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Development and transmission of *Cowdria ruminantium* by *Amblyomma* males transferred from infected to susceptible sheep

KOCAN (K.M.), NORVAL (R.A.I.), DONOVAN (P.L.). Développement et transmission de *Cowdria ruminantium* par des mâles d'*Amblyomma* transférés de moutons infectés à des moutons sensibles. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 183-188

Des mâles d'*Amblyomma* ont été testés comme vecteurs de *Cowdria ruminantium*, agent causal de la cowdriose. Les mâles ont été nourris sur des moutons infectés expérimentalement avec *C. ruminantium* et ont ensuite été transférés à des moutons sensibles. Dans une première expérience, *A. hebraeum* et le stock Palm River de *C. ruminantium* ont été utilisés, une deuxième expérience a été faite avec le stock de *Cowdria* Kiswani et *A. variegatum*. Des tiques ont été récoltées quotidiennement pendant toute la durée de chaque expérience, coupées en deux et préparées pour examen par microscopie classique et électronique, afin d'étudier le développement de *C. ruminantium* dans leurs tissus. Dans les deux expériences les tiques ont transmis *Cowdria* à un mouton sur deux. A l'examen microscopique quelques colonies ont été trouvées dans des cellules intestinales, mais aucune n'a été observée dans les glandes salivaires. Les deux espèces de tiques étaient infectées par *Rickettsia conorii*, comme en témoignait l'existence de rickettsies dans les noyaux et le cytoplasme de cellules des glandes salivaires.

Mots clés : Ovin - Cowdriose - *Cowdria ruminantium* - Tique - *Amblyomma hebraeum* - *Amblyomma variegatum* - *Rickettsia conorii* - Transmission des maladies.

INTRODUCTION

Cowdria ruminantium is the tick-borne agent of heartwater disease in cattle. This organism is one of several rickettsias that live and multiply only within membrane-bound colonies within host cell cytoplasm. Other rickettsias that develop within colonies include *Anaplasma, Ehrlichia* and *Coxiella* (22). *Cowdria* can be transmitted to cattle or sheep transstadially by nymphal and adult ticks (3). Transovarial transmission has been demonstrated once experimentally (4), but does not appear to occur readily. Although LOUNSBURY (14) was unable to demonstrate intrastadial transmission of *C. ruminantium* by male *A. variegatum* infected as adults, NORVAL et al. (1990) demonstrated intrastadial transmission using *Amblyomma hebraeum* males (16). Transmission of heartwater occurred when small numbers of males were transferred from infected to susceptible hosts, and the same males transmitted *C. ruminantium* repeatedly to successive, susceptible hosts.

Colonies of *C. ruminantium* were first described by COWDRY in 1925 in *A. hebraeum* ticks (5) and were later confirmed in this tick species in 1985 (2) and 1991 (6). KOCAN *et al.* (1987) described similar colonies of *C. ruminantium* in *A. variegatum* (9). In these studies, colonies of the rickettsia were seen within midgut epithelial cells and occasionally within the gut lumen. Recently, colonies of *C. ruminantium* were also described in salivary glands of *A. hebraeum* nymphae that were experimentally infected with the rickettsia as larvae (10). The morphology of *C. ruminantium* in tick gut and salivary glands was similar.

The importance of intrastadial infection and transmission of a similar rickettsia, *Anaplasma marginale,* by male *Dermacentor andersoni* was described recently (18, 19). Highest infection rates were demonstrated in males exposed as adults ; the ticks remained infected throughout their life and were able to reattach and transmit *A. marginale* to successive, susceptible cattle (11, 12, 20). Most notable in these studies was the demonstration of *A. marginale* in salivary glands of male ticks exposed as adults. In contrast, when male ticks were exposed to *A. marginale* as nymphs, colonies were not evident with microscopy, even though homogenates of salivary glands were infective for cattle and were proven to contain *A. marginale* DNA using a *Anaplasma*-specific DNA probe (13).

The experimental design from the *Anaplasma* studies was applied to the present experiments on *C. ruminantium* to confirm intrastadial transmission of *C. ruminantium* to sheep by male *Amblyomma* sp. exposed as adults. A primary objective of this study was to determine whether intrastadial infection of male ticks enhanced development of *C. ruminantium* in tick salivary glands.

MATERIALS AND METHODS

Agent

Two isolates of *Cowdria ruminantium* were used for these studies : the Palm River stock from Zimbabwe and the Kiswani isolate from Kenya. The inoculum used for infection of sheep was *Cowdria*-infected endothelial cell cultures.

