MOLECULAR DETECTION OF PIROPLASMS IN CATTLE AND ITS ASSOCIATED TICKS IN SELECTED DISTRICTS OF KELANTAN

NURUL IZZATI BINTI SOKERI

DOCTOR OF VETERINARY MEDICINE

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MOLECULAR DETECTION OF PIROPLASMS IN CATTLE AND ITS ASSOCIATED TICKS IN SELECTED DISTRICTS OF KELANTAN

BY

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CERTIFICATION

This is to certify that we have read this research paper entitled '**Molecular Detection of Piroplasms In Cattle and Its Associated Ticks in Selected Districts of Kelantan**' by Nurul Izzati binti Sokeri, and in our opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course DVT55204 – Research Project.

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ABSTRACT

An abstract of the research paper presented to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, in partial requirement on the course DVT55204 – Research Project.

Abstract: Piroplasm is a tick-borne pathogens infecting a wide variety of vertebrate hosts, especially cattle. This study aims to investigate piroplasm infection in cattle in selected districts of Kelantan through molecular detection and its transmission from ticks to cattle. Sample of blood were collected from 23 cattle and ticks were collected from 13 infested cattle. Detection of piroplasm from blood samples was done using Polymerase Chain Reaction (PCR). The results revealed the presence of piroplasm DNA in 22 out of the 23 (95.7%) cattle tested from the blood sample. Based on the results of PCR, *Babesia* spp. was found more prevalent than *Theileria* spp. No mix infection was detected from the blood samples. Risk factor of tick infestation on cattle analysis determined there was no significance with infection of piroplasm in cattle, but might be due to small sample size that was unable to represent the whole population. Detection of piroplasm from this study may provide valuable data for developing targeted control measures, such as tick control programs, vaccination strategies, and quarantine protocols to prevent the further spread of piroplasm in cattle.

Keywords*: *Piroplasm, Ticks, Cattle, Kelantan, Transmission, PCR

ABSTRAK

Abstrak: *Piroplasma ialah patogen bawaan sengkenit yang menjangkiti pelbagai jenis perumah vertebrata, terutamanya lembu. Kajian ini bertujuan untuk menyiasat jangkitan piroplasma dalam lembu di daerah terpilih dalam Kelantan melalui pengesanan molekul dan penentuan penularan piroplasma daripada sengkenit kepada lembu. Sampel darah dikumpul dari 23 ekor lembu dan sengkenit dikumpul dari 13 ekor lembu yang dijangkiti. Pengesanan piroplasma dari sampel darah dilakukan menggunakan Polymerase Chain Reaction (PCR). Keputusan menunjukkan kehadiran DNA piroplasma dalam 22 daripada 23 (95.7%) lembu yang diuji dari sampel darah. Berdasarkan keputusan PCR, Babesia spp. didapati lebih lazim daripada Theileria spp. Tiada jangkitan campuran dikesan dari sampel darah. Analisis faktor risiko sengkenit pada lembu menentukan bahawa tidak ada signifikannya dengan jangkitan piroplasma pada lembu, tetapi mungkin disebabkan oleh saiz sampel yang kecil yang tidak dapat mewakili keseluruhan populasi. Pengesanan piroplasma daripada kajian ini mungkin memberikan data berharga untuk membangunkan langkah kawalan yang disasarkan, seperti program kawalan sengkenit, strategi vaksinasi, dan protokol kuarantin untuk mencegah penyebaran piroplasma dalam lembu.*

Keywords*: *Piroplasma, Sengkenit, Lembu, Kelantan, Penularan, PCR

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My Orange Utan (Hafisah, Fatin, Jannah)

Vetsaster

Everyone

DEDICATIONS

I would like to express my deepest gratitude to my family and friends who have been unwavering pillars of support throughout this journey of completing my final year project. Their encouragement, understanding, and belief in my capabilities have been invaluable.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
CDC	Centers for Disease Control and Prevention
CFT	Complement Fixation Test

DNA	Deoxyribonucleic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FAT	Indirect Fluorescent Antibody Test
G	Gauge
PCR	Polymerase Chain Reaction
WOAH	World Organisation for Animal Health

