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EXPERIMENTAL TRANSMISSION OF CRIMEAN-CONGO HEMORRHAGIC FEVER VIRUS BY WEST AFRICAN WILD GROUND-FEEDING BIRDS TO *HYALOMMA MARGINATUM RUFIPES* TICKS

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Abstract. *Hyalomma (H.) marginatum rufipes* ticks commonly infest birds and are potential vectors of Crimean-Congo hemorrhagic fever (CCHF) virus in west Africa. An experimental model for investigating the role of birds in the CCHF virus transmission cycle was developed. Following CCHF virus inoculation, antibodies were detected by enzyme-linked immunosorbent assay in one red-beaked hornbill and one glossy starling, but not in two laughing doves and six domestic chickens. None of the birds showed a detectable viremia. *Hyalomma marginatum rufipes* larvae were placed on three red-beaked hornbills and one glossy starling. These birds were then inoculated with CCHF virus ($10^{1.5}$ 50% mouse intracerebral lethal doses). Virus transmission to larvae or nymphs was obtained and seroconversions in birds were recorded. Virus was also detected in 90% of the individually tested nymphs, as well as in adults. The virus was then successfully transmitted by adult ticks to rabbits and the engorged females were allowed to oviposit. Progeny larvae were placed on another group of birds and one of three birds showed seroconversion. The cycle of transmission of virus between ticks and aviremic ground-feeding birds represent a potential reservoir and amplification mechanism of CCHF virus in west Africa.

The tick-borne viral zoonosis, Crimean-Congo hemorrhagic fever (CCHF), has a widespread distribution, and is focally endemic in Senegal.[1,2] The understanding of the transmission cycle of the virus remains incomplete. Numerous potential reservoirs have been described.[2] The role of birds as potential host reservoirs has been considered, but their ability to transmit the virus is not clear.[3] In two areas where CCHF virus was isolated from ticks collected on tagged animals and IgM antibodies to CCHF were found in serosurveys in ungulates, the role of birds in the CCHF virus cycle was investigated. Antibodies (IgG) to CCHF were detected by enzyme-linked immunosorbent assay (ELISA) in some ground-feeding birds: long tailed glossy starlings (*Lamprotornis caudatus*), red-beaked hornbills (*Tockus erythrorhynchus*), and in one blue-helmet guinea-fowl (*Numidia meleagris*) (Zeller HG and others, unpublished data). Birds serve as hosts for infected *Hyalomma (H.) marginatum rufipes, H. impeltatum, H. truncatum,* and *Amblyomma variegatum* ticks.[4,5] *Hyalomma marginatum rufipes* ticks appeared to be the most commonly infected species in Senegal, as revealed by the results of CCHF virus isolation from ticks collected in northern Senegal and the Bandia area (Thies region) from small ruminants, cattle, and camels (26 isolates in 1992).[6] *Hyalomma marginatum rufipes* typically exhibits a biphasic ditrophic cycle. Immature forms most often parasitize birds or lagomorphs, while adults usually infest ungulates.[5] Engorged larvae molt on the host producing nymphs that feed on the same host. In 1992, one CCHF virus strain was isolated from two *H. marginatum rufipes* nymphs collected on a red-beaked hornbill in the Bandia area (Zeller HG and others, unpublished data).

To understand the precise role of birds in the natural transmission cycle of CCHF virus, red-beaked hornbills and glossy starlings were tested for viremia, antibody response, and their ability to transmit CCHF virus to *H. marginatum rufipes* ticks.

MATERIALS AND METHODS

The CCHF viral strain HD 49199, isolated from a fatal human case in Mauritania in 1989, was used after three passages in suckling mice.[7]

Wild birds were captured by nets, bled, and tested for the presence of CCHF antibodies prior to the experiments. They were confined in a pro-

