

# DETECTION OF *BABESIA BOVIS* IN CATTLE BY PCR-ELISA

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**Abstract.** We established a new highly sensitive method, PCR-ELISA, for the detection of *Babesia bovis* in cattle for farms in Thailand. The detection of around  $2.4 \times 10^{-8}\%$  parasitemia (equivalent to 1 infected erythrocyte per 2 ml) was achieved by PCR amplification followed by the ELISA detection of a biotin tagged gene. When comparing the sensitivity of PCR-ELISA with the microscopic method, our PCR-ELISA method is more sensitive than thin blood smears by at least 1,000 times. The established PCR-ELISA also showed high specificity to *B. bovis* with no cross reaction to other endemic parasites except for *A. marginale*. Regarding the detection threshold for *B. bovis*, the PCR-ELISA method could detect parasites inoculated into splenectomized calves at least 1 week earlier than the thin blood microscopic method. The PCR-ELISA method is a valuable screening technique for *B. bovis* and applicable for the routine detection of carrier states and automated analysis.

## INTRODUCTION

The bovine babesiosis is a tick-borne disease of cattle caused by an intra-erythrocytic protozoan parasite of the genus *Babesia*, namely *Babesia bovis* (*B. bovis*) and *Babesia bigemina* (*B. bigemina*). These parasites cause substantial monetary loss to the cattle industry in Thailand, as well as other developing countries in Asia, Africa, South America and many islands of the Caribbean and South Pacific (Thompson, 1979; McCosker, 1981; Bram, 1983; Brockelman, 1989). *B. bovis* is more virulent than *B. bigemina* and the babesiosis caused by *B. bovis* is less sensitive to some babesiacidal compounds making it difficult to cure the infected animals (Brockelman and Tan-ariya, 1991; Tan-ariya and Sarathaphan, 1991). The disease is clinically manifested by anemia, fever, hemoglobinuria, marked splenomegaly and hepatomegaly, and sometimes death. In Thailand, *B. bovis* causes mainly babesiosis in cattle or dairy cows, which

leads to heavy economic losses in animal production. Animals that survive from *B. bovis* infection generally become carriers of the parasite and serve as reservoirs for transmission (Mahoney, 1969). Vaccination of animals with live attenuated vaccines has been used to control babesiosis (Mahoney, 1967; De Vos and Bock, 2002). However, the disadvantages of vaccines containing parasites derived from the blood of animals are well known, including the risk of reactions or contamination, and sensitization against blood groups. Because of this and other limitations of vaccines, sensitive methods for the detection of *B. bovis* carriers or subclinical cattle are very important. Generally, the standard method used to diagnose *B. bovis* is by the detection of *Babesia* parasites in a Giemsa-stained thin blood smear film examined by microscopy. Using this method, it is very difficult to detect the parasites in cases of low parasitemia. The current methods for the diagnosis of bovine babesiosis are techniques of molecular biology, particularly polymerase chain reaction (PCR) and DNA hybridization (McLaughlin *et al*, 1986; Jasmer *et al*, 1990; Fahrimal *et al*, 1992; Petchpoo *et al*, 1992; Calder *et al*, 1996; Salem *et al*, 1999). The detection of the PCR product by gel electrophoresis and Southern blot hybridization has limitations

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in the number of samples that can be analyzed at one time. Polymerase chain reaction coupled with enzyme linked immunosorbent assay (PCR-ELISA) is a high potential method, which has been used for the diagnosis of many diseases (He *et al*, 1993; Gale *et al*, 1996; Luk *et al*, 1997; Masake *et al*, 2002). This method has not been reported to have been used for the diagnosis of *B. bovis* infection.

As an initial step to apply the PCR-ELISA method to the detection of *B. bovis* for large scale screening and in an attempt to develop alternative diagnostic methods for carriers of babesiosis, we evaluated the reliability, sensitivity, and specificity of PCR-ELISA and compared it with microscopic techniques and gel electrophoresis.