LIST OF SYMBOLS

%	Percentage
<	Less than

1. CHAPTER 1 INTRODUCTION

Piroplasm is an intracellular haemoprotozoan parasite. Piroplasms get their name due to the pear-shaped form that can be discovered to be nestled inside erythrocytes of infected animals. Piroplasm belongs to the order of Piroplasmida, phylum Apicomplexa. Piroplasm causes immense socio-economic impact, resulting in restriction of the development of the livestock industry (Hassan et al., 2018) due to the fact that piroplasmosis infection in livestock animals especially cows can cause serious or life-threatening consequences resulting in mortality (Schnittger et al., 2022). Piroplasms are transmitted primarily by ixodid ticks' bite, constituting a significant tick-borne disease that affects a wide range of vertebrate hosts (Homer et al., 2000).

According to Homer et al. (2000), cows are commonly infected by the genera *Babesia* and *Theileria,* resulting in bovine babesiosis and theileriosis, respectively. Common *Babesia* species that infect cattle are *B*. *bovis* and *B*. *bigemina*. From high fever and ataxia to anorexia and anemia, the disease presents a range of symptoms that can rapidly progress, often leading to severe consequences such as mortality. Moreover, the manifestation of haemoglobinuria also can be observed in cows affected with babesiosis as the parasite causes significant destruction of a substantial quantity of these red blood cells (Mandeep Singh Bal et al., 2016).

Theileriosis, is an important piroplasmid protozoan disease that infects erythrocytes with high morbidity and mortality of the infected cattle (Ma et al., 2020). The economically important species of *Theileria* are *T. parva, T. annulata*, and *T. orientalis* (WOAH, 2020). The typical signs of bovine theileriosis, including fever, enlargement of superficial lymph nodes, anemia, and respiratory distress.

There is limited study being conducted on the cattle piroplasmosis cattle in Malaysia. Based on a study conducted to detect *Theileria orientalis* infection among cattle in Peninsular Malaysia, the overall prevalence obtained was 49.76% (Ola-Fadunsin et al., 2020). The limited studies conducted on piroplasmosis in Malaysia underscore the need for comprehensive investigations to understand the prevalence, diversity, and impact of these parasites on the local cattle population particularly in specific geographic regions. This dissertation focuses on exploring the dynamics of tick-mediated piroplasm transmission, with a primary emphasis on the context of the selected districts in Kelantan, Malaysia.

By analyzing data obtained from the detection of piroplasm infections in Kelantan cattle resulting from tick bites, this study aspires to provide significant insights that can guide the development of strategies for preventing, controlling, and managing diseases within the local livestock industry.

1.1. Problem Statement

Due to the lack of comprehensive studies on the detection of piroplasm in cattle in Malaysia, this hinders our understanding of piroplasm prevalence and transmission dynamics in cattle populations to emphasize the urgency of detecting piroplasm in cattle. By conducting this study, cattle that are infected with piroplasm in Kelantan can be detected through molecular detection using PCR. The data collected from this study can be analyzed for development of strategies for preventing, controlling, and managing piroplasmosis through ticks within the local cattle in Kelantan.

1.2. Research Question

i. Is cattle in Kelantan infected with piroplasm ?

ii. Does cattle infested with ticks in Kelantan have piroplasmosis?

1.3. Research Hypothesis

H_{01}: No cattle infected with piroplasm in Kelantan are detected through molecular detection.

H_{A1}: Cattle infected with piroplasm in Kelantan are detected through molecular detection.

H_{02}: Cattle infested with ticks are positive for piroplasm.

H_{A2}: Cattle infested with ticks are negative for piroplasm.

H_{03}: Ticks unable to transmit piroplasm to cattle.

H_{A3}: Ticks able to transmit piroplasm to cattle.

1.4. Research Objective

i. To detect piroplasm in cattle from selected farms in Kelantan.

ii. To detect piroplasm in ticks from tick infested cattle.

2. CHAPTER 2 LITERATURE REVIEW

2.1. Piroplasm

Piroplasm is an intracellular haemoprotozoan parasite infecting a wide variety of vertebrate hosts, especially cattle. Piroplasm belongs to the order of Piroplasmida, phylum Apicomplexa. *Babesia*, *Theileria*, and *Cytauxzoon* are common genera under the order of Piroplasmida with veterinary relevance. These haemoprotozoan are known as piroplasm due to the pear-shaped or piriform characteristics when infecting the erythrocytes as can be seen from **Figure 1** (Saari et al., 2018).

