University of Veterinary Medicine Hannover

Diagnostic approaches for zoonotic hemorrhagic fever viruses of the order Bunyavirales in livestock

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Manuscripts extracted from the doctorate project:

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International Symposium on Zoonoses Research, Berlin, Germany, 2019:" *Ngari virus, a natural Bunyamwera and Batai virus reassortant*" (Poster presentation)

List of abbreviations

µg	Microgram
µl	Microliter
ABTS	2. 2′-azino di-ethylbenzothiazoline sulphonic acid
Arbovirus	Arthropod-borne virus
BATV	Batai virus
BUNV	Bunyamwera virus
CCHFV	Crimean-Congo hemorrhagic fever virus
CO_2	Carbon dioxide
CT	Cycle thresholds
CVV	Cache Valley virus
Cy3	Cyanine 3
DIC	Disseminated intravascular coagulation
E. coli	*Escherichia coli*
ELISA	Enzyme linked immunosorbent assay
FITC	Fluorescein isothiocyanate
FMDV	Foot-and-Mouth Disease virus
FLI	Friedrich-Loeffler-Institut
Gc	Glycoprotein c (-terminal)
Gn	Glycoprotein n (-terminal)
HRPO	Horseradish peroxidase
ICTV	International Committee on Taxonomy of Viruses
IgG	Immunoglobulin G
IgM	Immunoglobulin M

(I)IFA	(Indirect) immunofluorescence assay
kDa	Kilo Dalton
L segment	Large segment
M	Molar
mg	Milligramm
min	Minute
ml	Milliliter
mRNA	Messenger Ribonucleic acid
M segment	Medium segment
ND_{50}	50% neutralization dose
nm	Nanometre
Np	Nucleoprotein
NSm	Nonstructural protein, encoded on the medium segment
NSs	Nonstructural protein, encoded on the small segment
NRIV	Ngari virus
OD405	Optical density at 405nm
OIE	World Organization for Animal Health
ORF	Open reading frame
PBS	Phosphate buffered saline
PBST	PBS containing 0.1% Tween
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
pH	Potential of hydrogen
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid

RNP	Ribonucleoprotein
RT-qPCR	Quantitative real-time reverse transcriptase polymerase chain reaction
RVFV	Rift Valley fever virus
s	Second
SDS	Sodiumdodecyl-sulfate
Se	Sensitivity
S	segment Small segment
SNT	Serum neutralization test
Sp	Specificity
spp.	Species
S/P%	Sample-to-positive-ratio
$TCID_{50}$	50% tissue culture infective dose
UTR	Untranslated region
VNT	Virus neutralization test

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1 Introduction

Ngari virus (NRIV) is a natural reassortant which resulted from a coinfection of Bunyamwera virus (BUNV) and Batai virus (BATV) (Briese et al. 2006). All three viruses are members of the Bunyamwera serogroup within the genus *Orthobunyavirus* of the family *Peribunyaviridae* in the order Bunyavirales (Elliott 2014). Interestingly, this reassortment led to an increased virulence, which is associated with hemorrhagic fever in humans and ruminants (Dutuze et al. 2018). Other causative agents of hemorrhagic fever disease amongst others are Rift Valley fever virus (RVFV) and Crimean-Congo hemorrhagic fever virus (CCHFV), both also members of the order Bunyavirales. Viruses of the order Bunyavirales characteristically comprise a tri-segmented (S, M and L segments), enveloped negative-strand RNA genome (Briese et al. 2006). Hereby, the S segment encodes the N and NSs proteins, the M segment encodes the two glycoproteins (Gn and Gc) and the NSm protein, and the L segment codes for the RNA-dependent RNA-polymerase (Elliott 2014). These zoonotic arboviruses are transmitted by hematophagous arthropods, primary by mosquitoes. Only CCHFV is transmitted by ticks and is even believed to cause the most widespread tick-borne viral infection of humans (Bente et al. 2013). The geographic distribution of the *Orthobunyaviruses*, RVFV and CCHFV overlap in sub-Saharan Africa.

NRIV has been retrospectively isolated twice during concurrent RVFV outbreaks, in Kenya and Somalia in 1997-1998 and in Mauritania in 2010 (Bowen et al. 2001; Eiden et al. 2014). Since the diagnostic approach was primarily based on clinical presentation and since the laboratory diagnosis of NRIV is complex, its prevalence may be underestimated (Dutuze et al. 2018). Hence, the aim of the first study was to establish molecular and serological assays for NRIV to investigate its presence in livestock population and possible cocirculation with RVFV in Mauritania. Serum samples from small ruminants were collected during a confirmed RVFV outbreak and analyzed by PCR, serum neutralization tests (SNT) and indirect ELISA. Unfortunately, the close relationship between NRIV and its parental viruses complicates the unambiguous serological detection. The first study illustrates to what extent the *Orthobunyaviruses* are serologically distinguishable.

Evidence of RVFV and CCHFV circulation can be found in countries in northern Africa such as Egypt, and on the Arabian peninsula (Bente et al. 2013; Linthicum et al. 2016). In Egypt, the first outbreak of RVFV was reported in 1977. Till today, it remains the largest RVFV epizootic with an estimated number of 200,000 human infections, almost 600 deaths, and high economic losses in livestock (Bird et al. 2009). Further outbreaks in a lesser extent occurred in 1993/94, 1997 and most recently in 2003 (Ahmed Kamal 2011). The continuous import of infected livestock, especially camels from Sudan, without sufficient quarantine measurements was assumed as the main source for the

introduction of RVFV into Egypt (Ahmed Kamal 2011). Likewise, CCHF viral genomes were detected in ticks infested on camels that were imported from Sudan and Somalia (Chisholm et al. 2012). It remains unclear, whether the ticks were infected and infested the camels before or after importation. Nevertheless, the importation of livestock from countries where CCHFV is endemic, poses a potential risk for the introduction of the virus and its spread throughout Egypt. Hence, the aim of the second study was designed to monitor the prevalence status of RVFV and CCHFV in the livestock population in Egypt and to evaluate the virus introduction risk from neighboring countries into Egypt.

Generally, global livestock trade increases the risk of introduction of viral pathogens from endemic to pathogen-free regions, e.g. in Europe. Additionally, climate warming favours the expansion of competent vectors and thus affect the geographical distribution of arboviruses. Currently, only BATV of the here mentioned arboviruses has so far been detected in Germany (Ziegler et al. 2018). Traditionally, detection of BATV in ruminants was conducted by PCR, hemagglutination inhibition test immunoblot, immunofluorescence assay and virus neutralization test (Jöst et al. 2011; Ziegler et al. 2018; Hofmann et al. 2015; Medlock et al. 2007). All these serological tests are sensitive methods for antibody detection but require the use of live virus and therefore require compliance of a higher safety standard. Hence, the study was implemented to evaluate the use of an indirect ELISA based on the recombinant Gc ELISA as screening assay for BATV monitoring studies in Germany.

In summary, the here presented work provides diagnostic assays for Ngari virus and its parental viruses in order to allow investigations on the distribution of these three *Orthobunyaviruses* and the infection status of ruminants. Additionally, potential cocirculation with other hemorrhagic fever viruses namely RVFV and CCHFV is highlighted.

2 Literature review

2.1 Classification

In total, the order *Bunyavirales* comprises 12 different families including the family of *Peribunyaviridae*, *Phenuiviridae* and *Nairoviridae* (ICTV, 2019). Within the family *Peribunyaviridae,* 97 species are classified into four genera of which the genus *Orthobunyavirus* is the largest one comprising 88 species. Three of these species, *Ngari orthobunyavirus*, *Batai orthobunyavirus* and *Bunyamwera orthobunyavirus*, are part of the present thesis. The species *Rift Valley fever phlebovirus* belongs to the genus *Phlebovirus,* one of 19 genera within the family *Phenuiviridae.* In total, the genus *Phlebovirus* consists of 60 individual species. A total of 17 species have been placed among three genera in the family of *Nairoviridae*, whereby the species *Crimean-Congo hemorrhagic fever orthonairovirus* is a member of the genus *Orthonairovirus*. A brief taxonomic overview of the order *Bunyavirales* is shown in Figure 1 displaying only a selection of corresponding families, genera and species.

Order	Family	Genus	Species
Bunyavirales	Nairoviridae	Orthonairovirus	Crimean-Congo hemorrhagic fever orthonairovirus
	Peribunyaviridae	Orthobunyavirus	Batai orthobunyavirus
			Bunyamwera orthobunyavirus
			Ngari orthobunyavirus
	Phenuiviridae	Phlebovirus	Rift Valley fever phlebovirus

Figure 1. Taxonomy of members of the order *Bunyavirales.*

2.2 Morphology and genome characterization

Viruses of the order *Bunyavirales* vary in their morphology. However, virions generally are spherical or pleomorphic, 80–120 nm in diameter, and display surface glycoprotein spikes which are integrated in a lipid bilayered envelope (King 2012). Virion envelopes are derived from cellular Golgi membranes, or occasionally from cell surface membranes (King 2012). The surface spikes consist of two glycoproteins called Gn and Gc (King 2012). In *Orthobunyaviruses* the glycoprotein spikes comprise trimers of Gn–Gc heterodimers and form a unique tripod-like arrangement (Elliott 2014). The icosahedral particles of *Phleboviruses* are arranged in pentamers and hexamers (Sherman et al. 2009), and classical nairovirions are spiked with heterodimeric glycoprotein projections (Kuhn et al. 2016).

The helical nucleocapsids of the *Bunyavirales* are 2–2.5 nm in diameter, and 200–3000 nm in length (King 2012). They comprise one each of the three molecules of negative or ambisense single stranded RNA segments designated L (large), M (medium) and S (small) segment (King 2012).

The S segment codes for the nucleocapsid (N) protein, and in some *Orthobunyaviruses* and *Phleboviruses* also for a non-structural protein, NSs (Briese et al. 2013). Both the *Orthobunyavirus* proteins are encoded in the same mRNA and are translated after alternative initiation at different AUG codons (Fuller et al. 1983). In contrast, the *Phlebovirus* S segments are ambisense, with the N protein encoded in genome complementary RNA while the NSs protein is translated from a subgenomic genome-sense RNA (King 2012). The N protein is the main protein that is produced in infected cells, it is highly immunogenic and elicits complement-fixing antibodies (Elliott 2014). N protein encloses all three RNA segments into ribonucleoprotein complexes (RNP), and interacts with the viral polymerase (Briese et al. 2013). In contrast to the N protein, the amino acid sequence of NSs tends to be more variable between different viruses (Thomas et al. 2004). The NSs protein is essential for the viral replication cycle and is the major virulence factor (Eifan et al. 2013). Thereby, alternating replication in mammalian and mosquito cells is necessary to maintain the virulence factor as passages in single hosts lead to large nucleotide deletion and reduced infectivity (Moutailler et al., 2011). For *Orthobunyaviruses* and *Phleboviruses*, the NSs has been shown to suppress the interferon response (King 2012). The RVFV NSs protein is unique among the *Bunyavirales* as it forms a filamentous structure in the nucleus of infected cells, which is unexpected for a virus replicating in the cytoplasm (Pepin et al. 2010).

The M segment generates a polyprotein, which is co-translationally cleaved to give mature Gn and Gc glycoproteins named according to their relative proximity to the amino or carboxy terminus of the precursor (King 2012). Additionally, *Orthobunyaviruses* and some *Phleboviruses* encode a nonstructural protein (NSm) in

the genome complementary RNA (King 2012). *Nairoviruses* even encode two proteins: a single precursor polyprotein is processed by cotranslational cleavage into precursors to both Gn and Gc. Posttranslational cleavage of preGn yields a mucin-rich product and a glycoprotein GP38, whereas cleavage of preGc removes a polypeptide from its C-terminus and yields a mature Gc (King 2012). The M segment gene products have a major influence on vector competence, which is by definition the ability of a particular arthropod species to transmit the virus (Elliott 2014). Moreover, the two glycoproteins Gc and Gn are responsible for viral attachment and acid-activated penetration (Dutuze et al. 2018), and elicit hemagglutinating and neutralizing antibodies (King 2012). Additionally, they play a crucial role in virus assembly and budding (Overby et al. 2007; Shi et al. 2007). NSm suppresses virus-induced apoptosis and contributes to pathogenesis (Pepin et al. 2010).

The L segment codes for the large RNA-dependent RNA polymerase (RdRp or L protein), which is needed for RNA replication and RNA transcription (Briese et al. 2013). The L segment of *Nairoviruses* is nearly twice the size of those of other *Bunyavirales*. Its single open reading frame (ORF) is more than 12,000 nucleotides in length and encodes a polyprotein of nearly 4000 amino acids (Bente et al. 2013). In addition to polymerase activity, the L protein has an endonuclease activity that cleaves cellular messenger RNAs to initiate transcription of viral messenger RNAs (Briese et al. 2013). For *Orthobunyaviruses*, the L protein is considered the major determinant of neuroattenuation (Endres et al. 1991).

The three genomic RNA segments are encapsidated by the N protein to form RNP complexes that associate with the L protein and are contained within the viral envelope (Elliott 2014). The terminal nucleotides of each RNA segment are base-paired forming non-covalently closed, circular RNAs (King 2012). The RNA genome is flanked by 3´ and 5´ UTRs (untranslated region) which vary among the *Bunyavirales* in length and sequence (Elliott 1997). The UTRs function as promotors and are also required for the encapsidation of the genomic RNA by the N protein, the termination of mRNA transcription and packaging of the RNP into virus particles (Osborne and Elliott 2000; Kohl 2006). Interestingly, deletion of internal sequences in the UTRs of BUNV resulted in attenuation of virus replication and loss of cytopathogenicity in mammalian cell culture, but its mechanism remains unclear (Mazel-Sanchez and Elliott 2012).

Sequence analysis revealed that NRIV is a natural reassortant resulting from co-infection of BUNV and BATV, as NRIV contains the M segment of BATV combined with the S and L segments from BUNV (Briese et al. 2006; Gerrard et al. 2004). The nucleotide sequence homologies between BUNV and NRIV S segments and L segments, and between BATV and NRIV M segment are 93%, 97–98%, and 89–95%, respectively (Briese et al. 2006; Gerrard et al. 2004).

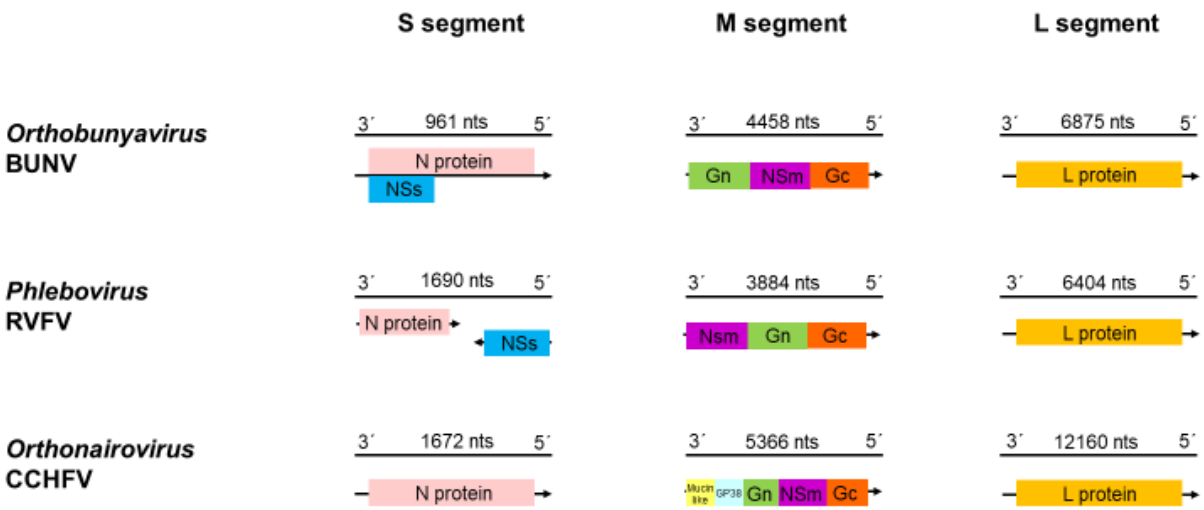

Figure 2. Coding strategies of genome segments of Bunyamwera virus, Rift Valley fever virus, and Crimean-Congo hemorrhagic fever virus.

Apparently, most recognized reassortants of *Orthobunyaviruses* and of other bunyaviruses possess L and S RNA segments derived from one virus and the M RNA from another one (Briese et al. 2013). It may indicate that a closer match between polymerase, N protein and RNA segments is needed than for the glycoproteins, whose interactions may rely on more universally conserved mechanisms (Briese et al. 2013). The event of a reassortment is more likely, the more closely related the viruses are (Iroegbu and Pringle 1981). However, the phenomenon of superinfection resistance usually prevents the co-infection by closely related viruses. Only in cases where the second virus infects rapidly after the first one, the superinfection resistance may be bypassed (Iroegbu and Pringle 1981).
Furthermore, geographic distances between viruses and vectors prevent reassortment (Briese et al. 2013). However, changing ecologic conditions or transportation of a relatively benign bunyavirus to a suitable econiche could produce reassortants with a closely related, endemic, and relatively benign bunyavirus (Briese et al. 2013). As in case of NRIV, reassortment of benign viruses might result in progeny virus that is not at all benign, but shows an increase in pathogenicity (Briese et al. 2013).

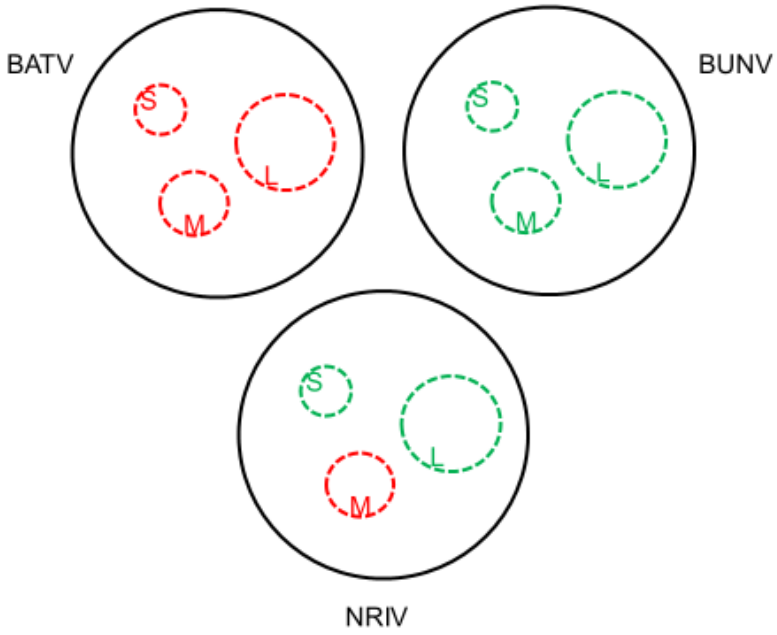

Figure 3. Schematic depiction of BATV, BUNV, and the reassortant NRIV.

2.3 Epidemiology

Ngari virus (NRIV) was discovered in 1979 in southeastern Senegal by isolation from *Aedes simpsoni* mosquitoes (Zeller et al. 1996). In the following years, the virus was detected in further mosquito species in Senegal, Burkina Faso, Central African Republic and Madagascar (Zeller et al. 1996). In 1993, NRIV was isolated for the first time in two patients in Dakar (Senegal) revealing its potential pathogenicity in humans (Zeller et al. 1996). The association of NRIV with severe disease was supported in 1988 in Sudan during a malaria outbreak, when 195 human patients were assayed for arboviruses of which 7% showed IgM antibodies and 61% IgG antibodies against NRIV (Nashed et al. 1993). Similarly, in Kenya and Somalia an outbreak of hemorrhagic fever was diagnosed as RVFV, but revealed in 14 out of 70 tested patients infection with NRIV (Bowen et al. 2001). Both events were preceded by unusually heavy rain and flood promoting mosquito breeding (Braack et al. 2018). The co-infection with RVFV was repeatedly observed in 2010 in Mauritania, when NRIV was isolated from two goats during an ongoing RVFV outbreak (Eiden et al. 2014). Thus, the spread of NRIV has so far been limited to sub-Saharan Africa.

Bunyamwera virus (BUNV) is also widely spread in sub-Saharan Africa. It was first isolated in 1943 in Semliki Forest in Uganda from Aedes mosquitoes during yellow fever surveillance (Smithburn et al. 1946) and in 1955 from *Aedes circumluteolus* mosquitoes in KwaZulu-Natal Province in South Africa (Kokernot et al. 1958). In the same province, BUNV was obtained from a mosquito catcher presenting with severe

headache, neck stiffness and fever (Kokernot et al. 1958), while a serosurvey for several arboviruses in the same area detected neutralizing antibodies in 54% of tested humans (Smithburn et al. 1959). Moreover, high immunity levels indicated BUNV to be common in the human population in the Okavango Basin of Botswana and Caprivi Region of Namibia (Kokernot et al. 1965). Other African countries, where BUNV is considered endemic now are Senegal, Guinea, Ivory Coast, Nigeria, Cameroon, Central African Republic, Kenya, and Madagascar (Wertheim et al. 2012). Recently, the virus was as well obtained in Ruanda from five cows with abortion (Dutuze et al. 2020). Furthermore, Cache Valley virus (CVV) was classified as BUNV strain, which expands overall North America (Calisher et al. 1986) towards South America (Tauro et al. 2009a). In Argentina, additional BUNV strains were isolated from birds and horses displaying neurological symptoms (Tauro et al. 2009b; Tauro et al. 2015).

Batai virus (BATV) is considered one of the most widespread *Orthobunyavirus* (Briese et al. 2006). It was first isolated from *Culex gelidus* collected in Malaysia in 1955 (Karabatsos 1985). Antigenically identical “Čalovo” virus was then isolated from *Anopheles maculipennis* s.l. mosquitoes collected in South Slovakia (Danielova et al. 1978) and West Ukraine (Vinograd et al. 1973). Moreover, evidence for circulation in Europe exists in Norway, Sweden, Finland, Slovakia, the Czech Republic, Croatia, Serbia, Bosnia, Montenegro, Italy, Hungary, Romania, Austria, Portugal, and Belarus (Medlock et al. 2007). In Germany, the virus was repeatedly isolated from mosquitoes and ruminants in recent years (Jöst et al. 2011; Scheuch et al. 2018; Ziegler et al. 2018; Hofmann et al. 2015). Other strains of BATV are ‘Chittoor’ which were detected in *Anopheles barbirostris* in India and in *Aedes curtipes* in Sarawak (Hubálek 2008). Intriguingly, BATV is not widely reported in Africa, having only been found in Uganda (Briese et al. 2006) and recently in Ruanda (Dutuze et al. 2020). However, the closely related Ilesha virus is widespread in Africa (Hunt and Calisher 1979a; Morvan et al. 1994). Virus isolation and serological evidence are reported in Cameroon, Central African Republic, Nigeria, Senegal, Uganda, Madagascar, Ghana and Niger (CDC 2020).

The occurrence of acute and highly fatal disease in exotic wool sheep in Rift Valley in Kenya in 1913 may have been due to an infection with Rift Valley fever virus (RVFV) (Davies 2010). However, the virus was not discovered and characterized until 1931, when approximately 4,700 lambs and ewes on a single farm in the Great Rift Valley of Kenya deceased suddenly within one month (Daubney et al. 1931). The next major epizootic occurred in South Africa in 1951 affecting livestock (abortion in 500,000 ewes and sudden death of 100,000 sheep), and as well as humans with acute illness, at first thought to be influenza (Swanepoel and Coetzer 2004; Gear 1951). Since that time, recurrent epidemics have been reported in South Africa, Zimbabwe, Mauritania, Senegal, Zambia, Namibia, Sudan, Tanzania, and Somalia (Bird et al. 2009). The massive epidemic-epizootic in Egypt in 1977 affecting humans and livestock marked the first time the virus was recognized outside sub-Saharan Africa (Meegan 1979). It

remains the largest outbreak, with extensive losses among livestock, an estimated 200,000 human infections and almost 600 deaths (Bird et al. 2009). Further Egyptian outbreaks in a lesser extent occurred in 1993/94, 1997 and most recently in 2003 (Kamal 2011). The continuous import of infected livestock, especially camels from Sudan, without sufficient quarantine measurements was assumed as the main source for the introduction of RVFV into Egypt (Kamal 2011). In 1979, RVFV was isolated for the first time outside of continental Africa in Madagascar where the virus is now endemic (Pepin et al. 2010). In 2000, RVFV could even emerge on the Arabian Peninsula (CDC 2000). A devastating outbreak in Saudi Arabia and Yemen caused at least 245 deaths among 2,000 infected humans and fatal diseases in thousands of goats and sheep (Bird et al. 2009). Genetic analyses revealed the close relationship between the Saudi Arabia and Yemen viruses and those circulating earlier in East Africa which suggested that the virus was introduced into Saudi Arabia and Yemen from eastern Africa possibly by commercial trade of livestock (Shoemaker et al. 2002). Nowadays, RVFV is circulating widely throughout Africa, as demonstrated by reports on virus isolations and serological evidence from more than 30 African countries (Linthicum et al. 2016). Most recent reports describe sporadic outbreaks in humans on the French Island Mayotte and Sudan (WHO 2019) and in livestock in Libya, Rwanda, South Sudan, and Uganda (OIE 2020).

Crimean-Congo hemorrhagic fever virus (CCHFV) was first discovered as Crimean hemorrhagic fever virus (CHFV) in 1944, when Soviet troops re-occupying Crimean Peninsula developed an acute febrile illness with a high incidence of bleeding and shock (Bente et al. 2013). Subsequently, a variety of cases of febrile disease throughout Soviet Union and Bulgaria were identified as CHFV infections (Chumakov et al. 1970; Papa et al. 2004). In 1969, the Crimean strain was shown to be identical with the Congo virus isolated in Democratic Republic of the Congo (Casals 1969). Since then, the agent is designated CCHFV. Nowadays, the known geographic distribution of CCHFV ranges from western China through southern Asia and the Middle East to southeastern Europe and throughout most of Africa (Bente et al. 2013). Hereby, it corresponds closely with the distribution of its main vector, i.e. members of the *Hyalomma* tick genus (Shayan et al. 2015). In Europe, Hungary and Romania are considered the northernmost extension of CCHFV with reports on detection of CCHFV-specific antibodies in sheep (Bente et al. 2013). Antibodies were as well detected in bats in France and in Portugal (Hoogstraal 1979). However, no indigenous case of CCHF has been reported in Europe west of the Balkan (Bente et al. 2013). First evidence of the virus in Spain was reported, when RNA was isolated from *Hyalomma lusitanicum* recovered from wild deer (Estrada-Peña et al. 2012). Due to its close relationship to African strains, it was postulated that it has been introduced by migratory birds (Estrada-Peña et al. 2012). Turkey represents a special case in CCHF epidemiology, as it developed from a country free of human infections to the ‘‘epicenter’’ of the disease, with more than 1000 confirmed cases per year (Bente et

al. 2013) and a mean fatality rate of approximately 5% (Ozkaya et al. 2010). Serosurveys in the Anatolian region detected specific antibodies in almost 80% of domestic animals (Gunes et al. 2009) and in 10% of humans (Bodur et al. 2012). In Africa, most cases of CCHFV have been reported in South Africa, where it was shown to circulate among a variety of wild and domestic animals such as scrub hares, giraffe, rhinoceros, and domestic dogs (Bente et al. 2013; Shepherd et al. 1987b). Moreover, ostriches were identified as only avian host for CCHFV, when CCHFV induced disease occurred in workers on ostrich farms and subsequently antibodies were detected in about one fourth of animals on that very farms (Shepherd et al. 1987a). In Egypt, viral RNA or specific antibodies were mostly reported in ticks or livestock imported from CCHFV endemic countries such as Sudan, Somalia, and Kenya (Chisholm et al. 2012; Morrill et al. 1990). In Sudan, sporadic human cases and nosocomial outbreaks have been described repeatedly in the past few years (Aradaib et al. 2011; Aradaib et al. 2010; Elata et al. 2011; Rahden et al. 2019; Bower et al. 2019).

2.4 Transmission

As arthropod-borne viruses, NRIV, its parental viruses BATV and BUNV, and RVFV as well as CCHFV are transmitted by hematophagous insects. Hereby, mosquitoes function as the main vectors, only in case of CCHFV ticks are primarily responsible for transmission. These viruses are capable of alternately replicating in vertebrates and arthropods, and generally are cytolytic for vertebrate host cells, but cause little or no cytopathogenicity in invertebrate host cells (King 2012).
A variety of mosquito species carrying the three *Orthobunyaviruses* has been discovered by molecular virus diagnostics. NRIV has been isolated from mosquitoes such as *Aedes argentepunctatus*, *Ae. minutus*, *Ae. vexans*, *Ae. mcintoshi*, *Ae. simpsoni*, *Ae. vittatus, Ae. neoafricanus, Anopheles coustani, An. pretoriensis, An. pharoensis, An. mascarensis, Culex bitaeniorhynchus, C. tritaeniorhynchus, C. antennatus*, and *C. poicilipes* (Zeller et al. 1996). Additionally, infection studies revealed that *Anopheles gambiae Giles* is competent vector for both, NRIV and BUNV (Odhiambo et al. 2014). In Argentina, BUNV was as well isolated from *Ochloretatus scapularis* (Tauro et al. 2015). However, *Aedes aegypti* is considered the primary vector for BUNV transmission (Odhiambo et al. 2014). Mosquito vectors for BATV are *Anopheles maculipennis s.l*, *An. claviger, Coquillettidia richiardii, Culex pipiens, Ochlerotatus punctor, Oc. communis,* and *Aedes vexans* in Europe and additionally *An. barbirostris* and *Ae. curtipes* in Asia (Hubálek 2008). Susceptible mammal species for all three *Orthobunyaviruses* are small ruminants (Eiden et al. 2014; Ziegler et al. 2018; Chung et al. 1990). Further vertebrate hosts for BUNV and BATV are cattle, horses, pigs and several bird species (Hubálek 2008; Tauro et al. 2015; Tauro et al. 2009b). Hereby, the risk of BATV transmission has been correlated with distribution of

migratory birds which may explain its wide geographic distribution across Europe and Asia (Hubálek and Halouzka 1996; (Hubálek 2008).

