Characterization of *Anaplasma phagocytophilum* Major Surface Protein 5 and the Extent of Its Cross-Reactivity with *A. marginale* 

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Major surface protein 5 (Msp5) of *Anaplasma marginale* is highly conserved in the genus *Anaplasma* and the antigen used in a commercially available competitive enzyme-linked immunosorbent assay (cELISA) for serologic identification of cattle with anaplasmosis. This study analyzes the degrees of conservation of Msp5 among various isolates of *Anaplasma phagocytophilum* and the extent of serologic cross-reactivity between recombinant Msp5 (rMsp5) of *Anaplasma marginale* and *A. phagocytophilum*. The msp5 genes from various isolates of *A. phagocytophilum* were sequenced and compared. rMsp5 proteins of *A. phagocytophilum* and *A. marginale* were used separately in an indirect ELISA to detect cross-reactivity in serum samples from humans and dogs infected with *A. phagocytophilum* and cattle infected with *A. marginale*. Serum samples were also tested with a commercially available competitive ELISA that uses monoclonal antibody ANAF16C1. There were 100% sequence identities in the msp5 genes among all of the *A. phagocytophilum* isolates from the United States and a horse isolate from Sweden. Sheep isolates from Norway and dog isolates from Sweden were 99% identical to one another but differed in 17 base pairs from the United States isolates and the horse isolate. Serologic cross-reactivity was identified when serum samples from cattle infected with *A. marginale* were reacted with rMsp5 of *A. phagocytophilum* and when serum samples from humans and dogs infected with *A. phagocytophilum* were reacted with rMsp5 of *A. marginale* in an indirect-ELISA format. Serum samples from dogs or humans infected with *A. phagocytophilum* did not cross-react with rMsp5 of *A. marginale* when tested with the commercially available cELISA. These results suggest that rMsp5 of *A. phagocytophilum* is highly conserved among United States and European isolates and that serologic distinction between *A. phagocytophilum* and *A. marginale* infections cannot be accomplished if rMsp5 from either organism is used in an indirect ELISA.

The order *Rickettsiales* represents obligate intracellular bacteria that reside in vacuoles of eukaryotic cells, with the potential to cause fatal tick-transmitted diseases in humans and several mammalian species. Recent genetic studies reorganized some species within the order *Rickettsiales*, between the families *Rickettsiaceae* and *Anaplasmataceae* (11). Based on these studies, three organisms, formerly known as *Ehrlichia phagocytophila*, *Ehrlichia equi*, and the HGE (human granulocytic ehrlichiosis) agent, were unified as a single species and moved to the genus *Anaplasma*. These three organisms are now reclassified as *Anaplasma phagocytophilum*, the causative agent of human granulocytic anaplasmosis, an emerging tick-borne disease (6, 11).

*A. phagocytophilum* has been detected worldwide, particularly in North America and Europe as well as in South America, South America, and Asia; it infects humans, horses, ruminants, cats, dogs, and a variety of wildlife species, including rodents, deer, and carnivores (4, 9, 12, 14, 15, 16, 19, 21, 22, 23, 24, 25, 26, 27, 28, 29, 33, 34, 36, 39, 40, 41, 42). Clinical signs of infection, although differing with the species of host and the virulence, include fever, anorexia, anemia, thrombocytopenia, leukopenia, neurological signs, hepatic inflammation, abortions, and even fatalities in a small percentage of mammalian hosts. Current serologic diagnosis is most often based on an indirect immunofluorescent antibody (IFA) test that uses whole, cultured organisms as a test antigen.

The serologic diagnosis of *Anaplasma marginale* infection is based on a commercially available competitive inhibition enzyme-linked immunosorbent assay (cELISA) developed in the mid-1990s (17, 38). This highly sensitive and specific assay uses recombinant Msp5 (rMsp5) as a diagnostic antigen, along with horseradish peroxidase (HRP)-conjugated monoclonal antibody ANAF16C1, which binds to an epitope specific for Msp5 of *A. marginale* (37).

