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Research Article

Development of Multiplex PCR for Simultaneous Detection of *Trypanosoma evansi* and Equine Piroplasma Infection in Horses

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ABSTRACT

The identification of equine piroplasmosis and surra disease in infected horses currently presents a significant challenge. Clinical symptoms and blood smears are commonly used to diagnose these two diseases. The objective of this study was to develop a multiplex amplification assay capable of simultaneously identifying all three blood protozoa (equine piroplasma and *Trypanosoma evansi*) in a single test. The primer pairs used for detecting *T. equi, B. caballi*, and *Trypanosoma evansi* were ema-2-t (587 bp), Bc-134 (429 bp), and Rotat 1.2 VSG (151 bp), respectively. The multiplex PCR assay was subsequently evaluated for its detection limit, sensitivity percentage, and specificity using single PCR as the reference standard. The multiplex PCR method demonstrated a sensitivity of 100% for detecting both *Theileria equi* and *Trypanosoma evansi*, with all positive samples confirmed by a single PCR. It also achieved 100% specificity for both the parasites. However, for *Babesia caballi*, while the sensitivity remained at 100%, the specificity was reduced to 66%, indicating some limitations in accurately identifying negative samples. For the multiplex PCR assay, the minimum detectable concentrations were 0.01 ng/µL for both *T. equi* and *B. caballi*, whereas *Trypanosoma evansi* exhibited a detection threshold of 1 ng/µL. When multiplex PCR assays were used to screen blood samples obtained from horses in selected districts of Bogor, only a single sample showed a positive result for *T. equi*. The sensitivities and specificities achieved by multiplex PCR and single PCR were comparable. Therefore, the multiplex PCR method developed in this study can be effectively applied to diagnose piroplasmosis and surra in horses.

1. Introduction

In Indonesia, the equine sector is promising and of significant economic importance. According to data, the horse population has increased by an average of 20,000 heads each year during the last five years (BPS 2018). The highest population of horses can be found in several regions of Indonesia, including South Sulawesi, East and West Nusa Tenggara, as well as the western and central parts of Java. In addition, horses are considered non-food things as well. Horse meat and milk are used as an alternate source of protein in several provinces, including East and West Nusa Tenggara (Asih & Akbar 2014). It is critical to enhance horse productivity by improving livestock health and parasitic infection prevention. Several studies have shown that parasite infections may reduce the weight of horses by 25-50 percent (Hutardo & Gilardo 2018).

Horses are often infected with blood parasites, such as *Trypanosoma evansi, Theileria equi*, and *Babesia caballi*. Surra, or trypanosomiasis, is a parasitic infection caused by the protozoan *Trypanosoma evansi* (*T. evansi*). In 2011, trypanosomiasis emerged in East Sumba, killing 4,268 livestock and 1,608 horses and causing 167.5 billion rupiahs in economic damage. Consequently, surra has been added to Indonesia's list of strategic infectious animal diseases. *Theileria equi* and *Babesia cabali* cause piroplasmosis. According to previous reports, this condition results in severe

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economic losses. Furthermore, yearly losses have ranged from 13.9 to 18.7 billion US dollars (Hutardo & Gilardo 2018).

Detecting piroplasmosis and surra infection in horses has proven to be challenging. These infections are often diagnosed using blood smears, serological tests, and molecular assays. However, these two diseases are still diagnosed on the basis of clinical symptoms and the procedure employed to evaluate blood smears (Singh *et al.* 2004; OIE 2014). The blood smear testing technique has limitations that can cause diagnostic errors, especially at low parasitemia levels (Quintao-Silva and Ribeiro 2003; Krause 2003; Eghianruwa and Oridupa 2018). Furthermore, the morphology of *B. caballi, T. equi*, and *T. evansi* is difficult to differentiate, especially in co-infections, which is a limitation of this technique. Misdiagnosed diseases may result in horse mortality, high medical costs, treatment resistance, and a fall in production and reproduction rates, contributing to a loss in the horse population (Rothschild and Knowles 2007; Jittapalapong *et al.* 2009; Wise *et al.* 2013).