The isolation of RVFV from mosquito species of two genera (*Eretmapodites* and *Aedes*) caught in the Semliki Forest in Uganda in 1944 established RVFV as arthropod-borne virus (Smithburn et al. 1948). Subsequent virus isolations from mosquitoes collected during epizootics in other African countries revealed 53 potentially competent species in eight genera within the family Culicidae (*Aedes, Culex, Anopheles, Eretmapodites, Mansonia, Coquillettidia, Anopheles,* and *Eumelanomyia*) (Linthicum et al. 2016). Although, mosquitoes are considered the only important biological vectors of RVFV, experimental infection indicate that several other arthropods, including phlebotomine, sandflies and ticks, may be able to mechanically transmit the virus (Linthicum et al. 1989; Hoch et al. 1984; Hoch et al. 1985). The unusually large range of vectors capable of transmitting RVFV enabled the virus to move outside traditionally endemic areas to Madagascar and Egypt, and even out of Africa to the Arabian Peninsula (Gerdes 2004). Studies revealing vector competence among mosquito species in North America and Europe (i.e. *Aedes albopictus, Ae. canadensis, Ae. Detritus, Culex pipiens,* and *Cx territans*) demonstrate that competent vectors are available for the establishment of epizootics and perhaps enzootics, if RVFV should be introduced to the North American and European continent (Birnberg et al. 2019; Brustolin et al. 2017; Moutailler et al. 2008; Lumley et al. 2018; Gargan et al. 1988). In semiarid regions of eastern and southern Africa the ecology of RVFV involves two cycles: the enzootic cycle and the epizootic or epidemic cycle (Bird et al. 2009) Thereby, the development of an epizootic or epidemic depends on climatic events such as the El Niño Southern Oscillation, which can lead to heavy precipitation in East Africa (Linthicum et al. 1987). During the enzootic cycle with normal amounts of rainfall, RVFV is maintained by transovarial transmission in *Aedes* mosquitoes (Linthicum et al. 1985), with low-level circulation in wildlife (e.g. African buffaloes) and in livestock (Linthicum et al. 2016; LaBeaud et al. 2011; Rissmann et al. 2017b). Heavy rainfall and consecutive flooding of breeding habitats of floodwater *Aedes* species result in massive hatching of infected mosquito eggs causing a shift from enzootic to epizootic or epidemic cycle (Linthicum et al. 1985). The transovarially infected mosquitoes then feed on susceptible livestock such as sheep and goats which in turn infect secondary bridge mosquito vectors such as *Culex* or *Anopheline* species (Bird et al. 2009). Humans eventually get infected by mosquito bites (Tantely et al. 2015), but main route of transmission is thought to be the direct or indirect contact with infected livestock (Anyangu et al. 2010). Hereby, possible infection routes are exposure to infectious aerosols, handling of aborted fetal materials or infected tissue and body fluids, or percutaneous injury during slaughtering or necropsy of viremic animals (Bird et al. 2009). Additionally, the consumption of raw milk, as it happened during the epidemic in Mayotte, is considered to be a potential risk factor for RVFV infection (Pepin et al. 2010).

CCHFV is maintained exclusively in ixodid ticks which remain infected throughout their several-year lifetime and therefore are the true natural reservoir (Bente et al. 2013). There is no evidence that mosquitoes or any other arthropod species are competent vectors for CCHFV (Bente et al. 2013). Among ticks, *Hyalomma* spp. are considered most competent (Papa et al. 2017). *Hyalomma marginatum* is the main vector in the area from Kosovo to Pakistan, and most commonly recovered from humans and animals in CCHFV endemic regions of Turkey (Özdarendeli et al. 2010). In Africa, commonly found vectors of CCHFV are *H. marginatum rufipes* and *H. truncatum* (Rechav 1986). Other thermophilic tick species shown to be competent vectors are *Rhipicephalus* ticks in Turkey, Greece and Albania and *Dermacentor marginatus* ticks in Turkey (Yesilbag et al. 2013; Papadopoulos and Koptopoulos 1978; Papa et al. 2010; Papa et al. 2011). Members of *Hyalomma*, *Dermacentor*, and *Rhipicephalus* genera are capable of transstadial transmission of CCHFV from larva to nymph and to adult (Logan et al. 1989; Gonzalez et al. 1992). Transovarial transmission to the offspring occurs within some of the species in these genera (Shayan et al. 2015). Furthermore, venereal transmission has been reported among some vector species (Shepherd et al. 1991; Wilson et al. 1991). Finally by cofeeding, the virus in tick saliva can spread directly to other ticks feeding nearby on uninfected, nonviremic animals (known as “nonviremic” transmission) (Nuttall and Labuda 2004; Bente et al. 2013). *Hyalomma* ticks are ‘‘multi-host’’ ticks, which quest and feed as larvae and nymphs actively on rodents, hares, hedgehogs, ground-feeding birds and other small animals, whereas adults feed on sheep, cattle and other large mammals (Hoogstraal 1979). The identification of mammalian hosts of CCHFV has been based largely on the detection of virus specific antibodies in field serum samples. Only seven reports on experimental infection of wild and domestic animals with CCHFV have been published in English language literature revealing that sheep, calves, scrub hares and ostriches became viremic, and in some cases transmitted the virus to feeding ticks (Bente et al. 2013). Since infected mammals show no overt clinical signs, the only evidence for circulating virus is the occurrence of disease in human ‘‘sentinels’’ (Bente et al. 2013). Moreover, humans represent accidental “dead-end” hosts in the transmission cycle of CCHFV who are not a source of infection for ticks (Bente et al. 2013). Apart from transmission by tick bite, humans can get infected by direct contact with infected animal blood exposing farmers, abattoirs workers, and veterinarians to a higher risk of infection (Shayan et al. 2015). In addition, person-to-person transmissions can occur due to direct or indirect contact with infected patients, primarily in hospital settings (Yolcu et al. 2014; Elata et al. 2011).

2.5 Clinical signs and pathology

All five viruses (BUNV, NRIV, BATV, RVFV, and CCHFV) have been reported to infect humans and other mammals. NRIV is associated with severe and fatal hemorrhagic fever in humans and small ruminants similar to RVFV infection, as seen in the outbreaks in Kenya and Somalia in 1998-1999 and in Mauritania in 2010 (Gerrard et al. 2004; Bowen et al. 2001; Eiden et al. 2014; Jäckel et al. 2013b). However, the course of disease and pathology of NRIV infection has not yet been investigated.

Infection with BUNV causes mild flu-like symptoms in humans, such as fever, headache, joint pain, and rash (Kokernot et al. 1958). However, in immunosuppressed patients the infection may progress to severe encephalitis (Southam and Moore 1951). In Argentina, BUNV was isolated from two horses that developed fatal neurological disease (Tauro et al. 2015). The closely related CVV (Cache Valley virus) is associated with severe disease in ruminants characterized by embryonic and fetal death, stillbirths, and multiple congenital malformations (McConnell et al. 1987; Rodrigues 2011; Chung et al. 1990). Humans are rarely infected, but severe headache, nausea, vomiting, fatigue, encephalitis, and multiorgan failure have been reported in patients (Cambell et al. 2006; Wilson et al. 2017). Infection studies in rhesus (*Macaca mulatta*) and cynamolgus (*M. irus*) monkeys revealed lack of gross pathological signs and relatively low virus levels in the tissues of vital organs (Schwartz and Allen 1970). However, higher levels of virus extracted from the liver than from the blood or other tissues indicates that BUNV has a greater affinity to the liver than to any other organ in this host (Schwartz and Allen 1970).

BATV has been repeatedly isolated from ruminants without corresponding reports of clinical signs (Liu et al. 2014; Yanase et al. 2006; Lambert et al. 2014). Only one study from India reported mild disease in sheep and goats due to infection with the Chittor strain (Singh and Pavri 1966). However, a German captive harbour seal that was naturally infected with BATV died of meningoencephalomyelitis. Evidence of virus replication was detected in Purkinje cells, neurons, enterocytes, and lymphocytes in peripheral tissues (Jo et al. 2018). In humans, infection with BATV has been associated with influenza like symptoms including fever, bronchopneumonia, tonsillitis, and gastritis (Sluka 1969). Antigenically similar Ilesha virus was described as the cause of at least seven human cases of febrile illness with rash or even hemorrhagic fever including one fatal case in Africa (Woodall 1969).

Typical hallmark of RVFV epizootics is the sudden appearance of high number of abortions (“abortion storms”) among ruminants which differ from many other common infectious etiologies of abortion such as Q fever (Coxiella burnettii), chlamydiosis, salmonellosis, listeriosis or toxoplasmosis (Pepin et al. 2010). Clinical apparent infections mainly occur in sheep, goats, cattle, and camel, whereby the clinical symptoms vary among animal species and ages (Bird et al. 2009). Young lambs are highly susceptible to RVFV infection which results in death within 24 to 72 hours in

approximately 90 to 100% of cases (Erasmus and Coetzer 1981). Adult sheep are less susceptible with mortality rates of 10 to 30%, but high abortion rates of up to 100% prevail in pregnant ewes (Swanepoel and Croetzer 2004). Clinical symptoms are fever, lethargy, nasal discharge, hematemesis, and bloody diarrhoea (Erasmus and Coetzer 1981). Gross and histopathological findings revealed widespread organ involvement with necrotic hepatitis accompanied by jaundice and multiple hemorrhages, retinitis, and encephalitis (Ikegami and Makino 2011). Goats are also highly susceptible to infection and exhibit similar clinical symptoms (abortion, lethargy, inappetence) (Swanepoel 1994). However, they appear to be more refractory to severe disease than sheep (Bird et al. 2009). A mortality rate of approximately 48% in kids was reported during an epizootic in Mauritania (Nabeth et al. 2001). Apart from a lower mortality (10% to 70% in calves, and 5% to 10% in adults), the characteristics of severe RVFV infections in cattle are similar to those in sheep (Bird et al. 2009; Nabeth et al. 2001). The pathogenicity in camels has not yet been sufficiently investigated (Abdallah et al. 2015). Besides from abortions in pregnant camels, an infection normally appears to stay clinically inapparent (Abdallah et al. 2015). A single study on the Mauritanian outbreak in 2010 reported hemorrhagic septicemia and severe respiratory distress among camels (El Mamy et al. 2014). Although, camels are rather resistant to RVFV, their importance in transmission of the virus may not be neglected. Epidemiological studies on the Egyptian outbreak in 1977 suggested the importation of viremic animals, especially camels, as the main source of infection (Ab d El-Rahim et al. 1999). In humans, infection with RVFV is usually asymptomatic or cause an influenza-like syndrome. However, patients may develop a more severe disease including hepatitis, retinitis, encephalitis, and hemorrhagic disease with fatal outcome in 1-2% of infections (Pepin et al. 2010).

CCHFV infection in susceptible animals such as cattle, sheep, scrub hares and ostriches remain clinically inapparent (Bente et al. 2013). In humans, infection with CCHFV usually causes only a nonspecific disease, but can lead to fatal hemorrhagic fever with mortality rates ranging from 2 to 30% (Weidmann et al. 2016). Mortality rates of nosocomial infections are often much higher than those acquired naturally through tick bites and may be related to the level of viremia (Shayan et al. 2015). Typically, CCHFV infection undergoes four distinct phases: incubation, prehemorrhagic, hemorrhagic, and convalescent (Hoogstraal 1979). The incubation period depends on the route of exposure. When infected by tick bite the period lasts from one to five days, whereas it lasts from five to seven days following a transmission via infected tissue or blood (Ergönül 2006). Hereby, substances in tick saliva might influence the acceleration of viral dissemination (Kocáková et al. 1999; Jones et al. 1992). The prehemorrhagic phase is characterized by nonspecific symptoms. Petechial rash of skin, conjunctiva and other mucous membranes, and bleeding from the gastrointestinal and urinary tracts initiate the hemorrhagic phase (Swanepoel et al. 1989). In fatal cases, death occurs due to hemorrhage, multi-organ failure and shock (Bente et al.

2013). The hemostatic failure and skin rash is caused by endothelial infections which have an important role in CCHF pathogenesis (Schnittler and Feldmann 2003). The endothelial damage stimulates platelet aggregation and degranulation and consequently activates the intrinsic coagulation cascade (Ergönül 2006). Further on, activation of coagulation may contribute to the development of disseminated intravascular coagulation (DIC) and multiorgan failure (Shayan et al. 2015). For surviving patients, recovery begins approximately 15 to 20 days after the onset of illness and can take as long as one year (Ergönül 2006). During convalescence the patients might experience weaknesses, headache, dizziness, loss of hair, poor appetite and vision, and memory loss (Swanepoel et al. 1989). However, no such complications have been described in more recent articles from Turkey and Iran (Bakir et al. 2005; Mardani and Kreshtkar-Jahroumi 2007).

2.6 Diagnostics

Diagnostic tests for *Orthobunyaviruses* in general are limited (Dutuze et al. 2018). Detection of nucleic acids by polymerase chain reaction (PCR) allows the differentiation between BATV, NRIV, and BUNV, but multiple genes are required and possible co-infections may falsify the result or on the contrary stay undetected (Dutuze et al. 2020). Serological assays like complement fixation, plaque reduction neutralization and hemagglutination inhibition assays were used to group the *Orthobunyaviruses* into serogroups (Elliott 1969; Hunt and Calisher 1979a). Hence, these tests are capable to distinguish between viruses of different complexes, but cross-reactivity prevents differentiation within serogroups (Hunt and Calisher 1979a). In addition, virus neutralization tests (VNT) and immunofluorescence assays (IFA) were performed for BATV prevalence studies (Medlock et al. 2007; Lambert et al. 2014; Hofmann et al. 2015; Ziegler et al. 2018) which are sensitive methods for antibody detection, but their ability to definitively identify BATV as the viral agent has not yet been assessed.

Laboratory diagnosis of RVFV ideally consists of a combination of serological and molecular assays (Mansfield et al. 2015). For reporting of RVFV in animals, the OIE requires laboratory confirmation by at least two positive results either for virus/viral RNA and antibodies or for IgM and IgG with increase of antibody titer in paired serum samples (OIE 2019). Nucleic acid based molecular tests allow a rapid detection of viral RNA during the viremic phase. A wide range of sensitive assays are available including quantitative realtime PCR (Bird et al. 2007; Drosten et al. 2002; Garcia et al. 2001), multiplex PCR-based macroarray assay (Venter et al. 2014), nested RT-PCR methods (Sall et al. 2002), RT Loop-mediated isothermal amplification (RT-LAMP) (Le Roux et al. 2009), and recombinase polymerase amplification (RPA) (Euler et al. 2012). Commonly used serological assays are the serum neutralization test (SNT) and the

enzyme-linked immunosorbent assay (ELISA). The highly sensitive SNT is considered the gold standard assay, but requires live virus and therefore can only be performed in high biosecurity facilities (OIE 2019). However, a novel SNT that is based on an avirulent RVF strain can be performed safely outside biosafety level 3 (BSL-3) facilities (Wichgers Schreur et al. 2017). A great variety of ELISAs have been developed using either whole cell lysate derived from infected cells or non-infectious recombinant protein as antigen (Mansfield et al. 2015). The ELISAs are capable of detecting either IgM antibodies indicative for recent infections (Rissmann et al. 2017a) and/or IgG antibodies which may be detectable for several years (van Jansen Vuren et al. 2007; Paweska et al. 2007; Jäckel et al. 2013a). Additionally, the immunofluorescence assay (IFA) can be performed for detection of specific RVFV antibodies in a wide range of host species (Mroz et al. 2017a; Rissmann et al. 2017a). Alternative techniques such as agar gel immunodiffusion (AGID), radioimmunoassay, complement fixation, and hemagglutination inhibition assay (HIA) are possible, but no longer used (OIE 2019).

Virus isolation was long considered the “gold standard” for CCHFV diagnostic (Bente et al. 2013). However, CCHFV is highly pathogenic for humans, and therefore must be handled in BSL-4 containment (Shayan et al. 2015), which is not available in CCHFV endemic countries, except of South Africa (Bente et al. 2013). Alternative diagnostics such as real-time RT-PCR are difficult to develop due to high genetic variability among virus strains (Shayan et al. 2015). This issue has been resolved by designing primers that target a highly conserved regions in the S segment (Garrison et al. 2007). Hence, RT-PCR should now be considered the method of choice for rapid, sensitive, and specific laboratory diagnostic (Drosten et al. 2003). Viral RNA is detectable during the viremic phase in the first 7-10 days of illness. Detection of IgM antibodies is possible from day seven after the onset of disease, shortly thereafter as well the detection of IgG antibodies (Shepherd et al. 1989). The level of IgM specific antibodies will decline within four months post-infection, whereas IgG remains detectable for at least 5 years (Ergönül 2006). Thus, acutely ill patient should be tested for both viral RNA and IgM, if both tests are available. Nevertheless, RT PCR remains the most reliable method, since an antibody response might fail to develop in severely ill patients (Bente et al. 2013). Moreover, the viral load might give an indication of the course and severity of the disease (Bente et al. 2013). In serology, a variety of assays is based on the recombinant N of CCHFV which is recognized as the predominant antigen that induces a high immune response in most bunyavirus infection (Shayan et al. 2015). Currently available assays and commercial kits are listed in Mertens et al. (2013).

2.7 Vaccination and treatment

No vaccines or specific antiviral agents are available against *Orthobunyaviruses*, since there has been little interest in research on antiviral drugs or vaccines against human *Orthobunyaviruses* due to a perceived limited market (Elliott 2014).
Currently, there are four available veterinary RVFV vaccines to protect ruminant populations: inactivated virus vaccines, the Smithburn live-attenuated virus vaccine, the Clone-13 live-attenuated virus vaccine, and the MP-12 live-attenuated virus vaccine (Mansfield et al. 2015). Formalin-inactivated vaccines are considered safe, but have the disadvantage to require three initial inoculations and annual booster immunizations (Harrington et al. 1980; Barnard 1979; Barnard and Botha 1977). Thus, inactivated vaccines are impractical for livestock use in RVFV endemic areas (Bird et al. 2009). However, for human use, a formalin-inactivated vaccine (TSI-GSD-200) was used in the mid-1970s to protect laboratory personnel from accidental exposure (Eddy et al. 1981). TSI-GSD-200 was proven safe and provided good long-term immunity, but is no longer produced and in limited supply (Pittman et al. 1999; Bird et al. 2009). The live-attenuated Smithburn vaccine provides protection against RVFV by single inoculation, and therefore became the preferred vaccination strategy of livestock throughout Africa since 1950 (Bird et al. 2009). However, it is reported to cause abortions and teratologic effects (Botros et al. 2006), and has the potential for reversion to virulence (Ahmed Kamal 2011). The Clone-13 is a naturally attenuated strain containing a large deletion in the main virulence factor, the NSs (Muller et al. 1995). No abortions has been observed in experimental vaccine trials (Dungu et al. 2010), and the risk of reversion is considered unlikely (OIE 2019). Another vaccine was generated by passaging a human RVFV strain in the presence of chemical mutagens (Caplen et al. 1985). The resulting virus, designated as MP-12, was reported to cause minimal teratogenic effects among ruminants in vaccination trials (Morrill et al. 1997; Hunter et al. 2002). More recently, vaccine candidates have been developed by use of viral subunits, recombinant virus vectors, viral cDNA, or recombinant live-attenuated RVFV containing deletions of virulence genes (Bird et al. 2009). These candidates may exhibit various advantages such as the ability to differentiate infected from vaccinated animals (DIVA), or the suitability for field use due to their thermostabilized form (Hartman 2017). There is no specific treatment available for RVFV infection other than supportive care (Bird et al. 2009). The antiviral agent ribavirin was administered to patients during the 2000 outbreak in Saudi Arabia due to promising in-vitro experiments and its limited in-vivo efficacy against other hemorrhagic fever viruses (Hartman 2017). However, the therapy was quickly stopped when it was correlated with an increased likelihood of neurological disease (Hartman 2017). Recombinant human interferon-γ can prevent severe disease in Rhesus macaques infected prior to infection, but its efficacy has not yet been evaluated in humans or other susceptible

hosts (Bird et al. 2009). Additionally, the broad-spectrum Favipiravir showed promising efficacy in a highly lethal rat model (Caroline et al. 2014).

Supportive therapy including administration of thrombocytes, fresh frozen plasma, and erythrocyte preparations is most essential for treatment of CCHFV infected patients who develop hypotension and hemorrhage (Ergönül 2006). Potential antiviral drugs were screened for their activity against CCHFV which revealed that ribavirin inhibited viral replication, whereas ribamidine and three other drugs (6-azauridine, selenazofurin, and tiazofurin) showed less or no significant antiviral activity (Paragas et al. 2004). Moreover, ribavirin inhibited viral replication in newborn mice (Tignor and Hanham 1993) and STAT-1 KO mice (Bente et al. 2010). Observational and experimental studies in the past decade generally stated a beneficial effect of ribavirin therapy when initiated in early phase (Mardani et al. 2003; Ergönül et al. 2004; Alavi-Naini et al. 2006). However, a systematic review of 21 of these studies concluded that the current data are compatible with an effect but are heavily confounded (Soares-Weiser et al. 2010). At least, no serious adverse effects that required the discontinuation of therapy have been observed (Soares-Weiser et al. 2010). An alternative attempt for CCHFV treatment with immune globulin is used in Bulgaria, though its efficacy has still not been confirmed in a randomized clinical trial (Christova et al. 2009; Papa et al. 2004; Vassilenko et al. 1990). As well, a study in Turkey described clinical improvement after hyperimmune globulin therapy, but here a control group was missing for comparison (Kubar et al. 2011). Finally, the newly identified molecule MxA, which is a member of the interferon-induced GTPases, was shown to inhibit CCHFV replication in vitro, but its in vivo efficacy remains to be demonstrated (Andersson et al. 2004). The first vaccine against CCHFV was developed in 1970 in the Soviet Union (Tkachenko et al. 1970). Though, the formalin-inactivated mouse-brain vaccine was demonstrated to induce neutralizing antibodies, its protective efficacy was not assessed (Tkachenko et al. 1971). In Bulgaria a similar vaccine is in use to protect high-risk groups from infection (Papa et al. 2004). However, its protective efficacy is only based on the decreased incidence of CCHFV in the country since beginning of the vaccination programme (Todorov et al. 2001). The vaccine was demonstrated to elicit cellular and humoral responses, but only low levels of neutralizing antibodies (Mousavi-Jazi et al. 2012). A more recently developed multivalent DNA vaccine against RVF, tick-borne encephalitis, Hantaan and CCHF viruses failed to induce an immune response in mice (Spik et al. 2006). In contrast, mice orally immunized with tobacco plants expressing the CCHFV Gn and Gc developed antibodies to the virus, but further assessments are necessary (Ghiasi et al. 2011).

3 Materials and Methods

The following section provides an overview of materials and methods used in this work. Detailed descriptions are found in the corresponding sections of manuscripts I-III.

3.1 Serological assays

In manuscripts I and II a commercial competition ELISA from ID Vet based on the nucleoprotein Np (ID Vet, Montpellier, France) was used for detection of RVFV specific antibodies. To detect recent infection, samples were further tested in a commercial IgM capture ELISA (ID Vet, Montpellier, France) in manuscripts I and II. As the ID Vet IgM capture ELISA is not established and evaluated for analysing camel sera, an indirect IgM in-house ELISA for camelids was deployed in manuscript II (Rissmann et al. 2017a). Positive and inconclusive results were verified by serum neutralization test (SNT) using the RVFV MP-12 strain (OIE 2020) in manuscripts I and II. Additional confirmation was performed with an adapted commercial immunofluorescence assay (IIFA) (Euroimmun, Lübeck, Germany) (Jäckel et al. 2013b). The serological investigation for CCHFV-specific antibodies in manuscript II consisted of a commercial double-antigen sandwich ELISA (ID Vet, Montpellier, France), as well as a modified commercial CCHF IgM/IgG IFA (Euroimmun, Lübeck, Germany) as conformational test (Mertens et al. 2015). In manuscript I and partially in manuscript III, indirect ELISAs based on the glycoprotein Gc of each Ngari virus (NRIV), Batai virus (BATV), and Bunyamwera virus (BUNV) were established in comparison to serum neutralization tests (SNT) using homologous virus strains.

3.2 PCR

In manuscript I, for the detection of RVFV RNA a RT-qPCR was used along with the Quanti Tect® Probe RT-PCR Kit (Qiagen, Hilden, Germany). The qRT-PCR for NRIV, BATV, and BUNV in manuscript I was carried out according to an adapted multiplex qRT-PCR protocol (Wernike et al. 2015) using the AgPath-ID™ One-Step RT-PCR Kit (Applied Biosystems, Foster City, USA).The qRT-PCR for RVFV in manuscript II was carried out according to an adapted multiplex qRT-PCR protocol (Wernike et al. 2015) using the AgPath-ID™ One-Step RT-PCR Kit (Applied Biosystems, Foster City, USA). The applied PCR protocol allows the simultaneous detection of RVFV, Foot and Mouth Disease Virus (FMDV) and NRIV. A second multiplex real-time RT-PCR was performed which simultaneously detect all six genotypes of CCHFV (Sas et al. 2018b)

using the QuantiTect Probe RT-PCR Kit (Qiagen, Hilden, Germany). In manuscript III, BATV genome was detected using the Quanti Tect® Probe RT-PCR Kit (Qiagen, Hilden, Germany) (Jöst et al. 2011).

3.3 Extraction and purification kits

In manuscripts I and II, RNA was obtained for subsequent PCR from serum samples using the NucleoMag® VET Kit (MACHEREY-NAGEL, Düren, Germany). In manuscript III, RNA isolation was performed using TRIzol LS Reagent (Life Technologies, Carlsbad, USA) and Viral RNA Mini Kit (Qiagen, Hilde, Germany) in combination.

3.4 Sequencing

In manuscript I, viral RNA was extracted from cell culture and subjected to a next-generation sequencing (NGS) workflow (Wylezich et al. 2018) using 150-cycle NextSeq550 Reagent Kits v2.5 (Illumina, San Diego, CA) on a NextSeq550 platform (Illumina, San Diego, CA) or the Ion Torrent S5 chemistry (ThermoFisher Scientific, Waltham, MA, USA) on an Ion Torrent S5 XL platform (ThermoFisher Scientific, Waltham, MA, USA).

3.5 Protein expression

In manuscripts I and III, synthetic genes were produced and cloned into E. coli expression vector pET21 by Eurofins Genomics (Ebersberg, Germany). Expression of the recombinant glycoproteins Gc of each NRIV, BATV and BUNV, and purification by Nickel chelating agarose were carried out under denaturing conditions (Jäckel et al., 2013).

4 Manuscript I

Cocirculation of Orthobunyaviruses and Rift Valley fever virus in Mauritania, 2015

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4.1 Abstract

Ngari virus (NRIV) has mostly been detected during concurrent outbreaks of Rift Valley fever virus (RVFV). Both are zoonotic hemorrhagic fever viruses displaying the same clinical picture in humans and small ruminants. To investigate whether NRIV and its parental viruses, Bunyamwera virus (BUNV) and Batai virus (BATV), played a role during the Mauritanian RVFV outbreak in 2015/16, we analyzed serum samples from sheep and goats from central and southern regions of Mauritania by qRT-PCR, serum neutralization test and ELISA. 41 of 458 samples exhibited neutralizing activity against NRIV, nine against BATV and three against BUNV. In addition, complete virus genomes from BUNV from two sheep as well as two NRIV isolates from a goat and one sheep were obtained. No RVFV derived viral RNA was detected, but 81 seropositive animals including 22 IgM positive individuals were found. Of these specimens, 61 samples showed antibodies against RVFV and at least against one of the three Orthobunyaviruses. An indirect ELISA based on NRIV/BATV and BUNV derived Gc protein was established as a complement to SNT which showed high performance in terms of NRIV but reduced sensitivity and specificity in terms of BATV and BUNV. Moreover, we observed high serological cross-reactivity between NRIV and BATV in all assays. Taken together, the data indicate the cocirculation of at least

BUNV and NRIV in the Mauritanian sheep and goat population, which is exposed to RVFV.

4.2 Introduction

Ngari virus (NRIV), Bunyamwera virus (BUNV) and Batai virus (BATV) are members of the Bunyamwera serogroup in the genus Orthobunyavirus of the family Peribunyaviridae. They are characterized by a tri-segmented (S, M and L segments) enveloped negative-strand RNA genome. The S segment encodes the N and NSs proteins, the M segment encodes the two glycoproteins Gn and Gc and the NSm protein, and the L segment codes for the RNA-dependent RNA-polymerase (Elliott 2014). Sequence analysis showed that NRIV is a natural reassortant resulting from co-infection of BUNV and BATV, as NRIV possesses the M segment of BATV combined with the S and L segments from BUNV (Briese et al. 2006). This reassortment probably led to an increased virulence, which is associated with hemorrhagic fever in humans and ruminants (Dutuze et al. 2018). In contrast, infection with BATV or BUNV is reported to cause only mild flu-like disease in humans (Dutuze et al. 2018). Susceptible vertebrate hosts for BUNV and BATV are ruminants, horses and birds (Hubálek 2008). The BATV strain Chittoor in India caused mild unspecific disease in sheep and goats (Singh and Pavri 1966), whereas in Europe no disease association in ruminants was described yet (Ziegler et al. 2018). Nevertheless, a German captive harbour seal which died from encephalitis was tested positive for BATV infection (Jo et al. 2018). In North America the BUNV strain Cache Valley virus (CVV) is associated with congenital abnormalities in sheep and other ruminants (Chung et al. 1990; Edwards 1994). Moreover, in Argentina BUNV was determined as causative agent for fatal encephalitis and abortion in horses (Tauro et al. 2015).

So far, NRIV was isolated only from sub-Saharan Africa, whereas BATV is found almost worldwide. Its distribution ranges from Malaysia towards Asian Russia and India and in Europe from Scandinavia towards Italy and Romania (Dutuze et al. 2018). In Africa, the virus was described as Ilesha virus in Sudan, Cameroon, Nigeria, Uganda, and Central Africa (Dutuze et al. 2018; Hubálek 2008) and most recently in Ruanda (Dutuze et al. 2020). BUNV was primarily isolated in several sub-Saharan African countries, such as Uganda, Tanzania, Mozambique, Nigeria, Guinea, South Africa, Democratic Republic of Congo, Senegal, Guinea, Ivory Coast, Nigeria, Cameroon, Central African Republic, Kenya, Uganda, South Africa, Madagascar, and Ruanda (Dutuze et al. 2018; Dutuze et al. 2020). Moreover, strains of BUNV have been discovered in North America towards Mexico and Argentina (Dutuze et al. 2018). However, the distribution of these viruses might be underestimated, since diagnostic capabilities available for Orthobunyaviruses in general are limited and the diagnostic approach was primarily based on clinical presentation (Dutuze et al. 2018). Hence,

three outbreaks of NRIV were first mistaken for more common etiologies of hemorrhagic fever, but afterwards identified as NRIV outbreak. This happened in Sudan in 1988, when an outbreak of hemorrhagic fever was clinically diagnosed as malaria, but afterwards two isolates of NRIV were found and IgM antibodies against NRIV were detected in 7% of patients (Briese et al. 2006). Likewise, NRIV has been isolated twice during concurrent Rift Valley fever virus (RVFV) outbreaks, in Kenya and Somalia in 1997-1998 and in Mauritania in 2010 (Bowen et al. 2001; Eiden et al.). RVFV, a member of the Bunyaviridae family in the Phlebovirus genus, is a zoonotic mosquito-borne virus of major concern in entire Africa (from Egypt towards South Africa) and could even emerge in the Arabian Peninsula in 2000 (Linthicum et al. 2016). Clinical symptoms of an infection with RVFV, closely resemble the symptoms caused by NRIV infection: in mild cases human patients show flu-like febrile illnesses, whereas in 1-2% of the cases the infection can develop to severe hemorrhagic fever, encephalitis, and retinitis (Chevalier 2013). RVFV and NRIV have not only similarities in their clinical manifestations, but seem to share also the same life cycle, i.e. use similar arthropod vectors and vertebrate hosts (Dutuze et al. 2018). Outbreaks of RVFV characteristically coincide with episodes of unusually heavy rains and extensive flooding in arid areas, e.g. as observed in Sudan in 1988 and in Somalia/Kenya in 1997-1998 during the NRIV outbreaks (Briese et al. 2006). Heavy rainfall leading to subsequent flooding induce mass hatching of infected Aedes eggs and to a Culex species propagation, all of which then infect humans and ruminants (Linthicum et al. 2016). Likewise, NRIV, BATV and BUNV are transmitted by mosquitoes (Dutuze et al. 2018). For transmission of BUNV *Aedes aegypti* might be the primary vector (Odhiambo et al. 2014). Additionally, in Argentina BUNV was isolated from *Ochloretatus scapularis* (Tauro et al. 2015). Infection studies revealed that *Anopheles gambiae* Giles is a competent vector for both, BUNV and NRIV (Odhiambo et al. 2014). Furthermore, NRIV was isolated from various mosquito species in Senegal such as *Aedes argentepunctatus, Ae. minutus, Ae. vexans, Ae. mcintoshi, Ae. simpsoni, Ae. vittatus, Ae. neoafricanus, Anopheles coustani, An. pretoriensis, An. pharoensis, An. mascarensis, Culex bitaeniorhynchus, C. tritaeniorhynchus, C. antennatus*, and *C. poicilipes* (Zeller et al. 1996). European mosquito species transmitting BATV are *Anopheles maculipennis s.l, An. claviger, Coquillettidia richiardii, Culex pipiens, Ochlerotatus punctor, Oc. communis*, and *Aedes vexans* (Hubálek 2008).

