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A RECOMBINANT ANTIGEN FROM THE HEARTWATER AGENT (*COWDRIA RUMINATIUM*) REACTIVE WITH ANTIBODIES IN SOME SOUTHEASTERN UNITED STATES WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*), BUT NOT CATTLE, SERA

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ABSTRACT: Recombinant baculovirus techniques were used to express the 260 amino acid carboxyterminal portion of the 32 kilodalton (kDa) major antigenic protein (MAP 1) of *Cowdria ruminantium*, the heartwater agent, as a fusion protein. The recombinant MAP 1 was fused to an aminoterminal independently antigenic octapeptide sequence (FLAG® peptide). Recombinant MAP 1 was used as an immunoblotting antigen to evaluate numerous reference antisera against organisms of the tribe Ehrlichieae. Monoclonal and polyclonal *C. ruminantium* antibodies, monoclonal anti-FLAG® ascites, and antisera to *Ehrlichia canis* and *Ehrlichia chaffeensis* reacted with this antigen. Twelve of 79 sera collected 1980 to 1992 from southeastern U.S. white-tailed deer (*Odocoileus virginianus*) were also unexpectedly immunoblot-positive to MAP 1. These 12 deer sera had, as a group, significantly ($P < 0.01$) greater anti-*E. chaffeensis* titers (previously determined) than the sera from MAP 1 immunoblot-negative deer living in the same areas. None of the 262 sera from cattle living in the same areas were immunoblot-positive to MAP 1. All of an additional 50 cervine sera from Michigan (USA), 72 bovine sera from northern U.S. cattle, and 72 sera from Puerto Rican cattle were also immunoblot-negative to MAP 1. Sera from African sheep which were falsely seropositive to authentic MAP 1 were also immunoblot-positive to the recombinant MAP 1. Unidentified *Ehrlichia* spp. capable of serologic crossreactivity with the heartwater agent appear to be present in some southeastern U.S. white-tailed deer but not cattle. These or related *Ehrlichia* spp. may also be found elsewhere in the world in non-cervine species.

Key words: Heartwater, serology, Ehrlichiae, white-tailed deer, cattle, *Odocoileus virginianus*, *Cowdria* spp.

INTRODUCTION

Cowdria ruminantium is the tick-borne etiologic agent of heartwater, a severe rickettsial disease of ruminants (Rikihisa, 1991). Species of *Amblyomma* ticks capable of transmitting heartwater are widely distributed throughout many of the world's temperate and tropical regions, including southeastern and southcentral areas of the United States (Walker, 1987). *Cowdria ruminantium* has never been detected within the United States and is presently found only in subsaharan Africa and on some Caribbean islands (Perreau et al., 1980). This pathogen retains the potential for extensive geographic spread, however, via its introduction into additional *Amblyomma* spp. populations (Walker, 1987). The current lack of a highly specific serodiagnostic test for heartwater hinders efforts to contain geographically and then eradicate the disease from affected wildlife and livestock populations (Du Pleiss et al., 1993).

Based on serologic and molecular genetic analyses, *C. ruminantium* is related to several members of the Tribe Ehrlichieae, including *Ehrlichia bovis*, *Ehrli-*

chia ovina, Ehrlichia equi, Ehrlichia canis, Ehrlichia chaffeensis, and the WSU 86-1044 agent (Dame et al., 1992; Jongejan et al., 1993). A 32-kilodalton (kDa) *C. ruminantium* major antigenic protein (MAP 1) has been used as an antigen for heartwater serodiagnosis and provokes perhaps the strongest and most enduring serologic response to *C. ruminantium* infection (Jongejan et al., 1991; Rossouw et al., 1990). At least some of the serologic cross-reactivity between *C. ruminantium* and other Ehrlichieae, however, results from conserved epitopes of MAP 1 (Jongejan et al., 1993). These shared epitopes underlie much of the heartwater serodiagnostic specificity problem in ruminant livestock populations (Mahan et al., 1993). We report here the expression of a recombinant MAP 1 fusion protein and an analysis of its serodiagnostic specificity with respect to the cattle and white-tailed deer populations of the southeastern U.S., the region considered most vulnerable to the entrance of *C. ruminantium* from the Caribbean region into continental North America (Walker, 1987).

