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Babesia bovis Merozoite Surface Antigen 2 Proteins Are Expressed on the Merozoite and Sporozoite Surface, and Specific Antibodies Inhibit Attachment and Invasion of Erythrocytes

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The *Babesia bovis* merozoite surface antigen 2 (MSA-2) locus encodes four proteins, MSA-2a$_1$, -2a$_2$, -2b, and -2c. With the use of specific antibodies, each MSA-2 protein was shown to be expressed on the surface of live extracellular merozoites and coexpression on single merozoites was confirmed. Individual antisera against MSA-2a, MSA-2b, and MSA-2c significantly inhibited merozoite invasion of bovine erythrocytes. As tick-derived sporozoites also directly invade erythrocytes, expression of each MSA-2 protein on the sporozoite surface was examined and verified. Finally, statistically significant inhibition of sporozoite binding to the erythrocytes was demonstrated by using antisera specific for MSA-2a, MSA-2b, and MSA-2c. These results indicate an important role for MSA-2 proteins in the initial binding and invasion of host erythrocytes and support the hypothesis that sporozoites and merozoites use common surface molecules in erythrocyte invasion.

During the first steps of erythrocyte invasion, *Babesia* and *Plasmodium* species use molecules located on the parasite surface coat to bind the host cell. Consistent with this role, antibodies against surface molecules inhibit parasite invasion of the host erythrocyte (2–4, 6, 10, 21). In *Babesia bovis*, the merozoite surface bears at least five proteins that belong to the variable merozoite surface antigen (VMSA) family (5, 8). Members of the VMSA family are defined by an amino-terminal hydrophobic signal sequence, a hydrophilic central region, and a conserved carboxy-terminal region containing a glycosylphosphatidylinositol anchor signal sequence and a 54-bp 3′ untranslated region that has been proposed elsewhere to be involved in regulating gene expression (5). The merozoite surface antigen 1 (MSA-1) localizes to the surface of both merozoites and tick-derived sporozoites (7, 18–20). In contrast to the requirement for an exoerythrocytic stage occurring after mosquito transmission of *Plasmodium* sp., *B. bovis* sporozoites directly invade erythrocytes following tick-borne transmission (11, 12). Notably, antibody against MSA-1 inhibits sporozoite attachment to erythrocytes in vitro, as well as blocking merozoite invasion (10, 18).

Unlike MSA-1, which is encoded by a single-copy gene, the four MSA-2 proteins, MSA-2a$_1$, -2a$_2$, -2b, and -2c, are encoded by tandemly arranged genes within an 8.3-kb genomic locus (5). MSA-2a$_1$ and MSA-2a$_2$ are closely related with 90% identity in amino acid sequence—consequently, polyclonal sera, as well as monoclonal antibody 23/70.174, bind both MSA-2a$_1$ and MSA-2a$_2$ (5). In contrast, MSA-2a$_1$ and MSA-2a$_2$ have, respectively, 54 and 64% identity with MSA-2b and 25 and 26% identity with MSA-2c. MSA-2b and MSA-2c have 25% identity. These structural differences among MSA-2a, MSA-2b, and MSA-2c are reflected by the presence of unique B-cell epitopes on each protein and the absence of cross-reactive antibody (5). Adsorption of polyclonal serum containing antibody to each MSA-2 protein with recombinant MSA-2a abolishes reactivity to MSA-2a but not to MSA-2b or MSA-2c, adsorption with recombinant MSA-2b abolishes reactivity to MSA-2b but not to MSA-2a or MSA-2c, and adsorption with recombinant MSA-2c abolishes reactivity to MSA-2c but not to MSA-2a or MSA-2b (5). Thus, if expressed on the parasite surface, individual MSA-2 proteins present unique targets for antibody inhibition. The goal of the present study was to determine if the MSA-2 proteins are expressed on the surface of merozoites and sporozoites and to test whether antibody specific to each MSA-2 protein blocks *B. bovis* binding and invasion of erythrocytes.

MSA-2a, initially described as Bo44, has previously been shown to be expressed on the surface of live, extracellular merozoites (13, 20). To determine if the other proteins were similarly expressed in merozoites, an immunofluorescence assay on live parasites was performed. The MSA-2a$_1$, -2b, and -2c recombinant His-tagged fusion proteins were purified from pBAD/thio-TOPO (Invitrogen, Carlsbad, Calif.)-transformed *Escherichia coli* with Ni^{2+}-charged His-bind resin (Novagen, Inc.) as described in detail elsewhere (5). Sera specific to each purified MSA-2 recombinant protein were generated by immunization of mice as previously reported (5). The specificity of each antiserum for MSA-2a, -2b, or -2c was confirmed by immunoblotting against *B. bovis* (Mo7 clone)-infected erythrocytes (23). The anti-MSA-2a$_1$ serum bound only the two proteins of the predicted size for MSA-2a$_1$ and MSA-2a$_2$ in the lysate of *B. bovis*-infected erythrocytes (Fig. 1A, lane 2). The anti-MSA-2b serum reacted with only a single protein corresponding to the size of MSA-2b (Fig. 1A, lane 4), and the anti-MSA-2c serum bound only a single protein of the appropriate size (Fig. 1A, lane 6). Monospecific rabbit antisera were obtained by immunizing two rabbits with 30 μg of purified recombinant MSA-2b or -2c emulsified in saponin followed by three booster immunizations at 2-week intervals with 30 μg of

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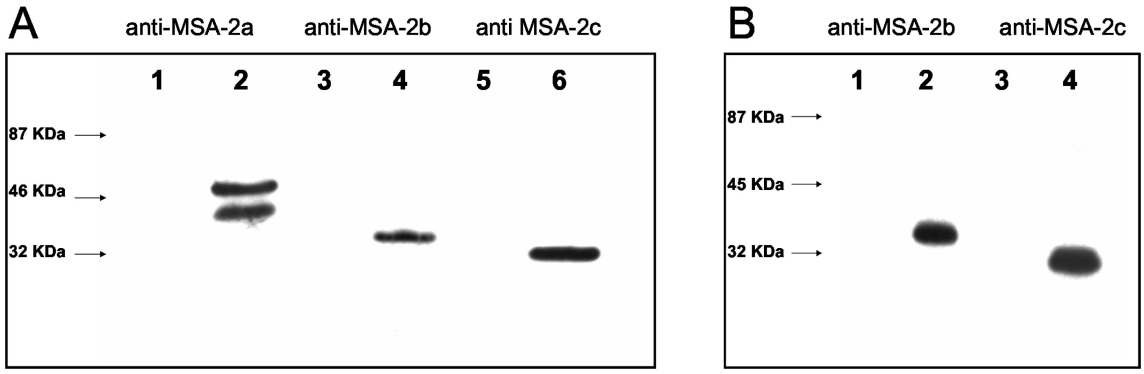