Mauritania experienced the most recent RVFV outbreak in 2015/16, affecting sheep and goats (OIE) as well as humans (Boushab et al. 2016). To investigate the prevalence of RVFV and possible coinfection with NRIV, BATV, and BUNV in the small ruminant population, 492 serum samples of goats and sheep were collected in 2015 in southwestern Mauritania and analyzed by qRT-PCR and serum neutralization tests (SNT) specific for each virus, as well as by a commercial RVFV enzyme-linked immunosorbent assays (ELISA). Additionally, we implemented ELISAs based on the glycoprotein Gc of each NRIV, BATV, and BUNV.

4.3 Materials and methods

4.3.1 Sample collection and workflow

A total number of 492 apparently healthy sheep and goats were sampled in eight different governorates of Mauritania (Inchiri, Chargui, Tagant, Assaba, Trarza, Guidimaka, Brakna, Gharbi) in 2015 (Table 1). Serum samples were separated and kept frozen at -20°C for further investigations.
After transportation to Germany, all samples were first tested in a quantitative RT-PCR detecting NRIV, BUNV and BATV genome simultaneously, and a second quantitative RT-PCR detecting RVF genome. For serological testing, the sera were inactivated with phosphate buffered saline and 0.1% Tween-20 (PBST) (1:1) and incubated at 56°C for 30 minutes. To detect antibodies against NRIV, BATV, and BUNV, the samples were tested in serum neutralization tests (SNT) using homologous virus strains. Additionally, indirect ELISAs based on the glycoprotein Gc of each virus were established to detect Gc specific antibodies. The serological investigation for RVFV-specific antibodies consisted of a commercial competition ELISA (ID Vet, Montpellier, France) using the nucleoprotein (NP) as antigen. To detect recent infections, samples showing positive and inconclusive results in the ELISAs, were further tested with the RVF ID Vet IgM capture ELISA (ID Vet, Montpellier, France). ELISA positive sera were confirmed by serum neutralization test which is generally accepted as gold standard in serological diagnostic of RVFV (Rissmann et al. 2017b). In case of divergent results in the ELISAs and SNT, the final assessment was achieved by an adapted commercial immunofluorescence assay (IIFA) (Euroimmun, Lübeck, Germany).

4.3.2 Real-time RT-PCR (qRT-PCR)

RNA isolation was performed using the Nucleo®Mag VET Kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. The samples were processed in a pool of five samples. As internal extraction control, a MS2 Bacteriophage was added to each serum pool before the extraction process based on the primers MS2F (5`-CTC TGA GAG CGG CTC TAT TGG T-3´) and MS2R (5`-GTT CCC TAC AAC GAG CCT AAA TTC-3´) and MS2P (HEX-TCAGACACGCGGTCCGCTATAACGA-BHQ1) (Ninove et al. 2011). The qRT-PCR for RVFV was carried out according to an adapted multiplex qRT-PCR protocol (Wernike et al. 2015) using the AgPath-ID™ One-Step RT-PCR Kit (Applied Biosystems, Foster City, USA). The protocol allows the simultaneous detection of RVFV, Foot and Mouth Disease Virus (FMDV) and Ngari/Bunyamwera virus (NRIV/BUNV) and uses the following primers: RVF-forw (5`-TGA AAA TTC CTG AGA CAC ATG G-3´) , RVF-rev (5`-CTT CCT TGC ATC ATC TGA TG-3´) and RVFV probe

(FAM-CAATGTAAGGGGCCTGTGTGGACTTGTG-BHQ1) for RVFV, FMD-IRES-4.1F (5`-TAA CAW GGA CCC RCS GGG CC-3´), FMD-IRES-4R (5`-TGA AGG GCA TCC TTA GCC TG-3´) and FMD-IRES probe (Texas Red-CAT GTG TGC AAY CCC AGC ACR G-BHQ2) for FMDV. For detection of NRIV/ BUNV S segment novel primers/probes were used: Bunyam_F (5`-GCT GGA AGA TTA CTG TAT ATA ATA C-3´), Bunyam_R (5`-CAA GGA ATC CAC TGA GGC GGT G-3´) and Bunyam_P (HEX-AAC AAC CCA GTT CCT GAC GAT GGT C-BHQ2). The final concentrations were 0,4µM (primer) and 0,08µM (probe) correspondingly using the AgPath-ID™ One-Step RT-PCR Kit (Applied Biosystems, Foster City, USA). For each reaction 2.5 µl of RNA, 5 pmol of both forward and reverse primer and 0.625 pmol of the probe were used in a total volume of 12.5 µl. PCR reaction condition was used as follows: 48 °C for 10 min, 95 °C for 10 min and 44 cycles at 95 °C for 15 s, and 60 °C for 45 s. Finally, a qRT-PCR assay targeting the BATV S segment (Jöst et al. 2011) was performed.

4.3.3 Sequencing

VeroE6 cells were inoculated with PCR positive serum samples and assayed for virus replication and cytopathic effects. The extracted viral RNA was subjected to a next-generation sequencing (NGS) workflow (Wylezich et al. 2018). They were normalized, sampled and sequenced using 150-cycle NextSeq550 Reagent Kits v2.5 (Illumina, San Diego, CA) on a NextSeq550 platform (Illumina, San Diego, CA) or the Ion Torrent S5 chemistry (ThermoFisher Scientific, Waltham, MA, USA) on an Ion Torrent S5 XL platform (ThermoFisher Scientific, Waltham, MA, USA). The obtained full-length recovered genome sequences of BUNV (accession no. MT731755 - MT731757) and NRIV (accession Nr. MT747972 - MT747974 and MT747975 - MT747977) were submitted to GenBank.

4.3.4 Serum neutralization test

The SNT for detecting BATV neutralizing antibodies was performed by using BATV strain 53.2 (acc. no. HQ455790, kindly provided by J. Schmidt-Chanasit, BNITM Hamburg, Germany) as already described by Seidowski et al. (2010) and Ziegler et al. (2015) Modifications were made by using Vero E6 cells (Collection of Cell Lines in Veterinary Medicine, Friedrich-Loeffler-Institut, Germany) and applying an incubation time of 6 days. Similarly, the SNT for detecting NRIV and BUNV neutralizing antibodies were performed by using a Mauritanian NRIV strain (accession nos. KJ716848–716850) and BUNV ATCC® VR87™ (kindly provided by J. Schmidt-Chanasit, BNITM Hamburg, Germany). Briefly, virus concentration of 100 $TCID_{50}$/well was added to each sample running in duplicate at a starting serum dilution of 1:10. Cytopathic effects were

seen 4-6 days post infection. The SNT for detecting RVFV neutralizing antibodies was performed as described in the OIE Terrestrial Manual 2014 (OIE, 2008). Briefly, 100 TCID50 of RVFV (MP-12 vaccine strain) were added to serial two-fold diluted sera and Vero 76 cells (Collection of Cell Lines in Veterinary Medicine, Friedrich-Loeffler-Institut, Germany) were added in a dilution of $3x10^5$ to each well. Plates were incubated at 37°C, 5% CO_2 for 6 days. The neutralizing antibody titer of the samples was defined as the 50% neutralization dose (ND_{50}). Positive result for neutralizing antibodies was confirmed when the ND_{50} were 10 or higher.

4.3.5 Recombinant glycoprotein Gc

Synthetic genes encompassing the domains GI and GII of the glycoprotein Gc were produced by Eurofins based on a partial BATV sequence (accession Nr. HQ455791, nucelotide position 601-1650), the corresponding NRIV sequence (accession Nr. KJ716849, position 1552-2505) and on BUNV sequence (accession Nr. M11852, position 1479-2534). The corresponding proteins sequences are aligned in Fig S1. All three sequences were optimized for expression in E. coli. The sequence codes for putative domains I and II of glycoprotein Gc and were cloned into E. coli expression vector pET21 a using 5' BamHI and 3' XhoI restrictions sides and expressed in BL21-Lys cells. Expression of the recombinant proteins and purification by Nickel chelating agarose were carried out under denaturing conditions as described previously (Jäckel et al., 2013). Finally, the proteins were dialyzed against 0.05 M carbonate-bicarbonate buffer pH 9.6 and checked in SDS-PAGE and Coomassie-staining.

4.3.6 Indirect (NRIV/BATV/BUNV) Gc ELISA

For serological testing by the ELISAs the serum samples were inactivated with PBST (1:1) at 56°C for 30 minutes. The three indirect inhouse ELISAs are based on a partial recombinant glycoprotein Gc of either NRIV or BATV or BUNV which were used for coating immunoplates in a dilution of 2µg/ml in 0.05 M carbonate-bicarbonate buffer pH 9.6 (100 µl diluted antigen per well). Protocol parameters, dilutions, optimal reagent concentrations and the selection of immunoplates were determined by standard checkerboard titration and choosing the combination with the highest difference in OD value between positive and negative control. After incubation of the coated immunoplates at 4 °C overnight, plates were washed three times with 300 µl washing buffer containing phosphate-buffered saline (PBS) pH 7.2 and 0.1% Tween 20. After blocking with 200 µl/well 10% skim milk powder (DIFCO™) diluted in PBS for 1 h at 37 °C in a moist chamber, ruminant field sera diluted 1:20 for NRIV ELISA and 1:10 for BATV and BUNV ELISA in PBS containing 2% skim milk were added in duplicate to

the plates. As positive control, polyclonal hyperimmune sheep serum were diluted 1:20 for NRIV ELISA and 1:10 for BATV and BUNV ELISA, respectively. A volume of 100 µl of each sample and control was added to the plates. After incubation at 37 °C for 1 h in a moist chamber plates were again washed three times with washing buffer. A volume of 100 µl per well of horseradish peroxidase (HRPO) conjugated Protein G (Calbiochem) diluted 1:5000 in dilution buffer was then added to the plates and incubated again for 1h as described before. After a final washing step, 100 µl per well of 2,20-azinodiethylbenzothiazoline sulfonic acid substrate (ABTS, Roche, Mannheim, Germany) was added and plates were incubated for 30 min at room temperature in the dark. The reaction was stopped by adding 1% sodium dodecyl sulphate (SDS) and the optical density (OD value) was determined at 405 nm. In case of BUNV ELISA, blocking milk and dilution buffer of ID Vet were used following the same protocol as described above. The results were expressed as percentage of the positive control serum (PP value) using the following formula: (mean OD of duplicate test serum/median OD of duplicate positive control) *100. Cut-off value, sensitivity and specificity of the indirect ELISAs were determined in correlation to the corresponding SNT results using the receiver operating characteristic analysis (ROC analysis) with regard to the criterion ‘‘Maximization of sensitivity and specificity’’. Calculations were performed using the program ‘‘R’’. Finally, to determine the accordance between the SNTs and the ELISAs among each other, the kappa coefficient was calculated using the program ‘‘R’’.

4.3.7 ID Vet RVF competition ELISA and ID Vet RVF IgM capture ELISA

All samples were analyzed in the commercial ID Screen® RVFV competition multispecies ELISA (ID Vet, Montpellier, France) according to manufacturer’s instructions. Both IgG and IgM antibodies are detected indistinguishably. Samples with a percentage of inhibition lower than 40% were defined as positive. Percentage inhibition values between 40% and 50% were considered as inconclusive and those higher than 50% as negative. Samples that gave positive or inconclusive results in the competition ELISA, were further tested with the ID Screen® Rift Valley Fever IgM capture ELISA (ID Vet, Montpellier, France) according to the manufacturer’s description. Samples with a sample-to-positive-ratio higher than 50% were defined as positive. Sample-to-positive-ratios between 40% and 50% were considered as inconclusive and those lower than 40% as negative.

4.3.8 Indirect immunofluorescence

Samples that gave divergent results in ELISAs and SNT, were further analyzed with a RVF in-house immunofluorescence assay (IIFA) according to a previously published protocol (Jäckel et al. 2013b) using the commercial RVFV immunofluorescence slides from Euroimmun (Lübeck, Germany). The detection of antibodies was realized with species-specific Cy3 labelled secondary antibodies (donkey anti-sheep, donkey anti-goat) in a 1:200 dilution.

4.3.9 Statistical analysis

The estimated prevalence and 95% confidence intervals (95% CI) were calculated using the calculation tool of Epitools (https://epitools.ausvet.com.au/ciproportion).

4.4 Results

Among the 492 serum samples tested consecutively for NRIV, BUNV, BATV and RVFV by qRT-PCR assays, 10 samples were tested positive for BUNV/NRIV derived RNA. Subsequent sequencing revealed two NRIV isolates and two BUNV isolates (Table 2). Positive serum samples derived from one goat and three sheep from the governorates Trarza, Guidimaka and Brakna. No RVFV nor BATV genome was detected (Table 2).

From four animals, full length virus genome sequences of the S-, M-, and L-segment were recovered. Phylogenetic analysis of complete genomes revealed that two isolates clustered to Ngari virus and two isolates to the Bunyamwera virus group (Figure 6). NRIV was isolated from one goat of the Trarza region and from a sheep of the Guidimaka region. Both isolates show high similarity among each other with 4, 32 and 31 nucleotide differences corresponding to S-, M-, and L-segment. In addition, they exhibited high similarity to a NRIV isolate recovered from small ruminant of the Adrar region in 2010. Bunyamwera virus was isolated from two sheep of the Brakna region. The sequences were identical indicating the co-infection of two individuals from one flock with the same isolate. The strain showed highest similarity to isolates from Kenia (43 nucleotide differences), from Dakar (161 nucleotide differences) and the BUNV prototype (279 nucleotide differences) regarding S, M and L segment.

Due to low sample volume, only 458 out of 492 sera were comprehensively analyzed in the serological tests including SNT and indirect ELISA. Each sample was screened consecutively by a NRIV, BATV and BUNV SNT, correspondingly. Sera showing a ND_{50} higher than 1:30 were retested to determine the endpoint values.

Neutralizing antibodies to more than one virus in a sample were attributed to a specific infection if an individual ND_{50} was four times higher to one of the viruses compared to the other(s).

Thus, in 41 out of 458 samples NRIV specific antibodies were found which gave a total prevalence of 9.0% (Table 3). BATV specific antibodies were found in nine samples (prevalence of 2.0%), and BUNV specific antibodies in only three samples (prevalence of 0.7%). In 97 sera, similar antibody titers against NRIV and BATV were found (prevalence of 21.2%), whereas only one sample was positive for NRIV as well as BUNV antibodies and another one for BATV as well as BUNV antibodies each corresponding to a prevalence of 0.2%. Finally, 62 sera were tested positive in all three Orthobunyavirus SNTs leading to a prevalence of 13.5%. Interestingly, the NRIV positive samples showed the highest values with antibody titers of up to 1:2560. In BATV based SNTs only one sample reached a titer of 1:960. Regarding BUNV specific SNTs, the titer did not exceed 1:40, but mostly ranged between 1:10 and 1:20. The geographical distribution of seropositive samples is depicted in Figure 5.

To further characterize these sera, we tested them in a newly established glycoprotein Gc ELISA for NRIV, BATV and BUNV respectively. The resulting OD values were compared to the results of the homologous SNT, which is used as reference and gold standard. Goat and sheep samples were evaluated separately by ROC analysis to determine specificity and sensitivity (Figure 7).

The NRIV Gc based ELISA displayed a specificity of 93.8% and 77.6% for goats and sheep, respectively, and a corresponding sensitivity of 86% and 79.5%. BATV Gc based ELISA exhibited a specificity of 83.2% (goat) and 78.4% (sheep), and a sensitivity of 84.7% and 89.8%, respectively. In the case of the BUNV Gc based ELISA, significantly lower values for a specificity (72.9% and 59.8%) and sensitivity (48.7% and 73.3%) were found. By using the calculated cut-offs, the NRIV Gc based ELISA detected 13 positive samples, the BATV Gc based ELISA 24 seropositive specimens and the BUNV Gc based ELISA 37 samples. 92 specimens showed reactivity in all three ELISAs, whereas 87 samples were positive in the NRIV/BATV based ELISA only. 15 samples showed reactivity against NRIV and BUNV Gc and 14 samples reactivity against BUNV/BATV Gc.

The degree of accordance between the serological assays of the three viruses was calculated by the kappa coefficient. Between SNT specific for NRIV and SNT specific for BATV for both goat and sheep derived samples the coefficient revealed an almost perfect agreement ($\kappa = 0.90$, p-value < 2.2e-16, 95% CI 0.84 – 0.96, and $\kappa = 0.86$, p-value < 2.2e-16, 95% CI 0.80 – 0.93). Between NRIV Gc based ELISA and BATV Gc based ELISA (for goat and sheep) the kappa coefficient shows a substantial agreement ($\kappa = 0.73$, p-value < 2.2e-16, 95% CI 0.64 – 0.82, and $\kappa = 0.71$, p-value < 2.2e-16, 95% CI 0.62 – 0.80). In contrast, the comparison of BUNV Gc ELISA or specific SNT only show fair to moderate agreement with BATV and NRIV serological tests (data not shown).

Finally, all samples were analyzed for antibodies against RVFV with a commercial species independent ELISA which revealed 84 antibody positive samples. 81 of these sera were verified by SNT corresponding to a prevalence of 17.7% (Table 3). Highest antibody titers were found in the regions Tagant, Assaba, and Brakna (29.4%, 53.7%, and 42.9%, respectively). Since the competition ELISA does not distinguish between IgG and IgM antibodies, all seropositive samples were tested by the IDVet IgM capture ELISA. Hereby, 22 samples revealed RVFV IgM antibodies resulting in a prevalence of 4.8%, of which 16 samples were collected in Assaba alone. The distribution of RVFV IgM positive and NRIV and BUNV positive samples is shown in Figure 4.

4.5 Discussion

The cocirculation of RVFV and NRIV was described in Kenya and Somalia in 1997 - 1998 (Bowen et al. 2001) and in Mauritania in 2010 (Eiden et al.). In Kenya, 23% of the hemorrhagic fever cases were diagnosed as infected with RVFV and 27% as infected with NRIV (Bowen et al. 2001). In Mauritania, an outbreak of RVFV caused 63 human infections (El Mamy et al. 2011) and 57 infected small ruminants out of 93 tested animals (Jäckel et al. 2013b). Moreover, Dutuze et al. observed the cocirculation of RVFV with BUNV and BATV in ruminants in Ruanda (Dutuze et al. 2020). In Mauritania, the most recent outbreak of RVFV started in September of 2015 and was resolved in March 2016 (OIE 2020). In this temporarily and spatially limited outbreak, 31 human patients suffered from a variety of symptoms, amongst which were also hemorrhagic and neurological manifestations (Boushab et al. 2016). Furthermore, in three regions in southern Mauritania (Brakna, Tagant, and Assaba) a total of 19 infected sheep and goats were detected and stamped out as part of outbreak control (OIE 2020). In these three and additional five regions (Brakna, Tagant, Assaba, Inchiri, Chargui, Trarza, Guidimaka, and Gharbi), our project partners collected 492 samples of sheep and goats before and at the beginning of the outbreak in the year of 2015. The serum samples were investigated comprehensively for RVFV and the Orthobunyaviruses NRIV, BATV, and BUNV to reveal if these viruses cocirculate in Mauritania again.

Viral RVFV RNA was not detected, but 17.7% of the investigated animals showed IgG/IgM antibodies. In comparison, in 2012/13 during inter-epidemic phase a much lower prevalence of 3.8% in 497 investigated small ruminants was reported in Mauritania (Rissmann et al. 2017a). Thus, we clearly observed an increase of the overall RVFV antibody incidence in the small ruminant population in 2015. If the regions are viewed separately, the tested animals from Brakna, Tagant, and Assaba developed the highest antibody incidence (29.4%, 53.7%, and 42.9%, respectively). These were the three regions which were affected in the RVFV outbreak in 2015 (OIE 2020). The OIE reported the first RVFV positive case in Assaba in mid of October (OIE

2020). At the same time, we collected serum samples from 32 sheep of which 16 samples showed RVFV specific IgM antibodies underlining the circulation of the virus in Assaba. The goat-derived samples from Assaba were taken one month earlier and tested positive for IgM antibodies in only one case. Likewise, in the other investigated regions only few IgM positive animals were detected. Therefore, the overall prevalence for IgM antibodies was just 4.8%. As already mentioned, viral RVFV RNA was not detected, but the molecular analysis indicated the presence of NRIV RNA in ten samples collected in Brakna, Trarza, and Guidimaka. The subsequent sequencing revealed two positive samples for NRIV and two positive samples for BUNV from one goat and three sheep.

Both NRIV- and both BUNV-positive samples were negative for RVFV RNA as well as negative for RVFV specific antibodies. However, a total of 61 NRIV, BATV and/or BUNV seropositive samples also revealed antibodies against RVFV, indicating a co-circulation of these viruses in the animal population in Mauritania. Because the RVFV sequence is highly divergent to the deduced *Orthobunyavirus* sequences, cross-reactivity is highly unlikely but needs to be substantiated.

The serological investigation for *Orthobunyaviruses* detected NRIV-specific antibodies in 41 out of 458 tested samples corresponding to a prevalence of 9.0%, BATV-specific antibodies in nine samples lead to a prevalence of 2.0%, and BUNV-specific antibodies in only three sera (prevalence of 0.7%). However, the results need to be interpreted carefully, since for a large part of seropositive samples an unambiguous determination was not possible. In a total of 161 samples, the SNT of at least two viruses detected neutralizing antibodies. In most cases, the SNT based on NRIV and the SNT based on BATV showed undistinguishable high antibody titers (97 samples corresponding to a prevalence of 21.2%). A total of 62 samples were positive in all three SNTs (prevalence of 13.5%). Thereby, the antibody titer in the BUNV specific SNT did not exceed a titer of 1:40, but mostly ranged between 1:10 and 1:20. In contrast, in BATV and NRIV based SNTs the antibody titer reached up to 1:1280 or even 1:2560. Apparently, BUNV is less immunogenic and induces hardly neutralizing antibodies. At least, BUNV cross-reacts less with NRIV and BATV than the latter among themselves. A limited cross-reaction between BUNV and BATV was already described by Hunt and Calisher, who investigated the antigenic relationship of 23 strains of Bunyamwera serogroup viruses by plaque reduction neutralization test (Hunt and Calisher 1979). A further indication for a low neutralizing activity of BUNV is that twice as many samples are positive in the BUNV Gc based ELISA than in SNT based on BUNV (158 ELISA-positive and 67 SNT-positive samples). That also explains the low sensitivity and specificity of the ELISA calculated in correlation to the SNT (48.7% for sheep and 73.3% for goats, and 72.9% for sheep and 59.8% for goats, respectively). The low prevalence of BUNV seropositive animals determined by the SNT is surprising, since we proved the presence of BUNV in the animal population by isolating sequences from two sheep, and therefore expected to detect a stronger immune response in the investigated animals. As well

as the SNT, the ELISAs do not allow an unambiguous differentiation between NRIV, BATV, and BUNV for every sample. Of 458 samples, 87 samples were equally positive in the NRIV Gc based ELISA and the ELISA based on BATV Gc, whereas a total of 37 samples exhibited antibodies against only one of the viruses. The strong agreement of NRIV and BATV based SNTs and ELISAs is confirmed by the kappa coefficient, which for the SNT is $\kappa = 0.90$ (for goats) and $\kappa = 0.86$ (for sheep), and for the ELISA $\kappa = 0.73$ (for goats) and $\kappa = 0.71$ (for sheep). The high cross-reactivity among the serological tests impedes to determine whether only one of the viruses induced the antibody response or whether a coinfection existed. However, since no BATV RNA was isolated, but only NRIV and BUNV RNA, the antibodies that were detected by the BATV based SNT and ELISA were probably caused by an infection with NRIV. Neutralizing antibodies are induced by the surface glycoproteins which are encoded by the virus M segment (Briese et al. 2006). The M segment of NRIV closely resembles that of BATV, showing only 11% and 5% differences in nucleotide or deduced amino acid sequence, respectively. This limited divergence is mainly observed in the N-terminal portion of Gc around the conserved potential trypsin cleavage site (Briese et al. 2006).

Thus, the specific differentiation between NRIV and BATV antibodies by Gc ELISA or SNT is not possible, but the strong accordance among the assays offers the advantage to use the BATV SNT instead of the NRIV SNT and hereby enables to work in a lower biosafety containment facility.

Overall, we could demonstrate that NRIV and BUNV were cocirculating in Mauritania during the RVFV outbreak in 2015/16. Prevalence studies for NRIV, BATV and BUNV are complicated by high cross-reactivity, especially between NRIV and BATV serological assays. Future attempts might consider establishing tests that are based on the nucleoprotein Np to distinguish BATV and NRIV induced antibodies.

4.6 Tables and Figures

Table 1. Samples ordered by species and region.

Species	Region	N° of samples	Age	Date of sample collection
goat	Inchiri	50	1-5	February 2015
sheep		29	1-4	November 2015
goat	Chargui	41	1-10	July 2015
sheep		40	1-5	July 2015
sheep	Gharbi	81	1-7	August 2015
not reported		4	not reported	October 2015
goat	Tagant	54	1-3	September + October 2015
sheep		5	not reported	October 2015
goat	Assaba	28	1-6	September 2015
sheep		32	1-5	October 2015
goat	Trarza	12	1-4	October 2015
sheep		19	1-3	October 2015
goat	Guidimaka	40	1-4	September 2015
sheep		34	1-4	September 2015
goat	Brakna	9	3-6	September 2015
sheep		13	3-5	September 2015
not reported		1	not reported	September 2015

Table 2. Summary of results from multiplex qRT-PCR (detecting NRIV, BATV, and BUNV) and sequencing.

Sample ID	Species	Region	CT Multiplex (BUNV S)	CT Singleplex (BUNV S)	sequencing
MR 393/15 SR	goat	Trarza	no CT	34.69	NRIV
MR 410/15 SR	sheep	Guidimaka	32.33	32.04	
MR 411/15 SR	sheep	Guidimaka	26.96	26.72	NRIV
MR 471/15 SR	sheep	Brakna	28.86	28.82	
MR 478/15 SR	sheep	Brakna	27.19	26.85	BUNV
MR 479/15 SR	sheep	Brakna	30.51	30.21	
MR 487/15 SR	sheep	Brakna	27.4	25.05	BUNV
MR 492/15 SR	goat	Brakna	no CT	34.47	
MR 495/15 SR	sheep	Brakna	30.48	28.16	
MR 496/15 SR	sheep	Brakna	33.98	31.21	

Table 3. Serological analysis of the Mauritanian serum samples using serum neutralization test (SNT). (A) NRIV, BATV (B), BUNV (C), and RVFV (D) specific antibodies.

A)

Species / Region	N° of samples	NRIV VNT positive					
		goat	sheep	not reported	total	prevalence (%)	95% CI
Inchiri	77	2	1	0	3	3.9	1.3 - 10.8
Chargui	80	1	5	1	7	8.8	4.3 - 17.0
Gharbi	83	0	5	0	5	6.0	2.6 - 13.3
Tagant	51	0	0	0	0	0.0	0.0 - 7.0
Assaba	54	3	0	0	3	5.6	1.9 - 15.1
Trarza	28	1	1	0	2	7.1	2 - 22.7
Guidimaka	71	11	6	0	17	23.9	15.5 - 35.0
Brakna	14	2	2	0	4	28.6	11.7 - 54.7
total	458	20	20	1	41	9.0	6.7 - 11.9

B)

Species / Region	N° of samples	BATV VNT positive					
		goat	sheep	not reported	total	prevalence (%)	95% CI
Inchiri	77	0	0	0	0	0.0	0.0 - 4.8
Chargui	80	2	2	0	4	5.0	2.0 - 12.1
Gharbi	83	0	0	1	1	1.2	0.2 - 6.5
Tagant	51	0	0	0	0	0.0	0.0 - 7.0
Assaba	54	1	0	0	1	1.9	0.3 - 9.8
Trarza	28	0	1	0	1	3.6	0.6 - 17.7
Guidimaka	71	2	0	0	2	2.8	0.8 - 9.7
Brakna	14	0	0	0	0	0.0	0.0 - 21.5
total	458	5	3	1	9	2.0	1.0 - 3.7

C)

Species / Region	N° of samples	BUNV VNT positive					
		goat	sheep	not reported	total	prevalence (%)	95% CI
Inchiri	77	0	0	0	0	0.0	0.0 - 4.8
Chargui	80	0	0	0	0	0.0	0.0 - 4.6
Gharbi	83	0	2	0	2	2.4	0.7 - 8.3
Tagant	51	1	0	0	1	2.0	0.4 - 10.3
Assaba	54	0	0	0	0	0.0	0.0 - 6.6
Trarza	28	0	0	0	0	0.0	0.0 - 12.1
Guidimaka	71	0	0	0	0	0.0	0.0 - 5.1
Brakna	14	0	0	0	0	0.0	0.0 - 21.5
total	458	1	2	0	3	0.7	0.2 - 1.9

D)

Species / Region	N° of samples	RVFV VNT positive					
		goat	sheep	not reported	total	prevalence (%)	95% CI
Inchiri	77	1	3	0	4	5.2	2.0 - 12.6
Chargui	80	1	0	0	1	1.3	0.2 - 6.8
Gharbi	83	0	11	2	13	15.7	9.4 - 25.0
Tagant	51	14	1	0	15	29.4	18.7 - 43.0
Assaba	54	9	20	0	29	53.7	40.6 - 66.3
Trarza	28	4	0	0	4	14.3	5.1 - 31.5
Guidimaka	71	6	3	0	9	12.7	6.8 - 22.4
Brakna	14	3	3	0	6	42.9	21.4 - 67.4
total	458	38	41	2	81	17.7	14.5 - 21.4

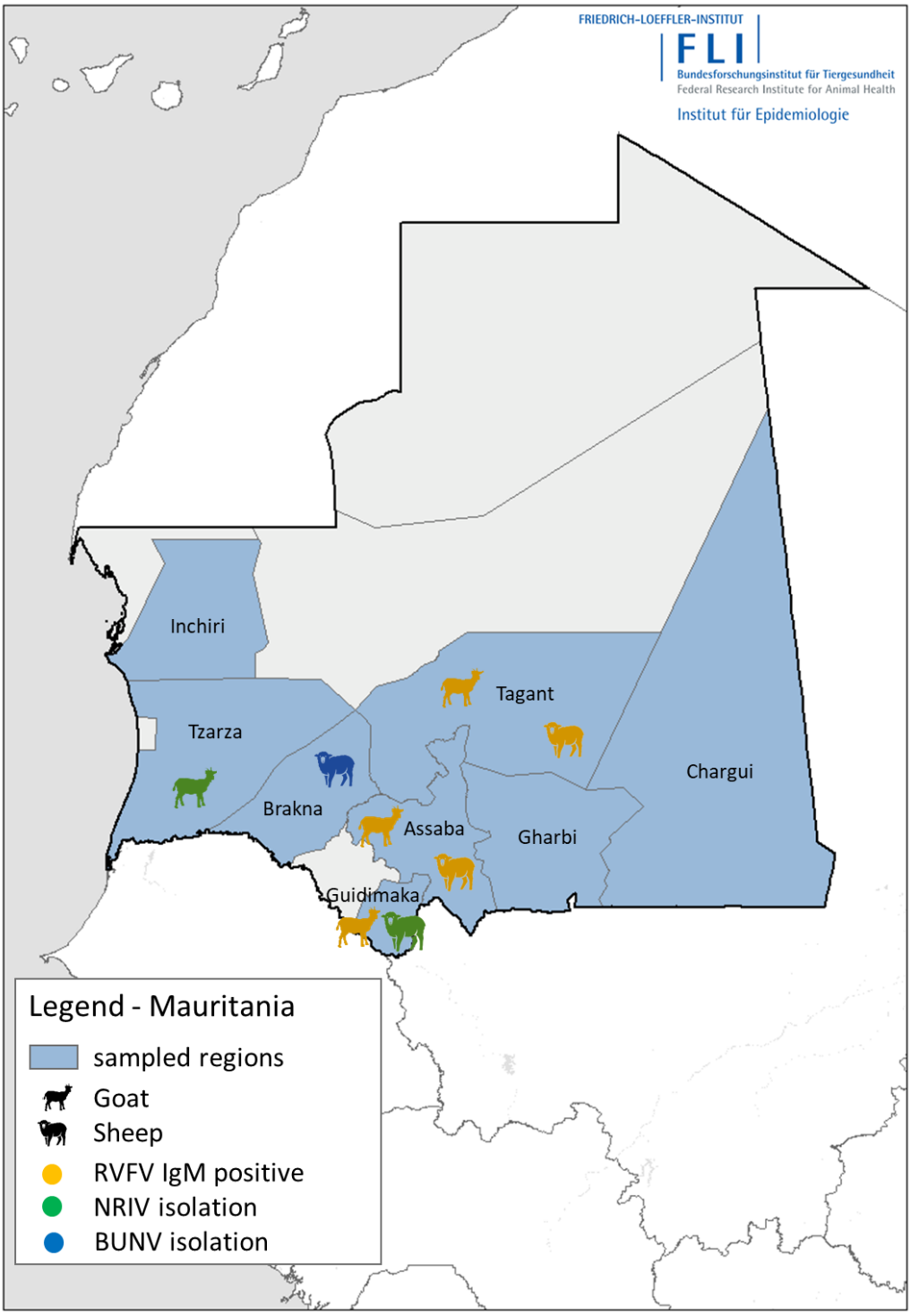


Figure 4. Geographical distribution of RVFV IgM positive sheep and goats and isolation sites of NRIV and BUNV in Mauritania.

