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Specific and Highly Sensitive Primers for PCR Detection of *Babesia bovis*

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ABSTRACT: Various portions of the published sequence of *Babesia bovis* mitochondrial DNA were selected for detection of *B. bovis* by PCR-based methods. Five primer pairs, namely P_1, P_2, P_3, P_4 and P_5, were designed from the gene sequence. Only P_4 and P_5 yielded PCR products of *B. bovis* DNA but not of bovine DNA. The PCR products are 120 and 181 bp for P_4 and P_5, respectively. The specificity of PCR was examined using DNA template from various haemoparasites: *B. bovis*, *Trypanosoma spp*., *B. bigemina*, *Theileria spp*., *Anaplasma marginale* and *A. centrale*. P_5 was specific for *B. bovis* whereas P_4 showed cross-amplification with *A. centrale* DNA. The P_5 primer pair, which was designed from the region closed to mitochondrial large subunit ribosomal DNA of *B. bovis*, showed sensitivity equivalent to the Fahrimal's primers but with higher amount of PCR product. The better result of PCR using this primer pair was confirmed by the duplex PCR when amplification of the apocytochrome *b* region was performed concurrently. Our study provides an alternative choice for PCR detection of *B. bovis* and demonstrates another region within mitochondrial DNA which is also the valuable target for specific amplification of the parasite DNA.

KEYWORDS: *Babesia bovis*, mitochondrial DNA, specific amplification, high sensitivity.

INTRODUCTION

Babesia bovis is an intraerythrocytic protozoan parasite and is a causative agent of bovine babesiosis. The haemoparasites enter the bovine host as sporozoites during tick feeding which invade erythrocytes, grow and develop into merozoites. The erythrocytes burst, releasing the merozoites to invade further erythrocytes. Repeated cycles of development of the parasites result in destruction of a large number of erythrocytes. The common clinical manifestations of this disease are mild to severe anemia, stress and weakness that can lead to death of the animals.[1] Frequently, animals, which recover from a primary acute infection either naturally or after chemotherapy, remain persistently infected and become carriers of the parasite. As carriers, the parasitemia is often too low to be detected by microscopy. Thus, highly sensitive and specific diagnostic tools are required to detect the carrier animals.[1-3] Currently, detection of *B. bovis* in carrier cattle is routinely conducted by thin or thick blood smear, stained with Wright's or Giemsa's stain, and examined under a light microscope. In addition, serological methods such as complement fixation (CF) test, indirect fluorescent antibody (IFA) test, indirect haemagglutination (IHA) test and enzyme linked immunosorbent assay (ELISA) have been established to detect this parasite.[4-6] However, serology is indirect and may not distinguish between past exposure and current infections. To obtain an accurate diagnosis, PCR-based techniques have been developed for specific and sensitive detection of the parasite DNA. The primers used were designed from the apocytochrome *b*, small subunit rRNA (SSrRNA) and β-tubulin gene sequences of *B. bovis*.[2,3,7]

In this report, we have designed 5 primer pairs from the published mitochondrial DNA sequence of *B. bovis* (GenBank accession no. AF053002) for PCR-based detection of *B. bovis*. One pair of these primers was found to be specific for *B. bovis* DNA. The sensitivity of PCR-based techniques using this primer pair was also higher than those previously reported. Our study provides an alternative tool that is useful for the detection of *B. bovis*.

Materials and Methods

Primer Design

Five oligonucleotide primer pairs were designed from the partial sequence of mitochondrial DNA of *B. bovis* (AF053002) published in the GenBank database. The position of the predicted region obtained from amplification using each primer pair is shown in Fig 1. The nucleotide sequences, lengths and melting temperatures of the designed primers P_1 to P_5 and the Fahrimal's primers[7] P_6, as well as predicted sizes of the PCR fragments are also illustrated (Table 1).

