Development of a Rapid Immunochromatographic Test for Simultaneous Serodiagnosis of Bovine Babesioses Caused by *Babesia bovis* and *Babesia bigemina*

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Abstract. With the objective of developing a simpler diagnostic alternative, a rapid immunochromatographic test (BoICT) was constructed for the simultaneous detection of *Babesia bovis*- and *Babesia bigemina*-specific antibodies using *B. bovis* recombinant merozoite surface antigen-2c and *B. bigemina* recombinant rhoptry-associated protein-1. The BoICT selectively detected specific antibodies to *B. bovis* and *B. bigemina*. All sera from cattle infected with other protozoan parasites (i.e., *Cryptosporidium parvum*, *Neospora caninum*, and *Theileria orientalis*) showed negative results in the BoICT. The relative sensitivity and specificity for detecting antibody to *B. bovis* were 96.7% (29 of 30) and 91.3% (73 of 80), respectively. This indicates that the BoICT is useful for fast field diagnostic assessment of bovine babesioses without the use of a laboratory equipment.

**INTRODUCTION**

Bovine babesiosis, especially caused by *Babesia bovis* or *Babesia bigemina*, is an important protozoan disease from both veterinary and economic viewpoints. It is estimated that bovine babesiosis may endanger half a billion cattle worldwide.1 The infections are generally characterized by fever, listlessness, anorexia, dehydration, and progressive hemolysis.2 Several serologic methods have been standardized for the diagnosis of babesiosis, such as the indirect fluorescent antibody test (IFAT), the enzyme-linked immunosorbent assay (ELISA), and the immunochromatographic test (ICT), and extensively used in epidemiologic studies.3–11 Compared with other serologic tests, the ICT is a simple and rapid method, which makes it suitable for clinical and field applications. It relies on migration of liquid across the surface of a nitrocellulose membrane and because of its convenience has been developed for a variety of applications over the past decade.12–14 Assays using this format take approximately 15 minutes to complete and require only a small volume (<200 μL) of the tested serum on the test strip.3,15–18

*Babesia bovis* and *B. bigemina* are transmitted by the same tick vector, e.g., *Rhipicephalus* (Boophilus) microplus, *R. annulatus*, and *R. geigyi*.2,18 Previous studies have reported the simultaneous prevalence of both parasites in cattle.2,19 However, none of the serologic methods enabled simultaneous detection of specific antibodies to *B. bovis* and *B. bigemina*. Therefore, a simpler test capable of simultaneously detecting two types of antibodies in an ICT is needed. A rapid and accurate assay for differential diagnosis between *B. bovis* and *B. bigemina* would be extremely valuable in testing clinical specimens, not only for diagnostic purposes and controlling the diseases, but also for better understanding of the epidemiology of these organisms.2,6

We have developed two ICTs (BoICT and BiICT) for the individual detection of *B. bovis*- or *B. bigemina*-specific antibodies by placing either of the recombinant C-terminal portions of the rhoptry-associated proteins-1 (rRAP-1/CTs) of these parasites on a test strip.3 Because *B. bovis* and *B. bigemina* rRAP-1/CTs were available for the development of two kinds of diagnostic ICTs, we attempted to combine these two rRAP-1/CTs on a test strip and evaluate the activity of the simultaneous serodiagnosis of both infections using a dual ICT. However, the sensitivity and specificity of the dual ICT was <80% compared with values for the separate tests, in which values were insufficient for diagnostic applications (Kim CM and others, unpublished data). Accordingly, to increase the sensitivity and specificity of the dual ICT, we replaced *B. bovis* rRAP-1/CT with recombinant merozoite surface antigen-2c (rMSA-2c), which was also known to be a potential antigen for serologic diagnosis of *B. bovis*,20 and then evaluated its use in the dual ICT.

In the present study, recombinant antigens of *B. bovis* MSA-2c and *B. bigemina* RAP-1/CT were prepared as antigens for the dual ICT (BoiICT). We then evaluated the BoiICT for simultaneous serodiagnosis of two bovine babesioses.

