Evidence for widespread infection of African bats with Crimean-Congo hemorrhagic fever-like viruses

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Crimean Congo hemorrhagic fever virus (CCHFV) is a highly virulent tick-borne pathogen that causes hemorrhagic fever in humans. The geographic range of human CCHF cases largely reflects the presence of ticks. However, highly similar CCHFV lineages occur in geographically distant regions. Tick-infested migratory birds have been suggested, but not confirmed, to contribute to the dispersal. Bats have recently been shown to carry nairoviruses distinct from CCHFV. In order to assess the presence of CCHFV in a wide range of bat species over a wide geographic range, we analyzed 1,135 sera from 16 different bat species collected in Congo, Gabon, Ghana, Germany, and Panama. Using a CCHFV glycoprotein-based indirect immunofluorescence test (IIFT), we identified reactive antibodies in 10.0% (114/1,135) of tested bats, pertaining to 12/16 tested species. Depending on the species, 3.6%–42.9% of cave-dwelling bats and 0.6%–7.1% of foliage-living bats were seropositive (two-tailed t-test, \( p = 0.0447 \) cave versus foliage). 11/30 IIFT-reactive sera from 10 different African bat species had neutralizing activity in a virus-like particle assay. Neutralization of full CCHFV was confirmed in 5 of 7 sera. Widespread infection of cave-dwelling bats may indicate a role for bats in the life cycle and geographic dispersal of CCHFV.

Crimean Congo hemorrhagic fever virus (CCHFV; Genus Nairovirus, Family Bunyaviridae) is a tick-borne pathogen that has caused more than 10,000 documented human infections worldwide, with a case fatality proportion of approximately 10–30%\(^2\). Clinical symptoms range from acute febrile illness to severe hemorrhagic fever\(^1\). To date, the majority of CCHFV cases have been documented in Asia (in particular Russia, Iran, Georgia, Turkey) and Southeast Europe (Greece)\(^1\). In Africa, approximately 100 human CCHFV cases have been reported, primarily in Mauritania and South Africa\(^2\)–\(^4\). Serological surveys on animals, however, suggest enzootic circulation of CCHFV in different parts of Africa\(^5\)–\(^6\). The Americas have not reported any autochthonous CCHFV cases\(^1\)–\(^2\).

Within the genus Nairovirus CCHFV represents one of at least seven serogroups. Nairoviruses, classified in serogroups Hughes, Dera Ghazi Khan and Sakhalin, are associated with birds. Viruses in the serogroups Thiafora and Qalyub were identified in shrews and rodents, respectively. Serogroups CCHFV as well as Nairobi sheep disease virus (NSDV) are associated with ungulates\(^2\). Only CCHFV, NSDV and the NSDV-related Dugbe virus\(^1\) are considered human pathogens, while all other nairoviruses are restricted to certain vertebrate host taxa\(^7\).

CCHFV is known to be transmitted by ticks of the genera Hyalomma and Rhipicephalus\(^2\)–\(^6\),\(^8\). The geographic distribution of CCHFV coincides well with the presence of ticks\(^9\). However, closely related CCHFV lineages occur in geographically distant regions that do not follow the habitat connectivity of tick hosts. Several studies suggested that tick-infested migratory birds may be responsible for the spatial distribution of CCHFV\(^9\)–\(^11\).
Bats (Order: Chiroptera) represent the only migratory flying mammals. Bats are commonly infested with soft and hard ticks12–14, and were recently shown to carry nairoviruses7,14,15. Phylogenetic analyses as well as serological studies based on cross-reactivity in complement fixation tests suggest that all identified bat-associated nairoviruses belong to two novel serogroups that are distantly related to CCHFV7. One representative, termed Leopards Hill virus (LPHV), was successfully isolated from samples of the African bat species Hipposideros gigas (H. gigas). Phenotypic characterization revealed that LPHV causes hemorrhagic disease in mice7. Whether LPHV may be pathogenic for other mammals including humans is unknown.