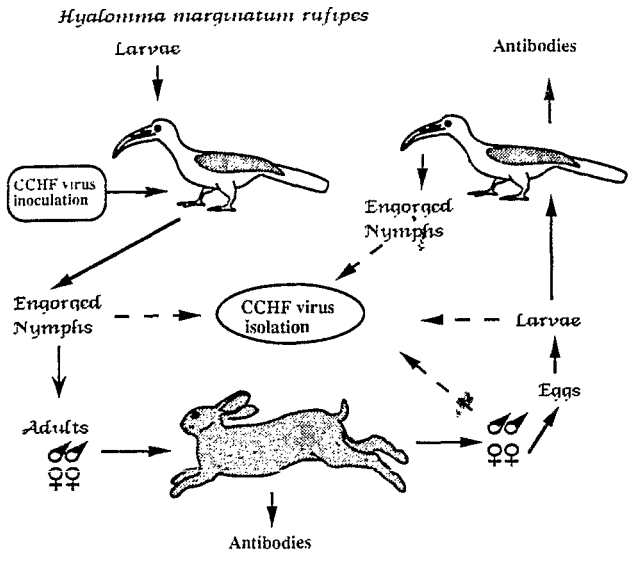

FIGURE 1. Diagram of experimental transmission of Crimean-Congo hemorrhagic fever (CCHF) virus from bird to *Hyalomma marginatum rufipes* immature ticks, and transstadial and transovarial transmission of the virus.

tected animal holding facility in individual cages and given a daily supply of food and water.

Six domestic chickens, two laughing doves, one red-beaked hornbill, and one glossy starling were inoculated intraperitonally with CCHF virus ($10^{1.5}$–$10^{3.5}$ 50% mouse intracerebral lethal doses [LD_{50}]). Blood was collected by wing vein puncture daily from days 2 to 10 and viremia was checked by intracerebral inoculation into 1–2-day-old suckling mice and inoculation of Vero cell cultures.[8,9] Birds were studied for CCHF antibody response by an antibody-capture ELISA.[10,11] Sera were diluted 1:100. Peroxidase-labeled, affinity-purified goat antibodies to chicken IgG and turkey IgG (Kirkergaard and Perry, Gaithersburg, MD) were used for antibody detection in birds.

Experimental transmission. Experimental transmission was performed using three hornbills (H1, H2, and H3), one starling (S4), and three chickens (C5, C6, and C7). Birds were tested for CCHF antibody status prior to experiments. Immature ticks were obtained from an *H. marginatum rufipes* tick colony raised in the laboratory and initiated from eggs of an engorged female collected in Tessekre (Ferlo region) from a cow. Larvae, nymphs, and adults were previously tested for CCHF virus. Larvae were placed on the head of each bird. Virus was inoculated the same day into chichen C5 three days after infection with larvae into hornbills H1

TABLE 1

*Crimean-Congo hemorrhagic fever virus viremia and antibody response in birds**

Bird species	No.	Inoculum (log LD_{50})	Viremia	Antibodies
Chicken	4	3.5	–	–
	1	2.5	–	–
	1	1.5	–	± day 10
Red-beaked hornbill	1	2.5	–	+
Glossy starling	1	1.5	NT	+

* LD_{50} = 50% lethal dose; NT = not tested.

and H2, starling S4, and chicken C6, and 10 days later for nymphal infection into hornbill H3 and chicken C7.

Engorged nymphs dropped off the birds. They were collected and allowed to molt. Virus isolation was attempted; nymphs were homogenized with a mortar and pestle in 1 ml of diluent (Hanks' balanced medium, 5% bovine albumin), centrifuged, and the supernatant was inoculated into suckling mice. Individual titrations were done as previously described.[8] Blood samples from birds were collected for CCHF antibody detection.

Adult ticks derived from some of the molted nymphs were allowed to feed on rabbits. Engorged females were collected and held at 25°C and a relative humidity of 75% in individual vials until egg laying was completed. Male ticks were detached with forceps. Male and female ticks were tested for CCHF virus, and rabbits were tested for CCHF antibodies. Eggs of engorged females were kept for hatching. Fractions of the larval progeny were tested for the presence of virus (100 larvae per pool). The remaining larvae were allowed to feed on seronegative birds. Engorged nymphs were collected and tested for CCHF virus and birds were tested for presence of CCHF antibodies (Figure 1).

RESULTS

Viremia was not detected in the various bird species tested from days 2 to day 10 after virus inoculation. Antibodies to CCHF virus were not detected in chickens and laughing doves, but a significant antibody response was obtained in the red-beaked hornbill and the glossy starling (Table 1). Four months later, antibodies were still detectable in these birds. A low dose ($10^{1.5}$ LD_{50}) of CCHF virus was enough to induce an antibody response.