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Tick propagation

Amblyomma hebraeum and *A. variegatum* were raised in colony for several generations at the University of Florida/USAID Heartwater Project, Harare, Zimbabwe. *Amblyomma variegatum* were originally collected from the Trafalgar Farm, Zimbabwe. Larval and nymphal ticks were fed on rabbits, collected after engorgement and allowed to molt in a humidity chamber until the male ticks were used for these studies.

Sheep

Twelve 6-month old sheep, previously unexposed to *C. ruminantium*, were used for these studies. Four sheep were experimentally infected with 2 ml *C. ruminantium*-infected tissue culture supernatant and used for feeding-exposure of male ticks. A brain smear was prepared after death from sheep inoculated with infected cell cultures and examined with light microscopy for colonies of *C. ruminantium to* confirm infection. Four sheep were used for the second tick feeding to test for tick transmission of heartwater. Two susceptible sheep were inoculated with 2 ml uninfected tissue culture supernatant and used for feeding of uninfected (control) ticks. Two susceptible sheep were used for the second feeding of these control ticks.

Exposure of ticks

Amblyomma hebraeum or *A. variegatum* males (250 per sheep) were placed in cotton cells glued to the sheep 4 days after the sheep was inoculated with infected and control cultures. The ticks were allowed to feed for 5 days after which they were removed and placed in a humidity chamber.

Experimental design

These experiments were done in two trials. Trial 1 tested transmission of the Palm River stock of *C. ruminantium* by *A. hebraeum* males. In Trial 2, transmission of the Kiswani stock was tested by *A. variegatum.* In each trial, two sheep were infected with *C. ruminantium* for exposure of ticks to the agent and two susceptible sheep were used for the second feeding of these exposed ticks (fig. 1). Two additional sheep were used for the first and second feeding of control (uninfected) ticks. The ticks were allowed to feed for 5 days on *Cowdria*-infected sheep, held for 5 days in a humidity chamber and then were allowed to feed on susceptible sheep for 10 days. Feeding periods of control ticks on susceptible sheep were the same as those for the feeding of infected ticks.

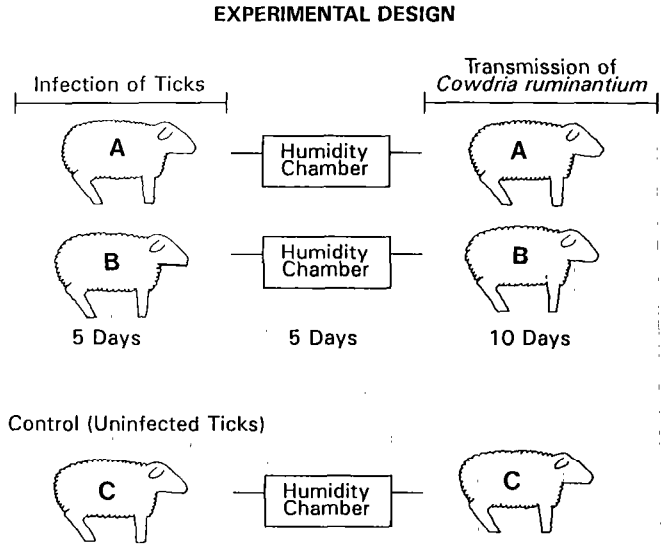

Figure 1 : Experimental design for study of intrastadial transmission of Cowdria ruminantium *by male ticks to sheep.*

Collection of ticks for electron microscopy

Five infected and three control ticks were collected and fixed for light and electron microscopy (LM and EM) before placement of ticks on sheep, on each day the ticks fed on the infected or control sheep, while held in the humidity chamber for 5 days and throughout the 10 days of the second tick feeding on susceptible sheep (total, 21 days). Each tick was cut in half with a razor blade, separating the right and left sides, placed in a 1.5 ml Eppendorf tube filled with 2 % glutaraldehyde in 0.1 M sodium cacodylate buffer and processed according to the procedures of KOCAN *et al.* (8). Thick sections (1 µm) were prepared

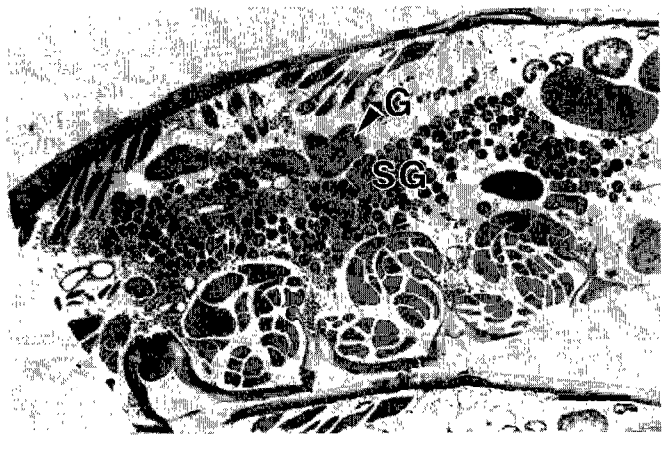

Photo 1 : A 1 µm plastic cross section of a half tick that includes gut (G) and salivary glands (SG). Bar = 460 µm.

