from shelter dogs in Selangor (n = 71) and pet dogs in Johor Bahru (n = 169) were collected. Conventional polymerase chain reaction (PCR) was used to detect *Mycoplasma* 16S rRNA. Overall, 21.7% of the tested samples were positive, with a higher prevalence among the shelter dogs (45.1%) than pet dogs (11.8%). The Mhc was the predominant species detected, with only one case of CMhp. Risk factors associated with *Mycoplasma* infection in shelter dogs included urban areas, and the presence of rodents, and wild animals, but no significant associations with tick infestations were detected. These findings necessitate the importance of *Mycoplasma* transmission dynamics among Malaysian dog populations to assist in the implementation of control measures.

Keywords: 16S rRNA; *Candidatus Mycoplasma haematoparvum; Mycoplasma haemocanis*; pet dogs; shelter dogs.

INTRODUCTION

Canine haemotrophic mycoplasmosis represents a serious risk to canine health worldwide. *Mycoplasma haemocanis* (Mhc) and *Candidatus Mycoplasma haematoparvum* (CMhp) are two haemopathogens affecting dogs. The erythrocytes of infected dogs are negatively impacted, resulting in morphological changes with a shorter lifetime. Infected dogs may experience severe clinical symptoms, such as fever, anorexia, thrombocytopenia, lethargy, and sometimes even lethality (Nascimento *et al*., 2012; Garden *et al*., 2019).

Although the precise mode of canine haemoplasma transmission remains uncertain, infected *Rhipicephalus sanguineus* sensu lato tropical lineage, now known as *Rhipicephalus linnae* ticks, are associated with the transmission because of their blood-feeding behaviour. (Kaewmongkol *et al*., 2017). However, other transmission routes, including aggressive dog interactions and potential horizontal transmission, have been proposed (Barker *et al*., 2010; Willi *et al*., 2010). Notably, cases of canine haemoplasma transmission through blood transfusions have been documented (Kim *et al*., 2020).

In Southeast Asia, the prevalence of *Mycoplasma* in dogs in Thailand and Cambodia has been reported at 19.9% and 9.9% for Mhc and 2.9% for CMhp, respectively (Inpankaew *et al*., 2016; Liu *et al*., 2016). However, in Malaysia, information regarding the prevalence of haemotrophic *Mycoplasma* in dogs is lacking, with existing studies focusing solely on feline populations (Yasmin *et al*., 2022; Zarea *et al*., 2022). Thus, this study aims to bridge the knowledge gap by molecularly detecting haemotrophic *Mycoplasma* and identifying epidemiological risk factors associated with transmission among dogs in Malaysia.

MATERIALS AND METHODS

Sample collection

A total of 240 blood samples were collected, comprising 71 samples from shelter dogs in Selangor and 169 samples from pet dogs collected at the Pet Expo event by the Small Animal Clinic in Johor Bahru. Approximately 2-4 mL of blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes (BD Vacutainer) from each dog via cephalic venepuncture. The samples were then sent to

Table 1. The 16S rRNA primers used in this study

	Primers	Approximate amplicon size	Annealing temperature	Reference
Genus-specific primer	myco-F (5'-ACGAAAGTCTGATGGAGCAATA-3') myco-R (5'-ACGCCCAATAAATCCGRATAAT-3')	170–193	60°C	Kewish *et al.* (2004)
	Htm-F (5'-ATACGGCCCATATTCCTACG-3') Htm-R (5'-TGCTCCACCACTTGTTGTTCA-3')	595–618	60°C	Criado-Fornelio *et al.* (2003)
Species-specific primer	Mhf-F1 (5'-GACTTTGGTTTCGGCCAAGG-3') Mhf-R3 (5'-CGAAGTACTATCATAATTATCCCTC-3')	393	52°C	Berent *et al.* (1998)
	CMhp-F (5'-GGAGAATAGCAATCC GAAAGG-3') CMhp-R (5'-GCATTTACCCCACCAA CAAC-3')	1062	57°C	Alves *et al.* (2014)

the Parasitology Laboratory, Faculty of Veterinary Medicine, UPM, and stored at -20°C until further use.

A set of three-section questionnaires was prepared for the owners or staff to determine the risk factors associated with the prevalence of hemoplasmas in dogs. The possible risks analysed were sex, age, tick infestation status, and the presence of rodents and other wild animals in the shelters. Demographic information about the dogs that tested positive for haemotrophic *Mycoplasma* was retrieved from clinical records.

