Conformational Dependence and Conservation of an Immunodominant Epitope within the Babesia equi Erythrocyte-Stage Surface Protein Equi Merozoite Antigen 1

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Equi merozoite antigen 1 (EMA-1) is an immunodominant Babesia equi erythrocyte-stage surface protein. A competitive enzyme-linked immunosorbent assay (ELISA), based on inhibition of monoclonal antibody (MAb) 36/133.97 binding to recombinant EMA-1 by equine anti-B. equi antibodies, detects horses infected with strains present throughout the world. The objectives of this study were to define the epitope bound by MAb 36/133.97 and quantify the amino acid conservation of EMA-1, including the region containing the epitope bound by MAb 36/133.97. The alignment of the deduced amino acid sequence of full-length EMA-1 (Florida isolate) with 15 EMA-1 sequences from geographically distinct isolates showed 82.8 to 99.6% identities (median, 98.5%) and 90.5 to 99.6% similarities (median, 98.9%) between sequences. Full-length and truncated recombinant EMA-1 proteins were expressed and tested for their reactivities with MAb 36/133.97. Binding required the presence of amino acids on both N- and C-terminal regions of a truncated peptide (EM1-L2) containing amino acids 1 to 98 of EMA-1. This result indicated that the epitope defined by MAb 36/133.97 is dependent on conformation. Sera from persistently infected horses inhibited the binding of MAb 36/133.97 to EMA-1.2 in a competitive ELISA, indicating that equine antibodies which inhibit binding of MAb 36/133.97 also recognize epitopes in the same region (the first 98 residues). Within this region, the deduced amino acid sequences had 85.7 to 100% identities (median, 99.0%), with similarities of 94.9 to 100% (median, 100%). Therefore, the region which binds to both MAb 36/133.97 and inhibiting equine antibodies has a median amino acid identity of 99.0% and a similarity of 100%. These data provide a molecular basis for the use of both EMA-1 and MAb 36/133.97 for the detection of antibodies against B. equi.

Babesia equi is a tick-borne parasite of the phylum Apicomplexa that infects erythrocytes and lymphocytes (17, 20). Following infection, the clinical syndrome is characterized by fever and hemolytic anemia. Acute disease usually resolves and leads to persistent, life-long infection (20). The parasite is distributed worldwide, and its prevalence is directly related to the distribution of ticks capable of transmission (4). The principal significance of B. equi is its impact on the international movement of horses. Several countries, including the United States, restrict the entrance of horses that are serologically positive for Babesia spp. (5).

Since 1969, the complement fixation test (CFT) has been used as the official assay for the detection of horses infected with B. equi or B. caballi (2, 3). However, several limitations of CFT, including low sensitivity, have been described, and a number of different diagnostic methods have been proposed to improve the detection of carrier horses (1, 15, 22–24). A competitive enzyme-linked immunosorbent assay (cELISA), based on inhibition of monoclonal antibody (MAb) 36/133.97 binding to equi merozoite antigen 1 (EMA-1) by equine anti-B. equi antibodies, has been developed (13). This assay has shown improved performance in the detection of anti-B. equi antibodies compared to those of CFT (10, 15) and immunofluorescence assays (18, 21). The performance of the cELISA depends on the immunodominance, structure, and conservation of the epitope recognized by both MAb 36/133.97 and equine antibodies against EMA-1. EMA-1 is a surface-exposed, immunodominant protein expressed during the B. equi erythrocyte stage (9, 12). MAb 36/133.97 is a mouse immunoglobulin G1 that binds to EMA-1 (14). Binding of MAb 36/133.97 to EMA-1 is inhibited by sera of horses from all 19 countries tested, which suggests conservation of the epitope defined by MAb 36/133.97 (13, 14).

