UNIVERSITI PUTRA MALAYSIA

# MOLECULAR DETECTION OF *MYCOPLASMA* SPP. AND RISK FACTORS IN DOGS FROM ANIMAL SHELTER IN SELANGOR

NUR AZIRA BINTI MOHD ZAHIR

lp
FPV 2022 33

# MOLECULAR DETECTION OF *MYCOPLASMA* SPP. AND RISK FACTORS IN DOGS FROM ANIMAL SHELTER IN SELANGOR

**NUR AZIRA BINTI MOHD ZAHIR**

**A project paper submitted to the**
**Faculty of Veterinary Medicine, Universiti Putra Malaysia**
**In partial fulfillment of the requirement for the**
**DEGREE OF DOCTOR OF VETERINARY MEDICINE**
**Universiti Putra Malaysia**
**Serdang, Selangor Darul Ehsan.**

**December 2022**

@COPYRIGHT UPM

It is hereby certified that I have read this project paper entitled “Molecular detection of *Mycoplasma* spp. And risk factors in dogs from animal shelter in Selangor”, by Nur Azira binti Mohd Zahir and in my/our* opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4901 - Project.

**NOR AZLINA BINTI ABDUL AZIZ**
**DVM (UPM), PHD (BRISTOL)**
Senior Lecturer,
Faculty of Veterinary Medicine Universiti Putra Malaysia
(Supervisor)

**2022/2023**

@COPYRIGHT UPM

**DEDICATION**

To

my parents, family and friends

who made all of this possible,

for their endless encouragement and patience

And also to

Everyone who gave me

support, strength and courage

@COPYRIGHT UPM

## ACKNOWLEDGEMENT

I would like to express my deep and sincere gratitude to all those who have helped me throughout my Final Year Project journey. First and foremost, I would like to thank my supervisor, Dr Nor Azlina and co-supervisors, Dr Azalea Hani and AP Dr Malaika for their valuable guidance, enthusiasm and knowledge in this parasitology field. I learnt so much from their insightful comments in all the time of writing my first thesis. Their continuous patience and motivation really made me believe in myself to present and finish this project well. Not to forget the laboratory staff, especially Quincie, Puan Maizatul and Encik Rashid who helped me during the PCR procedures and identification of *Mycoplasma* spp.

I also would like to take this opportunity to thank my parents and family members for giving me endless support and care. Your support really meant a lot that made me able to try giving the best as I could in my life.

Last but not least, my precious thanks to my beloved friends that I have known since foundation study, Azren, Maria, Ikha, Natasha and Sofia for giving your opinions, mental support and happiness along my journey in this veterinary school. I am going to treasure the sweet memories of us until we graduate and work as a veterinarian. In addition, a big thanks to everyone who involved directly or indirectly along with my time of finishing this thesis as one of the requirements as a veterinary student.

@COPYRIGHT UPM

**CONTENTS**



@COPYRIGHT UPM

**LIST OF TABLES** **Page**



@COPYRIGHT UPM

**LIST OF FIGURES** **Page**



@COPYRIGHT UPM

## ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4901 -Projek.

**PENGESANAN MOLEKUL *MYCOPLASMA* SPP DAN FAKTOR RISIKO DALAM ANJING DARI PERLINDUNGAN HAIWAN DI SELANGOR**

**Oleh**

**Nur Azira binti Mohd Zahir**

**2022**

**Penyelia: Dr. Nor Azlina binti Abdul Aziz**

Mycoplasma hemotropik anjing adalah penyakit bakteria bawaan kutu pada anjing yang diandaikan disebarkan oleh kutu *Rhipicephalus sanguineus*. Hemoplasma yang biasa dilaporkan, *Mycoplasma haemocanis* dan *Candidatus Mycoplasma haematoparvum* melekat pada permukaan eritrosit perumah vertebrata. Penyakit ini selalunya menyebabkan simptom ringan melainkan anjing yang immunokompromi atau organ limpa yang sudah dibuang dijangkiti yang boleh membawa kepada anemia yang teruk. Kajian ini bertujuan untuk mengesan kehadiran spesies *Mycoplasma* dalam anjing liar yang diselamatkan melalui amplifikasi Reaksi Rantai Polimerase (PCR) konvensional dan pemeriksaan mikroskopik sapuan darah nipis yang diwarnakan Giemsa. Sampel darah dikumpul melalui vena di bahagian lengan dari empat pusat perlindungan (n = 71) di Selangor. Pengesanan molekul spesies *Mycoplasma* dilakukan dengan amplifikasi gen 16SrRNA menggunakan primer khusus genus dan spesies. Sampel positif telah dihantar untuk penjujukan dan

@COPYRIGHT UPM

BLASTsearch dalam NCBI GenBank untuk pengesahan spesies. Sebanyak 32 anjing (45.1%) positif *Mycoplasma haemocanis*. Menurut kajian ini, tempat perlindungan yang berada di kawasan bandar dan kurang terdedah kepada tikus dan haiwan liar lebih berkemungkinan dijangkiti spesies *Mycoplasma* (p-value<0.05). Walau bagaimanapun, tiada perkaitan yang signifikan antara serangan kutu dan jangkitan mycoplasma (nilai p = 0.753, $\chi 2$ = 0.099) walaupun ia dikatakan sebagai vektor bagi parasit darah ini. Tiada signifikan juga didapati antara ciri intrinsik anjing iaitu jantina (nilai p = 0.338, $\chi 2$ = 0.918) dan umur (nilai p = 0.631, $\chi 2$ = 0.230). Kajian ini mendedahkan pengesanan *Mycoplasma haemocanis* dalam kalangan anjing yang diselamatkan dari tempat perlindungan yang berbeza di Selangor.

