Potential Value of Major Antigenic Protein 2 for Serological Diagnosis of Heartwater and Related Ehrlichial Infections

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Cowdria ruminantium is the etiologic agent of heartwater, a disease causing major economic loss in ruminants in sub-Saharan Africa and the Caribbean. Development of a serodiagnostic test is essential for determining the carrier status of animals from regions where heartwater is endemic, but most available tests give false-positive reactions with sera against related Ehrlichia species. Current approaches rely on molecular methods to define proteins and epitopes that may allow specific diagnosis. Two major antigenic proteins (MAPs), MAP1 and MAP2, have been examined for their use as antigens in the serodiagnosis of heartwater. The objectives of this study were (i) to determine if MAP2 is conserved among five geographically divergent strains of C. ruminantium and (ii) to determine if MAP2 homologs are present in Ehrlichia canis, the causative agent of canine ehrlichiosis, and Ehrlichia chaffeensis, the organism responsible for human monocytic ehrlichiosis. These two agents are closely related to C. ruminantium. The map2 gene from four strains of C. ruminantium was cloned, sequenced, and compared with the previously reported map2 gene from the Crystal Springs strain. Only 10 nucleic acid differences between the strains were identified, and they translate to only 3 amino acid changes, indicating that MAP2 is highly conserved. Genes encoding MAP2 homologs from E. canis and E. chaffeensis also were cloned and sequenced. Amino acid analysis of MAP2 homologs of E. chaffeensis and E. canis with MAP2 of C. ruminantium revealed 83.4 and 84.4% identities, respectively. Further analysis of MAP2 and its homologs revealed that the whole protein lacks specificity for heartwater diagnosis. The development of epitope-specific assays using this sequence information may produce diagnostic tests suitable for C. ruminantium and also other related rickettsiae.

Heartwater, or cowdriosis, a rickettsial disease of domestic and wild ruminants, is caused by the etiologic agent Cowdria ruminantium, which is transmitted by ticks belonging to the genus Amblyomma. Heartwater is responsible for mortality levels as high as 90% in susceptible ruminants and has been reported throughout sub-Saharan Africa and on several Caribbean islands (22). Animals surviving the disease may have persistent infections; thus, they are capable of serving as reservoirs for transmission to ticks (1).

Major antigenic protein 1 (MAP1) of C. ruminantium was found to be antigenically conserved in nine strains of C. ruminantium from Africa and the Caribbean (12) and was used in a competitive enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies (13). However, further studies revealed that MAP1 (complete, recombinant, or truncated) also reacts with sera from ruminants from heartwater-free areas (14, 15, 18) and with several members of the genus Ehrlichia (10). Furthermore, significant variability in nucleotide sequences of MAP1 among four African and two Caribbean strains ranged from 0.6 to 14.0% (20). These nucleic acid differences translated to amino acid substitutions, deletions, or insertions at three hypervariable regions of the gene (20). Demonstration of homology in MAP1 coding sequences between C. ruminantium and Ehrlichia spp. and divergence in hypervariable regions between strains suggest that MAP1 may not be a suitable candidate for specific serodiagnosis of heartwater. Thus, current serological tests for cowdriosis lack specificity, i.e., false-positive sera react with the whole C. ruminantium organism and the reported immunoreactive MAP1 (8).

The gene encoding another major antigenic protein (MAP2) was isolated, cloned, and expressed in Escherichia coli (17). Sera obtained from cattle, sheep, and goats infected with C. ruminantium reacted with this recombinant MAP2 (17). MAP2 is 55.5% identical at the amino acid level to major surface protein 5 (MSP5; 19 kDa) (17, 25) of Anaplasma marginale. A competitive ELISA using a monoclonal antibody against MSP5 has been evaluated and shown to be sensitive and specific for the diagnosis of Anaplasma infections in acute or persistently infected cattle (16).

In this study, we examine the potential value of MAP2 as an antigen for the serodiagnosis of heartwater. First, we report the cloning and sequencing of map2 genes from several C. ruminantium strains of differing geographic origins. Second, we determine if, as with MAP1, false-positive sera react with MAP2. Finally, we determine whether MAP2-like proteins are present in Ehrlichia canis and Ehrlichia chaffeensis (causative agent of human monocytic ehrlichiosis), which are closely related to C. ruminantium according to 16S rRNA studies (6, 24), to evaluate whether epitope-specific tests are required for diagnosis of these rickettsiae.