## MATERIALS AND METHODS

Parasites used in this study included *B. bovis*, *B. bigermina*, *Anaplasma centrale* (*A. centrale*), *Anaplasma marginale* (*A. marginale*), and *Trypanosoma evansi* (*T. evansi*). *B. bovis* was subinoculated into splenectomized calves as described previously (Callow and Mellors, 1966). *B. bovis* ( $7.5 \times 10^7$ ) in 1 ml of whole blood were subcutaneously inoculated into the splenectomized calf after it was shown to be healthy by examination, body temperature 38.2°C, red blood cell count  $7.34 \times 10^6/\text{mm}^3$ , and no antibody titer to *B. bovis* detected by the indirect fluorescent antibody technique (IFA). Routine checking for body temperature, red blood cell count, packed cell volume (PCV) and thin blood smear were taken every other day after inoculation until the first parasite was found on the thin blood smear. Fourteen days after inoculation, before the calf succumbed to death, the body temperature rose to 40.6°C, the PCV dropped to 8%, and the parasitemia was 1%. The other blood specimens were collected from cattle naturally infected with four types of parasites (*B. bigermina*, *A. centrale*, *A. marginale*, *T. evansi*). Parasites in the whole blood were stored in liquid nitrogen until used. The percentage of parasitemia was obtained from the middle of the thin blood smear stained with Giemsa's stain and examined in a standard way, which usually has RBC  $\geq 1,000$  cells per field at high magnification. The number of parasites in

twenty microscope fields examined at high magnification was counted. This number was expressed as a percentage of the total erythrocytes in the twenties field.

DNA of *B. bovis* as well as other parasites was isolated using a high pure PCR template preparation kit (Boehringer Mannheim Co, Germany). The purified *B. bovis* DNA from the inoculum of the splenectomized calf was used to determine the sensitivity and specificity of the method. The pure DNA of *B. bovis* isolated from  $1.5 \times 10^7$  parasites / 200  $\mu\text{l}$  of infectious blood was diluted in sterile water and used for the PCR template.

A pair of nucleotide primers from the Bo6 sequence of the *B. bovis* Mexican strain (5'-GGGTTTATAGTCGGTTTTGT-3' and 5'-ACCATTCTGGTACTATATGC-3') which responded to the apocytochrome *b* gene were synthesized and the primers were biotinylated at their 5' end (GIBCO BRL, USA). The PCR condition was described before with minor modifications (Fahrimal *et al*, 1992). Namely, 10 ng of *B. bovis* DNA was added to 100  $\mu\text{l}$  of a PCR mixture composed of 1  $\mu\text{M}$  of each biotinylated primer, 200 mM of dATP, dCTP, and dGTP, 130 mM dTTP, 70 mM Dig-11-dUTP and 4 U of *Taq* DNA polymerase (Boehringer Mannheim, Germany) in 10 mM Tris-HCl buffer, pH 8.3 containing 6 mM  $\text{MgCl}_2$  and 50 mM KCl. The PCR was taken through 30 cycles in a DNA thermal cycler (Cyclogene Cambridge). Conditions for amplification were: first cycle of 5 minutes at 94°C, (denature), 2 minutes at 55°C, (annealing), 5 minutes at 72°C (extension), followed by 28 cycles of 1 minute at 94°C, 2 minutes at 55°C, 5 minutes at 72°C followed by 1 cycle of 1 minute at 94°C, 2 minutes at 55°C, 7 minutes at 72°C. A buffer was used as control for checking the contamination of the PCR product.

## Detection of PCR products

**ELISA.** A microtiter plate (96 well plate; maxisorp<sup>TM</sup>, Nunc, Kamstrup, Denmark) was coated with 50  $\mu\text{l}$  of streptavidin (10  $\mu\text{g}/\text{ml}$ ) per well in 0.06 M carbonate buffer, pH 9.6 at 4°C, overnight. Unsaturated binding sites were blocked with 3% (w/v) skim milk in TST buffer (40 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05%