MATERIALS AND METHODS

Reference antisera (Table 1) were obtained to the following organisms: *C. ruminantium* (Crystal Springs isolate, caprine origin; polyvalent Gardel, Kumm, Kwanyanga, and Mali isolates, caprine origin), *Anaplasma marginale* (bovine and cervine origin), *E. canis* and *Ehrlichia platys* (canine origin), *E. chaffeensis* (canine, human, and cervine origin), *Ehrlichia risticii* (equine origin), and the WSU86-1044 agent (caprine and bovine origin). Monoclonal anti-MAP 1 antibody 4F10B4 (raised against Welgevonden isolate of *C. ruminantium*) (Jongejan et al., 1991) and monoclonal anti-FLAG® fusion peptide antibody (Knappik and Pluckthun, 1994) were also used, as were three heartwater false-positive ovine sera from Zimbabwe (Mahan et al., 1993). The false-positive sera were so defined because they had reacted by western blot with native MAP 1 but originated from animals which had been reared in known heartwater-free regions (Mahan et al., 1993). Blood samples from these animals were also incapable of seroconverting sentinel animals upon inoculation, and were negative for heartwater by nucleic acid probe methods as well

TABLE 1. Origins of reference and field sera evaluated by immunoblot and their reactivities with baculovirus-expressed recombinant major antigenic protein 1 (MAP 1)

Serum types and sources	Sera positive/ sera tested
Anti-MAP 1 4F10-B4 monoclonal antibody[a],	1/1
Anti-FLAG® peptide monoclonal antibody[b]	1/1
Heartwater convalescent sera, ovine, Zimbabwe[c]	3/3
Heartwater convalescent sera, caprine, United States[d]	4/4
Heartwater false-positive sera, ovine, Zimbabwe[c]	3/3
Heartwater negative sera, ovine, Zimbabwe[c]	0/3
Ehrlichia equi serum, equine[e]	0/1
Ehrlichia platys serum, canine[e]	0/1
Ehrlichia risticii sera, equine[f]	0/10
Ehrlichia chaffeensis sera, canine, human, cervine[g]	1/8
Ehrlichia canis sera, canine[e,h]	2/2
Ehrlichia ewingii serum, canine[h]	0/1
WSU 86-1044 sera caprine, bovine[i]	0/2
Anaplasma marginale sera bovine, cervine[j,k]	0/12
Field origin sera, cervine, southeastern United States[k]	12/79
Field origin sera, cervine, Michigan[l]	0/50
Field origin sera, bovine, United States[f]	0/262
Field origin sera, bovine, Puerto Rico[f]	0/72
Field origin sera, bovine, northern United States[f]	0/72

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(Mahan, et al., 1993). Sera were collected from 79 white-tailed deer (*Odocoileus virginianus*) killed between 1986 and 1993 in a broadly dispersed pattern across the southeastern states (25°55′ to 35°57′N, 80°07′ to 98°29′W) of Florida, Louisiana, Alabama, Georgia, Tennessee, and South Carolina (USA), (Keel et al., 1995; Lockhart et al., 1996). Thirteen of these deer were seropositive to *A. marginale* (Keel et al., 1995), and 35 of the others were seropositive to *E. chaffeensis* by indirect immunofluorescence (Lockhart et al., 1996). Sera were also obtained from 50 Michigan (USA) deer collected during the period 1980 to 1992 (41°43′ to 45°51′N, 82°25′ to 86°38′W). Two hundred sixty-two sera were collected at slaughter from cattle in the same southern states, together with an additional 72 bovine sera from Puerto Rico (17°55′ to 18°31′N, 65°40′ to 67°16′W), 72 bovine sera from the Texas gulf coast (25°54′ to 30°57′N, 93°55′ to 99°30′W), and 72 bovine sera from the Pacific northwestern (43°00′ to 49°00′N, 111°20′ to 124°12′W), north central (43°00′ to 49°00′N, 88°00′ to 103°41′W), and Rocky Mountain regions (41°00′ to 43°00′N, 104°59′ to 114°31′W) of the United States. The latter three regions were areas, unlike the others, in which heartwater-competent species of *Amblyomma* spp. ticks have not been found. Sera were collected during 1992 and 1993 from both beef and dairy cattle, and were obtained on different dates and from different locations to maximize dispersion of sample origins.

The baculovirus (BV)-insect cell protein expression system was used to express recombinant MAP 1. This eukaryotic protein expression system has an advantage in the production of serodiagnostically useful antigens relative to the simpler common *Escherichia coli* expression systems because it performs a wider range of post-translational protein processing functions than the latter systems (O'Reilly et al., 1994). The BV-insect cell host expression system also avoids the contamination of recombinant protein products with normal *E. coli* proteins. Antibodies to those proteins are abundant in the sera of all normal mammals and often interfere with serologic tests involving recombinant antigens (O'Reilly et al., 1994).