FIG. 1. Specificity of anti-MSA-2 murine and rabbit sera used in expression analysis. Normal, uninfected erythrocytes (lanes 1, 3, and 5) or *B. bovis*-infected erythrocytes (lanes 2, 4, and 6) were electrophoresed on sodium dodecyl sulfate-containing polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with 1:500 dilutions of murine sera specific for MSA-2a, -2b, or -2c (A) or rabbit sera specific for MSA-2b or -2c (B). Molecular size markers are on the left of each panel.

recombinant protein in the same adjuvant. The rabbit anti-MSA-2b serum reacted with only a single protein corresponding to the size of MSA-2b (Fig. 1B, lane 2), and the anti-MSA-2c serum bound only a single protein of the appropriate size (Fig. 1B, lane 4). The clear differences in molecular size of the MSA-2 proteins and the reactivity of each anti-recombinant serum only with the expected protein demonstrated the specificity of the sera and confirm the previous data showing lack of cross-reactivity among MSA-2a, MSA-2b, and MSA-2c (5). None of the murine or rabbit sera bound uninfected erythrocytes (Fig. 1A, lanes 1, 3, and 5, and 1B, lanes 1 and 3).

Merozoites were obtained from in vitro cultures of the Mo7 clone of *B. bovis*. Cultures containing free merozoites were centrifuged twice at 400 × *g* for 10 min at 4°C to pellet erythrocytes and intracellular parasites. The supernatant containing free merozoites was centrifuged at 958 × *g* for 30 min, and the merozoites were resuspended in VYM solution (24). Merozoites (5×10^6) were incubated for 30 min at room temperature with specific antisera at a 1:100 dilution in VYM solution with 0.1% casein. Cells were washed three times with VYM and incubated with a goat anti-mouse immunoglobulin G (IgG) (for MSA-2a) or goat anti-rabbit IgG (for MSA-2b and -2c) conjugated with tetramethyl rhodamine isothiocyanate (Zymed Laboratories, San Francisco, Calif.) at a 1:100 dilution in VYM–0.1% casein. To demonstrate specific labeling of live merozoites with intact surface membranes, 20 μg of 6-carboxyfluorescein diacetate (CFDA)/ml was added to the suspension and incubated for 30 min at room temperature (16). Merozoites were then washed three times with VYM. Resuspended merozoites were examined in wet mounts, and three images were taken with the AxioCam digital camera with phase-contrast or specific band-pass sets of filters for rhodamine and fluorescein. Multichannel images were composed by using AxioVision software version 3 (Carl Zeiss, Thornwood, N.Y.). As a positive control for binding, monoclonal antibody 23/10.36.18 against MSA-1 was used (20). As negative antibody controls, merozoites were incubated with the secondary antibodies alone, a murine antiserum against recombinant His-tagged merozoite surface protein 2 (MSP-2) OpAG3 from *Anaplasma marginale*, or a rabbit antiserum against recombinant MSP-1 from *A. marginale*.

Antibodies directed against MSA-2a, -2b, and -2c bound the surface of live merozoites (Fig. 2A to C). The pattern of expression was similar for each MSA-2 protein, with a multifocal distribution on the membrane of free merozoites or merozoites attached to ghost erythrocytes. Labeling was also observed on erythrocyte membranes with all three MSA-2 antibodies (data not shown). The labeling pattern observed on free merozoites and erythrocytes suggests shedding of the antigens from the surface membrane. Shedding of *B. bovis* merozoite surface proteins has been previously reported, and analysis of shed proteins in the exoantigen fraction of cultured merozoites includes MSA-$2a_1$ (14). Positive merozoite surface labeling was observed with the control anti-MSA-1 monoclonal antibody (Fig. 2D) but not when merozoites were incubated with the unrelated anti-*A. marginale* antibodies (Fig. 2E) or the secondary antibodies alone (data not shown).

Next, to examine if MSA-2 proteins were coexpressed on the same live merozoite, a triple-color immunofluorescence assay was performed. For this technique, purified merozoites were incubated with one of the following three combinations of antisera: murine anti-MSA-2a plus rabbit anti-MSA-2b, murine anti-MSA-2a plus rabbit anti-MSA-2c, and murine anti-MSA-2c plus rabbit anti-MSA-2b. The bound primary antibodies were then labeled with two secondary antibodies, a goat anti-mouse IgG conjugated with rhodamine (Santa Cruz Biotechnology, Santa Cruz, Calif.) and a goat anti-rabbit IgG conjugated with Alexa Fluor 350 (Molecular Probes, Eugene, Oreg.). CFDA was added to the merozoite suspensions, and the assay was performed as described above. Wet mounts were analyzed, and four images were taken with phase contrast or sets of filters for fluorescein, rhodamine, and Alexa Fluor 350. As negative controls, merozoites were incubated with the two secondary antibodies only or with the mouse antiserum against recombinant MSP-2 OpAG3 plus the rabbit antiserum against recombinant MSP-1, both proteins from *A. marginale*. Multichannel images with individual filters for rhodamine and Alexa Fluor 350 as well as the combined images showed positive labeling with all three combinations of antisera: MSA-2a plus MSA-2b (Fig. 3A to C), MSA-2a plus MSA-2c (Fig. 3E to G), and MSA-2b plus MSA-2c (Fig. 3I to K). Antibodies to at least two MSA-2 proteins bound a single merozoite, indicating that