Multiplex PCR techniques have been developed to identify *B. caballi* and *T. equi*, however, a similar method for detecting *T. evansi* has not yet been developed. Therefore, the objective of this study was to develop a multiplex PCR assay capable of identifying three blood-borne protozoan parasites, *B. caballi, T. equi,* and *T. evansi*, in a single test. This method is being developed to provide practical, rapid, and accurate results.

2. Materials and Methods

2.1. Sample Collection and DNA Extraction

Blood samples were obtained from 25 horses in Bogor, West Java, Indonesia. Approximately 3 ml of blood was drawn from each horse and stored in ethylenediaminetetraacetic acid-containing vacuum tubes. Giemsa-stained thin blood smears from sampled animals were examined microscopically. DNA was extracted from the blood samples using the Genomic DNA Mini Kit Blood (Geneaid) following the manufacturer's protocol. The extracted DNA was quantified using a Biocompare NanoDrop® ND-1000 Thermo Scientific before storage at -20°C until further use. *Theileria equi* and *Babesia cabali* DNA used as positive controls in this study were sourced from a previous study (Nugraha *et al.* 2018). *Trypanosoma* evansi DNA was isolated from field-infected animals and verified by microscopic and PCR-based molecular analyses (unpublished data).

2.2. Specific Primers Design

The target genes of ema-2t, Rotat 1.2 VSG, and Bc-134 were selected to develop multiplex PCR. The Bc-134 primers were designed from accession No. AB095267, using Primer 3 Software. The ema-2 and Rotat 1.2 VSG primers were used from the previous study (Salim *et al.* 2011; Nugraha *et al.* 2018). Primers used in this study are listed in Table 1.

2.3. Polymerase Chain Reaction (PCR) Analyses

Single PCR analyses were optimized and conducted to detect the DNA of individual parasites, whereas multiplex PCR was standardized and used to identify equine piroplasma and *Trypanosoma evansi* simultaneously. DNA samples that tested positive for *Theileria equi* and *Babesia caballi*, acquired from our earlier research (Nugraha *et al.* 2018), and *Trypanosoma evansi* were obtained from infected animals in the field and confirmed by microscopic and molecular examinations using PCR (unpublished data). In both single and multiplexed PCR tests, genomic DNA was used as the positive and negative control.

2.4. Single PCR Assays

A single PCR was conducted in a total volume of 20 µL. The reaction mixture comprised 1X My HS Taq Buffer (Bioline, Meridian Bioscience), 2 U of My HS Taq DNA Polymerase (Bioline), 0.4 µM of each primer (forward and reverse, Table 1), one µL of DNA template, and nuclease-free water (Qiagen) to reach the final volume. The singleplex PCR assays were as follows these cycling conditions: 1 minute initial denaturation at 95°C, 10 s annealing at 55°C, 1 minute

Table 1. Primer sequences used in this study

Primers	Primer sequences	Size (bp)	References
Rotat 1.2 VSG-F	5`-CTGAAGAGGTTG GAAATGGAGAAG-3`	151	Salim *et al.* 2011
Rotat 1.2 VSG-R	5`-GTTTCGGTGGTT CTGTTGTTGTTA-3`		
Ema-2t-F	5`-CGTTGTCACTCT CGGAGC-3`	587	Nugraha *et al.* 2018
Ema-2t-R	5`-TGACCCAGGAAT CACCAG-3`		
Bc 134-F	5`-AACGGCCGCTTT ACCATC-3`	429	Present study
Bc-134-R	5`-GCTTAATTTGGC ACCACC-3`		

extension at 72°C, and 90 seconds final extension at 72°C. The resulting PCR products were analyzed on a 1.5% agarose gel containing Florosafe DNA Stain (1st BASE, Singapore), subjected to electrophoresis at 100 V for 30 minutes, and visualized under UV light. Single infections were identified by amplified products of approximately 596 bp for *Theileria equi*, 429 bp for *B. caballi*, and 151 bp for *Trypanosoma evansi*.