Kata kunci: *Mycoplasma haemocanis*; 16S rRNA; kutu; anjing perlindungan; anemia

@COPYRIGHT UPM

## ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4901- Project.

**MOLECULAR DETECTION OF *MYCOPLASMA* SPP AND RISK FACTORS IN DOGS FROM ANIMAL SHELTER IN SELANGOR**

**by**

**Nur Azira binti Mohd Zahir**

**2022**

**Supervisor: Dr. Nor Azlina binti Abdul Aziz**

Canine hemotropic mycoplasma is a tick-borne bacterial disease in dogs which most likely to be transmitted by *Rhipicephalus sanguineus* ticks. Commonly reported hemoplasma, *Mycoplasma haemocanis* and *Candidatus Mycoplasma haematoparvum* adhere to the surface of erythrocytes of the vertebrate hosts. This disease often causes mild symptoms unless immunocompromised or in splenectomized dogs where infection can lead to severe anemia. This study was aimed to detect the presence of *Mycoplasma* spp in rescued dogs through conventional Polymerase Chain Reaction (PCR) amplification and microscopic examination of Giemsa-stained thin blood smear. Blood samples were collected via cephalic venipuncture from dogs in four shelters (n = 71) in Selangor. Molecular detection of *Mycoplasma* spp was performed by amplification of 16SrRNA gene using genus and species-specific primer. Positive samples were sent for sequencing and BLASTsearch in NCBI GenBank for species confirmation. A total of 32 dogs (45.1%) were positive for *Mycoplasma haemocanis*. According to this study, shelters that are in urban areas and have

@COPYRIGHT UPM

less exposure to rodents and wild animals are more likely to be infected with *Mycoplasma* spp (p-value<0.05). However, there is no significant association between tick infestation and mycoplasma infection (p-value = 0.753, $\chi 2 = 0.099$) although tick is believed to be the vector for this blood parasite. No significant associations found between intrinsic characteristics of dogs which are sex ($\chi 2 = 0.918$, df=1, p-value = 0.338) and age ($\chi 2 = 0.230$, df=1, p-value = 0.631). The present study revealed high detection of *Mycoplasma haemocanis* among rescued dogs from different shelters in Selangor.

Keywords: *Mycoplasma haemocanis*; 16S rRNA; ticks; shelter dogs; anemia

@COPYRIGHT UPM

## 1.0 INTRODUCTION

Canine tick-borne haemopathogen (TBH) diseases which include ehrlichiosis, babesiosis and anaplasmosis are well recognized in our country which could threaten the health and welfare of the animal and human itself (Konto et al, 2017; Prakash et al., 2018). They have been incriminated to be transmitted by *Rhipicephalus sanguineus sensu lato* (s.l.) tick as it is the most common tick infesting dogs in Malaysia which suggest to be the natural vector of various disease (Harrus et al., 2011; Solano-Gallego 2011). In addition to that, dogs that are frequently exposed to ticks are more susceptible to many tick-borne protozoa and bacteria diseases (Kaewmongkol et al., 2017). This can be supported with the presence of abundant arthropod vectors, pet animals, and varied hygiene management practices among households that contribute to canine blood parasite infection as one of the prevalent diseases to be reported every year. Furthermore, the hot and wet tropical climate in Malaysia is an ideal environment for the breeding of arthropods throughout the year.

However, another type of bacteria that is relevant in dogs as other TBH which is canine hemotropic hemoplasma caused by *Mycoplasma* spp. Canine mycoplasmosis is relatively less pathogenic and alarming as compared to other species of mycoplasmosis in our country. This is due to the presence of more pathogenic TBH such as *Babesia* spp, *Anaplasma* spp and *Ehrlichia* spp which cause more significant clinical evidence of acute haemolytic anemia in dogs. Mycoplasmosis could cause anemia particularly in immunocompetent dogs. However, this disease is as important as other TBH since effective treatment of anemia is to acknowledge and address the main cause of the problem which is the agent along with the supportive treatments. Clinically proven antibiotics that used to treat the infected animal are doxycycline, tetracycline and erythromycin. However, erythromycin

©COPYRIGHT UPM

could control acute infection without clearing up the infection (Messick et al., 2003). These antibiotics are commonly prescribed to dogs with other TBH infections. Once the clinical symptoms are resolved, the animal could be the source of infection to other susceptible dogs as they will become chronic carrier (Messick et al., 2004)

In Malaysia, the last report regarding the prevalence of Mycoplasma spp in dogs was done by Goh (2013) in Selangor and the other study by Lim in Perak (2007). Low detection of this parasite infection in both studies are highly influenced by the diagnostic method used which was direct smear evaluation. Both studies were thesis and until now, there is no published data on the prevalence of mycoplasmosis in dogs in Malaysia Diagnosis of tick-transmitted diseases requires a combination of direct microscopic evaluation of infective organisms, microbial culture and PCR assay. However, PCR alone is particularly appropriate in this case although this method is unable to distinguish between acute and chronic state of infection.

Canine mycoplasmosis is generally being underestimated by veterinary practitioners due to the lack of awareness. Apart from that, limited information available regarding the number of dogs infected with *Mycoplasma* spp at animal shelters in Selangor and dogs in general in Malaysia. Previous studies have lacked information regarding the epidemiological factors such as age, gender, breed, localities, hygiene practices, the use of arthropod repellent and exposure to wildlife as part of natural vectors in causing the disease. Thus, the objectives of this study are :

i. To detect the presence of *Mycoplasma* spp in dogs from animal shelter in Selangor

ii. To correlate the possible epidemiological factors and the presence of *Mycoplasma* spp in dogs from animal shelter in Selangor

@COPYRIGHT UPM

## 2.0 LITERATURE REVIEW

Canine hemotrophic mycoplasma is a tick-borne bacterium formerly classified as Haemobartonella and Eperythrozoon species that adhere to the surface of erythrocytes of the vertebrate hosts (Nascimento et al., 2012). This pleomorphic bacterium is only 0.5 to 1.5 micrometers in diameter which makes them appear similar to artifacts, basophilic stippling, siderotic inclusion, and Howell-Jolly bodies (Harvey., 2001; Vaden et al.,2015). They might appear singly, pairs, clusters or even in chains which canine *Mycoplasma* spp could resemble violin-bow shape appearance (Nascimento et al., 2012; Procop et al., 2020). Mycoplasmosis generally has been reported worldwide with a prevalence varying from 0.5% to 40% in all animal species (Novacco et al., 2010).

