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Molecular and seroepidemiological survey of *Babesia bovis* and *Babesia bigemina* infections in cattle and water buffaloes in the central region of Vietnam

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Abstract. In the present study, a total of 137 blood samples were collected from cattle and water buffaloes in central region of Vietnam and tested using nested polymerase chain reaction (nPCR), enzyme-linked immunosorbent assay (ELISA) and indirect fluorescent antibody test (IFAT) to determine the molecular and serological prevalence of *Babesia bovis* and *Babesia bigemina*. In cattle, the prevalence of *B. bovis* and *B. bigemina* was 21.3% and 16.0% by nPCR, 73.4% and 42.6% by ELISA and 60.6% and 59.6% by IFAT, respectively, whereas those of water buffalos were 23.3% and 0% by nPCR, 37.2% and 9.3% by ELISA and 27.9% and 18.6% by IFAT, respectively. IFAT and ELISA detected a higher number of infected cattle and water buffaloes than nPCR totally. Statistically significant differences in the prevalence of the two infections were observed on the basis of age. Overall, the current data suggest high incidence of *B. bovis* and *B. bigemina* infections in the central region of Vietnam, which is needed to develop comprehensive approach to the modern surveillance, diagnosis and control of bovine babesiosis.

INTRODUCTION

Bovine babesiosis is a major tick-borne disease of cattle caused by intra-erythrocytic protozoa of the genus *Babesia* (Bock *et al*., 2004). *Babesia bovis* and *Babesia bigemina* are the most common species affecting bovines in tropical and subtropical areas, where the only know vector is the one-host tick *Rhipicephalus microplus* (Montenegro-James 1992, Figueroa *et al*., 1998). Infections with these parasites can result in anemia, fever, hemoglobinuria, icterus and high morbidity leading to serious economic losses encountered in cattle farming operations. Therefore, epidemiological surveys using sensitive and specific diagnostic tools are essential to obtain reliable data for the disease surveillance and control.

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 approaches, including the indirect fluorescent antibody test (IFAT) and the enzyme-linked immunosorbent assay (ELISA) are capable of

detecting the antibody in carrier animals (Bose *et al.*, 1990; Araujo *et al.*, 1998). The early and accurate diagnosis of infected animals is critical to prevent the transmission of babesiosis. Therefore, the simultaneous application of two approaches, such as IFAT, ELISA and nPCR is more robust for the diagnosis of animals with latent infections.

Vietnam is a subtropical country located in Southeast Asia and is characterized by high temperature and humidity throughout the year. In the recent decade, the livestock industry in Vietnam has developed rapidly. Normally, local farmers use antiparasitic drugs for treatment of cattle but not for water buffaloes. The present study aimed to investigate the prevalence of *B. bovis* and *B. bigemina* infections in cattle and water buffalos in the central region of Vietnam by using nested PCR (nPCR), ELISA and IFAT.

MATERIALS AND METHODS

Samples

A total of 137 blood samples were collected from apparently healthy cattle (N = 94) from three farms in Quang Tri province and nine farms in Thua Thien-Hue province, and water buffaloes (N = 43) from five farms in Thua Thien-Hue province of Vietnam during the dry season between February and March 2010. During clinical examination, data on animals (e.g. provenance, travel history, age, breed, gender and history of previous tick infestation) were obtained from the owners. Approximately 5 ml of whole blood with EDTA were collected from the jugular vein of cattle and water buffalo. The samples were centrifuged at 500 × g for 15 min to pellet the cells, and the plasma removed and stored frozen at -30! until used in ELISA and IFAT. The genomic DNA was immediately extracted from the cell pellets using a commercial kit (QIAamp DNA Blood Mini-Kit, Germany) according to the manufacturer's instructions.

Parasites

Babesia bovis Texas and *B. bigemina* Argentina strain were used in this study. The *in vitro* cultures of *B. bovis* and *B. bigemina* were grown in bovine erythrocytes at 37! in humidified CO_2 (5%) and O_2 (5%) incubator (BIO-LABO, Japan) using a microaerophilous stationary-phase culturing system (Levy & Rustic, 1980; Vega *et al.*, 1985). When the parasitemias reached 5-10%, the cultured parasites were harvested for IFAT slide preparation and DNA extraction.

IFAT

The plasma was tested for *B. bovis* and *B. bigemina* antibody activity by the IFAT. To prepare the antigen slides, the *B. bovis*- and *B. bigemina*-infected erythrocytes were coated on indirect fluorescent antibody test (IFAT) slides (Matsunami Glass Ind., Ltd, Osaka, Japan), and fixed in absolute acetone for standard IFAT observation (Boonchit *et al.*, 2006). All plasma samples and appropriate positive and negative control samples were detected at a 1:100 dilution in phosphate buffered saline with 4% fetal bovine serum. The slides were observed under oil immersion at 1000 x magnification using a fluorescent microscope (E400 Eclipse, Nikon, Kawasaki, Japan).

