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Short communication

First molecular identification of *Anaplasma marginale* in Horses from Kelantan, Malaysia

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Abstract

This study aimed to detect the presence of *Anaplasma* spp. in blood samples from equine stables in Kelantan, Malaysia. Blood samples were collected from 117 horses from different equine stables from Kelantan. Microscopic examination was conducted using the Giemsa staining method followed by conventional polymerase chain reaction (PCR) to confirm the *Anaplasma* spp. Out of 117 samples, two (1.71%) exhibited the coccobacillus shape characteristic of *Anaplasma* spp. under microscopic examination, while one sample (0.85%) was confirmed as *Anaplasma* spp. through PCR analysis. The confirmed sample was then sent for 16S rRNA gene sequencing, which revealed 99.86%–100% similarity to *Anaplasma marginale*. The sequence was submitted to GenBank under the accession number PQ804341. Phylogenetic analysis showed that PQ804341 was closely related to *Anaplasma marginale* strains detected in horses from the Philippines and cattle from Thailand. These findings confirm the presence of *Anaplasma marginale* in horses in Kelantan region even in the absence of clinical signs for anaplasmosis in horses. Appropriate molecular diagnostic and screening is crucial to prompt implementation of prevention and control measures. The close relationship among *Anaplasma marginale* and other zoonotic *Anaplasma* should raise concern for further study on potential risk to humans, transmission rate which closely related among infected host and tick.

Keywords: *Anaplasma marginale*, Conventional PCR, Phylogenetic analysis.

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INTRODUCTION

The equine industry in Malaysia has recorded a yearly increase in the number of horses, which is mainly attributed to the involvement in equestrian activity since the 20th Century. Participation of horses in racing, the army, sports and the police industry has led to an increasing pattern in this population (Hussain et al., 2016). The recent figures indicated that there are about 4.12 thousand horses in 2022, and estimated number of ponies at around 5 thousand in the East Coast Peninsular Malaysia and Sabah according to Darmansah et al. (2017). Selangor and Kuala Lumpur being the central gathering place for Horse-based activities and Malaysia has 5 places that are active for turf clubs which are specific for horse racing. They are Selangor Turf Club, Perak Turf Club, Penang Turf Club, Royal Sabah Turf Club and Sarawak Turf Club (Hanis et al., 2020). Equine industry has a main role in conducting the equestrian sports, tourism, veterinary services, reproductivity, agriculture production that has a potential economic growth in Malaysia.

Anaplasmosis is a tick-borne disease that shows some nonspecific clinical signs, such as sudden onset of fever, lethargy, depression and reluctance to move (Bakken and Dumler, 2015). This causes worries among veterinarians since anaplasmosis can be transmitted to other horses and humans through biting infected ticks. Equine granulocytic anaplasmosis, known as a tick-borne disease affect horses worldwide, lead by *Anaplasma phagocytophilum.* These symptoms show on the early infection in horses; sudden onset of fever, limb edema and anorexia (Bogdan et al., 2024). Equine granulocytic anaplasmosis is a zoonotic infection, which means it does affect human (human granulocytic anaplasmosis) and also affect ruminants (Woldehiwet, 2008).

Ixodes tick, particularly *Ixodes scapularis*, are recognized as the primary vectors responsible for transmitting *Anaplasma phagocytophilum*, the causative agent of anaplasmosis. The use of polymerase chain reaction (PCR) as a molecular diagnostic tool has proven highly effective for detecting *Anaplasma phagocytophilum*, with studies from various regions, including the United States, reporting a PCR-positive prevalence of approximately 8% in horses (M'ghirbi et al., 2012). Current treatment for equine granulocytic anaplasmosis (EGA) typically involves the administration of oxytetracycline at a dosage of 7 mg/kg once daily, with treatment durations ranging from seven to ten days or more, depending on the severity of infection (Pusterla and Madigan, 2013). Supportive care, which is essential for recovery, can impose a significant financial burden due to the prolonged duration of therapy and associated veterinary costs.

