

Detection of Bovine Hemoparasite Infection Using Multiplex Polymerase Chain Reaction

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ABSTRACT A rapid and specific multiplex polymerase chain reaction has been developed for the diagnosis of hemoparasitic infection of bovine blood by five of the most common protozoan hemoparasites. This method relied on the detection of the presence of genomic DNA from the five different bovine hemoparasites isolated from red blood cells. Infection was detected by a single multiplex PCR reaction containing five pairs of oligonucleotide primers whereby each primer pair was specific for each parasite species. These primer sets amplify 160, 257, 446, 689 and 1,100 bp fragments for *Anaplasma marginale*, *Trypanosoma evansi*, *Babesia bovis*, *Babesia bigemina* and *Theileria* sp, respectively. The PCR products derived from each parasite species were visualized in ethidium bromide-stained agarose gels, thus allowing the rapid identification of any, or all, of the five bovine parasites, if present, in a single amplification reaction. This multiplex PCR was sensitive with the ability to detect the presence of as little as 10-1 pg of parasite DNA. The primers used in this multiplex PCR also showed highly specific amplification of each respective parasite DNA without the presence of non-specific and non-target PCR products. This multiplex PCR system was used to analyze 35 bovine blood samples for the presence of parasitic infection and the results were compared with detection of hemoparasites by light microscopic examination after Giemsa staining of thin film blood smears. A comparison of the two detection methods revealed that 86% of specimens showed concordant diagnoses with both techniques. However, 14% of the samples examined showed single or multiple infection by multiplex PCR analysis, but such infection was not detected by microscopy. The simplicity and rapidity of this specific multiplex PCR method make it suitable for large-scale epidemiological studies and for follow-up of drug treatments.

KEYWORDS: *Anaplasma marginale*, *Trypanosoma evansi*, *Babesia bovis*, *Babesia bigemina*, *Theileria* sp, bovine, hemoparasites, PCR, polymerase chain reaction.

INTRODUCTION

The protozoan parasites *B. bovis*, *B. bigemina*, *Theileria* sp, *A. marginale* and *T. evansi* are known to cause serious diseases of livestock, especially cattle.¹ The common clinical manifestations of these diseases are mild to severe anemia, stress and weakness which lead to the death of the animals. Abortion is often the endpoint in the case of pregnant animals. Animals that recover from a primary acute attack become carriers of the respective protozoan parasites. Such carriers are important contributors to the infectious agents within ticks.

A number of diagnostic methods for the detection of the presence of parasitic infection are currently available. The classical and simple microscopic techniques most commonly used are very efficient for the identification of parasites in bovine blood

but require an experienced and skilful person to make the diagnosis.² The ELISA technique has been widely used for the diagnosis of protozoan infection on the basis of the specific binding between antigen and antibody.³ However, the cost of each test is very high and can become prohibitive. Sensitive DNA-based methods such as DNA hybridisation and PCR are generally limited to the detection of genomic DNA derived from a single infectious agent.

In 1993, Figueroa and colleagues⁴ developed the technique of multiplex PCR for the simultaneous detection, by PCR amplification, of purified genomic DNA extracted from *B. bovis*, *B. bigemina* and *A. marginale*. We report an extension of this technique for the detection of up to five protozoan hemoparasites in crude blood samples using five sets of oligonucleotide primers that are each specific for a single parasite species (*A. marginale*, *T. evansi*, *B.*

bovis, *B. bigemina* and *Theileria* sp). The multiplex PCR developed was able to detect the presence of up to five different parasites, if present, in a single reaction. The rapidity and sensitivity of this technique make it suitable for large-scale epidemiological surveys to determine the frequency of parasitic infection in certain regions of transmission and for the assessment of drug treatments for such infections.