MATERIALS AND METHODS

Origins of C. ruminantium strains, E. canis, and E. chaffeensis. Three strains of C. ruminantium from Zimbabwe (Crystal Springs, Highway, and Palm River [4]).
TABLE 1. Primers designed or selected for amplification of the genes encoding the MAP2 homologs of \textit{E. canis} and \textit{E. chaffensis}

| Primers | Nucleic acid sequence a (5’ to 3’) |
|---------|----------------------------------|
| AB249   | AACCTCTATTATTACCTA                |
| AB251   | AAAAAAGACTAAGAAAGAC              |
| AB252   | GGTCTTATCACTACCTGTC              |
| AB254   | GAAAYCTCCAAAYCTC                |
| AB346   | CTTTTGATTTGTCATGTTG             |
| AB347   | GACTTTAGATCATCCAGATC            |
| AB351   | GACGAGAGATTTTGGTGGGATCAT        |
| AB353   | GATATCATCACCTAAT                 |
| AB371   | GAGCACTATGGAATGCTACT            |
| AB602   | GCTATCATTGTCGCAATAGG            |
| AB608   | TACNNAGTNNNNCCNNNCTT            |
| AB620   | AAACCTCTAGGACGTATTT            |
| AB623   | GCTATTTGTTGTGGCCTGG            |
| AB624   | GCTATTGGCGGCTCATTTCA          |

a M = A or C; N = A, C, G, or T; R = A or G; S = G or C; Y = C or T.

Amplication of the map2 genes of \textit{C. ruminantium} strains of different geographic origins and of the map2-like genes of \textit{E. chaffeensis} and \textit{E. canis}. Primers AB349 and AB251, corresponding to flanking regions of the map2 gene (open reading frame [ORF] 1) of \textit{C. ruminantium} clone pFS2.17, were synthesized. Primers for PCR amplification of \textit{E. canis} and \textit{E. chaffensis} were selected by using information from one of the sequences: (i) sequences determined after alignment of conserved regions of the map2 gene of \textit{C. ruminantium} map2 (A. margarita); (ii) sequences obtained from newly sequenced regions of the map2-like genes of \textit{E. canis} or \textit{E. chaffensis}, and (iii) primers developed to sequence the pFS2.12 clone of the Crystal Springs strain of \textit{C. ruminantium} (17).

TABLE 2. Primer combinations and optimal temperatures for amplification of the genes encoding the MAP2 homologs of \textit{E. canis} and \textit{E. chaffensis}

| Primer combination | Denaturing (min) | Denaturing (°C) | Annealing (min) | Annealing (°C) | Extension (min) | Extension (°C) |
|--------------------|-----------------|----------------|-----------------|----------------|----------------|----------------|
| AB249-AB251        | 40              | 94            | 1               | 59             | 1              | 72             |
| AB252-AB284        | 40              | 94            | 1               | 59             | 1              | 72             |
| AB346-F17          | 1.5             | 94            | 1               | 59             | 1              | 72             |
| AB347-AB146        | 1.5             | 94            | 1               | 59             | 1              | 72             |
| AB602-AB608        | 40              | 94            | 1               | 59             | 1              | 72             |
| AB660-AB262        | 47              | 94            | 1               | 59             | 1              | 72             |
RESULTS

Analysis of the map2 genes of C. ruminantium. Five geographically differing strains of C. ruminantium were examined in order to determine the conservation of MAP2. The map2 genes from the four strains were amplified with primers AB249 and AB251 (Table 1), resulting in an amplicon of approximately 0.74 kb (Fig. 1). The map2 genes from the strains were cloned by PCR cloning methods, and restriction enzyme analysis was performed and compared with that for the previously reported map2 gene of the Crystal Springs strain. Restriction map analysis of the map2 genes demonstrated similarities between Palm River, Um Banein, and Antigua and similarities between Crystal Springs and Highway (Fig. 2). EcoRV, XbaI, and HincII restriction sites were found in all strains analyzed. The AccI restriction site was observed in the Crystal Springs and Highway strains only. Likewise, the Palm River, Um Banein, and Antigua strains had identical restriction enzyme maps and contained an additional HincII site.