Anaplasma marginale, belonging to the family Anaplasmataceae, is an intracellular, coccobacillus gram-negative bacteria known as an erythrocyte parasite which shows an increasing pattern that leads to economic and animal health concerns in tropical and subtropical countries (Hairgrove et al., 2015). Since *Anaplasma phagocytophilum* are the main causative agent of equine granulocytic anaplasmosis, increasing concern regarding the cross-species transmission or tick vector exposure among horses and cattles (Chankong et al., 2021) on the identification of *Anaplasma marginale* in equine blood samples remain questionable (Qi et al., 2022).

Tick-borne pathogens such as *Anaplasma marginale* are well documented in livestock, but studies on the prevalence of equine anaplasmosis in Southeast Asia remain limited. Furthermore, the ecology of ticks associated with horses is still understudied. While the molecular detection of *Anaplasma marginale* in cattle has been reported in Malaysia, there is no published report on its prevalence in equine blood samples. Therefore, this study aimed to detect the presence of *Anaplasma marginale* in blood samples collected from equine stables in Kelantan, Malaysia. By investigating the presence and genetic diversity of *Anaplasma* spp. in horses, this study sought to fill an important gap in regional knowledge.

MATERIALS AND METHODS

Ethical concern & approval

This study was conducted in accordance with the ethical standards and animal ethics approval from the Institutional Animal Care and Use Committee Universiti Malaysia Kelantan (IACUC, FPV) on 20 March 2023 (Approval code: UMK/FPV/ACUE/PG/001/2023). Owner consent was obtained from each stable before the sampling using a consent form.

Animal & sample collection

This project was conducted around 2023-2024 which to ensure comprehensive data collection and analysis. It was conduct in a few districts in Kelantan (Bachok, Tumpat, Pasir Puteh, Machang, Kuala Krai, Kota Bharu, Tanah Merah, Pasir Mas). A total of 117 blood samples of apparently healthy horses (42 males and 75 females) were collected from various districts in Kelantan. Figure 1 shows the districts where the blood samples had been collected. Blood samples were collected using EDTA tube and kept under 4°C chiller until further analyses.

