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# Serological and molecular study of Crimean-Congo Hemorrhagic Fever Virus in cattle from selected districts in Uganda

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ABSTRACT

*Background:* Crimean-Congo Hemorrhagic Fever (CCHF) is a severe tick-borne viral hemorrhagic disease caused by Crimean-Congo Hemorrhagic Fever Virus (CCHFV) that poses serious public health challenges in many parts of Africa, Europe and Asia.

*Methods:* We examined 500 cattle sera samples from five districts for CCHFV antibodies using in-house and commercially available (IDVet) ELISA, Immunofluorescent assay (IFA) and Real-time polymerase chain reaction (RT-PCR).

*Results:* 500 cattle (73.8 % females) were analyzed; CCHFV seropositivity was 12.6 % (n = 63) and 75.0 % (n = 375) with the in-house and IDVet ELISAs, respectively. Seropositivity was associated with geographical location, increasing age, being female, and having a higher tick burden. Twenty four out of the 37 (64.8 %) were seropositive for CCHFV using IFA and all were negative for virus on RT-PCR. The IFA results were more comparable to IDVet ($\kappa_{coefficient} = 0.88$, p = <0.01) than to in-house ($\kappa_{coefficient} = 0.32$, p = 0.02).

*Conclusions:* Our study confirmed the presence and high prevalence of anti−CCHF antibodies in cattle based on three methods from all the five study districts, confirming presence and exposure of CCHFV. Given the zoonotic potential for CCHFV, we recommend a multidisciplinary public health surveillance and epidemiology of CCHFV in both animals and humans throughout the country.

*Abbreviations:* ABTS, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); BSA, bovine serum albumin; CCHF, Crimean-Congo Hemorrhagic Fever; CCHFV, Crimean-Congo Hemorrhagic Fever Virus; CI, Confidence interval; $CO^2$, Carbon dioxide; DAPI, 4′,6-Diamidino-2-Phenylindole; DMEM, Dulbecco's modified Eagle's medium; DRC, Democratic Republic of the Congo; EDTA, Ethylenediaminetetraacetic acid; ELISA, Enzyme-linked immunosorbent assay; IFA, Immunofluorescent assay; IgG, Immunoglobulin G; IgM, Immunoglobulin M; MOI, Multiplicity of infection; NaCl, Sodium chloride; OD, Optical density; ODSum, Sum of optical density; OR, Odds ratio; PBS, Phosphate buffered saline; R&D, Research and Development; RNA, Ribonucleic acid; RT-PCR, Real Time Polymerase Chain Reaction; RT-PCR, Reverse transcriptase polymerase chain reaction; SST, Serum separation tubes; UNCST, Uganda National Council for Science and Technology; UVRI, Uganda Virus Research Institute; WHO, World Health Organization.

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## 1. Introduction

Crimean-Congo Hemorrhagic Fever (CCHF) is one of the most medically important tick-borne diseases of humans, manifesting as a fever, or general malaise, but often as a severe non-specific hemorrhagic fever with up to 30 %, or higher, case fatality in hospitalized patients (Bente et al., 2013; Ergönül, 2006; Shayan et al., 2015). It is caused by Crimean-Congo Hemorrhagic Fever Virus (CCHFV), an enveloped, negative-sense RNA *Orthonairovirus* belonging to the *Nairoviridae* family (Abudurexiti et al., 2019). The Ixodid ticks are known to be the natural reservoirs, in which CCHFV is enzootically maintained via vertical and horizontal transmission. The virus circulates in several wild and domestic animals without causing overt disease, albeit with infectious viremia and a humoral response (Spengler et al., 2016b). Ixodid ticks are recorded in Uganda including *Hyalomma* species (Balinandi et al., 2020), which are thought to be the principal reservoir of the viral infections to humans through tick bites (Bente et al., 2013; Gargili et al., 2017). Humans are also occupationally exposed to CCHFV, especially abattoir workers through contact with blood and fluids of infected livestock, and frontline health workers who are exposed to the body fluids of infected patients (Bente et al., 2013).

Over the last few decades, the public health importance of CCHF has increased due to its epidemic potential (Mertens et al., 2013; Pigott et al., 2017), and its potential use in bioterrorism (Christian, 2013; Sidwell and Smee, 2003), yet safe or effective curative or prophylactic countermeasures are still lacking. Consequently, it was recently listed by World Health Organization (WHO) as one of the emerging infectious diseases that require accelerated efforts in Research and Development (R&D), including public health preparedness for outbreaks (Mehand et al., 2018). At present, it is a priority disease in many countries; all human outbreaks are mandatorily reported under the International Health Regulations (2005) as events that constitute a potential Public Health Emergency of International Concern (Kasolo et al., 2013; Kebede et al., 2010; Mamuchishvili et al., 2015; Anna. Papa, 2017). Indeed, since 2013, Uganda initiated an enhanced national-wide Laboratory-based surveillance system for viral hemorrhagic fevers in humans, including CCHF (Borchert et al., 2014; Shoemaker et al., 2018). The surveillance system has led to timely detection of sporadic CCHF outbreaks in humans (Balinandi et al., 2018; Kizito et al., 2018; Shoemaker et al., 2018), most of them occurring in the central region of Uganda.

To further understand the occurrence of CCHF outside the currently known range of human cases within Uganda, we performed a serological and molecular survey of CCHF using cattle samples collected from selected districts that are located outside the known range of human cases. The approach of using serological surveillance in animals to elucidate the circulation of CCHF in areas is highly recommended (Spengler et al., 2018, 2016) and is presently utilized in several Euro-Asian countries such as Italy, Romania and India (Ceianu et al., 2012; De Liberato et al., 2018; Mourya et al., 2015). This approach is also recommended in the roadmap for achieving the WHO's R&D blueprint for CCHF (Mehand et al., 2018). With the high burden of undiagnosed acute febrile illnesses in Uganda (Lamorde et al., 2018), such data is required to localize CCHF foci in order to inform advocacy and prioritization of intervention strategies such as enhanced community surveillance, public mobilization and laboratory capacity strengthening in the hotspot areas. Since significant differences in the accuracy of different CCHF diagnostic tests was recently identified (Escadafal et al., 2012; Spengler et al., 2016b; Vanhomwegen et al., 2012), the use of combination testing is recommended (Anna Papa et al., 2015). Thus, to increase the reliability of our study findings, we used a combination of assays, in parallel, that included an in-house ELISA, a commercially-available ELISA, an immunofluorescent assay (IFA) and RT-PCR.