EMA-1 is an orthologue of Theileria major merozoite and piroplasm surface antigens (mMPSA) (12). In Theileria spp., both variable and conserved regions within mMPSA have been reported (6, 11). Because variation in EMA-1 may affect its use in a diagnostic test, in this study we quantified the variations in EMA-1 and characterized the epitope defined by MAb 36/133.97. The results demonstrated that the epitope reactive with MAb 36/133.97 is located within the first 98 residues of EMA-1.
and is dependent on conformation. Sequence comparison revealed that the region where the MAB 36/133.97 binds is more conserved than the overall protein among *B. equi* isolates. This study provides a molecular basis for the use of EMA-1 and MAB 36/133.97 in a cELISA for detection of anti-*B. equi* antibodies.

**MATERIALS AND METHODS**

Database searching and sequence analysis. Databases were searched with the BLAST program through the National Center for Biotechnology Information. EMA-1 sequences from 16 isolates of *B. equi* recovered worldwide and published in GenBank were aligned by using the AlignX program from the Vector NTI Suite (Informax, Inc., Bethesda, Md.). The degree of homology was calculated by comparing each individual amino acid sequence with that deduced from the sequence of the Florida isolate (GenBank accession number A6A2370).

Synthetic peptide and dot immunoblotting. The peptide ASGAAYVFDQELSI representing the EMA-1 sequence from amino acids 27 to 39, which was previously described by others as containing the epitope described by others as containing the epitope defined by MAB 36/133.97 (7), was synthesized and tested for recognition by MAB 36/133.97. The peptide was synthesized with an ABI model 431A synthesizer by using 9-fluorenlymethoxy carbonyl chemistry (Laboratory for Biotechnology and Bioanalysis I, Washington State University, Pullman). Lyophilized peptide was dissolved in water and tested for MAB 36/133.97 binding in a nitrocellulose dot blot assay. The dot immunoblotting procedures were adapted from standard protocols (19). Briefly, nitrocellulose (Pierce, Rockford, Ill.) was spotted with the peptide (from 10⁻² to 10⁻⁴ μg/well) in a filtration manifold (Easy-Titer; Pierce). Affinity-purified native EMA-1 (10⁻⁵ to 10⁻⁷ μg/well) (9) was used as a positive control, and an irrelevant peptide (10⁻⁵ to 10⁻⁴ μg/well) consisting of residues 146 to 159 of a bovine prion protein (8) was used as a negative control. After the membrane was blocked with 5% bovine serum albumin and then 5% milk, the membrane was incubated with MAB 36/133.97 (5 μg/ml) and antibody binding was detected with horseradish peroxidase-labeled donkey anti-murine immunoglobulin G (Jackson Immunoresearch Laboratories, Inc., West Grove, Pa.) followed by enhanced chemiluminescence (NEL Life Science Products, Boston, Mass.). To ensure that the test peptides bound to the nitrocellulose membrane, a duplicate filter was stained with ponceau S (Sigma Chemical Co., St. Louis, Mo.). The duplicate membrane was stained with 0.1% ponceau S in 1% acetic acid for 30 s, briefly destained with water, and visualized for the presence of peptides.

The ability of the synthetic peptide to inhibit the binding of MAB 36/133.97 to native EMA-1 was also evaluated by dot immunoblotting. In this experiment, affinity-purified native EMA-1 (0.05 μg/well) was used as the antigen and MAB 36/133.97 (5 μg/ml) was adsorbed with the synthetic peptide, with 50 to 0.3 μg per reaction. MAB 36/133.97 was also adsorbed with native EMA-1 (from 0.25 to 0.003 μg per reaction) and the prion peptide (from 50 to 0.3 μg per reaction) as positive and negative controls, respectively.

