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Transmission of Crimean-Congo haemorrhagic fever virus from experimentally infected sheep to *Hyalomma truncatum* ticks

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SUMMARY

Crimean-Congo haemorrhagic fever (CCHF) virus was inoculated into West African sheep that were simultaneously infested with adult *Hyalomma truncatum* ticks. Certain sheep developed a viraemia and antibodies, indicating virus infection and replication; however, the length and magnitude of the viraemia and serological responses corresponded to the animals' immunological status. Tick attachment and feeding was not influenced by sheep infection. CCHF virus infection was acquired by 11-33 % of female and 0-60 % of male ticks. Infection in the ticks did not influence their feeding success, as judged by weight at drop-off, and the weight of eggs produced by infected and non-infected ticks was similar. Transovarial transmission of CCHF virus was demonstrated in 2 of 12 (17 %) egg batches from infected female ticks, but in none of 19 egg batches from ticks that tested negative for CCHF virus. Our results suggest that under certain ecological conditions, sheep may serve to amplify CCHF virus in nature through horizontal transmission and that the maintenance cycle also may be influenced by transovarial transmission to the next generation of ticks.

Key-words: Viraemia, CCHF virus, Haemorrhagic fever, Arbovirus, Tick; Africa, Sheep, *Hyalomma truncatum*, Transmission, Ecology, Antibodies.

INTRODUCTION

Crimean-Congo haemorrhagic fever (CCHF) virus (family *Bunyaviridae*, genus *Nairovirus*) is a virus transmitted enzootically by Ixodid ticks that may cause severe disease in humans (Hoogstraal, 1979). Remarkably widespread in its geographic distribution, CCHF virus is found throughout much of the southern Soviet Union, southern Europe, central Asia, the Middle East

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and Africa. It occurs in 3 of the world's 7 major biotic zones (Watts *et al.*, 1988). Equally astonishing is the diversity of potential tick vectors and vertebrate reservoirs: at least 31 species from 9 genera of Ixodid and Argasid ticks have been found to harbour CCHF virus (Camicas *et al.*, 1990), and more than 20 different vertebrate species have been found to be naturally infected with the virus (Watts *et al.*, 1988). Human disease, which is often severe and sometimes fatal (*e.g.* Swanepoel *et al.*, 1987) has been recognized from more than a dozen countries on 3 continents (Watts *et al.*, 1988).

Despite an abundance of research documenting the widespread distribution of CCHF virus, its possible vectors and potential vertebrate reservoirs, our understanding of the transmission cycle(s) remains incomplete.

Factors that influence zoonotic transmission of CCHF virus include the density of competent vector ticks and the relative abundance of vertebrates that serve as tick hosts or potential virus reservoirs (Hoogstraal, 1979). Sustained transmission is found only where species of *Hyalomma* ticks are present, and epizootic or epidemic transmission is believed to occur during periods of increased abundance of these ticks.

We recently documented such epizootic transmission in northern Senegal that corresponded temporally with a sudden increase in the abundance of adult *H. truncatum* and *H. impeltatum* (Wilson *et al.*, 1990a). Similarly, we have demonstrated a spatial correlation between the relative abundance of *Hyalomma* ticks and the prevalence of IgG antibodies against CCHF virus there (Wilson *et al.*, 1990b). Finally, the feeding patterns, host preferences and ecological associations of Ixodid ticks in West Africa (Morel, 1969) suggest that *Hyalomma* spp. are most likely to serve as enzootic vectors (Camicas *et al.*, 1990).

Vertebrate hosts for most of the Ixodid ticks considered to be likely vectors include small mammals or birds for the larval and nymphal stages, and large mammals for adult ticks. Domestic ungulates are the principal hosts of most adult *Hyalomma* ticks, and the prevalence of antibodies against CCHF virus in these animals indicates that they also are frequently infected (Watts *et al.*, 1988; Gonzalez *et al.*, 1990; Wilson *et al.*, 1990a, b). Because sheep are among the most abundant tick-infested animals in many regions where CCHF virus circulates, they may serve a role in horizontal transmission of the virus.

Accordingly, we studied whether adult ticks feeding on sheep viraemic with CCHF virus may be infected, and whether that infection may be transmitted transovarially to the next generation of ticks.