## 2. Materials and methods

### 2.1. Study areas and design

This study was carried out using a cross-sectional design by collecting ticks and blood samples from cattle in five districts of Uganda between September and November 2017. The 5 districts of Kasese, Hoima, Gulu, Soroti and Moroto were selected as study areas based on the mapping of the Ugandan agro-ecological zones (Drichi and National Biomass, 2003) and the broad direction of the 'Cattle Corridor' zone (Fig. 1). Importantly, none of these districts had a history of human CCHF at the time this study was conducted.

Briefly, Kasese and Moroto districts have a semi-arid climatic environment and represent the extreme ends of the Ugandan livestock farming borders. In addition, both districts have large expanses of land under wildlife conservation, so interactions between domestic and wild animals is common. Soroti and Gulu districts lie within a semi-moist zone with scattered mixed agricultural practices. Hoima lies within low to medium altitudes where extensive and commercialized agricultural livestock farming is practiced.

A multi-stage sampling strategy was used in which two sub-counties were selected from each district based on environmental diversity and differences in animal management practices. Thereafter, we randomly selected one parish from each sub-county based on the sampling frame provided by the local administrators. From each parish, five villages were identified based on geographical spread. Five households with cattle were then selected from each village based on convenience and willingness of the farmer to participate in the study. Finally, from each household, two animals were selected for collection of ticks and blood, based on either a random, purposive or convenience choice.

### 2.2. Household, herd management and animal data collection

We administered a structured questionnaire to collect information on CCHF predisposing risk factors such as management practices (e.g. herd size, methods of acaricide application, types of acaricides used, and animal movement) and individual animal attributes (e.g. age, sex, tick burden, animal's previous and current health status). Herd management information was obtained by interviewing the owners, whereas animal characteristics and risk factor attributes were assessed by a trained veterinary officer, who was part of the field investigation team.

### 2.3. Serological investigation for CCHF in cattle

From each selected animal, about 4 mL of whole blood was collected from the jugular or tail vein into Vacutainer Serum Separation Tubes (BD Vacutainer® SST™, Oakville, ON). Serum aliquots were processed by centrifugation on the same day, taking into consideration all the necessary biosafety precautions. Thereafter, all samples were stored in liquid nitrogen until they were delivered to Uganda Virus Research Institute (UVRI), Entebbe, Uganda, where they were kept at −80 °C until testing. The UVRI hosts the National Reference Laboratory for the surveillance and diagnostics of viral hemorrhagic fevers in Uganda and routinely tests for Ebola, Marburg, Rift valley fever and CCHF (Balinandi et al., 2018; Shoemaker et al., 2018)

Currently, there are no 'gold standard' or widely available and approved assays for the serological detection of CCHF in animal samples. Thus far, many studies have used in-house developed protocols, or modified protocols that were developed for human samples (Adam et al., 2013; Maiga et al., 2017; Schuster et al., 2016). In this study, we used a recently developed CCHF double antigen multi-species commercial ELISA kit (IDScreen®, CCHF Double Antigen Multispecies, IDVet, Grabels, France) for the detection of antibodies against CCHF in serum of cattle (Sas et al., 2018). According to the manufacturer, the CCHF double antigen ELISA has a specificity of 100 % and sensitivity of 99 % based on 268 positive reference sera used for validation. This ELISA kit is

based on recombinant N-protein of the IbAr10200 CCHFV strain and is safe for working under standard biosafety conditions. In addition, we used an in-house anti−CCHFV specific Immunoglobulin G (IgG) ELISA that was previously developed for human CCHF diagnosis (Bryan et al., 1996), but has since been modified and used elsewhere for over 20 years in similar animal CCHF serosurveys (Adam et al., 2013; Ibrahim et al., 2015; Mariner et al., 1995).

For the purposes of research and assay development, we further tested a proportion of randomly selected samples with an in-house immunofluorescent assay (IFA) and RT-PCR. The IFA was previously developed and used on human samples (Burt et al., 1994) but is being customized for animal samples, together with the PCR assay (unpublished), at Karolinska Institute and Karolinska University Hospital laboratories, Sweden. Both methods were used in this study.

#### 2.3.1. The ID screen® CCHF double antigen multi-species (IDVet) ELISA procedure

All test procedures were performed according to the manufacturer's instructions (Sas et al., 2018). Briefly, 30 µL of each test sample or control was prediluted with 50 µL of the kit diluent in a separately available 96-well Microtiter dilution or processing plate (Thermo Scientific) before the mixture was transferred to the antigen coated plate and incubated for 45 min at 25 °C. After a washing procedure, 50 µL of conjugate was added to each test well, followed by incubation for 30 min at 25 °C. A second washing procedure was then performed and 100 µL of substrate was added, followed by incubation for 15 min at 25 °C before stopping the reaction. An optical density value for each test sample or control was determined spectrophotometrically at 450 nm. Thereafter, determination of cut-off for sero-positivity or -negativity for CCHF was performed according to the kit criteria.

