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Spherical Body Protein 4 Is a New Serological Antigen for Global Detection of *Babesia bovis* Infection in Cattle▽

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Five *Babesia bovis* recombinant proteins, including merozoite surface antigen 2c (BbMSA-2c), C-terminal rhoptry-associated protein 1 (BbRAP-1/CT), truncated thrombospondin-related anonymous protein (BbTRAP-T), spherical body protein 1 (BbSBP-1), and spherical body protein 4 (BbSBP-4), were evaluated as diagnostic antigens to detect the infection in cattle. The recombinant proteins were highly antigenic when tested with experimentally *B. bovis*-infected bovine serum in Western blot analysis. Furthermore, five antisera that had been raised against each of the recombinant proteins reacted specifically with the corresponding authentic protein, as determined in Western blot analysis. Next, enzyme-linked immunosorbent assays (ELISAs) using these recombinant proteins were evaluated for diagnostic use, and the sensitivity and specificity of each protein were demonstrated with a series of serum samples from experimentally *B. bovis*-infected cattle. Furthermore, a total of 669 field serum samples collected from cattle in regions of *B. bovis* endemicity in seven countries were tested with the ELISAs, and the results were compared to those of an indirect fluorescent antibody test (IFAT), as a reference. Among five recombinant antigens, recombinant BbSBP-4 (rBbSBP-4) had the highest concordance rate (85.3%) and kappa value (0.705), indicating its reliability in the detection of specific antibodies to *B. bovis* in cattle, even in different geographical regions. Overall, we have successfully developed an ELISA based on rBbSBP-4 as a new serological antigen for a practical and sensitive test which will be applicable for epidemiologic survey and control programs in the future.

Bovine babesiosis is an economically important tick-borne disease in tropical and subtropical areas of the world (3). The disease is caused by hemoprotozoan parasites of the genus *Babesia*, namely, *B. bovis*, *B. bigemina*, *B. beliceri*, *B. divergens*, *B. major*, *B. ovata*, *B. occultans*, and *B. jakimovi* (28). Among them, *B. bovis* and *B. bigemina* are the most important species, and they are usually found together in most areas of endemicity (22, 31). Although *B. bovis* and *B. bigemina* are phylogenetically related and transmitted by *Rhipicephalus* (*Boophilus*) *microplus*, they cause remarkably different diseases in cattle (3). Infection by *B. bovis* is more severe than that of *B. bigemina*, due to the sequestration of infected erythrocytes in the microcapillaries of the kidneys, lungs, and brain, resulting in organ failure and systemic shock that leads to death. Cattle that survive *B. bovis* infection generally become carriers of the parasite and serve as reservoirs for transmission to other animals (3). Thus, highly sensitive and specific diagnostic tools are required to detect the carrier animals and to differentiate this infection from other closely related ones. Such diagnostic tests must lead to a better understanding of the protozoan epidemiology, providing useful information for disease management and control strategies (10).

A large number of serological tests have been developed for the detection of specific antibodies to bovine *Babesia* parasites for epidemiological surveys as well as for the identification of carrier animals (2, 7, 30). Among these assays, the indirect immunofluorescent antibody test (IFAT) is the most sensitive, but cross-reactivity with other *Babesia* spp., subjective interpretation, and low throughput have limited its usefulness (8). In contrast, enzyme-linked immunosorbent assays (ELISAs) can be used as a routine diagnostic test or as a screening test for epidemiologic studies. Although a number of diagnostic ELISAs have been developed, several problems regarding the sensitivity and specificity remain, related mostly to the characteristics and preparation of the antigens. For example, crude antigens have been used to detect the antibodies to *Babesia* parasites, but the poor purification quality of the antigen and the potential cross-reactivity with other protozoan parasites have impeded their application. Such crude antigens frequently contain host cell components, which may affect the accuracy of test results by increasing the nonspecific background (7–9, 21, 29). On the other hand, recombinant proteins derived from the parasites could become alternative sources of antigens, allowing a better standardization of the tests with high specificity and sensitivity (7, 8). Despite the potential advantages of using recombinant antigens in serological tests, their sensitivity needs to be significantly improved (29). Therefore, further research to identify new antigen makers is extremely desirable.

Babesia parasites are defined by the common characteristic structures of the apical complex, which consists of rhoptries, micronemes, and spherical body organelles. Proteins derived from these organelles, coupled with the membrane component of parasites, are believed to have critical functions in parasite

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survival and growth (34). These proteins include (i) variable merozoite surface antigens (VMSAs) (4), which are believed to play key roles in the initial attachment of merozoites and sporozoites during their invasion (34), (ii) microneme and rhoptry proteins, including apical membrane antigen 1 (AMA-1), thrombospondin-related anonymous protein (TRAP), and rhoptry-associated protein 1 (RAP-1), which seem to be involved in the formation of a tight junction between merozoites and erythrocytes (RBC) (11), and (iii) spherical body proteins (SBPs), which have roles in stabilizing the environment after invasion and in aiding parasite growth (14, 26). Importantly, they have been suggested as promising candidates for developing subunit vaccines or diagnostic antigens (34).