Parasites

B. bovis Thai strain was inoculated into a splenectomized calf kept in a tick-free environment. Whole blood was collected when a high parasitaemia was detected. The number of infected erythrocytes/total red blood cells (RBCs) was determined by using thin blood smear. This figure was then multiplied by RBC count/ml to obtain the number of infected erythrocytes/ml. *B. bigemina, Trypanosoma* spp., *Theileria* spp., *Anaplasma marginale* and *A. centrale* were collected from cattle in field surveys in Thailand. The parasite infections were detected from thin blood smears and stained with Giemsa's stain.

DNA Isolation

Red blood cell (RBC) pellets were prepared from EDTA-blood samples by centrifugation (3,000 g for 10 min) and removal of the plasma. The RBC pellets were washed three times with phosphate-buffered saline (PBS) and then added with 50% glycerol in PBS to the original volumes. The samples were divided into 200 µl aliquots and snap-frozen at –70 °C until used. DNA was isolated following the protocol of High Pure PCR Template Preparation Kit (Boehringer Mannheim, Germany). Two hundred microlitres of the RBC sample were added with 200 ml of binding buffer (6 M guanidine-HCl, 10 mM urea, 10 mM Tris-HCl, 20% Triton X-100, pH 4.4) and subsequently with 40 µl of 20 mg/ml proteinase K. The sample was mixed immediately, incubated for 30 min at 72°C and mixed well with 100 µl of isopropanol while still warm. The mixture was then applied to a High Pure filter tube connected on a collection tube. After centrifugation at 3,600 g for 10 min, the flowthrough was discarded. The DNA trapped on the filter was washed once with 500 µl of inhibitor removal buffer (20 mM Tris-HCl, pH 6.6, containing 5 M guanidine-HCl and 40% ethanol) and twice with 500 µl of washing buffer (2 mM Tris-HCl, pH 7.5, containing 20 mM NaCl and 80% ethanol), each time followed by centrifugation at 3,600 g for 10 min. The High Pure filter tube was inserted in a 1.5 ml microcentrifuge tube and DNA was eluted by centrifugation at 3,600xg for 1 min, with 200 µl of elution solution (10 mM Tris-HCl, pH 8.5) prewarmed to 70°C. The DNA solution was adjusted to a final concentration of about 1 ng/µl. The DNA was used fresh or stored at –70°C for further analysis.

For sensitivity test, the RBC samples with known amount of infected erythrocytes were serially diluted with RBCs before subjected to the extraction of DNA. In specificity test, the number of each parasite was adjusted to approximately $5x10^4$ infected erythrocytes (or parasites)/ml before DNA extraction.

PCR Amplification

The reaction mixture (50 µl) contained about 5 ng total DNA, 2.5 mM $MgCl_2$, 0.5 µM the primers, 100 µM each dNTPs, and 2.5 units of *Taq* DNA polymerase (Promega, U.S.A.). The PCR was carried out in a Cyclogene Dri-Block®cycler [Techne (Cambridge) Ltd. and Techne Inc.] for 30 cycles. Each cycle normally

Table 1. Primers used for amplification of *B. bovis* mitochondrial DNA. P_1 – P_5 are new designed primers whereas P_6 is Fahrimal's primers. F and R stand for forward and reverse primers, respectively.

Primer Set	Primer sequence	Length (nt)	Melting temp. (°C)	Predicted size of PCR fragment (bp)	Optimized annealing temp. (°C)	Optimized [Mg^{2+}] (mM)	Presence of PCR product from:	
							B. bovis DNA	bovine DNA
P_1	F 5'-AGGGATTGTGGTACTCA-3'	17	50	676	48	1	+	+
	R 5'-GTGATAGATGTCCACGA-3'	17	50					
P_2	F 5'-GCAAACTCCAATGCA-3'	15	44	189	43	2	—	+
	R 5'-GATGTCCACGATCTG-3'	15	44					
P_3	F 5'-ATCACAGATCGTGGACA-3'	17	50	577	48	2	—	+
	R 5'-GGTGGGAGGTTCATAC-3'	16	50					
P_4	F 5'-ATGGAAACCCAGTTGTAGGA-3'	20	58	120	48	2	+	—
	R 5'-AAACAATTGAGGAGCGTCTG-3'	20	58					
P_5	F 5'-ATGGAAACCCAGTTGTAGGA-3'	20	58	181	58	2	+	—
	R 5'- ATGATTGTACGCTGTTTCGTA -3'	21	58					
P_6	F 5'-GGGTTTATAGTCGGTTTTGT-3'	20	56	711	55	2.5	+	—
	R 5'-ACCATTCTGGTACTATATGC-3'	20	56					

