Evaluation of sequential coinfection with *Anaplasma phagocytophilum* and *Anaplasma marginale* in cattle

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**Objective**—To determine whether sequelae of infection differed among single versus double infection with *Anaplasma phagocytophilum* or *Anaplasma marginale*, with and without tick salivary extract, in cattle.

**Animals**—Eighteen 13-month-old steers.

**Procedures**—Treatment groups of 3 cattle each included *A marginale* inoculated ID followed on day 35 by *A phagocytophilum* without tick saliva, *A phagocytophilum* followed on day 10 by *A marginale* without tick saliva, *A marginale* followed on day 35 by *A phagocytophilum* with tick saliva, *A phagocytophilum* followed on day 10 by *A marginale* with tick saliva, tissue culture control injection, and tick saliva control injection. Infection was monitored via clinical observations, CBC, serologic testing, and PCR analysis of blood and tissues.

**Results**—Infected cattle had significantly reduced weight gain. Anemia occurred 25 to 32 days after *A marginale* infection, which was attenuated by tick saliva. Parasitism was greater if cattle had not previously been inoculated with *A phagocytophilum*. Nine of the 12 treated cattle had positive results of PCR analysis for *A phagocytophilum* from at least 1 blood sample. Five tissue samples had positive results of PCR analysis for *A phagocytophilum*; PCR results for *A marginale* were positive in spleen, lung, lymph node, heart, and ear skin of infected cattle.

**Conclusions and Clinical Relevance**—Results indicated an important biological interaction between *A marginale* and *A phagocytophilum* infection as well as with tick saliva in disease kinetics and severity in cattle, which may be important for interpretation of diagnostic tests and management of disease in areas where both pathogens occur. (Am J Vet Res 2008;69:1171–1178)

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Cattle may be infected with 2 bacterial species in the genus *Anaplasma*: *Anaplasma marginale* and *Anaplasma phagocytophilum*. *Anaplasma marginale* invades erythrocytes; leads to extravascular hemolysis; and can result in anemia, icterus, fever, and weakness. Primary infections are the most severe, after which infection persists for the life of the animal as cyclic, but often subclinical, bacteremia. The mechanism for persistence is the sequential expression of variants of the MSP2 and MSP3 antigens, with subsequent host antibody response. This disease causes major production loss in tropical, subtropical, and western American cattle. In the western United States, *A marginale* infection occurs primarily in cattle on the range. Disease also is reported in southern and central Europe. Transmission routes include ticks, particularly *Dermacentor* spp, as well as mechanical transmission by biting flies and fomites (iatrogenically).
**Anaplasma phagocytophilum** (formerly *Ehrlichia phagocytophila* in European hooftstock; *Ehrlichia equi* in North American horses; and the human granulocytic ehrlichiosis, now human granulocytic anaplasmosis or HGA agent) infects granulocytes of humans, cattle, dogs, sheep, deer, horses, and rodents.  Development of clinical signs of so-called pasture fever in cattle varies, depending on whether the cattle are infected with strains from the United States or Europe. In Europe, *A phagocytophilum* is transmitted by *Ixodes ricinus* ticks and is associated in cattle with fever as high as 40°C, reluctance to move, tachypnea, cough, nasal discharge, leukopenia, and thrombocytopenia. To the authors’ knowledge, no reports of clinical illness in cattle infected with strains of *A phagocytophilum* from the United States have been published. *Anaplasma phagocytophilum* also causes disease in humans, along with nonspecific clinical signs and hematologic changes including fever, headache, and thrombocytopenia. Cases of human anaplasmosis are reported with increasing frequency, and infection is common in Californian horses and dogs.  

The pathogenesis of disease associated with *Anaplasma* spp infection is influenced by the tick vector as it attaches to host skin, feeds, and inoculates the animal with the bacteria. During feeding, hard ticks including *Ixodes* spp and *Dermacentor* spp secrete bioactive salivary molecules into the skin to promote host bleeding and reduce antitick inflammation. Saliva may have complement, cytokine, and antibody inhibitors; histamine-binding proteins; leukocyte modulators; and anti-hemostatics. Thus, inoculation of tick-borne pathogens directly into the skin in the presence of tick saliva is likely to induce local changes in the dermis, and these changes may modulate the early pathogenesis of infection. *Anaplasma phagocytophilum* infection is in itself immunosuppressive; thus, coinfection with 2 *Anaplasma* spp may modulate immunopathologic sequelae of infection, resulting in either enhancement of morbidity, increased mortality rate, or a cross-protective effect.  