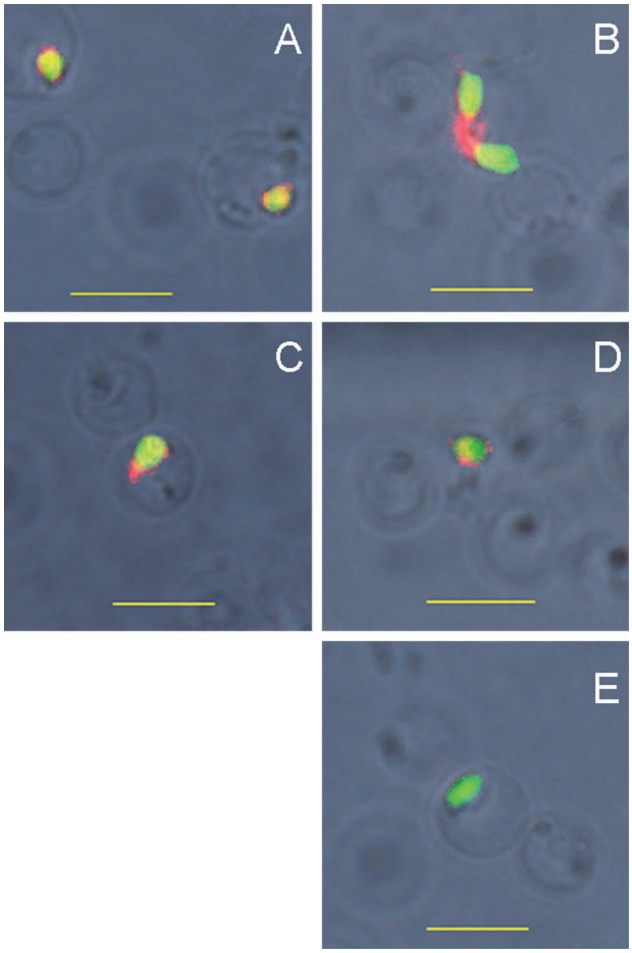


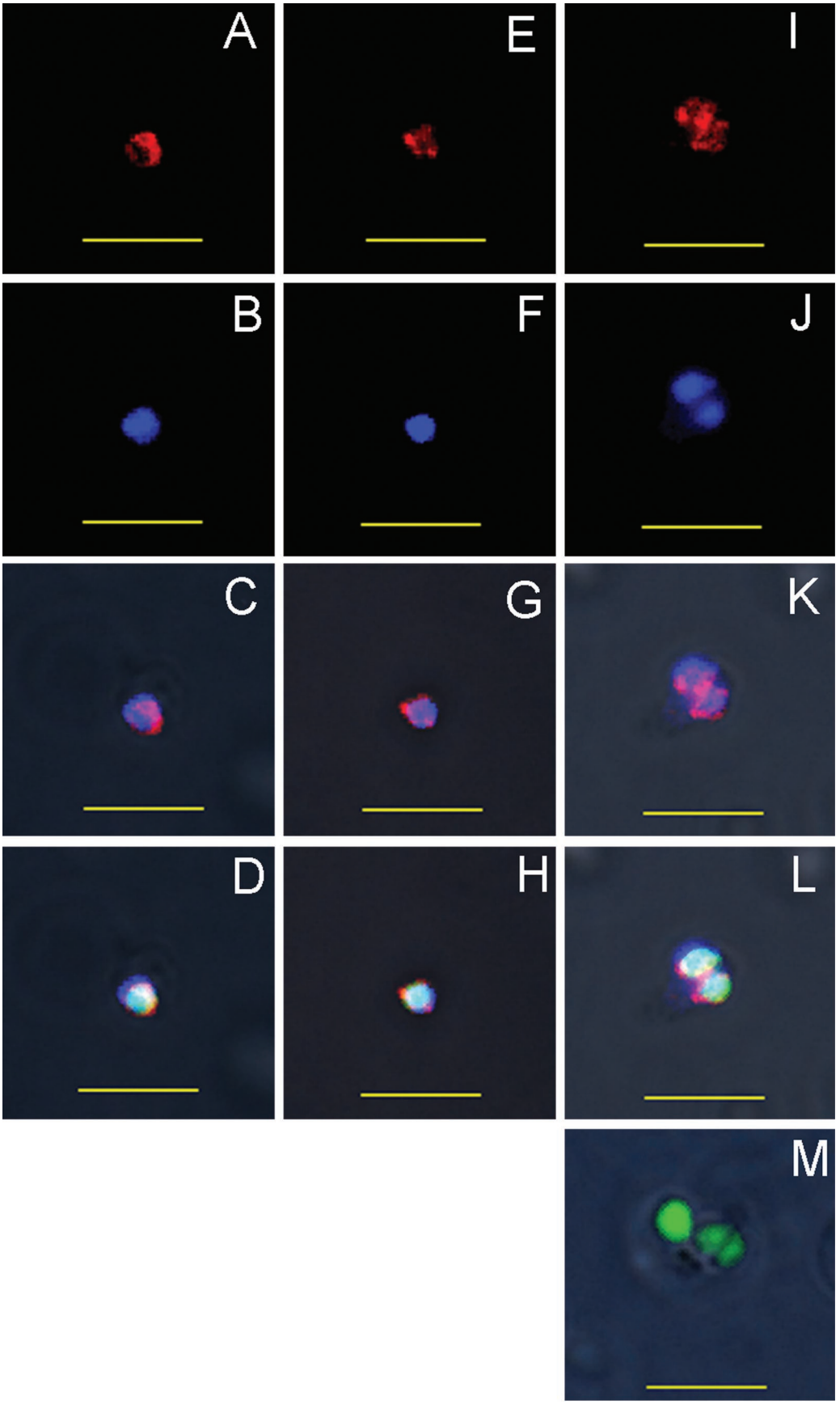
FIG. 2. Expression of MSA-2 proteins on the surface of live *B. bovis* merozoites. Merozoites were incubated with antisera for MSA-2a (A), -2b (B), or 2c (C) and then with a secondary antibody labeled with tetramethyl rhodamine isothiocyanate, which emits a red fluorescence. Merozoite viability was determined with CFDA, which emits a green fluorescence on live cells. Labeling was observed with the positive-control monoclonal antibody 23/10.36.18 against MSA-1 (D) but not with antisera against *A. marginale* (E). Bars = 5 μm.

there is coexpression of MSA-2 proteins in free merozoites and that there is not steric interference between any combinations of two specific anti-MSA-2 antibodies. Since the MSA-2a antiserum binds both MSA-2a$_1$ and -2a$_2$ (Fig. 1A), whether the antigens detected by the MSA-2a antiserum on the surface of merozoites were either MSA-2a$_1$, -2a$_2$, or both was not determined. Examination using immunoblots has shown that MSA-2a$_1$ and -2a$_2$ are detected at equivalent levels in merozoite protein extracts (5), suggesting that the two antigens are similarly expressed. Viability was confirmed by visualizing the merozoites with an additional set of fluorescein filters (Fig. 3D, H, and L). No labeling was observed with the secondary antibodies alone (data not shown) or the negative-control *A. marginale* antisera (Fig. 3M).

