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A RETROSPECTIVE SEROLOGIC SURVEY FOR *ANAPLASMA* SPP. INFECTION IN THREE BIGHORN SHEEP (*OVIS CANADENSIS*) POPULATIONS IN CALIFORNIA

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ABSTRACT: Using an indirect immunofluorescence assay, we determined the prevalence of *Anaplasma*-reactive antibody in three herds of bighorn sheep, each a different subspecies and occupying a different habitat in California (USA). Antibodies to *Anaplasma* spp. were identified in none of twenty California bighorn (*Ovis canadensis californiana*) sampled from the Mt. Baxter herd, 11 of 17 peninsular bighorn (*O. canadensis cremnobates*) sampled in the Santa Rosa Mountains, and all 20 desert bighorn (*O. canadensis nelsoni*) sampled at Old Dad Peak/Kelso Mountains. Based on an assay and an adsorption technique, the titers most likely were due to *Anaplasma ovis*. The presence and species of tick vectors in each of the habitats, and the presence or absence of deer or livestock were identified as factors potentially influencing seroprevalence of antibodies.

Key words: Anaplasmosis, bighorn sheep, *Ovis canadensis*, indirect immunofluorescence, serologic survey, *Anaplasma* spp.

INTRODUCTION

Anaplasmosis is an infectious, intraerythrocytic, rickettsial disease of wild and domestic ruminants. Anaplasmosis in cattle, caused by *Anaplasma marginale*, causes substantial economic loss and can produce severe clinical disease characterized by anemia, icterus, depression, anorexia and often death (Weinman and Ristic, 1968). Although the current prevalence of anaplasmosis in the United States is not known, McCallon (1976) found the highest prevalence in cattle occurred in California (USA). In California, black-tailed deer (*Odocoileus hemionus columbianus*) are commonly infected with *A. marginale* with antibody prevalence often approaching 65% (Behymer et al., 1989). Mule deer (*O. hemionus hemionus*) also are naturally infected (Renshaw et al., 1977). Infected deer rarely have overt signs of disease but serve as reservoirs of infection for cattle (Howarth and Hokama, 1973). White-tailed deer (*O. virginianus*), elk (*Cervus elaphus*), pronghorn antelope (*Antilocapra americana*) and bison (*Bison bison*) are susceptible to experimental *A. marginale* infection but their status as natural reservoirs is less well understood (Ristic and Watrach, 1961; Kreier and Ristic, 1963; Howe et al., 1964; Zaugg, 1987). Domestic sheep and goats both are susceptible to *A. ovis*, but the disease is more severe in goats (Splitter et al., 1956; Kimberling, 1988). Experimental *A. ovis* infections in white-tailed deer (Kreier and Ristic, 1963), mule deer (Zaugg, 1988), pronghorn antelope (Zaugg, 1987) and bighorn sheep (*Ovis canadensis*) (Tibbitts et al., 1992) have been described. Natural *A. ovis* infections have not been reported from wild ruminants except for desert bighorn sheep from which we recently recovered an *A. ovis* isolate in southern California (Goff et al., 1993).

Because bighorn sheep are susceptible to clinical anaplasmosis (Tibbitts et al., 1992) and since *A. ovis* has been isolated from that species in southern California

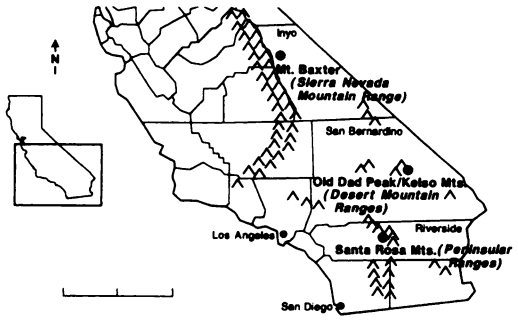

FIGURE 1. The locations of three bighorn sheep herds examined for immunofluorescent antibodies to anaplasmosis in California. Bar = 160 km.

