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## CHARACTERIZATION OF AN *ANAPLASMA OVIS* ISOLATE FROM DESERT BIGHORN SHEEP IN SOUTHERN CALIFORNIA

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**ABSTRACT:** To understand the role of bighorn sheep (*Ovis canadensis*) in the epidemiology of anaplasmosis, we recovered a field isolate from a suspected enzootic area in southern California (USA). Whole blood was collected from three desert bighorn sheep (*Ovis canadensis nelsoni*) and inoculated into a susceptible splenectomized domestic sheep, calf and a susceptible spleen-intact bighorn sheep. No infection occurred in the calf, but a detectable infection developed in both the domestic sheep and bighorn sheep 24 days after inoculation. The infection in both domestic and bighorn sheep resulted in severe clinical disease but was resolved with the use of tetracycline. Using monoclonal antibodies and DNA probes, we confirmed that the isolate was *Anaplasma ovis*.

**Key words:** Anaplasmosis, *Anaplasma ovis*, bighorn sheep, *Ovis canadensis*.

### INTRODUCTION

Anaplasmosis is an infectious disease of ruminants caused by members of the intraerythrocytic rickettsial genus *Anaplasma* (Blood et al., 1979). Both *A. marginale* and *A. ovis* infect several species of wild ruminants throughout the world (Kuttler, 1984). However, a complete understanding of the epizootiology of anaplasmosis, including the role of wild ruminants, is lacking. Some North American wild ruminant species may support infections of both *A. marginale* and *A. ovis* while others are susceptible to only one or the other. Mule deer (*Odocoileus hemionus hemionus*) and white-tailed deer (*O. virginianus*) are susceptible to experimental infections with both *A. marginale* and *A. ovis* (Ristic and Watrach, 1961; Kreier and Ristic, 1963; Howe et al., 1964; Zaugg, 1988). This also may be the case for pronghorn antelope (*Antilocapra americana*) (Howe et al., 1964; Zaugg, 1987). Only *A. marginale* infects bison (*Bison bison*) (Zaugg, 1986) and perhaps elk (*Cervus elaphus*) (Renshaw et al., 1979), although presumptive

evidence exists for *A. ovis* infection as well (Post and Thomas, 1961). We experimentally exposed two spleen-intact bighorn sheep (*Ovis canadensis canadensis*) to *A. marginale*, with equivocal results; neither developed clinical disease, but infected erythrocytes were detected from one and specific antibodies were detected by immunofluorescence from both (W. Goff, unpubl.). In contrast, bighorn sheep are fully susceptible to *A. ovis* (Tibbitts et al., 1992).

The situation in free-ranging wild ruminant populations is less clear. Natural *A. marginale* infections of mule deer and black-tailed deer (*Odocoileus hemionus columbianus*) have been established (Boynton and Woods, 1933; Christensen et al., 1960; Howe and Hepworth, 1965). Evidence for natural infections of *A. marginale* in other native wild ruminant species or *A. ovis* infections in any wild ruminant species is lacking. Serologic evidence has also proven difficult due to the incompatibility of the complement-fixation and card agglutination assays with wild ruminant sera (Howe and Hepworth, 1965;

Magonigle et al., 1981). Indirect immunofluorescence has shown some promise, but fails to distinguish between *A. marginale* and *A. ovis* infections (Tibbitts et al., 1992). In the present study, we describe a field isolate of *A. ovis* obtained from bighorn sheep (*Ovis canadensis nelsoni*) residing in an area of southern California (USA) where a high seroprevalence of *Anaplasma* antibody has been found (Jessup et al., 1993).

#### MATERIALS AND METHODS

A 4-mo-old Holstein calf (C-454) and a yearling mixed breed domestic sheep (S-005) were splenectomized. After a 2-wk post-surgical recovery period, the calf and domestic sheep were transferred to individual indoor isolation facilities. In addition a wild-caught, spleen-intact Rocky Mountain bighorn sheep (BH-1-H) was similarly housed and each animal was provided water, mineral salt and high quality alfalfa hay and pellets *ad libitum*. Each animal was restrained by hand for obtaining blood samples and for inoculation.

