University of Veterinary Medicine Hannover

Novel serological and molecular assays for Crimean‑Congo hemorrhagic fever virus infections and their application in prevalence studies on sub‑Saharan African countries

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To my family

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Manuscripts extracted from the doctorate project:

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2. Sas MA, Mertens M, Isselmou E, Reimer N, Mamy BOEL, Doumbia B, Groschup MH – Widespread Crimean-Congo Hemorrhagic Fever Virus Infections in Cattle in Mauritania; *Vector-Borne and Zoonotic Diseases* (under review).

3. Sas MA, Mertens M, Kadiat JG, Schuster I, Pongombo CPS, Maloba AGK, Groschup MH – Serosurvey for Crimean-Congo hemorrhagic fever virus infections in ruminants in Katanga province, Democratic Republic of the Congo; *Ticks and Tick-Borne Diseases* (under review).

4. Mertens M, Sas MA, Abel Wade A, Abah S, Namegni RP, Schuster I, Scheuch D, Staubach Ch, Unger H, Souley A, Groschup MH – Crimean-Congo hemorrhagic fever virus in Cameroon; this manuscript was prepared for submission.

5. Sas MA, Vina-Rodriguez A, Mertens M, Eiden M, Emmerich P, Chaintoutis SC, Mirazimi A, Groschup MH – A one-step multiplex real-time RT-PCR for the universal detection of all CCHFV genotypes; *Journal of Virological Methods* (to be submitted).

LIST OF ABBREVIATIONS

µg	microgram
µl	microliter
AGDP	agar gel diffusion precipitation
arbovirus	arthropod-borne virus
BATV	Batai virus
bp	base pair
BSL	biosafety level
BUNV	Bunyamwera virus
CAR	Central African Republic
CCHF	Crimean-Congo hemorrhagic fever
CCHFV	Crimean-Congo hemorrhagic fever virus
CCS	cell culture supernatant
cRNA	complementary RNA
Cy5	cyanine 5
DR Congo	Democratic Republic of the Congo
D-SN	diagnostic sensitivity
D-SP	diagnostic specificity
EBOV	Ebola virus
ELISA	enzyme linked immunosorbent assay
ER	endoplasmatic reticulum
FAM	carboxyfluorescein
Gc	C-terminal glycoprotein
Gn	N-terminal glycoprotein

GPC	glycoprotein precursor
G-protein	glycoprotein
HAZV	Hazara virus
HRP	horseradish peroxidase
IFA	immunofluorescence assay
IFN	interferon
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
kGy	kilo grey
KO	knock out
LOD	limit of detection
L-protein	large protein
L-segment	large segment
m	meter
ml	milliliter
mRNA	messenger RNA
M-segment	medium segment
nm	nanometer
NS_M	non-structural M-protein
NS_S	non-structural S-protein
N-protein	nucleocapsid protein
OD	optic density
PCR	polymerase chain reaction
pmol	picomole

PR China	People’s Republic of China
Pre_{Gc}	Gc precursor protein
Pre_{Gn}	Gn precursor protein
RdRp	RNA dependent RNA polymerase
RNA	ribonucleic acid
ROC	receiver operating characteristic
RTC	relative threshold cycle
RT-qPCR	quantitative reverse transcription polymerase chain reaction
ss(-)RNA	single stranded RNA of negative polarity
S-segment	small segment
STAT	Signal transducer and activator of transcription
TCS	tissue culture supernatant
TNF	tumor necrosis factor
UUKV	Uukuniemi virus
vRNA	viral RNA

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1 INTRODUCTION

Crimean-Congo hemorrhagic fever virus (CCHFV) belongs to the genus *Nairovirus*, one of five genera within the family *Bunyaviridae*. CCHFV is an arthropod-borne virus (arbovirus) transmitted by hard-bodied ticks many of which belong to the genus *Hyalomma*. Previous studies have shown that the virus occurrence correlates with the presence of *Hyalomma* ticks in Europe as well as in parts of Africa and Asia. Unfortunately, the CCHFV distribution and vector preferences in Africa are not very well studied.

Ticks are both vector and reservoir for CCHFV. Tick bites are the most likely route of infection for humans and animals. Other transmissions e.g. via contact to blood, other body fluids and tissues of infected animals or human patients are also possible and concern professional risk groups in particular. Humans can develop severe hemorrhagic fever with case fatality rates ranging from 5% (Turkey) up to 80% (People's Republic of China (PR China)), depending on the virus strain, general awareness of the population and quality of the public healthcare system. Treatment options for CCHFV are currently very limited and no therapy is available. CCHFV infections do not cause clinical signs in most animals, but the CCHFV antibody status in animals can be used as indicator for the presence of this virus in a country or area.

One major aim of this study was to develop validated reliable serological diagnostic methods for the detection of CCHFV infections in ruminants, which can also be used in African laboratories. The method of choice in CCHFV serology is enzyme linked immunosorbent assay (ELISA). While commercial kits are available for detecting human CCHFV-specific immunoglobulins only, no validated CCHFV‑ELISAs were available for animals until to date. Likewise an indirect immunofluorescence assay (IFA) was commercially available only for detecting human antibodies, but not for use in animals. In order to reach this goal, a commercial ELISA and IFA were modified to work with cattle sera from African countries. Moreover, a particular ELISA was developed which is suitable for application especially under tropical conditions. This assay shows an even improved diagnostic sensitivity (99%), whilst maintaining its diagnostic specificity (99%).

Another major aim of this thesis was the development of a novel real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) for reliable detection of all six currently known genotypes. RT-qPCR is a very important and time saving diagnostic technique for direct virus detection, especially since it does not require to work with highly pathogenic CCHFV (biosafety level (BSL) 4). The new RT-qPCR is both helpful for detecting human and animal CCHFV infections and molecular data can hence be used for epidemiology and risk assessment. RT-qPCR kits are commercially available from several companies but many have not been evaluated yet. Most of the assays detect a number of CCHFV strains but also miss some highly divergent CCHFV strains, such as the Greek strain AP92. Therefore, the primary aim of a newly developed RT-qPCR in this study was to detect all known genotypes of CCHFV with a high analytical sensitivity. This aim was reached by designing a broad range multiplex RT-qPCR employing 7 different primer sets (6 specific and 1 degenerated sets) and two different carboxyfluorescein (FAM) probes for the specific detection of CCHFV genome sequences. This assay was eventually validated using a broad set of synthetic ribonucleic acids (RNA) and full bunyaviruses.

The serological assays were subsequently used on more than 3,000 ruminant sera, which were collected in Cameroon, the Democratic Republic of the Congo (DR Congo), Mali and Mauritania in order to determine the CCHFV seroprevalence rates in these countries as a proxy for the putative infection risks for the human population. The lack of knowledge about CCHFV becomes especially apparent in Africa. Many African countries have not been surveyed for a long time or even at all. No data or clinical observations of Crimean-Congo hemorrhagic fever (CCHF) cases exist for Cameroon until to date. Therefore, testing cattle sera from Cameroon was of special interest for this study. Furthermore, it is a country with highly diverse vegetation zones, some of which providing very suitable vector habitats. In the DR Congo, natural CCHFV infections were detected in humans twice, but no seroprevalence study in ruminants has ever been conducted (even not after the last human case in 2008). Katanga province in DR Congo has most suitable habitats for the main vector (tree savanna), which is different to the rest of the country (60% covered by rain forest). Mali and Mauritania are neighboring countries with a common border and similar very to extreme arid climatic zones. For both countries, virus circulation has been shown before but the current distribution of CCHFV was unclear, as seroepidemiological studies have not been conducted in recent

years. Most tested cattle samples originated from the South of both countries since the Northern parts are more or less desert. In all four countries CCHFV-specific antibodies were detected. Prevalence rates were highest in Mauritania, Mali and Northern Cameroon, which are characterized by dry climate and less dense vegetation. CCHFV genomes could eventually be found in a small scale study by using the new RT-qPCR in *Hyalomma* ticks collected in Cameroon emphasizing the high virus circulation in this country.

Altogether, study results were giving a precise description of the CCHFV presence and distribution in Cameroon, DR Congo, Mali and Mauritania and provided up-to-date data for CCHFV risk assessments for these countries.

2 LITERATURE REVIEW

2.1 History and naming of CCHFV

Determining the first description of CCHF cases is difficult, as the symptoms are in many cases flu-like and non-pathognomonic and relevant diagnostic methods were developed just recently. However, there are indications that a human case in Persia described in the medical handbook Zakhirah-I Khvarazm'Shahi ("Treasure dedicated to the king of Khwarazm") dating back to 12th century may have referred to CCHF (Hoogstraal, 1979). Similar clinical diseases were also reported in Uzbekistan at about the same time (Hoogstraal, 1979). However, the first verified outbreak occurred between 1944 and 1945 on the war-torn and abandoned Crimean Peninsula where about 200 soviet soldiers contracted a febrile illness (Whitehouse, 2004). M. P. Chumakov acknowledged the importance of this disease as well as the connection to a tick vector. He named the virus that he managed to isolate in 1967 from new-born mouse brains, "Crimean hemorrhagic fever" virus (Hoogstraal, 1979). Shortly before that, the isolation of a so-called "Congo virus" was achieved; the sample originated from a boy from Stanleyville in Belgian Congo (today Kisangani, DR Congo) who died in 1956 from a disease formerly unknown or unrecognized on the African continent (Simpson et al., 1967, Woodall et al., 1967). In 1969 it was shown that the "Crimean hemorrhagic fever" virus and the "Congo virus" were indistinguishable from each other (Casals, 1969) so that both viruses were merged under the new name Crimean-Congo hemorrhagic fever virus.

2.2 Epidemiology

Evidence for the presence of CCHFV has meanwhile been reported in around 50 countries in Africa, Asia and Europe (Hoogstraal, 1979, Whitehouse, 2004, Bente et al., 2013). However, this number most likely does not reflect the complete global distribution of this virus. There are still countries where CCHFV has not been investigated and global climate change may facilitate further spreading of the virus. The occurrence of CCHFV correlates with its main vectors' (*Hyalomma* spp.) distribution and is limited up to 46° N (latitude North) (Whitehouse, 2004, Hubalek and Rudolf, 2012).

2.2.1 Europe

First evidence of CCHFV was given in 1944 by Chumakov et al. regarding the outbreak on the Crimean Peninsula (Hoogstraal, 1979). Subsequently, the circulation of CCHFV was also verified in other Oblasts (districts) of the Soviet Union (Hoogstraal, 1979). More recent reports about CCHFV and tick vectors in the Soviet Union are exclusively available in Russian language, which also is true for current reports from the Russian Federation. Fortunately, most epidemiological studies (including Russian reports) published before 2007 were summarized by Butenko and Karganova in the book "Crimean-Congo Hemorrhagic Fever – A Global Perspective". The authors reported over one thousand registered CCHF cases between 1948 and 2005 (Butenko and Karganova, 2007). Recently it has been shown that the number of cases is even increasing (Bente et al., 2013). The strains from the European part of Russia detected so far cluster phylogenetically with strains from Albania, Bulgaria, Kosovo and Turkey (Burt and Swanepoel, 2005, Drosten et al., 2002, Hewson et al., 2004, Yashina et al., 2003b, Yashina et al., 2003a). However, it stands to reason that an Asian strain is also circulating in Russia, as strains from the neighboring country Kazakhstan cluster with the strains from Tajikistan, Uzbekistan and China (Butenko and Karganova, 2007). In Moldavia, CCHFV was isolated from ticks in 1973/74 while an active virus circulation in the neighboring country Romania was verified 40 years later (Hoogstraal, 1979, Ceianu et al., 2012). Hungary is the northernmost country, where CCHFV circulates. There, Horvath detected CCHFV-specific antibodies in animal and human sera already in 1975 (Hoogstraal, 1979). A human case in 2004 and the circulation of vector ticks indicated that CCHFV is still present in the country (Hornok and Horvath, 2012). In Kosovo, CCHF cases occur sporadically and the virus was also isolated in the aftermath of the first outbreak in the 1950ies (Papa et al., 2002b, Drosten et al., 2002, Duh et al., 2008). More than half a century later, the initial description in neighboring Macedonia was successful, where 15% of the tested cattle sera contained specific antibodies against CCHFV (Mertens et al., 2015). In Albania, CCHFV was detected in 1986 and outbreaks were reported sporadically (Papa et al., 2002a). A recent paper reported a seroprevalence of CCHFV-specific antibodies in 23% of the tested domestic ruminants and therefore indicated the continuing circulation of the virus in the country (Schuster et al., 2016b). Bulgaria and Turkey are well-known CCHFV hotspots in Europe. About 1,500 cases have been detected in Bulgaria between 1953 and 2008 and

prevalence studies in ruminants have confirmed areas of elevated risk of infection (Papa et al., 2004, Barthel et al., 2014, Mertens et al., 2016b). An even higher case incidence is true for Turkey, where over 1,000 cases are reported annually (Ergonul, 2006, Mertens et al., 2016b). Human cases mainly originated from the Central Anatolian provinces, where very high seroprevalence rates were reported also for cattle (Gunes et al., 2009, Albayrak et al., 2012b, Mertens et al., 2016b). Most Turkish isolates cluster with isolates from Southern Russia and the Balkans, however, some isolates that cause a mild form of CCHF seem to be closely related to the AP92 strain from Greece (Midilli et al., 2009). This Greek strain was already isolated in 1975 by Papadopoulos and Koptopoulos from a *Rhipicephalus bursa* tick (Hoogstraal, 1979). Phylogenetically AP92 is very different from other European strains and is also considered to be apathogenic. Hence, it is not surprising that no human case has been reported in Greece until 2008. The CCHFV strain responsible for this first human case in 2008 was closer related to the Balkan strains than to AP92 (Papa et al., 2010). Until recently, no indigenous human CCHFV infections have been reported from southwestern Europe, yet, CCHFV-specific antibodies were detected in France and Portugal already decades ago (Hoogstraal, 1979, Filipe et al., 1985). A CCHFV circulation in Spain was eventually verified in 2010 by the first virus isolation from a *Hyalomma lusitanicum* tick and it was most recently proved by the occurrence of an indigenous human case (with a subsequent infection of a health care worker) in 2016, (ECDC, 2016, Estrada-Pena et al., 2012).

2.2.2 Asia

Hoogstraal summarized the CCHFV status of many Asian countries in 1979. The virus was shown to be present in Turkmenistan, Uzbekistan, Kirgizstan, Tajikistan, Armenia, Azerbaijan, Iran, Afghanistan, Pakistan and in India (Hoogstraal, 1979). In the PR China, the first CCHFV outbreak had also occurred in 1965 but it was not detected until 20 years later in a retrospective study in humans, sheep and ticks (Yen et al., 1985). The case fatality rate (80%) stated in this paper is one of the highest reported on all three continents (Yen et al., 1985). In Pakistan, CCHFV was first isolated from a *Hyalomma* tick in the 1960s (Begum et al., 1970). Since then, many outbreaks and nosocomial infections have occurred (Burney et al., 1980, Altaf et al., 1998, Athar et al., 2003). A similar problem with nosocomial infections became evident also in India (Mishra et al., 2011, Yadav et al., 2016), where an index CCHF

case was diagnosed in 2011 which led to six secondary infections (including three fatalities) as both the population and the healthcare sector were not sufficiently informed about CCHF (Mourya et al., 2012). Just recently, all currently known Indian CCHFV strains were classified. The respective report revealed that all novel Indian strains cluster with strains from Pakistan, Iran and Afghanistan, while the older isolates cluster with the Chinese strains (Singh et al., 2016). Studies from the last decade clearly identified a CCHFV infection risk for the human population in Afghanistan. Not only was the seroprevalence high in Afghan ruminants but also antibodies were detected in 4% of 800 tested Afghan National Army recruits (Mustafa et al., 2011, Todd et al., 2016). Furthermore, a documented CCHFV outbreak occurred in Afghanistan in 2008 (Mofleh and Ahmad, 2012). In Iran, the first human case was confirmed about 20 years after CCHFV antibodies were detected (Saidi et al., 1975). Since then, human cases have been reported from most provinces (Chinikar et al., 2004, Chinikar et al., 2010b, Chinikar et al., 2010a, Sharififard et al., 2016). In Iraq, CCHFV both was isolated and CCHFV-specific antibodies were detected in the 1980s (Tantawi et al., 1981, Tantawi et al., 1980). Due to the politically instable situation, investigations into CCHFV in Iraq were hindered for a long time. In 2002, Majeed et al. summarized the reported human cases between 1990 and 2010 and detected an increase of human CCHF cases. However, this increase might have been caused by an improvement of the reporting activity (Majeed et al., 2012). A case fatality rate of 36% was detected among confirmed cases (Majeed et al., 2012). The United Arab Emirates sporadically report CCHFV outbreaks connected to hospital or abattoir work (Suleiman et al., 1980, Khan et al., 1997). It was also reported that imported and indigenous cattle as well as camels were CCHFV-specific antibody positive (Khan et al., 1997). Apparently, the virus may be present in the United Arab Emirates, usually without causing CCHF symptoms or disease. Two human cases were reported recently (Mohamed Al Dabal et al., 2016). In the neighboring country Oman, CCHFV is also circulating and human cases occur sporadically (Al-Zadjali et al., 2013).

2.2.3 Africa

The CCHF prevalence in most African countries is still obscure. While countries such as South Africa report well-defined CCHF cases, the CCHF incidence in other countries is unclear. The first South African case was reported in 1981 (Gear et al., 1982) and a

nosocomial outbreak occurred in 1985, which led to an extensive study of CCHF (van Eeden et al., 1985b, van Eeden et al., 1985a, van de Wal et al., 1985, Shepherd et al., 1985, Joubert et al., 1985). Further insights into CCHFV in birds could also be gained because of South Africa's history of ostrich domestication. In 1984, a slaughterer working at an ostrich abattoir contracted a CCHFV infection (Swanepoel et al., 1987). Investigations about CCHFV in birds revealed that they are usually refractory to CCHFV but ostriches can develop a short viremia and hence transmit the virus (Swanepoel et al., 1998, Shepherd et al., 1987). Swanepoel noticed that seroprevalence rates in cattle correlate with the distribution of *Hyalomma* ticks in South Africa (Swanepoel et al., 1987), a finding which was also reported for European, Asian and other African countries. This has led to the conclusion that *Hyalomma* ticks are the main vector for CCHFV (Hoogstraal, 1979, Whitehouse, 2004). Far less is known about the distribution of CCHFV in Central African countries. Even though the virus was detected in DR Congo for the first time on the African continent, no further studies were conducted in DR Congo thereafter (Simpson et al., 1967, Woodall et al., 1967). Just one further incidental case was reported from the country in 2008 and molecular analysis showed that the new strain was almost identical to the first Congolese isolate and one isolate from Uganda (1958) (Grard et al., 2011). CCHFV has also been detected in ticks in the East African country Ethiopia but seroepidemiological studies were never performed to estimate the true risk of infection (Hoogstraal, 1979). In Tanzania, seroprevalence studies revealed a low risk for the human population (Hoogstraal, 1979). However, the results are outdated and were obtained by using the agar gel diffusion precipitation (AGDP) test, which is less sensitive than todays detection methods. More recently seroprevalence studies were performed in Kenya, which revealed that the CCHFV risk was significant in the human population (23% tested immunoglobulin G (IgG) positive) and one human case was described in 2002 (Lwande et al., 2012, Dunster et al., 2002). In Madagascar, CCHFV was isolated in 1985 from ticks (Mathiot et al., 1988). This Malagasy strain clusters with strains from the United Arab Emirates and Pakistan but is less closely related to other African strains (Andriamandimby et al., 2011, Rodriguez et al., 1997). A seroprevalence study in slaughterhouse workers from all over the country showed that the infection risk was low even within this risk group (Andriamandimby et al., 2011). More information is available for the West African countries Senegal and Mauritania. A quite detailed serosurvey from Wilson et al. highlighted that the prevalence in sheep and humans

was higher in the North than in the South of Senegal (Wilson et al., 1990). It was not surprising that corresponding studies in Mauritania gave similar results, as the South Mauritanian vegetation is quite similar to the one present in North Senegal, both offering excellent environmental conditions for *Hyalomma* spp. ticks (Nabeth et al., 2004b, Nabeth et al., 2004a, Gonzalez et al., 1990). Sporadic CCHF cases are reported from both countries and the latest human case in Mauritania was reported in 2015 (Gonzalez et al., 1990, Nabeth et al., 2004b, Nabeth et al., 2004a, Kleib et al., 2016). With regard to Mali, first indications for a CCHFV circulation were provided in 2005 when specific antibodies were detected in humans from Baguineda (Traore et al., 2005) and the virus was eventually isolated from *Hyalomma* ticks (Zivcec et al., 2014). However, no overarching prevalence study has been performed yet. One of the most recent CCHFV discoveries was made in Sierra Leone when 675 human samples of suspected yellow fever cases were tested for CCHFV-specific antibodies and 13 sera (2%) were found to be positive (O'Hearn et al., 2016). CCHFV also circulates in North Africa. In Sudan, the current activity of the virus was proven through seroprevalence studies in animals, by sporadic human cases and by a larger outbreak most recently (Aradaib et al., 2011, Adam et al., 2013, Ibrahim et al., 2015). Studies conducted in Egypt also showed circulation of CCHFV but the risk for the population appears to be lower than in other African countries (Hoogstraal, 1979, Mohamed et al., 2008). It has been postulated that CCHFV was imported to Egypt through animals infested with infected ticks (Morrill et al., 1990, Chisholm et al., 2012).

2.3 **Classification of CCHFV**

CCHFV belongs to the genus *Nairovirus* within the family *Bunyaviridae*. The CCHFV classification is shown in Figure 2.1. The first known member of the *Bunyavirus* family was Bunyamwera virus (BUNV), which was isolated in Uganda in the 1950ies (Smithburn et al., 1946). To date, more than 350 virus species are grouped into five genera: *Hantavirus*, *Nairovirus*, *Orthobunyavirus*, *Phlebovirus* and *Tospovirus* (ICTV). All *Bunyaviridae* are able to infect animals except of the plant specific members of the genus *Tospovirus*. The *Nairovirus* genus is organized into seven serogroups and includes arboviruses, which are transmitted by different tick species (Casals and Tignor, 1980, Zeller et al., 1989). The

non-human pathogenic Hazara virus (HAZV) is the only other virus belonging to the CCHF serogroup with exception of CCHFV.

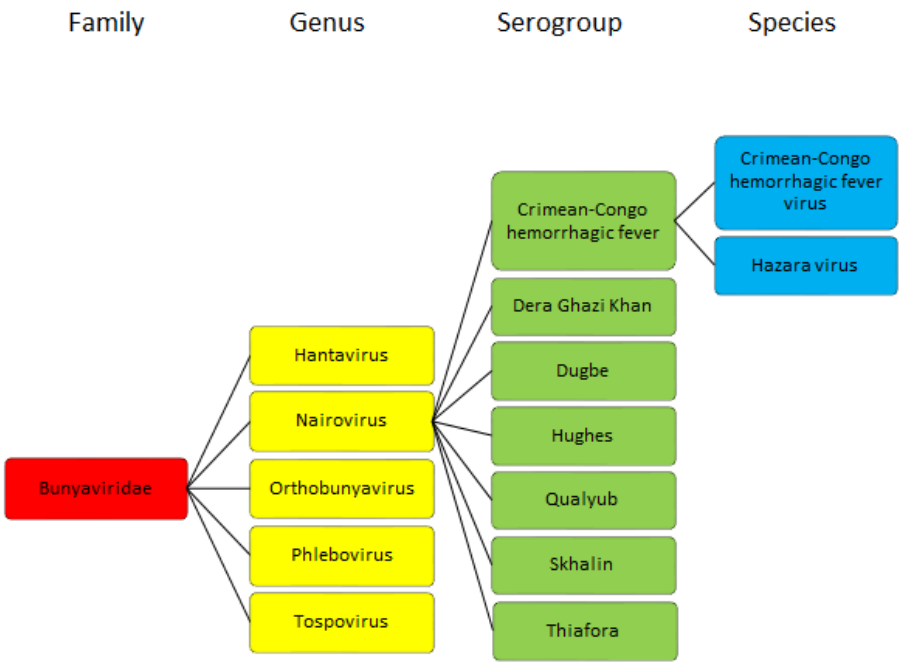

Figure 2.1 Classification of CCHFV

2.4 Morphology and genome characterization

The spherical CCHF virion has a diameter of approximately 100 nm (Korolev et al., 1976, Porterfield et al., 1975). The two glycoproteins of CCHFV are embedded in a lipid bilayer (Porterfield et al., 1975). Within the virus particle, the ss(-)RNA genome is divided into three segments. Each segment has the same complementary 5'-UCUCAAAGA and 3'-AGAGUUUCU non-coding endings, which border the open reading frame and form a panhandle structure through base-pairing within the strand (Elliott et al., 1991, Flick et al., 2002, Clerx-Van Haaster et al., 1982, Raju and Kolakofsky, 1989). This genetic information is conserved between all *Nairoviruses* and serves as promotor for the viral RdRp (Bergeron et al., 2010, Devignot et al., 2015). A schematic illustration of the CCHFV genome is shown in Figure 2.2.