In dogs, two common *Mycoplasma* species have been reported which are *Mycoplasma haemocanis* and *Candidatus Mycoplasma haematoparvum* which can cause acute haemolytic anemia and assumed to be transmitted by *Rhipicephalus sanguineus* ticks (Kaewmongkol et al., 2017). They utilize the erythrocyte's metabolism by scavenging the nutrients which leads to diminished erythrocyte morphology and lifespan. Overtime, exacerbation of anemia occurs during both acute and chronic disease (Nascimento et al., 2012).

Healthy dogs can get the infection through tick vector, traumas, fomites, blood transfusion, and vertical and horizontal transmission based on experimental transmission in previous work (Willy et al., 2010; Lashnits et al., 2019; Barbosa et al., 2021). There was a positive correlation of dogs infested with ticks and mycoplasma infection (Barbosa et al., 2021) but there were some studies which failed to prove the associations (Vieira et al.,

@COPYRIGHT UPM

2015). The gender of the animal and presence of wild animals were also among the risk factors in contracting the disease (Barbosa et al., 2021).

The most prevalent clinical manifestation of canine mycoplasmosis is hemolytic anemia ranging from asymptomatic, slight lethargy and death (Cataldo et al., 2020; Rosanna et al., 2020). Commonly presented clinical signs are tachypnea, depression, weakness, anorexia, weight loss, pale mucous membrane, dehydration, icterus and splenomegaly (Kewish et al., 2004). Most cases of canine hemoplasma revealed the disease remains latent and subclinical (Messick et al, 2002). However, the dogs which immunosuppressed, splenectomized or concurrently affected with other blood parasites or any infectious diseases could have more severe sign for example severe anemia, mild azotemia and hepatitis (Kemming et al., 2004; Sykes et al., 2004; do Nascimento et al., 2012, Kaewmongkol et al., 2017).

In Malaysia, the studies on mycoplasmosis in dogs were presented in 2007 and 2013 from the thesis. Both studies used evaluation of thin blood smear in identifying the blood parasites with the results of two and one positive cases respectively (Lim et al, 2007; Goh et al, 2013). Another more reliable diagnostic method is Polymerase Chain Reaction (PCR) which has proven to have greater sensitivity, specificity, and robustness for detecting this hemoplasma as compared to microscopy evaluation (Tasker., 2002). This organism also displays cyclical parasitemia where immediate blood film examination is needed to make a true diagnosis (Procop et al., 2020). Thus, microscopic examination with a combination of molecular technique could be ideal to avoid false negative evaluation.

@COPYRIGHT UPM

## 3.0 MATERIALS AND METHOD

### 3.1 Sample collection

Blood samples were obtained from 71 rescued dogs of all ages and both genders from a total of four shelters around Selangor with different management practices (Appendix 1). A set of questionnaires with 3 sections was prepared to be asked from the owner or staff available to relate the management practices and other possible risks with the presence of *Mycoplasma* spp in dogs. The blood samples were collected from randomly chosen dogs via cephalic venipuncture at a volume of 2-4 ml into EDTA (ethylene-diamine-tetra-acetic acid) tubes. A brief physical examination including body condition scoring, dehydration status, capillary refill time, mucous membrane assessment and manual inspection of ectoparasites infestation were performed before the blood sampling procedure. Medication status of the dogs was recorded to know their current health status and relate to the ongoing treatment given with the blood parasite infection. Two different techniques were used to detect the *Mycoplasma* spp in blood which are through microscopical examination of thin blood smear and molecular detection by conventional PCR based on 16S rRNA sequence.

### 3.2 Thin blood smear

A small drop of blood obtained from an EDTA tube placed at the far end of a clean glass slide. A 22 x 22mm coverslip held at 30º then used to produce the smear. The smear was left air-dry on a drying rack with the smear side facing down. The slide was then fixed with ethanol for 2 minutes before staining with Giemsa stain for 30 minutes The slide was

@COPYRIGHT UPM

examined under a light microscope at x1000 magnification (oil immersion) for detection of the blood parasite on the surface of erythrocytes.

### 3.3 DNA extraction, PCR assays and sequencing

DNA from the blood samples were extracted using an available commercial kit, NucleoSpin® Blood Genomic DNA Purification kit (Macherey-Nagel,Germany). Highly pure DNA was then eluted with 100 µL elution buffer and stored in the freezer (− 20 ◦C) until the molecular analysis was conducted. Two primers used were genus specific primer (*Mycoplasma* spp-Myco-F and Myco-R) and species-specific primer (*Mycoplasma haemocanis*-MhF-F1 and Mhf-R3) in the C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, USA) with different cycling specification as indicated in Table 1. Molecular detection of *Mycoplasma* spp was then performed using the technique of conventional PCR in the Biorad Gel Doc XR+ Imaging System Applications (Bio-Rad Laboratories, USA) for visualization of the DNA gel electrophoresis result. The 16S rRNA-based PCR amplifies a 170-bp and 190-bp fragment, and 393- bp fragment for *Mycoplasma* spp and *Mycoplasma Haemocanis* respectively. Samples which came out negative for species specific primer but positive for genus specific primer were sent to BLAST identity search to identify and compare with known sequences in the NCBI GenBank.