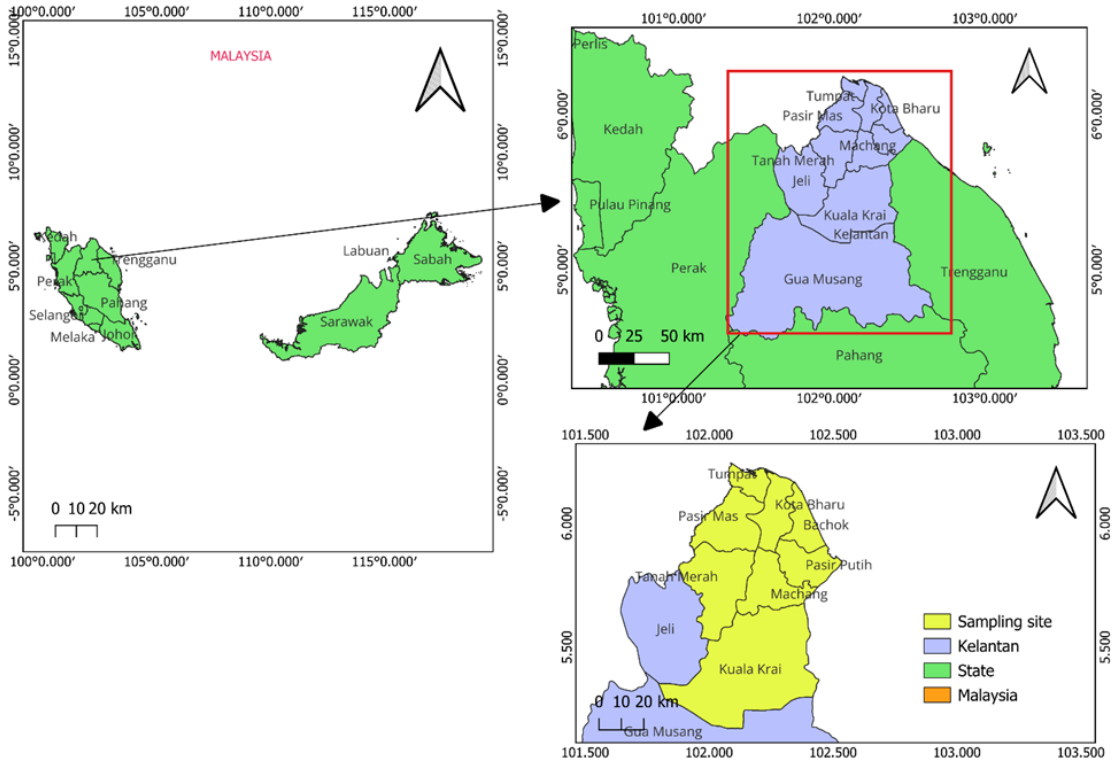

Figure 1 Equine field sampling in Kelantan. The highest number of samples, 45, was obtained from Bachok, followed by 25 from Machang. Additionally, 12 samples were collected from Tanah Merah, 10 from Tumpat, 8 each from Pasir Puteh and Kota Bharu, 6 from Pasir Mas, and 3 from Kuala Krai.

Microscopic examination

A blood smear was further conducted using the Giemsa-staining method. Dilution of 10% buffer solution between fixed thin smears and Giemsa stain were stained and left to stay for 20 min, then followed by washing using raw water for excessive stain removal. Slides were air dried and observed using light microscope for the presence of *Anaplasma* inclusion.

DNA extraction

DNA extraction was conducted according to the manufacturer protocol (PrimeWay Genomic DNA Extraction Kit). Quality control of each sample was carried out by initially running a plain 1% agarose gel stained with Green Midori nucleic acid stain to access DNA integrity and check for contamination, followed by spectrophotometric analysis using the NanoPhotometer P360 (IMPLEN) to determine DNA purity and concetration. Samples were considered valid if the A260/A280 ratio ranged between 1.8 and 2.0, and the A260/A230 ratio was above 2.0 (Lucena-Aguilar et al., 2016). To confirm the specificity of the PCR primers (AnaplsppF/AnaplR3), a BLAST search was performed using NCBI to ensure an exact match with *Anaplasma* spp. The PCR conditions were tested and optimized, ensuring that both positive and negative controls produced the expected results without amplifications in non-target or no-template controls. Sequencing can also serve as an additional quality control step to further verify the accuracy and specifity of the amplified products.

Molecular detection of *Anaplasma* spp.

A conventional PCR assay which amplifies the partial 16S rRNA gene of *Anaplasma* spp. were used. *A. marginale* DNA from Apical Scientific (Malaysia) validated by sequencing (Accession no. KX987327.1) was used as a positive control, and distilled water was used as the negative control for each run. Polymerase chain reaction amplification was carried out using specific primer set for *Anaplasma* spp. F: 5'-AGAAGAAGTCCCGGCAAACT-3'and R: 5'-GAGACGACTTTTACGGATTAGCTC-3' based on 16S rRNA gene with an expected amplicon size of ~800 basepair (bp) (Zobba et al., 2014). It was performed in a total volume of 25 µl for per reaction by using 12.5 µl of mastermix, 1 µl for each forward and reverse primer, 5 µl DNA template and 5.5 µl nuclease free water. Mastermix GoTaq® DNA Polymerase was supplied in 2X Green GoTaq® Reaction Buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 3mM MgCl2. For *Anaplasma* spp. detection, 16S rRNA gene was amplified in an initial denaturation at 94°C for 3 min followed by 30 cycles at 94°C for 30 sec (denaturation), 50°C for 30 sec (annealing) and 72°C for 60 sec (extension) with a final extension step for 72°C for 10 min. In 16S rRNA gene amplification only positive and negative control were used. Using 1.5% agarose gel in 0.5 TAE buffer, visualized the pcr products in the green midori advance stain and gel doc.

