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Parasitological and Molecular Detection of Babesiosis in Cattle and Buffalo in West and Central Java

D H Sawitri[1,*], A H Wardhana[1], F Ekawasti[1], and D A Dewi[2]

[1]*Indonesian Research Centre for Veterinary Science, Indonesian Agency for Agricultural Research and Development, Bogor, Indonesia*
[2]*Polytechnic of Agriculture Development Yogyakarta, Magelang-Indonesia*
[*]*Corresponding author. Email: dyah.haryuningtyas@gmail.com*

ABSTRACT
Babesia sp are the intra-erythrocytic protozoan parasite causes Bovine babesiosis. *Babesia bovis* and *Babesia bigemina* are two species that commonly infest cattle. A conventional parasitological technique is commonly used to diagnose Babesia sp. This technique, however, has limitations in subclinical infections. Polymerase chain reaction (PCR) has a high sensitivity and can identify parasites at much lower concentrations. The goal of this study was to detect *B. bovis* and *B. bigemina* in blood cattle and buffaloes from fields using the conventional Giemza Staining Thin Blood Smear method (GSTBS). In addition, the study compared the effectiveness of a single and duplex PCR in detecting parasites. One hundred and ninety-eight blood samples from cattle and buffalo were collected in Bogor (West Java), Pemalang, and Brebes (Central Java). The parasite's existence was determined using a thin blood smear. Furthermore, the PCR assay was employed with primers Bovar 2A and Bg3/4 specific for *B. bovis* and *B. bigemina*, yielding amplified products of 166 and 689 bp, respectively. Based on parasitological findings, 14 of 198 blood samples (7.1%) tested positive for Babesia sp. The results of a single PCR demonstrated that 21,21% (42/198) of the samples were positive for *B. bovis*, whereas duplex PCR successfully identified *B. bovis* and *B. bigemina* in 72 samples (36,36%). This finding indicated that duplex PCR is more efficient and cost-effective than GSTBS and single PCR for surveying babesiosis in epidemiological studies.

Keywords*: Babesia bovis, Babesia bigemina, single PCR, duplex PCR, Thin blood smear.*

1. INTRODUCTION

Tick–borne diseases (TBD) are a hemoprotozoa illness that poses a substantial threat to cattle production in tropical and subtropical countries, as well as having a considerable economic impact on farming communities. (1). Bovine babesiosis is one of these TBD caused by hemoprotozoa apicomplexa *Babesia bigemina* and *Babesia bovis* (family Babesiidae, order Piroplasmida). Bovine babesiosis is highly pathogenic, causing chronic to severe infections in cattle and buffaloes (2). This pathogen is mainly transmitted by *Rhipicephalus* (Boophilus) *micro-plus* tick (3). *Rhipicephalus* (Boophilus) *micro-plus* infects around 80% of bovines globally, resulting in annual economic losses approximately to 3,000 million dollars (4). Pathogens acting as agents of TBD are frequently found simultaneously in single host (5,6). Common clinical manifestation of this diseases are fever, anemia, hemoglobinuria, weakness, decrease milk production, nervous sign and occasionally death (7). A single species parasite did not produce any distinctive symptoms (8). Calves between the ages of 9 and12 months of age are generally resistant, but calves under the age of 6 month of age are relatively susceptible (9). Symptoms of babesiosis in newborn calves were failure to suckle, a high fever, coffee-colored urine, jaundice, and deep shallow respiration (10). Cattle that recover from the first infections become carriers, which contribute significantly to infectious agent transmission via ticks and flies. Survived animals typically become carriers with low level parasitemia, which continually stimulates specific antibodies and protect the animals against the recurrence of infection (11–13).