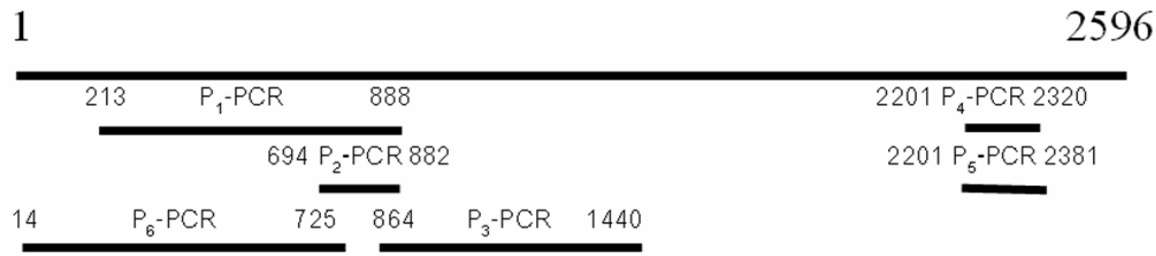

Fig 1. Positions of predicted regions obtained from amplifications using the designed primer pairs. The upper most line represents partial sequence of *B. bovis* mitochondrial DNA. The other lines illustrate predicted regions of DNA obtained from the amplification using different primer pairs: P_1-PCR to P_5-PCR from the primer pairs P_1 to P_5, while P_6-PCR from Fahrimal's primers P_6.

consisted of 1 min of denaturation at 94 °C (6 min for the first cycle), 2 min of annealing, and 3 min of extension at 72 °C, with additional 7 min at 72 °C after the last cycle. Only for P_5 primer pair, the periods of annealing and extension were reduced to 1 min and 30 sec, respectively. The annealing temperature for each primer set is shown in Table 1.

Duplex PCR

The combination of two primer pairs, P_5 and P_6, was used to perform duplex PCR. The PCR amplifications were conducted using conditions described for P_5 or P_6, except that the second primer pair was included in the reaction mixture.

Analysis of PCR Products by Agarose Gel Electrophoresis

The PCR products were electrophoresed on an agarose gel in TAE buffer.[8] Lambda DNA/*Hin*dIII markers (Promega, U.S.A.) or DNA marker V (Boehringer Mannheim, Germany) were used as standard size markers. Ten microlitres of PCR products were separated by electrophoresis at 70 volts (7 volts per cm). The DNA fragments were stained with SYBR green I (Sigma U.S.A.) and then visualized by transillumination with UV light.

Analysis of PCR Products by Southern Blot Hybridization

Digoxigenin (DIG)-labeled probes were prepared by using the above amplification procedures with modifications. 28% of the concentration of dTTP was replaced with DIG-11-dUTP (Boehringer Mannheim, Germany) and the extension time was increased to 1 min for P_5 and 5 min for P_6. The DIG-labeled products of P_5 and P_6 were employed as probes to detect the unlabeled PCR products of P_5 and P_6, respectively, by Southern blot hybridization.

After electrophoretic separation of the amplified DNA fragments, the agarose gel was incubated (2×15 min) in denaturation solution (0.5 M NaOH, 1.5 M NaCl) under agitation. The gel was rinsed once with sterile distilled water and soaked (2×15 min) in neutralization solution (0.5 M Tris-HCl, pH 7.5, containing 3 M NaCl). The DNA was then electrically transferred onto a MagnaCharge nylon membrane (Micron Separations Inc.). After a brief rinse with 6× SSC, the membrane was baked at 80 °C for 2 h and stored dry or immediately used for prehybridization (68 °C, 1 h) in 5× SSC supplemented with 1%(v/v) blocking solution (Boehringer Mannheim), 0.1%(w/v) N-lauroylsarcosine and 0.02%(w/v) SDS. Following hybridization at 68 °C for 16-18 h with approximately 25 ng/ml of DIG-labeled probe in the prehybridization solution, the membrane was washed in 2x SSC containing 0.1%(w/v) SDS (2x15 min) and then in 0.5x SSC containing 0.1%(w/v) SDS (2×15 min). Hybridization was detected with DIG-High Prime DNA Labeling and Detection Starter Kit I (Boehringer Mannheim) as recommended by the manufacturer.