With this in mind, we validated five ELISAs with different recombinant proteins, including merozoite surface antigen 2c (BbMSA-2c) (19), C-terminal rhoptry-associated protein 1 (BbRAP-1/CT) (6), thrombospondin-related anonymous protein (BbTRAP) (11), spherical body protein 1 (BbSBP-1) (16), and spherical body protein 4 (BbSBP-4) (M. A. Terkawi, F. J. Seuseu, P. E. Wibowo, N. X. Huyen, M. Aboulaila, N. Yokoyama, X. Xuan, and I. Igarashi, submitted for publication), and then evaluated their potential application for the diagnostic detection of specific antibodies to *B. bovis*. The results indicate the promising use of an ELISA with rBbSBP-4 antigen as a global diagnostic marker for the detection of *B. bovis* infection.

MATERIALS AND METHODS

Parasites. The Texas strain of *B. bovis* and the Argentina strain of *B. bigemina* were continuously cultured with bovine erythrocytes (RBC) by using a microaerophilous stationary-phase culturing system (1, 15, 20). The cultured parasite was harvested when the parasitemia reached 8 to 10%.

Expression and purification of recombinant proteins and production of polyclonal antibodies. The DNA fragments encoding *B. bovis* merozoite surface antigen 2c (BbMSA-2c; GenBank accession number AY052542) (19), the C-terminal region of rhoptry-associated protein 1 (BbRAP-1/CT [388 to 490 amino acids {aa}]; AF030062) (5, 6), full-length or truncated thrombospondin-related anonymous protein (BbTRAP or BbTRAP-T [321 to 561 aa]; AY486102) (11), spherical body protein 1 (BbSBP-1; AAC37226) (16), and spherical body protein 4 (BbSBP-4; AB594813) (Terkawi et al., submitted) were amplified from a *B. bovis* cDNA phage expression library by standard PCRs using gene-specific primers (Table 1). The amplified DNA fragments were cloned into a pGEX-4T1 plasmid vector (Amersham Pharmacia Biotech, Madison, CA) using the suitable restriction enzyme sites and then expressed as glutathione *S*-transferase (GST) fusion genes in the *Escherichia coli* DH-5α 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) and then analyzed by SDS-PAGE and Western blot analysis, as described previously (26).

Thereafter, each of the antisera for recombinant proteins was prepared in 6-week-old BALB/c mice ($n = 5$) according to the standard protocol (25). Briefly, mice were intraperitoneally (i.p.) immunized with 100 μg of each recombinant protein emulsified in Freund's complete adjuvant (Sigma, St. Louis, MO). Two boosters were given i.p. using 50 μg of the same proteins emulsified in Freund's incomplete adjuvant (Sigma) at 14-day intervals. Sera were collected 2 weeks after the last booster and then checked for the production of specific antibodies using the IFAT and Western blotting.

SDS-PAGE and Western blotting. The expressed recombinant proteins were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with subsequent Coomassie blue staining, while their antigenicities were confirmed with Western blot analysis. To identify the authentic parasite proteins, the *B. bovis*-infected RBC were obtained from the *in vitro* culture, washed three times with cold phosphate-buffered saline (PBS), and then lysed with 0.25% saponin. The pellet was washed four times with cold PBS, resuspended in PBS, disrupted three times by a freeze-thaw cycle in liquid nitrogen, and then sonicated in an ice slurry (Terkawi et al., submitted). The protein concentrations of lysates were determined by a bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL) and finally stored at −80°C until use. The extracted proteins were separated in 12% SDS-PAGE and then electroblotted onto a nitrocellulose membrane (32). The membrane was blocked with 0.05% Tween 20 in PBS (PBS-T) plus 5% skimmed milk and probed with the indicated protein-specific primary antibodies. After the membrane was washed with PBS-T, a secondary antibody, horseradish peroxidase (HRP)-conjugated anti-bovine or -mouse immunoglobulin G (IgG) antibody (Bethyl Laboratories, Montgomery, TX), was applied. Finally, reacted bands were visualized using a solution containing 3-diaminobenzidine tetrahydrochloride (DAB) and H_2O_2 (Dojindo, Tokyo, Japan).

TABLE 1. Gene-specific primers for amplifying *BbMSA-2c*, *BbRAP-1/CT*, *BbTRAP-T*, *BbSBP-1*, and *BbSBP-4*

Gene	Oligonucleotide primer[a]
BbMSA-2c	5′-CGGAATTCATGGTGTCTTTAACATAATAACC-3′
	5′-TAGCGGCCGCGAATGCAGAGAGAACGAAGTAGCAG-3′
BbRAP-1/CT	5′-ACGGATCCGAGTTTTTCAGGGAA-3′
	5′-ACCTCGAGAAACTCATGTATGAT-3′
BbTRAP-T	5′-AGAATTCGAACCAAGCCGTGCTACACCG-3′
	5′-ACTCGAGCTATTGTTTTTCGCCCTCGTAG-3′
BbSBP-1	5′-GCGAATTCAACGAAGCTGAGGTATCTCAG-3′
	5′-GGCTCGAGTTAGTCTAGCATCTGTATTTT-3′
BbSBP-4	5′-AGGAATTCGAGGAGGAGGAAACTGATGAG-3′
	5′-GCCTCGAGTTATTCCTCAATGTCGGCTGT-3′

[a] Each oligonucleotide primer includes restriction enzyme sites at the 5′ end (underlined), which are EcoRI and NotI for *BbMSA-2c*, BamHI and XhoI for *BbRAP-1/CT*, and EcoRI and XhoI for *BbTRAP-T*, *BbSBP-1*, and *BbSBP-4*.

