Molecular and serological prevalence of *Babesia bovis* and *Babesia bigemina* in water buffaloes in the northeast region of Thailand

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**A R T I C L E   I N F O**

Article history:
Received 10 September 2010
Received in revised form 14 January 2011
Accepted 18 January 2011

**Keywords:**
*Babesia bovis*
*Babesia bigemina*
Nested PCR
ELISA
IFAT
Water buffaloes
Epidemiology
Thailand

**A B S T R A C T**

Bovine babesiosis is a tick-transmitted hemoprotozoan disease that is mainly caused by *Babesia bovis* and *Babesia bigemina* and is characterized by significant morbidity and mortality worldwide. The disease is widespread in the northeastern region of Thailand, where an increasingly large part of the livestock is composed of water buffaloes. The present study was therefore conducted to investigate the epidemiological distribution of *B. bovis* and *B. bigemina* in water buffaloes in the northeastern region of Thailand. A total of 305 buffalo blood samples were randomly collected from five provinces and simultaneously analyzed by the nested PCR (nPCR) assay, ELISA, and IFAT techniques. The overall prevalence of *B. bovis* and *B. bigemina* was 11.2% and 3.6% by nPCR, 14.7% and 5.9% by ELISA, and 16.8% and 5.6% by IFAT, respectively. The high concordance between the molecular and the serological detection tests revealed the specificity and sensitivity of the diagnostic assays used for the detection of infection as well as the endemic stability status of the parasites in the surveyed areas. Statistically significant differences in the prevalence of the two infections were observed on the basis of age and location but not gender. Our data provide valuable information regarding the epidemiology of *B. bovis* and *B. bigemina* infection in water buffaloes in the northeastern region of Thailand which will likely be very beneficial for management and control programs of this disease.

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1. Introduction

Bovine babesiosis is a tick-borne disease of cattle which is caused by the protozoan parasites of the genus *Babesia*, order Piroplasmida, phylum Apicomplexa and is generally characterized by significant morbidity and mortality worldwide (McCosker, 1981). *Babesia bovis* and *Babesia bigemina* are known as the most important species because they are highly prevalent in tropical and subtropical regions of the world and cause enormous economic losses in the livestock industry (Bock et al., 2004; OIE, 2008). Although *B. bovis* and *B. bigemina* are phylogenetically related and transmitted by the same tick vector, *Rhipicephalus microplus*, they cause remarkably different diseases in cattle (Kuttler, 1988; Bock et al., 2004). The *B. bovis* infection is often more severe and results in higher mortality among susceptible cattle, most probably due to the ability of parasitized erythrocytes to sequester in microcapillaries of the kidneys, lungs, and brain, with consequent organ failure and systemic shock that lead to death.

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doi:10.1016/j.vetpar.2011.01.041
Babesiosis is traditionally diagnosed by the identification of the parasites in stained blood smear using microscopic examination; however, this method is not sensitive enough to detect the parasites in subclinical or chronic stage of infection. Recently, molecular approaches based on nucleic acids such as polymerase chain reaction (PCR) assays offer greater sensitivity and specificity over the existing diagnostic tests (Fahrimal et al., 1992; Figueroa et al., 1993). On the other hand, serological tests, including the indirect fluorescent antibody test (IFAT) and the enzyme-linked immunosorbent assay (ELISA) are capable of detecting the antibody in carrier animals; therefore, they are often used for surveillance and export certification (Weiland and Reiter, 1988; Bose et al., 1990; Araujo et al., 1998). Consequently, a combined PCR/ELISA approach could provide a powerful tool for epidemiological investigations with high accuracy in the diagnosis of Babesia infection (Goo et al., 2008).

Thailand is a developing agricultural country located in Southeast Asia, where the development of livestock industry has been hampered by the high occurrence of tick-borne diseases, particularly bovine babesiosis (Ahantarig et al., 2008; Iseki et al., 2010). Water buffaloes are predominantly distributed in the northeastern part of the country, posing an important source of various human needs, such as meat, horns, hides, milk and milk products, leather, land plowing, and transportation of people and crops (Somparn et al., 2004). Due to the fact that water buffaloes are raised together with cattle, among which bovine babesiosis is highly prevalent (Iseki et al., 2010), they might be potential carriers for Babesia parasites. However, no report has been published regarding babesiosis prevalence among water buffaloes in Thailand; therefore, research on epidemiological surveys of bovine babesiosis in water buffaloes is expected to be beneficial for reducing the economic losses of the livestock industry. With this in mind, we investigated the molecular and serological prevalence of B. bovis and B. bigemina infection in water buffaloes in the northeastern region of Thailand using the nested PCR assay, ELISA, and IFAT.

