Aus dem Friedrich-Loeffler-Institut

eingereicht über das Institut für Mikrobiologie und Tierseuchen
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Epidemiology of Crimean-Congo haemorrhagic fever virus in ticks and livestock in Balochistan, Pakistan

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
Khushal Khan Kasi
Tierarzt aus Quetta, Pakistan

Berlin 2020
Journal-Nr.: 4191

mbvberlin | mensch und buch verlag

Aus dem Friedrich-Loeffler-Institut

eingereicht über das Institut für Mikrobiologie und Tierseuchen

des Fachbereichs Veterinärmedizin

der Freien Universität Berlin

Epidemiology of Crimean-Congo haemorrhagic fever virus in ticks and livestock in Balochistan, Pakistan

Inaugural-Dissertation

zur Erlangung des Grades eines

Doktors der Veterinärmedizin

an der

Freien Universität Berlin

vorgelegt von

Khushal Khan Kasi

Tierarzt aus Quetta, Pakistan

Berlin 2020

Journal-Nr.: 4191

Gedruckt mit Genehmigung des Fachbereichs Veterinärmedizin

der Freien Universität Berlin

Dekan:	Univ.-Prof. Dr. Jürgen Zentek
Erster Gutachter:	Prof. Dr. Franz Josef Conraths
Zweiter Gutachter:	Univ.-Prof. Dr. Ard Nijhof
Dritter Gutachter:	Univ.-Prof. Dr. Marcus Doherr

Deskriptoren (nach CAB-Thesaurus):
Sheep, Goats, Cattle, Crimean-Congo hemorrhagic fever, Crimean-Congo hemorrhagic fever virus, Blood serum, Risk factors, Hyalomma, Rhipicephalus, Polymerase chain reaction, Epidemiology, Balochistan, Pakistan

Tag der Promotion: 16.03.2020

Bibliografische Information der *Deutschen Nationalbibliothek*
Die Deutsche Nationalbibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliografie; detaillierte bibliografische Daten sind im Internet über <https://dnb.de> abrufbar.

ISBN: 978-3-96729-043-1
Zugl.: Berlin, Freie Univ., Diss., 2020
Dissertation, Freie Universität Berlin
D188

Choriner Str. 85 - 10119 Berlin
verlag@menschundbuch.de – www.menschundbuch.de

To my parents

Dr. Muhammad Azam Kasi

&

Jamila Bano Kasi

Table of Contents

List of Figures

List of Tables

Abbreviations

AIC	Akaike information criterion
AST	Aspartate
ALT	alanine aminotransferases
BSL	biosafety laboratory
°C	degree Celsius
CAR	Central African Republic
CCHFV	Crimean-Congo Haemorrhagic Fever Virus
CHF	Crimean Haemorrhagic Fever
CCHF	Crimean-Congo Haemorrhagic Fever
cRNA	complementary RNA
CI	confidence interval
DNA	deoxyribonucleic acid
df	degrees of freedom
DRC	Democratic Republic of the Congo
ELISA	enzyme-linked immunosorbent assay
EGFP	enhanced green fluorescent protein
G_n	N-terminal glycoprotein
G_c	C-terminal glycoprotein
ID	identity
IL	interleukin
IgM	immunoglobulin M
IgG	immunoglobulin G
IFA	immunofluorescence assay
L segment	large segment
L-protein	large protein
M segment	medium segment
mRNA	messenger RNA
m	meter
mg	milligram
ml	milliliter

nm	nanometer
n	number
N	nucleocapsid
N-protein	nucleocapsid protein
No.	Number
nt	Nucleotide
OTU	ovarian tumour-like protease motif
OR	odds ratio
PBS	phosphate buffer solution
PLTs	Platelets
pmol	Picomole
p-value	probability value
Q	Quartile
RNA	ribonucleic acid
RdRp	RNA-dependent RNA polymerase
rpm	rotations per minute
Ref	Reference
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	quantitative reverse transcription polymerase chain reaction
RNP	ribonucleoprotein
S segment	small segment
SKI-1	subtilisin kexin isozyme-1
S1P	site-1 protease
TNF	tumour necrosis factor
UAE	United Arab Emirates
uv	ultraviolet
vif	variance inflation factor
vRNA	viral RNA
X^2	Pearson`s Chi-squared test
µl	microliter

