An Eperythrozoon-like organism affecting camelids was first reported in 1990 by 2 groups: 1 in Colorado and 1 in Kentucky.1,2 This organism, now known as CMhl, is most closely related to Mycoplasma suis, Mycoplasma wenyonii, Mycoplasma ovis, Mycoplasma hemophilus, and Mycoplasma hemominutum, which affect swine, cattle, sheep, opossums, and cats, respectively.3,4 These and other hemotropic organisms reclassified as mycoplasmas represent a unique group of mycoplasmas, sometimes known as haemoplasmas.5 The hemotropic mycoplasmas are small bacteria that lack a cell wall. These organisms have not been successfully cultured in vitro, which complicates the study of their life cycle and the pathophysiologic processes of infection. They are generally <1.0 µm in diameter and usually appear as coccoid, rod-shaped, or ring-shaped organisms that are located on, but do not penetrate, the erythrocyte plasma membrane. They easily detach from erythrocytes, so that if blood smears are not made soon after blood is collected, organisms may be in the background where they resemble stain precipitate or debris. For many years, identification of organisms in blood smears was the primary method of diagnosis of infection, but detachment of haemoplasmas from erythrocytes and the cyclic nature of the bacteremia as well as the low numbers of organisms often present make this method insensitive.

Infection is often associated with mild to marked anemia in stressed, immunosuppressed, and debilitated camelids, but low numbers of organisms have also been identified in apparently healthy camelids. Clinical signs in

### Use of a polymerase chain reaction assay to study response to oxytetracycline treatment in experimental Candidatus Mycoplasma haemolamae infection in alpacas

**Susan J. Tornquist, DVM, PhD; Lisa J. Boeder, BS; Christopher K. Cebra, VMD, MS; Joanne Messick, VMD, PhD**

**Objective**—To develop a PCR assay for Candidatus Mycoplasma haemolamae (CMhl) infection in alpacas and use it to study the efficacy of oxytetracycline treatment and development of a subclinical carrier state.

**Animals**—8 healthy adult alpacas.

**Procedures**—Alpacas initially had negative results for CMhl in blood samples via PCR assay and were experimentally infected with CMhl; 4 were treated with oxytetracycline, and 4 were not treated. All were monitored regularly via PCR assay, blood smear examination, PCV, rectal temperature, and physical examination. At 6 months after treatment, all alpacas were immunosuppressed by administration of dexamethasone and tested for CMhl.

**Results**—7 of 8 alpacas had positive PCR assay results 4 to 6 days after experimental infection. When organisms were detectable on a blood smear, they were seen 2 to 6 days after positive results of PCR assay. Infection was often associated with mild anemia that was usually transient. No alpacas became hypoglycemic. Oxytetracycline treatment was not associated with faster clearance of organisms or resolution of anemia, and 4 of 4 treated alpacas still had positive results of PCR assay when immunosuppressed 6 months later; 0 of 3 nontreated alpacas had positive results of PCR assay following immunosuppression. Transient fever was detected in 3 alpacas during immunosuppression.

**Conclusions and Clinical Relevance**—The PCR assay was more sensitive than blood smear examination for detection of infection. Clinical signs, anemia, and fever were not necessarily associated with infection. Oxytetracycline administration did not consistently clear CMhl infection. Although treated with oxytetracycline, infected alpacas remained chronic carriers. (Am J Vet Res 2009;70:1102–1107)

### Abbreviations

<table>
<thead>
<tr>
<th>CMhl</th>
<th>Candidatus Mycoplasma haemolamae</th>
</tr>
</thead>
<tbody>
<tr>
<td>PID</td>
<td>Postinfection day</td>
</tr>
</tbody>
</table>

**Acknowledgments**

Received June 25, 2008.
Accepted November 12, 2008.

From the Departments of Biomedical Sciences (Tornquist, Boeder) and Clinical Sciences (Cebra), College of Veterinary Medicine, Oregon State University, Corvallis, OR 97331; and the Department of Veterinary Comparative Pathobiology, School of Veterinary Medicine, Purdue University, West Lafayette, IN 47907 (Messick).

Supported by the Alpaca Research Foundation.


Address correspondence to Dr. Tornquist.

---

08-06-0203r.indd   1102
8/24/2009   9:10:09 AM

Unauthenticated | Downloaded 06/09/22 02:33 PM UTC
infected camelids may include chronic weight loss, signs of depression, decreased fertility, lethargy, and acute collapse and, rarely, death.\textsuperscript{3,6,9} The presence of organisms in the circulation and consistent clinical signs are often associated with shipping, movement of camelids from one premise to another, parturition, concurrent illness, or other infections.\textsuperscript{1,2,7,8} It is likely that many infected camelids never have clinical signs of the infection.