The detection of MSA-2b and MSA-2c expression on the surface of live merozoites, in addition to previously demonstrated MSA-2a expression (13, 20), and the confirmation of coexpression on individual merozoites led us to test whether antibody against MSA-2 proteins could block merozoite invasion of erythrocytes. A merozoite neutralization assay was performed according to a protocol previously established (10). Specific anti-MSA-2 sera were obtained following the immunization of two calves each with 50 μg of the individual recombinant proteins emulsified in saponin. The calves were boosted three times at 2-week intervals, and the specificity of the antibodies was tested by immunoblotting and immunofluorescence. Each bovine serum reacted with *B. bovis* merozoites as examined by immunofluorescence (data not shown) and specifically bound the recombinant expressed MSA-2 protein in immunoblots. The sera from the two MSA-2a$_1$-immunized calves, B65 and B66, bound only the two proteins of the predicted size for MSA-2a$_1$ and MSA-2a$_2$ in the lysate of *B. bovis*-infected erythrocytes (Fig. 4A, lanes 2 and 4). The sera from the two MSA-2b-immunized calves, B63 and B68, reacted with only a single protein corresponding to the size of MSA-2b (Fig. 4B, lanes 2 and 4), and the sera from calves immunized with recombinant MSA-2c, B62 and B69, bound only a single protein of the appropriate size (Fig. 4C, lanes 2 and 4). As controls, antisera were obtained by immunizing calves with recombinant MSA-1 or ovalbumin by using the same adjuvant and schedule as those for the MSA-2 antisera (9, 17). Each antiserum was heat inactivated, diluted 1:5 in complete M199 medium, and incubated with 10^6 live merozoites for 30 min at 4°C. Antibody-exposed merozoites were then added to an equal volume of 1.5% bovine erythrocytes in complete medium, and the cultures were incubated in 96-well plates at 37°C in a 5% CO_2 atmosphere. Microscopic examination of Giemsa-stained smears prepared from each well at 5 and 48 h was used to determine the number of infected erythrocytes from a total of 2,000 cells. The results were analyzed by one-way analysis of variance and Fisher's pairwise comparisons by using the Minitab13 computer program (Minitab Inc., State College, Pa.).

Results for merozoites incubated for 5 h with each of the bovine antisera specific for MSA-2a, MSA-2b, and MSA-2c showed a significant reduction in the number of infected erythrocytes compared to those for merozoites incubated with medium alone or with the negative-control antiserum against ovalbumin (Fig. 5). There were no significant differences between sera from individual animals immunized with the same MSA-2 antigen. The percentage of inhibition of erythrocyte

FIG. 3. Coexpression of MSA-2 proteins on the surface of live *B. bovis* merozoites. Merozoites were incubated with mouse anti-MSA-2a plus rabbit anti-MSA-2b (A to D), mouse anti-MSA-2a plus rabbit anti-MSA-2c (E to H), or mouse anti-MSA-2c plus rabbit anti-MSA-2b (I to L), and then with a rhodamine-labeled anti-murine IgG antibody (red fluorescence) and an Alexa Fluor 350-labeled anti-rabbit IgG antibody (blue fluorescence). Merozoite viability was determined with CFDA, which emits a green fluorescence on live cells. Images are phase contrast and the following channels: individual channel for rhodamine (A, E, and I), individual channel for Alexa Fluor 350 (B, F, and J); combined channels for rhodamine and Alexa Fluor 350 (C, G, and K); and combined channels for rhodamine, Alexa Fluor 350, and fluorescein (D, H, L, and M). Labeling was not observed when merozoites were incubated with a combination of a mouse antiserum against *A. marginale* recombinant MSP-2 OpAG3 plus a rabbit antiserum against MSP-1 (M). Bars = 5 μm.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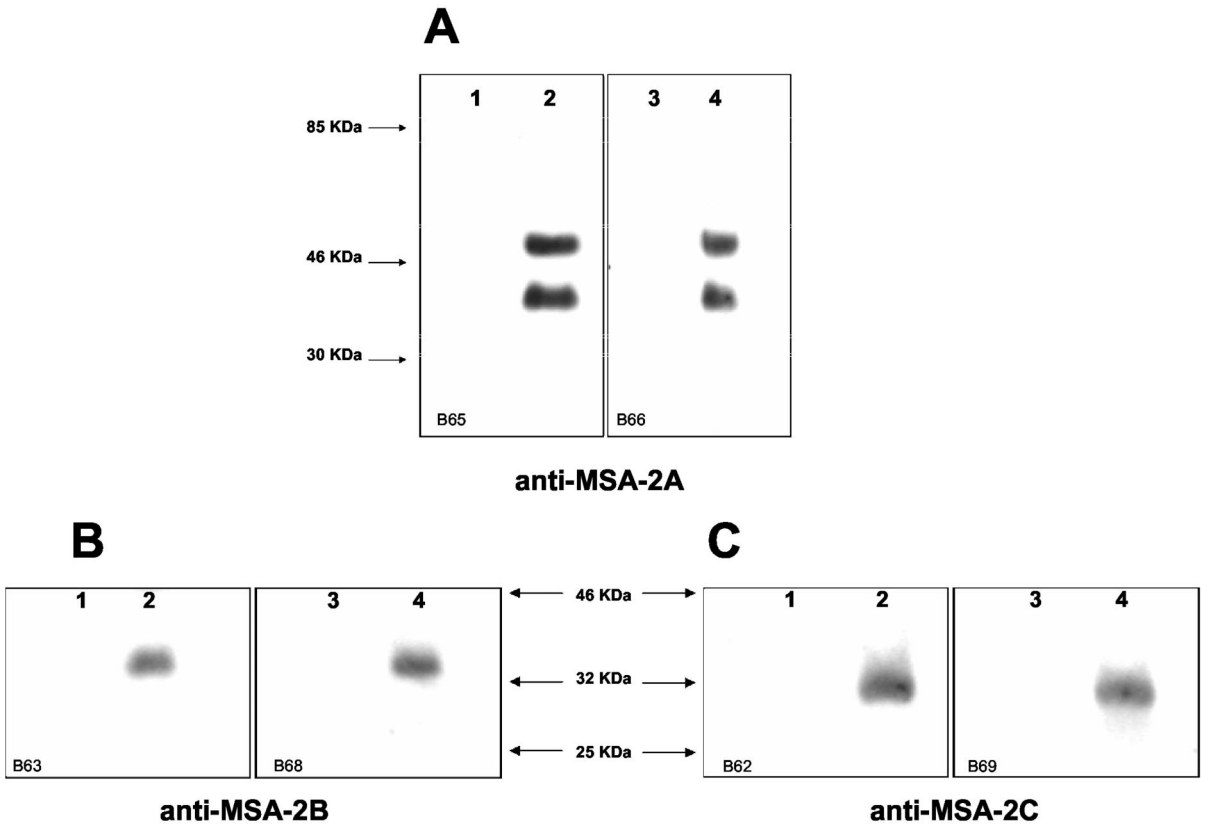