Even before the recent reclassification within the family *Anaplasmataceae*, the msp5 gene was known to be highly conserved among all *Anaplasma* species, which, at that time, in-
cluded A. marginale, A. centrale, and A. ovis (17). Based on 16S rRNA gene sequence similarity, A. phagocytophilum and A. platys were placed within the same family (11).

In this study, we investigate the conservation of the msps gene among various geographic isolates of A. phagocytophilum through cloning and sequencing of msps5, and we examine the level of cross-reactivity and the potential value of rMsp5 orthologs of A. phagocytophilum and A. marginale as test antigens for serodiagnosis of anaplasmosis.

MATERIALS AND METHODS

Source of A. phagocytophilum DNA. A. phagocytophilum genomic DNA samples were extracted from three individuals naturally infected with A. phagocytophilum (New York State) and from a human isolate that was in culture (NY18/ET2), three ovine isolates (Norway), two canine isolates (Sweden), two wood rat samples (California), and one equine isolate (Sweden).

Amplification of the msps5 gene of A. phagocytophilum. Primers which corresponded to the sequences encoding the predicted translated and processed proteins of the msps5 gene were synthesized by Biotecnologies Inc., The Woodlands, TX. Forward primer ARAR28 (5'-ACTGTGTTTCTCGGGA TCTCAGTATAGTAA 3') and reverse primer ARAR29 (5'-AGAATTTAAAG CTATTTACGAAATACAAA 3') were designed for in-frame insertion of amplicons into the pTrcHis2-TOPO vector (Invitrogen Corporation, Carlsbad, CA). The N terminus of the mature protein, without the peptide signal sequence, corresponds to nucleotide 46 of the open reading frame. Amplification was performed using Pfu DNA polymerase (Strategene, La Jolla, CA). Briefly, 10 ng/µl of genomic DNA was amplified using 0.5 µM each of primers ARAR28 and ARAR29 and 1.00 U of Pfu polymerase in 5 mM deoxynucleoside triphosphates, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, and 1.5 mM MgCl2. PCR assays were performed at 94°C for 3 min, followed by 10 cycles of denaturing at 94°C for 15 s, annealing at 43°C for 1 min, and extension at 72°C for 2 min. This was followed by 25 cycles of denaturing at 94°C for 15 s, annealing at 45°C for 1 min, and extension at 72°C for 2 min. A final extension step at 72°C was performed for 7 min. Amplicons were analyzed by gel electrophoresis on a 1% agarose gel in 1× TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM disodium EDTA).