In diverse host-pathogen systems, including trypanosomes and spirochetes, the original presentation of antigens by dermal dendritic cells can have an important influence on the nature of the subsequent immune response. Late events in anaplasmosis, when clinical disease occurs, depend critically on a cascade of interdependent host-pathogen interactions starting as early as the first few hours after the pathogen is inoculated into the skin and antigen is presented by dendritic cells. The reticuloendothelial system is important in the pathogenesis of erythrocytic anaplasmosis because infected cattle develop autoantibodies, and anemia is a result of splenic removal of antibody-tagged erythrocytes. Thus, splenectomized cattle, which are often used in studies of anaplasmosis in cattle, have an altered disease outcome, compared with natural disease. The present study used nonsplenectomized steers, ensuring that the important effects of interactions between rickettsial-infected cells and cells in the spleen were present.  

Despite the high likelihood that cattle in Europe and western North America could be infected with both *A marginale* and *A phagocytophilum*, little is known about how coinfection with the 2 pathogens could change the risk and course of disease and how tick bites could influence the outcome of infection. In particular, coinfection with the 2 *Anaplasma* spp could increase severity of both infections. The objective of the study reported here was to evaluate responses to sequential inoculation of *A marginale* and *A phagocytophilum* in yearling cattle and compare the effect of inoculation with and without a tick salivary gland extract.

**Materials and Methods**

**Animals**—Eighteen 13-month old Black Angus steers were obtained from a site in Nevada from which anaplasmosis was reportedly absent and were screened for exposure to *A marginale* and *A phagocytophilum* by use of serologic and PCR testing; all cattle had negative results for both pathogens. Cattle were housed in a single pen in a cement-floored feedlot, and the experiment took place during late winter before ticks appeared. The study was reviewed and approved by the University of California, Davis, Animal Care and Use Committee.

**Bacterial isolates**—The *A marginale* strain, designated South Idaho, was derived from a naturally infected animal in Caldwell, Idaho. Bovine blood with a rickettsemia of 42% was collected into bottles containing 4 U of heparin/mL and centrifuged at 1,700 × g for 30 minutes. The plasma and buffy coat were removed, and the blood was washed 3 times with PBS solution. To prepare stabilates, 10 mL of 31.2% dimethyl sulfoxide in filter-sterilized PBS solution was added to 10 mL of packed and washed erythrocytes, the tube was mixed briefly by inversion, and the sample was frozen in liquid nitrogen. The stabilate was thawed quickly prior to inoculation, and each steer received 3 mL of stabilate. The granulocytic strain was *A phagocytophilum* Webster, a human origin isolate from the blood of an infected patient in northwestern Wisconsin, which was cultured in human promyelocytic leukemia (HL-60) cells. The inoculum for each animal consisted of 1 mL of 1 × 10^7 HL-60 cells in 1 mL of tissue culture fluid, with 70% of cells infected with *A phagocytophilum* as judged on the basis of visual inspection of Wright-stained samples.

**Tick salivary extracts**—Adult *Ixodes pacificus* and *Dermacentor occidentalis* were collected by flagging vegetation in western Yolo County, Calif. Adult ticks were fed on New Zealand White rabbits for 4 days; removed with fine forceps; and washed sequentially in bleach, 70% ethanol, and PBS solution. The salivary glands of 9 live adult female *D occidentalis* were dissected by use of sterile techniques, homogenized, and suspended in 1 mL of PBS solution. Glands from 9 live adult female *I pacificus* were treated comparably. Gland extracts were used fresh. To make inoculum for infection experiments, 111 µL of *I pacificus* salivary extract was added to each 1 mL of *A phagocytophilum* inoculum, 111 µL of *D occidentalis* salivary extract was added to each *A marginale* inoculum, and a mixture of 55.5 µL of *I pacificus* extract and 55.5 µL of *D occidentalis* extract was added to 1 mL of HL-60 cell culture control.