Bovine sera. Positive serum samples were collected from cattle experimentally infected with *B. bovis* ($n = 25$) or *B. bigemina* ($n = 30$) (National Institute of Animal Health, Tsukuba, Ibaraki, Japan) or with *Theileria orientalis* ($n = 6$) (Obihiro University of Agriculture and Veterinary Medicine, Japan), while non-*Babesia*-infected control sera ($n = 50$) were obtained from healthy cattle that had been bred at Obihiro University of Agriculture and Veterinary Medicine, Washington State University (Pullman, WA), and Texas A&M University (College Station, TX) (19). Field bovine sera were collected from Brazil ($n = 108$), Ghana ($n = 80$), China ($n = 100$), Thailand ($n = 100$), Mongolia ($n = 81$), South Korea ($n = 100$), and Hokkaido, Japan ($n = 100$) (1, 5, 18).

ELISAs. Standard enzyme-linked immunosorbent assays (ELISAs) were performed in the present study as described previously (25). Briefly, 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 μl of each recombinant protein at a concentration of 2 μg/ml per well in a coating buffer (50 mM carbonate-bicarbonate buffer, pH 9.6). The plates were washed once with 0.05% Tween 20-PBS (PBS-T) and then incubated with 100 μl of a blocking solution (3% skim milk in PBS) for 1 h at 37°C. After the antigen-coated wells were washed once with PBS-T, they were incubated with 50 μl of the serum samples diluted 1:100 with the blocking solution for 1 h at 37°C. The plates were washed six times with PBS-T and then incubated with 50 μl of HRP-conjugated sheep anti-bovine IgG antibody (Bethyl) diluted 1:4,000 with the blocking solution for 1 h at 37°C as a secondary antibody. The plates were washed six times as described above, and 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), and 0.01% of 30% H_2O_2] was then added to each well. After incubation for 1 h at room temperature (RT), the optical density (OD) was measured with an MTP-500 microplate reader (Corona Electric, Tokyo, Japan) at a wavelength of 415 nm. The cutoff points were calculated by the receiver operating characteristic (ROC) analysis with MedCalc statistical software (version 11.4; http://www.medcalc.be) for each recombinant protein with 50 non-*Babesia*-infected bovine sera (12, 13).

IFAT. The *B. bovis*-infected RBC were coated on indirect fluorescent antibody test (IFAT) slides (Matsunami Glass Ind., Ltd., Osaka, Japan), dried, and then fixed in absolute acetone for 20 min for standard IFAT observation (4). Briefly,

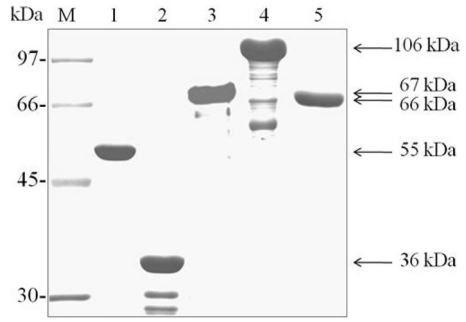

FIG. 1. Successful expression of recombinant proteins in *E. coli*. Twelve percent SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of recombinant protein stained with Coomassie blue. Lanes: M, molecular mass marker; 1, rBbMSA-2c; 2, rBbRAP-1/CT; 3, rBbTRAP-T; 4, rBbSBP-1; 5, rBbSBP-4. The size of each recombinant protein is indicated on the right.

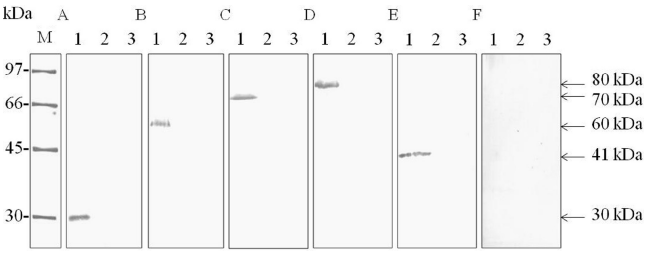

FIG. 3. Western blot analysis of authentic proteins with antisera against different recombinant proteins. BbMSA-2c (A), BbRAP-1 (B), BbTRAP (C), BbSBP-1 (D), and BbSBP-4 (E) were detected on the lysate of *B. bovis* parasites (lanes 1) but not on *B. bigemina* (lanes 2) and noninfected bovine RBC (lanes 3). The reactivity of the lysates was also examined with anti-GST serum (F) as a negative control.