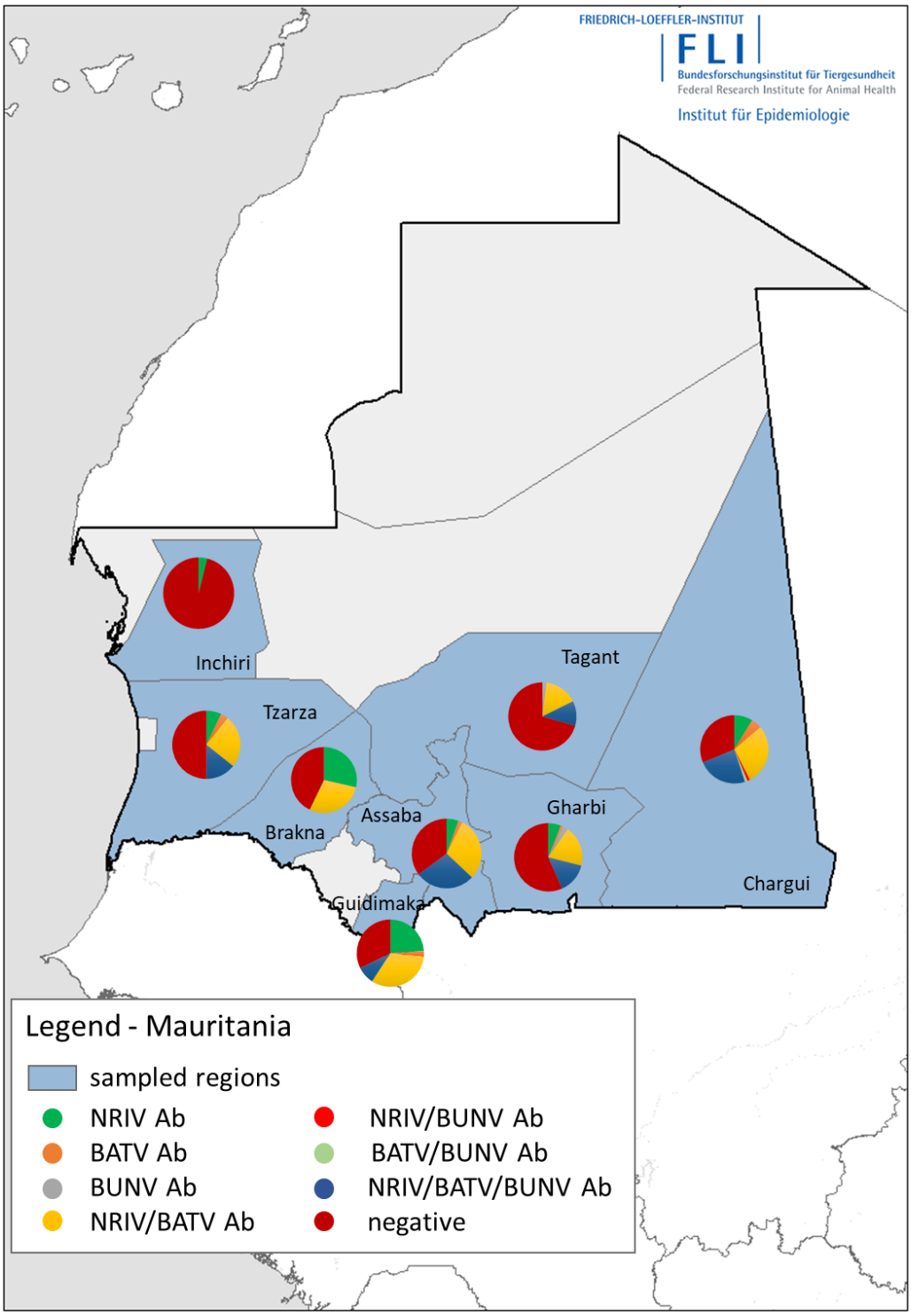
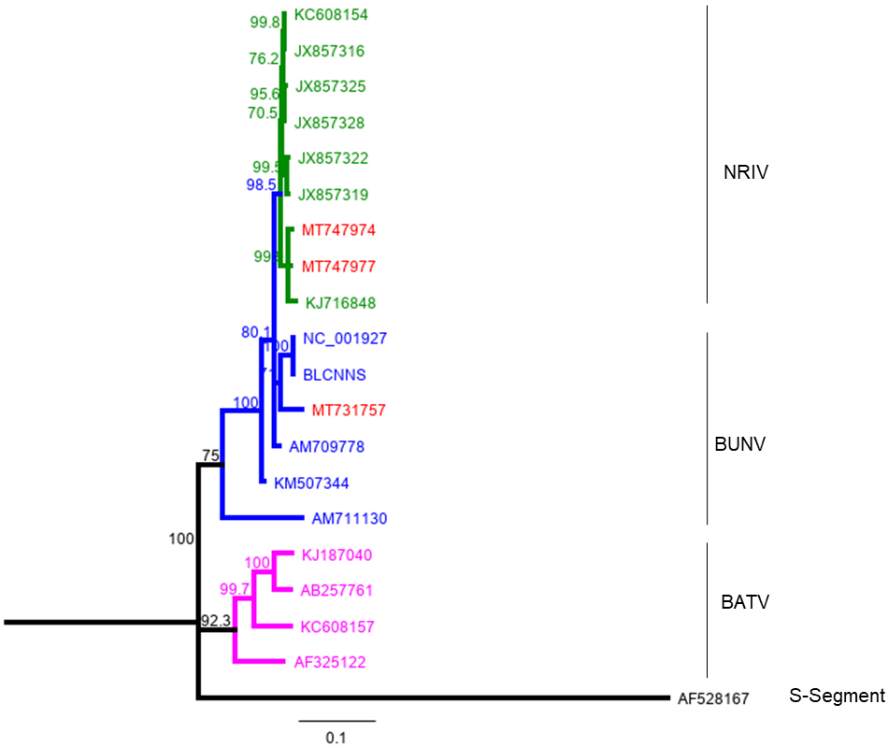


Figure 5. Geographical distribution of specific and unspecific antibodies against NRIV, BATV, and BUNV in the investigated Mauritanian small ruminants.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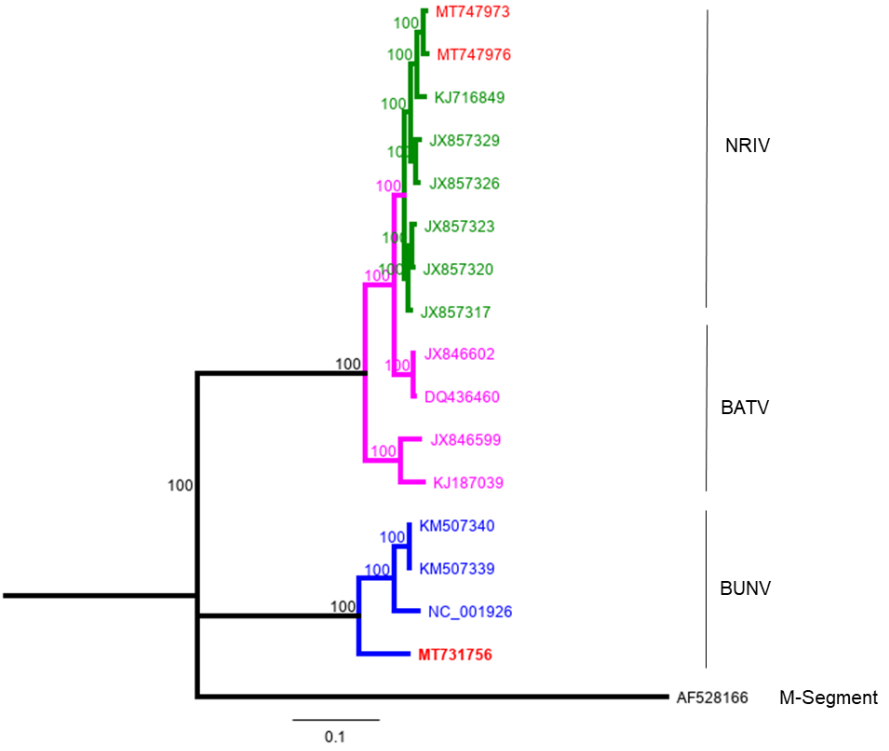
NRIV
BUNV
BATV
S-Segment
0.1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BUNV
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0.1

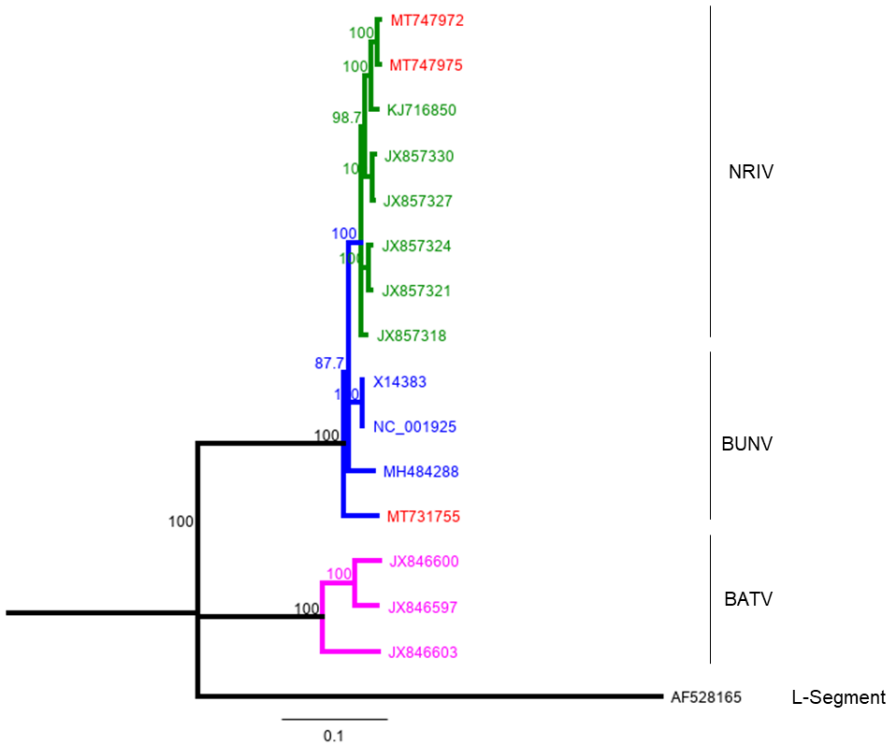
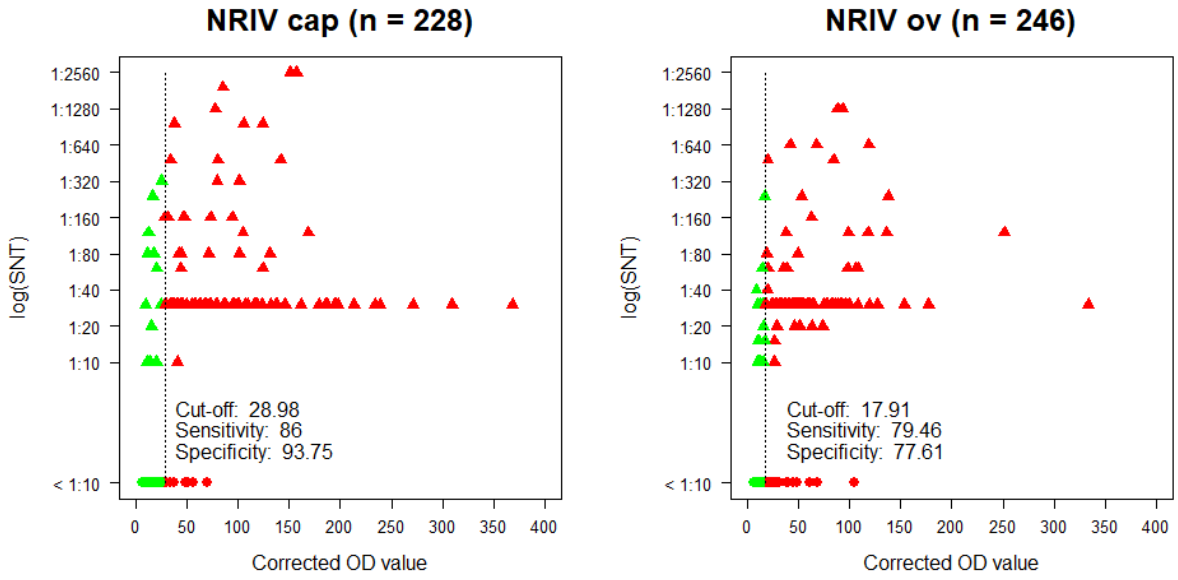


Figure 6. Phylogeny of isolated BUNV and NRIV sequences for each segment (S, M, and L-segment).



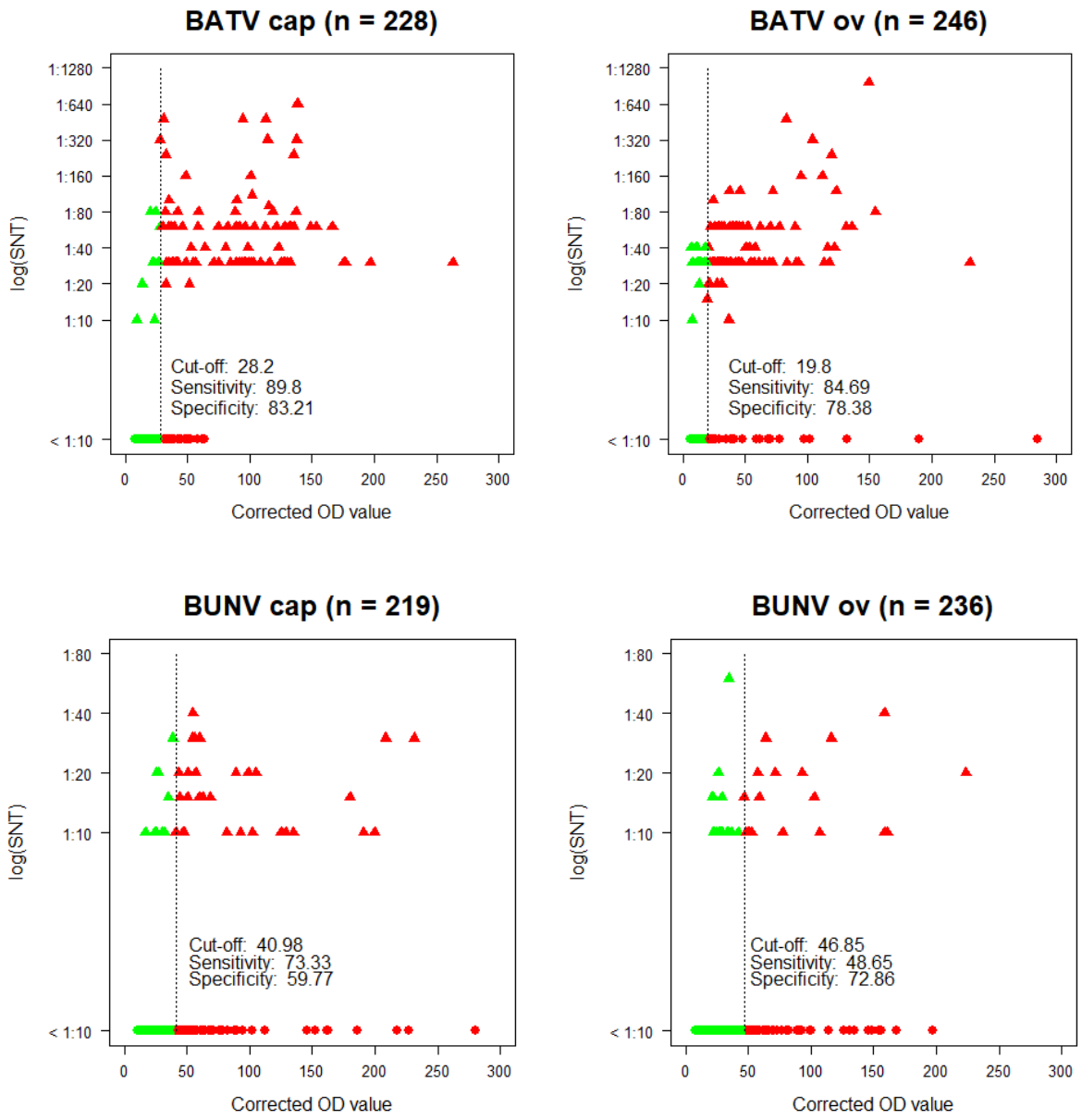


Figure 7. Corrected OD values of the ELISA in relation to neutralization titers (log (SNT)) of the SNT showing cut-off, sensitivity and specificity for each species (cap= goat, ov=sheep) and each virus. Green dots: SNT and ELISA negative samples. Green triangles: SNT positive, but ELISA negative samples. Red triangles: SNT and ELISA positive samples. Red dots: SNT negative, but ELISA positive samples.

4.7 Acknowledgments

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5 Manuscript II

Prevalence of Rift Valley fever virus and Crimean-Congo hemorrhagic fever virus in livestock in Egypt

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5.1 Abstract

Rift Valley Fever virus (RVFV) and Crimean-Congo hemorrhagic fever virus (CCHFV) are zoonotic arthropod-borne viral diseases with great impact on public health system. Egypt represents a Rift Valley fever endemic country with high outbreak risk. Outbreaks of CCHFV are frequently reported in neighbouring countries like Sudan. Therefore, a continuous surveillance of the Egyptian livestock is an important prerequisite to assess the current status of RVFV and CCHFV. A total of 1254 serum samples collected from sheep, cattle, camels and buffaloes from 13 different governorates of Egypt including also a camel importation quarantine station in the Nile region north to the Sudanese border were therefore subjected to a comprehensive molecular and serological analysis. No viral RNA was detected by quantitative reverse transcriptase PCR, however an overall seroprevalence for RVFV and CCHFV-specific antibodies was 6.6% and 24.4%, respectively. Especially, serum samples derived from the camels which were imported from Sudan revealed high antibody levels against both viruses (16% for RVFV and 85.6% for CCHFV) illustrating the close epidemiological interconnection between both countries.

5.2 Introduction

Rift valley fever virus (RVFV) and Crimean-Congo hemorrhagic fever virus (CCHFV) are zoonotic arboviruses and belong to the order *Bunyavirales*. RVFV, a member of the genus *Phlebovirus* within the family *Phenuiviridae*, is transmitted by mosquitoes and causes zoonotic disease among humans and livestock (Ikegami and Makino 2011). In sheep, goats and to a lesser extend also in cattle and camels, RVFV infections are causing abortion storms and acute deaths in newborns. The majority of infected humans develop only a self-limiting febrile illness, whereas in 1-2 % of patients, the infection leads to a more severe disease characterized by hepatitis, retinitis, encephalitis and hemorrhagic fever (Chevalier 2013). RVFV is transmitted by more than 30 different mosquito species belonging to 6 genera (primarily Aedes and Culex) (Hubálek et al. 2014). In Egypt, Culex pipiens is the most common mosquito species and functions as the main vector of RVFV (Hanafi et al. 2011). Besides, Cx. antennatus was identified as an important secondary vector which can be found in every governorate of Egypt (Gad et al. 1987).
However, the main infection route for humans is the direct or indirect contact with infected livestock, especially during slaughtering, butchering and necropsy (Chevalier et al. 2010). Hence, certain professional groups like veterinarians, health personnel, farmers, and abattoir workers are at higher risk of infection (Bird et al. 2009). RVFV is widespread in Africa and could emerge in the Arabian Peninsula in 2000 (Linthicum et al. 2016). In Egypt, the first outbreak of RVFV was reported in 1977. Till today, it remains the largest one with an estimated number of 200,000 human infections, almost 600 deaths, and high economic losses in livestock (Bird et al. 2009). Further outbreaks in a lesser extent occurred in 1993/94, 1997 and more recently in 2003 (Ahmed Kamal 2011). The continuous import of infected livestock, especially camels from Sudan, without sufficient quarantine measurements was assumed as the main source for the introduction of RVFV into Egypt (Ahmed Kamal 2011). Outbreaks of RVF in livestock do not only lead to severe economic losses, but also put the human population at risk, since it has been observed that human epidemics are preceded by epizootics in livestock (McElroy et al. 2009). Therefore, monitoring of the livestock population is one important approach in a comprehensive RVFV control strategy (FAO 2018). Several studies conducted in the past demonstrated inter-epidemic RVFV circulation in Egypt. In 2009, Ramadan detected a moderate RVFV antibody titer in different livestock species (20% in sheep, 17% in goats, 5% in cattle and 11% buffaloes) in Dakhalia governorate (Ramadan 2009). A survey from Marawan et al. in 2012 showed related prevalence rates in sheep, goats, camels, cattle and buffaloes (19%, 11%, 0%, 20% and 10%, respectively) (Marawan et al. 2012). In 2014 and 2015, Mroz et al. reported a much lower seroprevalence in sheep (0.5%) and goats (0%) living in the upper Nile delta, whereas the antibody titer in buffaloes, camels and cattle was in acccordance with the previous reports (5.9%, 3.2% and 12.6%, respectively) (Mroz et al. 2017a;

Mroz et al. 2017b). In a most recent study, El Bahgy tested camels originating from Sudan and camels of local breed for RVFV antibodies: the overall seroprevalence was 13.5%, whereby the antibody levels in imported camels were twice as high than in local breed (16.6% and 8.7%, respectively) (El Bahgy et al. 2018).

Crimean-Congo hemorrhagic fever virus, a member of the genus *Nairovirus* in the family *Bunyaviridae*, causes the most widespread tick-borne viral infection of humans (Bente et al. 2013). Its distribution ranges from western China through southern Asia and the Middle East to southeastern Europe and throughout most of Africa (Bente et al. 2013). It corresponds closely with the distribution of the main vector, the members of the Hyalomma genera (Shayan et al. 2015). In Egypt, six tick species are present which are reported to be vectors for CCHFV: *Hyalomma a. anatolicum*, *H. marginatum rufipes*, *H. impeltatum*, *Rhipicephalus sanguineus*, *R. turanicus*, and *Boophilus annulatus* (Darwish et al. 1978). Humans become infected with the virus through tick bites or contact with blood or other body fluids of infected CCHF patients or animals (Bente et al. 2013). A wide variety of animals (e.g. cattle, sheep, camels, hares and ostriches) are susceptible and develop a transient viremia, without showing any signs of illness (Bente et al. 2013). In contrast, infection in humans can cause high fever, headache, fatigue, myalgia, abdominal pain, nausea, vomiting, diarrhea, hemorrhages from various body sites and mortalities in severe cases. Reported mortality rates vary widely from two to 30% (Weidmann et al. 2016). In Egypt, CCHF viral genomes were detected in ticks infested on camels which were imported from Sudan and Somalia (Chisholm et al. 2012). Other studies reported the presence of antibodies against CCHFV in humans and livestock: the first seroepidemiological survey in Egypt reported prevalence rates of 0.2% in humans from Qena and Assiut governorates, and 8.8% in camels, 13.9% in cattle, 3.1% in buffaloes and 18.2% in sheep from an abattoir in Cairo (Darwish et al. 1977). No clear information was given whether animals were imported into Egypt or if they were locally bred. In contrast, Morrill et al. stated that 600 indigenous cattle and sheep from Aswan governorate were tested serologically negative, but 14% of camels imported from Sudan and Kenya were CCHFV-antibody-positive (Morrill et al. 1990) The low prevalence rate in humans was confirmed a few years later in Nile Delta governorate (1.1%) (Darwish et al. 1994). The most recent study in 2004, described low seroprevalence in cattle (3.83%), water buffaloes (0.38%), sheep (6.3%), and goats (1.14%) (Mohamed et al. 2008). Unfortunately, the origin of the sampled animals was not explicitly mentioned.

The present study is designated to investigate the current prevalence status of RVFV and CCHFV in the livestock population in different localities of Egypt using a comprehensive molecular and serological analysis. Moreover, to evaluate the risk for the introduction of CCHFV and RVFV from neighbour countries into Egypt, sera from imported camels from Sudan to a quarantine station in the South of Egypt were analyzed.

5.3 Materials and Methods

5.3.1 Sample collection and workflow

A total number of 1254 apparently healthy animals including sheep, buffaloes, camels and cattle were sampled in 13 different governorates of Egypt (Sohag, Assiut, Al Fayum, Matrouh, Aswan, Giezeh, Kafr-El-Sheikh, Dakahliya, Sharkia, Menoufia, Beni-Suef, Ismailia and Gharbia) in 2017 (Table 4). Serum samples were aseptically separated and kept frozen at -20°C for further investigations. After transportation to Germany, all samples were first tested under BSL-3 conditions in a quantitative RT-PCR detecting RVF genome and a second RT-PCR detecting CCHFV genome.
For serological testing under BSL-2 conditions, the sera were inactivated with 1:1 PBS Tween (phosphate buffered saline) at 56°C for 30 minutes. Due to low sample volumes, only 912 out of 1254 samples (82 sheep, 219 camel, 529 cattle, and 82 buffaloes) were available for serological tests.
Sera were run in the ID Vet competition ELISA (ID Vet, Montpellier, France) detecting both RVFV IgM and IgG antibodies. To detect recent infections, positive samples were further tested with the RVF ID Vet IgM capture ELISA (ID Vet, Montpellier, France). For camel sera, the commercial RVF IgM ELISA could not be applied. These samples were therefore analyzed using an indirect in-house IgM ELISA for camelids (Rissmann et al. 2017a). Sera that were inconclusive in the competition ELISA were additionally tested by serum neutralization test (SNT) using the RVFV MP-12 strain (OIE, 2008). Moreover, an additional confirmation of selected samples was performed with an adapted commercial immunofluorescence assay (IIFA) (Euroimmun, Lübeck, Germany) (Jäckel et al. 2013b).
The serological investigation for CCHFV-specific antibodies consisted of a commercial multi-species double-antigen sandwich ELISA (ID Vet, Montpellier, France) and a modified commercial CCHF IgM/IgG IFA (Euroimmun, Lübeck, Germany) (Mertens et al. 2015) as conformational test for all camel derived sera and ELISA-positive tested cattle sera.

5.3.2 Quantitative reverse transcriptase (RT) PCR

RNA isolation was performed using the Nucleo®Mag VET Kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. Pools of five samples were processed respectively. As internal extraction control, a MS2 Bacteriophage was added to each serum pool before the extraction process based on the primers MS2F 5`-CTC TGA GAG CGG CTC TAT TGG T-3´ and MS2R 5`-GTT CCC TAC AAC GAG CCT AAA TTC-3´ (Ninove et al. 2011). The qRT-PCR for RVFV was carried out according to an adapted multiplex qRT-PCR protocol (Wernike et al. 2015) using the AgPath-ID™ One-

Step RT-PCR Kit (Applied Biosystems, Foster City, USA). The applied PCR protocol allows the simultaneous detection of RVFV, Foot and Mouth Disease Virus (FMDV) and Ngari Virus (NRIV) using the following primers: RVF-forw 5`-TGA AAA TTC CTG AGA CAC ATG G-3´ and RVF-rev 5`-CTT CCT TGC ATC ATC TGA TG-3´ for RVFV, FMD-IRES-4.1F 5`-TAA CAW GGA CCC RCS GGG CC-3´and FMD-IRES-4R 5`-TGA AGG GCA TCC TTA GCC TG-3´ for FMDV, and Bunyam F 5`-GCT GGA AGA TTA CTG TAT ATA ATA C-3´ and Bunyam R 5`-CAA GGA ATC CAC TGA GGC GGT G-3´ for NRIV (Bird et al. 2007; Wernike et al. 2015). For each reaction 2.5 µl of RNA, 5 pmol of both forward and reverse primer and 0.625 pmol of the probe were used in a total volume of 12.5 µl. As positive control, RNA of the vaccine strain MP-12 for RVFV and HS062-08 for FMDV, and cell culture supernatant for NRIV in a 10^{-3} concentration were added on each plate. PCR reaction condition was used as follows: 48 °C for 10 min, 95 °C for 10 min and 44 cycles at 95 °C for 15 s, and 60 °C for 45 s.

For detection of CCHFV genomes an one-step multiplex real-time RT-PCR was carried out as described by Sas et al. using 14 primers and two probes which simultaneously detect all six genotypes of CCHFV (Sas et al. 2018b). Briefly, for each reaction 2.5 µl of RNA, 7.5 pmol of each CCHF-deg primer, 0.5 pmol of each genotype –specific CCHF-primer and 1.5 pmol of each CCHF-probe were used in a total reaction volume of 12.5 µl along with the QuantiTect Probe RT-PCR Kit (Qiagen, Hilden, Germany). PCR reaction conditions were applied as follows: 50 °C for 30 min, 95 °C for 15 min, 44 cycles at 95 °C for 10 s, 55 °C for 25 s, and 72 °C for 25 s (Sas et al. 2018b).

