Studies on the development of diagnostic methods and chemotherapeutics of babesiosis

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Contents

Abbreviations

A aa: Amino acid

AMA-1: Apical membrane protein 1

AV: Atovaquone

B BC-48: 48 kDa rhoptry protein

BLAST: Basic local alignment search tool

BLASTn: Basic local alignment search tool for nucleotide

*big*ICT: ICT for *Babesia bigemina* detection

*bov*ICT: ICT for *Babesia bovis* detection

bp: Base pair

BSA: Bovine serum albumin

BW: Body weight

B. bigemina: *Babesia bigemina*

B. bovis: *Babesia bovis*

B. caballi: *Babesia caballi*

B. divergens: *Babesia divergens*

B. jakimovi: *Babesia jakimovi*

B. major: *Babesia major*

B. microti: Babesia microti

B. occultans: *Babesia occultans*

B. orientalis: *Babesia orientalis*

B. ovata: *Babesia ovata*

B. venatorum: *Babesia venatorum*

C CCK-8: Cell counting kit-8

cDNA: Complementary deoxyribonucleic acid

CI: Combination index value

D DA: Diminazene aceturate

DMEM: Dulbecco's modified eagle's medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

dNTP: Deoxyribose nucleoside triphosphates

DPBS: Dulbecco's phosphate-buffer saline

dual-ICT: ICT for *Babesia bovis* and *Babesia bigemina* detections

E EC_{50}: Half maximum of effective concentration

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

G GST: Glutathione *S*-transferase

H HIV: human immunodeficiency virus

Hsp: Heat shock protein

Hsp90: Heat shock protein 90

I IC_{50}: Half maximum of inhibitory concentration

ICT: Immunochromatographic test

iELISA: Indirect Enzyme-linked immunosorbent assay

IFAT: indirect fluorescent antibody test

IgG: Immunoglobulin G

ITS: Internal Transcribed Spacer region

K kDA: Kilo Dalton

L LAMP: loop-mediated isothermal amplification

M MDBK: Madin-Darby bovine kidney

MEM: Minimum essential medium eagle

MSAs: Merozoite surface antigens

MSA-2c: Merozoite surface antigen 2c

N nM: Nanomolar

nPCR: Nested polymerase chain reactions

P PBS: Phosphate buffer saline

PCR: Polymerase chain reactions

PEG: Polyethylene glycol

R RAP-1a: Rhoptry-associated protein 1a

RAP1/CT17: Truncated C-terminal rhoptry-associated protein 1a

RBCs: Red blood cells

rRNA: Ribosomal ribonucleic acid

RT-PCR: Real time polymerase chain reactions

R. annulatus: *Rhipicephalus annulatus*

R. microplus: *Rhipicephalus microplus*

R. decoloratus: *Rhipicephalus decoloratus*

R. evertsi: *Rhipicephalus evertsi*

S *R. geigyi*: *Rhipicephalus geigyi*

SBPs: Spherical body proteins

SBP-4: Spherical body protein 4

Se: Sensitivity

T Sp: Specificity

TRAP-1: Thrombospondin related anonymous protein 1

T. equi: *Theileria equi*

17-AAG: Tanespimycin, 17-N-allylamino-17-demethoxygeldanamycin

17-dmag: Alvespimycin, 17-dimethylaminoethylamino-17-demethoxy geldanamycin

General introduction

1. Introduction

Babesia, the causative agent of babesiosis in humans and a wide range of animals, was reported for the first time in the red blood cells (RBCs) of cattle with hemoglobinuria by Romanian scientist Viktor Babes (Babes, 1988). In the USA, *B. bigemina*, the causative agent of Texas fever in cattle, was found in 1893 together with the discovery of the tick as the vector (Smith and Kilborne, 1893). So far, more than one hundred *Babesia* species have been reported, some creating a huge burden on the livestock industry and threatening the lives of susceptible humans. *B. bovis*, *B. bigemina*, *B. divergens*, *B. ovata*, *B. jakimovi*, *B. major*, and *B. occultans* have been found to infect cattle. The first two species are spread worldwide, while *B. divergens* and *B. major* infect cattle in Europe. *B. ovata* is found in Asia, while *B. jakimovi* and *B. occultans* have been found in Siberia and Africa, respectively. The pathogenic species in water buffalo is *B. orientalists*, which is found in Asia. *B. caballi* and *Theileria equi* (formerly *B. equi*) are the causative agents of equine piroplamosis and are distributed worldwide. Currently, babesiosis is emerging in humans. Human babesiosis is caused by *B. microti*, found mainly in the US. Several countries, such as Japan, Taiwan, and Europe, also have reported cases. In Europe, *B. divergens* and *B. venatorum* are the causative agents of human babesiosis. Furthermore, several uncharacterized species have been reported sporadically throughout the world (Vannier and Krause, 2012). *Babesia* spp. is transmitted by tick vectors. The genera of *Rhiphicephalus (Boophilus)*, *Ixodes*, *Haemaphysalis*, *Hyalomma*, and *Dermacentor* are the capable vectors. The distribution of *Babesia* spp. depends on the presence of their tick vectors in the area.

Clinical symptoms vary according the *Babesia* species. The major symptoms of *Babesia* spp. infection include acute or chronic hemolytic anemia, jaundice, and hemoglobinuria as a result of the altering and multiplication of parasites inside host RBCs. In cattle, *B. bovis* is the most important species because it causes severe disease in susceptible animals. It was also found to accumulate in the microvasculature of blood vessels, causing severe multiple organ failure in the host (Gohil *et al*., 2010). *B. microti* infection is usually asymptomatic in healthy humans; however, severe hemolytic anemia can be found in patients with cancer, human immunodeficiency virus (HIV) infection, or who receive immunosuppressive drugs or anti-cytokine therapy; newborns, and people older than 50 (Vannier and Krause, 2012).

Several challenges arise regarding the current approaches to the control of babesiosis (Mosqueda *et al*., 2012). Despite the fact that *Babesia* parasites have been found for more than a century and cause a significant burden on the livestock industry, the distribution of the disease remains as wide as the occurrence of their tick vectors. The first challenge is a vaccine for the prevention of parasite infection. Only several countries are vaccinating against babesiosis, and it is based on a live attenuated vaccine with variable effectivity and that is harmful to adult cattle. Chemotherapeutics is the second challenge. Only two drugs, diminazene aceturate and imidocarb dipropionate, are being used in domestic animals. However, the emergence of resistant parasites and issues of toxicity have limited their use in Europe, Japan, and the USA. The last challenge is the diagnostic method. Microscopic, serologic, and molecular methods with various sensitivities and specificities are available. However, methods with preferable criteria, such as being inexpensive, rapid, and reliable, are still in research and not available commercially. Ongoing efforts to overcome the challenges are being carried by only a few laboratories in the world that engage in babesiosis research.

2. Diagnosis of babesiosis

Many diagnostic methods are currently available for the detection of *Babesia* spp. They have been practiced widely and have variable sensitivities and specificities based on direct or indirect detection. The diagnostic method used also depends on the availability of trained personnel, equipment, and each laboratory's budget.

Microscopic observation of Giemsa-stained thin blood smears is the most commonly employed method for detecting *Babesia* parasites in many labs. In the acute phase, microscopy is a useful method. It can directly detect *Babesia* parasites at different stages of their life cycle. However, this method is less sensitive and not useful in animals with low parasitemia or chronic infections. The sensitivity of the microscopic method can be slightly increased using thick blood smears.

Immunological methods are sensitive for the detection of antibodies against *Babesia* spp. Several immunological diagnostic methods have been developed including the indirect fluorescent antibody test (IFAT), the enzyme-linked immunosorbent assay (ELISA), and the immunochromatographic test (ICT). IFAT is based on the binding of antibodies to coated antigens on a glass slide. Secondary antibodies (host antibodies) conjugated with fluorochromes were added to the slide for parasite observation by fluorescence microscopy. IFAT is sufficiently sensitive to detect parasite antibodies; however, it is not rapid, has problems with cross reaction, and requires an experienced microscopist to perform. The ELISA method has been evolved in its detection of antigens from crude antigen based to recombinant protein based. The recombinant protein has proven to increase the sensitivity and specificity of the ELISA method. For *B. bovis*, indirect ELISA (iELISA) and competitive ELISA (cELISA) are available in several laboratories. iELISA is a method with robust performance and high sensitivity and specificity. A number of recombinant proteins have been developed and evaluated

using iELISA. So far, SBP-4 of *B. bovis* is the protein with the most potential for use in iELISA (Terkawi *et al*., 2011a). A recent study reported that a strategy of modifying the coating improved the sensitivity and specificity of SBP-4-based ELISA (Chung *et al*., 2017). ELISAs for other species are also available that employ species-specific recombinant proteins with variable sensitivities and specificities. Furthermore, ICT strips for the detection of various *Babesia* species have recently been developed (Verdida *et al*., 2005; Kim *et al*., 2006; Jia *et al*., 2007; Luo *et al*., 2011). ICT is considered a pen-side diagnostic method that offers many advantages, such as being rapid, cheap, and easy to perform. However, ICT strips for the detection of *Babesia* spp. are still being researched and are not yet available commercially. The further development of ICT for the direct detection of parasite antigens directly from blood is possible. The advancement of a diagnostic method for *Plasmodium* parasites could benefit the current development of ICT for *Babesia* parasites.

Molecular methods such as polymerase chain reaction (PCR), nested PCR, real-time PCR, and loop-mediated isothermal amplification (LAMP) for the detection of *Babesia* parasites have also been developed. These methods offer reliable detection with high sensitivity and specificity. The PCR targeting of several specific genes such as merozoite surface antigens (MSAs), spherical body proteins (SBPs), and rhoptry-associated proteins (RAPs) has been reported to be effective in diagnosing *B. bovis.* Several other *Babesia* spp. genes have been specifically targeted by PCR, such as RAP-1a and apical membrane protein 1 (AMA-1) of *B. bigemina*, RAP-1 and 48 kDa rhoptry protein (BC-48) of *B. caballi*, and merozoite antigen-1 of *T. equi* (Munkhjargal *et al.*, 2013; Mtshali and Mtshali, 2013). Real-time PCR (RT-PCR) is more sensitive than PCR. While amplifying the DNA fragment, the method also quantifies the amplified DNA and calculates the copy number of the sequence. However, the method is expensive for application in routine surveillance or clinical cases, and it is only available

in well-equipped laboratories. Furthermore, LAMP is a specific, highly efficient, and rapid method. Detection is based on the amplification of nucleic acid at a constant temperature that targets different sequences. Its sensitivity is higher than that of PCR and is suitable for laboratories with limited resources and equipment.

3. Measures for controlling babesiosis

Measures for controlling babesiosis include tick control, chemoprophylaxis, and vaccination. Combining all control measures is an ideal method of tackling the babesiosis problem.

Tick control: Ticks are the specific vector of *Babesia* parasites. Effectively controlling the tick population might reduce the distribution of babesiosis. Acaricide and tick vaccine are common practices of controlling tick populations (Pegram *et al*., 2000). Many acaricides are available from several classes, such as organophosphates (coumaphos and diazinon), organochlorides (lindane and dieldrin), carbamates (carbaryl), pyrethrins (cypermethrin and permethrin), macrocyclic lactones (avermectins and milbemycins), and formamidines (amitraz). However, tick resistance to acaricides has been widely reported due to the lack of control practices (Abbas *et al*., 2014). Tick vaccines reduced the tick population. To date, the only commercially available tick vaccine in the American market is Gavac^{TM} (Guerrero *et al*., 2012). Recently, several vaccine candidates are being developed (Oldiges *et al*., 2016).

Chemoprophylaxis: Diminazene aceturate and imidocarb dipropionate are the current options for treating babesiosis in domestic animals (Mosqueda *et al.*, 2012). For human babesiosis, a combination of atovaquone and azithromycin is the first choice (Krause *et al*., 2000). However, the

issue of toxicity and the development of resistant parasites indicate the necessity of continuing efforts to develop new options for treating babesiosis.

Vaccination: Several countries are practicing vaccination against babesiosis based on a live-attenuated vaccine. This vaccine is relatively effective in calves under one year of age; however, it is harmful to susceptible adult cattle. Recently, a recombinant subunit vaccine has been reported to have a highly protective effect in mice (Gimenez *et al*., 2016). However, the vaccine is still in the research stage and needs improvement.

4. Objectives of the present study

Developing a diagnostic method with high sensitivity and specificity is important to support the current surveillance of babesiosis. The method is also expected to be cheap, easy to perform, and rapid. These criteria can be achieved by ICT, which is predicted to be the future diagnostic method for *Babesia* parasites. However, the development of ICT is quite intriguing, as the detection antigen should be chosen carefully to ensure its sensitivity and specificity, and the optimum condition should be assessed precisely to ensure its stability. Furthermore, field assessment of the diagnostic method is necessary to ensure its practical benefit when applied in routine surveillance.

In Indonesia, several studies based on microscopic and serological methods have shown that bovine babesiosis is endemic in the country. However, the data were limited and not supported by the sensitive diagnostic method. Genetic characterization was also absent. It is necessary to provide the data of the disease distribution and to support effective control measures.

The extensive worldwide distribution of babesiosis in domestic animals and the emergence of human babesiosis indicate the importance of developing effective control measures against the disease.

The drug choices available for babesiosis are limited and commercially only available in several countries due to many issues. Therefore, continuing efforts to evaluate potential compounds against the disease are of paramount importance.

This study aims to address several issues described above and to answer questions related to the recent development of control measures against babesiosis. The first chapter describes the evaluation of the ICT method based on SBP-4 of *B. bovis* and RAP1/CT17 of *B. bigemina* for the serological detection of bovine babesiosis in Western Java, Indonesia. In the second chapter, the coverage area of bovine babesiosis detection is expanded to several islands in the archipelago. Employing molecular and serological methods, the distribution of bovine babesiosis in the country is reported. The last chapter is an evaluation of 17-dmag, an Hsp90 inhibitor, for its inhibitory effect on the growth of *Babesia* and *Theileria* parasites *in vitro* and *in vivo*.

Chapter 1

Evaluation of immunochromatographic test (ICT) strips for the serological detection of *Babesia bovis* and *Babesia bigemina* infection in cattle from Western Java, Indonesia

1-1. Introduction

Babesiosis is an intraerythrocytic protozoan disease caused by *Babesia* parasites that are transmitted by infected ticks, usually *Rhipicephalus* and *Ixodes*, in domesticated or wild animals and humans (Homer *et al.*, 2000; Vannier *et al*., 2015). The disease has several clinical symptoms such as fever, anemia, jaundice, and hemoglobinuria in the acute phase but are asymptomatic in the chronic phase, whereas the host becomes a carrier for subsequent infections. Among the species, *B. bovis* and *B. bigemina* are considered to be the most important to infect cattle, leading to a significant economic burden in the livestock industries in many countries (Bock *et al*., 2004).

The immunochromatographic test (ICT) strip is widely used in human medicine and is considered a pen-side diagnostic tool because it is easy to use and inexpensive and can produce a result within 15 minutes. In 2008, Kim *et al*. reported a study of a system for dual detection of antibodies against *B. bovis* and *B. bigemina* infection on one ICT strip. The system employed recombinant protein MSA-2c of *B. bovis* and RAP1/CT17 of *B. bigemina* as detection antigens (Kim *et al*., 2008). In another study, several recombinant proteins from *B. bovis* with high antigenicity were evaluated and *Bbov*SBP-4 was proposed as the most potent antigen in the serological detection of *B. bovis* infection in cattle (Terkawi *et al*., 2011a). However, the rapid and reliable diagnostic method such as ICT strips utilizing this

protein had not been reported before. Therefore, in the current study, we prepared and evaluated the ICT strips employing *B. bovis* SBP-4 and *B. bigemina* RAP1/CT17. Together with ELISA, the performance of the ICTs was assessed for the detection of antibodies against *Babesia* parasites in cattle serum samples collected in Western Java, Indonesia.

1-2. Materials and methods

Parasites and the preparation of recombinant protein *B. bovis* SBP-4 and *B. bigemina* RAP1/CT17. *B. bovis* (Texas strain) and *B. bigemina* (Argentina strain) were cultured continuously using a microaerophilic culture system (Timms *et al*., 1983; Vega *et al*., 1985). The cultures were used to synthesize cDNA for the expression of *B. bovis* spherical body protein 4 (SBP-4) and *B. bigemina* C-terminal rhoptry-associated protein (RAP1/CT17) in accordance with the method described previously (Kim *et al*., 2008; Terkawi *et al*., 2011b). Briefly, PCR using oligonucleotide primer 5'-AGGAATTCGAGGAGGAGGAAACTGATGAG-3' and 5'-GCCTCGAGTTATTCCTCAATGTC GGCTGT-3' was used to amplified *B. bovis* SBP-4 from the cDNA. Underlined letters indicated the EcoRI and XhoI restriction site linkers, respectively. *B. bigemina* RAP1/CT17 was amplified using PCR with oligonucleotide primers 5'-CCGGAATTCCTGGTCCCCGAAGAGCAC-3' and primer 5'-ATAAGAATGCGGCCGCTTACGCATCTGAATCATCTG-3'. Underlined letters showed the EcoRI and NotI restriction site linkers, respectively. The amplified fragments were cloned and expressed in *E. coli*, followed by purification using glutathione-Sepharose 4B beads (GE healthcare, UK). The GST was cleaved from recombinant protein by thrombin and bound to glutathione-Sepharose 4B beads (GE healthcare, UK). The remaining thrombin in the protein was removed by HiTrap Benzamidine FF (High Sub, GE Healthcare, UK) following manufacturer's protocol. The

concentration of obtained recombinant proteins was measured and stored at -30°C for further procedures.

Production of polyclonal antibody and total IgG purification. The previously described protocol was followed to produce polyclonal antibodies (Leenaars *et al*., 2005 and Terkawi *et al*., 2011a). Briefly, one hundred µg/ml of *B. bovis* SBP-4 or *B. bigemina* RAP1/CT17 in PBS (pH 7.2) emulsified with an equal volume of Freund's complete adjuvant (Sigma-Aldrich, USA) was injected intraperitoneally into five six-week-old Balb/c mice (CLEA, Japan). Three boosters were injected in all mice with the same recombinant protein emulsified in Freund's incomplete adjuvant (Sigma Aldrich, USA) at 14 days interval after the initial injection. Subsequently, blood was collected by cardiac puncture on day ten after the last booster, and the total IgG was purified from serum using HiTrap Protein G HP (GE Healthcare Bio-Science AB, Sweden) in accordance with manufacturer'r protocol. Total IgG was stored at -30°C until ICT preparation.

Preparation of immunochromatographic test strips. Three types of ICT strips were prepared for the detection of antibodies against *B. bovis* and *B. bigemina* using *B. bovis* SBP-4 (*bov*ICT), *B. bigemina* RAP1/CT17 (*big*ICT), and combination of both proteins (dual-ICT). The optimum conjugation between proteins and gold colloid (20 nm; British BioCell Int., UK) was determined by mixing at a pH range from 4.5 to 8.5 and a concentration from 5 to 100 µg/ml. The best condition was indicated by the absence of color change from red to blue or gray after adding 100 µl of 10% sodium chloride. Other factor to consider was the highest absorbance at 520 nm, and most conjugated-material was sediment at the bottom of the tube after centrifugation at 11,200 g for 30 min (Burns *et al*., 2006; Moon *et al*., 2012). Subsequently, the gold-conjugated antigen was prepared in accordance with the previous procedure (Huang *et al*, 2006). Briefly, the recombinant protein was mixed gently with 4 ml

of gold colloid particles at the optimal concentration and pH. The mixture was incubated at room temperature for 10 min. Subsequently, to block and stabilize the conjugation, polyethylene glycol 20,000 (PEG) and bovine serum albumin (BSA) were added to the mixture at the final concentration of 0.05% and 1%, respectively. Subsequently, the mixture was precipitated by centrifugation at 11,400 g for 30 min. After removing the supernatant, the precipitated was then dissolve in PBS containing 0.5% BSA and 0.05% PEG by sonication. Second centrifugation was performed and after the removal of supernatant, the precipitated was then resuspend and adjusted until the optical density of 520 nm was 3 with PBS containing 0.5% BSA and 0.05%. The mixture was further diluted in 10 mM Tris-HCl (pH 8.2) containing 10% sucrose, sprayed onto glass fiber (Standard 17, GE Healthcare, UK), and dried overnight.

The optimum condition of capture membranes was determined by dispensing a range of protein concentrations and total IgG from 0.05 to 1 mg/ml onto a nitrocellulose membrane (Immunopore RP, GE Healthcare, UK). Following this step, the proteins were dispensed to the membrane using a BioDot XYZ3060 Dispense Platform (BioDot, Inc., USA) with the distance of 4 mm, except between purified IgG in dual-ICT with only 1 mm of length. Afterward, the nitrocellulose membrane was dried at 50^{o}C for 30 min and followed by blocking for 30 min using a solution containing sodium hydroxide, boric acid, and casein at final concentrations of 0.06%, 0.31%, and 0.5%, respectively. Following the blocking step, membranes were washed with 50 mM of Tris-HCl, pH 7.4, containing 0.5% sucrose and 0.05% sodium cholate, for 30 minutes and dried overnight at room temperature (Huang *et al.*, 2006).

ICT strips were prepared by attaching the sample pad and absorption pad (CF4, Whatman, UK), gold-conjugated antigen, and capture membrane in a plastic adhesive backing (AR9020, Adhesive

Research, Ireland). Using a CM4000 guillotine cutter (BioDot, Inc., USA), the assembled sheets were cut into 3-mm-wide strips and stored in a sealed container with a dehumidifier.

Validation and evaluation of ICT performance. Determination of the relative sensitivity (Se) and specificity (Sp) of ICT strips was carried out using serum samples kept in our laboratory and confirmed by IFAT, ELISA, and PCR as seropositive to *B. bovis* (n=50), *B. bigemina* (n=20), and negative (n=50). To determine the detection limit of the strip, a positive serum control was diluted in PBS (1:1, 1:5, 1:10, 1:25, 1:50, 1:100, 1:200, v/v). Furthermore, serum samples with antibodies positive for pathogen that might be co-infected, such as *Anaplasma* sp. (n = 3), *Trypanosoma evansi* (n = 3), and *Theileria orientalis* (n = 3), were used to determine the analytical specificity of the ICT strips.

The evaluation of ICTs was performed in 991 cattle serum samples randomly collected from three provinces in Western Java, Indonesia. Twenty microliters of serum sample was diluted in an equal amount of PBS and dropped onto the sample pad of ICT strip. After 15 min, the strips were observed for the appearance of bands in the control and the test line. The presence of a band in both the control and test lines indicated a positive result, while the absence of the band in the test line was considered negative. The result was found to be invalid if no band appeared in the control line. The ICT examination was repeated twice for each sample.

Enzyme-linked immunosorbent assay (ELISA). ELISA plates (Nunc, Thermo Fisher USA) were coated with 50 µl of *Bbov*SBP-4 at a concentration of 6.7 µg/ml or *Bbig*RAP1/CT17 at 3.7 µg/ml in a carbonate-bicarbonate buffer 50 mM, pH 9.6, incubated overnight at 4^{o}C. Indirect ELISA was performed in duplicated for each serum sample from cattle in Western Java, Indonesia by following the previous protocol (Boonchit *et al*., 2002). Three confirmed negative sera were included on each

plate. The cutoff value was determined as the mean of absorbance from the negative control in all plates used in the study and added by three standard deviations. A serum sample was considered to be seropositive if the value was above the cutoff.

Statistical analysis. Estimations of the true prevalence and the 95% confidence interval (CI 95%) of the diagnostic methods were analyzed using EpiTools epidemiological calculators (Sergeant, 2017). The agreement between ELISA and ICT method was analyzed using kappa statistics. Agreement was stated as poor (<0.20), fair (0.21–0.40), moderate (0.41–0.60), good (0.61–0.80), or very good (0.81–1.00) (Fleiss *et al*., 2013).

1-3. Results

Optimization of ICT strips. The optimum condition for all components were determined to ensure maximum performance. First, the condition for protein-gold conjugation was determined by mixing a range of pH values and protein concentrations with gold colloid. The absence of color changing was observed at the pH 4.5 to 6. Furthermore, at the pH 5, the highest absorbance at 520 nm was achieved by the conjugation of gold colloid with 30 µg/ml for *Bbov*SBP-4 and 40 µg/ml for *Bbig*RAP-1/CT17 (Moon *et al*., 2012). Second, we determined the optimum concentration of proteins and total IgG to be dispensed onto the cellulose membrane as a test and control line. The concentration of 0.5 mg/ml was chosen for each component due to the strength of the band that appeared after addition of positive serum control. All of the optimum conditions were used for further production of the ICT strips.

Validation of ICT strips. The validation of ICT strips, such as the relative sensitivity (Se) and specificity (Sp), was evaluated using confirmed positive and negative serum controls that were kept

in our laboratory. The *bov*ICT detected 47 out of 50 positive control sera, while three false positives were detected from 50 negative controls (Se = 94%, Sp = 94%). The *big*ICT detected 18 out of 20 positive serum controls and four false positives from 50 negative controls (Se = 90%, Sp = 92%). The Se and Sp for dual-ICT in the *B. bovis* test line were 90% and 88%, while those for the *B. bigemina* test line were 85% and 88%, respectively. To determine the cross reaction between ICTs, all of the positive serum controls of *B. bovis* and *B. bigemina* were applied to their ICT strip counterparts. *Bov*ICT detected three false positives from 20 *B. bigemina* positive sera (15%), and *big*ICT detected five false positives from 50 *B. bovis* positive sera (10%). Dual-ICT obtained a similar result. ICT strips were also evaluated with positive serum controls of several associated diseases, and the positive band only appeared for the target diseases. All results were observed by the naked eye. Furthermore, the lowest serum dilution from a positive sample that can be determined by all ICTs was 1:50 v/v in PBS. Therefore, the dilution at an equal amount of serum sample in PBS was used for further ICT detections to achieve their best performance. The ICT strips were also evaluated with positive serum controls of several associated diseases, and none of the diseases showed a positive band in the test line.

Antibody detection of *Babesia* parasite infection in cattle serum samples. The ICT strips evaluations were conducted for the serological detection of *B. bovis* and *B. bigemina* infection in 991 cattle serum samples from Western Java, Indonesia. Seropositive samples of *B. bovis* infection was detected in 243 cattle (24.5%) by dual-ICT, ranging from 7.5 to 55.6%, and the positive rate of *B. bigemina* infection was 226 (22.8%), ranging from 8.3% to 41.7% in different sampling locations. Details of the seroprevalence of *B. bovis* and *B. bigemina* for each sampling location are shown in Fig. 1, Table 1, and 2. Using *bov*ICT, the seropositivity of *B. bovis* was identified in 251 serum samples (25.3%), and *big*ICT detected antibody to *B. bigemina* in 240 serum samples (24.2%). ELISA

examination showed a slightly higher positive rate for both species, while 281 and 269 serum samples (28.4% and 27.1%) were seropositive for *B. bovis* and *B. bigemina*, respectively. Furthermore, the co-infection between both species in the sampling locations was 7.9, 9.0, and 11.4% by dual-ICT, single-ICT, and ELISA, respectively.

Agreement between ELISA and ICT for the detection of bovine babesiosis. To determine the agreement between ELISA and ICT, data from both methods were analyzed using kappa statistics. The kappa values between *bov*ICT and *bov*ELISA, dual-ICT and *bov*ELISA, *big*ICT and *big*ELISA, and dual-ICT and *Bbig*ELISA were more than 0.7, indicating satisfactory agreement between all methods. Also, the kappa values between single and dual-ICT for both *Babesia* species were more than 0.9. This indicates that the results from both ICT were not significantly different and showed the potential application of dual-ICT since the system could detect and differentiate the infection of *B. bovis* and *B. bigemina* (Table 3).