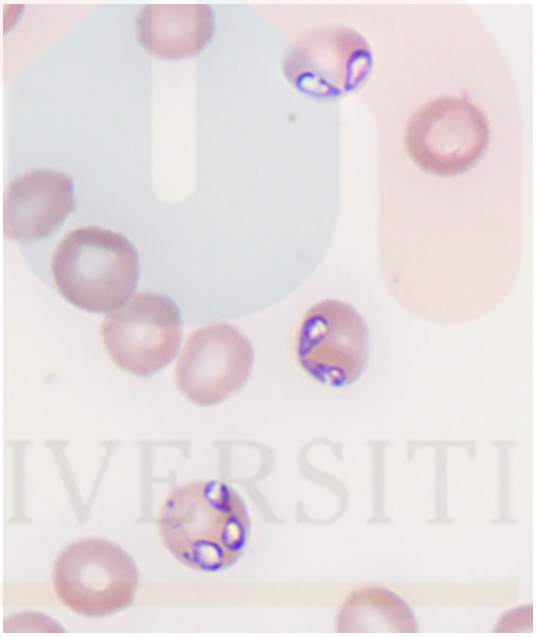

Figure 1: Merozoite stages of Babesia canis in erythrocytes of blood smear stained with Giemsa (Saari et al., 2018)

Piroplasm parasites are transmitted exclusively through vectors of the Ixodiade family which are known as hard ticks (Mehlhorn and Schein, 1993; Votypka et al., 2017) such as *Haemaphysalis* and *Rhipicephalus*.

Piroplasm life cycle consists of three phases; schizogony, gametogony and sporogony. Schizogony is asexual multiplication of the piroplasm in the vertebrae host. Gametogony is sexual reproduction inside the tick midgut. The gametes unite and divide to produce sporozoites. The infective sporozoites invade the vertebrae host through secretion of saliva from tick bite during blood-feeding and undergo asexual reproduction. All parasites of the group Piroplasmida reproduce asexually inside the blood cells of the vertebrate host (Jalovecka et al., 2018). They multiply and develop into merozoites. The merozoites are then released into the bloodstream where they invade healthy erythrocytes through recognition of specific receptors on the surface of erythrocytes by proteins on the surface of the merozoites. The intraerythrocytic merozoites appear singly as small round, and in pairs as pear-shaped (pyriform) hence its name, piroplasm.

Typically, Giemsa-stained blood smears are used to diagnose piroplasmosis; however due to the low levels of parasitemia, this method cannot be used to identify parasite carriers or cattle that have recovered from acute infections and develop subclinical infections (Hassan et al., 2018). According to Omer et al (2011), serological methods such as indirect fluorescent antibody tests (FAT) and complement fixation tests (CAT), enzyme-linked immunosorbent assays (ELISA) are also utilized in detecting piroplasmosis because the methods are highly

sensitive and easy to standardize but it may give of false negative results because of its low specificity in detecting carrier, thus influencing the test efficacy.

2.2. Clinical manifestations of piroplasm protozoa in cattle

Bovine babesiosis is a tick-borne disease of cattle caused by protozoan parasites of the genus *Babesia* of order Piroplasmida (WOAH, 2023). A number of species are known to infect cattle; *B. bovis*, *B. bigemina*, *B. divergens* and *B. occultans*. They are transmitted primarily by ixodid *Rhipicephalus* spp. Ticks especially in subtropical and tropical regions. Babesiosis is economically important as it has a huge impact on livestock health. *Babesia bovis* infections are characterized by hemolysis (piroplasm stage) causing general circulatory shock, high fever, anorexia, and nervous signs such as ataxia and teeth grinding (Bal et al., 2016). When the nervous symptoms of cerebral babesiosis develop, the outcome is almost always fatal. Anemia and haemoglobinuria may appear later in the course of the disease. Infected cattle will develop a life-long immunity against reinfection with the same species. The recovered cattle may be weak and in reduced condition.