DNA extraction and PCR assays

DNA from the blood samples was extracted using a commercial kit, the PrimeWay Genomic DNA Extraction Kit (1st Base, Singapore). The extracted DNA was eluted in 100 µL of elution buffer and stored at -20°C. Polymerase chain reactions (PCR) amplification of haemotrophic *Mycoplasma* targeting 16S rRNA was performed using genus and species-specific primers (Table 1). All PCR reactions were carried out in a C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, USA). The amplified products were electrophoresed (400 W/80 V) on a 1.5% agarose gel (MyAgarose™) with tris-acetic acid-EDTA (TAE) buffer and stained with RedSafe™ nucleic acid staining solution (20,000x). After one hour, the gel was visualised under a UV transilluminator (GeneDoc™, Bio-Rad Laboratories, USA).

Sequencing and phylogenetic analysis

Positive amplicons of haemotrophic *Mycoplasma* from dog blood (*n* = 12) were sequenced using the cycle sequencing technology (dideoxy chain termination/cycle sequencing) on ABI PRISM 3730xl Genetic Analyser (Applied Biosystems, USA). The sequences obtained were compared to the closely related sequences in the GenBank database and aligned using DAMBE5 (Xia, 2018). MEGA X was applied for phylogenetic relationship determination using a maximum likelihood (ML) algorithm (Kumar *et al*., 2018). At least 1,000 replicates were used to estimate each species' bootstrap values.

Statistical analysis

Pearson's chi-square test was used to determine the risk factors associated with haemotrophic *Mycoplasma* infection in dogs using Microsoft Excel (2021). The parameters were estimated at 95% confidence intervals, and $p < 0.05$ indicates statistical significance.

RESULTS

Haemotrophic *Mycoplasma* was detected in 52 of 240 (21.7%) dogs, with 32 (45.1%) shelter dogs and 20 (11.8%) pet dogs. There is a significantly higher prevalence of *Mycoplasma* in shelter dogs Selangor compared to pet dogs in Johor ($\chi^2 = 32.11$, df=1, *p*-value < 0.01). Among the positive cases, only 1 pet dogs tested positive for CMhp.

M. haemocanis morphology is observed as cocci in the chain at the periphery of the erythrocytes (Figure 1), while *Candidatus* M. *haematoparvum* appears quite similar to *Candidatus* M. *haemominutum* (Foley & Pedersen, 2001) under blood smear with the presence of single or double small cocci anchored to the erythrocytes (Sykes *et al*., 2005).

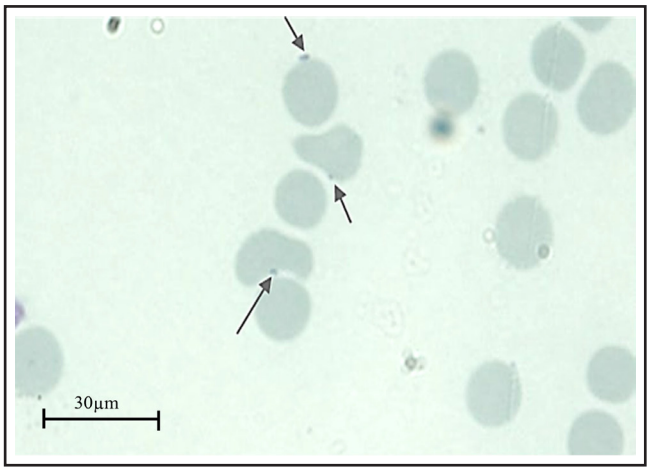

Figure 1. Microscopic visualization of *Mycoplasma haemocanis* on Giemsa-stained thin blood smear slide.

Demographic data for only 6 of 20 positive haemotrophic *Mycoplasma* cases were recorded in the veterinary clinic's data system, as presented in Table 2. The majority of the infected dogs within this subset are males, constituting 83.3% (N=5/6) of the sample group. In contrast, females make up a smaller portion, at 16.7% (N=1/6). The highest percentage of infected dogs falls within more than one- year-old dogs, categorising them as adults, followed by young dogs. A significant 83.3% (N=5/6) of the infected dogs are of purebred origin. The data also suggests that a significant proportion of infected dogs have not undergone deworming, 66.67% (N= 4/6) and are mostly managed indoors, 83.3% (N = 5/6). The significant association between demographic information and haemotrophic *Mycoplasma* was unable to be demonstrated due to limited records available for pet dogs. However, demographic patterns for positive dogs in this study revealed that males, adults, and purebreds had a greater risk of infection (Table 4).