**Experimental inoculation**—On day 0, cattle were inoculated 1 ID in the right lateral cervical region with either *A phagocytophilum*, *A marginale*, an HL-60 cell nega-
tive control injection, or a tick salivary extract control injection. Cattle were randomly allocated into the treatment groups of 3 cattle each as follows: Am/Ap NS, Ap/Am NS, Am/Ap S, Ap/Am S, TCC, and TSC. Work with cattle was overseen by the campus veterinarian of the University of California, Davis, and approved by the university’s institutional animal care and use committee.

**Clinical observations and sample collection**—Clinical observations were made at the time of inoculation and at 3-day intervals until the end of the experiment on day 50. Observations consisted of determination of weight and a physical examination by a veterinarian for any lesions at the infection site, attitude, hydration, capillary refill time, rectal temperature, respiratory rate and character, and heart rate. On each of the observation days, 3 mL of blood was collected via the coccygeal vein into EDTA for CBC, serologic testing, and PCR assay. Cattle were slaughtered on day 50 by use of a captive bolt gun, and all internal organs were examined visually for abnormalities by a veterinarian. Samples of spleen, heart, lung, mesenteric lymph node, and liver were harvested within 1 hour after slaughter. Half of each tissue sample was placed directly onto ice for transfer to the laboratory and freezing at –20°C for PCR assay, and the other half was fixed in neutral-buffered 10% formalin for histologic examination. The remaining tissue was considered to have positive results if they had a cycle threshold value < 40 and characteristic amplification plots. *Anaplasma marginale* PCR assay for the MSP4 gene was performed as described,20 with modifications. Each 50-μL reaction contained 10 pmol of primers MSP45 and MSP43, 1.5 mM MgCl₂, 0.2 mM dNTPs, 1× Taq DNA reaction buffer, 1.25 units of Taq DNA polymerase, and 31.75 μL of water. Reaction conditions were as described.20 Samples were evaluated via UV transillumination of 1% agarose gels.

**Statistical analysis**—Statistical analysis was performed with a commercially available software program.1 Average daily gain for each animal was calculated as final weight minus initial weight divided by days in trial. A 1-way ANOVA was used to compare gain among groups, with the method of least significant difference used for post hoc comparisons. Changes in Hct, MCV, RDW, and total WBC count were graphed, and differences among treatments were analyzed by use of ANOVA. The PPE was also graphed, and differences were analyzed by use of ANOVA. For all tests, P ≤ 0.05 was considered significant.

**Results**

Cattle in the control groups did not have any clinical changes, alterations in RBC or WBC values, seroconversion to either pathogen, or histopathologic abnormalities. They did not have positive PCR assay results in blood or tissue at any point during the study. Cattle in the infected treatment groups did not have clinical evidence of morbidity; that is, there were no apparent signs of depression, weakness, ataxia, pallor, or increased reperfusion time of mucous membranes. However, cattle in all infected treatment groups had significantly lower average daily weight gain than cattle in the control groups (Table 1).

Cattle in all infected treatment groups had alterations in a variety of blood values (Figure 1). The Hct decreased 25 to 32 days following *A. marginale* infection in all *A. marginale*–infected treatment groups. The onset and degree of anemia were significantly attenuated in cattle in infection groups that also included tick saliva, compared with those in treatment groups that did not include tick saliva. Increases in MCV and RDW occurred shortly after onset of anemia, app-

