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Molecular Identification of Babesia bigemina from Cattle and Buffaloes in Bogor District

(Identifikasi Molekular Babesia bigemina dari Sapi dan Kerbau di Kabupaten Bogor)

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ABSTRAK

Peternakan merupakan industri penting yang memberikan kontribusi signifikan terhadap sektor pertanian di Indonesia. Babesiosis adalah penyakit yang ditularkan melalui vektor caplak dan disebabkan oleh parasit darah genus Babesia. Penyakit ini telah menyebabkan kerugian ekonomi yang sangat signifikan. Deteksi molekular babesiosis memiliki sensitivitas dan spesifisitas yang tinggi. Namun, saat ini, deteksi babesiosis menggunakan Polymerase Chain Reaction (PCR) di Indonesia belum banyak digunaknan, khususnya di Kabupaten Bogor. Tujuan penelitian ini adalah untuk mengidentifikasi Babesia bigemina pada sapi dan kerbau di Kabupaten Bogor dengan teknik molekular. Sebanyak 27 sampel darah dikoleksi yang terdiri atas 22 sampel sapi dan 5 sampel darah kerbau. Sampel diwarnai dengan Giemsa 10%, selain itu sampel darah diekstraksi dan dilanjutkan dengan nested PCR. Hasil dari pemeriksaan apusan darah menunjukkan 81,48% positif terhadap Babesia spp, sedangkan hasil nested PCR 11,11% menunjukkan positif terhadap B. bigemina. Berdasarkan jenis hewan sapi dan kerbau positif terhadap B. bigemina, masing-masing sebesar 9,09% dan 20%. Hasil penelitian ini memberikan informasi dasar mengenai tingkat infeksi Babesia bigemina di kabupaten Bogor berdasarkan metode molekuler. Oleh karena itu, program pencegahan dan pengendalian terhadap infeksi parasit darah pada peternakan sapi dan kerbau sangat perlu dilakukan.

Kata kunci: Babesia bigemina; babesiosis; Bogor; kerbau; PCR; sapi

INTRODUCTION

Livestock can be defined as domesticated terrestrial animals farmed for meat, milk, eggs, hides, feathers, and fibers, among other goods and services. Livestock includes animals such as cattle, buffaloes, goats, sheep, pigs, and poultry (Abigarl *et al.* 2023). The cattle and dairy industries have experienced significant evolutions to enhance productivity and intensification (Tona 2023). The increasing demand for animal-derived food in developing countries, particularly red meat, is projected to double by 2050. However, disease-infected animals often experience reduced production of animal products, contributing to the lack of high-quality protein source.

One of the common diseases which portrays a significant threat to

livestock-based economies is babesiosis. Babesiosis is a worldwide emerging tick-borne disease caused by blood parasites of the genus *Babesia* (Avenant *et al.* 2021). It affects many mammals including cattle and humans as opportunistic hosts (Gray *et al.* 2010). Bovine babesiosis is primarily caused by *Babesia bovis* and *B. bigemina*, affecting cattle (*Bos taurus*) and water buffaloes (*Bubalus bubalis*) (WOAH 2020).

The severity of bovine babesiosis depends on the Babesia species and hosts' immune state. *B. bovis* is more pathogenic than *B. bigemina* (Suarez *et al.* 2019). Sequestration of *B. bovis* infected RBCs in internal organs and brain leads to severe cerebral babesiosis (Giglioti *et al*. 2021). The mortality rate of *B. bigemina* without treatment is relatively lower at 30%, compared to *B. bovis* between 70% to 80% due to associated neurological symptoms (Jaimes-Dueñez *et al.* 2018).

One of the current primary detection methods of Babesia infection is microscopic examination of blood smears with Giemsa stain (Nugraheni *et al.* 2023). Serological tests such as the indirect fluorescent antibody test (IFAT) and enzyme-linked immune-sorbent assay (ELISA) can identify antibodies in the carrier animals. However, the drawback of the serological methods is that antibodies can still be found years after the infection has recovered and no current infection. Thus, it is not favourable to determine the prevalence of babesiosis precisely (Mahmoud *et al.* 2015).

Molecular diagnostic techniques such as polymerase chain reaction (PCR) has high sensitivity and specificity in detecting babesiosis (Al-Hosary 2017). It produces copies of a particular deoxyribonucleic acid (DNA) from two short chains of oligodeoxynucleotides, known as primer through a polymerase-dependent repetitive thermal reaction (Putra *et al*. 2020). However, the use of PCR to detect babesiosis in cattle and buffaloes is not commonly used in Indonesia, especially in the Bogor district. Thus, the aim of this research is to carry out molecular identification of *B. bigemina* in cattle and buffaloes from several farms in the Bogor district.

