A chronological review of experimental infection studies on the role of wild animals and livestock in maintenance and transmission of Crimean-Congo hemorrhagic fever virus

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ABSTRACT

This article provides a definitive review of experimental studies on the role of wild animals and livestock in the maintenance and transmission of Crimean-Congo hemorrhagic fever virus (CCHFV), the etiologic agent of Crimean-Congo hemorrhagic fever (CCHF), beginning with the first recognized outbreak of the human disease in the Crimea in 1944. Published reports by researchers in the former Soviet Union, Bulgaria, South Africa, and other countries where CCHF has been observed show that the virus is maintained in nature in a tick-vertebrate-tick enzootic cycle. Although wild and domestic animals infected with CCHFV do not develop visible illness, they provide blood meals for large numbers of ticks during the period of viremia and may serve as amplifying hosts. Human disease most commonly results from the bite of an infected tick, but may also follow exposure to the blood and tissues of slaughtered infected animals. Viremia in birds is rare; birds likely contribute to the epidemiology of CCHF by carrying infected ticks across wide areas. Many aspects of animal involvement in the maintenance and spread of CCHFV are still poorly understood, due to complex tick vector and vertebrate host factors that remain to be elucidated. This article is the second in a series of reviews of more than 70 years of research on CCHF, summarizing important findings, identifying gaps in knowledge, and suggesting directions for future research.

1. Scope of this review

This article reviews experimental studies of the role of wild and domestic animals in the maintenance and transmission of Crimean-Congo hemorrhagic fever virus (CCHFV) that have appeared in the research literature since the first recognized outbreak of the disease in 1944. It is the second article in a series that aims to provide a definitive account of research on Crimean-Congo hemorrhagic fever (CCHF), which has not been thoroughly examined since the monumental monograph by the noted acarologist Harry Hoogstraal was published in 1979 (1). The first paper in this series reviewed seroepidemiologic studies of the geographic distribution, host range, and frequency of CCHFV infection of wild and domestic animals (2). An article now in preparation will review experimental studies of the role of ticks in the maintenance of CCHFV and its transmission to animals and humans, and a fourth paper will summarize attempts to replicate important features of CCHF through experimental infection of laboratory animals. Readers are also referred to a general review of the epidemiology, viral genetic diversity, and other features of CCHF that appeared in 2013, and to a recent review of experimental molecular studies of the virus (3,4).

2. Background: maintenance and transmission of CCHFV

CCHF was first recognized in the Crimean region of the former Ukrainian SSR, when more than 200 cases of a severe febrile illness occurred from 1944–1945 in soldiers working to restore agricultural production in farmland abandoned during the German occupation (5). Mikhail Chumakov, who had a few years previously

69 characterized the maintenance cycle of tick-borne encephalitis in the Soviet Far
70 East, led the team of scientists sent to investigate the outbreak. An association of
71 what was then-called Crimean disease with tick bites was quickly recognized, and a
72 viral etiology was established through the inoculation of filtered tick extracts into
73 military volunteers and psychiatric patients undergoing fever therapy (6,7).

74 The Crimean outbreak exemplified the major features of CCHF. The disease
75 occurred exclusively in the agricultural steppe, and no cases were reported in towns
76 or wooded areas. The outbreak coincided with a period of unusually high tick
77 numbers in the spring and early summer of 1944, and most patients reported a tick
78 bite prior to illness. Although a large number of soldiers became ill, there was no
79 evidence of person-to-person transmission. As described in the 1945 expedition
80 report (5), more than 3000 blood-sucking arthropods were collected, but the most
81 common parasite was the tick *Hyalomma marginatum* , which is now recognized to
82 be the principal vector of CCHF in Russia and adjacent areas.

83 In nature, ticks are the primary reservoir of CCHFV (family *Bunyaviridae*;
84 genus *Nairovirus*) (3). Nairoviruses are a genetically highly diverse group.
85 Congruence between tick and nairovirus phylogenies supports coevolution of the
86 viruses and their tick hosts; virus genetic relationships correlated well with tick
87 host associations (8). CCHFV circulates silently in a tick-vertebrate-tick enzootic
88 cycle (1,3,9), in which animals play a key role in maintaining and transmitting
89 CCHFV by serving as hosts to a number of vector-competent tick species (Figure 1).
90 Virus is maintained in ticks by transstadial and transovarial transmission, and less
91 efficiently by venereal transmission (9). Tick life cycles are dependent on animal

hosts, and are defined by the number of hosts fed on while developing through the three parasitic life stages (larva, nymph, adult) prior to the adult female dropping off the host for oviposition after the final blood meal (Figure 2). Over 30 tick species, including one-, two- and three-host species, have been implicated in CCHFV ecology (1,10). One-host ticks (e.g., *Rhipicephalus annulatus*, *Rhipicephalus decoloratus*, *Rhipicephalus microplus*) remain on one host, typically a large mammal like a cow, during all life stages. Two-host ticks (e.g., *H. marginatum* complex of species, *Hyalomma detritum*, *Hyalomma anatolicum*) feed and remain on the first host, typically a small mammal, during the larval and nymphal life stages, and then drop off and attach to a different host, usually a larger mammal, as adults for a final blood meal. Three-host ticks (e.g., *Ixodes ricinus*, *Haemaphysalis punctata*, *Dermacentor marginatus*) drop off and reattach to a new host during each life stage, generally using small mammals for the two initial stages, and large mammals for the final meal. Consideration of tick and vertebrate host ecology in CCHFV is complex. While some vector species may locally exhibit a narrow host range (e.g., *Hyalomma dromedarii* for camelids and *Hyalomma aegyptium* for *Testudo* species tortoises (11))), most have a wide host range; for example, *I. ricinus* has > 300 putative hosts, including many birds and lizards (12–14).

Because wild and domestic animals infected with CCHFV do not show signs of illness, serological surveys have been the principal source of information to identify species exposed to the virus and to monitor areas with natural virus transmission. However, tests performed on serum collected from animals do not provide data on characteristics and kinetics of infection. Experimental infection

115 studies have been performed in a variety of wild and domestic animals to assess
116 susceptibility to infection, duration of viremia, antibody response, absence or
117 presence of clinical disease, and respective contribution of animal species as hosts
118 in CCHFV maintenance and transmission. Data from these studies are useful in
119 assessing the risk of CCHF emergence in new geographic areas, in focusing One
120 Health countermeasures (15) for reducing endemic viral transmission (e.g., by
121 targeted acaricide treatment), assessing risk in cases of direct human exposure to
122 potentially infected animal tissues and body fluids (16–18), and identifying gaps in
123 knowledge and direct future research efforts.

124 Notably, these clinical infection studies and almost all of the important work
125 on CCHFV were performed after the application of the pivotal newborn white mouse
126 (NWM) isolation technique (19,20). Attempts to recover the etiologic agent were
127 largely unsuccessful for more than two decades after the Crimean outbreak. In
128 1964–1965, CCHFV isolated from the blood of patients was successfully passaged in
129 tissue culture; passage was confirmed by using the fluorescent antibody method and
130 interference phenomenon using vesicular stomatitis virus and Chikungunya virus
131 (21,22). However, in the late 1950s and 1960s, arbovirus researchers discovered
132 that many viruses are more successfully propagated through intracranial infection
133 of NWM (19,20). In efforts to isolate virus in animals prior to use of the NWM
134 technique, disease was often attributed to the presence of a contaminating organism
135 [e.g., studies in cats (5,23)]. The introduction of this simple but highly sensitive
136 method revolutionized CCHFV research by providing a means to isolate and

quantitate virus recovered from human patients, animals, and ticks, and facilitating the development of serologic tests and other research tools (1,24–27).

3. Experimental infection in wild mammals

In general, studies described below and summarized in Table 1 suggest that CCHFV replicates and produces a short viremic period (~7 days) in small mammals, followed by development of anti-CCHFV antibodies. Although experimental data in these species are limited, the potential role of small vertebrates as CCHFV reservoirs appears to be minimal. However, they remain important hosts for ticks and likely aid in virus amplification and transmission from infected to naïve co-feeding ticks. These studies are limited to small mammals, and although serological data do exist [as detailed in Spengler *et al.* (2)], to our knowledge, no experimental studies have been performed in larger wild mammalian species. Studies using larger wild mammal species would provide important information on contributions to CCHFV ecology of other hosts with diverse behavior and exposure to tick vectors.

3.1. Hares

Small mammals provide blood meals for two- and three-host ticks like *D. marginatus* and *Hyalomma asiaticum*. The initial report from the Crimean outbreak implicated the European hare (*Lepus europaeus*) as central in maintaining CCHFV; the *L. europaeus* population reportedly exploded during the original CCHF outbreak in the Crimea (1). Indeed, hares are widely distributed and are still considered to be primary hosts of several CCHFV vectors in many CCHF-endemic areas in Europe.

Moreover, of the 11 reported isolates of CCHFV from animals, three were from *L. europaeus* in the Crimea region of Ukraine (28). An overgrowth of vegetation due to cessation of farming, and an expansion of the hare population (and the associated abundance of ticks) were described during several CCHF outbreaks, including the initial Crimean CCHF outbreak and outbreaks in the former Soviet Union, Bulgaria, Kosovo, and Turkey (29–31).