The prevalent of Babesiosis reported were 19% in Egypt (7); 7,25% in China (14); 11,1- 12,5% in Thailand (15); 36,1% in India (16) and 17-20% in Pakistan (2). According to Noor et al. (17), the examination by using GSTBS on cattle blood sample collected from several locations in Indonesia showed positive results for piroplasmosis including Babesia sp. Australia, Kenya, Zimbabwe, Tanzania, South Africa, China, India, Indonesia, and the Philippines are predicted to lose 0.6 to

57.2 million US dollars each year due to babesiosis and anaplasmosis (18). Babesiosis is commonly diagnosed using parasitological techniques in Giemza staining thin blood smear (GSTBS) (16). This conventional method has a number of drawbacks. It takes time, is dependent on the number of parasites in the blood, and is difficult to differentiate parasite species due to their similar morphology (19). According to OIE (20), this method is only useful during the acute stage of the disease, when the number of intraerythrocytic parasites is usually sufficient to detect microscopically. In subclinical and chronic infection, more advanced and sensitive techniques such as nucleic acid base detection methods should be used to identify parasites with low parasitemia levels (21,22).Additionally, multiplex PCR will make it more economical and efficient to detect multiple hemoprotozoa simultaneously in a single reaction (16).

The rapidity and sensitivity of this technique are suitable for epidemiological studies aimed at determining the prevalence of parasitic infections in a given area associated with transmission and evaluating parasitic infection treatment (8).A single reaction requiring a less complex method and a lower cost is required to detect multiple pathogens for the diagnosis and surveillance of TBD in endemic regions (8). The aims of this study were to detect *B. bovis* and *B. bigemina* in the blood of cattle and buffalo collected from the field using the parasitological method (thin blood smear) and molecular methods (single and duplex PCR).

2. MATERIAL AND METHODS

2.1. Ethical statement

All procedures in this study adhered to the ethical guidelines for the use of animal samples established by the Animal Welfare Committee of the Indonesian Agricultural Research and Development Agency. (No: Balitbangtan/ BBLITVET/Rm/01/2017).

2.2. Blood sample Collection

The survey collected 198 samples of animal blood (buffalo and cow) in the Kunak-Bogor district (West Java Province), Pemalang and Brebes districts (Central Java Province). The samples were taken from randomly selected male and female cattle and buffalo, both adults and calves, that appeared to be in good health. Blood samples up to 3 ml were collected from the jugular vein and placed in tubes containing EDTA for DNA extraction and preparation of thin blood smears. The blood samples were stored at −20◦C till further analysis.

2.3. Conventional Method (Giemza staining thin blood smear (GSTBS))

Thin blood smears were made immediately after peripheral blood collection. The blood smears were dried in the air, fixed in methanol absolute for 5 minutes, stained with Giemsa 10% for 30 minutes, and washed with distilled water. Using a binocular microscope with an oil-immersion lens, the stained blood smear was analyzed for the presence of *B. bovis* and *B. bigemina* (100 x magnification). A positive sample was defined as having piroplasm levels more than or equal to 1 (23).

2.4. DNA Extraction

Genomic DNA was extracted at the Laboratory of Molecular Parasitology, Indonesian Research Centre for Veterinary Science, Bogor, according to the manufacturer's instructions using a commercial DNA extraction kit (Geneaid, Taiwan). The extracted DNA samples were stored at −20 °C pending further genetic analysis.

2.5. Primers for Single and Duplex-PCR.

The oligonucleotide primers used to establish single or duplex-PCR for *B. bovis* (Bovar 2A) and *B. bigemina* (Bg3/4) were designed to target the **multi-copy VESA–1a gene** for *B. bovis* and a small subunit ribosomal RNA sequence for *B. bigemina*, as described below (Table 1).

2.6. Optimization Single and Duplex PCR

The primer sequences reported by Wuyts et al and Ellis et al (24,25) were utilized to amplify *B. bovis* and B. *bigemina* DNA using PCR methods. Positive control PCR optimization was performed on bovine / buffalo DNA samples detected via thin blood smear. PCR amplification was performed using Bioline (England) consisting of 5x My Taq Reaction Buffer 10 µl; My Taq HS DNA polymerase 1 µl, forward and reverse primers 2 µl each (10 µM); 2 µl DNA template (50-100ng) and 8 µl PCR grade water with a total reaction volume of 25 µl.