Tween 20) (He *et al*, 1993). The plates were then washed with TST buffer followed by the addition of samples (4  $\mu$ l of PCR product diluted in 50  $\mu$ l of PBS, which contained 2  $\mu$ g/ml of sheared salmon sperm DNA) to each well. The plate was incubated at 37°C, for 1 hour. After the reaction, the plate was subjected to wash with TST buffer five times. Then 50  $\mu$ l of a 1:2,500 dilution of anti-Dig (Fab) conjugated with alkaline phosphatase (Boehringer Mannheim, Germany) was added to each well and incubated at 37°C for 30 minutes. The plate was washed three times with TST buffer, twice with TS buffer (40 mM Tris-HCl, pH 7.4, 150 mM NaCl) and then equilibrated with detection buffer for 30 seconds. Substrate solution (1 mg/ml of p-nitrophenyl phosphate in 0.05M carbonate buffer, pH 9.8, 0.005 M MgCl<sub>2</sub>) was added and the color was developed by adding 100  $\mu$ l of 1 M NaOH per well. The absorbance was measured at 405 nm using an ELISA reader (Labsystems Muttiskan MS).

**Agarose gel electrophoresis.** Four microliters of each PCR-amplified solution was loaded onto each lane of 1% agarose gel. Electrophoresis of amplified DNA was carried out in TBE buffer (90 mM Tris-borate, 2 mM EDTA) at a constant voltage of 100 V for 2 hours. Then, the gel was stained by ethidium bromide and photographed (Sambrook *et al*, 1989). Hind III-digested Lamda DNA was used as a marker for determining the sizes of the amplified DNA bands.

**Southern blot hybridization.** The electrophoretically separated DNA was blotted from gel by capillary transfer onto a nylon membrane (Boehringer Mannheim, Germany). The DNA was denatured by 0.5 M NaOH, 1.5 M NaCl. DNA was then crosslinked onto the membrane by exposure to UV light. The immobilized DNA membrane was placed into a prehybridization solution (5xSSC, 0.1% lauroylsarcosine, 0.02% SDS, 1% blocking reagent) and incubated at 68°C for 1 hour. The hybridization reaction was then carried out in the fresh hybridization solution containing the denature probe at 68°C, overnight. The membrane was washed twice in 2xSSC containing

0.1% SDS at room temperature for 15 minutes and twice in 0.5xSSC containing 0.1% SDS at 68°C for 15 minutes. The membrane was then blocked by blocking solution (Boehringer Mannheim, Germany) for 30 minutes. The diluted solution of 1:5,000 of anti-Dig conjugated with alkaline phosphatase was added and incubated for 30 minutes at room temperature. Excess antibody was removed from the membrane by washing twice with maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5), and then the membrane was equilibrated in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM MgCl<sub>2</sub>, pH 9.5) for 2 minutes. The substrate solution (NBT and BCIP, Boehringer Mannheim, Germany) was added and allowed to form the blue color for 16 hours. The reaction was stopped by washing the membrane with sterile water for 5 minutes.

**Light microscopic examination.** A thin blood smear of blood samples was fixed with methanol for 1 minute and stained with Giemsa solution for 30 minutes, then the parasites were determined by light microscopic examination.

## RESULTS

### Specificity of PCR reaction

There are many parameters in PCR that influence the specificity, fidelity and yield of the desired product (Innis and Gelfand, 1990). In this study the optimal concentrations of those PCR parameters are 5 ng/ml of *B. bovis* Thai isolated DNA, 2.5 mM of MgCl<sub>2</sub>, 0.5  $\mu$ M of each primer, and 1 U of *Taq* DNA polymerase in a mixture of 50  $\mu$ l (data not shown). Several optimization parameters for the ELISA were also studied. We found that 1  $\mu$ g of the biotinylated PCR products labeled-DIG (diluted in 50  $\mu$ l of dilution buffer) was sufficient for this ELISA and the optimum incubation time was at 37°C for 60 minutes. A 1:2,500 dilution of anti-Dig conjugated with alkaline phosphatase was enough to bind to digoxigenin in the PCR products. The suitable time for signal development color was 60 minutes with 100  $\mu$ l of 1 mg/ml of pNPP (data not shown).