Partial 16S rRNA sequencing and phylogenetic analysis

All positive PCR products with AnaplsppF/AnaplR3 primers were sent to Apical Scientific (Selangor, Malaysia) for Sanger sequencing. The sequenced product was manually trimmed and submitted to the NCBI GenBank database, where it was assigned an accession number. Multiple sequence alignment and nucleotide identity comparisons were performed using EMBL's Clustal Omega and Nucleotide BLAST, respectively, to determine the percentage identity between sequences. Multiple sequence alignment and phylogenetic analysis were conducted using MEGA 11 software (Tamura et al., 2021), employing the Maximum Likelihood method based on the General Time Reversible model. To estimate the genetic diversity among *Anaplasma* spp. sequences obtained in this study, analyses were performed using the Maximum Composite Likelihood model (Tamura et al., 2004). This analysis involved one positive nucleotide sequence, and the phylogenetic tree was constructed using 1000 bootstrap replications. The scale value of 0.02 indicates the genetic distance, representing the number of nucleotide substitutions per site. Reference sequences of the 16S rRNA gene of *Anaplasma* spp. were taken from GenBank for phylogenetic comparison. Inclusion criteria required the sequences to be at least 800 bp long, free from ambigious bases,

annotated with host and location metadata. A total of 26 sequences were selected, including samples from horse, cattle, tick, deer across Asia and Europe.

RESULTS

Demographic data

The highest number of samples, 45, was obtained from Bachok, followed by 25 from Machang. Additionally, 12 samples were collected from Tanah Merah, 10 from Tumpat, 8 each from Pasir Puteh and Kota Bharu, 6 from Pasir Mas, and 3 from Kuala Krai. The demographic data were presented in Table 1.

Table 1 Demographic data of animal & sample collection in Kelantan horses.

Type of variable	Category	Frequency (n=117)	Percentage
Sex	Male	42	35.9%
	Female	75	64.1%
Age	Young	31	26.5%
	Adult	69	59%
	Old	17	14.5%

Type of variable	Category	Frequency (n=117)	Percentage
Breed	Paddy	21	17.9%
	Arabian	42	35.9%
	Thoroughbred	25	21.4%
	Siamese	25	21.4%
	Others	4	3.4%
Districts	Bachok	45	38.5%
	Kota Bharu	8	6.8%
	Kuala Krai	3	2.6%
	Machang	25	21.4%
	Pasir Mas	6	5.1%
	Pasir Puteh	8	6.8%
	Tanah Merah	12	10.3%
	Tumpat	10	8.5%

Microscopic examination

Two (2) out of 117 blood smear slides showed the presence of *Anaplasma* spp. near the margin of the equine erythrocyte (Figure 2). A coccus-shaped organisms to suggest *Anaplasma* spp. were identified under the microscope. In Giemsa-stained thin film blood smears, *Anaplasma* spp. that infect horse appear as dense, dusky purple, inclusion bodies ranging from 0.3 to 1 µm. Since microscopy give a quick diagnostic observation and low sensitivity rate, using higher molecular techniques is preferable.

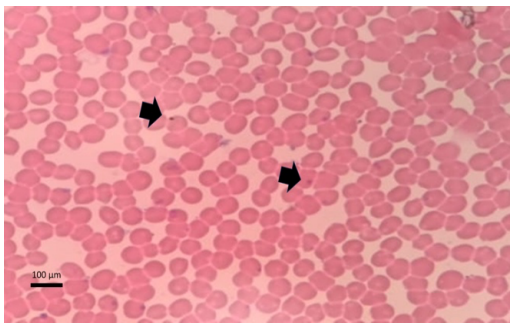

Figure 2 Detection of *Anaplasma marginale* was performed using Giemsa-stained blood smears. The organism, appearing as small coccoid to bacillary structures, was observed within the erythrocyte. Two samples tested positive for the presence of the parasite.

Molecular detection of *Anaplasma* spp.

Out of the two blood samples that showed the presence of *Anaplasma* spp., one sample (0.85%) was positive *Anaplasma* spp. using the PCR technique. The positive samples showed a single band to the expected size for *Anaplasma* spp. (~800 bp) (Figure 3).