1-4. Discussion

Babesia bovis SBP-4 appeared to be the best potential recombinant proteins for the serological detection of *B. bovis* after being evaluated among other antigenic proteins such as MSA-2c, RAP-1/CT, TRAP-1, and SBP-1. This protein is released into the cytoplasm from an infected erythrocyte at the late stage of infection, which enables early interaction with the host immune system once the parasites escape to infect another erythrocyte. Additionally, the protein is distinct from the other proteins of Apicomplexan parasites. It had been applied for the serological detection of antibodies against the infection of *B. bovis* by ELISA in many countries (Ibrahim *et al*., 2013; Li *et al*., 2014; Terkawi *et al*., 2011a). *Bbig*RAP1/CT17 is used for serological diagnosis of *B. bigemina*. The

combination of this protein with *B. bovis* MSA-2c has been developed in ICT strips (Kim *et al*., 2008). However, in the recent study, the antigenicity of *Bbov*MSA-2c was much lower than that of *Bbov*SBP-4 due to the high genetic diversity of this gene sequence among *B. bovis* strains in different areas of the world (Terkawi *et al*., 2011a). Therefore, in the present study, we selected *Bbov*SBP-4 and *Bbig*RAP1/CT17 as detection antigen for the preparation of ICT strip to be more applicable in field settings and can be used widely.

Three types of ICT strips were prepared including *bov*ICT, *big*ICT, and dual-ICT. The validation data showed these ICTs have a high specificity and sensitivity for the antibody detection of *B. bovis* and *B. bigemina* in cattle. However, the sensitivity of ICTs was slightly lower than those of ELISA. The rate of positive samples detected by ICTs decreased and some samples that have high titer antibody in ELISA, were negative in ICTs. The differences might be due to the non-specific reaction between the antigen and antibody (Terkawi *et al*., 2013). Unlike ELISA, which requires hours to obtain the result, ICT was more superior regarding the time for obtaining the result in only 10 to 15 min.

The ICTs were further evaluated using field serum samples from Western Java, Indonesia. The ability of this system to detect antibody of *B. bovis* and *B. bigemina* in the area was satisfied, despite in lower positive rate compared with ELISA. The result indicated SBP-4 gene of *B. bovis* and RAP1 gene of *B. bigemina* are conserved in the area. To our knowledge, this is the first report of the serological detection of bovine babesiosis in Western Java using serological method such as ICT strips. It will be the foundation for further study in utilizing these genes for diagnostic method and possibility to be used in the routine surveillance. The ICT strips could be a solution in situations with limited equipment or poorly trained personnel available. Further development and optimization of ICT strips for the detection of bovine babesiosis are necessary to increase their sensitivity and specificity along

with the stability of the strips to avoid a decrease in sensitivity at ambient temperatures. In this study, all of the ICT results were observed by the naked eye. Since some limitations are associated with naked-eye observation, further developments are necessary, such as the incorporation of an electronic reader for color development or adaptation of the quantitative assay system.

As we determined in this study, ICT strips for bovine babesiosis could be useful and convenient in the field settings. Satisfactory agreement between the ICT and ELISA indicated the potential of the method to be applied routinely in the surveillance of *B. bovis* and *B. bigemina*. Furthermore, bovine babesiosis is endemic in Western Java, Indonesia, and efficient control strategies are needed to reduce economic losses due to the disease.

1-5. Summary

Three types of immunochromatographic test (ICT) strips were prepared for the detection of an antibody response against spherical body protein 4 (SBP-4) of *Babesia bovis* (*bov*ICT), C-terminal-truncated rhoptry-associated protein 1 (rRAP1/CT17) of *B. bigemina* (*big*ICT), and the combination of both proteins (dual-ICT). The evaluation of their performance was conducted using a confirmed positive and negative serum panel for *B. bovis* and *B. bigemina*. Together with ELISA, the ICT strips were applied to determine the seroprevalence of bovine babesiosis in Western Java, Indonesia. Among 991 serum samples, 28.4%, 25.3%, and 24.5% of cattle were detected to be seropositive to *B. bovis* infection using ELISA, *bov*ICT, and dual-ICT, respectively. *B. bigemina* seropositive was detected in 27.1%, 24.2%, and 22.8% of samples using ELISA, *big*ICT, and dual-ICT, respectively. The comparison of ICT strips and ELISA results using field serum samples showed good agreement with Kappa values > 0.7 between all methods. The application of ICT strips is preferable in the field

situations where rapid diagnosis is required. Furthermore, the data showed the current seroprevalence of bovine babesiosis in Western Java, Indonesia, and efficient control strategies are needed to reduce economic losses due to the disease.

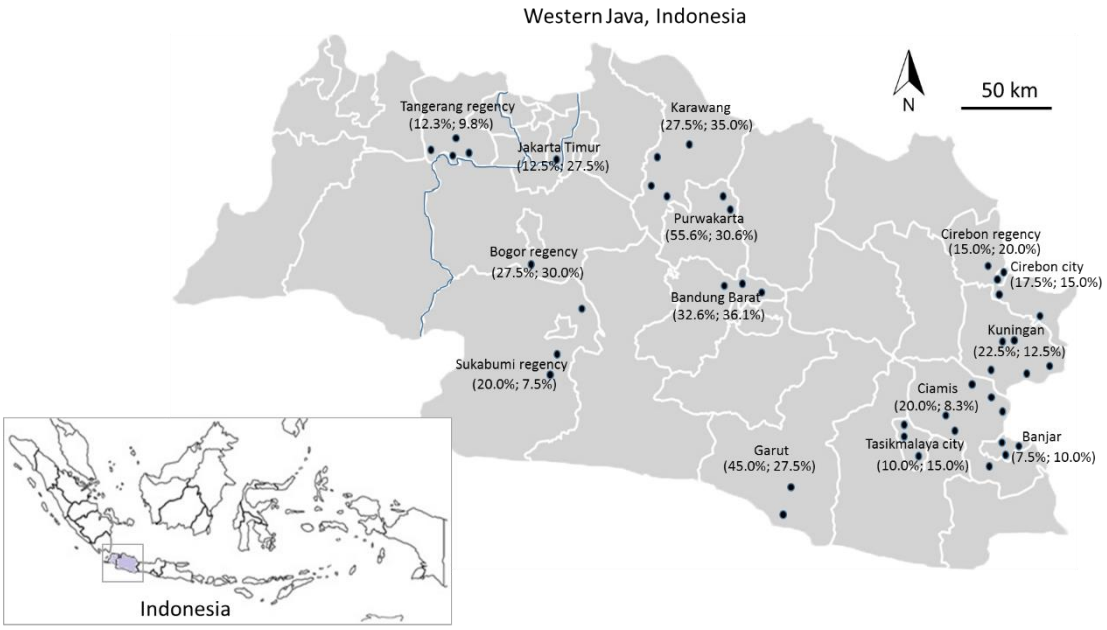

Fig. 1. Geographical distribution of sample locations in this study. The names of regencies/cities are displayed on the map. Black circles indicate the site of sample collections. The percentages in parentheses under the location show the respective seropositive rates of *Babesia bovis* and *Babesia bigemina* using dual-ICT.

Table 1. Serological detection of *Babesia bovis* and *Babesia bigemina* using ELISA in cattle from Western Java, Indonesia

Regency/City	n	*B. bovis*			*B. bigemina*		
		Positive[a]	%	(95% CI)[b]	Positive	%	(95% CI)
Banjar	40	0	0.0	(0-0.88)	6	15.0	(7.1-29.1)
Tasikmalaya City	40	4	10.0	(4-23.1)	8	20.0	(10.5-34.8)
Bandung Barat	313	118	37.7	(32.5-43.2)	127	40.6	(35.3-46.1)
Jakarta Timur	40	4	10.0	(4-23.1)	13	32.5	(20.1-48)
Ciamis	120	24	20.0	(13.8-28)	10	8.3	(4.6-14.7)
Garut	40	16	40.0	(26.3-55.4)	11	27.5	(16.1-42.8)
Tangerang Regency	122	20	16.4	(10.9-24.0)	17	13.9	(8.9-21.2)
Sukabumi Regency	40	10	25.0	(14.2-40.2)	10	25.0	(14.2-40.2)
Bogor Regency	40	14	35.0	(22.1-50.5)	13	32.5	(20.1-48)
Cirebon City	40	6	15.0	(7.1-29.1)	6	15.0	(7.1-29.1)
Cirebon Regency	40	7	17.5	(8.7-31.9)	9	22.5	(12.3-37.5)
Kuningan	40	11	27.5	(16.1-42.8)	10	25.0	(14.2-40.2)
Karawang	40	17	42.5	(28.5-57.8)	14	35.0	(22.1-50.5)
Purwakarta	36	30	83.3	(68.1-92.1)	15	41.7	(27.1-57.8)
Total	991	281	28.4	(25.6-31.2)	269	27.1	(24.5-30.0)

[a] A positive serum sample was determined if the mean of absorbance from duplicate repetition was above the cutoff value.

[b] 95% CI, 95% confidence interval

Table 2. Serological detection of *Babesia bovis* and *Babesia bigemina* using single and dual-ICT in cattle from Western Java, Indonesia

Regency/City	n	Single ICT						Dual-ICT					
		B. bovis			*B. bigemina*			*B. bovis*			*B. bigemina*		
		Positive[a]	%	(95% CI)[b]	Positive	%	(95% CI)	Positive	%	(95% CI)	Positive	%	(95% CI)
Banjar	40	3	7.5	(2.6-19.9)	5	12.5	(5.5-26.1)	3	7.5	(2.6-19.9)	4	10.0	(4-23.1)
Tasikmalaya City	40	4	10.0	(4-23.1)	6	15.0	(7.1-29.1)	4	10.0	(4-23.1)	6	15.0	(7.1-29.1)
Bandung Barat	313	107	34.2	(29.2-39.6)	120	38.3	(33.1-43.8)	102	32.6	(27.6-38.0)	113	36.1	(31.0-41.6)
Jakarta Timur	40	5	12.5	(5.5-26.1)	13	32.5	(20.1-48)	5	12.5	(5.5-26.1)	11	27.5	(16.1-42.8)
Ciamis	120	24	20.0	(13.8-28)	10	8.3	(4.6-14.7)	24	20.0	(13.8-28)	10	8.3	(4.6-14.7)
Garut	40	20	50.0	(35.2-64.8)	11	27.5	(16.1-42.8)	18	45.0	(30.7-60.2)	11	27.5	(16.1-42.8)
Tangerang Regency	122	15	12.3	(7.6-19.3)	12	9.8	(5.7-16.4)	15	12.3	(7.6-19.3)	12	9.8	(5.7-16.4)
Sukabumi Regency	40	8	20.0	(10.5-34.8)	5	12.5	(5.5-26.1)	8	20.0	(10.5-34.8)	3	7.5	(2.6-19.9)
Bogor Regency	40	11	27.5	(16.1-42.8)	12	30.0	(18.1-45.4)	11	27.5	(16.1-42.8)	12	30.0	(18.1-45.4)
Cirebon City	40	6	15.0	(7.1-29.1)	6	15.0	(7.1-29.1)	6	15.0	(7.1-29.1)	6	15.0	(7.1-29.1)
Cirebon Regency	40	6	15.0	(7.1-29.1)	8	20.0	(10.5-34.8)	6	15.0	(7.1-29.1)	8	20.0	(10.5-34.8)
Kuningan	40	9	22.5	(12.3-37.5)	7	17.5	(8.7-31.9)	9	22.5	(12.3-37.5)	5	12.5	(5.5-26.1)
Karawang	40	11	27.5	(16.1-42.8)	14	35.0	(22.1-50.5)	11	27.5	(16.1-42.8)	14	35.0	(22.1-50.5)
Purwakarta	36	21	58.3	(42.2-72.9)	11	30.6	(18-46.9)	20	55.6	(39.6-70.5)	11	30.6	(18-46.9)
Total	991	**251**	**25.3**	(22.7-28.1)	**240**	**24.2**	(21.7-27.0)	**243**	**24.5**	(21.9-27.3)	**226**	**22.8**	(20.3-25.5)

[a] A positive serum sample was determined if clear band appeared at the test line of the ICT strip from duplicate repetition.

[b] 95% CI, 95% confidence interval

Table 3. The agreement between ELISA and ICT for the serological detection of *Babesia bovis* and *Babesia bigemina* in cattle from Western Java, Indonesia

	Kappa Value	95% CI	Agreement
*bov*ICT and *bov*ELISA	0.756	0.710 – 0.802	Good
dual-ICT and *bov*ELISA	0.741	0.694 – 0.789	Good
*big*ICT and *big*ELISA	0.786	0.742 – 0.830	Good
dual-ICT and *big*ELISA	0.756	0.708 – 0.803	Good
*bov*ICT and dual-ICT	0.981	0.967 – 0.995	Very good
*big*ICT and dual-ICT	0.961	0.940 – 0.981	Very good

CI, confidence interval

Chapter 2

Molecular and serological detection of bovine babesiosis in Indonesia

2-1. Introduction

Bovine babesiosis is a huge threat to the livestock industry as it is associated with direct economic losses like loss of body weight and milk productions and death of animals, and indirect costs of prevention and treatment. Common *Babesia* species that infect cattle are *B. bovis*, *B. bigemina*, and *B. divergens*. Tick vectors such as *Rhipicephalus microplus*, *R. annulatus*, and *R. geigyi* can transmit *B. bovis* and *B. bigemina*, while *R. decoloratus* and *R. evertsi* can only transmit *B. bigemina*. *B. divergens* is usually transmitted by *Ixodes ricinus*. The distribution of tick vectors in many parts of the world correspond well with the presence of the parasite (Bock *et al*., 2004; Gohil *et al*., 2013). During infected ticks feeding blood on cattle, sporozoites enter the host's blood circulation and invade into the red blood cells (RBCs) to undergo asexual replication. The developmental stage in RBCs involves a few morphological changes from round trophozoites to binary fission trophozoites and then merozoites. Mature merozoites rupture the cells and subsequently infect new RBCs. Ticks ingest some merozoites during blood feeding, and sexual replication is initiated in the tick midgut. The parasites are transmitted transovarially to the tick eggs to continue the life cycle (Homer *et al*., 2000; Mosqueda *et al*., 2012).

Babesia parasites create and stabilize their intracellular environment to make it suitable during their life cycle. It involves the releasing of numerous molecules by apical complex of parasite in all

stage of asexual replication including cell invasion, intracellular developments, and egress from the cell. Exploitation of released molecules, produced by apical complex of parasite, has been shown to be an effective tool for the development of diagnostic methods and as the drug target in chemotherapeutic development (Gohil *et al*., 2010). The spherical body protein (SBP-4) of *B. bovis* is found in the spherical bodies, a component of apical complex. The protein is characterized by its abundance in the cytoplasm in the late stage of intracellular infection and released into blood circulation. Therefore, it has a greater possibility of reacting with the host immune systems during rupture of infected cells and has potential as serological diagnostic target (Terkawi *et al*., 2011b). The *B. bovis* SBP-4 gene is conserved among isolates from different geographic areas and has a low homology with other apicomplexan parasites, indicating the suitability of the gene as a specific target for molecular diagnosis (Ruef *et al*., 2000a; de Vries *et al*., 2006). Correspondingly, the utilization of *B. bovis* SBP-4-based serological and molecular diagnostic methods has been applied in several studies (Terkawi *et al*., 2012; da Silva *et al*., 2013; Ibrahim *et al*., 2013; Moumouni *et al*., 2015). The rhoptry-associated protein 1 (RAP-1) is a conserved gene family in many *Babesia* species and plays an important role during parasite invasion (Perkins, 1992; Suarez *et al*., 1998). An enzyme-linked immunosorbent assay (ELISA) using this gene has been developed to detect antibodies of *B. bovis* and *B. bigemina*; however, the cross-reactivity was apparently high (Boonchit *et al*., 2002). A previous study determined the high specificity of the truncated C-terminal RAP-1 for the serological detection of *B. bigemina* (Boonchit *et al*., 2006). For molecular detection, RAP-1a is a highly conserved gene among *B. bigemina* isolates that has been utilized in several studies (Terkawi *et al*., 2012; Ibrahim *et al*., 2013; Moumouni *et al*., 2015). Above all, the application of specific and sensitive diagnostic

methods is necessary to accurately determine the presence of *Babesia* parasites during routine surveillance.

In Indonesia, bovine babesiosis was first reported in 1896, and the disease was later found to be endemic in the country (Soekardono, 1989). In 1993, a study employing ELISA for the detection of *B. bovis* antibodies in cattle serum samples showed prevalences as high as 96% in the islands of Sumatera, Kalimantan, Sulawesi, Sumba, and Timor (Sukanto *et al*., 1993). The country also imports live cattle from several countries to meet domestic demands. Based on the microscopic observation of blood smears, one study reported that the average prevalence of babesiosis in cattle imported from Australia was 10.5% (Dewi, 2009). A recent study using a similar method showed a higher prevalence of babesiosis at 42.9% and a moderate rate of mixed infection of *Anaplasma* sp. and *Theileria* sp. The imported cattle did not show any clinical symptoms upon arrival. However, they could be the source of the subsequent infection for other cattle (Tefi, 2015). Indonesian cattle are not vaccinated against babesiosis, and the nation-wide surveillance program conducted by Veterinary Diagnostic Centers is based on microscopic observation. Therefore, studies to determine the distribution and genetic characterization of the *Babesia* parasites in the country are necessary to provide critical data for the development of effective measures for controlling the disease.

The main purpose of this study was to determine the distribution of bovine babesiosis in a wide geographical area across Indonesia. It also aimed to evaluate the potency of diagnostic methods such as indirect ELISA (iELISA), immunochromatographic test (ICT), and nested PCR (nPCR) to be applied in field surveillance in the country. Another objective was to characterize the genetic diversity of Indonesian isolates based on the SBP-4 gene of *B. bovis*, the RAP-1a gene of *B. bigemina*, and internal transcribed spacer (ITS) region of rRNA of *B. bovis* and *B. bigemina*.

2-2. Materials and methods

Parasites and the preparation of recombinant protein *B. bovis* SBP-4 and *B. bigemina* RAP-1/CT17. *B. bovis* (Texas strain) and *B. bigemina* (Argentina strain) were cultured continuously using a microaerophilic culture system (Timms *et al*., 1983; Vega *et al*., 1985). The cultures were used to synthesize cDNA for the expression of *B. bovis* spherical body protein 4 (SBP-4) and *B. bigemina* C-terminal rhoptry-associated protein (RAP1/CT17) in accordance with the method described in the Chapter 1. Genomic DNA for positive control in PCR amplification was also obtained from the cultures.

Study areas and blood sample collections. A total of 487 blood samples were randomly collected and divided into tubes, with and without anticoagulant, from clinically healthy cattle. Samples were collected from March to April 2016 in sixteen locations in Indonesia: Mandailing Natal (n = 32), Tapanuli Selatan (n = 28), Padang Mangateh (n = 60), Tangerang Regency (n = 18), Bogor Regency (n = 16), Karawang (n = 21), Indramayu (n = 10), Lamongan (n = 40), Jombang (n = 40), Tabalong (n = 60), Bulukumba (n = 74), Dompu (n = 17), Lombok Timur (n = 16), Kupang (n = 19), Manggarai Timur (n = 19), and Malaka (n = 17), as shown in Fig. 2. The breeds of cattle included Bali cattle (n = 207), Filial Ongole (n = 99), Brahman crossed (n = 88), Pesisir cattle (n = 60), and Taurine cattle (n = 33). The cattle were divided into three age groups including young (< 2 years), adult (2 – 4 years), and old (> 4 years). Serum samples were aliquoted into microcentrifuge tubes and transported to the laboratory with ice packs and stored at -30^{o}C before serological detections. The genomic DNA was purified from 200 µl of each blood sample using the QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. The DNA samples were stored at -30^{o}C until they were molecularly examined.

Serological assays for the detection of antibody responses against *B. bovis* and *B. bigemina.*

Three types of ICT strips were prepared including *bov*ICT, *big*ICT, and dual-ICT. The ICTs were based on the recombinant proteins *B. bovis* SBP-4, *B. bigemina* RAP1/CT17, and a combination of both proteins as detection antigens as described in Chapter 1. ICT examinations were performed twice on each serum sample. A 20-microliter serum sample was diluted with an equal amount of phosphate buffer saline (PBS) and applied to the sample pad of the ICT strip. The sample was determined to be seropositive if the clear band appeared in both the control and the test line after 15 min. For the iELISA, the recombinant proteins of *B. bovis* SBP-4 and *B. bigemina* RAP-1/CT17 were also used as detection antigens (*bov*ELISA and *big*ELISA). The sensitivity, specificity, and cut-off value had been determined in a previous study (Terkawi *et al*., 2011c). iELISA, performed according to the protocol described elsewhere, was performed on all serum samples in duplicate (Boonchit *et al*., 2006).

PCR screening for the detection of *B. bovis* SBP-4 and *B. bigemina* RAP-1a genes. PCR assays targeted the *B. bovis* SBP-4 and *B. bigemina* RAP-1a genes. The sets of primers, both for PCR and nPCR, were designed in accordance with the previously published work, as shown in Table 4 (Terkawi *et al*., 2011c; Cao *et al*., 2012). Amplification was carried out using 10 µl of the reaction mixture, which contained 1 µl of each sample or control as a DNA template, 1 µl of 10x Ex Taq buffer, 0.8 mM of dNTPs, 1 µM of both forward and reverse primers, and 0.1 U of Ex Taq polymerase (Takara Bio, Shiga, Japan) and brought to the total volume with distilled water. The PCR conditions for detecting *B. bovis* are as follows. The initial denaturation was at 94^{o}C for 1 min, followed by 35 cycles of denaturation at 94^{o}C for 30 s, annealing at 65^{o}C for 1 min, extension at 72^{o}C for 1 min, and final extension at 72^{o}C for 7 min. The conditions for nPCR were similar except the annealing temperature at 53.5^{o}C. For *B. bigemina*, the initial denaturation was at 95^{o}C for 5 min, followed by 35 cycles of

denaturation at 94ºC for 1 min, annealing at 55ºC for 1 min, extension at 72ºC for 1 min, and final extension at 72ºC for 10 min. Similar conditions were applied in nPCR for *B. bigemina* detection. The reaction mixture of the nPCR included the amplicon from the first PCR as a DNA template and the amplified fragments were analyzed using 1.5% agarose gel. The gel was subsequently stained with ethidium bromide and visualized under UV illumination.

Genetic characterization of *B. bovis* SBP-4, *B. bigemina* RAP-1a, and ITS region of *B. bovis* and *B. bigemina*. Positive DNA samples in the PCR screening were selected randomly for the amplification of ITS region of *B. bovis* (n = 9) and *B. bigemina* (n = 8). The sets of primers for the amplification of ITS1-5.8s rRNA gene-ITS2 regions from *B. bovis* and *B. bigemina* were shown in the Table 1 (Cao *et al.*, 2012). The reaction mixture contained 1 µl of each sample, 2 µl of 5x SuperFi buffer, 0.8 mM of dNTPs, 1 µM of both forward and reverse primers, and 0.2 U of Platinum SuperFi DNA polymerase (Invitrogen, Carlsbad, CA, USA), and brought to the total volume of 10 µl with distilled water. The PCR conditions for the amplification of *B. bovis* and *B. bigemina* ITS regions were as follows. The initial denaturation was at 98ºC for 30 s, followed by 35 cycles of denaturation at 98ºC for 10 s, annealing at 60.2ºC for 10 s, extension at 72ºC for 30 s, and final extension at 72ºC for 5 min. For the nPCR, similar conditions were used except annealing temperature at 64.9ºC and 63.3ºC for *B. bovis* ITS and *B. bigemina* ITS, respectively. The amplified fragments were analyzed using 1.5% agarose gel.

Gel purifications were conducted to the amplicon of *B. bovis* ITS, B. *bigemina* ITS regions, *B. bovis* SBP-4 (n = 16), and *B. bigemina* RAP-1a (n = 13) using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany). Subsequently, the purified fragments were cloned into the pCR™ 2.1-TOPO® TA vector (Invitrogen, Carlsbad, CA, USA) and transformed into *Escherichia*

coli TOP 10 competent cells (Invitrogen, Carlsbad, CA, USA). Two single colonies from each *E. coli*-transformed clone were selected and amplified with pCR™ 2.1-TOPO® TA vector primers. Sequence analysis was performed in both forward and reverse directions using a BigDye® Terminator v3.1 Cycle Sequencing Kit and an ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Bioinformatics analysis. All obtained sequences were analyzed using the Basic Local Alignment Search Tool for nucleotides (BLASTn) (NCBI Resource Coordinators, 2017) and pairwise sequence alignment by EMBOSS Needle (Li *et al*., 2015) to find the homologs in the database. The typical nucleotide sequence from each isolate was then processed for further multiple sequence alignments using Clustal Omega (Li *et al*., 2015). Finally, the aligned sequences were analyzed by the maximum likelihood estimation method to construct a phylogenetic tree using MEGA 7.0.21 software (Kumar *et al*., 2016).

Statistical analysis. The strength of agreement between ELISA, *bov*ICT, *big*ICT, and dual-ICT was evaluated using Kappa statistics (Fleiss *et al*., 2013). Significant differences ($p < 0.05$) of *B. bovis* and *B. bigemina* detections between sampling locations, cattle breeds, age of cattle, and the expected conditional probability of mixed infections were analyzed using a chi-square test utilizing EpiTools epidemiological calculators (Sergeant, 2017). Furthermore, DNA sequence polymorphism analysis was conducted to determine the number of haplotype (h), nucleotide diversity (π), and average number of nucleotide differences (k) using DnaSP version 5 (Librado and Rozas, 2009).

2-3. Results

Serological detection of *B. bovis* and *B. bigemina*. The serological detections in this study were based on ELISA and ICT systems to detect specific antibodies against *B. bovis* and *B. bigemina* in cattle. ELISA and ICT examinations revealed a high positive rate of antibody response against *B. bovis* (Table 5). Among all sampling locations, only two sites, Lamongan and Jombang, had a positive rate as low as 10%. Other locations even reached 100% positive rates, such as in Manggarai Timur and Malaka. In total, 340 (69.8%), 317 (65.1%), and 307 (63.0%) of serum samples were positive for *B. bovis* antibody when using *bov*ELISA, *bov*ICT, and dual-ICT, respectively. The positive rates of *B. bigemina* infections were lower than those of *B. bovis*. We found no positive case in Jombang and Lamongan by *big*ELISA and *big*ICT; however, 1 (2.5%) serum sample from Lamongan was seropositive by dual-ICT. From the total samples, *big*ELISA, *big*ICT, and dual-ICT detected positive antibody responses to *B. bigemina* in 134 (27.5%), 130 (26.7%), and 127 (26.15%) serum samples, respectively. Mixed infections by both parasites were detected in 125 (25.7%), 113 (23.2%), and 109 (22.4%) samples, respectively. Analysis of expected conditional probability of mixed infection (Table 9a, 9b, and 9c) showed that the observed number of cattle with both *B. bovis* and *B. bigemina* infections were not significantly different with the expected number in all sampling locations. The events only occurred by chance and were not influenced by additional factors. Furthermore, agreement between ELISA and ICT was analyzed using Kappa statistics (Table 6). For *B. bovis*, the Kappa values between *bov*ICT, dual-ICT, and *bov*ELISA ranged from 0.743 to 0.927, which indicated satisfactory agreement between the methods. Similar results were also determined for *B. bigemina* with the Kappa values between *big*ICT, dual-ICT, and *big*ELISA ranged from 0.748 to 0.888.