1. Introduction

Crimean-Congo haemorrhagic fever is a widespread, tick-borne zoonotic disease caused by CCHF virus (CCHFV). CCHFV is transmitted and maintained in nature by hard ticks (Ixodidae), mainly species of the genus Hyalomma. Therefore, ticks are considered to be reservoir and vector of CCHFV (Mourya et al., 2012; Sonenshine and Roe, 2014; Spengler et al., 2016a) as the virus persists in them throughout their life span (Gargili et al., 2017). Within the tick population, CCHFV can be transmitted transovarially, transstadially, venereally and through co-feeding (Logan et al., 1989; Gonzalez et al., 1992). Farm animals represent an important part of the tick-vertebrate-tick cycle (Spengler et al., 2016b). They are considered to be amplifying hosts with transient viremia, although they show no clinical signs (Gargili et al., 2017).

CCHF is a highly infectious disease with a case-fatality ratio between 5% and 80% in humans (Spengler et al., 2016a; Sas et al., 2018a). Humans get infected by the bite of infected ticks, through contact with viremic blood or tissue of infected animals or nosocomially by contact with an infected patient (Papa et al., 2018; Spengler and Estrada-Peña, 2018). The incubation period in humans is 3-7 days, depending on the route of infection (Whitehouse, 2004). The course of infection includes the incubation phase, a pre-haemorrhagic, a haemorrhagic phase and finally convalescence, if the patient survives. The observed clinical signs include fever, nausea, vomiting, petechiae haemorrhages on the skin and mucous membranes, bleeding from natural orifices, multi-organ failure and shock. Death may occur within 5-14 days (Hoogstraal, 1979; Bente et al., 2013).

CCHF was reported for the first time in 1944 on the Crimean Peninsula, and later in 1956 in the Belgian Congo (Democratic Republic of the Congo) (Whitehouse, 2004; Chamberlain et al., 2005; Messina et al., 2015; Spengler et al., 2018). The infection is endemic in Asia, Africa, south-western and south-eastern Europe (Messina et al., 2015; Mourya et al., 2015; Papa et al., 2015). In Asia, its distribution is from Middle Eastern to Far Eastern countries (Bente et al., 2013). It is endemic in Pakistan and its neighbouring countries; China, Afghanistan, Iran, and India (Sun et al., 2009; Chinikar et al., 2010; Mostafavi et al., 2012; Mourya et al., 2015).
In Pakistan, the first case was identified in 1976. Cases are reported from almost all parts of the country, however, the south-western province i.e., Balochistan is considered to be highly endemic with multiple outbreaks until today (Alam et al., 2013a; Ansari et al., 2018).

In Pakistan, Agriculture is a sector that makes a major contribution to the national economy of the country and a large proportion of the population is involved in livestock-related practices (Agricultural Census Organization, 2006). Moreover, in Balochistan compared to other parts of the country, livestock (especially sheep and goats) breeding is a major source of income for the native people (Government of Balochistan, 2016). Most people lack awareness regarding the zoonotic threat of CCHF, when they are in close contact with animals, and, most importantly, the presence of the tick vector on livestock and pastures (Atif et al., 2017). Recently, a study showed that *Hyalomma* ticks play a major role in infesting livestock in Pakistan. Ticks may harbour and transmit a large variety of pathogens in Pakistan (Rehman et al., 2017).

CCHF cases are often reported among agricultural and livestock workers (Lugaj et al., 2014b). The detection of CCHFV-specific antibodies allows estimating the seroprevalence in livestock and illustrates CCHFV circulation in particular geographical areas of Pakistan, including the province of Balochistan, and a possible risk for human infections in these areas (Spengler et al., 2016a; Ansari et al., 2018). In Pakistan, the epidemiological aspects of CCHFV infections in livestock and wild animals have not been studied extensively so far (Atif et al., 2017). Therefore, considering the importance of the disease, this study aimed at conducting a comprehensive epidemiological investigation of CCHFV infections among sheep and goats, both by serology and by collecting ticks from infested livestock and testing them for CCHFV in the province of Balochistan, Pakistan. Based on these results, the potential risk and protection factors for the infection of livestock with CCHFV identified with the ultimate goal of reducing the risk for human infection with this dangerous virus.