2. Materials and methods

2.1. Parasites

The Texas strain of B. bovis and the Argentina strain of B. bigemina were continuously cultured with bovine erythrocytes (RBC) using a microaerophilous stationary-phase culturing system (Levy and Rustic, 1980; Vega et al., 1985). When the parasitemia reached 5–10%, the cultured parasites were harvested for DNA or RNA extraction.

2.2. Field blood samples

Field samples of blood from water buffalo (n = 305) were collected from different farms in five provinces (Ubon Ratchathani, Roi Ed, Buriram, Surin, and Srisakhet) in the northeastern region of Thailand in January 2010. Blood was collected from the caudal or jugular vein of individual water buffaloes with EDTA or without, incubated at room temperature (RT) for 1 h, and then centrifuged at 3000 rpm for 15 min. Blood smears for each sample were made, stained by Giemsa stain and finally checked by microscopic examination. The sera were collected and then stored at −20°C until use. The genomic DNA samples were extracted from the whole blood using a commercial kit (QiAamp DNA Blood Mini-Kit, Madison, WI, USA) according to the manufacturer’s instructions.

2.3. Expression and purification of recombinant proteins

The DNA fragments encoding B. bovis spherical body protein-4 (BbSBP-4, GeneBank accession number AB594813) and the B. bigemina C-terminal region of rhoptry-associated protein 1 (BbgRAP-1a/CT: 390–480 aa, GeneBank accession number M60878) were amplified from the B. bovis and B. bigemina CDNA, respectively, using standard PCR assays as previously described (Kim et al., 2008; Altangerel et al., 2009; Terkawi et al., 2011). The amplified DNA fragments were subcloned into a pGEX-4T1 plasmid vector (Amersham Pharmacia Biotech, Madison, CA, USA) using suitable restriction enzyme sites and then expressed in Escherichia coli BL21 strain (Amersham Pharmacia Biotech). The recombinant proteins were purified from the soluble fractions of E. coli lysates using Glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). The purified recombinant proteins were then analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) using 12% acrylamide gels and stained with Coomassie blue as previously described (Laemmli, 1970).

2.4. ELISA

The standard enzyme-linked immunosorbent assay (ELISA) was performed as previously described (Terkawi et al., 2007). Briefly, 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 µl of each recombinant antigen (rBbSBP-4 and rBbgRAP-1a/CT) and rGST (negative control) at a concentration of 2 µg/ml per well in a coating buffer (a 50 mM carbonate–bicarbonate buffer, pH 9.6). The plates were washed once with 0.05% Tween 20–PBS (PBST) and then incubated with 100 µl of a blocking solution (3% skim milk in PBS) for 1 h at 37°C. After washing once with PBST, the antigen-coated wells were incubated with 50 µl of serum samples diluted 1:100 with a blocking solution for 1 h at 37°C. The plates were washed six times with PBST and then incubated with 50 µl of horseradish peroxidase (HRP)-conjugated sheep anti-bovine immunoglobulin G (IgG) (Bethyl Laboratories, USA) diluted to 1:4000 with the blocking solution for 1 h at 37°C as a secondary antibody. The plates were washed six times as indicated above...
and then 100 μl of a substrate solution (0.1 M citric acid, 0.2 M sodium phosphate, 0.3 mg/ml of 2,2′-azide-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma, St. Louis, MO, USA), and 0.01% of 30% H₂O₂) added per well. After incubation for 1 h at RT, the optical density (OD) was measured by an MTP-500 microplate reader (Corona Electric, Tokyo, Japan) at a wavelength of 415 nm. The OD values of GST-background values were subtracted from those of rBbSBP-4 or BbigRAP-1/CT. The cut-off values were calculated based on 50 non-Babesia-infected bovine sera (Terkawi et al., 2011) by the receiver operating characteristic (ROC) analysis with MedCalc statistical software (version 11.4; http://www.medcalc.be).