Cloning and sequencing of A. phagocytophilum msps5. Amplicons were incubated at 72°C for 10 min in 1.0 U of Taq DNA polymerase in order to produce amplicons with the 3' A overhangs needed for ligation into the TOPO vector (Invitrogen Corporation). Cloning was performed according to the manufacturer's recommendations. Recombinant plasmids were transformed into Escherichia coli (One Shot cells; Invitrogen Corporation), and transformants were grown on Luria-Bertani (LB) agar plates in the presence of ampicillin (50 µg/ml). Colonies were selected and incubated in LB broth in the presence of ampicillin (50 µg/ml) overnight at 37°C with vigorous shaking. Plasmid DNA was extracted by a rapid miniprep method (43), reconstituted in Tris-EDTA buffer (pH 8.0) containing 1.0 µg/ml of DNase-free RNase, and analyzed on a 1% agarose gel. Recombinant clones containing the msps5 orthologs of A. phagocytophilum were digested with restriction enzyme EcoRI to ensure the correct orientation of the insert in the plasmid vector. Digested DNA was analyzed on a 1% agarose gel. The DNA sequences of both strands of the 582-bp insert of pTrcHis2-TOPO K1 were determined by the DNA Sequencing Core Laboratory at the University of Florida, Gainesville, FL. The DNA sequences of the msps5 genes from various geographic isolates of A. phagocytophilum were determined for both strands, using forward and reverse primers based on vector sequences in flanking regions. DNA sequences were compared by Seqweb (Genetics Computer Group, Madison, WI). A. phagocytophilum recombinant Msp5 production and purification. Transformed cells containing the msp5 gene ortholog of A. phagocytophilum were incubated with vigorous shaking at 37°C in Luria-Bertani broth containing 50 µg/ml ampicillin overnight. A tenfold dilution of this overnight culture was added to Terrific Broth medium (32) containing 500 mM glycolycine (13) and incubated with vigorous shaking to an optical density at 600 nm of 1.0. Protein production was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside. Cells were incubated with vigorous shaking at 27°C for an additional 16 h. Recombinant proteins were purified by immobilized metal affinity chromatography (ProBond resin; Invitrogen Corporation) and incubated under native, nondenaturing conditions, using solutions with pH levels of 7.8, 6.0, and 5.5 and eluting at pH 4.0, according to the manufacturer's recommendations. Fractions containing the rMsp5 ortholog were identified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and staining with Coomassie blue. The rMsp5 protein contained a C-terminal polyhistidine tag for purification by affinity chromatography and a C-terminal Myc tag for protein identification in immunoblot assays. The authenticity of the rMsp5 ortholog of A. phagocytophilum was evaluated by Western immunoblot analysis using an HRP-conjugated anti-Myc antibody (Invitrogen Corporation) and pre- and postinoculation sera from a dog experimentally infected with A. phagocytophilum.

Antibodies and antisera. Monoclonal antibody ANA16C1 (cELISA anaplasma antibody test kit; VMRD, Pullman, WA) was used as a positive control for the A. marginale rMsp5 ortholog in immunoblot assays. WAT24A1, a monoclonal antibody against trypanosome surface antigen, was used as a negative control in immunoblot assays and in an indirect ELISA with rMsp5 of A. phagocytophilum and rMsp5 of A. marginale. Horseradish peroxidase-labeled anti-Myc antibody (anti-Myc [C-terminal]-HRP; Invitrogen Corporation) was used as a positive control for the A. phagocytophilum rMsp5 ortholog in immunoblot assays.

Alkaline phosphatase-conjugated rabbit anti-dog immunoglobulin G (whole molecule; Sigma Chemical Co., St. Louis, MO), alkaline phosphatase-conjugated goat anti-human immunoglobulin G and immunoglobulin M (whole molecule; Jackson ImmunoResearch Laboratories, West Grove, PA), alkaline phosphatase-conjugated rabbit anti-bovine immunoglobulin G (whole molecule; Sigma Chemical Co.), and alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin G (whole molecule; Sigma Chemical Co.) were used as secondary antibodies in indirect ELISAs.

Twenty pre- and postinfection serum samples were collected from 0 to 94 days from each of two dogs experimentally infected with the NY18 strain of A. phagocytophilum (3). In addition, five serum samples from dogs naturally infected with A. phagocytophilum (one from the University of Florida and four from the Vetsuisse Faculty of Berne, Switzerland) were evaluated for antibodies to A. phagocytophilum. A. marginale. Nine postinfection serum samples from three dogs experimentally infected with a Swedish isolate of A. phagocytophilum were tested for antibodies to rMsp5 of A. phagocytophilum, using an indirect ELISA.

Seventeen serum samples from dogs naturally infected with Ehrlichia canis and 10 serum samples from dogs naturally infected with Anaplasma platys were tested for antibodies to rMsp5 of A. phagocytophilum.

Thirty-three preinfection and postinfection serum samples were obtained from 29 cattle experimentally infected with A. marginale. The cattle were inoculated with different geographical strains of A. marginale, including a Missouri isolate (n = 8), a South Dakota isolate (n = 1), a Virginia isolate (n = 3), and a Florida isolate (n = 17). Infection was confirmed by microscopic visualization of organisms in peripheral blood smears and by detection of antibodies to rMsp5 of A. marginale (cELISA anaplasma antibody test kit; VMRD). These samples were evaluated for antibodies to rMsp5 of A. phagocytophilum, using an indirect ELISA.