Molecular detection of *B. bovis* and *B. bigemina.* Nested PCR detection targeted the highly conserved regions of *B. bovis* SBP-4 and *B. bigemina* RAP-1a genes. The negative controls consisted of genomic DNA from healthy cattle, which had been kept in our laboratory and the elution buffer that used during DNA extraction, did not show any amplification in the first PCR or the nested PCR. The positive rate of *B. bovis* infection was more than 30% in most sampling locations, except in Indramayu, Padang Mangateh, and Dompu (Table 5). In contrast, the infection rates of *B. bigemina* in most locations were lower than 30%, except in Padang Mangateh, Lombok Timur, Manggarai Timur, Malaka, and Mandailing Natal. In total, *B. bovis* and *B. bigemina* were detected in 247 (50.7%) and 93 (19.1%) of cattle DNA samples, respectively. Mixed infections by both parasites were detected in 52 samples (10.7%). Similar with serological detections, analysis of expected conditional probability of mixed infection (Table 9d) showed that the event only occurred by chance and were not affected by other factors.

Comparison of detections by ELISA, ICT, and nPCR. We also compared the results of the different methods used in this study (Table 7). The positive rate of ICT was lower than that of ELISA, but the correlation between method was high ($p < 0.05$). A high correlation was also determined on the detection of *B. bigemina* by nPCR ($p < 0.05$). On the other hand, the correlation between ELISA and nPCR on the detection of *B. bovis* was low ($p = 0.707$). Both ELISA and nPCR detected positive *B. bovis* in 174 (35.7%) samples, while 166 (34.1%) samples were detected as positive by ELISA only, 73 (15.0%) samples tested positive by nPCR only, and 74 (15.2%) samples tested negative by both methods. For *B. bigemina*, only 36 (7.4%) samples were positive by both methods, while 98 (20.1%) samples were positive by ELISA only, 57 (11.7%) samples were positive by nPCR only, and 296 (60.8%) samples remained negative by both methods. Additionally, high titer antibodies in ELISA

were more likely to be positive by nPCR. Some early infections of *B. bovis* were detected in Jombang, Lamongan, Bulukumba, and Lombok Timur, as indicated by the higher nPCR positive rate than for those with ELISA. Furthermore, a higher nPCR positive rate of *B. bigemina* was observed in Padang Mangateh, Lombok Timur, Kupang, Mandailing Natal, Jombang, and Lamongan.

Effect of breeds and age groups of cattle on *B. bovis* and *B. bigemina* infections. The rates of *B. bovis* infection detected by ELISA, *bov*ICT, dual-ICT, and nPCR were significantly different ($p < 0.05$) among cattle breeds (Fig. 3A). Pesisir cattle exhibited the highest positive rate of *B. bovis* using ELISA (98.3%), *bov*ICT (85%), dual-ICT (85%), but the lowest using nPCR (21.7%). Brahman crossed cattle showed the lowest positive rate of *B. bovis* infection among other breeds using ELISA (25%), *bov*ICT (23.9%), and dual-ICT (22.7%), but a higher rate was detected by nPCR (40.9%). The rates of *B. bigemina* infection detected by all diagnostic methods were also significantly different ($p < 0.05$) between cattle breeds (Fig. 3B). The highest positive rate was found in Pesisir cattle (43.3%, 41.7%, 41.7%, and 46.7%) and the lowest was found in Brahman crossed cattle (12.5%, 9.1%, 10.2%, and 4.5%) using ELISA, *bov*ICT, dual-ICT, and nPCR, respectively. The age of cattle also affected the rates of *B. bovis* infection using ELISA, *bov*ICT and dual-ICT ($p < 0.05$). However, the difference was not significant when using nPCR (Fig. 3C). Young cattle were likely to have less infection compared with older cattle. For *B. bigemina*, the age of cattle significantly affected the rate of infection using ELISA, *big*ICT, and dual-ICT ($p < 0.05$) as shown in Fig. 3D. Similar with *B. bovis*, no significant difference was observed on the detection using nPCR. Adult cattle at the age from 2 to 4 years have the highest infection rate of *B. bigemina* compared with young and old groups using ELISA, *big*ICT, and dual-ICT, but the lowest using nPCR.

Sequence analysis. All obtained sequences from each sample were aligned to acquire the representative sequence. Nucleotide BLAST analysis of the sequences was homologous with respective gene targets of *B. bovis* SBP-4, *B. bigemina* RAP-1a, and *B. bovis* and *B. bigemina* ITS regions in the database. The results confirmed the specificity of our nPCR assay for both *Babesia* species. For *B. bovis* SBP-4, the identities among isolates in this study and database ranged from 96.0% to 100%. Thirteen isolates with a product size of 503 bp shared 99.0 to 100% identities with isolates from Mongolia, Egypt, Texas, Brazil, Syria, Thailand, and Mexico. Two isolates from Dompu and Malaka with a product size of 512 bp and an isolate from Mandailing Natal (503 bp) shared 96.0 to 97.0% identities with isolates from the African continent. The multiple sequence alignment of the *B. bovis* SBP-4 gene sequences among sixteen Indonesian isolates revealed 57 nucleotide substitutions, of which 31 of them affected the modifications of 23 amino acids (aa). Most of the amino acid modifications were identified in Malaka, Dompu, and Mandailing Natal. The percentage identities among Indonesian *B. bovis* SBP-4 isolates ranged from 93.5 to 100% (Table 10a). The constructed phylogenetic tree of *B. bovis* SBP-4 gene sequences showed that 13 Indonesian isolates were among other isolates from across continents in clade 1 (Fig 6). Clade 2 was formed by isolates from Dompu and Malaka, while isolates from Mandailing Natal joined the third clade, together with isolates from African countries.

Indonesian *B. bigemina* RAP-1a isolates consisted of two types. Type 1 included seven Indonesian isolates, mostly from the western part of Indonesia, and shared 99.0 to 100% identities with isolates from across continents including Asia, South America, and Africa. In type 2, six Indonesian isolates, mostly found in the Nusa Tenggara area, shared 98 to 99% identities with sequences from Australia. The multiple sequence alignment of Indonesian *B. bigemina* RAP-1a gene

sequence showed 31 nucleotide substitutions, of which 26 substitutions affected the modifications of 24 aa. The percentage identities among Indonesian *B. bigemina* RAP-1a isolates ranged from 94.4 to 100% (Table 10b). The phylogenetic tree of *B. bigemina* RAP-1a formed three clades (Fig 7). Clade 1 was filled by seven Indonesian isolates (Lamongan, Karawang, Jombang, Bulukumba, Padang Mangateh, Mandailing Natal, and Bogor) together with isolates from Brazil, Thailand, Syria, Benin, Egypt, Puerto Rico, the Philippines, Uruguay, Kenya, Turkey, and Argentina. Four Indonesian isolates (Tabalong, Tangerang, Lombok Timur, and Manggarai Timur) were branched into clade 2, together with the isolates from Australia. The last clade was specifically formed by isolates from Kupang and Malaka.

Further characterizations were conducted to ITS regions of *B. bovis* and *B. bigemina*. The nucleotide sequences of Indonesian *B. bovis* ITS1-5.8s rRNA-ITS2 isolates in this study had a range of length from 487 to 508 bp. The sequences shared 91.0 to 96.0% identities with isolates from Thailand, Australia, Brazil, South Africa, and Mexico in the BLAST analysis. Among Indonesian isolates, the percent identities were ranged from 81.4 to 99.6% (Table 10c). Phylogenetic analysis incorporating other 25 isolates from several countries revealed seven genotypes of Indonesian *B. bovis* ITS isolates (Fig. 4). For *B. bigemina*, the length of ITS1-5.8s rRNA nucleotide sequences ranged from 495 to 498 bp. Nucleotide BLAST analysis revealed the high identities of Indonesian *B. bigemina* ITS isolates with the isolates from South Africa, Australia, China, Thailand, and Brazil. The percent identities between *B. bigemina* ITS sequences from Indonesia ranged from 93.5 to 98.8% (Table 10d). Unlike *B. bovis*, *B. bigemina* ITS isolates from Indonesia was less diverse in the phylogenetic analysis (Fig. 5).

Indonesian *B. bovis* SBP-4, *B. bigemina* RAP-1a, *B. bovis* ITS, and *B. bigemina* ITS isolates were further analyzed for the diversity of nucleotide sequences and nucleotide differences (Table 8). The highest nucleotide diversity (π) was determined in *B. bovis* ITS (0.079) with 35.889 of average number of nucleotide differences compared with *B. bigemina* ITS (π = 0.032 and k = 15.786), *B. bigemina* RAP-1a (π = 0.028 and k = 10.590), and *B. bovis* SBP-4 (π = 0.018 and k = 9.142).

The accession number of sequence nucleotides. Nucleotide sequences of *B. bovis* SBP-4 (n = 16), *B. bigemina* RAP-1a (n = 13), *B. bovis* ITS1-5.8s rRNA-ITS2 (n = 9), and *B. bigemina* ITS1-5.8s rRNA (n = 8) were submitted to GenBank. The sequences can be retrieved with accession numbers KY484510 to KY484534, KY562845 to KY562848, and MF664377 to MF664393.

2-4. Discussion

The economic losses to the livestock industry due to bovine babesiosis are underestimated in Indonesia. A nationwide surveillance program that relies upon microscopic observation of Giemsa-stained blood smears leads to the overlooking of the infection. Usually, the prevalence of *Babesia* spp. detected by microscopy was lower than 5% in many locations in Indonesia. Under those circumstances, sporadic outbreaks typically occurred in some locations of the country and usually went unreported. In Indonesia, the predominant *Babesia* species that infect cattle are *B. bovis* and *B. bigemina*. It is assumed that they are both are transmitted by the common tick vector *R. microplus*, which displays a widespread distribution in the country (Sahara *et al*., 2015). A tick eradication program is absent in the country; however, farmers sometimes use acaricide to control tick infestations in their cattle. Seeing that, a program to control the disease should be supported by a more sensitive method of providing reliable data so as to avoid financial losses by farmers.

In this study, we reported the distribution of bovine babesiosis in a wide geographic area of Indonesia. Employing serological methods such as ELISA and ICT strips and molecular methods such as nPCR, we determined the rate of *B. bovis* and *B. bigemina* infections in cattle blood samples collected from sixteen locations across the country. We chose recombinant proteins of *B. bovis* SBP-4 and *B. bigemina* RAP-1/CT17 as detection antigens in our ELISA and ICT methods. Both proteins showed a high sensitivity and specificity in the detections of specific antibodies against *B. bovis* and *B. bigemina* (Kim *et al*., 2008; Terkawi *et al*., 2011a; Guswanto *et al*., 2017). For nPCR, we chose *B. bovis* SBP-4 and *B. bigemina* RAP-1a genes as detection targets. Each gene is specific for and conserved in each *Babesia* species, and there is low homology between them (Ruef *et al*., 2000a; Boonchit *et al*., 2006; Terkawi *et al*., 2011b). A combination of these diagnostic methods will allow to reveal the presence of a chronic or acute infection in the studied samples. In the early stage of *Babesia* infections, the parasites appear in the red blood cells of host and can be detected by microscopy or DNA detection by PCR. However, antibodies against the parasites take days or weeks to produce and last for months during the chronic phase even though the parasites disappeared from red blood cells. In this phase, serological method such as ELISA or ICT would be more useful as diagnostic method (Mosqueda *et al*., 2012).

The rates of *B. bovis* infection differed significantly among sampling locations. Serological detections showed a high positive rate of *B. bovis* infection in most sampling locations, except two locations, Lamongan and Jombang, exhibited a low rate of positive antibodies. In contrast, molecular detection revealed a higher positive rate of *B. bovis* infection in these two locations. The predominant cattle breed in our samples from Lamongan and Jombang was Brahman crossed. This breed is known to be more resistant to the effect of *B. bovis* infection which reduces the level of immunity in

population. If the herd are exposed by high number of infected ticks, the possibility of new introduction of *B. bovis* is high (Zintl *et al*., 2005). The areas should avoid introducing of new cattle from the endemic area since the herds in these locations are susceptible to infection. Other locations with high positive rate of *B. bovis* in both diagnostic methods might develop the enzootic stability because the cattle were asymptomatic during sample collections. The risk of outbreak is high if the susceptible animals are introduced into those locations. Hence, farmers and veterinary service organizations should be aware of this condition.

B. bigemina had a lower infection rate if compare with *B. bovis* in Indonesian cattle. In all sampling locations, the seropositivity of *B. bovis* was higher than that of *B. bigemina* by ELISA and ICTs, except in Bogor and Indramayu. These two locations exhibited an equal positive rate of antibodies for both *Babesia* species. Molecular examinations of DNA samples also revealed higher positive rates of *B. bovis* than of *B. bigemina*, except in samples from Indramayu, Dompu, and Padang Mangateh. In Indramayu and Dompu, the positive rate for both *Babesias* was equal, while in Padang Mangateh, the positive rate of *B. bigemina* was higher than that of *B. bovis*. This divergence might be caused by a different distribution of tick vectors in these geographic areas. In Indonesia, *R. microplus*, the common vector of both *B. bovis* and *B. bigemina*, is widespread across the archipelago and has been reported in several islands in Indonesia, such as Sumatera, Java, Kalimantan, Sulawesi, Bali, Lombok, and Timor (Sahara *et al*., 2015). Other tick species that might transmit *Babesia* parasites, such as *R. annulatus* and *R. geigyi* that exclusively transmit *B. bovis* and *R. decoloratus* and *R. evertsi* that transmit only *B. bigemina,* have not reported the presence of these tick species in Indonesia. Therefore, further studies are necessary to determine which tick species are present in the country

possibly allowing to provide the reason for the significant difference between *B. bovis* and *B. bigemina* infection rates between different locations.

The ELISA, ICT, and nPCR in this study employed the same gene regions. We found a high correlation between ELISA result with *bov*ICT and dual-ICT on *B. bovis* SBP-4 antibody detection ($p < 0.05$), however, there was no correlation between ELISA and nPCR ($p = 0.707$). For *B. bigemina*, the correlations of ELISA result with *big*ICT, dual ICT, and nPCR were high ($p < 0.05$). Our results regarding the correlation between methods were similar with the previous study using the same gene regions in Syria, Thailand, and Egypt (Ibrahim *et al*., 2013; Terkawi *et* al., 2011c; Terkawi *et al*., 2012), except for nPCR of *B. bovis*. The results also showed that, the positive rates by nPCR for both *Babesia* species were lower than those of ELISA and ICTs. The present of antibodies for a long period in the blood circulation of host, even after the parasites were already cleared might explain the discrepancies between those methods (Carrique *et al*., 2000).

The breeds of cattle also affected the rate of positive samples. Among the breeds of cattle, Pesisir cattle had the highest positive rate of *B. bovis* and *B. bigemina* infections using ELISA and ICTs. This breed also showed the highest positive rate for *B. bigemina* infection, but the lowest for *B. bovis* using nPCR. We also reported the rate of *B. bovis* and *B. bigemina* infection in Bali cattle. Our study is the first report of *Babesia* infection in Pesisir and Bali cattle. It is speculated that Bali cattle is more resistant to *Babesia* infection, but our results showed that positive rates of *B. bovis* was high using serology and molecular detections. Further evaluation of *Babesia* infection in Bali cattle is necessary since the breed is Indonesian native cattle and had been distributed in many part of the country from their origin location due to their well adaptation to tropical climate and high meat productions. Age groups of cattle also affect the positive rate of *B. bovis* and *B. bigemina*. Young

cattle (age < 2 years) had a lower positive rate of *B. bovis* and *B. bigemina* infections compared with older cattle. The difference might be caused by the higher innate immune response of cattle at the young age (Zintl *et al*., 2005; Terkawi *et al*., 2012).

The genetic characterization of Indonesian *B. bovis* SBP-4 and *B. bigemina* RAP-1a isolates showed a high homology with other isolates in many countries. This result further supports the usefulness of this gene as a specific target for the PCR screening of *B. bovis*. Indonesia is a transcontinental country that is divided into two biogeographic realms, including the Indomalayan and Australasian realms. Each realm is considered to share a common fauna (Olson and Dinerstein, 1998). However, this does not affect the distribution of *B. bovis* in the country. A significant influence was observed in the distribution of *B. bigemina*. Most *B. bigemina* RAP-1a gene sequences from the western part of Indonesia shared high identities with isolates from the Indomalayan realm. The high identities of those sequences were also observed with published RAP-1a sequences from other continents, such as Africa and South America. On the other hand, the *B. bigemina* RAP-1a gene sequences from the Nusa Tenggara area (Lombok Timur, Manggarai Timur, Kupang, and Malaka), which belongs to the Australasian realm, were more similar to the Bond strain from Australia. Although Tangerang is located in the western part of Indonesia, the RAP-1a gene isolate from this location was identical with isolates from the Nusa Tenggara areas because the sample was collected from cattle imported from Australia. Hence, the strategy for controlling the bovine babesiosis in these locations will benefit from the advancement of the Australian approach to tackling the problem of the disease. Furthermore, effective control measures should be applied to cattle importation to avoid the new introduction of the disease into susceptible herds.

Unlike *B. bovis* SBP-4 gene, the high genetic diversity was observed in the nucleotide sequences of Indonesian *B. bovis* ITS region. At least seven different strains of *B. bovis* were distributed in the country. On the other hand, the diversity of *B. bigemina* ITS was not as high as *B. bovis*. Only three difference strains were observed in our study. The results were in agreement with other studies using *B. bovis* ITS region as target in Thailand (Cao *et al*., 2012; Jirapattharasate *et al*., 2017). Since *B. bovis* has a higher pathogenicity than *B. bigemina*, the correlation of *B. bovis* different strains with their pathogenicity could be explore and characterize in the future study.

Our study provided the current distribution of bovine babesiosis in Indonesia. The disease is endemic in the country, and effective control strategies are necessary to reduce economic losses. We found that Jombang and Lamongan, while endemicity was very low serologically, currently acquired new infections of bovine babesiosis from other locations. The potency of the outbreaks is high in those locations. We also demonstrated the suitability of our diagnostic methods for use in routine *Babesia* detections in the country. This study presents a basis to explore the epidemiology of bovine babesiosis and the distribution of the tick vector in this country.

2-5. Summary

Bovine babesiosis, mainly caused by *Babesia bovis* and *B. bigemina*, is a huge threat to the livestock industry. In Indonesia, the current distribution of the disease is unknown due to the lack of scientific study. In the present study, 487 blood samples were collected from cattle with different breeding and age groups in a broad geographic area across the archipelago. The presence of antibodies and current infections of *B. bovis* and *B. bigemina* were determined using enzyme-linked immunosorbent assay (ELISA), immunochromatographic test (ICT), and nested PCR (nPCR) targeting *B. bovis* SBP-4 and

B. bigemina RAP-1a genes. Sequence analysis was performed to the amplicon of *B. bovis* SBP-4, *B. bigemina* RAP-1a, and internal transcribed spacer (ITS) region of ribosomal RNA of both *Babesia* species. In total, *B. bovis* positives were detected by ELISA, single-ICT, dual-ICT, and nPCR in 340 (69.8%), 317 (65.1%), 307 (63.0%), and 247 (50.7%) samples, respectively. For *B. bigemina*, the positive samples were detected in 134 (27.5%), 130 (26.7%), 127 (26.1%), and 93 (19.1%), respectively. Furthermore, mixed infections were found in 125 (25.7%), 113 (23.2%), 109 (22.4%), and 52 (10.7%) samples, respectively, which occurred only by chance and were not influenced by additional factors. The obtained nucleotide sequences of *B. bovis* SBP-4 and *B. bigemina* RAP-1a genes showed a high homology with other isolates from different countries. Further nucleotide sequence analysis using ITS region showed a great genetic diversity of *B. bovis* isolates among sampling locations, but a less diversity was found in *B. bigemina* ITS isolates. These data revealed the current distribution of *B. bovis* and *B. bigemina* infection in cattle in Indonesia. The rate of infection varied among sampling locations, cattle breeds, and age groups. Furthermore, *B. bovis* ITS isolates from Indonesia were found to be more diverse genetically than *B. bigemina* ITS isolates. The data presented in this study is necessary to develop an effective strategy for controlling the disease in the country.

Fig. 2. Geographical distribution of the sampling locations

A total of 487 blood samples were collected from clinically healthy cattle from sixteen locations across the Indonesian archipelago: Mandailing Natal (n = 32), Tapanuli Selatan (n = 28), Padang Mangateh (n = 60), Tangerang Regency (n = 18), Bogor Regency (n = 16), Karawang (n = 21), Indramayu (n = 10), Lamongan (n = 40), Jombang (n = 40), Tabalong (n = 60), Bulukumba (n = 74), Dompu (n = 17), Lombok Timur (n = 16), Kupang (n = 19), Manggarai Timur (n = 19), and Malaka (n = 17).

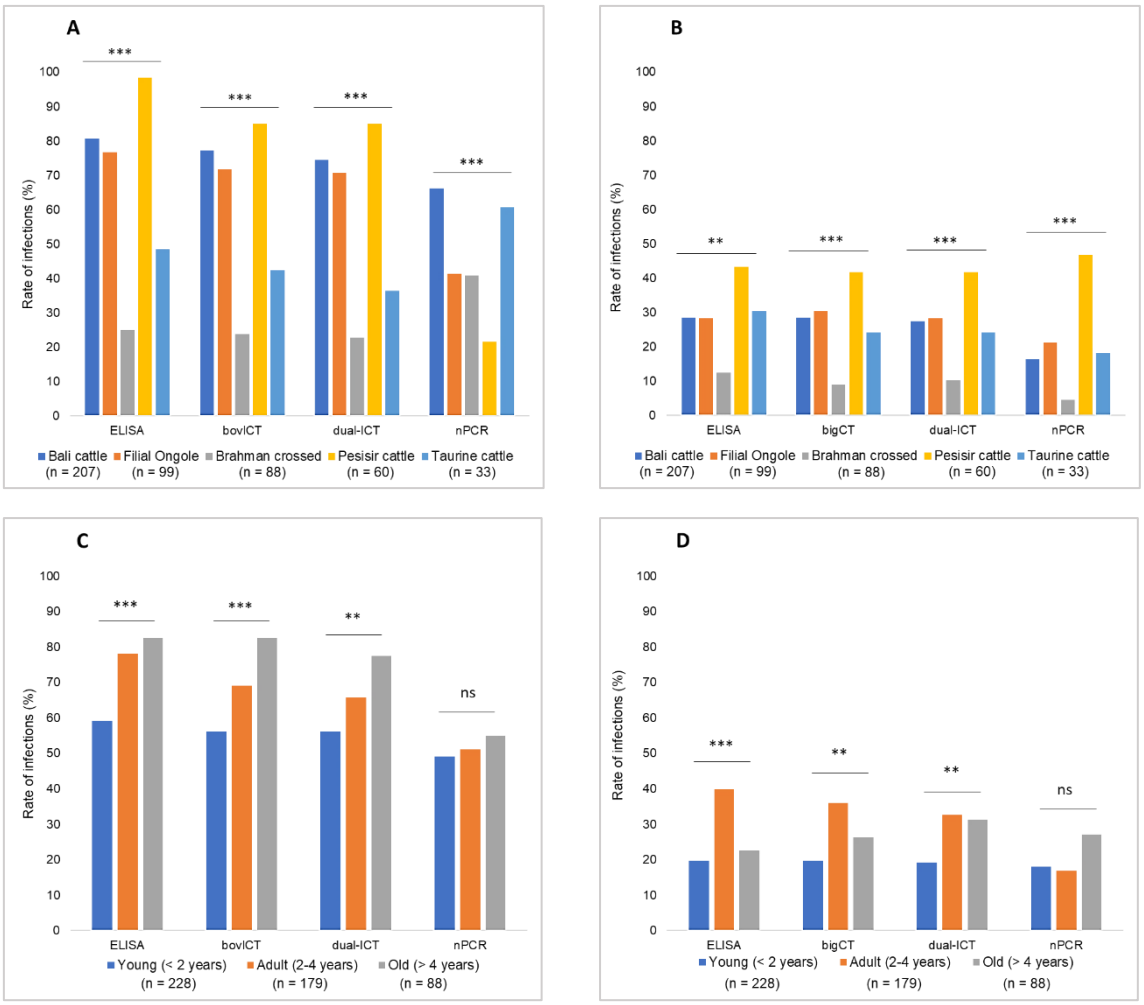

Fig. 3. Comparison of positive rate between the breeds and age groups of cattle. (A) and (B) are the effect of breeds on the infection of *B. bovis* and *B. bigemina*, respectively. (C) and (D) are the effect of age groups on the infection of *B. bovis* and *B. bigemina*, respectively. The data were analyzed using chi-square test. *p* value ≥ 0.05 (not significant, ns), *p* value = 0.01 to 0.05 (significant, *), *p* value = 0.001 to 0.01 (very significant, **), and *p* value < 0.001 (extremely significant, ***).

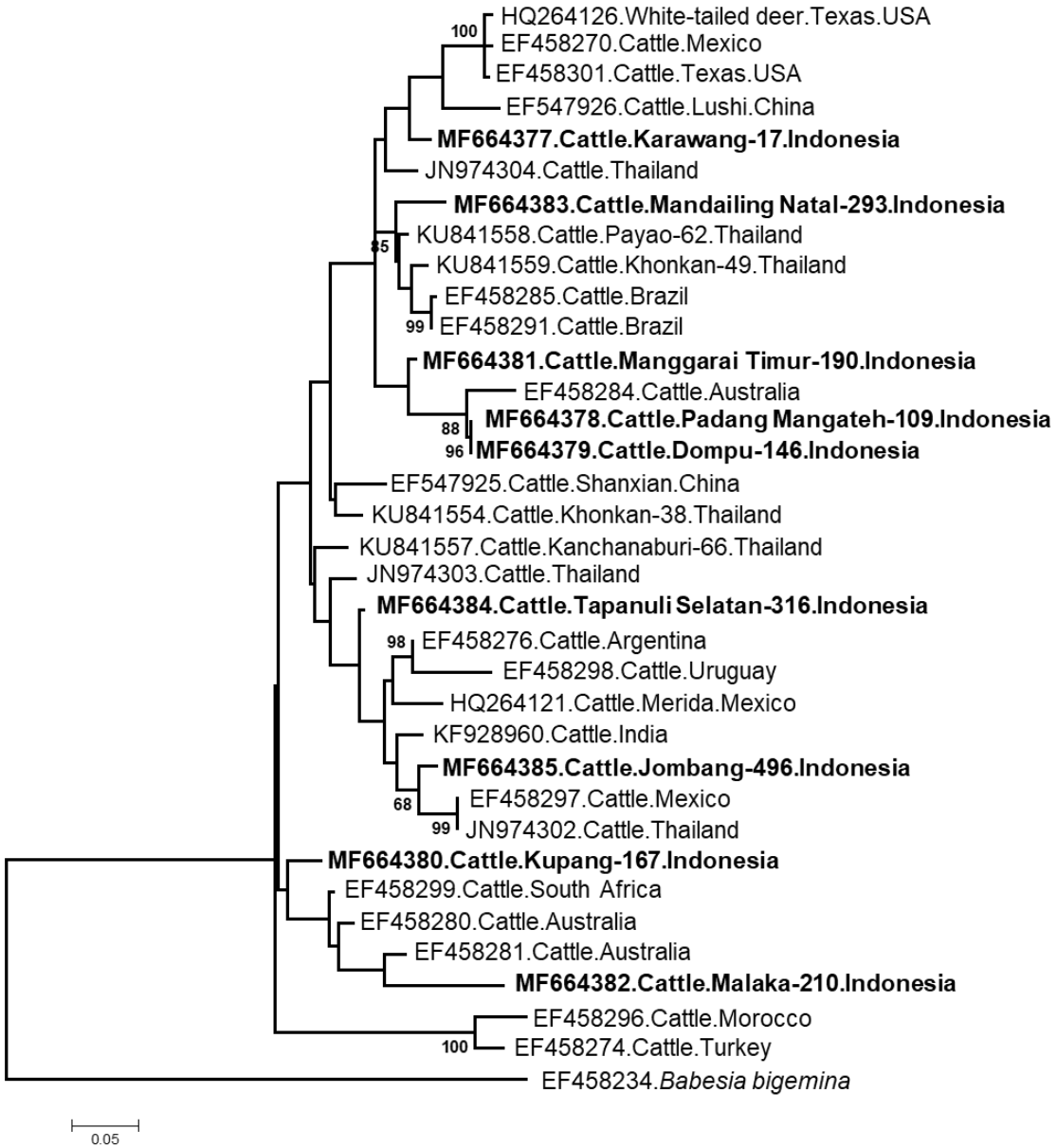

Fig. 4. Phylogenetic tree of *Babesia bovis* ITS1 partial sequence, 5.8s rRNA complete sequence, and ITS2 partial sequence from Indonesian isolates and other sequences from GenBank database. The maximum likelihood method based on the Kimura 2-parameter model with 1000 bootstrap replicates, available in MEGA ver.7, was used to determine the evolutionary history [30, 40]. All positions containing gaps and missing data were eliminated. Indonesian isolates are indicated by bold font.