2.5. IFAT

The B. bovis- and B. bigemina-infected RBC were coated on indirect fluorescent antibody test (IFAT) slides (Matsumani Glass Ind., Ltd, Osaka, Japan), dried, and then fixed in absolute acetone for 20 min for standard IFAT observation (Boonchit et al., 2006). Briefly, a 10 μl field serum sample diluted in PBS (1:100) was applied as the first antibody on the fixed smears and then incubated for 1 h at 37 °C in a moist chamber. After washing with PBS three times, the fluorescein isothiocyanate (FITC)-conjugated sheep antobody on the fixed smears and then incubated for 1 h at 37 °C. The slides were washed three times with PBS and then examined using a fluorescent microscope (E400 Eclipse, Nikon, Kawasaki, Japan).

2.6. Nested PCR

The genomic field DNA samples of water buffaloes were analyzed by nested PCR (nPCR) (Figueroa et al., 1993) for screening of B. bovis and B. bigemina using the developed primers specific to B. bovis BbSBP-4 and B. bigemina BbigRAP-1a genes (Table 1). Each PCR reaction was performed in a 10 μl volume containing 1 μl of the extracted DNA template (0.25 μg), 1 μl of each primers (10 pmol), 1 μl of 10× Ex buffer, 1 μl of dNTP (200 μM), 0.1 μl of Ex Taq polymerase (Takara, Otsu, Shiga, Japan), and adequately distilled water. The thermocycling conditions. The nested PCR products were subjected to electrophoresis in 1% agarose gel and then visualized under an ultraviolet light after staining with ethidium bromide.

2.7. DNA sequencing

To further confirm and validate the nPCR results, randomly selected positive samples of B. bovis and B. bigemina (3 samples for each parasite) were sequenced. In detail, 25 μl of the PCR products was purified from the agarose gel using a QIAquick Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany) and then ligated into a pGEM-T vector (Promega, Madison, WI, USA) for subsequent transformation in the E. coli DH5α-competent cells. A positive inserted plasmid was purified from a single clone using a QiAprep Spin Miniprep Kit (QIAGEN) and then sequenced using a Dye Terminalsequence comparison.

2.8. Statistical analysis

The kappa coefficient was calculated to evaluate the agreement among the nPCR assay, ELISA, and IFAT. The chi-square test was used to evaluate significant differences (P < 0.05) of infection rate in animals of different genders, ages, and locations (http://faculty.vassar.edu/lowry/VassarStats.html).

3. Results

3.1. The determination of cut-off values for ELISA antigens

The genes encoding BbSBP-4 and BbigRAP-1/CT were successfully expressed as soluble GST-fusion proteins in E. coli with molecular weights of 66 and 35 kDa, respectively (data not shown). The obtained recombinant proteins (rBbSBP-4 and rBbigRAP-1/CT) were used as ELISA antigens to detect the antibody to the infections of B. bovis and B. bigemina in water buffaloes. Using 50 negative bovine sera, the cut-off OD values of rBbSBP-4 and rBbigRAP-1/CT were determined to be 0.11 and 0.10, respectively.

Table 1

<table>
<thead>
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<th>Genes</th>
<th>Assays</th>
<th>Oligonucleotide primers</th>
<th>Product sizes</th>
</tr>
</thead>
<tbody>
<tr>
<td>BbSBP-4</td>
<td>PCR</td>
<td>5'-AGTTGTGGAGGAGGCTATA-3'</td>
<td>907 bp</td>
</tr>
<tr>
<td></td>
<td>nPCR</td>
<td>5'-TCCTCTCGGCTTTTCTT-3'</td>
<td>503 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-GAAATCCCTGGCTCAGAC-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-TCCTGATAAACAGTCAA-3'</td>
<td></td>
</tr>
<tr>
<td>BbigRAP-1a</td>
<td>PCR</td>
<td>5'-GACTGTGAAAACTTTAC-3'</td>
<td>879 bp</td>
</tr>
<tr>
<td></td>
<td>nPCR</td>
<td>5'-TCCTCTAGCACGCTCTTG-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-AGCGTTGTTTCCGAGGAG-3'</td>
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<td></td>
<td></td>
<td>5'-TGTTGCTTTGCCAGGAC-3'</td>
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</tbody>
</table>
3.2. Detection of B. bovis and B. bigemina infection in water buffaloes

