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Sexual and transovarian transmission of Crimean-Congo haemorrhagic fever virus in *Hyalomma truncatum* ticks

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SUMMARY

Male *Hyalomma truncatum* ticks were inoculated with Crimean-Congo haemorrhagic fever (CCHF) virus, hypostomectomized and then allowed to mate with uninfected females feeding on a naive rabbit. After mating, CCHF virus was reisolated from 2 out of 3 males tested and from 4 of 6 mated, engorged females (titre $\geqslant$ 2.2 log LD_{50}/ml). Vertical transmission was then demonstrated by virus reisolation from a portion of 2 of the 6 batches of eggs laid by the positive females. From these 2 positive egg batches, 6 larvae pools were tested with successful virus reisolation from one. Attempts to reisolate CCHF virus from 15 nymph pools of this positive batch of larvae were unsuccessful.

Virus reisolation from gonopore-closed female *H. truncatum* which cofed with preinfected males demonstrated transmission in the absence of copulation. Rabbits that served as bloodmeal sources seroconverted after infestation by infected male ticks. However, CCHF virus was not reisolated from 3 gonopore-closed, engorged females, nor from their eggs, after feeding with hypostomectomized preinfected males.

Transmission of CCHF virus during mating or cofeeding of adult *H. truncatum*, and subsequent transovarial transmission, appear to represent additional mechanisms of infection in the tick population, and may contribute to the maintenance of transmission in nature.

Key-words: Bunyaviridae, CCHF virus, Tick; *Hyalomma truncatum*, Sexual and transovarian transmission, Cofeeding, Africa.

INTRODUCTION

Crimean-Congo haemorrhagic fever (CCHF) virus, from the CCHF serogroup (genus *Nairovirus*, family *Bunyaviridae*), is a tick-transmitted arbovirus that is pathogenic to humans (Watts *et al.*, 1988). It has been reported in sub-saharan Africa, southern and central Europe, central Asia and the Middle East (Hoogstraal, 1979).

The maintenance cycle of CCHF virus is still not fully understood particularly in austral and

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western Africa (Watts *et al.*, 1988). Fundamental knowledge of the mechanisms of replication and transmission is a prerequisite for understanding the virus ecology. We previously reported on CCHF virus replication in adult *Hyalomma truncatum* and *Amblyomma variegatum* ticks (Gonzalez *et al.*, 1991), two major vectors of CCHF virus in West Africa (Camicas *et al.*, 1990); little is known, however, about transmission.

Classically, CCHF virus is acquired by an arthropod during a bloodmeal from a viraemic host. However, many infected hosts apparently exhibit a brief, sometimes undetectable, viraemia (Gonzalez *et al.*, 1989b). Other mechanisms may play a role in increasing the rate of CCHF virus infection of ticks in nature. Transovarial transmission to the next generation and direct transmission from male to female during mating ("sexual transmission") are two examples.

The present paper reports on experiments to study these mechanisms of CCHF virus transmission in *H. truncatum*.

MATERIALS AND METHODS

Virus strain

CCHF virus strain HD49199, isolated in 1988 from a fatal human case in Mauritania, was used after the third passage in mouse brain (Gonzalez *et al.*, 1990). As previously described, virus titration was done at several dilutions (undiluted to 10^{-6}) by intracranial inoculation into suckling mice (Gonzalez *et al.*, 1991). A viral suspension titrating 6.5 log LD_{50}/ml, was stored at −70°C. This was diluted (0.1) in Hanks' medium for inoculation.

Tick colony and infection

Adult *H. truncatum* were reared from immature ticks obtained from a single engorged female tick removed from a sheep in Yonoféré, Sénégal; a subset of eggs was tested and found to be virus-free (Wilson *et al.*, 1991). Our method of inoculation was based on those used by Lee and Kemp (1970) and Stelmaszyk (1975): adult male ticks were inoculated by the intra-anal route with a volume of 2 µl of the diluted CCHF virus supension (Gonzalez *et al.*, 1989a). They were then placed on a laboratory rabbit for 11 days before hypostomectomy and the introduction of females.

Tick hypostomectomy and genital sealing

Adult males were immobilized on their backs using a cork board and overlapping pins that secured but did not damage the tick. After the palps were spread apart, the hypostome was cut using opthalmic scissors. The chelicera were untouched. Formation of a haemolymph clot was immediate. Specimens were inspected after hypostomectomy and the quality of the section was confirmed by a classical scanning electron microscopy (Kruger and Hamilton-Atwell, 1988) (fig. 1).

For 6 female ticks, their genital aperture was sealed using "Cyanolit" glue: they were held with forceps while a drop of glue was placed over the genital slit and allowed to harden.