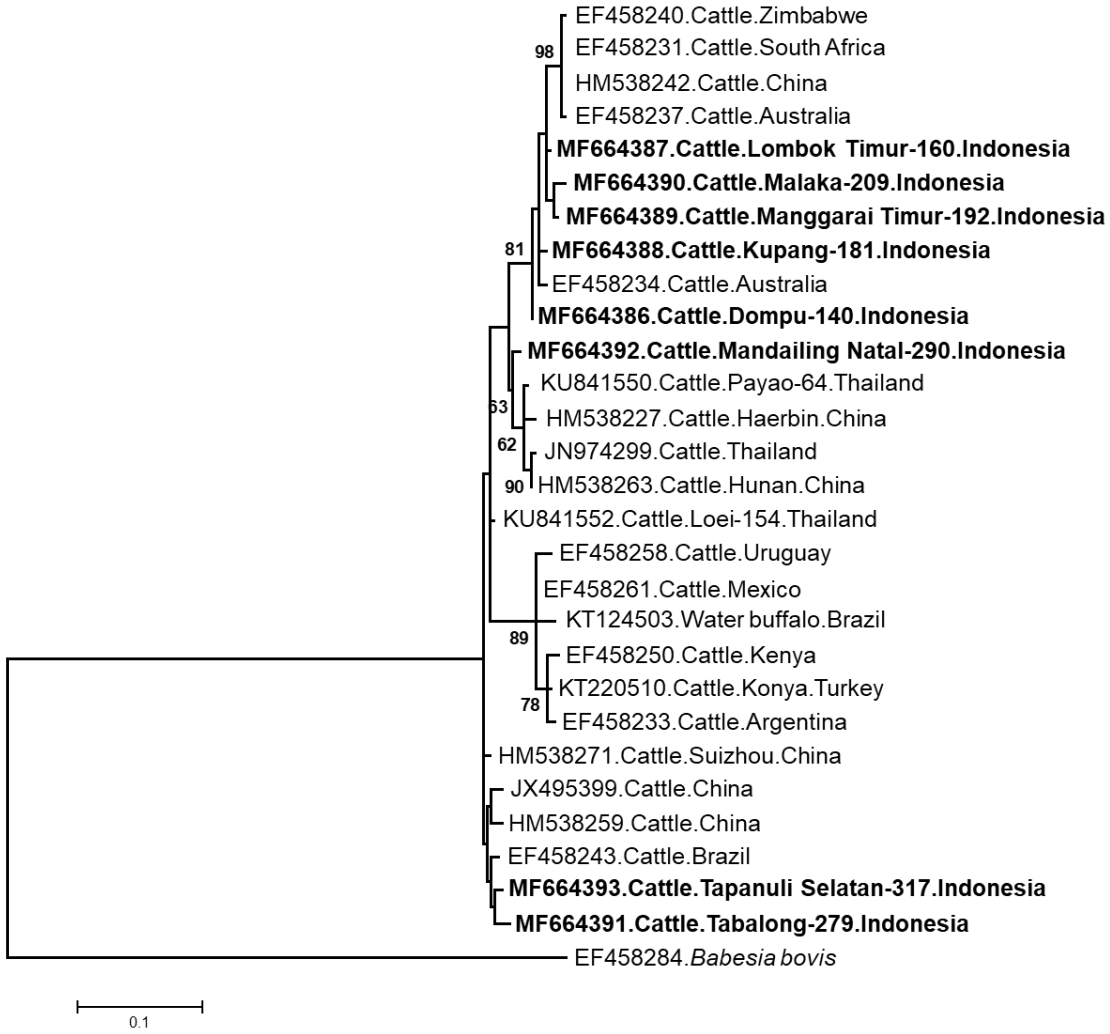

Fig. 5. Phylogenetic tree of *Babesia bigemina* ITS1 partial sequence and 5.8s rRNA partial sequence from Indonesian isolates and other sequences from GenBank database. The maximum likelihood method based on the Kimura 2-parameter model with 1000 bootstrap replicates, available in MEGA ver.7, was used to determine the evolutionary history [30, 40]. All positions containing gaps and missing data were eliminated. Indonesian isolates are indicated by bold font.

Fig. 6. Phylogenetic tree of *Babesia bovis* SBP-4 gene sequences. The maximum likelihood method based on the Kimura 2-parameter model with 1000 bootstrap replicates, available in MEGA ver.7, was used to determine the evolutionary history (Kimura, 1980; Kumar *et al.*, 2016). All positions containing gaps and missing data were eliminated. Indonesian *B. bovis* SBP-4 sequences are indicated by diamonds.

Fig. 7. Phylogenetic analysis of *Babesia bigemina* RAP-1a gene sequences. The maximum likelihood method based on the Kimura 2-parameter model with 1000 bootstrap replicates, available in MEGA ver.7, was used to determine the evolutionary history (Kimura, 1980; Kumar *et al*., 2016). All positions containing gaps and missing data were eliminated. Indonesian *B. bigemina* RAP-1a sequences are indicated by diamonds.

Table 4. Target genes and primers used in this study (Terkawi *et al*., 2011c; Cao *et al*., 2012)

Species	Target gene	Methods	Primer name	Oligonucleotide sequences (5' > 3')	Product size (bp)
B. bovis	SBP-4	PCR	bov-SBP-4-F	AGTTGTTGGAGGAGGCTAAT	907
			bov-SBP-4-R	TCCTTCTCGGCGTCCTTTTC	
		nPCR	bov-SBP-4-nF	GAAATCCCTGTTCCAGAG	503
			bov-SBP-4-nR	TCGTTGATAACACTGCAA	
B. bigemina	RAP-1a	PCR	big-RAP-1a-F	GAGTCTGCCAAATCCTTAC	879
			big-RAP-1a-R	TCCTCTACAGCTGCTTCG	
		nPCR	big-RAP-1a-nF	AGCTTGCTTTCACAACTCGCC	412
			big-RAP-1a-nR	TTGGTGCTTTGACCGACGACAT	
B. bovis	ITS region	PCR	bov-ITS-F	CGTCCCTGCCCTTTGTA	815
			bov-ITS-R	TATTTTCTTTTCTGCCGCTT	
		nPCR	bov-ITS-nF	CACCACCAGTGGAAGCAC	545
			bov-ITS-nR	TTGTGCCCCATGGACACT	
B. bigemina	ITS region	PCR	big-ITS-F	CGTCCCTGCCCTTTGTA	1,041
			big-ITS-R	TATTTTCTTTTCTGCCGCTT	
		nPCR	big-ITS-nF	AGTGGTCGGGACTCGTC	495
			big-ITS-nR	AGTACCGCGTGCGAGCAG	

Table 5. ELISA, ICTs, and nPCR results of *Babesia bovis* and *Babesia bigemina* in all sampling locations

Sampling Locations	No. of samples	Positive rate (%)											
		B. bovis				*B. bigemina*				Mixed infection			
		*bov*ELISA	*bov*ICT	dual-ICT	nPCR	*big*ELISA	*big*ICT	dual-ICT	nPCR	*bov/big*-ELISA	*bov/big*-ICT	dual-ICT	nPCR
Karawang	21	18 (85.7)	17 (81.0)	17 (81.0)	7 (33.3)	9 (42.9)	8 (38.1)	8 (38.1)	2 (9.5)	9 (42.9)	8 (38.1)	8 (38.1)	1 (4.8)
Tangerang	18	16 (88.9)	13 (72.2)	14 (77.8)	11 (61.1)	15 (83.3)	14 (77.8)	12 (66.7)	1 (5.6)	14 (77.8)	11 (61.1)	10 (55.6)	0 (0.0)
Bogor	16	13 (81.3)	10 (62.5)	11 (68.8)	7 (43.8)	13 (81.3)	10 (62.5)	10 (62.5)	2 (12.5)	10 (62.5)	6 (37.5)	7 (43.8)	2 (12.5)
Indramayu	10	4 (40.0)	4 (40.0)	4 (40.0)	2 (20.0)	4 (40.0)	4 (40.0)	3 (30.0)	2 (20.0)	2 (20.0)	2 (20.0)	2 (20.0)	0 (0.0)
Padang Mangateh	60	59 (98.3)	51 (85.0)	51 (85.0)	13 (21.7)	26 (43.3)	25 (41.7)	25 (41.7)	28 (46.7)	26 (43.3)	21 (35.0)	21 (35.0)	6 (10.0)
Dompu	17	16 (94.1)	15 (88.2)	15 (88.2)	3 (17.6)	10 (58.8)	10 (58.8)	11 (64.7)	3 (17.6)	10 (58.8)	9 (52.9)	10 (58.8)	1 (5.9)
Lombok Timur	16	9 (56.3)	8 (50.0)	8 (50.0)	14 (87.5)	1 (6.3)	2 (12.5)	2 (12.5)	6 (37.5)	1 (6.3)	2 (12.5)	2 (12.5)	5 (31.3)
Kupang	19	17 (89.5)	18 (94.7)	17 (89.5)	17 (89.5)	2 (10.5)	3 (15.8)	3 (15.8)	5 (26.3)	2 (10.5)	3(15.8)	3 (15.8)	4 (21.1)
Manggarai Timur	19	19 (100.0)	19 (100.0)	19 (100.0)	18 (94.7)	7 (36.8)	7 (36.8)	7 (36.8)	7 (36.8)	7 (36.8)	7 (36.8)	7 (36.8)	7 (36.8)
Malaka	17	17 (100.0)	17 (100.0)	17 (100.0)	14 (82.4)	7 (41.2)	8 (47.1)	8 (47.1)	6 (35.3)	7 (41.2)	8 (47.1)	8 (47.1)	5 (29.4)
Tabalong	60	50 (83.3)	47 (78.3)	40 (66.7)	30 (50.0)	14 (23.3)	11 (18.3)	8 (13.3)	6 (10.0)	13 (21.7)	10 (16.7)	6 (10.0)	5 (8.3)
Mandailing Natal	32	29 (90.6)	27 (84.4)	27 (84.4)	14 (43.8)	9 (28.1)	10 (31.3)	10 (31.3)	12 (37.5)	9 (28.1)	9 (28.1)	9 (28.1)	8 (25.0)
Tapanuli Selatan	28	27 (96.4)	24 (85.7)	23 (82.1)	14 (50.0)	9 (32.1)	11 (39.3)	11 (39.3)	5 (17.9)	8 (28.6)	10 (35.7)	9 (32.1)	3 (10.7)
Bulukumba	74	38 (51.4)	42 (56.8)	39 (52.7)	51 (68.9)	8 (10.8)	7 (9.5)	8 (10.8)	6 (8.1)	7 (9.5)	7 (9.5)	7 (9.5)	5 (6.8)
Lamongan	40	4 (10.0)	2 (5.0)	2 (5.0)	18 (45.0)	0	0	1 (2.5)	1 (2.5)	0	0	0	0 (0.0)
Jombang	40	4 (10.0)	3 (7.5)	3 (7.5)	14 (35.0)	0	0	0	1 (2.5)	0	0	0	0 (0.0)
Total	487	340 (69.8)	317 (65.1)	307 (63.0)	247 (50.7)	134 (27.5)	130 (26.7)	127 (26.1)	93 (19.1)	125 (25.7)	113 (23.2)	109 (22.4)	52 (10.7)

Table 6. Agreement between serological methods

Diagnostic methods	Kappa Value	95% CI[a]	Agreement[b]
*bov*ICT and *bov*ELISA	0.744	0.688 - 0.800	Good
dual-ICT and *bov*ELISA	0.743	0.687 - 0.798	Good
*bov*ICT and dual-ICT	0.927	0.896 - 0.957	Very good
*big*ICT and *big*ELISA	0.841	0.793 - 0.889	Very good
dual-ICT and *big*ELISA	0.748	0.689 - 0.808	Good
*big*ICT and dual-ICT	0.888	0.847 - 0.929	Very good

[a] 95% confidence interval

[b] Agreement was analyzed using kappa statistics and stated as poor (< 0.20), fair (0.21–0.40), moderate (0.41–0.60), good (0.61–0.80), or very good (0.81–1.00) (Fleiss, 2013).

Table 7. Comparison of the results summary of ELISA, ICT, and nested PCR

Species		ELISA	*bov*ICT/*big*ICT			Dual-ICT			Nested PCR		
			(+)	(-)	*p* value	(+)	(-)	*p* value	(+)	(-)	*p* value
B. bovis	(+)	340 (69.8)	303 (62.2)	37 (7.6)	< 0.05	299 (61.4)	41 (8.4)	< 0.05	174 (35.7)	166 (34.1)	0.707
	(-)	147 (30.2)	14 (2.9)	133 (27.3)		8 (1.6)	139 (28.5)		73 (15.0)	74 (15.2)	
	Total	487	317 (65.1)	170 (34.9)		307 (63.0)	180 (37.0)		247 (50.7)	240 (49.3)	
B. bigemina	(+)	134 (27.5)	121 (24.8)	13 (2.7)	< 0.05	110 (22.6)	24 (4.9)	< 0.05	36 (7.4)	98 (20.1)	< 0.05
	(-)	353 (72.5)	9 (1.8)	344 (70.6)		17 (3.5)	336 (69.0)		57 (11.7)	296 (60.8)	
	Total	487	130 (26.7)	357 (73.3)		127 (26.1)	360 (73.9)		93 (19.1)	394 (80.9)	

Table 8. Nucleotide polymorphisms analysis of *B. bovis* and *B. bigemina* isolates from Indonesia

Target gene	*n*	No. of sites	*h*	*hd*	π	*k*
B. bovis SBP-4	16	512	12	0.942	0.018	9.142
B. bigemina RAP-1a	13	412	10	0.949	0.028	10.590
B. bovis ITS region	9	538	8	0.972	0.079	35.889
B. bigemina ITS region	8	500	8	1.000	0.032	15.786

n, number of sequences; h, number of haplotype; *hd*, haplotype diversity; π, nucleotide diversity; *k*, average number of nucleotide differences

Table 9a. Expected conditional probability of mixed infections using ELISA

No	Locations	No. of sample	Positive sample			Observed				Expected				Chi square	*p* value	Bonferroni-corrected *p*-value	Bonferroni-corrected significance
			B. bovis *bov* ELISA	*B. bigemina* *big* ELISA	Mixed *bov/big* ELISA	*bov +* *big +*	*bov +* *big -*	*bov -* *big +*	*bov -* *big -*	*bov +* *big +*	*bov +* *big -*	*bov -* *big +*	*bov -* *big -*				
1	Karawang	21	18	9	9	9	9	0	3	7.7	10.3	1.3	1.7	2.62	0.11	0.0031	ns
2	Tangerang	18	16	15	14	14	2	1	1	13.3	2.7	1.7	0.3	1.8	0.18	0.0031	ns
3	Bogor	16	13	13	10	10	3	3	0	10.6	2.4	2.4	0.6	0.85	0.36	0.0031	ns
4	Indramayu	10	4	4	2	2	2	2	4	1.6	2.4	2.4	3.6	0.28	0.6	0.0031	ns
5	Padang Mengatas	60	59	26	26	26	33	0	1	25.6	33.4	0.4	0.6	0.778	0.378	0.0031	ns
6	Dompu	17	16	10	10	10	6	0	1	9.4	6.6	0.6	0.4	1.52	0.22	0.0031	ns
7	Lombok Timur	16	9	1	1	1	8	0	7	0.6	8.4	0.4	6.6	0.83	0.36	0.0031	ns
8	Kupang	19	17	2	2	2	15	0	2	1.8	15.2	0.2	1.8	0.26	0.61	0.0031	ns
9	Manggarai Timur	19	19	7	7	7	12	0	0	7	12	0	0	-	-	0.0031	ns
10	Malaka	17	17	7	7	7	10	0	0	7	10	0	0	-	-	0.0031	ns
11	Tabalong	60	50	14	13	13	37	1	9	11.7	38.3	2.3	7.7	1.193	0.275	0.0031	ns
12	Mandailing Natal	32	29	9	9	9	20	0	3	8.2	20.8	0.8	2.2	1.295	0.255	0.0031	ns
13	Tapanuli Selatan	28	27	9	8	8	19	1	0	8.7	18.3	0.3	0.7	2.19	0.14	0.0031	ns
14	Bulukumba	74	38	8	7	7	31	1	35	4.1	33.9	3.9	32.1	4.692	0.03	0.0031	ns
15	Lamongan	40	4	0	0	0	4	0	36	0	4	0	36	-	-	0.0031	ns
16	Jombang	40	4	0	0	0	4	0	36	0	4	0	36	-	-	0.0031	ns
	Total	487	340	134	125	125	215	9	138	93.6	246.4	40.4	106.6	48.316	<0.0001		

Critical value: 0.05 Corrected critical value: 0.0031

Number of tests: 16

ns: not significant

bov: *B. bovis*

big: *B. bigemina*

Table 9b. Expected conditional probability of mixed infections using single ICT

No	Locations	No. of sample	Positive sample			Observed				Expected				Chi square	*p* value	Bonferroni-corrected *p*-value	Bonferroni-corrected significance
			B. bovis bov-ICT	*B. bigemina big*-ICT	Mixed *bov/big*-ICT	*bov* + *big* +	*bov* + *big* -	*bov* - *big* +	*bov* - *big* -	*bov* + *big* +	*bov* + *big* -	*bov* - *big* +	*bov* - *big* -				
1	Karawang	21	17	8	8	8	9	0	4	6.5	10.5	1.5	2.5	3.04	0.08	0.0031	ns
2	Tangerang	18	13	14	11	11	2	3	2	10.1	2.9	3.9	1.1	1.27	0.26	0.0031	ns
3	Bogor	16	10	10	6	6	4	4	2	6.2	3.8	3.8	2.2	0.07	0.79	0.0031	ns
4	Indramayu	10	4	4	2	2	2	2	4	1.6	2.4	2.4	3.6	0.28	0.6	0.0031	ns
5	Padang Mengatas	60	51	25	21	21	30	4	5	21.2	29.8	3.8	5.2	0.034	0.855	0.0031	ns
6	Dompu	17	15	10	9	9	6	1	1	8.8	6.2	1.2	0.8	0.07	0.79	0.0031	ns
7	Lombok Timur	16	8	2	2	2	6	0	8	1	7	1	7	2.29	0.13	0.0031	ns
8	Kupang	19	18	3	3	3	15	0	1	2.8	15.2	0.2	0.8	0.2	0.66	0.0031	ns
9	Manggarai Timur	19	19	7	7	7	12	0	0	7	1	0	0	-	-	0.0031	ns
10	Malaka	17	17	8	8	8	9	0	0	8	9	0	0	-	-	0.0031	ns
11	Tabalong	60	47	11	10	10	37	1	12	8.6	38.4	2.4	10.6	1.255	0.263	0.0031	ns
12	Mandailing Natal	32	27	10	9	9	18	1	4	8.4	18.6	1.6	3.4	0.35	0.55	0.0031	ns
13	Tapanuli Selatan	28	24	11	10	10	14	1	3	9.4	14.6	1.6	2.4	0.4	0.53	0.0031	ns
14	Bulukumba	74	42	7	7	7	35	0	32	4	38	3	29	5.891	0.015	0.0031	ns
15	Lamongan	40	2	0	0	0	2	0	38	0	2	0	38	-	-	0.0031	ns
16	Jombang	40	3	0	0	0	3	0	37	0	3	0	37	-	-	0.0031	ns
	Total	487	317	130	113	113	204	17	153	84.6	232.4	45.4	124.6	37.195	<0.0001		

Critical value: 0.05 Corrected critical value: 0.0031

Number of tests: 16

ns: not significant

bov: *B. bovis*

big: *B. bigemina*

Table 9c. Expected conditional probability of mixed infections using dual-ICT

No	Locations	No. of sample	Positive sample			Observed				Expected				Chi square	*p* value	Bonferroni-corrected *p*-value	Bonferroni-corrected significance
			B. bovis dual-ICT	*B. bigemina* dual-ICT	Mixed dual-ICT	*bov* + *big* +	*bov* + *big* -	*bov* - *big* +	*bov* - *big* -	*bov* + *big* +	*bov* + *big* -	*bov* - *big* +	*bov* - *big* -				
1	Karawang	21	17	8	8	8	9	0	4	6.5	10.5	1.5	2.5	3.04	0.08	0.0031	ns
2	Tangerang	18	14	12	10	10	4	2	2	9.3	4.7	2.7	1	0.64	0.42	0.0031	ns
3	Bogor	16	11	10	7	7	4	3	2	6.9	4.1	3.1	1.9	0.02	0.89	0.0031	ns
4	Indramayu	10	4	3	2	2	2	1	5	1.2	2.8	1.8	4.2	1.27	0.26	0.0031	ns
5	Padang Mengatas	60	51	25	21	21	30	4	5	21.2	29.8	3.8	5.2	0.034	0.855	0.0031	ns
6	Dompu	17	15	11	10	10	5	1	1	9.7	5.3	1.3	0.7	0.22	0.64	0.0031	ns
7	Lombok Timur	16	8	2	2	2	6	0	8	1	7	1	7	2.29	0.13	0.0031	ns
8	Kupang	19	17	3	3	3	14	0	2	2.7	14.3	0.3	1.7	0.42	0.52	0.0031	ns
9	Manggarai Timur	19	19	7	7	7	12	0	0	7	12	0	0	-	-	0.0031	ns
10	Malaka	17	17	8	8	8	9	0	0	8	9	0	0	-	-	0.0031	ns
11	Tabalong	60	40	8	6	6	34	2	18	5.3	34.7	2.7	17.3	0.288	0.591	0.0031	ns
12	Mandailing Natal	32	27	10	9	9	18	1	4	8.4	18.6	1.6	3.4	0.35	0.55	0.0031	ns
13	Tapanuli Selatan	28	23	11	9	9	14	2	3	9	14	2	3	0	0.97	0.0031	ns
14	Bulukumba	74	39	8	7	7	32	1	34	4.2	34.8	3.8	31.2	4.357	0.037	0.0031	ns
15	Lamongan	40	2	1	0	0	2	1	37	0	2	1	37	0.05	0.82	0.0031	ns
16	Jombang	40	3	0	0	0	3	0	37	0	3	0	37	-	-	0.0031	ns
	Total	487	307	127	109	109	198	18	162	80.1	226.9	46.9	133.1	38.29	<0.0001		

Critical value: 0.05 Corrected critical value: 0.0031

Number of tests: 16

ns: not significant

bov: *B. bovis*

big: *B. bigemina*

Table 9d. Expected conditional probability of mixed infections using nPCR

No	Locations	No. of sample	Positive sample			Observed				Expected				Chi square	*p* value	Bonferroni-corrected *p*-value	Bonferroni-corrected significance
			B. bovis nPCR	*B. bigemina* nPCR	Mixed nPCR	*bov* + *big* +	*bov* + *big* -	*bov* - *big* +	*bov* - *big* -	*bov* + *big* +	*bov* + *big* -	*bov* - *big* +	*bov* - *big* -				
1	Karawang	21	7	2	1	1	6	1	13	0.7	6.3	1.3	12.7	0.28	0.6	0.0031	ns
2	Tangerang	18	11	1	0	0	11	1	6	0.6	10.4	0.4	6.6	1.66	0.2	0.0031	ns
3	Bogor	16	7	2	2	2	5	0	9	0.9	6.1	1.1	7.9	2.94	0.09	0.0031	ns
4	Indramayu	10	2	2	0	0	2	2	6	0.4	1.6	1.6	6.4	0.62	0.43	0.0031	ns
5	Padang Mengatas	60	13	28	6	6	7	22	25	6.1	6.9	21.9	25.1	0	0.97	0.0031	ns
6	Dompu	17	3	3	1	1	2	2	12	0.5	2.5	2.5	11.5	0.62	0.43	0.0031	ns
7	Lombok Timur	16	14	6	5	5	9	1	1	5.2	8.8	0.8	1.2	0.15	0.7	0.0031	ns
8	Kupang	19	17	5	4	4	13	1	1	4.5	12.5	0.5	1.5	0.62	0.42	0.0031	ns
9	Manggarai Timur	19	18	7	7	7	11	0	1	6.6	11.4	0.4	0.6	0.62	0.43	0.0031	ns
10	Malaka	17	14	6	5	5	9	1	2	4.9	9.1	1.1	1.9	0.01	0.94	0.0031	ns
11	Tabalong	60	30	6	5	5	25	1	29	3	27	3	27	2.963	0.085	0.0031	ns
12	Mandailing Natal	32	14	12	8	8	6	4	14	5.2	8.8	6.8	11.2	4.1	0.04	0.0031	ns
13	Tapanuli Selatan	28	14	5	3	3	11	2	12	2.5	11.5	2.5	11.5	0.24	0.62	0.0031	ns
14	Bulukumba	74	51	6	5	5	46	1	22	4.1	46.9	1.9	21.1	0.633	0.426	0.0031	ns
15	Lamongan	40	18	1	0	0	18	1	21	0.4	17.6	0.6	21.4	0.84	0.36	0.0031	ns
16	Jombang	40	14	1	0	0	14	1	25	0.4	13.7	0.6	25.4	0.55	0.46	0.0031	ns
	Total	487	247	93	52	52	195	41	199	47.2	199.8	45.8	194.2	1.241	0.265		

Critical value: 0.05 Corrected critical value: 0.0031

Number of tests: 16

ns: not significant

bov: *B. bovis*

big: *B. bigemina*

Table 10a. Percent identity of *Babesia bovis* sbp4 gene sequence among Indonesian isolates determined by Clustal Omega alignment (Li *et al*., 2015)