Cloning and expression of recombinant full-length and truncated EMA-1. Full-length and truncated *ema*-1 genes were cloned and expressed in *Escherichia coli* and used to identify the EMA-1 region bound by MAB 36/133.97. To generate truncated EMA-1 proteins, original plasmid pEMA-1 (13), which contains the full-length *ema*-1 gene (Florida isolate), was used as a template in a PCR with site-specific forward and reverse primers (Table 1). The following fragments were generated and ligated to the pBluescript SKM (Stratagene, La Jolla, Calif.) plasmid vector: *ema*-1.2, a 394-bp fragment representing residues 1 to 98 from EMA-1; *ema*-1.3, a 331-bp fragment representing residues 1 to 78; and *ema*-1.5, a 197-bp fragment representing amino acid residues 38 to 98. For cloning, *E. coli* DH5α was transformed with the plasmids containing the full-length and truncated EMA-1 genes, and the recombinant clones were selected on plates containing ampicillin (19). The nucleotide sequence of each plasmid was checked by double-strand sequencing by primer extension with BigDye terminator chemistry on an ABI Prism 377 XL-96 instrument (PE Applied Biosystems, Foster City, Calif.). The expression of the EMA-1 proteins in all constructs was checked by Western blotting with recombinant *E. coli* lysates and polyclonal anti-EMA-1 equine antibodies, as described below.

**Antigen preparations.** *E. coli* clones containing full-length or truncated *ema*-1 recombinant plasmids were processed for use as antigens in Western blotting assays and cELISA. To obtain recombinant EMA-1 lysates, transformed bacteria were cultured for 16 h at 37°C and 225 rpm in 100 ml of Luria-Bertani medium (1% tryptone, 0.5% yeast extract, 1% NaCl) containing ampicillin (100 μg/ml). Then, 2 mM isopropyl-β-D-thiogalactopyranoside was added to the cultures, and the cultures were incubated for an additional 3 h to induce protein expression. The cells were harvested by centrifugation at 4,000 × *g* for 10 min and lysed by resuspension in 50 mM Tris (pH 8.0) containing 2% sodium dodecyl sulfate (SDS) and sonication (two cycles of 10 s at 100 W). The lysates were centrifuged at 12,000 × *g* for 10 min at 4°C, and the supernatant containing the recombinant expressed proteins was collected and stored at 4°C. For some cELISA experiments, the SDS was used to disrupt the *E. coli* was replaced by NP-40 at a final concentration of 1%.

**Horse sera.** Reference positive and negative sera, previously defined by cELISA (13), were used in the present study. Negative sera were from uninfected horses kept at a breeding herd maintained at Washington State University, Pullman. Positive sera were from horses persistently infected with *B. equi* by either natural or experimental inoculation.

**Western blotting.** Immunoblots were used to confirm expression of EMA-1 subclones by recombinant *E. coli* and to evaluate their reactivities to MAB 36/133.97. Antigens (5 μg) were resolved by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and tested for their reactivities with both MAB 36/133.97 (2 μg/ml) and equine antibodies (serum diluted 1:100) by previously described techniques (13). Before use in Western blotting assays, horse sera were incubated for 3 h at room temperature with 1 volume of *E. coli* lysate (prepared as described above for antigen preparation) to adsorb anti-*E. coli* antibodies. Antigens expressed from *E. coli* containing pEMA-1 or pBluescript SKM were used as positive and negative controls, respectively. cELISA. The ability of equine antibodies to block the reactivity of MAB 36/133.97 against the full-length and truncated EMA-1.2 proteins was evaluated by cELISA. The cELISA was performed as described previously (13), with the exception that the antigens used were prepared as described above. Each individual serum sample (including positive and negative reference sera) was tested in duplicate. Results were expressed as percent inhibition based on the following formula: 100 × ([mean OD₄₀₅ for normal serum control – mean OD₄₀₅ for tested serum sample]/mean OD₄₀₅ for normal serum control), where OD₄₀₅ is the optical density at 405 nm. Sera from six uninfected horses were used as normal serum controls for calculation of percent inhibition. Analysis of variance was used to determine the level of significance of the differences observed between inhibition by uninfected and *B. equi*-infected horse sera in cELISA. Statistical analysis was performed with NCSS 2001 software (Number Cruncher Statistical Systems, Kaysville, Utah), and a P value ≤0.01 was considered significant.