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 the slides were washed with PBS three times, fluorescein isothiocyanate (FITC)-conjugated sheep anti-bovine IgG antibody (Bethyl Laboratories, Montgomery, TX) was applied as a secondary antibody (1:250), and incubation proceeded for 1 h at 37°C. Propidium iodide (PI) (Molecular Probes) was used to stain the parasite's nuclei (26). After the glass slides were washed with PBS twice, they were mounted by adding 10 μl of a 50% (vol/vol) glycerol-PBS solution and covering them with glass coverslips and examined using a fluorescent microscope (E400 Eclipse; Nikon, Kawasaki, Japan).

Statistical analysis. The results of ELISAs were compared with those of the IFAT to calculate the percentages of agreement, the sensitivity and specificity (18), and the kappa values; thus, the strength of agreement between the ELISA and the IFAT was considered the kappa value: fair (0.21 to 0.40), moderate (0.41 to 0.60), and substantial (0.61 to 0.8) (http://faculty.vassar.edu/lowry/VassarStats.html).

RESULTS

Production of recombinant proteins. The genes encoding BbMSA-2c, BbRAP-1/CT, BbTRAP-T, BbSBP-1, and BbSBP-4 were successfully expressed as soluble GST fusion proteins in *E. coli* with molecular masses of 55, 36, 66, 106, and 67 kDa, respectively (Fig. 1). Sera collected from cattle experimentally infected with *B. bovis* specifically reacted to all the recombinant proteins but not to the control GST protein in Western blot analysis (data not shown), suggesting their high antigenicity with the infected sera. Because full-length BbTRAP resulted in a low yield in the soluble fraction, only rBbTRAP-T was used for further validation (data not shown). Thereafter, five different antisera raised against each recombinant protein were used to identify the native proteins derived from *B. bovis*. Confocal microscopic observation of the IFAT demonstrated strong reactivity of each antiserum with both intra- and extraerythrocytic parasites of *B. bovis* (Fig. 2). Western blot analysis of *B. bovis* lysate probing these antisera revealed 30-, 60-, 70-, 80-, and 41-kDa proteins of BbMSA-2c, BbRAP-1, BbTRAP, BbSBP-1, and BbSBP-4, respectively (Fig. 3, lanes 1). All sizes of the authentic *B. bovis* proteins were consistent with the expected molecular weights for each mature protein. In contrast, these antisera did not cross-react with the lysate of *B. bigemina*-infected erythrocytes or normal bovine erythrocytes (Fig. 3, lanes 2 and 3, respectively).

Application of the recombinant proteins for serological diagnoses. The specificity and sensitivity of rBbMSA-2c, rBbRAP-1/CT, rBbTRAP-T, rBbSBP-1, and rBbSBP-4 were evaluated in a standard ELISA with experimentally infected and negative-control bovine sera. The cutoff OD values were determined to be 0.14, 0.20, 0.217, 0.173, and 0.11, respectively, for rBbMSA-2c, rBbRAP-1/CT, rBbTRAP-T, rBbSBP-1, and rBbSBP-4 using ROC analysis with 50 negative-control sera (Fig. 4). Notably, the ELISAs based on these recombinant proteins succeeded in clearly differentiating between *B. bovis*-infected sera and either the negative-control sera or B. *bigemina*-infected and *T. orientalis*-infected sera. All 25 *B. bovis*-

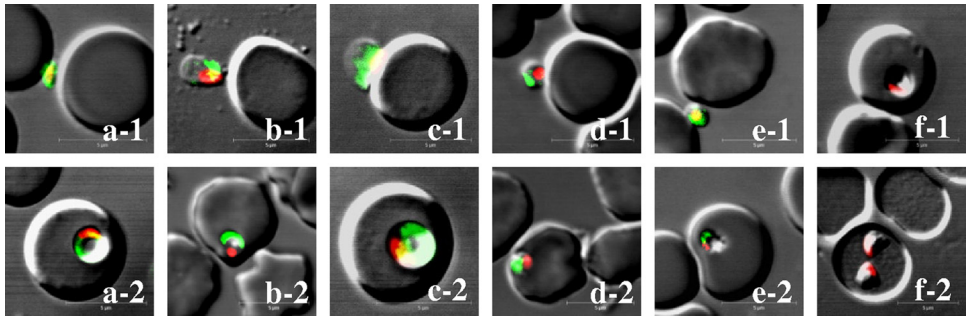

FIG. 2. Reactivity of each antiserum with *B. bovis* parasites by the IFAT. Confocal laser microscopic observation of extracellular (a-1 to e-1) and intracellular *B. bovis* parasites (a-2 to f-2). Thin blood smears of *B. bovis*-infected RBC fixed with absolute methanol were probed with each antiserum: a, anti-rBbMSA-2c; b, anti-rBbRAP-1/CT; c, anti-rBbTRAP-T; d, anti-rBbSBP-1; e, anti-rBbSBP-4; f, anti-GST. Specific immunofluorescent reaction (green) and nuclear staining (red) were observed.