The large diversity of viruses in bats16–20 together with previous findings of bat nairoviruses7,14,15 encourage a wider and more systematic assessment of the endemicity of nairoviruses in bats. Previous studies were predominantly based on nucleic acid detection and virus isolation, which can only detect viraemic animals. While we do not know the expected frequency of nairovirus viraemia in free-ranging bats, field studies on other RNA viruses with a viraemic infection pattern, including hepadnaviruses19, hepaciviruses17, filoviruses21–23, and bunyaviruses24,25, indicate very low virus detection rates. Unless very large sample collections are studied, virus detection by PCR lacks the power to reflect the host species range of a given virus, in particular because of a lack of negative predictive value27,18. Serological techniques are better suited for host range studies because seroprevalence is less sensitive to temporal and spatial variation of infection activity26. Serology is also more suitable for the limited sample size that can be achieved in bat-related studies, as most bat species are too small to be bled without destruction.

In the present study we screened 1,135 bat serum samples comprising 16 bat species (migratory and non-migratory) from five different countries for the presence of CCHF-like viruses. The sample was focused on African bat species as nairoviruses have been predominantly detected in the Old World bats14–16. We applied a staged set of serological assays including a recombinant immunofluorescence test (IIFT), a novel pseudotype neutralization assay27 as well as full virus neutralization tests conducted under biosafety level 4 conditions. Additional molecular screening involved two modified generic nairovirus RT-PCRs based on previously established protocols28–30.

Results

Bats carry CCHFV GP-reactive antibodies. From 2003 through 2011 bats were sampled in Congo, Gabon, Ghana, Germany and Panama. The sample included 1,135 blood or serum specimens from 16 bat species pertaining to six different bat families for the presence of CCHF-like viruses. The sample was focused on African bats as nairoviruses have been predominantly detected in the Old World bats14–16. We applied a staged set of serological assays including a recombinant immunofluorescence test (IIFT), a novel pseudotype neutralization assay27 as well as full virus neutralization tests conducted under biosafety level 4 conditions. Additional molecular screening involved two modified generic nairovirus RT-PCRs based on previously established protocols28–30.

Detection of CCHFV neutralizing antibodies in African bat species. As antibody cross-reactivity between CCHFV and viruses from the related NSDV serogroup cannot be completely ruled out by IIFT31, specific virus neutralization tests (NT) were done. NT can prove previous infection with CCHFV because there is no cross-neutralization between serogroups31. Because NTs require relatively large volumes of serum that cannot be obtained from most bat species, only for 30 of the 114 IIFT-positive sera covering 10/12 bat species, NT assays could be performed. In addition, 10 CCHFV IIFT-negative samples with sufficient volume, representing all 10 assessed bat species, were tested. Sera were tested at a 1:100 dilution in a 96-well format using a recently established CCHFV virus-like particle (VLP)-based NT37. Out of 30 IIFT-positive sera, 11 showed significant neutralizing activity defined as 80% reduction of luciferase luminescence signal (Fig. 2B, Supplementary Table 2). None of the 10 IIFT-negative control sera had neutralizing activity (Fig. 2B). In parallel, all 40 (30 IIFT-positive, 10 IIFT-negative) bat sera were tested in a Rift Valley fever (RVF) VLP-based NT36, all with negative results, ruling out nonspecific neutralization activity in bat sera (Fig. 3, Supplementary Table 2).

For 7 of 11 CCHF VLP-positive sera, enough material was available to conduct additional neutralization tests by a full virus CCHFV neutralization test under biosafety level 4 conditions. Endpoint titration by IIFT in these samples revealed high reciprocal titers between 160 and 1,280 (Table 2, Fig. 2A). Sera were thus titrated in 2-fold serial dilutions in a range of 1:40–1:1,280. The test confirmed full virus neutralizing activity in 5 of 7 sera, with reciprocal titers ranging between 40 (lowest testing dilution due to lack of serum) and 160 (Table 2).

