Studies of the Role of *Dermacentor occidentalis* in the Transmission of Bovine Chlamydial Abortion

HARLAN D. CALDWELL AND E. LEE BELDEN

Division of Microbiology and Veterinary Medicine, University of Wyoming, Laramie, Wyoming 82070

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*Dermacentor occidentalis* nymphaal ticks were successfully infected with the agent of bovine chlamydial abortion. Multiplication of the agent was shown to occur during nymphaal engorgement, but chlamydial isolations were not made from nymphaal ticks after detachment or during subsequent metamorphosis. Primary tissue cultures of nymphaal viscera infected in vitro or in vivo failed to demonstrate chlamydial replication.

The natural mode of transmission of bovine chlamydial abortion is unknown (5, 8). Chlamydial abortion occurs primarily in cattle from the foothills and mountain ranges of California (3, 6), with sporadic occurrences reported in the intermountain regions of the United States. The geographical restriction in incidence of the disease in California implies that transmission is by an arthropod vector. Recently, chlamydial isolates with a pathogenic similarity to bovine chlamydial abortion isolates have been recovered from the Pacific Coast tick *Dermacentor occidentalis* and from native mammals from a sylvatic environment in California (1). These findings suggest the presence of a reservoir host carrying the chlamydial agent, with *D. occidentalis* acting as a vector.

To assess the possible role of a biological vector transmission of bovine chlamydial abortion, a study was undertaken to determine whether *D. occidentalis* would support the growth or viability of the chlamydial agent responsible for bovine chlamydial abortion.

**MATERIALS AND METHODS**

Feeding and maintenance of *D. occidentalis* ticks. Ticks, initially isolated in California, were fed on New Zealand White rabbits whose backs and sides had been shaved. Feeding capsules were attached by an adhesive (Bull Cement, 3-M Co., St. Paul, Minn.) and tape. Capsules were placed on the rabbits 24 hr prior to the addition of ticks to ensure that volatile substances from the cement did not interfere with tick attachment. Individual rabbits were used only once for tick feeding.

Dissection of ticks and preparation of primary tick tissue cultures. The procedure for dissection and culturing was essentially that described by Yunker and Cory (10) and Martin and Vidler (6). Molting nymphaal ticks held 7 days after their detachment from rabbits were used. Ticks were surface disinfected by immersion in 3% hydrogen peroxide and 70% ethyl alcohol for 3 and 5 min, respectively. Sterile cotton swabs were used to remove large, visible, contaminating materials on the nymphal cuticle while in the alcohol wash. Ticks were immobilized by partially embedding their ventral surfaces in sterile paraffin. The exposed dorsal surface of the ticks was then covered with Hanks balanced salt solution (HBSS) containing dihydrostreptomycin, vancomycin (Vancocin HCl, supplied by Eli Lilly and Co., Greenfield, Ind.), and amphotericin B, at concentrations of 50, 100, and 0.25 μg/ml, respectively. An incision was made around the periphery of the tick’s body by using a precision-grade micro-dissecting knife. The dorsal muscle attachments were severed, and the dorsal cuticle of the tick was removed. The contents of the body cavity were removed with a precision-grade micro-dissecting forceps and immediately transferred to HBSS. The dissections were done with sterile instruments under a dissecting microscope at a magnification of ×40.

In vitro inoculation of *D. occidentalis* primary tick cultures with chlamydiae. Tick tissue culture medium consisted of HBSS with 0.5% lactalbumin hydrolysate, 10% heat-inactivated fetal bovine serum, 10% whole chicken egg ultrafiltrate (Microbiological Associates, Inc., Bethesda, Md.), 10 μg of bovine plasma albumin per ml (fraction V; Sigma Chemical Corp., St. Louis, Mo.), 100 μg of vancomycin per ml, 100 μg of dihydrostreptomycin per ml, and 0.25 μg of amphotericin B per ml.