Percent identity	Divergence																	
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**		
1	100.0	1.2	6.6	6.0	6.0	6.0	5.4	5.4	5.4	5.4	5.4	4.8	4.8	4.8	4.8	4.8	**1**	KY484534.Dompu-146
2	98.8	100.0	6.6	6.6	6.6	6.6	6.0	6.0	6.0	6.0	6.0	5.4	5.4	5.4	5.4	5.4	**2**	KY562845.Malaka-210
3	93.5	93.5	100.0	6.0	6.0	6.0	5.4	5.4	5.4	5.4	5.4	4.8	4.8	4.8	4.8	4.8	**3**	KY484530.Mandailing Natal-293
4	94.0	93.4	94.0	100.0	2.4	2.4	1.8	1.8	1.8	1.8	1.8	1.2	1.2	1.2	1.2	1.2	**4**	KY562847.Jombang-479
5	94.0	93.4	94.0	97.6	100.0	2.4	1.8	1.8	1.8	1.8	1.8	1.2	1.2	1.2	1.2	1.2	**5**	KY484524.Bogor-44
6	94.0	93.4	94.0	97.6	97.6	100.0	1.8	1.8	1.8	1.8	1.8	1.2	1.2	1.2	1.2	1.2	**6**	KY484523.Tangerang-30
7	94.6	94.0	94.6	98.2	98.2	98.2	100.0	1.2	1.2	1.2	1.2	0.6	0.6	0.6	0.6	0.6	**7**	KY562846.Tabalong-231
8	94.6	94.0	94.6	98.2	98.2	98.2	98.8	100.0	1.2	1.2	1.2	0.6	0.6	0.6	0.6	0.6	**8**	KY484533.Lamongan-452
9	94.6	94.0	94.6	98.2	98.2	98.2	98.8	98.8	100.0	1.2	1.2	0.6	0.6	0.6	0.6	0.6	**9**	KY484527.Lombok Timur-162
10	94.6	94.0	94.6	98.2	98.2	98.2	98.8	98.8	98.8	100.0	0.0	0.6	0.6	0.6	0.6	0.6	**10**	KY484522.Karawang-3
11	94.6	94.0	94.6	98.2	98.2	98.2	98.8	98.8	98.8	100.0	100.0	0.6	0.6	0.6	0.6	0.6	**11**	KY484525.Indramayu-70
12	95.2	94.6	95.2	98.8	98.8	98.8	99.4	99.4	99.4	99.4	99.4	100.0	0.0	0.0	0.0	0.0	**12**	KY484526.Padang Mangateh-109
13	95.2	94.6	95.2	98.8	98.8	98.8	99.4	99.4	99.4	99.4	99.4	100.0	100.0	0.0	0.0	0.0	**13**	KY484528.Kupang-170
14	95.2	94.6	95.2	98.8	98.8	98.8	99.4	99.4	99.4	99.4	99.4	100.0	100.0	100.0	0.0	0.0	**14**	KY484529.Manggarai Timur-188
15	95.2	94.6	95.2	98.8	98.8	98.8	99.4	99.4	99.4	99.4	99.4	100.0	100.0	100.0	100.0	0.0	**15**	KY484531.Tapanuli Selatan-337
16	95.2	94.6	95.2	98.8	98.8	98.8	99.4	99.4	99.4	99.4	99.4	100.0	100.0	100.0	100.0	100.0	**16**	KY484532-Bulukumba-364
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**		

Table 10b. Percent identity of *Babesia bigemina* rap-1a gene sequence among Indonesian isolates determined by Clustal Omega alignment (Li *et al*., 2015)

Percent identity	Divergence														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**		
1	100.0	0.7	0.5	1.0	1.0	0.7	0.7	3.6	3.6	3.6	4.4	5.6	5.1	**1**	KY484521.Jombang-480
2	99.3	100.0	0.2	0.7	0.7	0.5	0.5	3.6	3.6	3.6	4.4	5.6	5.1	**2**	KY484510.Karawang-6
3	99.5	99.8	100.0	0.5	0.5	0.2	0.2	3.4	3.4	3.4	4.1	5.3	4.9	**3**	KY484520.Lamongan-448
4	99.0	99.3	99.5	100.0	0.5	0.2	0.2	3.4	3.4	3.4	4.1	5.3	4.9	**4**	KY484519.Bulukumba-366
5	99.0	99.3	99.5	99.5	100.0	0.2	0.2	3.4	3.4	3.4	4.1	5.3	4.9	**5**	KY484512.Padang Mangateh-84
6	99.3	99.5	99.8	99.8	99.8	100.0	0.0	3.2	3.2	3.2	3.9	5.1	4.6	**6**	KY484518.Mandailing Natal-290
7	99.3	99.5	99.8	99.8	99.8	100.0	100.0	3.2	3.2	3.2	3.9	5.1	4.6	**7**	KY562848.Bogor-43
8	96.4	96.4	96.6	96.6	96.6	96.8	96.8	100.0	0.0	0.0	0.7	1.9	1.5	**8**	KY484511.Tangerang-38
9	96.4	96.4	96.6	96.6	96.6	96.8	96.8	100.0	100.0	0.0	0.7	1.9	1.5	**9**	KY484513.Lombok Timur-160
10	96.4	96.4	96.6	96.6	96.6	96.8	96.8	100.0	100.0	100.0	0.7	1.9	1.5	**10**	KY484515.Manggarai Timur-192
11	95.6	95.6	95.9	95.9	95.9	96.1	96.1	99.3	99.3	99.3	100.0	2.7	2.2	**11**	KY484517.Tabalong-239
12	94.4	94.4	94.7	94.7	94.7	94.9	94.9	98.1	98.1	98.1	97.3	100.0	1.5	**12**	KY484514.Kupang-181
13	94.9	94.9	95.2	95.2	95.2	95.4	95.4	98.5	98.5	98.5	97.8	98.5	100.0	**13**	KY484516.Malaka-209
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**		

Table 10c. Percent identity of *Babesia bovis* ITS region sequence among Indonesian isolates determined by Clustal Omega alignment (Li *et al*., 2015)

Percent identity	Divergence								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1	100	11.3	12.2	14.1	18.2	17.0	17.8	16.3	16.3
2	88.7	100	8.3	13.2	18.6	17.7	16.4	16.6	16.8
3	87.8	91.7	100	8.4	18.1	13.6	17.5	13.8	14.0
4	85.9	86.8	91.6	100	17.1	12.3	15.4	14.0	14.2
5	81.8	81.4	81.9	82.9	100	8.9	16.4	18.1	18.1
6	83.0	82.3	86.5	87.7	91.1	100	17.4	10.7	10.7
7	82.2	83.7	82.6	84.7	83.6	82.6	100	11.0	10.9
8	83.7	83.4	86.2	86.0	81.9	89.3	89.1	100	0.4
9	83.7	83.2	86.1	85.8	81.9	89.3	89.1	99.6	100
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**

1	MF664384.Tapanuli Selatan-316
2	MF664385.Jombang-496
3	MF664377.Karawang-17
4	MF664383.Mandailing Natal-293
5	MF664380.Kupang-167
6	MF664381.Manggarai Timur-190
7	MF664382.Malaka-210
8	MF664378.Padang Mangateh-109
9	MF664379.Dompu-146

Table 10d. Percent identity of *Babesia bigemina* ITS region sequence among Indonesian isolates determined by Clustal Omega alignment (Li *et al*., 2015)

Percent identity	Divergence 1	2	3	4	5	6	7	8		
1	100.0	6.5	6.1	5.7	5.7	4.8	4.1	3.4	**1**	MF664392.Mandailing Natal-290
2	93.5	100.0	2.2	2.6	3.0	3.0	4.3	4.7	**2**	MF664387.Lombok Timur-160
3	93.9	97.8	100.0	2.4	2.0	2.8	4.9	4.9	**3**	MF664390.Malaka-209
4	94.3	97.4	97.6	100.0	2.0	1.6	4.1	4.3	**4**	MF664388.Kupang-181
5	94.3	97.0	98.0	98.0	100.0	1.2	4.1	4.3	**5**	MF664389.Manggarai Timur-192
6	95.2	97.0	97.2	98.4	98.8	100.0	4.1	3.8	**6**	MF664386.Dompu-140
7	95.9	95.8	95.1	96.0	96.0	96.0	100.0	2.4	**7**	MF664393.Tapanuli Selatan-317
8	96.6	95.3	95.1	95.8	95.8	96.2	97.6	100.0	**8**	MF664391.Tabalong-279
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**		

Chapter 3

17-dmag, an Hsp90 inhibitor, inhibits the growth of *Babesia* and *Theileria* parasites *in vitro* and *in vivo*

3-1. Introduction

The chemoprophylaxis agents against babesiosis in livestock industries remain inadequate. After decades of use, diminazene aceturate and imidocarb dipropionate are still the first choices for the treatment of animals (Oliveira and de Freitas, 2015; Suarez and Noh, 2011). However, several problems such as the development of a resistant parasite, toxicity, drug residues, and withdrawal issues constrained the use of these drugs in many countries (Kuttler, 1980; Coldham *et al*., 1995; Baldissera *et al*., 2016b). Furthermore, the drugs are not approved for human medicine. The preferable treatment of babesiosis in humans is the combination of atovaquone and azithromycin due to their low side effects (Krause *et al*., 2000). Yet if atovaquone is used as a single drug, *Plasmodium* rapidly developed a resistance to it (Korsinczky *et al*., 2000). Another report showed the relapse of *Babesia gibsoni* due to the change in amino acid in the mitochondrial cytochrome B that led to the reduced effectiveness of atovaquone (Matsuu *et al*., 2006). Therefore, continuous efforts to discover and develop new effective drugs against babesiosis are important.

Heat shock proteins (Hsps), which are present in most eukaryotes and prokaryotes, are involved in stabilizing their client proteins to enable appropriate functions during a stress or non-stress response (Kumar *et al*., 1990; Ruef *et al*., 2000b). Heat shock protein 90 (Hsp90), one of the Hsp classes, is conserved among organisms (Chen *et al*., 2006). Due to its important role in supporting the cellular mechanism, this protein has been targeted in combating cancer cells in humans (Kim *et al*., 2009). In

protozoan parasites, the protein has been reported to regulate the cellular processes in zoonotic protozoan parasites such as *Plasmodium*, *Toxoplasma*, *Trypanosoma*, and *Leishmania* (Banumathy *et al*., 2003; Angel *et al*., 2013). Furthermore, several studies have shown the effectiveness of Hsp90 as a drug target in infectious diseases (Pizarro *et al*., 2013; Gillan *et al*., 2014).

The first inhibitor found specifically to bind Hsp90 was geldanamycin, which was isolated from the bacterium *Streptomyces hygroscopicus*. The inhibition of geldanamycin on Hsp90 led to the degradation of its client proteins. However, geldanamycin has poor solubility, induces liver damage, and is toxic to the erythrocyte (Jilani *et al*., 2013). Many analogs have been developed on the basis of geldanamycin, such as 17-AAG (tanespimycin, 17-N-allylamino-17-demethoxygeldanamycin) and 17-dmag (alvespimycin, 17-dimethylaminoethylamino-17-demethoxy geldanamycin). 17-AAG has lower hepatoxicity as compared with geldanamycin. It has been evaluated in cancer studies and, in protozoan parasites, showed growth inhibition on the rodent model of malaria (Pallavi *et al*., 2010). However, like geldanamycin, 17-AAG also has poor solubility, and its clinical trial with regard to cancer has been halted. On the other hand, 17-dmag is water soluble and has been evaluated on the *in vitro* culture and mouse model of *Trypanosoma brucei*. The result showed a very low half maximum effective concentration (EC_{50}) on the parasite and a high selectivity index over mammalian cells, resulting in great potency of this compound as an antitrypanosomal agent (Meyer and Shapiro, 2013).

This study aimed to evaluate the inhibition of 17-dmag on the growth of bovine *Babesia* (*B. bovis* and *B. bigemina*), bovine *Babesia* that also is known to infect humans (*B. divergens*), equine piroplasm parasites (*B. caballi* and *T. equi*), and rodent *Babesia* that also infects humans (*B. microti*). The evaluations were performed using *in vitro* cultures of all of those species, but using the mouse model

for *B. microti*. The cytotoxicity of 17-dmag was also evaluated using mammalian and mouse embryonic fibroblast cell lines.

3-2. Materials and methods

Parasites. *Babesia* parasite cultures were maintained for a long time using a microaerophilic stationary-phase culture system (Levy and Ristic, 1980; Avarzed *et al*., 1997; Igarashi *et al*., 1998). Briefly, bovine *Babesia* were grown in bovine red blood cells (RBCs) in a specific complete medium for each species. The medium for *B. bovis* (Texas strain) contained GIT medium supplemented with 10% bovine serum, while the medium for *B. bigemina* (Argentina strain) and *B. divergens* (German strain) was Medium 199 and RPMI 1640 medium, respectively, supplemented with 40% bovine serum (Lengauer *et al*., 2006). *B. caballi* (USDA strain) was grown using equine RBCs in GIT medium supplemented with 10% equine serum. *T. equi* (USDA strain) was grown in equine RBCs in an M199 medium supplemented with 40% equine serum and hypoxanthine (MP Biomedicals, USA) at a final concentration of 13.6 μg/ml. All media included 60 U/ml penicillin G, 60 μg/ml streptomycin, and 0.15 μg/ml amphotericin B (Sigma-Aldrich, USA). The cultures were incubated at 37°C in a humidified chamber with an atmosphere of 5% CO_2, 5% O_2, and 90% N_2.

B. microti (Munich strain) was recovered from -80ºC stock and infect to 2 six-week female Balb/c mice (Clea, Japan). The parasitemia was monitored every two days. After parasitemia reached approximately 30%, mice were euthanized, and the blood was collected by cardiac puncture to initiate the experiment (Goo *et al*., 2010). The animal experiment was conducted in accordance with the Regulations for Animal Experiments of Obihiro University of Agriculture and Veterinary Medicine, Japan (Accession numbers 28-111-2, 28-110, and 1417-2).

Reagents and chemicals. 17-dmag (DM, Focus Biomolecules, USA), diminazene aceturate (DA, Sigma-Aldrich, Japan), and atovaquone (AV, Sigma-Aldrich, Japan) were diluted in DMSO to make a 10-mM stock solution and stored at -30ºC until use. A lysis buffer containing tris-HCl (130 mM; pH 7.5), EDTA (10 mM), saponin (0.016%; w/v), and Triton X-100 (1.6% v/v) was prepared beforehand and stored at 4°C. Prior to fluorescence evaluations, the lysis buffer was mixed with 0.2 µl/ml of SYBR Green I (10,000x, Lonza, USA).

Effect of 17-dmag on bovines, equine erythrocytes, and healthy mice. Prior to the subculturing of parasites, bovine and equine RBCs were incubated with 1 µM 17-dmag for 3 h. The RBCs were washed three times with PBS and mixed with *B. bovis* and *T. equi* parasitized RBCs to obtain 1% parasitemia (infected RBCs). Subsequently, 100 µl of infected RBCs was added to 900 µl of complete medium in a 24-well plate. The untreated erythrocytes were used as the control. The growth of parasites was monitored daily using microscopic observation of a Giemsa-stained blood smear for 4 days. Furthermore, the effect of 17-dmag on healthy mice was evaluated using 8-week-old BALB/c mice. Groups 1 and 2, consisting of three mice each, received an intraperitoneal injection of 17-dmag at a concentration of 15 and 30 mg/kg BW, respectively, for five days. One group (3 mice) remained untreated as a healthy control. The changes in body weight and hematology profiles, including RBC, hematocrit, and hemoglobin, were observed for 20 days.

The growth inhibition assay of 17-dmag and combination with diminazene aceturate and atovaquone *in vitro*. The assays were conducted in accordance with the protocol described previously (Guswanto *et al*., 2014; Rizk *et al*., 2015). Briefly, the cultures of *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi* with a parasitemia of 8%, 3%, 15%, 4%, and 9%, respectively, were harvested and adjusted to 1% parasitemia before the inhibition assay. The sixty inner wells of 96-well plate were

used in the assay, while the peripheral wells were filled with sterile distillate water to reduce the evaporation during incubation. To each well in triplicate, 2.5 µl of infected RBCs (5 µl for *B. divergens*, *B. caballi*, and *T. equi*) was added and mixed with a culture medium containing the drug to the total volume of 100 µl. 17-dmag, DA, and AV were dissolved in the culture medium at various concentrations using 2-fold dilution. A culture medium containing DMSO at the final concentration of 0.2% was added to the well with infected RBCs as a positive control and to the well with uninfected RBCs as a negative control. Furthermore, a drug combination assay was conducted in parallel with the single drug assay at a constant ratio in accordance with the recommendation of the Chou–Talalay method (Chou, 2006). In the same plate with the single-drug inhibition assay, two-drug combinations (DA+DM, DA+AV, and DM+AV) at concentrations of 0.25 x IC_{50}, 0.5 x IC_{50}, 1 x IC_{50}, 2 x IC_{50}, and 4 x IC_{50} were added to the well containing infected RBCs in triplicate. The plate was placed in a humidified incubator with 5% CO_2, 5% O_2, and 90% N_2. After four days, 100 µl of a lysis buffer containing 2x SYBR Green I was added to each well and mixed, then incubated at room temperature, avoiding direct light exposure. After incubation for 6 h, fluorescence values were measured using a fluorescence spectrophotometer (Fluoroskan Ascent, Thermo Scientific, USA), with the excitation and emission wavelength at 485 nm and 518 nm, respectively. The fluorescence data were subtracted from the negative control and used to calculate the half maximum of inhibition concentration (IC_{50}). The experiment was repeated three times.

Cell cultures. Madin-Darby bovine kidney (MDBK) and mouse embryonic fibroblast (NIH/3T3) cells were cultured continuously at 37ºC in a humidified incubator with 5% CO_2. MDBK and NIH/3T3 were maintained in 75-cm^2 culture flasks with Minimum Essential Medium Eagle (MEM, Gibco, Life Technologies, USA) and Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Life

Technologies, USA), respectively. Each medium was supplemented with 10% fetal bovine serum, 0.5% Pen/Strep (Gibco, Life Technologies, USA), and 1% glutamine. The medium was changed every 3 to 4 days and incubated until approximately 80% confluent. The cells were free from mycoplasma contamination after being checked by staining with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich, USA). The detachment of cells from the culture flask was done using TrypLE^{TM} Express (Gibco, Life Technologies, USA) after washing two times with Dulbecco's phosphate-buffered saline (DPBS). Subsequently, the counting of viable cells was carried out using a Neubauer improved C-Chip (NanoEntek Inc., Korea) after staining with 0.4% trypan blue solution.

Cytotoxicity assay of 17-dmag, diminazene aceturate, and atovaquone on MDBK and NIH/3T3 cell lines. The drug-exposure viability assay was performed in accordance with the recommendation of Cell Counting Kit-8 (CCK-8, Dojindo, Japan). The assay was carried out using a 96-well plate at 37ºC in a humidified incubator with 5% CO_2. One hundred microliters of cells at a density of 5 x 10^4 cells/ml was seeded per well and allowed to attach to the plate for 24 h. Ten microliters of 2-fold drug dilutions was added to each well to a final concentration of 0.312 to 40 µM in triplicate. The wells with only a culture medium were used as blanks, while the wells containing cells and a medium with 0.4% DMSO were used as a positive control. The exposure of drugs was carried out for 24 h, followed by the addition of 10 µl of CCK-8. The plate was further incubated for 3 h, and the absorbance was measured at 450 nm using a microplate reader.

The growth inhibition assay using a *B. microti*-infected mouse model. The inhibitory effect of 17-dmag was also determined using a *B. microti* mouse model according to the previous protocol (Goo *et al*., 2010). Briefly, 40 female BALB/c mice aged 8 weeks were divided into 8 groups (5 mice/group). All mice received an intraperitoneal injection of 1 x 10^7 of *B. microti*–infected RBCs,

except 1 group remains uninfected. The experiment was conducted twice, so resulted data were the average from 10 mice in each group. The growth of *B. microti* was observed from blood smears after staining with Giemsa using a light microscope. Subsequently, the drug treatment was initiated after the parasitemia reached approximately 1%. Group 1 was treated intraperitoneally with 4% DMSO in saline water as a control. Groups 2–4 received an intraperitoneal injection of 30 mg/kg BW of DM, one of 25 mg/kg BW of DA, and oral administration of 20 mg/kg BW of AV, respectively. Groups 5–7 were treated with a combination of DM+DA (15+15 mg/kg BW), DM+AV (15+10 mg/kg BW), and DA+AV (15+10 mg/kg BW) with a route similar to that of single drug, respectively. The uninfected group was used as healthy control. The drug treatment was conducted for 5 days. The rate of parasitemia, body weight, and hematology profiles was monitored for 60 days.

Statistical analysis. The half maximum of inhibition concentration (IC_{50}) of 17-dmag, DA, and AV was calculated from the percent of inhibition on the *in vitro* growth of all tested species using nonlinear regression (curve fit), available in GraphPad Prism (GraphPad Software Inc., USA). Combination index (CI) values for the drug combination were calculated using Compusyn software (Chou, 2006). Since the chemotherapy effect is preferable at the highest inhibition, the degree of synergism was determined as the weighted average of CI values using the formula $((1 \times IC_{50}) + (2 \times IC_{75}) + (3 \times IC_{90}) + (4 \times IC_{95}))/10$ (Chou, 2006). The differences between groups regarding parasitemia, hematology profiles, and body weight in the *B. microti*–infected mouse model were analyzed using Student's *t* test, available in GraphPad Prism software. The difference was considered significant if the P value was less than 0.05.

3-3. Results

17-dmag inhibits the growth of *Babesia* and *Theileria* parasites *in vitro*. The preliminary evaluation of 17-dmag was conducted to know its effect on host RBCs. Bovine and equine RBCs were incubated with 17-dmag (1 µM) for 3 h prior to the subculture of *B. bovis* and *T. equi*. Microscopic observation and parasitemia counting showed that the growth of *B. bovis* and *T. equi* did not differ significantly between 17-dmag-treated RBCs and normal RBCs for either species. Evaluation was also conducted using healthy mice. Mouse body weight was reduced slightly in the group with 30 mg/kg BW on day 10 post treatment but was not significantly different compared to the control group, while the group with 15 mg/kg BW did not show any effect (Fig. 12). For the growth inhibitory effect, the assay was conducted on five species: *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*. The result showed that 17-dmag inhibited the growth of all species tested (Fig. 8, 9, and 10). The half maximum inhibitory concentration (IC_{50}) of 17-dmag for *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi* was 77.6 ± 2.9, 62.4 ± 1.9, 173.0 ± 10.9, 88.5 ± 9.6, and 307.7 ± 7.2 nM, respectively (Table 11). In this study, 17-dmag was more effective than DA for *B. bovis*, *B. bigemina*, and *B. divergens*. Furthermore, 17-dmag was more effective for *B. bigemina* and *B. caballi* when compared with the IC_{50} of AV (Table 11).

Combination of 17-dmag with diminazene aceturate and atovaquone on *in vitro* cultures. The combination of 17-dmag with DA and AV was expected to enhance its inhibitory effect while reducing the toxicity (Fig. 11). Five dilutions of 17-dmag, as recommended by the Chou-Talalay method (Chou, 2006), were combined at a constant ratio with DA or AV. The percent of inhibition of a single drug and each combination was analyzed using Compusyn software to generate the combination index (CI) value, dose reduction index, and CI value at IC_{50}, IC_{75}, IC_{90}, and IC_{95}. The

weighted average CI values showed that the combination of 17-dmag with DA was synergistic on *B. bovis*, *B. caballi*, and *T. equi*, while with AV was synergistic on *B. divergens* and *B. caballi* (Table 12). Other species showed additive effects, and none of the combinations showed antagonism.

Toxicity of 17-dmag on MDBK and NIH/3T3 cell lines. 17-dmag showed an inhibitory effect, similar to that of currently used drugs, DA and AV, on the *in vitro* culture of *Babesia* and *Theileria* parasites. The effect of 17-dmag on the host was evaluated using mammalian cells and mouse fibroblast cell lines. The half maximal effective dose (EC_{50}) of 17-dmag on MDBK cells was 8.8 ± 2 µM, while that on NIH/3T3 cells was 15.5 ± 4 µM. The results showed that 17-dmag is more toxic than DA and AV. In a separate assay, DA and AV at the concentration of 40 µM did not show any inhibition on MDBK and NIH/3T3 cell viability. The selectivity index, defined as the ratio of EC_{50} cell lines to the IC_{50} parasite, is shown in Table 11. The high selectivity index is favorable due to the low toxicity to host cells. Even though 17-dmag inhibited the viability of MDBK and NIH/3T3 cell lines, the drug was active in inhibiting the growth of *Babesia* and *Theileria* parasites with an IC_{50} far below the EC_{50} against the cells. Therefore, the generated selectivity index was high. Despite the high selectivity index, DA and AV were still superior to 17-dmag according to their inhibition on host cell viability. For this reason, a drug combination of DA or AV with 17-dmag might be a suitable strategy to retain the effectiveness of 17-dmag while reducing its toxicity.

17-dmag inhibits the growth of *B. microti* using a mouse model. The inhibitory effect of 17-dmag was further evaluated on the growth of *B. microti*. The experiment was carried out using a mouse model, since the *in vitro* culture of *B. microti* is not well established. In the DMSO control group, the growth of *B. microti* increased rapidly and reached the highest parasitemia at 45.6 ± 10.5% on day 9 post infection (p.i.). The parasitemia decreased gradually on the following days. On day 60, *B. microti*

was still detected in 4 mice. *B. microti* in the 17-dmag 30 mg/kg BW group reached peak parasitemia (4.7 ± 2.0%) on day 10 p.i., and no parasites were detected via microscopy from day 28 p.i. A similar inhibition was observed with the DA (25 mg/kg BW) and AV (20 mg/kg BW) groups, but the parasite disappeared from blood smears on day 24 (Fig. 13). However, 17-dmag seems a slow-acting drug compared with DA and AV, because it postponed the peak of parasitemia even though it had an inhibitory effect. The combination of 17-dmag (15 mg/kg BW) with DA (15 mg/kg BW) and AV (10 mg/kg BW) also showed an inhibitory effect comparable with that of a single treatment at a higher dose (Fig. 13). Furthermore, the infection of *B. microti* reduces the hematocrit, RBCs, and hemoglobin concentration in mouse blood, as observed in the DMSO control group on day 9 p.i. (Fig. 14). In contrast, the drug treatments only slightly reduce those profiles when compared with the healthy group. A similar pattern was observed for the body weight of mice.

3-4. Discussion

Treatment of babesiosis remains a significant challenge, especially in the livestock industries. Although diminazene aceturate and imidocarb dipropionate are still effective, current reports showed the development of resistance in *Trypanosoma evansi* in cattle and a relapse of *B. gibsoni* in dogs (Witola *et al*., 2005; Baldissera *et al*., 2016a; Rajapakshage *et al*., 2012). To date, many inhibitors have been reported to be effective against *in vitro* or *in vivo* growth of *Babesia* and *Theileria* parasites such as triclosan (Bork *et al*., 2003), gossypol (Bork *et al*., 2004), nerolidol (AbouLaila *et al*., 2010a), epoxomicin (AbouLaila *et al*., 2010b), artesunate (Goo *et al*., 2010), epigallocatechin gallate (AbouLaila *et al*., 2010c), fusidic acid (Salama *et al*., 2013), allicin (Salama *et al*., 2014), miltefosine (AbouLaila *et al*., 2014), enoxacin (Omar *et al*., 2016), gedunin and nimbolide (Guswanto *et al*., 2014),

bestatin (Aboge *et al*., 2015), and clofazimine (Tuvshintulga *et al*., 2016); however, all of them are in research and still far from clinical trials. Furthermore, the emergence of human babesiosis brings a new urgency to find an effective inhibitor of babesiosis. Atovaquone, which is effective on *Plasmodium*, was also found to inhibit the growth of *Babesia* parasites (Vial and Gorenflot, 2006). Since this drug was reported to be less toxic to host cells, its combination with azithromycin is currently suggested for the treatment of human babesiosis. However, the development of a resistant parasite limited the used of atovaquone. Therefore, the evaluation of a new potent compound against *Babesia* and *Theileria* parasites will provide more options for the treatment of the disease both in humans and domestic animals.