Enzyme-linked Immunosorbent Assay for Biotinylated DIG-Labeled PCR Products (PCR-ELISA)

The PCR amplifications using the P_5 primer pair were conducted as described above except that the 5' end of the forward primer was biotinylated and *Taq* DNA polymerase was added to the reaction mixture after predenaturation at 99°C for 10 min. The ELISA procedure was modified from that previously described.[9] The wells were coated, for 24 h at 4°C, with 50 µl /well of 5 µg/ml streptavidin in PBS (1.5 mM disodium hydrogen phosphate, 8 mM potassium dihydrogen phosphate, 137 mM sodium chloride, 2.5 mM potassium chloride, pH 7.4). The plate was washed twice with TBST (TBS containing 0.05% Tween 20), then added with 100 µl of blocking solution (3% w/v skim milk in TBST) and incubated for 30 min at room temperature. Following five washings with TBST, the wells were added with 0.5 µl of the biotinylated DIG-labeled PCR products diluted to 50 µl in PBS. After incubation for 1 h at 37°C, the wells were washed five times with TBST, then added with 50 µl of alkaline

phosphatase-linked anti-DIG antibody diluted 1:1,250 in the blocking solution and incubated at 37°C for 30 min. The wells were washed three times with TBST, three times with TBS (40 mM Tris-HCl, pH 7.4, containing 150 mM NaCl) and then incubated with 50 µl of detection buffer (50 mM carbonate buffer, pH 9.8, containing 5 mM $MgCl_2$) at room temperature for 30 sec. The detection buffer was discarded prior to addition of 100 µl of chromogenic substrate for alkaline phosphatase (1 mg/ml p-nitrophenyl phosphate in the detection buffer). After incubation at 37°C for 30 min, the absorbance was measured at 405 nm using a Labsystems Multiscan MS Microplate Reader (Labsystems).

The detection cut-off was identified as the mean plus five times of standard deviation of the PCR-ELISA using DNA isolated from parasite-free bovine blood.[9] The background was set to zero using the well without the biotinylated DIG-labeled PCR products. All samples detected on PCR-ELISA were performed in duplicate.

Results

PCR Product of *B. bovis* DNA

The predicted size of PCR fragment from each primer pair and the summarization of amplification results are shown in Table 1. When amplifications were performed with *B. bovis* DNA, all primer pairs except P_2 and P_3 yielded PCR products. However, PCR products were also obtained from bovine DNA by using P_1, P_2 or P_3 but not P_4, P_5 or P_6 (Fahrimal's primers). Therefore, P_4 and P_5 were the only designed primers which could distinguish between *B. bovis* DNA and bovine DNA, and were selected for further study on species specificity. The sizes of PCR products were close to the predicted