FIG. 4. Specificity of anti-MSA-2 bovine sera used for neutralization of merozoites and sporozoites. Normal, uninfected erythrocytes (lanes 1 and 3) or *B. bovis*-infected erythrocytes (lanes 2 and 4) were electrophoresed on sodium dodecyl sulfate-containing polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with a 1:500 dilution of sera from calves immunized with recombinant MSA-2a (A), -2b (B), or -2c (C). Molecular size markers are indicated by arrows.

invasion with the positive-control MSA-1 antiserum was 75%, which is similar to that previously reported (71%) (10). The percentages of inhibition for antiserum against each MSA-2 protein were as follows: for MSA-2a, 60 and 47%; for MSA-2b, 51 and 38%; and for MSA-2c, 34 and 42%. Although all six antisera against MSA-2 proteins inhibited erythrocyte invasion, compared to the negative-control antiserum, only one of the antisera (against MSA-2a) blocked invasion at a level comparable to that of the MSA-1 antiserum control. These results show that antibodies against each of the MSA-2 antigens inhibit early erythrocyte invasion and support a role for MSA-2 antibodies in immunity against the merozoite stage. In all the published experiments using monospecific antisera, it has not been possible to achieve 100% inhibition of merozoite invasion (9, 10, 22). To determine if the combination of antibodies against all the known VMSA proteins would effect complete inhibition, merozoites were cultured with an equal volume (5 μl) of each of the sera against MSA-1, MSA-2a, MSA-2b, and MSA-2c, the combination of which resulted in a final serum dilution of 1:10. At 5 h, the combined antibodies reduced the number of infected erythrocytes to a level comparable to that of the anti-MSA-1 control antiserum (73% inhibition) (Fig. 5). However, the combination did not completely block the invasion of erythrocytes. This lack of complete inhibition by the pooled antibodies does not appear to reflect steric hindrance among the antibodies, as the antisera to MSA-2a, MSA-2b, or MSA-2c do not interfere with the binding of MSA-1 antibodies (data not shown) or the other MSA-2 antibodies (Fig. 3). At this point we cannot conclude whether there are alternative ligands besides MSA-1 or MSA-2 that are involved in erythrocyte binding and invasion, or whether the reduced concentration of each antiserum in the mixture was insufficient to effectively bind all the ligands on the merozoite surface. At 48 h the MSA-2 antisera still induced a significant inhibition of the number of infected erythrocytes compared with the control groups. The percentages of inhibition for each MSA-2 antiserum were as follows: for MSA-2a, 46 and 38%; for MSA-2b, 38 and 34%; and for MSA-2c, 26 and 25%. The control anti-MSA-1 serum induced 52% inhibition, identical to that achieved by the combined antisera.

Tick-derived *Babesia* sp. sporozoites invade the same target cell, the erythrocyte, as do the merozoites. It has been hypothesized that merozoites and sporozoites may have very similar surface coats and use common molecules to attach to and invade erythrocytes (18). Having demonstrated that MSA-2 antigens are expressed on the surface of live merozoites and that specific antibodies inhibit erythrocyte invasion, we tested sporozoites for MSA-2 protein expression during initial erythrocyte attachment by an immunocytochemistry protocol. Sporozoites were obtained from infected *Boophilus microplus* ticks by a previously described protocol (18). Live sporozoites were cultured with erythrocytes in complete medium for 5 h to