This study showed that 17-dmag inhibits the growth of *Babesia* and *Theileria* parasites *in vitro*. Its ability to bind Hsp90 causes a significant reduction in parasite survival. Hsp90 is conserved in most organisms, and *Babesia* and *Theileria* parasites are no exception. There are some isoforms of Hsp90 in *Babesia bovis*, according to reports on full genome sequences (Brayton *et al*., 2007). These proteins protect organism during physical and non-physical stress, enabling the proper synthesis and stabilization of its client proteins. Targeting Hsp90 is especially relevant for cancer treatment because many Hsp90-related client proteins are involved in the progression of cancer cells (Miyata *et al*., 2013). However, recent studies showed that the inhibition of parasite Hsp90 could disrupt the stage differentiation of parasites such as *Toxoplasma gondii* (Echeverria *et al*., 2005), *Plasmodium falciparum* (Banumathy *et al*., 2003), *Eimeria tenella* (Péroval *et al*., 2006), *Trypanosoma brucei* (Meyer and Shapiro, 2013), and *Theileria annulata* (Kinnaird *et al.*, 2017) that lead to the halt of their growth. In agreement with those previous studies, 17-dmag might be attached to the binding domain

of *Babesia* and *Theileria* parasites Hsp90, causing insufficient binding of the client proteins to Hsp90 and disrupting the normal growth of parasites.

Hsp90 is also conserved in the host cells, which means that the binding of the inhibitor to the host's Hsp90 is possible. For this reason, the inhibitor should specifically bind to the parasite Hsp90 to avoid toxicity to the host. The basis of Hsp90 as a drug target in *Plasmodium falciparum* was the increasing ATPase activities in this species as compared with human Hsp90. The high stress conditions during parasite invasion of host cells might significantly increase the activity of their Hsp90 (Pallavi *et al*., 2010). Our study on MDBK and NIH/3T3 cells showed that the selectivity index of 17-dmag was quite high among the species tested. This means that 17-dmag was more likely to bind to *Babesia* and *Theileria* Hsp90 than host Hsp90. Like *Plasmodium falciparum*, *Babesia* and *Theileria* parasites experience high stress during their invasion of host cells. Further study on the biochemical characterization of the Hsp90 of *Babesia* and *Theileria* is required to better understand the potency of this protein as drug target.

The potency of 17-dmag as an inhibitor was further evaluated in a combination study using DA and AV. The cytotoxicity assay showed that 17-dmag was more toxic than DA and AV. The combination study aimed to enhance the potency of 17-dmag while reducing the dose that led to reduced toxicity. The result demonstrated that the combination of 17-dmag with DA or AV was synergism in some species while others were additive. Therefore, the combination of 17-dmag with DA or AV might have potential as a novel regime for treating piroplasms in animals.

The potent effect of the combination of 17-dmag with DA or DM was also observed on the growth of *B. microti* in the mouse model. The combination of 17-dmag with DA or AV at a half dose showed a growth inhibition comparable to that with the single drugs at a full dose. Hematology profiles such

as RBC count, hematocrit, and hemoglobin were also improved compared with the DMSO control group, in a similar manner to that of parasitemia and mouse body weight. Taken together, 17-dmag is a potent anti-*Babesia* drug as confirmed by the inhibition of six species, *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, *B. microti*, and *T. equi*, both *in vitro* and *in vivo*. Even though 17-dmag showed toxicity in the host cells, its combination with diminazene aceturate and atovaquone could improve the effect while reducing the toxicity.

3-5. Summary

Hsp90 is an important chaperone protein that stabilizes its client proteins to enable appropriate functions during a stress or non-stress response. This protein is a potential drug target to suppress the invasion of a protozoan parasite. In this study, 17-dmag was evaluated for its inhibitory effect on five *in vitro* cultures of *Babesia* and *Theileria* species including *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi* and on the growth of a *B. microti*–infected mouse model. 17-dmag showed an inhibitory effect in all of the species tested. The half maximum of inhibition concentration (IC_{50}) of 17-dmag on the *in vitro* growth of *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi* was 77.6 ± 2.9, 62.4 ± 1.9, 173.0 ± 10.9, 88.5 ± 9.6, and 307.7 ± 7.2 nM, respectively. The toxicity assay on MDBK and NIH/3T3 cell lines showed that 17-dmag affected the viability of cells with half maximum effective concentrations (EC_{50}) of 15.5 ± 4 μM and 8.8 ± 2 μM, respectively. Since the IC_{50} was much lower on the parasites than on the host, the selectivity index was high in all tested species. Furthermore, the two-drug combination of 17-dmag with diminazene aceturate (DA) and atovaquone (AV) showed synergism or addition *in vitro*. In the mouse model, 17-dmag at a concentration of 30 mg/kg BW effectively inhibited the growth of *B. microti*. Additionally, a combination at the half dose of 17-dmag

and DA showed a comparable inhibition if used at the full dose. Taken together, this indicates that 17-dmag is a potent drug for treating babesiosis. Its toxicity can be reduced by combining it with other effective drugs, such as DA and AV. The data warrant further evaluation of 17-dmag as an anti-*Babesia* and as a treatment option in combination with atovaquone for the treatment of human babesiosis.

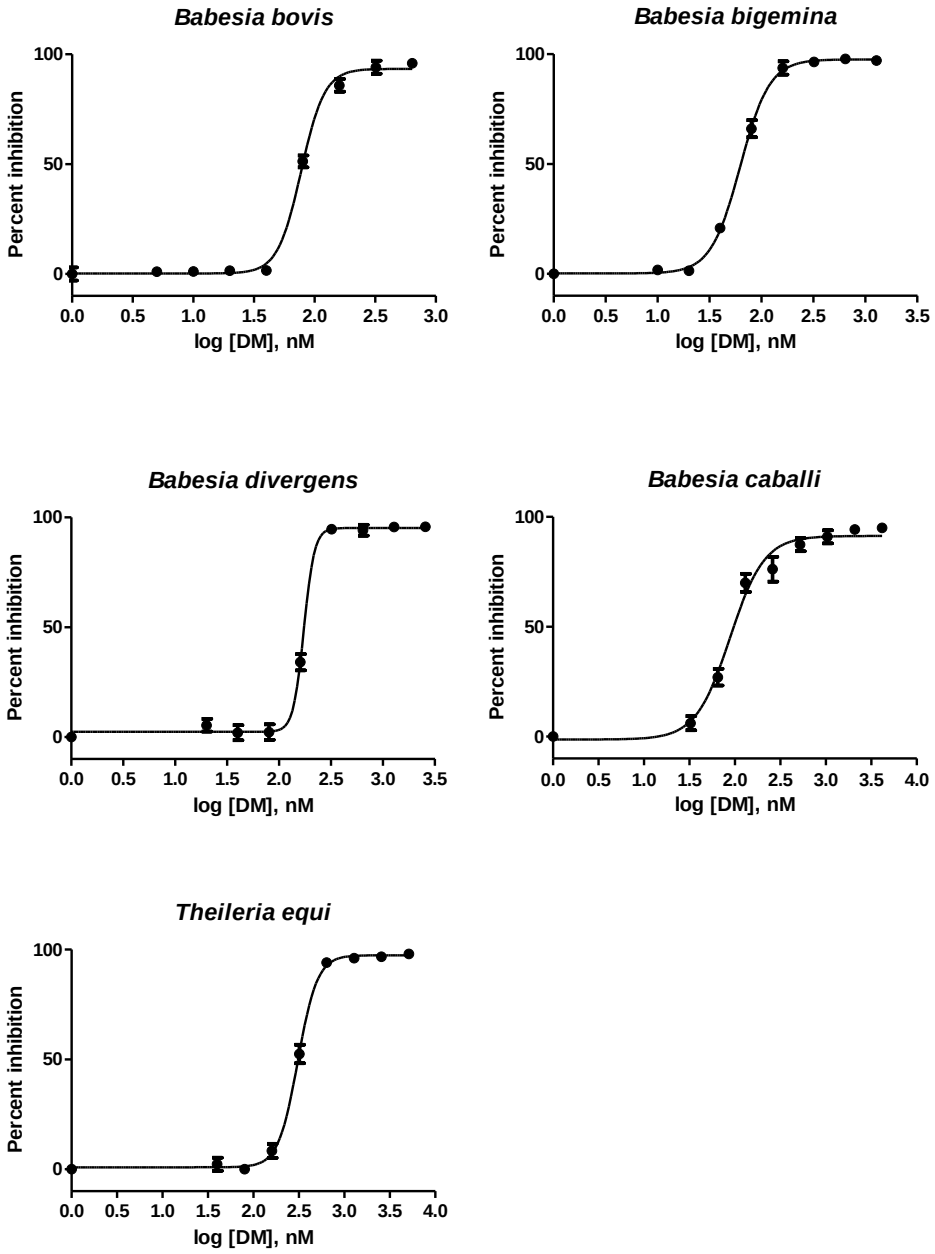

Fig. 8. Dose response curve of 17-dmag on the *in vitro* growth of *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*. Each parasite culture was exposed to various concentrations of 17-dmag for 96 h, and the inhibition was determined using an SYBR Green-based fluorescence assay. The percentage of inhibition is expressed as the percentage of inhibited parasites compared with the positive control (untreated wells) after subtracting the negative control (uninfected RBCs). The values from triplicate experiments are shown.

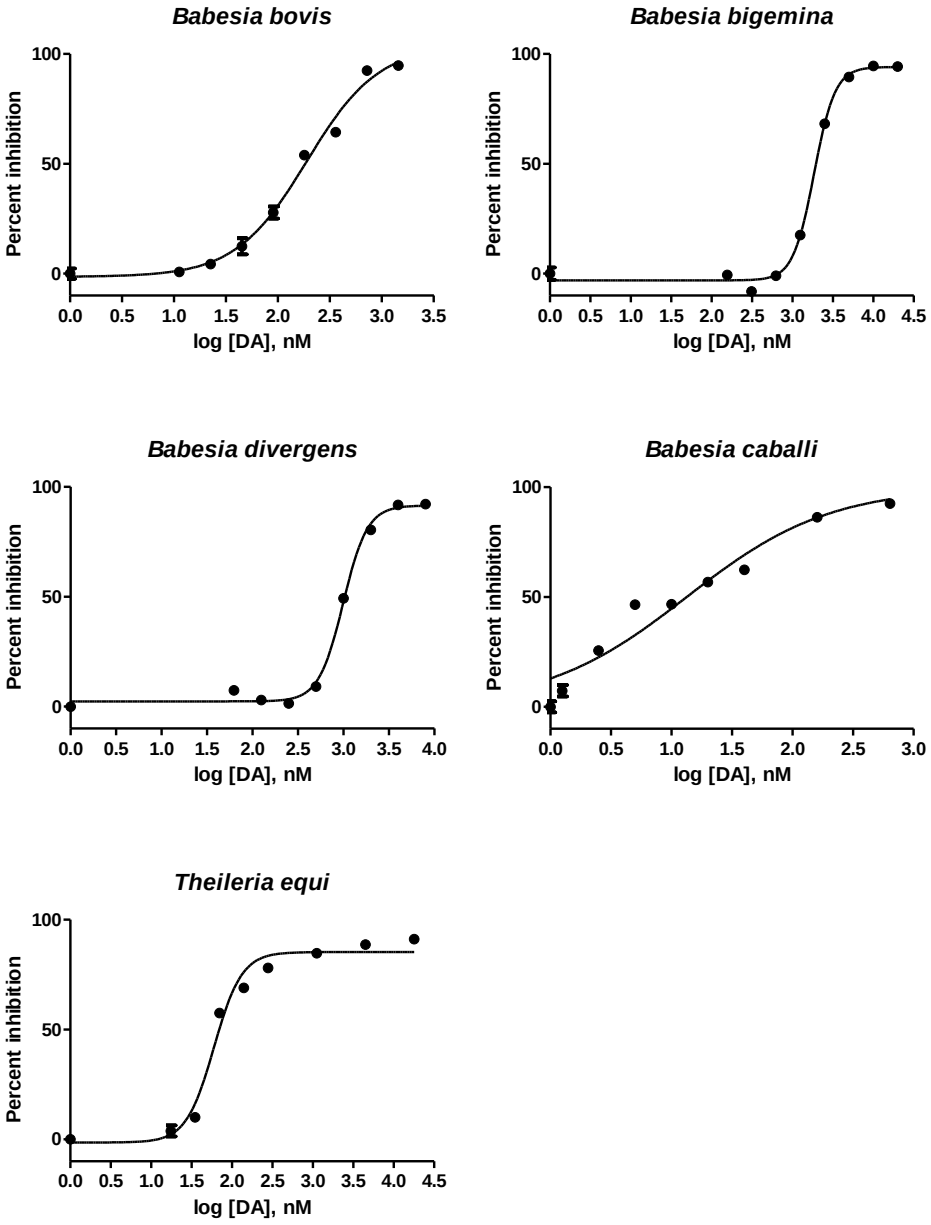

Fig. 9. Dose response curve of diminazene aceturate on the *in vitro* growth of *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*. Each parasite culture was exposed to various concentrations of diminazene aceturate for 96 h, and the inhibition was determined using an SG-based fluorescence assay. The percentage of inhibition is expressed as the percentage of inhibited parasites compared with the positive control (untreated wells) after subtracting the negative control (uninfected RBCs). The values from triplicate experiments are shown.

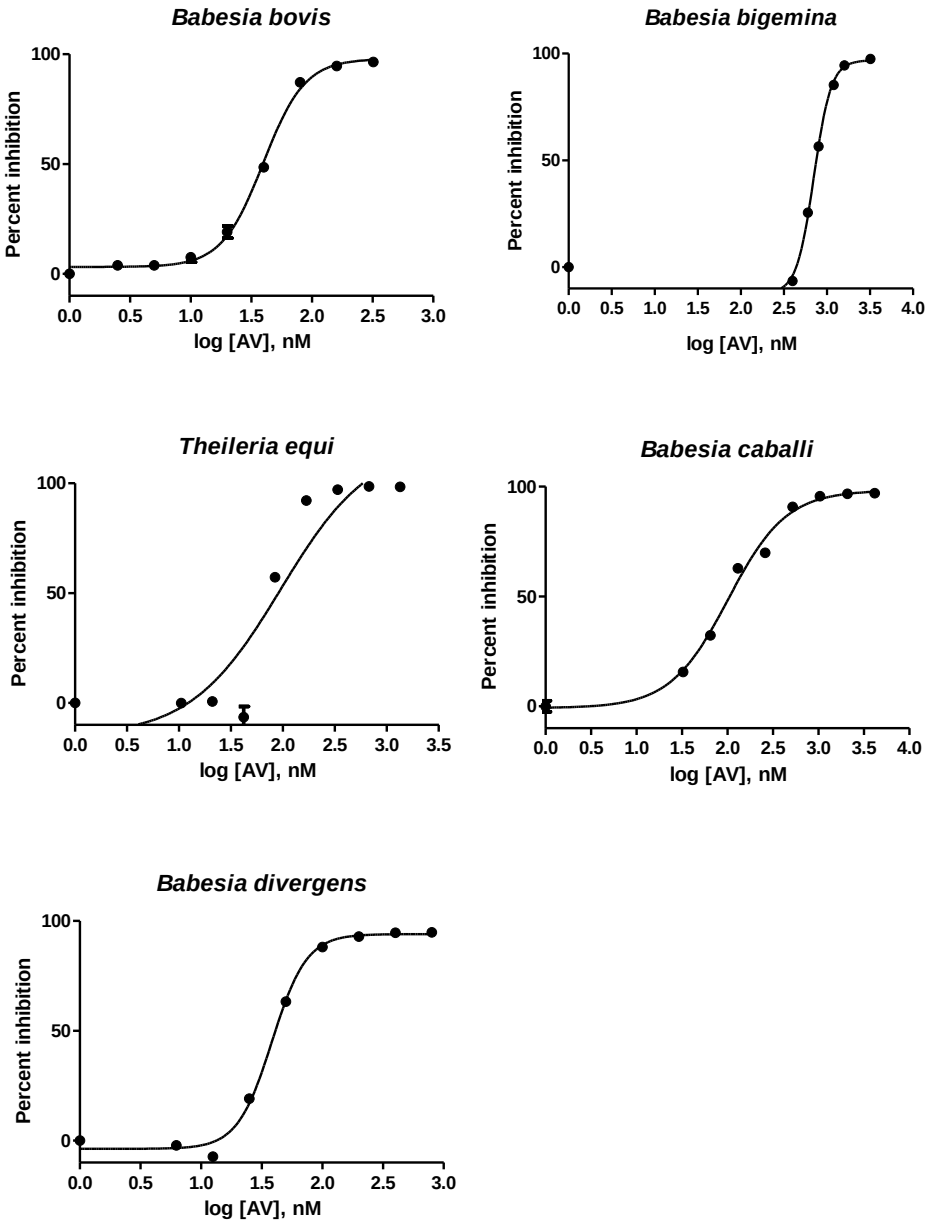

Fig. 10. Dose response curve of atovaquone on the *in vitro* growth of *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*. Each parasite culture was exposed to various concentrations of atovaquone for 96 h, and the inhibition was determined using an SG-based fluorescence assay. The percentage of inhibition is expressed as the percentage of inhibited parasites compared with the positive control (untreated wells) after after subtracting the negative control (uninfected RBCs). The values from triplicate experiments are shown.

Table 11. Selectivity index of 17-dmag, diminazene aceturate, and atovaquone

Drug	Parasites	IC_{50} (nM)[a]	EC_{50} (µM)[b]		Selectivity[c]	
			NIH/3T3	MDBK	NIH/3T3	MDBK
17-dmag	*B. bovis*	77.6 ± 2.9	15.5 ± 4	8.8 ± 2	200	113
	B. bigemina	62.4 ± 1.9			248	141
	B. divergens	173.0 ± 10.9			90	51
	B. caballi	88.5 ± 9.6			175	99
	T. equi	307.7 ± 7.2			50	29
DA	*B. bovis*	189.8 ± 42.1	> 40	> 40	> 211	> 211
	B. bigemina	1852 ± 104			> 22	> 22
	B. divergens	991.2 ± 50.8			> 40	> 40
	B. caballi	13.4 ± 3.6			> 2985	> 2985
	T. equi	59.9 ± 8.5			> 668	> 668
AV	*B. bovis*	39.7 ± 2.4	> 40	> 40	> 1008	> 1008
	B. bigemina	706.1 ± 38.7			> 57	> 57
	B. divergens	38.4 ± 2.2			> 1042	> 1042
	B. caballi	101.9 ± 14.1			> 393	> 393
	T. equi	95.0 ± 65			> 421	> 421

[a)] Half maximum inhibition concentration of each drug on *in vitro* culture of parasites. The value was determined from the dose response curve using non-linear regression (curve fit analysis). The values are means of triplicate experiments.

[b)] Half maximum effective concentration of each drug on cell lines. The value was determined from the dose response curve using non-linear regression (curve fit analysis). The values are means of triplicate experiments.

[c)] The ratio of EC_{50} on cell lines to each species IC_{50}. High numbers are favorable.

Table 12. Combination index (CI) values of two-drug combinations of 17-dmag, diminazene aceturate, and atovaquone

Parasites	Drug combinations[a]	CI values at				Weighted average CI values[b]	Degree of synergism[c]
		IC_{50}	IC_{75}	IC_{90}	IC_{95}		
B. bovis	DM+DA	0.868	0.853	0.842	0.838	0.845	Moderate synergism
	DM+AV	1.255	1.051	0.884	0.787	0.916	Nearly additive
	DA+AV	0.725	0.725	0.726	0.727	0.726	Moderate synergism
B. bigemina	DM+DA	1.353	1.194	1.054	0.968	1.077	Nearly additive
	DM+AV	1.181	1.073	0.983	0.930	1.000	Nearly additive
	DA+AV	1.054	1.006	0.969	0.949	0.977	Nearly additive
B. divergens	DM+DA	1.764	1.235	0.867	0.684	0.957	Nearly additive
	DM+AV	1.384	1.024	0.759	0.618	0.818	Moderate synergism
	DA+AV	1.248	0.900	0.652	0.525	0.710	Moderate synergism
B. caballi	DM+DA	0.693	0.503	0.387	0.332	0.419	Synergism
	DM+AV	1.085	0.789	0.574	0.463	0.624	Synergism
	DA+AV	0.290	0.288	0.303	0.321	0.306	Synergism
T. equi	DM+DA	1.168	0.786	0.591	0.503	0.653	Synergism
	DM+AV	1.224	1.033	0.872	0.778	0.902	Nearly additive
	DA+AV	0.925	0.652	0.526	0.472	0.569	Synergism

Abbreviations: *B. bovis*, *Babesia bovis*; *B. bigemina*, *Babesia bigemina*; *B. divergens*, *Babesia divergens*; *B. caballi*, *Babesia caballi*; *T. equi*, *Theileria equi*; CI value, combination index value; IC_{50}, 50% inhibition concentration; DM, 17-dmag; DA, diminazene aceturate; AV, atovaquone

a) Two-drug combination of 17-dmag, diminazene aceturate, and atovaquone at concentrations of approximately 0.25 x IC_{50}, 0.5 x IC_{50}, IC_{50}, 2 x IC_{50}, and 4 x IC_{50} (constant ratio).

b) The higher inhibition is preferable thus the weighted average CI value was calculated with the formula ((1 x IC_{50}) + (2 x IC_{75}) + (3 x IC_{90}) + (4 x IC_{95}))/10.

c) The degree of synergism was determined based on the following ranges: 0.3–0.7 (synergism); 0.7–0.85 (moderate synergism); 0.85–0.90 (slight synergism); 0.90–1.10 (nearly additive); 1.10–1.20 (slight antagonism); 1.20–1.45 (moderate antagonism); 1.45–3.3 (antagonism).

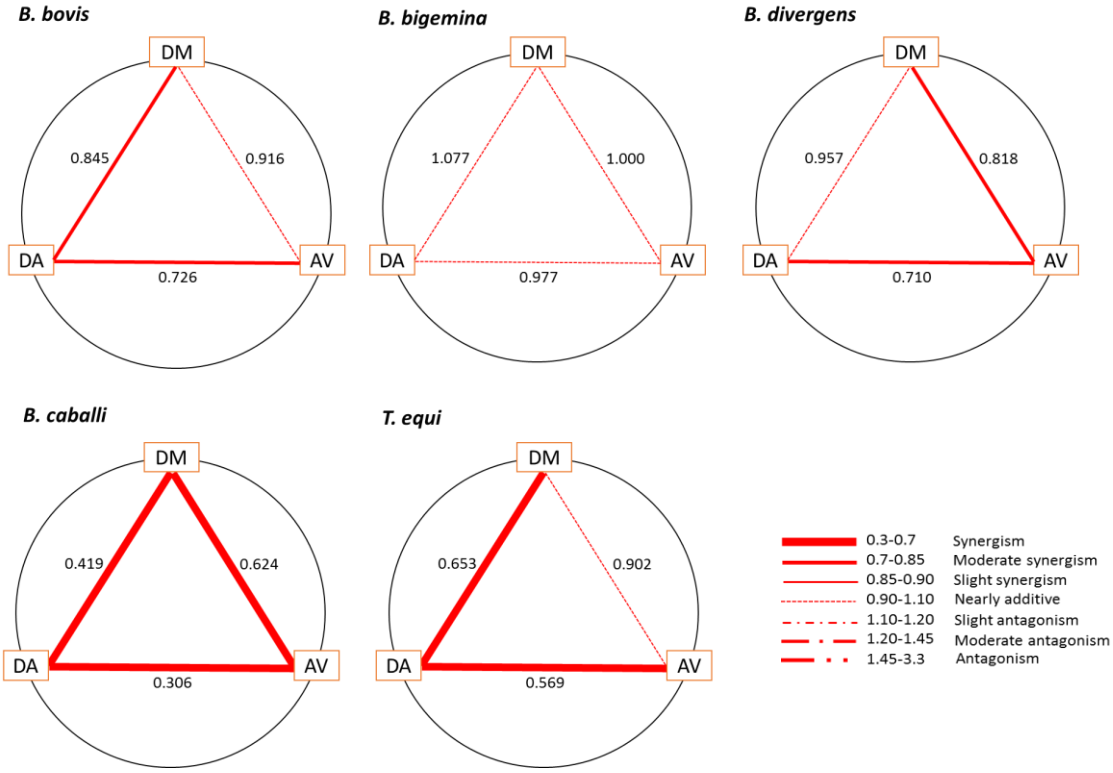

Fig. 11. Polygonogram of the degree of synergism for two-drug combinations of 17-dmag (DM), diminazene aceturate (DA), and atovaquone (AV) based on the weighted average of CI values at IC_{50}, IC_{75}, IC_{90}, and IC_{95}.

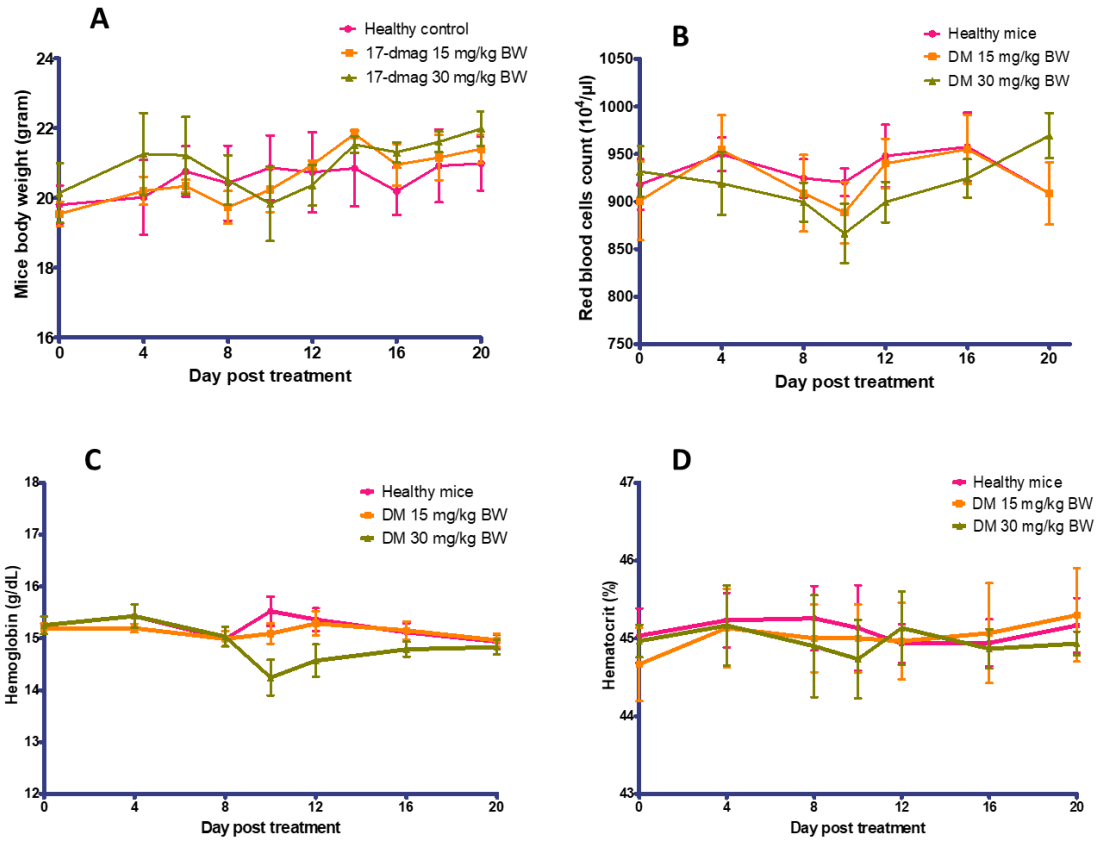

Fig. 12. Effect of 17-dmag on healthy mice in terms of body weight (A), RBC count (B), hemoglobin (C), and hematocrit (D). Treatment started at day 0 and continued for 5 days. Each group contained three mice. A p value < 0.05 is considered significant.