The specificity of the PCR was examined

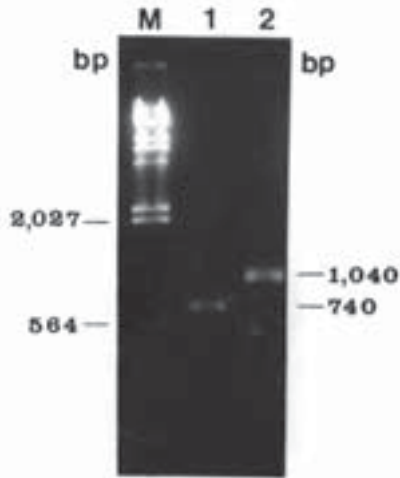


Fig 1—Specificity of the PCR method. Ethidium bromide stained agarose gel electrophoresis of PCR products from *B. bovis* with non-incorporated digoxigenin (lane 1), and incorporated digoxigenin (lane 2). Lane M lambda *Hind* III DNA digested marker.

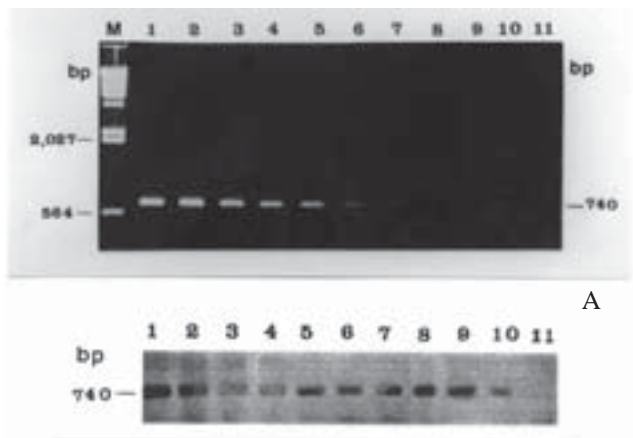
with the DNA extracted from *B. bovis* inoculated splenectomized calf erythrocyte. The results (Fig 1) showed that the bands of the expected size of apocytochrome *b* gene (740 bp for unincorporated digoxigenin and 1,040 bp when digoxigenin was incorporated into the DNA chain) were obtained. These results coincided with previous results (Fahrimal *et al*, 1992).

**Determination of the sensitivity of PCR-ELISA**

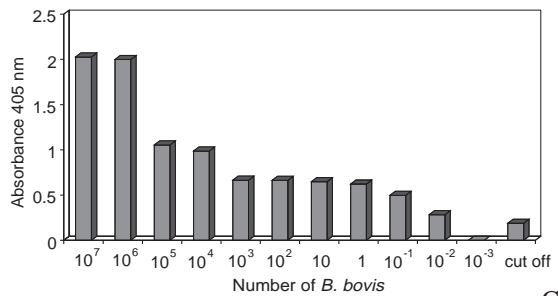
Ten-fold serial dilutions of *B. bovis* infected erythrocytes from the inoculated splenectomized calf ( $10^7$  to  $10^{-3}$  parasites) were added to 0.2 ml of normal bovine blood. The DNA was then extracted from each diluted sample for testing the sensitivity of the PCR method. As shown in Fig 2, agarose gel electrophoresis was sensitive in detecting parasite DNA with equivalence to 1 parasite. The PCR-ELISA and Southern blot hybridization could detect parasite DNA at about  $10^{-2}$  parasites with equivalence to a parasitemia of  $2.4 \times 10^{-8}\%$ .

**The species specificity of the PCR**

Other hemoparasites can be endemic with *B. bovis*, including *B. bigermina*, *A. marginale*, and *T. evansi*. Their DNA and leukocyte DNA were examined. Five nanograms of DNA from each species was amplified by PCR and then the PCR prod-



A  
B



C

Fig 2—Detection of sensitivity. The detection of sensitivity of PCR, followed by agarose gel electrophoresis (A), Southern blot hybridization (B), PCR-ELISA (C). Lane M is lambda *Hind* III DNA digested marker. Lanes 1-11 are the number of parasites diluted tenfold from  $10^7$ - $10^{-3}$  in bovine blood 200  $\mu$ l. The cut-off of ELISA is 0.189 at 405 nm.

ucts were analyzed by agarose gel electrophoresis and the ELISA method. While the amplified product detected was *B. bovis* DNA, no other PCR product was generated when the DNA from other hemoparasites or leukocytes was tested (Fig 3).