Rabbit infestation with ticks

Naive rabbits were infested only once. Fur on their backs was cut and the remaining hair removed using a depilatory cream (Veet, Reckitt & Colman). One day later, an open-ended aluminium tube (30 mm diameter) was glued at one opening to the naked skin using a nonallergenic glue (Pattex, Henkel). Ticks were fed by placing them into the tube, which then was covered by a cloth mesh.

Male ticks were given access to a rabbit for 11 days before being hypostomectomized, in order to start spermatogenesis. The day after hypostomectomy, they were again placed into a feeding chamber on a naive rabbit in the presence of non-infected females. Six to 8 specimens were used for each trial. Several trials in which males had not been inoculated were conducted as controls to detect any other means of viral transmission. Following the detachment of ticks, males were tested for virus and females were maintained in a humid chamber until egg laying was complete.

Virus assay and antigen-capture

Individual adult ticks, pools of ~ 50 larvae, and pools of ~ 50 nymphs were ground in Hanks' medium (10 % w/v) then centrifuged (10,000 *g* for

CCHF = Crimean Congo haemorrhagic fever.
ELISA = enzyme-linked immunosorbent assay.
TOT = transovarian transmission.

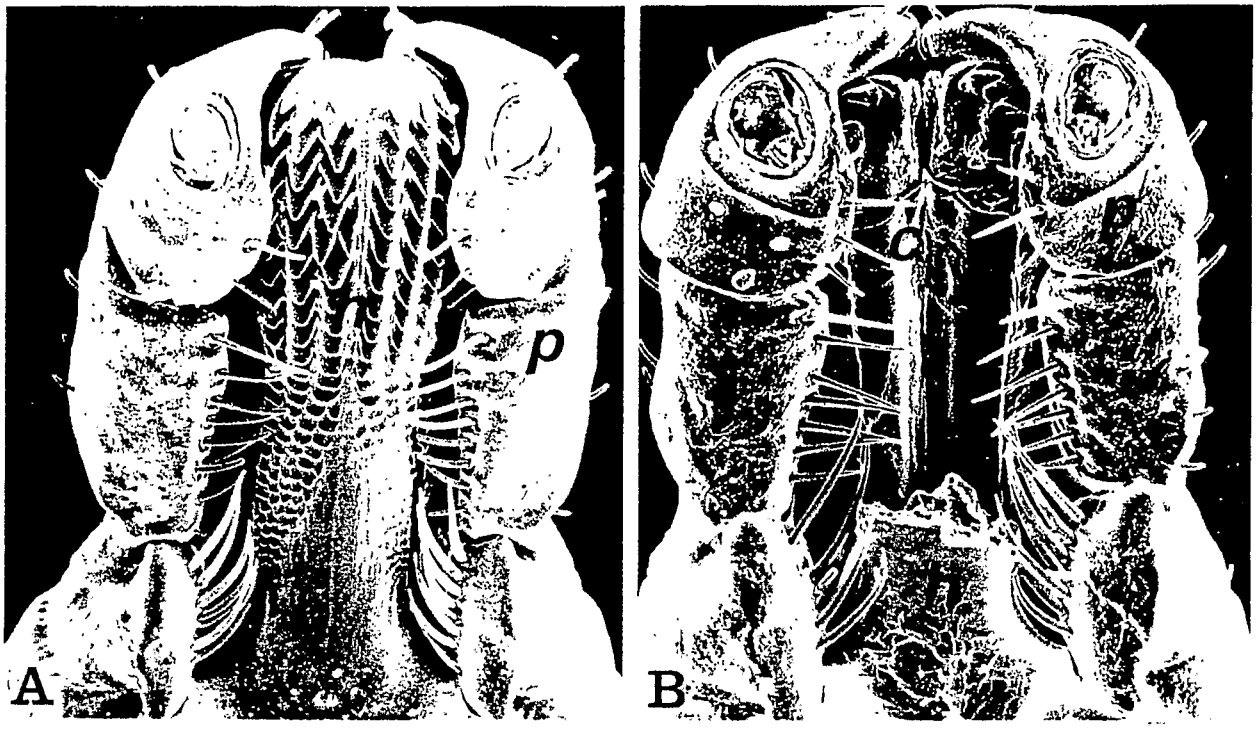

Fig. 1. Scanning electron microscopy of the mouth parts of a male *H. truncatum* tick.

Before (A) and after hypostomectomy (B); mouth parts are presented on their ventral aspect showing hypostome (h), chelicera (c) and palp (p). Magnification ×230.

