

An *Ehrlichia* Strain from a Llama (*Lama glama*) and Llama-Associated Ticks (*Ixodes pacificus*)

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An ehrlichia was identified in the blood of a diseased llama (*Lama glama*). Sequencing of its 16S rRNA gene showed the ehrlichia to be closely related to members of the *Ehrlichia phagocytophila* genogroup. The agent was also found in a pool of ticks (*Ixodes pacificus*) collected at the llama site.

Granulocytic ehrlichiosis caused by rickettsiae of the *Ehrlichia phagocytophila* genogroup has been reported to occur in equines, ruminants, dogs, and humans (1, 26). In California, equine granulocytic ehrlichiosis (EGE) caused by *Ehrlichia equi* occurs during the late fall, winter, and spring and is most probably transmitted by *Ixodes pacificus* ticks (13, 17, 24). The clinical picture typically includes fever, lethargy, distal limb edema, thrombocytopenia, and petechiation. Ruminant granulocytic ehrlichiosis (also called tick-borne fever or pasture fever) caused by *E. phagocytophila* occurs only in Europe and Asia and is transmitted by *Ixodes ricinus* (6, 25). The disease is characterized by fever, lethargy, loss of milk production, and in some cases abortion. Fever, lethargy, and thrombocytopenia are typical signs of granulocytic ehrlichiosis in dogs (12). Human granulocytic ehrlichiosis (HGE) is a recently described disease entity with symptoms of headache, myalgia, chills, and varying combinations of leukopenia, anemia, and thrombocytopenia (1). The causative ehrlichia (the HGE agent) is probably transmitted by *Ixodes* ticks (23) and can produce granulocytic ehrlichiosis in horses (2, 14, 16, 19) and dogs (12, 14) as well.

Llamas (*Lama glama*) are camelids of the suborder Tylopoda and so are taxonomically removed from other species known to be susceptible to granulocytic ehrlichiosis (8, 17). Llamas were domesticated in the Andes Mountains of South America, where they remain a valuable source of food, fiber, and fuel for indigenous peoples. In North America, llamas have grown in popularity as pack animals and pets and so are of increasing veterinary importance (8). It is estimated that between 100,000 and 120,000 llamas can be found in North America today (9). Remarkably few rickettsial diseases are known to occur in llamas, being limited almost exclusively to eperythrozoonosis (10, 21, 27). Here we report the identification of an ehrlichia in a llama suffering from an illness we interpret as granulocytic ehrlichiosis.

Case report. In late March 1996, an 11-year-old castrated male llama residing in El Dorado County, California, was examined for an illness of 3 days' duration. Clinical signs related by the owner were nonspecific in nature and included

partial anorexia, slight ataxia, and lethargy. Upon physical examination the llama was recumbent, with a rectal temperature of 102°F, a pulse rate of 52 beats per min, a respiratory rate of 24 breaths per min (these values being within relatively normal limits and not unusual for sick llamas), very pale mucous membranes, and decreased motility of stomach contents. An incidental finding was a 3-cm caseous abscess at the base of the tail that yielded no growth on culture. Hematologic examination revealed mild lymphopenia, monocytosis, eosinophilia, and the presence in neutrophils of rare cytoplasmic inclusion bodies characteristic of *E. equi* (17). The latter finding prompted consideration of a diagnosis of granulocytic ehrlichiosis. Following treatment with oxytetracycline, the llama improved rapidly and made an uneventful recovery.

The affected animal was housed with four other llamas of various ages in an enclosure composed primarily of drylot with a perimeter of weeds and other vegetation. The llama had not recently left the enclosure. None of the other llamas was symptomatic at the time, although one reportedly had exhibited similar signs in January 1994, with a mild band neutrophilia as the only hematologic abnormality. The site is located approximately 55 miles east of Sacramento in the Sierra Nevada foothills, an area where EGE has been known for many years to be enzootic and which is hospitable to *I. pacificus* (13, 17, 18). The surrounding area is a montane forest with black oak, madrone, and ponderosa pine as the predominant trees and an understory of poison oak, at an elevation of approximately 2,000 ft.