MATERIALS AND METHODS

Virus

The strain of CCHF virus (Dak H49199) used in these experiments was originally isolated in 1988 from a fatal human case in Rosso, Mauritania (Gonzalez *et al.*, 1990). Following 3 consecutive intracerebral mouse passages, the titre was log 6.5 LD_{50}/ml. Virus identification was performed on ground mouse brains using a previously described antigen capture (Saluzzo and Leguenno, 1987). Confirmation of the identity of virus was made by the CF test at the World Health Organization Reference Center for Arboviruses at the Pasteur Institute in Dakar.

CCHF = Crimean-Congo haemorrhagic fever.
CF = complement fixation.
dr = dram.
ELISA = enzyme-linked immunosorbent assay.
i.c. = intracranial(ly).
i.p. = intraperitoneal(ly).
LD_{50} = lethal dose, 50 % endpoint.
NB = newborn.
OD = optical density.
PBS = phosphate-buffered saline.
p.i. = post-infestation.
RH = relative humidity.
s.c. = subcutaneous(ly).
TOT = transovarial transmission.

Ticks

Adult *H. truncatum* Koch (Hoogstraal, 1956) were obtained by rearing them from the eggs of a single engorged female tick removed from a sheep in Yonofere, Senegal. Eggs were allowed to hatch, and immatures were fed on a captive hare *(Lepus whytei)* producing a cohort of sibling adult ticks that were of uniform age and condition. These unfed adults were weighed using an electronic balance (Sartorius, type H51-F1) and held in individual vials at 25°C and 75 % RH until being placed on the sheep. A subsample of these ticks was tested and found negative for arboviruses.

Sheep

Four female sheep of the West African "Peuhl-Peuhl" breed from a herd maintained at the Pasteur Institute farm near Dakar were studied: 3 were infected and 1 served as a control. One of the 3 test sheep had been infected about 4 months previously by this same CCHF virus strain and showed high-titred IgG. The second test sheep, a 3-month-old offspring of the first, also tested weakly antibody-positive, probably as the result of maternal antibody (Sawyer *et al.*, 1977). The third test sheep and the control sheep never had been infected and showed no evidence of antibody. During these experiments, the sheep were confined to an animal holding room at the Pasteur Institute in Dakar.

Infection and transmission

Two days prior to virus inoculation, each sheep was infested with 30 male and 30 female *H. truncatum*. An elastic cloth stockenette bag, cut at the ends, was glued to the base of the shaved tails of each sheep. The ticks were placed in the bag which was then sealed and they were allowed to attach. Sheep were inoculated *i.p.* with $10^{5.5}$ LD_{50} of CCHF virus. All sheep were bled daily by venipuncture, their temperature was taken and their general condition noted. Beginning 4 days p.i., the tail bags were examined daily; engorged female ticks that had naturally finished feeding were removed. Male ticks, which remain attached to sheep for a longer time, were detached by forceps at 16 days p.i.

Fed ticks were weighed, and individual engorged females were held at 25°C and 75 % RH in 3.5 dr plastic vials closed with a fine mesh cap until egg laying ended. Tick bodies subsequently were ground individually in Hanks' solution, centrifuged and inoculated into NB mice or cell culture. Tick eggs were treated similarly: a sample of about 100 eggs per batch were ground and inoculated into cell culture.

Serologic assays

Sera were tested blindly for evidence of anti-CCHF virus IgG using a direct ELISA test (Niklasson *et al.*, 1984) modified slightly by adding a saturated solution of PBS with 0.05 % Tween-20 and 1 % non-fat bovine milk. "Immulon II" 96-well plates (Dynatech Laboratories, Alexandria, VA) were coated with diluted CCHF virus hyperimmune mouse ascitic fluid. CCHF virus (Dak H49199) in crude NB mouse brain was heat-inactivated at 60°C for 1 h and then added. Test sera, diluted 1/400, followed by sheep-specific anti-IgG conjugated with horseradish peroxidase (Biosys, Compiègne, France) were used to detect the IgG. A chromogenic substrate (Ortho-tolidine, Sigma, LaVerpillière, France) was added for colorimetry. All plates included a control of crude NB mouse brain without CCHF virus antigen. Differences in OD between the test and control wells were measured at 450 nm using an automatic reader (Multiscan MCC/340, Flow Laboratories, Irvine, Scotland). Sera were considered positive if the OD was greater than 2 times that of the control.