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Daily gain</th>
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<tbody>
<tr>
<td>Ap/Am NS</td>
<td>0.99 ± 0.27</td>
</tr>
<tr>
<td>Am/Ap NS</td>
<td>0.65 ± 0.27</td>
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<tr>
<td>Ap/Am S</td>
<td>0.87 ± 0.07</td>
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<tr>
<td>Ap/Am S</td>
<td>0.86 ± 0.38</td>
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<tr>
<td>TCC</td>
<td>1.30 ± 0.16</td>
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<tr>
<td>TSC</td>
<td>1.75 ± 0.17</td>
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Table 1—Mean ± SE daily gain (kg/d) of control steers and steers infected with *Anaplasma marginale* and *Anaplasma phagocytophilum*. **
approximately 35 days after A marginale challenge (Figures 2 and 3). The experiment was not conducted long enough to determine relative differences in magnitude between cattle pretreated and not pretreated with A phagocytophilum. The presence of tick saliva resulted in significantly lower MCV in cattle in both treatments, compared with that in cattle lacking saliva. There was a small peak in RDW in the Am/Ep NS group on day 8, followed by peaks in the Am/Ep NS and Am/Ep S groups on day 35. Peaks for the Ap/Am S and Ap/Am NS cattle were not fully within the time scale of the experiment, but their maximum values were significantly higher than those in the Am/Ep cattle. In both cases, cattle that were inoculated with saliva had significantly lower RDW than did the comparable treatment group without saliva. Platelet counts were transiently decreased in the Am/Ep NS and Am/Ep S groups on day 22, followed by rebound thrombocytosis (although not outside the reference range) on days 34 to 37 after inoculation (Figure 4). If cattle had been pretreated with A phagocytophilum, there was often greater thrombocytopenia, compared with nonpretreated groups on day 42 (ie, 32 days after A marginale inoculation). No significant abnormalities were detected in leukocyte counts.

No A phagocytophilum morulae were observed during CBCs. In contrast, A marginale organisms were observed in cattle from 22 days after infection to the end of the experiment, with values ranging from 2% to 70% of erythrocytes infected (Figure 5). Cattle initially infected with A marginale had a significantly greater PPE of A marginale than cattle initially infected with A phagocytophilum.

At necropsy, no lesions were grossly visible in any animal. Histologically, the heart, lungs, spleen, and lymph nodes were all grossly normal. In 4 calves, the liver had mild periportal pyogranulomatous inflammation (all 3 calves in the Am/Ep S treatment and 1 calf in the Ap/Am S treatment group), and 1 calf (Am/Ep NS treatment group) had focal hepatic abcesses.

All cattle that initially received A marginale seroconverted on the basis of results of the A marginale cELISA, with cattle that were coinoculated with tick saliva typically seroconverting on day 6 and those lacking tick saliva seroconverting on day 8. There was serologic cross-reactivity between the ELISA for A marginale and the IFA for A phagocytophilum. Thus, cattle became seropositive to A phagocytophilum approximately 22 days after infection with A marginale, but before they had been inoculated with A phagocytophilum. Calves in the 2 treatment groups initially infected with A marginale remained seropositive throughout the trial.

When cattle were initially inoculated with A phagocytophilum, they consistently seroconverted (IFA test) on day 6, although titers were low. After these calves were infected with A marginale, they began to seroconvert to A marginale 6 days later. In this case, calves that were inoculated without tick saliva typically seroconverted before those inoculated with tick saliva. Calves in these 2 treatment groups also remained seropositive throughout the trial.

Nine of the 12 cattle had a positive PCR assay result for A phagocytophilum from at least 1 blood sample. The positive result was obtained at 3 days after infection (n = 1 calf), 6 days after infection (6), or 22 days after infection (2). Four calves had 5 or more blood samples with positive results of the PCR assay. Only 9 tissue samples had PCR assay positive results for A phagocytophilum: lung (n = 1), spleen (1), liver (1), and heart (2). All but one of the tissue samples came from a calf that had at least 1 blood sample with positive results.
Results of the *A. marginale* PCR assay were positive in most spleen, lung, lymph node, heart, and ear skin samples from infected, but not control, cattle, with the following exceptions: heart and lymph node samples yielded negative results in 1 *Am/Ap* steer that received salivary extract and 1 that did not, whereas the ear skin sample also yielded negative results in these cattle as well as in 1 *Ap/Am* steer that did not receive saliva.