TABLE 2

Experimental transmission of Crimean-Congo hemorrhagic fever (CCHF) virus from birds to Hyalomma marginatum rufipes *larvae or nymphs and results of transstadial and transovarial transmission**

	Hornbill			Starling	Chicken		
	H1	H2	H3	S4	C5	C6	C7
Day of larval infestation	0	0	0	0	0	0	0
CCHF virus (log LD_{50})	1.5	1.5	1.5	1.5	3.5	3.5	3.5
Day of inoculation	3	3	10	3	0	3	9
CCHF antibodies	NT	NT	+	+	–	–	–
Day of detection	14†	14†	25	25	24	18	25
No. of nymphs collected	150	180	16	8	19	18	18
Day(s) of collection	14	14	17–21	14–19	7–18	14–20	12–16
No. positive/no. tested (nymphs)	8/10	10/10	2/3	NT	0/19	0/18	0/18
Transstadial transmission							
Rabbit:	R1		R2		R3		R4
Antibody detection	+		+		+		+
No. of adults collected	8		5		3		5
No. positive/no. tested	1/1		NT		3/5		4/5
Transovarial transmission							
No. of egg-laying ticks	8		3		6		0
No. of positive larvae/no. tested‡	9/37		2/15		12/30		
Hornbill:	H11		H21		H31		
Antibody detection	+		NT§		–		
No. of positive nymphs/no. tested	0/4		1/7		0/9		

* NT = not tested.
† Died.
‡ 100 larvae/pool.
§ Died on day 12.

Chickens C5, C6, and C7, which received a $10^{3.5}$ LD_{50} CCHF virus inoculum, did not transmit the virus to *H. marginatum rufipes* larvae or nymphs (Table 2). The dose inducing an antibody response, $10^{1.5}$ LD_{50} of CCHF virus, was inoculated into hornbills H1, H2, and H3, and starling H4. Larvae did not drop off the birds after engorgement but instead molted on the host and remain attached until the engorged nymph stage (duration: two weeks or more). Hornbills H1 and H2 died on day 14 postinoculation, possibly as a result of the heavy tick infestation (> 150 nymphs). All nymphs still attached were collected from the cadavers. Virus was isolated from 17 of 17 pools of nymphs (10 nymphs/pool) from hornbills H1 and H2. In addition, CCHF virus was detected in 18 (90%) of 20 individually titrated nymphs (mean titer $10^{2.8}$ LD_{50}) (Table 3). The two virus-negative nymphs were unfed. In adult ticks, titers were higher ($10^{3.1–3.8}$ LD_{50}) after they fed on rabbits. Only a few nymphs were collected from hornbill H3.

Virus was transmitted to rabbits by adult ticks emerging from molted nymphs derived from hornbill H1 (rabbits 1 and 11), H2 (rabbit 2), H3 (rabbit 3) and starling S4 (rabbit 4) and induced an antibody response (Table 2). Virus titers were similar in nymphs isolated from hornbill H2 and in adults after feeding on a rabbit. Three of four male adults isolated from hornbill H3 and tested after feeding on rabbit 3 were positive, with a low CCHF virus titer ($10^{0.91}$ LD_{50}).

Larvae derived from engorged females were tested for the presence of virus (Table 2). Virus was recovered from 24.3% of the larvae isolated from hornbill H1 and rabbits R1a and R1b, 13.3% from hornbill H2 and and rabbit R2, and 40.0% from hornbill H3 and rabbit R3. Three hornbills (H11, H21, and H31) were infested with the remaining larvae isolated from H1-R1, H2-R2, and H3-R3, respectively. Seroconversion for CCHF virus was detected in one hornbill (H1), but no virus was isolated from the four nymphs collected. Virus was isolated from one (14.3%) of seven nymphs isolated from hornbill H21, which died prior to testing for antibody response.

Few nymphs dropped off the glossy starling

S4 (Table 2). Five months after molting, two males and two females were allowed to feed on rabbit R4, which showed seroconversion. Titers of CCHF virus in these two males were $10^{3.75}$ and $10^{5.17}$ LD_{50}. The two partially engorged females were allowed to feed on another rabbit with two uninfected males. Virus was not recovered from one male and from the larval progeny.