3.1.1. Former Soviet Union

Initial investigations of hares were performed on animals trapped on a hunting farm in the Ust'-Donets region (Russia, Rostov Region). Perelatrov *et al.* (1972) infected six 1–2-year-old naïve European hares (*L. europaeus*) with 1 mL of 10^5 NWM LD₅₀ of strain Sudarkina CCHFV (32). (NWM LD₅₀ refers to the virus dose lethal to 50% of infected NWM.) Infection was performed via intracardiac, subcutaneous (SC), or intradermal (ID) inoculation, with two animals infected by each inoculation route. After infection, no clinical signs were noted during the 2-year observation period. Viremia was investigated in whole or diluted blood. Detection and magnitude of viremia varied by route of inoculation: with SC inoculation, viremia was lowest and was detected at 1, 2, and 5 days post infection (dpi); intracardiac inoculation produced intermediate viremia levels detectable 2, 4, 5, and 9 dpi, but not 6, 7, and 8 dpi; and ID inoculation resulted in the highest viremia levels detectable 1–10 dpi. Why viremia was detected intermittently is not clear, but may be explained by the sensitivity of assays used in those experiments.

Antibodies were detected by complement fixation (CF) beginning 7 dpi and throughout an extended observation period (28–45 dpi) (32).

3.1.2. Africa

In accordance with the above study, Shepherd *et al.* (1989) reported a comparable viremia in scrub hares (*Lepus saxatilis*) inoculated SC with CCHFV strain SPU 4/81. Viremia was detected 1–7 dpi, with highest virus titers (4.2 log₁₀ NWM LD₅₀/mL) detected between 2–5 dpi (33). Along with vector studies and knowledge of host factors like foraging behavior and geographic distribution, these limited experimental infection investigations support the hypothesis that hares can replicate CCHFV, and provide antibody data for interpreting serological studies.

3.2. Ground squirrels

Some CCHFV-transmitting tick species, like *D. marginatus*, occasionally feed on ground squirrels and hedgehogs. These mammals are suitable hosts for the instar life stages of many tick species, as they spend significant time rummaging through leaf litter and brush where immature ticks are present. Two types of ground squirrels have been experimentally infected, and findings were comparable in both studies.

3.2.1. Former Soviet Union

Blagoveshchenskaya *et al.* (1972) infected fifty 1–1.5-month-old susliks (*Spermophilus pygmaeus*) intracranially (IC), or intramuscularly (IM) and SC with

strains Sudarkina and Hp-15 Zhr (34). Clinical signs were reported as absent in “most” animals, with no further details provided. Irrespective of inoculation method, only slight immunomorphological changes were observed in the reticulohistocytic system, mainly lymph nodes and spleen, with peak changes observed 5–7 dpi. These organs appeared normal after 30–40 dpi, and morphological changes were absent in other organs and tissues. CCHFV was detected in the blood and parenchymal organs of all animals 2–7 dpi (up to 10^2 NWM LD₅₀), but not later (34).

3.2.2. Africa

In 1989, SPU 4/81 strain infections of Cape ground squirrels (*Xerus inauris*) by Shepherd *et al.* led to viremia beginning 2–8 dpi and lasting 7 days. Anti-CCHFV antibodies were detected 8 dpi by reverse passive hemagglutination inhibition (RPHI), with maximum titer detected 512 dpi (33).

3.3. Hedgehogs

In contrast to ground squirrel investigations, experimental studies on the susceptibility of two hedgehog species to CCHFV yielded varying results, suggesting that susceptibility to CCHFV and infection dynamics may vary even between related species.

3.3.1. Former Soviet Union

Blagoveshchenskaya *et al.* (1972) infected seven European hedgehogs (*Erinaceus europaeus*) and two long-eared hedgehogs (*Hemiechinus auritus*) SC with

strain Sudarkina, and no clinical signs were observed in any of the animals (35).
Viremia was detected in *H. auritus* 2–6 dpi, peaking at 4–6 dpi, but no viremia was
detected in *E. europaeus*. Noteworthy, *H. auritus* is common in Central Asia in
regions endemic for CCHFV, while *E. europaeus* is found in Europe in regions that
are generally free of CCHFV.

3.3.2. Africa

Shepherd *et al.* (1989) infected African hedgehogs (*Atelerix frontalis*) with
strain SPU 4/81. Viremia was not detected, but anti-CCHFV antibodies were
detected by RPHI starting 8 dpi and reaching a maximum titer at 256 dpi (33).

3.4. Small rodents

3.4.1. Africa

Shepherd *et al.* (1989) investigated infection in several small wild rodent
species inoculated SC with $10^{6.0}$ – $10^{6.4}$ LD₅₀ of strain SPU 4/81(33). Viremia ranging
1.5–4.0 log₁₀ LD₅₀/mL and lasting 3–7 days was detected in white-tailed rats
(*Mystromys albicaudatus*), red veld rats (*Aethomys chrysophilus*), bushveld gerbils
(*Gerbilliscus leucogaster*), and four-striped grass mice (*Rhabdomys pumilio*). Viremia
was not detected in highveld gerbils (*Gerbilliscus brantsii*), southern multimammate
mice (*Mastomys coucha*), multimammate mice (*Mastomys natalensis*), and Namaqua
gerbils (*Desmodillus auricularis*). Antibodies were detected in all species tested
(sampled up to ≥ 35 dpi), with the exception of *R. pumilio*, which may have been due
to limited sampling in that species (up to 8 dpi).

4. Experimental infection in livestock

Several CCHFV tick vectors feed on a variety of domestic animals, and may also be synanthropic and ecologically associated with humans. Large domestic animals are known to support a high tick burden and bring ticks into close proximity to farmers. They can also expose humans to CCHFV directly through viremic blood during slaughtering (36–40). Seroepidemiological investigations have shown that a wide spectrum of domestic species is exposed to CCHFV (2), but experimental infection studies have been markedly more limited. Although small in number, experimental infections have been performed in several livestock species, including ruminants (cattle, sheep) and equids (horses, donkeys); these studies are summarized in Table 2.

Similar to data in small mammals, these studies show that livestock and equids develop brief periods of viremia and anti-CCHFV antibodies ~1 week post-inoculation. Additionally, maternal transfer of anti-CCHFV antibodies was demonstrated in sheep. In cattle, the absence of detectable viremia in all samples tested for an individual or at particular times post-inoculation may be a true reflection of absent or intermittent viral circulation, but more likely is an artifact of detection limits. These studies also further support the absence of clinical signs in the majority of animals tested, with the exception of sheep and horses. In the horse study, due to the nature of the inoculum, clinical signs (fever) were very likely associated with non-specific reaction to the inoculum matrix rather than the virus infection. Whether CCHFV infection or the inoculum caused the fever in sheep is less

clear, as the febrile period corresponded with the viremic period. While these data help to provide a better understanding of infection in domestic species, they each include only a small number of animals, providing challenges for interpretation, and must be broadly considered.

4.1. Cattle

4.1.1 Former Soviet Union

Zarubinsky *et al.* (1976) experimentally infected calves with the Sudarkina CCHFV strain (41). Two 6-month-old calves were infected either IM or SC, and two 2-month-old calves were infected both SC and ID. Clinical disease was not observed in any of the animals, and viremia was not detected in the 6-month-old calves; however, CCHFV was isolated from the blood of one of the 2-month-old calves 3 and 7 dpi, supporting the possibility of virus transmission to humans from exposure to fluids of infected animals (16–18). Anti-CCHFV antibodies, detected by agar-gel diffusion precipitation (AGDP) performed 5–20 dpi in samples from the 6-month-old calves, were seen at 13 and 17 dpi in the SC-infected and the IM-infected calf, respectively; antibodies were detected by CF only in the IM-infected animal (17 dpi). Additionally, antibodies were investigated in the 2-month-old calves using the indirect hemagglutination inhibition (IHI) test 5–35 dpi (42); antibody titers of 1:10 were detected in 1 calf on days 9, 14, and 20 after infection, increasing to 1:80 at 35 dpi.

4.1.2 Africa

In 1970, Causey *et al.* isolated CCHFV from cattle, a goat, and a hedgehog; these remain the majority of reported isolates obtained directly from animals. Additionally, two White Fulani (Bunaji) breed calves, 9 and 10 months of age, were inoculated with the goat isolate (IbAn 7620) (43). One calf was inoculated SC with $8.3 \log_{10} \text{LD}_{50}$ (low dose animal), and the other calf was inoculated both SC ($7.9 \log_{10} \text{LD}_{50}$) and ID ($7.1 \log_{10} \text{LD}_{50}$) (high dose animal). Five days prior to inoculation, feeding capsules of laboratory-reared *Hyalomma rufipes* ticks were attached to each animal (transmission data, further discussed in Section 6.2). Prior to the study, ticks and calves all tested negative for virus and neutralizing antibodies to CCHFV, respectively. After inoculation, both calves developed mild illness, characterized by dullness, lassitude, and decreased appetite. Viremia was detected 2 and 5 dpi in the low-dose calf, and 1–5 dpi in the high-dose calf. Viremia was determined by inoculation of suckling mice, and CCHFV presence was confirmed by complement fixation (43).