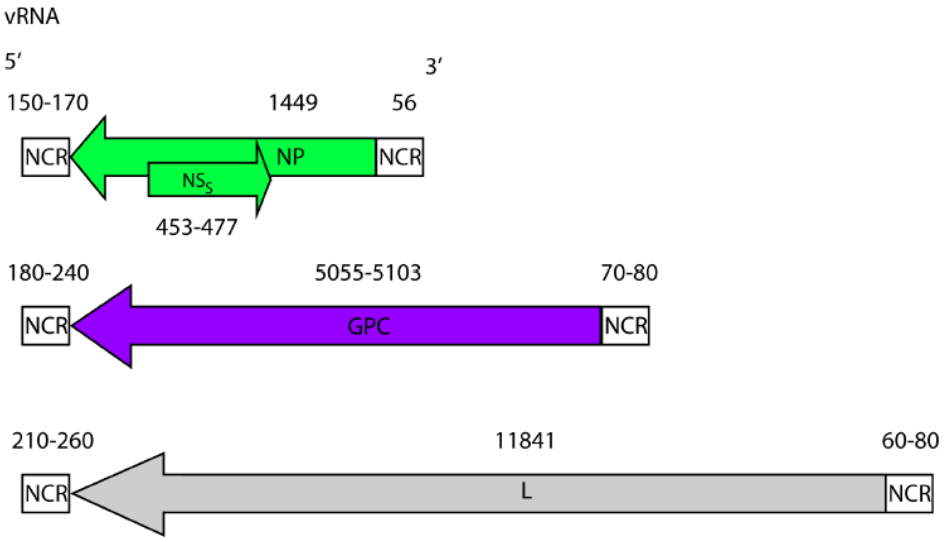

Figure 2.2 Schematic illustration of the CCHFV genome

The ss(-)RNA is divided into the small (~1.6 kb), the medium (~5.4 kb) and the large (~12.1 kb) segment, encoding the nucleoprotein (NP), the glycoprotein precursor (GPC) and the RNA dependent RNA polymerase (L) respectively. In positive orientation the small segment also encodes the non-structural S-protein (NS_S). The nucleotide length of all regions is based on full length sequences available in GeneBank. Reprinted from Zivcec et al. (Zivcec et al., 2016).

The S-segment of the tripartite RNA genome encodes the N-protein, which mainly encapsidates the RNA and links to the large protein (L-protein) to form Ribonucleoprotein complexes (Levingston Macleod et al., 2015). It has just recently been described that the small segment (S-segment) also encodes the non-structural S-protein (NS_S) in opposite orientation to the nucleocapsid protein (N-protein) (Barnwal et al., 2016). The same publication suggested the NS_S to have an apoptotic function. A comparison of the S-segment of different CCHFV strains revealed a diversity of 20% on the nucleotide level and 8% on the amino acid level (Deyde et al., 2006).

The medium segment (M-segment) encoded GPC is co-translationally cleaved into the Pre_{Gn}- and Pre_{Gc}- and a non-structural M-protein (NS_M); the final maturation takes place in the Golgi, where also three secreted non-structural proteins are split off (Sanchez et al., 2002, Sanchez et al., 2006, Altamura et al., 2007). The correct processing of the glycoprotein regulates the production of infectious particles (Bergeron et al., 2007). Additionally, Gn and Gc induce neutralizing antibodies and are responsible for CCHFV attachment (Flick and Whitehouse, 2005). The M-segment shows the highest diversity with a nucleotide variation of

31% and an amino acid variation of 27%. The high diversity of the M-segment stands to reason and is most likely caused by varying selection mechanisms, i.e. adaptation to a combination of different tick and vertebrate cells, different natural cycles and the hosts immune system (Deyde et al., 2006).

The large segment (L-segment) encodes the L-protein, which is one of the largest (3944 amino acids) of all *Bunyaviridae* (Honig et al., 2004). Its endonuclease domain and RdRp motifs are responsible for messenger RNA (mRNA) transcription and replication (Honig et al., 2004, Kinsella et al., 2004). The N-terminal region (first 600 amino acids) has *Bunyavirus* untypical sequences. The function of the ovarian tumor like protease detected in this part is unfortunately not fully understood yet but it is clear that it is dispensable for the RNA dependent RNA polymerase (RdRp) function (Honig et al., 2004, Bergeron et al., 2010). The L-segment shows a nucleotide variation of 22% and an amino acid variation of 10% (Deyde et al., 2006).

2.5 **Replication**

The life and replication cycle of CCHFV has not entirely been determined yet, but several critical points were revealed in the last years. The Gc-protein seems to play the leading role in the binding to a so far unknown host cell receptor. This was concluded since Gc-specific monoclonal antibodies have the ability to neutralize virus particles and prevent a CCHFV infection (Bertolotti-Ciarlet et al., 2005). CCHFV uses a clathrin-mediated endocytosis mechanism for host cell entry (Simon et al., 2009, Garrison et al., 2013). Also, the host cell molecule nucleolin was described to be important for CCHFV entry (Xiao et al., 2011). Nucleolin was also identified to have functional interactions with other viruses (Ueno et al., 2004, Tayyari et al., 2011). Additional positive influence factors for viral entry are cholesterol and a low pH (Simon et al., 2009, Garrison et al., 2013). Following the fusion of the CCHFV envelope with the host cell membrane, the ss(-)RNA segments are transcribed into mRNA. The host cell mRNA has to be cleaved by the viral endonuclease to obtain 5’capped primers (cap-snaching) (Devignot et al., 2015, Bergeron et al., 2010). The viral genome replication on the other hand is not influenced by cap-snaching (Devignot et al., 2015, Bergeron et al., 2010).

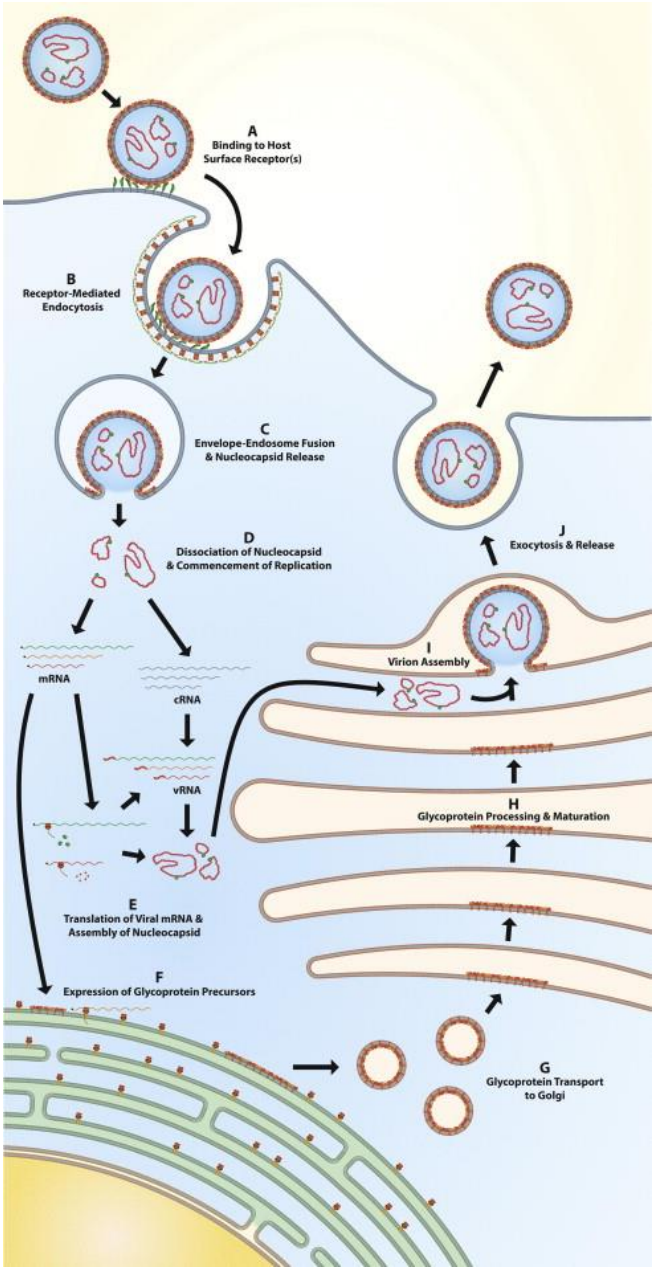

Figure 2.3 Replication of CCHFV

(A) The virion binds to an unknown receptor on the host cell. After endocytosis (B) the viral envelope and the endosome membrane merge and release the three nucleocapsids into the cytoplasm (C). They dissociate and are transcribed by the RNA dependent RNA polymerase (RdRp) into messenger RNA (mRNA) and complementary RNA (cRNA) (D). The cRNA serves as template for the new virus RNA (vRNA). The mRNA of the S- and the L-segment are translated and the new nucleocapsid proteins, RdRp and vRNA, assemble (E). The glycoprotein precursor is translated in the endoplasmatic reticulum (ER) (F). Subsequently, the first cleavage to PreGn and PreGc takes place in the ER. The glycoproteins are afterwards transported to the Golgi (G) for further processing and maturation (H). New virions are assembled (I) and the particle is released through exocytosis (J). Reprinted from Bente et al. (Bente et al., 2013).

The synthesis of the glycoproteins in CCHFV is much more complex compared to other *Bunyaviridae* (Sanchez et al., 2002). The GPC is synthesized and N-glycosylated in the endoplasmatic reticulum (ER); this is crucial for correct protein folding and trafficking to the Golgi (Bertolotti-Ciarlet et al., 2005, Erickson et al., 2007). Furthermore, the first cleavage into Pre_{Gn}, Pre_{Gc} and NS_M takes place in the ER (Altamura et al., 2007). These proteins are subsequently trafficked to the Golgi to be O-glycosylated (Shi et al., 2010). In the Golgi, Pre_{Gn} is cleaved again and a non-structural secreted product (GP160/85) is separated from the

N-terminus (Vincent et al., 2003). Budding of the virus particles takes place in the Golgi or the *trans*-Golgi network as this is the accumulation area of the glycoproteins; also, the N⁻proteins gather in the nearby perinuclear region (Andersson et al., 2004, Bertolotti-Ciarlet et al., 2005, Bergeron et al., 2007). Finally, the newly assembled virion is released from the host cell through exocytosis. The replication cycle of a CCHFV particle is outlined in Figure 2.3.

2.6 **Transmission**

2.6.1 Ticks as vector and reservoir

As it was already suspected in the 1940s, ticks play an important role in the transmission of CCHFV (Chumakov, 1947, Hoogstraal, 1979). Today, it is known that only hard-bodied ticks (Ixodidae) are vectors for CCHFV, as soft-bodied ticks (Argasidae) do not get infected (Shepherd et al., 1989b). Ixodid ticks are both vector and reservoir for CCHFV, as the virus is circulating stably in the tick population. Vertical transmission (transovarial) was shown for many tick species like *Hyalomma marginatum*, *Hyalomma truncatum*, *Hyalomma rufipes*, *Rhipicephalus evertsi* and *Amblyomma variegatum* (Turell, 2007, Gonzalez et al., 1992, Faye et al., 1999a, Faye et al., 1999b, Zeller et al., 1994a). This transmission route is very efficient as the reproductive organs, next to the salivary glands, reach the highest virus titers and as thousands of (infected) eggs are deposited by female ticks (Nuttall et al., 1994, Dickson and Turell, 1992). The other transmission routes within the tick population – veneral, transstadial and co-feeding – belong to the concept of horizontal transmission (Gonzalez et al., 1992, Jones et al., 1987, Logan et al., 1989). Co-feeding is an excellent way to circumvent the problem of CCHFV unsusceptible animals or of low virus blood titers in animals (Jones et al., 1987, Nuttall and Labuda, 2004). The transition of CCHFV from the saliva of an infected tick to another tick feeding in its vicinity is enhanced by special contents of the ticks' saliva. Infected ticks do not clear the virus but presumably stay infectious all their life (Logan et al., 1989). The transmission within the tick population is shown in Figure 2.4.

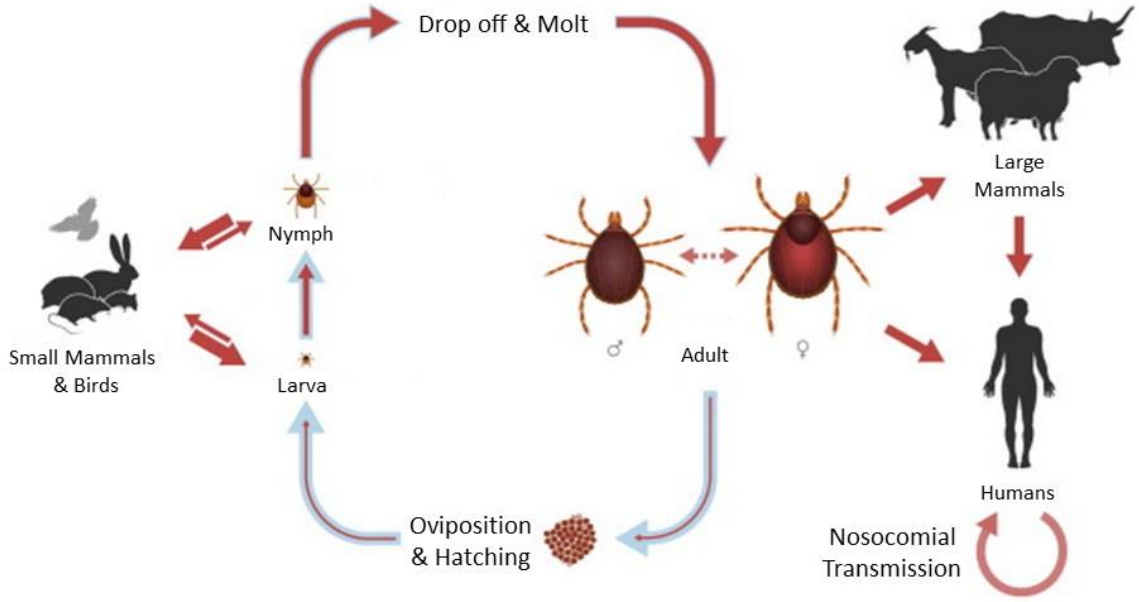

Figure 2.4 CCHFV transmission in the tick population, the tick-vertebrate-tick cycle and between vertebrates

The course of the tick lifecycle is indicated with blue arrows. The solid red arrows show the transmission possibilities while the thickness of the arrow indicates the transmission efficiency. CCHFV can circulate stably in the tick population through vertical (transovarial) and horizontal (veneral, transstadial, co-feeding) transmission. Veneral transmission is indicated with the dashed arrow. Larvae and nymphs prefer to take their blood meal from small mammals and birds. Adult ticks seek large mammals out for feeding and mating. Humans can get infected through tick bites, crushing ticks or through contact to tissue ore body fluids of viremic animals or CCHF patients. Reprinted (with small adaptations) from Bente et al. (Bente et al., 2013).

2.6.2 Tick-vertebrate-tick cycle

In a balanced natural environment CCHFV circulates silently in a tick-vertebrate-tick cycle like other tick-borne pathogens and is rarely involving humans. Larvae ingest their blood meal and either stay in place to molt on the first host or fall off and molt afterwards. Nymphs of two-host ticks remain on their host after molting and ingest a second blood meal from the same animal (Apanaskevich and Olicer (Jr.), 2014). Nymphs of three-host ticks are seeking another animal for their blood meal and for further maturation (Apanaskevich and Olicer (Jr.), 2014). *Hyalomma* ticks, the main vector of CCHFV, are “hunting ticks”; they pursue their host for up to 400 m. This also facilitates CCHFV spread to a new organism. Larvae and nymphs prefer small mammals like mice, hedgehogs and hares, but they also take their blood meal from birds (Hoogstraal, 1979, Watts et al., 1988). Most birds are not susceptible to CCHFV but can transport infected ticks over very long distances and therefore contribute to

the virus distribution (Shepherd et al., 1987, Zeller et al., 1994a, Swanepoel et al., 1998). Adult ticks take their blood meal from large mammals, preferably ruminants. Animals do not show clear signs of illness after CCHFV infection although they develop a viremia that lasts approximately one week (Causey et al., 1970, Gonzalez et al., 1998, Shepherd et al., 1991). However, humans can become infected in endemic areas by tick bites when working/staying outdoors or by direct contact with infected animals (esp. livestock) or products thereof or when they come into contact with clinically affected patients. Therefore human infections are most commonly seen in risk groups such as farmers, slaughterers and health care workers. The tick-vertebrate-tick cycle is visualized in Figure 2.4.

2.6.3 Direct transmission between vertebrates

Apart from tick bites and crushing/squeezing of ticks, there is also a possibility of CCHFV transmission between vertebrates (Figure 2.4.) (Hoogstraal, 1979). While virus transmissions to humans during slaughter or treatment of viremic animals are possible but happen not so often, nosocomial human infections are more frequent (Altaf et al., 1998, Mourya et al., 2012, van Eeden et al., 1985a). Particular risk factors for nosocomial infections are insufficient or underdeveloped healthcare and hygiene standards in endemic countries. High viral loads of up to 10^{10} copies/ml in blood of fatal human cases might be another reason (Duh et al., 2007). However, most reports refer to unprotected contact to blood, other body fluids or tissue of viremic patients (Altaf et al., 1998, Ergonul, 2006, Bodur et al., 2010, van Eeden et al., 1985b).

2.7 **CCHF in humans**

2.7.1 Course of infection and clinical features

The incubation times of CCHF are depend on the route of virus entry three days after a tick bite and six days after blood or tissue contact (Swanepoel et al., 1987). The pre-hemorrhagic period directly follows with unspecific flu-like symptoms; most common disorders are fever (unspecific pattern), headache and myalgia (Swanepoel et al., 1987, Hoogstraal, 1979). In some cases, CCHF is cleared after the pre-hemorrhagic period. The hemorrhagic period develops rapidly and lasts for 2-3 days. No correlation was observed between the height of the

fever and the severity of the hemorrhages. Petechiae and large hemorrhages are often seen in mucosa and skin. Most frequent bleeding sites are the nose, the gastrointestinal tract, the urinary tract and the respiratory tract (Ergonul, 2006, Hoogstraal, 1979). Severe cases are characterized by disseminated intravascular coagulation and circulatory shock (Ergonul, 2007, Swanepoel et al., 1989). On organ level, hepato- and splenomegaly are most common pathological changes in CCHF patients (Hoogstraal, 1979, Ergonul et al., 2004, Bakir et al., 2005). Elevated aspartate and alanine aminotransferase levels, decreased platelet counts, decreased fibrinogen and prolonged thromboplastin times are typical severity predicting biochemical and blood count parameters (Swanepoel et al., 1989, Ergonul et al., 2006a). Patients can survive the hemorrhagic period and eliminate the virus but very long convalescence periods that include memory loss, poor vision and bradycardia were reported (Hoogstraal, 1979). Fatality rates in CCHF vary between 5% in Turkey and up to 80% in China (Yilmaz et al., 2009, Yen et al., 1985). This variation probably depends on education, individual and general awareness, effectiveness of the public healthcare system, sensitivity of the notification system (only the severe cases are detected) and the circulating virus strain (Mertens et al., 2013). The number of CCHFV infections is usually relatively high in countries with a lower awareness about CCHF combined with a lack of knowledge of effective protection measures (Mertens et al., 2013).

2.7.2 Pathogenesis

The role of a pro-inflammatory cytokine response is the best investigated pathogenic mechanism in CCHFV infections. Different studies reported elevated serum levels of interleukin (IL)-6, IL-10, tumor necrosis factor (TNF)-α and interferon (IFN)-γ especially in severe cases (Ergonul et al., 2006b, Papa et al., 2006, Saksida et al., 2010). In addition, CCHFV replicates in human monocyte dendritic cells, which can release these pro-inflammatory cytokines (Connolly-Andersen et al., 2009). The findings are supported by studies in knock out (KO) mice. STAT1-KO (signal transducer and activator of transcription) mice develop elevated levels of IL-6, IL-10, TNF-α and IFN-γ after CCHFV infection. Also, the susceptibility of IFN-KO mice points out the importance of this antiviral mechanism (Bente et al., 2010, Bereczky et al., 2010). Therefore, CCHFV seems to induce similar changes in humans as Ebola virus (EBOV). EBOV studies indicated the induction of

pro-inflammatory cytokines and also intrinsic coagulation and platelet aggregation to be the reason of the subsequent vascular dysfunction (Mahanty and Bray, 2004, Schnittler and Feldmann, 2003). A further mediator may also be nitric oxide (Simon et al., 2006). Furthermore, it was shown that CCHFV directly interferes with endothelial cells and can affect the vascular function (Burt et al., 1997, Connolly-Andersen et al., 2011). Elevated levels of natural killer cells and of the ratio of cytotoxic T cells to lymphocytes, which may trigger the pathogenesis, are seen in CCHF patients (Yilmaz et al., 2008, Akinci et al., 2009). Macrophages are described to play a crucial role in EBOV induced hemorrhagic fevers and they also are target cells of CCHFV (Geisbert et al., 2003, Burt et al., 1994). Additionally, hemophagocytosis by cytokine hyperactivated monocytes and macrophages was observed in CCHF patients (Tasdelen Fisgin et al., 2008). In severe CCHF cases, IgG and immunoglobulin M (IgM) concentrations were reduced or not detectable at all (van Eeden et al., 1985b, Shepherd et al., 1989c, Burt et al., 1994). While this highlights the importance of the humoral response, the mechanism of the immunoglobulin reduction remains unclear.

2.8 **Prevention and treatment**

The first step for CCHF prevention in humans is to increase the current lack of awareness by running monitoring programs to discover endemic countries/areas and to subsequently implement public health protection measures in these areas. Early virus detection in ticks, small mammals and ruminants therefore helps to prevent human cases and define risk areas (Vorou, 2009). In CCHFV endemic areas, humans should avoid tick habitats and remove attached ticks as quickly as possible. When exposure is possible, people at risk should be wearing adequate personal protective equipment including gowns, gloves, masks and goggles, e.g. when handling body fluids and tissues of potentially infected animals or human patients (Ergonul, 2007). In the laboratory, full BSL 4 equipment is required for work with infectious virus. The CCHFV exposure risk of abattoir workers in endemic countries can reduced by using repellents two weeks before slaughter to keep the animals free of ticks (Swanepoel et al., 1998).

The current treatment for human CCHF cases is rather supportive by substituting blood, plasma, thrombocyte and erythrocyte losses (Leblebicioglu et al., 2012, Keshtkar-Jahromi et

al., 2011). The benefit of ribavirin therapy was debated in different studies and is advisable in early stages of the infection (Mardani et al., 2003, Ergonul et al., 2004, Alavi-Naini et al., 2006, Tasdelen Fisgin et al., 2009, Koksal et al., 2010). The use of hyperimmune serum derived from recovering patients needs further investigations (van Eeden et al., 1985b, Kubar et al., 2011). Also, the simultaneous use of immunoglobulin and type I interferon was discussed controversially (Papa et al., 2004, Vassilenko et al., 1990, Andersson et al., 2006, Karlberg et al., 2010). A formalin inactivated mouse brain vaccine against CCHFV was developed in the Soviet Union and a similar vaccine is still used for risk groups in Bulgaria today (Papa et al., 2004, Bente et al., 2013). However, the argument that this vaccine protects efficiently against CCHFV infection is solely based on the fact that CCHFV has never been observed in vaccinated persons (Bente et al., 2013). It is difficult to validate the efficacy of CCHFV vaccines unfortunately, as the necessary challenge studies require a suitable animal model and must be carried out under BSL 4 conditions.

2.9 **Detection**

CCHFV diagnosis improved significantly since the discovery of the virus. While first cases had to be confirmed by virus isolation, inoculation in newborn mice, reverse passive hemagglutination inhibition, AGDP and complement fixation assays, much quicker, more reproducible, sensitive and specific assays are used today. To date, CCHF diagnosis in humans relies especially on IgG- and IgM-ELISAs and on RT-pPCR (Table 2.1). However, for testing animal samples only few in-house ELISA were published, which usually were not properly validated.

CCHFV specific IgG and IgM antibodies are detectable about one week after infection in human and animal sera (Shepherd et al., 1989c). IgM antibodies have been detected even as early as day five but they decline after the third week of infection and are undetectable after four months, while IgG antibodies were shown to be detectable even five years post infection (Tang et al., 2003, Shepherd et al., 1989c, Ergonul, 2006). However, antibodies might not be detectable in severe acute human cases at all (van Eeden et al., 1985b, Shepherd et al., 1989c, Burt et al., 1994). This problem can be solved by polymerase chain reaction (PCR) detection in parallel to serology. The commercial VectorBest ELISAs are most often used even

although sensitivities range only from 80% (IgG) to 88% (IgM) (Vanhomwegen et al., 2012). The indirect VectorBest CCHFV-IgG-ELISA was also adapted to animal sera and showed even better diagnostic sensitivities (D-SN) than the original version, without any loss of specificity (D-SP) (Schuster et al., 2016b, Mertens et al., 2015). A small number of in-house CCHFV-ELISAs have also been developed for animal seroprevalence studies in the past (Burt et al., 1993, Qing et al., 2003, Garcia et al., 2006, Mertens et al., 2015, Schuster et al., 2016a, Schuster et al., 2016b). Animals of highest interest in these studies were cattle, sheep, camels and goats. They represent the species with the highest number of sampled animals (Spengler et al., 2016a).

While the first in-house ELISAs for CCHFV utilized the whole virus preparations as antigens, novel in-house ELISAs use recombinant N-protein antigens, which can be produced outside of BSL 4 laboratories (Mertens et al., 2015, Schuster et al., 2016a, Schuster et al., 2016b). The competitive ELISA allows the animal species independent antibody testing, but requires larger serum volumes.