The mode of transmission is not known, although a biting insect vector is suspected as it is in the other haemoplasma infections.\textsuperscript{3,5} There is evidence supportive of in utero infection because the organism has been detected in cria as young as 24 hours of age and diagnosed in early neonatal death in young cria.\textsuperscript{6,9} However, infected dams do not always give birth to cria with detectable infection, nor, in the authors’ experience, is the organism apparently consistently passed to cria in colostrum.

Treatment of clinically affected camelids with oxytetracycline often decreases clinical signs, improves anemia, and clears organisms from being detectable on blood smears.\textsuperscript{1,7} It is not known whether oxytetracycline clears the organisms entirely, given that blood smear examination is such an insensitive diagnostic method. In addition, the bacteremia is cyclic and thus the number of organisms in the blood may decrease below a detectable value at the time of sampling, but then rebound hours or days later. There has been speculation that camelids may be chronic, subclinical carriers following treatment or if they are not treated.

Similar challenges with diagnosis and assessment of treatment effectiveness in other hemotropic mycoplasma infections have been addressed by the development of PCR assays to detect these infections.\textsuperscript{3,10–13} A PCR assay is a sensitive and specific diagnostic technique that is particularly valuable in detection of subclinical infections.

The purpose of the study reported here was to develop a PCR assay for CMhl infection in alpacas and use it to study the efficacy of oxytetracycline treatment and development of a subclinical carrier state. The hypothesis was that treatment of CMhl-infected alpacas with oxytetracycline results in clearance of detectable organisms on blood smears, but persistence of PCR assay–detectable chronic infection that may recur despite when the alpacas are immunosuppressed.

**Materials and Methods**

**PCR assay—**All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Oregon State University. Blood from a naturally infected alpaca from Oregon was collected into tubes containing EDTA. The DNA was extracted from 200 µL of the blood by use of a commercial extraction system that captures sample DNA with a matrix material in a spin column while lysing cells in the sample.\textsuperscript{9} After contaminants were washed away, the purified sample DNA was eluted into the buffer solution from the kit. Initial amplification was accomplished by use of the following universal primers for the eubacterial 16S rRNA gene: forward primer Fh1 \textsuperscript{5}′–AGG GGT CGA CAG AGT TTG ATC CTG GCT–3′ and reverse primer Rhf2 \textsuperscript{5}′–CGC GGA TCC GCT ACC TTG TTA CGA CTT–3′. Each 50-µL of reaction mixture included 0.2 µM of each primer, 0.5 U of Taq polymerase/µL, 1.5 mM MgCl\textsubscript{2}, 0.2 mM deoxynucleoside triphosphate mix, and 2 µL of template. To ensure there was no DNA contamination during the extraction process, a negative control consisting of all of the reagents without a DNA sample was included.

Amplifications were performed in an automated thermal cycler\textsuperscript{4} and consisted of an initial 10-minute incubation at 94°C; 32 cycles at 94°C for 1 minute, 50°C for 1 minute, and 72°C for 2 minutes; and a final elongation step at 72°C for 7 minutes. The purified amplicon was cloned into an expression vector\textsuperscript{6} and sequenced in both directions with an automated sequence detection system.\textsuperscript{1} This DNA had 97% sequence homology with GenBank sequence accession AF306346, the sequence for CMhl.

The CMhl species-specific primers were selected to amplify a 318-bp sequence within the universal primer sequence that was unique to the hypervariable region of the CMhl 16S rRNA region as identified in the GenBank accession.\textsuperscript{3} These primers were as follows: forward primer \textsuperscript{5}′–TAG ATT TGA AAT AGT CTA AAT TAA–3′ and reverse primer \textsuperscript{5}′–AAT TAG TAC AAT CAC GAC AGA ATC–3′. The same reaction mixture and thermal cycler times and temperatures as described for the universal primer amplification were used for the CMhl-specific amplification. The amplified products were detected via electrophoresis on 1.5% (wt/vol) agarose gel containing 1 ng of ethidium bromide/mL in Tris-acetate-EDTA buffer. A 100-bp DNA ladder was included as a DNA size standard. The identity of the amplicon was confirmed by use of restriction fragment length polymorphism analysis.

A positive control sample processed by use of the same extraction kit was extracted at the same time as test samples. The positive control sample was EDTA-anticoagulated blood from a naturally infected alpaca with large numbers of typical CMhl organisms in blood smears. A negative control sample consisting of reaction mixture with no template DNA was included. Specificity was revealed by failure of the primers to amplify related *Mycoplasma* spp, including *M. suis*, *Mycoplasma haemofelis*, *Mycoplasma hemocanis*, and *Mycoplasma genitalium*.