(Goff et al., 1993), we were interested in determining the prevalence of anaplasmosis in selected bighorn sheep herds in California. For this retrospective study, we chose serum samples from three herds of bighorn sheep typical of the three recognized subspecies in California, each from distinct ecological areas with different livestock grazing and wildlife management practices. Because the serologic tests most often used for the diagnosis of anaplasmosis in domestic livestock (complement fixation (CF), card agglutination (CA)) have not proven reliable with most wild ruminant sera (Howe and Hepworth, 1965; Magonigle et al., 1981), we used an indirect immunofluorescence (IIF) assay modified for use with bighorn sheep (Tibbitts et al., 1992).

MATERIALS AND METHODS

Study animals

Eighteen to 20 serum samples from adult females (2 to 8 yr old) from the largest herds of each of three subspecies of bighorn sheep were selected from frozen, cataloged, sera archived by the California Department of Fish and Game (CDFG), Wildlife Investigations Laboratory (WIL). Locations of the three herds studied are shown in Figure 1.

Sera from eight adult animals sampled in March 1986 and 12 adult animals from March 1988 were selected from the Mt. Baxter population (36°51'N, 118°21'W) of California bighorn sheep (*O. canadensis californiana*), a state-listed threatened subspecies. The habitat types, elevations and aspects used by these bighorn have been described previously (Wehausen, 1980). A barrier fence separated a cattle grazing allotment from areas used by bighorn sheep. Cattle rarely breached this barrier and no significant contact was reported for many years. There were no domestic sheep within this range but the bighorn population shared both winter and summer ranges with migratory mule deer. Ixodid ticks inhabiting the eastern slope of the Sierra Nevada that were potential vectors of *Anaplasma* spp. included *Dermacentor albipictus*, *D. andersoni*, and *D. variabilis* (Lancaster et al., 1968; Furman and Loomis, 1984). During capture and sampling of bighorn sheep at Mt. Baxter in March 1986, ticks removed from the head and ears of six bighorn were identified as *D. albipictus* (E. C. Loomis, pers. comm.).

Twenty sera from adult animals from the Old Dad Peak/Kelso Mountains population (34°46'N, 115°49'W) of *O. canadensis nelsoni* were collected from October 1987 through January 1988. Elevations, habitats and habitat use patterns for these bighorn have been described (Bleich, 1993). There was substantial overlap between cattle and bighorn, and both species are sympatric in the flatter terrain (Bleich et al., 1987). There are no domestic sheep in this area. Mule deer are rarely seen in these mountain ranges. The primary potential vector for this region is *D. hunteri* (D. Stiller and W. Boyce, unpubl.). Although *D. occidentalis*, a proven natural vector of *A. marginale* (Osebold et al., 1962; Christensen and Howarth, 1966) and an experimental vector of *A. ovis* (D. Stiller, unpubl.) may be found in some areas of the Mojave Desert, its presence in this particular ecosystem is unlikely (Furman and Loomis, 1984). During captures of bighorn sheep at Old Dad Peak in December 1989 and 1992, ticks were removed from the ears of 65 individual animals and all were identified as *D. hunteri* (W. Boyce, unpubl.).

Sera from 12 bighorn sampled in 1981 and six bighorn sampled in 1983 from the Santa Rosa Mountains (33°25'N, 116°13'W) were tested for *Anaplasma* antibodies; *Ovis canadensis cremnobates*, a state-listed threatened subspecies of bighorn sheep, also is a candidate for federal listing as an endangered subspecies. The elevations and habitats used by these bighorn have been described (Jones et al., 1957). Contact between cattle and bighorn sheep probably occurred infrequently and there was no contact with domestic sheep. Resident mule deer inhabited the chaparral and forested areas above the bighorn habitat, and also occurred sympatrically with bighorn sheep at elevations around 1,400 m. *Dermacentor occidentalis* and *D. hunteri* in this region (Furman and Loomis, 1984) potentially could transmit *Anaplasma*; ticks removed from four free-ranging bighorn sheep

captured for sampling in the Santa Rosa Mountains in the spring of 1983 were identified as *D. hunteri* (E. C. Loomis, pers. comm.).

