

Molecular Detection and Phylogenetic Analysis of *Theileria orientalis* in Australian-Imported Buffaloes in Jambi Province, Indonesia

Diah Adhenia Ernis Putri Mustikaingtyas¹, Tabitha Andrea Putri¹, Aan Awaludin², Apinya Arnuphapprasert³, Sarwo Edy Wibowo⁴, Dwi Priyowidodo⁵, and Yudhi Ratna Nugraheni^{5*}

¹Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta, Indonesia

²Livestock Study Program, Department of Animal Science, Politeknik Negeri Jember, Jember, Indonesia

³Faculty of Veterinary Science, Rajamangala University of Technology Srivijaya, Thailand

⁴Department of Animal Health, Faculty of Animal Science, Jambi University, Jambi, Indonesia

⁵Department of Parasitology, Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta, Indonesia

Abstract. Indonesia routinely imports buffalo to meet domestic meat demand, however outbreaks of theileriosis caused by *Theileria orientalis* have been reported in Vietnam among imported buffalo. Early detection is crucial in Jambi Province, which receives large consignments of buffalo meat. Theileriosis is an intracellular protozoan infection caused by *Theileria* sp., significantly affects livestock health and productivity. Information on detection and genetic diversity of *Theileria* sp. in Indonesian buffalo remains scarce. This study aimed to identify *Theileria* sp. from imported buffalo arriving in Jambi and to analyze their phylogenetic relationship with isolates from other regions using microscopic examination, PCR, and 18S rRNA sequencing. A total of 38 blood samples were examined microscopically and tested by PCR targeting the 18S rRNA gene, followed by Sanger sequencing for phylogenetic analysis. Microscopic examination did not reveal intraerythrocytic piroplasms, whereas PCR confirmed infection in two samples, yielding an prevalence of 5.26%. Phylogenetic reconstruction using the maximum likelihood method demonstrated that Jambi isolates shared high nucleotide homology and clustered with *T. orientalis* from Thailand and *T. buffeli* from Myanmar, Vietnam, and India, supported by a bootstrap value of 85%. These findings suggest the possibility of transboundary dissemination of *T. orientalis* genotypes through livestock trade. This study contributes to understanding the distribution and genetic relationship of *T. orientalis* in buffalo imported into Indonesia and provides useful insights for epidemiological studies.

1 Introduction

The demand for animal protein in Indonesia continues to rise annually, driven by population growth and increasing public awareness of nutritional needs [1]. Meat remains one of the primary protein sources, yet domestic production is insufficient to meet national demand,

* Corresponding author: yudhi.ratna.n@mail.ugm.ac.id

resulting in a deficit of 294.55 thousand tons in 2024 [2]. To address this gap, the Indonesian government introduced a buffalo meat import policy in 2016, sourcing primarily from Australia, which has since become the major supplier of both beef and buffalo. Buffalo is considered a promising alternative protein source, as its protein content (20–23.3%) is higher than that of beef (19–20%) [1][3].

Despite these benefits, animal trade poses risks of pathogen introduction. Outbreak of theileriosis caused by a virulent genotype of *Theileria orientalis* in cattle imported from Australia to Vietnam, underscoring the importance of early detection [4]. This is particularly critical in Jambi Province, a key entry point for buffalo meat, where theileriosis has been reported as the most prevalent hemoparasitic disease, accounting for 72% of 1,891 blood samples examined.

According to the World Organisation for Animal Health (WOAH), theileriosis is recognized as a major livestock disease. It is caused by obligate intracellular protozoa of the genus *Theileria*, transmitted by ixodid ticks in humid environments. The genus comprises pathogenic and non-pathogenic species with distinct clinical consequences. Pathogenic species such as *T. parva*, *T. annulata*, and *T. lestoquardi* are associated with severe disease, whereas non-pathogenic species including *T. mutans*, *T. velifera*, and *T. orientalis* generally cause subclinical or mild infections [5]. In the Asia–Pacific region, *T. orientalis* is frequently detected in cattle and water buffalo without overt clinical signs [6]. Nevertheless, fatal outbreaks have been reported in Australia, New Zealand, India, and Vietnam, characterized by hemolytic anemia, abortion, and mortality [7]. These outbreaks are thought to be linked to virulent *T. orientalis* genotypes, which are widely distributed in cattle and buffalo populations [8].