Theileriae are intracellular protozoa belonging to the phylum Apicomplexa, the order Piroplasmida and the genus *Theileria*. The economically important species of Theileria that are able to infect cattle are *T. parva, T. annulata*, and *T. orientalis* (WOAH, 2020). *Theileriae* are transmitted by ixodid ticks, and have complex life cycles in both vertebrate and invertebrate hosts (A. Ica et al., 2007). Young cattle,

cows in late-pregnancy and recently calved cows are most likely to be affected. Infected cattle will be anemic, pyrexic, swelling of lymph nodes, lethargic and weak (Ma et al., 2020).

2.3. Hard tick (Family: Ixodidae)

Hard ticks are external hematophagous parasites that feed on blood of vertebrate hosts. Taxonomic classification of hard ticks is in the class Arachnida, order Ixodida and family Ixodidae. According to Zanet et al. (2020), hard ticks are the main parasitic vectors of diverse infectious pathogens due to its wide host range and tendency to feed on several hosts during their lifetime, infecting both humans and animals worldwide. Ixodid tick species in the genera Ixodes such as *Dermacentor*, *Amblyomma*, *Haemaphysalis*, *Hyalomma*, and *Rhipicephalus* (Horak et al., 2002, pp. 27–54). Ixodida feed on the blood slowly and stay attached to the host for a long time. This permits adequate time for the pathogens to be transmitted to susceptible hosts.

2.4. Life cycle of hard tick

The basic four developmental forms of hard ticks are egg, larva, nymph and adult. Both nymphs and adults have eight legs whereas the larvae have just six legs. Only one blood meal is taken during each of the four life stages.

According to Grigoryeva and Shatrov (2022), larvae hatch from an egg batch and crawl randomly in search of a small host. It attaches to the suitable host and the

feeding time for larvae is generally short. The larva becomes engorged after a blood meal, drops off and molts to become a nymph. The duration of the larval stage may vary from 3 to 13 days.

After molting, the nymph attaches to larger hosts than before for a blood meal. After several days of feeding it drops off and molts into adult ticks. Adult ticks may live up to a year or more in search of suitable hosts. Male and female adults seek out a third host, feed, mate, and drop off to the ground to oviposit.

Ticks that feed on the same host species at each stage are referred to as one-host ticks, whereas those that feed on different hosts each time are called three-host ticks. Three-host ticks drop off and reattach to a new host during each life stage, until finally the adult females lay their batch of eggs.

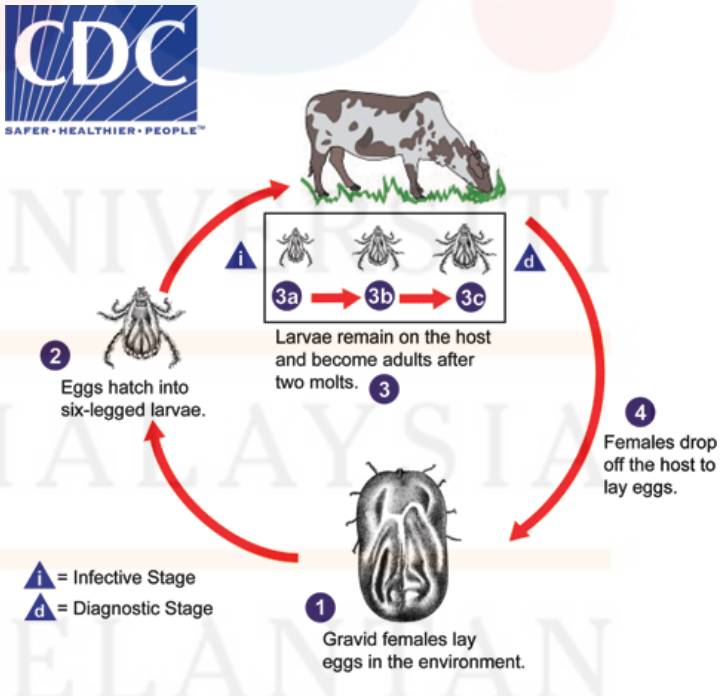

Figure 2: Life cycle of hard tick. (CDC - DPDx - Ticks, 2024)

2.5. Molecular detection of piroplasm using PCR

Detection of antigen using molecular-based methods has improved the accuracy of diagnosing and identifying the causative agents that infect the animals. Polymerase chain reaction (PCR) is one of the molecular techniques used to amplify a single copy of a piece of target DNA to generate copies of a particular DNA sequence. DNA polymerase needs a specific primer to synthesize new DNA copies by adding nucleotides onto a preexisting 3'-OH group to generate an extended region of double stranded DNA.