Serology

An indirect immunofluorescent (IIF) assay was used to detect serum antibodies to *Anaplasma* spp.. The assay was modified for use with wild ruminant sera, particularly bighorn sheep, as described by Tibbitts et al. (1992). Both *A. marginale* and *A. ovis* antigens were included, and control sera included both negative and positive (*A. marginale* and *A. ovis*).

The *A. marginale*-specific antisera included serum with a specific IIF antibody titer of 1/6,400 and cross-reaction titer with *A. ovis* antigen of 1/3,200 from a yearling Holstein steer (C-250) experimentally infected with an Idaho isolate of *A. marginale* (Zaugg et al., 1986); it also included serum with an *A. marginale* titer of 1/3,200 and cross-reaction titer to *A. ovis* antigen of 1/1,600 from a yearling spleen-intact male bighorn sheep (BH-Y-45) inoculated with the Idaho *A. marginale* isolate.

The *A. ovis*-specific antisera included serum with an IIF titer of 1/3,200 and cross-reaction titer with *A. marginale* antigen of 1/3,200 from an adult domestic sheep (S-87287) experimentally infected with an Idaho (USA) isolate of *A. ovis* (Stiller et al., 1989); it also included serum with an *A. ovis* IIF titer of 1/3,200 and cross-reaction titer with *A. marginale* antigen of 1/3,200 from a spleen-intact male bighorn lamb (BH-W-717) inoculated with the Idaho *A. ovis* isolate. In addition, serum was obtained from a domestic sheep (S-005) experimentally infected with an *A. ovis* isolate from bighorn sheep resident in the Old Dad Mountains (Goff et al., 1993). Serum was stored at −20 C until used.

Adsorption protocol

A modification of the procedure by Palmer and McGuire (1984) was used for purification of both *A. marginale* and *A. ovis* initial body (antigen adsorbent). Briefly, 4 l of pooled blood were obtained from two splenectomized calves infected at >30% parasitized erythrocytes (PPE) with the Idaho isolate of *A. marginale*. Three liters of pooled blood from two domestic sheep were obtained when both had Idaho isolate *A. ovis* infections of >20 PPE. The cattle and sheep blood were treated identically, but separately. Pooled blood was washed three times with phosphate buffered saline (PBS), pH 7.2, removing buffy coat cells each time. The washed infected red blood cells (RBC) were stored frozen at −70 C as 12 ml aliquots until used. Each aliquot was handled separately. An aliquot was thawed at 37 C for 5 min, adjusted to 40 ml with PBS and washed twice by centrifuging at 27,000 × *g* for 30 min at 4 C. The pellet then was suspended in 10 ml distilled water and allowed to stand at 21 C for 15 min. Thirty ml of PBS was added and the material again centrifuged at 27,000 × *g* for 15 min at 4 C. The pellet was resuspended in 20 ml of PBS and sonicated for 2 min at 50 watts relative output with a microtip probe (Sonifer model W140D, Branson Sonic Power Co., Danbury, Connecticut, USA). The sonicate was centrifuged at 1,500 × *g* for 15 min and washed once with PBS. The pellet was resuspended in 10 ml of distilled water and allowed to stand for 5 min. The material was adjusted to 40 ml with PBS and washed twice at 1,500 × *g* for 15 min at 4 C, and the final pellet resuspended in 1 ml of Puck's Saline-G (Palmer and McGuire, 1984), transferred to microcentrifuge tubes and stored at −70 C until used as a purified initial body adsorbent.