**RESULTS**

Comparison of EMA-1 proteins from isolates of *B. equi* recovered worldwide. The degree of homology between the predicted amino acid sequence of EMA-1 (272 residues) from the reference Florida isolate and 15 other published sequences from independent isolates of *B. equi* showed identities of 82.8 to 99.6% (median, 98.5%) and similarities of 90.5 to 99.6% (median, 98.9%) (Table 2). The alignment of all 16 EMA-1 proteins revealed that most of the variation among the sequences is a result of amino acid substitutions scattered

**TABLE 1. Primers used to amplify truncated *ema*-1 gene by PCR with plasmid pEMA-1**

<table>
<thead>
<tr>
<th><em>ema</em>-1 fragment</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ema</em>-1.2</td>
<td>5'-AGGGAAACAAAAAGCT-3'</td>
<td>5'-TCAGGTCMTGACAGCAGTA-3'</td>
</tr>
<tr>
<td><em>ema</em>-1.3</td>
<td>5'-AGGGAAACAAAAAGCT-3'</td>
<td>5'-TCAGGTCMTGACAGCAGTA-3'</td>
</tr>
<tr>
<td><em>ema</em>-1.5</td>
<td>5'-TGAATTCATGACCAGCT-3'</td>
<td>5'-TCAGGTCMTGACAGCAGTA-3'</td>
</tr>
</tbody>
</table>

*pEMA-1, pBluescript SKM (Stratagene) containing the full-length *ema*-1 gene.*
TABLE 2. Degree of homology between the deduced amino acid sequence of the reference EMA-1 from Florida isolate and EMA-1 sequences from 15 isolates of B. equi recovered worldwide.

<table>
<thead>
<tr>
<th>EMA-1 amino acids</th>
<th>% Identity (median)</th>
<th>% Similarity (median)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–272&lt;sup&gt;a&lt;/sup&gt;</td>
<td>82.8–99.6 (98.5)</td>
<td>90.5–99.6 (98.9)</td>
</tr>
<tr>
<td>1–98&lt;sup&gt;a&lt;/sup&gt;</td>
<td>85.7–100.0 (99.0)</td>
<td>94.9–100.0 (100.0)</td>
</tr>
</tbody>
</table>

<sup>a</sup> The GenBank accession number of the Florida isolate is AAA72370. The GenBank accession numbers of the other 15 isolates are as follows: AAC38827, AF255730-1, BAA32975, BAA32976, BAA32977, BAA32978, BAA32979, BAA32981, BAA32982, BAA32983, BAA32984, BAA32985, BAA32986, BAA32987, and BA096134.
<sup>b</sup> Full-length EMA-1.
<sup>c</sup> Region where the MAb 36/133.97 epitope is located.

Throughout the protein, and a hypervariable region is not present (Fig. 1).

**Binding of MAb 36/133.97 to peptides.** To test whether the EMA-1 sequence from amino acids 27 to 39 contained the epitope recognized by MAb 36/133.97, the peptide VVDFQLESI<sup>39</sup> was tested for binding to MAb 36/133.97 by dot immunoblotting. Although MAb 36/133.97 binds to native EMA-1 at 10<sup>–3</sup> µg per reaction, there was no reaction against this peptide or the irrelevant peptide used as a negative control when both were tested at amounts ranging from 10<sup>–3</sup> to 10<sup>–4</sup> µg per reaction. Peptides were shown to be bound to the nitrocellulose membrane by pseudo S staining (data not shown). Also, MAb 36/133.97 absorbed with this peptide (50 to 0.3 µg)-bound native EMA-1 by dot immunoblotting, whereas the reactivity of MAb 36/133.97 was inhibited by adsorption with native EMA-1 at 0.03 µg per reaction. These results indicated that the linear sequence of EMA-1 from amino acids 27 to 39 is not recognized by MAb 36/133.97.

**Cloning and expression of recombinant full-length and truncated EMA-1.** Full-length and truncated ema-1 genes were cloned and expressed as fusion proteins in E. coli. Each of the recombinant ema-1 sequences was shown to be in frame with the β-galactosidase promoter by nucleic acid sequencing. The deduced amino acid sequence of the protein obtained from each clone included 44 and 18 vector-encoded residues on the N terminus and C terminus of EMA-1, respectively. The orientations of the genes encoding full-length and truncated recombinant proteins relative to the predicted hydropathicity (16) of EMA-1 are shown in Fig. 2.