Based on Kumar (2022), initial screening of samples using piroplasmid-specific PCR is cost-effective and efficient method for identifying parasite species, due to the enormous number of parasite species. Various regions of DNA were targeted for molecular identification of *Theileria* and *Babesia* parasites but the most common target is 18S rRNA.

3. CHAPTER 3 MATERIALS AND METHODS

The study was conducted to detect piroplasm in cattle from selected farms in Kelantan through molecular detection technique which is Polymerase Chain Reaction (PCR).

3.1. Study Area

This study was conducted in cattle farms of selected districts in Kelantan which were Tumpat, and Kota Bharu. Whole blood and tick samples were collected from cattle of selected farms for piroplasm detection.

3.2. Study Population

A total of 23 cattles were sampled from 4 farms located in Tumpat and 3 farms in Kota Bharu in Kelantan.

3.3. Selection Criteria

3.3.1. Inclusion criteria

The inclusion criteria of this study was simple random selection of cattle but with no specific age, sex or breed from any cow farm that was located in the selected districts of Kelantan (Tumpat and Kota Bharu).

3.3.2. Exclusion criteria

Cattle with history of treatment with anti-parasitic drugs less than two weeks prior to sampling were excluded from sampling.

3.4. Blood Sampling

Blood samples were collected aseptically from the jugular or coccygeal vein of each cattle into 5 ml tubes containing disodium ethylene diamine tetraacetic acid (EDTA) as an anticoagulant. A sterile vacutainer was used to collect the blood from each cattle. The blood samples were transported in an ice box that was filled with ice packs to the laboratory. The samples were stored at 4°C prior to analysis.

3.5. DNA extraction of blood sample

The DNA was extracted from 200 µl of each collected blood sample using gSYNC™ DNA Extraction Kit according to the manufacturer's protocol. The extracted DNAs were kept at −20°C before the molecular analysis through PCR.

3.6. Detection of piroplasms by Polymerase Chain Reaction (PCR) from blood sample

PCR reaction mixture was prepared. PCR amplification was performed for all samples and followed by agarose gel electrophoresis. Primers used and sequences were stated in **Table 1.** Nuclease-free water (NFW) was used as a negative control. Positive control of *Babesia* sp. And *Theileria* sp. were used and the resulting PCR products were compared with positive control to confirm the detection of piroplasm.

3.7. Ticks Collection

The cattle was securely restrained and any ticks that were found were carefully removed from the hair or skin surfaces where they had been attached. The ticks were kept in 70% ethanol.

Table 1

Primer Sequence and PCR Cycling Conditions

Primer	Sequence	Target gene	Expected size (bp)	PCR cycling conditions
Bec-UF1	5'-GTTGATCCTGCCAGTAGTCA-3'	"Catch-all" (*Babesia* spp. and *Theileria* spp.)	876 *Babesia* spp. 913 *Theileria* spp.	1. Initial denaturation at 95◦C for 5 mins 2. Denaturation at 95◦C for 1 min 3. Annealing at 60.5◦C for 1 min Elongation at 72◦C for 1 min 4. Final extension at 72 ◦C for 10
Bec-UR	5'-CGGTATCTGATCGTCTTCGA-3'			

				min

Bec-UF1 = universal forward primers; Bec-UR =universal reverse primer.

3.8. Data Analysis

Data was entered on the spreadsheet of Microsoft Excel. The statistical analysis was performed using the IBM SPSS software (version 26). Pearson's chi square was used to test potential correlations between piroplasm infection in cattle with presence of ticks on the cattle. For statistical significance, P-value < 0.05 (confidence level of 95 percent) were used.