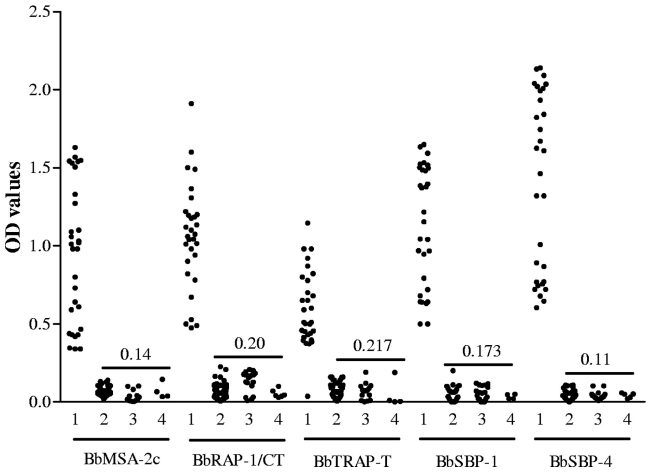

FIG. 4. Reactivity of ELISA using recombinant proteins with bovine sera. Lane 1, experimentally *B. bovis*-infected sera; lane 2, experimentally *B. bigemina*-infected sera; lane 3, noninfected bovine sera; lane 4, *Theileria orientalis*-infected sera. The cutoff of each recombinant protein is indicated by a bar.

infected serum samples tested had higher ODs than the cutoff value, contrary to the 30 *B. bigemina*-infected sera and 6 *Theileria orientalis*-infected sera, which had ODs lower than the cutoff values (Fig. 4). Although all recombinant proteins demonstrated good performance as ELISA antigens capable of identifying the experimental infection, rBbSBP-4 seemed to be the best due to its high OD values with *B. bovis*-infected sera and its lower OD values with normal bovine sera or *B. bigemina*- and *T. orientalis*-infected sera. Of note, rBbTRAP-T showed low OD values with *B. bovis*-infected sera. Furthermore, many samples were near the cutoff, and one sample was below, which indicates the low potential of rBbTRAP-T as a diagnostic antigen.

The diagnostic performances of these recombinant proteins were also evaluated with 669 field samples collected from areas of *B. bovis* endemicity, including Brazil (108 samples), Ghana (80 samples), China (100 samples), Thailand (100 samples), and Mongolia (81 samples), and areas where *B. bovis* is nonendemic, including South Korea (100 samples) and Hokkaido, Japan (100 samples). In addition, their results were compared to those of the IFAT, as a reference test (Table 2). None of the antigens showed a reaction, as tested using ELISAs and the IFAT with serum samples derived from areas where *B. bovis* is nonendemic (data not shown). On the other hand, the specificity and sensitivity of ELISAs with serum samples derived from the areas of endemicity ranged from 54.85% (rBbTRAP-T) to 96.01% (rBbSBP-4) and from 23.45% (rBbTRAP-T) to 96.43% (rBbSBP-4), respectively (Table 3). Next, the results were statistically compared to IFAT data. The agreement (con-

TABLE 2. Summary of ELISA results with recombinant proteins and IFAT results with field sera collected from different areas of *B. bovis* endemicity

Site or IFAT result	No. of IFAT samples	No. of ELISA samples									
		MSA-2c		RAP-1/CT		TRAP-T		SBP-1		SBP-4	
		+	−	+	−	+	−	+	−	+	−
Brazil											
+	80	52	28	35	45	10	70	74	6	78	2
−	28	2	26	0	28	1	27	8	20	4	24
Total	108	54	54	35	73	11	97	82	26	82	26
Ghana											
+	41	24	17	13	28	16	25	27	14	32	31
−	39	6	33	3	36	1	38	8	31	8	9
Total	80	30	50	16	64	17	63	35	45	40	40
China											
+	36	22	14	21	15	3	33	22	14	34	2
−	64	9	55	6	58	1	63	9	55	6	58
Total	100	31	69	27	73	4	96	31	69	40	60
Thailand											
+	57	45	12	34	23	10	47	38	19	51	6
−	43	16	27	16	27	2	41	14	29	11	32
Total	100	61	39	50	50	12	88	52	48	62	38
Mongolia											
+	29	17	12	18	11	9	20	16	13	18	11
−	52	11	41	10	42	4	48	6	46	10	42
Total	81	28	53	28	53	13	68	22	59	28	53
Overall											
+	243	160	83	121	122	48	195	177	66	213	30
−	226	44	182	35	191	9	217	45	181	39	187
Total	469	204	265	156	313	57	412	222	247	252	217

TABLE 3. The specificity and sensitivity of ELISAs and comparison of ELISA and IFAT results

Parameter	BbMSA	BbRAP-1CT	BbTRAP-T	BbSBP-1	BbSBP-4
Specificity (%)	85.3	72.3	54.85	91.49	96.01
Sensitivity (%)	83.95	64.2	23.45	91.35	96.43
Concordance (%)	72.9	66.53	56.5	76.33	85.3
Kappa value	0.461	0.339	0.153	0.528	0.705

cordance) between the IFAT and the ELISA was calculated to be 56.5% (rBbTRAP-T), 66.53% (rBbRAP-1CT), 72.9% (rBbMSA-2c), 76.33% (rBbSBP-1), and 85.3% (rBbSBP-4), and the kappa values were determined to be 0.153, 0.339, 0.461, 0.528, and 0.705, respectively (Table 3). These results revealed that rBbSBP-4 is the best diagnostic antigen assayed in the ELISA for the detection of a specific antibody to *B. bovis* among five antigens examined.

