Characterization of the Major Antigenic Protein 2 of *Ehrlichia canis* and *Ehrlichia chaffeensis* and Its Application for Serodiagnosis of Ehrlichiosis‡

Tamece T. Knowles,1 A. Rick Alleman,2* Heather L. Sorenson,2 David C. Marciano,1 Edward B. Breitschwerdt,3 Shimon Harrus,4 Anthony F. Barbet,1 and Myriam Bélanger1†

Departments of Pathobiology1 and Physiological Sciences,2 College of Veterinary Medicine, University of Florida, Gainesville, Florida 32610; Department of Companion Animal and Special Species Medicine, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina 27606; and School of Veterinary Medicine, The Hebrew University of Jerusalem, Rehovot, Israel 76100

Received 11 September 2002/Returned for modification 27 November 2002/Accepted 11 March 2003

Canine monocytic ehrlichiosis, caused by *Ehrlichia canis* or *Ehrlichia chaffeensis*, can result in clinical disease in naturally infected animals. Coinfections with these agents may be common in certain areas of endemicity. Currently, a species-specific method for serological diagnosis of monocytic ehrlichiosis is not available. Previously, we developed two indirect enzyme-linked immunosorbent assays (ELISAs) using the major antigenic protein 2 (MAP2) of *E. chaffeensis* and *E. canis*. In this study, we further characterized the conservation of MAP2 among various geographic isolates of each organism and determined if the recombinant MAP2 (rMAP2) of *E. chaffeensis* would cross-react with *E. canis*-infected dog sera. Genomic Southern blot analysis using digoxigenin-labeled species-specific probes suggested that map2 is a single-copy gene in both *Ehrlichia* species. Sequences of the single map2 genes of seven geographically different isolates of *E. chaffeensis* and five isolates of *E. canis* are highly conserved among the various isolates of each respective ehrlichial species. ELISA and Western blot analysis confirmed that the *E. chaffeensis* rMAP2 failed to serologically differentiate between *E. canis* and *E. chaffeensis* infections.

Canine monocytic ehrlichiosis, caused by *Ehrlichia canis*, is a debilitating tick-borne disease that has been reported throughout the United States and most of the world. Infection with *E. canis* may result in extensive morbidity and mortality (18). Nonspecific clinical and hematological abnormalities, such as dyspnea, anemia, leukopenia, and thrombocytopenia, are often observed during *E. canis* infections (14). Infection with *Ehrlichia chaffeensis*, the causative agent of human monocytic ehrlichiosis, is also known to occur in dogs (14). Although natural infections with *E. chaffeensis* have been observed in dogs with clinical findings consistent with ehrlichiosis, a causal relationship has not been clearly established (5).

The immunofluorescent-antibody (IFA) test is the method most widely used to diagnose *E. chaffeensis* and *E. canis* infections and is generally considered to be the “gold standard” for serologic diagnosis (20). However, the results are often subjective and nonspecific due to cross-reacting epitopes between species (5, 19). In addition, the IFA test is time-consuming, requires cultivation and purification of the whole organism, and cannot test multiple samples simultaneously. The use of recombinant proteins may improve test specificity because of a lack of cross-reaction with proteins common to several species of organisms, such as heat shock proteins (20).

There have been several reports evaluating recombinant antigenic proteins for the diagnosis of *E. canis* and *E. chaffeensis* infections including P28 (22) and P120 (21) of *E. chaffeensis* and P30 (16) and P43 (15) of *E. canis*. Some of these antigens have been shown to contain species-specific epitopes (15, 21, 23). Recently, the recombinant major antigenic protein 2 (rMAP2) of *E. canis* and that of *E. chaffeensis* were evaluated to determine the potential of each as a serodiagnostic test antigen (1, 2). Both assays were able to accurately distinguish *E. canis*-infected dogs from noninfected and *E. chaffeensis*-infected people from noninfected people.

The map2 genes of *E. canis* and *E. chaffeensis* have significant homology to the map2 of *Ehrlichia ruminantium* (4) and the msp5 of *Anaplasma marginale* (17). The map2 of *E. ruminantum* and the msp5 of *A. marginale* have been shown to be single-copy genes that are highly conserved among the geographically different isolates of each respective species (4, 17).