2.5. The Optimization of Multiplex PCR Assay

A multiplex PCR reaction of 20 µL, designed for the simultaneous identification of equine piroplasma and *Trypanosoma evansi*, was optimized. This involved experimenting with various annealing temperature gradients (49–64°C), adjusting the PCR cycle numbers (25-40), and modifying oligonucleotide concentrations (0.3-0.5 µM). The final multiplex PCR assay comprised 1X My HS Taq Buffer (Bioline, Meridian Bioscience), 2 U of My HS Taq DNA Polymerase (Bioline), 0.3 µM of both forward and reverse primers (Table 1), one µL of DNA template, and nuclease-free water (Qiagen) to reach a total volume of 20 µL. The PCR protocol included an initial denaturation for 1 minute at 95°C, annealing for 10 s at 55°C, extension for 1 minute at 72°C, and a final extension for 90 s at 72°C. The resulting PCR products were subjected to electrophoresis on a 1.5 % agarose gel containing Gel dyed Midori Green Direct (Genetics, Japan) at 100 V for 30 min, followed by UV light visualization. Amplified products for simultaneous detection were observed at approximately 587, 429, and 151 bp for *Theileria equi, B. caballi*, and *Trypanosoma evansi*, respectively.

2.6. Determining the Detection Threshold of Single and Multiplex PCR Assay

To prepare the DNA mixture for this study, we quantified the concentration of the extracted DNA using a Biocompare-NanoDrop® ND-1000 Thermo Scientific spectrophotometer. Following the measurement, each sample was diluted with nuclease-free water to achieve a concentration of 10 ng/µL. To establish the detection limits for both single and multiplex PCR, we prepared serial 10-fold dilutions of each control DNA, ranging from 10 to 0.0001 ng/µL. To simulate mixed infections of *T. equi, B. caballi*, and *T. evansi*, we combined 10 µL of each stock DNA solution in a 1:1:1 ratio within a 1.5 mL microtube and homogenized the mixture. To assess the detection threshold for mixed infections using both single and multiplex PCR, we created a 10-fold dilution series of the combined control DNA, ranging from 10 to 0.0001 ng/µL.

2.7. Evaluating Multiplex PCR's Sensitivity and Specificity

To determine the sensitivity and specificity percentages in this research, we conducted a comparison between single PCR (considered the gold standard) and multiplex PCR results. This comparison was performed using positive DNA samples that were serially diluted tenfold, ranging from 10 ng/µL to 0.0001 ng/µL. The sensitivity calculation was performed using the following equation (Parikh *et al.* 2008):

$$\text{Sensitivity} = \frac{a}{a + b} \times 100\%$$

Where:
a : findings of true positive numbers
b : findings of false negative numbers

$$\text{Specificity} = \frac{d}{c + d} \times 100\%$$

Where:
c : findings of false positive numbers
d : findings of true negative numbers

2.8. Validation of Multiplex PCR

Multiplex PCR was validated by analyzing DNA extracted from equine blood samples obtained in an earlier study. A total of 25 horse blood specimens were evaluated using both individual and multiplex PCR techniques. The outcomes of the two methods were compared. Additionally, samples that showed positive results for *T. equi* through multiplex PCR were also confirmed to be positive by nested PCR (Nugraha *et al.* 2018) and subsequently sequenced.

2.9. Nested PCR (nPCR) Assays

To detect *T. equi*, nested PCR assays targeting EMA-2 were employed (EMA-2 nPCR). The primers used in this assay have been previously described by Nugraha *et al.* (2018). The PCR reaction mixture, totaling 20 µL, consisted of 1X My HS Taq Buffer (Bioline, Meridian Bioscience), 2 U of My HS Taq DNA Polymerase (Bioline), 0.4 µM of each forward and reverse primer (Table 1), one µL of DNA template, and nuclease-free water (Qiagen) to reach the final volume. Single PCR assays were conducted under the following cycling conditions: initial denaturation for 1 minute at 95°C, annealing for 10 s at 55°C, extension for 1 minute at 72°C, and final extension for 90 s at 72°C. In the subsequent PCR cycle, one microliter of the initial PCR product was placed in a fresh tube

containing an identical reaction mixture, except that the inner forward and reverse primers replaced the original primers. The second round of PCR employed the same cycling parameters as those in the first round. The PCR products were subjected to agarose gel electrophoresis and visualized under ultraviolet (UV) light. Bands appearing at approximately 221 bp were interpreted as positive for *T. equi*.