#### 2.3.2. In-house anti−CCHFV specific IgG ELISA procedure

The procedures used in the in-house assay for the detection of anti−CCHF specific IgG in serum were as previously described and used in similar studies (Adam et al., 2013; Bryan et al., 1996; Mariner et al., 1995). Reagents used in this procedure: CCHF capture antibody, antigen and serum controls, were supplied by the Special Pathogens Branch of the US Centers for Disease Control and Prevention (CDC), as part of its efforts to develop UVRI's capacity to diagnose viral hemorrhagic fevers (Borchert et al., 2014; Shoemaker et al., 2018). Briefly, 96-well Microtiter plates (Thermo Electron Corporation, Milford, MA) were coated with 100 µL/well of a mouse-derived CCHF capture antibody that was prediluted 1 in 1000 with serum diluent (5% w/v goat skim milk in PBS; pH = 7.4). After an overnight incubation at 4 °C, plates were washed 3 times with 250 µL/well of wash buffer (PBS containing 0.1 % Tween-20 v/v), followed by addition of 100 µL/well of a CCHF antigen in the upper half of the plate, and a mock (control) antigen in the lower half of the plate. After 1 h of incubation at 37 °C, plates were washed with 250 µL/well of wash buffer before 33 µL of serum diluent were delivered to all wells. Thereafter, test or control sera were prediluted by adding 42 µL to 1000 µL (or 1 in 25) of serum diluent before 33 µL of each diluted sample or control was delivered in duplicates to the plates, with one part added to the antigen half and another to the control half. These aliquots were then subjected to serial 4-fold titrations on the plate, thus making the first and last dilutions in both the antigen or control halves a 1 in 100 and 1 in 6,400, respectively. After a 1 h incubation at 37 °C, plates were washed and 100 µL of rabbit anti-bovine IgG conjugated with horseradish peroxidase (KPL, Gaithersburg, MD) were added to the test wells at a dilution of 1 in 1000 and incubated for 1 h at 37 °C. The plates were then washed and incubated for 30 min at 37 °C with 100 µL/well of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) substrate (KPL, Gaithersburg, MD), before they were read spectrophotometrically at 490 nm. A sum optical density (ODSum) for each test serum was obtained by adding the differences between the OD values of the control antigen-coated wells from their corresponding CCHF-antigen-coated wells. A positive diagnosis for CCHF IgG in the respective test serum was scored if its ODSum was ≥0.95.

#### 2.3.3. Immunofluorescent assay (IFA)

After performing the IDVet and in-house ELISA assays, a sub-set of 37 samples were randomly selected within the groups; 1) highly positive on both (n = 5); 2) medium positive on both (n = 5); 3) highly positive by IDVet ELISA and negative by in-house ELISA (n = 6); 4) medium positive by IDVet ELISA and negative by in-house ELISA (n = 5); and 5) weakly positive by IDVet ELISA and negative by in-house ELISA (n = 5). Samples that were negative on both assays were also included as negative controls (n = 10). In addition, the only sample that was positive by in-house ELISA and negative by IDVet ELISA was included in test samples.

The IFA method we used in this investigation is generally as previously described by Burt et al. (1994), with slight modifications. Briefly, Vero cells (ATCC, Middlesex, UK) were seeded in a T75 flask for 24 h in Dulbecco's Modified Eagle's Medium (DMEM; Thermofisher) supplemented with 1% non-Essential Amino-Acid (Thermofisher), 10 mM Hepes (Thermofisher) and 10 % fetal bovine serum (FBS). After 24 h of incubation at 37 °C in 5% $CO_2$, cells were infected with CCHFV isolate (Ibar10200 strain, GenBank Accession Number NC005302) in DMEM (without FBS) for 1 h at 37 °C and 5% $CO_2$ at a Multiplicity of infection (MOI) of 1. Thereafter, the inoculum was removed and 10 mL of fresh DMEM 10 %FBS was added. After 24 h of post-infection, cells were detached by incubating for 5 min at 37 °C using 3 mL/well of trypsin (in 0.25 % EDTA, v/v). Thereafter, 40 µL/well of cell soup were seeded in 12-wells microscope slides (Novakemi, Sollentuna, Sweden). Cells were incubated for 4 h at 37 °C in a wet chamber to allow attachment. Once cells were attached, the medium was removed and the cells were immediately washed in NaCl, then rinsed by immersing in a room-temperature water bath before being fixed using pure acetone for 30 min. Slides were then dried and kept at −80 °C until used. To test samples, slides were washed once with PBS and further incubated with 20 µL of 5% IgG-free BSA (Jackson ImmunoResearch, PA, USA) for 1 h at 37 °C. Cells were then washed 3 times and incubated with 20 µL of serum sample prediluted 1–2 in 0.2 % IgG-free BSA for 1 h at 37 °C. Thereafter, cells were washed 3 times with PBS and then incubated for 1 h at 37 °C with Alexa Fluor 488-conjugated AffiniPure Rabbit Anti-Bovine IgG (1/800; Jackson ImmunoResearch, PA, USA) diluted in 0.2 % IgG-free BSA. Cells were washed 3 times with PBS and allowed to dry. Slides were then mounted using ProLong Diamond Antifade Mountant containing DAPI (Thermofisher). Slides were sealed with CoverGrip Coverslip Sealant (Biotium, CA, USA) and then dried for 15 min. Fluorescence was detected using a fluorescence microscope (Leitz Laborlux K). When no signal was detected, samples were considered as negative. If a strong signal was detected, samples were considered as strongly positive or weakly positive with a weak signal.

### 2.4. RT-PCR targeting CCHFV

Real-time polymerase chain reaction (RT-PCR) was performed in order to detect any CCHF viral particles in the serum samples. To this end, 200 µL of each serum sample were inactivated by addition of 600 µL of Trizol (Thermofisher). Ribonucleic acids (RNA) were then extracted using Directzole RNA extraction kit (Zymo Research, CA, USA). Thereafter, RT-PCR was performed using a TaqMan Fast Virus 1-step Master Mix (Thermofisher) and primers and probes for amplification of a segment of the CCHFV L-sequence (Forward: GCCAACTGTGACKGTKTTCTAYATGCT; Reverse 1: CGGAAAGCCTATAAAACCTACCTTC; Reverse 2: CGGAAAGCCTATA AAACCTGCCYTC; Reverse 3: CGGAAAGCCTAAAAAATCTGCCTTC; Probe : FAM-CTGACAAGYTCAGCAAC-MGB) on a LightCycler® (Roche) under the following conditions: reverse transcription, 10 min at 50 °C; denaturation, 2 min at 95 °C; 45 cycles of amplification (at 95 °C for 10 s, 60 °C for 40 s). Any sample with a sigmoid curve was interpreted as positive, otherwise it was considered to be negative.