Under cold anaesthesia, male ticks were plunged into fixative (2.5 % glutaraldehyde, 0.05 M cacodylate buffer) then dehydrated. Specimens were examined in a "JEOL 35CF" (Japan Electronic Optical Laboratory) scanning electron microscope.

10 min). The supernatant was assayed for virus reisolation by intracranial inoculation into suckling mice (Swiss mice strain, Pasteur Institute of Dakar). Tick egg batches (~ 1,000 eggs) were ground in Hanks' medium (50 % w/v), centrifuged (10,000 $g \times 10$ min) and the supernatant assayed for virus in Vero cell culture.

Virus detection and identification were done by a previously described, antigen-capture ELISA test performed with crude mouse brain or infected cell supernatant (Saluzzo and LeGuenno, 1987; Gonzalez *et al.*, 1991).

Serology

Blood samples from rabbits were obtained by cardiac puncture one day before and 15 days after infestation. Serological tests were done using a direct ELISA (Niklasson *et al.*, 1984) which was modified slightly (Wilson *et al.*, 1990). Sera were diluted 1/400 and revealed by a caprine anti-rabbit immunoglobulin conjugated with horseradish peroxidase (Cappel, Weschester, PA 19380).

RESULTS

Different experiments showed that there was CCHF virus transmission from male to female *H. truncatum* ticks either by copulation or by cofeeding (table I). In a mating experiment (exp. 1), CCHF virus was isolated at a titre > 3.2 log LD_{50}/ml from 2 of the 3 surviving hypostomectomized males, demonstrating successful introduction of the virus. Four of 6 females that mated with these males showed virus titres > 2.2 log LD_{50}/ml. Transovarian transmission (TOT) was observed by virus isolation from 2 of the 4 egg batches that were

Table I. Transmission of CCHF virus by mating between infected adult male *H. truncatum* ticks.

Exp. no.	Transmission studied	Tick pre-infection	Tick treatment: Hypostomectomy (male)	Tick treatment: Gonopore occlusion (female)	Virus isolates (no. tested): Adult Males	Adult Females	F_1 Eggs [a]	F_1 Larvae [a]	F_1 Nymphs [b]	Rabbit seroconversion: Male pre-feeding	Rabbit seroconversion: Female and male cofeeding
1	Mating	Males	Yes	No	2 (3) [c]	4 (6) 0 (2) [e]	2 (4) [d] 0 (4) [e]	1 (6) [d] None [f]	0 (15) [d] None	+	+
2	Cofeeding	Males	No	Yes	3 (3)	3 (3)	None	None	None	+	+
3	Control	Males	Yes	Yes	3 (3)	0 (3)	None	None	None	+	−
4	Control	Females	Yes	Yes	0 (3)	3 (3)	None	None	None	−	+
5	Control	None	No	Yes	0 (3)	0 (3)	None	None	None	−	−

[a] Each observation was pooled from the eggs of one female.
[b] Nymphs from positive pools of larvae.
[c] A fourth tick that was dead tested negative.
[d] Pools from previous stage that tested positive.
[e] Pools from previous stage that tested negative.
[f] Not produced/not alive.

produced by the 4 virus-positive females (table I). From these positive egg batches, 6 larval pools were tested, and virus was isolated from one of them at a titre of 3.7 log LD_{50}/ml. Attempts to isolate the virus from 15 nymph pools derived from the larvae of the batch that tested positive were negative. The rabbits fed upon by preinfected, hypostomectomized males cofeeding with females also had seroconverted 15 days postinfestation (antibody titre > 1,600), presumably from infection via the female ticks.

In the cofeeding experiment (exp. 2), involving hypostome-intact males and gonopore-closed females, each female tick that was associated with an inoculated male became infected. The rabbit seroconverted, demonstrating virus transmission from the male ticks. Apparently these gonopore-closed female ticks became infected by feeding on the rabbit.

In one control experiment (exp. 3) involving preinfected, hypostomectomized males and gonopore-closed females, no virus was isolated from the engorged females. All male ticks tested positive but the rabbit on which males and females were placed did not seroconvert (table I). In another control (exp. 4), 3 preinfected females, which tested positive after feeding, did not transmit CCHF virus to 3 hypostomectomized males in the same capsule; the rabbit, however, seroconverted. Finally (exp. 5), the virus was not isolated from non-infected, hypostome-intact male ticks, nor from the non-infected gonopore-closed female ticks. The rabbit had not seroconverted when tested 15 days after infestation.

All rabbits that served in the initial "prefeeding" exposure of preinfected male ticks seroconverted following infestation (antibody titre > 800). No rabbits prefed upon by infected males showed evidence of CCHF antibody.