2. Literature review

2.1 Discovery and naming of CCHFV

The earliest suspected Crimean-Congo haemorrhagic fever (CCHF) infection in a human being was reported in the 12th century in Tadzhikistan. It was reportedly caused by the bite of a louse or a tick and resulted in clinical signs such as vomitus, severe haemorrhages with blood in sputum, the gums, in the abdominal cavity, the rectum and in urine. A disease with similar clinical signs at the same time was also described in other parts of Central Asia (Hoogstraal, 1979). The assumption that the patients suffered from CCHF was purely based on the fact that potential vectors of CCHF had attacked the affected people and developed related signs and symptoms, as no definite diagnostic tools existed at that time.

The Crimean haemorrhagic fever (CHF) outbreak in 1944-1945 on the war-torn and devastated Crimean Peninsula was brought to the attention of modern medical science, when around 200 Soviet soldiers became infected with CHF virus (Hoogstraal, 1979). In 1967, CHF virus was isolated for the first time from a patient (Drosdov) through inoculation of new-born mice by Chumakov and his team at the Institute of Poliomyelitis and Viral Encephalitides Moscow. This opened a new gateway for researchers towards the characterization of the virus and production of reagents (antigens and antibodies) for serological studies to identify CHF virus foci across different geographical areas (Whitehouse, 2004). In 1969, Casal (1969) showed that the CHF virus Chumakov had isolated from a patient in the USSR was antigenically similar to the 'Congo virus' that had been retrieved in Belgian Congo (now Democratic Republic of the Congo) in 1956 (Simpson et al., 1967; Woodall et al., 1967). Therefore, Casals et al. (1970) suggested CHF-Congo virus as a common name, but Hoogstraal (1979) coined the term 'Crimean-Congo haemorrhagic virus' (CCHFV) and since then this name has been used in the literature around the world.

2.2 Classification of CCHFV

CCHFV is a member of the genus *Orthonairovirus* in the family *Nairoviridae* (Figure 1). According to the most recent scientific view on virus taxonomy issued by the International Committee on Taxonomy of Viruses (ICTV), the *Orthonairovirus* along with *Shaspivirus* and *Striwavirus* represent the three genera in the family *Nairoviridae*. The genus *Orthonairovirus* has 14 species, while *Shaspivirus* and *Striwavirus* have only one species each (ICTV, 2018). The species belonging to the genus *Nairovirus* (currently named as *Orthonairovirus* by ICTV), are transmitted

by ticks (either by argasid or ixodid ticks). In this genus, three species are known to be pathogenic to humans, i.e. CCHFV, Nairobi sheep disease virus and Dugbe virus (Whitehouse, 2004).