Shepherd *et al.* (1991) inoculated two cows SC with CCHFV strain SPU 4/81 to assess transmission to ticks. The cows were viremic 5–8 dpi or on 6 dpi. In accordance with the previous studies, no clinical signs were reported (44). Tick transmission data are reviewed below (Section 6.2).

4.2. Sheep

4.2.1 Former Soviet Union

Kondratenko *et al.* (1968) identified cattle, sheep, and goats as the main hosts of adult ixodid tick vectors of CCHFV in Rostov Oblast [Kondratenko *et al.*

1968, in (41)] . Thus, in addition to experimental infection of cattle (See 4.1), Zarubinsky *et al.* (1976) experimentally infected five lambs (2–2.5 months old) SC and ID with the Sudarkina strain (41). No clinical disease was observed in any of the animals, but CCHFV was isolated from the blood of all lambs (4–8 dpi in lamb 1; 4–5 dpi in lamb 2; 6–8 dpi in lamb 3; 4–6 dpi in lamb 4; and 4–7 dpi in lamb 5). AGDP analysis of lamb sera 5–35 dpi indicated the presence of precipitating antibodies at 21 dpi (lambs 1, 2, 3, and 5) and 35 dpi (lamb 4).

4.2.2 Africa

To understand the impact of infection on sheep and their ability to replicate virus, Gonzalez *et al.* (1998) infected 10 adult (> 1 year old) naïve sheep intraperitoneally (IP; $n = 2$) or via CCHFV-infected *Hyalomma truncatum* ticks ($n = 8$) with CCHFV strain HD 49199 (45). Fever ($39.7^{\circ}\text{C} \pm 0.3$) was observed in the sheep beginning at 3 dpi, peaking around 1°C above normal for 5 days and declining within 36 h thereafter. Viremia was detected in all infected animals and correlated with fever, starting at 3 dpi, peaking at 5 dpi ($4.7 \log \text{LD}_{50}/\text{mL}$), and lasting 4–7 days total. Interestingly, no differences were noted between IP and tick bite-infected sheep. Renal and liver functions were assessed in a subset of the infected sheep; in all sheep, a slight but significant increase was observed in aspartate aminotransferase levels for 3 to 10 days starting at 3 dpi, but no changes were observed in azotemia, creatininemia, or alanine aminotransferase. Five of the infected sheep were pregnant ewes, of which four normally delivered healthy lambs and one spontaneously aborted. No virus was isolated from the aborted fetus,

suggesting unrelated etiology of the abortion. Enzyme-linked immunosorbent assays (ELISA) of non-immune sheep samples showed an IgM response 7 dpi, followed by an IgG response one day later.

Wilson *et al.* (1991) used $10^{5.5}$ LD₅₀ of strain Dak H49199 inoculated IP to challenge sheep with varying levels of previous exposure to CCHFV (46). The group found that viremia corresponded to previous exposure: naïve-infected sheep in the study developed the highest and most prolonged viremia detected 2–6 dpi, and high titers of IgM beginning 6 dpi, followed by elevated IgG. Similar patterns of viremia to those in naïve-infected animals were reported in a separate study by Shepherd *et al.* (1991) in sheep [$n = 3$] inoculated SC with SPU 4/81; in that study, viremia was detected at 2–6 dpi or at 4–7 dpi (44). To test the effect of intermediate CCHFV exposure, Wilson *et al.* used the offspring of a previously exposed sheep; the offspring had weak pre-infection IgG as a result of maternal transfer. After experimental infection, this offspring developed low viremia 3–6 dpi, weakly elevated IgM beginning 9 dpi, and IgG levels that slowly increased over the study period. The sheep that had been previously inoculated had high levels of CCHFV IgG prior to re-challenge. Re-challenge resulted in undetectable viremia, slightly elevated IgM titers, and persistence of high levels of IgG (46).

4.3. Equids

4.3.1 Former Soviet Union

The role of equids in maintenance and transmission of CCHFV is not well described, but serological data support natural exposure to virus (2). Experimental

infection studies were performed in both horses and donkeys to investigate the possibility of producing antibodies for putative antibody-based human therapeutics. Milyutin *et al.* (1969) infected two 3-year-old mares intravascularly (IV) and IM, or IM and SC, with CCHFV strain Sudarkina. Hyperthermia as high as 40.3°C, somnolence, and weakness were observed at 1–2 dpi, before the animals' condition normalized (47). Increased erythrocyte sedimentation rate, indicating non-specific inflammation, and an insignificant leukocytosis were observed. No infectious virus could be detected in blood or urine at 2 dpi or at any later time points investigated (3–7, 9, 11, 13, 15, and 20 dpi). Modest serological response was detected by AGDP, with titers up to 1:32 in the CF assay (47).

Based on concerns about anaphylactic reactions in humans to antibodies produced from horse blood sera, Rabinovich *et al.* (1972) subsequently investigated anti-CCHFV antibody production for therapeutic use in donkeys via experimental infection (48). Two donkeys (4 and 5 years old) were inoculated SC with 15 mL of CCHFV (6.1 log LD₅₀/0.02 mL). Animals were observed for 10 dpi, and no changes in temperature or blood analysis parameters were detected. Low-level viremia was observed 4, 6, and 7 dpi, indicated by “some” disease in NWM (titer was not reported). CF detected anti-CCHFV antibodies in the donkeys at 8 and 13 dpi. Antibody titers increased after a second inoculation of the donkeys 15 days after the primary inoculation. Neutralizing activity of the serum from donkeys was comparable to the activity observed in sera from inoculated horses, but the report does not make clear whether the donkey sera were collected after the initial or the secondary inoculation. No precipitating antibodies were detected (48).

5. Experimental infection in birds

The role of birds is the least well-understood aspect of CCHFV maintenance and transmission. Despite extensive investigations, the absence of serological evidence of CCHFV-specific antibodies in most avian species sampled suggests that most birds are predominantly refractory to CCHFV infection. However, resistance is not uniform, as there is serological evidence of exposure in some species (2). In the original outbreak in Crimea in 1944, Grobov collected immature *H. marginatum* ticks from wild birds and domestic fowl, considering birds as the putative reservoir of the then-unknown infectious agent (49). Birds were suspected as important in virus maintenance due to their extremely high tick infestation rates and infestation indexes (calculated as the [no. ticks/no. infested animals] × [% of infected animals]). The infestation index of immature *H. marginatum* in eight species of ground-feeding birds in the Volga Delta (collected 1963–1964) ranged 3.3–68.9 (50). The highest infestation index was in rooks (*Corvus frugilegus*); hundreds of *H. marginatum* have been noted to feed on a single rook in CCHFV-endemic regions (51). Indeed, CCHF cases in the Volga River floodplains and delta (Astrakhan and Rostov Oblasts) epidemiologically linked birds, specifically tree-roosting *C. frugilegus* hosting immature *H. marginatum* ticks near cattle pastures, to disease occurrence (1). In addition, virus has been isolated from ticks feeding on birds (52).

Overall, reports to date show that birds may be important tick blood meal hosts and implicated in wide-range transportation of tick reservoirs, but very rarely in CCHFV amplification (Table 3). The absence of detectable viremia was reported in

experimental studies of both wild and domestic avian species, with ostriches as a notable exception, emphasizing the importance of not overgeneralizing CCHFV infection dynamics in animal taxa. Ostriches can host a variety of tick species involved in CCHFV transmission (1), develop detectable and sustained viremia, and have been epidemiologically associated with human infection (53). As viremia appears overwhelmingly absent in most birds, they likely introduce infected ticks into new territories by carrying feeding immatures that were transovarially infected from adult ticks. However, anti-CCHFV antibody production has been reported independently of detectable viremia, and in addition to ostriches, persistent anti-CCHFV antibodies were detected in a red-beaked hornbill (*Tockus* sp.) and glossy starling (*Lamprotornis* sp.). While viremia may have been missed due to assay sensitivity or timing issues, studies have shown that CCHFV or tick-borne encephalitis virus can be transmitted from one co-feeding tick to the next in the absence of detectable viremia in the host (9,54,55). The concept of threshold viremia therefore might not apply to tick-borne viruses and might not be the hallmark feature for identifying hosts involved in CCHFV maintenance. Investigating the contribution of threshold viremia in CCHFV transmission is warranted.

5.1. Former Soviet Union

The earliest reported experimental investigations into the role of birds were performed by Berezin *et al.* (1969 – 1971), but never published [as described in (1)]. Rooks (*C. frugilegus*) and rock doves (*Columba livia*) were experimentally

inoculated with CCHFV. No clinical signs were observed, no virus was isolated, and no serological evidence of viremia was found [data reviewed in (56–58)].