Based on the association between risk factors and the detection of Mhc in shelter dogs, variables such as area and, the presence of rodents and, wild animals, e.g., for examples foxes, show significant associations (Table 3).

Table 3. Demographic data of infected shelter dogs in Selangor

Epidemiological factor	Detection (%)	*p*-value	χ^2
Sex			
Male	9/24 (37.5%)	0.338	0.918
Female	23/47 (48.9%)		
Age			
<1 year	3/6 (50%)	0.631	0.230
> 1 year	29/65 (44.6%)		
Area			
Urban	13/20 (65%)	0.034*	4.489
Suburban	19/51 (37.3%)		
Presence of rodents			
Yes	19/51 (37.3%)	0.034*	4.489
No	13/20 (65%)		
Presence of wild animals			
Yes	13/39 (33.3%)	0.027*	4.862
No	19/32 (59.4%)		
Tick infestation			
Yes	6/13 (46.2%)	0.753	0.099
No	26/58 (44.8%)		

*Significant (p<0.05).

Table 4. Demographic data of pet dogs with positive haemotrophic *Mycoplasma*

Epidemiological factor	Detection (%) (N=6)
Sex	
Male	5 (83.3%)
Female	1 (16.7%)
Age	
<1 year	1 (16.7%)
> 1 year	5 (83.3%)
Breed	
Pure Breed	5 (83.3%)
Cross Breed	1 (16.7%)
Deworming	
Yes	2 (33.3%)
No	4 (66.7%)
Management	
Indoor	5 (16.7%)
Outdoor	1 (83.3%)

The *Candidatus* M. haematoparvum observed in the current study has 100% similarity to the sequence from Argentina (KY117662), and 99% similarity to Mhc from Cuba (MZ221174), Chile (KY117659), and South Korea (MT345534) (Kim *et al*., 2020; Roblejo-Arias *et al*., 2022). Accession numbers for the *M. haemocanis* sequences are PQ133358 – PQ133362, and PQ149013 - PQ149018, while for *Candidatus* Mycoplasma haematoparvum is PQ149020. Molecular identification of nucleotide sequences for both organisms was supported by the distinct separation of species-specific clades inferred from the phylogenetic analyses (Figure 2).

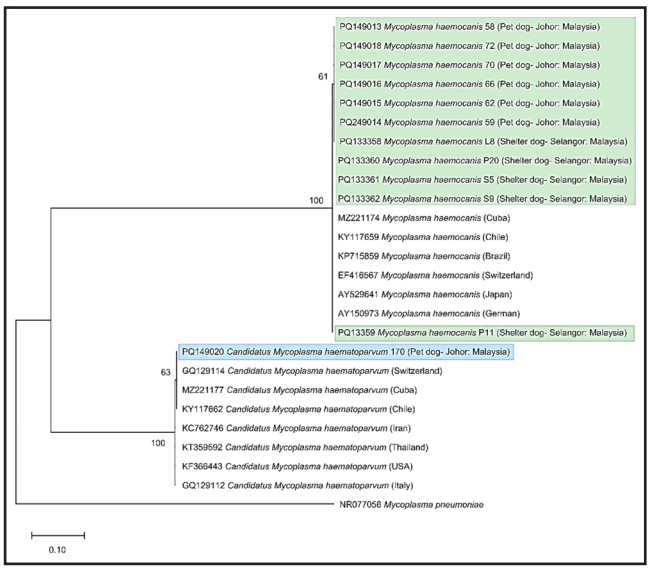

Figure 2. Phylogenetic relationship of *Mycoplasma haemocanis* (highlighted in green) and *Candidatus* Mycoplasma haematoparvum (highlighted in blue) sequences isolated in this study to other *Mycoplasma* spp. based on a partial sequence of the 16S rRNA gene. The analyses were performed using maximum likelihood method. A homologous sequence from *Mycoplasma pneumonia* (accession number: NR077056) was used as the outgroup.

DISCUSSION

Molecular detection in this study revealed a significant prevalence of haemotrophic *Mycoplasma* in shelter dogs than in pet dogs. This difference could be attributed to the shelter setting, which typically houses packs of rescued dogs, increasing the potential transmission of haemotrophic *Mycoplasma* through vectors or direct contact (Birkenheuer *et al*., 2002; Ayoob *et al*., 2010). Group housing can be stressful for these dogs, increasing their susceptibility to infections (Brennan *et al*., 2008). In contrast to pet animals living in hygienic environments, hygiene practices and animal management standards in kennels are often less stringent (Paradies *et al*., 2007; Brennan *et al*., 2008; Novacco *et al*., 2010). This suggests that environmental factors and living conditions could play a role in the prevalence of haemotrophic *Mycoplasma* infections in dogs. Further research is needed to explore these specific risk factors associated with haemotrophic *Mycoplasma* infections in different dog populations in Malaysia.