Table 1. Primer duplex PCR for *B. bovis* and *B. bigemina*

Hemoprotozoa		Primer	Size	Reference
B. bovis	Bovar-2A	5′ - CAA GCA TAC AAC CAG GTG G – 3′	166	(24)
		5′- ACC CCA GGC ACA TCC AGC TA– 3′		
B.bigemina	Bg3/4	5′-TAGTTGTATTTCAGCCTCGCG–3′	689	(25)
		5′-AACATCCAAGCAGCTAHTTAG–3′		

Table 2. Giemza Staining Thin Blood Smear, single and duplex PCR of *B. bovis and B. bigemina*

No	Breed	N	Sample positive		
			GSTBS *Babesia sp*	Single PCR *B. bovis*	Duplex PCR *B.bovis and B.bigemina*
1	Cow	141	9	33	53
2	Buffalo	57	5	9	19
	TOTAL	198	7.07% (14/198)	21.21% (42/198)	36.36% (72/198)

Table 3. The result of PCR duplex of *B. bigemina , B. bovis and co infection* both in cattle and buffaloes

No	Breed	N	Babesiosis	*B.bovis*	*B. bigemina*	Mix *B. bigemina* dan *B. bovis*
1	Cow	141	53	33	4	16
2	Buffalo	57	19	9	7	3
	TOTAL	198	36.36% (72/198)	21.21% (42/198)	5.55% (11/198)	9.59% (19/198)

The PCR conditions used was initial denaturation at 95°C for 3 minutes was followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 56°C for 15 seconds, extension at 72°C for 30 seconds, and the final extension at 72°C for 10 minutes. Electrophoresis on a 2% agarose gel was used to visualize the PCR results.

3. RESULT AND DISCUSSION

Babesiosis is one of vector-borne diseases that is prevalent throughout the tropics and subtropics, including Indonesia. According to the findings of this study, Babesiosis appears to be endemic in a number of Indonesian areas. Blood smear examination with giemza staining on buffalo and cattle blood samples collected in the field (Bogor, Brebes, and Pemalang) revealed 14 positive Babesia sp. samples out of 198 (Table 2). In this study, parasitemia was detected in blood samples using GSTBS at a level of 0.001–0.002% parasitized erythrocytes. Babesia species was identified based on its pyriform shape (Figure 1). Clinical symptoms were not detected in any of the animals sampled in the present study. Infested livestock tend to be subclinical. This is because it has a low parasitemia value (0,001-0,002%). However, it is necessary to be aware that when an animal's immune is compromised, these parasites will multiply rapidly and cause serious illness. Although cattle infected with babesia rarely cause clinical symptoms, but they are economically detrimental because of their morbidity and decrease productivity in domestic animals (26,27). According to Perry et al. (1998) stated which a state of “Endemic Stability” in Babesiosis where the relationship between parasite, vector, host and environment were interrelated that clinical symptom occur rarely or not at all need to be consider. Although babesia infections in cattle are uncommon, they are economically damaging due to their morbidity and decreased output in domestic animals (26,27).

3.1. Specificity of PCR Primers

Positive control PCR amplified *B. bovis* and *B. bigemina* were identified using DNA extracted from field blood samples that tested positive for parasites on both blood smear and PCR examination. For *B. bovis* and *B. bigemina*, the specific primers detected expected fragments of size 166 bp and 689 bp, respectively (Figure 2). To detect *B. bovis* and *B. bigemina* simultaneously, a duplex PCR was designed by combining two pairs of specific primers, Bovar 2A (166bp) for B. bovis and Bg3/4 (689bp) for *B. bigemina* in a single tube reaction. Using a specific primer for one parasite species, no PCR results from any other parasite species were produced. species. Additionally, the duplex PCR method using both sets of primers generates two bands from *B. bovis* and *B. bigemina* that are the same length as the one-round PCR. The PCR amplification of parasite isolates from various districts is consistent, as all isolates were amplified using the same set of primers.z