Epidemiological investigations of theileriosis in buffalo in Indonesia remain scarce, with most studies focusing on cattle, goats, and sheep. However, buffalo infections may have equally significant implications for livestock health and farm productivity. Conventional diagnostic methods, particularly microscopic examination of blood smears, remain widely used to identify intraerythrocytic developmental stages of the parasite [9]. Yet, their sensitivity is limited, especially in subclinical carrier animals with low parasitemia. Furthermore, diagnostic accuracy depends on the expertise of laboratory personnel in recognizing diverse parasite morphologies. In contrast, molecular techniques such as polymerase chain reaction (PCR) and DNA sequencing provide higher sensitivity and specificity, enabling precise detection and genotypic identification of *Theileria* species.

Given the limited research on hemoparasitic diseases in buffalo in Indonesia, the present study was designed to detect and analyze the phylogeny of *Theileria* sp. in Australian-imported buffaloes at slaughterhouses in Jambi Province. This study is expected to provide valuable insights into the epidemiology of theileriosis in buffalo and contribute to improved disease surveillance and control strategies

2 Material and Methods

2.1 Ethical Approval

The study was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Universitas Gadjah Mada (Ethical Clearance No. 17/EC-FKH/Int.2024).

2.2 Sampling site and study design

Blood samples were obtained from the Australian-imported buffaloes at Jambi Province slaughterhouse between 2019 until 2021. Blood smear examination, DNA extraction of blood

parasite, amplification of 18S rRNA gene, and electrophoresis were performed at Department of Parasitology, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia. DNA Sequencing of blood parasites was conducted in Integrated Research and Testing Laboratory, Universitas Gadjah Mada (LPPT UGM), Yogyakarta, Indonesia.

2.3 Sample collection

A total of 38 blood samples were randomly collected from clinically healthy (asymptomatic) buffaloes imported from Australia at Jambi Province slaughterhouse. The sample size was determined based on the availability of imported buffaloes presented for slaughter during the study period, therefore the number of samples was limited. Blood samples were drawn from the jugular vein and collected into 3 mL ethylenediaminetetraacetic acid (EDTA)-coated tubes that prepared for blood smear examination. The remaining EDTA blood samples were stored at -20°C until DNA extraction. All 38 serum samples were extracted simultaneously in a single batch.

2.4 Microscopic blood smear examination

Thin blood smear was prepared on a glass slide and fixed with methanol for 3 minutes, followed by staining with 10% Giemsa solution for 20 minutes. The slide was then rinsed with water and examined under oil immersion at 100× magnification using a microscope (Optika B-150POL-B, Italy) to observe the presence of intraerythrocytic piroplasms of *Theileria* sp. Each slide was examined in 10 microscopic fields at 100x magnification.

2.5 Molecular diagnosis

PCR amplification targeting the 18S rRNA gene was performed using PanpiroF forward and reverse primers as describe by Nguyen [8]. The reaction followed the GoTaq® Green Master Mix protocol (Promega Corporation, 2012) in a total volume of 22 µL, consisting of 12.5 µL GoTaq® Green Master Mix (2X), 1 µL each of forward and reverse primers, 7.5 µL sterile distilled water, and 1 µL of DNA template. Amplification was carried out in a Labcycler 48 Thermocycler (SensoQuest) under standard cycling conditions comprising pre-denaturation, denaturation, annealing, extension, and final extension. The reaction produced a 1221 bp amplicon as shown in Table 1, and the temperature optimization is presented in Table 2.

Table 1. Primers for piroplasm used in this study

Target gene	Primer name	Primer nucleotide sequence (5' → 3')	Target size
Piroplasms 18S rRNA	PanpiroF <i>Forward</i>	GCAAATTACCCAATCCTGACACAGG	1221
	PanpiroF <i>Reverse</i>	CCGAATAATTCACCGGATCACTCG	

PCR products were analyzed by 1.5% agarose gel electrophoresis in 1× TAE buffer containing FluoroSafe DNA stain (3 µL per 50 mL gel). Each 5 µL PCR product was mixed with 1.5 µL loading dye and loaded alongside a DNA ladder and positive and negative controls. Electrophoresis was carried out at 100 V for 30 min, and DNA bands were visualized under a UV transilluminator. Samples showing a single, distinct band of the expected size were considered positive and selected for sequencing. The PCR positive samples were sequenced using the Sanger method at the Integrated Research and Testing

Laboratory (LPPT), Universitas Gadjah Mada (UGM), to determine the nucleotide sequences.