Blood samples were obtained from three clinically normal, adult desert bighorn sheep caught by net-gunning (Kock et al., 1987). The animals were members of a free-ranging population of bighorn sheep inhabiting the Old Dad/Kelso Mountains, which are surrounded by the Mojave desert and range from 400 to 1,600 m in elevation (35°06'N, 115°51'W). The habitat consists primarily of scrub vegetation (Martens and Baldwin, 1983). Sparse vegetation and limited water result in a forage use pattern by bighorn sheep that is not distinctly seasonal. Mule deer occur rarely. There is no contact between cattle and bighorn sheep at the higher elevations, but the two share forage at the lower elevations. There is no contact between domestic sheep and bighorn sheep. The principal tick with vector potential for *A. ovis* found on bighorn sheep in this area is *Dermacentor hunteri* (W. Boyce, unpubl.). This tick can transmit both *A. marginale* and *A. ovis* (D. Stiller, unpubl.).

Each blood collection included 100 ml in acid-citrate-dextrose (135 mM dextrose, 75 mM sodium citrate dihydrate, 3 mM citric acid monohydrate) (ACD) to be used for inoculation, 10 ml in EDTA to be used for extraction of DNA, and 10 ml without additive for serum. The blood was sent chilled to the U.S. Department of Agriculture, Agricultural Research Service Animal Disease Research Unit laboratory in Pullman, Washington (USA) and was received within 24 hr of collection.

Whole blood for inoculation was washed three times in phosphate buffered saline (0.15 M NaCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 3 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.2) (PBS) to remove buffy coat leucocytes. The erythrocytes then were suspended to 15% packed cell volume (PCV) in Puck's Saline-Glucose (PSG) (Palmer et al., 1982), pooled, then divided into three aliquots of 40 ml each, and placed on ice until inoculated. Each of the three aliquots was individually inoculated intravenously into animals C-454, S-005 and BH-1-H. A pre-inoculation blood sample from each recipient animal was obtained and additional samples were taken three times each week for 60 days. From each blood sample, 5 ml was added to a tube containing sodium heparin as anticoagulant for determining PCV and preparation of a thin blood smear to be stained with Giemsa, 5 ml was added to a tube containing EDTA for extraction of DNA, and 10 ml was used to extract serum. The PCV was determined on a micro-capillary centrifuge, model MB (International, Boston, Massachusetts, USA). Infection was recorded as the percent infected erythrocytes (PPE) after counting a minimum of 3,000 Giemsa-stained cells. When recipient animals developed infection accompanied by a severe anemia, tetracycline (LA-200, Pfizer Incorporated, New York, New York) was administered intramuscularly at 25 mg/kg body weight.

For the indirect immunofluorescence (IIF) assay (Tibbitts et al., 1992), we used erythrocytes infected with well characterized, tick-transmissible Idaho (USA) isolates of *A. ovis* (Stiller et al., 1989) and *A. marginale* (Zaugg et al., 1986) as antigens. Fluoresceinated recombinant Protein-G (Zymed, San Francisco, California) was used as secondary Immunoglobulin G (IgG) detecting reagent as described by Tibbitts et al. (1992). Control antisera were obtained from the same animals as those from which antigen had been obtained. Both *A. ovis* and *A. marginale* IIF antigen slides were used to evaluate sera obtained from each of the experimental animals throughout the 60-day period as well as sera obtained from the blood donor bighorn sheep. When monoclonal antibody (MoAb) was employed as the primary reagent, fluoresceinated rabbit anti-mouse IgG (Zymed) was used in place of Protein-G. No monoclonal antibody specific for *A. ovis* is yet available, but several exist that recognize antigens specific to *A. marginale* (McGuire et al., 1984). An *A. marginale*-specific monoclonal antibody (A-22B1) (McGuire et al., 1984) that recognizes an isolate-common determinant was used with antigen blood smears prepared from infected experimental animals.