The species specificity of PCR-ELISA was also analyzed. The results showed a few signals in ELISA for *A. marginale*, however, the absorbance from *B. bovis* showed a different intensity. When the incubation time for the color reaction was changed to 30 minutes the intensity of color was eliminated. However, cross-reaction with *A. marginale* in PCR-ELISA was observed.

**Detection threshold of PCR-ELISA**

The blood sample from the experimental splenectomized calf was used to check the detec-

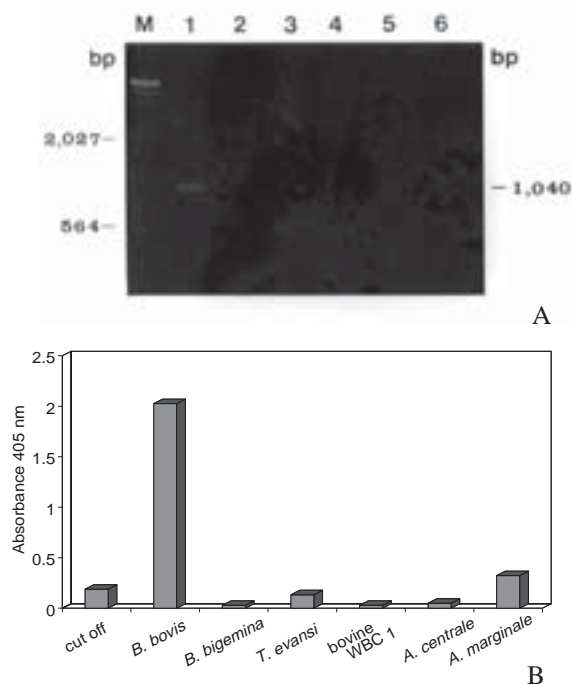


Fig 3—Species specificity of PCR-ELISA. PCR followed by agarose gel electrophoresis (A), ELISA (B). Lane M: lambda *Hind* III DNA digested marker; lane 1 *B. bovis*; lane 2: *B. bigemina*; lane 3: *T. evansi*; lane 4: bovine WBC DNA; lane 5: *A. centrale*; lane 6: *A. marginale*.

tion threshold of the PCR-ELISA. Blood samples were collected from the calf at two days prior to subinoculation, and at the days 0, 3, 5, 10, 12, and 14 after inoculation. From our results, the PCR-ELISA method detected parasites day 3 after inoculation of the splenectomized calf. On the other hand, the thin blood smear technique detected the parasite on day 12 after inoculation (Fig 4).

#### Applicability of the PCR-ELISA to the detection of *B. bovis* in blood samples

The PCR-ELISA was used to detect blood samples of naturally infected cattle from farm and splenectomized calves, which were maintained under tick free conditions. The results of PCR-ELISA were compared with the immunology method, thin blood smear method, and PCR-gel electrophoresis. The results are shown in Table 1. PCR-ELISA had good sensitivity and specificity in detecting two samples of carrier cattle

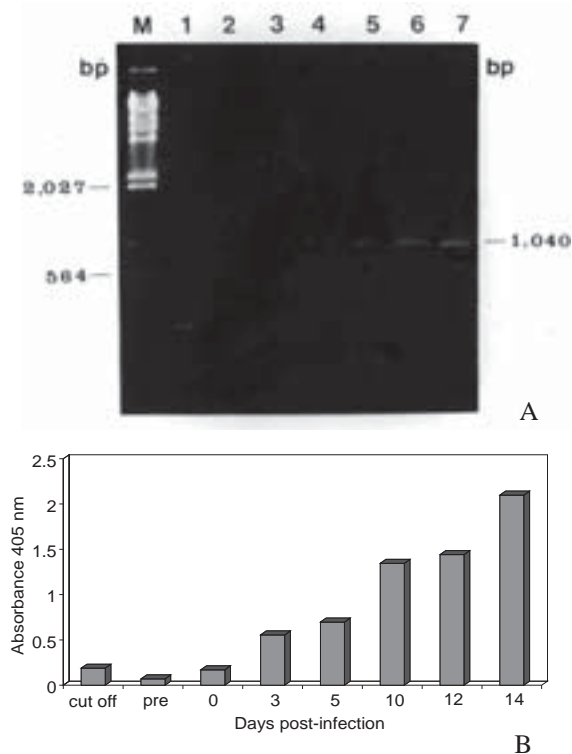


Fig 4—Detection of the parasite DNA of *B. bovis* in splenectomized calf. PCR followed by agarose gel electrophoresis (A), ELISA (B). Lane M: lambda *Hind* III DNA digested marker; lane 1: two day prior to inoculation; lane 2: the day of inoculation; lanes 3-7: 3-14 day after inoculation to splenectomized calf respectively.

from six naturally infected samples for which the blood smear method and PCR-gel electrophoresis showed negative results. The splenectomized calf showed all negative for PCR-ELISA.