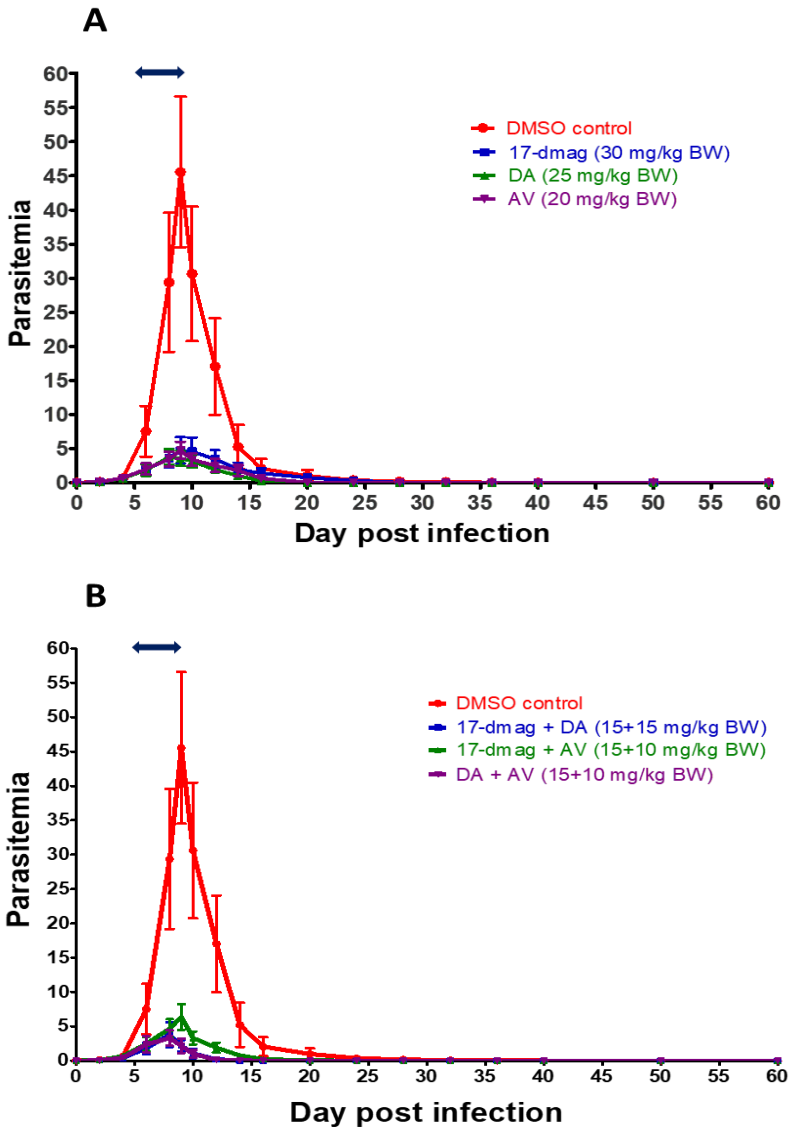

Fig. 13. The effect of drug treatment on the growth of *B. microti* in mice. (A) Treatment with DMSO as the control, 17-dmag at 30 mg/kg BW, diminazene aceturate at 25 mg/kg BW, and atovaquone at 20 mg/kg BW. (B) Treatment with a combination of 17-dmag and diminazene aceturate at 15 mg/kg BW each; 17-dmag and atovaquone at 15 and 10 mg/kg BW, respectively; diminazene aceturate and atovaquone at 15 and 10 mg/kg BW, respectively; and DMSO as a control. The drug treatment was conducted from day 5 to day 9 post infection (left–right arrow). The data were the mean and standard deviation from two separate experiments (5 mice/group).

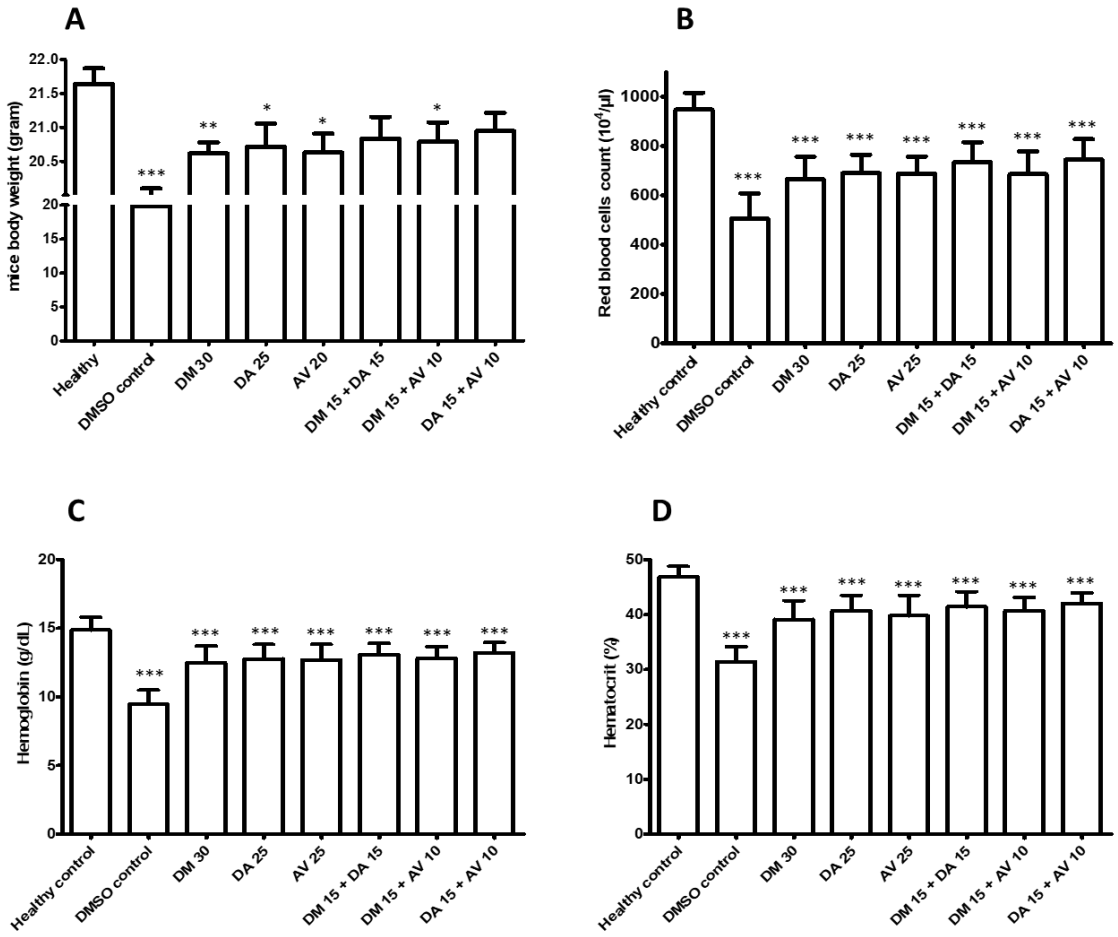

Fig. 14. Body weight and hematology profiles of mice on day 10 post infection. Mice body weight (A), red blood cell count (B), hemoglobin (C), and hematocrit (D). Data are the means from 10 mice in each group. The differences are marked with * if the P value is less than 0.05.

General discussion

These studies were trying to answer challenges in the current approach to the control of babesiosis, such as a reliable diagnostic method and an effective chemotherapeutic. In chapter 1, ICTs were evaluated for the detection of a *B. bovis* and *B. bigemina* antibody. Recombinant protein *Babesia bovis* SBP-4 and *Babesia bigemina* RAP1/CT17 were selected as detection antigens for the preparation of an ICT strip. These recombinant proteins have been used in several studies and showed a better performance among other proteins as detection antigens in ELISA. Subsequently, three types of ICT strips were prepared: *bov*ICT, *big*ICT, and dual-ICT. The ICTs showed a high sensitivity and specificity for antibody detection in the validation study using a panel of negative and positive controls of *B. bovis* and *B. bigemina*. If compared with ELISA, the sensitivity and specificity of ICTs were slightly lower but in good agreement. To evaluate the ICTs in the field sample, a panel of cattle serum samples from Western Java, Indonesia, was collected and tested for the antibodies of *B. bovis* and *B. bigemina*. The results showed that the average seroprevalence of *B. bovis* and *B. bigemina* in the area was quite high and revealed the endemicity of the disease.

This study used the ELISA method for the comparison with ICT. ELISA enables the detection of a high number of samples in one run as compared with ICT. It is also more sensitive than ICT because it only needs small amount of serum sample. However, ELISA is not fast, needs standardized method and trained personnel to perform, and needs more expensive equipment. In contrast, ICT is faster, easier, less costly and does not require trained personnel. Therefore, ICT strips could be useful and convenient in field settings for the serological detection of bovine babesiosis.

Despite the absence of scientific research on the genetic characterization of *B. bovis* and *B. bigemina* in Western Java, Indonesia, this study showed that the SBP-4 and RAP1 genes are conserved by those species, respectively. As evidence, the ICTs could recognize the antibodies of SBP-4 and RAP1 gene in the samples. This study is the first seroprevalence report of bovine babesiosis in the area and can be a foundation for further research on *Babesia* parasites. Regarding the ICT development, further adaptation of the quantitative detection of ICTs based on colorimetry or an electronic device is necessary to tackle the shortcoming associated with naked-eye observation.

In chapter 2, the coverage area of study was extended to several islands of Indonesia. The detection methods included ELISA and ICT for serological detection and nested PCR for molecular detection. The results were expected to reveal the current distribution of bovine babesiosis in the country as well as the genetic characterization based on the SBP-4 gene of *B. bovis* and the RAP-1a gene of *B. bigemina*. Usually, the outbreak of babesiosis is unreported. The nationwide surveillance program is based on microscopic observation of Giemsa-stained blood smears, leading to the underestimation of its impact on the livestock industry. This study showed that bovine babesiosis is endemic in most locations in Indonesia. Only two locations, Jombang and Lamongan, showed a low seropositive rate for both *B. bovis* and *B. bigemina*. However, nPCR results revealed some positive samples of *B. bovis* and *B. bigemina*, indicating introduction of the disease from an endemic area. These two locations might be at risk for an outbreak of bovine babesiosis.

The application of serological and molecular methods could differentiate between chronic and early infections according to the results of ELISA, ICT, and nPCR for each sample. Most infections found in this study were at the chronic stage, indicated by the higher positive rate of ELISA/ICT compared with nPCR. The samples that were positive by nPCR but negative by ELISA/ICT were

considered new infections. If this occurs in a susceptible herd, the risk of outbreak is high. Therefore, effective control measures should be conducted for prevention, and the introduction of cattle from endemic areas should be avoided. Furthermore, the location with endemic stability should be on the lookout for the disruption of the condition. Regarding genetic characterization, Indonesian isolates of the SBP-4 gene of *B. bovis* and the RAP-1a gene of *B. bigemina* showed a high homology with other isolates from around the world. This suggests the suitability of these diagnostic methods for application in broad geographic areas of the world. In addition, tick distribution is the main factor in *Babesia* transmission. In Indonesia, *Rhipicephalus microplus*, the vector of *B. bovis* and *B. bigemina*, is widespread. Effective control of that tick species could reduce the distribution of bovine babesiosis.

In chapter 3, 17-dmag was evaluated for its inhibitory effect on the *in vitro* growth of *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi* and on the growth inhibition assay of *B. microti* in the mouse model. The results showed that 17-dmag was effective to inhibit all of the species tested. Its combination with diminazene aceturate and atovaquone increased the effect at a lower dose. 17-dmag is known as an Hsp90 inhibitor. It binds specifically to the binding region of Hsp90 and prevents the attachment of Hsp90 client proteins so that they lose their activities that lead to abnormality of the cells. Hsp90 inhibitors were more attractive in the cancer research due to the high expression of Hsp90 in the cancer cells as compared with normal cells. In the treatment of protozoan parasites, Hsp90 is also a potential drug target because the parasite expression of Hsp90 is higher than Hsp90 expression in the host.

17-dmag showed a toxic effect on the viability of MDBK and NIH/3T3 cell lines. However, the inhibition of 17-dmag was much stronger on the parasite than that of those cell lines. Therefore, the selectivity index was high. The combination of 17-dmag with diminazene aceturate or atovaquone

could reduce the toxicity, because the combination of those drugs effectively inhibits the growth of parasite at lower concentrations. Overall, 17-dmag is a potent anti-*Babesia* drug, and its combination with DA or AV might have potential as a novel regime for treating piroplasms in wide range of piroplasms in animals.

General summary

Babesia parasites have been found for more than a century and cause a significant burden in the livestock industry and emerging disease in humans. The distribution of the disease remains as wide as the occurrence of their tick vectors. This study aimed to evaluate a reliable diagnostic method, such as immunochromatographic test (ICT) strips, for serological detection of bovine babesiosis in field samples. In an extended sampling coverage in Indonesia, ICT strips, ELISA, and nested PCR were employed for the detection of *B. bovis* and *B. bigemina* in cattle. Another objective of this study was to evaluate the inhibitory effect of 17-dmag alone and in combination with diminazene aceturate and atovaquone on the growth of *Babesia* and *Theileria* parasites *in vitro* and *in vivo*.

In chapter 1, three types of immunochromatographic test (ICT) strips were prepared for the detection of an antibody response against the spherical body protein 4 (SBP-4) of *Babesia bovis* (*bov*ICT), the C-terminal-truncated rhoptry-associated protein 1 (rRAP1/CT17) of *B. bigemina* (*big*ICT), and a combination of both proteins (dual-ICT). The evaluation of their performance was conducted using a confirmed positive and negative serum panel for *B. bovis* and *B. bigemina*. Together with ELISA, the ICT strips were applied to determine the seroprevalence of bovine babesiosis in Western Java, Indonesia. Among 991 serum samples, 28.4%, 25.3%, and 24.5% of cattle were detected to be seropositive for *B. bovis* infection using ELISA, *bov*ICT, and dual-ICT, respectively. *B. bigemina* seropositivity was detected in 27.1%, 24.2%, and 22.8% of samples using ELISA, *big*ICT, and dual-ICT, respectively. The comparison of ICT and ELISA results using field serum samples showed good agreement with Kappa values were more than 0.7. The application of ICT strips is preferable in field situations where rapid diagnosis is required. Furthermore, the data showed the current seroprevalence

of bovine babesiosis in Western Java, Indonesia, and efficient control strategies are needed to reduce economic losses due to the disease.

In chapter 2, an extended sample collection was conducted in several islands of Indonesia. Subsequently, molecular and serological detections of bovine babesiosis were performed on 487 blood samples. The presence of antibodies and current infections of *B. bovis* and *B. bigemina* were determined using an enzyme-linked immunosorbent assay (ELISA), immunochromatographic test (ICT), and nested PCR (nPCR) targeting *B. bovis* sbp4 and *B. bigemina* rap-1a genes. Among 487 samples, ELISA, single-ICT, dual-ICT, and nPCR detected positive *B. bovis* in 340 (69.8%), 317 (65.1%), 307 (63.0%), and 247 (50.7%) of the samples, respectively. For *B. bigemina*, 134 (27.5%), 130 (26.7%), 127 (26.1%), and 93 (19.1%) samples were positive, respectively. Furthermore, mixed infections were found in 125 (25.7%), 113 (23.2%), 109 (22.4%), and 52 (10.7%) samples, respectively. The obtained nucleotide sequences of *B. bovis* sbp4 and *B. bigemina* rap-1a genes in this study showed a high homology with other isolates from many countries, and the identities among Indonesian isolates were 94 to 100%. These data revealed the current distribution of *B. bovis* and *B. bigemina* infection in cattle in Indonesia, and we found that the positive rate of *B. bovis* is high in most sampling locations while *B. bigemina* is lower. The data presented in this study are necessary to develop an effective strategy for controlling the disease in that country.

In chapter 3, an Hsp90 inhibitor, 17-dmag, was evaluated for its inhibitory effect on five *in vitro* cultures of *Babesia* and *Theileria* species, *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*, followed by the growth inhibition assay of a *B. microti*–infected mouse model. 17-dmag showed the growth inhibition in all of the species tested. The half maximum of inhibition concentration (IC_{50}) of 17-dmag on the *in vitro* growth of *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi* was 77.6

± 2.9, 62.4 ± 1.9, 173.0 ± 10.9, 88.5 ± 9.6, and 307.7 ± 7.2 nM, respectively. The toxicity assay on MDBK and NIH/3T3 cell lines showed that 17-dmag affected the growth of cells with half maximum effective concentrations (EC_{50}) of 15.5 ± 4 µM and 8.8 ± 2 µM, respectively. Since the IC_{50} was much lower on the parasites, the selectivity index was high in all tested species compared to the host. Furthermore, the two-drug combination of 17-dmag with diminazene aceturate (DA) and atovaquone (AV) showed synergism or addition *in vitro*. In the mouse model, 17-dmag at the concentration of 30 mg/kg BW effectively inhibited the growth of *B. microti*. Moreover, the combination of a half dose of 17-dmag and DA showed a comparable inhibition if used at the full dose. Taken together, this indicates that 17-dmag is a potent drug in treating babesiosis. Its toxicity can be reduced by combining it with other effective drugs, such as DA and AV. The data warrant further evaluation of 17-dmag as an anti-*Babesia* and as a treatment option in combination with atovaquone for the treatment of human babesiosis.

To sum up, these studies are reporting the current development of diagnostic methods for bovine babesiosis. The ICTs in this study, even though their sensitivity is slightly lower than that of ELISA, are useful for the detection of *B. bovis* and *B. bigemina* antibodies, especially in field applications. These data also reveal the current distribution of bovine babesiosis in cattle in Western Java, and the extent sampling areas in Indonesia. The results indicate that bovine babesiosis might be a significant economic burden on the Indonesian livestock industry. The development of effective control strategies for bovine babesiosis in that country could be benefited by the data provided in this study. Furthermore, 17-dmag is a potent anti-*Babesia* drug. It specifically inhibits the growth of *Babesia* and *Theileria* parasites both *in vitro* and *in vivo*. Its toxicity could be reduced by combination therapy. The

development of diagnostic methods and chemotherapeutics for bovine babesiosis is still in progress, and further improvements are necessary to combat this important disease.

和文要旨

バベシアは１世紀以上前に発見され、世界的に畜産業に大きな経済的被害を与えており、またヒトにも感染する。バベシア病はダニによって媒介されるので、ダニが生息する地域に認められる。本研究の目的の一つは、野外の牛バベシア病に対する信頼できる診断法の評価である。具体的には、イムノクロマトグラフィー法(ICT)、ELISA、nested PCR(nPCR)を用いてインドネシアの牛の *B. bovis* と *B. bigemina* を検出する事である。もう一つの目的は、*in vitro* および *in vivo* のバベシアとタイレリアに対する 17-dmag の単独および dimina zene aceturate と atovaquone との併用による増殖抑制効果の評価である。

第１章では、抗体検出のために *B. bovis* の spherical body たんぱく質４（SBP-4）、*B. bigemina* の C 末端欠損 rhoptry たんぱく質（rRAP1/CT17）、両たんぱく質を用いた３種類のイムノクロマトグラフィー、（bovICT）、（bigICT）および（dual-ICT）のストリップを作製した。これら特異性は、*B. bovis* と *B. bigemina* 陽性および陰性血清パネルを使って確認された。更に、ELISA と ICT を用いて、インドネシアの西ジャワで採取された 991 の血清サンプルを検査した。その結果、ELISA、bovICT および dual-ICT により、それぞれ 28.4%、25.3%、24.5%のサンプルが *B. bovis* 感染陽性であった。また、*B. bigemina* では、それぞれ、27.1%、24.2%、および 22.8%のサンプルが陽性であった。これらの結果により、迅速な結果が求められる野外診断では ICT が適している事が示唆された。

第２章では、調査地域をインドネシアの他の島々にも広げ、487 の血清サンプルについて、血清および遺伝子診断法を実施した。血清診断法は ELISA、ICT を、遺伝子診断法は sbp4 遺伝子と rap-1a 遺伝子を標的とした nPCR を用いて行った。その結果、*B. bovis* では ELISA、bovICT および dual-IC、nPCR により、それぞれ 340（69.8%）、317（65.1%）、307（63.0%）、247（50.7%）サンプルが陽性であった。また、*B. bigemina* ではそれぞれ 134（27.5%）、130（26.7%）、127（26.1%）、93（19.1%）サンプルが陽性反応を示した。またこれらの方法によりそれぞれ、125（25.7%）、113（23.2%）、109（22.4%）、52（10.7%）のサンプルにおいて混合感染が認められた。nPCR で得られた *B. bovis* の sbp4、 *B. bigemina* の rap-1a 遺伝子の DNA 配列は、他の多くの国で得られている 配列と高い相同性を示し、またインドネシアで得られた遺伝子の配列は 94～100％の相同性を示した。また、インドネシアの *B. bovis* の陽性率は *B. bigemina* よりも高かった。

第３章では、熱ショックたんぱく質 90 の阻害剤である 17-dmag の増殖抑制効果を、５種類の培養バベシアとタイレリア（*B. bovis*、*B. bigemina*、*B. div*

ergens、*B. caballi*、および *T. equi*）と *B. microti* 感染マウスモデルを用いて行った。17-dmag の IC_{50} は、*B. bovis*、 *B. bigemina*、 *B. divergens*、 *B. caballi*、 *T. equi* に対し、それぞれ 77.6、62.4、173.0、88.5、および 307.7 nM であった。また、17-dmag の MDBK と NIH/3T3 細胞に対する毒性（EC50）は、それぞれ 15500 と 8780 μM で IC_{50} より遥かに高く、選択性インデックスは宿主細胞に比べてのすべての原虫で高かった。さらに、*B. microti* 感染マウスに 30 mg/kg の 17-dmag を腹腔投与したところ、非治療群と比較して有為に低い赤血球寄生率が認められた。さらに半分の用量の 17-dmag と diminazene aceturate を併用して治療したところ、単独で用いた場合と同じ治療効果が認められた。

相乗あるいは相加効果が認められた。以上の結果により、17-dmag がバベシア病の治療薬として有望であり、他の薬剤と併用する事により、副作用の軽減も期待される。また、併用療法は人バベシア病の治療薬として検討する価値がある。

以上を要約すると、本研究は牛バベシア病の診断法と治療法の開発について検討した。ICTs は、ELISA より感度が少し低くても、*B. bovis* と *B. bigemina* の抗体検出の野外応用に有用である。これらの研究により、西ジャワおよびインドネシア全体の牛バベシア病の現在の分布やバベシア原虫の遺伝子情報が明らかになった。これらのバベシア病に関する研究成果は、インドネシアの畜産業の重要な経済的損失であり、今後効果的な制御戦略の確立に役立つ事が期待される。さらに、17-dmag は、in vitro と in vivo でバベシアとタイレリアの増殖抑制効果を示し、有効な抗バベシア薬となる可能性を有しており、他の薬剤との併用により治療効果を高め、副作用を軽減する事が期待される。今後、更に牛バベシア病の診断方法およびバベシア症のための化学療法薬剤の開発を推進する事が、この重要なバベシア病の制圧に必要である。

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References

Abbas, R.Z., Zaman, M.A., Colwell, D.D., Gilleard, J., Iqbal, Z., 2014. Acaricide resistance in cattle ticks and approaches to its management: The state of play. Vet. Parasitol. 16, 6–20.

Aboge, G.O., Cao, S., Terkawi, M.A., Masatani, T., Goo, Y., AbouLaila, M., Nishikawa, Y., Igarashi, I., Suzuki, H., Xuan, X., 2015. Molecular characterization of *Babesia bovis* M17 leucine aminopeptidase and inhibition of *Babesia* growth by bestatin. J. Parasitol. 101, 536–541.

AbouLaila, M., Batadoj, D., Salama, A., Munkhjargal, T., Ichikawa-Seki, M., Terkawi, M.A., Yokoyama, N., Igarashi, I., 2014. Evaluation of the inhibitory effects of miltefosine on the growth of *Babesia* and *Theileria* parasites. Vet. Parasitol. 204, 104–110.

AbouLaila, M., Nakamura, K., Govind, Y., Yokoyama, N., Igarashi, I., 2010a. Evaluation of the *in vitro* growth-inhibitory effect of epoxomicin on *Babesia* parasites. Vet. Parasitol. 167, 19–27.

AbouLaila, M., Sivakumar, T., Yokoyama, N., Igarashi, I., 2010b. Inhibitory effect of terpene nerolidol on the growth of *Babesia* parasites. Parasitol. Int. 59, 278–282.

AbouLaila, M., Yokoyama, N., Igarashi, I., 2010c. Inhibitory effects of (-)-epigallocatechin-3-gallate from green tea on the growth of *Babesia* parasites. Parasitology 137, 785–791.

Adjou Moumouni, P.F., Aboge, G.O., Terkawi, M.A., Masatani, T., Cao, S., Kamyingkird, K., Jirapattharasate, C., Zhou, M., Wang, G., Liu, M., Iguchi, A., Vudriko, P., Ybañez, A.P., Inokuma, H., Shirafuji-Umemiya, R., Suzuki, H., Xuan, X., 2015. Molecular detection and characterization of Babesia bovis, Babesia bigemina, Theileria species and Anaplasma marginale isolated from cattle in Kenya. Parasit. Vectors 8, 496. doi:10.1186/s13071-015-1106-9.

Avarzed, A., Igarashi, I., Kanemaru, T., Hirumi, K., Omata, Y., Saito, A., Oyamada, T., Nagasawa, H., Toyoda, Y., Suzuki, N., 1997. Improved *in vitro* cultivation of *Babesia caballi*. J. Vet. Med. Sci. 59, 479–481.

Babes, V., 1988. Sur l'hémoglobinurie bactérienne du bœuf. C. R. Acad. Sci. 107, 692–694. [French]

Baldissera, M.D., Gonçalves, R.A., Sagrillo, M.R., Grando, T.H., Ritter, C.S., Grotto, F.S., Brum, G.F., da Luz, S.C.A., Silveira, S.O., Fausto, V.P., Boligon, A.A., Vaucher, R.A., Stefani, L.M., da Silva, A.S., Souza, C.F., Monteiro, S.G., 2016a. Effects of treatment with the anti-parasitic drug diminazene aceturate on antioxidant enzymes in rat liver and kidney. Naunyn-Schmiedeberg's Arch. Pharmacol. 389, 429–438.

Baldissera, M.D., Grando, T.H., Souza, C.F., Cossetin, L.F., Sagrillo, M.R., Nascimento, K., da Silva, A.P., Dalla Lana, D.F., da Silva A.S., Stefani L.M., Monteiro, S.G., 2016b. Nerolidol nanospheres increases its trypanocidal efficacy against *Trypanosoma evansi*: New approach against diminazene aceturate resistance and toxicity. Exp. Parasitol. 166, 144–149.