2.10. Sequence and Phylogenetic Analysis

Samples that tested positive for *T. equi* were sequenced and examined using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/blast.cgi). Subsequently, the obtained sequences were aligned with sequences from GenBank using the MEGA software version 7 for comparative analysis.

3. Results

3.1. The Findings of Single PCR Using Both Single and Mixed DNA Templates, Along with Annealing Optimization of Multiplex PCR

Our results indicate that single PCR successfully amplified from both single and mixed DNA templates, corresponding accurately to each of our gene targets, as shown in Figure 1, panel A. The multiplex PCR was optimized using several methods involving annealing temperature and cycle number of PCR. The annealing temperature was tested at four different temperatures: 48, 51, 52, and 55°C. They indicated that the optimized annealing temperature for multiplex PCR was 55°C, with a total of 35 cycles (Figure 1).

3.2. The Detection Threshold of Single and Multiplex PCR

Single and multiplex PCR were compared to evaluate the detection thresholds of the multiplex method. For multiplex PCR, the minimum detectable concentrations were determined to be 0.01 ng/µL for both *T. equi* and *B. caballi*, whereas *Trypanosoma evansi* had a detection limit of 1 ng/µL (Figure 2 & Table 2).

3.3. Comparison of the Sensitivity Percentage of Single PCR Compared with Multiplex PCR

The multiplex PCR technique demonstrated 100% sensitivity, as all four samples that tested positive were subsequently verified using a single PCR, which was considered the reference standard for this comparison. Additionally, the specificity was 100%, as two samples were identified as negative by a single PCR (Table 2). These findings suggest that multiplex PCR is highly effective in detecting Theileria equi without producing false-positive or false-negative results. For Babesia caballi, multiplex PCR demonstrated a sensitivity of 100%, successfully identifying all three positive samples confirmed by a single PCR. However, the specificity was lower at 66%, as only two out of the three negative samples were accurately identified by a single PCR (Table 3). This indicates that multiplex PCR is reliable for detecting *B. caballi* in positive samples. In the detection of *Trypanosoma evansi*, multiplex PCR again achieved a sensitivity of 100%, with both positive samples confirmed by a single PCR. The specificity was also 100%, as all four test negatives