### 2.5. Compilation of human CCHF data

We obtained data on the geographical locations of previously reported human CCHF cases in Uganda from literature (Balinandi et al., 2018; Kizito et al., 2018; Messina et al., 2015; Shoemaker et al., 2018) as well as from the national Viral Hemorrhagic Fevers database at UVRI. They were mapped to show their distribution with reference to the location of the study areas, as shown in Fig.1. The intention was to show whether the presence or absence of human CCHF cases in the areas could be related to our study findings.

### 2.6. Data analysis

Data were analyzed using multiple logistic mixed models; modelling the prevalence of CCHF as determined by the in-house ELISA and the IDVet ELISA, respectively. For IFA and RT-PCR generated data, only a simple Cohen's kappa comparison between assays was done (Altman, 1991; Kwiecien et al., 2011), as these assays are 'prerelease' and still undergoing development.

Geographical location, tick burden estimates on the animal at time of sampling, as well as the sex and age of the animal, were used as explanatory variables. The hierarchical structure of data collection was accounted for by including the random factors subcounty and household within subcounty. In an additional analysis, the variable 'Assay agreement', defined as the case when both methods showed concordant positive or negative results, was modelled using the same explanatory variables and the same model structure as above. This variable coincided substantially with when positive results were registered for the in-house method.

Odds ratios including 95 % confidence intervals were computed. Results were considered significant at the 5% level.

## 3. Results

A total of 500 cattle (73.8 % females and 26.2 % males) were sampled from 250 herds across the 5 study districts. Almost 90 % of the animals were obtained from the indigenous breeds of Uganda: Zebu (64.8 %) and Ankole (29.7 %). The average herd size was 25 animals, with varying animal management practices: free-range or communal grazing (62.4 %), tethering (32.0 %) and paddocking (14.0 %). The animals' median age was 3.5 years (Range = <1 - 12 years) with females generally being older than males (median age was 4 years for females vs. 2 years for males). There was an average of 61 ticks per animal. Only 8.0 % of herd owners said their animals often graze with wild animals such as elephants, impala and zebra. The main baseline characteristics of the sampled animals are summarized in Table 1.

The overall seropositivity for CCHFV antibodies was 12.6 % (n = 63; 95 % CI: 9.9–15.9) with the in-house ELISA and 75.0 % (n = 375; 95 % CI: 70.9–78.7) with IDVet ELISA respectively. We found higher seropositivity for CCHFV antibodies using commercial IDVet ELISA (Kasese, 90 %; Soroti, 83 %; Gulu, 80 %; Hoima, 74 % and Moroto, 48 %) compared to the in-house ELISA (Kasese, 14 %; Soroti, 18 %; Gulu, 13 %; Hoima, 14 % and Moroto, 4%). Further, all samples except one that were positive on in-house ELISA were also positive with the commercial IDVet ELISA kit. Thus, a significant difference in animal CCHF seroprevalence levels between the studied districts in both the in-house assay ($\chi^2 = 10.27$, $p = 0.036$) and IDVet kit ($\chi^2 = 27.50$, $p = <0.001$) was observed. Geographical locations for cattle that were positive for both in-house and the commercial IDVet assays are geospatially presented in Fig.1.

Map of Uganda showing the location of study districts and Crimean-Congo Hemorrhagic fever (CCHF) serological outcomes using the IDVet (A) and in-house (B) ELISA, in relation to the occurrence of previous human cases of CCHF (Source: this map was created using open source data in ArcGIS software, v10.2, Environmental Systems Research Institute, Inc., Redlands, CA, USA). *Data was obtained from National Viral Hemorrhagic Fevers database at Uganda Virus Research Institute, Entebbe, Uganda and others (Balinandi et al., 2018; Kizito et al., 2018; Shoemaker et al., 2018); **Additional data was obtained from the global compendium of human CCHF cases (Messina et al., 2015)

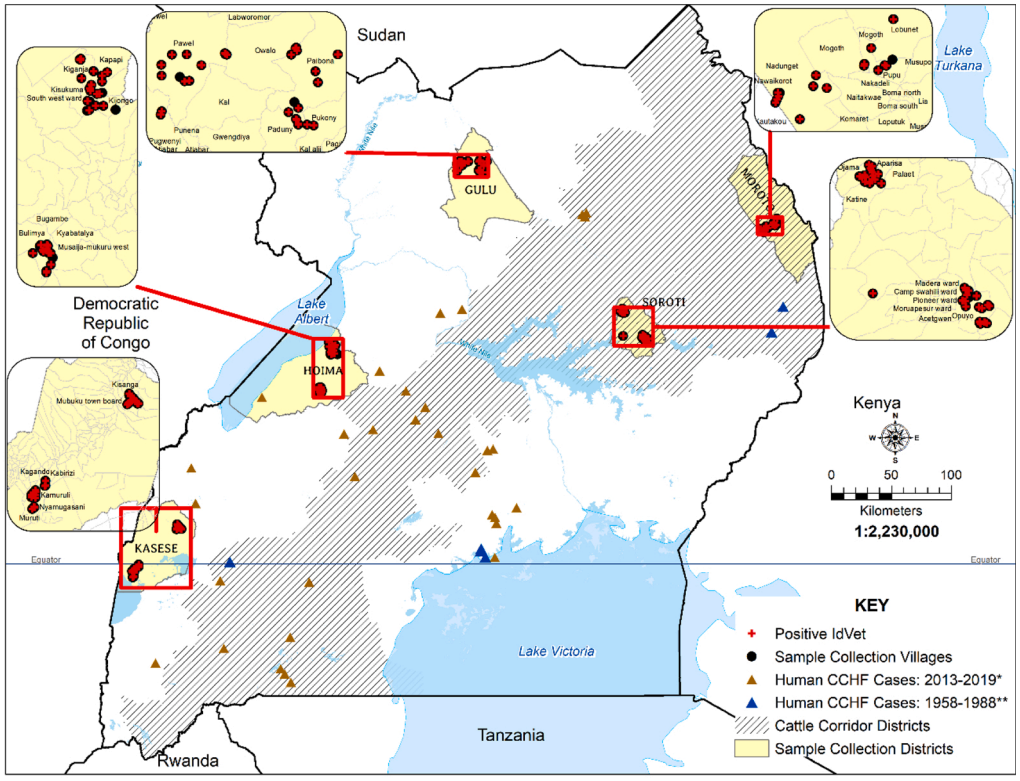
Using a multiple logistic regression analysis as shown in Table 2, animal CCHF seropositivity (based on where there was Assay