IgM antibodies were detected by immunocapture ELISA (Saluzzo and LeGuenno, 1987). Plates were coated with a sheep anti-μ-chain-specific antibody. The test serum, followed by CCHF viral antigen, was then added. The detecting antibody was a high-titred mouse ascitic fluid prepared against CCHF virus antigen. Anti-mouse immunoglobulin, conjugated with horseradish peroxidase and the chromogenic substrate, was added as above. Evaluation and criteria for positivity were as for IgG.

Virologic assays

Virus reisolation was attempted by i.c. inoculation of NB mice and/or by inoculation of Vero cells, using undiluted and 10-fold-diluted sera. Virus identification in cell culture was made by indirect immunofluorescent antibody test on Vero cells, using polyclonal and monoclonal antibodies.

An antigen capture ELISA (Saluzzo and LeGuenno, 1987) was also employed to test for the presence of CCHF viral antigen in NB mouse brain and cell culture supernatant. Plates were coated first with anti-human μ-chain-specific IgM antibody, followed by human sera with high-titre IgM. The test serum was added next and then a high-titred anti-CCHF virus monoclonal IgG was added to bind to any antigen captured from the test serum. An anti-mouse IgG, conjugated with horseradish peroxidase, and the chromogenic substrate, were used for colorimetry.

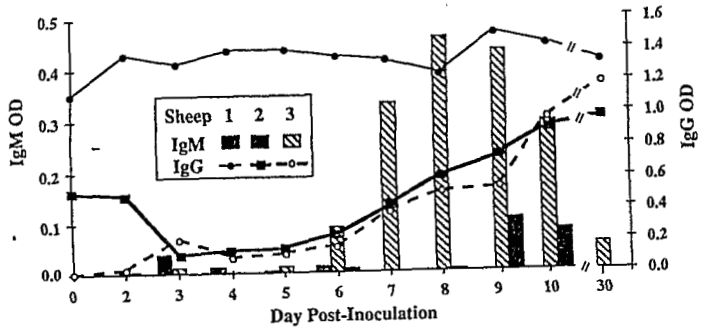

Fig. 1. Antibody response of 3 sheep inoculated with CCHF virus.
Bars indicate IgM OD values (ELISA) and lines indicate IgG OD.

RESULTS

Sheep serology and viraemia

Inoculated sheep exhibited high-titred antibodies to CCHF virus, although the temporal pattern differed with their past exposure (fig. 1). The previously exposed sheep (no. 1) produced a slightly elevated IgM titre (1/100) and maintained high IgG (1/10,000). Its offspring (no. 2) showed weakly elevated IgM (1/100) beginning 9 days p.i. and an increase in IgG that developed slowly over the period of study. The naive sheep (no. 3) developed high-titre IgM (1/1,000) beginning 6 days p.i. followed by elevated IgG. No antibody was detected in the control sheep (no. 4).

Viraemia duration (0-6 days) and magnitude (0-3 × 5 LD_{50}/ml) also corresponded to previous exposure (table I). Viraemia was undetectable in the previously exposed sheep, mild and brief in its offspring, elevated and longer-lasting in the naive sheep, and non-existent in the control.

Table I. Virological and serological results of CCHF virus inoculation of sheep and of transmission to adult *H. truncatum* ticks feeding on them.

	Results from sheep							
	Inoculated with CCHF virus						Non-inoculated	
	no. 1 (ad. female)		no. 2 (juv. female)		no. 3 (juv. female)		no. 4 (ad. female)	
Sheep								
Preinfection IgG	Strong		Weak		Negative		Negative	
Origin of antibody	Previous infection		Maternal transfer		—		—	
Virus inoculated	5×10^6 LD_{50}		5×10^6 LD_{50}		5×10^6 LD_{50}		None	
Viraemia	Undetectable		Weak		Strong		Undetectable	
Day p.i.	—		3-6		2-6		—	
Ticks								
Sex	M	F	M	F	M	F	M	F
No. infested	30	30	30	30	30	30	30	30
No. attached	29	27	29	30	29	28	21	24
No. tested	9	18	10	21	10	27	7	14
% infected	0 %	11 %	30 %	14 %	60 %	33 %	0 %	0 %

Adult tick feeding and infection

A total of 108 male (90 %) and 109 female (91 %) ticks attached to the sheep and were removed alive for further study. The rate of attachment per sheep varied from 70-97 % for male and 80-100 % for female ticks, but no systematic differences in tick feeding among sheep were evident (table I). Engorged female ticks began detaching 6 days p.i., with similar drop-off patterns (fig. 2). All female ticks that attached took a blood-meal although their mean weights at detachment differed among sheep (table II). Male tick weights were similar.