from the tick halves, stained with Mallory's stain (17) for 2 min at 60° C and were examined by LM for colonies of *C. ruminantium*. Cross sections of entire tick halves enabled examination of all tick tissues (photo 1). Just prior to fine sectioning, a 1 µm section was prepared for LM photography. Ultrathin (silver-gold reflective) sections were cut with a Sorvall MT-5000 ultramicrotome and a Diatome diamond knife. The sections were collected on 300-mesh copper grids, stained with uranyl acetate and lead citrate (21) and observed and photographed with a JOEL 100 CX electron microscope operated at 80 kV.

RESULTS

Infection of ticks and transmission of C. *ruminantium*

The four sheep used for infection of ticks died from heartwater disease 7-12 days post-inoculation (table I). In both trials, ticks transferred from sheep A died and therefore failed to transmit *C. ruminantium* to susceptible sheep. The ticks that fed on sheep B reattached and transmitted *C. ruminantium* to susceptible sheep during a second feeding (table I), causing fatal heartwater disease.

TABLE 1 Transmission of Cowdria ruminantium *(Palm River and Kiswani stock) by* Amblyomma *spp. males transferred from infected to susceptible sheep.*

Ticks	Tick feeding	
	Exposure	Transmission
Trial 1 :	Transmission of the Palm River stock of *C. ruminantium* by *A. hebraeum* male ticks	
Group A*	Sheep died 9 days PI	—
Group B	Sheep died 10 days PI	Sheep died 18 days PI
Trial 2 :	Transmission of the Kiswani stock of *C. ruminantium* by *A. variegatum* male ticks	
Group A*	Sheep died 12 days PI	—
Group B	Sheep died 7 days PI	Sheep died 10 days PI

* *Ticks died before infestation of the second sheep.*

Morphology of C. *ruminantium* tick tissues

Small numbers of colonies of *C. ruminantium* were seen with LM and EM in midgut epithelial cells of *A. hebraeum* and *A. variegatum* (photos 2, 3, 4). The morphology of organisms within colonies was similar to that described previously. *Cowdria* colonies were not seen in salivary glands with LM or EM. Organisms that appeared to be *Rickettsia conorii* were seen within the cytoplasm and nucleus of cells surrounding the collecting

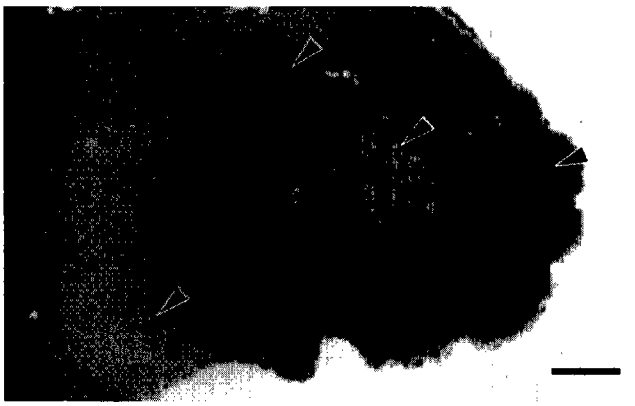

Photo 2 : Colonies of Cowdria ruminantium *(arrows) in midgut cells of* Amblyomma hebraeum *in a 1 µm plastic section stained with Mallory stain. Bar = 4,5 µm.*