The expression of EMA-1 proteins of the predicted size by each recombinant clone was demonstrated by Western blotting with the recombinant E. coli lysate as antigen and serum from a horse persistently infected with B. equi. Equine antibodies reacted with recombinant proteins EMA-1, EMA-1.2, EMA-1.3, and EMA-1.5 (Fig. 3B, lanes 1 to 4, respectively). The extra bands detected by the horse serum in recombinant lysates probably represent alternative transcription or translation of EMA-1 proteins, aggregation with E. coli or EMA-1 products, or degradation of expressed proteins. Low background reactivity was obtained when horse serum was reacted with the E. coli lysate, indicating that serum absorption removed most of the anti-E. coli antibodies (Fig. 3B, lane 5).

**Binding of MAb 36/133.97 to EMA-1.** The binding of MAb 36/133.97 to EMA-1 and truncated proteins was evaluated by Western blotting. As shown in Fig. 3A, MAb 36/133.97 bound to recombinant EMA-1 (lane 1) and EMA-1.2 (lane 2) proteins, but did not bind to the EMA-1.3 (lane 3), EMA-1.5 (lane 4), or E. coli (lane 5) proteins. The minor bands detected in lanes 1 and 2 were likely the result of limited degradation of EMA-1 products. Although no reaction of MAb 36/133.97 against EMA-1 and EMA-1.5 has been demonstrated, these proteins were expressed, as demonstrated by reaction with equine antibodies (Fig. 3B, lanes 1 to 4). These data show that the epitope recognized by MAb 36/133.97 is within the EMA-1.2 truncated protein, which represents the first 98 amino acids of full-length EMA-1. In addition, MAb 36/133.97 did not bind to EMA-1.3 or EMA-1.5, from which amino acids from the EMA-1.2 C terminus (amino acids 78 to 98) and N terminus (amino acids 1 to 38) were removed. This indicates that both the N-terminal region (some or all of the first 38 residues) and some or all of the last 20 residues in the C-terminal region of the EMA-1.2 truncated protein are necessary for MAb 36/133.97 binding, which is compatible with the conformational dependence of the epitope.

The degree of amino acid homology within the region that contained the epitope defined by MAb 36/133.97 (truncated protein EMA-1.2) was also analyzed. The sequences of this region from six isolates were 100% identical to that of EMA-1 from the Florida strain. The amino acid identities of the proteins analyzed ranged from 85.7 to 100% (median, 99.0%), and the similarities of the amino acid sequences of the proteins analyzed, considering conserved substitutions, ranged from 94.9 to 100.0% (median, 100.0%) (Table 2). This analysis shows that the degree of homology within the region to which MAb 36/133.97 binds is higher than that in the full-length EMA-1.

**Inhibition of MAb 36/133.97 binding.** To test whether horse antibodies which inhibit binding of MAb 36/133.97 to EMA-1 also recognize epitopes in the same region to which the MAb binds, a cELISA was performed with EMA-1 and EMA-1.2 as antigens and sera from B. equi-infected horses. Antigens prepared with NP-40 or SDS were tested for MAb 36/133.97 binding. The MAb bound to EMA-1–SDS, EMA-1–NP-40 and EMA-1.2–SDS but did not bind to EMA-1.2–NP-40, suggesting that NP-40 affects the protein conformation, thus abrogating MAb binding. On the basis of these results, the cELISA was conducted with EMA-1 and EMA-1.2 in SDS. Positive sera were able to significantly (P < 0.01) block MAb 36/133.97 binding to both EMA-1 and EMA-1.2 compared to the ability of the sera from uninfected horses to bind to the proteins (Table 3). These results indicate that both MAb 36/133.97 and horse antibodies recognize epitopes within the first 98 amino acids of EMA-1.