MATERIALS AND METHODS

Collection of parasites and preparation of purified parasite DNA

B. bovis, *A. marginale* and *Theileria* sp. were collected in Thailand. Each parasite was used separately to infect experimentally splenectomized calves. Blood samples (50 ml) were obtained by venipuncture of the jugular vein and parasites were isolated by CF-11 column chromatography.⁵ The eluate, containing intact red blood cells (RBC), was collected and transferred to 50 ml centrifuge tubes prior to centrifugation (700 x g, 10 min). The pellet, containing intact parasites and both parasitised and non-parasitised RBC (volume 9-10 ml), was suspended in 17.5 ml of 1% (w/v) NaCl and the volume was immediately made up to 50 ml with sterile water. The suspension was allowed to stand on ice for 15 min with occasional gentle agitation. The released parasite suspension was centrifuged (1,500 x g, 20 min) and the resulting pellet was washed in PBS (0.1 M Na₂HPO₄, 0.1 M NaH₂PO₄, 0.01% NaCl, pH 7.4), recentrifuged at 3,000 rpm and resuspended in 0.85 M NaCl, 10 mM Tris, 10 mM EDTA (pH 8) containing proteinase K (100 µg/ml). After proteinase K digestion (14-16 h, 50°C), parasite chromosomal DNA was isolated and purified by phenol/chloroform extraction and ethanol precipitation.⁶ The DNA pellets were dissolved in TE buffer (10 mM Tris-HCl, pH 7.4 and 1 mM EDTA, pH 8.0) and stored at 4°C until required.

T. evansi was obtained from blood collected from Ladkrabang herds in Bangkok, Thailand. The isolated parasites were used to infect mice for 3 days prior to blood collection and subsequent parasite isolation using DEAE 52 column chromatography.⁷ The eluate containing intact parasites was collected and centrifuged at 3,000 rpm for 10 min. The pellet was resuspended in 1 ml PBS buffer containing 1% (w/v) SDS and proteinase K (100 µg/ml). Parasite DNA was further isolated and purified as described above.

B. bigemina DNA was isolated in Florida, USA, and was a gift of Prof Chariya Brockelman, Mahidol University.

Blood collection and extraction of parasite DNA for multiplex PCR analysis

The samples of tailprick blood were randomly collected from 35 cattle in the endemic area of southern and north-eastern Thailand. The collected blood was used directly for DNA extraction. When a number of blood samples were to be analyzed, 10 ml of infected blood was added directly into 25 ml of 0.002% (w/v) SDS solution until the red blood cells were completely disrupted. This mixture was then heated at 95°C for 10 mins prior to centrifugation (13 000 rpm, 10 min). An aliquot (25 µl) of this boiled solution, containing parasite DNA, was used for PCR amplification. The boiled samples, derived from this procedures, containing parasite DNA, could be stored at -20°C for 14 days without compromising the PCR.⁸

Detection of protozoan infection by microscopic examination

A thin blood film was prepared from each blood sample at the time of collection. The presence of protozoan parasites was determined by light microscopic examination of Giemsa-stained blood smears. The packed cell volume (PCV) was determined for each blood sample and used as a rough indication of the presence of anemia due to infection.

Oligonucleotide primers

Specific oligonucleotide primers of the parasites and their sequences were shown in Table 1. Primers for the amplification of *A. marginale* (Set A; Am3 and Am4) DNA were derived from the sequence of the gene encoding the surface protein⁴. Primers TR3 and TR4 (Set B), used for the amplification of *T. evansi* DNA, were derived from repetitive nucleotide sequences.⁹ Primers Bb1 and Bb2 (Set C), used for the amplification of *B. bovis* DNA, were derived from the sequence of the gene encoding the enzyme carbamoyl phosphate synthetase II.¹⁰ Primers for the amplification of *B. bigemina* (Set D; Bg3 and Bg4) and *Theileria* sp. (Set E; 989 and 990) DNA were based on their small subunit ribosomal RNA sequences.^{11,12}

Multiplex PCR amplification

Purified parasite DNA (10 ng) derived from each of the 5 parasites was added to a 50 µl reaction mixture containing 10 mM Tris-HCl, pH 9.5, 50 mM KCl, 0.01% (w/v) gelatin, 2.1 mM MgCl₂, 350 µM each dNTP, 1 µM each of primers Am3 and Am4, 0.07 µM each of TR3 and TR4, 2.0 µM each of Bg3 and Bg4, 0.3 µM each of Bb1 and Bb2, 0.7 µM each

of 989 and 990 and 2 U of *Taq* polymerase (Perkin Elmer Cetus). The mixture was overlaid with paraffin oil and PCR was started with an initial denaturation step (95°C, 5 min) followed by 30 cycles of denaturation (95°C, 45 s), annealing (50°C, 45 s) and extension (72°C, 45 s). After amplification, 0.3-0.4 volume of each sample was electrophoresed in a 1.0% agarose gel and visualized under ultraviolet light.