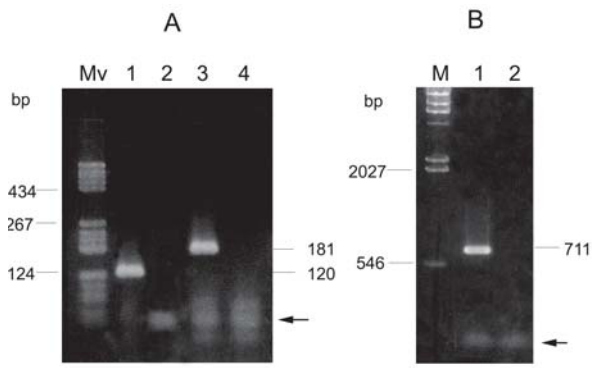

Fig 2. SYBR green I stained gels illustrating the PCR products of DNA extracted from *B. bovis* infected and uninfected bovine blood. (A) Analyses of amplification products were performed on 4% agarose gel for PCR products amplified with P_4 (lane 1: *B. bovis* infected blood, lane 2: uninfected bovine blood) and P_5 (lane 3: *B. bovis* infected blood, lane 4: uninfected bovine blood). Lane Mv represents DNA marker V. The 120 bp PCR product of P_4 and 181 bp PCR product of P_5 are also indicated. (B) Amplification products of P_6 were analysed on 1.5% agarose gel for PCRs using, *B. bovis* infected blood (lane 1) and uninfected bovine blood (lane 2). Lane M represents lambda DNA/*Hin*dIII markers. The PCR product is shown at 711 bp. An arrow (→) indicates primer dimer.

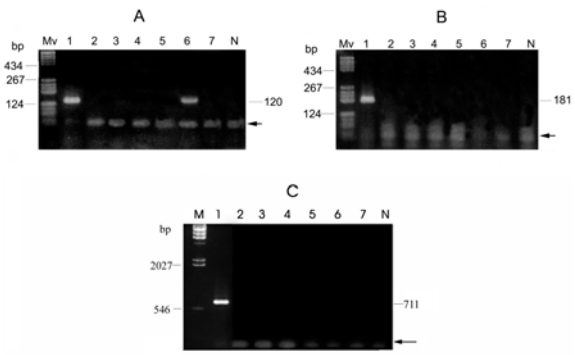

Fig 3. SYBR green I stained gels of PCR products demonstrating species specificity of the reactions using P_4 (A), P_5 (B) and P_6 (C) primers. The separation was conducted on 4% gel for the products amplified with P_4 and P_5, and 1.5% gel for the products amplified with P_6. Individual DNA samples were from *B. bovis* (lane 1), *Trypanosoma* spp. (lane 2), *B. bigemina* (lane 3), *Theileria* spp. (lane 4), *A. marginale* (lane 5), *A. centrale* (lane 6), and uninfected bovine blood (lane 7). Lanes Mv and M contain DNA marker V and lambda DNA/*Hin*dIII markers, respectively. Lane N represents no template DNA (negative control). An arrow (→) indicates primer dimer.

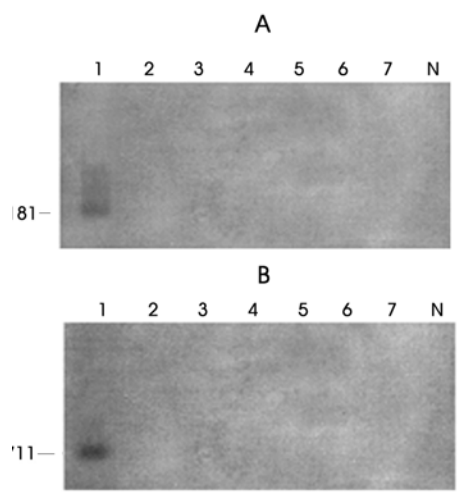

Fig 4. Southern blot hybridization of PCR products demonstrating species specificity of the reactions using P_5 (A) and P_6 (B) primers. Lanes, 1, *B. bovis*; 2, *Trypanosoma* spp.; 3, *B. bigemina*; 4, *Theileria* spp.; 5, *A. marginale*; 6, *A. centrale*; 7, uninfected bovine blood and N, no template DNA (negative control).

ones, which are 120 and 181 bp for P_4 and P_5 respectively, compared with the 711 bp fragment from the PCR with P_6 (Fig 2).

Species Specificity of P_4 and P_5

P_4 and P_5 were examined for their specificity in comparison with P_6. DNA templates were isolated from bovine blood infected with *B. bovis* or the other parasites that can be endemic along with *B. bovis* in Thailand, including *Trypanosoma* spp., *B. bigemina, Theileria* spp., *A. marginale* and *A. centrale*. PCR amplifications were conducted in parallel with DNA template from parasite-free bovine blood. PCR product of P_5 as well as P_6 were obtained only from the reactions using *B. bovis* DNA while no product was generated from bovine DNA or the other parasite DNAs (Fig 3). Similar results were obtained from PCR containing P_4 except that cross-amplification with *A. centrale* DNA was observed. The specificity of PCR with P_5 compared to P_6 was confirmed by Southern blot analysis using each DIG-labeled product as the probe (Fig 4).