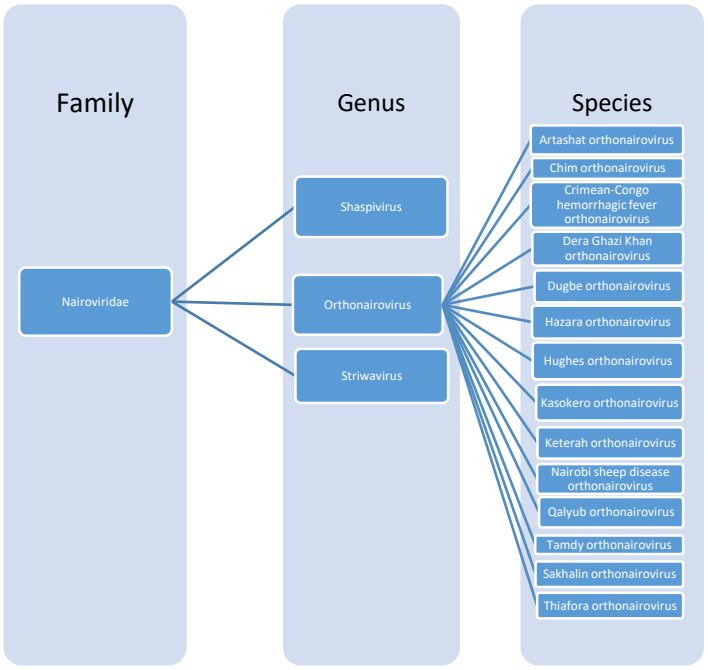

Figure 1. Taxonomic classification of CCHFV by the International Committee on Taxonomy of Viruses (ICTV, 2018).

2.3 Structure and genome of CCHFV

The CCHF virion has a spherical shape and a diameter of approximately 100 nm (Figure 2) (Porterfield et al., 1975; Korolev et al., 1976). Its lipid bilayer envelope with a thickness of approximately 5-7 nm is derived from the host cell. Protruding glycoproteins (G_n and G_c), which form 8-10 nm long spikes, are embedded into the lipid envelope (Whitehouse, 2004). The protrusions enable the virion to bind to its host cell receptors. Infected hosts usually form neutralizing antibodies against the glycoproteins Gn and Gc (Bente et al., 2013).

The genome of CCHFV consists of segmented negative sense single-stranded RNA. The small (S), medium (M) and large (L) segments encode the nucleocapsid (N), the glycoprotein precursor and the RNA-dependent RNA polymerase (RdRp), respectively (Morikawa et al., 2007). Each genome segment has complementary 5'-UCUCAAAGA and 3'-AGAGUUUCU terminal sequences, forming a stable panhandle structure and closed non-covalently circular RNA

molecules through intra-strand base-pairing. This characteristic is conserved among all nairoviruses. The terminal base pairs serve as functional promoters for the viral RdRp (Bente et al., 2013).

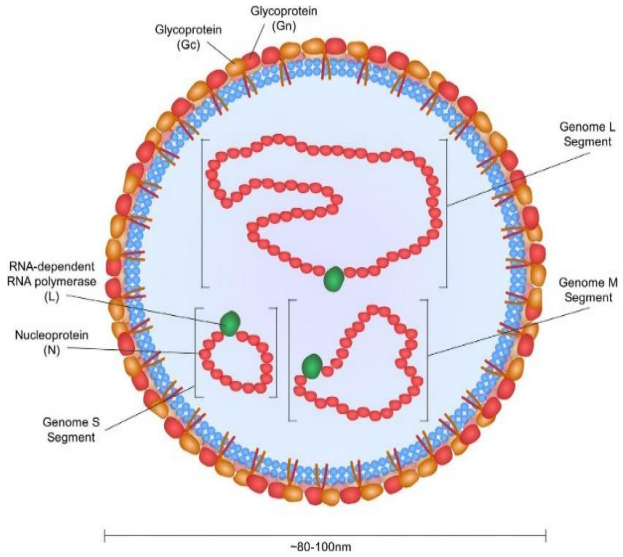

Figure 2. Portrait of the CCHF virion. The glycoproteins (Gn and Gc) are embedded into the outer lipid envelope and protrude as spikes. The RNA genome consists of a small (S), a medium (M) and a large (L) segment. It is encapsidated together with the nucleoprotein (N) and the RNA-dependent RNA polymerase (RdRp). Reprinted from Bente et al. (2013).

2.3.1 Small (S) segment

The S segment of the RNA genome encodes the nucleocapsid protein (N-protein). The N-protein forms the viral ribonucleoprotein complex (RNP) by binding the RNA genome. The RNP and L-protein (RNA-dependent RNA polymerase) interaction initiates the viral replication (Macleod et al., 2015). The N-protein, considered as an important antigen, it induces a strong immune response in the host (Dowall et al., 2016). It consists of a large globular domain and a protruded conserved caspase-3 cleavage site. The globular domain is probably responsible for RNA-binding (Carter et al., 2012), while role of the caspase-3 cleavage site is unclear. In the later stages of infection, the N-protein is cleaved in apoptotic cells (Dowall et al., 2016). When S segment sequences of CCHFV strains from different geographical areas were compared, a variability of 20% at the nucleotide and 8% at the amino acid level was observed (Deyde et al., 2006).