An aliquot (25 µl) of boiled blood sample, derived from either of the two procedures described above, were used in the multiplex PCR reaction. PCR was carried out in a 50 µl reaction volume and the PCR products were analysed as described above.

Specificity of primers

Primers were examined for their specificity of amplification of each individual parasite species by amplification of DNA samples of individual parasite species as well as from a mixture of DNA samples (derived from the five hemoparasites) with each set of primers. Bovine DNA was used as a negative control for each pair of primers.

Sensitivity of detection

The sensitivity of detection of each primer was determined by PCR amplification of 10-fold (100 ng-1 pg) serial dilutions of each purified DNA samples (*A. marginale*, *T. evansi*, *B. bovis*, *B. bigemina* and *Theileria* sp.). The PCR condition was accomplished according to those of multiplex PCR as

described above except that each pair of primers was used for each test. After PCR amplification, 25 µl aliquot of the resulting PCR products was applied to 1.0 % agarose gel electrophoresis and the PCR products were analyzed as previously described.

RESULTS

Specificity of PCR primers

Upon PCR amplification of each individual DNA sample (*A. marginale*, *T. evansi*, *B. bovis*, *B. bigemina* and *Theileria* sp.) using their specific primers led to the detection of the expected fragments of size 160, 257, 446, 689 and 1,100 bp, respectively (Fig 1). Each set of primers was found to be specific for the respective parasite DNA and amplification of non-target DNA samples did not lead to the production of PCR products when other parasite or bovine DNA samples were used. Primers specific for one parasite species did not produce PCR products from any of the other parasite species.

Sensitivity of the multiplex PCR system

Single PCR was performed to determine the sensitivity of detection of each hemoparasite DNA. Such an analysis showed that each set of primers could detect the presence of as little as 10 pg of each parasite DNA except those of *T. evansi* showed the minimal level of detection at 1 pg of parasite DNA (Fig 2).