### 3.4 Statistical analysis

The presence and absence of mycoplasmosis were recorded in a Microsoft Excel spreadsheet. Then, statistical analysis was performed using chi square test to determine the

@COPYRIGHT UPM

correlation of possible risks on *Mycoplasma* spp infection in dogs. The possible risks analyzed were sex, age, tick infestation status, and presence of rodents and other wild animals into the shelters. The parameters were estimated at 95 % confidence intervals and a p-value of 0.05 or less ($p < 0.05$) was used to indicate a statistically significant difference.



@COPYRIGHT UPM

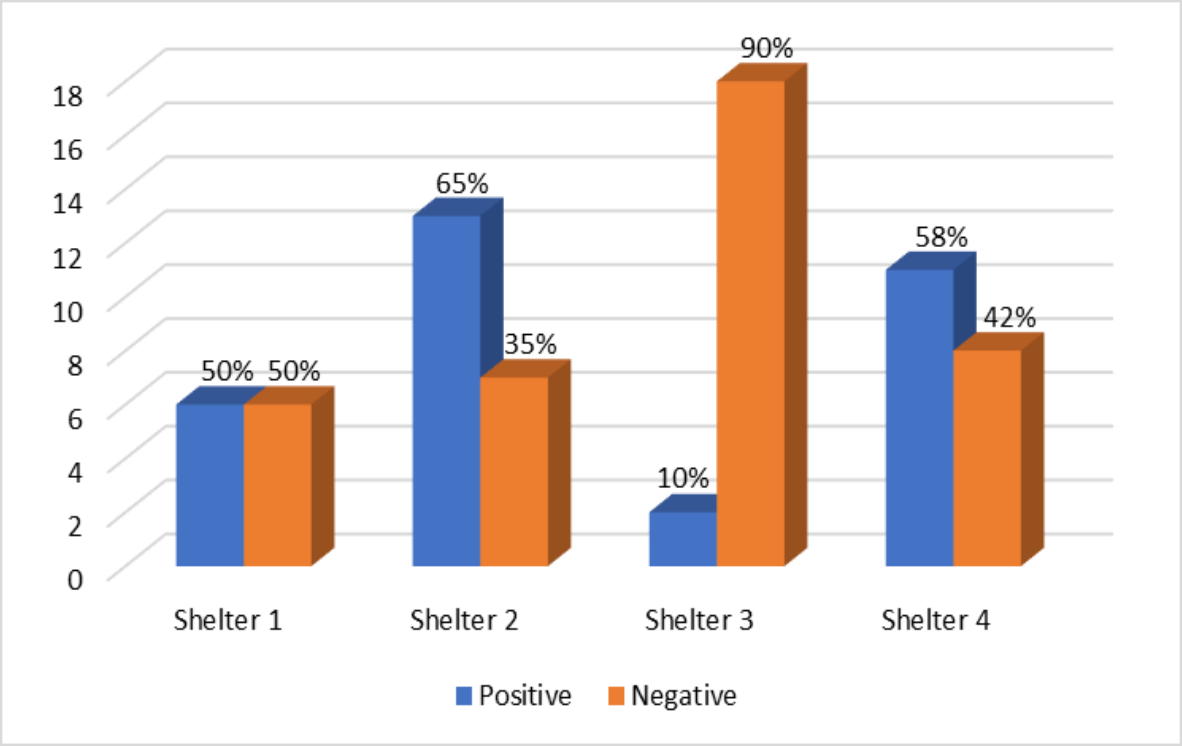
Table 1: Primer sets and cycling condition for PCR detection of *Mycoplasma* spp.

| | Genus specific primer | Species specific primer |
|---|---|---|
| Primer sequence (5′ –3′ ) | myco-F (5′-ACGAAAGTCTGATGGAGCAATA-3′) | Mhf-F1 (5′-GACTTTGGTTTCGGCCAAGG-3′) |
| | myco-R (5′-ACGCCCAATAAATCCGRATAAT-3′) | Mhf-R3 (5′-CGAAGTACTATCATAATTATCCCTC-3′) |
| Initial denaturation | 95 ◦C, 10 min | 94 ◦C, 10 min |
| Denaturation | 95 ◦C, 20 s | 94 ◦C, 45 s |
| Annealing | 60 ◦C, 1 min | 52 ◦C, 45 s |
| Extension | 72 ◦C, 1 min | 72 ◦C, 1 min |
| Final extension | 72 ◦C, 7 min | 72 ◦C, 7 min |
| Total cycles | 40 | 40 |
| Reference | Kewish et al. (2004) | Berent et al. (1998). |