**Discussion**

This experiment revealed an important biological interaction between *A. marginale* and *A. phagocytophilum* infection as well as with tick saliva, with regard to disease kinetics and severity in cattle. These findings may be important for the interpretation of diagnostic tests and management of disease in cattle in areas where both pathogens occur, such as in the western United States and southern and central Europe. For example, if diagnostic testing for *A. marginale* were to occur in an area where *A. phagocytophilum* is present, false-positive interpretation of test results could result, given the cross-reactivity observed with *A. phagocytophilum*, which could lead to inappropriate disease management if clinical decisions were based on those test results. Infection with *A. marginale* causes considerable clinical signs and production loss, although management strategies help mitigate against extensive mortality rates. These include movement of young cattle (which are more resistant to severe clinical disease) onto endemically infected rangeland and use of a state-licensed live vaccine and an experimental killed vaccine. Most severe signs are seen in cattle that have primary infection when > 2 years of age. Severely affected cattle develop hemolytic anemia; icterus; reduction in milk production and rumination; lethargy; and, possibly, aggressiveness because of cerebral hypoxia.

Clinical granulocytic anaplasmosis appears to be common in Europe and is typically associated with moderate to severe clinical disease in sheep and cattle. In contrast, infection is uncommon in American cattle. Moreover, experimental infection with European cattle strains, but not European human or American horse or human-origin strains, reproduces natural severe disease. In 6 cattle, in which 2 served as control animals, 2 received a Californian equine isolate, and 2 received a midwestern human isolate, none developed clinical signs or hematologic abnormalities or had positive results of PCR assay, although all seroconverted. Thus, we expected that any impact of granulocytic anaplasmosis on cattle in the present study would likely be via modulation of the more severe clinical impact of erythrocytic anaplasmosis.

Cattle in the present study were exposed to anaplasmosis via a simulated tick exposure. Some prior experimental studies of *A. marginale* have relied on IV inoculation of splenectomized cattle, which causes reduced ability to clear the pathogen from circulation. Inoculation into the dermis could differ with regard to early pathogenesis, particularly in the presence of tick saliva. Ticks inject an array of anti-inflammatory and immunomodulatory chemicals during feeding to prevent host blood clotting, platelet aggregation, natural killer cell activity, and Th1 cytokine responses. Unfortunately, cattle that received *A. marginale* followed by *A. phagocytophilum* developed acute disease shortly before they were slaughtered, making it impossible to compare the recovery phase of the different treatment groups. Additionally, the use of a mixed salivary extract (ie, half from each *D. occidentalis* and *I. pacificus*) in the
control cattle precluded us from being able to evaluate the effect of specific tick species on the outcome of exposure to the extract. Nevertheless, we could identify significant differences in some aspects of the outcomes of infection in cattle exposed to tick saliva and pathogens, compared with those that were not. In particular, changes in Hct, MCV, and RDW associated with *A. marginale* infection were delayed and attenuated in cattle exposed to tick saliva. One possible mechanism could be that immunomodulatory effectors in the saliva created a milieu in which *A. marginale* had reduced access to target cells. Alternatively, given that many clinical outcomes of erythrocytic anaplasmosis are immune mediated, early anti-inflammatory effects caused by tick saliva could have delayed the onset of clinical signs. Also, we used young cattle, and in older cattle, immunologic sequelae may be more pronounced. Thus, an important target of future research would be to evaluate short-term immunomodulation in the dermis after *A. marginale* is inoculated in the presence of tick saliva in both older and younger cattle.

There were considerable hematologic but lesser clinical sequelae of *A. marginale* infection in the present study. Cattle infected with either or both pathogens had failure of weight gain, and there was fever in *A. marginale*-infected cattle, probably caused by hemolysis. Anemia was extensive, possibly because of a large inoculum dose, the effect of salivary mediators, or ID inoculation. Despite the extent of anemia and thrombocytopenia, cattle did not develop lethargy, changes in capillary refill time, or mucous membrane color. Concurrent with the anemia, erythrocyte parasitism was extensive, typically affecting as many as 70% of RBCs, but as many as 100% in a few cattle. These findings suggested more severe erythrocytic anaplasmosis than in some earlier studies. For example, in splenectomized cattle after 42 days, there was a peak parasitemia in 48% of the erythrocytes and a peak reduction in PCV by 74%.24