In this study, we further characterize the MAP2s of *E. canis* and *E. chaffeensis* to define MAP2’s suitability as a diagnostic test antigen by determining if the protein is encoded by a single-copy gene and if this gene is highly conserved within each species. In addition, the *E. canis* rMAP2 enzyme-linked immunosorbent assay (ELISA) showed no cross-reactivity when serum from dogs infected with *Anaplasma platys*, *Ehrlichia ewingii*, *Neorickettsia risticii*, *Rickettsia rickettsii*, *Bartonella vinsonii*, *Mycoplasma haemocanis*, and *Neospora caninum* was tested (2). However, cross-reactivity studies using the rMAP2s of *E. canis* and *E. chaffeensis* have not been performed. In this study, we also evaluate the potential for serologically distin-
guising between E. canis and E. chaffeensis infections using the rMAP2.

MATERIALS AND METHODS

Origins and cultivation of E. chaffeensis and E. canis isolates. Chromosomal DNA from four canine E. canis isolates was used (Florida, DJ [North Carolina], 5), Jelleybean [Virginia], and Israel no. 611 [13]). Three human E. chaffeensis isolates from Florida, but from different counties (Wakulla, Liberty, and Osceola), were obtained from Roman Reddy (Kans State University, Manhattan, Kans.). DNA from three canine E. chaffeensis cases from North Carolina (Shasta, Cherokee, and Jack) (5) was also used. E. chaffeensis Wasuka, Liberty, and Osceola were cultivated in the DH82 canine macrophage cell line in Eagle’s minimal essential medium containing 2 mM -glutamine and 10% fetal bovine serum at 37°C with 5% CO₂ (9). E. canis Oklahoma was cultivated as previously described (2). Uninfected DH82 cells were cultivated under the same conditions as those for the infected macrophages.

Ehrlichia purification and DNA preparation. DH82 cells containing either E. chaffeensis or E. canis were harvested at 90 to 100% infectivity and purified by differential centrifugation as previously described (8). Briefly, the organisms were purified from host cell components by centrifugation through 15% meglumine diatrizoate and diatrizoate sodium (RenoCal-76; Bracco Diagnostics, Inc., Princeton, NJ) or phosphate-buffered saline (PBS; 5.2 mM KH₂PO₄, 8.1 mM Na₂HPO₄, 116 mM NaCl [pH 7.0]) at 22,000 × g for 20 min. Final pellets were resuspended in buffer 1 from the Qiagen, Inc. (Valencia, Calif.) genomic DNA buffer set containing 100 μg of RNase/ml. Genomic DNAs of purified E. chaffeensis and E. canis, as well as DNA from uninfected DH82 cells, were purified with the Qiagen genomic DNA purification kit according to the manufacturer’s instructions.