5.3.3 ID Vet RVF competition ELISA and ID Vet CCHF double-antigen sandwich ELISA

For serological testing for RVFV-specific antibodies, the serum samples were heat inactivated with 1:1 PBS Tween (phosphate buffered saline) at 56°C for 30 minutes. A total of 912 samples were analyzed in the commercial ID Screen® RVFV competition multispecies ELISA (ID Vet, Montpellier, France) according to manufacturer's instructions. Both IgG and IgM antibodies are detected indistinguishably. Samples with a percentage of inhibition lower than 40% were defined as positive. Percentage inhibition values between 40% and 50% were considered as inconclusive and those higher than 50% as negative.

CCHF-specific antibodies were detected by the commercial ID Vet CCHF double-antigen sandwich ELISA (ID Vet, Montpellier, France) which was established for testing sera from cattle, small ruminants, camels and other species by Sas et al. (2018a). Samples with a S/P percentage (S/P% = OD of the sample/ OD of positive control x 100) below or equal to 30% were classified as negative and a value over 30% as positive (Sas et al. 2018a).

5.3.4 ID Vet RVF IgM capture ELISA and indirect RVF IgM in-house ELISA for camelids

Sera from cattle, sheep and buffaloes that gave positive or inconclusive results in the competition ELISA were further tested with the ID Screen® Rift Valley Fever IgM capture ELISA (ID Vet, Montpellier, France) according to the manufacturer's description. Samples with a sample-to-positive-ratio higher than 50% were defined as positive. Sample-to-positive-ratios between 40% and 50% were considered as inconclusive and those lower than 40% as negative. As the ID Vet IgM capture ELISA cannot be used for analyzing camel sera, an indirect IgM in-house ELISA for camelids was deployed as previously described (Rissmann et al. 2017a). Briefly, ELISA plates were coated with recombinant RVFV NP or with buffer only. After blocking, the serum samples in a dilution of 1:25 were added to a well coated with NP and to another with buffer only. For detection, a 1:1000 goat anti-camelid IgM antibody and an additional 1:5000 secondary rabbit anti-goat antibody conjugated with horseradish-peroxidase (Dianova) were added. After visualization with 2.2′-azino di-ethylbenzothiazoline sulphonic acid (ABTS, Roche, Mannheim, Germany), the reaction was stopped with 1% sodiumdodecyl-sulfate, and the plates were read at 405 nm. For final analysis, a corrected OD405 was the difference of the OD value of the coated and uncoated wells respectively (ΔOD405). The analysis of the results was conducted in accordance with the ID Vet IgM ELISA.

5.3.5 Indirect immunofluorescence

Selected samples were further analyzed with a RVF in-house immunofluorescence assay (IIFA) according to a previously published protocol (Jäckel et al. 2013b) using the commercial RVFV immunofluorescence slides from Euroimmun (Lübeck, Germany). The detection of antibodies was realized with species-specific Cy3 labelled secondary antibodies (donkey anti-sheep, donkey anti-goat, goat anti-bovine) in a 1:200 dilution. For testing the camel sera, an additional secondary antibody, goat anti-lama unlabelled in a 1:100 dilution, was necessary.
All of the camel sera and ELISA-positive tested cattle sera were analyzed in a commercially available CCHF IFA (Euroimmun, Lübeck, Germany) which was adapted for use in ruminants and camels as described before by Mertens et al. (2015). Species-specific IgG-FITC labelled secondary antibodies (goat anti-bovine, rabbit anti-sheep, donkey anti-goat) were used in a dilution of 1:320, 1:1000 and 1:40, respectively. For detecting antibodies in camel sera, an additional secondary antibody, goat anti-lama, was applied in a dilution of 1:200.

5.3.6 RVF serum neutralization test

To verify the results from the RVF competition ELISA, the samples that gave an inconclusive result were also analyzed by serum neutralization test (SNT) performed as described in the OIE Terrestrial Manual 2014 (OIE, 2008). Briefly, 100 $TCID_{50}$ of RVFV (MP-12 vaccine strain) were added to serial two-fold diluted sera and Vero 76 cells (Collection of Cell Lines in Veterinary Medicine, Friedrich-Loeffler-Institut, Germany) were added in a dilution of $3x10^5$ to each well. Plates were incubated at 37°C, 5% CO_2 for 6 days. The neutralizing antibody titer of the samples was defined as the 50% neutralization dose (ND_{50}). Positive result for neutralizing antibodies was confirmed when the ND_{50} values were 10 or higher.

5.3.7 Biostatistical analysis

Prevalence rates were calculated using the calculation tool of Epitools (https://epitools.ausvet.com.au/ciproportion).

5.3.8 Ethics statement

This study was carried out in strict accordance with the guidelines of the Egyptian Network of Research Ethics Committees (ENREC), which complies with the international laws and regulations regarding the ethical considerations in research. All efforts were made to minimize animal suffering and to reduce the number of animals used.

5.4 Results

A total of 1254 animals (98 sheep, 279 camels, 777 cattle, and 100 buffaloes) from 13 governorates in Egypt (Figure 8) were first tested by qRT-PCRs for RVF and CCHF genomes and none of these samples were found positive essentially.
Due to low sample volume, only 912 samples could be screened serologically for RVFV and CCHFV antibodies thereafter.
Out of 912 samples analyzed in a competition RVFV ELISA, 57 (6.2%) samples were positive for RVFV antibodies and nine samples (1%) gave inconclusive results. Out of these nine samples, three specimens were determined positive in a subsequent serum neutralization test. Therefore, the corresponding overall seroprevalence for RVFV-specific antibodies in the Egyptian livestock population was 6.6% (Table 5). Selected samples were additionally confirmed in the RFV IgG specific in-house IIFA (Figure 9).

It should be noted that the sampled animals had no history of previous vaccination against RVFV.
Taken by species, out of 82 tested buffaloes, 17 (20.7%) were found to be seropositive. All positive tested samples were obtained from Fayoum and Sharkia governorates. The 529 serologically tested cattle were sampled from five Egyptian governorates. In three of these governorates (Fayoum, Assiut and Sohag), seven animals were found to be RVFV antibody positive resulting in a prevalence of 1.3%. All of the 219 serologically tested camels were originally imported from Sudan and sampled in Aswan governorate. The analysis revealed 35 positive sera corresponding to a seroprevalence of 16%. One out of 82 serum samples from sheep was positive for RVFV-specific IgM antibodies indicating a recent infection. The seropositive sheep was a 2-years-old female sheep located in Aswan governorate in the south of Egypt.
A total of 628 sera samples were tested serologically for CCHFV-specific antibodies using a double-antigen sandwich ELISA. Furthermore, all samples of camels and ELISA-positive cattle sera were verified by CCHF IFA (Figure 10). The serological investigation for CCHFV-specific antibodies revealed an overall seroprevalence of 28.8% (196 positive out of 665 tested samples). Interestingly, all of the sheep and buffalo samples were negative in the double-antigen sandwich ELISA, whereas 18 out of 392 (4.6%) tested cattle sera were antibody-positive. Out of these 18 samples, 13 sera were confirmed also by the CCHFV IFA resulting in a prevalence of 3.3%. All positive samples were collected from animals from Fayoum, Assiut, Ismailia and Sohag governorates. Due to low sample volumes, only 174 out of the original 219 camel sera could be analyzed in the double-antigen ELISA and in the CCHFV IFA. Samples were rated positive only when both tests were in agreement. Hence, 149 out of 174 camel sera were antibody positive for CCHFV which corresponds to a local prevalence of 85.6%. None of the animals were vaccinated against CCHFV, since there is no vaccine available.

5.5 Discussion

Continuous monitoring of livestock population is a mandatory tool of the RVFV infection control in endemic countries to detect first evidence for a potential outbreak (FAO 2018). Former studies investigated the seroprevalence of RVFV in Egyptian livestock by testing mainly samples that were collected in the Nile Delta from where in previous RVFV outbreaks the epidemics initially originated. However, only about 200 km of the 1400 km stretch of the Nile flowing through Egypt belong to the river delta. Therefore also more southerly located Nile River areas like in Fayoum, Beni-Suef, Assiut and Sohag should not be neglected as RVFV reservoirs (Napp et al. 2018). These areas pose a high risk, since they offer the optimal living conditions for the mosquitoes. Therefore, the present study was dedicated to evaluate the current RVF situation in

livestock in Egypt in both the Nile Delta and in the more upstream Nile river governorates.

Antibodies against RVFV were found in all species, but sheep and cattle showed lower RVFV prevalences (1.2% and 1.3%, respectively) compared to camels and buffaloes (16% and 20.7%, respectively).

The here analyzed sheep samples were collected in six governorates from northern (Matrouh, Giezeh and Gharbia) to southern Egypt (Assiut, Sohag and Aswan). One sample that was taken in Aswan governorate next to the border to Sudan was tested positive for RVFV IgM antibodies, but showed a negative result in the qRT-PCR. The detection of RVFV genome is only possible for up to 14 days post infection, whereas IgM antibodies are detectable as early as four days after infection leaving a short time-frame of 10 days for concurrent detection of viral RNA and IgM antibodies (Pepin et al. 2010). Since the 2-year-old sheep was born and raised in Egypt and not vaccinated against RVFV, a natural infection with a virulent local RVFV strain can be assumed. As source of infection, various scenarios are possible, e.g. infection by an endemic circulating virus or via contact with imported animals from Sudan. Our findings are in agreement with former studies conducted in 2014 and 2015 by Mroz et al. showing a low prevalence of only 0.5% in sheep in the Nile delta region (Mroz et al. 2017b). In contrast, earlier studies from 2009 and 2012 stated a prevalence of 20% and 19%, respectively (Marawan et al. 2012). Unfortunately, no further information about the tested animals are available to identify, whether higher age and therefore a higher cumulative RVFV exposure risk and/or vaccination might be the explanation for these divergent prevalence rates.

Our investigation revealed also a low antibody titer against RVFV in cattle serum samples (1.3%). The majority of here tested cattle were sampled in the governorates of Fayoum and Assiut. In previous studies, non-vaccinated cattle from the governorates in the Nile Delta gave slightly higher (7.9% and 19.5%, respectively) prevalences (Mroz et al. 2017a; Marawan et al. 2012). The difference between the prevalence levels might be explained by regional differences of the collection sites. In the Nile Delta north to Cairo, the majority of the animal markets trade livestock between different governorates, whereas in the south of Cairo mainly local markets are used which trade animals from the same district (Napp et al. 2018). Trading animals over greater distances is linked with higher RVFV spreading risks.

In the present study, 20.7% of the tested buffalo specimens were found to carry RVFV-specific antibodies. All positive tested animals were sampled from two small holdings in the governorates of Fayoum and Sharkia. In the former studies the RVFV prevalence in buffaloes ranged from 5.9% to 10% and 11%, respectively (Mroz et al. 2017a; Marawan et al. 2012). However, Mroz et al. noticed a difference in antibody titers in animals on large farms and individual small buffalo holdings ranging from 0% to 22%, respectively (Mroz et al. 2017b). This could be due to lower hygiene standards in small holdings, which accelerates the spread of pathogens and the transmission to contact

animals. The presence of antibodies against RVFV in buffaloes during inter-epidemic periods indicates undetected circulation of RVFV in the buffalo population and underlines their potential role as amplifying hosts (Beechler et al. 2015; Mroz et al. 2017a; Mroz et al. 2017b; Jori et al. 2015; LaBeaud et al. 2011).

The camel sera analyzed in the present study were taken from imported animals from Sudan which resided in a quarantine station in Aswan province. After quarantine of three days, camels are usually transported to animal markets and slaughterhouses throughout Egypt (Napp et al. 2018). In this study, the presence of antibodies against RVFV in the investigated camels was 16%. The result is in line with a recent study conducted by El Bahgy et al. showing a prevalence of 16.6% in imported camels from Sudan (El Bahgy et al. 2018). Former surveys conducted by Marawan et al. and Mroz et al. stated a much lower antibody titer of 0% and 3.2%, respectively (El Bahgy et al. 2018; Mroz et al. 2017b). Marawan et al. provided no further information about the origin of the sampled camels. The results of Mroz et al. differ from the present findings, which might be explained by evaluating the age of the sampled animals. In the previous study, specimens were taken from camels of 2-7 years, whereas here 8 to 15-years-old camels were tested for RVFV. The association between the infection with RVFV and higher age has been already described before and is caused by the long time exposure of older camels to mosquito vectors (Olaleye et al.; Sindato et al. 2015; Abdallah et al. 2015, 2015).

In the present study, antibodies against CCHFV were only found in cattle and camels resulting in a prevalence of 3.3% and 85.6%, respectively. The sampled cattle were all born and bred in Egypt, whereas all of the camels were imported from Sudan to Aswan governorate. All of the tested sheep and buffalo samples were serologically negative for CCHFV. However, the number of analyzed sheep and buffalo samples in our study was low (63 sheep and 36 buffalo sera, 95% CI 0.00 - 5.75 and 0.00 - 9.64, respectively). Furthermore, our results might not display the CCHFV infection status in sheep and buffaloes in whole Egypt, since only selected governorates were investigated.

CCHFV is endemic in Sudan which is an important trading partner in regards to the import of animals like camels and cattle to Egypt (Ibrahim et al. 2015; Adam et al. 2011; Suliman et al. 2017). Besides of CCHFV antibody-positive camels and cattle, nosocomial outbreaks and sporadic cases of infections in humans have been reported in the some regions in Sudan, namely Kordufan, North Kordufan and Dafur (Aradaib et al. 2010; Aradaib et al. 2011; Elata et al. 2011; Bower et al. 2019; Rahden et al. 2019). This underlines the correlation between seroprevalence in livestock and seroprevalence in humans and the role of livestock in the transmission cycle of CCHFV in humans (Bente et al. 2013). Furthermore, the trade of animals increases the risk for the introduction of pathogens to non-endemic countries by importing infected animals or vectors infested on imported animals (Chisholm et al. 2012).

Former seroepidemiological surveys on CCHFV in Egypt reported low to negative prevalence rates. In 1977, Darwish et al. determined a seroprevalence of 3.1% in buffaloes and 18.2% in sheep (Darwish et al. 1977). In 1990, Morrill et al. stated that 400 native sheep from Aswan governorate were tested serologically negative (Morrill et al. 1990) The most recent study from 2004 described low antibody titers of 0.4% in water buffaloes and 6.3% in sheep (Mohamed et al. 2008). Considering all these data, the prevalence of CCHFV in sheep and buffaloes in Egypt is very low - also compared to other countries. Reports about sheep from Iran, Pakistan, Albania, Romania and Macedonia described prevalence rates of 16.2%, 19%, 46%, 27.8% and even 75%, respectively (Saghafipour et al. 2019; Kasi et al. 2020; Schuster et al. 2016; Ceianu et al. 2012). Buffaloes from India showed a seroprevalence of 25.4% and were infested by ticks carrying CCHFV RNA (Mourya et al. 2014).

Similarly, only a small number of cattle in Egypt are found to be CCHFV-antibody-positive. Our analysis revealed a prevalence of 3.3%, which is in line with a study from 2004 reporting 3.8% positive cattle from Sharkia governorate (Mohamed et al. 2008). An older survey from 1977 stated a higher prevalence rate of 13.9% (Darwish et al. 1977), which was not confirmed in 1990, when Morrill et al. tested 200 cattle sera as CCHFV antibody negative (Morrill et al. 1990). In other African countries, much higher seroprevalences in cattle were reported: in Mali 66% (Maiga et al. 2017) and in Mauritania 67% (Sas et al. 2017). The current low prevalence rates in the native livestock population in Egypt suggest that although CCHFV may be sporadically circulating at low levels, it is not endemic and does not yet play a major role.

In contrast, the analysis of the camel specimens yielded a high seroprevalence of 85.6%. All of the animals were imported from Sudan to a quarantine station in Aswan governorate. Investigation on CCHFV prevalence in camels in Egypt are scarce. The first seroepidemiological survey reported an antibody titer of 8.8% without clearly stating whether the animals were imported into Egypt or if they were locally bred (Darwish et al. 1977). In 1990, 14% of camels imported from Sudan and Kenya were CCHFV antibody positive (Morrill et al. 1990). In Sudan, one study about CCHFV was conducted which revealed a seroprevalence of 21.3% (Suliman et al. 2017). The great discrepancy to our result of 85.6% might be due to the fact that the camels in our study were of older age ranging from 8 to 15 years. Suliman divided his animals into a group of young (< one year) and old camels (> one year) without further stating the exact age (Suliman et al. 2017). As former serosurveys could show, seropositivity is more common in adult than in young animals (Bente et al. 2013). Moreover, antibody positivity depends on the occurrence of tick vectors, which might be regionally different in Sudan. In Egypt, the isolation of CCHFV genome was succeeded once in Hyalomma ticks which were collected from slaughtered livestock in an abattoir in Giezeh governorate (Chisholm et al. 2012). CCHFV positive ticks were found on four camels originally from Sudan and one camel from Somalia (Chisholm et al. 2012). None of the indigenous animals in this study harbored infected ticks. Still, it is not possible to

conclude whether the ticks acquired the infection in Egypt or in the country of origin. Unfortunately, blood samples of the camels were not taken for a comprehensive CCHFV analysis. The presence of tick vectors for CCHFV in Egypt was proven already long ago (Darwish et al. 1978), but so far there is no evidence for a CCHFV circulation in ticks in Egypt. Therefore, further studies are needed in this regard and to determine whether the ticks cohabitate with livestock.

In summary, results obtained from the present study indicate that the highest risk of introduction of RVFV and CCHFV into Egypt is by the import of infected animals from Sudan. All imported bovines are slaughtered at the quarantine facility, which reduces the risk of spreading eventually imported pathogens. In contrast, imported camels may be transported to slaughterhouses or animal markets after quarantine (Napp et al. 2018) which may contribute to the virus spread even though camels are clinically unsuspicious (Ahmed Kamal 2011; Bente et al. 2013).

5.6 Tables and Figures

Table 4. Samples ordered by species and region. Camels originated from Sudan were sampled in Aswan governorate.

Species	Age (in years)	Region	Number of samples
Sheep	1.5	Matrouh	15
	<1	Matrouh	10
	2	Aswan	9
	1.5	Sohag	14
	<1	Sohag	7
	2	Assiut	14
	1.5	Giezeh	18
	<1	Gharbia	11
Buffalo	2-3	Giezeh	4
	2-3	Beni Suef	7
	2-3	Fayoum	21
	3-4	Sharkia	9
	2-3	Sharkia	10
	2-3	Dakahliya	20
	3-4	Kafr-El-Sheikh	6
	2-3	Kafr-El-Sheikh	9
	3	Menoufia	6
	3-4	Menoufia	5
	2-3	Menoufia	3
Camel	8-9	Originally from Sudan	76
	10-15	Originally from Sudan	14
	9-10	Originally from Sudan	68
	12-14	Originally from Sudan	28
	8-9	Originally from Sudan	93
Cattle	2.5	Sohag	86
	1.5	Assiut	263
	1.5	Ismailia	79
	2	Ismailia	65
	4-5	Fayoum	284

Table 5. Serological analysis of the Egyptian serum samples (A) for RVFV with ID Vet competition ELISA, ID Vet IgM capture ELISA, indirect IgM in-house ELISA for camelids(*) and (B) for CCHFV with ID Vet double-antigen sandwich ELISA and Euroimmun IIFA.

(A)

	N° of samples	IgM/IgG competition ELISA		IgM capture ELISA	IgG/IgM ELISA + SNT	Prevalence (%)	95% CI
		positive	inconclusive	positive	positive		
Sheep	82	1	0	1	1	1.2	0.2 - 6.6
Buffalo	82	17	1	0	17	20.7	13.4 - 30.7
Cattle	529	7	3	0	7	1.3	0.6 - 2.7
Camel	219	32	5	0*	35	16.0	11.7 - 21.4
total	912	57	9	1	60	6.6	5.2 - 8.4

(B)

		double-antigen sandwich ELISA	IIFA	ELISA + IIFA	Prevalence (%)	95% CI
		positive	positive	positive		
Sheep	63	0	not tested	0	0.0	0.0 - 5.8
Buffalo	36	0	not tested	0	0.0	0.0 - 9.6
Cattle	392	18	13	13	3.3	2.0 - 5.6
Camel	174	164	153	149	85.6	79.7 - 90.1
total	665	196	166	162	24.4	21.3 - 27.8

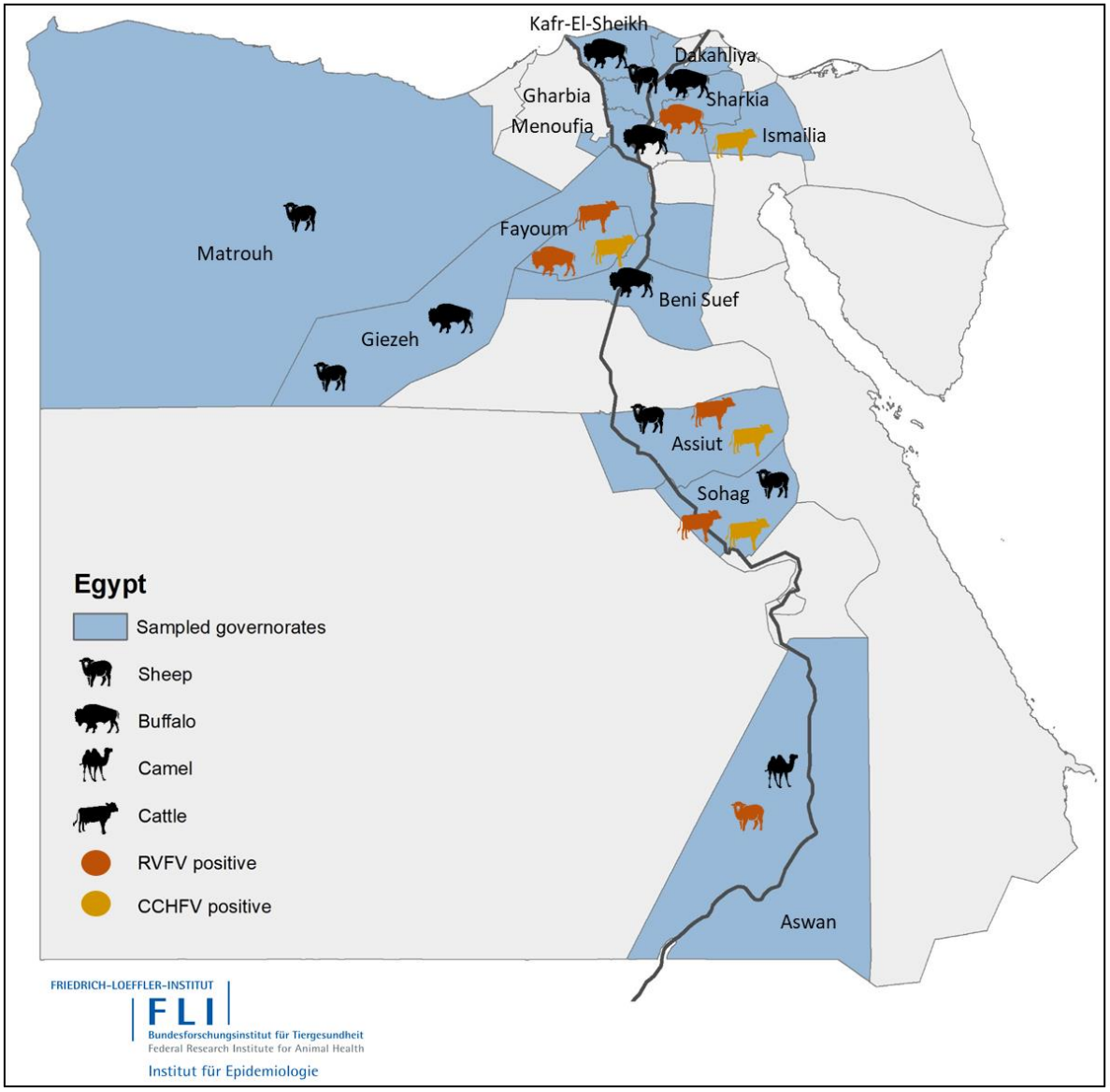

Figure 8. Geographical distribution of RVFV- and CCHFV-antibody positive livestock in Egypt.

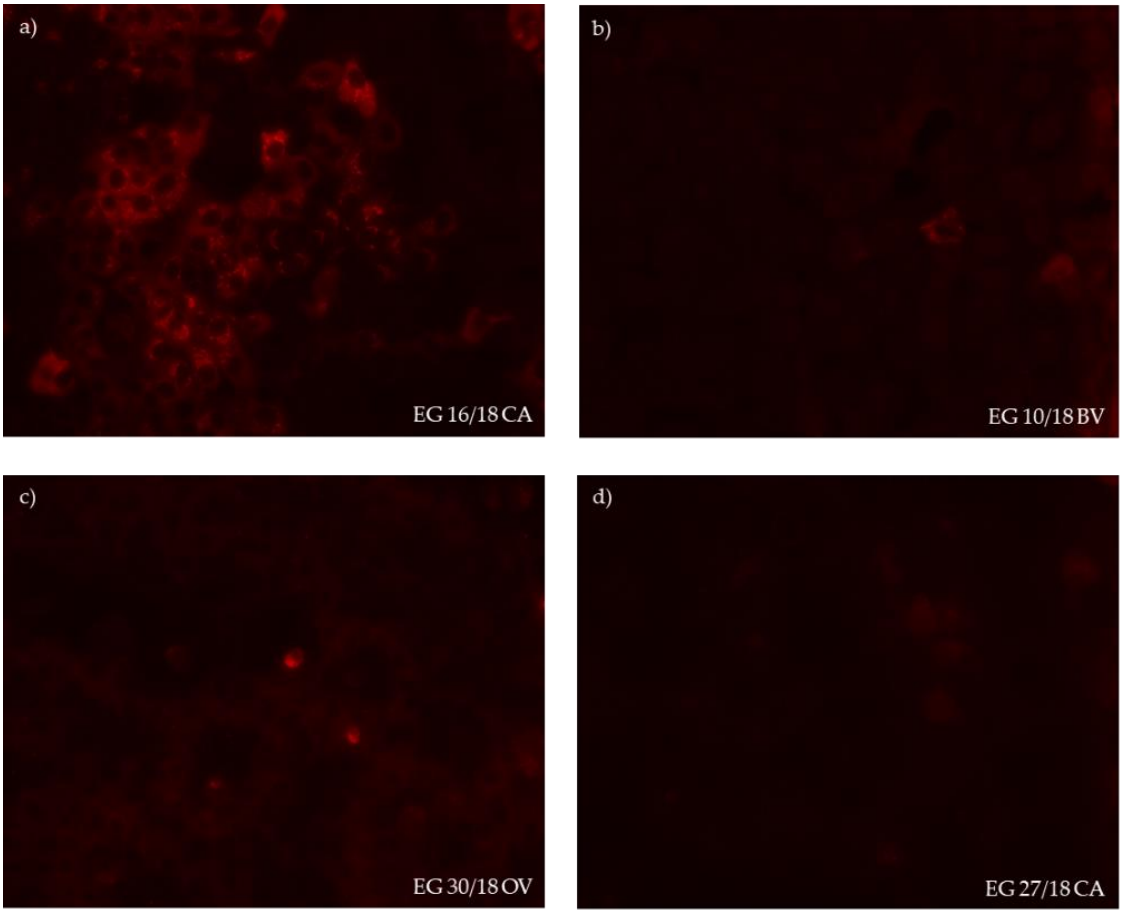

Figure 9. Immunofluorescence staining of selected serum samples using commercial Rift Valley fever virus slides (Euroimmun, Lübeck); (a-c) positive staining of camel (CA), cattle (BV), and sheep (OV) sera; (d) negative sample.

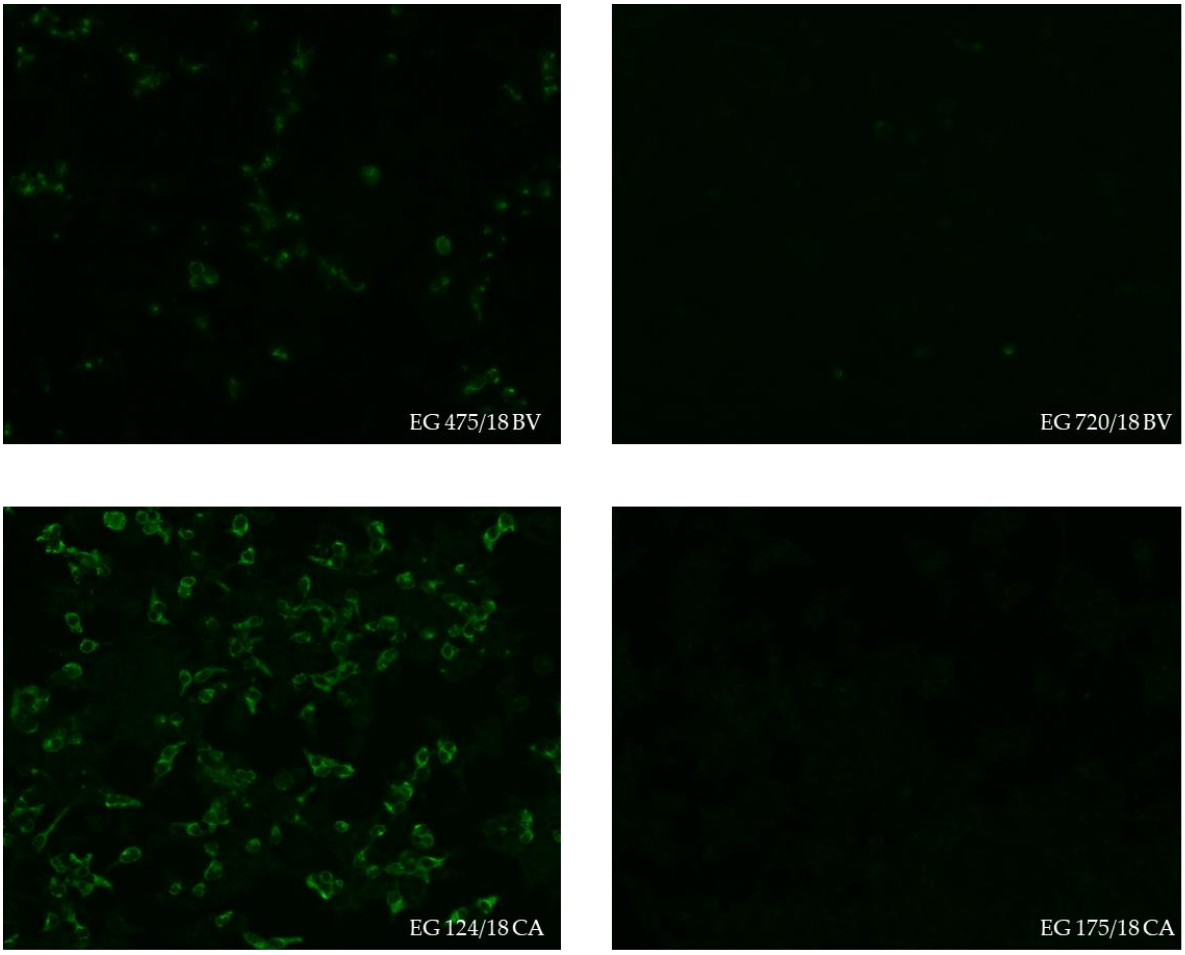

Figure 10. Immunofluorescence staining of selected serum samples using commercial Crimean-Congo hemorrhagic fever virus slides (Euroimmun, Lübeck); (a-b) positive staining of cattle (BV) and camel (CA), and (c-d) negative samples.