**Table 1**
Animal Baseline Characteristics and Crimean Congo Hemorrhagic Fever Serological Outcomes, Uganda, 2017.

| | | | In-house Assay | | IDVet Assay | | Assay Agreement | |
|---|---|---|---|---|---|---|---|---|
| Animal Characteristics | | Frequency (%) | Negatives (%) | Positives (%) | Negatives (%) | Positives (%) | Negatives (%) | Positives (%) |
| District | Moroto | 100 (20.0) | 96 (96.0) | 4 (4.0) | 52 (52.0) | 48 (48.0) | 51 (51.0) | 2 (2.0) |
| | Gulu | 100 (20.0) | 87 (87.0) | 13 (13.0) | 20 (20.0) | 80 (80.0) | 20 (20.0) | 13 (13.0) |
| | Hoima | 100 (20.0) | 86 (86.0) | 14 (14.0) | 26 (26.0) | 74 (74.0) | 26 (26.0) | 14 (14.0) |
| | Kasese | 100 (20.0) | 86 (86.0) | 14 (14.0) | 10 (10.0) | 90 (90.0) | 10 (10.0) | 14 (14.0) |
| | Soroti | 100 (20.0) | 82 (82.0) | 18 (18.0) | 17 (17.0) | 83 (83.0) | 17 (17.0) | 18 (18.0) |
| Sex | Male | 130 (26.2) | 125 (96.2) | 5 (3.8) | 43 (33.1) | 87 (66.9) | 43 (33.7) | 5 (3.8) |
| | Female | 366 (73.8) | 309 (84.4) | 57 (15.6) | 82 (22.4) | 284 (77.6) | 81 (22.1) | 55 (15.0) |
| Breed | Zebu | 320 (64.8) | 101 (31.6) | 219 (68.4) | 101 (31.6) | 219 (68.4) | 100 (31.2) | 32 (9.7) |
| | Ankole | 147 (29.7) | 20 (13.6) | 127 (86.4) | 20 (13.6) | 127 (86.4) | 20 (13.6) | 22 (15.0) |
| | Others[a] | 27 (5.5) | 21 (77.8) | 6 (22.2) | 4 (14.8) | 23 (85.2) | 4 (14.8) | 6 (22.2) |
| Age (Yrs.) | ≤5 | 400 (80.6) | 351 (87.7) | 49 (12.3) | 116 (29.0) | 284 (71.0) | 115 (28.8) | 47 (12.0) |
| | >5 | 96 (19.4) | 83 (86.5) | 13 (13.5) | 9 (9.4) | 87 (90.6) | 9 (9.4) | 13 (13.5) |
| Animal Management practices[b] | Communal grazing | 312 (62.4) | 269 (86.2) | 43 (13.8) | 84 (26.9) | 228 (73.1) | 83 (26.6) | 41 (13.1) |
| | Tethering | 160 (32.0) | 142 (88.8) | 18 (11.2) | 36 (22.5) | 124 (77.5) | 36 (22.5) | 18 (11.2) |
| | Paddocking | 70 (14.0) | 62 (88.6) | 8 (11.4) | 14 (20.0) | 56 (80.0) | 14 (20.0) | 8 (11.4) |
| Health Condition | Good | 391 (78.8) | 347 (88.7) | 44 (11.3) | 108 (27.6) | 283 (72.4) | 107 (27.3) | 43 (11.0) |
| | Sickly | 105 (21.2) | 87 (82.9) | 18 (17.1) | 17 (16.2) | 88 (83.8) | 17 (16.2) | 17 (16.2) |
| Body weight (Kgs.) | ≤100 | 77 (15.5) | 72 (93.5) | 5 (6.5) | 33 (42.9) | 44 (57.1) | 32 (41.5) | 4 (12.5) |
| | 101 - 300 | 254 (51.2) | 226 (89.0) | 28 (11.0) | 75 (29.5) | 179 (70.5) | 75 (29.5) | 28 (11.0) |
| | >300 | 165 (33.3) | 136 (82.4) | 29 (17.6) | 17 (10.3) | 148 (89.7) | 17 (10.3) | 28 (17.0) |
| Rectal temperature | <37.0 | 37 (8.7) | 33 (89.2) | 4 (10.8) | 4 (10.8) | 33 (89.2) | 4 (10.8) | 4 (10.8) |
| | 37.0 – 39.0 | 354 (83.3) | 311 (87.9) | 43 (12.1) | 101 (28.5) | 253 (71.5) | 100 (28.2) | 41 (11.6) |
| | >39.0 | 34 (8.0) | 29 (85.3) | 5(14.7) | 10 (29.4) | 24 (70.6) | 10 (29.4) | 5 (14.7) |
| Tick burden Estimate | =<50 | 400 (81.1) | 356(89.0) | 44 (11.0) | 109 (27.2) | 291 (72.8) | 108 (27.0) | 42 (10.5) |
| | 51 - 100 | 77 (15.6) | 65 (84.4) | 12(15.6) | 13 (16.9) | 64 (83.1) | 13 (16.8) | 12 (15.6) |
| | >100 | 16 (3.2) | 10(62.5) | 6 (37.5) | 2 (12.5) | 14 (87.5) | 2 (12.5) | 6 (37.5) |

[a] Cross and Exotic breeds.
[b] Some herds practiced a combination of animal management methods.