To determine sensitivity of the assay, a plasmid with a known single gene copy number was constructed by use of a 219-bp amplicon from the conventional PCR assay. Serial 10-fold dilutions were prepared with 5 µL of DNA and amplified via real-time PCR assay to generate a standard curve.\textsuperscript{5} A purified preparation of organism DNA from a known infected alpaca was determined to contain \(6.8 \times 10^7\) copies of CMhl in 5 µL via regression analysis from the real-time PCR standard curve. This purified DNA was subjected to serial 10-fold dilutions, and the conventional PCR assay was performed on each dilution. On the basis of this dilution series, the lower detection limit of the conventional PCR assay was determined to be 28 copies.

Following development, the assay was performed by use of the procedure described. Following extraction with the capture column kit, DNA samples were eluted into buffer and frozen at –20°C until amplification. The assay was used to detect the presence of CMhl in a large
number of blood samples from llamas and alpacas from a wide variety of geographic locations in the United States, Canada, Australia, and the United Kingdom to ensure that it was not specific to CMh1 found in only certain locations.

**Experimental infections**—Because the hemotropic mycoplasmas, including CMh1, have not been successfully cultured in vitro, a source of infectious organism was first developed. A healthy adult alpaca gelding was splenectomized, then infected via IV transfusion of 35 mL of blood from a naturally infected alpaca at the Oregon State University Veterinary Teaching Hospital. Infection of the splenectomized alpaca was confirmed via positive results of PCR assay (day 9) and by detection of the organism on blood smears (day 13). This alpaca was closely monitored and did not become febrile, anemic, or anorectic or have signs of depression during the study. After its blood was used for infection of the study alpacas, the splenectomized alpaca was treated with oxytetracycline# at 20 mg/kg, SC, every third day for 5 treatments; CMh1 was undetectable in its blood via PCR assay after the second treatment.

Eight healthy adult alpacas from the Oregon State University College of Veterinary Medicine herd were infected for this study. They included 7 males and 1 female and ranged in age from 1.5 to 8 years. Their weights ranged from 37 to 82 kg. All 8 alpacas were determined to be healthy on the basis of results of physical examinations, CBCs, and serum biochemical panels. No alpacas had positive results for CMh1 via blood smear examination or PCR assay prior to the study.

Infections took place in the veterinary teaching hospital where the alpacas were housed together, but separately from other camelids. Two hundred eighty milliliters of blood was obtained from the previously infected, splenectomized alpaca via jugular venipuncture and collection into a container with sterile acid–sodium citrate. The splenectomized alpaca had 1 or more CMh1 organisms on approximately 80% of its erythrocytes, and the RBC count was 11.2 × 10^6 cells/μL at the time of infection. Thirty-five milliliters of this blood was immediately transfused into each recipient alpaca via IV injection into a jugular vein. Following transfusion, the alpacas were evaluated by observation of respiratory rate, mucous membrane color, alertness, and gait to ensure that no adverse transfusion reactions occurred.

For the next week, alpacas were examined daily with rectal temperatures measured and 1-mL blood samples collected into tubes containing EDTA for evaluation of PCV, total protein concentration (via refractometer), and blood smears and development of a PCR assay for CMh1. These examinations and samplings continued every other day for an additional 3 weeks. At 4 weeks after infection, the examinations and sampling were conducted once a week until the study was completed. At 10 days after infection, a serum sample was obtained from each alpaca for a biochemical profile performed on an automated chemistry analyzer. The PCR assays were performed as described. Confirmed infected blood and distilled water were included in each assay as positive and negative control samples, respectively. Blood smears were stained with Wright-Giemsa stain and examined by one of the authors (SJT).

The 8 alpacas were allocated randomly into 2 groups of 4 alpacas each in the treatment group and the control group. At PID 7, 4 of the alpacas began receiving long-acting oxytetracycline (20 mg/kg, SC, q 72 h, for 3 treatments). Four control alpacas received injections of sterile saline (0.9% NaCl) solution at the corresponding times and volumes of injection.

After the oxytetracycline treatment regimen was completed, blood samples were collected once a week from each alpaca for an additional 6 months. At the end of this period, all alpacas were immunosuppressed by IV administration of dexamethasone (2 mg/kg, q 24 h, for 3 days). During the 3 days of immunosuppression, the alpacas had daily physical examinations and blood samples were obtained for evaluation of PCV, total protein concentration, and blood smears and development of a PCR assay for CMh1.