Samples of the initial body preparations were checked before and after freezing for viability and antigenicity using a dual fluorescence method (Goff et al., 1988). Briefly, initial bodies were incubated in antiserum C-250 for *A. marginale*, and antiserum S-87287 for *A. ovis*; they then were washed and incubated in either rhodamine-conjugated anti-bovine or anti-ovine IgG (Kirkagaard and Perry, Gaithersburg, Maryland, USA) to detect antigenicity. After an additional wash, the initial bodies were incubated in diluted (10 μg/ml) 6-carboxy fluorescein-diacetate (Boehringer Mannheim, Indianapolis, Indiana, USA) to detect viability. Following a final wash, samples were evaluated under epifluorescence microscopy using appropriate filters.

For each serum, two of the one ml tubes of initial body adsorbent were thawed rapidly, combined, and centrifuged at 15,000 × *g* at 4 C for 10 min in a microcentrifuge. The supernatant was discarded and 60 μl of serum added. The serum and adsorbent were mixed well and incubated at 37 C for 1 hr (primary adsorption). The material was centrifuged again and serum removed. Ten μl was saved for IIF analysis and the remainder was added to another pellet prepared from 1 ml of adsorbent. The material was mixed well and allowed to stand at 4 C overnight (secondary adsorption). The process was repeated for a third time at 37 C for 1 hr (tertiary adsorption). The serum collected after the third adsorption was evaluated by IIF against both antigens. Two aliquots of each serum were adsorbed, one against *A. marginale* initial bodies and the other against *A. ovis* initial bodies.

RESULTS

All sera were initially screened with the IIF assay at 1:100 serum dilution. Titration

TABLE 1. Indirect immunofluorescence (IIF) tests for antibodies to *Anaplasma* spp. in California bighorn sheep (*Ovis canadensis*), 1981 to 1988.

Subspecies	IIF serology Number positive/total (range of titers)[a]
O. c. nelsoni	20/20 positive (1:100 to ≥1:6,400)
O. c. cremnobates	11/18 positive (1:100 to ≥1:6,400)
O. c. californiana	0/20 (not done)

[a] Each serum was screened and titrated against both *Anaplasma marginale* and *A. ovis* antigens.

of sera from each of the positive herds representing weak, moderate and strong reactions against both *A. marginale* and *A. ovis* antigens failed to identify which species were likely involved due to extensive cross-reactivity (Table 1).

Calf (C-250) and domestic sheep (S-87287) control sera were used initially to establish the optimal parameters for adsorption. Despite the sera having titers >1:1,000 to both antigens, the three phase adsorption procedure resulted in a complete removal of cross-reactive antibody and only a one or two dilution decrease in homologous titer (Table 2). However, this was not the case with control bighorn antisera. The *A. marginale* adsorbent removed all antibody reactivity from BH-Y-45 antisera, and while the *A. ovis* adsorbent removed cross-reactive antibody, it also removed most of the homologous antibody reactivity (Table 2). The *A. ovis* adsorbent removed cross-reactive antibody from BH-W-717 antisera and most of the homologous antibody reactivity. Like BH-Y-45, cross-reactive antibody was removed from BH-W-717 with the *A. marginale* adsorbent, as well as most of the specific *A. ovis* antibody reactivity (Table 2).

Both the *A. marginale* and *A. ovis* adsorbents removed all the *A. marginale* antibody reactivity from the four field sera tested (Table 2). Similar to that of the defined bighorn antisera, the *A. marginale* adsorbent failed to remove all the *A. ovis* reactivity, but did reduce the titer >10-fold (Table 2). Results of adsorption of serum from domestic sheep (S-005) were similar to those of S-87287, where a con-

TABLE 2. Results of indirect immunofluorescence (IIF) following immunoadsorption of defined and field antisera with *Anaplasma marginale* and *A. ovis* initial bodies.