Tick tissue cultures were prepared from viscera of 7-day metamorphosing, engorged, nymphaal *D. occidentalis* ticks. Six viscera were placed in each of eight 30-ml polystyrene tissue culture flasks con-
taining 4.5 ml of culture medium. The viscera were incubated at 30 C for 24 hr after which the culture medium was withdrawn and replaced with 4.5 ml of fresh medium containing 20 µg of diethylaminoethyl (DEAE)-dextran per ml. The cultures were then incubated for 90 min at 30 C. A partially purified suspension (E. L. Belden, Ph.D. thesis, University of California, Davis, 1970) of the chlamydial isolate EBA 59-795 (9) was diluted to concentrations of 10^4.5, 10^4, and 10^3 50% chicken embryo lethal doses (ELD₅₀)/ml in 4.5-ml volumes of tick tissue culture medium. Each dilution was used to replace the medium containing DEAE-dextran in two of the eight primary cultures. The remaining two control cultures received 4.5 ml of culture medium only. Cultures containing dilutions of the organism were incubated for 3 hr at 30 C. The medium was then replaced with 4.5 ml of fresh culture medium, and the cultures were reincubated at 30 C. Sampling of the culture medium was done prior to infecting cultures and every day thereafter for 15 days. Cultures were sampled by removing 0.5 ml of medium. The sampled volume was replaced with 0.5 ml of fresh medium. The samples were stored in screw-cap vials at -70 C until they were assayed by inoculating 0.1 ml of the undiluted sample into six 7-day embryonating eggs. Viscera from one tick at each dilution were removed every 3 days postinoculation for 15 days and stored at -70 C. Viscera were homogenized in 1 ml of culture medium with a glass tissue grinder, and the homogenate was assayed by inoculating it undiluted in 0.1-ml amounts into embryonating eggs. The approximate ELD₅₀ values of the inocula were determined by calculating the average day of death (ADD) of inoculated eggs and extrapolating the infectivity from a standard curve (2).

Infection of feeding nymphal ticks with chlamydiae. Unfed D. occidentalis nymphal ticks were placed in two feeding capsules secured to a rabbit and allowed to attach. Twenty-four hours after attachment, intravenous injections of chlamydiae were administered. Three inoculations of 2 × 10^5.5 ELD₅₀ each were given in the right marginal ear vein at times 0, 1.5, and 3 hr. The objective of the series of inoculations was to create a chlamydiaemia in the host which would provide a more natural route for infection of ticks. To insure that a chlamydiaemia was produced, the rabbit was bled in the left marginal ear vein during and after the period of inoculations. Samples were taken at 0.5-hr intervals for 4 hr and then at 6, 18, 24, and 48 hr after the initial intravenous injection was administered. Blood samples were collected with a heparinized syringe and needle. The samples were diluted 1:5 in HBSS and inoculated immediately into six 7-day embryonating eggs. The ADD of inoculated eggs was calculated, and their approximate ELD₅₀ determined.

Four D. occidentalis nymphal ticks were removed from the rabbit on days 1, 2, and 3 postinoculation, surface-disinfected, and homogenized in 2.0 ml of HBSS. The homogenates were inoculated undiluted into embryonating eggs in 0.1-ml amounts. Embryos that died were examined for chlamydial developmental forms by Macchiavello staining of both impression smears and homogenate suspensions of the yolk sac membrane.

Additional D. occidentalis nymphal ticks previously exposed to chlamydiae by intravenous inoculations of the agent into their host were divided into two experimental groups. Group I engorged nymphal ticks were held for 7 days in cotton-plugged glass vials at 26 to 28 C. The ticks were dissected and prepared for in vitro cultivation. Six metamorphosing viscera were transferred to a 30-ml polystyrene culture flask containing 4.5 ml of culture medium. Twelve flasks containing six viscera each were prepared. Six flasks were incubated at 33.5 C and six at 30 C. Control viscera, fed on uninculturated rabbits, were incubated at both temperatures. Flasks at both incubation temperatures were further divided for sampling. Three flasks were used exclusively for visceral sampling, and the remaining three flasks were used for sampling of the culture medium. Two tick viscera were sampled every 3 days for 21 days, placed in 0.5 ml of fresh culture medium, and stored for no more than 5 days at -70 C before being assayed. Culture medium was assayed by inoculating the pooled undiluted medium in six 7-day embryonating eggs. One of the two sampled viscera from each sampling time was homogenized in 1 ml of culture medium and assayed for infectivity. The second sampled viscera was crushed onto a microscope slide, stained with Macchiavello stain, and examined for developmental forms of the organism.

Group II nymphal ticks were studied as intact metamorphosing nymphal ticks as compared to tick tissues cultivated in vitro (group I). Engorged ticks were separated according to their incubation temperatures. One lot of ticks was incubated in cotton-plugged glass vials at 33.5 C in a closed, humid jar. The second lot was incubated at 26 to 28 C under identical conditions. Control ticks fed on uninculturated rabbits were also incubated at both temperatures. Four ticks from each lot were sampled at 3-day intervals until molting was complete and were stored at -70 C for no more than 5 days prior to being assayed. The remaining two ticks were used for Macchiavello staining. Four ticks from each sampling time were pooled, surface-disinfected, homogenized with 2.0 ml of HBSS, and assayed for infectivity in six 7-day embryonating eggs.