The IFA is another serological assay that is less commonly applied than ELISA. The reason for this may be that reading IFA slides requires considerable experience. Two IFAs are commercially available to test human sera currently (Euroimmun, Lübeck, Germany). Quite recently, this assay was adapted and validated for use in different animal species (Mertens et al., 2015, Schuster et al., 2016b).

Many decades ago, recovery of virus material in newborn mice was considered to be the "gold standard" in CCHFV diagnosis. Apart from the animal welfare issue, a BSL 4 laboratory was and still is required for such infection studies. To date, RT-qPCR should be considered the "standard diagnostic method" as it is faster, more sensitive and much safer (Bente et al., 2013). RT-qPCR is a very useful tool for the diagnosis of acute human infections and for the detection of infected ticks (Gunes et al., 2011). A major problem exists with CCHFV detection because of the genetic diversity of the virus (Deyde et al., 2006). Hence, it is not surprising that first RT-qPCRs could not detect virus strains of all lineages (Burt and Swanepoel, 2005, Duh et al., 2006, Wolfel et al., 2007, Escadafal et al., 2012). The AP92 strain from Greece caused most problems because of its high phylogenetic divergence to other CCHFV strains. An international quality assessment of molecular CCHFV detection

revealed that many laboratories “performed suboptimal” (Escadafal et al., 2012) with regard to detecting this virus. However, a direct conclusion of assay quality was impossible as different PCR kits were used. A recently developed RT-qPCR uses a highly conserved part of the 5’-non coding region of the S-segment of CCHFV. This novel PCR technique detected strains of all lineages of CCHFV. In contrast, Jääskeläinen et al. developed a RT-qPCR with a very short run time (68 minutes) that ensures high sensitivity (100%) and specificity (97%) (Jaaskelainen et al., 2014). Only the commercial RT-qPCR from Altona Diagnostic runs faster. However, the diagnostic sensitivity of this commercial test is far lower (80%) (Vanhomwegen et al., 2012).

Table 2.1 Commercial diagnostic tests

Assay	Target species	Company
IgM ELISA	Human	VectorBest (Russia), BDSL (Scotland), Gentaur* (Belgium), NovaTec Immundiagnostica* (Germany), Alpha Diagnostic* (USA, TX), Creative Diagnostics* (USA, NY)
	Mouse	Alpha Diagnostic* (USA, TX)
IgG ELISA	Human	VectorBest (Russia), Gentaur* (Belgium), NovaTec Immundiagnostica* (Germany), Alpha Diagnostic* (USA, TX), Creative Diagnostics* (USA, NY)
	Bovine, rabbit, mouse	Alpha Diagnostic* (USA, TX)
IgM IFA	Human	Euroimmun (Germany)
IgG IFA	Human	Euroimmun (Germany)
Antigen capture ELISA	Virus	VectorBest (Russia)
RT-qPCR	Virus	Altona Diagnostics (Germany), Liferiver Bio-Tech (USA, CA), Gentaur* (Belgium)

* non-validated assay and for research purposes only

3 MATERIALS AND METHODS

This section is restricted to overarching approaches and generally used materials and methods. Detailed descriptions of the experimental setups and the specific materials and methods used can be found in the individual Manuscripts I-V.

3.1 **Samples**

Sera and ticks for the epidemiological studies (Manuscript I, II, III, IV) were collected by collaborating partners from the governmental veterinary services (Central veterinary laboratory bovine serum bank Mali, Laboratoire National Vétérinaire (LANAVET) Cameroon, Centre National d'Elevage et de Recherches Vétérinaires (CNERV) Mauritania, Laboratoire Vétérinaire de Lubumbashi (LVL) and Université de Lubumbashi (UniLu) DR Congo) in the context of national monitoring programs respectively. Sampling guidelines were established and used by all partners to ensure representative serum sample panels. Briefly, a village was defined as a sampling site where three to five farms should be sampled. For every region three to five sampling sites should be investigated. Sample numbers of at least 500 sera per animal species and year representing an equal distribution over the country/province were optimal, but could not be achieved in all cases. Political instability, inadequate infrastructure and local outbreaks of epidemics affected sampling and shipment to Friedrich-Loeffler-Institut (FLI). Tick samples presented in Manuscript IV were used as proof for the current CCHFV presence in Cameroon. 109 ticks from one area in Cameroon were considered sufficient for this purpose. The samples for assay evaluation and validation derived from previous studies in other countries (e.g. Turkey, Bulgaria) or were provided directly by collaborating partners. Detailed descriptions of the samples used are given in the material and methods section of the individual manuscripts.

3.2 **Inactivation**

Many high contagious, zoonotic and/or transboundary diseases are still endemic in Africa to date. Therefore, all sera were inactivated by γ-irradiation with a dose of approximately 30 kGy (Synergy Health Radeberg GmbH, Radeberg, Germany) prior to their further use. The

success of this inactivation technique was demonstrated for different viruses and bacteria before (Miekka et al., 2003, Nims et al., 2011). It was also shown that irradiation doses of around 30 kGy have only a limited effect on proteins (Miekka et al., 2003, Nims et al., 2011). Consequently, the serological results are not influenced by this treatment. Cell culture supernatants (CCS) and tissue culture supernatant (TCS) of CCHFV were received in AVL buffer (Qiagen, Hilden, Germany). Additionally, they were heated at 70 °C for 20 minutes prior to their use. This procedure was chosen in concordance with recent results on EBOV inactivation (Smither et al., 2015). Both CCS of HAZV and Cameroonian ticks were treated with TRIzol (Invitrogen, Carlsbad, CA, USA) (900 µl TRIzol + 100 µl HAZV CCS, 1 ml TRIzol + 1 tick). The inactivation of various pathogens with TRIzol was demonstrated by Blow et al. (Blow et al., 2004). Batai virus (BATV), BUNV and Uukuniemi virus (UUKV) CCSs were received already heat-inactivated in AVL buffer.

3.3 **RNA extraction**

RNA for RT-qPCR evaluation was extracted by collaborating partners in general and was provided as described in Manuscript V. In Manuscript IV RNA from 109 ticks from Cameroon was purified after homogenization and phenol-chlorophorm extraction using the RNeasy Mini Kit (Qiagen) as described in Manuscript IV. HAZV CCS was extracted following the same phenol-chlorophorm extraction protocol and the manufacturer's instructions of the RNeasy Mini Kit. RNA was extracted with the QIAamp Viral RNA Mini Kit (Qiagen) from CCSs (Greece and Turkish CCHFV isolate, UUKV, BATV, BUNV), TCSs (Afghan and Senegalese CCHFV isolate) and sera (cattle, sheep, goats) according to the manufacturer's instructions.

3.4 **Diagnostic approach (serological tests)**

All cattle, goat and sheep sera were initially analyzed with species specific in-house CCHFV-IgG-ELISAs using a flow chart for seroepidemiological studies (Mertens et al., 2009). All samples were furthermore tested with adapted species specific commercial CCHFV‑IgG‑ELISAs (VectorBest). Inconclusive results were subsequently clarified by

using species adapted specific CCHFV-IgG-IFAs (Euroimmun) (Figure 3.1). All serological assays are described in Manuscript I, II, III and IV in detail.

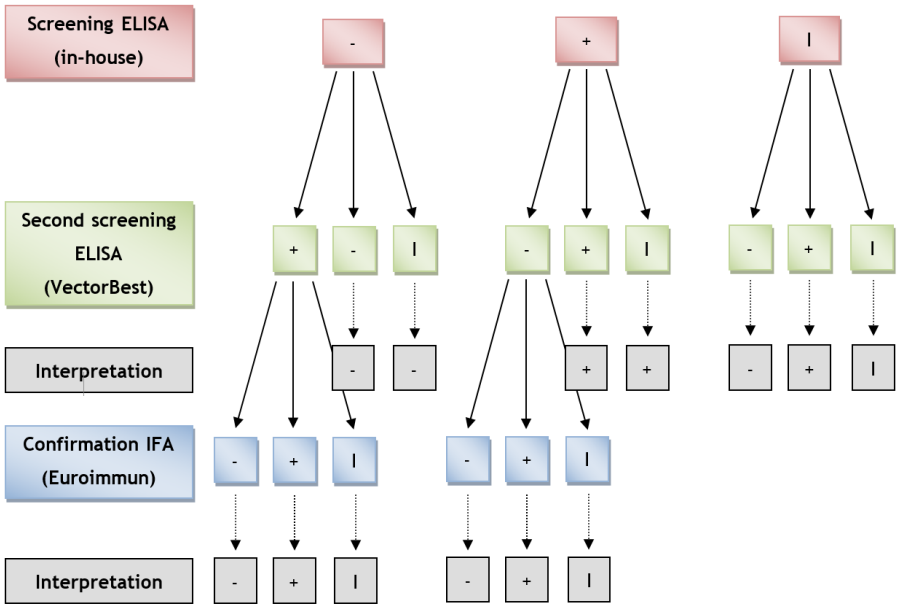

Figure 3.1 Diagnostic approach for CCHFV serology

Diagnostic approach for the complete serological analysis of ruminant samples. I: inconclusive

3.5 **RT-qPCR**

The development and evaluation of a multiplex real-time RT-qPCR for the detection and quantification of all known CCHFV genotypes is described in Manuscript V. The presented primer set (Manuscript V) was used to test Cameroonian tick RNA using the QuantiTect SYBR Green RT-PCR Kit (Qiagen) (Manuscript VI). Samples with a specific signal were retested with the Probe RT-qPCR (Manuscript V) and sequenced with a 3130 Genetic Analyzer (Applied Biosystems/Life Technologies, Waltham, MA, USA) (Manuscript IV). Sequence alignment was performed in BLAST (National Center for Biotechnology Information, Bethesda, MD, USA).

4 MANUSCRIPT I: SEROSURVEY OF CRIMEAN-CONGO HEMORRHAGIC FEVER VIRUS IN CATTLE, MALI, WEST AFRICA

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5 MANUSCRIPT II: WIDESPREAD CRIMEAN-CONGO HEMORRHAGIC FEVER VIRUS INFECTIONS IN CATTLE IN MAURITANIA

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Sas MA, Mertens M, Isselmou E, Reimer N, Mamy BOEL, Doumbia B, Groschup MH. Widespread Crimean-Congo Hemorrhagic Fever Virus Infections in Cattle in Mauritania.

5.1 **Abstract**

5.1.1 Background

Crimean-Congo hemorrhagic fever virus (CCHFV) was detected for the first time in Mauritania in 1983 and several CCHFV outbreaks were reported in the following years. The last human case was diagnosed in 2015. However, no recent data exist about the prevalence of CCHFV in animals, although it is already described that prevalence studies in animals serve as good risk indicators. CCHFV can cause a severe hemorrhagic fever with a high case fatality rate in human. Therefore, a precise risk assessment on the basis of updated data is very important. This paper gives an overview about the current CCHFV prevalence in cattle in Mauritania.

5.1.2 Methods and findings

A seroprevalence study was carried out using 495 cattle sera from Mauritania which were collected in year 2013. The sera were analyzed by an in-house CCHFV-IgG-ELISA. As second screening test, an adapted commercial CCHFV-IgG-ELISA was performed. Inconclusive sera were additionally tested by a modified commercial CCHFV-IgG-IFA. All assays showed high diagnostic sensitivity (>95%) and specificity (>98%). The overall prevalence of CCHFV-specific antibodies found in Mauritanian cattle was 67%, ranging from 56% to 90% in different provinces.

5.1.3 Conclusion

This study shows a very high CCHFV-specific antibody prevalence in cattle in Mauritania. It is the highest seroprevalence detected in Mauritania so far. This strengthens the hypothesis that CCHFV is a serious and ongoing threat for public health in Mauritania.

5.2 **Introduction**

Crimean-Congo hemorrhagic fever (CCHF) is a potentially fatal infectious disease in humans caused by a zoonotic arbovirus (arthropod-borne virus), which belongs to the *Nairovirus* genus in the family *Bunyaviridae*. CCHFV is predominantly transmitted by ticks of the genus *Hyalomma*. Within the tick population vertical (transovarial) and horizontal (sexual,

transstadial) transmission takes place (Logan et al., 1989, Gonzalez et al., 1992). Also, transmission between ticks via cofeeding is possible (Logan et al., 1989). These ticks therefore function as vector as well as reservoir for CCHFV (Whitehouse, 2004). Next to tick bites, origins of human infections are unprotected contact with blood, other body fluids and tissues of viremic animals or humans (Hoogstraal, 1979). Therefore, risk groups are health care workers, abattoir workers, veterinarians and farmers (Ergonul, 2006, Whitehouse, 2004). The case fatality rate in human CCHF cases ranges from 5% in Turkey up to 80% in China (Yilmaz et al., 2009, Yen et al., 1985). This variation probably depends on education, individual and general awareness, effectiveness of the public health system, sensitivity of the notification system (only the severe cases are detected) and the circulating virus strain (Mertens et al., 2013). Number of CCHFV infections are usually quite high in countries with low awareness about CCHF combined with a lack of knowledge of effective protection measures (Mertens et al., 2013).

In contrast to humans, animals do not seem to develop obvious clinical symptoms and only a seroconversion is observed after a viremia of up to two weeks (Gunes et al., 2011, Causey et al., 1970, Gonzalez et al., 1998). However, the detection of CCHFV-specific antibodies in animals is a good indicator for CCHF virus circulation in a region and can be used to define risk areas.

CCHFV was already found in many countries of Africa, Asia and Europe (Hoogstraal, 1979). In Africa, CCHFV has been detected for the first time in Belgian Congo (today Democratic Republic of Congo) in 1956 (Simpson et al., 1967, Woodall et al., 1967). Since then CCHFV was discovered in a number of African countries including Mauritania and its neighbor countries Senegal and Mali (Saluzzo et al., 1985a, Hoogstraal, 1979, Traore et al., 2005). Only a few incidental seroprevalence studies were carried out in Mauritania in humans and animals in the context of human CCHF cases in 1983, 1988 and 2003 (Saluzzo et al., 1985a, Gonzalez et al., 1990, Nabeth et al., 2004a), indicating overall prevalence rates of 9% to 32% in ruminants (Gonzalez et al., 1990, Nabeth et al., 2004a, Saluzzo et al., 1985b).

Given the ongoing occurrence of CCHF cases in humans in Mauritania we have determined the seroprevalence in 495 sera obtained from bovines from different regions in Mauritania in 2013. Sera were screened by an in-house CCHFV-IgG-ELISA, an adapted commercial

CCHFV-IgG-ELISA (VectorBest, Novosibirsk, Russia) and a newly modified commercial CCHFV-IgG-IFA (Euroimmun, Lübeck, Germany).

5.3 **Materials and methods**

5.3.1 Ethic statement

Serum samples were collected according to fundamental ethical principles for diagnostic purposes in the framework of a governmental program for the national surveillance called "Mauritanian epidemiological survey network (REMEMA)" from 1998. This program focusses mainly on: Contagious bovine pleuropneumonia, Rift-Valley fever, foot and mouth disease, peste des petits ruminants, rabies and pasteurellosis in camels.

5.3.2 Study site and serum collection

Mauritania has a size of 1,030,000 km^2, of which 90% are located within the Sahara. Most of the human population and livestock are living in the south. For the current prospective study serum samples were collected in 2013 at different locations within six out of the thirteen regions of Mauritania (Assaba (n=80), Gorgol (n=66), Guidimaka (n=51), Hodh el Chargui (n=50), Hodh el Gharbi (n=65) and Nouakchott (n=183)) by the Mauritanian State Veterinary Laboratory, the Centre National de l'Élevage et de Recherches Vétérinaires (CNERV). The serum samples were not specifically collected for our study but for routine diagnostic monitoring on the Mauritanian livestock by CNERV. 222 out of 495 cattle sera originated from two slaughterhouses, Nouakchott abattoir (n=183) and Hodh el Gharbi abattoir (n=39), which were sampled in the course of the normal slaughtering process and assayed by CNRRV also for the purpose of getting insight into the general infection status of the national cattle herd.

5.3.3 Reference sera

The negative reference serum panel consisted of 113 cattle sera from Germany. Sera were considered to be negative as neither an established *Hyalomma marginatum* population nor human CCHF cases nor the virus itself are present in Germany. Positive reference sera were collected in Albania in 2013 (n=15) and in Macedonia in 2011 (n=20). These sera were

considered to be positive if the commercial CCHFV-IgG-ELISA and the commercial CCHFV-IgG-IFA were positive.

5.3.4 Commercial CCHFV-IgG-IFA

The commercial CCHFV-IgG-IFA adapted for ruminant sera (Mertens et al., 2015) was used to test the gamma irradiated cattle sera at 1/20 serum dilutions in Tris buffered saline with 0.1% Tween 20 (TBST). Goat anti-bovine IgG-FITC (Sothern Biotech, Birmingham, USA) diluted at 1/200 in TBST, containing 0.005% Evans Blue was used as detection system.

5.3.5 Sero-epidemiological investigation

Samples were tested by the in-house CCHFV-IgG-ELISA (Mertens et al., 2015) according to a flow chart for the "Interpretation of ELISA and immunoblot test results" (Mertens et al., 2009). Additionally all sera were tested in the commercial CCHFV-IgG-ELISA (Mertens et al., 2015). Samples were considered to be positive if they were reactive in both ELISAs or equivocal in one ELISA and positive in the other. Inconclusive results were clarified by commercial CCHFV-IgG-IFA.

5.4 **Results**

5.4.1 Adaptation of the commercial CCHFV-IgG-ELISA for testing West African cattle sera

The commercial CCHFV-IgG-ELISA is designed for human sera and was previously adapted for testing sera from European cattle (Mertens et al., 2015). As background staining reactions were slightly higher when sera from African cattle breeds were tested, a new cut-off value was defined and validated. In the newly adapted assay OD values of 0.6 or lower were considered to be "negative" and OD values over 0.6 classified to be "positive".

For this assay validation 113 negative sera from Germany and 51 positive sera from Mauritania were used (Table 5.1). Due to the absence of a gold standard in CCHFV serology, sera from Mauritania were defined as positive if they were reactive in the in-house CCHFV‑IgG‑ELISA and in the CCHFV‑IgG‑IFA.

All negative sera were “true negative” and just one positive defined serum showed “false negative”. Over all, this lead to a diagnostic sensitivity (D-SN) of 98% and a diagnostic specificity (D-SP) of 100% (Table 5.2).

5.4.2 Modification and validation of the commercial CCHFV-IgG-IFA

The previously adapted commercial CCHFV-IgG-IFA (Mertens et al., 2015) was validated using sera from Mauritania (n=50), Albania (n=15), Germany (n=48) and Macedonia (n=20). Sera were considered ‘positive’, if positive in the in-house CCHFV-IgG-ELISA and in the adapted commercial CCHFV-IgG-ELISA (Figure 5.1).

Four samples gave a “false negative” result, one sample gave a “false positive” result and one sample gave an “equivocal” result (Table 5.1) so that the diagnostic sensitivity and specificity were 95% and 98% respectively (Table 5.2).

5.4.3 Seroprevalence study

333 (67%) out of 495 sera from Mauritania tested positive for CCHFV-specific antibodies. 51 positive results (64%) were found in Assaba region, 37 (56%) in Gorgol region, 38 (75%) in Guidimaka region, 45 (90%) in Hodh el Chargui region, 17 (65%) in Hodh el Gharbi region, 23 (59%) in Hodh el Gharbi abattoir and 122 (67%) in Nouakchott abattoir. The regional prevalence rates were therefore quite high and reached even up to 90% for Hodh el Chargui. Detailed results are shown in Figure 5.2 and Table 5.3.

5.5 **Discussion**

In this study we investigated the prevalence of CCHFV in cattle in different regions of Mauritania. For this purpose, it was necessary to modify and validate the commercial CCHFV-IgG-IFA and to validate the commercial CCHFV-IgG-ELISA. Both modified assays showed a high diagnostic sensitivity and specificity using defined positive and negative reference sera.

It is quite remarkable that using these assays we found a surprisingly high (67%) overall seroprevalence for the presence of CCHFV antibodies in cattle in Mauritania. In an earlier study from the 90ties seroprevalence rates in similar Mauritanian regions were found to be up

to 26% and on average 18% (Gonzalez et al., 1990). A newer study, from Nabeth et al., included a relatively small number of samples (n=97) from just two regions. It focused on animals from Nouakchott and Brakna in 2004 in direct surrounding of infected humans and showed a CCHFV-specific antibody prevalence of 18% (Nabeth et al., 2004a). As no data were collected about Brakna region in the present study, a direct comparison is difficult.

The sera from Nouakchott and part of the sera from Hodh El Gharbi were collected at the slaughterhouse and it was not possible to prove their primary origin. However, it is likely that these cattle came from surrounding pastry regions. Also, it is difficult to check the validity of the results of the former studies as only little or no data were given in regards to the D-SN and D-SP of the ELISAs used.

Therefore our data now indicate that the majority of Mauritanian cattle have been infected by CCHFV, yielding seroprevalence even higher than those found in high endemic regions in Turkey where about one thousand clinical human cases are seen every year of which up to 50 are of fatal outcome (Mertens et al., 2016b). Cattle in slaughterhouses show similar high prevalence like the rest of the tested regions. This indicates the especially high risk for workers in this field.

5.6 **Conclusion**

The high CCHFV-specific antibody prevalence in Mauritania indicate an active virus circulation in the human environment. This means an elevated CCHFV exposure risk especially for humans living and working outdoors in areas, where *Hyalomma* ticks reside, e.g. a large part of the pastoral and agricultural population in Mauritania. Surprisingly, human CCHF cases are reported in Mauritania only sporadically and it is not clear why this number is so low. The most likely option may be a massive underreporting of cases, as the human population is inadequately informed about this potentially fatal disease. Education of the medical personnel and risk groups like veterinarians, slaughterhouse workers and farmers is recommended in areas with a high seroprevalence. Another reason for the discrepancy between detected prevalence rates and reported human cases might be the circulation of a low or avirulent CCHFV strain. This can only be clarified through further characterization of

circulating virus strains, prevalence studies in humans and a better detection and reporting of human cases.

Taken together the here presented study will help to stress the importance of CCHFV infections in Mauritania and the need to implement public health measures for the prevention and diagnosis of CCHF.

5.7 **Tables**

Table 5.1 Validation results of the commercial CCHFV-IgG-ELISA and-IFA for the reference sera.

		Positive reference sera	Negative reference sera
Commercial CCHFV-IgG-ELISA	Positive	50	0
	Negative	1	113
Commercial CCHFV-IgG-IFA	Positive	80	1
	Negative	4	47
	Inconclusive	1	0

Table 5.2 Diagnostic sensitivity and specificity of the commercial tests.

	Diagnostic sensitivity in %	Diagnostic specificity in %
Commercial CCHFV-IgG-ELISA	98 (90-100)	100 (97-100)
Commercial CCHFV-IgG-IFA	95 (88-99)	98 (89-100)

The confidence interval (95%) is shown in brackets.

Table 5.3 Results of different seroepidemiological studies in ruminants in Mauritania.