©COPYRIGHT UPM

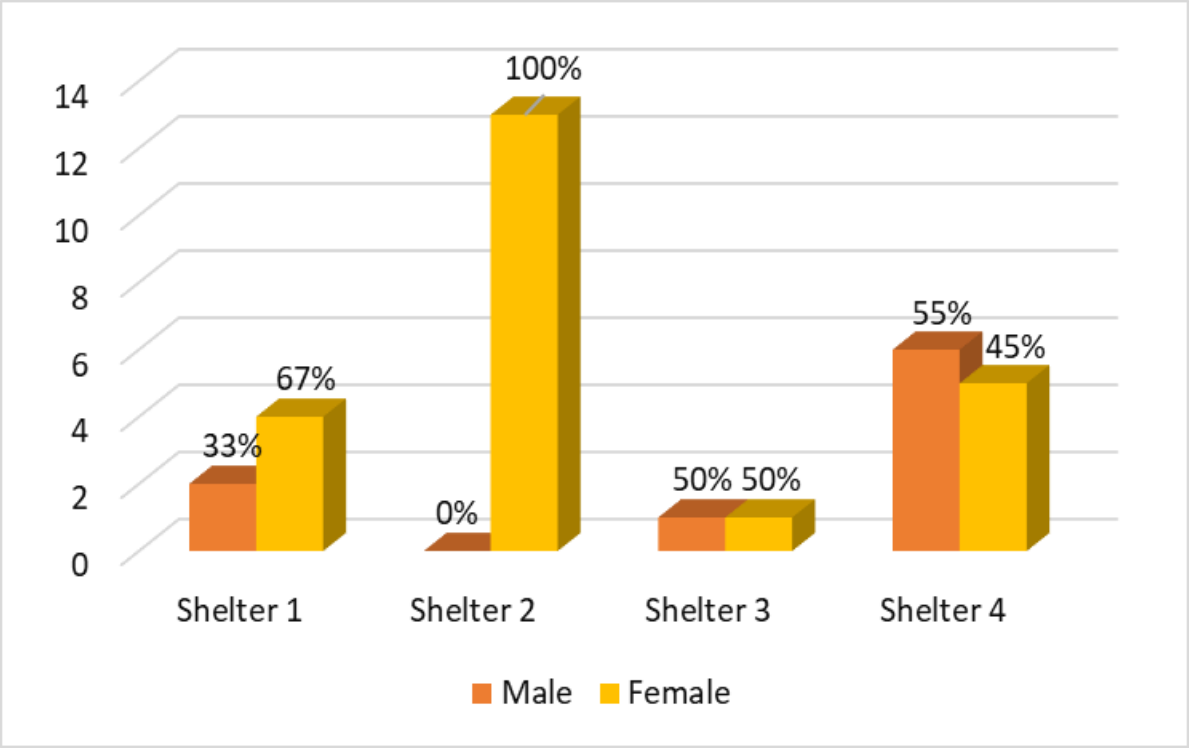
## 4.0 RESULTS

Figure 1: *Mycoplasma haemocanis* infection among dog shelters in Selangor




A total of 32 dogs out of 71 (45.1%) rescued dogs were detected with *Mycoplasma* spp. Shelter 2 had the highest number of detections with 13 out of 20 dogs (65%) were positive (Figure 1). Half of the dogs in shelter 1 (50%; N=6/12) were positive with mycoplasmosis while more than half in shelter 4 (58%; N=11/19) dogs were detected positive. Low detection was observed in Shelter 3 (10%; N=2/20) where only two dogs were observed to be positive for mycoplasmosis. Based on the sequencing result, all representative samples (N=10) were 99% similar to *Mycoplasma haemocanis* from Cuba and South Korea with accession number of MZ221174.1 and MT345534.1 respectively.

Figure 2: *Mycoplasma haemocanis* infection between genders from dog shelters in Selangor




According to gender, higher detection of *Mycoplasma haemocanis* observed in female dogs in shelter 1 (67%; N=4/6) and shelter 2 (100%; N=13/13) (Figure 2). Shelter 3 shared the same percentage of both genders with 1 dog (50%) each while more than half of positive dogs in shelter 4 were male dogs (55%; N=6/11)

@COPY

Table 2: Detection of *Mycoplasma haemocanis infection* in relation with characteristics that may be associated with the infection in dogs

| Epidemiological factor | Detection (%) | p-value | χ2 |
|---|---|---|---|
| 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%) | | |

@COPYRIGHT UPM

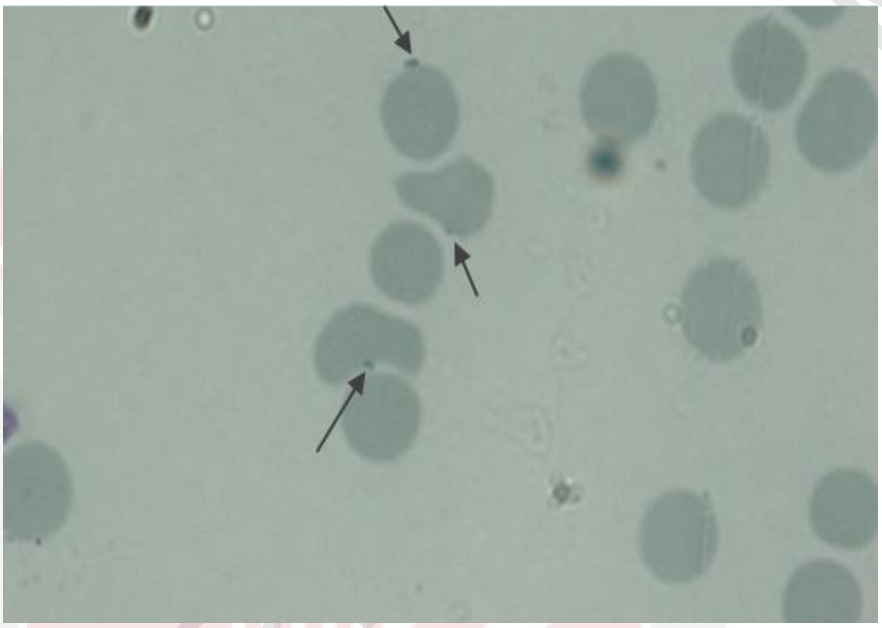
| | | | |
|---|---|---|---|
| Tick infestation | | | |
| Yes | 6/13 (46.2%) | 0.753 | 0.099 |
| No | 26/58 (44.8%) | | |

χ2: Chi-square test; *= p<0.05

Based on the association of risk factor and detection of *Mycoplasma haemocanis*, area, presence of rodents and presence of wild animals showed significant association (Table 2). Other variables which were sex, age and tick infestation status were not significantly associated with mycoplasmosis (p-value>0.05).