5.2 Africa

In later investigations to assess viral pathogenicity in wild birds, Shepherd *et al.* (1987) inoculated six adult and 20 3-week-old blue-helmeted guinea fowl (*Numida meleagris*) with strain SPU 4/81, and measured viremia and antibody responses (59). Adult guinea fowl were inoculated SC, IM, and IV ($6.7 \log_{10} \text{LD}_{50}/\text{mL}$); 3-week-old guinea fowl were inoculated IM and SC with half the volume of the same virus suspension. Viremia was assessed 1–9 dpi, and was detected only intermittently in a small subset of the adult and 3-week-old animals (0–20% of the birds tested per day). Most of the young birds were refractory to infection based on antibody assessment: antibodies were detected in 8 of 20 serum samples taken 7–21 dpi, and in 5 of 16 birds bled on day 28. Antibodies were detected in adult birds starting on 5 dpi by immunofluorescence (IF) and on 6 dpi by RPHI; maximum titers were observed 8 and 9 dpi, and RPHI and IF antibody titers dropped to undetectable levels by 49 dpi in most of the experimentally infected guinea fowl. These studies highlighted the transient nature of the antibody response in birds, and by using a variety of techniques (immunodiffusion, RPHI, and IF) to detect antibodies, they demonstrated that the immunodiffusion test is unsuitable for use in serological surveys to detect CCHFV antibodies in birds (59–61).

Zeller *et al.* (1994) experimentally inoculated laughing doves (*Spilopelia senegalensis*; $n = 2$), a red-beaked hornbill (*Tockus* sp.; $n = 1$), and a glossy starling

457 (*Lamprotornis* sp.; $n = 1$) IP with strain HD 49199 ($10^{1.5}$ – $10^{3.5}$ NWM LD₅₀) (62).
458 Viremia was not detected in any of the birds from 2–10 dpi, though the glossy
459 starling was not tested. Antibodies to CCHFV were not detected in the laughing
460 doves, but a significant antibody response was seen in the red-beaked hornbill and
461 the glossy starling despite the absence of detectable viremia. Anti-CCHFV antibodies
462 were still detectable in these birds 4 months later (62). This suggests that the
463 existing assays were unable to detect viremia in these species that viremia was
464 acutely transient, or that antibody development may be exclusive of viremia.

465 The embryonated chicken egg has been successfully infected and used for
466 propagation of CCHFV (63); experimentally infected chickens (*Gallus gallus*
467 *domesticus*) do not appear susceptible to infection. In parallel to inoculations in
468 guinea fowl (see above), Shepherd *et al.* (1987) inoculated 6 adult chickens,
469 detecting no viremia and no antibodies by RPHI test in sera collected up to day 28
470 (59). Zeller *et al.* (1994) inoculated 6 chickens IP with a series of challenge doses of
471 CCHFV strain HD 49199 ($10^{3.5}$ LD₅₀, $n = 4$; $10^{2.5}$ LD₅₀, $n = 1$; $10^{1.5}$ LD₅₀, $n = 1$). Again,
472 viremia was not detected in any of the animals 2–10 dpi, and antibody-capture
473 ELISA detected no anti-CCHFV antibodies (62).

474 While chickens appear refractory to infection, studies by Swanepoel *et al.*
475 (1998) following an outbreak of CCHF in workers on an ostrich farm (53), reported
476 viremia 1–4 dpi (maximum at 2 dpi) in experimentally infected ostriches. Anti-
477 CCHFV antibodies were detected by compliment ELISA in 1 of 9 ostriches at 5 dpi,
478 and by 13 dpi all animals had seroconverted. CCHFV was detected in spleen, liver,
479 and kidneys up to 5 dpi, 1 day after it was no longer detectable in blood. No infective

virus was detected in muscle samples, but viral RNA was detected by reverse transcription-polymerase chain reaction (RT-PCR) in muscle from an ostrich sacrificed on day 3 pi (64).

6. Experimental vector transmission studies

CCHFV is only transiently associated with the vertebrate host but persists in ticks; mammals remain viremic for no more than a few weeks (usually ~ 1 week), but ticks undergo life-long infection. As a result, ticks are considered the vector and the long-term reservoir for the virus in nature (1,65–67). Compared to mosquitoes, ticks feed for a long period of time and need to immunomodulate the bite site using biologically active molecules secreted in tick saliva. Tick-borne pathogens have been known to exploit these mechanisms. Certain molecules in arthropod saliva have been shown to promote pathogen transmission to the vertebrate host, a phenomenon termed saliva-assisted transmission (SAT). SAT is well documented in transmission of several tick-borne pathogens (68–70), but has only been demonstrated in two CCHFV studies (62,71). SAT factors appear to differ among tick vector species, and might depend on the vertebrate host species on which the tick feeds, indicating the need for the right tick-host pairing in experimental studies. Additionally, SAT can lead to higher viremia levels in infected animals than produced by traditional inoculation, further emphasizing the importance of using true vector transmission in experiments in addition to traditional needle inoculation. Elucidating the properties of circulation and preservation of CCHFV in its natural foci and the possibility of transstadial and transovarial transmission in

ticks was prioritized in early experimental studies. Several studies have investigated CCHFV infection via tick inoculum and virus transmission to ticks from inoculated animals.

6.1. Transmission from ticks to animals

Transmission of CCHFV from infected ticks to mammals was investigated in cattle, sheep, and guinea pigs. Initial reports of tick transmission by Lee and Kemp were based on limited experimental infection studies in calves. Susceptible 6-month-old (~100 kg) White Fulani calves ($n = 2$) were inoculated using adult *H. rufipes* ticks that were infected with strain IbAn 7620 (72) as nymphs. The inoculation process was noted to cause trauma in the ticks, considerable seepage of body fluid and inoculum from the puncture sites was observed, and it was unknown what amount of virus suspension was actually administered, or to what tissue sites. Although testing subsets of the ticks indicated low infection success, the first calf was inoculated by placement of 11 inoculated ticks left to feed for 14 days; viremia was detected in the calf on days 2 and 7 post exposure. In addition, progeny of one female tick that fed on this calf contained viable CCHFV, supporting vertical CCHFV transmission in ticks. The second calf was used to subsequently examine transmission from larvae of an adult female tick that had fed on the first calf. This female tick was allowed to feed on a rabbit and molt before being placed onto the second calf, which did not develop viremia. However, while virus was detected in larvae from the infected female, virus was not confirmed in any of the nymphs tested, so little can be concluded from the absence of viremia in the second calf (72).

Shepherd *et al.* (1989) infected groups of adult *H. rufipes*, *H. truncatum*, and *Rhipicephalus evertsi mimeticus* intracoelomically with strain SPU 4/81, and allowed the ticks to feed on susceptible sheep 56–97 days later. CCHFV was successfully transmitted to the sheep (73). Later, four pools of larvae from each species of the infected ticks described above were allowed to feed on susceptible guinea pigs (73), and 1,357 larvae were subsequently removed from the guinea pigs. Virus was not isolated from the larvae, and anti-CCHFV antibodies were not detected in the sera of guinea pigs collected 21 dpi. Although transstadial and transovarial transmission have been reported for these species [*H. rufipes* (72); *H. truncatum* (46,73); *R. evertsi mimeticus* (73)], the lack of virus in the larvae suggests that transovarial transmission did not occur in these studies and the larvae used were likely not infected, thus prohibiting assessment of CCHFV transmission by ticks to the guinea pigs in this report. Tick transmission may not be well modeled via experimental (intracoelomical) infection, as artificial infection did not lead to detectable infection in progeny, whereas natural infection (i.e., by allowing ticks to feed on infected vertebrates) in studies described above did. This supports the influence of tick host factors on transmission that is not modeled by some experimental approaches.

While not demonstrated by the above study, transmission from ticks to guinea pigs does occur, and was shown in earlier studies by Berezin *et al.* (1971), who investigated virus persistence in *H. marginatum* ticks at 4°C (74). Ticks maintained for 7–9 months at low temperatures were able to transmit CCHFV to guinea pigs; the hosts developed viremia 11 and 12 days post attachment, and antibodies were detected in surviving animals 21 days following tick bites, further

supporting long-term CCHFV persistence in the tick vector. Notably, in these animals the infection course was described as severe and frequently fatal (74), whereas clinical signs have not been reported in other studies of experimental infection of guinea pigs (75). Thus, transmission via natural tick vector rather than by needle inoculation may produce more severe illness; experimental infection by tick transmission should therefore be considered in CCHFV studies.

6.2. Transmission from animals to ticks

Experimental transmission from inoculated animals to ticks has been demonstrated in a variety of species: cattle, sheep, susliks (*S. pygmaeus*), long-eared hedgehogs (*H. auritus*), hares (*L. saxatilis*), and laboratory rabbits. Causey *et al.* (1970; see Section 4.1) investigated CCHFV transmission from ticks to cattle by placing *H. rufipes* ticks on two calves that were inoculated with IbAn 7620 five days after tick placement. One of the calves developed a local purulent reaction at the feeding site. The second calf supported the tick infestation well: tick feeding was observed 1 day prior to inoculation. A single engorged female dropped off 11 dpi, oviposited 4 days later, and was positive for CCHFV 18 days after dropping (24 days after viremia was last detected in the calf). Virus was not detected in the resulting larvae tested 27 days after oviposition (43).

