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NOTE

Parasitology

Serological and molecular surveys of *Babesia bovis* and *Babesia bigemina* among native cattle and cattle imported from Thailand in Hue, Vietnam

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ABSTRACT. Serum and DNA from blood samples collected from Vietnamese yellow cattle (n=101) and cattle imported from Thailand (n=54) at a Vietnamese slaughter house were screened for *Babesia bovis* and *Babesia bigemina* infections by enzyme-linked immunosorbent assay (ELISA) and PCR. The positive rates determined by ELISA (*B. bovis* and *B. bigemina*) or PCR (*B. bigemina*) in the Vietnamese cattle were significantly higher than those found in Thai cattle. Some PCR-positive Vietnamese animals were ELISA-negative, whereas all PCR-positive Thai cattle were ELISA-positive, suggesting that the animals were infected in Thailand. Importing *Babesia*-infected cattle may lead to the introduction of new parasite strains, possibly compromising the development of anti-*Babesia* immune control strategies in Vietnam.

KEY WORDS: *Babesia bigemina*, *Babesia bovis*, cattle, Thailand, Vietnam

Hemoprotozoan parasites that infect cattle generally include species of *Babesia*, *Theileria* and *Trypanosoma*. Among bovine *Babesia* parasites, *Babesia bovis* and *Babesia bigemina*, which are economically significant pathogens, are widespread in tropical and sub-tropical regions worldwide [3]. Both parasite species are transmitted by tick vectors and cause severe clinical diseases in susceptible cattle [16]. *Babesia* sporozoites injected by the infected tick vector during its blood meal invade bovine red blood cells (RBCs) and then transform into merozoites that multiply asexually [12]. Merozoite egress from infected RBCs causes massive destruction of these cells within the blood vessels, leading to anemia and anemia-related clinical signs [3]. In addition, acute infection with *B. bovis* may result in respiratory and neurological syndromes via sequestration of infected RBCs in the capillary beds [9]. In both cases, the prognosis is poor when treatment of the disease is delayed.

Vietnam is an agricultural country. Although the livestock sector has the potential to greatly contribute to the Vietnamese national economy, the productivity of this industry remains low for various reasons, such as outbreaks of infectious diseases. Recent studies reported the detection of different species of hemoprotozoan parasites, including *B. bovis* and *B. bigemina*, in Vietnamese cattle and buffalo bred in different geographical regions of the country [17, 22, 24]. As the meat production is not sufficient to meet the domestic demand, Vietnam has recently started to import live cattle from several countries, including Thailand, for use in the meat industry. Notably, various hemoprotozoan parasite species, including *B. bovis* and *B. bigemina*, have been reported in cattle bred in Thailand [1, 7, 11, 13, 19]. However, the animals imported from Thailand have not been investigated for infections with *Babesia* parasites in Vietnam. Therefore, the objective of the present study was to investigate whether *B. bovis* and *B. bigemina* are present among cattle imported from Thailand by enzyme-linked immunosorbent assay (ELISA) systems and PCR assays.

Previously, ELISA systems based on recombinant forms of the rhoptry-associated protein-1 (RAP-1) were found to be useful

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Table 1. PCR primers used in the present study

Purpose	Parasite	Target gene	Primers (5′–3′)		Amplicon size (bp)	Reference
			Forward	Reverse		
Sequencing	*B. bovis*	RAP-1	AAGTTCATCGAGGATACTAACG	TCAGAGGTATCCGGCGGTGTC	741	This study
	B. bigemina	RAP-1	TTGATTATGAAGCACGTCTC	TTACGCATCTGAATCATCTG	522	This study
Protein expression[a)]	*B. bovis*	RAP-1	gcggatccAACTATCTGAAAGCCAATG	gccctcgagtcaAGCAATATTCTCGCCTAGG	300	[20]
	B. bigemina	RAP-1	gcggatccCCTCACTACCTTTCTAAGGC	gccctcgagtcaATCTTCATTTTTGGGGTCATC	300	[20]
Diagnostic PCR	*B. bovis*	RAP-1	CACGAGCAAGGAACTACCGATGTTGA	CCAAGGACCTTCAACGTACGAGGTCA	356	[10]
	B. bigemina	AMA-1	TACTGTGACGAGGACGGATC	CCTCAAAAGCAGATTCGAGT	211	[21]

a) Uppercase letters in the primer sequences indicate the regions corresponding to the template sequences. The restriction sites in the forward (*Bam*HI) and reverse (*Xho*I) primers are underlined.