@COPYRIGHT UPM

Figure 3: Microscopic visualization of *Mycoplasma haemocanis* on Giemsa-stained slide



*Mycoplasma haemocanis* morphology observed were cocci in chain at the peripheral of erythrocytes. All samples that were detected positive by PCR were confirmed by the presence of t *Mycoplasma haemocanis* morphology inside red blood cell in the blood smear. Blood samples with bright PCR bands had higher amounts of observed bacteria under the microscope compared to faint bands due to higher concentration of *Mycoplasma haemocanis* DNA in the blood samples.

@COPYRIGHT

## 5.0 DISCUSSION

The present study observed higher detection of mycoplasmosis of 45.1% (32/71) among dog shelters in Selangor with all mycoplasma species detected as *Mycoplasma haemocanis.* There is no presence of the other common canine *Mycoplasma spp* which is *Candidatus Mycoplasma haematoparvu*m in our work. Thailand, which is a neighboring country has identified both *Mycoplasma* spp among client own dogs using the same molecular technique (Kaewmongkol et al., 2017). The detection of *Mycoplasma* spp. from the current study were perform using molecular diagnosis of PCR due to the high sensitivity compared to thin blood smear analysis. The microscopical examination of the slides alone is not advisable to avoid false negative results. However, it is very beneficial to describe the morphology of the bacteria on RBCs for further reference. Based on the results, the clear bright bands on the agarose gel have a greater number of bacteria visualized in the blood smear evaluation as compared to the faint bands which suggestive of low *Mycoplasma haemocanis* DNA concentration in the samples. Thus, the appearance of the band could be suggestive of the burden of mycoplasma infection in dogs. Although further investigation and validation is needed to confirm the suggestion.

In present study, gender, age and tick infestation status were not significantly associated with hemoplasma infection. However, 71% (23/32) of positive cases were female. In addition, there is only female dogs were sampled from shelter 2 and highest detection of mycoplasmosis were from the same shelter. Thus, explain the high frequency difference observed between genders. The current study also practiced convenience sampling, a non-random method that could lead to sampling bias. However, gender association with

@COPYRIGHT UPM

presence of mycoplasmosis was undemonstrated in previous work in Brazil (Barbosa et al., 2021).

According to age, dogs aged more than one year old had more positive cases compared to less than one year old dogs with 90% (29/32) detection. However, according to previous work the correlation between the age and mycoplasmosis were inconsistent. In mediterranean countries, a significant correlation was noticed between young age (<1 year) and mycoplasmosis (Novacco et al, 2010). However, there was a study in Brazil that observed adult dogs (>1 year) were significantly associated with hemoplasma infection (Vieira et al., 2015). However, there were studies who failed to prove the association between age and infection status (Biondo et al., 2009; Roura et al., 2020; Tennant et al., 2011).

Out of 13 dogs sampled that were having ticks infestation, only 46% (6/13) of them were detected positive. Although the tick, *Rhipicephalus sanguineus,* is known to be a vector for this disease, there were studies which were unable to prove the association between the tick and the mycoplasma infection (Barker et al., 2010; Aktas and Ozubek., 2017). Furthermore, detection of TBHs pathogens in ticks is lower than pathogens detected in the blood of animals with the tick infestation (Sipin et al., 2020). Hence, further studies are required to prove that the brown dog tick is a natural and main vector for canine *Mycoplasma* spp (Barbosa et al, 2020).

Risk factors of area, exposure to rodents and wild animals are significantly associated with mycoplasmosis. Shelters that are 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

@COPYRIGHT UPM

be infected with *Mycoplasma* spp. 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. The other shelters that were not mentioned were located in suburban areas, and were exposed to rodents and other wild animals such as foxes. This present study echoes with findings by Barbosa (2020) in Brazil. However, for the presence of wild animals, there were studies in Brazil and Japan that discovered association of hemoplasma infection for both *Mycoplasma haemocanis* and *Candidatus Mycoplasma haematoparvum* in wild animals (Andre et al., 2011; Furtado et al., 2018; Sazaki et al., 2008). This in fact suggests that the transmission between foxes and dogs could occur with the involvement of arthropod vectors (Sazaki et al., 2008). The disease might perhaps be transmitted through horizontal transmission which most likely occurs via biting and fighting that could be related with male's dog behavior (Barker et al., 2010). In this study, presence of rodents is unassociated with *Mycoplasma* spp-positive cases, however in a study in Brazil, shelters with rodents' presence into the households showed high chances of dogs becoming infected (Barbosa et al., 2021). Abundant strains of 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 mycoplasma infection.

For shelter 2, only 1 dog was observed with tick infestation, but 13 dogs were detected positive with mycoplasmosis. This could be explained as in urban areas, there is a high population of stray dogs and pet dogs together with ticks in the environment as a vector which maintains the availability of the parasite. In this case, there is no involvement of rodents and wildlife as part of transmission of mycoplasmosis in the shelter.

@COPYRIGHT UPM

Most cases of canine mycoplasmosis only showed mild symptoms such as slight lethargy and gradual loss in appetite which could be unnoticed by the owner (Cataldo et al., 2020; Rosanna et al., 2020). In this study, only 1 dog with mycoplasmosis, which is from shelter 1, showed abnormal physical examination findings with capillary refill time more than three second, pale mucous membrane, body condition score of two out of three with dehydration status of seven percent. The particular dog was under two days of medication for tick fever which is doxycycline together with another three dogs. Doxycycline is one of the proven effective treatments for this blood parasite infection. Out of the three dogs mentioned, two dogs were positive for mycoplasmosis under a week and two days of medication with doxycycline. However, doxycycline treatment courses required daily doses up to 21 days to clear the infection. Therefore, although the animals were treated with doxycycline, they were still positive for mycoplasmosis.

@COPYRIGHT UPM

## 6.0 CONCLUSION

The present study detected higher presence of 45% *Mycoplasma haemocanis* in dogs from animal shelters in Selangor by using the molecular detection technique of PCR assay. No other canine *Mycoplasma* spp were detected.