Early investigations suspected the prominent role of ticks as the CCHFV reservoir, and animals were often used in experimental studies for understanding CCHFV maintenance in the tick while tangentially providing data on transmission from animals. Zgurskaya *et al.* (1971) were interested in interepidemic virus

survival via transovarial and transstadial transmission in ticks (76). European hares (*L. europaeus*) and long-eared hedgehogs (*H. auritus*), inoculated IV and IM, were found to be susceptible to experimental CCHFV infection and were used as CCHFV infection sources for *H. marginatum* ticks. Viremia was reported in *L. europaeus* until 15 dpi, with peak viremia of 3.6 log LD₅₀/0.02 mL at 4 dpi. Virus was detected in nymphs collected from both *L. europaeus* and *H. auritus*, and from adult ticks that molted from nymphs, confirming transmission from animals to ticks and transstadial transmission in *H. marginatum* ticks. The group went on to use animals to confirm that *H. marginatum* can preserve CCHFV following artificial overwintering conditions (45 days at 4°C) for 13 months and to demonstrate transovarial transmission by confirming infection via transmission, determined by antibody development, in guinea pigs and rabbits, respectively (76). Blagoveshchenskaya *et al.* (1975) also investigated transmission from infected hedgehog to ticks (35). After confirming hedgehog susceptibility to CCHFV (see Section 3), they demonstrated that the virus could be transmitted from *H. auritus* to *H. marginatum* ticks, but not to *R. rossicus* or *D. marginatus* ticks (35).

Later studies by Zgurskaya *et al.* (1975) investigated threshold levels of CCHFV for infecting *H. marginatum* by allowing ticks to feed on infected lagomorphs (hares, rabbits) (77). Animals were inoculated IV and IM with CCHFV (strain not reported). Viremia was determined in the animals and related to infection in associated blood-fed ticks. Threshold levels for CCHFV transmission from the lagomorphs to the ticks were estimated to be 2.7–3.6 log LD₅₀/0.02 mL (NWM inoculation); animals that developed this range of viremia ($n = 3$) transmitted

CCHFV to ticks, while no transmission was observed from animals ($n = 2$) with a viremia of 2.1–2.7 log LD₅₀/0.02 mL. These studies are limited to lagomorphs and investigated few animals and ticks, but highlight an important principle in maintenance and transmission that warrants additional investigation.

Zarubinsky *et al.* (1976) placed laboratory-reared *H. rufipes*, *Rhipicephalus rossicus*, and *D. marginatus* ticks on 2 experimentally infected 6-month-old calves 24 h after the calves were infected with CCHFV. No ticks became infected from feeding, but no viremia was detected in the calves, prohibiting any conclusions to be drawn from these investigations (41). However, a subsequent study of ticks that fed on viremic cattle infected with strain SPU 4/81 (44) confirmed transmission of CCHFV to adult ticks of five species (*H. truncatum*, *Rhipicephalus appendiculatus*, *R. e. evertsi*, *R. decoloratus*, and *Amblyomma hebraeum*); transmission to *H. rufipes* was not detected.

Additional transmission studies in livestock by Wilson *et al.* (1991) demonstrated that sheep may serve as amplification hosts for CCHFV, and that their relative involvement is influenced by prior exposure status. Female West African Peul-Peul sheep ($n = 4$) were infested with 30 male and 30 female *H. truncatum* ticks 2 days prior to virus inoculation. Three sheep were then inoculated IP with 10^{5.5} NWM LD₅₀ of CCHFV strain Dak H49199, and 1 sheep was used as an uninfected control. Beginning at 4 dpi, ticks were examined daily; engorged females that had naturally finished feeding were removed, and male ticks were detached by forceps at 16 dpi. Virus was detected in at least 1 tick that fed on each of the 3 inoculated sheep. Higher viremia corresponded to lower pre-exposure of the sheep to CCHFV

and to lower levels of previously acquired anti-CCHFV antibodies. Virus was detected in 11% of the female and 0% male ticks collected from the previously exposed sheep; in 14% of the female and 30% of the male ticks from the offspring of a previously exposed sheep; and in 33% of the female and 60% of the male ticks from naïve-infected sheep. Time of detectable viremia in the sheep corresponded to the time during which infected female ticks were detaching. Transovarial transmission was detected in 2 of 12 (17%) of egg batches from infected female ticks (46).

In a study by Shepherd *et al.* (1991), three sheep inoculated SC with a different human isolate (strain SPU 4/81) were fed on by adult *H. truncatum* (as in the previous study), *A. hebraeum*, *H. rufipes*, *R. e. evertsi*, *R. appendiculatus*, and *Rhipicephalus simus* ticks, and by larval or nymphal *A. hebraeum*, *R. e. evertsi*, and *R. simus* ticks. Four-hundred and forty-three pools of ticks were tested (adults; immatures; females after oviposition, and larvae from these eggs; and adults that had molted from engorged immatures); none yielded CCHFV despite detectable viremia ($10^{2.5} - 10^{3.2}$ LD₅₀/mL) in the sheep (44).

Shepherd *et al.* (1991) conducted additional transmission studies in small mammals; no CCHFV was isolated from engorged larvae of *A. hebraeum*, *H. truncatum*, or *R. e. mimeticus* that dropped off infected white rats (*M. albicaudatus*), nor from nymphs or adults of these species tested after molting (44). Similarly, no CCHFV was isolated from engorged *A. hebraeum*, *H. truncatum*, or *R. e. mimeticus* larvae that fed on three infected Duncan-Hartley guinea pigs, nor in *A. hebraeum* or *H. truncatum* nymphs tested after molting (44). These studies suggest that the tick-

host interaction is complex, and only certain combinations of hosts and ticks support CCHFV transmission.

6.3. Tick-borne transmission from vertebrate to vertebrate

In 1970–1971, Levi and Vasilenko investigated circulation and maintenance of CCHFV in the tick vector, finding evidence of transovarial transmission in *Hyalomma plumbeum* ticks and demonstrating the full vertebrate-tick-vertebrate transmission cycle. Ticks were fed on Belgian giant breed rabbits (*Oryctolagus cuniculus*) inoculated IV with CCHFV-infected plasma of living animals and high-titer liver suspensions, defined as $\sim 4.5\text{--}5.5 \log \text{LD}_{50}$ (CCHFV strain or animal source of plasma and liver suspensions were not reported). After 10 days (for adults) or 20 days (for larvae or nymphs) of feeding, the ticks were collected and tested immediately or in later stages of development. CCHFV was detected in all fed ticks and their offspring, confirming transstadial and transovarial transmission. Additionally, transovarially infected nymphs were placed on uninfected naïve rabbits that subsequently developed AGDP-detectable antibodies to CCHFV, with antibody titers positively correlating with infected tick burden (78).

In 1976, Kondratenko *et al.* investigated the importance of ixodid ticks in CCHFV transmission and maintenance by feeding 50–500 ticks (*H. plumbeum*, *R. rossicus*, or *D. marginatus*) on each of six susliks (*S. pygmaeus*; 2–3 months old) infected SC with a NWM brain suspension of strain Sudarkina (56 passages(79)). Subsets of engorged nymphs were tested; others were kept at 22–25°C and 60–80% humidity and allowed to molt to adults. Adults were maintained at 4°C, and tested

every 1–2 months for virus. Following artificial overwintering, adults were fed on 1.5–2 months old naïve susliks (*Spermophilus* sp.) or 6-week-old rabbits to investigate infection transmission to vertebrates and transovarial transmission in ticks. Ticks infected with CCHFV, irrespective of the developmental stage at feeding, transmitted virus to susliks and rabbits by feeding on them within 620 days (observation period) of initial infection.

Transmission between vertebrates via tick vector was later demonstrated in additional studies. Shepherd *et al.* (1991) demonstrated a cycle of hare-tick-sheep virus transmission; scrub hares (*L. saxatilis*) infected with SPU 4/81 transmitted CCHFV to *H. truncatum* and *H. rufipes* ticks, and sheep on which these ticks subsequently fed seroconverted (44). Notably, Zeller *et al.* (1994) reported CCHFV transmission from birds to ticks and then subsequent transmission from ticks to rabbits (62), despite extensive serological data suggesting that birds are refractory to infection (2). Experimental transmission studies followed initial experimental infections of birds (Section 5), and were performed using three red-beaked hornbills (*Tockus* sp.), three glossy starling (*Lamprotornis* sp.), and three domestic chickens (*G. g. domesticus*) by placing *H. rufipes* larvae on the head of each bird. The birds were inoculated with CCHFV at different points after larvae placement: same day (1 chicken), 3 days later (2 chickens, 2 hornbills, and 1 starling) to assess CCHFV transmission to larvae, or 10 days later (1 chicken and 1 hornbill) to assess transmission to nymphs. Virus isolation was performed on engorged nymphs that had dropped off the birds and were collected and allowed to molt. Adult ticks derived from some of the molted nymphs were then allowed to feed on rabbits. Male

and female adult ticks and progeny were tested for CCHFV, and rabbits were tested for anti-CCHFV antibodies. Chickens did not transmit the virus to *H. rufipes* larvae or nymphs. Larvae placed on the hornbills and starling did not drop off the birds after engorgement, but molted on the host and remained attached until the engorged nymph stage (≥ 2 weeks duration). Two of the hornbills died 14 dpi, likely due to heavy tick infestation (> 150 nymphs). Nymphs were collected from the cadavers and virus was isolated from all 17 pools of 10 nymphs each. Anti-CCHFV antibodies were detected in one hornbill and the starling. The adult ticks emerging from molted nymphs derived from the hornbills and starling transmitted CCHFV to rabbits, inducing an antibody response (62).