DISCUSSION

The global impact of bovine babesiosis has spurred an interest in developing effective diagnostic strategies that could lead to better management of the control of infection. *B. bovis* infection can be diagnosed directly by microscopic examination or PCR and indirectly with several serological methods (31). Although all of these tests have shortcomings, the serological tests, particularly the ELISA, seems to be the most practical and economical for epidemiological investigation (2, 7, 8). Indeed, the ELISA offers greater sensitivity, objectivity, and capacity for quick adaptation to test a large number of sera than any of the existing serological tests. The crude antigen prepared from merozoites has been utilized traditionally for serological detection; however, recombinant protein can be an alternative source, allowing better standardization of the test while reducing the cost of the massive production of the parasite (7, 9, 21). At this point, many recombinant antigens have been evaluated for diagnostic ELISAs; however, their sensitivities in diverse geographic areas have not produced perfect results. Therefore, further research on new antigens is extremely desirable for the development of a standard global assay. Within this context, we have validated five ELISAs utilizing deferent recombinant proteins and evaluated their usefulness for global application.

Although the ELISAs based on recombinant proteins differentiated clearly between experimentally *B. bovis*-infected and other closely related protozoan-infected bovine sera, their performances using field bovine sera varied, especially in comparison to IFAT results. Of note, rBbSBP-4 demonstrated the best performance, with high specificity, sensitivity, and agreement rates with the IFAT. These might be due to the fact that BbSBP-4 is a unique protein that has no significant homology with other apicomplexan parasites and is highly conserved among different geographic isolates from Asia, Africa, and South America (Terkawi et al., submitted). In addition, BbSBP-4 was found to shed within the cytoplasm of infected RBC in the late developmental stage of the parasites and was, subsequently, detected as an exoantigen in the supernatant of an *in vitro* culture (Terkawi et al., submitted). The secretion nature of BbSBP-4 may make the molecule very immunogenic during infection and allow direct interaction with host immune cells. On the other hand, rBbSBP-1 showed good diagnostic performance, with promising specificity and sensitivity for the detection of infection. Indeed, BbSBP-1 has been documented to be an immunodominant 80-kDa merozoite protein located in the spherical bodies, detected within the cytoplasmic face of the infected RBC membrane shortly after the invasion, and capable of eliciting the proliferation of both $CD4^+$ and $CD8^+$ T lymphocytes (16, 27). The better performance of rBbSBP-4 than of rBbSBP-1 in diagnosis might be due to the characteristics of the native protein which is found abundantly in the cytosol of infected host cells in the late stage of division of the parasites and, consequently, in the supernatant of the culture (Terkawi et al., submitted). This allows a direct and longer exposure to host immune cells (17). Moreover, the analyses of the determinant's composition based on an *in silico* epitope are necessary to understand antigenic attributes of these molecules. Further study including the combination of the two antigens on the basis of their B- and T-cell epitopes in the ELISA might be valuable, providing more accurate diagnostic tools for the detection of *B. bovis* infection.

A major membrane protein, BbMSA-2c, is a member of the complex VMSA genes and is known as a promising diagnostic antigen (19). Members of this family, however, exhibit high sequence variability among geographically distant strains (23). This might be the reason for the lower antigenicity of rBbMSA-2c for the detection of infection and might limit its potential for global diagnosis. The rhoptry-associated protein 1 (RAP-1) occurs in all *Babesia* species, has significant sequence homology with other apical complex proteins, and includes neutralization-sensitive B-cell and T-cell epitopes (24). BbRAP-1 is known as an exoantigen that is detected within the cytoplasm of infected RBC at the early stage of infection and persists shortly before disappearing at the ring stage (26, 27, 33). The full-length protein has been documented to be unsuitable for diagnosis due to the high conservation of 300 amino acids at the N-terminal region, which causes cross-reactivity with other *Babesia* spp. On the contrary, the C terminus presents distinct sequences with low identities with other *Babesia* RAP-1s and can serve as a species-specific diagnostic antigen (5, 6, 26). Therefore, the C terminus of BbRAP-1 has been used broadly as an antigen for serodiagnosis and epidemiological surveys, with high reliability (6, 18). However, our data have shown that this antigen had good performance with samples from Asia but not with those from Ghana and Brazil. In support of this, Boonchit et al. (5) have noted that rBbRAP-1CT has better performance for the detection of infection in Mongolian than Brazilian serum samples. The low sensitivity of rBbRAP-1CT is probably due to diversity in the sequences encoding the C-terminal region among geographically distant strains. Unexpectedly, rBbTRAP-T showed the lowest specificity and sensitivity with field serum samples, indicating that this antigen is not suitable for the development of a serological test. In a related study, rBgTRAP was noted as the best antigen for the detection of infection with *B. gibsoni* in canine sera (14, 26). The discrepancies in performance might be due to differences in the host and immune responses as well as the low identical homology between the genes in the species.