Production of recombinant proteins

The *B. bovis* spherical body protein-4 (BbSBP-4, GeneBank accession number AB594813) and the *B. bigemina* carboxyl-terminal region of rhoptry-associated protein 1 (BbigRAP-1a/CT: 390-480 aa, GeneBank accession number M60878) expression plasmids were constructed previously (Terkawi *et al.*, 2011). The rBbSBP-4 and rBbigRAP-1a/CT were expressed as glutathione *S*-transferase (GST) fusion protein in the *E. coli* BL21 strain and then purified using Glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). The purified recombinant proteins were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

ELISA

ELISAs were performed according to modified procedure described previously (Terkawi *et al.*, 2011). The absorbance was measured at 415 nm using microplate reader (Seac, Radim Company, Italy). ELISA data were determined on the base of mean optical

densities at a value of 415 nm (OD_{415}) for the recombinant antigens (BbSBP-4 or BbigRAP-1a/CT) subtracted from those of GST protein. The cutoff values were determined as the OD_{415} value for *B. bovis* or *B. bigemina* negative sera plus three standard deviations; and BbSBP-4: 0.019 and BbigRAP-1a/CT: 0.02 in cattle (n=22), and BbSBP-4: 0.013 and BbigRAP-1a/CT: 0.016 in buffalo (n=22). The negative sera were tested and confirmed negative by PCR and nested PCR of its corresponding whole blood samples.

Nested PCR

Genomic DNA samples were analyzed by nested PCR on the bases of primer sets targeting *B. bovis* SBP-2 and *B. bigemina* RAP-1a genes as previously described (AbouLaila *et al*., 2010; Cao *et al*., 2012). Genomic DNA purified from RBCs infected with *Babesia* parasites were used as positive control and DNA from non-infected RBC was used as negative control.

Sequencing

For confirmation of the nPCR results, three nPCR positive samples for *B. bigemina* or *B. bovis* were randomly selected and used as template in conventional PCR assay with small subunit ribosomal RNA (18S rRNA) gene sequences. Upper primer B-18S rRNA-F (5' TAC GGT GAA ACT GCG AAT GG 3') and lower primer B-18S rRNA-R (5' CAG ACT TGC GAC CAT ACT CC 3') were expected to generate a product of 920 bp for *B. bovis* and 955 bp for *B. bigemina*, respectively. PCR products were extracted from the agarose gel using QIAquick Gel Extraction Kit (QIAGEN GmbH, Germany), ligated into a pGEM-T Easy Vector (Promega, USA) and transformed into the *E. coli* DH5á-competent cells. A positive inserted plasmid was purified from a single clone using Plasmid Quick Pure (NucleoSpin, MACHEREY-NAGEL GmbH & Co. KG, Germany) and subsequently sequenced using a Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) with pGEM-T Easy Vector primers (pUC/M13). DNA sequences were determined using ABI PRISM 3100 genetic analyzer (Applied Biosystems, USA). Obtained sequences were analyzed using GenBank BLASTn analysis.

Statistical analysis

The proportions of agreement were calculated to evaluate the concordance between IFAT and ELISA (http://vassarstats.net/). The kappa coefficient was calculated to evaluate the agreement between the nPCR assay and ELISA or IFAT. Either the Chi-square or Fisher's exact text was used for the independent variables to determine the association between the individual factors with molecular and seropositivity to *B. bovis* and *B. bigemina*. The odds ratio (OR), 95% confidence interval, and p values were calculated separately for each variable. The results were considered significantly different when $p< 0.5$ (www.medcalc.org/calc/odds_ratio.php). Chi-square test was used to evaluate significant differences ($P<0.05$) of infection rate in animals of different genders and age groups (http://faculty.vassar.edu/lowry/VassarStats.html).

RESULTS

A total of 137 blood samples collected from cattle and water buffalos were checked for *B. bovis* and *B. bigemina* simultaneously using nPCR, ELISA and IFAT. For the molecular detection method, the nPCR assay identified *B. bovis* and *B. bigemina* in 30 (21.9%) and 15 (10.9%) samples, respectively. For the serological assays, the positive samples of *B. bovis* were 85 (62.0%) and 69 (50.4%) by ELISA and IFAT, respectively, whereas that of *B. bigemina* was 44 (32.1%) and 64 (46.7%), respectively. The proportion of agreement between ELISA and IFAT was 85.1% for *B. bovis* and 83.0% for *B. bigemina* in cattle, whereas in water buffalos, was 86.1% for *B. bovis* and 90.7% for *B. bigemina*. Moreover, the overall prevalence of *B. bovis* in cattle using nPCR, ELISA and IFAT was 21.3%, 73.4% and 60.6%, respectively whereas for *B. bigemina* it was 16.0%, 42.6% and 59.6%, respectively. Water buffalo samples indicated23.3%, 37.2% and 27.9% for *B. bovis* prevalence and 0%, 9.3% and 18.6% for *B. bigemina* prevalence by nPCR, ELISA and IFAT, respectively. In addition, mixed infections were detected in 34 cattle (36.2%) and 3 water buffalos (7.0%) by ELISA,