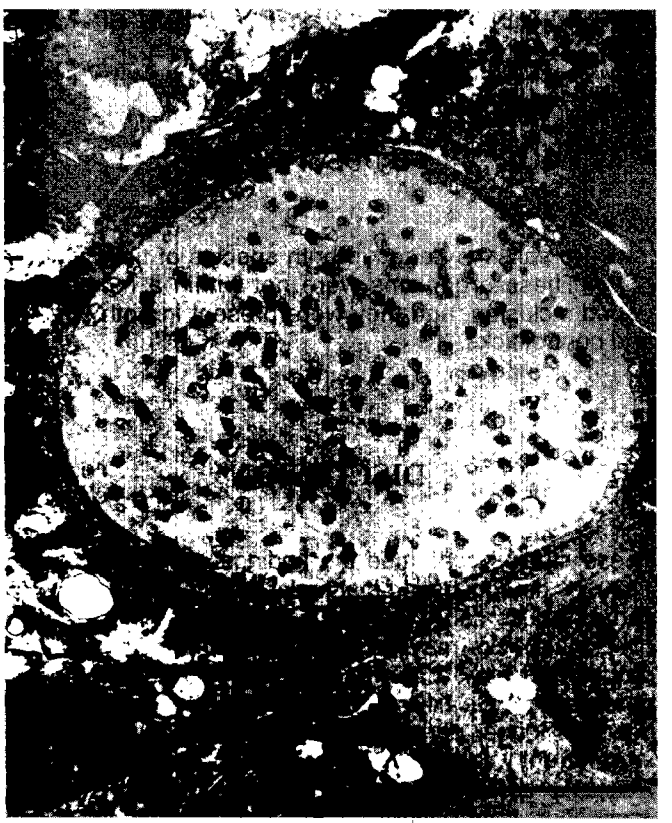

Photo 3 : An electron micrograph of a colony of Cowdria ruminantium *in a midgut epithelial cell of* Amblyomma hebraeum. *Bar = 9,25 µm.*

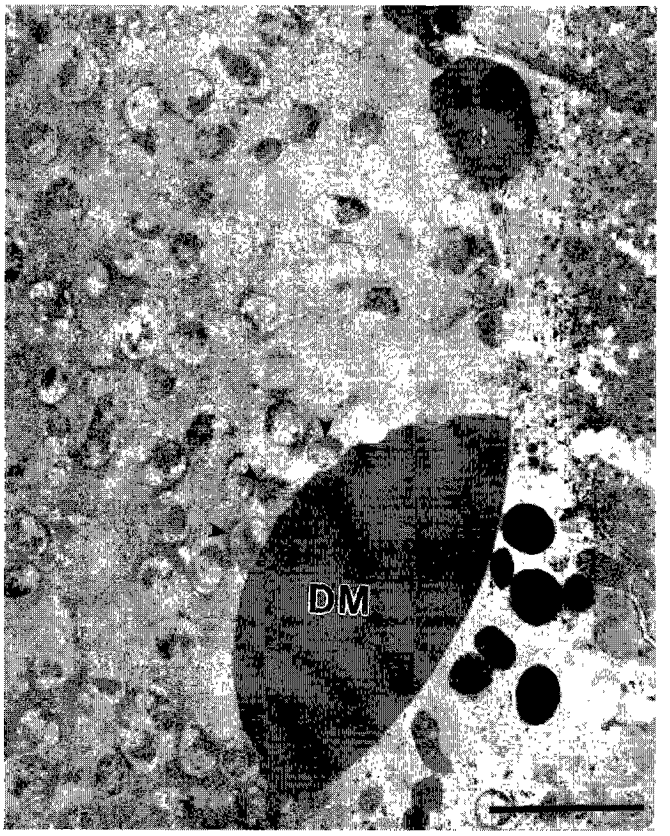

Photo 4 : A higher magnification of a colony of Cowdria ruminantium *in a midgut cell of* Amblyomma hebraeum. *The colony contains dense material (DM) to which some organisms are attached (small arrows). Bar = 18,5 µm.*

Photo 5 : Rickettsia conorii *(R) within the cytoplasm of a cell associated with salivary gland collecting ducts. Bar = 1,85 µm.*

ducts of salivary glands in both species of ticks (photos 5, 6). These organisms were not within a membrane-bound inclusion, and they were present in both infected and control ticks.

DISCUSSION

These studies confirmed the findings of NORVAL *et al.* 1990, by demonstrating that male *Amblyomma* spp. exposed as adults can transmit *C. ruminantium* intrastadially (16). Male ticks may be efficient vectors of *Cowdria* in nature and play a central role in the epidemiology of heartwater. Male ticks remain attached to their ruminant hosts for longer periods (up to eight months) where they intermittently feed and mate. The males attain sexual maturity after feeding approximately one week. Thereafter, they emit an aggregation-attachment pheromone (AAP) which can be detected by unfed adults and nymphs, contributing to the tick's selection of hosts and attachment sites (15). Males thus accumulate on suitable hosts, where they occur in clusters on the parts of the ruminants that are groomed least effectively.