Blood samples collected in EDTA were initially washed in PBS to remove buffy coat cells; the packed erythrocytes then were frozen at

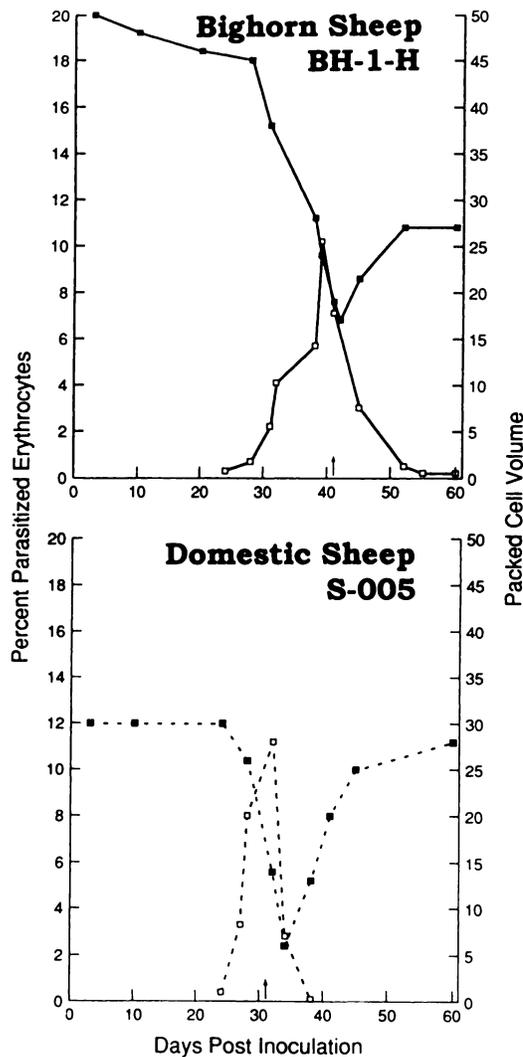


FIGURE 1. Clinical parameters during the course of an *Anaplasma* infection in a spleen-intact bighorn sheep and splenectomized domestic sheep. Both animals were inoculated with blood pooled from three desert bighorn sheep in an area of southern California with a high prevalence of *Anaplasma* antibodies. Closed squares represent packed cell volume (%). Open squares represent percent parasitized erythrocytes. Arrows indicate the day of treatment with tetracycline.

–20 C until used. After thawing, 0.5 ml of each sample was prepared for DNA hybridization, as described by Eriks et al. (1989). Control DNA was prepared from the Idaho isolates of *A. marginale* and *A. ovis*. The DNA probes pAM113 from an *A. marginale* genomic DNA library (Barbet et al., 1987) and pAO12A from an *A.*

*ovis* genomic DNA library (Shompole et al., 1989) were used under hybridization conditions described by Goff et al. (1988) and Shompole et al. (1989).

## RESULTS

Infected erythrocytes were detected from Giemsa-stained blood smears of both the domestic sheep (S-005) and the bighorn (BH-1-H) 24 days post-inoculation (PI). The parasitemia increased in conjunction with a decrease in PCV over the next several days (Fig. 1). A peak PPE of 11.2% occurred in S-005 on day 32 PI. In BH-1-H, the peak PPE of 10.2% did not occur until day 39 PI. When the PCV dropped to 17% (66% reduction from pre-inoculation level) on day 42 PI the bighorn sheep was given tetracycline. The acute infection began to resolve immediately with a slow return to a normal clinical condition. The domestic sheep also developed clinical disease as a result of the infection, and the acute episode was characterized by severe anemia with a PCV of 6%; this was an 80% reduction from the pre-inoculation level. As with the bighorn sheep, the animal recovered slowly following tetracycline treatment. In contrast, there was no evidence of infection in the calf (C-454) throughout the 60-day observation period.