Lack of evidence for CCHFV-related nucleotide sequences in bat serum samples. To identify CCHFV-related nucleotide sequences in serum samples, we combined three previously established generic RT-PCR protocols that were shown to detect all known nairoviruses28–30. Modifications were made to increase the sensitivity for the detection of CCHFV-related nucleotide sequences by applying low annealing temperatures and degenerated oligonucleotides. Viral RNA of CCHFV strain lbAr was used to test oligonucleotides from Lambert et al.29 in combination with primers from Wolff and colleagues30. The endpoint RT-PCR detection limit of an RT-PCR formulation that used the best combination of primers was between 8 and 80 copies per reaction. An
| Bat species                      | Country    | Site         | Year | Diet | Migratory* | IIFT CCHFV-GP n positive/tested (percentage) | CCHFV VLP assay | CCHFV NT |
|---------------------------------|------------|--------------|------|------|------------|---------------------------------------------|-----------------|----------|
| **Foliage-living bats**         |            |              |      |      |            |                                             |                 |          |
| *Artibeus jamaicensis*           | Panama     | BCI          | 2011 | F    | N          | 0/28 (0.0)                                  | ND               | ND       |
| *Artibeus lituratus*             | Panama     | BCI          | 2011 | F    | N          | 0/15 (0.0)                                  | ND               | ND       |
| Eidolon helvum                  | Ghana      | Kumasi Zoo   | 2009 | F    | Y          | 1/96 (1.0)                                  | 0/1 (0.0)        | ND       |
|                                |            |              |      |      |            |                                             |                 |          |
| Epomops franqueti               | Gabon      | Lepaka       | 2008 | F    | Y          | 2/44 (4.5)                                  | 0/2 (0.0)        | ND       |
|                                | Congo      | Lebango      | 2006 |      |            | 5/26 (19.2)                                  | 1/2 (50.0)       | 0/1 (0.0) |
|                                | Mbono      |              | 2006 |      |            | 0/29 (0.0)                                  | ND               | ND       |
| Hypsignathus monstrosus         | Gabon      | Cimetière    | 2005 | F    | Y          | 0/1 (0.0)                                   | ND               | ND       |
|                                | Ekata      |              | 2006 |      |            | 0/7 (0.0)                                   | ND               | ND       |
|                                | Hopital    |              | 2005 |      |            | 0/1 (0.0)                                   | ND               | ND       |
|                                | Lepaka     |              | 2008 |      |            | 0/13 (0.0)                                  | ND               | ND       |
|                                | Congo      | Lebango      | 2005 |      |            | 0/18 (0.0)                                  | ND               | ND       |
|                                |            |              | 2006 |      |            | 0/19 (0.0)                                  | ND               | ND       |
|                                | Mbono      |              | 2003 |      |            | 0/17 (0.0)                                  | ND               | ND       |
|                                |            |              | 2005 |      |            | 0/17 (0.0)                                  | ND               | ND       |
|                                |            |              | 2006 |      |            | 1/8 (12.5)                                  | 0/1 (0.0)        | ND       |
| Micropteropus pusillus          | Gabon      | Cimetière    | 2005 | F    | Y          | 1/4 (25.0)                                  | 0/1 (0.0)        | ND       |
|                                | Hopital    |              | 2005 |      |            | 0/4 (0.0)                                   | ND               | ND       |
|                                | Hopital S  |              | 2005 |      |            | 0/4 (0.0)                                   | ND               | ND       |
|                                | Keri       |              | 2005 |      |            | 0/5 (0.0)                                   | ND               | ND       |
|                                | Ngounie    |              | 2005 |      |            | 0/1 (0.0)                                   | ND               | ND       |
|                                | Congo      | Lebango      | 2005 |      |            | 0/1 (0.0)                                   | ND               | ND       |
|                                |            |              | 2006 |      |            | 0/2 (0.0)                                   | ND               | ND       |