PCR amplification and cloning of the map2 genes. Target DNA (10 to 200 ng) was amplified with 2.5 U of cloned Pfu polymerase (Stratagene, La Jolla, Calif.) in a mixture containing 0.5 μM concentration of each appropriate primer, 2 mM deoxyxycydosine triphosphates, 200 mM Tris-HCl (pH 8.8), 100 mM KCl, 100 mM (NH₄)₂SO₄, 1% Triton X-100, and 1 mg of nuclease-free bovine serum albumin (BSA)/ml. PCR amplification of the map2 genes from E. chaffeensis was performed with primers AB620 and AB623 (4) under the following conditions: 94°C for 5 min; 30 cycles of denaturing at 93°C for 30 s, annealing at 45°C for 1 min, and extension at 72°C for 2.5 min; and a final elongation step at 72°C for 10 min. Amplification of the E. canis map2 gene was performed as previously described (1) with primers AB620 and AB623 (4). Amplifiers were synchronized by gel electrophoresis on a 1% agarose gel in 1× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM disodium EDTA). The High Pure PCR purification kit (Roche Molecular Biochemicals, Indianapolis, Ind.) was used to purify PCR products for E. canis (Florida, Israel no. 611, DJ, and Jelleybean isolates) and E. chaffeensis (Liberty isolate) prior to sequencing. Tag DNA polymerase (Sigma Chemical Co., St. Louis, Mo.) was added to the E. chaffeensis Wasuka and Osceola PCR mixtures, followed by a 10-min incubation at 72°C, to prepare the amplon for ligation into the pCR2.1 TOPO TA vector (Invitrogen Corporation, Carlsbad, Calif.) by the addition of an A residue to the 3' ends. The altered 1.3-kb amplon was electrophoresed on a 1% low-melting-point agarose gel. The desired band was excised and melted at 65°C. The amplon was ligated into the pCR2.1 TOPO TA vector by following the manufacturer’s instructions and transformed into competent Escherichia coli (One Shot cells; Invitrogen Corp.). Transformants were grown on Luria-Bertani (LB) agar plates containing ampicillin (50 μg/ml) and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside/ml overnight. Blue and white selection was used to select colonies with an insert. White colonies were selected and incubated in LB broth with ampicillin (50 μg/ml) overnight at 37°C. Plasmids were recovered and purified with the Qiagen plasmid purification kit. Plasmids were digested with EcoRI and analyzed on agarose gel prior to sequencing.

DNA sequencing and analysis. The DNA sequences of both strands of the 1.3-kb inserts of the pCR2.1 TOPO TA vector and of the purified PCR products were determined by the DNA Sequencing Core Laboratory (Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville). Sequences were analyzed with UNIX and SeqWeb Genetics Computer Group (GCG) programs (University of Wisconsin, Madison) and aligned by the Pile-up method.

Southern blot analysis. Digoxigenin (DIG)-labeled DNA probes were generated by PCR amplification of the map2 gene by using purified DNA from E. chaffeensis clone K (1) and E. canis clone 8a (2). Primers AR3A and AR4A were used (1), and primers AR5A and AR6A were used for E. canis (Melton et al.). Genomic DNA of E. chaffeensis (Wasuka) or E. canis (Oklahoma) as well as control DH82 DNA (1 to 2 μg) was digested with restriction enzymes BamHI, EcoRI, Clal, and VspI for 1 h at 37°C. The purified unlabeled PCR product (600 bp) from E. chaffeensis clone K, or E. canis clone 8a was used as a positive control. The digested DNA was electrophoresed on a 1% agarose gel, transferred to a positively charged nylon membrane (Boehringer Mannheim, Indianapolis, Ind.) by capillary transfer, and hybridized with a map2 DIG-labeled probe diluted in a mixture containing 5% SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate), 2% blocking solution, 0.1% Na-lauroylsarcosine, 50% formamide, and 0.02% sodium dodecyl sulfate (SDS) hybridization solution. Hybridization was performed overnight at 37°C under low-stringency conditions. The membrane was washed twice for 5 min at room temperature in 2× SSC-0.1% SDS and twice for 15 min at 2× SSC at 0.5× SSC-0.1% SDS. The hybridized probes were detected with chemiluminescence and exposed to Hyperfilm (Amersham International plc, Little Chalfont, Buckinghamshire, England) at room temperature.

Sera. Twenty-five serum samples from the College of Veterinary Medicine, North Carolina State University, Raleigh, and the College of Veterinary Medicine, University of Florida, Gainesville, were tested for antibodies against the rMAP2 of E. canis and E. chaffeensis. Five of the 25 samples were IFA-negative sera from clinically healthy dogs during well-patient visits or preinfected sera from experimentally infected dogs. The remaining 20 samples were IFA positive. Eighteen of the IFA-positive samples were obtained from dogs experimentally infected with E. canis in a previous study performed at North Carolina State University (6). Two of the IFA-positive samples were obtained from naturally infected dogs that presented clinical signs consistent with canine ehrlichiosis. E. chaffeensis IFA-positive human serum was used as a positive control for the E. chaffeensis rMAP2. This sample was obtained from an individual with a history of tick exposure and clinical and laboratory findings consistent with human monocytic ehrlichiosis (1). A horseradish peroxidase (HRP)-labeled antihuman antibody (anti-His (C-term)-HRP, Invitrogen Co.) was used as a positive control for the rMAP2 immunoblotting.