Animal	Pre-adsorption titers		*A. marginale* adsorbent		*A. ovis* absorbant	
	A. marginale antigen	*A. ovis* antigen	*A. marginale* antigen	*A. ovis* antigen	*A. marginale* antigen	*A. ovis* antigen
C-250[a]	6,400[b]	3,200	200	400	1,600	Neg.
BH-Y-45[c]	1,600	800	Neg.	Neg.	100	Neg.
S-87287[d]	1,600	3,200	Neg.	1,600	100	200
BH-W-717[e]	3,200	3,200	Neg.	100	Neg.	100
S-005[d]	3,200	1,600	Neg.	400	Neg.	Neg.
OCC81006[f]	6,400	6,400	Neg.	200	Neg.	100
OCC81010[f]	1,600	6,400	Neg.	100	Neg.	100
OCN88001[g]	3,200	1,600	Neg.	100	Neg.	100
OCN88010[g]	1,600	6,400	Neg.	100	Neg.	100
OCC86026[h]	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.

[a] Calf experimentally infected with *A. marginale*.
[b] Reciprocal of final dilution positive by IIF; Neg. = negative.
[c] Bighorn sheep experimentally infected with *A. marginale*.
[d] Domestic sheep experimentally infected with *A. ovis*.
[e] Bighorn sheep experimentally infected with *A. ovis*.
[f] Bighorn sheep (*O. canadensis cremnobates*) from the Santa Rosa herd.
[g] Bighorn sheep (*O. canadensis nelsoni*) from the Old Dad Peak herd.
[h] Bighorn sheep (*O. canadensis californiana*) from the Mt. Baxter herd.

siderable amount of the original *A. ovis* titer remained after *A. marginale* adsorption (Table 2).

DISCUSSION

Cross-reactivity on the IIF assay between *A. marginale* and *A. ovis* precluded its use as a means of identifying the likely species of *Anaplasma* involved. For this reason, an adsorption protocol using both *A. marginale* and *A. ovis* antigen adsorbents was adopted. Unambiguous results of such a procedure would be either the complete removal of cross-reactive antibody with little effect on homologous titer, or the equal removal of both antigen reactive antibodies with both adsorbents; this latter case would be evidence that the antisera contained only cross-reactive antibody, and that the organism responsible for antibody induction was not homologous to either isolate used as assay antigen.

Adsorption of cross-reacting antibody was clearly demonstrated with antisera from calf C-250 and domestic sheep S-87287 experimentally infected with known *A. marginale* and *A. ovis* isolates, respectively. In addition, serum from domestic sheep S-005 that was experimentally infected with *A. ovis* isolated from desert bighorn sheep adsorbed similarly to that of domestic sheep S-87287. However, using either adsorbent, most homologous reactivity was removed from antisera of BH-Y-45 and BH-W-717. Similar results were obtained with the four field sera. Removal of antibody by both adsorbents was not entirely equal, some reactivity to *A. ovis* antigen always remained. Thus we conclude that there was something unique about bighorn sera that affected this adsorption procedure. Since some *A. ovis* reactivity remained after adsorption, we also inferred that the free-ranging bighorn sheep were infected with *A. ovis* or a very closely related organism. Also, we propose that cross-reactive antigenic determinants may be immunodominant in bighorn sheep.

There is additional evidence that these bighorn sheep were infected with *A. ovis*. Bighorn sheep are more susceptible to experimental infection with *A. ovis* than *A. marginale* (Tibbitts et al., 1992). Also, a known (Idaho) isolate of *A. ovis* was observed to be readily transmissible between captive bighorn sheep by *D. andersoni* male ticks (D. Stiller, unpubl.). Finally, *A. ovis* was isolated from desert bighorn sheep from Old Dad Peak (Goff et al., 1993). The presence of *A. marginale* in bighorn sheep, however, cannot be completely ruled out. Bighorn sheep BH-Y-45 used in this study as a source of specific *A. marginale* antiserum developed an infection after an intravenous inoculation of an Idaho isolate of *A. marginale*. The animal was wild-caught and under considerable stress which may have contributed to increased susceptibility; but the spleen was intact.

The prevalence of *Anaplasma* antibody in bighorn sheep varied widely among the three study populations. In free-ranging wild ruminants, insect and acarine vectors are the most likely means of transmission and complex interrelationships between domestic and wild mammal hosts and their common tick vectors influence the epidemiology of *Anaplasmosis*. Therefore, we hypothesize that the prevalence of *Anaplasma* antibodies in bighorn sheep largely depends on local ecological factors that influence the abundance and species of vectors (ticks), the competence of those vectors, and distribution of infected and susceptible hosts.