## DISCUSSION

We have developed a new method, PCR-ELISA, for the detection of a carrier status of Thai isolated *B. bovis* infection. The PCR product of *B. bovis* Thai isolated is slightly different from the PCR product (711 bp) amplified from *B. bovis* Mexican, Australia L and S and Texas strains DNA (Fahrimal *et al.*, 1992). This difference may be due to the polymorphism of the apocytchrome *b* gene. The incorporation of digoxigenin moieties into the PCR products caused the size of the

Table 1

Comparison of blood smears, antibody screening, agarose gel electrophoresis and PCR-ELISA for the detection of *B. bovis* infection in the field.

	Number of samples	Antibody screening	Blood smears technique	Number of positive samples	
				PCR followed by :	
				gel electrophoresis	ELISA
Splenectomized calves <sup>c</sup>	4	0 <sup>a</sup>	0	0	0
Cows in dairy farms	6	6 <sup>b</sup>	0	0	2

<sup>a</sup>The results of antibody screening for *B. bovis*, *B. bigermina*, and *A. marginale*, and blood smears for hemoparasites were conducted before removing the spleen; <sup>b</sup>The results of antibody screening for *B. bovis*, *B. bigermina*, *A. marginale*, *A. centrale* and *T. evansi*; <sup>c</sup>Splenectomized calves were maintained under tick free conditions.

product to increase from 740 to 1,040 bp on gel electrophoresis due to the mass retardation of digoxigenin.

The PCR-ELISA system described here has the operational advantage of requiring no PCR products purification before subjection to ELISA and of yielding the statistical validated negative cut-off point. Our PCR condition was found to be specific for the apocytochrome *b* gene of *B. bovis* when DIG-unlabeled PCR products are analyzed by gel electrophoresis and Southern blot hybridization. The PCR-ELISA did show a slight signal from *A. marginale*. This result may be due to the long extension time of the PCR condition, which is caused by the non-specific reaction generated from *Taq* DNA polymerase.

The high sensitivity of established PCR-ELISA lower than one parasite per sample may be due to the use of the extra chromosomal DNA of *B. bovis* as a template, which has greater than 100 copies per parasite genome (Salem *et al*, 1999). This PCR-ELISA also showed higher sensitivity than previous PCR base detection methods (Fahrimal *et al* 1992; Salem *et al*, 1999; Calder *et al*, 1996). Comparison of PCR-ELISA with thin blood smears for the detection of carrier or sub-clinical status of *B. bovis* revealed that the PCR-ELISA can detect the parasite at least 1 week earlier than thin blood smears. These results suggest that the PCR-ELISA method has the potential to detect parasite DNA in very early stages of infection, before the host animal shows

clinical symptoms. The PCR-ELISA is also applicable for the detection of the natural carrier status of *B. bovis*. It can distinguish the real status of infection while antibody screening does not always indicate the infection of parasite because the antibody has persisted for many years without the hemoparasite (Mahoney and Wright, 1973; 1976). Thirty-three percent of 6 *B. bovis*-positive antibody screens was detected by PCR-ELISA. This result indicates that PCR-ELISA can discriminate the active parasite in cattle from a previous infection.

In conclusion, the comparison of PCR-ELISA with the microscope technique revealed the PCR-ELISA to be more sensitive in the detection of carrier cattle. The PCR-ELISA can detect the early infection stage from three days after infection; this is very important in making decisions regarding treatment on the cattle farm. The major advantages of this technique are the high sensitivity for the detection of carrier status and that it allows many samples to be analyzed at one time. Besides these, it could be adapted to automatic analysis in the future.

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