Transmissions from ticks to vertebrates and from vertebrates to ticks, as well as a full vertebrate-tick-vertebrate transmission cycle, have been recapitulated in experimental studies. While results were often variable (e.g., tick transmission to 1 of 2 calves or sheep tested), these studies provide important proof of principle for the widely accepted viral enzootic cycle in nature, and challenge evidence suggesting a limited role of birds as susceptible hosts in CCHFV maintenance. Importantly, these limited data also suggest potential limitations of using traditional lab species in transmission studies, as rats and guinea pigs did not transmit virus to ticks in the laboratory setting.

7. What we have learned

Nearly 40 years after the publication of Hoogstraal's monograph (1), many aspects of the complex relationship between CCHFV, mammalian hosts, and tick

vectors still need to be elucidated. Clearly understanding the transmission and maintenance cycle of CCHFV is not a simple task, as CCHFV is widely disseminated, with a multitude of vertebrate and arthropod species involved. Importantly, however, we do broadly understand the major ecological contributors to maintenance and transmission of CCHFV necessary for general public health education, clinical awareness, and intervention strategy development (e.g., acaricide use).

Similar to interpretation of serological studies, where serology does not seem to adequately correlate with viremia or with the ability of the seropositive animal to infect feeding ticks, experimental infection data must not be over-interpreted. Limited data from experimental infections, without considering the full spectrum of ecological contributors like tick-host dynamics, may not accurately reflect relative roles of the tested animal in a natural transmission setting.

Despite limitations, experimental animal studies have been critical in providing CCHFV data to complement the numerous serological studies performed in animals, and provide key insight into all aspects of the hypothesized transmission cycle. While all mammals investigated appear almost susceptible to infection, the brief viremic period demonstrated in these studies does not support a role for mammals as reservoirs of CCHFV. Instead, the studies emphasize ticks as reservoirs for viral persistence in nature, with vertebrates providing a necessary blood meal for the tick reservoir, and serving as potential amplification hosts.

8. What we still do not know

The current knowledge of major ecological contributors to maintenance and transmission of CCHFV is necessary for ongoing interventions, but not sufficient. Similar to many other zoonotic agents, CCHFV causes little or no disease in its natural hosts, and thus is not typically a disease of veterinary concern. Nevertheless, CCHF merits significant veterinary consideration, as animal hosts are essential for the tick vector. Thus, animals have a critical role in viral maintenance and are potential sources of introducing both CCHFV and infected ticks into naïve geographic regions.

Reported viremia in vertebrates is short-lived, of variable intensity, and notably absent in several species tested in the laboratory [e.g., (41,44–46)]. However, vertebrate-to-tick transmission is highly efficient and thus naturally regulated, in part, by the brevity of the viremic period in vertebrates. Even if *Hyalomma* genus tick larvae and nymphs feed consecutively on the same host without molting on the ground, the overlap between host viremia development and the feeding of ticks in different stages must be finely tuned.

A narrow window of viremia may, however, be the origin of an amplification route. The onset of annual activity of *Hyalomma* spp. larvae and nymphs may last for several weeks, so not every hibernating tick stage is activated at the same time of the year (80). Therefore, each spring, the larvae initiate the life cycle by feeding on new hosts after temperatures reach a threshold to activate them after hatching. The time of larval feeding to nymphal feeding on the same host may last between 20 and 30 days. Therefore, feeding nymphs may overlap on the same host with newly collected larvae. If the nymphs are infected and transmit the virus to the host, and

the host develops viremia, newly acquired larvae could become heavily infected. Such a mechanism strongly suggests that the first groups of feeding larvae that develop into nymphs are transovarially infected, later spreading infection to newly acquired larvae that subsequently molt to nymphs. This hypothesis jointly considers the transovarial route of infection of the newly hatched larvae and the amplifying role of the hosts in infecting new larvae. The ability of the infected hosts to transmit CCHFV to ticks, and, therefore, the number of ticks infected from one single reservoir host, is unknown. These data are relevant in considering feasibility and effectiveness of interventions like vaccination of livestock. While putative livestock vaccination programs are largely cost prohibitive and challenging to implement due to the absence of disease in the host, proper consideration for these and other intervention strategies would require a more detailed understanding of virus transmission.

Susceptibility to CCHFV infection is not uniform. Almost all avian species investigated appear refractory to infection; serological studies report the absence of CCHFV antibodies in birds, and no viremia was detected in almost all experimentally infected avian species. There are two notable exceptions: low-level viremia was detected in blue-helmeted guinea fowl (*N. meleagris*) (59), and ostriches developed high-titer viremia and have been associated with human CCHFV cases (64). The role of birds in CCHFV maintenance and transmission is far from well understood. How detectable viremia translates into a role in transmission of CCHFV is unclear, as evidence of arbovirus transmission to ticks from non-viremic birds has been reported (81,82). Furthermore, the existence of cryptic transmission cycles, the

exchange of virus between two cycles when tick vectors feed on the same host (83),
is unknown in birds and needs to be investigated.

Considering the relatively low numbers of feeding immature ticks that birds
can transport, birds' ability to amplify the infection in ticks seem to be low. Birds,
however, should be considered as a very effective method of transporting ticks
during migratory flights, and appear to have a crucial role in distributing ticks over
wide geographic areas (84). Considering the time immature ticks spend feeding on
birds, birds probably do not directly transport infected ticks from an origin in Africa,
for example, to a destination in the Mediterranean region. Rather, many birds stop
multiple times while migrating to feed and rest, thereby transporting the ticks to
each rest stop, depositing them, and picking up new ones that are then deposited
farther along the route. While still unknown, this is probably what happens with
birds that follow the migratory route along the coast of western Africa, resting every
few kilometers and continually dropping and picking new ticks. To support this,
empirical evidence indicates that the molecular features of CCHFV found on the
western coast of Africa are similar to those of the strain found in southwestern
Spain, both points along the main western pathway followed by many migratory
birds from Africa to Europe (85).

The spread of CCHFV from its African ancestor to other areas of the world
appears to overlap in time with movements of livestock (86). While small- and
medium-sized wild mammals serve as hosts for large numbers of immature ticks,
domestic and wild ruminants have the potential to feed large numbers of adults
(Figure 2). As a result of the substantial tick burdens large vertebrates may support,

a large population of infected tick larvae may develop on a single host even if CCHFV transmission efficiency is low. About 500 females may simultaneously feed on a single animal, and each female may lay around 5,000 eggs (1). The data concerning the efficiency of transovarial infection from the female to the egg is unclear, but even with a transovarial transmission efficiency of ~1%, this scenario would generate 25,000 infected larvae from only one host that is not a reservoir, but just the food source for the females. Undoubtedly, the close proximity in the natural habitats of hosts for both immature (i.e., hares) and adult (i.e., ungulates) ticks would result in a self-fueled CCHFV prevalence in ticks. As several studies provide evidence of viremia in livestock, the overlap of feeding adult ticks on infected hosts could further increase the number of infected ticks, but this remains to be demonstrated.

9. Recommendations for future investigations

Overall, there are few independent experimental infection studies in wild and domestic animals. Key ecological questions were investigated in CCHFV experimental studies by experienced and innovative researchers in the field, but the data that exist have several flaws hampering interpretation and comparison. These limitations include: limited numbers of animals, complicating the varying results; variations in inoculum preparation and source (Table 4); unclear inoculum dose based on varying titration techniques over time; and potential confounding by unrelated disease, as was seen in studies by Shepherd *et al.* (44). These limitations are important to consider in designing future investigations. Transparency in

825 experimental design and data, like clearly reporting the virus strain, titer
826 techniques, and passage history is critical in future studies. How greatly passage
827 history of a virus may affect experimental outcome is unknown. In addition, early
828 studies were done with virus titrated in NWM. Sensitivity of NWM isolation has not
829 been compared to cell culture isolation. Studies on these fundamental questions are
830 critically lacking.

831 Future studies should continue to investigate the roles of wild vertebrate
832 hosts in amplification and transmission of virus, tick-transmission dynamics, and
833 domestic animal infection. Our current knowledge of CCHFV epidemiology has many
834 gaps. The resolution of these gaps needs intensive laboratory work in high-security
835 biosafety conditions, and may be limited by existing transmission model systems
836 (87). Domestic animals may directly spread CCHFV to humans. Additional
837 experimental infection studies in species with high risk of infection in nature and
838 frequent human interaction (i.e., livestock and other domestic species) can be used
839 to assess human risk of exposure and disease and are warranted. Ideally, future
840 studies would mirror natural infection by tick inoculation.