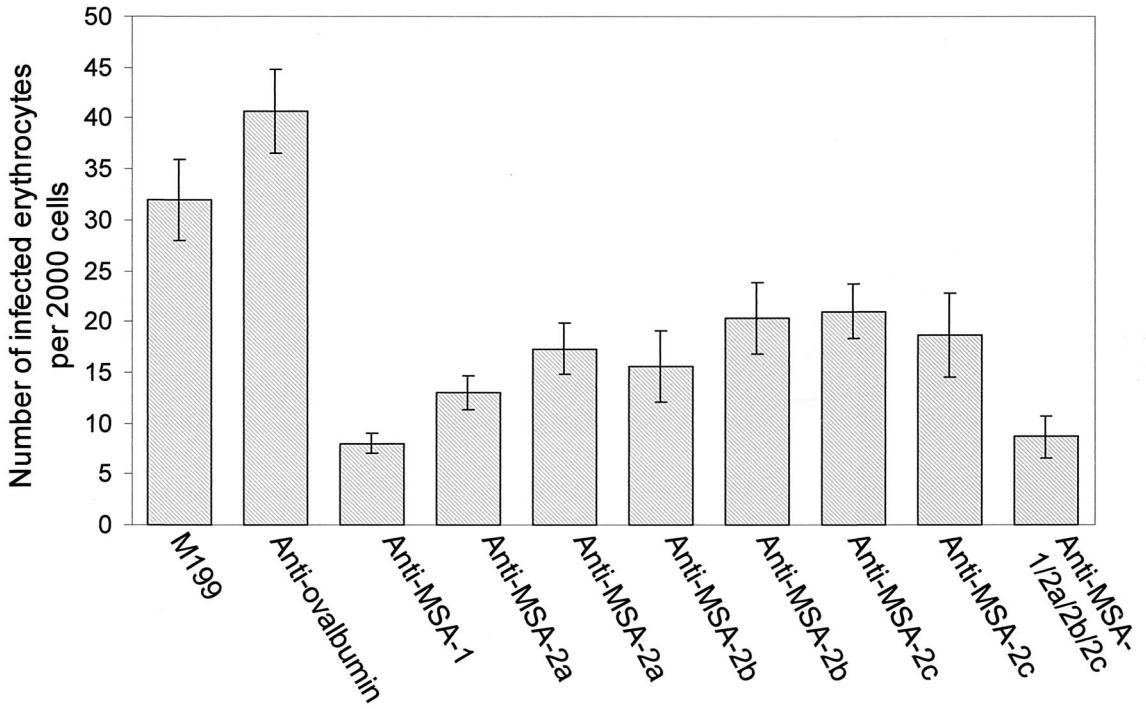

FIG. 5. Antibody-mediated inhibition of merozoite invasion. Shown are the total numbers of infected erythrocytes from 2,000 cells counted at 5 h after merozoite addition. Merozoites were incubated with M199 medium alone; with negative-control bovine antiserum against ovalbumin; with positive-control bovine antiserum against MSA-1; with two different antisera against MSA-2a, -2b, and -2c; or with the combined antisera (MSA-1, -2a, -2b, and -2c). Error bars indicate standard deviations of the results from triplicate cultures.

allow binding to erythrocytes prior to development into merozoites, which occurs at 8 h postinvasion (15). After this period, culture samples were taken, and smears were made on Probe-On slides (Fisher, Santa Clara, Calif.). The protocol for immunocytochemistry has been previously described (18). Briefly, smears were air dried for 2 h, fixed in methanol for 5 min, and rinsed in 125 mM Tris buffer containing 0.5% Triton X-100. After a 10-min blocking step using 5% goat serum at 37°C, the slides were incubated for 15 min at 37°C with a 1:100 dilution of murine monospecific antiserum to MSA-2a, -2b, or -2c. A goat anti-murine IgG biotin-labeled immunoglobulin (USA-HRP detection system; Signet Laboratories, Dedham, Mass.) was incubated for 10 min, and this was followed by the addition of streptavidin-horseradish peroxidase complex and incubation for 10 min at 37°C. The AEC chromogen (DAKO, Carpinteria, Calif.) was used to develop the reaction followed by filtered Mayer's hematoxylin as a counterstain. As a positive control, the monoclonal antibody 23/10.36.18 against MSA-1 was used. The murine antiserum against recombinant MSP-2 OpAG3 from *A. marginale* was used as a negative-control antibody. As a negative control for binding of tick cells, extracts from uninfected *B. microplus* larvae cultured in vitro with erythrocytes were used.

Specific MSA-2a, -2b, and -2c antisera recognized sporozoites bound to erythrocytes (Fig. 6A to C), and early intraerythrocytic stages (data not shown). Bound sporozoites were also recognized by the positive-control monoclonal antibody 23/10.36.18 against MSA-1 (Fig. 6D) but not by the negative anti-*A. marginale* antiserum (Fig. 6E). None of the MSA-2 antisera bound to erythrocytes cultured with uninfected larval extracts (Fig. 6F). The detection of MSA-2a, -2b, and -2c molecules in sporozoites attached to erythrocytes confirms that these proteins are also expressed in sporozoites. Similarly to MSA-1, MSA-2 expression by sporozoites and merozoites as well as inhibition of merozoite invasion by specific antisera suggested that sporozoite attachment to erythrocytes could be blocked by specific MSA-2 antibodies. To test this hypothesis, a sporozoite blocking assay was performed. One million live sporozoites were first incubated with a 1:5 dilution of a bovine antiserum specific for MSA-2a, -2b, or -2c for 30 min and then cultured with erythrocytes in vitro for 5 h. The number of sporozoites attached to the erythrocyte surface was recorded from 2,000 cells counted. Significantly fewer attached sporozoites were observed following incubation with antisera against MSA-2a, -2b, or -2c compared with sporozoites incubated with medium alone or the negative-control antiovalbumin serum (Fig. 7). There were no significant differences in the inhibition level between the two antisera for each MSA-2 protein. The percentages of inhibition of sporozoite binding to erythrocytes for each MSA-2 antiserum were as follows: for MSA-2a, 71 and 69%; for MSA-2b, 71 and 60%; and for MSA-2c, 71 and 69%. The positive-control antiserum against MSA-1 resulted in 77% inhibition, similar to that previously reported (68%) (18). Only one of the MSA-2 antisera (against MSA-2b) was significantly different from the positive-control antiserum against MSA-1.

Together, these results demonstrate the expression of MSA-2 proteins on the surface of both merozoites and sporozoites and show that specific antibodies block initial binding and subsequent erythrocyte invasion. To date, the surface coat

