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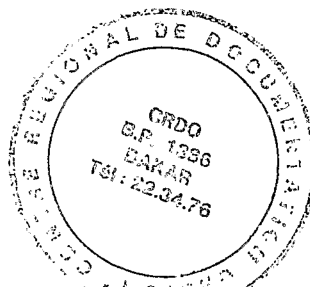

# Crimean-Congo haemorrhagic fever virus replication in adult *Hyalomma truncatum* and *Amblyomma variegatum* ticks

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SUMMARY

**The kinetics of the replication of the Crimean-Congo haemorrhagic fever virus (CCHFV) was studied in intra-anally inoculated adult *Hyalomma truncatum* and *Amblyomma variegatum* ticks. The virus was re-isolated by suckling mouse inoculation and revealed by antigen capture with ground ticks and indirect immunofluorescence of haemolymph. The virus was detected in ticks in the first hours post-inoculation (p.i.) and its replication was observed from 36 h p.i. onwards. Virus titre reached a maximum within 3-5 days then decreased slowly to a level of at 2 log $LD_{50}$/ml for several months until the end of observations.**

**Several specific, non-identified factors seem to favour CCHFV replication in *H. truncatum.* Long-term virus persistence seems to occur in CCHFV-infected adult ticks.**

*Key-words:* Crimean-Congo haemorrhagic fever virus, Replication, Tick; *Hyalomma truncatum, Amblyomma variegatum,* Persistence.

## INTRODUCTION

Crimean-Congo haemorrhagic fever virus (CCHFV) is a highly pathogenic human arbovirus which is transmitted by Ixodid ticks. Belonging to the CCHFV serogroup, genus *Nairovirus,* family *Bunyaviridae,* CCHFV is distributed over 3 continents in various ecosystems of subsaharan Africa, southern and central Europe, central Asia and the Middle East. The virus has been isolated from a wide variety of vertebrates and ticks, some of which have been shown to be efficient vectors. Nevertheless, the ecology of this virus still needs to be clarified, particularly in the austral and western regions of Africa (Hoogstraal, 1979; Peters and Leduc, 1984; Watts *et al.,* 1988).

Despite the documented occurence of viraemia in livestock, CCHFV transmission to ticks and the ability of various ticks to replicate and transmit the virus in nature, still remain unclear (Watts *et al.,* 1988). Maintenance of CCHFV re-

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quires competent vertebrate hosts and vectors to periodically replicate and transmit the virus. The intricate mechanisms that contribute to such competence must be studied at the different levels of viral ecology. Several reports on the ability of tick species to replicate and transmit the virus have been published (Lee and Kemp, 1970; Okorie and Fabiyi, 1980; Logan *et al.*, 1989; Sheperd *et al.*, 1989; Wilson *et al.*, 1990) but much more needs to be learned regarding interactions among a variety of vectors, hosts and even virus strains.

The goal of the present study was to examine aspects of CCHFV behaviour in two potential tick vectors from West Africa. We studied virus replication in *Hyalomma truncatum,* an important tick species in CCHFV ecology, and *Amblyomma variegatum,* another most common tick that feeds on a variety of hosts and is a potential vector of CCHFV (Camicas *et al.*, 1990).

## MATERIALS AND METHODS

### Virus and ticks

CCHFV (HD-49199) isolated in 1988 in Mauritania from a fatal human case (Gonzalez *et al.*, 1990), was used after a third passage in mouse brain. The viral suspension was stored at −70°C and was inoculated at a titre of 6.5 log $LD_{50}$/ml.

The adult *H. truncatum* used originated from a single engorged female tick removed from a sheep in Yonofere, Senegal, in 1989 (Wilson *et al.*, 1990). *A. variegatum* were reared from a female collected in 1986 in the Central African Republic (Gonzalez *et al.*, 1990). Adults from the 6th generation were used.

Inoculation of ticks, which has previously been described (Gonzalez *et al.*, 1989a) was based on the techniques of Lee and Kemp (1970) and Stelmaszyk (1975). Adult ticks were inoculated intra-anally with 7.0 µl of the CCHFV suspension diluted 1/10 in Hanks' medium and kept cool during inoculation on a bed of ice.

### Virus re-isolation and antigen capture

The development of virus titre in ticks was studied by re-isolation titration. Whole ticks, tested one-by-one (at least 3 per group), were ground in Hanks' medium (10 % w/v) and centrifuged (10,000 *g* for 10 min). The resulting supernatant was then assayed for virus by intracranial (i.c.) inoculation into suckling mice (Swiss mice strain from the Pasteur Institute, Dakar) at several dilutions (undiluted to $10^{-6}$) for virus titration.