Sensitivity of PCR Using P_5

Since P_5 produced specific amplification for *B. bovis* DNA, the minimal number of infected erythrocytes detectable by this PCR primer pair was then determined. Ten-fold serial dilutions of *B. bovis* infected erythrocytes collected from inoculated calf were prepared by addition of RBCs from uninfected cattle. The DNA solution obtained from each dilution was divided for separate analyses in the PCR with P_5 and P_6. Analyses

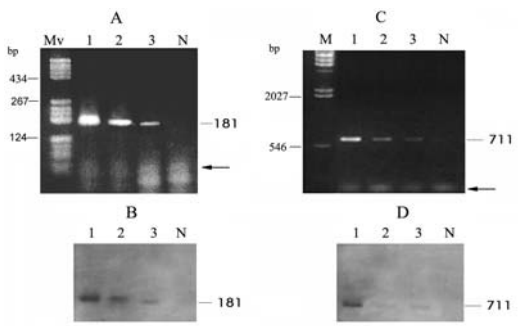

Fig 5. Sensitivity of the PCR using P_5 and P_6 primers determined from 10-fold serial dilutions of *B. bovis* infected blood. (A) SYBR green I staining after separation of PCR products on 4% gel and (B) Southern blot hybridization for the products amplified with P_5. (C) SYBR green I staining after separation on 1.5% gel and (D) Southern blot hybridization for the products amplified with P_6. Lanes Mv and M, are DNA marker V and lambda DNA/*Hin*dIII markers, respectively. Lanes 1-3 are 5×10^2, 5×10^1 and 5×10^0 infected erythrocytes/ml, respectively. Lane N represents no template DNA (negative control). An arrow (→) indicates primer dimer.

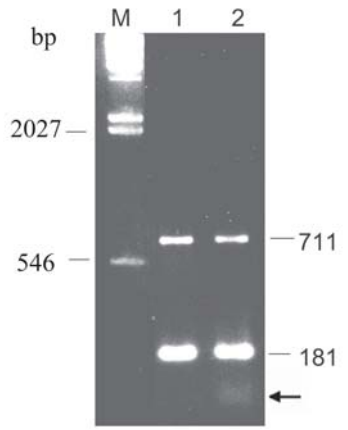

Fig 6. Amplification of *B. bovis* DNA by a duplex PCR using combination of P_5 and P_6 at two different conditions. The PCR products were separated on 2% gel and stained with SYBR green I, showing bands at 181 and 711 bp. Lane M is lambda DNA/ *Hin*dIII markers. Lanes 1 and 2 are PCR products performed at conditions optimal for P_5 and P_6, respectively. An arrow (→) indicates primer dimer.

of PCR products by agarose gel eletrophoresis and Southern blot hybridization showed minimal detection as low as 5 infected erythrocytes/ml sample for P_5 (Fig 5A and B, lane 3) and P_6 (Fig 5C and D, lane 3), corresponding to 1 infected erythrocyte/200 μl (working volume).

Amplification Products of Duplex PCR

The same PCR products of 181 and 711 bp, were obtained from both P_5 and P_6 conditions. The results also showed much higher intensity of the 181 bp band than the 711 bp band (Fig 6).

PCR-ELISA Using P_5

The PCR-ELISA using P_5 was performed since ELISA is a useful technique for mass screening. The specificity and sensitivity obtained by this technique were the same as those obtained from agarose gel electrophoresis and Southern blot. Only the PCR products of *B. bovis* DNA showed the reactivity on ELISA at higher level than the cut-off in specificity test (Fig 7A). The sensitivity test indicates the usefulness of P_5 in PCR-ELISA for diagnosis of *B. bovis* infection since the signal obtained from 5 infected erythrocytes/ml (1 infected erythrocyte/200 μl) is much higher than the cut-off value (Fig 7B).