A segment of Crystal Springs isolate *C. ruminantium* DNA was amplified using the polymerase chain reaction (PCR) (Mahan et al., 1994). This portion was located between nucleotides 556 and 1336 of the published MAP 1 gene sequence (Van Vliet et al., 1994). This DNA fragment encodes a 260 amino acid open reading frame and excludes the putative signal sequence. The amplified fragment was ligated into the *Eco* RI site of the fusion protein expression plasmid pFLAG® (Eastman Kodak Co., New Haven, Connecticut, USA) described by Knappik and Pluckthun (1994). The recombinant gene thus encoded a vector-contributed aminoterminal octapeptide tag (FLAG® peptide) fused in the correct reading frame to the sequence of the predicted mature MAP 1 polypeptide. A second round of PCR was conducted to amplify the FLAG®-MAP 1 sequence such that it was preceded by an initiation codon and an 18-nucleotide 5′ sequence containing a *Not* I restriction site. The resulting 823 base-pair (bp) fragment encoded a 271 amino acid polypeptide with a predicted molecular weight of 29.3 kDa. This fragment was ligated into the *Not* I site of pVL 1392, a baculovirus polyhedrin gene replacement plasmid (O'Reilly et al., 1994), to yield pBAC-MAP 1. The latter was cotransfected with baculoviral DNA into *Spodoptera frugiperda* (SF-9) insect cells (Invitrogen, Inc., San Diego, California USA) as described by Kitts et al. (1990). A plaque-purified recombinant baculovirus (MAP 1-BV) was verified by PCR to contain the recombinant MAP 1 gene. The MAP 1-BV-infected SF-9 cell monolayers were evaluated by immunoperoxidase assay (Katz et al., 1995) to confirm recombinant MAP 1 protein expression. Immunoblots of infected cell extracts (Sambrook et al., 1989), reference heartwater polyclonal and monoclonal antibodies, were used to confirm the heartwater-related identity of the recombinant FLAG® peptide-MAP 1 fusion protein (Fig. 1).

Sera were diluted 1:35 (v/v) with 50mM Tris-buffered saline, pH 7.5, (Sigma Chemical Co., St. Louis, Missouri, USA) prior to immunoblot analysis. Immunoblot antigen was a 0.1% Triton X-100 (Biorad, Inc., Hercules, California, USA) extract of SF-9 cell cultures infected 72 hr previously with MAP 1-BV at a multiplicity of infection (MOI) of 1. The method of extract preparation and subsequent denaturing polyacrylamide gel electrophoresis (SDS-PAGE), semi-dry electrophoretic transfer, and immunoblotting were performed as described by Katz et al. (1995), using 14% polyacrylamide gels (Novex, Inc., San Diego, California) and 0.2 μm nitrocellulose membranes (Schleicher and Schuell, Inc., Keene, New Hampshire, USA). Bound anti-MAP 1 or anti-FLAG® antibodies were detected using a protein G-horseradish peroxidase conjugate (Zymed, Inc., San Francisco, California) and 4-chloronaphthol substrate (Kirkegaard and Perry, Inc., Gaithersburg, Maryland, USA).

Association of positive immunoblot results with the species of origin of the sera was analyzed using the chi-square test, considering $P < 0.01$ as the criterion for significance (Snedecor and Cochran, 1967). Evaluation of indi-