Virus detection and identification were also attempted using a previously described antigen-capture (AC) ELISA carried out with crude mice brain or ground ticks. AC was used because of its high sensitivity and specificity (Saluzzo and Leguenno, 1987; Gonzalez *et al.*, 1989b). The assay was performed in 96-well microplates coated with an anti-sheep µ chain (Cappel Laboratories), then anti-CCHFV sheep IgM was added. Undiluted samples of crude mouse brain or freshly ground ticks in Hanks' medium were added and incubated overnight at 4°C. A high-titred anti-CCHFV hyperimmune mouse ascitic fluid, prepared with the HD-49199 CCHFV strain, was then added to bind to any antigen captured from the test sample. Anti-mouse IgG conjugated with horse radish peroxidase (Biosys) was used to reveal any bound IgG and a chromogenic substrate (ortho-tolidine, Sigma T3510) was then added for colorimetry. Differences in the optical density (OD) between the test and control wells were measured at 450 nm using an automatic reader (Multiskan MCC340, Flow Laboratories, Irvine, Scotland). Positive samples were defined as a ratio $\geqslant 2$ between the test sample OD value and the negative control (non-infected suckling mouse brain or non-infected ground tick).

### Immunofluorescent antibody (IFA) and haemolymph tests

A haemolymph test originally developed by Burgdorfer (1970) for rickettsiae was modified. A drop of exuded haemolymph obtained by sectioning a tick leg or hypostome was used to directly smear 10-spot "Teflon" slides. A classical indirect IFA test was done on dried smears after 10 min cold acetone fixation (Johnson *et al.*, 1981). Hyperimmune mouse ascitic fluid was used to bind CCHFV antigen which was revealed by fluorescein-labelled anti-mouse IgG (Cappel Laboratories). Ticks inoculated with Hanks' medium only served as controls for each test.

AC = antigen capture.
CCHFV = Crimean-Congo haemorrhagic fever virus.
ELISA = enzyme-linked immunosorbent assay.
i.c. = intracranial.
IFA = immunofluorescent antibody.
OD = optical density.
p.i. = post-inoculation.
SMI = suckling mouse inoculation.

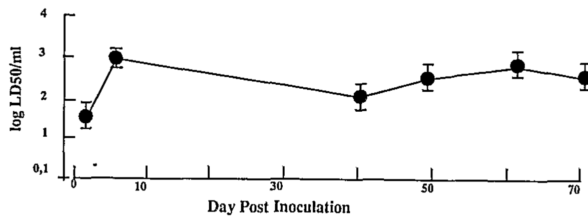


**Fig. 3.** Persistence of CCHFV in intracoelomically infected adult *A. variegatum* ticks (mean ± SE) tested by SMI (log $LD_{50}$/ml).

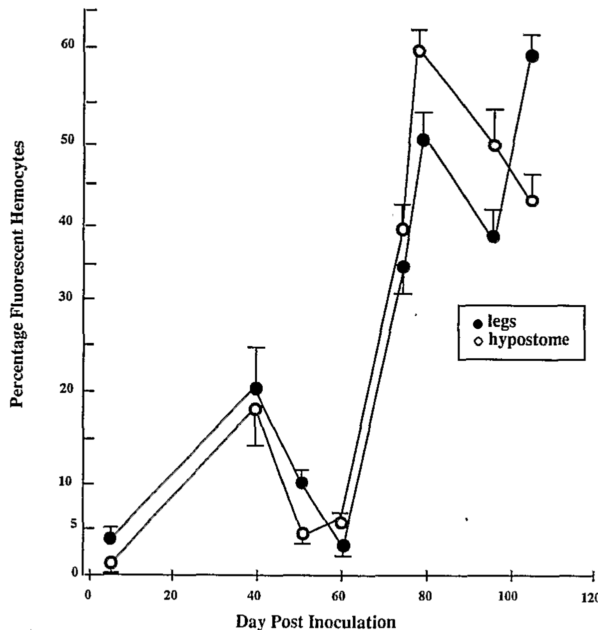


**Fig. 4.** Persistence of CCHFV titre in haemocytes of intracoelomically infected adult *A. variegatum* ticks tested by IFA.