2.3.2 Medium (M) segment

The M segment of the CCHFV genome encodes the polyprotein, which is further cleaved co-translationally by a signal peptidase into the PreGn and PreGc precursors, which are then cleaved to form the N-termini of Gn (and the mucin-GP38 domain) and Gc by the subtilisin kexin isozyme-1/site-1 protease (SKI-1/S1P) and the SKI-1-like protease, respectively. This process begins in the endoplasmic reticulum and terminates in the Golgi apparatus, the virus assembly site (Figure 3) (Altamura et al., 2007; Bergeron et al., 2007). SKI-1/S1P-deficient cells infected with CCHFV secrete nucleoprotein-containing particles without glycoproteins; hence they do not produce infectious virus, although the PreGn and PreGc normally accumulate in the Golgi apparatus (Bergeron et al., 2007). Gn and Gc as parts of CCHFV virion attach to the host cell receptors and induce neutralizing antibodies in infected hosts (Flick and Whitehouse, 2005). When M segment sequences of CCHFV strains from different geographical areas were compared, a variability of 31% at the nucleotide and 27% at the amino acid level were observed. In this segment, considerably higher mutation rates are observed, which might be due to selection for effective attachment to the cells of vertebrates, and arthropods in distinct natural cycles in different geographic area (Deyde et al., 2006).

2.3.3 Large (L) segment

The L segment (12164 nucleotides) of the CCHFV is around twice as large as that of other bunyaviridae. It comprised of a 5'-noncoding region (76 nucleotides), an open reading frame (11835 nucleotides), and a 3'-noncoding region (253 nucleotides). The open reading frame encodes the L-protein, which consists of 3944 amino acids and contains an ovarian tumour-like protease motif (OTU), a DNA topoisomerase domain, a zinc finger motif, a Leucine zipper motif and a polymerase catalytic domain (Honig et al., 2004). The L segment of CCHFV strains has a variability of 22% at the nucleotide and 10% at the amino acid level (Deyde et al., 2006).