E. canis and E. chaffeensis rMAP2 production and purification. Production, purification with imidazole and pH elution buffer (20 mM NaPO₄, 500 mM NaCl [pH 4.0]), and analysis by SDS-polyacrylamide gel electrophoresis of the rMAP2s of E. canis and E. chaffeensis were performed as previously described (1).

Indirect ELISA for cross-reactivity analysis. ELISAs were performed as previously described with purified rMAP2 from E. canis or E. chaffeensis as the test antigen (1, 2). Briefly, each well of polystyrene microtiter plates (Maxi Sorp; Nunc, Roskilde, Denmark) was coated with 100 μl of purified rMAP2 of E. canis or E. chaffeensis (2 μg/ml) in 0.1 M carbonate-bicarbonate buffer (pH 9.6; Sigma Chemical Co.), and microtiter plates were incubated overnight at 4°C. The wells were then washed four times with wash buffer containing 1× PBS and 0.5% (vol/vol) Tween 20 and blocked for 60 min at room temperature with 1% (wt/vol) bovine serum albumin in 1× PBS. The plates were washed four times as described above and incubated for 60 min at room temperature with test sera. Samples from 18 dogs experimentally infected with E. canis were tested simultaneously and in triplicate at a dilution of 1:500, with each rMAP2 as the antigen. Uninfected canine serum was used as a negative control for the E. canis rMAP2 ELISA. Uninfected human serum and serum from an E. chaffeensis-infected individual were used as negative and positive controls, respectively, for the E. chaffeensis rMAP2 ELISA. Alkaline phosphatase-conjugated goat anti-human immunoglobulin G (whole molecule; Sigma Chemical Co.) or alkaline phosphatase-conjugated rabbit anti-dog immunoglobulin G (whole molecule; Sigma Chemical Co.) was used as the secondary antibody at a dilution of 1:5,000 in 1% (wt/vol) BSA in 1× PBS with the substrate p-nitrophenylphosphate (1 mg/ml; Sigma Chemical Co.). Absorbance at 405 nm was measured with a Rainbow plate reader (Tecan U.S. Inc., Durham, N.C.). An absorbance reading of >0.70 was considered indicative of a positive reaction (11).

Western immunoblot analysis. Western immunoblotting was performed as previously described (1, 2) to verify the reactivity of sera experimentally infected with E. canis with the rMAP2s of E. canis and E. chaffeensis. Briefly, E. canis rMAP2 was dissolved in 3× sample buffer containing 7.5% β-mercaptoethanol and heat denatured at 100°C for 3 min prior to electrophoresis on SDS–10% polyacrylamide gels. E. chaffeensis rMAP2 was dissolved in 3× sample buffer without β-mercaptoethanol and was not heat denatured. Serum samples from dogs experimentally infected with E. canis were used at a dilution of 1:500 and reacted with rMAP2s of E. canis or E. chaffeensis. Noninfected human or dog serum was used as a negative control at a dilution of 1:300. The secondary antibody, HRP-conjugated goat anti-human or rabbit anti-dog immunoglobulin G (Sigma Chemical Co.), was used at a dilution of 1:15,000. The bound antibody was visualized by chemiluminescence using hyperfilm (Amersham International plc).
RESULTS

Sequencing and analysis of the map2 gene. From comparing sequence data, it was found that geographic isolates within each species differed at only a single nucleotide position. The map2 genes from three human isolates of E. chaffeensis (Arkansas [GenBank accession no. AF117731], Osceola, and Liberty) contained a thymine at position 243, while the Wakulla isolate had a cytosine at this position. The map2 genes from three canine cases of E. chaffeensis infection (Jack, Cherokee, and Shasta) were identical to those of the Arkansas, Osceola, and Liberty strains of the human isolate. A thymine was detected at position 549 in the Virginia and North Carolina E. canis isolates (Jellybean and DJ, respectively), while the Oklahoma (GenBank accession no. AF117730), Israel no. 611, and Florida strains had a cytosine at this position. None of the nucleotide substitutions observed within either species translated into any differences in amino acid sequences. A comparison of the MAP2 amino acid sequences of E. canis (Oklahoma) and E. chaffeensis (Arkansas) revealed that there is a 79% identity between the two proteins (Fig. 1). However, a 19-amino-acid peptide sequence of higher heterology between the two species was also identified (Fig. 1).