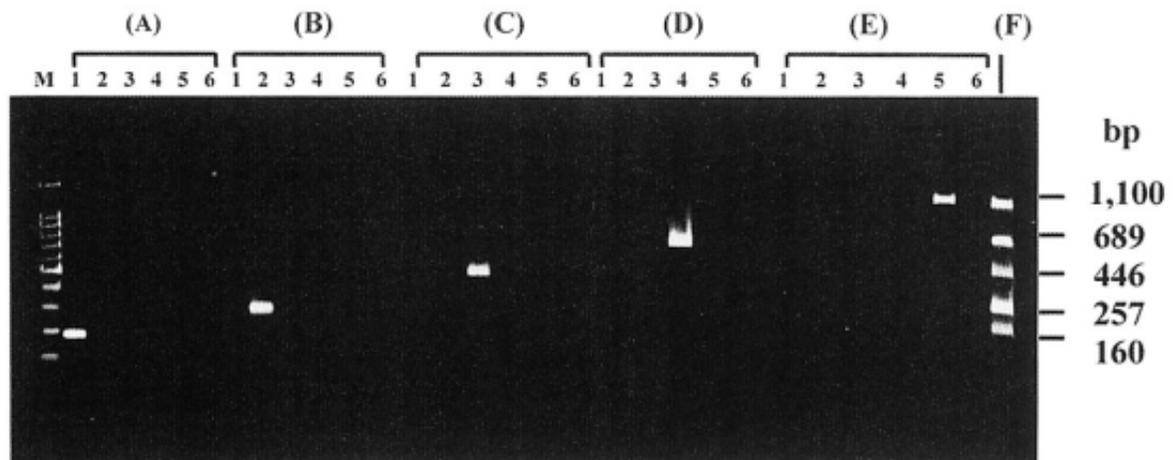


Fig 1. Specificity testing of primers using PCR amplification of individual DNA samples from *A. marginale* (lane 1), *T. evansi* (lane 2), *B. bovis* (lane 3), *B. bigemina* (lane 4), *Theileria* sp. (lane 5) and bovine DNA (lane 6). Primers specific for each parasite were indicated as follows; (A) Am3/Am4 for *A. marginale*; (B) TR3/TR4 for *T. evansi*; (C) Bb1/Bb2 for *B. bovis*; (D) Bg3/Bg4 for *B. bigemina*; (E) 989/990 for *Theileria* sp.; (F) cocktail of primers for amplification of mixed parasite DNA samples. Lane M contains the molecular weight size markers consisting of fragments of 100-1,500 bp. Upon using their specific primers, the expected fragments of size 160, 257, 446, 689 and 1,100 bp were obtained, respectively

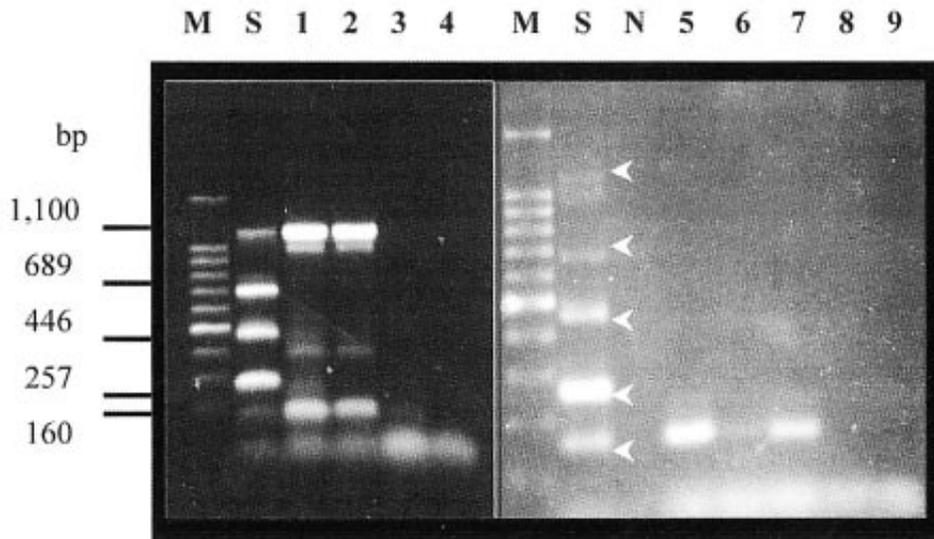


Fig 2. Sensitivity testing of the each individual PCR using each pair primers. PCR was separately carried out with the DNA derived from a) *A. marginale*; b) *T. evansi*; c) *B. bovis*; d) *B. bigemina* and e) *Theileria* sp. at a concentration of 100 ng (lane 1), 10 ng (lane 2), 1 ng (lane 3), 100 pg (lane 4) and 10 pg (lane 5) and 1 pg (lane 6). The PCR condition using for each reaction was based on those of multiplex PCR as described in Materials and Methods. Lane M contains the molecular weight size markers consisting of fragments of 100-1,500 bp.

Multiplex PCR of collected blood samples

Thirty five bovine blood samples were collected and tested using the multiplex PCR system (Fig 3) in parallel with Giemsa staining of thin film blood smears followed by light microscopy. The Multiplex PCR results revealed that mixed infection of *A. marginale*, *T. evansi* and *Theileria* sp. were occurred. Data obtained from the two methods was compared

Table 1. Oligonucleotide primers used in MPCR and their sequences.

Primers	Sequences (5' to 3')	Specific parasites
Set A		
Am3	GTGGCAGACGGGTGAGTAATG	<i>A. marginale</i> ⁴
Am4	CATGTCAAGAAGTGGAAGGT	
Set B		
TR3	GCGCGGATTCCTTGACAGACGA	<i>T. evansi</i> ⁷
TR4	TGCAGACACTGGAATGTACT	
Set C		
Bb1	TTGGTATTGTCTGGTCAT	<i>B. bovis</i> ⁶
Bb2	ACCACTGTAGTCAAACCTACC	
Set D		
Bg3	TAGTTGTATTTCAGCCTCGCG	<i>B. bigemina</i> ⁷
Bg4	AACATCCAAGCAGCTAHTTAG	
Set E		
989	AGTTTCTGACCTATCAG	<i>Theileria</i> sp. ⁸
990	TTGCCCTAAACTTCCTTG	

and is presented in Table 2. The data indicates that infection in 86% of the samples were in agreement using both multiplex PCR. The data also revealed that infection in 14 % of the samples could only be detected by the multiplex PCR protocol.