All sera from the three donor bighorn sheep had antibody that reacted to both *A. ovis* and *A. marginale* IIF antigens. Both S-005 and BH-1-H seroconverted on day 24 PI, with IIF antibody reactivity to both *A. ovis* and *A. marginale* antigens (Table 1).

The pAO12A probe first hybridized with DNA from the sample of both S-005 and BH-1-H collected on day 17 PI. On this day infected erythrocytes remained undetectable by stained blood smear evaluation. Intensity of the hybridization signal increased over the next several days in parallel with the increasing parasitemia as revealed by stained blood smears. As the acute infection resolved, there was a corresponding decrease in the hybridization signal intensity (Table 1). Throughout the

TABLE 1. Results of inoculation of desert bighorn sheep (*Ovis canadensis nelsoni*) blood into a spleen-intact bighorn, splenectomized domestic sheep and splenectomized calf, and analysis by indirect immunofluorescence (IIF) and nucleic acid hybridization.

| Recipient animal      | Day post-inoculation | Giemsa-stained blood smear (PPE) <sup>a</sup> | Indirect immunofluorescence ( <i>A. marginale</i> antigen/ <i>A. ovis</i> antigen titer) <sup>b</sup> | Nucleic acid probe hybridization |                             |
|-----------------------|----------------------|---|---|----------------------------------|-----------------------------|
|                       |                      |   |   | <i>A. marginale</i> probe pAM113 | <i>A. ovis</i> probe pAO12A |
| Bighorn sheep BH-1-H  | 3                    | neg. <sup>c</sup>                             | neg.  | neg.                             | neg.                        |
|                       | 17                   | neg.  | neg.  | neg.                             | +                           |
|                       | 20                   | <0.1  | neg.  | neg.                             | ++                          |
|                       | 24                   | 0.3   | 800/400   | neg.                             | ++                          |
|                       | 32                   | 4.1   | 800/400   | neg.                             | +++                         |
|                       | 39                   | 10.2  | 800/400   | neg.                             | ++++                        |
|                       | 45                   | 3.0   | 6,400/3,200   | neg.                             | ++++                        |
|                       | 60                   | 0.2   | 1,600/3,200   | neg.                             | +                           |
| Domestic sheep S-0005 | 3                    | neg.  | neg.  | neg.                             | neg.                        |
|                       | 17                   | neg.  | neg.  | neg.                             | +                           |
|                       | 20                   | <0.1  | neg.  | neg.                             | +                           |
|                       | 24                   | 0.4   | ND <sup>d</sup> /800  | neg.                             | ++                          |
|                       | 32                   | 11.2  | 400/800   | neg.                             | ++++                        |
|                       | 39                   | 0.1   | 400/800   | neg.                             | ++                          |
|                       | 45                   | <0.1  | 3,200/1,600   | neg.                             | +                           |
|                       | 60                   | neg.  | 3,200/1,600   | neg.                             | neg.                        |
| Calf C-454            | 3                    | neg.  | neg.  | neg.                             | neg.                        |
|                       | 17                   | neg.  | neg.  | neg.                             | neg.                        |
|                       | 20                   | neg.  | neg.  | neg.                             | neg.                        |
|                       | 24                   | neg.  | neg.  | neg.                             | neg.                        |
|                       | 32                   | neg.  | neg.  | neg.                             | neg.                        |
|                       | 39                   | neg.  | neg.  | neg.                             | neg.                        |
|                       | 45                   | neg.  | neg.  | neg.                             | neg.                        |
|                       | 60                   | neg.  | neg.  | neg.                             | neg.                        |

<sup>a</sup> Percent parasitized erythrocytes (PPE) based on the observation of at least 3,000 erythrocytes.

<sup>b</sup> Titer equals the reciprocal of the last dilution giving a positive reaction defined as: fluorescence intensity equal or greater to that of a weak positive control.

<sup>c</sup> neg., negative.

<sup>d</sup> Not done.

experimental period, the pAM113 probe failed to hybridize with any of the samples, indicating that the infection was not due to *A. marginale* (Table 1).