Southern blot analysis. Genomic DNA of E. canis and E. chaffeensis was cut with restriction enzymes VspI, BamHI, ClaI, and EcoRI. When digested genomic DNA was hybridized with a DIG-labeled species-specific map2 probe, single bands of 2.5, 12.2, and 5.0 kb were recognized in E. chaffeensis DNA digested with VspI, BamHI, and ClaI, respectively (Fig. 2). Similarly, single bands measuring 0.5, 12.2, and 11.0 kb were recognized by using the species-specific probe when E. canis DNA was digested with VspI, BamHI, and ClaI, respectively (Fig. 2). A single hybridized band was expected if a single-copy gene encoded the MAP2. These enzymes do not cut within the known sequence of the map2 gene. EcoRI digestion generated two fragments in both E. chaffeensis (12.2 and 3.9 kb) and E. canis (6.1 and 1.5 kb) (Fig. 2). This was also expected if a single-copy gene encodes the MAP2, since one internal EcoRI site is present in each of the genes.

Cross-reactivity analysis. All of the 18 serum samples from dogs experimentally infected with E. canis reacted strongly with the rMAP2s of E. canis and E. chaffeensis. Lanes 1 to 3 (counting from the left), E. canis rMAP2 reacted with the antihistidine control (lane 1). IFA-negative canine serum from an uninfected animal used as a negative control (lane 2), and IFA-positive immune serum from an experimentally infected dog used as a positive control (lane 3); lane 4, E. chaffeensis rMAP2 reacted with E. canis-infected serum. Molecular size standards (in kilodaltons) are given on the left.

FIG. 1. Alignment of amino acid sequences of E. canis (can) and E. chaffeensis (chaff) MAP2 proteins. Dashes, identical amino acids; boldface, region predicted to be an antigenic determinant by the PEPTIDE STRUCTURE program (GCG) (12).

FIG. 2. Genomic Southern blot analysis of E. chaffeensis (left) and E. canis (right) DNA with DIG-labeled map2 species-specific gene probes. Lanes map2, map2 gene amplified by PCR with species-specific primers (positive control); lanes DH82, uninfected DH82 macrophage cell DNA (negative control) cut with VspI. The other lanes show genomic DNA of E. chaffeensis or E. canis cut with BamHI, VspI, EcoRI, and ClaI. Numbers on the left of each panel indicate molecular sizes based on a 1-kb ladder (Invitrogen).

FIG. 3. Western blot analysis of cross-reactivity between serum from an E. canis-infected dog with rMAP2s of E. canis and E. chaffeensis. Lanes 1 to 3 (counting from the left), E. canis rMAP2 reacted with the antihistidine control (lane 1). IFA-negative canine serum from an uninfected animal used as a negative control (lane 2), and IFA-positive immune serum from an experimentally infected dog used as a positive control (lane 3); lane 4, E. chaffeensis rMAP2 reacted with E. canis-infected serum. Molecular size standards (in kilodaltons) are given on the left.
DISCUSSION

We have further characterized the MAP2 of E. chaffeensis and E. canis. Species-specific DIG-labeled map2 probes hybridized to the map2 genes in the E. chaffeensis and E. canis genomes. Southern blot analyses were performed under low-stringency conditions to ensure that the species-specific probes would identify all possible copies of the map2 gene, despite any sequence differences in the copies. The hybridization patterns observed using each restriction enzyme suggest that, like the map2 of E. ruminantium (4) and the msp5 of A. marginale (17), the map2 genes of E. chaffeensis and E. canis are single-copy genes. Since many organisms use multigene families to produce antigenic diversity, this indicates that there is not likely to be antigenic variation of map2 among different strains of the organism.