Cloned map2 genes were sequenced and compared with the known sequence of the Crystal Springs strain map2 gene (GenBank accession no. G289922) at both the nucleotide (Fig. 3) and amino acid (Fig. 4) levels. At both levels, Highway and Crystal Springs MAP2 sequences were identical. Palm River, Um Banein, and Antigua differed at nucleotides 111, 192, 319, 465, 483, 534, 553, and 594. Um Banein differed at nucleotide 170 from all other strains analyzed. Antigua differed at nucleotide 323 from all other strains analyzed. The DNA sequence changes at the AccI (5'-GTAGAC-3') and HincII (5'-GTCAC-3') sites are in agreement with the restriction map data. Of the 10 nucleotide differences, 7 do not change the encoded amino acid, but changes at nucleotides 319, 323, and 553 lead to 3 amino acid substitutions. Serine (amino acid 107) and threonine (amino acid 185) of Crystal Springs and Highway were both replaced by alanine in Palm River, Um Banein, and Antigua. Antigua differs from all others at amino acid 108, where a glycine has been substituted for an aspartic acid. The map2 gene for all strains of C. ruminantium is 627 bases. Thus, MAP2 is highly conserved among these five geographically differing strains examined.

Analysis of the map2-like genes of E. chaffeensis and E. canis. The map2-like genes were amplified by using several sets of PCR primers (Tables 1 and 2), cloned, and sequenced. The nucleotide and translated amino acid sequences were compared with the sequences of the C. ruminantium strains (Fig. 3 and 4). When the amino acid sequences are aligned, Ehrlichia proteins begin 4 amino acids downstream from the starting methionine of the ORF of MAP2 of C. ruminantium (Fig. 4). Although MAP2-related polypeptides in E. chaffeensis and E. canis are highly homologous to C. ruminantium MAP2, they were distinguishable by numerous substitutions throughout the polypeptide chain. Overall, the MAP2 homologs of E. canis and E. chaffeensis were 83.4 and 84.4% identical, respectively, to MAP2 of C. ruminantium (Crystal Springs strain). These data show that MAP2 homologs are present in two Ehrlichia spp., E. canis and E. chaffeensis, but that there is variability throughout the nucleic acid and amino acid sequences that may allow the development of epitope-specific serodiagnostic assays.

Immunoaassays demonstrating antigenic similarity between C. ruminantium and E. canis. C. ruminantium antiserum (from sheep 483) recognized an approximately 21-kDa protein in C. ruminantium and E. canis (Fig. 5a). More specifically, anti-recombinant MAP2 serum (from sheep 177) recognized MAP2 in C. ruminantium (lane A) and a MAP2 homolog in E. canis (lane B). Anti-E. canis serum also recognized MAP2 of C. ruminantium and E. canis (Fig. 5). These data confirm the sequence data and show that a MAP2 homolog which shares epitopes with MAP2 of C. ruminantium is expressed in E. canis.

Indirect ELISA for heartwater diagnosis using MAP2. Sera from sheep were tested to determine whether recombinant MAP2 is recognized by both anti-C. ruminantium and false-positive sera, as observed with MAP1 (18). Sera obtained from five animals experimentally infected with C. ruminantium tested positive at dilutions as low as 1:3,200 (Fig. 6). False-positive sera obtained from four animals from heartwater-free regions of Zimbabwe (18) reacted in a pattern similar to that of true-positive sera (Fig. 6). Immunoblot-negative sera

RESTRICTION ENZYME ANALYSIS OF MAP2 GENES

FIG. 2. Restriction maps of the map2 genes from C. ruminantium strains. Maps are drawn left to right, 5' to 3' with respect to the MAP2 coding sequence. Restriction enzymes used for mapping are shown. The restriction map of the Crystal Springs strain map2 gene is presented for comparison.
n53) consistently gave optical density values lower than the true-positive and false-positive sera at similar dilutions down to 1:12,800. Hence, an indirect ELISA using the complete recombinant MAP2 does not discriminate between true- and false-positive sera.

**DISCUSSION**

The isolation of genes encoding immunoreactive proteins of *C. ruminantium* may enable the development of diagnostic tests with improved sensitivity and specificity. The immuno-reactive protein must be conserved among *C. ruminantium* strains and yet sufficiently different from the homologs present in related species. MAP1 has been tested in various assays (10, 13) and shown to cross-react with anti- *Ehrlichia* sera and with sera from known *C. ruminantium*-free animals (18). A truncated MAP1 protein, MAP1B, has improved specificity but still reacts with some anti- *Ehrlichia* sera (14, 15, 23). Recent data showing (i) the presence of three hypervariable regions in MAP1 in different strains of *C. ruminantium* (20), (ii) recognition of the protein by sera from patients with *C. ruminantium* infection (17), and (iii) the development of a sensitive and specific indirect ELISA using purified MAP1 (16) suggest that MAP1 may be a useful diagnostic marker for *C. ruminantium* infection. The isolation of MAP2 and its homologs in other *C. ruminantium* strains may provide additional diagnostic markers for this important zoonotic disease.
nition of linear peptides representing these hypervariable regions (20) by sera from animals infected with *C. ruminantium* (19, 20), and (iii) the presence in *E. canis* and *E. chaffeensis* of MAP1 homologs which have sequences very similar to that of MAP1 of *C. ruminantium*, except in the hypervariable regions, suggest that MAP1 may not be an ideal diagnostic antigen.