|                                | Mbono      |              | 2005 |      |            | 0/12 (0.0)                                  | ND               | ND       |
|                                |            |              | 2006 |      |            | 1/67 (1.5)                                  | 0/1 (0.0)        | ND       |
| Myonycteris torquata            | Congo      | Lebango      | 2006 | F    | Y          | 3/91 (3.3)                                  | 0/3 (0.0)        | ND       |
|                                | Mbono      |              | 2006 |      |            | 0/9 (0.0)                                   | ND               | ND       |
| **Cave-dwelling bats**          |            |              |      |      |            |                                             |                 |          |
| Coleura afris                   | Gabon      | Batouala     | 2009 | I    | Y          | 5/12 (41.7)                                  | 2/3 (66.6)       | 1/1 (100.0) |
|                                | Faucon     |              | 2009 |      |            | 1/2 (50.0)                                  | 1/1 (100.0)      | 1/1 (100.0) |
| Hipposideros cf. caffer         | Gabon      | Batouala     | 2009 | I    | N          | 2/6 (33.3)                                  | 1/2 (50.0)       | 1/1 (100.0) |
|                                | Faucon     |              | 2009 |      |            | 1/30 (3.3)                                  | 0/1 (0.0)        | ND       |
|                                | Zadie      |              | 2009 |      |            | 0/12 (0.0)                                  | ND               | ND       |
| Hipposideros gigas              | Gabon      | Batouala     | 2009 | I    | Y          | 19/33 (57.6)                                 | 0/1 (0.0)        | ND       |
|                                | Faucon     |              | 2009 |      |            | 5/56 (8.9)                                  | 1/2 (50.0)       | 1/1 (100.0)† |
|                                | Zadie      |              | 2009 |      |            | 8/40 (20.0)                                  | 0/1 (0.0)        | ND       |
| Miniopterus inflatus            | Gabon      | Batouala     | 2009 | I    | Y          | 9/49 (18.4)                                  | 2/4 (50.0)       | 1/1 (100.0)† |
|                                | Faucon     |              | 2009 |      |            | 0/1 (0.0)                                   | ND               | ND       |
|                                | Zadie      |              | 2009 |      |            | 0/1 (0.0)                                   | ND               | ND       |
| Myotis dasycneme                | Germany    | Methorst     | 2008 | I    | Y          | 1/25 (4.0)                                   | ND               | ND       |
| Myotis daubentorii              | Germany    | Bavaria      | 2002 | I    | Y          | 0/1 (0.0)                                   | ND               | ND       |
|                                | Berlin     |              |      |      |            | 0/1 (0.0)                                   | ND               | ND       |
|                                | Hannover   |              | 2003 |      |            | 0/1 (0.0)                                   | ND               | ND       |
|                                | Hannover   |              | 2003 |      |            | 0/1 (0.0)                                   | ND               | ND       |
|                                | Natternburg|              |      |      |            | 0/1 (0.0)                                   | ND               | ND       |
|                                | ND         |              | 2003 |      |            | 0/1 (0.0)                                   | ND               | ND       |
|                                | Rehburg    |              | 2003 |      |            | 0/1 (0.0)                                   | ND               | ND       |
|                                | Wahlstorf  |              | 2009 |      |            | 1/18 (5.6)                                  | ND               | ND       |
|                                | Wunstorf   |              | 2003 |      |            | 0/3 (0.0)                                   | ND               | ND       |
| Nyctalus noctula                | Germany    | Bodenholz    | 2009 | I    | Y          | 0/24 (0.0)                                  | ND               | ND       |
| Rhinolophus spec.               | Gabon      | Faucon      | 2009 | I    | N          | 0/1 (0.0)                                   | ND               | ND       |
|                                | Mine       |              | 2009 |      |            | 0/15 (0.0)                                  | ND               | ND       |
| Rousettus aegyptiacus           | Gabon      | Batouala     | 2009 | F    | Y          | 8/15 (53.3)                                  | ND               | ND       |
|                                | Hopital    |              | 2005 |      |            | 1/60 (1.7)                                  | ND               | ND       |