Discussion

PCR-based methods have been shown to be very useful in the early detection of *B. bovis* infection. A number of primers were reported to create specific

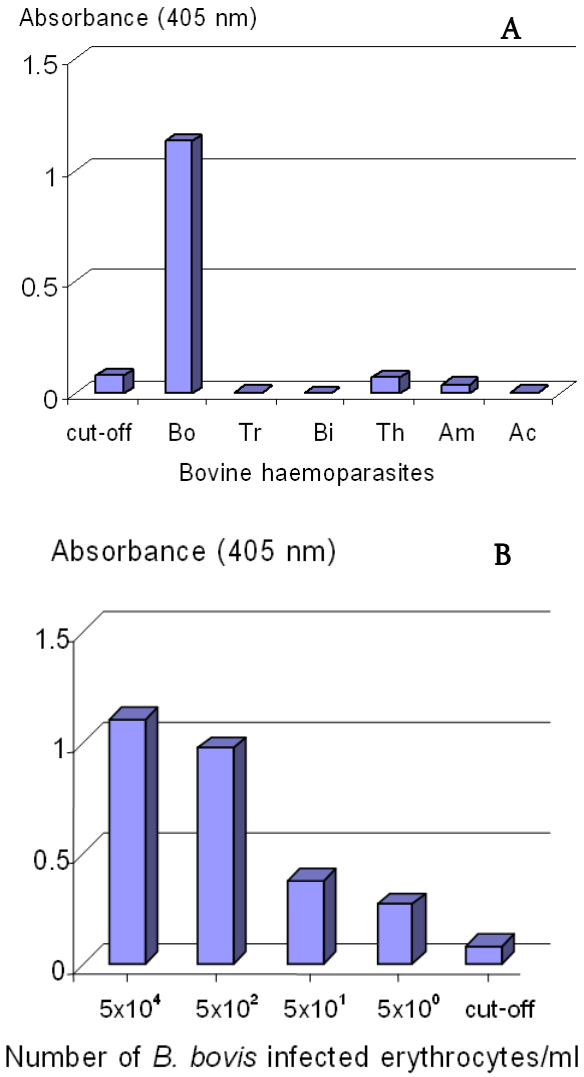

Fig 7. PCR-ELISA using P_5 primer. (A) The specificity of the reaction was determined among haemoparasites: *B. bovis* (Bo), *Trypanosoma* spp. (Tr), *B. bigemina* (Bi), *Theileria* spp. (Th), *A. marginale* (Am) and *A. centrale* (Ac). (B) The sensitivity for detection of *B. bovis* was determined from 10-fold serial dilutions of *B. bovis* infected blood: 5×10^4, 5×10^2, 5×10^1 and 5×10^0 infected erythrocytes/ml.