MATERIALS AND METHODS

The samples were collected in 2021 from several farms in the Bogor District while identification of *B. bigemina* was between December 2023 to January 2024 at the Protozoology Laboratory, Division of Parasitology and Medical Entomology, School of Veterinary Medicine and Biomedical Sciences, IPB University.

The tools used were Ethylenediaminetetraacetic acid (EDTA) tubes, Venoject® Multi-Sample Luer Adapter, cooler box,

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light microscope, 1.5mL Eppenford tube, P-200 micropipette, thermal cycler machine, PCR tubes, transilluminator, and electrophoresis gel machine. The materials were cattle and buffalo blood samples, absolute methanol, Giemsa 10%, distilled water, immersion oil, QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany), nuclease free water (NFW), 10× DreamTaq Buffer, 1× Tris Acetate EDTA (TAE), ViSafe Green Gel Stain (Vivantis Technologies, Malaysia), Deoxynucleoside triphosphate (dNTP), DreamTaq DNA polymerase, apocytochrome B target primers, 1.5% agarose gel, and 100 bp DNA ladder (Vivantis Technologies, Malaysia).

A total of 27 blood samples from 22 cattle and 5 buffaloes were collected from the jugular vein with 3ml from each animal, and inserted into an EDTA tube. The blood samples were placed in a cooler box, brought to the Protozoology Laboratory, and stored in a stored between 4°C to 8°C until further use.

Next, a drop of blood was placed on a microscope slide. The edge of another slide was placed at a 30 to 45° angle, pulled towards the blood drop and pushed across the first slide. Blood smears were left to dry and fixated with absolute methanol for 5 to 10 minutes (Adewoyin and Nwogoh 2014). The smears were left to dry then stained with Giemsa stain for 45 minutes. Lastly, the preparations were washed with aquadest, air dried, and viewed under a light microscope with magnification 1000× (Dyahningrum *et al*. 2019).

DNA was extracted from 200µl of whole blood samples using the QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

The primer used targets the apocytochrome b (cytb) genes of *B. bigemina*. During the first PCR round, the primers used were oBBig_mit_F with sequence 5`-TCCAACACCAAATCCTCCTA-3` and oBBig_mit_R with sequence 5`-CGTGGGTTTCGTTTTTGTAT-3`, amplifying the 394 base pairs(bp). In the second PCR round, the primers used were iBbig_mit_F with sequence 5`-AAGAGATACCATATCAGGGAA CCA-3` and iBbig_mit_R with sequence 5`-TTGGGCACTTCGTTATTTCC-3`, amplifying the 250bp.

Both the PCR amplification reactions utilised a total of 12.5 µl volume consisting of 2 µl of DNA template and 10.5 µl of master mix. The master mix included 1.3 µl of 1X DreamTaq buffer, 1.3 µl of 0.2mM dNTP, 0.6µl of 0.5µM each primer, 0.1µl DreamTaq DNA Polymerase, and 6.6µl of nuclease free water (Romero-Salas *et al.* 2016). *B. bigemina* DNA was used as the positive control while nuclease free water was used as the no template control (NTC).

The PCR machine was programmed to thermocycling conditions of PCR amplification. The

initial denaturation was set at 95°C for 3 minutes, denaturation at 95°C for 30 seconds, annealing at 53.9°C for 30 seconds while extension at 72°C for 30 seconds, and final elongation at 72°C for 7 minutes. Denaturation to final elongation stages were carried out for 30 cycles.

2µl of the amplicon was used as template for the second PCR amplification. Initial denaturation was set at 95°C for 3 minutes, denaturation at 95°C for 30 seconds, annealing at 55.7°C for 30 seconds, extension at 72°C for 30 seconds, and final elongation at 72°C for 7 minutes. The denaturation to final elongation stage were carried out for 30 cycles.

The PCR amplification results were visualised using agarose gel electrophoresis using 1.5% agarose gel and 1X TAE buffer at 100 volts for 30 minutes. Gel was stained with ViSafe Green Gel Stain (Vivantis Technologies, Malaysia), and was visualized under blue light emitting light diodes (LED). The DNA fragments size was determined using 100bp DNA ladder as a reference.