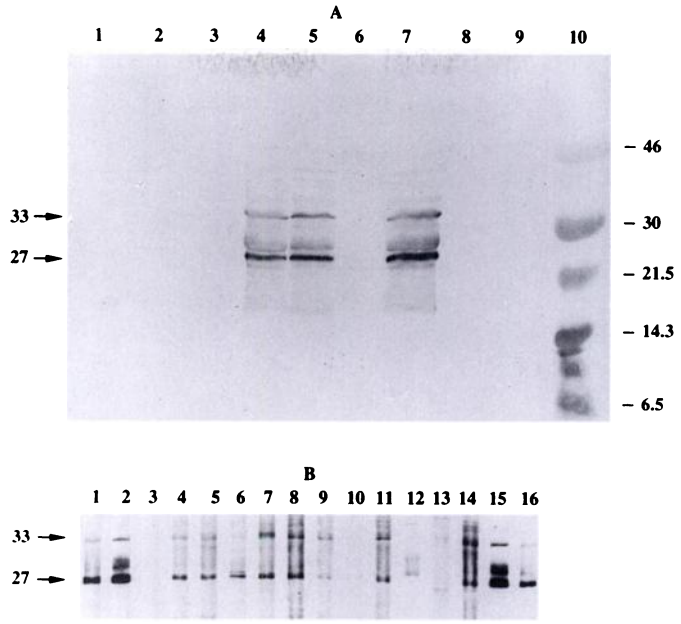

FIGURE 1. Panel A: Immunoblot analysis of *C. ruminantium* major antigenic protein (MAP 1) fusion protein expression in SF-9 cell cultures. Lanes 1 to 5: Cells grown in serum-free medium. Lane 1: Uninfected SF-9 cell homogenate. Lanes 2 and 3: Cell culture fluids taken 48 and 96 hr, respectively, after infection with recombinant MAP 1 baculovirus. Lanes 4 and 5: Cell pellets from same cultures used in Lanes 2 and 3 respectively. Lanes 6 to 9: Cells grown in medium containing 5% (v/v) fetal bovine serum. Lanes 6 and 7: Culture fluids and cell pellet, respectively, 72 hr after infection with recombinant MAP 1 baculovirus. Lanes 8 and 9: Culture fluids and cell pellet, respectively, 72 hr after infection with nonrecombinant wild type baculovirus as an additional negative control for specific identity of recombinant protein. Lane 10: Molecular size standards in kilodaltons (kDa). Blot is probed with a 1:100 dilution of convalescent sheep heartwater reference antiserum. Arrows in Panels A and B are used to denote major 27 kDa and 33 kDa forms of recombinant. Panel B: Immunoblot analysis of recombinant MAP 1 using reference and field-origin sera. Lane numbers refer to the different sera used as listed below. Lanes 1 and 16: Identical 4F10-B4 monoclonal antibody directed against the 260 amino acid MAP 1-specific portion of the recombinant protein. Lanes 2 and 15: Identical anti-FLAG® peptide monoclonal antibody directed against the vector-encoded octapeptide tag fused to the aminoterminal end of the recombinant protein. Lanes 1, 2, 15 and 16 provide confirmation of the antigenic identity of the recombinant MAP 1 fusion protein used in the immunoblot. They are placed as pairs on each end of the blot to serve as positional references. They also monitor the uniformity of vertical electrophoretic migration of antigen across the width of the gel which was used in preparing this immunoblot. Lane 3: Normal sheep serum. Lanes 4 and 5: Two different *E. canis* reference antisera. Lane 6: African sheep serum with false-positive reaction. Lanes 7, 8, 9 and 11: Sera from four southeastern U.S. white-tailed deer considered falsely positive to recombinant MAP 1. Lane 10: Normal white-tailed deer serum. Lanes 12 and 13: Bovine and cervine sera, respectively, reactive to residual SF-9 cell culture proteins. Lane 14: Human *E. chaffeensis* antiserum.

rect immunofluorescence (IFA) anti-*E. chaffeensis* geometric mean titer (GMT) differences between MAP 1 immunoblot-positive and immunoblot-negative groups of deer was conducted using Student's *t*-test at a $P < 0.01$ level of significance (Snedecor and Cochran, 1967).

RESULTS

Based on immunoblot analysis, SF-9 cells infected with MAP 1-BV expressed proteins were specifically immunoreactive with anti-MAP 1 and anti-FLAG® antibodies. Two dominant (27 kDa and 33 kDa) protein species were observed (Fig. 1A). This pattern was evident 36 hr after virus-cell culture inoculation, was stable thereafter for at least another 60 hr, and was unaffected by the presence or absence of fetal bovine serum in the cell culture media (Fig. 1A).

Cowdria ruminantium reference positive and negative sera reacted as expected; (Fig. 1B, Table 1). The ovine false-positive sera from Zimbabwe were also positive using the recombinant MAP 1 antigen (Fig. 1B, Table 1). All other reference antisera were negative to the recombinant antigen by immunoblot except for both *E. canis* antisera (canine origin) and one of eight *E. chaffeensis* antisera (human origin, IFA titer >25,000) (Table 1). However, 12 of the 79 southeastern U.S. deer sera were immunoblot-positive to recombinant MAP 1. The anti-*E. chaffeenis* geometric mean IFA titer (GMT) of these 12 cervine sera was 360, whereas the GMT of the other 67 southeastern deer sera was 100; this difference was significant (Student's *t*-test, $P < 0.01$). None of the 12 MAP 1 immunoblot-positive cervine sera had been found previously reactive to *A. marginale*. None of the sera from Michigan deer or the 406 bovine sera was positive by immunoblot to the recombinant MAP 1 antigen, although some reacted to SF-9 cell proteins of clearly different molecular weights (Fig. 1B, Lanes 12 and 13). The species association of MAP 1-positive immunoblot results with southeastern deer sera but not southeastern cattle sera was statistically significant (chi-square test; $P < 0.01$).