Significant association was found between urban areas and, less exposure to rodents and wild animals with *Mycoplasma haemocanis* infection in dogs in this study. However no other significant association was found between age, gender and tick infestation status.

@COPYRIGHT UPM

## 7.0 RECOMMENDATIONS

More dog shelters with different localities around Malaysia should be involved which are then classified into urban, suburban and rural areas in order to increase data precision and prove its correlation with canine mycoplasmosis. Other than that, blood sampling from different seasons which are dry and rainy season should be one of the possible risks since dry season has correlation with tick infestation which can transmit the disease. Random sampling in all selected shelters should also be done to reduce biases in the study whether it is related to gender, age or even health status.

Additional molecular diagnostic technique which is molecular identification of *Mycoplasma* spp in tick vectors collected from animal bodies should have been considered. The finding later will be useful to know whether the assumed tick is really a part of this disease epidemiology. Hence better control and management of the disease can be suggested. Last but not least, the information on the co-infection with other blood parasites through PCR assay or manual smear observation under the microscope should be included as part of the study to know how common and severe the co-infection would be in dogs in Malaysia.

@COPYRIGHT UPM

## 8.0 REFERENCES


Aktas, M., Ozubek, S.N. (2017). Molecular survey of haemoplasmas in shelter dogs and associations with Rhipicephalus sanguineus sensu lato. Med. Vet. Entomol., 31: 457–461.

Andre, M.R., Adania, C.H., Allegretti, S.M., Machado, R.Z. (2011). Hemoplasmas in wild canids and felids in Brazil. J. Zoo Anim. Med., 42: 342–347.

Barbosa, M. V., Paulino, P. G., Camilo, T. A., Martins, D., Paulis, L., Senne, N. A., Ramirez, O., Angelo, I. C., Massard, C. L., & Santos, H. A. (2021). Spatial distribution and molecular epidemiology of hemotropic Mycoplasma spp. and Mycoplasma haemocanis infection in dogs from Rio de Janeiro, Brazil. Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases., 87: 104660.

Barker, E.N., Tasker, S., Day, M.J., Warman, S.M., Woolley, K., Birtles, R., Georges, K.C., Ezeokoli, C.D., Newaj-Fyzul, A., Campbell, M.D., Sparagano, O.a.E., Cleaveland, S., Helps, C.R. (2010). Development and use of real-time PCR to detect and quantify Mycoplasma haemocanis and “Candidatus Mycoplasma haematoparvum” in dogs. Vet. Microbiol., 140: 167–170.

Biondo, A. W., Santos, A. P. D., Guimarães, A. M. S., Vieira, R. F. D. C., Vidotto, O., Macieira, D. D. B., ... & Messick, J. B. (2009). A review of the occurrence of hemoplasmas (hemotrophic mycoplasmas) in Brazil. Revista brasileira de parasitologia veterinária., 18: 1-7.

Di Cataldo, S., Hidalgo-Hermoso, E., Sacristán, I., Cevidanes, A., Napolitano, C., Hernández, C. V., Esperón, F., Moreira-Arce, D., Cabello, J., Müller, A., & Millán, J. (2020). Hemoplasmas Are Endemic and Cause Asymptomatic Infection in the Endangered Darwin's Fox (Lycalopex fulvipes). Applied and environmental microbiology., 86(12): 779-20.

Furtado, M.M., Taniwaki, S.A., Metzger, B., O’Dwyer, L.H., Paduan, K.D.S., J´ acomo, A.T. A., Porfírio, G.E.O., Silveira, L., Sollmann, R., Torres, ˆ N.M., Ferreira-Neto, J.S. (2018). First detection of feline hemoplasmas in free-ranging jaguars (Panthera onca). Vet. Microbiol., 214: 75–80.

Harrus, S., & Waner, T. (2011). Diagnosis of canine monocytotropic ehrlichiosis (Ehrlichia canis): an overview. The Veterinary Journal., 187(3): 292-296.

Harvey, J. W. (2001). Atlas of veterinary hematology., Philadelphia: WB Saunders Company.

Kaewmongkol, G., Lukkana, N., Yangtara, S., Kaewmongkol, S., Thengchaisri, N., Sirinarumitr, T., Jittapalapong, S., & Fenwick, S. G. (2017). Association of

Ehrlichia canis, Hemotropic Mycoplasma spp. and Anaplasma platys and severe anemia in dogs in Thailand. Veterinary microbiology., 201: 195–200.

Kemming GI, Messick JB, Enders G, et al. (2004). Mycoplasma haemocanis infection—a kennel disease? Comp Med., 54: 404–409.

Kewish, K. E., Appleyard, G. D., Myers, S. L., Kidney, B. A., & Jackson, M. L. (2004). Mycoplasma haemofelis and Mycoplasma haemominutum detection by polymerase chain reaction in cats from Saskatchewan and Alberta. The Canadian veterinary journal = La revue veterinaire Canadienne., 45(9): 749–752.

Koh, J. T. (2013). Blood parasites in dogs in Manjung, Perak. DVM thesis, Faculty of Veterinary Medicine. Universiti Putra Malaysia, Serdang

Konto, M., Tukur, S. M., Watanabe, M., Abd-Rani, P. A. M., Sharma, R. S. K., Fong, L. S., & Watanabe, M. (2017). Molecular and serological prevalence of Anaplasma and Ehrlichia sp. among stray dogs in East Malaysia. Trop Biomed., 34: 570-575.

Lashnits, E., Grant, S., Thomas, B., Qurollo, B., Breitschwerdt, E.B. (2019). Evidence for vertical transmission of Mycoplasma haemocanis, but not Ehrlichia ewingii, in a dog. J. Vet. Intern. Med., 33: 1747–1752.