MAP2 is an immunoreactive protein consistently recognized by sera from sheep, goats, and cattle infected with *C. ruminantium* (17). MAP2 is 55.5% identical to MSP5 of *A. marginale*. MSP5 is conserved in all the different isolates of *A. marginale* tested, and an epitope-specific assay using MSP5, which consistently identifies cattle both acutely and chronically infected with *A. marginale*, has been developed (16, 25). Examination of the MAP2 of five geographically diverse strains of *C. ruminantium* revealed a highly conserved protein with amino acid substitutions at only three positions (amino acid variability between 0 and 1.44%). This differs from the sequenced MAP1 of several strains, which was found to contain up to 10.0% variability in amino acid sequence (20). MAP2 meets two criteria for a suitable diagnostic antigen, immunoreactivity and conservation. However, further data showed reactivity between recombinant MAP2 and sera from known heartwater-free animals. Thus, like MAP1, MAP2 lacked specificity for a diagnostic test.

Because MAP2 reacted with false-positive sera, it was essential that we examine other closely related rickettsiae for the presence of MAP2 homologs in order to evaluate whether there is a basis for screening of anti-MAP2 monoclonal antibodies or the use of specific MAP2 regions to develop an epitope-specific assay. The existence of a MAP2 homolog in *E. canis* was demonstrated by immunoblotting total antigens of *C. ruminantium* and *E. canis* with an antiserum specific to recombinant MAP2.

The genes encoding MAP2 homologs in *E. canis* and *E. chaffeensis* were amplified and sequenced, and ORFs similar to the *map2* gene were translated into amino acids. The resulting MAP2 homologs had amino acid similarities of 83.41% (for

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**FIG. 4.** Comparison of the MAP2 and MAP2-like coding sequences at the amino acid level. The nucleotide sequences for five strains of *C. ruminantium* and two *Ehrlichia* species were translated. The complete sequence for the Highway strain is presented. The sequences for other strains and the *Ehrlichia* species are presented only when they differ from the Highway sequence. A dot indicates identity with the Highway sequence.

**FIG. 5.** (a) Immunoblot demonstrating antigenic similarity between *C. ruminantium* and *E. canis*. Lysates of *C. ruminantium* (lanes A) or *E. canis* (lanes B) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were reacted with anti-*C. ruminantium* antiserum (sheep 483), anti-rMAP2 antiserum (sheep 177), or anti-*E. canis* antiserum (Mukanya). (b) Indirect ELISA using dog sera. Serum from a dog infected with *E. canis* (positive) or from a healthy dog (negative) was tested in an indirect ELISA against recombinant MAP2.
The initial discrepancy between the MAP2 homologs of *C. ruminantium* and *Ehrlichia* appears to be the first methionine. The second methionine of *C. ruminantium* (4 amino acids from the first methionine) aligns with the first methionine of the *Ehrlichia* species, suggesting that it is probably the initiator methionine in MAP2. This is further supported by alignment of MAP2 with MSP5 of *A. marginale*; MSP5 also begins at the second methionine of *C. ruminantium*. This is a predicted ribosomal binding site 5' to the second methionine but 3' to the first methionine (Fig. 3). Thus, it appears that the reported amino acid sequence of MAP2 (17) may be incorrect and should actually be shorter by 4 amino acids at the N terminus. Like MSP5 of *A. marginale*, therefore, MAP2 may be useful in the diagnosis of animals infected with *C. ruminantium* if a similarly unique epitope can be defined. MAP2 is highly conserved among geographically different isolates and is recognized by sera from *C. ruminantium*-infected sheep, goats, and cattle. However, MAP2 is recognized antigenically by anti-*E. canis* antiserum, and false-positive sera and MAP2 homologs were identified from *E. canis* and *E. chaffeensis*; thus MAP2 as a whole protein lacks specificity for heartwater diagnosis. From this study, it was determined that development of a serodiagnostic assay using MAP2 may require the use of truncated peptides or monoclonal antibodies specific to unique sequences of MAP2 of *C. ruminantium*. Further, the availability of the sequences of MAP2 homologs in *E. chaffeensis* and *E. canis* may aid in the development of specific diagnostic tests for these species.

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