*Continued*
additional RT-PCR assay was developed based on a hemi-nested formulation using primers described in Honig et al., followed by a 2nd round RT-PCR step with novel primers. The sensitivity limit of this assay also ranged between 8 and 80 copies per reaction. Serum samples from Artibeus jamaicensis (n = 28), A. lituratus (n = 15, both from Panama) as well as Myotis dasycneme (n = 26, Germany) were not available for RT-PCR testing due to sample volume limitations (<25 μl). In the remaining 1,067 of 1,135 serologically-tested sera, no CCHFV RNA was found by both RT-PCR formulations. In parallel, these same bat serum sample-derived RNA extracts had successfully been used for the detection of novel paramyxo-, hepe-, hepadna- and flaviviruses, confirming that the material was appropriate for RNA virus testing.

Discussion
In this assessment of the potential host species range of CCHFV in bats we obtained strong serological evidence for bats constituting a putative host for CCHFV or a closely related virus belonging to the CCHFV serotype. Our failure to directly detect viral RNA may be caused by an overall low infection prevalence that precludes detection.
in spite of using sensitive RT-PCR assays on a rather large collection of serum samples from bats. We applied two generic pan-nairovirus RT-PCR assays that made use of low stringency amplification conditions to enable the detection of unknown CCHF-related viruses. These conditions led to a maximal 10-fold higher limit of detection (LOD: 8–80 copies/reaction) compared to previously established diagnostic CCHFV real time PCRs with LODs of 5–16 copies per reaction. However, as CCHFV-infected viraemic humans, for example, have viral loads up to $10^9$ viral RNA copies per ml, we assume that viraemic animals would have been identified if present. Nonetheless, we cannot rule out that bats may not experience a pronounced viraemia as observed for birds. Previous studies conducted by us and others have already shown that the detection frequency of viral RNA is generally very low (<3%).

In addition, sampling time point and/or type of sample may have prevented amplification. In humans it was shown that viremia can be very short (6 days). However, as CCHFV-infected viraemic humans, for example, have viral loads up to $10^9$ viral RNA copies per ml, we assume that viraemic animals would have been identified if present. Nonetheless, we cannot rule out that bats may not experience a pronounced viraemia as observed for birds.

As shown by Ishii et al., the bat-related nairovirus LPHV was primarily detected in lung samples. Therefore, destructive sampling involving the collection of organ tissue of designated bat species may be more promising for
nairovirus detection as virus might persist for prolonged periods in parenchymatous organs such as the lung or liver\textsuperscript{14,15}. In the context of this retrospective study we did not test different organs from bats because most species are protected and were sampled without destruction or samples were exhausted from previous experiments\textsuperscript{40,41}.

In spite of this limitation we consider the serological results from our study to be indicative of the presence of members of the CCHFV serogroup in bats. The current demarcation criteria for nairovirus taxa have to rely on the definition of serogroups as long as formal species definitions are not in place. Serogroups based on cross-reactivity in antibody affinity assays reflect phylogeny and can be used to classify novel nairoviruses as they are identified\textsuperscript{42,43}. For example, complement fixation assays (CFA) and IIFT were applied to discriminate shrew nairoviruses from CCHFV and classify them into a separate serogroup termed Thiafora\textsuperscript{44}. Phylogenetically, all members of the Thiafora serogroup form a monophyletic clade in sister relation to a clade containing all members of the CCHFV and NSDV serogroups\textsuperscript{7} (overview shown in Fig. 4). The NSDV serogroup contains NSDV (also named Ganjam virus), and Dugbe virus whose serological classification in one serogroup was again based on cross-reactivity in CFA and IIFT\textsuperscript{31}. NT enables further serological discrimination between taxa in serogroups, exemplified by the presence of cross-reactivity but absence of cross-neutralization between Dugbe and NSDV\textsuperscript{31}. These taxa might be referred to as two different serotypes within the NSDV serogroup (Fig. 4). The whole NSDV serogroup is discriminated from the CCHFV serogroup, its monophyletic sister taxon, based on absence of cross-reactivity in CFA and decreased IIFT endpoint titers\textsuperscript{31,43,45}. Further sub-differentiation into serotypes based on NT has not been observed within the CCHFV serogroup\textsuperscript{42,46}. Consequently, the combined reactivity in IIFT and NT suggests that bat species, tested in the present study, carry viruses which are less distinct from CCHFV than Dugbe virus is from NSDV.

Also the height of IIFT titers observed in our study, up to 1:1,280, corresponds to CCHFV-specific titers in experimentally infected wild mammals that reached endpoint titers of 128–1,024 by IIFT\textsuperscript{47}. We conclude that African bat species have been infected with CCHFV or a closely related nairovirus in the same serogroup and probably the same species.