**DISCUSSION**

EMAb and MAb 36/133.97 are used in a diagnostic test to identify anti-B. equi antibodies in horses. Therefore, we investigated whether variations in EMA-1 potentially affect the binding of both MAb 36/133.97 and equine antibodies inhibiting the binding of this MAb. It has been reported that the EMA-1 sequence from residues 27 to 39 is bound by MAb 36/133.97 and the sequence from residues 123 to 135 is bound by equine antibodies in a library of overlapping synthetic peptides covering the full-length protein (7). In this study, the peptide representing amino acids 27 to 39 from EMA-1 was
FIG. 1. Alignment of deduced amino acid sequences of EMA-1 from *B. equi* isolates recovered worldwide. The sequences were obtained from GenBank and aligned by use of the AlignX program from the Vector NTI Suite. The line indicates the region where the epitope defined by MAb 36/133.97 is located. Black boxes designate nonhomologous residues, and gray boxes designate conserved substitutions. Gaps are represented by dots.
not recognized by MAb 36/133.97. Although our data indicate that the epitope was located within amino acids 1 to 98, it was demonstrated that both the N- and the C-terminal regions of EMA-1.2 (amino acids 1 to 38 and 78 to 98, respectively) are required for binding. These results indicate that binding of MAb 36/133.97 is dependent on the protein structure, which is consistent with the conformational dependence of the EMA-1 epitope recognized by MAb 36/133.97. However, whether the amino acids in these distinct regions are juxtaposed to form the epitope or whether the accessibility to the epitope is dependent on the secondary structure provided by these regions is unknown.

Analysis of the hydropathicity of EMA-1 revealed that the sequence from residues 1 to 98 is located in a hydrophilic domain, which is in agreement with surface exposure. The surface location of the epitope recognized by MAb 36/133.97 has been demonstrated by immunofluorescence with *B. equi* merozoites (14). In addition, surface-exposed elements, consisting of hydrophilic residues forming turns and loops in the peptide, are strong candidates for antibody binding. By cELISA, it was also shown that equine antibodies associated with MAb 36/133.97 inhibition bound to the same region where the MAb 36/133.97 epitope is located (residues 1 to 98). These results, which differ from those reported previously (7), demonstrate that equine antibodies which inhibit binding of

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**TABLE 3. Inhibition by horse sera of MAb 36/133.97 binding to EMA-1 and EMA-1.2 recombinant proteins in a competitive inhibition ELISA**

<table>
<thead>
<tr>
<th>Horse serum infection status</th>
<th>EMA-1 Mean (SE) % inhibition</th>
<th>EMA-1.2 Mean (SE) % inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive (n = 31)</td>
<td>71.18 (3.48)</td>
<td>51.83 (5.87)</td>
</tr>
<tr>
<td>Negative (n = 6)</td>
<td>0.76 (7.92)</td>
<td>−6.08 (13.36)</td>
</tr>
</tbody>
</table>

*a The equation used to calculate percent inhibition is provided in Materials and Methods.*
MAb 36/133.97 also recognize epitopes outside residues 123 to 135.

The alignment of the predicted amino acid sequences of EMA-1 from the Florida isolate with sequences from geographically distinct isolates of B. equi revealed median identities of 98.5% in the full-length protein and 99.0% in the region where the MAb epitope is located. When conserved substitutions were not counted for the analysis, the median similarities between sequences were 98.9 and 100% for the full-length protein and the first 98 amino acids, respectively. Interestingly, although some variation has been detected in EMA-1, it does not seem to affect the binding of specific antibodies. Evidence for the presence of conserved epitopes in EMA-1 is that sera from horses from 19 countries are able to inhibit MAb 36/133.97 binding in the cELISA (15).

The results obtained in this study, in association with data reported previously (13, 15), support the conclusion that the amino acid substitutions within the region containing the epitope defined by MAb 36/133.97 are unlikely to affect the development of horse antibodies capable of inhibiting MAb 36/133.97 binding. Therefore, recombinant EMA-1 and MAb 36/133.97 can reliably be used in a cELISA for the diagnosis of B. equi infections in horses.

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