Thirty-five human serum samples from the Wadsworth Center, New York State Department of Health, Albany, NY, were evaluated for antibodies to the rMsp5 ortholog of A. phagocytophilum, and for antibodies to rMsp5 of A. marginale, by indirect ELISA and competitive ELISA, using a cELISA anaplasma antibody test kit (VMRD). These samples were obtained from patients who had clinical findings consistent with human granulocytic anaplasmosis and who had been previously diagnosed with A. phagocytophilum infection by demonstration of antibodies reactive with A. phagocytophilum (NY18 strain) by IFA testing and/or PCR analysis. Four serum samples from individuals naturally infected with Ehrlichia chafeensis were also tested for antibodies to rMsp5 of A. phagocytophilum.

Serum samples from uninfected, clinically healthy dogs, cattle, and humans were used to calculate cutoff values for postinfection sera at 1:100 and 1:300 dilutions for each indirect ELISA. These samples were tested by IFA for antibodies to A. phagocytophilum (canine and human) and for antibodies to A. marginale (bovine) by competitive ELISA, using a cELISA anaplasma antibody test kit (VMRD). These samples were obtained from patients who had clinical findings consistent with human granulocytic anaplasmosis and who had been previously diagnosed with A. phagocytophilum infection by demonstration of antibodies reactive with A. phagocytophilum (NY18 strain) by IFA testing and/or PCR analysis. Four serum samples from individuals naturally infected with Ehrlichia chafeensis were also tested for antibodies to rMsp5 of A. phagocytophilum.

The protein concentration of rMsp5 was determined by the Coomassie blue G dye-binding assay as previously described (30). The proteins were dissolved in a 3× sample buffer containing 0.1 M Tris (pH 6.8), 5% (wt/vol) SDS, 50% glycerol, and 0.0125% bromophenol blue, either with or without 7.5% β-mercaptoethanol. Samples were heat denatured at 100°C for 3 min prior to electrophoresis on 10% (wt/vol) SDS-polyacrylamide gels.

Immunoblot analysis. Approximately 3 µg of rMsp5 of A. marginale and A. phagocytophilum, as well as native A. phagocytophilum (NY18 strain) proteins, was loaded into each well and separated by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes (Hybond ECL; Amersham International PLC, Little Chalfont, Buckinghamshire, England) as described previously (1). The membranes were blocked for 1 h with...
FIG. 1. Nucleotide alignment of *Anaplasma phagocytophilum* msp5 from human (United States), equine (Sweden), wood rat (United States), canine (Sweden), and ovine (Norway) samples. Dashes indicate areas of homology, and asterisks indicate areas where sequence data are unavailable.

5% skim milk (wt/vol) in 1× phosphate-buffered saline (PBS) with 0.25% Tween 20 and washed with 1% (wt/vol) milk in 1× PBS with 0.25% Tween 20 as described previously (1). Membranes were probed with the anti-*A. marginale* Msp5 monoclonal antibody ANAF16C1 at a concentration of 0.01 μg/ml. The monoclonal antibody to trypanosome surface antigen, WAT24A1, was used as a negative isotype control at the same concentration. HRP-conjugated anti-Myc antibody was used as a positive control for rMsp5 of *A. phagocytophilum* at a dilution of 1:15,000. Preinfection sera and sera collected 69 days postinfection
from dogs experimentally inoculated with *A. phagocytophilum* (3) were used as negative and positive controls, respectively. Membranes were then washed with 1% (wt/vol) milk in 1× PBS as described previously (1) and reacted with a secondary antibody, horseradish peroxidase-conjugated anti-mouse immunoglobulin G (whole molecule; Sigma Chemical Co.), at a dilution of 1:15,000. Membranes were processed for enhanced chemiluminescence with detection reagents containing luminol (SuperSignal substrate; Pierce, Rockford, IL) as a substrate and were exposed to X-ray film (Hyperfilm-MP; Amersham International PLC) for visualization of the bound antibody.