The identification of high neutralizing antibody titers (up to 1:160 based on full virus neutralization) indicates that bats experience infections followed by seroconversion, which in case of humans is correlated with clearance of the virus and survival of infection\textsuperscript{48}. CCHFV might thus exemplify another highly pathogenic agent that is effectively controlled in bats.

As several cave-dwelling bat species migrate over various distances, bats might contribute to the geographic dispersal of CCHFV in similar ways as hypothesized for birds\textsuperscript{9–11}. Future studies should aim to clarify whether the CCHFV strains carried by bats or bat ticks are identical or distinct from strains associated with birds or bird ticks.

Interestingly, we found a predominance of CCHFV antibody-positive bats among cave-dwelling species in particular in the Batouala cave in Gabon. The specific habitat conditions in caves (high population density, host availability, humidity, moderate temperature variations) could potentially enable a virus amplification cycle between bats and ticks\textsuperscript{12,13,49}. Caves harbor many different arthropods including soft and hard ticks. Cave-dwelling
animals like ruminants, bird and bats are highly exposed to blood-sucking parasites. Whereas in case of humans and ruminants it is known that CCHFV is predominantly transmitted by hard ticks \(^2\), \(^6\), \(^8\) the role of soft ticks in the CCHFV transmission cycle is less clear. However, soft ticks have been found to carry bunyaviruses \(^50\) and evidence from CCHFV-endemic countries suggests that certain soft tick species (\textit{Ornithodoros lahorensis}, \textit{Argas reflexus}) \(^51\), \(^52\) may as well serve as vectors for CCHFV \(^51\), \(^52\). Soft ticks are long-lived and blood meals of soft ticks often take only a short period of time (minutes to hours) \(^53\). Consequently, bats may not be infested by soft ticks during sampling. The low rate of viremia in bats points to a persistence of the virus in ticks rather than bats, encouraging targeted investigations into the pathogen ecology of CCHFV based on cave-associated ticks that can be studied in proximity to bat roosts.

### Methods

#### Sampling sites, time points and ethics.

In total, \(N = 1,135\) bat serum specimens were collected in five countries (Congo, Gabon, Ghana, Germany, Panama) from 2003 through 2011. A detailed summary of sampling time points is shown in Supplementary Table 3. All animals were handled according to national and European legislation for the protection of animals (EU council directive 86/609/EEC). For all individual sampling sites, study protocols including trapping, sampling and testing of animals were approved by the responsible animal ethics committees as detailed below. Maximum efforts were made to leave animals unharmed or to minimize suffering.
of animals. All bats were caught with mist nets and blood was taken by vein or heart punctures by trained personnel in accordance with the approved guidelines of the respective authorities. Any surgical procedure was performed under sodium pentobarbital/ketamine anesthesia. Sampling, capturing and sample transport were done as described before\textsuperscript{17–20} under the following wildlife permits and ethical clearances: Panama (Ethics committee of the Smithsonian Tropical Research Institute (STRI); research permit: STR2563 (PI VC)- IACUC 100316-1001-18; ethics permit: IACUC 100316-1001-18; export permits: SEX/A-30-11, SEX/A-55-11, SEX/A-81-10, SEX-A-26-10); Ghana (Ministry of Food and Agriculture, Wildlife Division, Forestry Commission, Accra; research permit A04957; ethics permit: CHRPE49/09; export permit: state contract between Ghana and Germany; Congo/ Gabon (Ministry of Water and Forest, Libreville; ethics and export permit: 00021/MEEPA/SG/DGEF/DFC); Germany (Landesamt für Naturschutz und Umwelt (LANUV); ethics permit: LANU 314/5327.74.1.6).

**Indirect immunofluorescence test.** CCHFV antibody detection was performed by a modified, commercial, indirect IIFT (EUROIMMUN AG, Lübeck, Germany) according to previous protocols.\textsuperscript{18,54} EU90 cells (African green monkey kidney) were transfected with plasmids encoding the CCHFV GP (full length GPC precursor of strain IbAr10200). Sera were applied in a 1:40 dilution. Detection involved goat-anti bat IgG (Bethyl, Montgomery, AL, USA) diluted 1:1,000 and Dylight488 labeled donkey-anti goat IgG (1:100). Analysis was performed with a Motic Immunofluorescence microscope (Zeiss, Jena, Germany).