5.7 Acknowledgments

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6 Manuscript III

Serological and molecular investigation of Batai virus infection in ruminants from the State of Saxony-Anhalt, Germany, 2018

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6.1 Abstract

Arthropod-borne Batai virus (BATV) is an *Orthobunyavirus* widely distributed in European livestock and has been linked to febrile diseases in humans. In Germany, BATV has been found in mosquitoes, and antibodies have recently been detected in ruminants. We have therefore conducted a follow-up study in ruminants from Saxony-Anhalt, the most affected region in eastern Germany. A total of 325 blood samples from apparently healthy sheep, goats and cattle were tested using BATV-specific real-time polymerase chain reaction and serum neutralization tests. No viral RNA was detected, but the presence of antibodies was confirmed in sheep (16.5%), goats (18.3%) and cattle (41.4%) sera respectively. Sera were further analyzed by a glycoprotein Gc-based indirect ELISA to evaluate Gc-derived antibodies as a basis for a new serological test for BATV infection.

6.2 Introduction

Batai virus (BATV) is one of the most widespread members of the genus *Orthobunyavirus* within the family *Peribunyaviridae*. Its distribution ranges from

Malaysia to Asian Russia and India and in Europe from Scandinavia to Italy and Romania (Dutuze et al. 2018). In Africa, the virus was described as Ilesha virus in Sudan, Cameroon, Nigeria, Uganda, and Central Africa (Dutuze et al. 2018; Hubálek 2008).

The negative-strand RNA genome is segmented with small, medium and large (S, M, and L) segments and encodes four structural and two non-structural proteins: The S segment encodes the N and NSs proteins, the M segment encodes the two glycoproteins Gn and Gc and the NSm protein, and the L segment encodes the RNA-dependent RNA-polymerase. The glycoproteins and the non-structural NSm protein are encoded as precursor polyprotein that is co-translationally processed by host proteases to produce the three proteins (Elliott 2014). Especially, the glycoprotein Gc is supposed to be responsible for virus attachment and entry into vertebrate and invertebrate cells (Elliott 2014). Here, we describe the expression and purification of a BATV Gc subunit and the establishment of an indirect ELISA in order to subsequently screen ruminant sera for Gc specific antibodies.

As arthropod-borne virus, BATV is transmitted in a principally domestic animal-zoophilic mosquito cycle (Hubálek 2008). Vertebrate hosts are pigs, horses, ruminants and several bird species (Hubálek 2008). Infections with the Chittor strain in India were reported to cause mild diseases in sheep and goats (Singh and Pavri 1966). In contrast, in Europe no associated ruminant disease has been described, yet. However, a BATV infection was detected in a German captive harbour seal with manifested encephalitis (Jo et al. 2018). In humans, infection with BATV has been associated with influenza like symptoms including fever, bronchopneumonia, tonsillitis, and gastritis (Sluka 1969). Main vectors for BATV in Europe are mosquitoes of the species *Anopheles maculipennis* sensu lato, *Anopheles claviger, Coquillettidia richiardii* and less often *Ochlerotatus punctor* and *Ochlerotatus communis* (Hubálek 2008). Two molecular surveys in 2009 (Jöst et al. 2011) (in Southwest Germany) and 2012/2013 (in Northeast Germany) (Scheuch et al. 2018) revealed the presence of BATV in anopheline and culicine mosquitoes and also BATV antibodies were found in ruminants (Ziegler et al. 2018). The present study was implemented as follow up study of the previously mentioned surveys. For this purpose, a total number of 325 blood samples from sheep, goats and cattle were collected in Saxony-Anhalt in East Germany selecting flocks that showed high prevalence of BATV in the earlier study (Ziegler et al. 2018). Moreover, we evaluated an indirect ELISA for screening of BATV Gc specific antibodies in ruminants.

6.3 Materials and Methods

6.3.1 Sample collection

In the context of diagnostic testing, a total of 325 blood samples (serum and plasma) from apparently healthy sheep, goats, and cattle were provided by the “Landesamt für Verbraucherschutz” in Stendal, Germany. In detail, 60 goats from four flocks, 121 sheep from 11 flocks, and 144 cattle from 13 flocks were sampled in 2018 from throughout the whole state of Saxony-Anhalt.

6.3.2 Ethics statement

Blood samples were collected during obligatory monitoring schemes for other diseases by the “Landesamt für Verbraucherschutz” in Stendal, Germany and provided for our study.

6.3.3 Real-time RT-PCR (qRT-PCR)

RNA isolation from serum and plasma was performed using TRIzol LS Reagent (Life Technologies, Carlsbad, USA) and Viral RNA Mini Kit (Qiagen, Hilde, Germany) in combination following manufacturer`s instructions. The qRT-PCR for BATV was carried according to a previously published protocol using primers and probes which target a 99-nucleotide region of the S segment (Jöst et al. 2011). Additionally, as internal control system IC2 was included (Hoffmann et al. 2006), using duplex real-time PCR.

6.3.4 Serum neutralization test

All serum samples of sheep, goats and bovines were analyzed in a specific serum neutralization test (SNT) by using BATV strain 53.2 (acc. No. HQ455790, kindly provided by J. Schmidt-Chanasit, BNITM Hamburg, Germany). SNT was performed as described by Seidowski et al. (2010) and Ziegler et al. (2015) Modifications were made by using Vero E6 cells (Collection of Cell Lines in Veterinary Medicine, Friedrich-Loeffler-Institut, Germany) and applying an incubation time of 6 days. Briefly, virus concentration of 100 $TCID_{50}$/well was added to each sample running in duplicate at a starting serum dilution of 1:10. Cytopathic effects were seen 4-6 days post infection. The neutralizing antibody titer of the samples was defined as the 50% neutralization dose (ND_{50}). ND_{50} results of 10 or higher were qualified positive.

6.3.5 Recombinant glycoprotein Gc

A synthetic gene optimized for expression in E. coli was produced by Eurofins based on a partial BATV sequence (HQ455791) encompassing nucleotide position 601-1650. The sequence code for putative domains I and II of glycoprotein Gc was cloned into E. coli expression vector pET21a using 5' BamHI and 3' XhoI restrictions sites and expressed in BL21-Lys cells. Expression of the recombinant protein and purification by Nickel chelating agarose was carried out under denaturing conditions as described before (Jäckel et al. 2014). Finally, the protein was dialyzed against 0.05 M carbonate-bicarbonate buffer pH 9.6 and checked by SDS-PAGE and Coomassie-staining.

6.3.6 Indirect ELISA

The novel indirect inhouse ELISA is based on a partial recombinant BATV glycoprotein Gc which was used for coating immunoplates at a dilution of 2µg/ml in 0.05 M carbonate-bicarbonate buffer pH 9.6 (100 µl per well). Protocol parameters, dilutions, optimal reagent concentrations and the selection of immunoplates were determined by standard checkerboard titration and the combination with the highest positive to negative control difference (in OD values) was chosen. After overnight incubation of the coated immunoplates at 4 °C, plates were washed three times with 300 µl washing buffer containing phosphate-buffered saline (PBS) pH 7.2 and 0.1% Tween 20. After blocking with 200 µl/well 10% skim milk powder (DIFCO™) diluted in PBS for 1 h at 37 °C in a moist chamber, ruminant field sera in a dilution of 1:10 in PBS containing 2% skim milk were added in duplicates to the plates. As positive controls, polyclonal hyperimmune rabbit and sheep sera were diluted 1:20 and 1:10, respectively. 100 µl sera dilutions and controls were added to the plates. After incubation at 37 °C for 1 h in a moist chamber, plates were again washed three times with washing buffer. 100 µl per well of horseradish peroxidase (HRPO) conjugated Protein G (Calbiochem) diluted 1:5000 in dilution buffer was then added to the wells respectively and incubated again for 1h as described before. After a final washing step, 100 µl per well of 2,20-azinodiethylbenzothiazoline sulfonic acid (ABTS, Roche, Mannheim, Germany) substrate was added and plates were incubated for 30 min at room temperature in the dark. The reaction was stopped by adding 1% sodium dodecyl sulphate (SDS) and the optical density (OD) values were determined at 405 nm. The results were expressed as a percentage of the positive control serum (PP value) using the following formula: (mean OD of duplicate test serum/median OD of duplicate positive control) *100. Cut-off value, sensitivity and specificity of the indirect ELISA were determined in correlation to the SNT results using a receiver operating characteristic analysis (ROC analysis) with regard to the criterion ‘‘maximization of sensitivity and specificity’’. Calculations

were performed using the program ‘‘R’’ and the R package “OptimalCutpoints” (López-Ratón et al. 2014; R Core Team 2020).

6.3.7 Statistical analysis

The estimated prevalence and 95% confidence intervals (95% CI) were calculated using the calculation tool of Epitools (https://epitools.ausvet.com.au/ciproportion).

6.4 Results

A total of 325 ruminants (60 goats, 121 sheep, and 144 cattle) from Saxony-Anhalt in East Germany were tested by qRT-PCR for BATV genomes. No BATV-specific RNA was detected. The serological analysis was performed by a specific SNT and additionally by an indirect ELISA. 4 out of 144 blood samples from cattle, showed cytotoxic effects on the cells and therefore were not included in the data evaluation. Of the remaining 140 cattle samples, 58 specimen revealed neutralizing antibodies (seroprevalence of 41.4%) (Table 6). Thereby, in almost every investigated flock antibody-positive bovines were detected. The prevalence in sheep and goats were in similar range (16.5% and 18.3%, respectively). An indirect ELISA was implemented and resulting OD values were compared with results from SNT, which were used as reference and gold standard. 11 of 140 cattle sera were not tested in the ELISA, due to low sample volume. The highest correlation was achieved with a cut-off at PP value 18.2 for cattle, yielding a specificity of 80.5% and a sensitivity of 80.8%, for sheep cut-off at PP value 20.3, yielding a specificity of 73.3% and a sensitivity of 85,0%, and for goats a cut-off at PP value 27.9%, yielding a specificity of 83.7% and a sensitivity of 100% (Figure 11). Both assays classified 69 of the 310 analyzed sera as positive (Table 7). In addition, 51 sera were ELISA positive, but negative in SNT. Interestingly, 37 of these 51 samples had low PP values of ≤ 36%. Only 13 sera were positive in the SNT, but were found negative by the ELISA.

6.5 Discussion

The presence of BATV antibodies in cattle, goat, and sheep samples collected in the time period 2013 to 2016 in East Germany was demonstrated recently. High BATV seroprevalences were found in Saxony-Anhalt (36.4%, 38.8% and 44.7%, respectively (Ziegler et al. 2018). Thus, it was assumed that ruminants are susceptible for BATV and the virus is endemically circulating in Eastern Germany (Ziegler et al. 2018). Eventually the detection of viral RNA in anopheline and culicine mosquitoes in the very

same region confirmed this conclusion (Scheuch et al. 2018). In Southwestern Germany, BATV was detected in mosquitoes even earlier (Jöst et al. 2011). However, a subsequent seroprevalence study in ruminants only revealed three antibody positive bovines out of 548 tested animals (Hofmann et al. 2015) which indicates regional prevalence differences within Germany.

To further monitor the circulation in the most affected region in Eastern Germany, the present follow up study conducted a molecular and serological analysis in 18 ruminant flocks in Saxony-Anhalt (Figure 12). No viral RNA was detected which indicates the animals were not viremic during period of sampling.

So far, serological investigation for BATV were conducted by HI, VNT, plaque reduction neutralization tests (PRNT), and by immunofluorescence assay (IFA) which are sensitive methods, but require to cultivate live virus and therefore require higher biosafety standards (Medlock et al. 2007; Lambert et al. 2014; Hofmann et al. 2015; Ziegler et al. 2018). Hereby, we implemented an indirect ELISA based on the partial recombinant BATV glycoprotein Gc for BATV Gc-specific antibodies. ELISAs based on recombinant proteins are fast and reliable allowing testing of large numbers of samples for monitoring purposes. Compared to the SNT the here established ELISA exhibits suitable sensitivity and specificity levels for principal serology. Hence, we recommend to screen samples with the ELISA first, and to verify the positive samples in the SNT in a second step, especially samples with low OD nearby the cut-off value. In future, efforts could be made to increase the sensitivity and specificity of the ELISA and to reduce the application of SNT.

Antibodies against BATV were found in all species. Sheep and goats showed moderate BATV prevalences (16.5% and 18.3% respectively) whereas cattle had the highest antibody incidence (41.4%). Thus, in 2018 we observed a significantly lower seroprevalence than in the earlier study. Obviously, this could also be due to a sampling artefact by testing different sets of animals respectively. Interestingly, when ELISA positive samples were tested by SNT, the more recent samples gave lower antibody titers: in 2018 only few samples showed a titer of 1:640, but most of the titers did not exceed 1:120, whereas in 2013 to 2016 antibody titers were up to 1:2560 (Ziegler et al. 2018).

In other countries antibody titers detected by hemagglutination inhibition test ranged from 1% to 46% in bovines and ovines in Finland, Austria, Slovakia, Portugal, Romania and former Yugoslavia (Medlock et al. 2007). A recent study in northern Italy found nine antibody positive bovines out of 128 tested animals corresponding to a prevalence of 7.0% (Lambert et al. 2014). In humans the prevalence of hemagglutination inhibition (HI) antibody was generally very low (<1%) in Sweden, Finland, Germany, Austria, and former Yugoslavia, but reached 32% in southern Slovakia (Medlock et al. 2007). These previously published data along with the results of our study show that BATV is circulating widely in Europe in low to moderate extent.

Besides of mild flu-like illnesses in humans (Sluka 1969), infections with BATV seem to cause no clinical symptoms in most vertebrate hosts, and therefore may play a minor role for human and veterinary public health. However, a German grey harbor seal that suffered from fatal encephalitis was infected with BATV (Jo et al. 2018). Furthermore, infection with the closely related Cache Valley virus in America is associated with stillbirth and congenital abnormalities in ruminants (Chung et al. 1990) and encephalitis in humans (Sexton et al. 1997).

BATV contributed as parental segment donor in a natural reassortment event (Briese et al. 2006). Coinfection of BATV and Bunyamwera virus (BUNV) resulted in the generation of virulent progeny, so-called Ngari virus (NRIV), which caused two major hemorrhagic fever outbreaks in humans in Africa (Dutuze et al. 2018). Apparently, this reassortment led to an increase in pathogenicity. However, infection studies with NRIV and its parental viruses in ruminants are scarce or even missing (Dutuze et al. 2018). The reassortent NRIV which possesses the S and L segments from BUNV combined with the M segment of BATV probably evolved in coinfected mosquitoes (Dutuze et al. 2018), as mosquitoes can feed on different vertebrates that could be infected with distinct viruses (Elliott 2014). M segment gene products (Gc, Gn and NSm) have a major influence on vector competence (ability to efficiently transmit a virus) (Elliott 2014). Thus, the risk of NRIV transmission by autochthonous mosquitoes in Europe, which are demonstrated to be competent for BATV, should not be neglected and could be addressed by vector competence studies.

6.6 Tables and Figures

Table 6. Serological analysis of the ruminant blood samples for BATV with virus neutralization test (SNT).

species	flocks	tested animals	positive flocks	positive animals	Prevalence (%)	95% CI
goats	4	60	1	11	18.3	10.6 - 29.9
sheep	11	121	6	20	16.5	11.0 - 24.2
cattle	13	140	11	58	41.4	33.6 - 49.7
total	18	321	89	89	27.7	23.1 - 32.9

Table 7. Summary of SNT and indirect ELISA results for each species a) sheep, b) goats, c) cattle, and d) in total.

a) sheep

	ELISA positive	ELISA negative	total
SNT positive	17	3	20
SNT negative	27	74	101
total	44	77	121

b) goats

	ELISA positive	ELISA negative	total
SNT positive	11	0	11
SNT negative	8	41	49
total	19	41	60

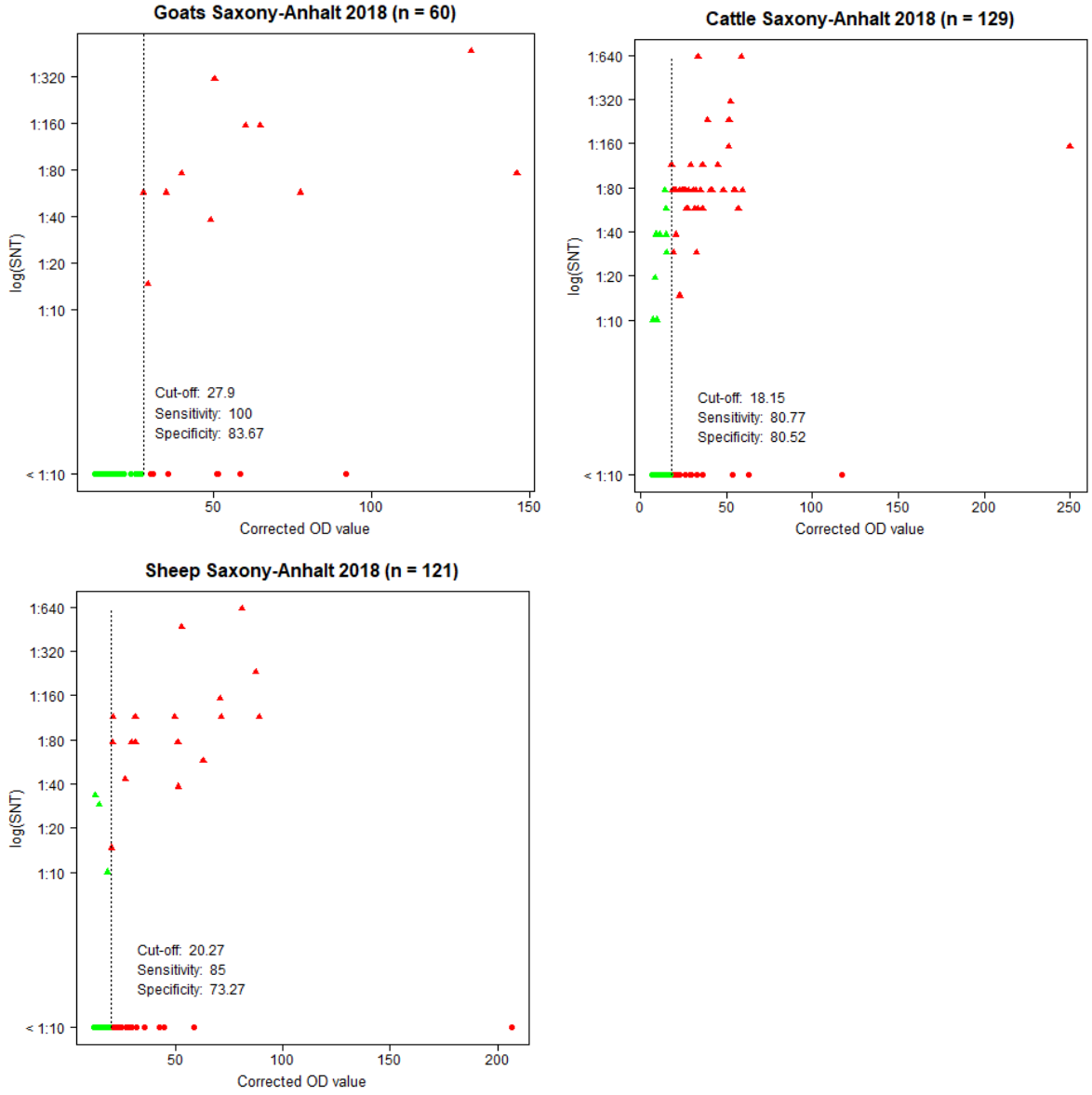
c) cattle

	ELISA positive	ELISA negative	total
SNT positive	41	10	51
SNT negative	16	62	78
total	57	72	129

d) all species

	ELISA positive	ELISA negative	total
SNT positive	69	13	82
SNT negative	51	177	228
total	120	190	310

Figure 11. Corrected OD values of the ELISA in relation to neutralization titers (log (SNT)) of the SNT showing cut-off, sensitivity and specificity for each species. Green dots: SNT and ELISA negative samples. Green triangles: SNT positive, but ELISA negative samples. Red triangles: SNT and ELISA positive samples. Red dots: SNT negative, but ELISA positive samples.



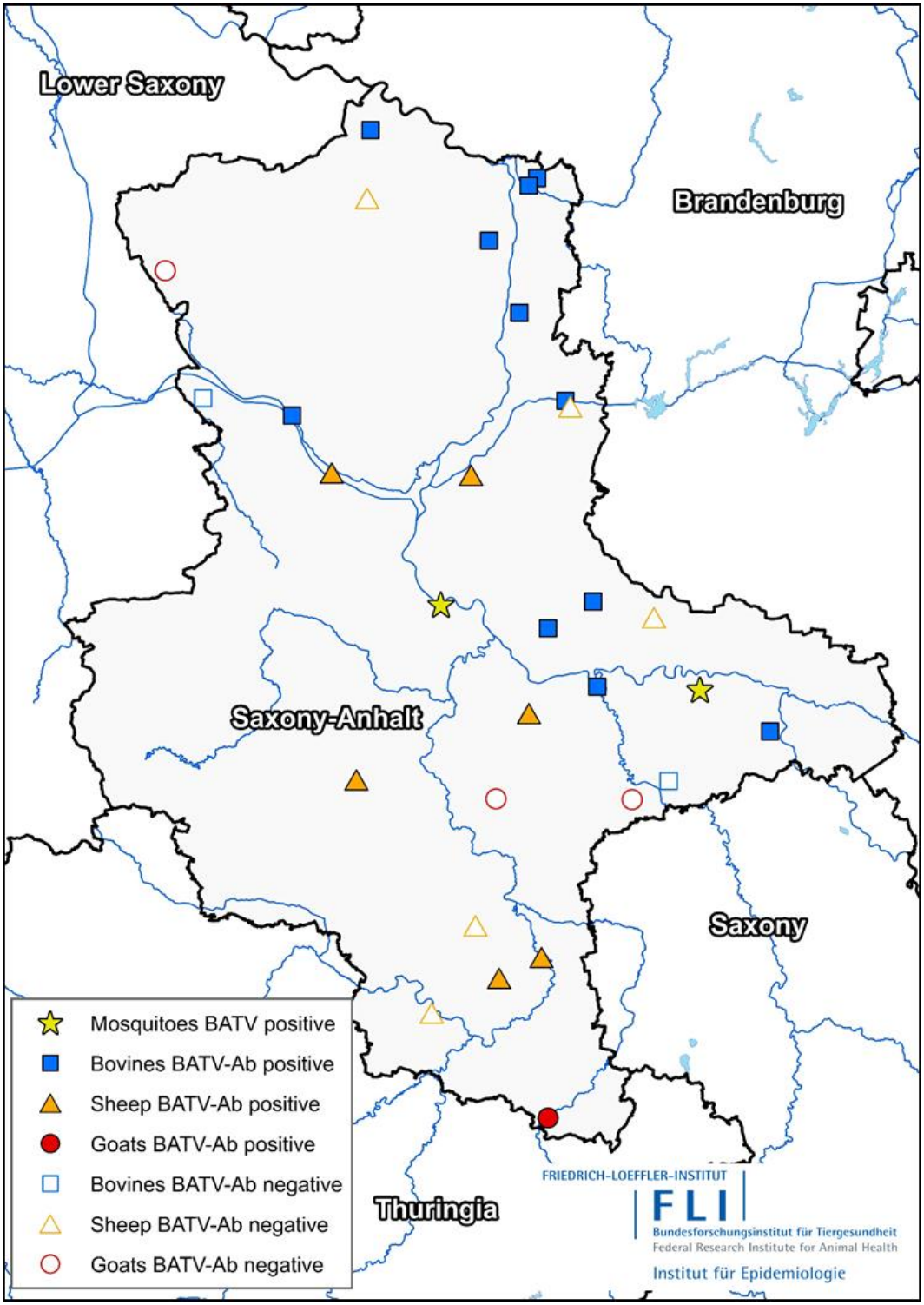


Figure 12. Geographical distribution of the investigated ruminant flocks in Saxony-Anhalt in combination with the serological results (BATV-Ab = BATV antibodies) and the sites with BATV-positive mosquitoes (virus or viral RNA).

6.7 Acknowledgments

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7 General discussion

Ngari virus (NRIV) emerged from a natural reassortment event through coinfection of Bunyamwera virus (BUNV) and Batai virus (BATV) (Briese et al. 2013a). Intriguingly, NRIV is more virulent than its parental viruses. While BATV and BUNV are described to cause no or only mild febrile illnesses, an infection with NRIV in humans and ruminants might evolve in hemorrhagic fever (Dutuze et al. 2018). Other hemorrhagic fever viruses amongst others are Rift Valley fever virus (RVFV) and Crimean-Congo hemorrhagic fever virus (CCHFV) which are also members of the order Bunyavirales. While BUNV, BATV, NRIV and RVFV are transmitted by hematophagous arthropods, CCHFV is transmitted by ticks.

CCHFV causes probably the most widespread tick-borne viral infection of humans (Bente et al. 2013). Apart from infection by tick bites, humans are infected also by contact with blood or other body fluids of infected patients or animals (Bente et al. 2013). Susceptible animals such as cattle, sheep, camels, hares and ostriches show no signs of illness, but only develop a transient viremia (Bente et al. 2013). In contrast, infections in humans are associated with high fever, headache, myalgia, vomiting, diarrhea, and hemorrhages.In severe cases the infection can lead to a fatal course, whereby reported mortality rates vary widely from 2 to 30% (Weidmann et al. 2016).

RVFV is transmitted by more than 30 mosquito species of 6 different genera (especially Aedes and Culex mosquitoes) (Hubálek et al. 2014). However, in most cases humans get infected by direct or indirect contact with infected livestock, especially during slaughtering, butchering and necropsy (Chevalier 2013). Therefore, professional groups like veterinarians, health personnel, farmers, and abattoir workers are at high risk of infection (Bird et al. 2009). Infected humans usually show only a self-limiting febrile illness, whereas in 1-2 % of patients, the infection may progress to a more severe disease characterized by hepatitis, retinitis, encephalitis and hemorrhagic fever (Chevalier 2013). In sheep, goats and cattle, RVFV infection induces abortion storms and/or acute deaths in newborns (Pepin et al. 2010). Outbreaks of RVF in livestock cause severe economic losses as well as pose a threat to the human population, since it has been noted that human epidemics are preceded by epizootics in livestock (McElroy et al. 2009). Therefore, continuous monitoring of the livestock population is a mandatory tool for the RVFV infection control in endemic countries to detect first evidence of potential outbreak events (FAO 2018). Outbreaks of NRIV, RVFV, and CCHFV have been reported throughout sub-Saharan Africa and the cocirculation of NRIV with RVFV as well as BATV and BUNV with RVFV have been observed (Bowen et al. 2001; Eiden et al.; Dutuze et al. 2020). The first concurrent outbreak of RVFV and NRIV was retrospectively noticed in Kenya and Somalia, when 23% of the hemorrhagic fever cases were diagnosed as RVFV and 27% as NRIV infections (Bowen et al. 2001). In Mauritania, NRIV was isolated from goats during a laboratory

confirmed RVFV outbreak in the human and livestock population (Eiden et al.; Rissmann et al. 2017a; Jäckel et al. 2013b). Since the clinical picture associated with a NRIV infection is indistinguishable to that of a RVFV infection, diagnostic assays are necessary to reveal the causative agent. However, the available diagnostic capabilities for NRIV and its parental viruses are limited (Dutuze et al. 2018). Hence, the aim of the first study was to provide molecular and serological assays for NRIV, BATV, and BUNV to investigate their presence in the livestock population and possible cocirculation with RVFV in Mauritania. Therefore, almost 500 serum samples from small ruminants were collected during a confirmed RVFV outbreak and analyzed by PCR, serum neutralization tests (SNT), a commercial RVFV ELISA and by newly established indirect ELISAs based on the recombinant glycoproteins Gc of each NRIV, BATV, and BUNV. No viral RVFV RNA was detected, but 17.7% of the investigated animals showed IgG/IgM antibodies. Hence, we observed an increase in the antibody incidence in 2015 compared to the inter-epidemic phase in 2012/13, when 3.8% of small ruminants revealed RVFV-specific antibodies (Rissmann et al. 2017a). If the regions are assessed separately, animals from Brakna, Tagant, and Assaba, in which the RVFV outbreak occurred in 2015, developed the highest antibody incidence (29.4%, 53.7%, and 42.9%, respectively). The first RVFV positive case was reported in Assaba in mid of October (OIE 2020). At the same time, we collected serum samples from 32 sheep of which 16 samples showed RVFV specific IgM antibodies underlining the ongoing virus circulation in Assaba. The goat-derived samples from Assaba were collected one month before the first reported RVFV case and only one sample was tested positive for IgM antibodies. Likewise, in the other investigated regions only few IgM positive animals were detected. Therefore, the overall IgM antibody prevalence was only 4.80%. No viral RVFV RNA was detected, but sequencing revealed two positive samples for NRIV and two positive samples for BUNV from one goat and three sheep. Both NRIV and both BUNV positive samples were negative for RVFV specific antibodies. However, a total of 61 NRIV, BATV and/or BUNV seropositive samples contained also antibodies to RVFV, indicating a cocirculation of these viruses in the animal population in Mauritania. As the RVFV genome sequence is highly distinct from to the *Orthobunyavirus* sequences, cross-reactivities are unlikely. The serological investigation for *Orthobunyaviruses* revealed a prevalence of 9.0% for NRIV-specific antibodies, a prevalence of 2.0% for BATV-specific antibodies, and a prevalence of 0.7% for BUNV-specific antibodies. However, the results should be interpreted carefully, since an unambiguous confirmation was not possible for many samples. In most cases, the SNT based on NRIV and the SNT based on BATV showed undistinguishable high antibody titers, whereby in the BATV based SNT the titer reached up to 1:1280 and in the NRIV SNT even up to 1:2560. In contrast, in the BUNV specific SNT the titer did not exceed 1:40, but mostly ranged between 1:10 and 1:20. BUNV seems to induce hardly neutralizing antibodies and to cross-reacts less with NRIV and BATV than the latter among themselves. A limited cross-reaction between

BUNV and BATV was already described for the plaque reduction neutralization test (Hunt and Calisher 1979b). The prevalence determined by the BUNV based SNT is surprisingly low, since we isolated two BUNV sequences and therefore expected to detect a stronger immune response in the investigated ruminants. The BUNV Gc based ELISA detected twice as many positive samples than the SNT based on BUNV which is also indicative for a low neutralizing antibody activity of BUNV. Neither the ELISAs allow an unambiguous differentiation between NRIV, BATV, and BUNV for every sample. As in the SNTs, a strong agreement was observed between the ELISA based on NRIV Gc and the ELISA based on BATV Gc. However, as no BATV RNA was isolated, but only NRIV and BUNV RNA, the antibodies that were detected by the BATV serological tests were probably caused by an infection with NRIV. Neutralizing antibodies are induced by the surface glycoproteins which are encoded by the virus M segment (Briese et al. 2013a). BATV is the M segment donor of NRIV showing only 11% and 5% differences in nucleotide or deduced amino acid sequence, respectively (Briese et al. 2006b). Hence, the close relationship between NRIV and its parental viruses complicates the unambiguous serological detection. On the other hand, the strong agreement among the assay results offers the advantage to use the BATV SNT instead of the NRIV SNT and hereby enables to work in a lower biosafety containment facility. Overall, the first study demonstrates that NRIV and BUNV were cocirculating in the small ruminant population during the Mauritanian RVFV outbreak in 2015/16. Prevalence studies for the *Orthobunyaviruses* are complicated by high cross-reactivity, especially between NRIV and BATV serological assays. Future attempts might consider establishing tests that are based on the nucleoprotein Np to distinguish BATV and NRIV induced antibodies.