Serological diagnosis of *Babesia* infection is usually based on the detection of the antibodies against asexual blood-stage parasites. Although the IFAT has been a reliable serological

test in recent decades, it is time-consuming and subjective. Additionally, it cannot be automated, and results are largely influenced by the degree of training of the technician. Recently, the competitive ELISA (cELISA) with the C terminus of the rhoptry-associated protein 1 has been validated and undergone interlaboratory validation under OIE standards (12, 13). Despite the high specificity and sensitivity of the cELISA with *B. bovis*-infected sera from different geographic regions, the assay has shown a limitation in detecting the early stage of infection. Indeed, the IFAT could detect specific antibodies in experimentally *B. bovis*-infected sera about 2 days before the cELISA (12). Moreover, Goff et al. (13) have noted that samples with low OD values (near the cutoff point) in the cELISA should be repeated for further confirmation. These may affect the accuracy of the diagnosis in epidemiological investigation, particularly with carrier animals that have low antibody titers. Therefore, there is a need to continue research on the development of effective diagnostic assays. In the present study, we attempted to develop an indirect ELISA for the diagnosis of *B. bovis* infection. Our results demonstrated the usefulness of an ELISA utilizing rBbSBP-4 for the serological detection of *B. bovis* infection in cattle from different geographic regions of the world. The ELISA with rBbSBP-4 provides a more practical and sensitive tool than the standard IFAT and thus will be applicable for epidemiological surveys and control of the disease. Further investigations including a direct comparison of this ELISA with the previously validated cELISA (13) are required to achieve better diagnosis of *B. bovis* infection in the future.