841 There is a need to assess the efficacy of transovarial infection in ticks from
842 infected hosts with detectable viremia. The ideal conditions would include
843 experimentally infecting candidate reservoir vertebrates (e.g., hares), allowing
844 immature ticks to feed on them while viremia is detectable, and then allowing the
845 resulting adult ticks to feed on naive livestock or wild deer. These investigations
846 would provide reliable data about the ability of a putative reservoir to transmit
847 infective CCHFV to feeding immature ticks. In addition to assessing the ability of the

848 resulting adults to infect livestock, non-infected ticks can be allowed to feed
849 alongside with the putatively infected female ticks, and CCHFV prevalence can
850 assessed in the hatching larvae from both sets of ticks. The potential of infection by
851 co-occurring ticks should not be discarded. In any case, the experiments should
852 clarify a pattern regarding the ability of infected animals to infect feeding ticks and
853 to confirm transovarial transmission under controlled conditions.

854 Recognizing the central role of ticks in CCHFV transmission and maintenance,
855 experimental infections, such as those proposed above, must be designed with
856 serological and tick ecology data in mind, selecting ticks compatible to hosts that can
857 be infected with CCHFV and are naturally found in the same habitats. Future studies
858 focused on the interplay between hosts and vectors will further our knowledge, and
859 may aid in identifying putative intervention strategies to interrupt natural CCHFV
860 transmission cycles. This work can help refine interventions and identify high-risk
861 scenarios for high-impact epizootics or virus introduction.

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879
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Table 1. CCHFV experimental infection in small mammals. None of the studies reported clinical signs in inoculated animals. LD₅₀ is indicative of NWM intracranial LD₅₀ levels. Abbreviations: AGPD, agar gel diffusion precipitation; CDC, the Centers for Disease Control and Prevention; CF, complement fixation; dpi, days post infection; IC, intracranial; ID, intradermal; IM, intramuscular; IP, intraperitoneal; NR, not reported; SC, subcutaneous. *Antibody was not detected in animals euthanized up to 8 dpi.

Common name	Taxonomic name (Class/Order)	Virus strain	Route/Dose	Viremia	Antibody response	Year	Ref.
European hedgehog	<i>Erinaceus europaeus</i> (Mammalia/Erinaceomorpha)	Sudarkina	SC, 10 ^{5.5} –10 ^{6.5} LD ₅₀ /0.02 mL (1.0 mL)	No viremia detected (5–13 dpi)	No (CF or AGPD, up to 1 month)	1975	(35)
Long-eared hedgehog	<i>Hemiechinus auritus</i> (Mammalia/Erinaceomorpha)	Sudarkina	SC, 10 ^{5.5} –10 ^{6.5} LD ₅₀ /0.02 mL (0.5 mL)	Viremia, 2–6 dpi (max 4–6 dpi)	No (CF or AGPD, up to 1 month)	1975	(35)
Long-eared hedgehog	<i>Hemiechinus auritus</i> (Mammalia/Erinaceomorpha)	NR	IV/IM	Viremia (details NR)	NR	1971	(76)
South African hedgehog	<i>Atelerix frontalis</i> (Mammalia/Erinaceomorpha)	SPU 4/81	SC, 10 ^{6.0} –10 ^{6.4} LD ₅₀	No viremia detected	Yes	1989	(33)
European hare	<i>Lepus europaeus</i> (Mammalia/Lagomorpha)	Sudarkina	Intracardiac, SC, or ID (<i>n</i> = 2, each), 10 ^{5.0} LD ₅₀	Intracardiac inoculation: viremia detected at 2, 4, 5, and 9 dpi (but not 6, 7, 8 dpi); SC inoculation: viremia at 1, 2, and 5 dpi; ID inoculation: at 1–10 dpi	Yes, CF antibody at 7 dpi; high CF Ab titers detectable 28–45 dpi (study period)	1972	(32)
European hare	<i>Lepus europaeus</i> (Mammalia/Lagomorpha)	NR	IV/IM, 8 log LD ₅₀ /0.02 mL (3 mL total)	Viremia up to 15 dpi (max of 3.6 log LD ₅₀ /0.02 mL at 4 dpi)	NR	1971	(76)
Hare (European?)	<i>Lepus</i> sp. (Mammalia/Lagomorpha)	NR	IV/IM	Viremia (3.6–3.2 log LD ₅₀ /0.02 mL at 4–6 dpi)	NR	1975	(77)

Scrub hare	<i>Lepus saxatilis</i> (Mammalia/Lagomorpha)	SPU 4/81	SC, $10^{6.0}$ – $10^{6.4}$ LD ₅₀	Viremia, 1–7 dpi (max 4.2 log ₁₀ LD ₅₀ /mL)	Yes	1989	(33)
Bushveld gerbil	<i>Gerbilliscus leucogaster</i> (Mammalia/Rodentia)	SPU 4/81	SC, $10^{6.0}$ – $10^{6.4}$ LD ₅₀	Viremia, 1–7 dpi (max 2.7 log ₁₀ LD ₅₀ /mL)	Yes	1989	(33)
Ground squirrel (little suslik)	<i>Spermophilus pygmaeus</i> (Mammalia/Rodentia)	Sudarkina, Hp-15 Zhr	IC, 10^5 LD ₅₀ ; or IM/SC, 10^6 LD ₅₀ IM	Virus isolated from blood and organs of all animals 2–7 dpi (but no later)	NR	1972	(34)
Cape ground squirrel	<i>Xerus inauris</i> (Mammalia/Rodentia)	SPU 4/81	SC, $10^{6.0}$ – $10^{6.4}$ LD ₅₀	Viremia 2–8 dpi (max 3.2 log ₁₀ LD ₅₀ /mL)	Yes	1989	(33)
Highveld gerbil	<i>Gerbilliscus brantsii</i> (Mammalia/Rodentia)	SPU 4/81	SC, $10^{6.0}$ – $10^{6.4}$ LD ₅₀	No viremia	Yes	1989	(33)
Southern multimammate mouse	<i>Mastomys coucha</i> (Mammalia/Rodentia)	SPU 4/81	SC, $10^{6.0}$ – $10^{6.4}$ LD ₅₀	No viremia	Yes	1989	(33)
Multimammate mouse	<i>Mastomys natalensis</i> (Mammalia/Rodentia)	SPU 4/81	SC, $10^{6.0}$ – $10^{6.4}$ LD ₅₀	No viremia	Yes	1989	(33)
Namaqua gerbil	<i>Desmodillus auricularis</i> (Mammalia/Rodentia)	SPU 4/81	SC, $10^{6.0}$ – $10^{6.4}$ LD ₅₀	NR	Yes	1989	(33)
Red veld rat	<i>Aethomys chrysophilus</i> (Mammalia/Rodentia)	SPU 4/81	SC, $10^{6.0}$ – $10^{6.4}$ LD ₅₀	Viremia, 1–3 dpi (max 4.0 log ₁₀ LD ₅₀ /mL)	Yes	1989	(33)
Four-striped grass mouse	<i>Rhabdomys pumilio</i> (Mammalia/Rodentia)	SPU 4/81	SC, $10^{6.0}$ – $10^{6.4}$ LD ₅₀	Viremia, 1 day duration (5 dpi), 1.5 log ₁₀ LD ₅₀ /mL	No*	1989	(33)
White-tailed rat	<i>Mystromys albicaudatus</i> (Mammalia/Rodentia)	SPU 4/81	SC, $10^{6.0}$ – $10^{6.4}$ LD ₅₀	Viremia, 1–6 dpi (max 3.7 log ₁₀ LD ₅₀ /mL)	Yes	1989	(33)

Table 2. CCHFV experimental infection in livestock. LD₅₀ is indicative of NWM intracranial LD₅₀ levels. Abbreviations: dpi, days post infection; IC, intracranial; ID, intradermal; IM, intramuscular; IP, intraperitoneal; IT, inoculated ticks; IV, intravenous; NAb, neutralizing antibody; NR, not reported; SC, subcutaneous. †Clinical signs associated with inoculum

Common name	Taxonomic name	Virus strain	Route	Viremia	Clinical signs	Antibody response	Year	Ref.
Cow	<i>Bos taurus</i> (White Fulani) (Mammalia/Artiodactyla)	IbAn 7620 (isolated from goat in this study)	SC (<i>n</i> = 1), SC/ID (<i>n</i> = 1)	Viremia 2 and 5 dpi in low dose inoculation; viremia 1–5 dpi in high dose inoculation	Yes, mild illness, dullness, lassitude, decreased appetite in both animals	NR	1970	(43)
Cow (calf) (2 mo, 6 mo)	<i>Bos taurus</i> (Mammalia/Artiodactyla)	Sudarkina	SC, IM, or SC/ID	Viremia	No	Yes	1976	(41)
Cow (calf)	<i>Bos taurus</i> (Mammalia/Artiodactyla)	K128-12, 3010, Nakiwogo, K67/67, IbAn 7620, JD 206, IbAr 10200	IC and/or peripheral	Viremia (2 to 5–7 dpi, titer 10 ^{2.5} –10 ^{4.5} LD ₅₀ /0.02 mL)	NR	Yes	1979	(75)
Sheep (Touabir and Peul-Peul breeds)	<i>Ovis aries</i> (Mammalia/Artiodactyla)	HD 49199	IP or IT	Viremia (range 0–9 dpi)	Yes, fever (range 0–9 dpi), hepatic dysfunction, abnormal blood cell count	Yes, and passive transfer to offspring	1998	(45)
Sheep	<i>Ovis aries</i> (Mammalia/Artiodactyla)	SPU 4/81	IT		Mild fever	Yes	1989	(73)
Sheep	<i>Ovis aries</i> (Mammalia/Artiodactyla)	K128-12, 3010, Nakiwogo, K67/67, IbAn 7620, JD 206, IbAr 10200	IC and/or peripheral	Viremia (day 2 to 5–7 dpi, titer 10 ^{2.5} –10 ^{4.5} LD ₅₀ /0.02 mL)	NR	Yes	1979	(75)