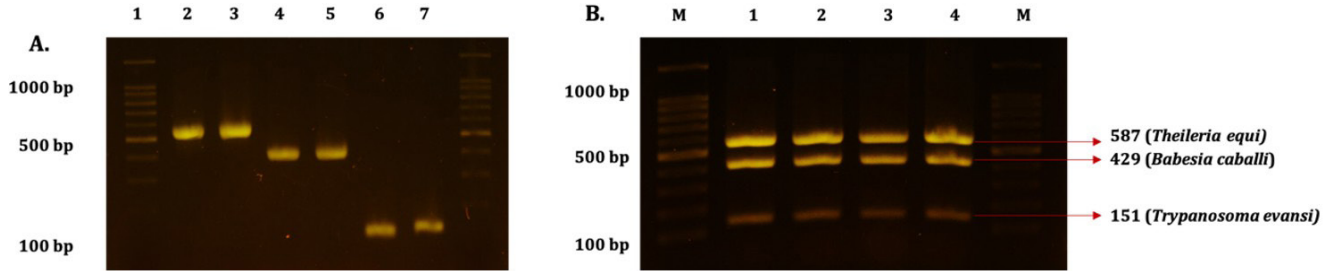
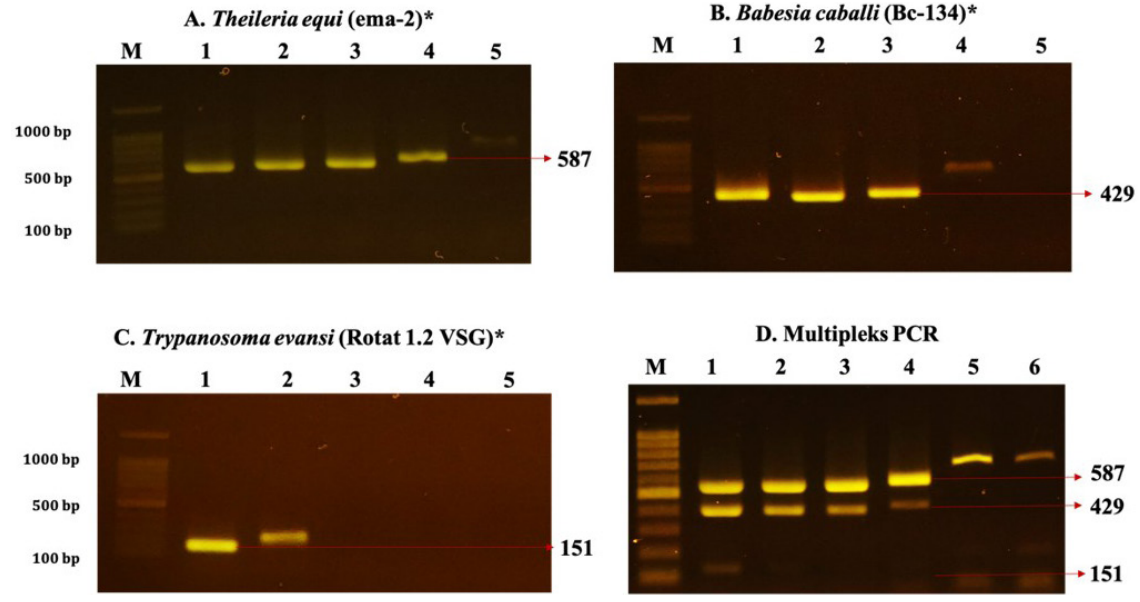


Figure 1. Findings from single PCR using both single and mixed DNA templates, along with annealing temperature optimization for multiplex PCR. Lane 2-3 (single DNA template of *T. equi*), lane 4-5 (single DNA template of *B. caballi*), and lane 6-7 (single DNA template of *Trypanosoma evansi*). (A) Single PCR results: lane 1: marker 100 bp; lane 2 (*T. equi*-ema2t), lane 4 (*B. caballi*-Bc-134) and lane 6 (*Trypanosoma evansi*-Rotat.1.2 VSG). Lanes 2, 4, and 6 show the results of single PCR using a single DNA template. Lanes 3, 5, and 7 show the results of a multiplex PCR using single DNA templates. (B) Multiplex PCR, lane M: marker, lane (1-4), gradients annealing temperature 48, 51, 53 and 55°C



*: Single PCR

Figure 2. Results of multiplex PCR electrophoresis using mixed DNA templates. DNA comprising all three parasite DNAs was employed as a template (*T. equi, B. caballi*, and *Trypanosoma evansi*). M: marker 100 bp; lane 1-6; mix DNA with concentrations of 10, 1, 0.1, 0.01, 0.001, and 0.0001 ng/µL (10 fold dilutions). (A-C) Single PCR; (A) *Theileria equi*-ema-2t; (B) *Babesia caballi*-Bc-134; (C) *Trypanosoma evansi*-Rotat.1.2-VSG, D (multiplex PCR)

Table. 2. The detection limit of single PCR and multiplex PCR in mixed infections

Parasite	DNA concentration (ng/µL)				
	10	10°	10^{-1}	10^{-2}	10^{-3}
Single PCR					
Theileria equi (ema-2)	+	+	+	+	-
Babesia caballi (Bc-134)	+	+	+	-	-
Trypanosoma evansi (Rotat 1.2 VSG)	+	+	-	-	-
Multiplex PCR					-
Theileria equi (ema-2)	+	+	+	+	
Babesia caballi (Bc-134)	+	+	+	+	-
Trypanosoma evansi (Rotat 1.2 VSG)	+	+	-	-	-