Virus was detected in 1 or more ticks that fed on each of the 3 inoculated sheep; however, the percentage of infected ticks differed markedly (table I). The previously inoculated sheep (no. 1) infected 11 % of the female and none of the male ticks tested. More of the ticks feeding on offspring (no. 2) were infected: 14 % of females and 30 % of males. The sheep that previously was naive to CCHF virus or antibodies (no. 3) infected 33 % of female and 60 % of male ticks tested. None of the ticks from the control sheep (no. 4) were infected.

The period of detectable viraemia corresponded to the time during which infected female ticks were detaching (fig. 2). Ticks which detached after the viraemia had become undetectable, with 1 exception, were not infected. Two of 18 ticks feeding on sheep no. 1, which never produced a detectable viraemia, were nevertheless infected.

The weights of fed ticks varied among the

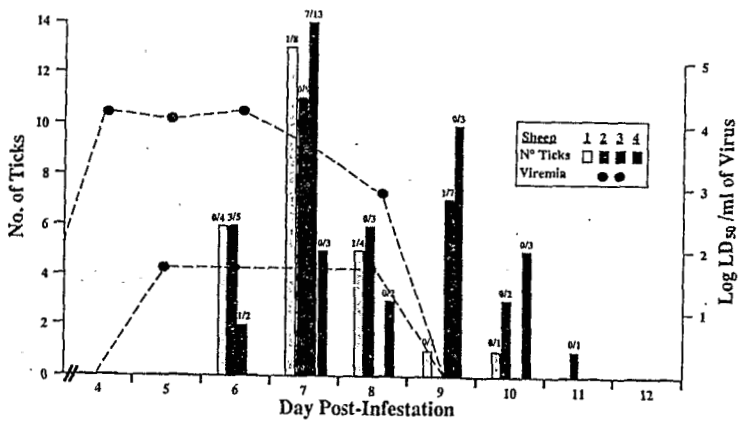

Fig. 2. Tick drop-off and viraemia from sheep inoculated with CCHF virus.
Bars indicate the number of engorged female *H. truncatum* detaching daily from each of 4 sheep. The number of infected ticks and of ticks tested are shown in parentheses. Sheep nos. 1-3 were inoculated with CCHF virus and no. 4 was a control. No viraemia was detected in no. 1 and no. 4.

Table II. Weights of adult *H. truncatum* ticks feeding on CCHF virus-infected and non-infected sheep.

	Weight [$\bar{X}$ (+/−SD) mg] of ticks or eggs infected no. 1 (ad. female)	infected no. 2 (juv. female)	infected no. 3 (juv. female)	Control no. 4 (ad. female)
Female ticks				
infected	512.9 (±9.6)	771.3 (±28.7)	828.2 (±52.1)	—
uninfected	523.3 (±158.7)	761.8 (±107.6)	804.5 (±56.4)	674.6 (±111.6)
Male ticks				
infected	—	14.5 (±0.9)	13.6 (±1.2)	—
uninfected	13.6 (±1.5)	15.2 (±0.6)	14.6 (±1.0)	14.9 (±1.2)
Egg batches				
infected	264.2	391.1	—	—
uninfected	153.6 (±54.2)	430.5 (±41.6)	449.3 (±41.1)	408.4 (±24.3)

sheep, but infected and uninfected tick weights were not significantly different (table II). Male ticks, which weighed 11.3 (±1.0) mg prior to attachment, ranged between 13.6 (±1.5) mg and 15.2 (±0.6) mg upon their removal. Female ticks, whose size and weight increases many-fold during feeding, weighed 13.9 (±0.9) mg prior to feeding and ranged between 512.9 (±9.6) mg and 828.1 (±52.1) mg at drop-off. Curiously, the weight of engorged females declined as the length of time to dropoff increased (fig. 3). No systematic differences between the weights of infected and non-infected ticks were found (table II).