Attempts at activating chlamydiae in adult ticks which were exposed to the organism as nymphs. To test the hypothesis that chlamydiae were present in tick tissue in an inactive form, adult D. occidentalis ticks which were exposed to chlamydiae as nymphs were fed on two different host species in an attempt to activate the agent. Fifty adult D. occidentalis ticks were placed in feeding capsules secured to a rabbit. The ticks were examined daily for attachment and on each of days 1, 2, 3, and 5 postattachment; four adult female ticks were removed. The four ticks from each sampling time were surface-disinfected and homogenized in 5.0 ml of...
HBSS. The homogenate was then divided into two equal volumes, one of which was centrifuged at 6,000 rev/min for 15 min at 4 C to pellet cell debris, and the other was left in its original state. The supernatant fluids from the centrifuged homogenate and the original homogenate were assayed by inoculating 0.1-ml amounts of the undiluted suspensions into six 7-day embryonating eggs. Controls consisted of homogenates from adult female ticks which had not been exposed to chlamydiae as nymphs.

The right shoulder areas of two, 8-month-old calves were shaved with electric clippers. Feeding capsules, identical to those used on rabbits, were then secured to the calves’ skin with adhesive and sutures. Approximately 50 adult ticks that had been exposed to chlamydiae as nymphs were placed in the attached feeding capsule on one calf, and 30 control or unexposed adults were placed in the capsule attached to the second calf. Ticks were examined daily for attachment, and on the second day of active feeding tick sampling was begun. Three adult, female ticks were removed after 2, 3, and 4 days of feeding and again after complete engorgement. Sampled ticks were surface-disinfected and homogenized in 5.0 ml of HBSS. The homogenate was then divided equally in volume; one portion was centrifuged to pellet cell debris, and the other left in its original state. The supernatant fluids from the centrifuged homogenate and the original homogenate suspension were then inoculated into separate groups of embryonating eggs. Control ticks were treated in an identical manner, but sampling numbers were reduced due to a limited number of feeding adult ticks.

Calves were bled for chlamydial isolation, leucocyte counts, and serology prior to the attachment of ticks. After the first day of tick attachment, calves were bled for 10 consecutive days and then on days 15 and 21 after tick attachment. Rectal temperatures of calves were taken before tick attachment and then in accordance with each bleeding. Sampled blood was inoculated undiluted in 0.1-ml amounts into 7-to 9-day embryonating eggs 1 hr after its collection. Serum samples were collected and diluted 1:5, and the relative anti-chlamydial titer was determined by the microtiter-complement fixation test (E. L. Belden, Ph.D. thesis).

Chlamydial isolations from the skin of calves were attempted 21 days after initial tick attachment. Feeding capsules were removed, and the area of skin where ticks had fed was washed with 70% ethyl alcohol. An elliptical incision of the skin was made down to the subcutaneous fascia, and the tissue was removed. For restraint, calves were administered 100 mg of proprionate-methyldichloride (Tranven, Diamond Laboratories, Des Moines, Iowa) intravenously. The outer skin of the excised tissue was removed and discarded. The remaining tissue was finely minced and then pulverized with a mortar and pestle in 2.5 ml of HBSS mixed with Alundum (Fisher Scientific Co., Fair Lawn, N.J.). The resulting suspension was transferred to a sterile test tube and placed at 4 C to allow the Alundum to settle from the suspension. A 0.1-ml amount of the undiluted suspension was then cultured in embryonating eggs.

## RESULTS

Isolation attempts from primary visceral cultures inoculated in vitro with chlamydiae. Chlamydial isolation attempts from both the sampled culture medium and tick viscera were unsuccessful. Inoculated embryos were killed 13 days postinoculation by placing them at 4 C. The inoculated embryos did not exhibit gross pathological changes characteristic of chlamydial infection. Macchiavello-stained yolk sac membrane homogenates were negative for elementary bodies. Three embryonating egg subpassages of pooled membrane homogenates were done before samples were designated negative for chlamydial isolations.

Isolations from nymphal ticks exposed to chlamydiae by intravenous injections of rabbits. The results of rabbit blood sampling are shown in Fig. 1. It was found that chlamydiae were cleared from the blood 6 hr after the initial inoculation. Homogenates of ticks removed from the rabbit on day 1 postinoculation of chlamydiae failed to kill inoculated embryos on the initial egg passage. Chlamydial isolations were not made after two additional egg subpassages. Homogenates of ticks removed from rabbits on days 2 and 3 postinoculation of chlamydiae.