DISCUSSION

By altering the ability of male *H. truncatum* to feed and/or of female ticks to become inseminated, we have demonstrated that CCHF virus can be sexually transmitted to females from experimentally preinfected males. Preinfected females, however, did not sexually transmit CCHF virus to hypostomectomized males. Virus apparently was not transmitted either to female ticks or to the host by leakage of saliva or other infected secretions. From these observations, it appears that the male spermatophore served as the means by which CCHF virus was transmitted to the female. These infected female ticks in turn became infectious for their rabbit hosts.

Moreover, the virus was vertically transmitted from the females through their eggs at least to the first developmental stage.

When sexual transmission was prevented, CCHF virus was passed horizontally from male to female ticks, apparently by cofeeding. Horizontal transmission in ticks may take place by several means. Classically, it occurs when vectors feed during the time that vertebrate hosts are viraemic. However, other mechanisms have been suspected, as host viraemia is often undetectable. CCHF virus transmission has been observed when non-infected ticks cofed with infected ones (Logan *et al.*, 1989). Even when viraemia is undetected, Thogoto virus transmission was observed by "distant" cofeeding on a non-viraemic host (Jones *et al.*, 1987). Another rare mode of transmission could occur during hyperparasitism of the infected females by males (Ntiamoa-Baidu, 1986 *in* Logan *et al.*, 1989). Recently, we demonstrated horizontal transmission of CCHF virus from a weakly immune non-viraemic preimmunized sheep to adult *H. truncatum* (Wilson *et al.*, 1991).

Vertical transmission of CCHF virus has been demonstrated by TOT (Lee and Kemp, 1970). However, rates of infection are low, ranging from 0-5 % of egg batches (Lee and Kemp, 1970; Logan *et al.*, 1989; Sheperd *et al.*, 1989). We previously observed on one occasion that 17 % of *H. truncatum* eggs were infected by TOT (Wilson *et al.*, 1991); a more thorough study of this phenomenon would be very useful. In the present study, CCHF virus appeared via TOT in a few larvae but not in nymphs. The variable rates of TOT must depend on numerous factors related to the biological characteristics of the virus as well as vector physiology.

In the present experiments, we have demonstrated 3 mechanisms that may influence the maintenance of CCHF virus in *H. truncatum* ticks: transmission while cofeeding, during mating and by TOT. The relative importance of each in nature remains unknown. Although laborious, studies of field-engorged females ticks and their offspring are needed to evaluate the natural frequency of TOT. Perhaps females infected during mating are more likely to produce infected eggs than those infected by cofeeding or as immature ticks. In such cases, sexual transmission of CCHF virus might be an important means by which CCHF virus is maintained in natural populations of *H. truncatum*.

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Transmission sexuelle expérimentale du virus de la fièvre hémorragique de Crimée-Congo chez la tique *Hyalomma truncatum*

Des mâles de *Hyalomma truncatum* ont été infectés expérimentalement avec le virus de la fièvre hémorragique de Crimée-Congo (CCHF). Après le repas sanguin servant à terminer la spermatogenèse, ils ont subi une hypostomectomie supprimant la transmission du virus par repas simultané (cofeeding). L'accouplement de femelles non infectées avec les mâles ainsi préparés a été réalisé sur un lapin séronégatif pour le virus CCHF.

La transmission sexuelle a été démontrée par l'isolement du virus à partir des femelles. De plus, la transmission transovarienne a été observée dans un tiers des pontes. Dans un cas le virus était réisolé des larves issues d'une de ces pontes positives. Le lapin hôte, infesté par la femelle infectée par voie sexuelle, a présenté une séroconversion nette au quinzième jour post-infestation. Enfin, nous avons observé l'infection simultanée lors du repas sanguin de femelles non infectées rendues inaptes à la copulation (gonopore obturé) avec des mâles infectés non hypostomectomisés. Les conditions expérimentales que nous avons réunies nous donnent à penser que la transmission sexuelle du virus à la femelle a lieu par l'intermédiaire du spermatophore infecté. La femelle s'infecte, réplique le virus et peut alors transmettre celui-ci verticalement et horizontalement.

Les tiques du genre *Hyalomma* apparaissent comme un vecteur essentiel du virus CCHF en Afri-

que. Leur rôle de vecteur efficace est la résultante de leur aptitude à s'infecter, répliquer et transmettre le virus CCHF. La transmission sexuelle, suivie d'une transmission transovarienne, pourrait participer au maintien du virus dans la nature, en augmentant le taux d'infection du vecteur.

Mots-clés: Bunyaviridae, Virus CCHF, Tique; *Hyalomma truncatum,* Transmission sexuelle et transovarienne, Cofeeding, Afrique.

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