The only contact between bighorn sheep in these study groups and other susceptible ruminant hosts were with cattle and mule deer. Mule deer are susceptible to experimental *A. ovis* infection (Zaugg, 1988) but mule deer in our study areas have not been tested for *Anaplasma* antibody. However, mule deer from other areas in California have been tested by IIF, and 40% of about 60 samples contained antibody reactive to *Anaplasma* antigens (W. Goff and D. Jessup, unpubl.). This is very similar to the overall prevalence of *Anaplasma* antibodies (34%) reported for three subspecies of

deer from California (Behymer et al., 1989). While mule deer could play a role in the epizootiology of anaplasmosis, we found *Anaplasma* antibody reactivity in 100% of bighorn sheep sera from Old Dad Peak where deer are seldom, if ever, found. In contrast, no evidence of *Anaplasma* antibody reactivity was found in sera from bighorn sheep from Mt. Baxter, an area where they share habitat with deer much of the year. The situation in the Santa Rosa Mountains is less clear. Although bighorn sheep and deer have some habitat overlap at the higher elevations, we know of no significant changes between 1981 and 1983 that would explain a decrease in apparent seroprevalence from 92% to 0%. The smaller sample size in 1983 may be a contributing factor.

Although cattle could act as a reservoir for *Anaplasma* spp., the amount of habitat overlap and the lack of interaction between these species makes this scenario unlikely. Moreover, serologic evidence and isolation of *A. ovis* from apparently healthy bighorn at Old Dad Peak support the hypothesis that *A. ovis* rather than *A. marginale* is the most likely *Anaplasma* organism present.

Bighorn sheep populations may maintain *Anaplasma* spp. without transmission from other ruminant species. The population of bighorn sheep with the highest antibody prevalence also was the most dense. Density may play a role in the prevalence of anaplasmosis within bighorn populations. It is interesting to note that *D. hunteri* was present in both areas where *Anaplasma* spp. antibodies were found and that *D. hunteri* can transmit both *A. marginale* and *A. ovis* experimentally (D. Stiller, unpubl.). Transmission also may follow blood feeding by many arthropods (Ewing, 1981; Marchette and Stiller, 1982; and Stiller et al., 1989).

Dermacentor albipictus experimentally transmits both *A. marginale* (Boynton et al., 1936) and *A. ovis* (D. Stiller, unpubl.). However, we found no evidence of anaplasmosis in the bighorn sampled at Mt. Baxter in 1986, the same year *D. albipictus* was found on them. In a study of cattle in high desert mountain ranges in Idaho, similar in climate, vegetation types, and potential vectors to the Mt. Baxter study area, Zaugg (1990) concluded it unlikely that wildlife served as reservoirs of anaplasmosis, and that the source of infection among cattle were the cattle themselves.

Our findings have significant wildlife management implications. Over the past 10 years the Old Dad Peak population has been one of the largest and most prolific herds of desert bighorn sheep in California, and over 200 bighorn have been relocated from this herd to areas of historic bighorn sheep use (Bleich et al., 1990; Torres and Bleich, 1993). Some of these areas have had existing remnant populations of bighorn sheep whose exposure status to anaplasmosis is not known. Since there is a potential for clinical disease due to anaplasmosis in bighorn sheep (Tibbitts et al., 1992), previously unexposed desert bighorn sheep may be at risk if exposed to carrier bighorn sheep in an environment where a competent vector population exists. Therefore, additional studies seem warranted to verify the geographic locations and species of potential tick vectors, to determine the prevalence of anaplasmosis in bighorn sheep herds throughout California, to clarify the migratory behavior of bighorn sheep and deer relative to seasonal tick activity, and to identify suitable tick micro- or macro-habitat.

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