Samples were taken from legs and hypostome of each tick (N = 3).

in *H. truncatum* slowly decreased on day 7 p.i. to a plateau (4.5 ± 5 log $LD_{50}$/ml) until day 12 p.i. (fig. 2). Antigen was first detected in *H. truncatum* haemocytes 4 h p.i. at a low level of cell infection (⩽ 1 %) and rate of infected specimen (2 to 4), then increased regularly to day 12 p.i. This corresponded with antigen detection by AC (fig. 2).

Changes in CCHFV titre in *A. variegatum* were different. Titres reached a plateau in 5 days and remained at a moderate level of 2 ± 5 log $LD_{50}$/ml up to 70 days p.i. (fig. 3). When we explored the distribution of the virus in *A. variegatum* infection in the leg and hypostome haemolymph increased in a similar way up to day 115 p.i. (fig. 4).

## DISCUSSION

The absence of a classical eclipse phase may have resulted from the nature of the test or the frequency of sampling. Perhaps the high titre of virus used in inoculation provided enough extracellular virions to hide an eclipse phase. A similar study of *H. truncatum* nymphs inoculated with CCHFV reported that there was continued viral detection by mouse inoculation but virus was not detectable by fluorescent focus assay 24 h p.i. (Shepherd *et al.*, 1989). Secondly, the highly sensitive ELISA may not have been capable of spotting a reduction in the virus concentration. Finally, a brief eclipse may have gone undetected if it had occured between samples.

CCHFV titre was always consistently higher in *H. truncatum* than in *A. variegatum*. Shepherd *et al.* (1989) observed by fluorescence focus assay that CCHFV disappeared in unfed *Amblyomma hebraeum* more than one month before it disappeared in *H. truncatum* and *Rhipicephalus evertsi mimeticus*. Similarly, *R. appendiculatus* appeared to be more susceptible than *A. variegatum* to infection with the Togoto virus (Davies *et al.*, 1990).

It is not surprising that the ability of a virus to replicate depends on the vector. Two major factors could influence intrinsic virus replication in ticks: (1) the gut barrier limiting passage of virus out of the gut and (2) the ability of the tick haemocytes, haemolymph or target organs to favour virus replication. Tick species vary in the composition of the haemolymph (Neitz *et al.*, 1978) and only a few of the more than 30 species that have been found to be naturally infected by CCHFV favour active virus replication (Hoogstraal, 1979; Camicas *et al.*, 1990). As a similar titre of viral inoculum was used in our study, it appears that *H. truncatum* favours the replication of CCHFV more than *A. variegatum*.

Despite exhibiting a lower titre, *A. variegatum* still remained infected with CCHFV

**Table I.** CCHFV infection in experimentally inoculated adult *H. truncatum* and *A. variegatum* ticks tested 5 days p.i.

| Method | Virus detected in: *H. truncatum* No. | % Positive | *A. variegatum* No. | % Positive |
|---|---|---|---|---|
| SMI | 21 | 100 | 15 | 100 |
| IFA | 17 | 59 | 28 | 75 |
| AC | 17 | 59 | 32 | 56 |

No. = number of individual ticks tested.

## RESULTS

CCHFV was re-isolated 5 days p.i. from 100 % of ticks tested by suckling mice inoculation (SMI), demonstrating effective replication (table I). Infection in ticks was detected less often by AC and IFA tests.

During the early phase of virus replication, the virus titre increased in *H. truncatum* but not in *A. variegatum* (fig. 1). We did not observe an eclipse phase in either species of tick. Following inoculation, the virus titre increased gradually to more than 6 log $LD_{50}$/ml at 100 h p.i. for *H. truncatum* but remained more-or-less constant for *A. variegatum*. Then, the virus titre

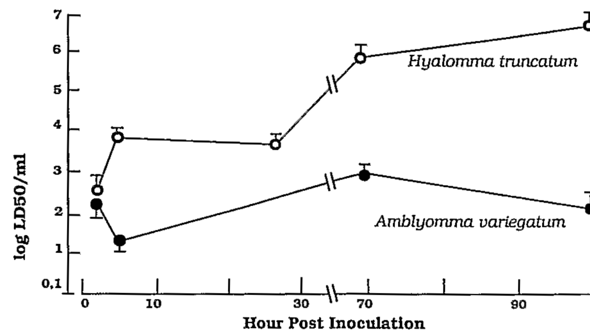


**Fig. 1.** Development of CCHFV titre (mean ± SE) in intracoelomically infected adult *H. truncatum* and *A. variegatum* (N = 3) tested by SMI (log $LD_{50}$/ml).