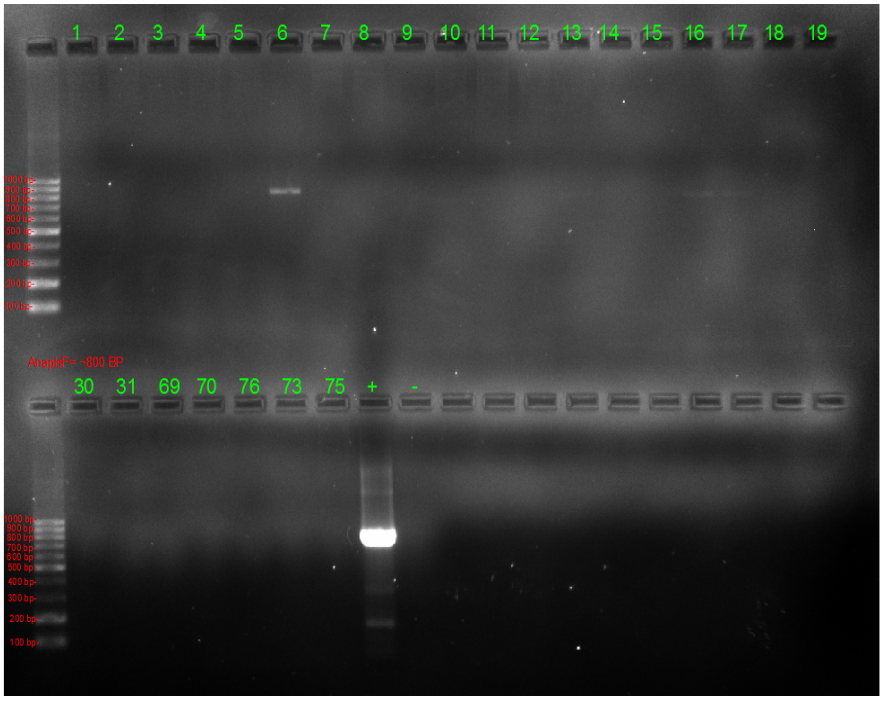

Figure 3 Gel electrophoresis of PCR products targeting the 16S rRNA gene of *Anaplasma* spp. was conducted using a 100 bp DNA ladder as a molecular size marker. The results revealed that sample No.6 produced a distinct band at the expected size of approximately 800 bp, indicating a positive amplification.

Partial 16S rRNA sequencing and phylogenetic analysis

Amplicons were sequenced to obtain representative sequences for *Anaplasma* spp. (n=1). Uncultured *Anaplasma* sp. 16S rRNA isolated from the current study (PQ804341) shared 99.86-100% identity with each other and 99.86-100% identical to *A. marginale* isolate from a Rhipicephalus microplus tick in China. *Anaplasma* 16S rRNA sequences from eight district genotypes were obtained from one PCR-positive sample (PQ804341). The sequence was most closely related to novel *Anaplasma* sp. (Uncultured *Anaplasma* sp.) isolated from horses in the Philippine.