Region	Present study (cattle)		Nabeth et al. 2004 (sheep, goat)		Gonzalez et al. 1990 (sheep)	
	n	Prev. in %	n	Prev. in %	n	Prev. in %
Assaba	80	64 (52-74)	-	-	252	18 (13-23)
Brakna	-	-	25	16 (5-36)	42	26 (14-42)
Gorgol	66	56 (43-68)	-	-	238	13 (9-18)
Guidimaka	51	75 (60-86)	-	-	153	9 (5-14)
Hodh El Chargui	50	90 (78-97)	-	-	331	22 (18-27)
Hodh El Gharbi region	26	65 (44-83)	-	-		
Hodh El Gharbi abattoir	39	59 (42-74)	-	-	-	-
Nouakchott region	-	-	72	18 (10-29)	-	-
Nouakchott abattoir	183	67 (59-73)	-	-	-	-
Trarza	-	-	-	-	203	24 (18-30)
Total	**495**	**67 (63-71)**	**97**	**18 (11-27)**	**1219**	**18 (16-21)**

n: Number of samples; Prev.: Prevalence; the confidence interval (95%) is shown in brackets

5.8 **Figures**

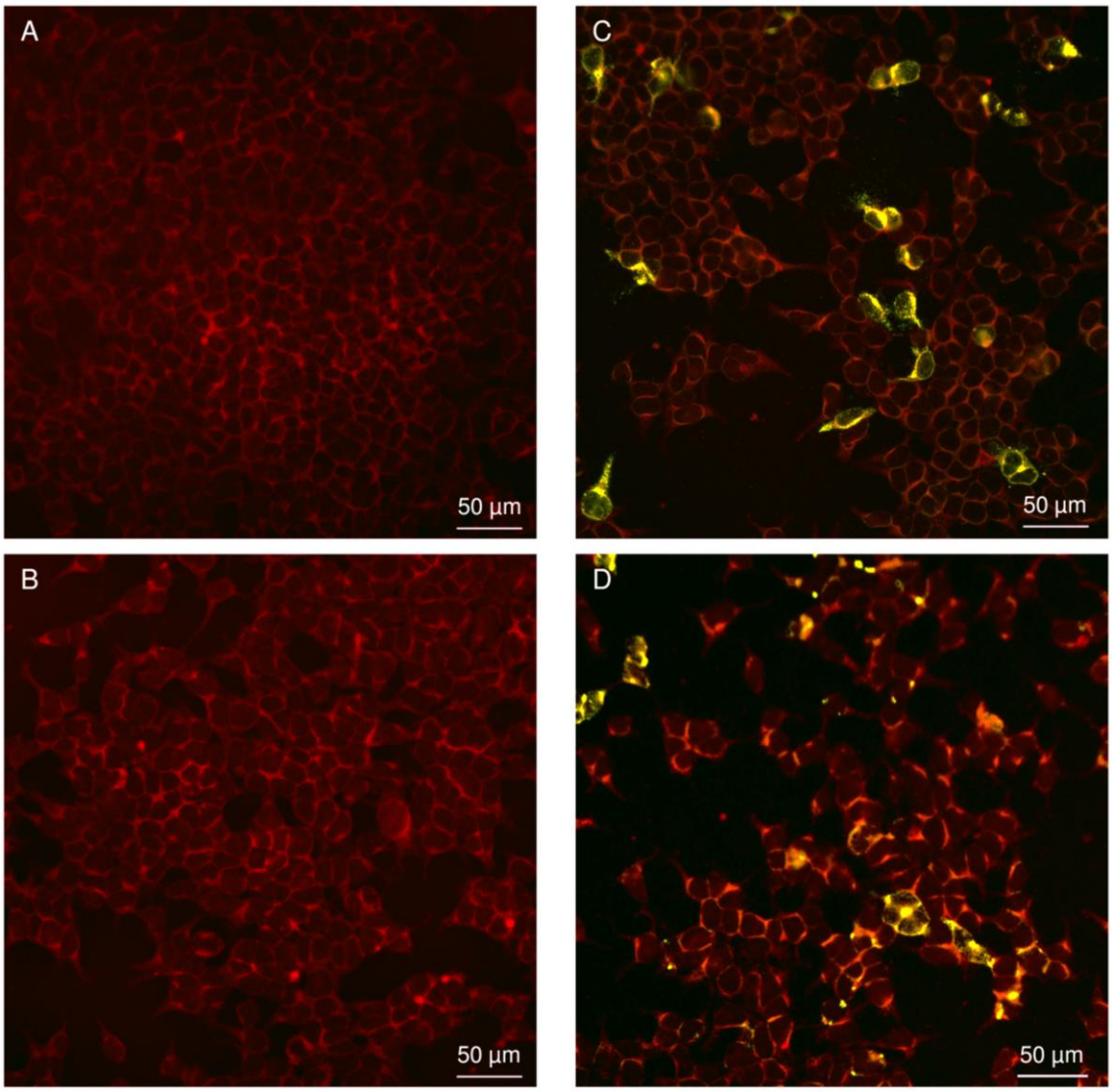

Figure 5.1 Commercial CCHFV-IgG-IFA.

The commercial CCHFV-IgG-IFA was performed with sera from Europe and Africa. Negative sera (Germany) show no fluorescence for glyco-protein (A) and nucleocapsid-protein (B) transfected cells, whereas positive sera (Mauritania) show fluorescence for glyco-protein (C) and nucleocapsid-protein (D) transfected cells.

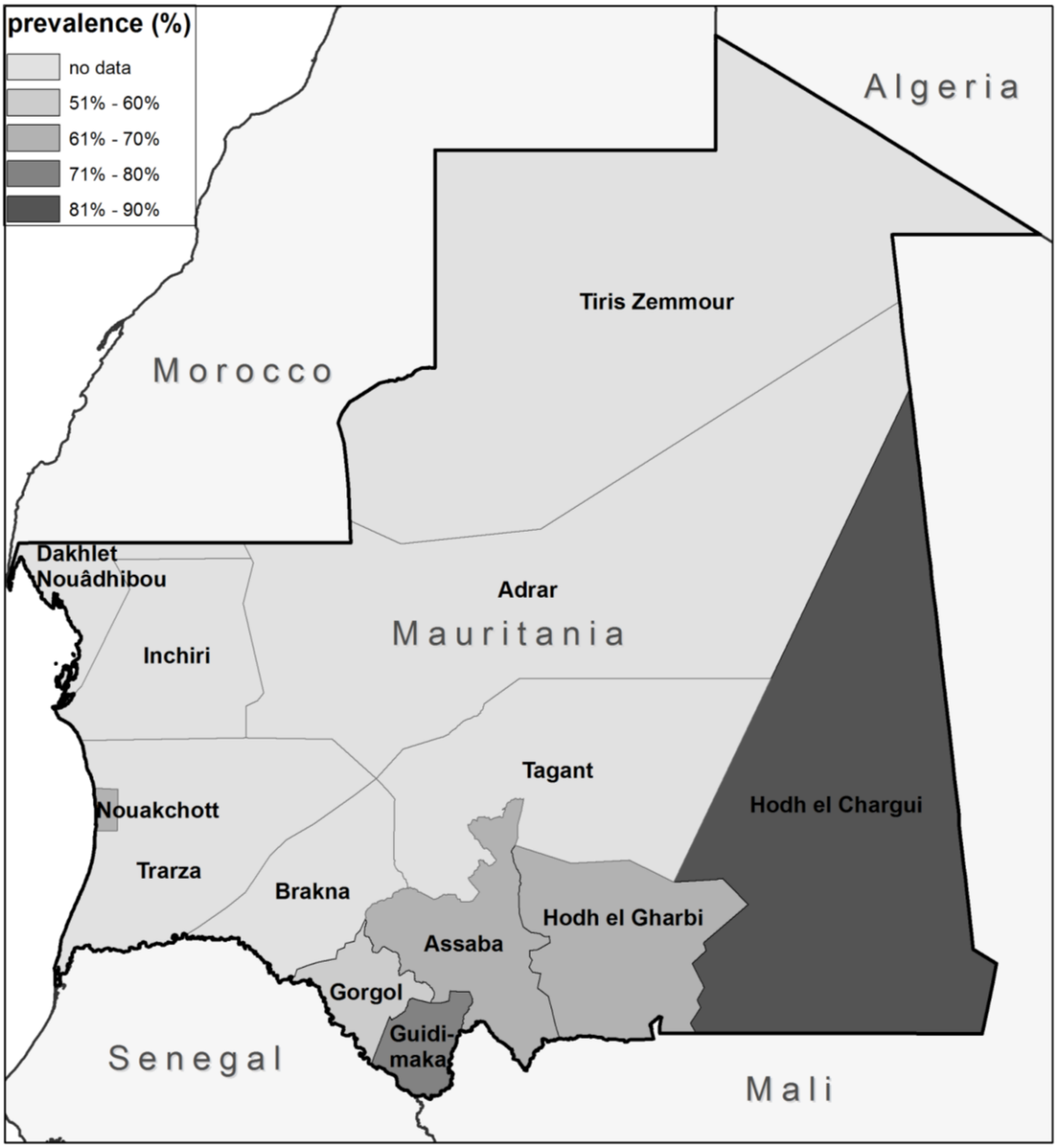

Figure 5.2 Map of Mauritania with sampled regions.

All sera from Nouakchott and 23 out of 40 sera from Hodh el Gharbi were collected at the slaughterhouse. All other cattle sera were collected at different farms.

5.9 **Acknowledgments**

This study was funded by the German Federal Foreign Office in the framework of the German Partnership Program for Excellence in Biological and Health Security (grant number 2513AA0374, URL: http://www.auswaertigesamt.de/EN/Aussenpolitik/Abruestung/BioChemie/Biosicherheitsprogramm.html).

6 MANUSCRIPT III: SEROSURVEY FOR CRIMEAN-CONGO HEMORRHAGIC FEVER VIRUS INFECTIONS IN RUMINANTS IN KATANGA PROVINCE, DEMOCRATIC REPUBLIC OF THE CONGO

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Sas MA, Mertens M, Kadiat JG, Schuster I, Pongombo CPS, Malobab AGK, Groschup MH. Serosurvey for Crimean-Congo hemorrhagic fever virus infections in ruminants in Katanga province, Democratic Republic of the Congo.

6.1 **Abstract**

Crimean-Congo hemorrhagic fever virus (CCHFV) has been detected in many African countries. Unfortunately, little is known about the current CCHFV situation in most of those countries including the Democratic Republic of the Congo (DRC). In over 50 years, three human CCHF cases have been detected in DRC but no seroepidemiological investigation was performed so far. To determine the prevalence of CCHFV-specific antibodies we tested 838 serum samples of cattle, goat and sheep from the southern province Katanga, DRC.

The detected seroprevalence in ruminants was 1.4% ranging from 0.2% to 3.4% between the two sampling sites. The low prevalence indicates only sporadic introduction of CCHFV into this part of the country. DRC is a very large country and the study was performed only at two locations in one province; therefore, the investigations can be only a starting point for further epidemiological activities.

6.2 **Introduction**

Crimean-Congo hemorrhagic fever virus (CCHFV) is a *Nairovirus* belonging to the family *Bunyaviridae.* CCHFV is predominantly transmitted by ticks of the genus *Hyalomma*. Other transmission pathways are unprotected contact with blood, other body fluids and tissues of viremic animals or human patients (Hoogstraal, 1979). In humans, CCHFV infections can cause a severe hemorrhagic disease (CCHF) with case fatality rates ranging from 5% in Turkey to 80% in China (Yilmaz et al., 2009, Yen et al., 1985). This variability probably depends on the circulating virus strain, awareness of the population and effectiveness of the public health system (Mertens et al., 2013). Unlike humans, infected animals do not show clinical signs but a one to two weeks' viremia and a seroconversion can be detected. The detection of antibody positive livestock correlates with the occurrence of human cases and hence can be used to identify CCHFV risk areas (Bente et al., 2013).

CCHFV has been detected in several countries of Africa, Europe and Asia. The first detection in Africa was in a 13-year-old boy from Kisangani with a poorly described clinical history in the Democratic Republic of the Congo (DRC; formerly Belgian Congo) in 1956; followed directly by a laboratory infection caused by the isolated strain of this boy (Simpson et al.,

1967, Woodall et al., 1967). The next human CCHF case in DRC was a 26-year-old man from Beruwe in 2008 (Grard et al., 2011).

Even though the country was partly name-giving and a few human cases have been reported decades ago, there have been no seroepidemiological studies carried out in DRC to date using ruminants as indicator species for CCHFV infections in the environment.

6.3 **Material and methods**

6.3.1 Serum samples

Serum samples were collected under the direction of the University of Lubumbashi and the Laboratoire Vétérinaire de Lubumbashi in year 2013. All cattle sera (n = 514) originated from a farm in Kamina, Katanga province. The goat (n = 186) and the sheep (n = 138) sera were collected at different sites in and around Lubumbashi. All samples were gamma-irradiated before serological investigation at FLI, Germany.

6.3.2 Serological analysis

All sera were analyzed by a species specific in-house CCHFV-IgG-ELISA and an adapted species specific commercial CCHFV-IgG-ELISA (Vector Best, Novosibirsk, Russia). In case of divergent results, samples were run in a species adapted commercial CCHFV-IgG-IFA (Euroimmun, Lübeck, Germany) to get to a final result.

The serological assays for cattle (Mertens et al., 2015) and for goat and sheep (Schuster et al., 2016b) were previously described. The cattle specific IFA was adapted for use in irradiated sera (Sas et al., In preparation-a).

6.4 **Results**

One of the 514 bovines tested CCHFV antibody positive (0.2% prevalence; 95% CI: 0% – 1.1%), whereas in eleven of 186 tested goats (5.9% prevalence; 95% CI: 3.0% - 10.3%) CCHFV-specific antibodies were detected. No sheep was positive for CCHFV-specific

antibodies. The overall prevalence for small ruminants was 3.4% (95% CI: 1.7% - 6.0%) in the Lubumbashi area (Table 6.1 and Figure 6.1).

6.5 **Discussion and conclusion**

DRC is eponymous in part for CCHFV as the first African cases were detected in Kisangani, Orientale province in 1956. However, the diagnosis of just three human cases in over 50 years raises the question, whether CCHF cases are just underreported or indeed occur only quite rarely.

Kisangani is located in the equatorial rain forest area as is Beruwe, North Kivu, where the CCHF case occurred in 2008 (Figure 6.1). The tropical rain forest is not the preferred habitat for *Hyalomma* ticks which are considered to function as the main vector for CCHFV. Indeed, *Hyalomma* ticks prefer rather grass and tree savanna and semiarid desert habitats and CCHF cases are usually linked to this type of clime and vegetation (Estrada-Pena et al., 2007). Therefore, the tree and grassland savanna regions of the Katanga province were chosen for this CCHF seroprevalence study in ruminants, in order to increase the chances for finding antibodies to CCHFV. Surprisingly, only low prevalence rates were found in cattle (0.2%), goat (5.9%) and sheep (0%) in the Katanga province. This shows that CCHFV is circulating albeit at a very low level so that the human CCHFV exposure risk might be fairly low. However, risk groups, i.e. health care personnel, slaughterhouse workers, veterinarians and farmers should still not neglect this diagnosis and risk. Since only animals from two different locations in one province were tested, it would be speculative to believe that the results reflect the situation for the whole country. A seroepidemiological study in cattle in neighboring Tanzania revealed similarly low (0.6% - 7.4%) prevalence rates (Hoogstraal, 1979), while a much higher seroprevalence (36.5%) was detected in cattle from Uganda (Spengler et al., 2016a). However, those studies were performed more than 40 years ago by the less sensitive agar gel precipitation test.

To corroborate our results now, vector studies should be carried out by collecting sucking ticks from mammals and assay their CCHFV infection status.

6.6 **Tables**

Table 6.1 Results of the seroepidemiological study in ruminants in DRC.

Location	Species	n	Positives	Prev. in %
Kamina	Cattle	514	1	0.2 (0-1.1)
Kafubu road (LA)	Small ruminant	29	1	3.4 (0.1-17.8)
Kamalondo (L)	Small ruminant	62	3	4.8 (1.0-13.5)
Kasumbalesa road (LA)	Small ruminant	98	7	7.1 (2.9-14.2)
Kasenga road (LA)	Small ruminant	135	0	0 (0-2.7)
Total		**838**	**12**	**1.4 (0.7-2.5)**

L: Lubumbashi; LA: Lubumbashi area; n: number of samples; Prev.: prevalence; the CI (95%) is shown in brackets

6.7 **Figures**

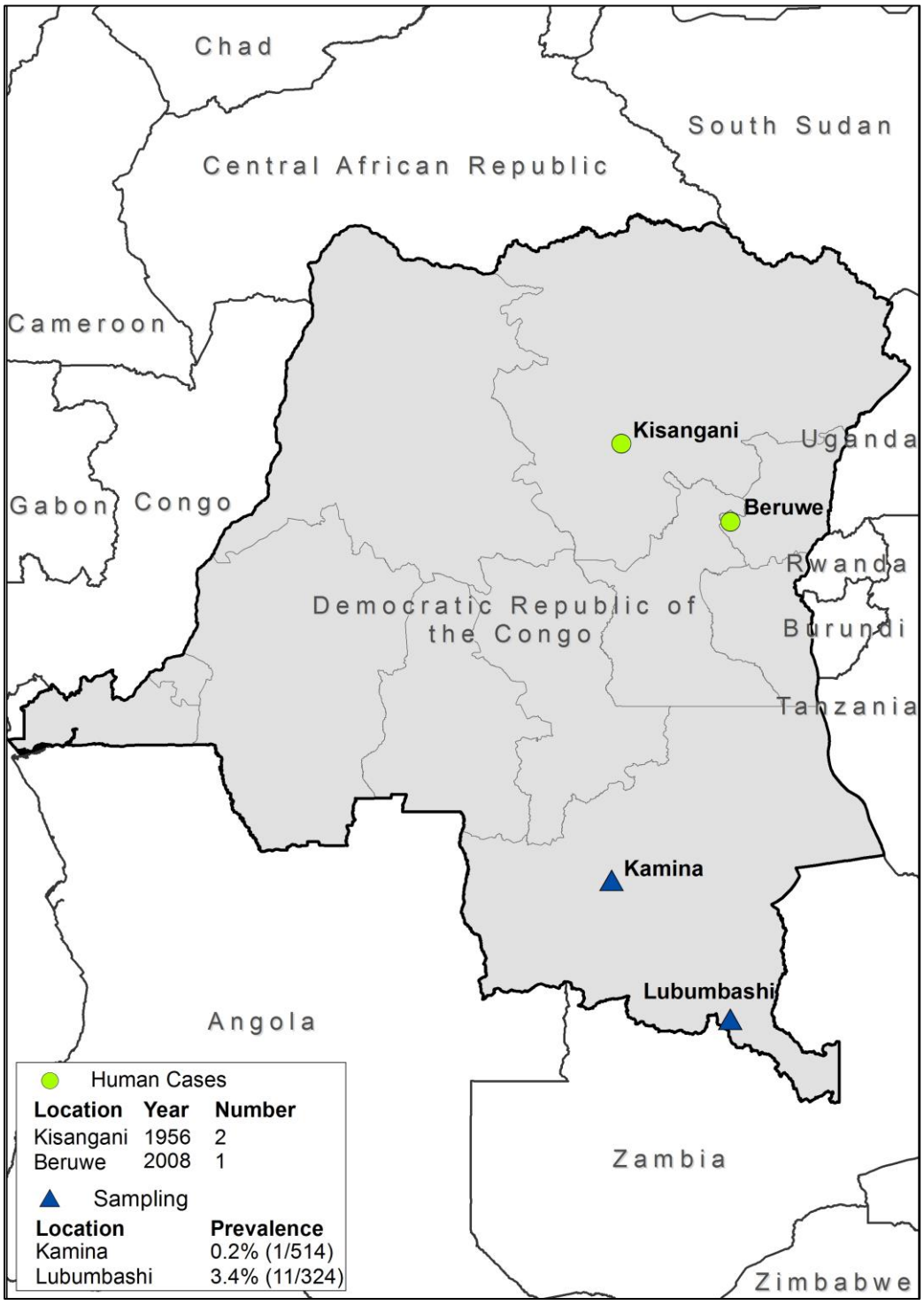

Figure 6.1 Map of DRC with human cases and sampling sites.

All sera from Kamina were collected at one farm. The sera from Lubumbashi were collected at different sampling sites in Lubumbashi area (Kafubu road, Kamalondo, Kasumbalesa road and Kasenga road). This Map was designed at FLI using ArcGIS 10.3.1.

6.8 **Acknowledgments**

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7 MANUSCRIPT IV: CRIMEAN-CONGO HEMORRHAGIC FEVER VIRUS IN CAMEROON

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This manuscript was prepared for submission.

7.1 **Abstract**

Crimean-Congo hemorrhagic fever virus (CCHFV) is a tick-borne zoonotic virus, which can induce a severe hemorrhagic fever with lethality rates of up to 80% in humans. The virus circulates in a tick-vertebrate-tick cycle. Infected animals show no clinical signs but may exhibit a viremia for up to two weeks and antibodies can be detected for up to several years. The screening of domestic ruminants for CCHFV-specific antibodies can be a good indicator for ongoing or previous CCHFV infections in an area. CCHFV is distributed in several countries in Southern Europe, Asia and Africa, but with few exceptions hardly any detailed information is available on particular incidence rates. If available, for most African countries most data on CCHFV are older than 20 years. Therefore, in the present work the CCHFV situation was analyzed in Cameroon by screening close to one thousand bovines coming from seven different national departments. Thereby, an overall seroprevalence of 74% was found. Moreover, in order to demonstrate the actual presence of CCHFV, 109 Hyalomma ticks were collected from cattle in high prevalence areas and CCHFV-RNA was detected in 7 of them. This result represents the first indication of the CCHFV circulation in Cameroon.

7.2 **Introduction**

Crimean-Congo hemorrhagic fever virus (CCHFV) is a vector-bone zoonotic pathogen circulating in a tick-vertebrate-tick cycle. CCHFV is found in Africa, Asia and Southern Europe and its presence correlates with and is limited by the distribution of vector-competent ticks (Whitehouse, 2004, Bente et al., 2013). Ticks, especially those of the genus Hyalomma, are the vector as well as natural reservoir of CCHFV (Bente et al., 2013). The virus is transmitted within the tick population horizontally (transstadial, venereal) and vertically (transovarial) (Logan et al., 1989, Gonzalez et al., 1992). CCHFV-RNA or proteins were detected in ticks from endemic areas of Turkey (20%), Kosovo (15%) and Bulgaria (15%) (Gunes et al., 2011, Duh et al., 2006, Panayotova et al., 2016). A broad spectrum of small and large mammals are involved in the infection cycle as amplifying hosts and as hosts for ticks needing a blood meal for their development as well as the egg development (Apanaskevich, 2004, Gargili et al., 2013, Shepherd et al., 1989a, Spengler et al., 2016a). Infected ticks transmit the virus to naïve animals and naïve ticks can be infected by sucking blood of a

viremic animal as well as by co-feeding (Logan et al., 1989). Infected animals do not show clinical signs, but can develop a viremia lasting up to 15 days and a seroconversion can be observed (Gonzalez et al., 1998, Spengler et al., 2016b). In Turkey, CCHFV-RNA was found in up to 9% of the tested ruminants in the endemic region (Albayrak et al., 2012a). While CCHFV-specific IgM antibodies are detectable for approximately one to two month, IgG antibodies persist for about three years (Gonzalez et al., 1998).

Human infections occur by tick bite, crushing of ticks or by unprotected contact with blood or body fluids of viremic animals or humans (Mertens et al., 2013). In contrast to animals, infection of humans can lead to a severe hemorrhagic disease (Crimean-Congo hemorrhagic fever (CCHF)) with fatality rates of up to 80% (Yen et al., 1985).

The information about presence or absence of CCHFV in a region or a country is essential for defining the need of implementing public health protection measures. Especially protecting health care workers is essential in this respect (Mertens et al., 2013). Since CCHFV-specific IgG antibodies can persist for several years in animals, the presence and distribution of CCHFV can be assessed by antibody screening of ruminants of a given area (Mertens et al., 2016a). Using this approach a current or recent CCHFV circulation was demonstrated for several new countries or regions in the last years (Mertens et al., 2015, Ceianu et al., 2012, Nemeth et al., 2013, Tuncer et al., 2014, Spengler et al., 2016a).

In Nigeria, the first detection of CCHFV was in 1964 and until 1968 a total of 34 CCHFV isolates were obtained from ticks and ruminants (Causey et al., 1970). In 1983, testing of the cattle population (n = 1164) for CCHFV-specific antibodies by Agar Gel Diffusion Precipitation (AGDP) test revealed a seroprevalence of 26% (Umoh et al., 1983). In the present study, the occurrence of CCHFV was investigated in neighboring Cameroon for the first time. Therefore, 980 cattle were tested for CCHFV-specific antibodies. For confirmation of the serological results, 109 ticks were tested for CCHFV-RNA by PCR.

7.3 **Materials and Methods**

In 2013, serum samples from 416 healthy cattle were randomly collected in five Cameroonian departments (Mayo Sava, Diamare, Benoue, Mayo Louti, Vina) and in 2014, another 564 sera

(departments Diamare, Benoue, Vina, Mefou-et-Akono and Dja-et-Lobo). Samples were tested for CCHFV-specific IgG antibodies by in-house and by commercial (VectorBest, Novosibirsk, Russia) Enzyme-linked Immunosorbent Assays (ELISAs) as well as by commercial Immunofluorescence Assay (IFA; Euroimmun, Lübeck, Germany). Assays were designed or respectively adapted for cattle sera (Mertens et al., 2015, Maiga et al., in preparation, Sas et al., In preparation-a). In-house and commercial ELISAs were used as parallel screening assays and samples were classified as positive if they were positive in both assays. In case of divergent results the IFA was used as confirmatory assay (Mertens et al., 2015). Seroprevalence rates with confidence intervals of 95% were calculated and compared using the Fisher Exact Test. A p-value lower than 0.05 ($p < 0.05$; 95% confidence interval; CI) was considered to be statistically significant.

In 2015, 109 ticks of the genus Hyalomma were collected from grazing cattle in the Nord region. The ticks were sent immersed in 98% ethanol to Friedrich-Loeffler-Institut, Germany. Each tick was homogenized with a 5 mm steal bead (Isometall, Pleidelsheim, Germany) using a TissueLyser II (Qiagen, Hilden, Germany) at 30 Hz for 2 min in 1 ml TRIzol (Invitrogen, Carlsbad, CA, USA). Following a phenol-chloroform extraction the viral RNA was purified using the RNeasy Mini Kit (QIAGEN) following the manufacturer's instructions. RNA was eluted with 50 µl RNAse free water and stored at -80 °C. The purified RNA was screened by SYBR Green real-time quantitative reverse transcription polymerase chain reaction (RT‑qPCR) using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), the QuantiTect SYBR Green RT-PCR Kit (QIAGEN) and a previously designed primer set (Sas et al., in preparation-b). The cycling conditions were: 50 °C for 30 min, 95 °C for 15 min, followed by 44 cycles at: 95 °C for 15 sec, 55 °C for 25 sec, 72 °C for 25 sec and 78 °C for 5 sec. The quantity was measured after each 55 °C, 72 °C and 78 °C step. Melt curve analysis was performed by denaturation at 95 °C for 1 min and a temperature gradient from 60 °C to 93 °C (0.1 °C each step for 5 sec). The PCR targeted a 181 nucleotide long sequence on the S-segment. Amplicons from samples giving a specific signal in the heat curve analysis and a CT value of < 34 were sequenced on a 3130 Genetic Analyzer (Applied Biosystems/Life Technologies, Waltham, MA, USA). Sequences were eventually aligned with CCHFV sequences obtained from the GenBank Database (National Center for Biotechnology Information, Bethesda, MD, USA).