**MATERIALS AND METHODS**

**Parasites.** The Texas T2B strain of *B. bovis*21 and the Argentine strain of *B. bigemina*22 were grown in purified bovine erythrocytes using a previously established continuous microaerophilous stationary-phase culture system.23 The parasites were used for DNA extraction as previously described.3

**Preparation of recombinant antigens.** In our previous study, *B. bigemina* rRAP-1/CT was prepared as a glutathione S-transferase (GST)-fusion protein and directly used for construction of BiICT because the fusion protein was not successfully cleaved into intact rRAP-1/CT and GST by thrombin protease.2 In the present study, a new recombinant C-terminal portion of the RAP-1 (rRAP-1/CT17) was designed (amino acids 326–480, GenBank accession no. M60878) and prepared by using oligonucleotide primers 5′-ccggaattcCTGGTCCCCGAAGAGCAC-3′ and 5′-ataa-gagtgaggcgctTTACGCAATGCAATCTCTTG-3′ (the lowercase letters show the EcoRI and NotI restriction site linkers, respectively) as described previously.3 The GST protein was removed from the fusion protein using thrombin protease (Amersham Biosciences Corp., Piscataway, NJ), and an rRAP-1/CT17 without GST was successfully obtained.

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Babesia bovis \(rMsa-2c\) was prepared with oligonucleotide primers 5'-cgaatctATGTTGTCTTTAAACATAATTGCC-3' and 5'-ataggattgccgGAATCGAGAGAAGACGA-GTATGCAG-3' (the lowercase letters show the EcoRI and NotI restriction site linkers, respectively) on the basis of the nucleotide sequence of the MSA-2c gene (GenBank accession no. AY052542), and 798 bases of a DNA fragment containing the MSA-2c gene was amplified from the extracted DNA of the cultured parasite by polymerase chain reaction. The amplified DNA product was digested with EcoRI and NotI, purified with a QIAquick gel extraction kit (Qiagen, Inc., Hilden, Germany), and ligated into the EcoRI and NotI sites of a pGEX-4T E. coli expression plasmid vector (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom). The resulting plasmid produced a recombinant MSA-2c fused with GST in the transformed E. coli BL21 strain, and non-fused rMSA-2c was successfully purified and cleaved as described previously.\(^7\)

Production of rabbit antibodies to \(rMsa-2c\) and \(rRAP-1/CT17\). Three-month-old female Japanese white rabbits (CLEA Japan, Tokyo, Japan) were immunized with 1 mg of \(rMsa-2c\) or \(rRAP-1/CT17\) protein mixed with an equal volume of TiterMax (CytRx Corporation, Norcross, GA) by multiple intradermal injections into their backs. Three booster immunizations were given at seven-day intervals. A week after the third booster immunization, sera were confirmed for the development of specific IgG titers (> 1/8,192) by ELISA\(^8\) and collected from immunized rabbits. Total IgG was purified from the sera with an Econo-Pac Protein A Kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s instructions.

Preparations of gold colloid-conjugated antigens and ICT strip. Gold colloid-conjugated antigens and an ICT strip were prepared as previously described\(^16\) with some modifications. Briefly, 200 µg/mL of purified rMsa-2c and rRAP-1/CT17 proteins were mixed gently with gold colloid (British BioCell International, Cardiff, United Kingdom), and the colloid-conjugated antigens were then sprayed on glass fiber (Schleicher and Schuell, Keene, NH). The assemblage of the ICT strip was then carried out as described previously with some modifications.\(^12\) Briefly, purified rabbit anti-MSA-2c IgG (2 mg/mL) and rMsa-2c (300 µg/mL) and rRAP-1/CT17 proteins (100 µg/mL) werejetted linearly on a nitrocellulose membrane as the procedural control, \(B.\) bovis, and \(B.\) bigemina test lines, respectively. The nitrocellulose membrane, as well as the conjugated pad, sample pad, and absorbent pad, was assembled on an adhesive card (Schleicher and Schuell) and then cut into 2-mm-wide strips using a BioJet 3050 quanti-dispenser (BioDot Inc., Irvine, CA). A single ICT for the individual detection of \(B.\) bovis- (BoICT) or \(B.\) bigemina-specific antibody (BiICT) was also prepared as previously described.\(^5\) The strips were stably stored with dehumidification in foil pouches at ambient temperature until use.

Enzyme-linked immunosorbent assay. The ELISA using either rMsa-2c (BoELISA) or rRAP-1/CT17 (BiELISA) protein diluted with carbonate buffer (pH 9.6) at a final concentration of 0.5 or 0.3 µg/mL, respectively, was performed as described previously.\(^3,5\) The optical densities were measured at a wavelength of 415 nm (\(OD_{415}\)) using an MTP-120 ELISA reader (Corona Electric, Ibaraki, Japan).