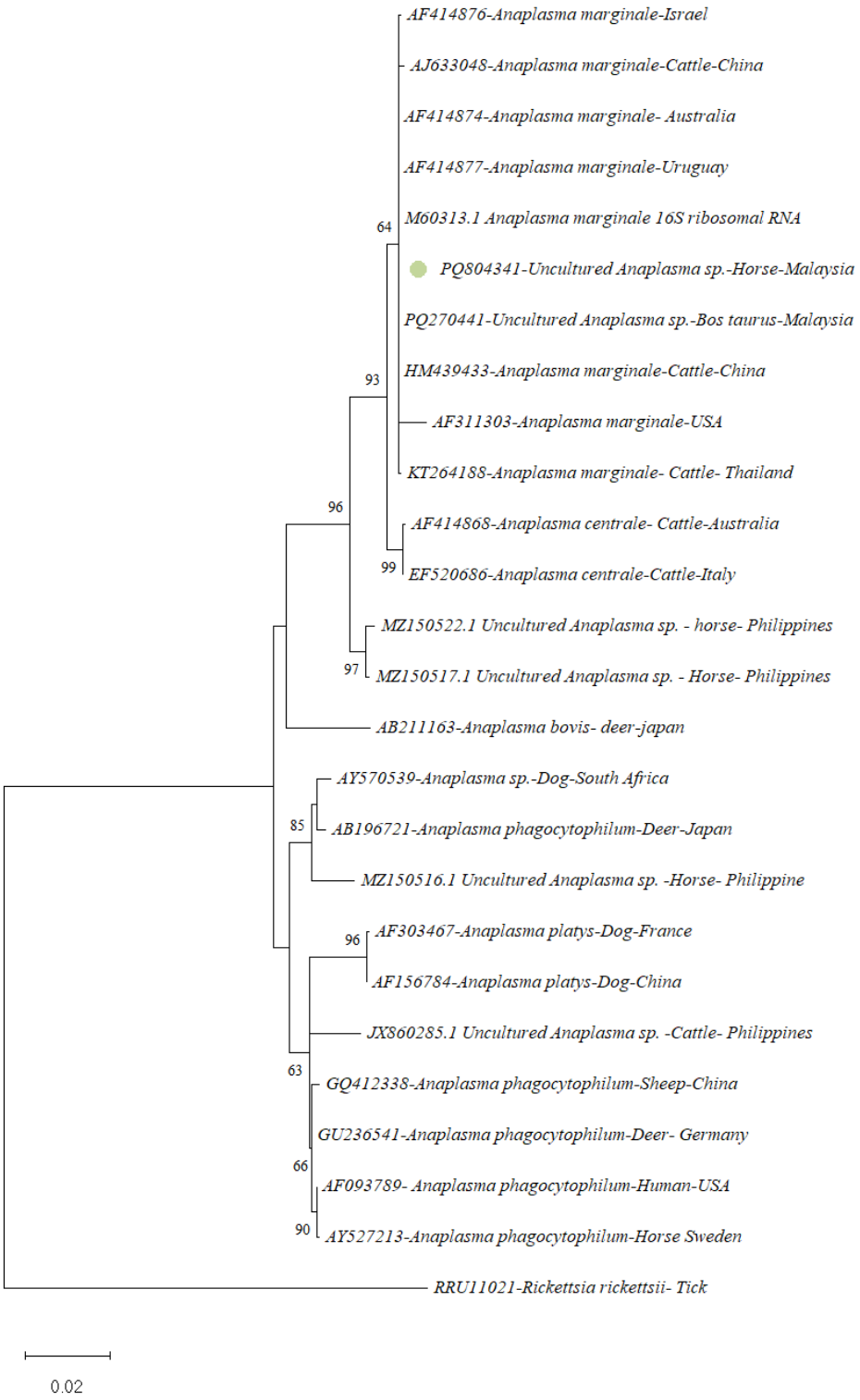

Figure 4 Molecular phylogenetic analysis based on partial (~800 bp) 16S rRNA gene sequence of *Anaplasma* spp. by maximum likelihood method. Sequences for species detected in the current study showed the strain name, the species, and the number of positive equines, followed by the accession number of GenBank. The green circles indicate *Uncultured Anaplasma sp.* identified in this study. The 16S rRNA gene sequence of *Rickettsia ricketssi* (RRU11021) was used as the outgroup. The scale bars indicate the phylogenetic distance.

DISCUSSION

This overall occurrence rate was reported at 0.85%, as this is the first report of *A. marginale* in horses. All the horses that positive with *Anaplasma* species infection appeared healthy and exhibited no clinical signs of anaplasmosis. This finding is similar from the previous study by Da Silva et al. (2018), which stated that *A. marginale* didn't show any acute clinical sign in goats.

The presence of *A. marginale* in Malaysian horses contrasts with previous studies in other livestock species. *A. marginale* was detected in peninsular Malaysia at 72.6% (n=759/1045; 95% CI = 69.9–75.3) of cattle (Ola-Fadunsin et al., 2018) and *Anaplasma* spp. was found in 30.9% (n=127/411) of goats (Tan Li, 2024). The variation could be attributed to geographical area and climate differences, which may lead to various biological vectors and affect the rate of transmission by pathogens (Rahman et al., 2022). In this study, female horses had higher odds (OR=0.326, 95% CI: 0.029-3.676) of being positive to *Anaplasma* spp. than males. However, the odds ratio for other characteristics could not be determined due to low prevalence of infection.