The blood smear microscopic images were analysed to determine the size of the parasite. The measurement was carried out using ImageJ application (https://imagej.nih.gov/ij/index.html)

RESULTS AND DISCUSSION

The 27 blood samples collected from the cows and buffaloes were identified using blood smear examination. Blood samples containing positive results for *Babesia spp.* observation can be seen in Figure 1. The shape of the *Babesia spp*. appeared to be oval to pear-shaped inside of erythrocytes. Based on the results, the size of *Babesia spp.* measured ranged between 1.84 and 2.76 µm. According to Jalovecka *et al.* (2018), *Babesia* are usually categorized by a piriform shape, but can also appear to be spherical, rod-like, or amoeboid shape. *Babesia spp.* can be single or paired form, with average size of 1.0 to 5µm. Based on the morphology, *Babesia spp.* can be divided into small *Babesia spp.* with length from 1.0 to 2.5µm and large *Babesia spp.* which are 2.5 to 5.0µm (Laha *et al.* 2015). Small *Babesia spp.* such as *B. bovis* usually appears in pairs or clusters and vacuolated rings are frequently seen. Large *Babesia spp.* such as *B. bigemina* typically contain large circular merozoites forming an acute angle (Alvarez *et al.* 2019).

The results of the blood smear examination on the presence of *Babesia spp.* can be seen in Table 2. Among the 22 cattle blood samples, 19 samples were positive. Among the 5 buffaloes blood samples, 3 were positive. The examination showed that 22 out of 27 blood samples were positive for *Babesia spp*., indicating a positive rate of 81.48%.

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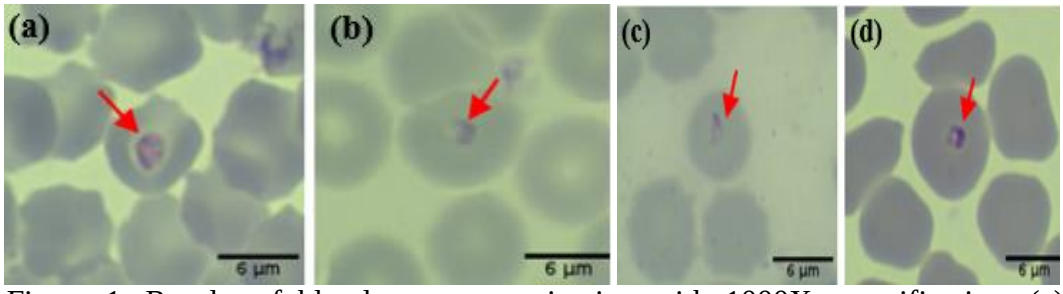

Figure 1. Results of blood smear examination with 1000X magnification. (a) *Babesia spp.* with length 2.35µm. (b) *Babesia spp.* with length 1.84µm. (c) *Babesia spp.* with length 2.76µm. (d) *Babesia spp.* with length 1.92µm. The red arrow indicates erythrocytes infected with piroplasm.

Table 1. Results of blood smear examination for *Babesia spp.* detection

Type of sample animal	Number of samples (n)	Examination results (n, %)	
		Positive	Negative
Cattle	22	19 (86.36)	3 (13.64)
Buffaloes	5	3 (60)	2 (40)
Total samples	27	22 (81.48)	5 (18.52)

Explanation: n= total positive / negative samples

Blood smear examination is generally simple and less time consuming than other detection methods (Meredith *et al*. 2021). It is sometimes regarded as gold standard for the diagnosis of babesiosis, especially during acute stages (J. Mosqueda *et al.* 2012). However, its limitations include low sensitivity as it relies on visual identification and babesiosis cases involving low parasitaemia during early infections may not be detected. Moreover, it is insufficient to accurately identify *B. bovis* and *B. bigemina* during mixed infections, especially in carrier or sub-clinical infections (Mahmoud *et al*. 2015).

The results of the second PCR amplification of nested PCR for the detection of *B. bigemina* can be observed in Table 3. 3 samples were tested positive, 2 were cattle while 1 was a buffalo.