Sera. Bovine sera used for the evaluation of ICTs were as follows: sequential bovine sera from experimentally infected cattle with \(B.\) bovis (\(n = 13, 0–93\) days post-infection) or \(B.\) bigemina (\(n = 13, 0–274\) days post-infection) provided by the National Institute of Animal Health (Tsukuba, Ibaraki, Japan); non-infected control sera (\(n = 10\)) obtained from healthy cattle that had been bred at Obihiro University of Agriculture and Veterinary Medicine in Japan, where no \(B.\) bovis and \(B.\) bigemina infections have been reported; Neospora caninum- (\(n = 5\)) and Cryptosporidium parvum–positive bovine sera (\(n = 5\)) diagnosed using ELISA\(^24,25\), Theilera orientalis–positive bovine sera (\(n = 5\)) diagnosed using a microscopic test and polymerase chain reaction using blood. For ICTs, 50 µL of the diluted serum with phosphate-buffered saline (1:1) was applied on the sample pad, followed by evaluation within 10 minutes at room temperature as described previously.\(^8\) The relative sensitivities and specificities of ICTs and ELISAs were determined with 30 \(B.\) bovis- or \(B.\) bigemina-positive field bovine sera or 50 \(B.\) bovis- and \(B.\) bigemina-negative field bovine sera that had been previously evaluated by IFAT and different ELISAs using recombinant \(B.\) bovis and \(B.\) bigemina antigens (RAP-1/CTs).\(^8\)

Statistical analysis. The strength of agreement between the BoiICT and single ICT was estimated by calculating the kappa statistic. Kappa statistic values > 0.75, 0.40–0.75, and < 0.40 represent excellent agreement, good to fair agreement, and poor agreement, respectively.\(^26\)

RESULTS

Evaluations of ELISAs using \(B.\) bovis \(rMsa-2c\) and \(B.\) bigemina \(rRAP-1/CT17\). To evaluate the usefulness of \(B.\) bovis \(rMsa-2c\) and \(B.\) bigemina \(rRAP-1/CT17\) proteins as serodiagnostic antigens, these proteins were subjected to the diagnostic ELISAs (BoELISA and BiELISA). The BoELISA for \(B.\) bovis infection detected antibodies in two negative sera and 29 \(B.\) bovis- and 2 \(B.\) bigemina-positive sera at an OD\(_{415}\) > 0.22 (Figure 1A), whereas 3 negative sera and 2 \(B.\) bovis- and 29 \(B.\) bigemina-positive sera showed positive responses in the BiELISA for \(B.\) bigemina infection at an OD\(_{415}\) > 0.18 (Figure 1B). The BoELISA and BiELISA showed identical relative sensitivity (96.7%, 29 of 30), and the relative specificities were 95% (76 of 80) and 93.8% (75 of 80) for the BoELISA and BiELISA, respectively, compared with data evaluated by previously described methods.\(^18\) We then examined the usefulness of the ELISAs by using sequential sera obtained from cattle experimentally infected with \(B.\) bovis or \(B.\) bigemina. The BoELISA and BiELISA detected antibodies to \(B.\) bovis and \(B.\) bigemina from 14 to 93 days and from 13 to 274 days post-infection, respectively.

Evaluation of the BoiICT for simultaneous serodiagnosis for \(B.\) bovis and \(B.\) bigemina infections. Figure 2A shows three types of detectable lines for procedural control and \(B.\) bovis and \(B.\) bigemina tests produced on the BoiICT after the application of various kinds of serum samples. The BoiICT can detect simultaneously, but selectivity, two kinds of specific antibodies to target parasites on a single test strip. We then examined the usefulness of BoiICT on the simultaneously diagnostic activity in sequential bovine sera from cattle infected with \(B.\) bovis or \(B.\) bigemina. The BoiICT detected the antibodies to \(B.\) bovis in the sera collected from 14 to 93 days post-infection (Figure 2B) and those to \(B.\) bigemina from 13 to 274 days post-infection (Figure 2C). In ad-
dition, no cross-reaction was observed between the *B. bovis*- and *B. bigemina*-infected positive sera, and all sera collected from cattle infected with other protozoan parasites (*Cryptosporidium parvum*, *Neospora caninum*, and *Theileria orientalis*) showed negative reactions in the BoiICT.