DISCUSSION

The outlined multiplex PCR procedure provides a highly specific, sensitive and rapid tool for the diagnosis of both single and mixed hemoparasitic infection. We have applied this technique to the detection of five protozoan parasites (*A. marginale*, *T. evansi*, *B. bovis*, *B. bigemina* and *Theileria* sp.) that are commonly found in bovine blood. Blood samples were collected and DNA was isolated by using the published methods for detection of human malaria in the

Table 2. A comparison of multiplex PCR with microscopic technique for the detection of protozoan parasites in 35 bovine blood samples.

Infectious agent	Number of Samples Blood smear (-) MPCR (-)	Number of Blood smear (+) MPCR (+)	Number of Blood smear (-) MPCR (+)
<i>Theileria</i> sp.	18	13	4
<i>A. marginale</i>	29	0	6
<i>T. evansi</i>	28	2	5

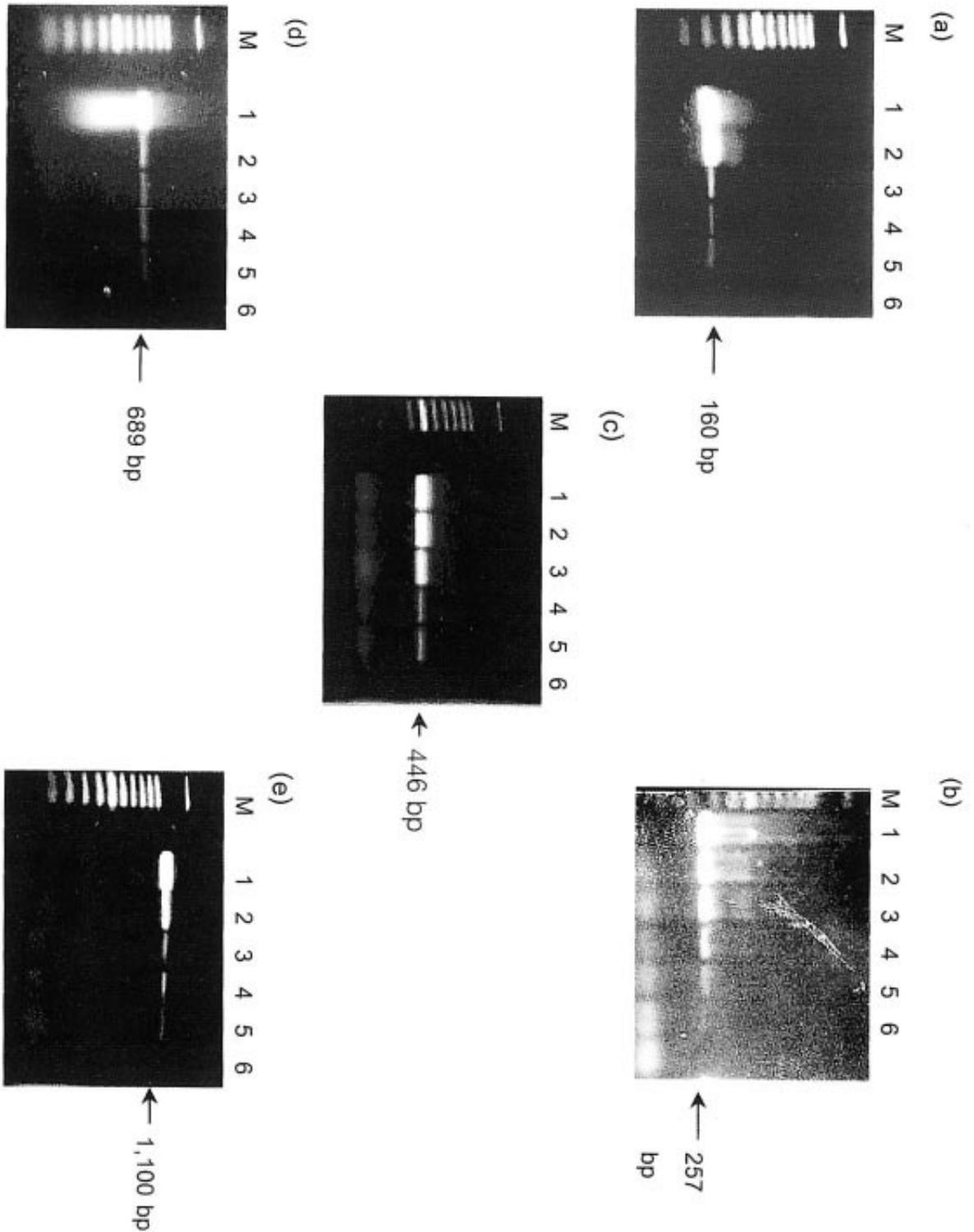


Fig 3. Multiplex PCR detection of hemoparasite DNA in collected bovine blood samples using a mixture of 5 primer sets. A positive control containing all 5 purified DNA samples is present in lane S. A negative control with the absence of all 5 purified DNA is presented in lane N. Products of the amplification of DNA isolated from blood samples randomly collected in different parts of Thailand are shown in lanes 1-9. The data shows both single and mixed infection of *Theileria* sp. and *A. marginale* in blood specimens. The multiplex PCR condition for these samples were used as described in Materials and Methods. Lane M contains the molecular size markers consisting of 100-1,500 bp fragments.

application of this multiplex PCR technique for an epidemiological survey of the frequency of hemoparasitic infection.⁸ The method involved the immediate treatment of an aliquot of each blood sample with 0.002% (w/v) SDS, to lyse the red blood cells and release the parasites, followed by heating at 95°C to release the parasite DNA. This procedure was suitable for the analysis of a large number of samples as the DNA isolated in this way could be stored for up to two week without significant loss of PCR sensitivity.⁸ We found that this method could be suitably applied for treatment of infected bovine blood specimens and was appropriated for the processing of a large number of samples. It is also convenient for packaging and transportation of samples when they cannot be processed immediately.