Table 2. ELISA and PCR results summary

Animal type	No. of samples	*B. bovis*				*B. bigemina*			
		ELISA		PCR		ELISA		PCR	
		No. positive	% (CI)	No. positive	% (CI)	No. positive	% (CI)	No. positive	% (CI)
Vietnamese yellow cattle	101	74	73.3 (63.9–80.9)	16	15.8 (10–24.2)	78	77.2 (68.1–84.3)	31	30.7 (22.5–40.3)
Thai cattle	54	23	42.6 (30.3–55.9)	3	5.6 (1.9–15.1)	30	55.6 (42.4–68.0)	4	7.4 (2.9–17.6)

CI, confidence interval.

for serological detection of *B. bovis* and *B. bigemina* infections [4, 5]. In the present study, genetic variations present in the *B. bovis* and *B. bigemina rap-1* sequences from Vietnam were analyzed before the development of RAP-1 antigen-based ELISAs. *Babesia bovis*- and *B. bigemina*-positive archived blood DNA samples sourced from Vietnamese cattle (*B. bovis*, n=8; *B. bigemina*, n=18) and buffalo (*B. bovis*, n=3; *B. bigemina*, n=2) [27] were used to amplify 741-base pair (bp) and 522-bp fragments of the N-terminal region of *rap-1* of *B. bovis* and *B. bigemina*, respectively, using the primer sets shown in Table 1. The gene fragments were then cloned into a PCR 2.1 vector (TOPO, Invitrogen, Carlsbad, CA, U.S.A.) and sequenced on an ABI PRISM 3700 genetic analyzer. The newly determined *B. bovis* and *B. bigemina rap-1* sequences were registered in GenBank (*B. bovis*, LC323157–LC323167; *B. bigemina*, LC323168–LC323188). Sequencing analyses revealed that the identity and similarity scores shared among the *rap-1* sequences of *B. bovis* (98.2–100 and 96.3–100%, respectively) and *B. bigemina* (97.5−100 and 96.5−100%, respectively) were very high, indicating that Vietnamese *B. bovis* and *B. bigemina rap-1* are highly conserved.

Subsequently, 300-bp gene fragments encoding 100 amino acids within the *B. bovis*- and *B. bigemina*-specific regions of *rap-1* sequences [4, 5] were PCR-amplified using the previously described primer sets and the PCR 2.1 vectors containing inserts from the Vietnamese *B. bovis* (LC323159) and *B. bigemina rap-1* (LC323168) gene sequences (Table 1) [20]. The PCR products were ligated into a pGEX-4T1 (*B. bovis*) or pGEX-6p2 (*B. bigemina*) plasmid vector (GE Healthcare, Little Chalfont, U.K.), and recombinant RAP-1 antigens were expressed as glutathione *S*-transferase-fusion proteins as described previously [20]. Finally, the glutathione *S*-transferase tag was cleaved using thrombin and PreScission protease (GE Healthcare) to purify the *B. bovis* and *B. bigemina* recombinant RAP-1 proteins (rRAP-1), respectively [20]. These rRAP-1 antigens were then used to develop ELISAs for the sero-diagnosis of *B. bovis* and *B. bigemina* infections. Briefly, ELISA plates were coated with 100 μl of 1 μg/m*l* *B. bovis* or *B. bigemina* rRAP-1 antigen in carbonate-bicarbonate buffer (Sigma-Aldrich, St. Louis, MO, U.S.A.) at 4°C overnight. The plates were then washed once with phosphate-buffered saline containing 0.5% Tween 20 (PBST), and then blocked with 1% bovine serum albumin (BSA; Sigma-Aldrich) (100 μl per well) at 37°C for 1 hr. After washing once with PBST, 100 μl of each serum sample diluted 1:100 in 1% BSA was added to the wells in duplicate, and the plate was incubated at 37°C for 1 hr. The ELISA plates were washed six times with PBST, and 100 μl of horseradish peroxidase-conjugated rabbit anti-bovine IgG (Bethyl Laboratories, Inc., Montgomery, TX, U.S.A.) diluted 1:4,000 in 1% BSA was added to each well. The ELISA plates were incubated at 37°C for 1 hr and washed six times with PBST. Next, 50 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Sigma-Aldrich) was added to each well. The plates were incubated in the dark for 10 min, and the reaction was stopped by adding 50-μl of TMB stop solution (Sigma-Aldrich). Finally, the optical density (OD) value was measured at 450 nm. A sample was considered positive if the OD value was higher than the cut-off value that had been determined as the sum of the mean OD values and 5 ×standard deviations of the five negative serum samples used in each plate (data not

shown) [20]. Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis showed that the Vietnamese *B. bovis* and *B. bigemina rap-1* sequences shared 98 and 97% minimum identity scores with the sequences reported globally. Therefore, although the ELISAs in the present study were developed based on gene sequences isolated in Vietnam, they can also detect specific antibodies against *B. bovis* and *B. bigemina* of Thai origin.