DISCUSSION

Crimean-Congo hemorrhagic fever virus is unable to replicate in chickens, as shown by the absence of viremia and antibody response and the failure of these birds to transmit the virus to *H. marginatum rufipes* immature ticks. Virus transmission to larvae/nymphs was obtained with the red-beaked hornbill and glossy starling, even though these birds had undetectable viremias. The detection of viremia using intracerebral inoculation of suckling mice was not sensitive enough to detect a low level of circulating virus in the blood, and tests were only performed once a day. The antigen-capture method as previously performed in the laboratory gave results similar to those with inoculation of suckling mice. It was not used due to the small volume of blood collected daily.[7,12] However, the antibody response indicated some viral replication that permitted the infection of ticks. The virus was subsequently transmitted transstadially to nymphs and adults and infected rabbits used as experimental hosts of the adult stage. Virus was recovered from the progeny derived from these ticks. It was transmitted to one hornbill, which then developed an antibody response. Transovarial transmission of CCHF virus was successful and larvae were able to infect another bird.

Previously, birds were not thought to be important reservoirs of CCHF virus because they did not develop high viremia. Russian investigators were unable to reisolate the virus and did not obtain serologic evidence of infection in rooks (*Corvus frugilegus frugilegus*) and rock doves (*Columba livia*).[3] In guinea fowls, viremia of low intensity was demonstrated, followed by a transient antibody response.[13] A case of CCHF in a worker who was infected while slaughtering ostriches on a farm in South Africa was reported. Antibodies to CCHF virus were detected in 23.9% (22 of 92) of the ostriches.[13]

The accepted World Health Organization def-

TABLE 3

Individual Crimean-Congo hemorrhagic fever virus titrations of Hyalomma marginatum rufipes *nymphs collected from hornbills and adults collected from rabbits**

Tick stage	Engorged	Hornbill 1			Hornbill 2			Hornbill 3		
		No. positive/ no. tested	Log LD_{50} titer	Range	No. positive/ no. tested	Log LD_{50} titer	Range	No. positive/ no. tested	Log LD_{50} titer	Range
Nymph	no	0/2								
Nymph	+	5/5	2.85	0.92–3.44	10/10	3.58	2.56–5.20	–		
Nymph	++	3/3	2.46	1.76–3.20	1/1	2.83	–	–		
Nymph	Total	8/10	2.70	0.92–3.44	11/11	3.51	2.56–5.20	–		
		Rabbit 1			Rabbit 2			Rabbit 3		
Male	1/1	3.80	–		4/4	3.08	2.71–3.90	3/4	0.91	0.38–1.44
Female†	1/3	1.71	–		2/2	3.12	3.09–3.24		None tested	

* LD_{50} = 50% lethal dose; + = partially engorged; ++ = fully engorged.
† After laying eggs

inition of an arbovirus states that arboviruses "multiply and produce viremia in the vertebrates".[14] A detectable viremia is necessary for infection of competent tick vectors. Birds have shown a nondetectable viremia that was dependent on the method used, suggesting a nonviremic transmission of CCHF virus.[15] Other modes of transmission, such as cofeeding or sexual transmission, have been described.[12, 16, 17] It has also been postulated that nonviremic transmission is mediated by factors secreted in the saliva of feeding ticks (saliva-activated transmission).[18] These observations indicate that vertebrates that do not develop any detectable viremia can serve as important maintenance and amplifying hosts of CCHF virus.

Hyalomma marginatum rufipes exhibits a biphasic ditrophic cycle with an average larval-nymphal infestation period of 12–21 days on birds. Virus infecting larvae persisted in adult ticks for a long time, as shown in the starling experiment.

Virus was recovered from at least 23 (0.28%) of 8,200 larvae tested. This transovarial transmission rate can explain the maintenance of the virus. The role of wild ground-feeding birds as amplifying hosts may induce the high rate of infected ticks collected on cattle. Virus has been isolated from immature ticks on viremic scrub hares, but not from adult ticks fed on viremic cattle.[19] Ungulates, as the most abundant tick-infested animals, may be involved in horizontal transmission, providing blood for transovarially infected eggs.

Wild ground-feeding birds such as the red-beaked hornbill and the glossy starling have a wide distribution in Africa. Hornbills are known to migrate locally and to travel long distances to their food supply.[20] Their widespread distribution in Africa correlates with the large distribution of CCHF virus. Previously, birds were considered refractory to CCHF virus infection, with the exception of ostriches in South Africa.[13] The effective transmission of CCHF virus from birds to immature *H. marginatum rufipes* ticks and from immature ticks to birds associated with other transmission factors such as cofeeding indicates a potential role of wild ground-feeding birds in the ecology of CCHF virus.

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