Table 2. Amplification cycles for PCR used in this study

Amplification stage	Temperature	Time	Cycle
Pre-denaturation	95°C	2 minutes	1
Denaturation	95°C	30 seconds	35
Annealing	51°C	30 seconds	35
Extension	72°C	45 seconds	35
Final extension	72°C	5 minutes	1

2.6 Statistic analysis

Statistical analysis was performed using the Epitools online statistical platform (<https://epitools.ausvet.com.au>). The binomial Wilson method was applied to calculate 95% confidence intervals ($P < 0.05$) for proportion estimates. This method is particularly suitable for small sample sizes or when the estimated proportions are close to 0 or 1.

2.7 Phylogenetic analysis

The obtained DNA sequences were aligned using BioEdit version 5.0.9 to generate a consensus sequence. Sequence alignment was performed with the ClustalW program to determine the level of nucleotide homology [10]. The resulting consensus sequences were compared with reference sequences of other *Theileria* species available in the GenBank database using the NCBI Basic Local Alignment Search Tool (BLASTn) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) as shown in Table 3.

Sequences showing the highest to lowest percentage identity in BLASTn results were downloaded for further analysis. The best-fit evolutionary substitution model and bootstrap analysis were determined using IQ-TREE version 1.16.2, while the phylogenetic tree was constructed based on the maximum likelihood method with 1,000 bootstrap replications and visualized using FigTree version 1.4 [11].

Table 3. Reference *Theileria* species used as comparators in the phylogenetic analysis

GenBank accession numbers	Species	Host	Location
MG757636	<i>T. orientalis</i>	Buffalo	Thailand
MG757646	<i>T. orientalis</i>	Buffalo	Thailand
MG757648	<i>T. orientalis</i>	Buffalo	Thailand
OR067896	<i>T. buffeli</i>	Buffalo	India
DQ104611	<i>T. buffeli</i>	Cow	China
LC602479	<i>T. buffeli</i>	Dog	Myanmar
HM538195	<i>T. sergenti</i>	Cow	China
FJ822144	<i>T. sergenti</i>	Cow	China
EU083804	<i>T. sergenti</i>	Cow	China
MF287924	<i>T. annulata</i>	Cow	India
KT367869	<i>T. annulata</i>	Cow	India
KP407013	<i>T. luwenshuni</i>	Sika deer	China
KP407014	<i>T. luwenshuni</i>	Roe deer	China
KP407015	<i>T. luwenshuni</i>	Roe deer	China
KP407019	<i>T. cervi</i>	Red deer	China
KP407020	<i>T. cervi</i>	Sika deer	China
KP407021	<i>T. cervi</i>	Sika deer	China
LC781922	<i>T. equi</i>	Horse	Mongolia

MG052917	<i>T. equi</i>	Horse	Brazil
KJ573374	<i>T. equi</i>	Horse	Brazil

3 Results

3.1 Microscope blood smear examination for *Theileria* sp.

Microscopic examination of Giemsa-stained blood smears was performed to identify the presence of *Theileria* sp. blood parasites. Examination of 38 blood smear samples revealed no intraerythrocytic piroplasms of *Theileria* sp.

3.2 PCR amplification results for *Theileria* sp.

Visualization of the electrophoresis results under a UV transilluminator revealed positive DNA bands in 2 out of 38 samples (KJ15 and KJ20) as shown in Figure 1. Based on these findings, the tested samples were confirmed to be positive for piroplasm infection. The prevalence of piroplasmosis in buffaloes at the Jambi slaughterhouse was calculated to be 5.26%.

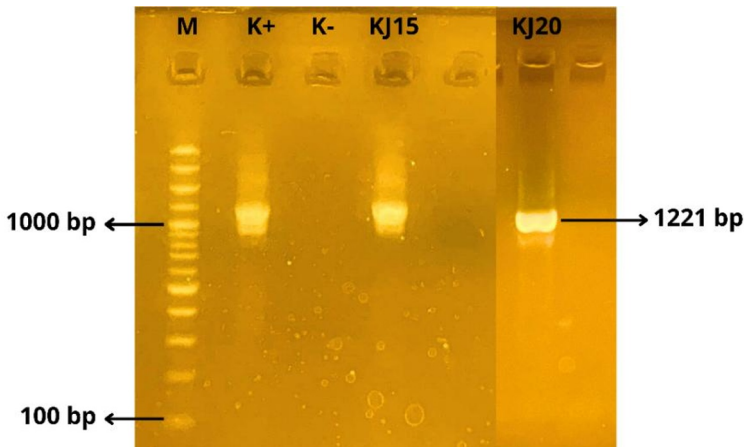


Fig. 1. Analysis of PCR products of the 18S rRNA gene of piroplasm. M = molecular size marker; K+ = positive control; K- = negative control; KJ15 = sample number 15; KJ20 = sample number 20.