A (IDVet assay positives)




B (In-house assay positives)

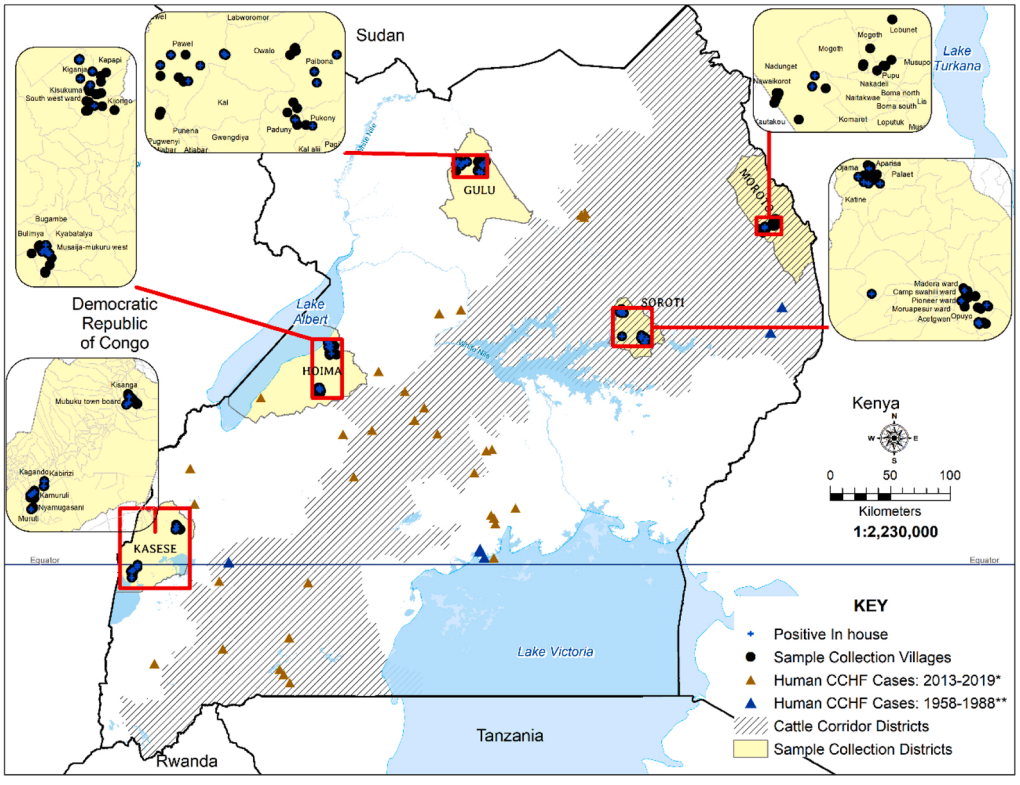



**Fig. 1.** A (IDVet assay positives), B (In-house assay positives).

**Table 2**
Multiple logistic regression analysis for association between Animal Characteristics and Crimean Congo Hemorrhagic Fever Serological Outcomes, Uganda, 2017.

| Variable | | Negative (%) | Positive (%) | Estimate | OR | CI (95 %) | p-value |
|---|---|---|---|---|---|---|---|
| A: In-house Assay | | | | | | | |
| Tick-burden[a] | | | | 0.01 | 1.01 | 1.003–1.021 | 0.01 |
| Age[a] | | | | 0.16 | 1.18 | 1.00–1.39 | 0.05 |
| Sex | Male | 125 (96.2) | 5 (3.8) | Ref | | | |
| | Female | 309 (84.4) | 57 (15.6) | 1.78 | 5.93 | 2.05–17.41 | <0.01 |
| District[b] | Moroto | 96 (96.0) | 4 (4.0) | Ref | | | |
| | Gulu | 87 (87.0) | 13 (13.0) | 1.33 | 3.78 | 1.02–14.27 | 0.05 |
| | Hoima | 86 (86.0) | 14 (14.0) | 1.55 | 4.71 | 1.33–16.96 | 0.02 |
| | Kasese | 86 (86.0) | 14 (14.0) | 0.75 | 2.12 | 0.58–7.82 | 0.26 |
| | Soroti | 82 (82.0) | 18 (18.0) | 1.92 | 6.82 | 1.87–24.76 | <0.01 |
| B: IDVet Assay | | | | | | | |
| Tick-burden[a] | | | | 0.01 | 1.01 | 0.999–1.024 | 0.08 |
| Age[a] | | | | 0.59 | 1.81 | 1.48–2.22 | <0.01 |
| Sex | Male | 43 (33.1) | 87 (66.9) | Ref | | | |
| | Female | 82 (22.4) | 284 (77.6) | 0.47 | 1.60 | 0.85–3.03 | 0.15 |
| District[b] | Moroto | 52 (52.0) | 48 (48.0) | Ref | | | |
| | Gulu | 20 (20.0) | 80 (80.0) | 1.40 | 4.07 | 1.58–10.46 | <0.01 |
| | Hoima | 26 (26.0) | 74 (74.0) | 1.79 | 5.96 | 2.40–14.79 | <0.01 |
| | Kasese | 10 (10.0) | 90 (90.0) | 2.31 | 10.06 | 3.20–31.60 | <0.01 |
| | Soroti | 17 (17.0) | 83 (83.0) | 2.50 | 12.12 | 4.35–33.80 | <0.01 |
| C: Assay Agreement | | | | | | | |
| Tick-burden[a] | | | | 0.01 | 1.01 | 1.003–1.019 | <0.01 |
| Age[a] | | | | 0.16 | 1.17 | 1.01–1.36 | 0.03 |
| Sex | Male | 43 (33.7) | 5 (3.8) | Ref | | | |
| | Female | 81 (22.1) | 55 (15.0) | 1.64 | 5.16 | 1.94–13.85 | <0.01 |
| District[b] | Moroto | 51 (51.0) | 2 (2.0) | Ref | | | |
| | Gulu | 20 (20.0) | 13 (13.0) | 1.52 | 4.57 | 1.21–17.42 | 0.02 |
| | Hoima | 26 (26.0) | 14 ((14.0) | 1.77 | 5.87 | 1.62–21.63 | <0.01 |
| | Kasese | 10 (10.0) | 14 (14.0) | 1.00 | 2.72 | 0.71–10.48 | 0.14 |
| | Soroti | 17 (17.0) | 18 (18.0) | 2.07 | 7.92 | 2.19–28.81 | <0.01 |