amplification of various regions within *B. bovis* genomic DNA. Among the previous reports, the amplification of a region in mitochondrial apocytochrome *b* gene[7] showed higher sensitivity than amplification of SSrRNA gene of *B. bovis*.[2] Such apocytochrome *b* gene derived primers also exhibited no cross-amplification with host DNA, while the detection of *B. bovis* β-tubulin gene[3] needed nested PCR to discriminate amplified fragments of host DNA. Although amplification of the apocytochrome *b* region shows higher sensitivity and specificity than the other regions for detection of *B. bovis* by PCR-based methods, high mutation rate of mitochondrial DNA[10] may cause problems. Other regions in this extrachromosomal DNA, which generate specific amplification for *B. bovis*, should be introduced in combination with the apocytochrome *b* region. In the present study, 5 primer pairs were designed from alignment of the mitochondrial DNA sequence (AF053002) of *B. bovis* with all sequences of *A. centrale, A. marginale, B. bigemina, Theileria* spp., *Trypanosoma* spp. and other regions of *B. bovis* published in GenBank. Four of them (P_1, P_2, P_3, P_4) showed cross-amplification with bovine DNA or other haemoparasite DNAs. Two primer pairs (P_2, P_3) unexpectedly generated amplification products of bovine DNA but not *B. bovis* DNA. The failure of these primers in amplifying *B. bovis* DNA possibly reflects variation or mutation in the target sequences of the Thai strain. Our emphasis was on one primer pair (P_5) which was found to create a specific PCR amplicon for *B. bovis* DNA. The sensitivity of PCR with P_5 was then determined in comparison with P_6 (Fahrimal's primers). The sensitivity of PCR using P_6 described herein was similar to that previously reported.[7] Surprisingly, detection by SYBR green I stain and Southern blot hybridization indicated that PCR product of P_5 produced greater signals than that of P_6 at the same blood dilution. The greater sensitivity of PCR product of P_5 over P_6 was confirmed by the duplex PCR. The product of P_5 could be seen in much higher amount than that of P_6, even in the conditions optimal for P_6. This may be caused by the length of the amplified regions. The region of P_5 is much shorter than that of P_6, which may lead to lower interference from folding and/or intrachain base pairing of the DNA template during the PCR process. It was additionally noted that detection of the PCR products on gel showed similar sensitivity to Southern blot analysis and PCR-ELISA. The high sensitivity of SYBR green I used in DNA staining[11] should account for this observation. Our study demonstrates that P_5, which was designed from the region close to mitochondrial large subunit ribosomal DNA of *B. bovis*, can be used as an alternative primer pair for *B. bovis* detection by PCR-based methods including PCR-ELISA in mass screening, according to its specificity and high sensitivity. PCR using P_5 alone or duplex PCR using this primer pair in combination with the Fahrimal's primers, as well as PCR-ELISA, will be very useful in early detection of *B. bovis*, that will help stop transmission of the disease.

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REFERENCES

1. Tananyutthawongese C, Saengsombut K, Sukhumsirichat W, Uthaisang W, Saratphan N and Chansiri K (1999) Detection of bovine hemoparasite infection using multiplex polymerase chain reaction. *ScienceAsia* **25**, 85-90.
2. Calder JAM, Reddy GR, Chieves L, Courtney CH, Littell R, Livengood JR and Norval RAI, et al (1996) Monitoring *Babesia bovis* infections in cattle by using PCR-based tests. *Journal of Clinical Microbiology* **34**, 2748-55.
3. Caccio S, Camma C, Onuma M and Severini C (2000) The β-tubulin gene of *Babesia* and *Theileria* parasites is an informative marker for species discrimination. *International Journal for Parasitology* **30**, 1181-5.
4. Kudamba C, Campbell RS, Paull NI and Holroyd RG (1982) Serological studies of babesiosis and anaplasmosis of cattle. *Australian Veterinary Journal* **59**, 101-4.
5. Blandino T, Alonoso M and Barrera M (1994) Detection of antibodies to *Babesia bovis* using an ELISA test. *Archives of Medical Research* **25**, 219-22.
6. Boonchit S, Xuan X, Yokoyama N, Goff WL, Wagner G and 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. *Journal of Clinical Microbiology* **10,** 3771-5.
7. Fahrimal Y, Goff WL and Jasmer DP (1992) Detection of *Babesia bovis* carrier cattle by using polymerase chain reaction amplification of parasite DNA. *Journal of Clinical Microbiology* **30,** 1374-9.
8. Sambrook J, Fritsch EF and Maniatis T (1989) Gel electrophoresis of DNA. In: *Molecular cloning: a laboratory manual, 2nd ed*, pp. 6.3-6.19. New York: Cold Spring Harbor Laboratory Press.
9. He Y, Coutlee F, Antoine PS, Voyer H and Elbaz AK (1993) Detection of polymerase chain reaction-amplified human immunodeficiency virus type 1 proviral DNA with a digoxigenin-labeled RNA probe and an enzyme-linked immunoassay. *Journal of Clinical Microbiology* **31,** 1040-7.
10. Wallace DC (1999) Mitochondrial diseases in man and mouse. *Science* **283,** 1482-8.
11. Kyo S, Takakura M, Tanaka M, Murakami K, Saitoh R, Hirano H and Inoue M (1998) Quantitative differences in telomerase activity among malignant, premalignant, and benign ovarian lesions. *Clinical Cancer Research* **4,** 399-405.