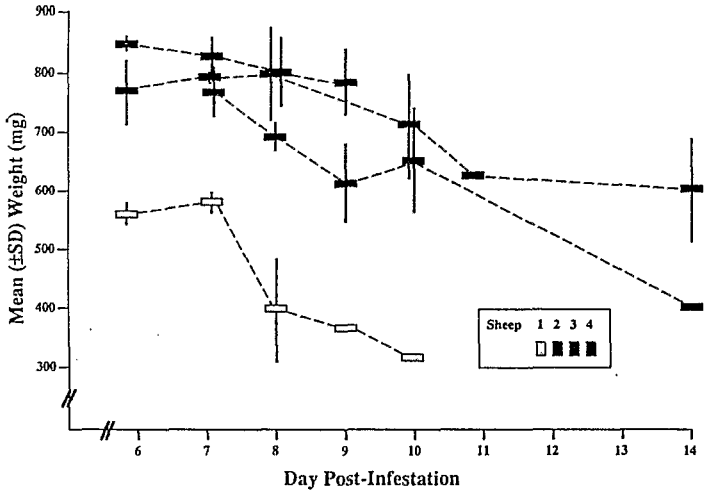

Fig. 3. Mean weight (±SD) of engorged female *H. truncatum* detaching from 4 sheep on day p.i.

Transovarial transmission

Eggs were laid by 105 of 109 (96 %) engorged females (3 engorged ticks died prior to oviposition and 1 was crushed in the tail bag). Individual eggs were not counted, but the weights of eggs laid by infected female ticks did not differ from those of uninfected ticks (table II). CCHF virus antigen was detected in 2 of 12 (17 %) egg batches from infected female ticks. Specifically, 1 of 2 and 1 of 3 egg batches from infected ticks of sheep no. 1 and no. 2, respectively, tested positive; all 7 egg batches from infected ticks of sheep no. 3 tested negative. None of 19 egg batches from female ticks testing negative were positive.

DISCUSSION

Adult *H. truncatum*, a potential enzootic vector of CCHF virus, became infected while feeding on sheep that were viraemic. Sheep were chosen as the vertebrate host for this experiment because they are heavily infested by numerous tick species in West Africa (Gueye *et al.*, 1986; Teel *et al.*, 1988; Gueye *et al.*, 1987, 1989a, 1989b), and because serological surveys have demonstrated that they are frequently infected by CCHF virus (Causey *et al.*, 1970; David-West *et al.*, 1974; Darwish *et al.*, 1977; Umoh *et al.*, 1983). *H. truncatum* ticks were studied because they are commonly infected by CCHF virus in nature (Camicas *et al.*, 1990) at prevalences that are elevated relative to other tick species (Camicas *et al.*, 1986).

Viraemia was detected in 2 sheep 3-6 days post-inoculation, indicating virus replication. This infection, however, did not produce disease in the sheep (Gonzalez *et al.*, unpublished results) a finding similar to that of Shepherd *et al.* (1989). Ticks that were attached to the sheep in our study during this viraemic period became infected. These results demonstrate that CCHF virus replicates in sheep and that they are capable of transmitting virus to feeding ticks.

The sheep that was previously exposed to CCHF virus (no. 1) and the offspring (no. 2) that had maternal antibody (*e.g.* Sawyer *et al.*, 1977) responded differently to infection. Furthermore, their efficiency in transmitting CCHF virus to feeding ticks also apparently differed. These 2 sheep with antibodies from prior infection produced low-titred or undetectable viraemia following inoculation. Only the naive sheep (no. 3) developed an elevated viraemia. In addition, more ticks feeding on the naive sheep became infected than those from the previously exposed sheep. Curiously, viraemia was not detected in sheep no. 1, yet 2 ticks feeding on this animal were found to be infected. These results suggest that immune hosts that are reinfected may not produce detectable viraemia, yet still may be capable of infecting vectors. Similar results involving such "non-viraemic" transmission of Thogoto virus from *Rhipicephalus appendiculatus* ticks to guinea pigs were reported by Jones *et al.* (1987), who later demonstrated a role of tick saliva in this system (Jones *et al.*, 1989). Nevertheless, the most efficient transmission of CCHF virus to feeding ticks occurred on sheep that had not been infected previously, and hence that developed the most intense and long-lasting viraemia.

Ticks successfully engorged on all sheep, and CCHF virus infection did not appear detrimental to their feeding, survival or reproduction. A high rate of attachment was observed on each sheep, demonstrating that feeding on the tails of these hosts was effective. The timing of female tick drop-off was similar among all sheep in this experiment, and was like that previously observed when *H. truncatum* fed on other uninfected sheep (T.M. Logan and M.L. Wilson, unpublished). Eggs were laid by most of the ticks from each sheep, indicating that mating had occurred. Interestingly, both female ticks, which consumed large quantities of host fluids, and males, whose fluid uptake was small, became infected. Whether some ticks were infected by the genital route was not determined in our study, although controlled observations to answer this question would be useful.