A total of 305 buffalo blood samples were randomly collected from five different provinces (Ubon Ratchathani, Roi Ed, Burirum, Surin, and Srisakhet) in the northeastern region of Thailand (Fig. 1) and simultaneously analyzed by the blood smears examination, nPCR assay, ELISA, and IFAT techniques. All blood smears were negative by microscopic examination (data not shown). The overall prevalence of B. bovis was 11.2%, 14.7%, and 16.8% by the nPCR assay, ELISA, and IFAT, respectively, whereas that of B. bigemina was 3.6%, 5.9%, and 5.6%, respectively. Mixed infections were detected only serologically in a total of 4 buffaloes (1.3%). Notably, of 34 B. bovis nPCR-positive samples, 27 and 24 were positive by ELISA and IFAT, respectively; on the other hand, of 11 B. bigemina nPCR-positive samples, 8 and 6 were positive by ELISA and IFAT, respectively (Table 2). Although these serological assays (ELISA and IFAT) detected higher positive rates of both parasites than the molecular detection method (nPCR assay), the results obtained from the nPCR assay were highly concordant with those of ELISA and IFAT, as determined by the kappa values, which were calculated to be 0.64 and 0.50 for B. bovis and 0.53 and 0.41 for B. bigemina, respectively.

Furthermore, three B. bovis- or B. bigemina-nPCR-positive field samples were selected for DNA sequencing of the BbSBP-4 and BbigRAP-1a genes, respectively. The amplified BbSBP-4 and BbigRAP-1a genes as expected sizes (907 bp and 879 bp, respectively) were purified and then ligated into a pGEM-T vector for subsequent sequencing. Sequence comparisons of the amplified BbSBP-4 isolated from the genomic DNA of field samples (GenBank accession nos. AB586125, AB594814 and AB594815) revealed high conservation of Thai isolates (data not shown), and they were similar to published B. bovis SBP-4 with 98–100% identity (GenBank accession no. AF021246). Likewise, the amplified BbigRAP-1a genes (879 bp) from DNA field samples (GenBank accession nos. AB586126, AB594816 and AB594817) were highly conserved (data not shown) and shared 96–97% identity to the published sequence of the BbigRAP-1a gene (GenBank accession no. M60878).

3.3. Comparison of B. bovis and B. bigemina prevalence in different buffalo populations

The results of an nPCR assay and ELISA were combined, and the overall prevalence of B. bovis and B. bigemina infections in water buffaloes in the northeastern region of Thailand was determined to be 17.5% and 6.9%, respectively (Table 2). Next, the prevalence of bovine babesiosis

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**Table 2**

Summary of the molecular and serological detection of B. bovis and B. bigemina using nPCR assay, ELISA, and IFAT. The results of nPCR were cross-tabulated with those of ELISA and IFAT.

<table>
<thead>
<tr>
<th>nPCR/ELISA</th>
<th>ELISA</th>
<th>IFAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>B. bovis</td>
<td>34</td>
<td>27</td>
</tr>
<tr>
<td>(−)</td>
<td>271</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>305</td>
<td>45</td>
</tr>
<tr>
<td>B. bigemina</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>(−)</td>
<td>294</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>305</td>
<td>18</td>
</tr>
</tbody>
</table>

*a* The frequencies of positive and negative samples as results of nPCR.

*b* The frequencies of positive and negative samples as results of ELISA and IFAT cross-tabulated with nPCR results.

*c* The frequencies of positive and negative samples of combined nPCR and ELISA results.
in different buffalo populations was compared on the basis of different ages, genders, and geographical regions, as shown in Fig. 2. Statistically significant differences were essentially observed in animals of different ages ($P = 0.0406$ and 0.0489, respectively). Young water buffaloes (1–5 years) showed higher rates of *B. bovis* and *B. bigemina* infections than older animals (>5 years) (Fig. 2A). Female water buffaloes showed slightly higher infection rates than males (Fig. 2B). *B. bovis*- and *B. bigemina*-infected buffaloes were found in all analyzed regions, although the data suggested significant differences in the distribution of infection among the different regions ($P < 0.0001$). The infection rates of *B. bovis* and *B. bigemina* were significantly higher in Roi Ed and Srisakhet Provinces than in the other provinces (Fig. 2C).