Blood buffy-coat cells obtained from the llama were strongly positive by the nested PCR (Fig. 1, lane L), which amplifies a 928-bp sequence of the 16S rRNA gene of *E. equi*/HGE agent (3, 19). A PCR-positive result with buffy-coat cells is considered diagnostic for granulocytic ehrlichiosis in horses and humans (1–3, 19, 24). For DNA sequencing, the universal eubacterial primers POMod and PC5 (28) were used to amplify a majority of the 16S rRNA gene. The amplified product was sequenced with a fluorescence-based automated sequencing system (ABI system model 373; Applied Biosystems, Foster City, Calif.). The resulting sequence was subjected to BLAST analysis of GenBank nucleic acid sequences and for similarity rank by using an established database (20). The sequences were aligned for maximum similarity with the closest identified 16S rRNA gene sequence by using PCGene version 6.5 (IntelGenetics, Inc., Mountain View, Calif.).

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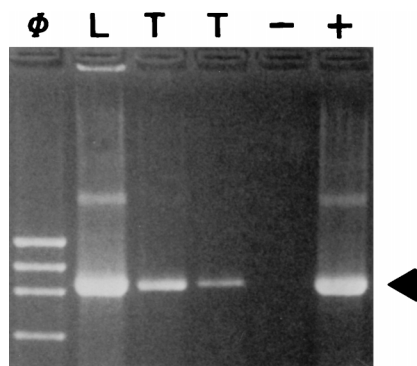


FIG. 1. *E. equi*/HGE agent nested PCR with DNA from blood buffy-coat cells of the affected llama (lane L) and with DNA derived from two llama-associated tick pools (lanes T). The arrowhead indicates the position of the 928-bp nested PCR product. Lane -, negative control (uninfected horse); lane +, positive control (horse experimentally infected with *E. equi*); lane ϕ , ϕ X174 replicative-form DNA digested with *Hae*III (molecular size marker).

Sequence data indicated that the ehrlichial 16S rRNA gene from the affected llama was very closely related (99.8 to 99.9% similarity) to those of *E. phagocytophila* genogroup members (Table 1). Of the four nucleotides at which differences were noted, three were shared with *E. equi* MRK, two were shared with the HGE agent, two were shared with *E. phagocytophila*, one was shared with a second strain of *E. equi*, and one was unique (A at position 1085).

Examination of ticks. A total of 123 unfed adult ticks were collected from the perimeter of the llama enclosure by standard flagging methods. These ticks included 120 western black-legged ticks (*I. pacificus*; 68 males and 52 females) and 3 Pacific coast ticks (*Dermacentor occidentalis*; 1 male and 2 females). The ticks were frozen (-70°C) and thawed, and the *I. pacificus* ticks were segregated by sex into 24 pools of ca. five ticks each. The three *D. occidentalis* ticks were combined into a single, separate pool.

Ticks were processed for PCR by a modification of previous methods (3, 4). Briefly, ticks in each pool were sectioned with sterile scissors and placed into 2-ml microtubes containing 300 μl of zirconia-silica beads (0.5-mm diameter; Biospec Products, Bartlesville, Okla.) and 400 μl of DNA extraction buffer (10 mM Tris [pH 8.0], 2 mM EDTA, 0.1% sodium dodecyl sulfate, 500 μg of proteinase K per ml). The ticks were mechanically disrupted with a Mini-BeadBeater (Biospec) at the high setting, with three bursts of 1 min each. The tubes were spun at top speed in a microcentrifuge for 30 s and incubated,

first at 56°C for 3 h and then at 97°C for 15 min. After pelleting, the supernatants were transferred to fresh sterile tubes. The DNA was precipitated by adding 3 to 4 volumes of ice-cold absolute ethanol and 100 μl of 3 M sodium acetate (pH 5.2) and keeping the tubes at -20°C for 24 to 48 h. The DNA was pelleted at top speed in a microcentrifuge for 15 min at 4°C and washed twice with ice-cold 70% ethanol. After being dried, the DNA was resuspended in 100 μl of DNase-free water or Tris-EDTA (TE) (pH 8.0).