7.4 **Results**

Summarizing the results of 2013 and 2014, testing of cattle for CCHFV-specific IgG antibodies revealed high seroprevalence for animals from the northern (Mayo Sava 69%, Diamare 87%, Mayo Louti 48%, Benoue 82%) and central regions (Vina 85%), while much lower prevalences were found for animals from the southern regions (Mefou-et-Akono 9%, Dja-et-Lobo 24%). Therefore the overall prevalence in north and central Cameroon (n = 923) was 77% (CI: 75-80), whereas the prevalence in the south (n = 57) was 21% (CI: 11-34). Altogether, the overall prevalence in cattle (n = 980) in Cameroon was 74% (CI: 71-77) (Table 7.1, Figure 7.1). The comparably lower prevalence in one northern district Mayo Louti (48%; CI: 40-56; n = 160) is unclear but may be caused by a sample selection artifact.

Finally, seven out of 109 Hyalomma ticks, collected from grazing cattle of the Benoue district were positive for CCHFV-RNA. The highest sequence homology was found with the respective frame of the S-segment of CCHFV strain IbAr10200 from Nigeria (Causey et al., 1970). This strain belongs to clade III together with strains from Mauritania, the Central African Republic, Sudan and South Africa (Bente et al., 2013). All positive ticks were identified as *Hyalomma truncatum*, which is the most common tick species in Cameroon (unpublished data).

7.5 **Discussion**

The high prevalence of CCHFV-specific antibodies in cattle (74%) in combination with the demonstration of CCHFV-RNA in ticks is a strong indication for a stable circulation of CCHFV in wildlife and in domestic animals especially in the northern and central regions of Cameroon. As only few ticks were collected, finding of CCHFV-RNA in them means rather a proof of principle, but a prevalence analyses was not possible. Anyhow, the finding of 6% positive ticks is quite high compared to 0.4% CCHFV positive individual ticks in Nigeria (Causey et al., 1970). It is uncertain, how these results compare to the results for endemic areas in Kosovo where an average CCHFV prevalence of 11% (Clades V, Europe 1) have been reported in individual *Hyalomma marginatum* ticks (Sherifi et al., 2014) and to 19%

CCHFV antigen positive *Hyalomma marginatum* tick pools collected from cattle in the high endemic area of Turkey (Gunes et al., 2011).

Anyhow, no human CCHF case was reported in Cameroon so far. It is unclear whether this means that the circulating strain is causing only very mild or no clinical symptoms, similar to the AP92 strain in Greece (Papa et al., 2014), or whether human cases are essentially underdiagnosed. The Cameroonian strains seem to form a cluster with the Nigerian IbAr10200 strain, whereby the data from the circulating strains in Nigeria are not up to date and from Cameroon full length sequences are missing (Causey et al., 1970). Anyhow, further studies are needed to characterize the circulating CCHFV strains in Cameroon and to reveal its infection cycle.

Interestingly, no seroprevalence differences were observed for the cattle cohorts, from which blood was collected in 2013 and in 2014, meaning that there was no obvious trend over time. Results for both years were almost identical for Diamare, and the difference for Benoue between 2013 (75%) and 2014 (86%) was not significant ($p = 0.5$).

The seroprevalence was significantly lower ($p < 0.01$) for the two southern districts as compared to the northern regions. Although sample numbers for the southern districts were quite low, the results obtained indicate that the risk of human exposure is much higher in the north. Altogether, these findings are in line with modeling data, which identified the north of Cameroon as a probable risk area for CCHFV (Messina et al., 2015). The reason for this gradient may result from the different climatic zones in Cameroon (Figure 7.1). Due to the impact of the Sahara, the northern part of Cameroon is much hotter and dryer than the south. This Sub-Saharan climate constitutes the preferred habitat of the CCHFV vector ticks, which prefer low to moderate levels of humidity and a long dry season during the summer months (Estrada-Pena et al., 2011, Whitehouse, 2004). The southern regions of Cameroon are dominated by rain forests forming a much more humid climate. A similar result was found in a previous study in Nigeria. The authors found the highest prevalences in cattle of the most northern sampling location (30%) and the lowest in the south (22%) (Umoh et al., 1983). Another reason for the low prevalence in the south of Cameroon might be the low density of cattle in the south compared to the northern and central regions (Figure 7.1). Cattle are important hosts for adult Hyalomma ticks, and higher densities of cattle support much bigger

tick populations and therewith an easier horizontal CCHFV transmission, especially by co‑feeding.

In conclusion, this is the first report about the circulation of CCHFV in Cameroon. The high antibody prevalence in cattle and the detection of CCHFV-RNA in ticks can mean a public health risk. As in other countries and following the suggestions of OIE, no animal trade restrictions apply. Since no human cases have been reported until now, further studies are needed to characterize the circulating CCHFV strain and to determine the prevalence of CCHFV-specific antibodies in humans. CCHFV infection risks can be reduced effectively by informing the public and by training health care workers in using adequate protection measures.

7.6 **Tables**

Table 7.1 Results of the seroepidemiological study in cattle of Cameroon (2013/2014)

Department	**Year**	**Number of Samples**	**Seroprevalence per Year (%)***	**Overall Seroprevalence (%)***
Mayo Sava	2013	36	69 (52-84)	69 (52-84)
Diamare	2013	35	86 (70-95)	87 (81-91)
	2014	181	87 (81-91)	
Mayo Louti	2013	160	48 (40-56)	48 (40-56)
Benoue	2013	109	75 (66-83)	82 (77-86)
	2014	175	86 (80-91)	
Vina	2013	76	76 (65-85)	85 (80-89)
	2014	151	89 (83-94)	
Mefou-et-Akono	2014	11	9 (0-41)	9 (0-41)
Dja-et-Lobo	2014	46	24 (13-39)	24 (13-39)
Total	**-**	**980**	**74** (71-77)	

* 95% confidence intervals shown in brackets

7.7 Figures

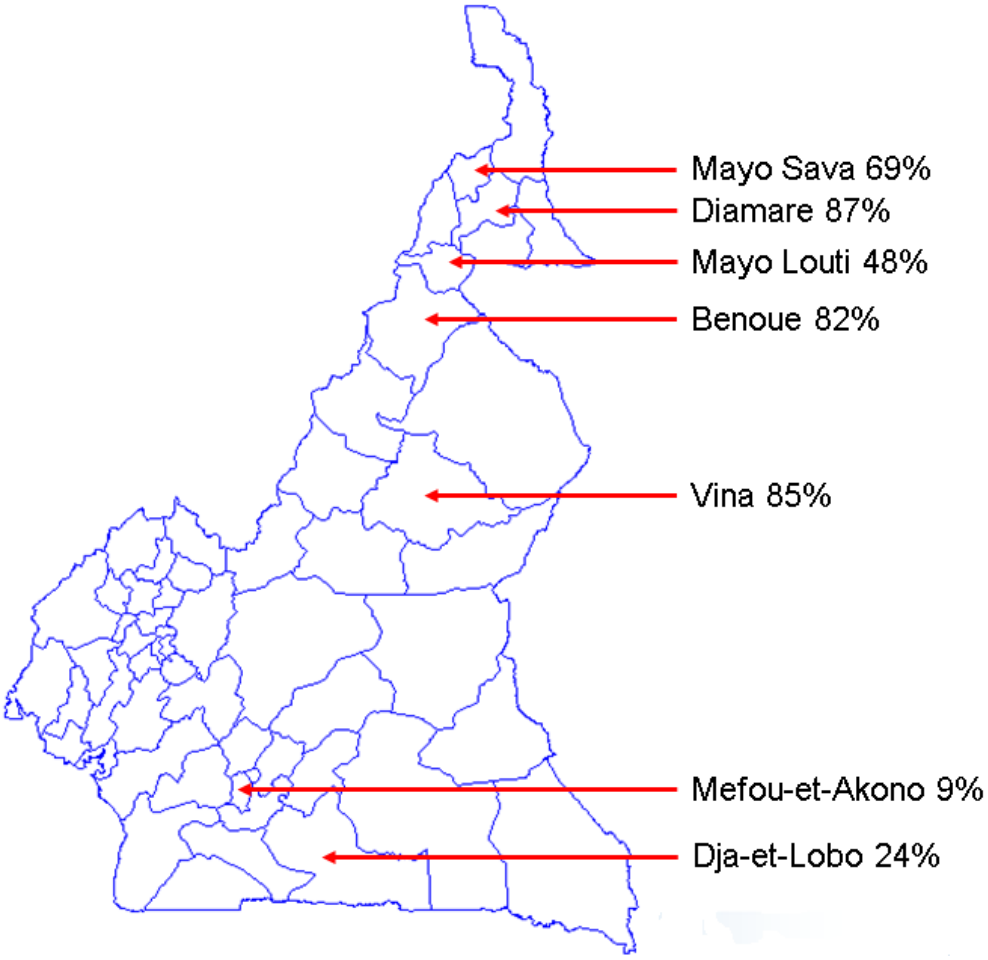

Figure 7.1 Distribution of CCHFV in Regions of Cameroon

Province names with prevalence: CCHFV-specific antibodies in cattle (%).

7.8 Acknowledgements

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8 MANUSCRIPT V: A ONE-STEP MULTIPLEX REAL-TIME RT-PCR FOR THE UNIVERSAL DETECTION OF ALL CCHFV GENOTYPES

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8.1 **Abstract**

Crimean-Congo hemorrhagic fever (CCHF) is a fatal disease in humans, which is endemic in many countries of Africa, Southern Asia and Southeastern Europe. It is caused by the Crimean-Congo hemorrhagic fever virus (CCHFV), which is an arthropod-borne virus (arbovirus) transmitted by ixodid ticks, mainly of the genus *Hyalomma*. Animals like hares, hedgehogs, cattle, camels and small ruminants can become infected without developing clinical signs. Seroconversion occurs after a short viremia of up to two weeks, and thus seroprevalence studies in ruminants can be used to reveal risk areas for the human population. Virus detection by real-time quantitative reverse transcription polymerase chain reaction (RT‑qPCR) is essential to prove an actual circulation of CCHFV in a country and is also used as diagnostic method for acute human CCHFV infections. In this study, we present a new universal one-step multiplex real-time RT-qPCR for the sensitive and specific detection of CCHFV. For this purpose, 14 new primers and 2 probes were simultaneously used to detect RNAs representing all six CCHFV genotypes. Additionally, a GC-mirrored sequence within the synthetic RNAs enables the discrimination between true positive samples and unintentional laboratory contaminations. CCHFV negative samples from different animal species and ten different members of the family *Bunyaviridae* were eventually tested to reveal the specificity of the new RT-qPCR. This is the first report of an all genotype encompassing RT-PCR-detection of CCHFV.

8.2 **Introduction**

Crimean-Congo hemorrhagic fever virus (CCHFV) belongs to the family *Bunyaviridae*, which includes more than 350 diverse viruses, grouped into five different genera (*Hantavirus*, *Nairovirus*, *Orthobunyavirus*, *Phlebovirus*, *Tospovirus*). Together with Hazara virus (HAZV) CCHFV forms a distinct serogroup within the *Nairovirus* genus.

Crimean-Congo hemorrhagic fever (CCHF) is a zoonotic disease with a potentially fatal outcome in humans. In contrast, CCHFV infections are asymptomatic in most animal species (Whitehouse, 2004). Clinical signs in humans are usually non-specific and vary from gastrointestinal to flu-like symptoms. Eponymous and characteristic hemorrhages occur just in severe cases and often directly precede the patient's death. Case fatality rates spanning

from 5 % in Turkey to 80 % in China (Yen et al., 1985, Yilmaz et al., 2009) have been reported. This broad variation might depend on awareness of the population, effectiveness of the public health system and the circulating virus strain (Mertens et al., 2013).

The most common transmission route of CCHFV is via tick bites. Ticks, predominantly *Hyalomma* spp., are not only the primary vectors, but also reservoirs, as the virus can circulate stably within the tick population (Logan et al., 1989, Gonzalez et al., 1992, Whitehouse, 2004). Arboviruses usually show low levels of genome diversity, perhaps since they have adapted to different vector and host species (Weaver, 2006). However, CCHFV does not follow this concept and shows a high nucleic acid diversity of 20 % in the small (S)-segment, 22 % in the large (L)-segment and even 31 % in the medium (M)-segment which comprise the viral genome (Deyde et al., 2006). Even on amino acid level the variation is still 8 % (S), 10 % (L) and 27 % (M) (Deyde et al., 2006). Thereby, CCHFV acts more like a typical RNA virus, being especially susceptible to mutations due to the high error rate of the RNA dependent RNA polymerase (Holland and Esteban, 1998).

CCHFV circulates in many countries of Southeastern Europe, Southern Asia and Africa (Hoogstraal, 1979), and strains can be grouped phylogenetically into six genotypes (Bente et al., 2013, Carroll et al., 2010). These genotypes can be assigned to different geographic areas: I – West Africa, II – Central Africa, III – South and West Africa, IV – Asia and Middle East, V – South and East Europe, VI – Europe (AP92-group) (Bente et al., 2013).

Over the last decade, real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) has become more and more important for CCHF diagnosis and research. It presents a reliable, fast and safe method and allows identification of the biosafety level 4 pathogen CCHFV, even in facilities with lower biosecurity levels. The predominant problem in CCHFV RT-PCR development has always been the high genetic diversity of this virus (Deyde et al., 2006). The most common approach to this issue is to include additional primers and probes, which target especially diverse virus strains, to cover all genotypes of CCHFV (Wolfel et al., 2007, Jaaskelainen et al., 2014). Another option is to target the forward primer to the 5' non-coding *Nairovirus* specific end of the S-segment (Atkinson et al., 2012). In contrast to that, our approach was to design specific primer sets for each of the six known CCHFV genotypes. Furthermore, one degenerate primer pair for the detection of all

genotypes and two probes were added. This is to our knowledge the first one-step multiplex real-time RT-qPCR using genotype-specific primer sets for the reliable and specific detection of all six genotypes of CCHFV. The new RT-qPCR system was tested and evaluated with six genotype-specific synthetic RNAs and corresponding inactivated virus strains, as well as with samples from different animal species and with other members of the family *Bunyaviridae*.

8.3 **Materials and methods**

8.3.1 Samples and RNA isolation

A total of 86 negative control samples derived from cattle (n = 10), goats (n = 10), sheep (n = 10), red deer (n = 10), fallow deer (n = 10), roe deer (n = 10), hares (n = 5), rabbits (n = 5), wild boars (n = 10) and ticks (5 *Ornithodoros moubata*, 1 *Ixodes ricinus*) were used to test non-specific interferences of the new RT-qPCR. All samples originated from Germany, a country currently free of CCHFV. Ten members of the family *Bunyaviridae* were used for specificity analysis (Table 8.1). The nearest tested relative to CCHFV was HAZV. CCHFV cell culture supernatants (CCS) and tissue culture supernatants (TCS) were used to verify the functionality of the new RT-qPCR. Four out of six CCHFV genotypes were represented (Table 8.2).

8.3.2 Primers and TaqMan probes

Primer and probe regions were selected after comparing all available S-segment sequences from the National Center for Biotechnology Information Database (NCBI). The most homologous section was chosen after sequence alignment and comparison in Geneious (version 9.1; Biomatters, Auckland, New Zealand) and *VisualOligoDeg* (https://github.com/qPCR4vir/VisualOliDeg) (Vina-Rodriguez et al., in preparation). For each of the six CCHFV genotypes, one specific primer pair targeting the same region was designed and synthesized. Additionally, one degenerate primer pair putatively detecting sequences of all genotypes was used. Two probes with a 5’ 6-Carboxyfluorescein (FAM) reporter dye and a 3’ Black Hole Quencher (BHQ1) to minimize intra-assay variability (Yang et al., 2009) were also selected. All primer and probe sequences are listed in Table 8.3.

8.3.3 Synthetic RNAs

Known S-segment sequences of CCHFV were used to design six synthetic RNAs (Figure 8.1), one for each genotype, using Geneious and *VisualOligoDeg*. All sequences were altered in the non-primer and non-probe binding region 5'-TGAGCTCTTTGCCGATGATTCTTT-3' (position 68-91) by mirror inversion of 10 GC-nucleotides. This created the new target site 5'-TCACGTGTTTCGGCATCATTGTTT-3' for the specific synthetic control probe (CCHF-CoProbe). The CCHF-CoProbe uses a cyanine 5 (Cy5)-fluorophore which emits outside the fluorescence range of FAM and enables the discrimination of true positive results and potential laboratory contamination. A 201 bp construct containing the synthetic CCHFV sequence and a T7 promotor was commercially cloned into a pEX-A2 vector (Eurofins, Ebersberg, Germany). The vector was linearized with BamHI (New England Biolabs, Ipswich, MA, USA) and transcribed into RNA using the Riboprobe Combination System – T3/T7 RNA Polymerase Kit (Promega, Madison, WI, USA) in accordance with the manufacturers' instructions. RNA was isolated without carrier RNA addition using the QIAmp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and quantified with the Quant-iT™ RNA Assay Kit (Thermo Fischer Scientific, Waltham, MA, USA), so copy numbers could be calculated. The synthetic RNA concentration was adjusted to 2×10^{10} copies/µl and subsequently a 10-fold dilution series (down to 2 copies/µl) was produced.

8.3.4 Real-time RT-qPCR

5 µl of RNA, 15 pmol of each CCHF-deg primer, 1 pmol of each genotype-specific CCHF‑primer and 3 pmol of each CCHF-probe (also CCHF-CoProbe) were used, along with the QuantiTect Probe RT-PCR Kit (Qiagen) in a total reaction volume of 25 µl. An *in vitro* transcript of enhanced green fluorescent protein (EGFP), named IC2-RNA, was used as an extraction control. EGFP-Mix 1 (5 pmol of each primer) and EGFP-HEX (3 pmol) were used for detection (Hoffmann et al., 2006). The real time RT-qPCR was performed with a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The cycling conditions used were as follows: 50 °C for 30 minutes (reverse transcription), 95 °C for 15 minutes (reverse transcriptase inactivation/Taq polymerase activation), followed by 44 cycles at 95 °C for 10 seconds (denaturation), 55 °C for 25 seconds (annealing) and 72 °C for 25 seconds (elongation). Fluorescence data were collected after each 55 °C step and analysis of

the fluorescence data was conducted with the CFX Manager software (Bio-Rad Laboratories, Hercules, CA, USA).

8.4 **Results and discussion**

In the present study we developed a one-step multiplex real-time RT-qPCR for the detection and quantification of all known genotypes of CCHFV. For this purpose, six genotype-specific synthetic RNAs were designed to test the performance of the new PCR system (Figure 8.1). The synthetic calibrator RNAs can be furthermore used for genotype-specific quantification.

Serial dilutions (in triplicate) were used to determine the limit of detection (LOD) for each genotype (Table 8.4). The analytical sensitivity for some of the genotypes was higher than others, as indicated by the differences in LODs. Already 2 copies/µl (threshold cycle (Ct) between 34.81 and 36.63) were detected (at least in one of three dilutions) for genotypes II (DR Congo), IV (Afghanistan), V (Kosovo) and VI (Greece), while the LODs for genotype III (Mauritania; mean Ct 34.44) and I (Senegal; mean Ct 32.93) were 200 copies/µl. The cut-off was set at Ct 39, since Ct values of higher magnitude were found to be non-specific.

Ct values were plotted on the y-axis against the log of the concentration (copies/µl) on the x-axis. The obtained linear standard curves over four logs were used to further analyze the performance of the RT-PCR and to reveal inter-genotype differences (Figure 8.2). All graphs showed efficiencies between 92.9 % and 105.2 %, which is indicated by the slopes of the standard curves, ranging from -3.20 to -3.50. R^2 had the desired value over 0.99 for four synthetic RNAs (genotypes II (DR Congo), III (Mauritania), IV (Afghanistan) and V (Kosovo)), and slightly lower values for genotypes I (Senegal) and VI (Greece). This is particularly unusual for genotype VI, as this primer set matches exactly the target sequence and genotype VI isolates are not very diverse by themselves. In general, the relative threshold cycle (RTC) method was used to demonstrate the effect of nucleotide changes on the amplification efficiency (Table 8.5) (Sikorsky et al., 2004). We chose genotype II (DR Congo) as reference sequence, due to the fact that the phylogeographic analysis indicated that CCHFV has evolved from Central/West Africa (Bente et al., 2013, Lukashev et al., 2016). In addition, genotype II (DR Congo) performed better than genotypes III (Mauritania) and I (Senegal), which are the two other genotypes present in Africa. The mean ΔCt is based

on the difference between the mean Ct of the respective synthetic RNA and the mean Ct of the reference genotype II (DR Congo) specific synthetic RNA. It functions as calculation basis for the RTC. The strongest effect of nucleotide changes to the amplification efficiency was seen for genotype III (Mauritania) (18 nucleotide changes) (Figure 8.2). However, nucleotide changes are not solely responsible for the decrease of the RTC efficiency. Genotype VI (Greece) has even 19 nucleotide changes within the same sequence and the effect was much smaller than for III (Mauritania). The nucleotide changes might alter the templates secondary structure, which hinders the RNA polymerase advancement and consequently reduces RTC efficiency (Sikorsky et al., 2007).

The evaluation of the RT-qPCR protocol by using synthetic RNAs has its limitations. Therefore, we also assayed different virus strains of CCHFV, which were propagated in CCSs and TCSs, using the new RT-qPCR as a proof of principle. The four CCHFV strains used belonged to different genotypes II (Dakar), IV (Afghanistan), V (Turkey) and VI (Greece). Even the Turkish strain, which was undetected by the former RT-PCR protocol – a modification of a previously described protocol (Wolfel et al., 2007) (data not shown) – was giving a clearly positive signal, although with lower RFU values (18 copies/µl).

The specificity of the assay was eventually assessed using ten bunyaviruses, belonging to the genera *Hantavirus* (Puumala virus, Tula virus), *Nairovirus* (Hazara virus), *Orthobunyavirus* (Akabane virus, Schmallenberg virus, Batai virus, Bunyamwera virus, Ngari virus) and *Phlebovirus* (Rift Valley fever virus, Uukuniemi virus). None of these viruses was detected by the developed RT-qPCR, which is demonstrating its high analytical specificity. Finally, an interference test was performed with RNAs extracted from tissues, blood and serum samples of different animal species and ticks. All samples were negative and no interference was observed.

8.5 **Conclusion**

This is the first report of a one-step multiplex real-time RT-qPCR, which utilizes specific primer sets for each of the six known CCHFV genotypes, to reliably detect all currently described CCHFV strains. The synthetic RNAs designed for this RT-qPCR enable the discrimination of true positive results and unintentional laboratory contaminations.

Additionally, the synthetic RNAs can be used as calibrators for genotype-specific quantification.

8.6 **Tables**

Table 8.1 Members of the family *Bunyaviridae* tested for the evaluation of analytical specificity.

Genus	Virus species	Strain
Hantavirus	Puumala virus[1]	Sotkamo
	Tula virus[1]	Moravia
Nairovirus	Hazara virus	JC 280
Orthobunyavirus	Akabane virus[2]	A347
	Schmallenberg virus[3]	BH80/11-4
	Batai virus[4]	53.2 Germany
	Bunyamwera virus[4]	VR-87
	Ngari virus[5]	Mauritania 2010
Phlebovirus	Rift Valley fever virus[6]	MP-12
	Uukuniemi virus[7]	-

[1] Kindly provided by Detlev H. Krüger (Charité Center Diagnostic Laboratory and Preventive Medicine, Institute of Virology, Berlin, Germany) and Rainer Ulrich (Institute of Novel and Emerging Infectious Diseases, FLI, Greifswald, Germany)
[2] Kindly provided by Peter Kirkland (Elizabeth Macarthur Agriculture Institute, New South Wales, Australia)
[3] Kindly provided by Kerstin Wernike (Institute of Diagnostic Virology, FLI, Greifswald, Germany) (Hoffmann et al., 2012)
[4] Kindly provided by Jonas Schmidt-Chanasit (Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany)
[5] (Eiden et al., 2014)
[6] Kindly provided by Richard Elliot (University of Glasgow, Centre for Virus Research, United Kingdom)
[7] Kindly provided by Manfred Weidmann (University of Stirling, Institute of Aquaculture, United Kingdom)

Table 8.2 CCHFV isolates tested as prove of principle.

Isolate	Genotype	Ct	Copies/µl
Dakar	II	17.28	811,800
Afghanistan	IV	16.04	6,036,000
Turkey	V	35.15	18
Greece	VI	21.50	118,000

Table 8.3 Primer and probe sequences of the CCHFV-specific RT-qPCR.

