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ISOLATION OF A CHLAMYDIAL AGENT FROM ROCKY MOUNTAIN BIGHORN SHEEP

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Abstract: A total of 53 clinical specimens from both healthy and diseased Rocky Mountain bighorn sheep *(Ovis canadensis)* were examined for *Chlamydia.* An agent consistently lethal for chicken embryos was recovered from a nasal swab taken from a normal ewe. This agent, designated BHS-15, possesses antigens which fix complement in the presence of anti-chlamydial serum, is susceptible to chlortetracycline, and is resistant to sodium sulfadiazine and streptomycin. Attempts to culture the isolate in quality control media, including blood agar, thioglycolate broth, and PPLO broth and agar were unsuccessful. A recommendation is made for classification of agent BHS-15 as a member of the species *Chlamydia psittaci.* The possible relationship of the isolate to the pneumonia complex in bighorn sheep is discussed.

INTRODUCTION

Bighorn sheep *(Ovis canadensis)* populations have been declining for some time. The encroachment of man and domestic livestock on land once used by the sheep for winter range has resulted in crowding of these herds which have accentuated several decimating factors. The presence of a pneumonia complex in the sheep has been shown to be of considerable importance in the reduction of bighorn sheep numbers.[2] Numerous infectious agents have been identified in association with pneumonias in these sheep and include lungworms in the genus *Protostrongylus,*[4] bacteria of the genera *Pasteurella, Corynebacterium,* and *Diplococcus*[5,11] mycoplasmas,[17] and viruses.[9,10]

Fifty-three samples collected from both free-ranging and captive bighorn sheep were presented by the CSU Wild Animal Disease Center to the virology lab of the CSU Diagnostic Laboratory to culture for *Chlamydia.* Studies with a *Chlamydia*-like agent isolated from a normal bighorn ewe are presented in this report.

MATERIALS AND METHODS

Sample Collection

Nasal swabs collected in the field were suspended in a transport medium of buffered saline containing no antibiotics and frozen on dry ice for transport to the laboratory. These swabs and tissue samples from sheep examined postmortem were frozen at -70 C until processing for *Chlamydia.*

Chlamydia Isolation

Thawed swabs were rinsed thoroughly in the transport medium and the fluid collected. Thawed tissues were homogenized with 10 ml of cold saline in a Ten Broeck grinder. Samples were processed by three cycles of differential centrifugation at 1200 × g for 30 min. After each cycle the middle 10 ml were harvested, added to an additional 10 ml of saline, and recentrifuged. The final 10

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ml of supernatant was harvested as inoculum.

Identical sets of six or seven day old specific pathogen free chicken embryos [a] were inoculated via the yolk sac with either undiluted inoculum or inoculum diluted 1:10. Uninoculated control eggs were also included with each set. Eggs were incubated at 37 C and candled daily for 12 days post inoculation. Embryos dying 3 to 12 days post inoculation with lesions typical of chlamydial infection and exhibiting elementary bodies in Gimenez-stained yolk sac smears were subpassaged.[15]

Stock Preparation

The isolate designated BHS-15 was cultured a total of 15 embryo pasages. Stocks were suspended in saline and frozen at -70 C between passages. A chlamydial agent isolated from the kidney of an aborted ovine fetus, and designated B-577[16] was used as a reference agent and for preparation of specific antisera. Calculations of chicken embryo lethal dose ($CELD_{50}$) were performed by the method of Reed and Muench[12] at the 12th and 15th passages of the isolate.

Complement Fixation Test

Group specific (GS) antigen for use in the complement fixation (CF) test was prepared by ether-extraction from BHS-15 infected yolk sac as described by Reed.[12] Uninfected yolk sacs and strain B-577 infected yolk sacs were prepared in the same manner for use as negative and positive control antigens respectively. Positive GS antisera was prepared by repeated injections of rabbits with ether-extracted GS antigen from strain B-577. Normal rabbit sera was used as a negative control. The CF test was performed using the microtiter method described by Schmidt.[13] Heat inactivated sera (56 C for 30 min.) were block titrated against ether-extracted antigen preparations. A two percent suspension of sheep red blood cells mixed with equal volumes of standardized antisheep hemolysin comprised the hemolytic system. The highest dilution of antigen and antisera which completely fixed two units of complement was considered the endpoint.

Antibiotic Sensitivities

Antibiotic sensitivities were performed by inoculating four sets of chicken embryos with serial tenfold dilutions of BHS-15. The first set then received 1 mg sodium sulfadiazine per embryo. The second received 150 μg streptomycin per embryo, and the third received 25 μg chlortetracycline per embryo. The fourth set remained untreated. Calculations of $CELD_{50}$ were performed for each set and compared.

RESULTS

One of fifty-three samples yielded an agent lethal for chicken embryos. The sample from which the isolate was recovered was a nasal swab collected from a five-year-old bighorn ewe showing no clinical signs of illness. This agent, designated BHS-15, produced a deep hyperemia and congestion of yolk sac blood vessels, and the yolk fluid was watery and bright yellow in color. In addition, the toes and legs of the embryo were hyperemic, and ecchymotic hemorrhages often were visible in the skin. Gimenez-stained impression smears of infected yolk sac showed the presence of numerous elementary bodies.