[a] analyzed as a continuous variable.
[b] A chi-square test comparison for CCHF seroprevalence between study districts was significantly different as follows; in-house assay ($\chi^2 = 10.27$, $p = 0.036$); IDVet Assay ($\chi^2 = 27.50$, $p = <0.001$) and Assay Agreement ($\chi^2 = 12.87$, $p = 0.012$).

agreement), was significantly associated with being female (OR = 5.16; p = <0.01), increasing age (OR = 1.170; p = 0.03), tick burden estimate (OR = 1.01; p = <0.01), and having been sampled from the districts of Gulu (OR = 4.57; p = 0.02), Hoima (OR = 5.87; p = <0.01) and Soroti (OR = 7.92; p = <0.01) compared to the district of Moroto.

Results from the IFA analyses were more in agreement with the IDVet assay than the in-house assay. Altogether, 24 out of the 37 samples tested on IFA were positive for CCHF viral antibodies. On the one hand, all IDVet results, except for two samples that had been categorized as medium and weakly positive, were reconfirmed by the IFA method (Cohens kappa $\kappa_{coefficient} = 0.88$, p = <0.01). On the other hand, discordance was observed in 14 samples when the in-house assay was compared with IFA ($\kappa_{coefficient} = 0.32$, p = 0.02); 13 samples that were initially negative on the in-house assay were positive on IFA, whereas the only sample that was positive by in-house and negative by IDVet was also negative on IFA. Using qRT-PCR, all the 37 samples tested on IFA were negative for CCHFV.

## 4. Discussion and conclusions

The main objective of this study was to establish the prevalence of CCHFV and viral antibodies in cattle from five selected districts of Uganda using both serological and molecular methods. Overall, we report seroprevalence for CCHF viral antibodies as 12.6 % and 75.0 %, using the in-house and IDVet assays, respectively, with cattle samples. Our findings present new information on CCHFV exposure in cattle in Uganda, where such high prevalence levels were previously unknown. Globally, seroprevalence for CCHF in domestic and wild animals ranging from less than 1%–80 %, has been reported, depending on the animal species being tested, the test method used and geographical location of the study area (Spengler, Bergeron, et al., 2016). In a recent study, Mangombi et al. (2020) found a 57.1 % CCHF seroprevalence level in cattle from Senegal. Similarly, in two separate studies done in Sudan, Adam et al. (2013) and Ibrahim et al. (2015) found seroprevalence for CCHF virus in cattle to be 7.0 % and 19.1 %, respectively. While Maiga et al. (2017) found a seroprevalence range of 13–95 % in Malian cattle. In brief, the CCHF seroprevalence levels observed in this study were comparable to findings from elsewhere.

From this study, seropositivity for CCHF was high and was associated with geographical (district) location, the sex, and age of the host, as well as the number of ticks found on the animal at the time of the study. The difference in geographical seroprevalence may be typical of vector-borne diseases where the intensity of the vector and other socio-ecological dynamics play a major role (Colwell et al., 2011; Morse, 1995). In this study, we simultaneously collected specimens of ticks from the same animals and we reported 8 *Rhipicephalus* species, 5 *Amblyomma* species and 2 *Hyalomma* species as being widely distributed in the same study areas (Balinandi et al., 2020). Notably, Moroto district which had the lowest CCHF seroprevalence in both assays, presented with the highest tick diversity and the only district in which *Hyalomma spp.* were recorded. Similarly, previous studies that involved extensive tick surveys in Uganda, did not find *Hyalomma spp.* outside the eastern and north-eastern regions (Matthysee and Colbo, 1987; Walker et al., 2014). Given that *Hyalomma spp.* are considered as the main tick vectors and natural reservoirs for CCHFV (Hoogstraal, 1979), although these ticks were not present in all study areas where CCHFV circulation was evident, we hypothesize that another tick vector may be involved in CCHF epidemiology in Uganda. Moreover, in a previous, albeit limited investigation, CCHFV was detected by PCR in *Rhipicephalus decoloratus* collected from central Uganda (Balinandi et al., 2018). This finding requires further investigation. In his review of global CCHF epidemiology, Hoogstraal previously reported the presence of CCHF virus in several tick genera (Hoogstraal, 1979), although available data on their vectorial capability for CCHFV remains limited (Gargili et al., 2017).

The relationship between CCHF seropositivity and other animal factors, such as increasing age, breed and tick burden, is consistently

reported and discussed elsewhere (Adam et al., 2013; Barthel et al., 2014; Ibrahim et al., 2015). However, from this present study, we observed that female animals had a significant higher seroprevalence than their male counterparts, contrary to observations in Senegal and Sudan (Adam et al., 2013; Wilson et al., 1990). Although male and female animals would be equally infected by ticks, the observed difference in CCHF seroprevalence levels between the female and male animals in this study can be attributed to several factors. First, there was a disproportionately high number of female cattle sampled in the study than male, which could have resulted from a selection bias that ultimately misconstrues findings in epidemiological studies (Delgado-Rodríguez and Llorca, 2004). Second, higher seroprevalence of CCHF in female cattle could be due to females being kept longer on the farms than males, perhaps for breeding purposes before they are sold off for meat. This is likely to increase their lifetime risk to tick bites and hence CCHF infection. In fact, as a consequence, this made it more difficult to separate the effect of age from sex in this study. As shown in Table 2, age, but not sex, was significant in the model for IDVet assay, while the opposite was true in the model for the in-house assay. Third, it is worth noting the recent reports about possible sexual transmission of CCHF among humans (Ergonul and Battal, 2014; Pshenichnaya et al., 2016), with no available data inferred to animal populations. However, if this is true for animals, it means one bull can potentially transmit CCHFV to several females, creating an unequal level of infection between the two sexes, as in this study.