3.3 Sequencing and phylogenetic analysis

Two PCR-positive samples were selected for sequencing. The obtained sequences showed 99.67% for nucleotide sequence identify *T. orientalis* and *T. buffeli* sequences deposited in NCBI GenBank by BLAST analysis. A phylogenetic tree was constructed based on the sequences obtained in this study and sequences retrieved from the GenBank database. The phylogenetic analysis revealed that sample KJ20 (20JAMBI) identified as *T. orientalis* clustered within the same clade as *T. orientalis* isolates from Thailand (MG757637, MG757649, and MG757645), *T. buffeli* from India (OR067896), *T. buffeli* from China (DQ104611), and *T. buffeli* from Myanmar (LC602479), with a bootstrap value of 85%.

4 Discussion

In the present study, we investigated the presence of *T. orientalis* infections using molecular detection and conducted phylogenetic analysis in Australian-imported buffaloes at the Jambi slaughterhouse.

Microscopic examination of blood smears in this study revealed no intraerythrocytic piroplasms. This may be attributed to the absence of clinical symptoms in the sampled buffaloes, which could explain the lack of detectable parasites in the examined samples. Animals with low parasitemia levels often do not exhibit clinical signs, making parasite detection through microscopy difficult. Although microscopic examination of blood smears remains a conventional method for identifying piroplasm stages within erythrocytes, it has several limitations, including low sensitivity, the need for skilled personnel, and the inability to detect subclinical infections or carrier animals. According to [9], microscopic examination alone cannot accurately identify *Theileria* species. In contrast, molecular techniques such as PCR have been shown to offer higher sensitivity and specificity for diagnostic purposes.

PCR amplification targeting the 18S rRNA gene revealed the presence of *T. orientalis* in two out of 38 blood samples (KJ15 and KJ20), yielding 1221 bp products as expected. The proportion of PCR-positive samples was analyzed using the binomial Wilson confidence interval, estimating the prevalence of piroplasmosis in buffaloes at the Jambi slaughterhouse to be 5.26%. The 95% confidence interval ranged from 1.46% to 17.29% (Table 4). Although the detected prevalence was relatively low, the wide confidence interval suggests uncertainty due to the limited sample size. Thus, further studies with larger sample numbers are required to obtain a more precise estimation.

Table 4. Prevalence of piroplasm infection in buffaloes at Jambi slaughterhouse based on PCR results

Test	Positive samples	Total samples	Prevalence	95% CI (lower limit)	95% CI (upper limit)
Piroplasma	2	38	0.0526	0.0146	0.1729

To further characterize the detected parasites, sequencing and phylogenetic analysis were conducted. Phylogenetic analysis revealed that the sequences obtained from Jambi were closely related to *T. orientalis* isolates from Thailand, *T. buffeli* from India, *T. buffeli* from China, and *T. buffeli* from Myanmar, with a bootstrap value of 85% (Figure 2). This finding confirms that the strain obtained from Jambi belongs to *T. orientalis*. The high genetic similarity between the Jambi isolates and reference sequences from other Asian regions reflects a shared evolutionary ancestry rather than indicating a recent transmission event or direct geographic origin. The *T. orientalis* complex has historically included *T. sergenti* and *T. buffeli* due to their high genetic similarity and frequent occurrence in mixed infections in cattle and buffalo, which are now considered genetically indistinguishable or closely related taxa. Although the samples were collected in Jambi, the buffaloes originated from Australia, making it difficult to determine the timing and location of infection. Infection may have occurred prior to importation or after arrival in Indonesia through exposure to local vectors; however, the lack of epidemiological data precludes definitive conclusions.