Blood samples were collected from September to December (2016) at a slaughter house located in Hue from 101 Vietnamese yellow cattle, including 93 males and 8 females, and 54 Brahman cattle (males) that had been imported from Thailand. All animals were apparently healthy during sampling. The Vietnamese animals were 1–6 years old (98 and 3 animals were 1–3.5 and 4–6 years old, respectively), while the Thai cattle were 1.5–3.5 years old. The period between the departure of cattle from Thailand and time of sampling was one week. Blood samples were subjected to serum separation and DNA extraction. Serum samples were analyzed by *B. bovis*- and *B. bigemina*-specific ELISA, while DNA samples were subjected to *B. bovis*- and *B. bigemina*-specific PCR assays [10, 21] as described previously (Table 1) [26]. The positive rates were analyzed by OpenEpi software (http://www.openepi.com/Proportion/Proportion.htm) to determine the 95% confidence intervals using a Wilson score interval [25]. The *P* values were calculated using an "N-1" χ^2 test [6, 18] (https://www.medcalc.org/calc/comparison_of_proportions.php). The rate difference was considered statistically significant when the *P* value was <0.05.

The ELISA positivity rates for *B. bovis* and *B. bigemina* in Vietnamese cattle (73.3 and 77.2%, respectively) were significantly higher than those in Thai cattle (42.6 and 55.6%, respectively) (Table 2). The *B. bigemina* PCR-positivity rate was also higher for Vietnamese cattle (30.7%) than for Thai cattle (7.4%), but a significant difference was not found for *B. bovis* PCR-positive rates between Vietnamese and Thai cattle (15.8 and 5.6%, respectively) (Table 2). Co-infections with *B. bovis* and *B. bigemina* were detected by ELISA and PCR in the parasite-infected Vietnamese (61 and 7 animals, respectively) and Thai cattle (17 and 1 animals, respectively). Of the 16 samples that were PCR-positive for *B. bovis* in Vietnamese cattle, 12 were positive by ELISA, whereas four samples that were positive by PCR were negative by ELISA. Similarly, of the 31 samples that were PCR-positive for *B. bigemina*, 22 were positive by ELISA, whereas nine PCR-positive samples were ELISA-negative. The PCR-positive but ELISA-negative results for these samples indicate that the animals likely became infected very recently and that the samples were collected before ELISA-detectable antibodies had been developed. In contrast, all Thai cattle that were *B. bovis*- and *B. bigemina*-positive by PCR were also positive by ELISA, suggesting that no recent infections occurred among Thai cattle. A previous study found that *B. bovis* antibodies in experimentally infected cattle were detectable by ELISA from days 10 to 14 post-infection [8]. In the present investigation, Thai animals were sampled one week after they left Thailand. Thus, the Thai cattle were likely infected before importation. However, additional studies are needed to test the animals for *Babesia* infections in Thailand and Vietnamese quarantine facilities before and after their importation to confirm this hypothesis.

Although the infection rates for *B. bovis* and *B. bigemina* were higher among Vietnamese cattle compared to in cattle imported from Thailand, the introduction of new parasite strains may complicate the development of immune control strategies in Vietnam. For example, genetic analyses of *B. bovis* merozoite surface antigen-1 (*msa-1*) demonstrated that the genotypic distribution of *B. bovis* differs between Vietnam and Thailand [15, 23, 27]. Previous studies showed that the immune profiles of *B. bovis*-infected cattle may differ in a strain-specific manner based on the genetic variation observed in merozoite surface antigens, such as *msa-1* [2, 14]. Thai cattle typically arrive in Hue 1–3 weeks after departing from Thailand. The animals graze occasionally during transportation and before slaughtering. Therefore, tick vectors in Vietnamese pasture may acquire *Babesia* infection from Thai cattle during blood meal. In addition, entry of *Babesia*-infected ticks together with imported cattle into Vietnam cannot be ruled out.

In conclusion, the present study, which analyzed *B. bovis* and *B. bigemina* infections in Vietnam using molecular and serological diagnostic tools, found that cattle imported from Thailand had been exposed to and still harbored these parasite species. Therefore, Thai cattle should be tested for *B. bovis* and *B. bigemina* positivity before importing them into Vietnam to ensure that only the *Babesia*-free animals are imported into the country.

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