There was a significant difference between the seroprevalence in the two assays; the IDVet assay seroprevalence level was 6 times higher than that of the in-house assay ($X^2 = 21.1$; $p = <0.001$). Further analysis using the validated IFA technique on a proportion of samples that had tested positive and negative on both in-house and IDVet Assays confirmed our results of CCHF seropositivity. We report higher concordance levels observed between the IFA and IDVet assays, than between the IFA and in-house assays. We suggest that the recently developed CCHF double antigen multi-species commercial ELISA kit (IDVet) has good performance, as reported by manufacturer and by our results, and hence can be used for the routine surveillance of CCHFV exposure in high risk areas to guide appropriate interventions. IFA also showed good performance considering the findings from this pilot study. However, it is not as affordable and feasible as ELISA for sero-surveillance, particularly in low-resource settings. According to the categorization of concordance values between assays by Altman (Altman, 1991), the comparison between the IDVet assay and IFA was 'Very good', while it was only 'Fair' when the in-house assay was compared to IFA. However, the usage of the in-house assay is still valuable especially where accessibility of commercial kits is difficult and expensive. In a previous evaluation, Papa et al. (2014) observed that the in-house assay presented with less colorimetric background and hence a reduced number of false positive results, compared to commercial ELISA. Our in-house assay uses a natural protein as a coating antibody while IDVet assay uses a recombinant protein, which might explain the differences in the detection levels between the two assays. Indeed, the differences in the binding affinities of these 2 types of proteins has been noted previously (Abdelbaset et al., 2017; Garcia-Corbeira et al., 1999). Also, as described in the methods section, the two ELISA assays used in this study had differing test sample volumes, with the in-house assay using only 1 μL of the sample compared to 30 μL in the IDVet assay. This 30-times difference in the sample volumes means that the amount of detectable antibodies available in each assay were not the same, with perhaps lower antibody concentrations failing to be detected in the in-house assay than with the IDVet assay. Given the relatively high concordance between the two assays that utilize high sample volumes (IDVet ELISA and IFA), this hypothesis requires further investigation. Finally, it is important to note that the high genetic (and potentially antigenic) variability of CCHFV among geographical regions, as has been observed with PCR, hampers the universality of most CCHF diagnostic kits (Bente et al., 2013; Vanhomwegen et al., 2012).

Nevertheless, we consider our results to be indicative of potentially widespread CCHFV in the study areas and probably other regions of the country. Evidence of CCHFV antibodies in cattle was apparent in all study areas. The lack of detectable or active virus circulation by RT-PCR among the studied animals, especially in a cross-sectional study design such as this one, can be attributed to the low and short duration of viremia that is associated with animal infections (Spengler et al., 2016b).

To the best of our knowledge, this study constitutes the first report of CCHFV seroprevalence in cattle using a combination of methods and covering a large geographical area of Uganda. This study covered a stretch of over 700 km across Uganda (between Kasese and Moroto districts) which means that several million people in these areas may be potentially at risk of CCHF infection through tick bites and other risky occupations. In fact, one case of human CCHF was identified in 2018 in the Kikuube area, which at the time of our sample collection in 2017, was being carved out of Hoima district into the current Kikuube district (this human case is showed in Hoima district in Fig. 1). However, we could not determine the reasons for the current lack of human CCHF outbreaks in the rest of the study areas other than poor public health surveillance, in addition to poor vigilance by the clinicians to identify suspected cases due to co-circulating pathogens such as malaria, which can mimic CCHF (Tarantola et al., 2006). Our study provides evidence of CCHFV antibodies in cattle sampled from five districts; an indication of the presence, as well as probably a longer history of infections of cattle with the virus. Therefore, we hope our data will stimulate multidisciplinary public health efforts that can enhance surveillance for CCHFV in both animal and human populations. Such interventions would be required for the identification of undiagnosed human cases and to determine the true incidence and spread of human CCHFV infections in Uganda. More especially, a great effort should focus on the identification of the natural reservoirs of CCHFV in Uganda, given that the ecological distribution of the known tick vector species does not necessarily overlap with the occurrence of human CCHF in the country.

## Ethics approval and consent to participate

This study was duly approved by the School of Veterinary Medicine & Animal Resources Research Ethics Committee of Makerere University, Kampala, Uganda (Approval Number SVARREC/03/2017) and Uganda National Council for Science and Technology (Approval Number UNCST A580). In addition, a written consent permitting the use of the collected information and animal samples for research purposes, was obtained from the animal owners through a questionnaire form that was approved as part of the data collection tools.

## Availability of data and materials

The datasets used during the current study are available at the SciLifeLab Data Repository at: https://doi.org/10.17044/scilifelab.13564364

## Author agreement

I hereby certify that all authors have seen and approved the final version of the manuscript being submitted. The article is the authors' original work, hasn't received prior publication and isn't under consideration for publication elsewhere.

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## CRediT authorship contribution statement

**Stephen Balinandi:** Conceptualization, Investigation, Writing - original draft, Visualization. **Claudia von Brömssen:** Formal analysis, Writing - review & editing. **Alex Tumusiime:** Investigation. **Jackson Kyondo:** Investigation. **Hyesoo Kwon:** Methodology, Investigation, Writing - review & editing. **Vanessa M. Monteil:** Methodology, Investigation, Writing - review & editing. **Ali Mirazimi:** Conceptualization, Resources. **Julius Lutwama:** Resources. **Lawrence Mugisha:** Conceptualization, Investigation, Resources, Writing - review & editing, Supervision, Project administration. **Maja Malmberg:** Conceptualization, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

## Declaration of Competing Interest

All authors declare no competing interest.

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