**Results**

Six alpacas had positive results for CMh1 via PCR assay at PID 4; 4 had positive results for organisms on blood smear examination 2 days later, one 4 days later, and one 6 days later. One alpaca had positive results of PCR assay at PID 6 and did not have detectable organisms on blood smears until the time of immunosuppression at 6 months after infection. One alpaca never had positive results via PCR assay or on a blood smear.

During the 6-month period of the study, 122 blood samples yielded positive results for CMh1 via PCR assay but negative results via blood smear examination. Twenty-four samples yielded positive results via both methods, and 2 samples appeared to have positive results on a blood smear but had negative results via PCR assay.

The 4 alpacas that received oxytetracycline were among the 6 that had positive results via PCR assay at PID 4. Two of these alpacas had negative results of PCR assay at PID 10 (1 at PID 11 and 1 at PID 12). Of the 3 nontreated alpacas that developed positive results, 1 had negative results at PID 10, 1 at PID 12, and 1 at PID 14.

Except for the alpaca that never had positive results, all alpacas sometimes had positive results and sometimes had negative results via PCR assay for the following 6 months with no common pattern. At the time of immunosuppression, all 4 of the treated alpacas developed positive results and none of the nontreated alpacas developed positive results (Figure 1).

Seven of the 8 alpacas became mildly anemic (PCV < 25%) within the 4 days following infection. The anemia lasted for 1 week or less for 3 alpacas, but persisted for approximately 1 month after infection in 4 alpacas. This group with prolonged anemia included 3 treated alpacas and 1 untreated alpaca. For most of the duration of the anemia, these alpacas had negative results for CMh1 via PCR assay. When immunosuppressed, 6 alpacas became anemic, including 3 treated and 3 nontreated alpacas. The PCVs from anemic alpacas ranged from 17% to 24%.

None of the alpacas was febrile (rectal temperature, > 38.9°C) following transfusion, except for 3 alpacas that became transiently febrile (rectal temperature, 39.4° to 40.3°C) for 1 to 2 days during the 3 days of
immunosuppression. These alpacas had positive results of PCR assay for CMhl during their febrile episodes, and 2 also had positive results on a blood smear. No serum biochemical abnormalities were detected, and specifically, no alpacas became hypoglycemic (glucose concentration, < 88 mg/dL).

**Discussion**

Development of PCR assays for the diagnosis of hemotropic mycoplasmas has facilitated both the diagnosis of infections and the study of the animals’ response to treatment. The increased sensitivity of the PCR assay for CMhl in comparison to blood smear examination has been useful in diagnosing and further characterizing the infection in camelids because it provides the ability to accurately identify early infections in infected camelids with no clinical signs or laboratory abnormalities. In addition to being used in the study reported here, it has been used in diagnostic testing and screening of > 4,000 camelid samples in the veterinary diagnostic laboratory.

Following experimental infection, CMhl was first detectable via PCR assay at PID 4 and detectable by PID 6 in most alpacas. Organisms were sometimes apparent on blood smears 2 to 6 days after positive results of PCR assay were detected, but sometimes never became detectable on blood smears from infected alpacas. This reflects both the higher sensitivity of the PCR assay and the fact that organisms can be difficult to distinguish from stain precipitate or other debris on a slide, especially when organisms are present in small numbers. The 2 samples in which the organisms appeared to be present on a slide but corresponding PCR assay results were negative likely represented false-positive results caused by stain precipitate or debris. During this study, it was observed that among samples collected within a 4-hour period, organisms seen on blood smears from infected alpacas could vary from large numbers to few or none. This suggested cyclicity of bacteremia, which was similar to that seen in other hemotropic mycoplasma infections. It also indicated that active infections are not limited to those associated with positive results on a blood smear.

The absence of clinical signs such as fever, signs of depression, lethargy, and anorexia in the alpacas in this study confirmed that infection in otherwise healthy camelids was not necessarily associated with either acute or chronic disease. Our experience with performing the PCR assay on large numbers of camelid blood samples also suggested that most animals with positive results did not have clinical signs. There is substantial variability in the presence of clinical disease in infections with the other hemotropic mycoplasmas. Cats that are acutely infected with *M hemofelis* most often have clinical signs such as fever, lethargy, and anorexia, whereas those infected with *Candidatus Mycoplasma hemominutum*, another feline hemotropic mycoplasma, may have minimal clinical signs. Clinical signs associated with infection with other hemotropic mycoplasmas.
depend on the animal's age, health status, and, probably, dose and virulence of infecting strain.  

Anemia is commonly associated with infection with most of the hemotropic mycoplasmas and is usually thought to be caused by hemolysis.  