More evidence of RVFV and CCHFV was found in countries in northern Africa such as Egypt, and on the Arabian Peninsula (Linthicum et al. 2016; Bente et al. 2013). In Egypt, the first outbreak of RVFV was reported in 1977, which till today remains the largest epidemic with an estimated number of 200,000 human infections, almost 600 deaths, and high economic losses in livestock (Bird et al. 2009). Smaller outbreaks occurred in 1993/94, 1997 and most recently in 2003 (Ahmed Kamal 2011). The import of infected livestock, especially camels from Sudan, was identified as the main source for virus introduction into Egypt (Ahmed Kamal 2011). Likewise, CCHF viral genome was detected in ticks infested on camels that were imported from Sudan and Somalia (Chisholm et al. 2012). It remains unclear, whether the ticks were infected and infested the camels before or after importation. The repeated serological evidence of CCHFV in Sudanese livestock indicates that CCHFV is endemic in this country which is an important trading partner of Egypt, e.g. importation of animals like camels and cattle (Ibrahim et al. 2015; Adam et al. 2011; Suliman et al. 2017). Besides of CCHFV antibody positive camels and cattle, nosocomial outbreaks and sporadic cases of infections in humans have been reported in the same regions in Sudan, namely Kordufan, North Kordufan and Dafur (Aradaib et al. 2011; Aradaib et al. 2010; Elata et

al. 2011; Bower et al. 2019; Rahden et al. 2019). That underlines that the seroprevalence in livestock correlates with the seroprevalence in humans and that livestock play a major role in the transmission cycle of CCHFV in humans (Bente et al. 2013). Hence, the aim of the second study was designated to monitor the prevalence status of RVFV and CCHFV in the livestock population in Egypt and to evaluate the risk for virus introduction from neighbour countries into Egypt and the occurrence of hemorrhagic diseases in livestock and human population.

For this purpose, a total number of 1254 apparently healthy animals including sheep, buffaloes, camels and cattle were sampled in 13 Egyptian governorates surrounding the Nile Delta and Nile River (Sohag, Assiut, Al Fayum, Matrouh, Aswan, Giezeh, Kafr-El-Sheikh, Dakahliya, Sharkia, Menoufia, Beni-Suef, Ismailia and Gharbia).The camels originated from Sudan and were imported to a quarantine station in Aswan. Serum samples were first tested in a quantitative RT-PCR for RVF genomes and a second RT-PCR detecting CCHFV genomes. The serological analysis for RVFV consisted of the ID Vet competition ELISA, the ID Vet IgM capture ELISA and an indirect in-house IgM ELISA for camelids (Rissmann et al. 2017a), the serum neutralization test (SNT) using the RVFV MP-12 strain (OIE 2019) for inconclusive samples, and as additional confirmation an adapted commercial immunofluorescence assay (IIFA). For detection of CCHFV-specific antibodies a commercial double-antigen sandwich ELISA and a modified commercial CCHF IFA were performed. Antibodies against RVFV were found in all species. Sheep and cattle showed lower RVFV prevalences (1.2% and 1.3%, respectively) compared to camels and buffaloes (16.0% and 20.7%, respectively). One sheep sample that was taken in Aswan governorate next to the border to Sudan was tested positive for RVFV IgM antibodies. A natural infection with a wild strain of RVFV in Egypt can be assumed here, since the 2-year-old sheep was born and bred in Egypt and not vaccinated against RVFV. Our findings are in line with former studies conducted in 2014 and 2015 showing a prevalence of 0.46% in sheep (Mroz et al. 2017b). Our current study revealed a lower prevalence of RVFV in cattle (1.3%) than reported in former studies (Mroz et al. 2017a; Marawan et al. 2012) (7.9% and 19.5%, respectively). The difference between the prevalence levels might be explained by regional differences of the collection sites. In the Nile Delta north to Cairo livestock on animal markets are traded between different governorates primarily, whereas livestock trade on markets in the south of Cairo is more local (i.e. same district) (Napp et al. 2018). Trading animals over greater distances poses a higher risk of spreading pathogens like RVFV over a larger region.

In our study, 20.7% of the tested buffalo sera contained RVFV-specific antibodies which compares to earlier results of 22% in buffaloes of small holding farms (Mroz et al. 2017b) while no RVFV specific antibodies were detected on large farms (Mroz et al. 2017b). This may have been caused by lower hygiene standards in small holding flocks, which promotes horizontal spreading to contact animals. Furthermore, repeated reports on the presence of RVFV antibodies in buffaloes during inter-epidemic periods

indicate an ongoing circulation of RVFV in the buffalo population and underline their potential role as amplifying hosts (Beechler et al. 2015; Mroz et al. 2017a; Mroz et al. 2017b; Jori et al. 2015; LaBeaud et al. 2011). Our serological investigation of camel sera resulted in a prevalence of 16.0%. The result is in accordance with a recent study conducted by El Bahgy et al. (El Bahgy et al. 2018) showing a prevalence of 16.6% in camels that were imported from Sudan as well. In contrast, Mroz et al. (2017b) described a much lower antibody prevalence (3.2%) for camels in the Nile delta. Moreover, these sera were taken from younger camels (2-7 years), whereas in this present study 8-15 year-old camels were tested. The association between the infection with RVFV and higher age has been already described before and is probably attributed to frequent exposure of older camels to mosquito vectors (Olaleye et al.; Sindato et al. 2015; Abdallah et al. 2015).

In our investigation, antibodies against CCHFV were only found in cattle and camels resulting in a prevalence of 3.3% and 85.6%, respectively, whereas the 63 sheep and 36 buffalo samples were negative for CCHFV. Clearly, the small number of samples tested cannot reflect the situation throughout Egypt. However, it indicates the minor role of sheep and buffaloes in the transmission cycle of CCHFV in Egypt, which has been already been observed before (Morrill et al. 1997; Darwish et al. 1977; Mohamed et al. 2008). Similarly, only a small number of the native Egyptian cattle are found to carry CCHFV antibodies which confirms an earlier report (3,8%) (Mohamed et al. 2008). The current low prevalence rates in the native livestock population in Egypt leads to the conclusion, that CCHFV might circulate only sporadically, but is not playing a major role. In contrast, the analysis of the camel specimens yielded a high CCHFV seroprevalence, but all animals originated from Sudan. Previous studies on imported camels into Egypt reported lower prevalence rates of 14% (Morrill et al. 1990) and 21.3% (Suliman et al. 2017). This might again be due to age effects: camels in the here presented study were of older age ranging from 8 to 15 years, while Suliman et al. divided the animals into a group of young (< one year) and old camels (> one year) without stating the exact age of the older group (Suliman et al. 2017). As several surveys could show, seropositivity is more common in adult than in young animals (Bente et al. 2013). Furthermore, antibody positivity depends on the presence of tick vectors, which might be regionally different in Sudan. In Egypt, the isolation of CCHFV genome was succeeded once in Hyalomma ticks which were collected from slaughtered camels originating from Sudan and Somalia (Chisholm et al. 2012). However,it is not possible to conclude whether the ticks acquired the infection in Egypt or in the country of origin. The occurence of tick vectors for CCHFV in Egypt was proven by Darwish et al. (1978), but so far there is no evidence that transmission of this virus occurred in Egypt. Therefore, further studies are necessary to investigate whether the regional occurrence of CCHFV vectors correlates with the presence of livestock stables and on that basis, to evaluate the risk of transmission and spread of CCHFV within the Egyptian livestock population.

Overall, the results obtained from the second study indicate that the highest risk of introduction of RVFV and CCHFV into Egypt is by the import of infected animals from Sudan. All imported bovines are slaughtered at the quarantine facility, which reduces the spreading risk for imported pathogens. In contrast, imported camels may be transported to slaughterhouses or animal markets after quarantine times (Napp et al. 2018). Although camels are quarantined for three days after coming from Sudan, the infection of animals might stay clinically undetected since the infection with RVFV in camels often remains unapparent and CCHFV infected animals in general show no clinical symptoms (Ahmed Kamal 2011; Bente et al. 2013). Besides, quarantine time of three days is very short. Camels could be still infectious, when they are released, since the viremic phase last for up to 14 days post infection (Pepin et al. 2010; Leblebicioglu et al. 2015). Moreover, a local virus circulation at the quarantine station is also possible.

To reduce the risk of introduction of pathogens into Egypt, strict compliance of quarantine measures should be mandatory containing a high standard of hygiene, protection of personnel, a well-planned RVFV vaccination program and treatment of animals with ascaricides against tick vectors of CCHFV. Furthermore, continuous monitoring of the livestock population is an important part of disease control and prevention, as an infection with CCHFV is clinically unapparent in animals and human RVFV epidemics are often preceded by epizootics in livestock.

In general, global livestock trade increases the risk of introduction of viral pathogens from endemic to pathogen-free regions, e.g. in Europe. Additionally, climate warming could favour the expansion of competent vectors and thus affect the geographical distribution of arboviruses (Chevalier 2013). BATV is the only member of the Bunyamwera serogroup which has been so far detected in Europe and even in Germany (Hubálek 2008). Virus isolation from anopheline and culicine mosquitoes and antibody detection in ruminants have proven the circulation of BATV in eastern as well as southwestern Germany (Hofmann et al. 2015; Jöst et al. 2011; Ziegler et al. 2018; Scheuch et al. 2018). Thereby, regional differences in prevalence rates within Germany became apparent. In the state of Baden-Württemberg, a seroprevalence of 0.55% was found in cattle (Hofmann et al. 2015), while the prevalence in Sachsen-Anhalt was 36.4% for cattle, 38.8% for goats, and 44.7% for sheep (Ziegler et al. 2018). Traditionally, detection of BATV in ruminants was conducted by PCR, hemagglutination inhibition test, immunoblot, immunofluorescence assay and virus neutralization test (Jöst et al. 2011; Hofmann et al. 2015; Ziegler et al. 2018; Medlock et al. 2007). These sensitive serological tests can only be carried out in laboratories with higher biosafety standards due to the use of cultivated live virus. Hence, the third study was implemented to further monitor the circulation of BATV in the most affected region in Eastern Germany and to evaluate the use of an indirect ELISA based on the recombinant BATV Gc as screening assay. Blood samples of 60 goats, 121 sheep, and 144 cattle were collected and analysed by qRT-PCR, serum neutralization test

(SNT), and the novel indirect ELISA. No viral RNA was detected which indicates the animals were not viremic when the blood was drawn. However, antibodies against BATV were found in all species revealing moderate prevalences in sheep and goats (16.5%, and 18.3% respectively), and highest prevalence in cattle (41.4%). Thus, in 2018 we observed a significant decrease of seropositive small ruminants compared to the former study (prevalence of 38.8% for goats, and 44.7% for sheep) (Ziegler et al. 2018). Interestingly, the antibody titer in the SNT was lower as well: in 2018 only few samples showed a titer of 1:640, but most of the titers did not exceed 1:120, whereas in the former study the antibody titers reached up to 1:2560 (Ziegler et al. 2018). The performance of the newly established indirect ELISA based on the BATV Gc was evaluated in correlation to the SNT. Sensitivity (Se) and specificity (Sp) of the indirect ELISA were calculated using the receiver operating characteristic analysis (ROC analysis) which revealed a sensitivities and specificities of 80.77% and 80.52%, 85% and 73.27% and 100% and 83.67% for cattle, sheep and goats respectively. A small number of sera did not react with recombinant Gc antigen in the indirect ELISA, but were positive in the SNT. The recombinant Gc used in the ELISA lacks the glycosylation which are frequently neutralizing antibody targets. Moreover, other viral proteins such as the glycoprotein Gn, and the non-structural proteins Nss and Nsm may also trigger neutralizing antibodies. Vice versa, a number of borderline Gc antigen positive sera were negative in the SNT. These results might be explained by the presence of non-neutralizing antibodies against Gc protein which was already shown for envelope glycoproteins of other viruses (Burton 2002). Hence, we recommend to screen the samples first in the indirect ELISA, and to verify the positive samples by the SNT in a second step. Thus, the indirect ELISA is a useful diagnostic tool for large scale serological surveys. In contrast to already existing serological tests, the recombinant Gc protein is produced under BSL2 conditions. Therefore, the indirect ELISA would be suitable for screening studies in countries lacking high biosafety standards.

Former studies investigating the presence of BATV specific antibodies in European ruminant and human population revealed a high variation in the prevalence. It ranges from 1% to 46% in bovines and ovines in Finland, Italy, Austria, Slovakia, Portugal, Romania and former Yugoslavia (Medlock et al. 2007; Lambert et al. 2014) and in humans from less than 1% to 32% in Sweden, Finland, Germany, Austria, former Yugoslavia, and southern Slovakia (Medlock et al. 2007). An infection with BATV remains clinically unapparent in ruminants and causes only mild febrile disease in humans (Sluka 1969) and therefore plays a minor role in human and veterinary public health. However, the close related Cache Valley virus in America is reported to cause stillbirth and congenital abnormalities in ruminants (Chung et al. 1990) and encephalitis in humans (Sexton et al. 1997). Moreover, BATV is the M segment donor for the more virulent progeny, NRIV. M segment gene products (Gc, Gn and NSm) have a major influence on vector competence (Elliott 2014), therefore competent vectors for BATV

in Europe might be competent for NRIV as well Hence, vector competence studies are advisable to evaluate the risk of NRIV transmission by European mosquitoes.

In summary, the here presented studies could demonstrate that NRIV and BUNV circulate in the small ruminant population in Mauritania and might have contributed to the occurrence of hemorrhagic fever cases during the confirmed RVFV outbreak in 2015. The import of livestock, especially camels from Sudan poses a risk of introduction and spread of RVFV and CCHFV in Egypt. In Germany, BATV specific antibodies were detected in sheep, goats, and cattle, whereby a decrease in prevalence in small ruminants was observed compared to a previous study in the investigated area. Indirect ELISAs based on the glycoprotein Gc of each NRIV, BATV, and BUNV were established which can be used for large seroprevalence studies. However, further attempts are necessary to successfully distinguish between NRIV and its parental viruses.

8 Summary

Diagnostic approaches for zoonotic hemorrhagic fever viruses of the order Bunyavirales in livestock

Nicole Cichon

The natural reassortant Ngari virus (NRIV) derived from the coinfection of Batai virus (BATV) and Bunyamwera virus (BUNV). The latter viruses are described to cause unapparent or mild febrile disease in humans and ruminants, while the infection with NRIV is associated with hemorrhagic fever. Other zoonotic arboviruses of the same order, the Bunyavirales, are Rift Valley fever virus (RVFV) and Crimean-Congo hemorrhagic fever virus (CCHFV). All mentioned viruses are found in Africa, where hemorrhagic fever outbreaks threaten the human population and cause high economic losses in livestock. NRIV has been repeatedly isolated during concurrent RVFV outbreaks. Since the clinical disease associated with an NRIV infection is indistinguishable to that of a RVFV infection, diagnostic assays are necessary to verify the causative agent. However, the available diagnostic capabilities for NRIV and its parental viruses are limited. Hence, the aim of the first study was to provide molecular and serological assays for NRIV, BATV, and BUNV to investigate their presence in livestock population and possible cocirculation with RVFV. The molecular and serological analysis of almost 500 serum samples from small ruminants from Mauritania revealed the presence of NRIV and BUNV during a confirmed RVFV outbreak. Our investigation found no viral RVFV RNA, but two BUNV and two NRIV isolates were extracted from one goat and three sheep. Antibodies against RVFV as well as NRIV, BATV and/or BUNV were detected in a great number of tested animals. However, due to high cross-reactivity among the serum neutralization tests as well as the ELISAs the unambiguous determination of specific NRIV, BATV or BUNV antibodies was not possible for every sample. Additionally, possible coinfection might have complicated the assessment. Nevertheless, our findings demonstrate that NRIV and BUNV circulate in the small ruminant population in Mauritania and might have contributed to the occurrence of hemorrhagic fever cases during the confirmed RVFV outbreak in 2015.
The distribution of NRIV is largely limited to sub-Saharan Africa. In contrast, evidence of RVFV and CCHFV can also be found in countries in northern Africa such as Egypt. Main source for the introduction of RVFV is assumed to be the import of infected livestock, especially camels from Sudan, without taking sufficient quarantine measures. Since the infection in adult animals usually remains inapparent, the continuous monitoring of the livestock population is an important part of disease control

and prevention. Hence, the aim of the second study was to monitor the prevalence status of RVFV and CCHFV in the livestock population in Egypt and to evaluate the risk for virus introduction from neighbouring countries into Egypt. No RVF and CCHF viral RNA was found in about 1200 sera from Egyptian sheep, cattle and buffaloes, and in camels which were imported from Sudan. However, RVFV specific IgM antibodies were detected in a single sheep. Highest number of RVFV IgG positive samples were found among camels and buffaloes. Camels also had the highest CCHFV prevalence among the tested animals. These findings underline the potential role of buffaloes as amplifying hosts for RVFV, and consider the risk of introduction of RVFV and CCHFV into Egypt is the import of infected animals from Sudan.

In general, global livestock trade increases the risk of introduction of viral pathogens from endemic to pathogen-free regions, e.g. in Europe. Only BATV of the here mentioned arboviruses has been detected so far in Europe and even in Germany. Especially, in Eastern Germany high prevalence rates in the ruminant population were observed. BATV is usually detected in ruminants by PCR, hemagglutination inhibition test, immunoblot, immunofluorescence assay and virus neutralization test. These serological tests necessitate the use of cultivated live virus and therefore require work under higher biosafety standards. Hence, the third study was implemented to further assess the circulation of BATV in the most affected region in East Germany especially by using of a newly developed indirect ELISA based on the recombinant BATV Gc. No viral RNA was detected in the blood samples of 60 goats, 121 sheep, and 144 cattle. The serology revealed moderate prevalence rates in sheep and goats and highest prevalence in cattle. The performance of the new indirect BATV Gc ELISA was compared to the SNT. Due to the moderate numbers of false positive samples, an initial screening of samples by the indirect ELISA and the verification of the positive sera by SNT can be recommended, which by itself is also a desirable approach under biosafety aspects.

9 Zusammenfassung

Diagnostik von zoonotischen hämorrhagischen Fieberviren der Ordnung Bunyavirales bei Nutztieren

Nicole Cichon

Die natürliche Reassortante Ngari-Virus (NRIV) entstand durch die Koinfektion von Batai-Virus (BATV) und Bunyamwera-Virus (BUNV). Letztere Viren verursachen bei Menschen und Wiederkäuern lediglich inapparente oder nur leicht fieberhafte Erkrankungen, während eine Infektion mit NRIV mit hämorrhagischem Fieber assoziiert sein kann. Alle genannten Viren kommen in Afrika südlich der Sahara vor, wo Ausbrüche von hämorrhagischen Fiebern die menschliche Bevölkerung bedrohen und hohe wirtschaftliche Verluste beim Viehbestand verursachen. Andere zoonotische Arboviren derselben Ordnung der Bunyavirales sind das Rift-Valley-Fieber-Virus (RVFV) und das Krim-Kongo-Hämorrhagisches-Fieber-Virus (CCHFV). NRIV wurde wiederholt während RVFV-Ausbrüchen isoliert. Da die klinische Krankheit, die mit einer NRIV-Infektion einhergeht, nicht von einer RVFV-Infektion zu unterscheiden ist, sind diagnostische Tests erforderlich, um die Infektionen zu unterscheiden. Die verfügbaren diagnostischen Methoden für NRIV und seine parentalen Viren sind jedoch begrenzt. Daher bestand das Ziel der ersten Studie darin, molekulare und serologische Assays für NRIV, BATV und BUNV zu erarbeiten, um deren Präsenz in der Nutztierpopulation und eine mögliche Kozirkulation mit RVFV zu untersuchen. Die molekulare und serologische Analyse von fast 500 Serumproben von kleinen Wiederkäuern aus Mauretanien ergab, dass NRIV und BUNV während eines bestätigten RVFV-Ausbruchs zirkulierten. Bei den Untersuchungen konnte keine RVFV-RNA nachgewiesen werden, aber zwei BUNV- und zwei NRIV-Isolate von einer Ziege und drei Schafen wurden gefunden. Sowohl Antikörper gegen RVFV als auch gegen NRIV, BATV und/oder BUNV wurden bei einer großen Anzahl der getesteten Tiere nachgewiesen. Aufgrund der hohen Kreuzreaktivitäten zwischen den Serumneutralisationstests sowie den ELISAs war es jedoch nicht möglich, die detektierten Antikörper in jeder Probe eindeutig zuzuordnen. Ferner könnten Koinfektionen vorgelegen haben. Dennoch zeigen die Ergebnisse, dass NRIV und BUNV in der kleinen Wiederkäuerpopulation in Mauretanien zirkulierten und zum Auftreten von Fällen von hämorrhagischem Fieber während des RVFV-Ausbruchs im Jahr 2015 beigetragen haben.
Die Verbreitung von NRIV ist auf Subsahara-Afrika beschränkt. RVFV und CCHFV hingegen wurden auch nördlich der Sahara, z.B. in Ägypten nachgewiesen. Als Haupteintragsquelle für RVFV wird die Einfuhr von infizierten Nutztieren, insbesondere

von Kamelen aus dem Sudan vermutet, die ohne ausreichende Quarantänemaßnahmen eingeführt werden. Da die Infektion bei erwachsenen Tieren in der Regel unauffällig bleibt, ist die kontinuierliche Überwachung des Viehbestandes ein wichtiger Teil der Krankheitsbekämpfung und -prävention. Daher war das Ziel der zweiten Studie, den Prävalenzstatus von RVFV und CCHFV in der ägyptischen Nutztierpopulation zu überwachen und das Risiko einer Erregereinfuhr aus Nachbarländern und das damit verbundene Auftreten von hämorrhagischen Erkrankungen zu beurteilen. Bei der Analyse von etwa 1200 Serumproben von ägyptischen Schafen, Rindern und Büffeln und von aus dem Sudan eingeführten Kamelen wurde keine RVFV-RNA nachgewiesen, aber bei einem Schaf RVFV-spezifische IgM-Antikörper. Die höchste Anzahl RVFV-IgG-positiver Proben zeigte sich bei Kamelen und Büffeln. Kamele wiesen auch die höchste CCHFV-Prävalenz unter den getesteten Tieren auf. Diese Befunde unterstreichen die potenzielle Rolle von Büffeln als amplifizierende Wirte für RVFV und deuten darauf hin, dass der Import infizierter Tiere aus endemischen Ländern wie dem Sudan das höchste Risiko für die Einschleppung von RVFV und CCHFV nach Ägypten darstellt.
Im Allgemeinen erhöht der weltweite Nutztierhandel das Risiko der Einschleppung von viralen Krankheitserregern aus endemischen in erregerfreie Regionen, z.B. in Europa. Von den hier erwähnten Arboviren wurde bisher nur BATV in Europa und sogar in Deutschland nachgewiesen. Insbesondere in Ostdeutschland wurden hohe Prävalenzraten in der Wiederkäuerpopulation beobachtet. Bisher wurde der Nachweis von BATV bei Wiederkäuern mittels PCR, Hämagglutinationstest, Immunoblot, Immunfluoreszenztest und Virusneutralisationstest durchgeführt. Bei all diesen serologischen Tests handelt es sich um sensible Methoden zum Antikörpernachweis, die jedoch die Verwendung lebender Viren und damit auch die Einhaltung eines höheren Sicherheitsstandards erfordern. Daher wurde die dritte Studie durchgeführt, um die Zirkulation von BATV in der am stärksten betroffenen Region in Ostdeutschland weiter zu überwachen und die Verwendung eines indirekten BATV Gc ELISAs als Screening-Assay für größere seroepidemiologische Studien zu evaluieren. In den Blutproben von 60 Ziegen, 121 Schafen und 144 Rindern wurde keine virale RNA nachgewiesen, was darauf hindeutet, dass die Tiere während der Probenahme nicht virämisch waren. Die Serologie ergab mittlere Prävalenzraten bei Schafen und Ziegen und die höchste Prävalenz bei Rindern. Der neu etablierte indirekte ELISA basierend auf dem rekombinanten BATV Gc wurde mit dem SNT verglichen. Aufgrund der geringen Anzahl falsch-positiver Ergebnisse empfehlen wir die Proben zunächst im indirekten ELISA zu screenen und positive Ergebnisse in einem zweiten Schritt im SNT zu verifizieren. Der indirekte ELISA ermöglicht damit eine schnelle und sichere Diagnostik für groß angelegte serologische Untersuchungen, der auch in Laboren ohne hohe Biosicherheitsstandards eingesetzt werden kann.

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11 Supplements

11.1 Manuscript I

Data file S1. Results (corrected OD value) of the indirect glycoprotein (Gc) ELISA based on NRIV (NRIV ELISA), BATV (BATV ELISA), and BUNV (BUNV ELISA) ordered by species (ov=ovine, cap=caprine, n.r.=not reported) and region. Antibody-positive samples are coloured.

Sample ID			Species	Region	NRIV ELISA	BATV ELISA	BUNV ELISA
MR	1	/15 SR	cap	Inchiri	16,24	48,00	31,87
MR	2	/15 SR	cap	Inchiri	11,08	26,19	47,31
MR	3	/15 SR	cap	Inchiri	11,00	33,08	77,11
MR	4	/15 SR	cap	Inchiri	9,45	21,97	16,04
MR	5	/15 SR	cap	Inchiri	9,89	42,69	25,39
MR	6	/15 SR	cap	Inchiri	11,34	57,82	25,49
MR	7	/15 SR	cap	Inchiri	9,21	33,81	21,44
MR	8	/15 SR	cap	Inchiri	14,74	57,93	42,33
MR	9	/15 SR	cap	Inchiri	15,72	23,46	30,85
MR	10	/15 SR	cap	Inchiri	13,57	25,88	21,71
MR	11	/15 SR	cap	Inchiri	10,05	24,26	20,06
MR	12	/15 SR	cap	Inchiri	11,66	21,13	27,58
MR	13	/15 SR	cap	Inchiri	17,45	35,76	14,34
MR	14	/15 SR	cap	Inchiri	6,24	27,76	18,84
MR	15	/15 SR	cap	Inchiri	11,52	28,18	29,47
MR	16	/15 SR	ov	Inchiri	9,48	16,47	28,30
MR	17	/15 SR	ov	Inchiri	11,52	39,66	33,63
MR	18	/15 SR	ov	Inchiri	12,47	19,54	13,19
MR	19	/15 SR	ov	Inchiri	19,36	29,05	24,89
MR	20	/15 SR	ov	Inchiri	8,70	13,76	23,19
MR	21	/15 SR	ov	Inchiri	12,59	38,96	59,26
MR	22	/15 SR	ov	Inchiri	12,62	77,47	57,76
MR	23	/15 SR	ov	Inchiri	10,68	16,89	32,41
MR	24	/15 SR	ov	Inchiri	8,58	14,09	24,46
MR	25	/15 SR	ov	Inchiri	10,45	47,68	14,93
MR	26	/15 SR	ov	Inchiri	13,82	19,57	29,79
MR	27	/15 SR	ov	Inchiri	11,56	19,03	12,42
MR	28	/15 SR	ov	Inchiri	10,09	15,86	15,66
MR	29	/15 SR	ov	Inchiri	9,43	17,08	16,35
MR	30	/15 SR	ov	Inchiri	8,68	14,06	19,17
MR	31	/15 SR	ov	Inchiri	8,47	12,29	16,26
MR	32	/15 SR	ov	Inchiri	8,58	11,91	38,02
MR	33	/15 SR	ov	Inchiri	9,86	16,70	10,90
MR	34	/15 SR	ov	Inchiri	12,93	19,55	17,17
MR	35	/15 SR	cap	Inchiri	13,43	32,71	145,52

MR	37	/15 SR	cap	Inchiri	13,55	44,63	37,17
MR	38	/15 SR	cap	Inchiri	10,78	22,77	21,52
MR	39	/15 SR	cap	Inchiri	18,70	52,53	53,58
MR	40	/15 SR	cap	Inchiri	48,42	36,65	35,01
MR	41	/15 SR	cap	Inchiri	9,81	23,18	52,67
MR	42	/15 SR	cap	Inchiri	17,42	51,04	44,02
MR	43	/15 SR	cap	Inchiri	28,86	62,35	30,25
MR	44	/15 SR	cap	Inchiri	12,58	31,11	32,35
MR	45	/15 SR	cap	Inchiri	12,65	38,61	25,22
MR	46	/15 SR	cap	Inchiri	27,79	18,31	52,57
MR	47	/15 SR	cap	Inchiri	21,57	63,48	57,93
MR	48	/15 SR	cap	Inchiri	19,51	26,76	52,05
MR	49	/15 SR	cap	Inchiri	10,87	17,11	50,22
MR	50	/15 SR	ov	Inchiri	13,69	6,69	11,98
MR	51	/15 SR	ov	Inchiri	14,06	6,68	10,87
MR	52	/15 SR	ov	Inchiri	16,06	7,17	10,76
MR	54	/15 SR	ov	Inchiri	26,76	6,89	21,10
MR	55	/15 SR	ov	Inchiri	14,55	6,65	16,04
MR	56	/15 SR	ov	Inchiri	12,94	7,04	9,28
MR	58	/15 SR	ov	Inchiri	25,68	7,42	30,53
MR	59	/15 SR	ov	Inchiri	14,89	7,25	14,91
MR	60	/15 SR	ov	Inchiri	15,20	7,11	90,47
MR	61	/15 SR	cap	Inchiri	16,54	8,85	16,94
MR	62	/15 SR	cap	Inchiri	14,55	8,96	11,25
MR	63	/15 SR	cap	Inchiri	16,38	10,42	19,12
MR	65	/15 SR	cap	Inchiri	20,13	36,21	60,62
MR	66	/15 SR	cap	Inchiri	27,63	23,27	64,15
MR	67	/15 SR	cap	Inchiri	17,79	18,12	29,23
MR	68	/15 SR	cap	Inchiri	40,96	36,27	31,61
MR	69	/15 SR	cap	Inchiri	16,62	12,05	24,54
MR	70	/15 SR	cap	Inchiri	21,60	14,13	33,49
MR	71	/15 SR	cap	Inchiri	15,65	7,77	90,25
MR	72	/15 SR	cap	Inchiri	30,25	20,02	61,76
MR	73	/15 SR	cap	Inchiri	16,95	16,38	34,73
MR	74	/15 SR	cap	Inchiri	16,15	10,50	39,26
MR	75	/15 SR	cap	Inchiri	17,67	13,18	35,93
MR	76	/15 SR	cap	Inchiri	14,68	8,07	17,59
MR	77	/15 SR	cap	Inchiri	16,13	9,45	46,35
MR	78	/15 SR	cap	Inchiri	14,60	8,41	16,33
MR	79	/15 SR	cap	Inchiri	18,18	12,53	15,83
MR	80	/15 SR	cap	Inchiri	14,08	8,55	19,69
MR	81	/15 SR	cap	Inchiri	13,05	9,28	12,13
MR	82	/15 SR	ov	Chargui	18,80	14,30	51,65
MR	83	/15 SR	ov	Chargui	45,03	47,41	80,26
MR	84	/15 SR	ov	Chargui	83,65	42,15	25,88
MR	85	/15 SR	ov	Chargui	77,12	43,38	52,68
MR	86	/15 SR	ov	Chargui	46,36	22,22	12,72