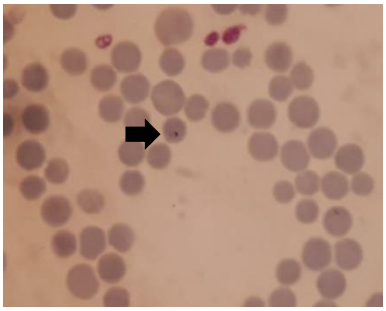
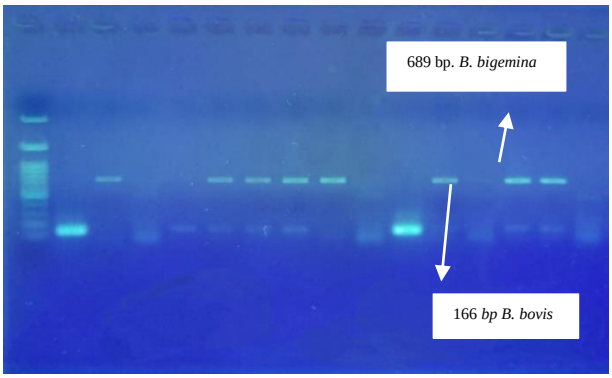


Figure 1. *Babesia sp*. on cattle thin blood smear Giemza staining from Bogor (100x magnification).

3.2. PCR analysis of the field blood samples

Babesia bovis is the most prevalent species in these three districts in Indonesia (Bogor, Pemalang and

Brebes). Meanwhile, *Babesia bovis* and *Babesia bigemina* are the predominant species in bovines in endemic areas in India (9,10), South Africa (29), Malaysia (30) and Thailand (31). The results of this study indicated that overall prevalence of Babesiosis in large ruminants at the three districts was 36.36 % when duplex PCR was used, compared to a lower 21.21 % when single PCR was applied and a much lower 7,07 percent when GSTBS was used (Table 2). *Babesia bovis* detection by single PCR is consistent with detection of *B. bovis* and *B. bigemina* by duplex PCR. In this study, the prevalence of single *B. bovis* infection (21.21%) was significantly higher than that of single *B. bigemina* infection (5.55%) or mixed infection (9.59%) (Table 3). This finding contrasted with those of Oliveira et al (32) and Adham et al. (33)who found that in all cattle groups, a higher positivity percentage was typically observed in cattle with mixed infection by more than one species, not only infected with *B. bovis* but also infected with *B. bigemina* concomitantly. A similar situation occurred in vectors in



cattle of all ages, with a higher positivity percentage as determined by multiplex PCR.

Figure-2. Gel electrophoresis of duplex PCR products of cattle and buffalo blood samples with primer *Bovar 2A* (166 bp) *for B. bovis* and Bg2/3 (689bp) for *B. bigemina* Lane 1 positive control; lane 3,9,12 *B. bovis* and *B. bigemina* negative, lane 4,10 *B.bovis* positive; lane 2,8,11 *B. bigemina* positive; lane 5,6,7,13,14 mix *B. bovis and B. bigemina* positive; lane 13,14 *B. bigemina* positive; lane 15 negative control. The amplified products prepared from GSTBS positive and negative blood samples from the field.