**Neutralization test using CCHFV virus-like particles.** Biosafe NT were done using CCHF VLP consisting of CCHFV structural proteins and a Renilla luciferase minigenome unable to produce infectious viral progeny. In parallel, a pGL3-\textsuperscript{Firefly} construct was applied as transfection control. VLPs were incubated for 1 h at 37 °C with antisera at a final dilution of 1:100. Cells were infected with serum-treated VLPs for 1 h at 37 °C. At 24 h post infection, luciferase activities were measured in cell lysates using the Dual-Luciferase Reporter Assay System (Promega, Mannheim, Germany). The same VLP methodology was described earlier for Rift Valley Fever virus (RVFV) and was used in parallel as a control antigen in this study.\textsuperscript{32}

**Neutralization test by CCHFV strain IbAr.** Sera reactive against CCHF VLP at dilution 1:100 were confirmed by quantitative CCHFV NT in a biosafety level 4 facility. Serial two-fold dilutions (from 1:40–1:1,280) of sera were incubated with ~1,000 focus-forming units (FFU) of CCHFV (IbAr strain, originally isolated from Gabon). Sera were applied in a 1:40 dilution. Detection involved goat-anti bat IgG (Bethyl, Montgomery, AL, USA) diluted 1:1,000 and Dylight488 labeled donkey-anti rabbit conjugate (Thermo Scientific, Braunschweig, Germany).

**Viral nucleic acid detection.** For nucleic acid detection viral RNA was extracted with the QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany). A combination of three previously established generic nairovirus PCR protocols were applied with modifications.\textsuperscript{28–30} Oligonucleotides from Lambert et al. \textsuperscript{29} were used in multiple combinations with primers described by Wößel and colleagues.\textsuperscript{30} Tenfold dilution series of viral RNA of CCHFV strain IbAr were used to determine the sensitivity of the PCR assays. Optimal amplification of CCHFV RNA was identified using the oligonucleotide combinations Nairo-F (ATG ATT GCS AAY AGS AYT TAA A) and Nairo-R (ACA GCA RTG SAT SGG SCC CCA YTT, 1st round) and cc1α-c-F (GTG CCA CTA ATG ATG CAC AAA AGG ATT CCA TCT) and Nairo-R (2nd round) in a hemi-nested PCR protocol. The use of the nucleoside inosine is indicated by the number 5. In a second PCR assay, which was based on the Honig et al.\textsuperscript{29} protocol, the published 1st round of PCR amplification by oligonucleotides Nairo-F and Nairo-R was followed by a newly introduced 2nd round PCR using oligonucleotides Nairo-Fnest (CCA AGA AGY GT3 AGR AY AAR GT) and Nairo-Rnest (TTG GCC CCC ACT TSG TRT TCR C5C C). The PCR protocol was as follows: 1st round PCR including reverse transcription 50 °C, 20 min, 94 °C 3 min, 10× (94 °C 15 s; annealing for 20 s at decreasing temperature starting at 60°C to 50°C, 72°C 35 s), 40× (94 °C 15 s, 50°C 20 s, 72°C 35 s). 2nd round PCR same condition as the 1st round without reverse transcription.

**Statistics.** A two-tailed t-test with equal variance (www.openepi.com) was used to correlate CCHFV seroprevalence in different bat species with designated features (age: adult/subadult versus juvenile; dietary: frugivorous versus insectivorous; gender: male versus female; migration: migratory versus resident; roosting: foliage versus cave-living; seasonality: wet versus dry). For all calculations only bat species with a positive CCHFV-IIFT result were included.

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**Author Contributions**

M.A.M., S.D., F.W., J.F.D., E.M.L. and C.D. designed and conceived the work. M.A.M., S.D., E.L., V.M.C., G.D.M., F.G.-R., T.B., P.V., P.E., V.M.C., M.T., S.O., J.F.D., F.W. and E.M.L. carried out the experiments and provided essential material. M.A.M. and C.D. wrote the main manuscript text. All authors reviewed the manuscript.

**Additional Information**

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