To ensure species specificity of amplification products, oligonucleotide primers were based on gene sequences that are known to be specific to the genome of each parasite. This eliminates cross-hybridisation of each primer pair to non-target sequences. In the case of known species of parasites such as *T. evansi* and *B. bovis*, primers were derived from the sequences of specific repetitive sequences or genes that are unique to each particular species. This strategy could not be applied to *Theileria* sp. which normally occurs as unclassified species in South-East Asian countries such as Thailand. Hence, primers that could detect the genus *Theileria* were employed for multiplex PCR. A pair of primers, 989 and 990, derived from sequences of the conserved regions of the small subunit ribosomal RNA genes of the genus *Theileria*, were utilised. In this case both pathogenic and non-pathogenic *Theileria* parasites will be detected. These primers can also detect the other known species of *Theileria* such as *T. annulata* and *T. parva* that normally exist in Europe and Africa. At present, there are no reports that these two pathogenic parasites are existed in Thailand. However, other primers need to be designed for specific detection of these parasites.

The data obtained (Table 2) indicate that the frequency of multiple or mixed parasitic infection, as determined by multiplex PCR analysis, was in the range of 2-20 % of the total tested population. The parasites most commonly detected in the bovine blood samples collected were *Theileria* sp., *T. evansi* and *A. marginale*. From the data, *Theileria* sp. is frequently found in infected blood samples but does not cause the death to animal. *B. bovis* and *B. bigemina* were found to rarely occur. The absence of *B. bovis* and *B. bigemina* may be due to the season in which collection was carried out and the incidence of their outbreak.

In conclusion, the multiplex PCR protocol developed for the simultaneously diagnosis of hemoparasitic infection in bovine blood has been able to detect the presence of mixed infections. The technique is rapid, reproducible and has a high specificity and sensitivity. It is therefore suitable for epidemiological surveys as it can diminish the cost, time and personnel required.

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