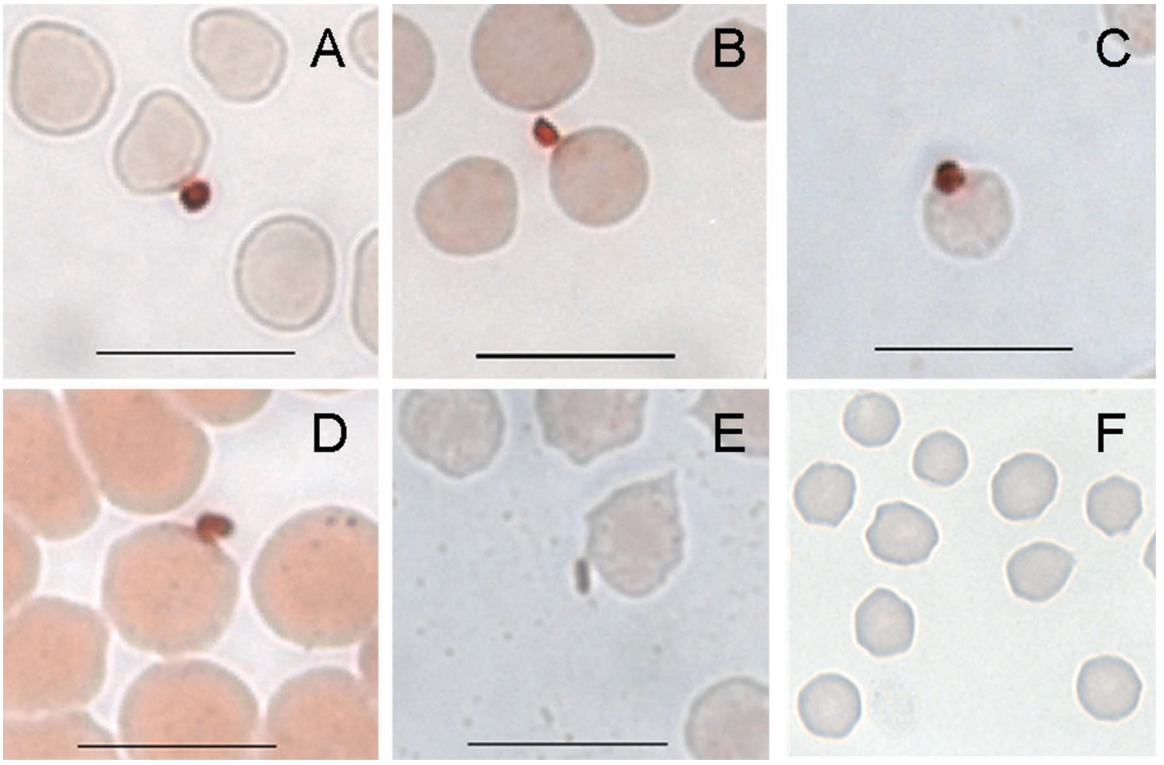

FIG. 6. Expression of MSA-2 proteins by sporozoites at the time of attachment to erythrocytes. Smears of erythrocyte cultures initiated with sporozoites were incubated with specific mouse antiserum against MSA-2a (A), MSA-2b (B), MSA-2c (C), monoclonal antibody 23/10.36.18 against MSA-1 (D), or negative-control mouse antiserum against *A. marginale* MSP-2 OpAG3 (E). Erythrocyte cultures treated identically but with the use of extracts from uninfected larvae were incubated with specific mouse antiserum against each MSA-2 protein; anti-MSA-2a is shown in panel F (magnification, ×63,000). The reaction was visualized with AEC, which results in brown staining. Bars = 10 μm.

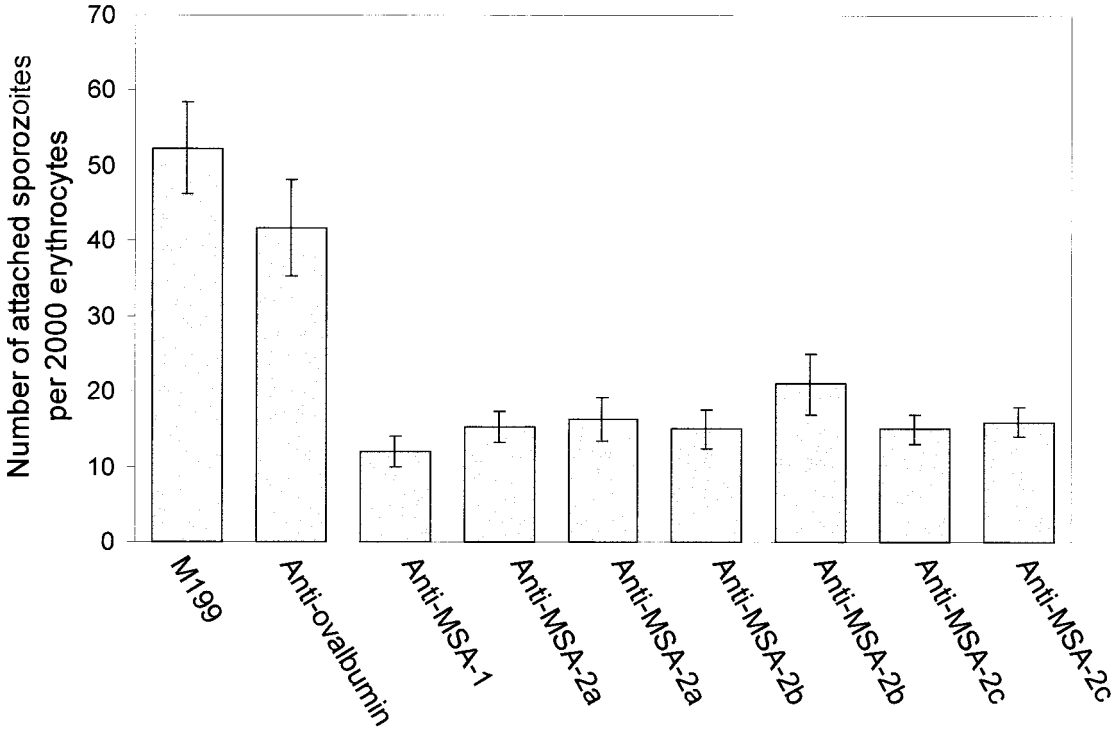

FIG. 7. Antibody-mediated inhibition of sporozoite attachment to erythrocytes. The total number of parasites attached to 2,000 erythrocytes was counted at 5 h after sporozoite addition. Sporozoites were incubated with M199 medium alone; with negative-control bovine antiserum against ovalbumin; with positive-control bovine antiserum against MSA-1; or with two different antisera against MSA-2a, -2b, or -2c. Error bars indicate standard deviations of the results from triplicate cultures.

and rhoptry antigens initially described for *B. bovis* merozoites also have been found in sporozoites, including MSA-1, MSA-2, and rhoptry-associated protein 1 (18). This is consistent with the sporozoite and merozoite having similar surface coats and a common mechanism for erythrocyte binding. The ability of both MSA-1 and MSA-2 antigens to elicit invasion-blocking antibodies underscores the importance of the VMSA proteins in erythrocyte binding and invasion. Ideally, a *Babesia* vaccine should elicit an immune response against sporozoites and merozoites (1). By targeting antigens present in both stages, it may be possible to prevent initial sporozoite invasion and the subsequent early cycles of merozoite multiplication.