**Indirect ELISA.** Polyethylene microtiter plates (MaxiSorp; Nunc, Roskilde, Denmark) were coated with 100 μl per well of purified rMsp5 ortholog of *A. phagocytophilum* (4 μg/ml) in 0.05 M carbonate-bicarbonate buffer, pH 9.6 (Sigma Chemical Co.), or with a purified rMsp5 ortholog of *A. marginale* (4 μg/ml) that was produced as previously described (2, 38) and 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 (BSA) in 1× PBS. The plates were washed four times as described above and incubated at room temperature with test sera at 1:100 or 1:300 dilution in 1% (wt/vol) BSA in 1× PBS (100 μl). The wells were again washed (four times) and incubated at room temperature for 60 min in the presence of alkaline phosphatase-conjugated goat anti-human immunoglobulin G and immunoglobulin M (whole molecule; Jackson ImmunoResearch Laboratories), alkaline phosphatase-conjugated rabbit anti-bovine immunoglobulin G (whole molecule; Sigma Chemical Co.), or alkaline phosphatase-conjugated rabbit anti-dog immunoglobulin G (whole molecule; Sigma Chemical Co.) at a dilution of 1:5,000 in 1% (wt/vol) BSA in 1× PBS. The wells were again washed (four times), and the substrate, 5-nitrophenylphosphate (1 mg/ml) in 0.05 M carbonate-bicarbonate buffer, pH 9.6 (Sigma Chemical Co., St. Louis, MO), was added at 100 μl per well and incubated for 90 min at room temperature. Absorbance was measured at 405 nm using a Tecan Rainbow plate reader (Tecan U.S. Inc., Durham, NC).

For each assay, serum samples from seven clinically healthy subjects (human, dog, or cow) were used to establish the cutoff values for determining whether a test sample was positive or negative. These samples were used at dilutions identical to those of the test sera each time an ELISA was performed. Similarly, in an indirect-ELISA format, rMsp5 of *A. marginale* and *A. phagocytophilum*, each at a concentration of 4 μg/ml, were reacted with the *A. marginale* Msp5 monoclonal antibody ANAF16C1. The monoclonal antibody to trypanosome surface antigen, WAT24A1, was used as a negative control in this assay.

**Competitive ELISA.** A cELISA anaplasma antibody test kit (VMRD) was used in cross-reactivity studies of serum samples from *A. phagocytophilum*-infected humans and dogs. The assay was performed according to the manufacturer’s recommendations. Serum samples with ≥30% inhibition were interpreted as positive for the presence of *Anaplasma* antibodies. The U.S. Department of Agriculture has approved this assay for the detection of *Anaplasma* antibodies, particularly in bovine serum samples (17, 37, 38).

**Statistical analysis.** Data analysis was performed using Microsoft Office Excel 2003 (Microsoft Corporation), SigmaStat for Windows Version 3.11 (Systat Software Inc.), and SigmaPlot 2004 for Windows Version 9.01 (Systat Software, Inc.). For each indirect ELISA, the cutoff value (absorbance reading) used to determine whether a test sample was positive or negative was established using the upper limit of the t distribution-based 99% confidence interval of the mean absorbance value for the negative-control samples. A sample was considered positive if the absorbance value was greater than the 99% confidence interval of the mean absorbance value of the negative controls.