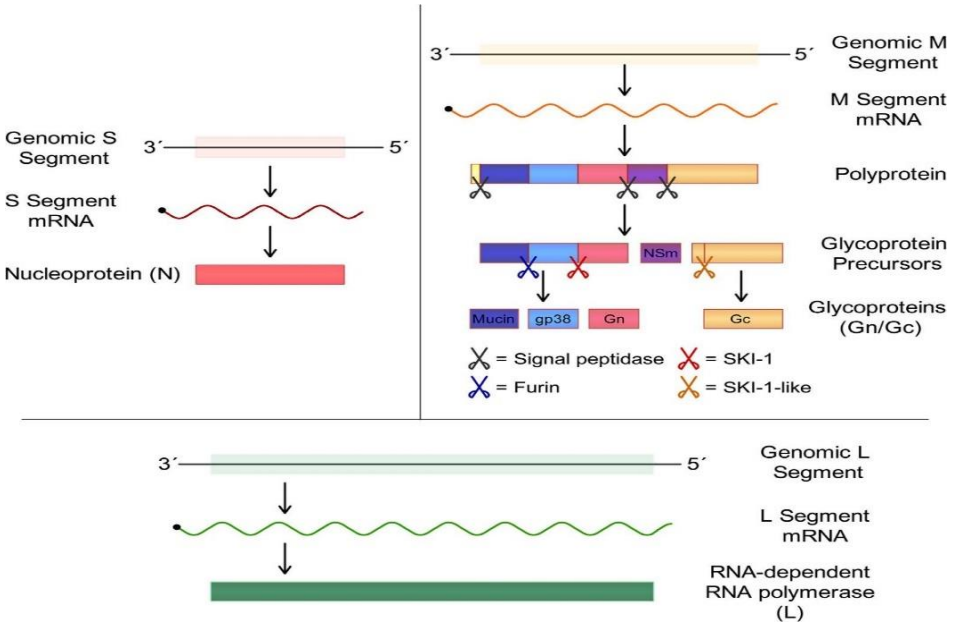

Figure 3. Processing of the S, M and L segments of CCHFV-encoded proteins. The process of transcription and translation from the S and L segments results in the nucleoprotein (N-protein) and RNA-dependent RNA polymerase, respectively. The M segment codes for a polyprotein that undergoes a process of cleavages and modifications, which finally lead to the generation of the glycoproteins Gn and Gc. Reprinted from Bente et al. (2013).

2.4 Replication

The host cell receptors specific for CCHFV attachment have not yet been unambiguously determined; however, a recent study revealed that the CCHFV surface glycoprotein Gc binds to a human host cell receptor known as nucleolin, a virus entry factor (Xiao et al., 2011). Earlier, a study by Bertolotti-Ciarlet et al. (2005) showed that Gc-specific monoclonal antibodies inhibited CCHFV infection in mice in a neutralization assay. In this case, CCHFV entered into the host cell through a clathrin-dependent endocytosis mechanism (Simon et al., 2009b). After entering the cytoplasm, the viral RdRp and its interaction with the viral genome segments give rise to the generation of complementary positive strands, which are then used as templates for the synthesis of negative strands (Bente et al., 2013). The virus is dependent on host cell microtubules for its process of internalization, virus assembly and finally egress (Simon et al., 2009a). A schematic view of the replication cycle of CCHFV is presented in Figure 4.