Microscopic examination identified two positive slides for *Anaplasma*, while molecular detection using polymerase chain reaction (PCR) confirmed only one positive case in the Bachok district. This discrepancy highlights the limitations of microscopic methods, which can yield false positives due to the subjective nature and variability in smear interpretation. In contrast, PCR is recognized for its higher sensitivity, specificity, and accuracy in detecting *Anaplasma* DNA, reducing the likelihood of false positives or negatives (Han et al., 2017). The use of the 16S rRNA gene as a molecular marker further enhances the reliability of PCR, as it is highly conserved and suitable for bacterial identification and comparative analysis (Bartoš et al., 2024). The identification of a positive case in horses, along with the highest infection rates in goats reported in Bachok and Tanah Merah (Tan Li, 2024), suggests the potential environmental or vector-related factors influencing *Anaplasma* transmission across host species in these regions.

The findings of this present study suggest that *Anaplasma sp.* (PQ804341) from Bachok, Kelantan, is genetically similar to *A. marginale*, sharing high identity scores (99.9%–100%) with this species. Notably, the Malaysian isolate exhibited more remarkable genetic similarity with *A. marginale* from the Philippines (MZ150522) and Thailand (KT264188) than with the Australian isolate (AF414874) (Lew et al., 2003), indicating potential regional genetic variations. Given the genetic proximity of this isolate to *A. marginale*, a known pathogen in cattle, further surveillance is crucial to assess its pathogenicity in horses and its potential role in equine anaplasmosis. Expanding investigations to other states in Malaysia with significant horse populations will help determine the disease's prevalence and epidemiology.

This study had several limitations. One of them concerns diagnostic confirmation. The use of 16S rRNA, known as a traditional method for bacterial identification, has limitations particularly in detecting novel pathogens and uncultured bacteria, and due to the incomplete accuracy of reference databases (Janda and Abbott, 2007). While it is reliable at the genus level, such as for identifying *Anaplasma* spp., it is less effective at distinguishing species like *A. marginale*. Therefore, the use of additional genetic markers such as msp4 and groEL is recommended in future studies. Another limitation is the small sample, which restricts the power of statistical analysis and may affect the reliability of the findings (Faber and Fonseca, 2014).

The PCR-positive results identified in this study indicate the active presence of *Anaplasma* spp. within the horse stable, likely due to tick exposure in the surrounding environment. This molecular detection suggests that tick survival and transmission may be influenced by the farm's ecological conditions. Active or subclinical infections in horses can disrupt their activity, compromise immunity, and affect overall fitness if not managed properly. Furthermore, the detection of *Anaplasma* spp. highlights the zoonotic potential of this pathogen in shared human-

animal environments, emphasizing the need for prompt and integrated cross-species surveillance.

The absence of clinical symptoms in infected horses highlights the challenge of diagnosing anaplasmosis based on clinical observations alone. Therefore, routine diagnostic protocols for equine blood parasites should incorporate PCR-based detection of *A. marginale* to enable early identification and case management. Further studies are needed to characterize the clinical manifestations of *A. marginale* infections in Malaysian horses. This provide a clearer understanding of its impact on equine health and inform the development of targeted control and prevention strategies.

CONCLUSION

Anaplasmosis presents with nonspecific symptoms, and infected animals often show no clinical signs, making diagnosis and treatment challenging. The detection of *A. marginale* in horses on the east coast of Malaysia highlights the need for an increased awareness regarding its primary host and potential impact. These findings provide crucial information on the presence of tick-borne disease (Anaplasmosis) and lead to awareness and implementation of diagnosis, efficient treatment strategies, and control programs to manage the new occurring of *Anaplasma* infections in Malaysian horses.

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AUTHOR CONTRIBUTIONS

NZIMR and **MAM** conceptualized the study. **MAA** performed the experiments and analyzed the data. **MSAR** and **RS** contributed to data interpretation. **MAA** wrote the original draft. **INAK** and **MDG** reviewed and edited the manuscript. **MFHR** and **GSH** supervised the project and resources. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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