4. CHAPTER 4 RESULTS

A total of 23 cattles were sampled from 4 farms located in Tumpat and 3 farms in Kota Bharu in Kelantan. The blood samples were detected for piroplasm using Polymerase Chain Reaction (PCR). Universal 'catch-all' primers (Bec-UF1and Bec-UR) were used to visualize that the DNA bands size was 876 bp and 913bp, meaning that the samples were positive for *Babesia* spp. and *Theileria* spp. respectively.

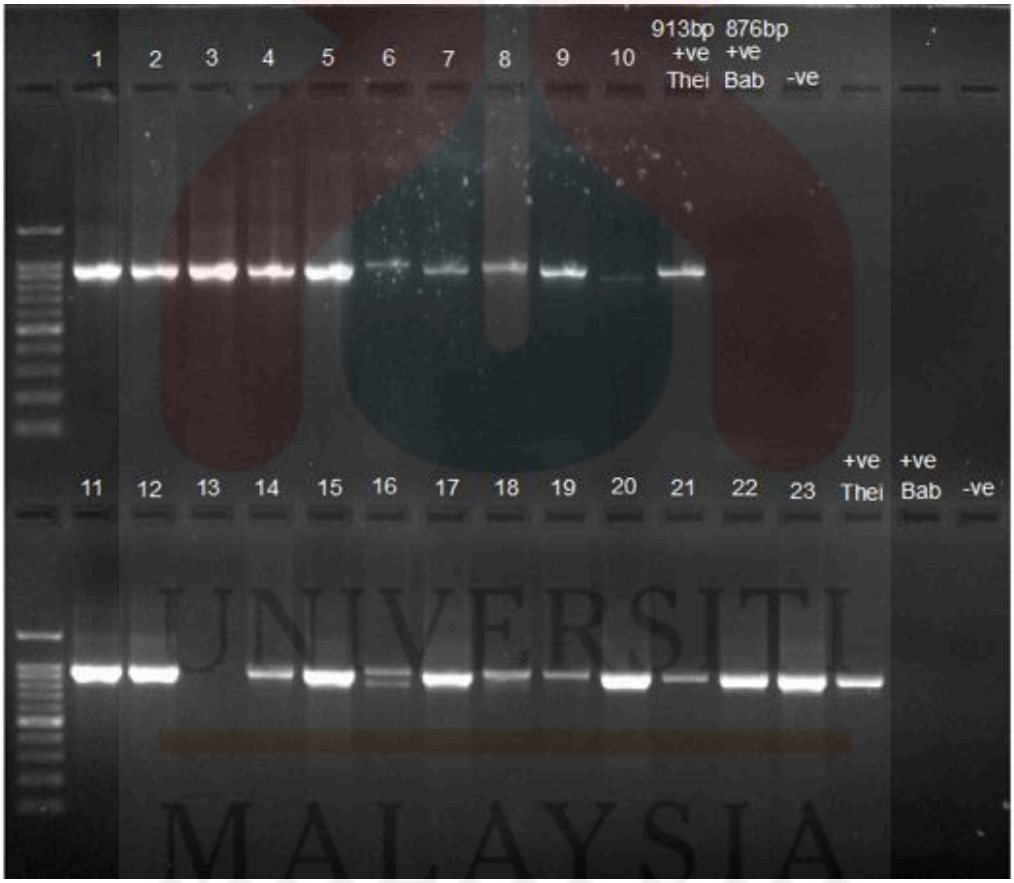

Figure 3: Gel electrophoresis of PCR products, using primers of Catch-all" (*Babesia* spp. and *Theileria* spp.): First lane: molecular marker; +ve Thei: positive control for *Theileria* sp. with 913bp; +ve Bab: positive control for *Babesia* sp. With 876bp; -ve: negative control for piroplasm

Ticks were collected only from 13 cattle. Piroplasm infections were detected in cattle at 95.7% (22/23) for blood samples as shown in **Table 2**.

Table 2:

PCR results for blood sample

	PCR		Total
	Positive (%)	**Negative (%)**	
Blood	22 (95.7)	1 (4.3)	23

Results in **Table 3** showed that among the positive cases irrespective of the species of piroplasm, presence of ticks on cattle upon presentation was not associated with the piroplasm infection in cattle (p-value > 0.05). Upon presentation of each cattle, 56.52% (13/23) of the cattle were found to have ticks on their bodies while 43.48% (10/23) of the cattle were without the presence of ticks.