Table 2. Results of nested PCR for *B. bigemina* detection

Type of sample animal	Number of samples (n)	Examination results (n, %)	
		Positive	Negative
Cattle	22	2 (9.09)	20 (90.91)
Buffaloes	5	1 (20)	4 (80)
Total samples	27	3 (11.11)	24 (88.89)

Explanation: n= total positive / negative samples

All the blood samples underwent the first PCR amplification using specific primers to confirm the results from the blood smear examination. PCR was conducted using specific designed primer pairs

which targets the apocytochrome b (CYTb) genes of *B. bigemina*, which are proteins found inside the mitochondrial membranes. This primer showed sensitivity of at least 1,000 times higher compared to targeted PCR (Romero-Salas *et al.* 2016). As seen in Figure 2(a) and 2(b), positive samples contained DNA bands size of 394 base pairs (bp) during first PCR examination and 250 bp during second PCR examination. Sample bands parallel to the positive control were considered positive for *B. bigemina*. Samples with a high level of parasitaemia which were detected previously served as positive controls.

The comparison between the positive rate of babesiosis using blood smear and nested PCR technique is seen in Table 4. Positive rate of babesiosis in the cattle and buffaloes using blood smear method was 81.48% while positive rate using nested PCR method was 11.11%.

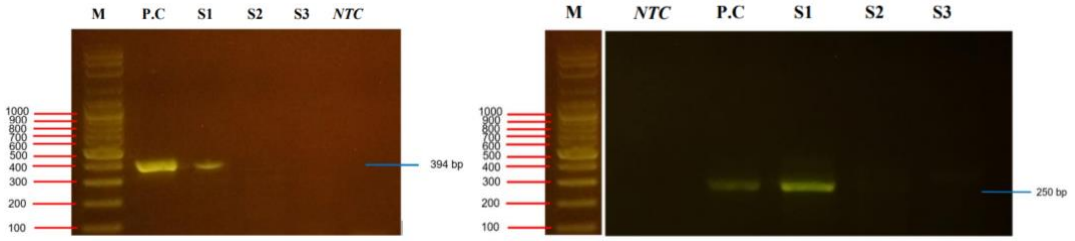

Figure 2. (a) Results of first PCR examination of nested PCR. M: Marker; P.C: Positive Control; S1: Sample 1; S2: Sample 2; S3: Sample 3, *NTC: No Template Control*; (b) Results of second PCR examination of nested PCR. M: Marker; *NTC: No Template Control*; P.C: Positive Control; S1: Sample 1; S2: Sample 2; S3: Sample 3.

Table 3. Comparison of infection rate of *B. bigemina* using blood smear examination and nested PCR

Type of sample animal	**Number of samples (n)**	**Positive rate (n, %)**	
		Blood smear	**Nested PCR**
Cattle	22	19 (86.36)	2 (9.09)
Buffaloes	5	3 (60)	1 (20)
Total	27	22 (81.48)	3 (11.11)

Explanation: n= total positive / negative samples

The positive rate from the molecular identification nested PCR method has a relatively lower value compared to the microscopic blood smear examination. This is because nested PCR methods are highly specific, thus only detecting babesiosis cases caused by specific causative agent. The primer pairs used in the nested PCR assay selectively bind to the species-specific CYTb gene of *B. bigemina*, located inside the mitochondria. Therefore, it only detects babesiosis cases caused by *B. bigemina*. This gene was selected as a target gene as

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mitochondrial encoded genes showed higher sensitivity compared to ribosomal DNA PCR (Ganzinelli *et al.* 2020). Unlike blood smear examination, the results may contain false positive results as the species of Babesia cannot be identified.

Furthermore, high sensitivity nested PCR allows detection of early infections, even before detectable antibody levels are produced (Goff *et al.* 2006). Babesiosis cases with DNA concentration as low as 0.1fg can be detected (Romero-Salas *et al*. 2016). As a result, nested PCR assay can significantly lower or eliminate false negative results. Therefore, it can identify babesiosis in chronic carrier animals which are asymptomatic (Ganzinelli *et al.* 2022). The onset of the chronic phase with low parasitaemia occurs approximately a week after the first symptoms are observed (Bock *et al*. 2004). Animals recovered from clinical babesiosis, having persistent infection have low parasitaemia and can continue living as carriers up to 22 months (Alvarez *et al.* 2019). In babesiosis cases caused by *B. bigemina*, even acute stage is sometimes indicated by low parasitaemia of less than 1% (Githaka *et al*. 2022). Thus, diagnostic methods such as the blood smear examination can lead to false negative results.

CONCLUSION

In conclusion, this study had identified cases of *B. bigemina* in cattle and buffaloes from several farms in the Bogor district by using molecular identification of nested PCR. The overall positive rate of babesiosis detected using nested PCR is 11.11%, including 9.09% in cattle and 20% in buffaloes.

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