Primer/probe	Sequence 5'→3'	Genome position[#]
CCHF-I-f	CAAGAGGCACTAAAAAAATGAAGAAGGC	1,068-1,095
CCHF-II-f	CAAGGGGYACCAARAAAATGAAGAAGGC	
CCHF-III-f	CAAGAGGTACCAAGAAAATGAAGAAGGC	
CCHF-IV-f	CAAGGGGTACCAAGAAAATGAAGAARGC	
CCHF-V-f	CAAGGGGGACCAARAAAATGAAAAAGGC	
CCHF-VI-f	CAAGGGGCACCAAGAAAATGAAGAAAGC	
CCHF-deg-f	CAAGGGGKACCAAGAAAATGAARAAGGC	
CCHF-I-r	GCAACAGGGATGGTTCCAAAGCAAAC	1,223-1,248
CCHF-II-r	GCYACRGGGATGGTTCCRAAGCAGAC	
CCHF-III-r	GCCACGGGGATTGTCCCAAAGCAGAC	
CCHF-IV-r	GCCACAGGGATTGTTCCAAAGCAGAC	
CCHF-V-r	GCAACAGGGATTGTTCCAAAGCAGAC	
CCHF-VI-r	GCTACAGGAATTGTCCCAAAGCAGAC	
CCHF-deg-r	GCMACAGGGATTGTYCCAAAGCAGAC	
CCHF-probe-1	6-FAM-ATCTACATGCACCCTGCYGTGYTGACA-BHQ1	1,172-1,198
CCHF-probe-2	6-FAM-TTCTTCCCCCACTTCATTGGRGTGCTCA-BHQ1	1,101-1,128

The degenerate bases are indicated in red. IUPAC ambiguity codes: K=G/T, M=A/C, R=A/G and Y=C/T.
[#]Genome position refers to the Congolese isolate 3010 (accession-no: DQ144418).

Table 8.4 Limit of detection, determined by testing serial dilutions of synthetic RNAs.

	I	II	III	IV	V	VI
	Senegal	**DR Congo**	**Mauritania**	**Afghanistan**	**Kosovo**	**Greece**
Copies/µl	Mean Ct (replicates detected)	Mean Ct (replicates detected)	Mean Ct (replicates detected)	Mean Ct (replicates detected)	Mean Ct (replicates detected)	Mean Ct (replicates detected)
200000	22.61 (3/3)	19.93 (3/3)	24.56 (3/3)	22.94 (3/3)	21.52 (3/3)	21.18 (3/3)
20000	26.39 (3/3)	23.73 (3/3)	27.93 (3/3)	26.01 (3/3)	25.59 (3/3)	24.87 (3/3)
2000	30.49 (3/3)	26.58 (3/3)	31.26 (3/3)	29.58 (3/3)	28.61 (3/3)	28.85 (3/3)
200	32.93 (3/3)	29.66 (3/3)	34.44 (3/3)	32.82 (3/3)	31.65 (3/3)	31.03 (3/3)
20	N/A	32.55 (3/3)	N/A	35.51 (3/3)	34.40 (3/3)	34.38 (3/3)
2	N/A	34.82 (1/3)	N/A	36.46 (1/3)	36.63 (1/3)	36.04 (1/3)

Table 8.5 Relative threshold cycle (RTC) to determine amplification efficiency per genotype.

	II	**I**	**III**	**IV**	**V**	**VI**
	DR Congo	**Senegal**	**Mauritania**	**Afghanistan**	**Kosovo**	**Greece**
Copies/µl	Ct	Ct	Ct	Ct	Ct	Ct
2×10^5	19,9	22,6	24,6	22,9	21,5	21,2
2×10^4	23,7	26,4	27,9	26,0	25,6	24,9
2×10^3	26,6	30,5	31,3	29,6	28,6	28,9
2×10^2	29,7	32,9	34,4	32,8	31,7	31,0
Slope	-3,50	-3,20	-3,29	-3,32	-3,34	-3,35
R^2	0,996	0,988	0,997	0,999	0,994	0,985
Mean ΔCt	0,0	-3,1	-4,6	-2,9	-1,9	-1,5
Mean RTC	1,0	0,11	0,04	0,14	0,27	0,35

Mean ΔCt is calculated as difference of the corresponding mean Ct values and the mean Ct values of II_DR Congo across all concentrations (copies/µl). Mean RTC = $2^{\text{meanΔCt}}$.

8.7 **Figures**

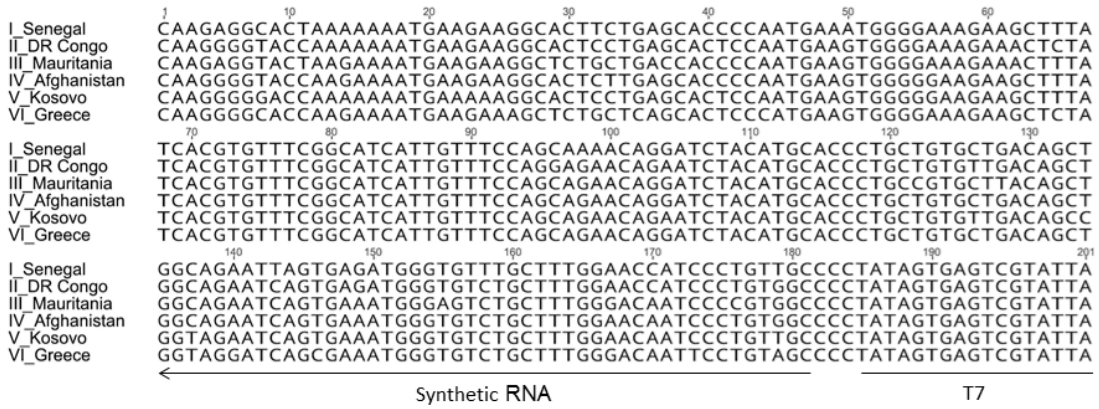
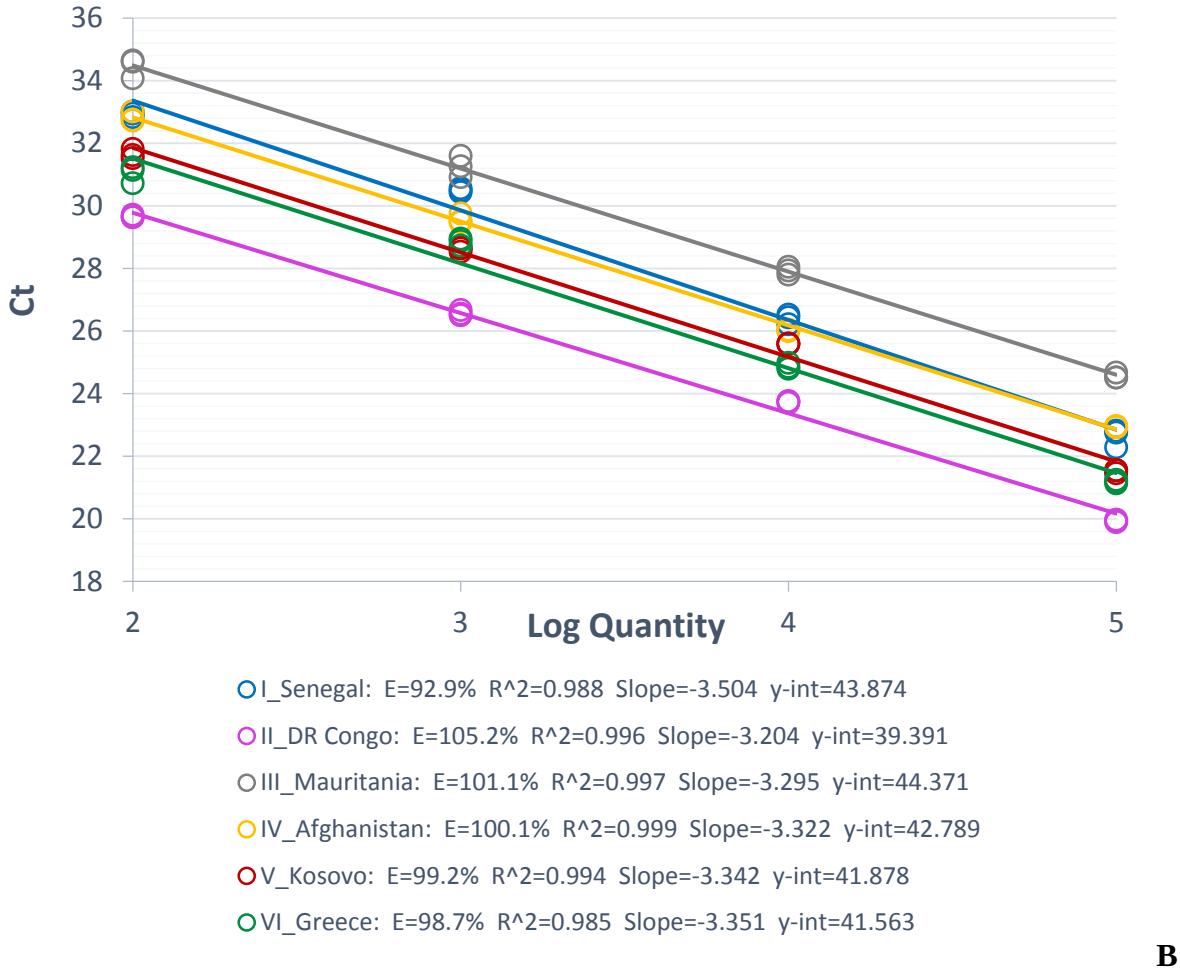


Figure 8.1 Nucleotide sequences of the synthetic RNAs
The synthetic RNA sequences were cloned into vector pEX-A2. The T7 promotor sequence was used for *in vitro* transcription. This figure was prepared with Geneious (version 9.1; Biomatters, Auckland, New Zealand).

A



B

Figure 8.2 Comparison of genotype-specific synthetic RNAs

(A) Genotype II - DR Congo is used as reference sequence. Nucleotides with mismatches to this reference are indicated for each genotype. This figure was prepared with Geneious (version 9.1;Biomatters Auckland, New Zealand) . (B) Ct values were plotted against the log of the concentration (copies/µl), to obtain the standard curve of each synthetic RNA template.

8.8 **Acknowledgements**

We are grateful to Jan Hendrik Forth (FLI) for the provision of RNA of ticks from the FLI insectary colonies. Wild boar samples were kindly provided by Sandra Blome (FLI) and hare and rabbit samples by Felicitas Hammerschmidt (Institute for Food Safety, Faculty of Veterinary Medicine, Ludwig Maximilian University Munich), which were obtained from hunted animals. All deer samples were kindly provided by Ulrich Schotte (Central Institute of the Bundeswehr Medical Service Kiel, Department of Veterinary Medicine, Kronshagen, Germany).

9 GENERAL DISCUSSION

CCHF is a potentially fatal disease in humans with reported case fatality rates ranging from 5% to 80% (Yilmaz et al., 2009, Yen et al., 1985). Highest fatality rates of 60% - 80% were reported in Iraq, the PR China and the United Arab Emirates (Tantawi et al., 1980, Al-Tikriti et al., 1981, Yen et al., 1985, Schwarz et al., 1997) which may reflect a general lack of awareness for CCHF in both the population and in healthcare personnel. Disease awareness enables to treat patients earlier and consequently increases the chances of survival. This works very well in Turkey with up to 1400 clinical human cases annually and a fatality rate of only 5% (Ergonul, 2006). CCHFV is mainly transmitted to humans via tick bites, especially of ticks of the *Hyalomma* genus, which serve as the main vector. Their distribution reaches up to a latitude of 46° N with mountainous areas (Alps, Balkan mountains) serving as natural barriers (Hornok and Horvath, 2012, EFSA, 2010). The most suitable habitat for *Hyalomma* ticks are areas with a warm clime and highly fragmented vegetation (grassland, bushland, forest, agricultural landscape) (Estrada-Pena et al., 2007). *Hyalomma* ticks are hunting ticks and thus prefer less dense vegetation as a habitat to pursue their hosts over ranges up to 400 m. CCHFV distribution directly correlates with the abundance of its vector. However, neither up-to-date vector nor CCHFV prevalence studies exist for many endemic regions, especially on the African continent. A first step towards a better understanding of CCHFV distribution is to investigate the seroprevalence in animals. For this purpose, robust and reliable serological assays that can be applied even under basic laboratory conditions are indispensable. In addition, molecular diagnostic methods have to be updated and validated on a regular basis to guarantee the reliable investigation of current virus circulation in a region (Bente et al., 2013). Although Africa provides most suitable tick habitats, the current CCHFV status of many countries is unknown or their real status is outdated. The sub-Saharan countries analyzed in this work were chosen because they represent different vegetation and climatic zones. Additionally, there were country-specific reasons that will be discussed for each country below.

9.1 **Serological diagnostic assays**

CCHF is a deadly disease, for which a immunoprophylaxis or curative treatment does not exist to date. Therefore, it is classified as BSL 4 agent and should be handled under high containment laboratory conditions only. First antibody detection assays used genuine virus as antigen (Burt et al., 1993, Mariner et al., 1995, Bryan et al., 1996, Rodriguez et al., 1997, Shepherd et al., 1989c). The difficulty in using these assays worldwide is that the antigen can only be produced in BSL 4-laboratories, which are not available in most CCHFV endemic countries. Therefore, recombinant protein systems are much better suited for the use in more commonly available lower category containment laboratories (BSL 1/2). All in-house ELISAs used in this study are based on recombinant His-tagged N-protein derived from the strain Kosovo Hoti (accession no.: DQ133507). Previous assays partly lacked validation results, showed a lack of sensitivity or were evaluated only with a very small sample panel (Marriott et al., 1994, Saijo et al., 2002, Dowall et al., 2012). The indirect FLI in-house CCHFV‑IgG‑ELISA for bovine sera is a highly sensitive and specific assay (D-SN 98 % and D-SP 99 %), which was initially developed for use in Balkan countries (Mertens et al., 2015, Mertens et al., 2016b, Schuster et al., 2016b). The assay time and the costs are reduced in indirect assays as less working steps and reagents are required (Dowall et al., 2012). In this current study, these assays was also employed for testing samples from Mauritania, DR Congo and Cameroon (sera collected in 2013) (see Manuscripts II, III and IV). Even though the FLI in-house ELISA works very well, it is difficult to be carried out in African laboratories lacking adequate technical equipment. This initial ELISA protocol was developed and validated to carry out all incubation steps in a CO_2 incubator with a water bath (Mertens et al., 2015). As such an incubator is not available in many African laboratories and the fact that the CO_2 incubation acidifies the buffer solutions over time, it was necessary to redevelop the CCHFV-IgG-ELISA protocol. The first change was therefore to carry out the coating step under normal atmospheric conditions (Manuscript I). This change made it necessary to screen several different ELISA plates, to choose another antigen concentration and a more suitable coating buffer. Finally, Greiner F immunoplates coated with 0.2 µg/well recombinant N‑protein, diluted in 100 µl coating buffer (PBS + 0.5% BSA, pH 9) and incubated overnight at 4 °C were found to give reliable results. Furthermore, the BSA concentration in the coating buffer was adapted to 0.5%. The other step of the protocol that had to be altered in the

adaptation process was the conjugate dilution, which was lowered to 1/1000 goat anti-bovine IgG-horseradish peroxidase (HRP) conjugate in conjugate dilution buffer. A more detailed description of the protocol is given in Manuscript I. The modified in-house indirect cattle CCHFV-IgG-ELISA was validated with a reference panel consisting of 504 characterized sera from Albania, Germany, Macedonia, Turkey and Mauritania. A receiver operating characteristic (ROC) based biometric analysis was performed in R, using a confidence level of 95%. Targeting a maximal diagnostic specificity and sensitivity a cut-off of 17% was determined. This ROC analysis revealed a diagnostic sensitivity and specificity of 99% and 97%, respectively. However, as the CCHFV-specific antibody positive and negative sample populations overlapped, two cut-offs (16% and 19%) were set as suggested in the "Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2016" (OIE, 2016). Applying the above modifications, the accuracy of the assay was elevated and the D-SN (99%) proved to be higher than in the original assay designed by Mertens et al. (98%). Both assays showed identical D-SP (99%). The new in-house indirect cattle CCHFV-IgG-ELISA was eventually validated by several collaborating partners in a small ring trial, which provided evidences that the assay is reproducible and easily established even in African laboratories. The first successful applications of the adapted in-house indirect cattle CCHFV-IgG-ELISA in seroepidemiological studies are shown in Manuscript I and IV.

The second screening test of the diagnostic approach described above (Manuscript II) was the commercially available (but species adapted) CCHFV-IgG-ELISA produced by VectorBest (Mertens et al., 2015, Schuster et al., 2016b). All African sera presented in Manuscript I to IV were screened once with this test. The benefit of this ELISA is that inactivated genuine virus (strain UZ10145) is used, on which the antigens are presented in a more native form and naturally glycosylated. The cattle adapted VectorBest ELISA protocol had to be cut-off adapted for the studies presented in Manuscript I to IV. The ROC based analysis with 164 sera from Germany and Mauritania identified a cut-off of 0.6 optic densities (OD) (Manuscript II) and comparable diagnostic sensitivity and specificity values were observed as for the original cattle adapted commercial CCHFV-IgG-ELISA (Mertens et al., 2015). The ELISA was successfully used in Manuscripts I to IV. An assay may need to be validated again, when applied on samples coming from another geographical region or animal population (OIE, 2016). So far, the adapted VectorBest assay was validated with sera from

Mauritania (Manuscript II), but more samples from different African countries should be included in the reference panel over time to improve the precision of the assay (OIE, 2016).

As there is no "gold standard" for the serological diagnosis of CCHF the diagnostic hierarchy characterized by two primary assays which both need to confirm the other in order to lead to a positive result followed by a third test (based on a different detection modus) is a very sensible approach to detect CCHFV antibodies with high specificity. The VectorBest ELISA is based on the Asian strain UZ10145 (genotype IV), which belongs to another genotype of CCHFV than the European strain Kosovo Hoti (genotype V) used in the in-house ELISA.

The species-adapted CCHFV-IgG-IFA provided by Euroimmun, which is based on proteins of the Nigerian stain IbAr10200 (genotype III), was used as third assay in all sero‑epidemiological studies presented in Manuscript I to IV. Analyzing IFA requires a trained objective visual judgement and therefore microscopic slide reading skills may affect the sensitivity and specificity (Dowall et al., 2012). Therefore, the species adapted CCHFV‑IgG-IFA of Euroimmun was not applied for the primary analysis, but rather used to clarify divergent results of the in-house and the species adapted VectorBest CCHFV‑IgG‑ELISAs. IFA protocols published before are based on CCHFV infected cells and hence require a BSL 4 laboratory (Johnson et al., 1983, Morrill et al., 1990). More recent publications report the use of transfected recombinant N-protein as antigen in IFAs (Saijo et al., 2002, Garcia et al., 2006). The advantage of the Euroimmun IFA is the use of three biochips per field. The biochips contain N-protein, GPC-protein (both from strain IbAr10200) and non-transfected cells. Hence, not only a negative control is available for each field but also antibodies against GPC and N are detected. This certainly improves the specificity of the assay. However, as recombinant antigen expression in transfected cells is usually lower than an infection induced antigen presentation, the IFA can have a lower diagnostic sensitivity and low or borderline positive sera may be missed. The applied diagnostic hierarchy therefore preferenced assay specificity over assay sensitivity, in order to avoid false positive final results. This may have led to missed positive samples, which were acceptable as the studies carried out with these assays were intended to provide data on population rather than individual animals CCHFV antibody statuses.

Although antigens from three different CCHFV strains were used, which represent three different genotypes, the assays may miss the antibodies elicited by more divergent CCHFV strains. Further studies are therefore needed employing also N-proteins from other CCHFV genotypes as well as from HAZV which belongs to the same serogroup.

9.2 **CCHFV RT-qPCR**

In the early days of CCHFV diagnostics, intracerebral inoculation of samples into newborn mice was considered as the "gold standard" for virus isolation. For animal welfare reasons this technique was eventually replaced by virus isolation on cultured cell monolayers, however, isolation rates were often considerably lower. Modern diagnostics use PCR-based protocols for CCHFV genome detection as proxy of infectivity. The major advantage is that PCR diagnosis can be carried out outside of BSL 4 laboratories using inactivated samples. To date, real-time RT-qPCR can be considered as the standard diagnostic method for CCHFV detection, because of its safety, speed and accuracy (Bente et al., 2013). However, first CCHFV real-time PCR approaches lacked the ability to cater for the high diversity of this virus species. The phylogenetically divergent strain AP92 from Greece proved especially hard to detect (Burt and Swanepoel, 2005, Duh et al., 2006, Wolfel et al., 2007, Escadafal et al., 2012). Therefore a recent approach used two reverse primers together with three probes (Jaaskelainen et al., 2014). Alternatively, a highly conserved part of the *Nairovirus* specific 5'-non coding region of the S-segment of CCHFV was targeted by the forward primer to amplify more strains (Atkinson et al., 2012). However, these results could not be reproduced in our laboratory. Hence, a new multiplex real-time RT-qPCR was designed using Geneious and VisualOligoDeg for the determination of the best primer and probe combinations (Manuscript V) (Vina-Rodriguez et al., in preparation). The S-segment was chosen as target template as it is less diverse and contains the largest amount of known sequences currently available in databases (Deyde et al., 2006). The multiplex system combines one degenerated universal primer pair, six genotype specific primer pairs and two probes for the detection of CCHFV. Additionally, a control probe for the detection of synthetic control RNA and a primer-probe mix for the detection of an internal control were used (Hoffmann et al., 2006). This approach enabled to detect CCHFV, to prove successful an efficient RNA extraction and

to reveal potential laboratory contaminations . Six synthetic RNAs were designed to test the PCR's performance and to serve as positive controls and calibrators for genotype specific quantification. A detection threshold of 2 copies/µl was achieved in one out of three replicates for four genotypes (II (DR Congo), IV (Afghanistan), V (Kosovo), VI (Greece)), however, the analytical sensitivity was lower for genotypes I (Senegal, 200 copies/µl) and III (Mauritania, 200 copies/µl). The limit of detection (LOD) differences could be traced back to nucleotide changes using relative threshold cycle (RTC) method. However, not only the number of nucleotide changes had an effect on the amplification efficiency. It is also suspected that nucleotide changes in general might alternate the template's secondary structure. This may hinder RNA polymerase advancement and subsequently reduce the amplification efficiency (Sikorsky et al., 2007). These results indicate that general amplification mismatches may exist between the different strains of CCHFV. In consequence, quantification should be performed with synthetic calibrator RNA of the same genotype to obtain the most precise result. In the standard evaluation curve, the efficiencies of all synthetic RNAs were within the frame of good reaction quality. However, R^2 was a bit too low for strains I (Senegal) and VI (Greece). This was especially surprising for strain VI (Greece) as the primer set fit the strain very well.

As the analysis of an RT-qPCR protocol using synthetic RNAs may be considered an artificial system with limited practical relevance, CCS and TCS of different natural CCHFV strains were tested with the novel RT-qPCR as a proof of principle. All four isolates belonging to four different genotypes including the Turkish genotype V isolate - previously undetectable using the former PCR protocol – gave a positive result for CCHFV. Furthermore, relatively low amounts of copy numbers could be detected in this sample (18 copies/µl). No interferences of 86 different negative samples (tissue, blood, serum, whole tick) with the primer-probe set were detected and cross-reactivity testing with ten different *Bunyaviridae* proved negative as well. Therefore, we conclude that the new multiplex RT-qPCR displays an excellent specificity for the detection of CCHFV.

109 *Hyalomma truncatum* ticks from Northern Cameroon (Manuscript IV) were eventually tested with the novel primer sets (Manuscript V) in order to demonstrate the current presence of CCHFV in the region. In doing so, a SYBR Green protocol was used for the amplification. The most common African genotype III was shown to be present in Cameroons neighboring country Nigeria (Causey et al., 1970). Therefore, it was likely that a putative Cameroonian

strain would also fall into genotype III. As described before, the new multiplex real-time RT-qPCR showed a limited detection of both genotype III and genotype I. SYBR Green PCR systems are usually more sensitive and are more suitable to detect low virus concentration or a previously undetected CCHFV strain. The disadvantage of the SYBR Green method is that this chemical binds to any double stranded DNA which may not specifically be RT-qPCR amplified CCHFV genome. Therefore, suspected samples were additionally sequenced with a 3130 Genetic Analyzer (Applied Biosystems/Life Technologies, Waltham, MA, USA) and tested with the Probe RT-qPCR protocol (Manuscript V). Using this approach 7 out of 109 ticks were CCHFV positive and RNA sequences clustered with genotype III. The highest homology was to the Nigerian strain IbAr10200, first described by Causey et al. (Causey et al., 1970).

The novel primer and primer set worked efficiently with SYBR Green (Manuscript IV) and the probe protocol (Manuscript V), respectively. However, it is imperative to confirm positive SYBR Green findings with the Probe RT-qPCR and/or sequencing (Manuscript IV). The new RT-qPCR protocol showed both good sensitivity and excellent specificity as compared to previously described methods (Manuscript IV). In order to include also newly discovered divergent strains regular sequence updates and analyses should be carried out to improve the quality of the PCR.