Other experimental efforts to demonstrate transmission of CCHF virus to *H. truncatum* also have suggested that this tick is a competent vector. Nymphal *H. truncatum* that were inocu-

lated intracoelomically replicated CCHF virus (Shepherd *et al.*, 1989). Similarly, larval *H. truncatum* that fed on infected NB mice replicated and transstadially transmitted CCHF virus (Logan *et al.*, 1989). Virus remained detectable in adult ticks for more that 200 days p.i. in these latter studies, suggesting a possible mechanism for interepidemic maintenance (Shepherd *et al.*, 1989).

Transovarial transmission (TOT) of CCHF virus by female *H. truncatum* ticks to their eggs was observed only twice in our experiment. None of the egg batches from female ticks testing negative were positive, suggesting that the method was specific. Experimental TOT of CCHF virus has been observed previously in *H. marginatum rufipes* (Lee and Kemp, 1970) and *H. m. marginatum* (Kondratenko *et al.*, 1970; Zgurskaya *et al.*, 1971; Levi and Vasilenko, 1972). However, the frequency of TOT of this virus in *Hyalomma* ticks apparently occurs rarely (Shepherd *et al.*, 1989). While some ticks may serve as both vector and reservoir to CCHF virus, unless TOT occurs with 100 % efficiency, another mechanism of amplification or reintroduction is necessary to maintain the virus. Since viraemia persists briefly in most vertebrates thus far studied (Watts *et al.*, 1988), amplifying, horizontal transmission may be limited to a short period of cofeeding by infected and uninfected ticks. Such cofeeding occurs among immature stages of certain *Hyalomma* ticks (Hoogstraal, 1956, 1979); however, infected adult ticks would be unlikely to transmit horizontally to these immatures, since the latter use different hosts (Hoogstraal, 1979). Thus, while sheep may be important in vector tick reproduction, their role in the transmission cycle of CCHF virus may simply be to provide blood for transovarially infected eggs.

The vector(s) of CCHF virus remain poorly defined despite evidence of infection in 6 other genera of ticks (Camicas *et al.*, 1990). Nevertheless, 1 or more *Hyalomma* species appear to be important (Hoogstraal, 1979; Watts *et al.*, 1988; Camicas *et al.*, 1986, 1990). Our studies in Senegal have demonstrated a temporal relationship between the abundance of *H. truncatum* and *H. impeltatum* and the epizootic transmission of CCHF virus (Wilson *et al.*, 1990a). Also, a spatial correlation between *Hyalomma* tick abundance and CCHF viral antibody in humans and sheep has been demonstrated (Wilson *et al.*, 1990b). Finally, we recently have completed a study demonstrating that being bitten by an adult male *H. truncatum* is a major risk factor for human CCHF virus infection in northern Senegal (Chapman *et al.*, 1991). Results of the present study further support the role of *H. truncatum* in the transmission of CCHF virus, and raise the possibility that sheep may serve as amplifying hosts in the enzootic cycle.

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Transmission expérimentale du virus CCHF du mouton à la tique *Hyalomma truncatum*

Le virus de la fièvre hémorragique de Crimée-Congo (CCHF) a été inoculé à des moutons d'Afrique occidentale simultanément infestés avec des tiques *Hyalomma truncatum* adultes. Certains moutons ont fait une virémie et produit des anticorps, signant l'infection et la réplication virale. Toutefois la durée et l'ampleur de la virémie et de la réponse sérologique correspondent à l'éventualité d'un contact préalable avec le virus. La fixation et le repas des tiques n'est pas influencés par l'infection des moutons. Le virus CCHF est transmis à 11-33 % des tiques femelles et à 0-60 % des tiques mâles. L'infection des tiques n'a pas d'influence sur leur aptitude à se gorger si l'on en juge par le poids des femelles gorgées et par le fait que le poids de la ponte est semblable entre les deux groupes. La preuve de la transmission transovarienne a été obtenue dans 2 pontes sur 12 (17 %) provenant de femelles infectées.

Nos résultats suggèrent que, dans certaines conditions écologiques, les moutons peuvent amplifier ce virus dans la nature, par transmission horizontale, et que le cycle d'entretien peut être influencé en partie par la transmission transovarienne à la génération de tiques suivante.

Mots-clés: Virémie, Virus CCHF, Fièvre hémorragique, Arbovirus, Tique; Afrique, Mouton, *Hyalomma truncatum*, Transmission, Ecologie, Anticorps.

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