To determine the relative sensitivity and specificity of the BoiICT compared data evaluated by previously described methods, 18–30 *B. bovis*- or 30 *B. bigemina*-positive sera or 50 *B. bovis*- and *B. bigemina*-negative sera were examined using the BoiICT, and the results were compared with those obtained with BoICT and BiICT, two tests that were developed for the individual diagnosis of either *B. bovis* or *B. bigemina* infection. The relative sensitivities of both BoICT and BiICT for detecting antibody to *B. bigemina* were 96.7% (29 of 30), and the relative specificities were 93.8% (75 of 80) and 92.5% (74 of 80), respectively (Table 2). When these results were compared statistically, the agreement between BoiICT and single ICTs was 98.2% (108 of 110) with a kappa statistic of 0.96 (*P* < 0.001).

**DISCUSSION**

In recent years, there have been several reports on the serodiagnosis of bovine babesiosis. However, these methods, such as the ELISA and IFAT, involve complex procedures that require laboratory materials, equipment, and trained personnel. In the present study, we developed a new rapid assay (BoiICT) using rMSA-2c and rRAP-1/CT17 an-
tigens for serodiagnosis. The obvious advantage of ICT is that this assay provides accurate results in 15 minutes or less. Another important aspect of the BoiICT developed in the present study, compared with other available tests, is its multiplex characteristic. With this approach, it was possible to simultaneously and selectively detect two kinds of specific antibodies to *B. bovis* or *B. bigemina* using one reaction strip. Simultaneous detection is extremely useful because these species have often shown an overlapping geographic distribution.7,8

Merozoite surface proteins of apicomplexan hemoparasites, such as *Babesia* and *Plasmodium* spp., often provide potential targets for the immune-mediated control.27–29 *Babesia bovis* MSA-2c is also exposed on the surfaces of both merozoites and sporozoites.30 Previous studies showed that *B. bovis* MSA-2c has a high degree of genetic and antigenic conservation among geographically distant strains.29 Thus, this antigen appears to be an adequate diagnostic candidate for the serodiagnosis of bovine babesiosis. The adaptation of rMSA-2c, instead of rRAP-1/CT, could lead to the successful development of an alternative test. Because a highly specific recombinant antigen in ELISA has showed promise for its use in the diagnostic ICT,15,16 the diagnostic potential of *B. bovis* rMSA-2c and *B. bigemina* rRAP-1/CT17 was first evaluated in ELISA prior to construction of the ICT. The developed ELISAs using the rMSA-2c and rRAP-1/CT17 proteins (BoELISA and BiELISA, respectively) showed high relative sensitivity (96.7%) and specificities (95% and 93.8%, respectively). Therefore, we applied these two proteins in developing a simultaneous ICT, BoiICT, for *B. bovis* and *B. bigemina* infections.

From the results of the BoiICT, the relative sensitivity and specificity for *B. bovis* infection were 96.7% and 91.3%, respectively, and those for *B. bigemina* were 96.7% and 92.5%, respectively. The results suggest that the BoiICT is sensitive and specific for serodiagnosis of both infections, as indicated in previous reported ICTs for various protozoan infections with sensitivities ranging from 72% to 100% and specificities ranging from 61% to 100%.15–17,31–33 In addition, no significant differences were observed in the sensitivities and specificities between the BoiICT and the BoELISA and those between the BiICT and the BiELISA, although discordant cases were observed in two sera between the BoiICT and the BoIELISA. These discordant sera showed high absorbance (OD$_{415}$ > 1.2) above the cutoff value against only one species of either *B. bovis* or *B. bigemina* in the ELISA. Therefore, non-specific reactions might influence the antigen or antibody cross-reactivity, and the BoiICT might result in a misdiagnosis.

For clinical use, the ICT strip must have high specificity to distinguish other infections of related pathogenic protozoa. The BoiICT could accurately differentiate the *B. bovis* or *B. bigemina* infections from other infections. Therefore, the present configuration of the BoiICT is useful for simultaneously detecting *B. bovis* and *B. bigemina*-specific antibodies under a variety of infective circumstances. Although the specificity of the BoiICT was slightly lower than that of single ICTs, the kappa value (0.96) demonstrated excellent agreement between them. Moreover, the BoiICT detected antibodies to *B. bovis* and *B. bigemina* from 14 and 13 days to the last days post-infection, respectively, without cross-reactivity. These results indicate that this rapid test could simultaneously detect the early stage to latent infection of *B. bovis* and *B. bigemina*.

In conclusion, the BoiICT is a simple and rapid method that provides accurate and simultaneous detection of *B. bovis* and *B. bigemina* infections, thereby saving time and eliminating the need for special training. This rapid test can therefore be practically implemented in epidemiologic surveys for bovine babesiosis, although evaluation on a larger scale with various cattle sera is still necessary.

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