Two haemotrophic *Mycoplasma* species were identified in this study using molecular techniques. This observation is consistent with the from Thailand, a neighbouring country, where both species were identified among pet dogs, with two dogs (2.47%) positive for Mhc (1.23%) one dog positive for CMhp (Kaewmongkol *et al*., 2017). In addition, the findings from this study are in line with results from Iran (Mhc, = 17.8%, CMhp = 7.02%), Trinidad (Mhc, = 4.9%, CMhp = 2.7%) and Turkey (Mhc, = 50%, CMhp = 21.6%), where Mhc is more prevalent than CMhp (Barker *et al*., 2010; Dolgun & Kırkan, 2023; Beus *et al*., 2024).

The risk factors for haemotrophic *Mycoplasma* infection include urban habitats and the presence of rodents. These findings are consistent with a previous study showing that dogs from households with rodents are more susceptible to Haemotrophic *Mycoplasma* infection (Barbosa *et al*., 2021). Haemotropic *Mycoplasma* has been detected in urban rats such as *Rattus* spp. (Conrado *et al.,* 2015; Gonnalves *et al.,* 2015; Hornok *et al.,* 2015), suggesting that rodents might serve as reservoirs. Further investigation is needed to fully understand the dynamics of transmission and the extent of risk posed by urban wildlife.

Risk factors such as area and, exposure to rodents and wild animals are significantly associated with mycoplasmosis. Dogs from shelters located in urban areas (p-value = 0.034), and unexposed to rodents (p-value =0.034) and wild animals (p-value =0.027) are more likely to be infected with haemotrophic *Mycoplasma.* In this study, only Shelter 2 was located in an urban area and unexposed to rodents and other wild animals. Another shelter that was unexposed to wild animals was Shelter 1. Meanwhile, the other shelters were located in suburban areas, and were exposed to rodents and other wild animals, such as foxes.

This study is congruent with the findings by Barbosa (2021) in Brazil. However, studies in Brazil and Japan discovered the association of haemoplasma infection for both *M. haemocanis* and *Candidatus* M. haematoparvum in wild animals (Andre *et al*., 2011; Furtado *et al*., 2018; Sasaki *et al*., 2008). The findings suggests that the transmission between foxes and dogs could occur with the involvement of arthropod vectors (Sasaki *et al*., 2008). The disease might perhaps be transmitted through horizontal transmission, which most likely occurs via biting and fighting that could be related to male dog's behaviour (Barker *et al*., 2010). In this study, the presence of rodents is not associated with haemotrophic *Mycoplasma* positive cases, however in a study in Brazil, shelters with rodents' in the households showed high chances of dogs becoming infected (Barbosa *et al*., 2021). Abundant strains of haemotrophic *Mycoplasma* are isolated from rodents, including *Mycoplasma haemuris* and *Mycoplasma coccoides* (Vieira *et al*., 2015), which might suggest rodents as a natural reservoir of *M. haemocanis* to dogs. However, this possible cross-species transmission needs to be studied and investigated properly to prove the association with the haemotrophic *Mycoplasma* infection.

The results showed that tick infestation was not a risk factor for haemotrophic *Mycoplasma* infection in dogs in this study. This finding challenges the assumption that ticks are vectors of haemotrophic *Mycoplasma* among dogs (Seneviratna *et al*., 1973). The presence of 26 infected dogs without tick infestations suggests alternative routes of transmission or reservoirs for haemotrophic *Mycoplasma.* It is crucial to explore these alternative pathways to understand the risk factors contributing to haemotrophic *Mycoplasma* infection among dogs in Malaysia.

This study represents the first report of haemotrophic *Mycoplasma* in dogs in Malaysia. It was more prevalent in shelter dogs than in pets, possibly due to living conditions and lower hygiene standards in the shelter environment. This study identified two haemotrophic *Mycoplasma* species using molecular techniques, with *M. haemocanis* predominating. Urban area and rodent presence are the risk factors, while tick infestation did not correlate with haemotrophic *Mycoplasma* infection in this study. These findings revealed a potential risk factor that urges comprehensive investigation to support control strategies and ensure the well-being of canine populations.

Conflict of Interests

The author declares that they have no conflict of interests.

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