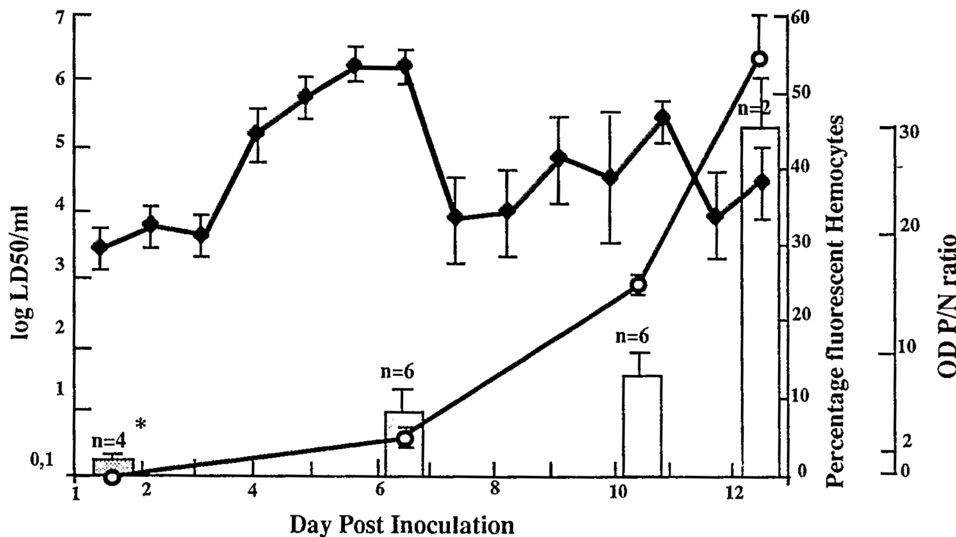


**Fig. 2.** CCHFV titres (mean ± SE) in intracoelomically infected adult *H. truncatum* ticks as measured by SMI (solid diamonds), IFA test (open circles), and AC (bar diagram).

Scales are log $LD_{50}$/ml (SMI), percentage of fluorescing cells (IFA) and OD ratios of positives to negatives (AC). Sample sizes N = 3 unless otherwise indicated (*).

4 months p.i. when tested by suckling mice inoculation. Similarly, long-term infection was seen in other studies with *H. truncatum* where virus was re-isolated 11 months p.i. with an average titre of 2.3 log $LD_{50}$/ml (J.P. Cornet *et al.*, unpublished data) and with *H. marginatum rufipes* 9 months p.i. at a titre of about 2.0 log $LD_{50}$/ml (Sheperd *et al.*, 1989). However, with time, the percentage of infected haemocytes remained lower with *A. variegatum* than with *H. truncatum*. The stability of CCHFV titre after about 15 days' intrinsic incubation suggests that a life-long, stable persistence of the virus may occur in certain susceptible adult ticks.

All ticks of both species were infected by intra-anal inoculation. The amount of total viral antigen detected by AC and the percentage of infected cells gradually increased. However, some specimens remained negative in both tests even though virus could be re-isolated in suckling mice. It is difficult to explain these intraspecific variations. Nevertheless, when virus was present, cells were infected at a high rate, and after 60 days p.i., 100 % of the haemocytes were infected. Thus, CCHFV replicated rapidly when these ticks were infected, reaching a plateau at a few days p.i. Ticks remained infected for several months, though virus replication varied among individuals and between species.

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**Réplication expérimentale du virus de la fièvre hémorragique de Crimée-Congo chez les imagos des tiques *Hyalomma truncatum* et *Amblyomma variegatum***

La cinétique de réplication du virus de la fièvre hémorragique de Crimée-Congo (CCHF) est observée chez les imagos de tiques *Hyalomma truncatum* et *Amblyomma variegatum* infectées *per anum*.

Le virus est réisolé par inoculation au souriceau nouveau-né et mis en évidence par capture de l'antigène sur la tique broyée et par immunofluorescence indirecte sur les hémocytes. Le virus est détecté chez la tique dans les premières heures post-inoculation (p.i.) et sa réplication observée dès la 36$^{e}$ h p.i. Le titre viral atteint son maximum en 3 à 5 jours puis il décroît lentement avant de se stabiliser (2 log $LD_{50}$/ml) pendant plusieurs mois d'observation.

Certains facteurs spécifiques non identifiés semblent favoriser une meilleure réplication du virus chez *H. truncatum*. La persistence à long terme du virus semble être la règle chez l'imago de tique infecté par le virus CCHF.

*Mots-clés:* Virus de la fièvre hémorragique de Crimée-Congo, Réplication, Tique; *Hyalomma truncatum, Amblyomma variegatum,* Persistance.

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