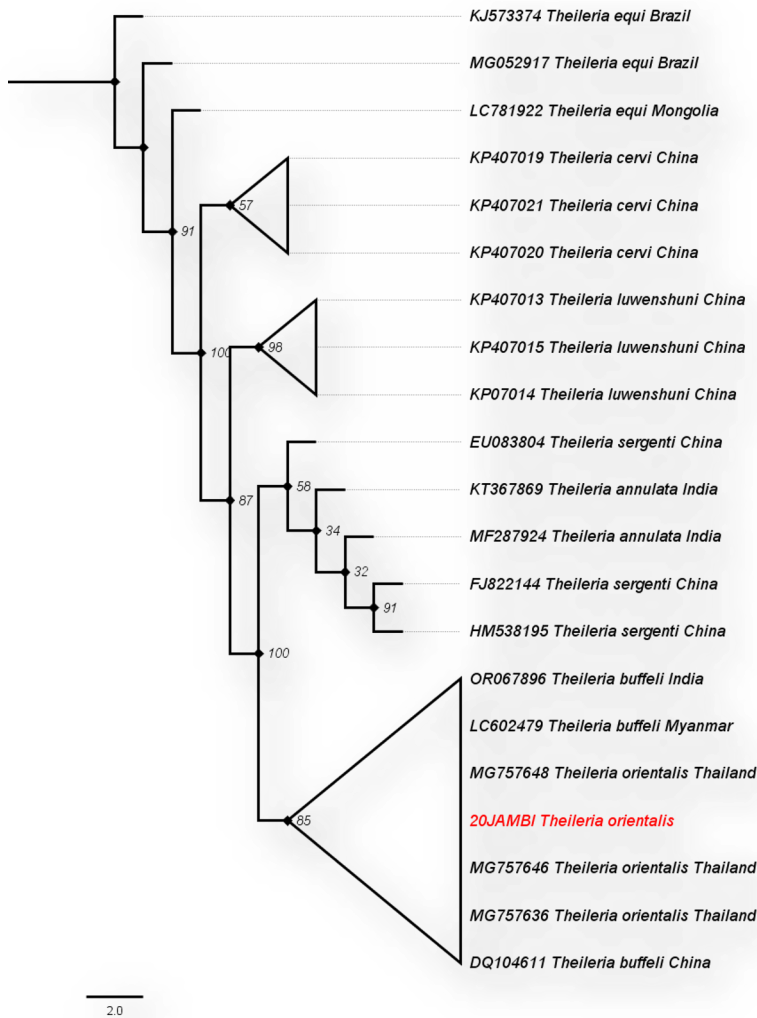


Fig. 2. Phylogenetic tree of 18S rRNA of *T. orientalis* in imported buffaloes isolated from Jambi Province. The phylogenetic tree was constructed using the Maximum Likelihood method with 1000 bootstrap replications.

Transmission of the *T. orientalis* complex is primarily associated with ticks of the genus *Haemaphysalis*, although *Rhipicephalus microplus* has also been implicated [4][12]. In Thailand, *R. microplus* has been identified as the principal vector responsible for the spread of theileriosis [14]. In addition to tick-borne transmission, mechanical transmission via blood-sucking flies (*Tabanidae*) and sucking lice has also been suggested [13]. Therefore, if local transmission occurred, both tick genera may potentially play a role. However, vector involvement was not assessed in this study.

Generally, cases of theileriosis in Indonesia do not exhibit severe clinical manifestations. Among animals that tested positive, many are likely carriers, reflecting endemic stability of the parasite. Livestock in such regions may have been exposed to the pathogen early in life, leading to mild or absent clinical signs [6]. Although asymptomatic, these animals can serve as reservoirs for ticks, posing a major challenge in piroplasmiasis control. When naïve animals without prior exposure are introduced into these endemic areas, they may develop

severe clinical disease, sometimes leading to death. Consequently, infection by this blood parasite can cause significant economic losses through mortality and decreased productivity in affected livestock.

This study has several limitations, including the small sample size, lack of detailed clinical data, and absence of vector sampling, which should be addressed in future studies. Further studies should include a larger number of samples collected from a wider geographical area in Indonesia to enable more comprehensive identification and phylogenetic analysis of *T. orientalis*. In addition, more detailed molecular characterization of *T. orientalis* in Indonesia is recommended using more specific DNA sequence regions, such as *mssl* α , *mssl4*, *mssl5*, and MPSP. Despite these limitations, this study provides baseline molecular evidence of *T. orientalis* infection in Australian-imported buffaloes slaughtered in Jambi.

Conclusion

This study provides the first molecular evidence of *T. orientalis* infection in Australian-imported buffaloes slaughtered in Jambi, Indonesia. While microscopic examination failed to detect intraerythrocytic piroplasms, PCR successfully identified *T. orientalis*, demonstrating the higher sensitivity of molecular diagnostics. Phylogenetic analysis showed that the detected isolates belonged to the *T. orientalis* complex and were genetically similar to isolates reported from other Asian regions. This genetic similarity reflects shared evolutionary ancestry within the *T. orientalis* complex and the limited availability of reference sequences from Australian buffaloes, rather than indicating recent transboundary dissemination or a definitive geographic origin of infection. The presence of *T. orientalis* in clinically healthy buffaloes highlights the role of carrier animals in maintaining endemic stability, emphasizing the need for continuous molecular surveillance and effective vector control.