while IFAT showed 39 cattle (41.5%) and 4 water buffalos (9.3%) had mixed infections. However, the mixed infections were only detected in 5 cattle (5.3%) by nPCR (Table 1).

Antibodies to *B. bovis* and *B. bigemina* were 69/94 (73.4%) and 40/94 (42.6%) by ELISA and 57/94 (60.6%) and 56/94 (59.6%) by IFAT in cattle, respectively, whereas those of water buffalos were 16/43 (37.2%) and 4/43 (9.3%) by ELISA and 12/43 (27.9%) and 8/43 (18.6%) by IFAT, respectively. The seropositives of *B. bovis* and *B. bigemina* in cattle were significantly higher than the seropositives of *B. bovis* and *B. bigemina* in water buffalos, both detected by ELISA and IFAT (Table 2 and 3). Furthermore, the prevalence of bovine babesiosis in cattle and water buffalo populations were compared on the basis of their age groups. Two age groups were found to have specific antibodies to either *B. bovis* or *B. bigemina*. The serological prevalence ranged between 50.0% and 65.9% for *B. bovis* infection and between 29.4% and 48.2% for *B. bigemina* infection detected by both ELSIA and IFAT in both cattle and water buffaloes. Although *B. bovis* appeared to be more seroprevalent than *B. bigemina*, this difference was not statistically significant among the two age groups of cattle and water buffaloes infected by either *B. bovis* or *B. bigemina*. *Babesia bigemina* DNA was not detected in cattle aged more than 5 years and in all age groups of water buffaloes. In addition, female and male cattle as well as water buffaloes had similar infection rates of *B. bovis* and *B. bigemina* (Table 2 and 3).

Randomly selected three *B. bovis*- and *B. bigemina*-nPCR positive field DNA samples were amplified by using primers specific for 18S rRNA. The PCR products were inserted into pGEM-T easy vector and positive clones were sequenced. Sequence comparisons among the 18S rRNA of *B. bovis* genes (GenBank accession no. JN714976; JN714977) revealed between 99% and 100% nucleotide identity with published *B. bovis* BBOV2 and BBOV3 (GenBank accession no. L19077.1; L19078.1). Likewise, the 18S rRNA of *B. bigemina* genes amplified (GenBank accession no. JN714975) showed 100% identity to *B. bigemina* Kunming isolate from China (GenBank accession no. AY603402.1).

DISCUSSION

Bovine babesiosis caused by *B. bovis* and *B. bigemina* threatens the livestock industry and leads to great economic losses in many Southeast Asian countries. In this study, molecular and serological tools were preformed for the first time using the nPCR, IFAT and ELISA to detect *B. bovis* and *B. bigemina* infection in cattle and water buffaloes in the central region of Vietnam. The recombinant *B. bovis* spherical body protein-4 (BbSBP-4) and the *B. bigemina* Carboxyl-terminal region of rhoptry-associated protein 1 (BbigRAP-1a/CT) used to detect antibodies against *B. bovis* and *B. bigemina* in cattle serum samples by ELISA and nPCR was used to amplify the conserved *B. bovis* pherical body protein-2 (BbSBP-2) and *B. bigemina* rhoptry-associated protein 1 (BbigRAP-1a) fragment in genomic DNA (AbouLaila *et al*., 2010; Cao *et al*., 2012; Terkawi *et al*., 2011). These previously reported methods have high specificity and sensitivity for their use in epidemiological surveys. The results of this study indicate that

Table 1. Results of nPCR, ELISA and IFAT of *B. bovis* and *B. bigemina* in central regions of Vietnam

Animals	Number	*B. bovis*			*B. bigemina*		
		nPCR	ELISA	IFAT	nPCR	ELISA	IFAT
Cattle	94	20 (21.3%)	69 (73.4%)*	57 (60.6%)*	15 (16.0%)	40 (42.6%)*	56 (59.6%)*
Water buffalo	43	10 (23.3%)	16 (37.2%)	12 (27.9%)	0	4 (9.3%)*	8 (18.6%)*
Total	137	30 (21.9%)	85 (62.0%)*	69 (50.4%)*	15 (10.9%)	44 (32.1%)*	64 (46.7%)*