MR	87	/15 SR	ov	Chargui	74,43	62,09	48,18
MR	88	/15 SR	ov	Chargui	20,76	16,53	50,21
MR	89	/15 SR	ov	Chargui	63,26	52,09	51,14
MR	90	/15 SR	ov	Chargui	16,15	11,95	33,29
MR	91	/15 SR	ov	Chargui	39,64	28,99	58,76
MR	92	/15 SR	ov	Chargui	21,27	8,06	25,52
MR	93	/15 SR	ov	Chargui	17,39	9,52	38,26
MR	94	/15 SR	ov	Chargui	16,64	10,01	56,78
MR	95	/15 SR	ov	Chargui	16,37	11,44	15,73
MR	96	/15 SR	ov	Chargui	51,76	36,98	38,17
MR	97	/15 SR	ov	Chargui	17,00	11,91	63,43
MR	98	/15 SR	ov	Chargui	52,42	42,10	55,80
MR	99	/15 SR	ov	Chargui	14,33	10,01	17,07
MR	100	/15 SR	ov	Chargui	61,37	40,10	92,43
MR	101	/15 SR	ov	Chargui	12,38	12,43	15,87
MR	103	/15 SR	ov	Chargui	16,17	14,01	23,67
MR	104	/15 SR	ov	Chargui	31,49	58,68	27,63
MR	105	/15 SR	ov	Chargui	14,82	11,19	36,32
MR	106	/15 SR	ov	Chargui	333,20	284,56	153,84
MR	107	/15 SR	ov	Chargui	127,56	68,47	223,65
MR	108	/15 SR	ov	Chargui	17,45	11,34	22,13
MR	109	/15 SR	ov	Chargui	15,13	11,21	14,99
MR	110	/15 SR	ov	Chargui	10,91	11,36	76,79
MR	111	/15 SR	ov	Chargui	18,23	189,83	156,45
MR	112	/15 SR	ov	Chargui	10,70	7,52	38,35
MR	113	/15 SR	ov	Chargui	15,31	62,32	27,76
MR	114	/15 SR	ov	Chargui	13,35	15,23	32,95
MR	115	/15 SR	ov	Chargui	177,19	131,21	26,86
MR	116	/15 SR	ov	Chargui	12,12	9,61	46,18
MR	117	/15 SR	ov	Chargui	28,92	97,24	49,37
MR	118	/15 SR	ov	Chargui	11,51	9,55	13,53
MR	119	/15 SR	ov	Chargui	10,16	7,50	14,90
MR	120	/15 SR	ov	Chargui	16,01	20,47	26,74
MR	121	/15 SR	ov	Chargui	45,67	23,93	29,30
MR	122	/15 SR	cap	Chargui	57,66	54,86	44,33
MR	123	/15 SR	cap	Chargui	40,58	38,52	87,68
MR	124	/15 SR	cap	Chargui	79,09	71,09	43,31
MR	125	/15 SR	cap	Chargui	58,56	41,14	68,48
MR	126	/15 SR	cap	Chargui	79,99	84,69	50,73
MR	127	/15 SR	cap	Chargui	38,25	38,91	46,51
MR	128	/15 SR	cap	Chargui	308,90	262,99	199,42
MR	129	/15 SR	cap	Chargui	24,86	34,65	62,24
MR	130	/15 SR	cap	Chargui	271,07	166,74	30,23
MR	131	/15 SR	cap	Chargui	57,04	32,70	35,23
MR	132	/15 SR	cap	Chargui	367,89	148,73	49,47
MR	133	/15 SR	cap	Chargui	195,69	135,19	279,83
MR	134	/15 SR	cap	Chargui	99,46	131,76	102,23

MR	135	/15 SR	cap	Chargui	44,51	36,74	42,21
MR	136	/15 SR	cap	Chargui	109,35	82,28	191,01
MR	137	/15 SR	cap	Chargui	119,09	45,75	62,58
MR	138	/15 SR	cap	Chargui	72,53	40,44	77,12
MR	139	/15 SR	cap	Chargui	132,26	96,43	64,88
MR	140	/15 SR	cap	Chargui	179,21	108,83	40,45
MR	141	/15 SR	cap	Chargui	46,21	22,79	59,85
MR	142	/15 SR	cap	Chargui	37,85	27,49	34,99
MR	143	/15 SR	cap	Chargui	117,75	99,11	134,89
MR	144	/15 SR	cap	Chargui	29,69	21,89	47,84
MR	145	/15 SR	cap	Chargui	54,70	33,24	54,56
MR	146	/15 SR	cap	Chargui	194,49	129,91	43,59
MR	147	/15 SR	cap	Chargui	94,49	96,79	81,99
MR	148	/15 SR	cap	Chargui	22,26	23,71	56,47
MR	149	/15 SR	cap	Chargui	63,30	37,26	29,05
MR	150	/15 SR	cap	Chargui	238,90	196,83	51,03
MR	151	/15 SR	cap	Chargui	14,39	9,57	88,92
MR	152	/15 SR	cap	Chargui	137,53	115,89	88,97
MR	153	/15 SR	cap	Chargui	96,73	101,23	162,26
MR	154	/15 SR	cap	Chargui	213,38	165,98	27,27
MR	155	/15 SR	cap	Chargui	123,80	127,74	98,78
MR	156	/15 SR	cap	Chargui	12,75	10,31	20,35
MR	157	/15 SR	cap	Chargui	20,67	16,73	226,61
MR	158	/15 SR	cap	Chargui	15,67	13,98	29,74
MR	159	/15 SR	cap	Chargui	41,54	27,98	40,83
MR	160	/15 SR	cap	Chargui	13,90	14,76	56,63
MR	161	/15 SR	cap	Chargui	10,69	8,05	47,15
MR	162	/15 SR	cap	Chargui	67,57	48,56	41,05
MR	163	/15 SR	ov	Gharbi	78,64	22,06	34,69
MR	164	/15 SR	ov	Gharbi	19,40	15,75	114,43
MR	165	/15 SR	ov	Gharbi	31,97	16,73	50,55
MR	166	/15 SR	ov	Gharbi	11,34	8,48	33,84
MR	167	/15 SR	ov	Gharbi	91,34	61,42	31,41
MR	168	/15 SR	ov	Gharbi	12,62	7,46	20,69
MR	169	/15 SR	ov	Gharbi	24,56	20,01	28,91
MR	170	/15 SR	ov	Gharbi	19,17	11,70	52,72
MR	171	/15 SR	ov	Gharbi	13,03	13,24	17,17
MR	172	/15 SR	ov	Gharbi	26,93	17,95	56,27
MR	173	/15 SR	ov	Gharbi	14,94	11,04	29,01
MR	174	/15 SR	ov	Gharbi	13,75	13,44	41,25
MR	175	/15 SR	ov	Gharbi	17,91	12,80	16,92
MR	176	/15 SR	ov	Gharbi	11,15	7,61	20,19
MR	177	/15 SR	ov	Gharbi	13,37	8,56	28,97
MR	178	/15 SR	ov	Gharbi	13,98	16,16	72,99
MR	179	/15 SR	ov	Gharbi	29,55	27,99	49,69
MR	180	/15 SR	ov	Gharbi	17,40	18,46	22,38
MR	181	/15 SR	ov	Gharbi	12,43	7,71	14,09

MR	182	/15 SR	ov	Gharbi	11,04	6,65	99,05
MR	183	/15 SR	ov	Gharbi	119,45	89,95	116,04
MR	184	/15 SR	ov	Gharbi	46,26	38,53	16,05
MR	185	/15 SR	ov	Gharbi	12,53	7,84	14,75
MR	186	/15 SR	ov	Gharbi	12,45	7,46	16,84
MR	187	/15 SR	ov	Gharbi	100,45	71,89	29,04
MR	188	/15 SR	ov	Gharbi	27,49	25,44	23,72
MR	189	/15 SR	ov	Gharbi	14,15	10,41	24,38
MR	190	/15 SR	ov	Gharbi	44,34	35,12	29,83
MR	191	/15 SR	ov	Gharbi	15,03	9,43	27,30
MR	192	/15 SR	ov	Gharbi	86,19	31,27	29,30
MR	193	/15 SR	ov	Gharbi	37,99	24,73	27,77
MR	194	/15 SR	ov	Gharbi	75,23	47,17	21,33
MR	195	/15 SR	ov	Gharbi	10,96	6,15	22,84
MR	196	/15 SR	ov	Gharbi	26,02	21,28	38,36
MR	197	/15 SR	ov	Gharbi	11,58	6,46	36,58
MR	198	/15 SR	ov	Gharbi	12,18	8,35	65,14
MR	200	/15 SR	ov	Gharbi	11,39	6,63	29,22
MR	201	/15 SR	ov	Gharbi	88,14	37,30	52,65
MR	202	/15 SR	ov	Gharbi	153,69	77,48	22,14
MR	203	/15 SR	ov	Gharbi	15,21	16,63	13,09
MR	204	/15 SR	ov	Gharbi	13,19	8,85	158,42
MR	205	/15 SR	ov	Gharbi	53,88	44,95	197,18
MR	206	/15 SR	ov	Gharbi	18,50	14,44	32,12
MR	207	/15 SR	ov	Gharbi	63,27	37,23	22,25
MR	208	/15 SR	ov	Gharbi	36,15	29,21	27,99
MR	209	/15 SR	ov	Gharbi	12,44	7,42	32,22
MR	210	/15 SR	ov	Gharbi	25,32	12,78	52,06
MR	211	/15 SR	ov	Gharbi	51,28	31,40	24,88
MR	212	/15 SR	ov	Gharbi	14,12	8,93	18,48
MR	213	/15 SR	ov	Gharbi	17,46	15,45	14,75
MR	214	/15 SR	ov	Gharbi	22,32	16,94	21,89
MR	215	/15 SR	ov	Gharbi	48,44	44,09	38,95
MR	216	/15 SR	ov	Gharbi	27,78	23,14	24,41
MR	217	/15 SR	ov	Gharbi	14,93	11,65	28,81
MR	218	/15 SR	ov	Gharbi	50,36	54,31	41,92
MR	219	/15 SR	ov	Gharbi	16,74	16,60	65,92
MR	220	/15 SR	ov	Gharbi	17,35	16,98	13,37
MR	221	/15 SR	ov	Gharbi	24,61	29,34	69,91
MR	222	/15 SR	ov	Gharbi	10,90	9,60	20,39
MR	223	/15 SR	ov	Gharbi	53,54	154,56	92,92
MR	224	/15 SR	ov	Gharbi	12,81	8,86	26,92
MR	225	/15 SR	ov	Gharbi	17,59	22,31	22,06
MR	226	/15 SR	ov	Gharbi	13,80	21,29	72,92
MR	227	/15 SR	ov	Gharbi	13,20	12,99	45,42
MR	228	/15 SR	ov	Gharbi	104,30	101,69	45,66
MR	229	/15 SR	ov	Gharbi	49,15	131,62	126,29

MR	230	/15 SR	ov	Gharbi	20,85	14,13	56,98
MR	231	/15 SR	ov	Gharbi	38,83	34,80	148,56
MR	232	/15 SR	ov	Gharbi	11,19	9,90	38,12
MR	233	/15 SR	ov	Gharbi	24,50	21,88	88,48
MR	234	/15 SR	ov	Gharbi	13,86	9,59	17,41
MR	235	/15 SR	ov	Gharbi	46,44	33,20	80,70
MR	236	/15 SR	ov	Gharbi	118,06	72,12	44,11
MR	237	/15 SR	ov	Gharbi	19,08	30,91	26,66
MR	238	/15 SR	ov	Gharbi	13,45	16,88	168,07
MR	239	/15 SR	ov	Gharbi	17,72	21,58	31,37
MR	240	/15 SR	ov	Gharbi	40,23	25,91	29,23
MR	241	/15 SR	ov	Gharbi	14,57	12,30	43,20
MR	242	/15 SR	ov	Gharbi	12,97	10,89	28,09
MR	243	/15 SR	ov	Gharbi	39,21	39,88	90,34
MR	244	/15 SR	cap	Tagant	146,54	128,54	24,97
MR	245	/15 SR	cap	Tagant	35,49	91,33	92,68
MR	247	/15 SR	cap	Tagant	20,10	22,18	19,26
MR	249	/15 SR	cap	Tagant	30,06	56,53	20,58
MR	250	/15 SR	cap	Tagant	20,45	19,26	128,91
MR	251	/15 SR	cap	Tagant	69,21	39,48	54,79
MR	252	/15 SR	cap	Tagant	161,59	121,42	81,58
MR	254	/15 SR	ov	Tagant	24,88	48,08	38,50
MR	255	/15 SR	ov	Tagant	17,78	17,78	57,89
MR	256	/15 SR	cap	Tagant	13,09	11,58	21,75
MR	260	/15 SR	ov	Tagant	177,58	93,12	21,11
MR	262	/15 SR	cap	Tagant	36,21	26,21	46,96
MR	263	/15 SR	cap	Tagant	197,61	175,24	55,74
MR	264	/15 SR	cap	Tagant	13,30	24,71	30,02
MR	265	/15 SR	cap	Tagant	107,20	89,20	16,95
MR	266	/15 SR	cap	Tagant	233,74	176,68	25,85
MR	267	/15 SR	cap	Tagant	12,82	14,13	27,78
MR	268	/15 SR	cap	Tagant	16,08	13,75	28,09
MR	269	/15 SR	cap	Tagant	100,72	93,50	29,64
MR	270	/15 SR	cap	Tagant	14,97	15,57	28,87
MR	271	/15 SR	cap	Tagant	185,81	127,21	33,91
MR	273	/15 SR	cap	Tagant	17,31	12,56	18,55
MR	274	/15 SR	cap	Tagant	15,36	11,58	12,75
MR	275	/15 SR	cap	Tagant	16,48	15,57	14,34
MR	276	/15 SR	cap	Tagant	12,75	9,10	17,72
MR	277	/15 SR	cap	Tagant	20,36	16,69	35,71
MR	278	/15 SR	cap	Tagant	29,02	20,10	41,93
MR	279	/15 SR	cap	Tagant	14,58	10,44	22,29
MR	280	/15 SR	cap	Tagant	25,96	14,64	21,26
MR	281	/15 SR	cap	Tagant	187,53	133,09	25,89
MR	283	/15 SR	cap	Tagant	12,14	14,59	29,29
MR	284	/15 SR	cap	Tagant	18,65	15,50	46,78
MR	285	/15 SR	cap	Tagant	51,14	39,11	46,50

MR	286	/15 SR	cap	Tagant	18,64	16,95	33,33
MR	287	/15 SR	cap	Tagant	12,60	12,32	27,09
MR	288	/15 SR	cap	Tagant	9,93	7,66	10,36
MR	289	/15 SR	cap	Tagant	10,58	10,13	18,30
MR	290	/15 SR	cap	Tagant	15,70	14,03	32,17
MR	291	/15 SR	cap	Tagant	13,76	26,09	17,94
MR	292	/15 SR	cap	Tagant	21,83	14,93	52,81
MR	293	/15 SR	cap	Tagant	11,93	14,62	28,29
MR	294	/15 SR	cap	Tagant	17,33	22,11	21,44
MR	295	/15 SR	cap	Tagant	25,87	23,51	21,71
MR	296	/15 SR	cap	Tagant	13,03	11,97	16,28
MR	297	/15 SR	cap	Tagant	18,10	19,99	14,27
MR	298	/15 SR	cap	Tagant	11,62	15,47	33,33
MR	299	/15 SR	cap	Tagant	13,65	14,36	19,73
MR	300	/15 SR	cap	Tagant	12,85	12,70	20,30
MR	301	/15 SR	cap	Tagant	14,74	12,14	28,45
MR	302	/15 SR	cap	Tagant	10,84	12,06	62,92
MR	303	/15 SR	cap	Tagant	13,37	11,29	20,71
MR	304	/15 SR	ov	Assaba	16,51	10,20	20,87
MR	305	/15 SR	ov	Assaba	48,48	39,95	24,39
MR	306	/15 SR	ov	Assaba	12,43	41,67	37,68
MR	308	/15 SR	ov	Assaba	24,07	28,38	40,91
MR	309	/15 SR	ov	Assaba	65,57	43,95	48,02
MR	310	/15 SR	ov	Assaba	41,45	29,41	36,91
MR	311	/15SR	ov	Assaba	17,95	14,07	34,10
MR	312	/15 SR	ov	Assaba	11,39	8,20	14,71
MR	314	/15 SR	ov	Assaba	17,80	13,73	21,08
MR	315	/15 SR	ov	Assaba	13,35	13,74	26,75
MR	316	/15 SR	ov	Assaba	15,30	13,27	21,69
MR	317	/15 SR	ov	Assaba	33,36	23,28	35,96
MR	319	/15 SR	ov	Assaba	59,34	44,84	33,32
MR	320	/15 SR	ov	Assaba	31,35	21,51	29,42
MR	321	/15 SR	ov	Assaba	32,66	30,63	16,03
MR	322	/15 SR	ov	Assaba	10,53	8,74	41,51
MR	323	/15 SR	ov	Assaba	19,06	11,34	20,21
MR	324	/15 SR	ov	Assaba	59,84	53,17	161,05
MR	325	/15 SR	ov	Assaba	14,59	18,69	40,73
MR	326	/15 SR	ov	Assaba	47,02	25,14	27,11
MR	327	/15 SR	ov	Assaba	47,37	27,80	16,56
MR	328	/15 SR	ov	Assaba	12,79	11,46	34,00
MR	329	/15 SR	ov	Assaba	31,48	33,13	34,09
MR	332	/15 SR	ov	Assaba	82,36	135,98	134,91
MR	335	/15 SR	ov	Assaba	32,94	37,50	21,71
MR	336	/15 SR	cap	Assaba	104,92	75,36	81,54
MR	337	/15 SR	cap	Assaba	145,82	95,45	58,46
MR	338	/15 SR	cap	Assaba	15,71	17,11	23,48
MR	339	/15 SR	cap	Assaba	116,03	103,16	125,00

MR	341	/15 SR	cap	Assaba	6,36	10,08	37,12
MR	342	/15 SR	cap	Assaba	87,87	96,65	70,36
MR	343	/15 SR	cap	Assaba	11,70	10,90	19,78
MR	344	/15 SR	cap	Assaba	12,81	11,20	39,94
MR	345	/15 SR	cap	Assaba	13,05	10,21	35,44
MR	346	/15 SR	cap	Assaba	16,11	26,82	55,42
MR	347	/15 SR	cap	Assaba	10,23	19,72	22,49
MR	348	/15 SR	ov	Assaba	10,29	10,64	17,46
MR	349	/15 SR	cap	Assaba	6,54	9,16	11,77
MR	350	/15 SR	cap	Assaba	137,86	132,71	46,42
MR	351	/15 SR	cap	Assaba	16,67	21,47	33,41
MR	352	/15 SR	cap	Assaba	94,73	88,29	57,01
MR	353	/15 SR	cap	Assaba	44,66	88,87	67,59
MR	354	/15 SR	cap	Assaba	19,64	14,28	32,47
MR	355	/15 SR	cap	Assaba	11,20	7,82	13,40
MR	356	/15 SR	cap	Assaba	10,27	8,40	12,63
MR	357	/15 SR	cap	Assaba	29,12	32,84	16,78
MR	358	/15 SR	cap	Assaba	50,03	52,68	54,19
MR	359	/15 SR	cap	Assaba	44,94	58,58	32,26
MR	360	/15 SR	cap	Assaba	72,91	74,75	30,30
MR	361	/15 SR	cap	Assaba	33,89	35,21	54,57
MR	362	/15 SR	ov	Assaba	69,47	70,50	42,06
MR	363	/15 SR	cap	Assaba	69,83	80,52	52,92
MR	364	/15 SR	cap	Assaba	12,30	12,69	27,87
MR	365	/15 SR	cap	Assaba	10,13	13,31	24,53
MR	366	/15 SR	ov	Trarza	12,81	8,82	19,30
MR	367	/15 SR	ov	Trarza	11,04	9,64	13,47
MR	368	/15 SR	ov	Trarza	16,32	19,80	62,78
MR	369	/15 SR	cap	Trarza	15,52	10,93	26,38
MR	370	/15 SR	cap	Trarza	34,71	16,38	53,27
MR	371	/15 SR	ov	Trarza	28,77	25,39	46,61
MR	372	/15 SR	ov	Trarza	10,72	7,38	57,43
MR	373	/15 SR	cap	Trarza	40,07	40,24	52,76
MR	374	/15 SR	ov	Trarza	20,44	16,93	35,75
MR	375	/15 SR	ov	Trarza	61,62	65,78	22,57
MR	376	/15 SR	ov	Trarza	16,02	13,04	22,87
MR	377	/15 SR	ov	Trarza	20,09	17,26	28,76
MR	378	/15 SR	cap	Trarza	32,01	28,20	40,98
MR	379	/15 SR	cap	Trarza	124,24	103,69	24,16
MR	380	/15 SR	ov	Trarza	11,09	9,71	19,08
MR	381	/15 SR	cap	Trarza	33,78	45,18	75,43
MR	383	/15 SR	cap	Trarza	62,48	63,89	43,98
MR	384	/15 SR	ov	Trarza	11,25	6,97	31,74
MR	385	/15 SR	ov	Trarza	55,66	50,29	26,26
MR	386	/15 SR	cap	Trarza	16,94	19,16	28,87
MR	387	/15 SR	ov	Trarza	11,30	8,57	35,13
MR	388	/15 SR	ov	Trarza	10,74	7,70	12,36

MR	389	/15 SR	ov	Trarza	10,89	7,37	20,91
MR	390	/15 SR	ov	Trarza	11,09	7,77	28,55
MR	392	/15 SR	cap	Trarza	73,40	51,65	112,47
MR	394	/15 SR	ov	Trarza	88,46	149,18	66,53
MR	395	/15 SR	cap	Trarza	12,09	9,61	12,20
MR	396	/15 SR	ov	Trarza	18,48	27,74	106,81
MR	397	/15 SR	ov	Guidimaka	27,68	32,07	66,08
MR	398	/15 SR	ov	Guidimaka	51,37	57,23	66,22
MR	399	/15 SR	ov	Guidimaka	92,54	83,63	130,90
MR	400	/15 SR	ov	Guidimaka	96,26	117,66	22,66
MR	401	/15 SR	ov	Guidimaka	20,03	45,18	31,84
MR	402	/15 SR	ov	Guidimaka	137,98	104,14	44,64
MR	403	/15 SR	ov	Guidimaka	13,72	13,38	37,19
MR	404	/15 SR	ov	Guidimaka	108,51	90,80	24,53
MR	405	/15 SR	ov	Guidimaka	136,17	113,35	82,33
MR	406	/15 SR	ov	Guidimaka	251,44	230,48	158,70
MR	407	/15 SR	ov	Guidimaka	39,31	52,19	38,20
MR	408	/15 SR	ov	Guidimaka	9,00	16,72	23,87
MR	409	/15 SR	ov	Guidimaka	7,22	18,09	54,13
MR	410	/15 SR	ov	Guidimaka	6,98	9,50	20,80
MR	413	/15 SR	cap	Guidimaka	42,24	41,96	38,88
MR	414	/15 SR	cap	Guidimaka	6,44	8,86	44,53
MR	416	/15 SR	cap	Guidimaka	37,87	48,53	65,04
MR	417	/15 SR	cap	Guidimaka	34,63	32,49	23,37
MR	418	/15 SR	cap	Guidimaka	13,17	35,06	40,08
MR	419	/15 SR	cap	Guidimaka	16,44	10,90	22,27
MR	420	/15 SR	cap	Guidimaka	44,58	45,72	27,85
MR	421	/15 SR	cap	Guidimaka	7,48	8,74	15,86
MR	422	/15 SR	cap	Guidimaka	13,72	15,32	35,61
MR	423	/15 SR	cap	Guidimaka	7,13	15,86	49,15
MR	424	/15 SR	cap	Guidimaka	101,37	94,55	20,76
MR	425	/15 SR	cap	Guidimaka	12,59	20,14	29,68
MR	426	/15 SR	cap	Guidimaka	20,51	13,52	33,13
MR	427	/15 SR	cap	Guidimaka	101,28	91,45	50,57
MR	428	/15 SR	cap	Guidimaka	8,25	20,98	152,22
MR	429	/15 SR	cap	Guidimaka	13,65	24,65	53,39
MR	430	/15 SR	cap	Guidimaka	9,78	22,73	68,64
MR	431	/15 SR	cap	Guidimaka	77,65	101,97	208,44
MR	432	/15 SR	cap	Guidimaka	47,94	89,72	26,97
MR	433	/15 SR	cap	Guidimaka	25,54	58,69	27,78
MR	434	/15 SR	cap	Guidimaka	17,07	29,97	56,33
MR	435	/15 SR	cap	Guidimaka	8,26	15,43	41,61
MR	436	/15 SR	cap	Guidimaka	73,22	115,17	65,12
MR	437	/15 SR	cap	Guidimaka	17,45	24,48	30,47
MR	438	/15 SR	cap	Guidimaka	7,20	21,56	217,71
MR	439	/15 SR	cap	Guidimaka	9,82	19,80	36,87
MR	440	/15 SR	cap	Guidimaka	18,19	30,92	38,76

MR	441	/15 SR	cap	Guidimaka	46,84	123,09	185,97
MR	442	/15 SR	ov	Guidimaka	13,51	13,60	61,54
MR	443	/15 SR	ov	Guidimaka	10,72	21,69	43,35
MR	444	/15 SR	ov	Guidimaka	9,15	18,59	21,24
MR	445	/15 SR	ov	Guidimaka	98,52	123,28	40,38
MR	446	/15 SR	ov	Guidimaka	20,33	24,48	45,84
MR	447	/15 SR	ov	Guidimaka	12,37	10,15	63,15
MR	448	/15 SR	ov	Guidimaka	37,85	37,50	22,90
MR	449	/15 SR	ov	Guidimaka	42,15	45,69	63,91
MR	450	/15 SR	ov	Guidimaka	15,13	26,20	50,41
MR	451	/15 SR	ov	Guidimaka	19,47	16,79	100,47
MR	452	/15 SR	ov	Guidimaka	10,34	6,79	7,71
MR	453	/15 SR	ov	Guidimaka	119,11	94,59	55,72
MR	454	/15 SR	ov	Guidimaka	106,65	69,40	9,30
MR	455	/15 SR	ov	Guidimaka	67,82	69,95	57,32
MR	456	/15 SR	ov	Guidimaka	108,34	121,66	46,85
MR	457	/15 SR	ov	Guidimaka	6,40	8,66	37,86
MR	458	/15 SR	cap	Guidimaka	7,24	10,85	39,11
MR	459	/15 SR	cap	Guidimaka	28,98	41,91	68,59
MR	460	/15 SR	cap	Guidimaka	151,08	137,90	180,07
MR	461	/15 SR	cap	Guidimaka	12,10	17,59	43,08
MR	462	/15 SR	cap	Guidimaka	157,69	138,54	162,00
MR	463	/15 SR	cap	Guidimaka	80,56	112,59	56,11
MR	464	/15 SR	cap	Guidimaka	131,13	137,17	60,46
MR	465	/15 SR	cap	Guidimaka	85,45	112,94	104,93
MR	466	/15 SR	cap	Guidimaka	142,05	135,38	54,56
MR	467	/15 SR	cap	Guidimaka	71,80	96,33	78,90
MR	468	/15 SR	ov	Guidimaka	84,61	112,13	62,32
MR	469	/15 SR	ov	Guidimaka	93,60	119,57	102,75
MR	470	/15 SR	ov	Guidimaka	50,05	40,03	71,62
MR	471	/15 SR	ov	Brakna	19,53	20,77	29,88
MR	472	/15 SR	ov	Brakna	63,19	116,23	145,58
MR	473	/15 SR	ov	Brakna	14,72	15,75	68,98
MR	474	/15 SR	ov	Brakna	35,26	58,09	77,43
MR	475	/15 SR	cap	Brakna	9,64	48,83	94,92
MR	476	/15 SR	cap	Brakna	168,42	153,45	102,41
MR	477	/15 SR	ov	Brakna	13,12	9,78	25,55
MR	479	/15 SR	ov	Brakna	20,54	13,30	50,61
MR	481	/15 SR	cap	Brakna	8,12	16,00	26,51
MR	482	/15 SR	cap	Brakna	80,06	118,69	47,04
MR	483	/15 SR	ov	Brakna	11,70	8,22	14,05
MR	486	/15 SR	ov	Brakna	17,29	31,15	38,90
MR	489	/15 SR	cap	Brakna	105,99	100,69	71,07
MR	494	/15 SR	cap	Brakna	124,53	114,37	231,31
MR	497	/15 SR	n.r.	Gharbi	7,07	12,65	21,20
MR	498	/15 SR	n.r.	Gharbi	15,42	39,76	39,85
MR	500	/15 SR	n.r.	Gharbi	15,97	21,99	129,68

Data file S2. Samples tested positive by RVFV IgM capture ELISA. Results are ordered by species (ov=ovine, cap=caprine, n.r.=not reported) and region.

Sample ID			**Species**	**Region**	**S/P%**
MR	252	/15 SR	cap	Tagant	65,44
MR	254	/15 SR	ov	Tagant	44,89
MR	268	/15 SR	cap	Tagant	53,55
MR	304	/15 SR	ov	Assaba	48,29
MR	306	/15 SR	ov	Assaba	138,34
MR	308	/15 SR	ov	Assaba	51,75
MR	309	/15 SR	ov	Assaba	150,20
MR	310	/15 SR	ov	Assaba	102,34
MR	311	/15 SR	ov	Assaba	136,78
MR	312	/15 SR	ov	Assaba	87,89
MR	314	/15 SR	ov	Assaba	94,80
MR	315	/15 SR	ov	Assaba	73,49
MR	317	/15 SR	ov	Assaba	149,80
MR	320	/15 SR	ov	Assaba	162,93
MR	325	/15 SR	ov	Assaba	81,83
MR	327	/15 SR	ov	Assaba	117,65
MR	328	/15 SR	ov	Assaba	114,11
MR	335	/15 SR	ov	Assaba	62,18
MR	358	/15 SR	cap	Assaba	58,30
MR	466	/15 SR	cap	Guidimaka	48,44
MR	498	/15 SR	n.r.	Gharbi	72,70
MR	500	/15 SR	n.r.	Gharbi	59,04

12 Authors ‘contribution

Manuscript I:
Preparation of proteins and characterization by Western blot. Establishment of ELISAs based on glycoprotein Gc of each NRIV, BATV and BUNV. Establishment of protocol for multiplex qRT-PCR. Investigation of the collected samples by ELISAs and differentiating SNTs for both BATV and BUNV. Data analysis (e.g. calculations of prevalence and CI) and interpretation of molecular and serological results. Writing first draft of the manuscript.

Manuscript II:
Collaboration with the providers of the blood samples. Establishment of protocol for multiplex qRT-PCR. Investigation of the collected samples (e.g. RNA extraction with subsequent qRT-PCR, ELISA, IIFA). Interpretation of the data together with the regional origin of the samples and interpretation of the molecular and serological results. Compilation and presentation of the results. Writing of the original draft of the manuscript.

Manuscript III:
Preparation of BATV glycoprotein Gc (expression, purification, dialysis and characterization by Western blot). Establishment of ELISA based on recombinant protein. Investigation of the collected samples by ELISA for BATV. Interpretation of the molecular and serological results and writing the original draft of the manuscript.

Martin H. Groschup and Martin Eiden supervised all studies scientifically.
Martin Eiden taught me the molecular diagnostic methods. Moreover, he performed sequencing and sequence analysis in first manuscript.
Ute Ziegler introduced me to the SNT and performed SNT for Ngari virus in first manuscript and SNT for Batai virus in third manuscript. Furthermore, she provided ruminant blood samples for third manuscript and supervised the third study.
Melanie Rissmann showed me practical methodology and interpretation of IIFA.
Franziska Stoek performed RVFV investigation for second manuscript.
Jana Schulz worked on calculation of ELISA parameters and preparation of geographical maps in first and second manuscript.
Anne Günther performed qRT-PCR in third manuscript.
Patrick Wysocki prepared geographical map in third manuscript.

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