The authors express their gratitude to the staff of the Ruminant Slaughterhouse (Rumah Pematangan Hewan Ruminansia) in Kasang Kota Karang, Jambi Province for their valuable assistance and cooperation during sample collection.

References

1. C. A. Fitri, D., A. Abubakar, F. Afiq, and H. Salami, Sifat fisikokimia dan organoleptik dendeng daging kerbau dengan persentase penggunaan enzim papain yang berbeda. *Jurnal Teknologi Hasil Peternakan*. **5**, 1 (2024)
2. Pusat Data dan Sistem Informasi Pertanian, Outlook Komoditas Peternakan Daging Sapi 2023, (Sekretariat Jenderal Kementerian Pertanian Republik Indonesia Jakarta, 2023)
3. I. Sumantri and H. S. Chang, Impact of imported Indian buffalo meat on red meat supply and demand in South Kalimantan, Indonesia, *IOP Conference Series: Earth and Environmental Science* (2021), 012033
4. H. Gebrekidan, R. Gasser, G. Baneth, D. Yasur-Landau, Y. Nachum-Biala, A. Hailu, A. Jabbar, Molecular characterization of *Theileria orientalis* from cattle in Ethiopia. *Ticks Tick Borne Dis*. **7**, 5 (2016)
5. Z. Ganaie, I. Shahardar, I. Bulbul, I. Wani, R. Shahardar, I. Maqbool, K. Bulbul, I. Allaie, Z. Wani, An overview of bovine theileriosis. *International Journal of Veterinary Sciences and Animal Husbandry*. **9**, 1 (2019)
6. I. C. B. Prado, L. Capuno, P. Collera, A. Cabralda, K. De Ramos, J. Bernardo, B. Divina, T. Masatani, T. Tanaka, R. Galay, Molecular detection and characterization of *Babesia*

- and *Theileria* in cattle and water buffaloes from Southern Luzon, Philippines. *Microorganisms*. **10**, 678 (2022)
7. K. Baghel, B. Saravanan, K. Jeeva, D. Chandra, K. Singh, S. Ghosh, A. Tewari, Oriental theileriosis associated with a new genotype of *Theileria orientalis* in buffalo (*Bubalus bubalis*) calves in Uttar Pradesh, India. *Ticks Tick Borne Dis*. **14**, 1 (2023)
 8. A. H. L. Nguyen, S. Tiawsirisup, and M. Kaewthamasorn, Low level of genetic diversity and high occurrence of vector-borne protozoa in water buffaloes in Thailand based on 18S ribosomal RNA and mitochondrial cytochrome b genes. *Infection, Genetics and Evolution*. **82** (2020)
 9. B. Kumar, B. R. Maharana, B. Thakre, N. N. Brahmbhatt, and J. P. Joseph, 18S rRNA gene-based piroplasmid PCR: an assay for rapid and precise molecular screening of *Theileria* and *Babesia Species* in animals. *Acta Parasitol*. **67**, 4 (2022)
 10. Suryadi, A. Yuniaty, and A. H. Susanto, Genetic diversity among three cultivars of peanut (*Arachis hypogea* L.) based on RAPD markers. *Scripta Biologica*. **4**, 1 (2017)
 11. D. I. Roslim and A. Fitriani, Barkoding DNA pada Tumbuhan Durik-Durik (*Syzygium* sp.) asal Riau menggunakan daerah gen ndhF. *J Bios Logos*. **11**, 1 (2021)
 12. P. Kakati *et al.*, Emergence of oriental theileriosis in cattle and its transmission through *Rhipicephalus (Boophilus) microplus* in Assam, India. *Vet World*. **8**, 9 (2012)
 13. J. G. Watts, M. C. Playford, and K. L. Hickey, *Theileria orientalis*: a review. *N Z Vet J*. **64**, 1 (2012)
 14. K. Thinnabut, R. Rodpai, O. Sanpool, W. Maleewong, and U. Tangkawanit, "Detection of *Theileria* in cattle ticks (*Rhipicephalus microplus*) (Canestrini, 1888) in upper-northeastern Thailand," *Acta Trop*. **260** (2024)