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REFERENCES

1. **Brown, W. C., and G. H. Palmer.** 1999. Designing blood-stage vaccines against *Babesia bovis* and *B. bigemina*. Parasitol. Today **15:**275–281.
2. **Chitnis, C. E., and M. J. Blackman.** 2000. Host cell invasion by malaria parasites. Parasitol. Today **16:**411–415.
3. **Cowman, A. F., D. L. Baldi, M. Duraisingh, J. Healer, K. E. Mills, R. A. O'Donnell, J. Thompson, T. Triglia, M. E. Wickham, and B. S. Crabb.** 2002. Functional analysis of *Plasmodium falciparum* merozoite antigens: implications for erythrocyte invasion and vaccine development. Philos. Trans. R. Soc. Lond. B Biol. Sci. **357:**25–33.
4. **Egan, A. F., P. Burghaus, P. Druilhe, A. A. Holder, and E. M. Riley.** 1999. Human antibodies to the 19kDa C-terminal fragment of *Plasmodium falciparum* merozoite surface protein 1 inhibit parasite growth in vitro. Parasite Immunol. **21:**133–139.
5. **Florin-Christensen, M., C. E. Suarez, S. A. Hines, G. H. Palmer, W. C. Brown, and T. F. McElwain.** 2002. The *Babesia bovis* merozoite surface antigen 2 locus contains four tandemly arranged and expressed genes encoding immunologically distinct proteins. Infect. Immun. **70:**3566–3575.
6. **Fox, B. A., P. Xing-Li, K. Suzue, T. Horii, and D. J. Bzik.** 1997. *Plasmodium falciparum*: an epitope within a highly conserved region of the 47-kDa amino-terminal domain of the serine repeat antigen is a target of parasite-inhibitory antibodies. Exp. Parasitol. **85:**121–134.
7. **Goff, W. L., W. C. Davis, G. H. Palmer, T. F. McElwain, W. C. Johnson, J. F. Bailey, and T. C. McGuire.** 1988. Identification of *Babesia bovis* merozoite surface antigens by using immune bovine sera and monoclonal antibodies. Infect. Immun. **56:**2363–2368.
8. **Hines, S. A., T. F. McElwain, G. M. Buening, and G. H. Palmer.** 1989. Molecular characterization of *Babesia bovis* merozoite surface proteins bearing epitopes immunodominant in protected cattle. Mol. Biochem. Parasitol. **37:**1–9.
9. **Hines, S. A., G. H. Palmer, D. P. Jasmer, W. L. Goff, and T. F. McElwain.** 1995. Immunization of cattle with recombinant *Babesia bovis* merozoite surface antigen-1. Infect. Immun. **63:**349–352.
10. **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.
11. **Hoyte, H. M. D.** 1961. Initial development of infections with *Babesia bigemina*. J. Protozool. **8:**462–466.
12. **Hoyte, H. M. D.** 1965. Further observations on the initial development of infections with *Babesia bigemina*. J. Protozool. **12:**83–85.
13. **Jasmer, D. P., D. W. Reduker, S. A. Hines, L. E. Perryman, and T. C. McGuire.** 1992. Surface epitope localization and gene structure of a *Babesia bovis* 44-kilodalton variable merozoite surface antigen. Mol. Biochem. Parasitol. **55:**75–83.
14. **Johnson, W. C., L. E. Perryman, and W. L. Goff.** 1997. *Babesia bovis*: identification of immunodominant merozoite surface proteins in soluble culture-derived exoantigen. Parasitol. Res. **83:**776–780.
15. **Mahoney, D. F., I. G. Wright, and P. J. Ketterer.** 1973. *Babesia argentina*: the infectivity and immunogenicity of irradiated blood parasites for splenectomized calves. Int. J. Parasitol. **3:**209–217.
16. **McElwain, T. F., L. E. Perryman, W. C. Davis, and T. C. McGuire.** 1987. Antibodies define multiple proteins with epitopes exposed on the surface of live *Babesia bigemina* merozoites. J. Immunol. **138:**2298–2304.
17. **McElwain, T. F., L. E. Perryman, A. J. Musoke, and T. C. McGuire.** 1991. Molecular characterization and immunogenicity of neutralization-sensitive *Babesia bigemina* merozoite surface proteins. Mol. Biochem. Parasitol. **47:**213–222.
18. **Mosqueda, J., T. F. McElwain, D. Stiller, and G. H. Palmer.** 2002. *Babesia bovis* merozoite surface antigen 1 and rhoptry-associated protein 1 are expressed in sporozoites, and specific antibodies inhibit sporozoite attachment to erythrocytes. Infect. Immun. **70:**1599–1603.
19. **Palmer, G. H., T. F. McElwain, L. E. Perryman, W. C. Davis, D. R. Reduker, D. P. Jasmer, V. Shkap, E. Pipano, W. L. Goff, and T. C. McGuire.** 1991. Strain variation of *Babesia bovis* merozoite surface-exposed epitopes. Infect. Immun. **59:**3340–3342.
20. **Reduker, D. W., D. P. Jasmer, W. L. Goff, L. E. Perryman, W. C. Davis, and T. C. McGuire.** 1989. A recombinant surface protein of *Babesia bovis* elicits bovine antibodies that react with live merozoites. Mol. Biochem. Parasitol. **35:**239–247.
21. **Siddique, A. B., N. Ahlborg, B. Wahlin Flyg, P. Perlmann, and K. Berzins.** 1998. Antibodies to sequences in a non-repeat region of *Plasmodium falciparum* antigen Pf155/RESA inhibit either cytoadherence or parasite growth in vitro. Parasitology **117:**209–216.
22. **Suarez, C. E., M. Florin-Christensen, S. A. Hines, G. H. Palmer, W. C. Brown, and T. F. McElwain.** 2000. Characterization of allelic variation in the *Babesia bovis* merozoite surface antigen 1 (MSA-1) locus and identification of a cross-reactive inhibition-sensitive MSA-1 epitope. Infect. Immun. **68:**6865–6870.
23. **Suarez, C. E., T. F. McElwain, I. Echaide, S. Torioni de Echaide, and G. H. Palmer.** 1994. Interstrain conservation of babesial RAP-1 surface-exposed B-cell epitopes despite *rap-1* genomic polymorphism. Infect. Immun. **62:**3576–3579.
24. **Vega, C. A., G. M. Buening, T. J. Green, and C. A. Carson.** 1985. In vitro cultivation of *Babesia bigemina*. Am. J. Vet. Res. **46:**416–420.

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