The quality of the prepared DNA was assessed with tick DNA primers 16S+2 and 16S-1 (5) in a single-round PCR (35 cycles, 48°C annealing temperature) prior to the nested PCR. Conditions for the nested PCR were identical to those for buffy-coat samples except that the number of cycles per round was increased from 35 to 40.

Of the 24 *I. pacificus* pools examined by nested PCR, 2 were found to be strongly positive (Fig. 1, lanes T). Both pools were composed of male ticks. The 16S rRNA gene sequence of the ehrlichia from one of the tick pools was identical to that of the llama agent. The second positive pool contained a 16S rRNA gene differing from that of the llama agent by a single base pair change (A in place of G at position 108). The *D. occidentalis* tick pool was negative by PCR.

Discussion. The case described here extends the range of species susceptible to infection with *E. phagocytophila* genogroup members to include a New World camelid, the llama. Similarities of 16S rRNA gene sequences, coupled with the animal's history and clinical signs, the detection of characteristic inclusion bodies in neutrophils, the clinical response to oxytetracycline, and the presence in the vicinity of ticks harboring the llama ehrlichia, led us to conclude that the disease of the affected llama was granulocytic ehrlichiosis.

The granulocytotropic ehrlichiae found in Swedish dogs and horses (14), in dogs from the upper midwestern United States (12), and in horses from Connecticut (16) have 16S rRNA gene sequences identical to that of the HGE agent, suggesting that they may all be the same organism. The sequences of 16S rRNA genes are known to vary in an orderly manner throughout the phylogenetic tree; hence, 16S rRNA genes represent desirable targets for PCR amplification and relatedness testing (28). The average substitution rate for 16S rRNA in eubacteria is approximately 1% per 50×10^6 years (22). Although this suggests that the divergence of the llama ehrlichia from other members of the *E. phagocytophila* genogroup occurred in the distant past, the impact that such minor base changes in 16S rRNA genes might have on the host range, infectivity, and pathogenicity of different ehrlichial strains is unknown.

Two adult *I. pacificus* pools from the perimeter of the llama enclosure were found to contain ehrlichiae, one of them identical in 16S rRNA gene sequence to the llama agent. The positive pool was composed of unfed adult ticks, which must have acquired the ehrlichiae during a previous life stage (nymph or larva) and thus probably acquired them from an animal other than the affected llama. Transstadial transmission of *E. equi* in *I. pacificus* has been shown to occur (24); thus, the more likely scenario is that ticks present in the llama enclosure were the source of infection for this llama, rather than vice versa. This hypothesis is made more tenable by the fact that all recognized members of the *E. phagocytophila* genogroup are known or suspected to be transmitted by ixodid ticks (12, 17, 23-26). The identity and significance of the second ehrlichia with the single base pair change in its 16S rRNA gene remain speculative.

We are unaware of any previous descriptions of ehrlichiosis or ehrlichiosis-like illness in llamas or any other New World camelids (8-10, 15, 27). Infestation with hard ticks is a com-

TABLE 1. Nucleotide differences in the 16S rRNA genes of *E. phagocytophila* genogroup ehrlichiae

DNA source	Nucleotide at position ^a :			
	33	84	886	1085
<i>Ehrlichia equi</i> ^b	C	A	— ^c	T
<i>Ehrlichia phagocytophila</i> ^d	T	A	— ^c	T
Llama ehrlichia	T	A	G	A
<i>Ehrlichia equi</i> MRK (11)	T	A	G	T
HGE agent	T	G	G	T

^a Numbers refer to positions in the 16S rRNA gene of the HGE agent (GenBank accession no. U02521).

^b California strain of *E. equi* (GenBank accession no. M73223).

^c No nucleotide corresponds to HGE position 886 in *E. equi* or *E. phagocytophila*.

^d GenBank accession no. M73220.

mon problem among llamas in the southwestern and mountain states, particularly in animals used for packing into wilderness areas (8, 15). Owing to the nonspecific nature of the clinical signs, the need to identify neutrophil inclusions as a clue to diagnosis, and the probability of tick transmission, it seems likely that granulocytic ehrlichiosis represents a previously unrecognized cause of illness among llamas in the United States. Considering that granulocytic ehrlichiosis has been reported to occur in large numbers of horses in South America (7), it is possible that its llama counterpart exists there as well.

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