Sheep (Peul-Peul Fulani breed)	<i>Ovis aries</i> (Mammalia/Artiodactyla)	Dak H49199	IP	Viremia (0–6 days), corresponding to previous CCHFV exposure	NR	Yes	1991	(46)
Sheep (lamb)	<i>Ovis aries</i> (Mammalia/Artiodactyla)	Sudarkina	SC/ID	Viremia	No	Yes	1976	(41)
Donkey	<i>Equus asinus</i> (Mammalia/Perissodactyla)	NR	SC	Viremia (low level, titer NR)	No	Yes	1972	(48)
Donkey	<i>Equus asinus</i> (Mammalia/Perissodactyla)	K128-12, 3010, Nakiwogo, K67/67, IbAn 7620, JD 206, IbAr 10200	IC and/or peripheral	Viremia (day 2 to 5–7 dpi, titer $10^{2.5}$ – $10^{4.5}$ LD ₅₀ /0.02mL)	NR	Yes	1979	(75)
Horse	<i>Equus caballus</i> (Mammalia/Perissodactyla)	Sudarkina	IV/IM or IM/SC	Viremia (low level, titer NR)	No†	Yes, high NAb	1969	(47)

Table 3. CCHFV experimental infection in birds. Reports of experimental infection in birds are limited to a few independent investigations. Viremia was undetectable in most species, with the notable exceptions of guinea fowl and ostriches. Clinical signs were not reported in any animals. Abbreviations: dpi, days post infection; IM, intramuscular; IP, intraperitoneal; IV, intravenous; NR, not reported; SC, subcutaneous.

Common name	Taxonomic name	Virus strain	Route	Viremia	Antibody response	Year	Ref.
Red-billed hornbill	<i>Tockus</i> sp. (Aves/Bucerotiformes)	HD 49199	IP	No viremia detected, but transmitted virus to naïve ticks	Yes	1994	(62)
Laughing dove	<i>Spilopelia senegalensis</i> (Aves/Columbiformes)	HD 49199	IP	No viremia	No	1994	(62)
Rock dove	<i>Columba livia</i> (Aves/Columbiformes)	NR	NR	No viremia	No	1969–1972	(56–58)
Chicken	<i>Gallus gallus</i> (Aves/Galliformes)	HD 49199	IP	No viremia	Yes (in 1 of 6 animals)	1994	(62)
Chicken	<i>Gallus gallus</i> (Aves/Galliformes)	SPU 4/81	SC/IM/IV	No viremia	No	1987	(59)
Blue-helmeted guinea fowl	<i>Numida meleagris</i> (Aves/Galliformes)	SPU 4/81	SC/IM/IV	3-week-old and adult birds similar, low-level viremia in 10–20% of animals; most refractory	Yes, beginning 5–6 dpi	1987	(59)
Glossy starling	<i>Lamprotornis</i> sp. (Aves/Passeriformes)	HD 49199	IP	No viremia	Yes	1994	(62)
Rook	<i>Corvus frugilegus</i> (Aves/Passeriformes)	NR	NR	No viremia	No	1969–1972	(56–58)
Ostrich	<i>Struthio camelus</i> (Aves/Struthioniformes)	SPU 4/81	SC	Viremia 1–4 dpi (max $10^{4.0}$ LD ₅₀ /mL at 2 dpi); virus detected in spleen, liver, kidney (up to 5 dpi)	Yes	1998	(64)

Table 4. CCHFV strains used in maintenance and transmission experimental infection animal studies. This table summarizes the variety of isolates that were used in the studies described, including CCHFV strains and passage history. When original isolation information was not reported, year of isolation is listed as $\leq$ the year in which the earliest published report of the isolate was found. Abbreviations: NR, not reported; SMB, suckling mouse brain passage or suspension; SSR, Soviet Socialist Republic. Some isolates (denoted with †) were obtained via experimental inoculation of ticks or mammals and re-isolation.

Year of isolation	Region	Strain	Source of isolate (reference)	Passage history	Ref.
1956	Congo	UG 3010	Human (75,89–91)	SMB	(75)
1958	NR	Nakiwogo	Human serum (91)	SMB	(75)
1965	Nigeria	IbAn 7620	Goat (75)	3–4× SMB	(72,75)
1966	Sokoto, Nigeria	IbAr 10200	<i>Hyalomma excavatum</i> tick collected from camel (43)	SMB	(75)
1967	Uzbek SSR	Khodzha (Hodzha)	Human (92)	29 or 41× SMB	(75) (92)
1968	Rostov Oblast	Sudarkina	Human (34,47)	16× SMB	(47)
1968	Rostov Oblast	Sudarkina	Human (34,47)	21–48× SMB	(34)
1968	Rostov Oblast	Sudarkina	Human (34,47)	42–49× SMB	(41)
1968	Rostov Oblast	Sudarkina	Human (34,47)	50× SMB	(32)
1968	Rostov Oblast	Sudarkina	Human (34,47)	56–58× SMB	(35)
1968	Astrakhan Oblast	Hp-16	<i>Hyalomma marginatum</i> ticks collected from rooks (74)	NR	(74)
1969	Russia	Hp-15	<i>Hyalomma marginatum</i> ticks (34)	6–7× SMB	(34)
≤ 1969	NR	K67/67	Human (75,93)	SMB	(75)
1970	Nigeria	Ar 43665†	<i>Hyalomma rufipes</i> ticks experimentally inoculated with IbAn 7620 (72)	IbAn 7620 (NR), 1× tick	(72)
1976	Pakistan	Congo, JD 206	<i>Hyalomma anatolicum</i> ticks (75)	NR	(75)
≤ 1979	Pakistan	Hazara (JC 280)	<i>Ixodes redikorzevi</i> ticks (75,94)	NR	(75)
≤ 1979	Nigeria	IbAn 7620†	<i>Atelerix albiventris</i> hedgehog experimentally infected with InAn 7620 (95)	3× SMB, 1× hedgehog	(95)
≤ 1979	NR	K128-12	<i>Hyalomma asiaticum</i> ticks (75)	SMB	(75)
1981	South Africa	SPU 4/81	Human, fatal (60)	2× cell culture, 3× SMB; 2× cell culture, 4× SMB	(33,44,73)
1984	South Africa	SPU 41/84	Human, non-fatal (33)	8× SMB	(33)
1988	Southern Mauritania	Dak H49199	Human, fatal (96)	3× SMB	(32,46,97)
1988	North-central Senegal	ArD49088	<i>Hyalomma impeltatum</i> ticks collected from sheep (98)	3× SMB	(97)

FIGURE LEGENDS

Figure 1. Life cycle of *Hyalomma* spp. ticks and vertical and horizontal transmission of Crimean-Congo hemorrhagic fever virus (CCHFV) (3). The course of the tick life cycle is indicated with blue arrows. Upon hatching, larvae find a small animal host for their first blood meal (hematophagy). Depending on the tick species, the larvae either remain attached to their host following engorgement and molt in place (two-host ticks) or drop off to molt (three-host ticks); this transition is marked by an asterisk. The nymphs then either continue to feed on the animal on which they molted (two-host ticks) or attach to a new small animal (three-host ticks). Upon engorgement, nymphs of all species drop off their host and molt into adults. Adult ticks then find a large animal for hematophagy, and mate while attached to the host. After taking a blood meal, the engorged females drop off and find a suitable location for ovipositing. During the tick life cycle, CCHFV can be transmitted between ticks and mammals (solid red arrows), and directly between ticks through co-feeding (dashed arrows). For each kind of virus transfer, the thickness of the arrow indicates the efficiency of transmission. Humans can become infected through the bite of an infected tick or through exposure to the body fluids of a viremic animal or an infected person.

Figure 2. Idealized life cycle of an *Ixodes* spp. tick with three hosts, showing the three stages (larva, nymph and adult) (14). The eggs incubate in ground litter (A), protected against stressful environmental conditions (e.g., water losses). When the larvae hatch, they climb the vegetation, questing for a host (A→B). Larvae feed on such hosts (B), and when engorged, drop off to the ground and molt to nymphs (B→C). The nymphs quest for a second host, feed, and molt again off the host (C→D). The resulting adults attach to a third host, feed, and mate, and the females drop off for ovipositing on the ground.

A

B

C

D