**Results.**

The open reading frame of the *msp5* gene of *A. phagocytophilum* is 582 bp in length. The nucleotide sequences of various geographic isolates, including samples from three humans from New York State, two wood rats from California, three sheep from Norway, and one horse and two dogs from Sweden, were compared to that of the cultured *msp5* NY18E2b strain of *A. phagocytophilum* (Fig. 1). All isolates from the United States and the horse isolate from Sweden displayed 100% nucleotide sequence identities in their *msp5* genes. The sheep isolates from Norway and the canine isolates from Sweden were 99% identical to one another (differing in 8 bp), but they differed from the U.S. isolates and the equine isolate from Sweden in a total of 20 bp. When the amino acid sequences of *msp5* of *A. phagocytophilum* and *Msp5* of *A. marginale* were compared, 65.1% sequence identity and 70.8% sequence similarity were found (3).

Indirect enzyme-linked immunosorbent assays using rMsp5 of *A. marginale* were performed on 45 *A. phagocytophilum*-positive canine serum samples at dilutions of 1:100 and 1:300. All but one serum sample tested positive for antibodies to rMsp5 of *A. marginale* (Table 1). These results suggest a serologic cross-reactivity of 98%.

Thirty-five human serum samples from patients naturally infected with *A. phagocytophilum* and showing clinical signs of human anaplasmosis were tested for antibodies to rMsp5 of *A. marginale* in indirect ELISAs. Twenty-nine of the 35 human serum samples tested positive at dilutions of both 1:100 and 1:300 when evaluated by the indirect ELISA using rMsp5 of *A. marginale*. An additional five human serum samples tested positive at a dilution of 1:100 but were negative at the higher dilution (Table 1). Those results suggest a serologic cross-reactivity of 97%.

Thirty-two bovine serum samples were obtained from cattle experimentally infected with *A. marginale* and were collected...

### Table 1. Cross-reactivities between *Anaplasma phagocytophilum* and *A. marginale*

| Source of serum samples | Total no. of serum samples tested | No. of positive serum samples | % Cross-reactivity |
|-------------------------|----------------------------------|------------------------------|-------------------|
| Dogs infected with *A. phagocytophilum* reacted with rMsp5 of *A. marginale* | 45 | 44 | 98% |
| Humans infected with *A. phagocytophilum* reacted with rMsp5 of *A. marginale* | 35 | 34 | 97% |
| Cattle infected with *A. marginale* reacted with rMsp5 of *A. phagocytophilum* | 32 | 24 | 75% |

* a Cross-reactivity of serum samples from dogs experimentally and naturally infected with *A. phagocytophilum* reacted with rMsp5 of *A. marginale*, using an indirect-ELISA format.

* b Cross-reactivity of serum samples from human patients naturally infected with *A. phagocytophilum* reacted with rMsp5 of *A. marginale*, using an indirect-ELISA format.

* c Cross-reactivity of serum samples from cattle experimentally infected with *A. marginale* reacted with rMsp5 of *A. phagocytophilum*, using an indirect-ELISA format.
Cross-reactivities between *Anaplasma phagocytophilum* and related rickettsial agents

| Source of serum samples                   | Total no. of serum samples tested | No. of positive serum samples | % Cross-reactivity |
|------------------------------------------|-----------------------------------|-------------------------------|--------------------|
| Dogs experimentally infected with *Ehrlichia canis* | 17                                | 17                            | 100                |
| Dogs naturally infected with *Anaplasma platys* | 10                                | 10                            | 100                |
| Humans infected with *Ehrlichia chaffeensis* | 4                                 | 4                             | 100                

*Cross-reactivity of serum samples from dogs experimentally infected with *Ehrlichia canis*, dogs naturally infected with *Anaplasma platys*, or humans infected with *Ehrlichia chaffeensis* and with rMsp5 of *A. phagocytophilum*, using an indirect-ELISA format.*
Based on these cross-reactivity experiments, infection is likely to be detected in serum samples from animals infected with *A. phagocytophilum*, *A. marginale*, *E. chaffeensis*, *E. canis*, or *O. prey* when the whole peptide of rMsp5 of *A. phagocytophilum* is used as the diagnostic-test antigen in an indirect-ELISA format. We conclude that the use of the whole peptide of rMsp5 of *A. phagocytophilum* as a test antigen cannot distinguish between infections with *Anaplasma* spp. or *Ehrlichia* spp.