### 4. Discussion

The combination of molecular and serological assays, including the nPCR assay and ELISA, provides a powerful tool for the accurate diagnosis and epidemiological investigation of *Babesia* infections (Bose et al., 1990; Figueroa et al., 1993). Bovine babesiosis caused by *B. bovis* and *B. bigemina* threatens the livestock industry, particularly, the productivity of cattle and buffaloes in many Asian countries, including Thailand (Ahantarig et al., 2008). In the present study, we investigated the epidemiology of *B. bovis* and *B. bigemina* infections in water buffaloes in the northeastern region of Thailand for the first time using the nPCR assay, IFAT, and ELISA. The results clearly indicated the presence of *B. bovis* and *B. bigemina* infections in water buffaloes that appeared healthy and showed no clinical signs. However, clinical cases of babesiosis have been commonly reported in water buffaloes and might be fatal in other areas, including China and India (Liu et al., 1997; Yao et al., 1997). The absence of clinical signs of babesiosis might suggest the high long-term endemicity of such infections in the studied areas and the frequency of asymptomatic chronic carriers. These animals might serve as reservoirs for the infections of cattle, since they are together in the pastures and continue to infect the tick vector (Homer et al., 2000; Bock et al., 2004).

The results of molecular and serological tests were highly concordant, revealing their reliability in the detection of babesial infections as well as the endemic stability status of the parasites in the surveyed areas. On the other hand, serological assays detected a higher number of infected animals than the nPCR assay; this could be explained by differences in the timing of the parasite presence and the antibody responses in the infected animals as well as the stage of infections. However, during chronic infections, antibodies remain in the circulating blood for a long period even after parasite clearance, while the parasite DNA is not detectable by nPCR (Carrique et al., 2000). Although the presence of parasites in the circulating blood revealed recent or acute infection in the present study, no clinical signs were observed in the water buffaloes. This is most probably due to the immunity developed by the host against parasite infection and the long history of epidemiology (de Vos and Potgieter, 1994; Homer et al., 2000). Next, the overall prevalence of *B. bovis* and *B. bigemina* infections was compared among different buffalo populations on the basis of gender, age, and location. The infection rate of bovine babesiosis was significantly higher in young water buffaloes, indicating their susceptibility to the infections. The differences on the basis of age are probably due to that old animals might have longer exposure to the pathogens, which causes them to develop a better protective immunity than young animals (Homer et al., 2000). On the other hand, the slightly higher rate of infection detected in female is most likely because the physiology of the female during pregnancy and lactation period, which are associated with hormonal and immunological changes (Khansari et al., 1990). Moreover, the significant differences in prevalence among the different provinces suggest a necessity of further study to investigate the geographic distribution of vector ticks and the farm management in the surveyed areas.
Bovine babesiosis was previously reported to be highly prevalent in Thai cattle (Nishikawa et al., 1990; Phrikhanahok et al., 2000), and recent study has also documented that the seropositivity was 73.8% and 69.1% for *B. bovis* and *B. bigemina* infections, respectively, in the northern part of Thailand (Iseki et al., 2010). However, the widespread *Rhipicephalus microplus*, recognized as a tick vector for both *B. bovis* and *B. bigemina* (Bock et al., 2004) in Thailand, might be the reason for the high rate of bovine babesiosis in cattle and buffaloes. In support with this, Jittapalapong et al. (2004) have noted that 45.6% of the ticks collected from cattle were positive to these parasites in an enzootic area of Thailand. In our study, the lower prevalence of the infections in water buffaloes, as compared to that in cattle, could be explained by differences in the nature of the host. Water buffaloes spend much of their day submerged in the muddy waters of Asia's tropical and subtropical forests (Somparn et al., 2004), which might decrease the opportunity of the tick vector to attach to the animals and transmit the infections. Additionally, the higher prevalence of *B. bovis* than *B. bigemina* in the water buffaloes in the surveyed areas of Thailand emphasizes the importance of further study on the geographic distribution of tick vector in Thailand.

In conclusion, this is the first epidemiologic study investigating the occurrence of *B. bovis* and *B. bigemina* in water buffalo in the northeastern region of Thailand using both molecular and serologic tests. These data provide important information about the incidence of *B. bovis* and *B. bigemina* infections in water buffaloes and will likely be very beneficial for management and control programs of the disease.

Conflict of interest statement

None of the authors of this work has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Acknowledgments

This work was supported by a grant from the Global COE Program (J02) and a Grant-in-Aid for Scientific Research (B-19405044), both from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, and a program for the Promotion of Basic Research Activities for Innovative Bioscience (PROBRMAIN), and the Faculty of Veterinary Medicine, Kasetsart University Research Development Institute (KURDI). The first and second authors were supported by a research grant fellowship from the Japanese Society for the Promotion of Science (JSPS) for young scientists, Japan, and by Japan International Cooperation Agency (JICA), respectively.

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