The conservation of the map2 gene was evaluated by comparing nucleotide sequences from seven geographically different isolates of E. chaffeensis and five isolates of E. canis. The map2 genes were highly conserved among the various isolates of each respective bacterial species. In fact, translated amino acid sequences for all isolates within each species were identical. The MAP2 proteins of E. ruminantium (4) and the MSP5 proteins of A. marginale (17) were also highly conserved among the various isolates within their respective species, suggesting that this protein may be important for the metabolic function and sustainment of the microorganisms.

The function of the MAP2s of E. chaffeensis and E. canis is still unknown. The translated amino acid sequence of MAP2 is homologous to that of the SCO2 protein precursor found in Rickettsia prowazekii (3) (E value: 2e-23; accession no. F71663 [Protein Identification Resource database]). The SCO2 protein of R. prowazekii is homologous to a protein located in the mitochondrial inner membrane of mammalian cells that is involved in mitochondrial organization as well as respiration. Defects in SCO2 result in decreased activity in cytochrome oxidase (7, 10). The fact that this function is vital to cell viability may explain why the ortholog of this gene is found in E. canis, E. chaffeensis, A. marginale, and E. ruminantium.

There is a high percentage of identity (79%) between the amino acid sequences of E. canis and E. chaffeensis MAP2s. Because this indicates a high probability of epitope sharing, cross-reactivity experiments were performed. All serum samples from dogs experimentally infected with E. canis contained antibodies that recognized the rMAP2 of E. chaffeensis. Western blot analysis confirmed that the E. canis-infected dog serum reacted strongly with both the E. canis and E. chaffeensis rMAP2 antigens, demonstrating that the rMAP2s of E. canis and E. chaffeensis have shared epitopes.

Previously we reported that the rMAP2 of E. chaffeensis contained structurally dependent epitopes that were destroyed by boiling, SDS, or β-mercaptoethanol (1). However, this work was done using serum samples from people naturally infected with E. chaffeensis. Subsequently, we reported that, when E. canis rMAP2 and serum from naturally and experimentally infected dogs were used, epitopes on the E. canis rMAP2 were easily identified on Western blots (2). In the present study, rMAP2 from E. chaffeensis was reacted with immune serum from a dog experimentally infected with E. canis. We conclude that there are shared epitopes between rMAP2s of E. canis and E. chaffeensis and that at least some of the epitopes recognized by serum from dogs infected with E. canis are not structurally dependent. Additionally, the immune response in dogs experimentally infected with large numbers of organisms may be of greater magnitude than that in people with natural infections with E. chaffeensis.

A small region in the MAP2 amino acid sequences (positions 32 through 50) of E. canis and E. chaffeensis was predicted to be an antigenic determinant by the PEPTIDE STRUCTURE program (GCG) (12). In addition, these segments are areas of concentrated heterology between the two proteins, with different amino acids in 11 of the 19 positions (42% homology).

In this study, we determined that a highly conserved single-copy gene encodes E. canis and E. chaffeensis MAP2s. The rMAP2s of E. chaffeensis and E. canis can be used for the serodiagnosis of canine ehrlichiosis but failed to serologically distinguish between infections. However, the sensitivity of the rMAP2 would not be affected by geographic location, as we did not detect any genetic variants. Future studies are needed to determine if epitope differences between E. chaffeensis and E. canis MAP2s could be targeted to develop a serologic assay that might rapidly distinguish between E. canis and E. chaffeensis infections. Such a test would be convenient, cost-effective, and useful for clinical diagnosis of ehrlichiosis. The prevalence of E. chaffeensis infection in dogs is currently unknown, and the development of such an assay could be a valuable tool for epidemiological studies to determine the importance of dogs as reservoirs for E. chaffeensis infections.

ACKNOWLEDGMENTS

This work was supported by a grant from the University of Florida, College of Veterinary Medicine, Department of Sponsored Research, UPN 01031471.

We thank Donald J. Forrester, John Harvey, and Patrick Meeus for critical review of the manuscript.

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