DISCUSSION

Cowdria ruminantium MAP 1 was expressed as a recombinant fusion protein in baculovirus-infected SF-9 insect cells and identified using monoclonal and polyclonal heartwater reference antibodies and a monoclonal anti-FLAG® peptide antibody. Two major recombinant protein species were observed. This was evidence that the baculovirus-insect cell system translated and post-translationally modified the recombinant protein molecule to different degrees of completion or along multiple metabolic pathways. Similar multiple expression products of a single gene have been observed in other baculovirus expression systems (Nene et al., 1995). The metabolic pathways for this phenomenon are not well defined (O'Reilly et al., 1994).

Cowdria ruminantium infection elicits a strong, prolonged serologic response to native MAP 1 (Mahan et al., 1993). The MAP 1 is the serologically dominant antigen common to all isolates of *C. ruminantium*, and this increases its potential diagnostic value (Jongejan and Thielmans, 1989). However, native MAP 1 possesses determinants shared with the homologous proteins of closely related Ehrlichieae (Jongejan et al., 1993). These epitopes are probably among those responsible for the false positive heartwater responses observed in epizootiologic investigations of Caribbean and African livestock believed to be heartwater-free (Jongejan et al., 1991; Mahan et al., 1993; Du Pleiss et al., 1994). Not surprisingly then, the recombinant MAP 1 antigen was reactive by immunoblot with an extremely high-titered (1:25,000) *E. chaffeensis* antiserum, with both *E. canis* antisera and with Zimbabwean sheep sera believed to be falsely immunoreactive to native MAP 1. The lack of recombinant MAP 1 reactivity with reference WSU 86-1044 hyperimmune sera was somewhat unexpected because these sera crossreact strongly with native whole *C. ruminantium* antigen (Dilbeck et al., 1990). Perhaps further recombinant subunit expression of MAP 1 gene fragments might yield a protein expressing only heartwater-specific serodiagnostic epitopes.

In contrast to earlier studies of African and Caribbean livestock (Du Pleiss et al., 1993; Mahan et al., 1993), we observed no false positive reactions between MAP 1 and antibodies in any of the 406 American and Puerto Rican cattle sera. Most of these cattle originated from the southeastern and gulf coastal United States, areas where *C. ruminantium* could become established in the resident *Amblyomma* spp. tick vector populations. Thus, the unknown serologically crossreactive Ehrlichieae present in some African and Caribbean livestock may be absent in U.S. cattle located in the southeastern states and Puerto Rico. If so,

the recombinant MAP 1 fusion protein reported here may be diagnostically useful for heartwater surveillance within the U.S. cattle population. Unexpectedly, 12 of the sera from deer located in the same southeastern areas reacted strongly with the recombinant MAP 1 by immunoblot. The anti-*E. chaffeensis* IFA titers of all the southeastern deer sera had been determined previously (Lockhart et al., 1996). We found that the anti-*E. chaffeensis* GMT of the 12 MAP 1 immunoblot-positive deer sera was significantly higher than the anti-*E. chaffeenis* GMT of the other 67 deer sera which were immunoblot-negative to MAP 1. Additional sampling of northern white-tailed deer populations would help determine if the absence of MAP 1 reactivity in Michigan deer sera was peculiar to that group of animals or was perhaps related to the regional absence of tick species capable of transmitting *E. chaffeensis* to white-tailed deer, such as *Amblyomma americanum*. Experimentally, *E. chaffeensis* infects white-tailed deer (Dawson et al., 1994a), and perhaps this or a closely related organism (Dawson et al., 1994b) was responsible for the observed MAP 1 cross-reactivity. Whether the significant serologic differences seen between southeastern U.S. cattle and deer in this study result from a species barrier to infection or to microgeographic, ecological, or management differences between the species remains a subject for further study. Future development of heartwater serodiagnostic tools for the United States should take into account possible differences in the Ehrlichieae found there compared with those found in Africa and the Caribbean. There may also be species barriers between cattle and deer or other wildlife species within the United States with respect to infections with as-yet-unidentified Ehrlichieae antigenically related to *C. ruminantium*.

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