Lim, C. W. (2007). Prevalence of blood parasites in canine pet and stray population: prevalence and effect on haematological parameters. DVM thesis, Faculty of Veterinary Medicine. Universiti Putra Malaysia, Serdang

Low, V. L., Prakash, B. K., Lim, Y. A. L., Tan, T. K., Vinnie-Siow, W. Y., Sofian-Azirun, M., & AbuBakar, S. (2018). Detection of Anaplasmataceae agents and co-infection with other tick-borne protozoa in dogs and Rhipicephalus sanguineus sensu lato ticks. Experimental and Applied Acarology., 75(4): 429-435.

Messick, J. B., Walker, P. G., Raphael, W., Berent, L., & Shi, X. (2002). 'Candidatus mycoplasma haemodidelphidis' sp. nov.,'Candidatus mycoplasma haemolamae'sp. nov. and Mycoplasma haemocanis comb. nov., haemotrophic parasites from a naturally infected opossum (Didelphis virginiana), alpaca (Lama pacos) and dog (Canis familiaris): phylogenetic and secondary structural relatedness of their 16S rRNA genes to other mycoplasmas. International journal of systematic and evolutionary microbiology., 52(3): 693-698.

Messick, J. B. (2003). New perspectives about Hemotrophic mycoplasma (formerly, Haemobartonella and Eperythrozoon species) infections in dogs and cats. Veterinary Clinics: Small Animal Practice., 33(6): 1453-1465.

Messick, J. B. (2004). Hemotrophic mycoplasmas (hemoplasmas): a review and new insights into pathogenic potential. Veterinary Clinical Pathology., 33(1): 2-13.

do Nascimento, N. C., Santos, A. P., Guimaraes, A. M., Sanmiguel, P. J., & Messick, J. B. (2012). Mycoplasma haemocanis--the canine hemoplasma and its feline counterpart in the genomic era. Veterinary research., 43(1): 66.

Novacco M, Meli ML, Gentilini F, Marsilio F, Ceci C, Pennisi MG, Lombardo G, Lloret A, Santos L, Carrapiço T, Willi B, Wolf G, Lutz H, Hofmann-Lehmann R. (2010). Prevalence and geographical distribution of canine hemotropic mycoplasma infections in Mediterranean countries and analysis of risk factors for infection. Vet Microbiol., 142: 276–284.

Procop, G. W., Church, D. L., Hall, G. S., & Janda, W. M. (2020). Koneman's color atlas and textbook of diagnostic microbiology., Jones & Bartlett Publishers.

Rosanna, Z., Andrea, C., Isabella, B., Francesca, S., Alberto, A., Teresa, A. & Luisa, P. (2020). Immune-Mediated Hemolytic Anemia Associated with Candidatus Mycoplasma Haematoparvum in a Splenectomized Dog in Italy. Acta Veterinaria.,70(2): 277-284.

Roura, X., Peters, I. R., Altet, L., Tabar, M. D., Barker, E. N., Planellas, M., ... & Tasker, S. (2010). Prevalence of hemotropic mycoplasmas in healthy and unhealthy cats and dogs in Spain. Journal of Veterinary Diagnostic Investigation., 22(2): 270-274.

Sasaki, M., Ohta, K., Matsuu, A., Hirata, H., Ikadai, H., & Oyamada, T. (2008). A molecular survey of Mycoplasma haemocanis in dogs and foxes in Aomori Prefecture, Japan. The journal of protozoology research., 18(2): 57-60.

Sipin, Q., Kamal, F. M., Watanabe, M., Rani, P. A. M. A., Low, V. L., & Aziz, N. A. A. (2020). Molecular detection of tick-borne haemopathogens in shelter dogs and Rhipicephalus sanguineus (sensu lato) ticks from Peninsular Malaysia. Comparative immunology, microbiology and infectious diseases., 73: 101563.

Solano-Gallego, L., & Baneth, G. (2011). Babesiosis in dogs and cats—expanding parasitological and clinical spectra. Veterinary parasitology., 181(1): 48-60.

Sykes JE, Bailiff NL, Ball LM, et al. (2004). Identification of a novel haemotropic mycoplasma in a splenectomized dog with hemic neoplasia. J Am Vet Med Assoc., 224: 1946–1951.

Tennant, K. V., Barker, E. N., Polizopoulou, Z., Helps, C. R., & Tasker, S. (2011). Real-time quantitative polymerase chain reaction detection of haemoplasmas in healthy and unhealthy dogs from Central Macedonia, Greece. Journal of Small Animal Practice., 52(12): 645-649.

Vaden, S. L., Knoll, J. S., Smith Jr, F. W., & Tilley, L. P. (2009). Blackwell's five-minute veterinary consult: laboratory tests and diagnostic procedures, canine and feline., Wiley-Blackwell.

Vieira, R. F. D. C., Vidotto, O., Vieira, T. S. W. J., Guimaraes, A. M. S., Santos, A. P. D., Nascimento, N. C., ... & Messick, J. B. (2015). Molecular investigation of hemotropic mycoplasmas in human beings, dogs and horses in a rural settlement in southern Brazil. Revista do Instituto de Medicina Tropical de São Paulo., 57: 353-357.

Tasker, S. L. M. R., & Lappin, M. R. (2002). Haemobartonella felis: recent developments in diagnosis and treatment. Journal of Feline Medicine and Surgery., 4(1): 3-11.

Willy, B., Novacco, M., Meli, M.L., Wolf-Jackel, G.A., Boretti, F.S., Wengi, N., Lutz, H., Hofmann-Lehmann, R. (2010). Haemotropic mycoplasmas of cats and dogs: transmission, diagnosis, prevalence, and importance in Europe. Schweiz. Arch. Tierheilkd., 152: 237–244.

@COPYRIGHT UPM

## APPENDICES

### Appendix 1: Four selected shelters with different management practices

Shelter 1

Shelter 2


@C UPM

Shelter 3

Shelter 4