The assay currently approved by the U.S. Department of Agriculture (USDA) for screening cattle for *A. marginale* infection (cELISA anaplasma antibody test kit; VMRD) is a competitive ELISA that utilizes a monoclonal antibody, ANAFA16C1. This antibody recognizes an epitope proposed to be specific for *A. marginale* (37). In our study, sera from humans or canines infected with *A. phagocytophilum* tested negative when the anaplasma antibody test kit was used. Our results indicate that when used in its proper format (e.g., the cELISA), the rMsp5 of *A. marginale* can accurately detect infection in cattle and can distinguish between infections with *A. phagocytophilum* and infections with *A. marginale*. This is of particular importance when cattle are tested in areas where these two infections coexist.

Recombinant Msp5 of *A. marginale* has been used as a diagnostic-test antigen in an indirect-ELISA format for surveying *A. marginale* infection in cattle in several countries (7, 8, 31, 35). Our data indicate that an indirect ELISA using rMsp5 of *A. marginale* cannot distinguish between infections with *A. phagocytophilum* and infections with *A. marginale*. Cattle infected with either organism will likely be seropositive, giving rise to a false representation of disease incidence in a particular area.

The serological cross-reactivity between *A. phagocytophilum* and *A. marginale* observed with the commercially available cELISA was previously evaluated (10). In that study, *A. phagocytophilum* infection was evaluated in various species. Cattle were experimentally infected with a Swiss strain of *A. phagocytophilum*, sheep with a British (Old Sourhope) strain of *A. phagocytophilum*, and horses with a human isolate (Webster strain) of *A. phagocytophilum* from Wisconsin (10). These animals were inconsistently seropositive in the cELISA that used monoclonal antibody ANAFA16C1. It was concluded that the epitope recognized by the monoclonal antibody is not species specific for *A. marginale*. These positive results for the cELISA appeared 4 weeks after inoculation of *A. phagocytophilum*-infected cattle and eventually converted to negative states by week 10.

In our study, cross-reactivity between sera from humans and dogs infected with *A. phagocytophilum* and rMsp5 of *A. marginale* was never observed when we used rMsp5 in its cELISA format. In addition, in a Western immunoblot analysis, monoclonal antibody ANAFA16C1 did not react with recombinant or native Msp5 of *A. phagocytophilum*, but it readily identified rMsp5 of *A. marginale*. Furthermore, in an indirect ELISA, the monoclonal antibody clearly reacted with rMsp5 of *A. marginale*; however, no reactivity was observed with the use of rMsp5 of *A. phagocytophilum*.

There could be several explanations for the discrepancy between our findings and those of the previous study (10). One explanation is that experimental infection, used in the previous study, does not mimic natural infection with *A. phagocytophilum* in terms of antibody response to *A. marginale* Msp5. In addition, as indicated in the earlier paper, coinfection with *A. marginale* and *A. phagocytophilum* could have been present in the original inoculum that was used to infect the animals, resulting in an altered antibody response. Another explanation for the inhibition of the monoclonal antibody in the cELISA, and a false-positive result, could be steric hindrance of the monoclonal antibody by other antibodies directed against epitopes adjacent to the one recognized by monoclonal antibody ANAFA16C1.

In summary, we demonstrated the high conservation of Msp5 of *A. phagocytophilum* among various isolates in the United States and Europe. This whole antigen has potential as a screening tool for anaplasmosis/ehrlichiosis when used in an indirect-ELISA format. Such a serological assay would provide a time- and cost-effective means for rapid diagnosis and implementation of therapy. Because of the cross-reactivity between the Msp5 orthologs of *A. phagocytophilum* and *A. marginale*, the commercially available cELISA should be used in epidemiological studies where distinctions between these two infectious agents in cattle are necessary.

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