![Fig. 1. Isolations of chlamydiae from the blood of an intravenously injected rabbit on which nymphal Dermacentor occidentalis were feeding. Inoculations were given immediately after bleeding at times 0, 1.5, and 3 hr.](http://iai.asm.org/)
mydiae killed embryos on the first egg passage. Dead embryos were examined, and elementary bodies were found in smears of yolk sac membrane homogenates. The recovery of chlamydiae from nymphaal ticks is shown in Fig. 2. Chlamydiae were not recovered at 24 hr but were recovered at 48 to 72 hr. Upon the completion of engorgement, 4 days after inoculations, chlamydiae could no longer be detected and apparently did not survive in the metamorphosing nymphaal tick. Similarly, chlamydiae were not detected in primary visceral cultures of nymphaal ticks prepared after completion of feeding. Three successive embryonating egg subpassages were made for all inocula before they were designated as negative.

Attempts at activating chlamydiae by feeding adult D. occidentalis ticks that were exposed to the agent as nymphs. Homogenates from adult ticks which were fed on rabbits or calves failed to demonstrate chlamydiae when cultured in embryonating eggs. Inocula were subpassaged a minimum of three times before being designated as negative. All attempts to detect the presence of chlamydial infection in calves which were exposed to adult ticks, previously infected as nymphs, were un-successful. Cultured blood for each sampling time was negative for chlamydial isolations after three successive egg subpassages. Blood leukocyte counts and rectal temperatures of both the control and principle calves were normal throughout the experimental period. Serum samples which were collected in accordance with each bleeding were negative for chlamydial antibody at a 1:5 dilution, as determined by the microtiter-complement fixation test. Negative results were obtained when chlamydial isolation was attempted from the skin of calves where adult ticks had previously fed.

DISCUSSION

Primary visceral cultures of D. occidentalis inoculated with the chlamydial isolate and incubated at 30 C failed to support the growth of the agent. The inability to infect tick viscera in vitro suggested that a more natural route of exposure to the organism might be required in order to produce an active infection in ticks. Nymphal ticks were infected when fed on chlamydic rabbits. Chlamydial isolations were not made from homogenates of ticks removed from the rabbit 24 hr postinoculation of the agent; however, isolations were made on days 2 and 3 postinoculation. The period elapsed before isolations were first made from feeding nymphal ticks was 48 hr. This is approximately the time required for chlamydiae to complete a single developmental cycle. This would indicate that the failure to isolate the agent from ticks sampled at 24 hr postinoculation was because the organism was undergoing development. Therefore, homogenates of tick samples on day 1 probably consisted of relatively non-infectious initial bodies without the presence of infectious elementary bodies. Sampling of ticks on days 2 and 3 would have allowed time for the completion of chlamydial development and the subsequent release of infectious elementary bodies. The possibility that chlamydial isolations were the result of mechanical surface contamination is unfavorable since blood isolations of chlamydiae were not made from the intravenously injected rabbit after 6 hr postinoculation of the agent, and isolations from nymphs were made only after 48 hr.

Viscera of nymphs fed on chlamydemic rabbits and cultured in vitro did not support chlamydial growth. Chlamydial isolations were also negative when nymphal ticks were sampled through metamorphosis. It should be remembered that an important property of chlamydiae is their dependence on the host for high energy metabolites (7). One might speculate
that the failure to isolate chlamydiae from the above conditions is related to the slow metabolic activities of arthropods. Competition for high energy-metabolites coupled with suboptimal incubation temperatures and possible nutrient deficiencies could very well prevent chlamydial replication. The fact that infectious elementary bodies were isolated from actively feeding nymphal ticks but not from ticks which had completed engorgement and detached from the rabbit would support this speculation. Ticks undoubtedly have an increase in metabolic rates during feeding as the body contents are continuously bathed with host blood. In addition, environmental temperatures are increased somewhat since the feeding tick is in close proximity with its host. Such conditions would theoretically produce changes in tick tissue which selectively increase the possibility of chlamydial development. Attempts at activating chlamydial infection in adult ticks infected as nymphs were unsuccessful. However, conditions for activation may be more complicated in nature.

This report does not prove that *D. occidentalis* is a biological vector for the transmission of bovine chlamydial abortion. However, the demonstration of at least one cycle of replication in the feeding nymph indicates that vector transmission is possible and may account for at least one mechanism of disease transmission, particularly in situations where interrupted tick feeding may occur. Since chlamydial replication was not directly demonstrated in tick tissues, the actual site of replication remains to be identified.

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**LITERATURE CITED**