Blood smears prepared from both S-005 and BH-1-H on the day of peak parasitemia were used as antigen in the IIF assay employing monoclonal antibodies. Both *A. ovis* and *A. marginale* IIF antigen slides were included as controls. The A22-B1 MoAb reacted with control *A. marginale* antigen as expected and failed to react with either the control *A. ovis* antigen or antigen slides prepared from each experimental animal. A polyclonal antiserum from a domestic sheep containing antibody to *A.*

*ovis* and an antiserum from a calf containing antibody to *A. marginale* reacted with the antigen prepared from both the infected domestic sheep and bighorn sheep. The reaction was similar to that commonly found with *Anaplasma* IIF serology, in which the cross-reactivity is extensive.

#### DISCUSSION

This is the first report of natural *Anaplasma* infections occurring in bighorn sheep. We previously demonstrated that bighorn sheep are susceptible to experimental infection with *A. ovis* (Tibbitts et al., 1992), and that bighorn sheep may support replication of *A. marginale*, although

not as efficiently as *A. ovis* (W. Goff, unpubl.). Therefore, it may be possible, under certain conditions, that bighorn sheep could be infected with either or both of these species. The strongest evidence implicating *A. ovis* as the isolate described in this paper was its failure to infect a splenectomized calf, while producing a typical *A. ovis*-like infection in both a splenectomized sheep (Splitter et al., 1956; Shompole et al., 1989) and bighorn sheep (Tibbitts et al., 1992) within the expected incubation period. At no time during the 60-day period of observation and testing was there any indication of an infection in the splenectomized calf. In addition, no detectable antibody reactivity developed during this time.

Currently, IIF is the only useful serologic assay for anaplasmosis in wildlife (Tibbitts et al., 1992; Jessup et al., 1993), and it has a major deficiency in that it is only genus specific. However, under conditions involving specifically adsorbed sera from bighorn sheep in this area (Jessup et al., 1993), the IIF assay provided additional serologic evidence that *A. ovis* is the likely species to be found associated with these bighorn sheep. There is need for an *A. ovis*-defined antigen assay that will allow for the discrimination between these two species. Because of this problem, we used other techniques to characterize this field isolate. Fortunately, highly specific *A. marginale* reagents were available, both MoAb and a DNA probe, that allowed us to rule out *A. marginale*. In addition, the probe derived from an *A. ovis* DNA sequence helped verify that the isolate was most likely *A. ovis*. This particular probe is lacking in specificity because there is some cross-hybridization with *A. marginale* DNA; however, it still is a useful epidemiologic tool when used in conjunction with the highly specific *A. marginale* probe.

Anaplasmosis is characterized by persistent infection (Zaugg et al., 1986). This also may occur in wild ruminants, but has not been extensively studied (Christensen

et al., 1960). From our experience, we suggest that a persistent infection follows the resolution, natural or tetracycline induced, of the acute infection. This is based on the detection of specific antibody with no reduction in titer for  $\leq 4$  mo following seroconversion. If this occurs in free-ranging populations, transmission probably occurs during active *Dermacentor* spp. tick activity. *Dermacentor* ticks are vectors of both *A. marginale* (Stiller et al., 1983) and *A. ovis* (Stiller et al., 1989; Kocan and Stiller, 1992). We recently found *D. Andersoni* to be a suitable vector for the experimental transmission of *A. ovis* from domestic sheep to bighorns, between bighorns, and back to domestic sheep (D. Stiller, unpubl.). *Dermacentor hunteri* ticks occur on bighorn sheep in this study area (W. Boyce, unpubl.) and can transmit both *A. ovis* and *A. marginale* under experimental conditions (D. Stiller, unpubl.). The origin of *A. ovis* infection in free-ranging bighorn sheep populations and in domestic sheep is not known. Currently, there is no evidence for a mandatory interaction between domestic sheep and bighorn sheep in the maintenance of infection within populations of either. This aspect of the epizootiology of the disease deserves further attention.

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