The main effect of *A. phagocytophilum* infection seemed to be modulation in changes associated with *A. marginale* infection. The mild granulocytic anaplasmosis observed in the present study was consistent with another report of infection with North American *A. phagocytophilum* strains and many human cases25,26 in which hematologic abnormalities are often minor. Sequential coinfection was hypothesized to exacerbate single infection via immunomodulation or to reduce infection severity (a vaccine-like effect). However, the observed effect of the coinfection was exacerbated hematologic abnormalities, compared with those attributable to either pathogen alone. Autoimmune changes have been reported in cats and humans with granulocytic anaplasmosis,27 with development of antinuclear or antiplatelet antibodies in 80% of human patients in 1 study.28 *Anaplasma phagocytophilum*-infected cattle, goats, and sheep are more susceptible to staphylococcal pyemia, *Pasteurella haemolytica* and *Chlamydia psittaci* infections, and parainfluenza-3 viral pneumonia.29–32 Typically, cattle with so-called pasture fever are neutropenic with defective neutrophil migration (diapedesis) and activity, lymphocytopenic, have reduced CD4+ and CD8+ counts, and have reduced T-cell lymphoproliferation in response to mitogens.33–36 *Anaplasma phagocytophilum* infection also impairs the host’s response to humoral challenge37 and prevents phagosome-lysosome fusion in neutrophils.38 Thus, in the present study, some immune-mediated sequelae of *A. marginale* infection may have been exacerbated by *A. phagocytophilum* preinfection (anemia and erythrocyte responses such as RDW and MCV); however, rates of *A. marginale* parasitism in erythrocytes were reduced after *A. phagocytophilum* infection, which suggested 2 differing interactive mechanisms between the pathogens.

To some extent, the immunologic interaction between the 2 pathogens also was reflected in the difficulties interpreting serologic results when cattle were coinfected. The MSP5 protein, which is the target of the cELISA licensed for the diagnosis of *A. marginale* in cattle, is reportedly 63% identical in amino acid sequence to the paralog from *A. phagocytophilum*, and some serologic cross-reaction by use of recombinant MSP5 has been reported among humans, cattle, sheep, and horses.39,40 Sheep, horses, and cattle that were experimentally infected with *A. phagocytophilum* seroconverted by use of the *A. marginale* cELISA, although the duration of seropositivity was limited, whereas cattle infected with *A. marginale* developed *A. phagocytophilum* IFA-positive results.41 However, dogs and horses naturally infected with *A. phagocytophilum* were not seropositive by use of the *A. marginale* cELISA.42 The value of serologic testing for *A. marginale* probably depends on the magnitude of the titer, prevalence of pathogens, and clinical signs. In a field study of cattle in Switzerland in which both *A. marginale* and *A. phagocytophilum* were present, *A. marginale* seropositivity was strongly statistically associated with anemia and there was excellent agreement between serologic and microscopic findings. These pub-
lished results and our findings make interpretation of serologic results problematic for cattle with coinfection. In contrast, PCR assay may be more specific than serologic testing, but results may be positive only during active infection. The finding of positive results of PCR assay for A. phagocytophilum was transient in blood and tissue, consistent with the lack of visible monulae in RBCs. However, almost all tissues yielded positive PCR assay results for *A. marginale* terminally.

Erythrocytic anaplasmosis remains a devastating disease for cattle in the United States, and granulocytic anaplasmosis is an important disease of cattle in Europe as well as a potential zoonotic disease in the United States. Opportunities for coinfection are common, yet immunopathologic interactions, mechanisms, and implications have received little attention. Other techniques to induce anaplasmosis, both erythrocytic and granulocytic, often rely on needle inoculation methods to study kinetics of infection in cattle; however, results of the present study have provided evidence that natural-1ID inoculation with tick salivary presents a good method for ongoing evaluation of *Anaplasma* spp. coinfection.


b. eELISA, VMRD, Pullman, Wash.
c. DNeasy tissue kit, Qiagen, Valencia, Calif.
d. ABI Prism 7700, Applied Biosystems, Foster City, Calif.
e. Applied Biosystems, Foster City, Calif.
f. SPSS, version 13.0 for Windows, SPSS Inc, Chicago, Ill.

References