By the third passage, BHS-15 produced 100% mortality in inoculated embryos. However, freezing of infected yolk material at -70 C produced a significant reduction in percentage of mortality; immediate inoculation of fresh embryos after harvesting was necessary to maintain consistent titers. At the tenth chicken embryo passage, the titer of the

[a] Larson Laboratory Eggs, Inc., Gowrie, Iowa 50543, USA.

inoculum was calculated to be $10^{1.9}$ and at the fifteenth passage, a $CELD_{50}$ titer of 2.3 was observed. A correlation between concentration of the inoculum and the time of death of embryos after inoculation was not observed until the fifteenth passage, at which time an inverse linear relationship was demonstrated (Fig. 1).

Ether-extracted GS antigen prepared from eggs infected with BHS-15 fixed two units of complement at a dilution of 1:16 in the presence of a 1:32 dilution of immune serum (Table 1).

The titer of the isolate was reduced by only one of the four antibiotics tested. A hundred-fold reduction in titer of the inoculum occurred in embryos treated with chlortetracycline as compared with untreated embryos (Table 2). Agent BHS-15 was completely resistant to the action of sodium sulfadiazine at the concentration tested.

DISCUSSION

The pathologic changes induced by isolate BHS-15 in chicken embryos and its morphology under the light microscope indicate that it is chlamydial in nature. Since chlamydial agents are known to be involved in domestic sheep pneumonias, further characterization was necessitated. The identification of the isolate as a *Chlamydia* is based on the following criteria: 1) Identification of elementary bodies in yolk sac impression smears; 2) analysis of the behavior of the isolate in chicken embryos; 3) tests for the presence of rickettsiae, myco-

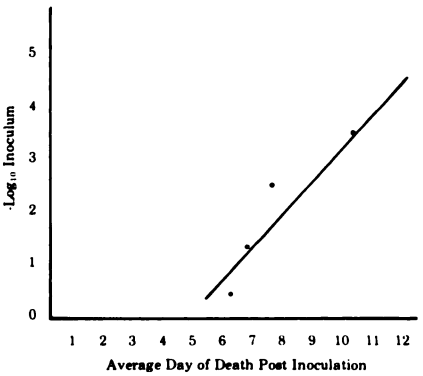

FIGURE 1. Correlation of inoculum dilution with average day of death of chicken embryos post inoculation.

TABLE 1. Demonstration of group specific chlamydial antigen by complement fixation.

Anti B-577 Serum Dilutions	BHS-15 Antigen Dilutions						B-577 Antigen Dilutions						Uninfected Antigen Dilutions					
	1:2	1:4	1:8	1:16	1:32	1:64	1:16	1:32	1:64	1:128	1:256	1:512	1:2	1:4	1:8	1:16	1:32	1:64
1:2	4	4	0	0	0	0	4	4	4	2	2	1	2	1	1	0	0	0
1:4	4	4	4	4	3	0	4	4	4	4	4	3	1	0	0	0	0	0
1:8	4	4	4	4	2	0	4	4	4	4	4	4	0	0	0	0	0	0
1:16	4	4	4	4	2	0	4	4	4	4	4	4	0	0	0	0	0	0
1:32	4	4	4	4	1	0	4	4	4	4	4	3	0	0	0	0	0	0
1:64	4	4	4	3	1	0	4	4	4	4	3	3	0	0	0	0	0	0
1:128	4	2	2	1	0	0	4	4	4	4	3	2	0	0	0	0	0	0
1:256	1	1	1	4	0	0	4	4	3	3	2	2	0	0	0	0	0	0

Degree of Complement Fixation: 4+ No hemolysis
3+ 25% hemolysis
2+ 50% hemolysis
1+ 75% hemolysis
0 Complete hemolysis

TABLE 2. Effect of antibiotics on infectivity of isolate BHS-15.

Treatment	Titer* of Inoculum
No treatment	2.32
Streptomycin (150 μg/embryo)	2.32
Sodium Sulfadiazine (1 mg/embryo)	2.38
Chlortetracycline (25 μg/embryo)	0.30

*Log_{10} $CELD_{50}$.

plasmas, bacteria, and viruses in the stock preparations; and 4) demonstration of group-specific antigen in ether-extracted yolk sac preparations of the isolate.[14]

Further, the isolate can be tentatively placed in the species *C. psittaci* because of its resistance to the action of sodium sulfadiazine. All mammalian strains of *Chlamydia*, other than those found only in man, are currently placed in this species.

Chlamydial agents have been reported in a wide variety of domestic and wild animal species, and appear to be as ubiquitous in the animal kingdom as are the viruses. It is not surprising to find that bighorn sheep also harbor this organism. Previous attempts to isolated chlamydia from bighorn sheep have not been reported.

The expansion of domestic animal herds and the recent successes in keeping captive bighorns has increased the possibility of bighorn exposure to chlamydial agents. Interspecies transfer of *C. psittaci* has been shown to occur.[1,7,8] Avian and mammalian strains of chlamydiae, those included in the *C. psittaci* group, appear to have a rather low host specificity.[6]

It is interesting to note that the agent characterized in this study was isolated from a clinically normal ewe. Possibly *Chlamydia* occur in bighorn sheep as part of the normal flora of the respiratory or gastrointestinal tracts and act as opportunists. Sub-clinical chlamydial infections are known to occur in many animal species and the intestinal tract of some ruminant species appears to be a natural habitat for *Chlamydia*.[3,19] However, without further data it would be difficult to estimate the prevalence in bighorn sheep. The need for information concerning the types of organisms that might be found in the normal or diseased bighorn is obvious. The results of the present study indicate chlamydiae also should be included and considered in future investigations of bighorn sheep disease.

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