Table 3:

PCR results of blood samples and its risk factor of tick presence upon presentation

		PCR of Blood (%)		Total	p-value
		Positive	Negative		
Presence of tick upon presentation	**Present**	13 (56.52)	0	13	0.244
	Absent	9 (39.13)	1 (4.35)	10	
Total		22	1	23	

For blood samples collected from cattle, 56.5% (13/23) of the samples were identified as positive for *Babesia* spp. and 43.4% (10/23) were positive for *Theileria* spp. *Babesia* spp. was found more prevalent than *Theileria* spp. No co-infection was detected from blood samples.

Table 4

Number of samples that tested positive for *Babesia* spp. and *Theileria* spp.

	Piroplasm positive		Total
	***Babesia* spp.**	***Theileria* spp.**	
Blood	13	10	23

5. CHAPTER 5 DISCUSSION

In this study, blood collected from cattle along with the ticks infesting the cattle were detected for piroplasm. Based on Haben Fesseha et al. (2022), the risk of babesiosis was significantly higher in tick-infested cattle than in cattle with absence of tick on the body. However, according to the result analysis, tick infestation in cattle was determined to be insignificant in the transmission of piroplasm in cattle in Tumpat and Kota Bharu of Kelantan, Malaysia. In this study, ticks that were unable to be found on the cattle could be due to the transformation from larva to nymph and then to an adult stage depending on the species that was infesting the cow. During this process, each stage of the tick feeds on different hosts. Following each blood meal, the tick detaches from the host, undergoes a metamorphic process to transition to the next stage, and subsequently seeks a different host for its next feeding. As a result, it is conceivable for a cow to harbor tick-borne pathogens even when there are no apparent signs of ticks on its body.

This could also be due to the abundance of infected ticks in specific geographical areas. According to Nooshin Mojahed et al., (2022), transmission of disease-causing agents by arthropod vectors are significantly influenced by weather and climate, influencing the spread and abundance of ticks. Consequently, this will affect the distribution patterns and occurrences of infections transmitted by tick (Dantas‐Torres, 2015). In regions where ticks harboring piroplasm are prevalent, there is an increased probability of cattle coming into contact with these vectors, thereby raising the risk of infection. Hence, continuous monitoring and surveillance is necessary to understand the intricate and dynamic characteristics and spread of ticks as vectors for piroplasm.

As only 23 blood samples and 13 tick samples from infested cattle were collected, the small sample size may not accurately represent the larger population of the sampling area. Consequently, this limitation restricted the scope of statistical analyses that could have been conducted. Future research needs to be done to ensure the inclusion of a sufficient number of positive cases.

Based on the results of PCR from blood samples, *Babesia* spp. was found more prevalent than *Theileria* spp. No mix infection was detected from blood samples. However, due to limited study being done to detect piroplasm in cattle, especially in Malaysia, the prevalence was unable to be determined. Most studies focused on associating the risk factors of piroplasm and sensitivity of piroplasm detection through different methods such as microscopic examination of blood smear, serology and molecular methods. Based on a study conducted on Kedah-Kelantan x Brahman cattle by Agina et al. (2021), *Theileria* infection is endemic in a cattle farm in Pahang. Hence, it is advisable for farm owners in Kelantan, Malaysia, to consider annual tick removal, and consistent health monitoring as measures to decrease the occurrence of piroplasm and tick infestation.

6. CONCLUSION

In conclusion, this study confirmed the presence of piroplasms in cattle through molecular detection of PCR, notably *Babesia* spp. being more prevalent than *Theileria* spp. In summary, employing molecular methods for detecting piroplasms in cattle through blood samples has yielded valuable insights into distribution of piroplasm in cattle in Kelantan. The findings highlight the significance of a dual approach, considering both the vertebrate host and the arthropod vector, to better understand the dynamics of piroplasm infections. This study's results contribute to the broader understanding of vector-borne diseases in cattle, laying the groundwork for the development of targeted strategies to effectively manage and alleviate the impact of piroplasmosis in livestock populations. However, further studies covering all districts of Kelantan should be conducted to generate and compare the prevalence data for the distribution of ticks as vectors in livestock.

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