- On the basis of other known or speculated pathogenic mechanisms of mycoplasmas, production of autoantibodies leading to immune-mediated destruction of erythrocytes and production of inflammatory cytokines or other mediators that can suppress bone marrow production of erythrocytes are other potential mechanisms of anemia.  

Anemia is the most common clinicopathologic abnormality seen in clinical cases of CMhl infection in camelids. However, infected camelids are not always anemic, and the anemia may be transient as in some alpacas in the present study. Observed PCVs were lower than the reference range for 1 week or less following infection in 3 of the alpacas. In contrast, the present study also revealed that there are alpacas that may appear to clear the organism but remain anemic, despite general good health and adequate diet. This finding supported the idea that CMHL-associated anemia is not caused by hemolysis. Erythrocyte morphology in acutely and chronically infected camelids is usually not typical of regenerative anemia, such as that often seen in hemoysis, in that substantial polychromasia, anisocytosis, reticulocytosis, and nucleated erythrocytes are often not seen. Iron deficiency has been reported in a small number of infected llamas.  

In a report of 4 CMHL-infected llamas with juvenile llama immunodeficiency syndrome, anemia in the immunodeficient llamas was marked, normocytic, normochromic, and regenerative and was more severe than anemia in noninfected but immunodeficient llamas. The 3 immune-competent, but CMHL-infected, llamas in that report did not have any hemorrhagic abnormalities, leading to the suggestion that infection is only associated with anemia in immune-compromised or debilitated camelids. Our findings did not support that hypothesis, although we certainly did see more marked and more clinically important anemia in stressed camelids and those with concurrent disease. It was interesting that prolonged, although generally mild anemia, developed in a few alpacas that were persistently infected and had positive results of PCR assay when immunosuppressed, but anemia also developed in 1 alpaca that rarely had positive results via PCR assay and did not have recrudescence when immunosuppressed.  

Infection with hemotropic mycoplasmas is sometimes associated with hypoglycemia, especially if bacteremia is severe.  

Hypoglycemia has been reported in some cases of CMHL infection in neonates and older camelids and is sometimes profound. It is not, however, a consistent feature of infection and is sometimes profound. It is not, however, a consistent feature of infection with the hemotropic mycoplasmas, but their reported efficacy varies greatly. Other antimicrobials such as enrofloxacin, azithromycin, and marbofloxacin reduce bacteremia, but none clear it, and they are not routinely used clinically. Many factors, including the strain of infecting hemoplasma, dosage and duration of treatment, and individual variability, likely contribute to the differences in results. Overall, there does not appear to be an antimicrobial regimen that effectively eliminates hemoplasma infections in a consistent manner. This is particularly apparent when treated animals appear to have eliminated an infection but are later immunosuppressed and found to be infected. There are conflicting opinions as to whether antimicrobial treatment is likely to create or avert a carrier state.  

In the study reported here, oxytetracycline was administered at a dose and schedule commonly used for naturally infected camelids. The treated alpacas had negative results of PCR assays within 3, 4, or 5 days after starting treatment. Two of the control (nontreated) alpacas had negative results of PCR assay in the same time frame, and the third had negative results 2 days later. These numbers were too small for meaningful statistical analysis, but suggested that otherwise healthy camelids with competent immune responses and no concurrent disease or stress can suppress bacteremia to less than detectable concentration without antimicrobial treatment. Those with suppressed immunity will most likely benefit from treatment, but this was not tested in the present study.  

The question as to whether oxytetracycline treatment facilitates or eliminates the carrier state was not resolved in this study because of the small sample size. However, all 4 of the oxytetracycline-treated alpacas had positive results of PCR assay when immunosuppressed at 6 months, in addition to intermittently having positive PCR assay results during that 6-month period. In contrast, the 3 infected control alpacas did not have detectable organisms when immunosuppressed at 6 months. All of these alpacas occasionally did have positive results of PCR assays when tested weekly, with the number of positive results ranging from 2 to 19. These findings suggested that a chronic carrier state may be common in treated and untreated camelids. In addition, results of this study indicated that having detectable organisms via PCR assay did not correlate closely with PCV, rectal temperature, or the presence of clinical signs.  

References  

haemodidelphidis’ sp. nov., Candidatus mycoplasma haemolamae’ sp. nov. and Mycoplasma haemocanis comb. nov. haemotrophic parasites from a naturally infected opossum (Didelphis virginiana), alpaca (Lama pacos) and dog (Canis familiaris): phylogenetic and secondary structural relatedness of their 16S rRNA genes to other mycoplasmas. Int J Syst Evol Microbiol 2002;52:693–698.