Because *B. bovis* and *B. bigemina* infections exhibit nearly identical clinical signs and frequently co-occur in suitable tropical climatic conditions, it is critical to use a sensitive and specific technique to detect both diseases concurrently in suspected animals (16). The current study demonstrated that the PCR method detected more positive samples than the thin GSTBS method. Duplex PCR, on the other hand, was more sensitive than single PCR or the conventional method. Additionally, several PCR-positive samples were found to be negative on blood smear (false negative), which could be due to the blood smear diagnostic method's inefficiency in distinguishing some Babesia species, which are frequently confused for other piroplasm or blood smear artifact. According to Terkawi et al. (29) the gold standard test for diagnosing Babesiosis is a direct method of identifying the parasite in the GSTBS, although this technique is insensitive during the subclinical and chronic phases of infection due to low parasitemia levels. Molecular method can detect and differentiate Babesia sp. when conventional methods are taken, the morphological characteristics of babesias are very similar, making differentiation difficult, even more particularly when co-infection occurs.

The detection of *B. bovis* and *B. bigemina* in blood samples using blood smears revealed approximately 0.001–0.002 % parasitized erythrocytes in this study. Oliveira et al. (32) estimated that the analytical sensitivity of PCR for *B. bigemina* was 0.003 % for *B. bovis* and 0.000017 % for *B. bigemina*. According to Liu et al. (34) The single-round Multiplex PCR of *B. bigemina* and *B. bovis* primers could detect 0.1 pg and 1 pg of DNA in the samples, respectively.

A previous study conducted by Mclaughlin et al. (35) demonstrated that the multiplex PCR was able to detect 1 pg and 10 pg of DNA respectively, comprising DNA from *B. bigemina* and *B. bovis* equal to the amount of DNA in 50 µl of 0.0001% and 0.001% parasite-infected erythrocytes. *Babesia sp*. was shown to be prevalent in all age groups of animals in this investigation. The incidence rate is lower in young animals than in older animals (Figure-3).

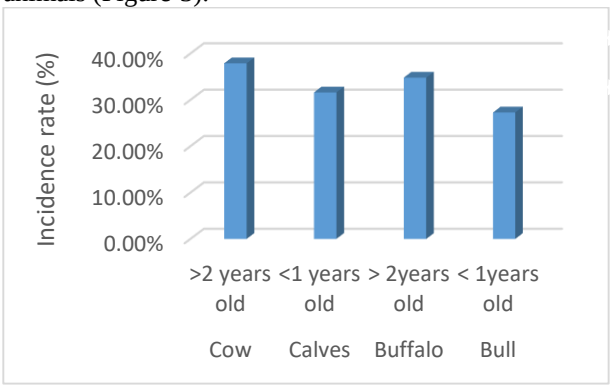


Figure-3. Distribution Babesiosis (*B. bigemina* and *B. bovis*) infection of cattle and buffalos based on animal age by using duplex PCR methods.

Overall, the highest prevalence of babesiosis in terms of age was detected by PCR in older animals (greater than two years of age) compared to younger animals (less than one year of age) on both cattle and buffalo. Calves between the ages of 9 and 12 months are generally resistant, but calves under the age of 6 months are relatively susceptible (9). Cattle in endemic areas, according to the OIE (36) become infected at an early age and develop long-lasting immunity. When young animals are exposed to ticks or susceptible cattle are introduced into these endemic areas, outbreaks can ensue. Disease

outbreaks may also result in the introduction of Babesia-infected ticks into previously tick-free areas.

4. CONCLUSION

DNA amplification technique employing duplex PCR results in a 1,6x increase in amplification efficiency over single PCR and a 5x increase in efficiency over microscopic method. In endemic area of study, the incidence of *B. bovis* infection is significantly higher than that of *B. bigemina* infection or co infection. Diagnosis Babesiosis can be diagnosed using parasitological examination (thin blood smear) in conjunction with polymerase chain reaction (PCR) methods to increase sensitivity. Duplex PCR is required to detect co-infection and to minimize false negatives, considering that infested animals rarely cause clinical symptoms but can act as a reservoir that has the potential to transmit the disease from one area to another.

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AUTHOR CONTRIBUTION

There is no conflict of interest in writing this manuscript. The main authors of the manuscript are DH Sawitri. AH Wardana, F Ekawasti and DA Dewi as co-authors. All authors had read and approved the final manuscript.

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