9.3 **Epidemiological Studies in sub-Saharan Africa**

Ruminant serum samples from DR Congo (n = 838), Cameroon (n = 980), Mali (n =1075) and Mauritania (n = 495) were tested for CCHFV-specific antibodies to determine the viral prevalence in different sub-Saharan countries. CCHFV-specific IgG antibodies are detectable for many years hence they can be used as indicators for the virus' circulation in a region (Mertens et al., 2013). However, an accumulation of IgG was also reported in older animals as they can be re-infected with the virus several times (Wilson et al., 1990). Ideally, animal age should always be considered when interpreting the human risk from ruminant seroprevalence studies. This was not possible for some samples discussed in this work, as many of them originated from previous studies or national surveillance monitoring (Mali, Mauritania, small ruminants from DR Congo). However, the age influence should be already

taken into count in the concept of future studies. The main focus of this work was to get a general insight into the CCHFV distribution in different sub-Saharan countries. The most important influence factor in virus occurrence proved to be the distribution of the vector tick. The fact that ticks play a crucial role in CCHFV transmission has been considered since the first outbreak on the Crimean Peninsula in 1944 (Chumakov, 1947, Hoogstraal, 1979). To date, the predominant vector of CCHFV has been identified as *Hyalomma* spp. ticks. *H. marginatum* is associated to the occurrence of CCHF in Europe. This is strongly supported by the northern distribution limit of *H. marginatum* which matches the CCHFV occurrences (Hoogstraal, 1979, Whitehouse, 2004, Mertens et al., 2013). Only limited data on tick species correlation is available for Africa even though it was shown already in early CCHFV research that CCHFV distribution is likely to be linked to *Hyalomma* ticks in Africa as well. (Wilson et al., 1990, Burt et al., 1996). *Hyalomma* ticks are "hunting" ticks and pursue their hosts for distances of up to 400 m. Hence, they prefer less dense vegetation. *Hyalomma* ticks also depend on long dry seasons with an annual accumulated temperature of more that 3000 ‑ 4000 °C to successfully produce a self-sustained tick population (Estrada-Pena et al., 2011).

9.3.1 CCHFV in Cameroon (Manuscript IV)

As no CCHFV research was done in this country before, it was of special interest to determine the CCHFV status of Cameroon. High probability of CCHF occurrence was predicted by Messina et al. and the need of CCHF surveillance was pointed out for Cameroon (Figure 10.1) (Messina et al., 2015). The virus was detected in the neighboring countries Nigeria and the Central African Republic (CAR) in the early beginnings of CCHFV research (Causey et al., 1970, Hoogstraal, 1979). A study targeting neutralizing antibodies in humans showed the relevance of serosurvey in Nigeria (David-West et al., 1974). In conclusion, it was long overdue that CCHFV was investigated in Cameroon, too. Cameroon combines many African vegetative zones and can give a wider insight into correlations of CCHFV with vegetation and African vector prevalence than other African countries. A high seroprevalence (74%) was detected in 980 cattle serum samples originating from different regions in Cameroon. A comparison of the prevalence in Northern and Southern Cameroon revealed a significant difference. The cattle tested in the North of the country proved 77%

CCHFV‐specific IgG antibody positive while the prevalence in the South of Cameroon was measured to be 21% only. Even though only 57 samples originated from the South of Cameroon and the relatively low prevalence could be partly a sampling artefact, a clear trend of CCHFV prevalence between Northern and Southern Cameroon that reflects already known habitat preferences of *Hyalomma* spp. becomes obvious. Cameroon displays a vegetation gradient including rain forest, moist forest, moist savanna, dry savanna and xeric shrublands from its Southern to Northern borders. The preferred habitats of *Hyalomma* ticks therefore can be found in the North of the country. Similar findings were also presented for Senegal (Wilson et al., 1990). Wilson et al. detected 75% specific-CCHFV antibodies in the northernmost sampling location (xeric shrublands) and 0% in the South (moist savanna) of Senegal. However, the extreme difference has to be critically discussed, as 75% prevalence were detected in only 35 samples and the IgG prevalence in the bordering bioclimatic zone was only 11.4% (n = 484).

Hyalomma spp. were found in many different habitats already. As an example contradicting the rule, both natural infections in the DR Congo mentioned above did originate from the moist/rain forest area in the North of the country. Once introduced into a suitable habitat with suitable nutritive conditions, the ticks may well be capable of establishing smaller but nevertheless stable populations in that local area. This may be the reason that CCHFV also occurs in Northern Cameroon, although the habitat is seemingly less suitable for a hunting tick.

For 238 cattle samples analyzed in this study in Cameroon, the age was stated with an average of 5.3 years (2 months to 22 years). Within these 238 samples, CCHFV was detected in animals of all age groups including juvenile cattle. This fact on its own already makes the current circulation of the virus in Cameroon obvious. To confirm these results, a RT-qPCR was performed using 109 *Hyalomma truncatum* ticks to unequivocally prove the virus presence in the country. All ticks originated from North Cameroon, where the highest prevalence was detected. 7 of these 109 ticks proved positive for CCHFV and sequencing revealed the highest similarity of the virus specimens with the Nigerian strain IbAr10200 (Causey et al., 1970). However, no information is available about the strains currently circulating in Nigeria.

To sum up, CCHFV is present in Cameroon and prevalence rates in cattle indicated a high risk for the human population in the North of the country. Further surveillance studies in ruminants are needed to get a better insight into habitat and clime correlations. Surveillance studies in humans should be performed to reveal the real threat for the human population in parallel. As already mentioned, the possibility of "silent" CCHFV circulation with only sporadic human cases is likely when the abundance of transmitting ticks and animals is balanced and could be the reason why CCHFV did not attract attention in Cameroon so far. Last but not least, an in-depth characterization of the circulating virus strain(s) is needed to assess the risk of CCHF for the Cameroonian population.

9.3.2 CCHFV in DR Congo (Manuscript III)

The annual average temperature in DR Congo is relatively high (25 °C) and only alternates up to 5 °C on the monthly level. This seems to be an optimal condition for the spreading of *Hyalomma* spp. However, other factors also influence the establishment of a self-sustaining tick population: ground coverage is an essential criterion and may be one of the limiting factors in DR Congo. Two third of the country are covered by rain forest and do not represent a suitable habitat for *Hyalomma* ticks. This also indicates that a relatively low occurrence of CCHF can be expected in this country as was already stated from Messina et al. (Figure 9.1) (Messina et al., 2015). In the South of the country, vegetation alters from moist savanna to dry forest to dry savanna, while the Center and the North is primarily covered by rain forest or moist forest. All samples analyzed in this study were collected in Katanga (the southernmost province of DR Congo) in order to increase the chance of finding antibodies specifically directed against CCHFV. As the DR Congo (2.3 million km2) is 6.6 times as large as Germany, it was not possible to cover different provinces. Already within Katanga province it was difficult to obtain samples from different regions, as the country has a poor infrastructure and traveling over far distances is very troublesome. Yet, it was possible to sample 514 cattle from one farm in Kamina and 324 small ruminants from different location in the greater Lubumbashi area. The seroprevalence in cattle from Kamina was lower (0.2%) than the seroprevalence detected in small ruminants from Lubumbashi (3.4%). However, the extremely limited number of sampling sites does not allow a conclusion about geographical influences. The results point towards a relatively low risk for human infection in these areas.

The scientifically sound assumption that this can be extrapolated to the situation in most parts of DR Congo however rest upon the vegetative and climatic conditions in the country. While Katanga province offers a suitable habitat for *Hyalomma* ticks to some degree, the large rain and moist forest areas in the DR Congo are unlikely to show a higher vector occurrence. It is furthermore supported by the fact that just two natural CCHFV infections have been reported in over half a century. Furthermore, in the neighboring country Tanzania, which has a dryer climate and has less dense vegetation, only 9% of the tested cattle (n = 1048) were seropositive (Hoogstraal, 1979). In Uganda, another neighboring country of the DR Congo, a higher seroprevalence (36%) was detected in cattle (Hoogstraal, 1979). However, the first seroprevalence results on CCHFV must be carefully evaluated as the detection was performed by AGDP, which lacks sensitivity. Prevalence in Tanzania may in fact prove to be higher when analyzed with more sensitive detection methods such as ELISA. Altogether, the detected prevalence in Katanga province of DR Congo is in concordance with the vegetative and climatic circumstances and can also be compared to CCHFV occurrence in neighboring countries. However, further research is required to cover all vegetative areas of the country, also moist savanna and moist/rain forest areas. A special focus of CCHF research in the DR Congo should rest upon vector and seroprevalence studies in the region where human cases have occurred before.

9.3.3 CCHFV in Mali and Mauritania (Manuscript I and II)

The seroprevalence rates detected in Mali (66%) and Mauritania (67%) can be compared to the results obtained from Northern Cameroon (Manuscript IV). These findings can easily be explained due to the fact that Southern Mali and also the southernmost regions of Mauritania share the same vegetative zones as Northern Cameroon (dry savanna and xeric shrublands with succulents). Messina et al. described the South of both countries as most suitable for CCHFV occurrence (Figure 9.1) (Messina et al., 2015). The warm arid clime in both countries provides excellent living conditions for *Hyalomma* spp. A further correlation to precipitation was already shown for both Mauritania and neighboring Senegal more than 25 years ago. In locations with higher average annual rainfall, CCHFV prevalence is significantly lower than in areas with lesser precipitation (Gonzalez et al., 1990, Wilson et al., 1990). Furthermore, Gonzalez et al. showed that CCHFV was present in all different habitable parts (not Sahara)

of Mauritania. However, this study is outdated and the necessity of a new seroprevalence study is obvious when comparing the seroprevalences detected in this study to the ones documented by Gonzalez et al. (Manuscript II). Sampling artefacts should be taken into count as one influence factor. It is possible that the sampled animals in our study (average age 6.8 years) were much older and that an accumulation of IgG leads to the apparent discrepancy in prevalence. However, this does not explain the relatively large difference between the overall prevalence (67%) detected in this study to the one presented by Gonzalez et al. (18%). Another influence factor may be changes of the Mauritanian fauna which occurred within the 25 years between both studies. The free roaming wild animals in Mauritania's savanna since have declined and the presence of commercially farmed livestock have significantly increased (Kirsch-Jung and von Urff, 2008). As a result, ticks eventually target cattle more often today than in 1990. Despite the multitude of possible factors, neither of these aspects provides a satisfying explanation for the stunning discrepancy between the seroprevalence rates reported 25 years ago and the current results of our study yet. Also no difference between the southernmost region and the rest of Southern Mauritania was confirmed in Manuscript II. It currently remains uncertain why CCHFV is distributed differently in Mauritania today and shows the urgent necessity of close monitoring programs in endemic countries to better understand the behavior of CCHFV.

In Mali, CCHFV-antibodies were first described in humans at the city of Baguineda (located near the capital Bamako in Southern Mali,) in 2005 (Traore et al., 2005). Zivcec et al. detected a CCHFV strain closely related to the ArD39554 strain first detected in Mauritania (98% sequence identity) (Zivcec et al., 2014). However, the ticks containing this CCHFV strain were collected at Daral cattle market and could have originated from an imported animal from Mauritania, Senegal or neighboring countries. The ultimate proof of current CCHFV circulation was provided by Safronetz et al., when 4.8% CCHFV-specific IgM antibodies were detected retrospectively (2009 - 2013) in samples from suspected yellow fever patients (Safronetz et al., 2016). Results presented in Manuscript I indicate, that the human risk for CCHFV infection is relatively high in Mali. Although some areas were represented by a relatively small panel of only 20 tested serum samples (Tombouctou, Kidal, Gao, Kayes and Sikasso), a general overall risk assessment for Mali is possible. Detected seroprevalence rates of the regions did not show a difference in samples from Northern and

Southern Mali but they could be linked to the cattle density in the country. Areas with high cattle density (9 bovines/km^2) are Sikasso (95%) and Mopti (69%) whereas significantly less cattle is held in Tombouctou (1-4 bovines/km^2), where a prevalence of 40% was detected. In conclusion, the overall prevalence detected in Mali (Manuscript I) is very similar to the one detected in Mauritania (Manuscript II). However, there is a discrepancy to results from the neighboring countries Senegal and Niger (Wilson et al., 1990, Mariner et al., 1995). The high probability of CCHF occurrence in Mali and Mauritania which was presumed in a previous model was confirmed by this study (Figure 9.1) (Messina et al., 2015). As mentioned also for Cameroon and the DR Congo, further virus characterization is urgently needed for strains detected in Mali and Mauritania, too, in order to put isolates into a phylogeographical context.

9.3.4 Epidemiological Conclusion

Our CCHFV seroprevalence studies in four sub-Saharan countries (Manuscript I, II, III, IV) revealed differences between climatic and vegetative zones of Africa. While humans in Mauritania (Manuscript II), Mali (Manuscript I) and especially the North of Cameroon (Manuscript IV) seem to have a high infection risk, the general population in Katanga province of the DR Congo (Manuscript III) is probably under a significantly smaller risk of contracting CCHF. Risk groups like healthcare personnel, abattoir workers and farmers should always we be or should be made aware of this highly virulent and potentially fatal disease. Furthermore, we were able to confirm Messina's predictions on the occurrence of CCHF in Africa for all four countries (Figure 9.1). This work was pivotal for Cameroon, where no CCHFV research has been performed before. The results for Mali and DR Congo are similarly important as they represent the first seroepidemiological work and risk assessment in both countries ever to be conducted. In Mauritania, our findings highlighted the urgency for establishing a regular surveillance program.

To sum up, public healthcare workers in the African countries investigated in our study must be trained in CCHFV diagnostic and overall risk groups require training in using right protection measures to prevent further unnecessary CCHF casualties. By conducting further investigation on smaller scale risk assessments it is possible to specify risk areas within a country. A future primary target of CCHFV research should be the systematic characterization of circulating virus strains and the investigation of their individual virulence.

The virulence of a strain is often speculated to be partly responsible for regional differences of human cases and case fatality rates, but this theory has only been clarified for the avirulent and low virulent strains of genotype IV.

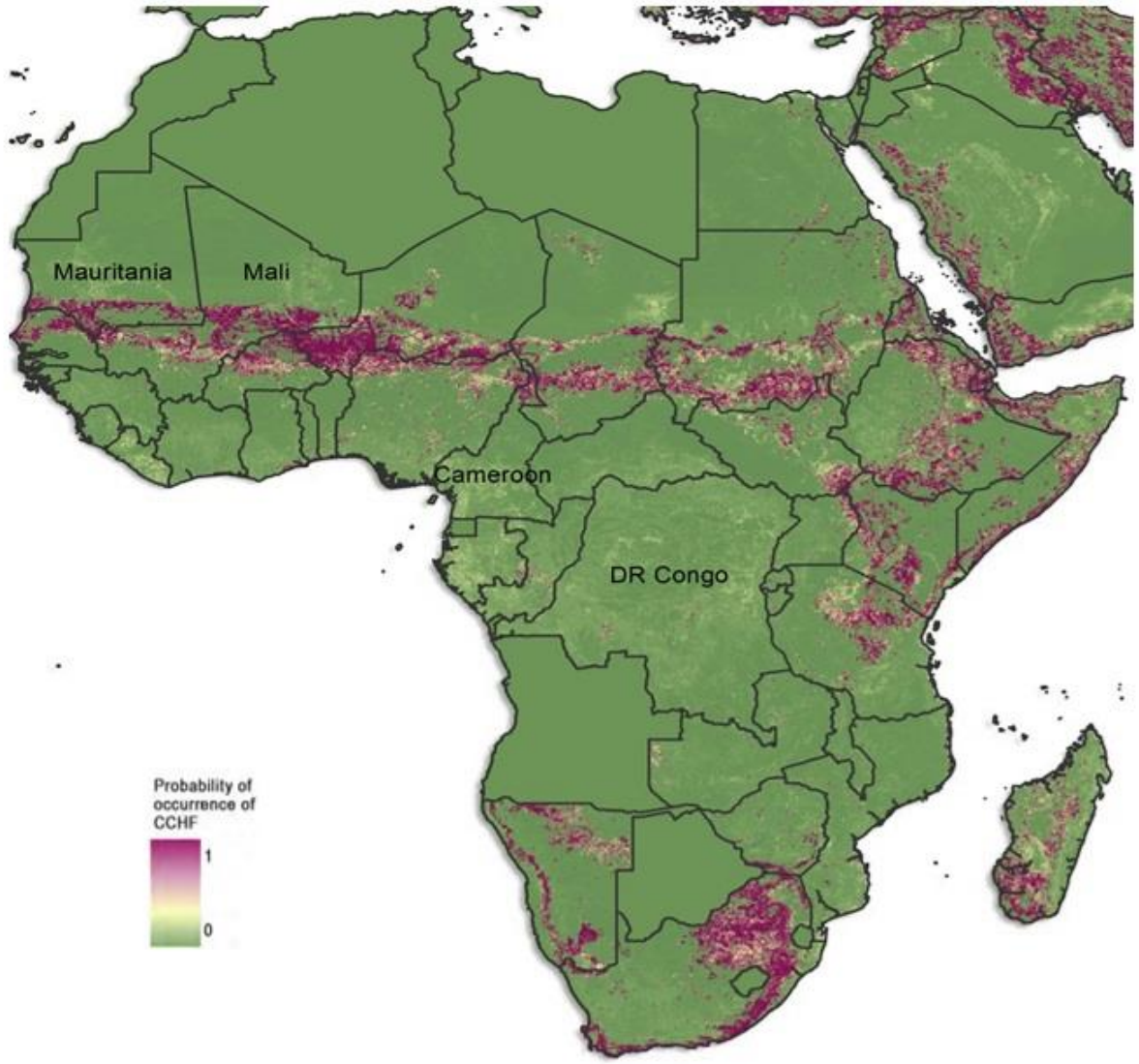


Figure 9.1 Predicted CCHF occurrence in Africa

Adapted from Messina et al. (Messina et al., 2015).

10 SUMMARY

Miriam Andrada Sas

Novel serological and molecular assays for Crimean-Congo hemorrhagic fever virus infections and their application in prevalence studies on sub-Saharan African countries

Crimean-Congo hemorrhagic fever (CCHF) is a fatal disease in humans with observed case fatality rates varying from 5% in Turkey to 80% in the People's Republic of China. This variability most likely depends on general awareness of the population, quality of the public healthcare system, sensitivity of the notification system and on the individual virus strain. The primary transmission route of Crimean-Congo hemorrhagic fever virus (CCHFV) is by tick bites. Therefore, the virus distribution on the African, Asian and European continent is closely linked to the main vector, ticks of the genus *Hyalomma*. *Hyalomma* spp. generally prefer a warm and dry climate with a fragmented and less dense vegetation. Hence, these ticks are not able to create a stable and self-sustaining population further north than 46° N. Other sources of infections are contact to blood, body fluids and tissues of viremic animals or human patients and by crushing infected ticks.

The detection of CCHFV-specific antibodies in ruminant sera is commonly used as indicator to reveal infection risk areas also for humans. Even though animals do not show clinical signs, they develop a stable IgG antibody titer that is detectable for many years after infection. CCHFV is best investigated in endemic areas in Europe. In contrast, research on the CCHFV prevalence on the African continent was fairly neglected for many decades and up-to-date information is only available for a few African countries. Therefore, the main goal of this thesis was to investigate the CCHFV prevalence in four different countries located in sub-Saharan Africa. For this purpose, a series of serological assays recently developed for testing ruminant sera from Southeastern Europe was employed. These assays included a commercial (species adapted) enzyme linked immunosorbent assay (ELISA) and an in-house CCHFV‑IgG-ELISA as well as a commercial (species adapted) immunofluorescence assay (IFA). The adaptation of these assays included protocol and cut-off changes to reach convincing diagnostic sensitivities (95% - 98%) and specificities (98% - 100%).

However, as the assays were supposed also to be run in less well equipped laboratories in Africa, a robust and highly reproducible indirect cattle in-house CCHFV-IgG-ELISA was developed. The extensive modifications in the novel CCHFV-IgG-ELISA did not only increase the robustness of the assay and simplify the procedure but also enabled testing sera under the technical standards currently available in African laboratories. Nonetheless this new assay had an excellent diagnostic sensitivity and specificity (both 99%) and was therefore used in the seroepidemiological studies in Mali and Cameroon (sera collected in 2014).

In parallel, a highly specific and sensitive multiplex CCHFV RT-qPCR using 12 genotype‑specific primers, 2 universal primers and 2 carboxyfluorescein probes for the reliable detection of all currently known CCHFV strains was newly designed and validated. This assay detects synthetic and native virus sequences of all six known genotypes and six calibrator RNAs enable the genotype-specific and precise quantification of CCHFV.

Serological assays were subsequently used to screen more than 3,000 ruminant sera from Mauritania, Mali, Cameroon and the Democratic Republic of the Congo (DR Congo), countries all situated in sub-Saharan Africa, and questions about the correlation of CCHFV prevalence with vegetation, climate and animal density were addressed. Ruminant sera were considered positive for CCHFV antibodies, if they were reactive in two independent ELISAs. In case of divergent results, the IFA was used for final diagnosis.

This approach revealed CCHFV infections in ruminants in all four countries Mauritania, Mali, Cameroon and DR Congo, which was a surprising result especially for Cameroon as this was the first demonstration of this infectious disease in this country. Highest prevalence rates were detected in Mauritania, Mali and North Cameroon, which is consistent with the vegetation and climatic conditions and the habitat preference of *Hyalomma* ticks. Conversely, medium to low prevalence rates were found in Southern Cameroon and the DR Congo, which was also in accordance with the suitability of local vegetation and clime for these arthropods. Moreover, cattle densities seemed also to correlate with the presence of CCHFV in an area. Most CCHFV-specific antibody positive cattle were detected in North Cameroon, where cattle density is much higher than in the South and a similar correlation was noticed for Mali. As this study revealed for the first time CCHFV infections in Cameroon, it was of crucial importance also to prove that the virus is circulating in this environment. For this purpose,

109 *Hyalomma* ticks were collected from infested bovines in a CCHFV highly endemic area in North Cameroon and were assayed for CCHFV genomes using a novel highly sensitive multiplex SYBR Green CCHFV RT-qPCR based on 14 different primers for the detection of all currently known CCHFV genotypes. Samples with a characteristic amplification were eventually reconfirmed using a multiplex CCHFV RT-qPCR, which had the same genotype specific primer sets and two additional CCHFV specific probes included. CCHFV was found in 7 of the 109 ticks and amplified genome sequences clustered phylogenetically into genotype III.

In summary, the work presented here highlights the importance of seroprevalence studies in ruminants as indicator animals and molecular diagnostic studies in ticks to determine, whether CCHFV is circulating in sub-Saharan countries/regions. The current data obtained for Cameroon, DR Congo, Mali and Mauritania allow carrying out risk assessments for human infections and therefore help to define whether public health protection measures (e.g. raising awareness for risk groups and the broader public) should be applied.

12 ZUSAMMENFASSUNG

Miriam Andrada Sas

Neue serologische und molekulare Tests zum Nachweis von Infektionen mit dem Krim-Kongo-Hämorrhagischen-Fieber-Virus und deren Anwendung in Prävalenzstudien in Ländern Subsahara-Afrikas

Krim-Kongo Hämorrhagisches Fieber (CCHF) ist eine beim Menschen tödlich verlaufende Erkrankung mit Letalitätsraten von 5% (Türkei) bis 80% (Volksrepublik China). Diese Variation hängt vom Kenntnisstand in der Bevölkerung über das Vorkommen dieser Infektionskrankheit, von der Qualität des Gesundheitswesens, von der Genauigkeit des Meldesystems und vom zirkulierenden Virusstamm ab. Das Krim-Kongo-Hämorrhagische-Fieber-Virus (CCHFV) wird vorrangig durch Zecken der Gattung *Hyalomma* übertragen und kommt in Afrika, Asien und Europa vor. *Hyalomma* spp. bevorzugen im Allgemeinen ein warmes und trockenes Klima und eine weniger dichte Vegetation. *Hyalomma* spp.-Populationen kommen deshalb natürlicherweise nur bis zum 46. nördlichen Breitengrad vor. Weitere Infektionsquellen für den Menschen sind der Kontakt zu Blut, Körperflüssigkeiten und Gewebematerial von virämischen Tieren oder auch infektiösen Menschen; auch das Zerdrücken von CCHFV-infizierten Zecken kann zur Infektion führen.

Obwohl Tiere in der Regel nach der Infektion keine klinischen Symptome zeigen, entwickeln sie einen stabilen Antikörpertiter, der noch viele Jahre nach der Infektion nachweisbar ist. Deshalb kann der CCHFV-Antikörpernachweis bei Wiederkäuern zur Feststellung von Risikogebieten verwendet werden. Am gründlichsten ist die CCHFV-Infektion in endemischen Gebieten in Europa und Kleinasien untersucht worden. Demgegenüber gibt es über das CCHFV-Vorkommen in Afrika kaum aktuelle Daten. Daher war das Hauptziel der hier vorgestellten Studien, das CCHFV-Vorkommen in verschiedenen Subsahara-Ländern (Mauretanien, Mali, Kamerun und Demokratische Republik Kongo (DR Kongo)) zu untersuchen. Hierzu wurden zunächst modifizierte serologische Tests angewendet, die zuvor zur Untersuchung von Wiederkäuerseren aus Südosteuropa entwickelt worden waren. Diese Testmethoden umfassten einen kommerziellen (Spezies-adaptierten) Enzym-gekoppelten-Immunadsorptionstest (ELISA) und einen im eigenen Labor entwickelten indirekten

CCHFV‑IgG-ELISA sowie einen (Spezies-adaptierten) indirekten Immunfluoreszenz-Test (IFA). Die Anpassung dieser Tests umfasste Veränderungen in den Protokollen und bei den Grenzwerten. Die so modifizierten Tests erreichten diagnostische Sensitivitäten von 95% ‑ 98% und Spezifitäten von 98% - 100%.