Small numbers of *C. ruminantium* were seen in gut cells in *Amblyomma* ticks exposed to either *Cowdria* stock and none were seen in salivary glands. Although COWDRY (5) did not observe *C. ruminantium* in salivary glands, BEZUIDENHOUT (1) found homogenates of salivary glands of prefed adults exposed as nymphae to be infective for sheep. Furthermore, colonies of *C. ruminantium* were described with LM and EM in approximately 15 % of the nymphal ticks studied that were exposed to *C. ruminantium* as larvae. YUNKER *et al.* (1993), using a *Cowdria*-specific DNA probe, demonstrated that, although salivary glands contained *Cowdria* DNA, the gut was the main site of replication of *C. ruminantium* in ticks (23). However, infected salivary glands were more frequently demonstrated in male ticks. Because intrastadial infection of male ticks allowed for amplification of *A. marginale* in salivary glands of *Dermacentor* sp. ticks, we applied the same experimental design to *Cowdria* in *Amblyomma* males. However, this method of exposure was not effective in increasing salivary gland infections ; colonies were not seen with LM or EM. It appears that the major site of infection of *Cowdria* in ticks is in midgut cells.

Photo 6 : Rickettsia conorii *(R) within the nucleus (Nu) of a cell associated with salivary gland collecting ducts. Bar = 1,85 µm.*

The *Amblyomma* ticks in this study were also infected with *Rickettsia conorii,* causative agent of boutonneuse fever (7). This organism is easily differentiated from *Cowdria* because it occurs free in the cell cytoplasm rather than in membrane-bound inclusions. Furthermore, *R. conorii* is one of the few rickettsias reported to occur within the cell nucleus. This rickettsia is transmitted from one tick generation to the next via the egg, thus resulting in persistent infection of ticks, even those reared in the laboratory for many generations.

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KOCAN (K.M.), NORVAL (R.A.I.), DONOVAN (P.L.). Development and transmission of *Cowdria ruminantium* by *Amblyomma* males transferred from infected to susceptible sheep. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 183-188

Male *Amblyomma* sp. were tested as vectors of *Cowdria ruminantium*, causative agent of heartwater disease. The males were allowed to feed on sheep experimentally infected with *C. ruminantium* and then were transferred to susceptible sheep to test for transmission of the rickettsia. The experiments were done in two trials. In the first trial, *A. hebraeum* were exposed to the Palm River stock of C. *ruminantium*, while in the second trial the Kiswani stock of *Cowdria* was tested with *A. variegatum*. Ticks were collected daily throughout each experiment, cut in half, and processed for light and electron microscopy to study development of *C. ruminantium* in tick tissues. In both trials, the male ticks transmitted *Cowdria* to one of two susceptible sheep. When ticks were examined with microscopy, a few colonies were found in gut cells while none were seen in salivary glands. Both species of ticks were infected with *Rickettsia conorii*, as evidenced by the occurrence of rickettsiae in the nucleus and cytoplasm of salivary gland cells.

Key words : Sheep - Heartwater - *Cowdria ruminantium* - Tick - *Amblyomma hebraeum* - *Amblyomma variegatum* - *Rickettsia conorii* - Disease transmission.

KOCAN (K.M.), NORVAL (R.A.I.), DONOVAN (P.L.). Desarrollo y transmisión de *Cowdria ruminantium* mediante *Amblyomma* : transferencia de machos de ovinos infectados hacia ovinos susceptibles. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 183-188

Se probaron los machos de *Amblyomma* sp. como vectores de *Cowdria ruminantium*, agente causal de la cowdriosis ("heartwater disease"). Los ácaros machos fueron alimentados a partir de ovejas infectadas experimentalmente con *C. ruminantium* y transportados a ovejas susceptibles con el fin de probar la transmisión de la rickettsia. Los experimentos se llevaron a cabo en dos etapas. La primera consistió en exponer *A. heabraeum* al stock de *C. ruminantium* de Palm River y el segundo ensayo examinó el stock Kiswani de *Cowdria* con *A. variegatum*. En los dos casos las garrapatas se recolectaron diariamente, se cortaron en mitades y se procesaron para el estudio en microscopio de luz y electrónico, con el fin de estudiar el desarrollo de *C. ruminantium* en los tejidos del ácaro. En ambos ensayos, el ácaro macho transmitió *Cowdria* a uno de los dos ovinos susceptibles. Cuando las garrapatas se examinaron con el microscopio, se encontraron algunas colonias en las células intestinales, pero no se observó ninguna en las glándulas salivales. Ambas especies de garrapatas se infectaron con *Rickettsia conorii*, como lo demostró la aparición de rickettsias en el núcleo y el citoplasma de las células de las glándulas salivales.

Palabras claves : Ovino - Cowdriosis - *Cowdria ruminantium* - Garrapata - *Amblyomma hebraeum* - *Amblyomma variegatum* - *Rickettsia conorii* - Transmisión de enfermedades.