* Values with superscripts are significantly different ($P < 0.05$)

Table 2. Molecular and serological prevalence of *B. bovis* infections in different age and gender of cattle and water buffaloes

Factor	nPCR					ELISA					IFAT				
	+/*n*	(%)	OR	95% CI	*p* Value	+/n	(%)	OR	95% CI	*p* Value	+/n	(%)	OR	95% CI	*p* Value
Species															
Cattle	20/94	21.3	0.8919	0.3763-2.1138	0.7950	69/94	73.4	4.6575	2.1581-10.0514	0.0001	57/94	60.6	3.9797	1.8163-8.7199	0.0006
Buffalo	10/43	23.3				16/43	37.2				12/43	27.9			
Age (years)															
<2 year	10/52	19.2	0.7738	0.3299-1.8150	0.5555	29/52	55.8	0.6530	0.3218-1.3248	0.2377	26/52	50.0	0.9767	0.4898-1.9477	0.9467
>2 year	20/85	23.5				56/85	65.9				43/85	50.6			
Gender															
Male	8/53	15.1	0.5010	0.2046-1.2269	0.1304	28/53	52.8	0.5305	0.2615-1.0764	0.0791	21/53	39.6	0.5421	0.2697-1.0898	0.0857
Female	22/84	26.2				57/84	67.9				46/84	54.8			

+, Number of positive animals; *n*, number of samples; OR, odds ratio; 95% CI, 95% confidence interval
Values were considered significantly different when $p < 0.05$

Table 3. Molecular and serological prevalence of *B. bigemina* infections in different age and gender of cattle and water buffaloes

Factor	nPCR					ELISA					IFAT				
	+/*n*	(%)	OR	95% CI	*p* Value	+/n	(%)	OR	95% CI	*p* Value	+/n	(%)	OR	95% CI	*p* Value
Species															
Cattle	15/94	16.0	16.9623	0.9907-290.4285	0.0508	40/94	42.6	7.2222	2.3866-21.8554	0.0005	56/94	59.6	6.4474	2.6969-15.4137	< 0.0001
Buffalo	0/43	0				4/43	9.3				8/43	18.6			
Age (years)															
<2 year	5/52	9.6	0.7979	0.2568-2.4793	0.6963	19/52	36.5	1.3818	0.6644-2.8740	0.3867	23/52	44.2	0.8511	0.4256-1.7023	0.6486
>2 year	10/85	11.8				25/85	29.4				41/85	48.2			
Gender															
Male	6/53	11.3	1.0638	0.3557-3.1815	0.9118	12/53	22.6	0.4756	0.2181-1.0371	0.0617	18/53	34.0	0.4248	0.2083-0.8664	0.0185
Female	9/84	10.7				32/84	38.1				46/84	54.8			

+, Number of positive animals; *n*, number of samples; 95% CI, 95% confidence interval
Values were considered significantly different when $p < 0.05$

a high presence of infection of cattle and water buffaloes in the central region of Vietnam and suggest that the surveyed areas can be considered endemic for *B. bovis* and *B. bigemina* infection.

IFAT and ELISA detected a higher number of infected cattle and water buffaloes than nPCR. The reason for these differences could be that the high number of cattle and water buffaloes continued to have the anti-*B. bovis* and *B. bigemina* antibodies in circulation for a long period of time after acute infection. Another reason may be that the blood samples were collected from apparently clinically healthy cattle and water buffaloes. The parasite concentrations in the blood possibly drop below the limit of detection of the nPCR (Zulfiqar *et al.*, 2012). Moreover, this study may not accurately reflect the epidemiological status of bovine babesiosis caused by *B. bovis* and *B. bigemina* in Vietnam, therefore a full epidemiological survey involving a large number of animals and covering the whole country is required.

The overall prevalence of *B. bovis* and *B. bigemina* infections was compared among the different ages of cattle and water buffalo populations. The infection rate of bovine babesiosis was significantly higher in young cattle and water buffaloes, indicating their susceptibility to the infections. The differences in prevalence rates observed among the different age groups could be due to longer exposure of older animals to the infection than in younger ones. Hence, they are able to develop a solid protective immunity to fight the infections as compared to younger animals (Homer *et al.*, 2000). Normally, *Babesia* infections closely correlate to tick bite. The ticks which were collected from Thua Thien Hue province in Vietnam have been identified mainly as *R. microplus* (Khukhuu *et al.*, 2011). In future, it might be of interest to investigate the incidence of *Babesia* infection in ticks in Vietnam.

Results of the present study provide molecular and serological evidence for the presence of *B. bovis* and *B. bigemina* in cattle and water buffaloes from the central region of Vietnam. These data will be of importance to help carry out a comprehensive and rationally designed plan to control and prevent these currently neglected diseases, at a regional level.

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