Da diese Tests jedoch auch in weniger gut ausgestatteten Laboren in Afrika bei tropischer Hitze verwendet werden sollten, wurde ein weiterer auch unter diesen Verhältnissen noch robuster indirekter CCHFV-IgG-ELISA für Rinder mit vergleichbarer diagnostischer Performance (Sensitivität und Spezifität jeweils 99%) entwickelt. Dieser ELISA wurde anschließend im Rahmen seroepidemiologischer Studien an Rinderseren aus Mali und Kamerun (2014 gesammelt) eingesetzt.

Parallel dazu wurde ein hochsensitiver Multiplex-CCHFV-RT-qPCR-Nachweis auf der Basis von 12 Genotyp-spezifischen und 2 universalen Primern entwickelt und validiert, der darauf abzielt alle aktuell bekannten CCHFV-Stämme zu detektieren. Dieser RT-qPCR-Nachweis wurde komplettiert durch die Verwendung zweier spezifischer Carboxyfluorescein-Sonden zum direkten CCHFV-Erregergenom-Nachweis. Ein solcher universeller Nachweis war bis dato angesichts der großen Sequenz-Variationen zwischen den CCHFV-Stämmen der sechs bekannten Genotypen nicht verfügbar. Mithilfe von sechs Kalibrator-RNAs ist ferner die genaue Genotyp-spezifische Quantifizierung von CCHFV möglich.

Die serologische Nachweismethoden wurden anschließend zur Untersuchung von über 3000 Wiederkäuerseren aus den in Subsahara-Afrika liegenden Ländern Mauretanien, Mali, Kamerun und DR Kongo verwendet. Wiederkäuerseren wurden als CCHFV-Antikörper-positiv erachtet, wenn sie in zwei unabhängigen ELISA-Systemen reaktive Ergebnisse erbrachten. Bei abweichenden Ergebnissen wurde eine IFA zur endgültigen Diagnose durchgeführt.

Die Untersuchungen zeigen, dass CCHFV-Infektionen in allen vier Ländern (Mauretanien, Mali, Kamerun und DR Kongo) vorkommen. Dies war insbesondere für Kamerun ein überraschendes Ergebnis, da es sich um den ersten Nachweis dieser Infektionskrankheit in diesem Land handelt. Die höchsten Prävalenzraten wurden in Mauretanien, Mali und Nordkamerun nachgewiesen. Dies stimmte mit den Vegetations- und klimatischen Bedingungen und den dort jeweils vorliegenden, von *Hyalomma*-Zecken präferierten

Habitaten überein. Umgekehrt lassen sich auch die deutlich niedrigeren Prävalenzen in Südkamerun und DR Kongo mit der (geringeren) Eignung der lokalen Vegetation und des lokalen Klimas für *Hyalomma*-Zecken erklären. Ggfs. steht auch die Viehdichte mit dem Vorkommen von CCHFV in einem Gebiet im direkten Zusammenhang. In Nordkamerun, wo eine deutlich höhere Viehdichte als im Süden des Landes vorzufinden ist, wurden deutlich mehr Rinder positiv auf CCHFV-spezifische Antikörper getestet als im Süden mit geringerer Rinderdichte. Eine ähnliche Korrelation wurde auch in Mali nachgewiesen. Da im Rahmen dieser Studie zum ersten Mal CCHFV-Infektionen in Kamerun detektiert werden konnten, war es wichtig, das Virus auch direkt nachzuweisen. Zu diesem Zweck wurden in einem CCHFV-hochendemischen Gebiet in Nordkamerun 109 *Hyalomma*-Zecken von Rindern abgesammelt und molekulardiagnostisch auf CCHFV untersucht. Hierzu wurde die hochsensitive Multiplex-SYBR–Green-CCHFV-RT-qPCR verwendet. Proben mit charakteristischer Amplifikation wurden mithilfe der Sonden-basierten Multiplex-CCHFV-RT-qPCR bestätigt. CCHFV konnte in 7 von 109 Zecken nachgewiesen werden, und die amplifizierte Genomsequenz konnte phylogenetisch als Genotyp III eingeordnet werden.

Zusammenfassend kann festgehalten werden, dass Wiederkäuer auch in den Subsahara-Ländern als ausgezeichnete Indikator-Tiere für CCHFV-Infektionen angesehen werden können und dass hierzu nun hochsensitive und -spezifische und gleichzeitig robuste ELISAs zur Verfügung stehen. Die hohe Seroprävalenz in Nordkamerun wurde durch den molekularen RT-qPCR-Nachweis des CCHFV-Genoms bestätigt. Die serologischen und molekulardiagnostischen Daten deuten auf ein weiträumiges und gleichzeitig hochgradiges Infektionsgeschehen in den Ländern Subsahara-Afrikas hin. Angesichts der vorliegenden Daten erscheint zumindest in bestimmten Regionen die Einführung von Aufklärungsaktionen für potenzielle Risikogruppen und auch der Allgemeinbevölkerung empfehlenswert.

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15 SUPPLEMENTARY

Supplementary file 1

Indirect cattle in-house CCHFV-IgG-ELISA: Calculation of the results.

	NC	PC	Serum 1
Ag+	A	A	A
Ag+	B	B	B
Ag-	C	C	C
Ag-	D	D	D

Ag+: coated with antigen; Ag-: coated without antigen; NC: Negative control; PC: Positive control

Calculation of the corrected OD value (subtracted background):

(A+B)/2 - (C+D)/2

Calculation of the final result (correlation to positive control):

(OD Serum 1) / (OD PC) x 100

Supplementary file 2

Indirect cattle in-house CCHFV-IgG-ELISA: Correlation between the pH of the coating buffer and antigen binding to the plate.

pH 7,5

TR1	TR2	FR1	FR3
1,783	1,423	0,569	0,247
100%	80%	32%	14%

pH 8,0

TR1	TR2	FR1	FR3
1,675	1,324	0,357	0,248
100%	79%	21%	15%

pH 8,5

TR1	TR2	FR1	FR3
1,650	1,140	0,409	0,249
100%	69%	25%	15%

pH 9,0

TR1	TR2	FR1	FR3
1,406	1,186	0,127	0,193
100%	84%	9%	14%

pH 9,5

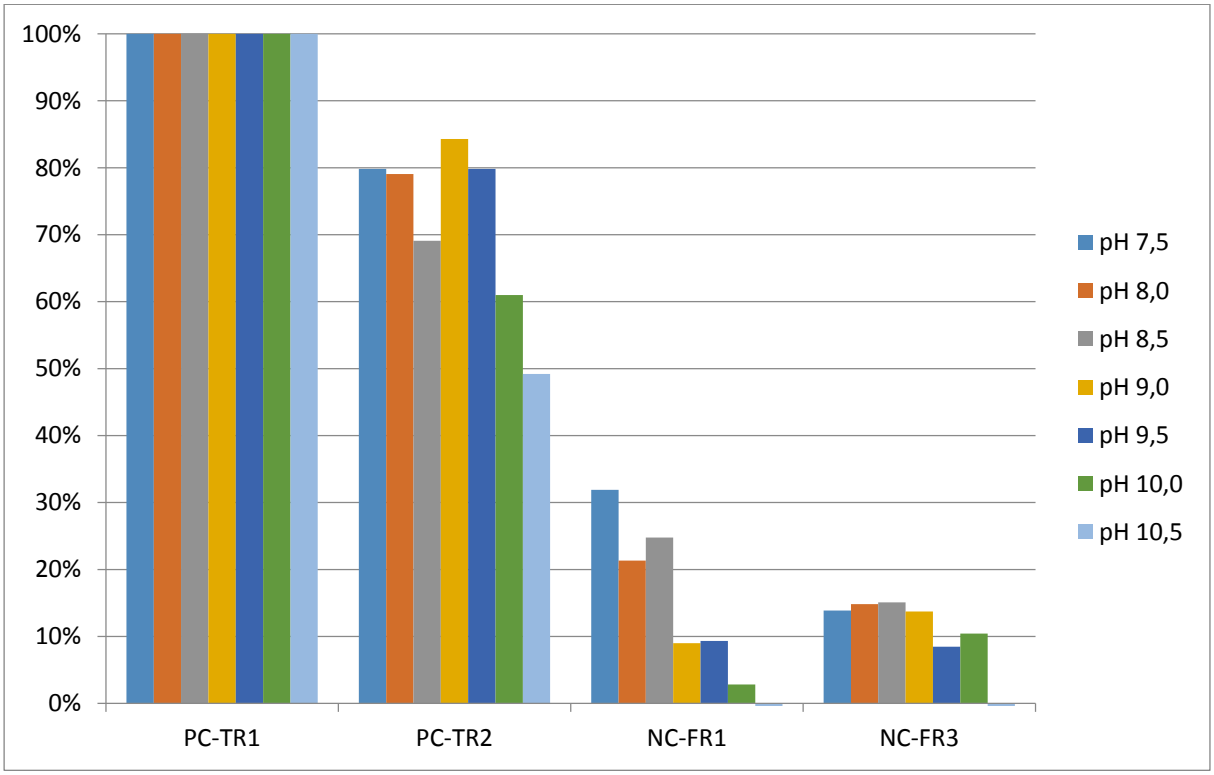
TR1	TR2	FR1	FR3
1,532	1,223	0,143	0,130
100%	80%	9%	8%

pH 10,0

TR1	TR2	FR1	FR3
0,940	0,573	0,027	0,098
100%	61%	3%	10%

pH 10,5

TR1	TR2	FR1	FR3
0,156	0,077	-0,011	-0,002
100%	49%	-7%	-1%



PC: Positive control; NC: Negative control; TR: Turkish serum; FR: French serum

Supplementary file 3

Indirect cattle in-house CCHFV-IgG-ELISA: Comparison of 0.5% and 1% BSA concentration in the coating buffer.

PBS + 0.5% BSA

TR1	TR2	FR7	D1/15	D005	D006	D007	D008	D009	D010
1,839	1,645	0,109	0,065	0,100	0,091	0,127	0,288	0,164	0,530
100%	89%	6%	4%	5%	5%	7%	16%	9%	29%

PBS + 1% BSA

TR1	TR2	FR7	D1/15	D005	D006	D007	D008	D009	D010
1,165	1,104	0,119	0,080	0,111	0,099	-0,228	0,205	0,121	0,480
100%	95%	10%	7%	10%	9%	-20%	18%	10%	41%

TR1: Positive control; FR7: Negative control; TR: Turkish serum; FR: French serum; D: German serum

Supplementary file 4

Indirect cattle in-house CCHFV-IgG-ELISA: Comparison of coating times.

Coating 3 days

1	**2**
0,713	3,095
0,756	3,250
0,661	0,632
0,544	0,594
2,872	1,571
2,529	1,605
0,795	0,567
0,688	0,647

D1/15	TR1 1/40
0,132	2,559
5%	100%
TR1 1/80	TR1 1/160
1,959	0,981
77%	38%

Coating overnight

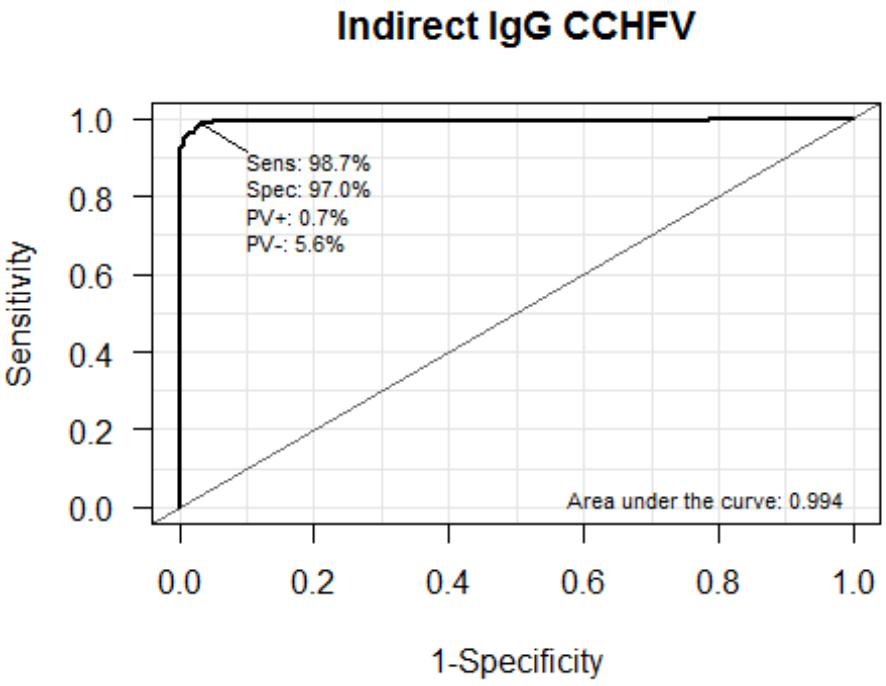
1	**2**
0,691	2,742
0,734	2,817
0,631	0,795
0,609	0,793
2,156	1,539
1,760	1,474
0,660	0,560
0,618	0,616

D1/15	TR1 1/40
0,093	1,986
5%	100%
TR1 1/80	TR1 1/160
1,319	0,918
66%	46%

D: German serum; TR: Turkish serum; D1/15: Negative control; TR1 1/40: Positive control

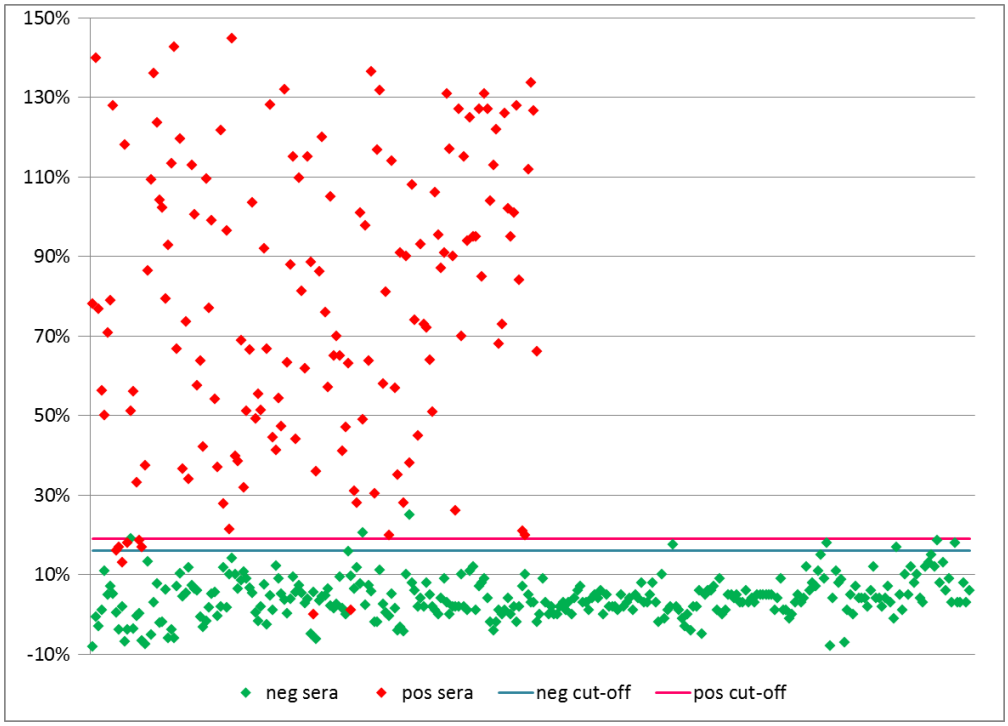
Supplementary file 5

Indirect cattle in-house CCHFV-IgG-ELISA: ROC analysis with a 95% confidence interval (detected cut-off 17%).



Supplementary file 6

Indirect cattle in-house CCHFV-IgG-ELISA: Validation with sera from Albania, Germany, Macedonia, Turkey and Mauritania.



Negative cut-off	**<16%**
Positive cut-off	**>19%**
True negative	294
False negative	3
False positive	2
True positive	193
Inconclusive	12
n	504
Diagnostic sensitivity	**99%**
Diagnostic specificity	**99%**
Inconclusive	**2%**

Supplementary file 7

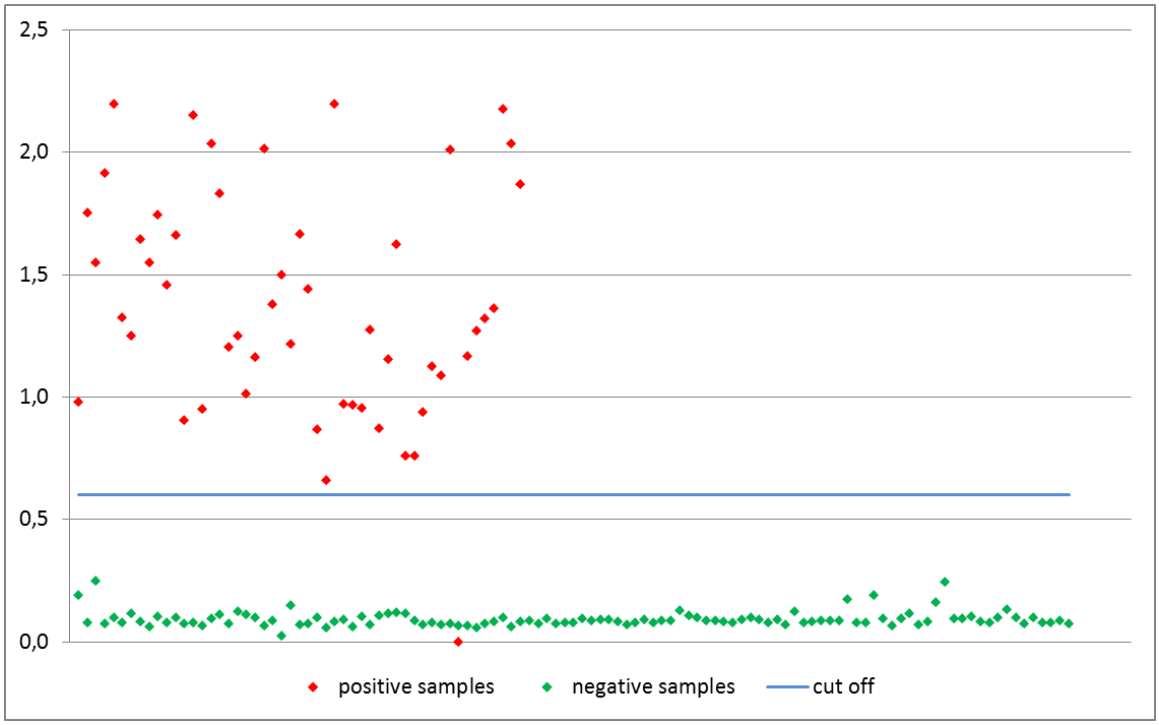
Indirect cattle in-house CCHFV-IgG-ELISA: Reproducibility analysis.

FLI characterization		Person 1		Person 2	
NC	-	0,151	8%	0,129	6%
PC	+	1,988	100%	2,195	100%
BG 651/11	+	0,480	24%	0,662	30%
D 19/12	-	0,126	6%	0,221	10%
D 24/12	-	0,155	8%	0,211	10%
BG 72/11	+	1,836	92%	1,849	84%
D 25/12	-	0,057	3%	0,311	14%
BG 649/11	+	2,641	133%	2,820	128%
BG 653/11	+	1,296	65%	1,375	63%
D 23/12	-	0,039	2%	0,241	11%
D 20/12	-	0,146	7%	0,103	5%
BG 650/11	+	0,347	17%	0,481	22%
D 27/12	-	-1,181	-59%	0,283	13%
D 22/12	-	-0,081	-4%	0,126	6%
BG 654/11	+	0,484	24%	0,661	30%
D 28/12	-	-0,080	-4%	0,180	8%
BG 66/11	+	1,179	59%	1,687	77%
BG 652/11	+	1,366	69%	1,277	58%
D 21/12	-	-0,182	-9%	0,290	13%
BG 648/11	+	2,457	124%	2,779	127%

NC: Negative control; PC: Positive control; D: German serum; BG: Bulgaria serum

Supplementary file 8

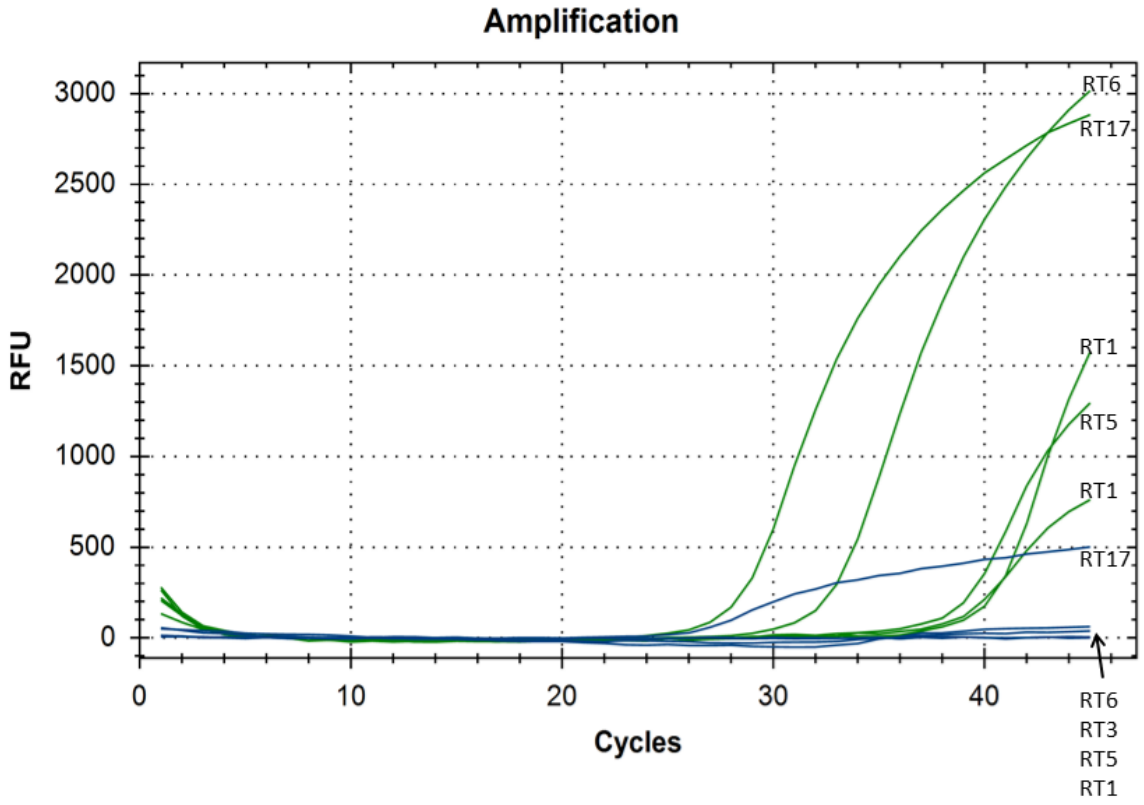
Commercial cattle adapted CCHFV-IgG-ELISA (Vector Best): Validation with sera from Mauritania and Germany.



Cut-off	**>0,6**
True negative	113
False negative	1
False positive	0
True positive	50
n	164
Diagnostic sensitivity	**98%**
Diagnostic specificity	**100%**

Supplementary file 9

CCHFV RT-qPCR: Comparison of the former RT-qPCR (adapted from Wölfel et al., 2007) with the protocol designed by Atkinson et al. (2012).



Green: former FLI RT-qPCR; Blue: RT-qPCR designed by Atkinson et al.; RT: Ring test sample from the "International External Quality Assessment of Molecular Detection of Crimean-Congo Hemorrhagic Fever Virus".

Supplementary file 10

CCHFV positive ticks: Results of the novel multiplex RT-qPCRs and Sequencing

Tick no.	Location	Ct (SYBR)	Ct (FAM)	Genotype	
MO 11	Maya Oulo	28.66	29.90	III	100Q, 98I (IbAr10200)
DM 6	Dembo	34.97	31.80	III	100Q, 98I (IbAr10200)
DM 40	Dembo	32.80	33.18	III	99Q, 98I (IbAr10200)
DYE 5	Dourbey	28.52	29.84	III	98Q, 95I (IbAr10200)
DYE 7	Dourbey	29.64	30.04	III	98Q, 95I (IbAr10200)
DYE 8	Dourbey	28.51	29.97	III	99Q, 99I (IbAr10200)
DYE 11	Dourbey	30.12	30.53	III	99Q, 98I (IbAr10200)

Ct: Threshold cycle; SYBR: Multiplex CCHFV RT-qPCR based on SYBR Green detection; FAM: Multiplex CCHFV RT-qPCR based on FAM-probe detection; Q: Query coverage; I: Identity; IbAr10200: Nigerian CCHFV strain

16 AUTHORS' CONTRIBUTIONS

Manuscript I:

Conception of the study:	Miriam Andrada Sas had contributed together with others under the lead of Ousmane Maiga.
Developed the assays:	Miriam Andrada Sas
Performed the experiments:	Miriam Andrada Sas had contributed together with others under the lead of Ousmane Maiga.
Wrote the manuscript:	Maiga O, Sas MA, Rosenke K, Kamissoko B, Mertens M, Sogoba N, Traore A, Sangare M, Niang M, Schwan TG, Maiga HM, Traore SF, Feldmann H, Safronetz D, Groschup MH

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Analyzed the data:	Sas MA, Vina-Rodriguez A, Mertens M, Eiden M, Emmerich P, Chaintoutis SC, Mirazimi A, Groschup MH
Wrote the manuscript:	Sas MA, Vina-Rodriguez A, Groschup MH

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