Table. 3. The sensitivity of single PCR compared to multiplex PCR for detecting *Theileria equi*

Multiplex PCR	Single PCR (gold standard)	
	Test positive	Test negative
Test positive	4	0
Test negative	0	2
Total	4	2
Sensitivity (%)	4/4 (100%)	
Specificity (%)		2/2 (100%)

were accurately detected by a single PCR (Table 4 & 5). These results indicate that multiplex PCR is highly effective for *Trypanosoma evansi* detection, similar to its performance with *Theileria equi*.

Table. 4. The sensitivity of single PCR compared to multiplex PCR for detecting *Babesia caballi*

Multiplex PCR	Single PCR (gold standard)	
	Test positive	Test negative
Test positive	3	1
Test negative	0	2
Total	3	3
Sensitivity (%)	3/3 (100%)	
Specificity (%)		(2/3) (66%)

Table 5. The sensitivity of single PCR compared to multiplex PCR for detecting *Trypanosoma evansi*

Multiplex PCR	Single PCR (gold standard)	
	Test positive	Test negative
Test positive	2	0
Test negative	0	4
Total	2	4
Sensitivity (%)	2/2 (100%)	
Specificity (%)		4/4 (100%)

3.4. Validation of Multiplex PCR

In the validation of horse field samples using multiplex PCR, a single sample out of 25 examined positive for *T. equi* in both the single and multiplex PCR assays (Table 6). All samples tested negative for the remaining two parasites. The sequence of *T. equi* in this study is shown together with other samples (Figure 3), in particular with Indonesian isolate (MG948458).

Table 6. The application of multiplex PCR using field samples

Parasites	Single PCR		Multiplex PCR		Total samples
	Positive (+)	Negative (-)	Positive (+)	Negative (-)	
Theileria equi	1	24	1	24	25
Babesia caballi	0	25	0	25	25
Trypanosoma evansi	0	25	0	25	25