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REFERENCES

1. **Altangerel, K., et al.** 2009. Evaluation of *Babesia bigemina* 200 kDa recombinant antigen in enzyme-linked immunosorbent assay. Parasitol. Res. **105:** 249–254.
2. **Araújo, F. R., et al.** 1998. Comparison between enzyme-linked immunosorbent assay, indirect fluorescent antibody and rapid conglutination test in detecting antibodies against *Babesia bovis*. Vet. Parasitol. **74:**101–108.
3. **Bock, R., L. Jackson, A. de Vos, and W. Jorgensen.** 2004. Babesiosis of cattle. Parasitology **129:**247–269.
4. **Bono, M. F., et al.** 2008. Efficiency of a recombinant MSA-2c-based ELISA to establish the persistence of antibodies in cattle vaccinated with *Babesia bovis*. Vet. Parasitol. **157:**203–210.
5. **Boonchit, S., et al.** 2002. Evaluation of an enzyme-linked immunosorbent assay with recombinant rhoptry-associated protein-1 antigen of *Babesia bovis* for the detection of specific antibodies in cattle. J. Clin. Microbiol. **40:**3771–3775.
6. **Boonchit, S., et al.** 2004. Improved enzyme-linked immunosorbent assay using C-terminal truncated recombinant antigens of *Babesia bovis* rhoptry-associated protein-1 for detection of specific antibodies in cattle. J. Clin. Microbiol. **42:**1601–1604.
7. **Böse, R., R. H. Jacobson, K. R. Gale, D. J. Waltisbuhl, and I. G. Wright.** 1990. An improved ELISA for detection of antibodies against *Babesia bovis* using either a native or a recombinant *B. bovis* antigen. Parasitol. Res. **76:**648–652.
8. **Böse, R., W. K. Jorgensen, R. J. Dalgliesh, K. T. Friedhoff, and A. J. de Vos.** 1995. Current state and future trends in diagnosis of babesiosis. Vet. Parasitol. **57:**61–74.
9. **de Echaide, S. T., et al.** 1995. Evaluation of enzyme-linked immunosorbent assay kit to detect *Babesia bovis* antibodies in cattle. Prev. Vet. Med. **24:**277–283.
10. **de Vos, A. J., and F. T. Potgieter.** 1994. Bovine babesiosis, p. 278–294. *In* J. A. W. Coetzer, G. R. Thomson, and R. C. Tustin (ed.), Infectious diseases of livestock. Oxford University Press, Cape Town, South Africa.
11. **Gaffar, F. R., A. P. Yatsuda, F. F. Franssen, and E. de Vries.** 2004. A *Babesia bovis* merozoite protein with a domain architecture highly similar to the thrombospondin-related anonymous protein (TRAP) present in *Plasmodium* sporozoites. Mol. Biochem. Parasitol. **136:**25–34.
12. **Goff, W. L., et al.** 2003. Competitive enzyme-linked immunosorbent assay based on a rhoptry-associated protein 1 epitope specifically identifies *Babesia bovis*-infected cattle. Clin. Diagn. Lab. Immunol. **10:**38–43.
13. **Goff, W. L., et al.** 2006. Validation of a competitive enzyme-linked immunosorbent assay for detection of antibodies against *Babesia bovis*. Clin. Vaccine Immunol. **13:**1212–1216.
14. **Goo, Y. K., et al.** 2008. *Babesia gibsoni*: serodiagnosis of infection in dogs by an enzyme-linked immunosorbent assay with recombinant BgTRAP. Exp. Parasitol. **118:**555–560.
15. **Hines, S. A., G. H. Palmer, D. P. Jasmer, T. C. McGuire, and T. F. McElwain.** 1992. Neutralization-sensitive merozoite surface antigens of *Babesia bovis* encoded by members of a polymorphic gene family. Mol. Biochem. Parasitol. **55:**85–94.
16. **Hines, S. A., et al.** 1995. Genetic and antigenic characterization of *Babesia bovis* merozoite spherical body protein Bb-1. Mol. Biochem. Parasitol. **69:** 149–159.
17. **Homer, M. J., et al.** 2003. Identification and characterization of putative secreted antigens from *Babesia microti*. J. Clin. Microbiol. **41:**723–729.
18. **Iseki, H., et al.** 2010. Seroprevalence of *Babesia* infections of dairy cows in northern Thailand. Vet. Parasitol. **170:**193–196.
19. **Kim, C. M., et al.** 2008. Development of a rapid immunochromatographic test for simultaneous serodiagnosis of bovine babesiosis caused by *Babesia bovis* and *Babesia bigemina*. Am. J. Trop. Med. Hyg. **78:**117–121.
20. **Levy, M. G., and M. Ristic.** 1980. *Babesia bovis*: continuous cultivation in a microaerophilus stationary phase culture. Science **204:**1218–1220.
21. **Machado, R. Z., et al.** 1997. An enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against *Babesia bovis* in cattle. Vet. Parasitol. **71:**17–26.
22. **McCosker, P. J.** 1981. The global importance of babesiosis, p. 1–24. *In* M. Ristic and J. P. Kreier (ed.), Babesiosis. Academic Press, Inc., New York, NY.
23. **Palmer, G. H., et al.** 1991. Strain variation of *Babesia bovis* merozoite surface-exposed epitopes. Infect. Immun. **59:**3340–3342.
24. **Suarez, C. E., et al.** 1991. Characterization of the gene encoding a 60-kilodalton *Babesia bovis* merozoite protein with conserved and surface exposed epitopes. Mol. Biochem. Parasitol. **46:**45–52.
25. **Terkawi, M. A., et al.** 2007. *Babesia gibsoni* ribosomal phosphoprotein P0 induces cross-protective immunity against *B. microti* infection in mice. Vaccine **25:**2027–2035.
26. **Terkawi, M. A., et al.** 2009. Molecular characterizations of three distinct *Babesia gibsoni* rhoptry-associated protein-1s (RAP-1s). Parasitology **136:** 1147–1160.
27. **Tetzlaff, C. L., A. C. Rice-Ficht, V. M. Woods, and W. C. Brown.** 1992. Induction of proliferative responses of T cells from *Babesia bovis*-immune cattle with a recombinant 77-kilodalton merozoite protein (Bb-1). Infect. Immun. **60:**644–652.
28. **Uilenberg, G.** 2006. *Babesia*—a historical overview. Vet. Parasitol. **138:**3–10.
29. **Waltisbuhl, D. J., I. G. Goodger, I. G. Wright, M. A. Commins, and D. F. Mahoney.** 1987. An enzyme-linked immunosorbent assay to diagnose *Babesia bovis* infection in cattle. Parasitol. Res. **73:**126–131.
30. **Weiland, G., and I. Reiter.** 1988. Methods for serological response to *Babesia*, p. 143–158. *In* M. Ristic. (ed.), Babesiosis of domestic animals and man. CRC Press, Inc., Boca Raton, FL.
31. **World Organization for Animal Health (OIE).** 2008. Chapter 2.3.8, Bovine babesiosis. *In* Manual of diagnostic tests and vaccines for terrestrial animals. OIE, Paris, France. http://www.oie.int/fr/normes/mmanual/A_00059.htm.
32. **Xuan, X., et al.** 2001. Expression of *Babesia equi* merozoite antigen 1 in insect cells by a recombinant baculovirus and evaluation of its diagnostic potential in an enzyme-linked immunosorbent assay. J. Clin. Microbiol. **39:**705–709.
33. **Yokoyama, N., et al.** 2002. Cellular localization of *Babesia bovis* merozoite rhoptry-associated protein 1 and its erythrocyte-binding activity. Infect. Immun. **70:**5822–5826.
34. **Yokoyama, N., M. Okamura, and I. Igarashi.** 2006. Erythrocyte invasion by *Babesia* parasites: current advances in the elucidation of the molecular interactions between the protozoan ligands and host receptors in the invasion stage. Vet. Parasitol. **138:**22–32.