Banumathy, G., Singh, V., Pavithra, S.R., Tatu, U., 2003. Heat shock protein 90 function is essential for *Plasmodium falciparum* growth in human erythrocytes. J. Biol. Chem. 278, 18336–18345.

Bock, R., Jackson, L., de Vos, A., Jorgensen, W., 2004. Babesiosis of cattle. Parasitology 129 Suppl., S247–S269.

Boonchit, S., Alhassan, A., Chan, B., Xuan, X., Yokoyama, N., Ooshiro, M., Goff, W.L., Waghela, S.D., Wagner, G., Igarashi, I., 2006. Expression of C-terminal truncated and full-length *Babesia bigemina* rhoptry-associated protein 1 and their potential use in enzyme-linked immunosorbent assay. Vet. Parasitol. 137, 28–35.

Boonchit, S., Xuan, X., Yokoyama, N., Goff, W.L., Wagner, G., Igarashi, I., 2002. Evaluation of an enzyme-linked immunosorbent assay with recombinant rhoptry-associated protein 1 antigen against *Babesia bovis* for the detection of specific antibodies in cattle. J. Clin. Microbiol. 40, 3771–3775.

Bork, S., Okamura, M., Boonchit, S., Hirata, H., Yokoyama, N., Igarashi, I., 2004. Identification of *Babesia bovis* L-lactate dehydrogenase as a potential chemotherapeutical target against bovine babesiosis. Mol. Biochem. Parasitol. 136, 165–172.

Bork, S., Yokoyama, N., Matsuo, T., Claveria, F.G., Fujisaki, K., Igarashi, I., 2003. Growth inhibitory effect of triclosan on equine and bovine *Babesia* parasites. Am. J. Trop. Med. Hyg. 68, 334–340.

Burns, C., Spendel, W.U., Puckett, S., Pacey, G.E., 2006. Solution ionic strength effect on gold nanoparticle solution color transition. Talanta 69, 873–876.

Cao, S., Aboge, G.O., Terkawi, M.A., Yu, L., Kamyingkird, K., Luo, Y., Li, Y., Goo, Y.K., Yamagishi, J., Nishikawa, Y., Yokoyama, N., Suzuki, H., Igarashi, I., Maeda, R., Inpankaew, T., Jittapalapong, S., Xuan, X., 2012. Molecular detection and identification of *Babesia bovis* and *Babesia bigemina* in cattle in northern Thailand. Parasitol. Res. 111(3), 1259–1266.

Carrique, J.J., Morales, G.J., Edelsten, M., 2000. Endemic instability for babesiosis and anaplasmosis in cattle in the Bolivian Chaco. Vet. J. 160, 162–164.

Chou, T.-C., 2006. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol. Rev. 58, 621-681.

Chung, C.J., Suarez, C.E., Bandaranayaka-Mudiyanselage, C.L., Bandaranayaka-Mudiyanselage, C.-B., Rzepka, J., Heiniger, T., Chung, G., Lee, S.S., Adams, E., Yun, G., Waldron, S.J., 2017. A

novel modified-indirect ELISA based on spherical body protein 4 for detecting antibody during acute and long-term infections with diverse *Babesia bovis* strains. Parasit. Vectors 10, 77. http://doi.org/10.1186/s13071-017-2016-9.

Coldham, N.G., Moore, A.S., Dave, M., Graham, P.J., Sivapathasundaram, S., Lake, B.G., Sauer, M.J., 1995. Imidocarb residues in edible bovine tissues and *in vitro* assessment of imidocarb metabolism and cytotoxicity. Drug Metab. Dispos. 23, 501–505.

da Silva, J.B., André, M.R., da Fonseca, A.H., de Albuquerque Lopes, C.T., da Silva Lima, D.H., de Andrade, S.J.T., Oliveira, C.M.C., Barbosa, J.D., 2013. Molecular and serological prevalence of *Babesia bovis* and *Babesia bigemina* in water buffaloes in the north region of Brazil. Vet. Parasitol. 197, 678–681.

de Vries, E., Corton, C., Harris, B., Cornelissen, A.W., Berriman, M., 2006. Expressed sequence tag (EST) analysis of the erythrocytic stages of *Babesia bovis*. Vet. Parasitol. 138, 61–74.

Dewi, R.S., 2007. Babesiosis in Australian cattle imported via Tanjung Priok. Master Thesis: Bogor Agricultural University. https://core.ac.uk/download/pdf/32341447.pdf. Accessed April 5, 2017 [Indonesian].

Echeverria, P.C., Matrajt, M., Harb, O.S., Zappia, M.P., Costas, M.A., Roos, D.S., Dubremetz, J.F., Angel, S.O., 2005. *Toxoplasma gondii* Hsp90 is a potential drug target whose expression and subcellular localization are developmentally regulated. J. Mol. Biol. 350, 723–734.

Fleiss, J., Levin, B., Cho-Paik, M., 2013. Statistical methods for rates and proportions. 3rd ed. Wiley-InterScience.

Gillan, V., O'Neill, K., Maitland, K., Sverdrup, F.M., Devaney, E., 2014. A repurposing strategy for Hsp90 inhibitors demonstrates their potency against filarial nematodes. PLoS. Negl. Trop. Dis. 8, e2699. https://doi.org/10.1371/journal.pntd.0002699.

Gimenez, A.M., Françoso, K.S., Ersching, J., Icimoto, M.Y., Oliveira, V., Rodriguez, A.E., Schnittger, L., Florin-Christensen, M., Rodrigues, M.M., Soares, I.S., 2016. A recombinant multi-antigen vaccine formulation containing *Babesia bovis* merozoite surface antigens MSA-2a1, MSA-2b and MSA-2c elicits invasion-inhibitory antibodies and IFN-γ producing cells. Parasit. Vectors 9, 577. http://doi.org/10.1186/s13071-016-1862-1.

Gohil, S., Herrmann, S., Günther, S., Cooke, B.M., 2013. Bovine babesiosis in the 21st century: Advances in biology and functional genomics. Int. J. Parasitol. 43 (2), 125–132.

Gohil, S., Kats, L.M., Sturm, A., Cooke, B.M., 2010. Recent insights into alteration of red blood cells by *Babesia bovis*: Moovin' forward. Trends Parasitol. 26, 591–599.

Goo, Y.K., Terkawi, M.A., Jia, H., Aboge, G.O., Ooka, H., Nelson, B., Kim, S., Sunaga, F., Namikawa, K., Igarashi, I., Nishikawa, Y., Xuan, X., 2010. Artesunate, a potential drug for treatment of *Babesia* infection. Parasitol. Int. 59, 481–486.

Guerrero, F.D., Miller, R.J., Pérez de León, A.A., 2012. Cattle tick vaccines: Many candidate antigens, but will a commercially viable product emerge? Int. J. Parasitol. 42, 421–427.

Guswanto, A., Allamanda, P., Mariamah, E.S., Munkjargal, T., Tuvshintulga, B., Takemae, H., Sivakumar, T., AbouLaila, M., Terkawi, M.A., Ichikawa-Seki, M., Nishikawa, Y., Yokoyama, N., Igarashi, I., 2017. Evaluation of immunochromatographic test (ICT) strips for the serological detection of *Babesia bovis* and *Babesia bigemina* infection in cattle from Western Java, Indonesia. Vet. Parasitol. 239, 76–79.

Guswanto, A., Sivakumar, T., Rizk, M.A., Elsayed, S.A.E., Youssef, M.A., ElSaid, E.E.S., Yokoyama, N., Igarashi, I., 2014. Evaluation of a fluorescence-based method for antibabesial drug screening. Antimicrob. Agents and Chemother. 58, 4713–4717.

Homer, M.J., Aguilar-Delfin, I., Telford, S.R., Krause, P.J., Persing, D.H., 2000. Babesiosis. Clin. Microbiol. Rev. 13, 451–469.

Huang, X., Xuan, X., Verdida, R.A., Zhang, S., Yokoyama, N., Xu, L., Igarashi, I., 2006. Immunochromatographic test for simultaneous serodiagnosis of *Babesia caballi* and *B. equi* infections in horses. Clin. Vaccine Immunol. 13 (5), 553–555.

Ibrahim, H.M., Adjou Moumouni, P.F., Mohammed-Geba, K., Sheir, S.K., Hashem, I.S.Y., Cao, S., Terkawi, M.A., Kamyingkird, K., Nishikawa, Y., Suzuki, H., Xuan, X., 2013. Molecular and serological prevalence of *Babesia bigemina* and *Babesia bovis* in cattle and water buffalos under small-scale dairy farming in Beheira and Faiyum Provinces, Egypt. Vet. Parasitol. 198, 187–192.

Igarashi, I., Njonge, F.K., Kaneko, Y., Nakamura, Y., 1998. *Babesia bigemina*: *In vitro* and *in vivo* effects of curdlan sulfate on the growth of parasites. Exp. Parasitol. 90, 290–293.

Jia, H., Liao, M., Lee, E., Nishikawa, Y., Inokuma, H., Ikadai, H., Matsuu, A., Igarashi, I., Xuan, X., 2007. Development of an immunochromatographic test with recombinant BgSA1 for the diagnosis of *Babesia gibsoni* infection in dogs. Parasitol. Res. 100 (6), 1381–1384.

Jilani, K., Qadri, S.M., Lang, F., 2013. Geldanamycin-induced phosphatidylserine translocation in the erythrocyte membrane. Cell. Physiol. Biochem. 32, 1600–1609.

Jirapattharasate, C., Adjou Moumouni, P.F., Cao, S., Iguchi, A., Liu, M., Wang, G., Zhou, M., Vudriko, P., Efstratiou, A., Changbunjong, T., Sungpradit, S., Ratanakorn, P., Moonarmart, W., Sedwisai,

P., Weluwanarak, T., Wongsawang, W., Suzuki, H., Xuan, X., 2017. Molecular detection and genetic diversity of bovine *Babesia* spp., *Theileria orientalis*, and *Anaplasma marginale* in beef cattle in Thailand. Parasitol. Res. 116(2), 751–762.

Jonsson, N.N., Bock, R.E., Jorgensen, W.K., 2008. Productivity and health effects of anaplasmosis and babesiosis on *Bos indicus* cattle and their crosses, and the effects of differing intensity of tick control in Australia. Vet. Parasitol. 155 (1–2), 1–9.

Kim, C., Alhassan, A., Verdida, R.A., Yokoyama, N., Xuan, X., Fujisaki, K., Kawazu, S., Igarashi, I., 2007. Development of two immunochromatographic tests for the serodiagnosis of bovine babesiosis. Vet. Parasitol. 148 (2), 137–143.

Kim, C.M., Blanco, L.B.C., Alhassan, A., Iseki, H., Yokoyama, N., Xuan, X., Igarashi, I., 2008. Development of a rapid immunochromatographic test for simultaneous serodiagnosis of bovine babesiosis caused by *Babesia bovis* and *Babesia bigemina*. Am. J. Trop. Med. Hyg. 78, 117–121.

Kim, Y.S., Alarcon, S.V., Lee, S., Lee, M.J., Giaccone, G., Neckers, L., Trepel, J.B., 2009. Update on Hsp90 inhibitors in clinical trial. Curr. Top. Med. Chem. 9, 1479–1492.

Kimura, M., 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.

Kinnaird, J.H., Singh, M., Gillan, V., Weir, W., Calder, E.D.D., Hostettler, I., Tatu, U., Devaney, E., Shiels, B.R., 2017. Characterization of HSP90 isoforms in transformed bovine leukocytes infected with *Theileria annulata*. Cell. Microbiol. 19, e12669. http://doi.org/10.1111/cmi.12669.

Korsinczky, M., Chen, N., Kotecka, B., Saul, A., Rieckmann, K., Cheng, Q., 2000. Mutations in *Plasmodium falciparum* cytochrome b that are associated with atovaquone resistance are located at a putative drug-binding site. Antimicrob. Agents and Chemother. 44, 2100–2108.

Krause, P.J., Lepore, T., Sikand, V.K., Gadbaw, J., Jr., Burke, G., Telford, S.R., Brassard, P., Pearl, D., Azlanzadeh, J., Christianson, D., McGrath, D., Spielman, A., 2000. Atovaquone and azithromycin for the treatment of babesiosis. N. Eng. J. Med. 343, 1454–1458.

Kumar, N., Zhao, Y., Graves, P., Perez-Folgar, J., Maloy, L., Zheng, H., 1990. Human immune response directed against *Plasmodium falciparum* heat shock-related proteins. Infect. Immun. 58, 1408–1414.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.

Kuttler, K.L., 1980. Pharmacotherapeutics of drugs used in treatment of anaplasmosis and babesiosis. J. Am. Vet. Med. Assoc. 176, 1103–1108.

Lengauer, H., Just, F.T., Edelhofer, R., Pfister, K., 2006. Tick infestation and the prevalence of *Borrelia burgdorferi* and *Babesia divergens* in cattle in Bavaria. Berl. Munch. Tierarztl. Wochenschr. 119, 335–341.

Levy, M.G., Ristic, M., 1980. *Babesia bovis*: Continuous cultivation in a microaerophilous stationary phase culture. Science 207, 1218–1220.

Li, W., Cowley, A., Uludag, M., Gur, T., McWilliam, H., Squizzato, S., Park, Y.M., Buso, N., Lopez, R., 2015. The EMBL-EBI bioinformatics web and programmatic tools framework. Nucleic Acids Res. 43 (W1), W580–584 http://doi.org/10.1093/nar/gkv279.

Li, Y., Luo, Y., Cao, S., Terkawi, M.A., Bich Lan, D.T., Long, P.T., Yu, L., Zhou, M., Gong, H., Zhang, H., Zhou, J., Yokoyama, N., Suzuki, H., Xuan, X., 2014. Molecular and seroepidemiological survey of *Babesia bovis* and *Babesia bigemina* infections in cattle and water buffaloes in the central region of Vietnam. Trop. Biomed. 31, 406–413.

Librado, P., Rozas, J., 2009. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics. 25, 1451-1452.

Luo, Y., Jia, H., Terkawi, M.A., Goo, Y.K., Kawano, S., Ooka, H., Li, Y., Yu, L., Cao, S., Yamagishi, J., Fujisaki, K., Nishikawa, Y., Saito-Ito, A., Igarashi, I., Xuan, X., 2011. Identification and characterization of a novel secreted antigen 1 of *Babesia microti* and evaluation of its potential use in enzyme-linked immunosorbent assay and immunochromatographic test. Parasitol. Int. 60 (2), 119–125.

Matsuu, A., Miyamato, K., Ikada, H., Okano, S., Higuchi, S., 2006. Short report: Cloning of the *Babesia gibsoni* cytochrome B gene and isolation of three single nucleotide polymorphisms from parasites present after atovaquone treatment. Am. J. Trop. Med. 74, 593–597.

Meyer, K.J., Shapiro, T.A., 2013. Potent antitrypanosomal activities of heat shock protein 90 inhibitors *in vitro* and *in vivo*. J. Infect. Dis. 208, 489–499.

Miyata, Y., Nakamoto, H., Neckers, L., 2013. The therapeutic target Hsp90 and cancer hallmarks. Curr. Pharm. Des. 19, 347–365.

Moon, J., Kim, G., Lee, S., 2012. A gold nanoparticle and aflatoxin B1-BSA conjugates based lateral flow assay method for the analysis of aflatoxin B1. Materials (Basel) 5, 634–643.

Mosqueda, J., Olvera-Ramirez, A., Aguilar-Tipacamu, G., Canto, G.J., 2012. Current advances in detection and treatment of babesiosis. Curr. Med. Chem. 19, 1504–1518.

Mtshali, M.S., Mtshali, P.S., 2013. Molecular diagnosis and phylogenetic analysis of *Babesia bigemina* and *Babesia bovis* hemoparasites from cattle in South Africa. BMC Vet. Res. 9, 154. http://doi.org/10.1186/1746-6148-9-154.

Munkhjargal, T., Sivakumar, T., Battsetseg, B., Nyamjargal, T., Aboulaila, M., Purevtseren, B., Bayarsaikhan, D., Byambaa, B., Terkawi, M.A., Yokoyama, N., Igarashi, I., 2013. Prevalence and genetic diversity of equine piroplasms in Tov Province, Mongolia. Infect. Genet. Evol. 16, 178–185.

NCBI Resource Coordinators., 2017. Database Resources of the National Center for Biotechnology Information. Nucleic Acids Res. 45. Database issue: D12–D17. http://doi.org/10.1093/nar/gkw1071.

Niu, Q., Liu, Z., Yu, P., Yang, J., Abdallah, M.O., Guan, G., Liu, G., Luo, J., Yin, H., 2015. Genetic characterization and molecular survey of *Babesia bovis*, *Babesia bigemina* and *Babesia ovata* in cattle, dairy cattle, and yaks in China. Parasit. Vectors 8, 518. http://doi.org/10.1186/s13071-015-1110-0.

Oldiges, D.P., Laughery, J.M., Tagliari, N.J., Leite Filho, R.V., Davis, W.C., da Silva Vaz, I., Termignoni, C., Knowles, D.P., Suarez, C.E., 2016. Transfected *Babesia bovis* expressing a tick GST as a live vector vaccine. PLoS Neglected Tropical Diseases 10 (12), e0005152. http://doi.org/10.1371/journal.pntd.0005152.

Oliveira, G.L.S., de Freitas, R.M., 2015. Diminazene aceturate—An antiparasitic drug of antiquity: Advances in pharmacology and therapeutics. Pharmacol. Res. 102, 138–157.

Olson, D.M., Dinerstein, E., 1998. The Global 200: A representation approach to conserving the Earth's most biologically valuable ecoregions. Conserv. Biol. 12, 502–515.

Omar, M.A., Salama, A., Elsify, A., Rizk, M.A., Al-Aboody, M.S., AbouLaila, M., El-Sayed, S.A.E., Igarashi, I., 2016. Evaluation of *in vitro* inhibitory effect of enoxacin on *Babesia* and *Theileria* parasites. Exp. Parasitol. 161, 62–67.

Pallavi, R., Roy, N., Nageshan, R.K., Talukdar, P., Pavithra, S.R., Reddy, R., Venketesh, S., Kumar, R., Gupta, A.K., Singh, R.K., Yadav, S.C., Tatu, U., 2010. Heat shock protein 90 as a drug target against protozoan infections: Biochemical characterization of Hsp90 from *Plasmodium falciparum* and *Trypanosoma evansi* and evaluation of its inhibitor as a candidate drug. J. Biol. Chem. 285, 37964–37975.

Pegram, R.G., Wilson, D.D., Hansen, J.W., 2000. Past and present national tick control programs: Why they succeed or fail. Ann. N. Y. Acad. Sci. 916, 546–554.

Perkins, M.E., 1992. Rhoptry organelles of apicomplexan parasites. Parasitol. Today 8, 28–32.

Péroval, M., Péry, P., Labbé, M., 2006. The heat shock protein 90 of *Eimeria tenella* is essential for invasion of host cell and schizont growth. Int. J. Parasit. 36, 1205–1215.

Pizarro, J.C., Hills, T., Senisterra, G., Wernimont, A.K., Mackenzie, C., Norcross, N.R., Ferguson, M.A.J., Wyatt, P.G., Gilbert, I.H., Hui, R., 2013. Exploring the *Trypanosoma brucei* Hsp83 potential as a target for structure guided drug design. PLoS. Negl. Trop. Dis. 7, e2492. https://doi.org/10.1371/journal.pntd.0002492.

Rajapakshage, B.K.W., Yamasaki, M., Hwang, S.-J., Sasaki, N., Murakami, M., Tamura, Y., Lim, S.Y., Nakamura, K., Ohta, H., Takiguchi, M., 2012. Involvement of mitochondrial genes of *Babesia gibsoni* in resistance to diminazene aceturate. J. Vet. Med. Sci. 74, 1139–1148.

Rizk, M.A., El-Sayed, S.A.E.S., Terkawi, M.A., Youssef, M.A., El Said, E.S.E.S., Elsayed, G., El-Khodery, S., El-Ashker, M., Elsify, A., Omar, M., Salama, A., Yokoyama, N., Igarashi, I., 2015.

Optimization of a fluorescence-based assay for large-scale drug screening against *Babesia* and *Theileria* parasites. PLoS One 10, e0125276. http://doi.org/10.1371/journal.pone.0125276.

Ruef, B.J., Dowling, S.C., Conley, P.G., Perryman, L.E., Brown, W.C., Jasmer, D.P., Rice-Ficht, A.C., 2000a. A unique *Babesia bovis* spherical body protein is conserved among geographic isolates and localizes to the infected erythrocyte membrane. Mol. Biochem. Parasitol. 105, 1–12.

Ruef, B.J., Ward, T.J., Oxner, C.R., Conley, P.G., Brown, W.C., Rice-Ficht, A.C., 2000b. Phylogenetic analysis with newly characterized *Babesia bovis* hsp70 and hsp90 provides strong support for paraphyly within the piroplasms. Mol. Biochem. Parasit. 109, 67–72.

Sahara, A., Prastowo, J., Widayanti, R., Kurniasih, Nurcahyo, W., 2015. Genetic relationship Indonesian *Rhipichephalus (Boophilus) microplus* tick based on internal transcribed spacer-2 sequence. J. Veteriner. http://ojs.unud.ac.id/index.php/jvet/article/view/15998. Accessed April 5, 2017 [Indonesian].

Salama, A.A., AbouLaila, M., Moussa, A.A., Nayel, M.A., El-Sify, A., Terkawi, M.A., Hassan, H.Y., Yokoyama, N., Igarashi, I., 2013. Evaluation of *in vitro* and *in vivo* inhibitory effects of fusidic acid on *Babesia* and *Theileria* parasites. Vet. Parasitol. 191, 1–10.

Salama, A.A., AbouLaila, M., Terkawi, M.A., Mousa, A., El-Sify, A., Allaam, M., Zaghawa, A., Yokoyama, N., Igarashi, I., 2014. Inhibitory effect of allicin on the growth of *Babesia* and *Theileria equi* parasites. Parasitol. Res. 113, 275–283.

Sergeant, E.S.G., 2017. Epitools epidemiological calculators. Ausvet Pty Ltd. http://epitools.ausvet.com.au/content.php?page=home. Accessed April 5, 2017.

Smith, T., Kilborne, F.L., 1893. Investigations into the nature, causation, and prevention of Texas or southern cattle fever, 8th and 9th Repts. Bur. Anim. Industr., U.S. Dept. Agric. 177–304.

Soekardono, S., 1989. Important protozoan diseases of animals in Indonesia (a review). Bul. Penel. Kes. http://ejournal.litbang.depkes.go.id/index.php/BPK/article/view/655. Accessed April 5, 2017.

Suarez, C.E., Noh, S., 2011. Emerging perspectives in the research of bovine babesiosis and anaplasmosis. Vet. Parasitol. 180, 109–125.

Suarez, C.E., Palmer, G.H., Hötzel, I., McElwain, T.F., 1998. Structure, sequence, and transcriptional analysis of the *Babesia bovis* rap-1 multigene locus. Mol. Biochem. Parasitol. 93, 215–224.

Sukanto, I.P., Payne, R.C., Partoutomo, S., 1993. Bovine babesiosis in Indonesia. Prev. Vet. Med. 16, 151–156.

Tefi, I.K., 2015. The study of blood parasites (*Anaplasma*, *Babesia*, and *Theileria*) and physiological profiles of imported cattle from Australia. Master Thesis: Bogor Agricultural University. http://repository.ipb.ac.id/handle/123456789/75273. Accessed April 5, 2017 [Indonesian].

Terkawi, M.A., Huyen, N.X., Wibowo, P.E., Seuseu, F., Aboulaila, M., Ueno, A., Goo, Y.K., Yokoyama, N., Xuan, X., Igarashi, I., 2011a. Spherical body protein 4 is a new serological antigen for global detection of *Babesia bovis* infection in cattle. Clin. Vaccine Immunol. 18, 337–342.

Terkawi, M.A., Seuseu, F.J., Eko-Wibowo, P., Huyen, N.X., Minoda, Y., Aboulaila, M., Kawai, S., Yokoyama, N., Xuan, X., Igarashi, I., 2011b. Secretion of a new spherical body protein of *Babesia bovis* into the cytoplasm of infected erythrocytes. Mol. Biochem. Parasitol. 178, 40–45.

Terkawi, M.A., Huyen, N.X., Shinuo, C., Inpankaew, T., Maklon, K., AbouLaila, M., Ueno, A., Goo, Y.K., Yokoyama, N., Jittapalapong, S., Xuan, X., Igarashi, I., 2011c. Molecular and serological

prevalence of *Babesia bovis* and *Babesia bigemina* in water buffaloes in the northeast region of Thailand. Vet. Parasitol. 78, 201–207.

Terkawi, M.A., Alhasan, H., Huyen, N.X., Sabagh, A., Awier, K., Cao, S., Goo, Y.K., Aboge, G., Yokoyama, N., Nishikawa, Y., Kalb-Allouz, A.K., Tabbaa, D., Igarashi, I., Xuan, X., 2012. Molecular and serological prevalence of *Babesia bovis* and *Babesia bigemina* in cattle from central region of Syria. Vet. Parasitol. 187, 307–311.

Terkawi, M.A., Kameyama, K., Rasul, N.H., Xuan, X., Nishikawa, Y., 2013. Development of an immunochromatographic assay based on dense granule protein 7 for serological detection of *Toxoplasma gondii* infection. Clin. Vaccine Immunol. 20, 596–601.

Timms, P., Dalgliesh, R.J., Barry, D.N., Dimmock, C.K., Rodwell, B.J., 1983. *Babesia bovis*: Comparison of culture-derived parasites, non-living antigen and conventional vaccine in the protection of cattle against heterologous challenge. Aust. Vet. J. 60, 75–77.

Tuvshintulga, B., AbouLaila, M., Davaasuren, B., Ishiyama, A., Sivakumar, T., Yokoyama, N., Iwatsuki, M., Otoguro, K., Omura, S., Igarashi, I., 2016. Clofazimine inhibits the growth of *Babesia* and *Theileria* parasites *in vitro* and *in vivo*. Antimicrob. Agents and Chemother. 60, 2739–2746.

Vannier, E., Krause, P.J., 2012. Human babesiosis. N. Engl. J. Med. 366, 2397–2407.

Vannier, E.G., Diuk-Wasser, M.A., Ben Mamoun, C., Krause, P.J., 2015. Babesiosis. Infect. Dis. Clin. North Am. 29, 357–370.

Vega, C.A., Buening, G.M., Green, T.J., Carson, C.A., 1985. *In vitro* cultivation of *Babesia bigemina*. Am. J. Vet. Res. 46 (2), 416–420.

Verdida, R.A., Xuan, X., Fukumoto, S., Huang, X., Zhou, J., Igarashi, I., Claveria, F.G., Nagasawa, H., 2005. Development of a practical immunochromatographic test with recombinant P50 for the diagnosis of *Babesia gibsoni* infection in dogs. Parasitology 131 (6), 769–774.

Vial, H.J., Gorenflot, A., 2006. Chemotherapy against babesiosis. Vet. Parasitol. 138, 147–160.

Witola, W., Tsuda, A., Inoue, N., Ohashi, K., Onuma, M., 2005. Acquired resistance to berenil in a cloned isolate of *Trypanosoma evansi* is associated with upregulation of a novel gene, TeDR40. Parasitology 131, 635–646.

Zintl, A., Gray, J.S., Skerrett, H.E., Mulcahy, G., 2005. Possible mechanisms underlying age-related resistance to bovine babesiosis. Parasite Immunol. 27, 115-120.