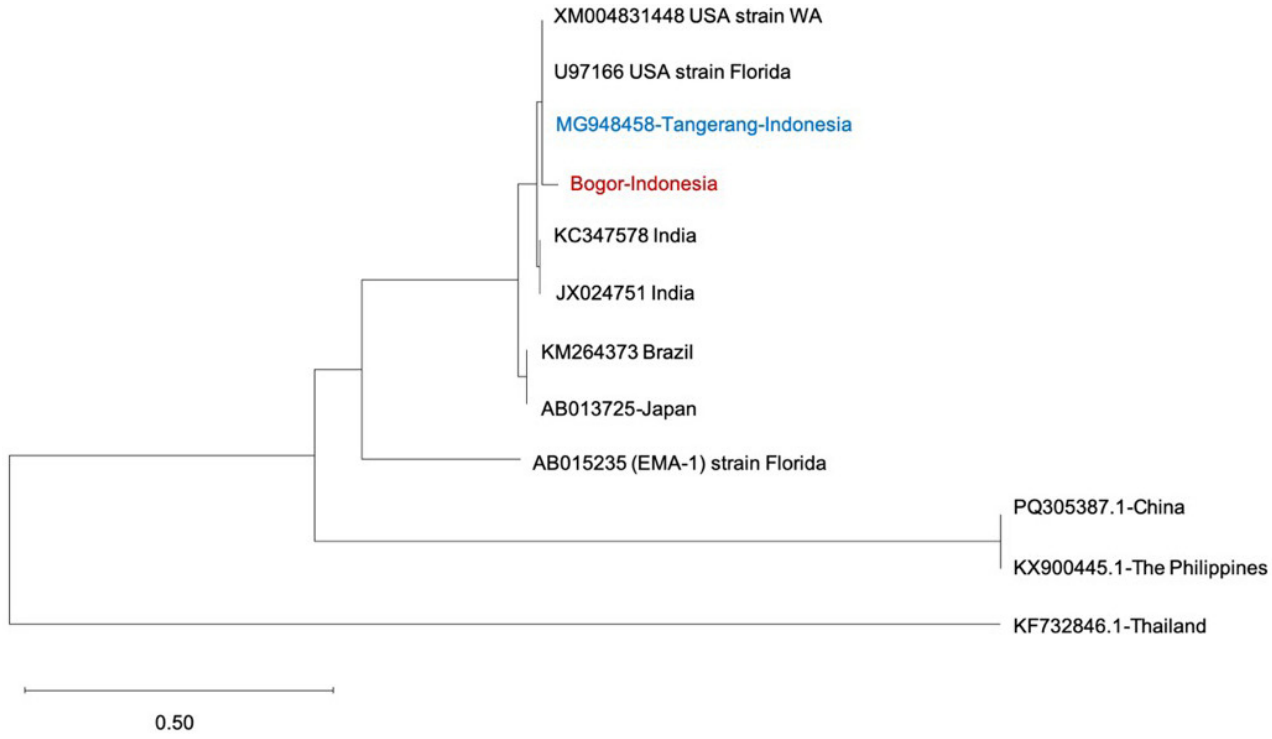


Figure 3. Analyses of phylogenetic relationships. Maximum-likelihood nucleotide sequences were constructed using the *T. equi* EMA-2 gene sequences obtained in this analysis and those identified in other regions

4. Discussion

Numerous studies have identified *T. evansi, T. equi*, and *B. caballi* using polymerase chain reaction (PCR) (Singh *et al.* 2004; Allsopp *et al.* 2007; Alhassan *et al.* 2005; Sawitri *et al.* 2015; Sumbria *et al.* 2015). However, these approaches involve sophisticated processes that are time-consuming and inefficient for routine diagnostics. Therefore, our approach could be used to screen horses for surra and piroplasmosis. Moreover, this study represents the first attempt to create a multiplex PCR method for the simultaneous detection of surra and piroplasmosis.

The findings of this study indicate that multiplex PCR exhibits exceptional sensitivity for detecting equine piroplasma, and *Trypanosoma evansi*. This confirmed the sensitivity and specificity of the technique for accurately identifying samples positive for these three pathogens. The multiplex PCR method demonstrated a sensitivity of 100% for detecting both *Theileria equi* and *Trypanosoma evansi*, with all positive samples confirmed by a single PCR. It also achieved 100% specificity for both the parasites. However, for *Babesia caballi*, while the sensitivity remained at 100%, the specificity was reduced to 66%, indicating some limitations in accurately identifying negative samples. This suggests the need for further evaluation to enhance its accuracy in distinguishing between positive and negative results. Validation using field samples revealed that in both single and multiplex PCR, only one sample was positive for *T. equi*. These results are consistent with earlier research, suggesting that *T. equi* has a relatively low occurrence in Indonesia (Nugraha *et al.* 2018). In 2018 investigation (Nugraha *et al.* 2018) reported seroprevalence rates of 6.4% and 2.1% for *T. equi* and *B. caballi*, respectively. Molecular analysis identified only one horse as positive for *T. equi*, whereas four horses were found to be positive for *B. caballi*. Additionally, the prevalence of *Trypanosoma evansi* in horses has been reported to be 2.2% based on serological tests, whereas the presence of parasites has been reported to be 25% (Apsari *et al.* 2024).

The multiplex PCR method introduced in this study could serve as a potential alternative screening approach to detect surra and piroplasma infections during the early phases of the disease. Therefore, this technique may help to prevent and manage surra and piroplasm infections, particularly in establishing a rapid, accurate, and cost-effective diagnosis. In conclusion, the multiplex PCR molecular approach established in this study may be used to diagnose the three blood protozoa simultaneously in a single test. The sensitivity and specificity of the single PCR and multiplex PCR were comparable. To further validate multiplex PCR, it is essential to use larger sample sizes or samples from other regions endemic to equine piroplasmosis and surra disease to increase the number of samples tested.

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