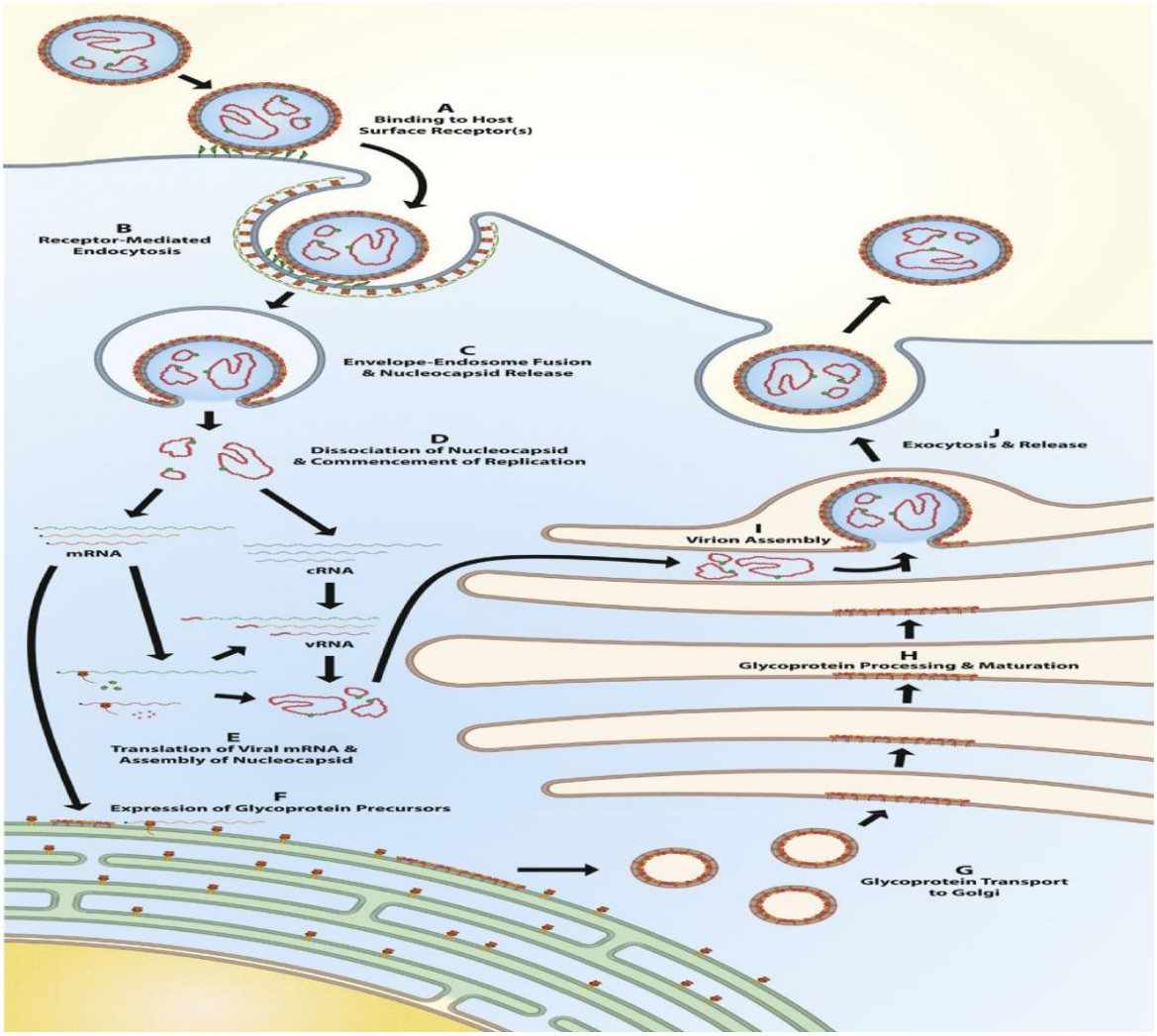

Figure 4. Scheme of the CCHFV replication cycle. (A) The virion binds to the host cell receptors, (B) endocytosis occurs, (C) and virion nucleocapsids are released into the cytoplasm by the fusion of the endosome membrane with the viral envelope. (D) The RNA-dependent RNA polymerase (RdRp) transcribes the viral genome dissociated from the nucleocapsids into messenger RNA (mRNA) and complementary RNA (cRNA). (E) The mRNA translates into viral protein and cRNA used as a template for vRNA production. Capsid protein, RdRp and vRNA associate to form new nucleocapsids. (F) In the endoplasmic reticulum, translation of the glycoprotein precursors occurs, (G) the glycoprotein precursors, are transported into the Golgi apparatus (H) for further processing. (I) Finally, new virions are generated and (J) exocytosis occurs. Reprinted from Bente et al. (2013).

2.5 Strains of CCHFV

The CCHFV strain classification is based on complete or partial sequences of the CCHFV S segment obtained from various geographical areas. Carroll et al. (2010) identified six lineages/clades, while Atkinson et al. (2012a), Atkinson et al. (2012b), Mild et al. (2010) and Bartolini et al. (2019) assigned virus isolates to 7 clades/lineages (Table 1, Figure 5). CCHFV is considered a genetically diverse arbovirus. The global livestock trade and migratory birds infested by potentially CCHFV-infected ticks may have caused a wide distribution of CCHFV around the world. This has apparently resulted in the accumulation of diverse sequences within the same geographical area, but also in the presence of similar strains in distant geographical regions Bente et al. (2013).

Table 1. Genetic lineages/clades of CCHFV, based on analysis of partial or complete S segment sequences. Reprinted from Bente et al. (2013).

Designation by Carroll et al. (2010)	Countries where isolated	Designation by (Atkinson et al., 2012a; Atkinson et al., 2012b)	Designation by (Mild et al., 2010)
I	Iran, South Africa, Senegal, Mauritania	Africa 3	6
II	South Africa, Namibia, DRC, Uganda	Africa 2	5
III	South Africa, Namibia, UAE, Senegal, Mauritania, Nigeria, Burkina Faso, CAR	Africa 1	3
IV	Iran, Pakistan, UAE, Madagascar, Oman, Iraq, China, Uzbekistan, Kasakhstan, Tadjikistan	Asia 1, Asia 2	1,2
V	Iran, Turkey, Greece, Russia, Bulgaria, Kosovo, Albania	Europe 1	4
VI	Greece, Turkey	Europe 2	7

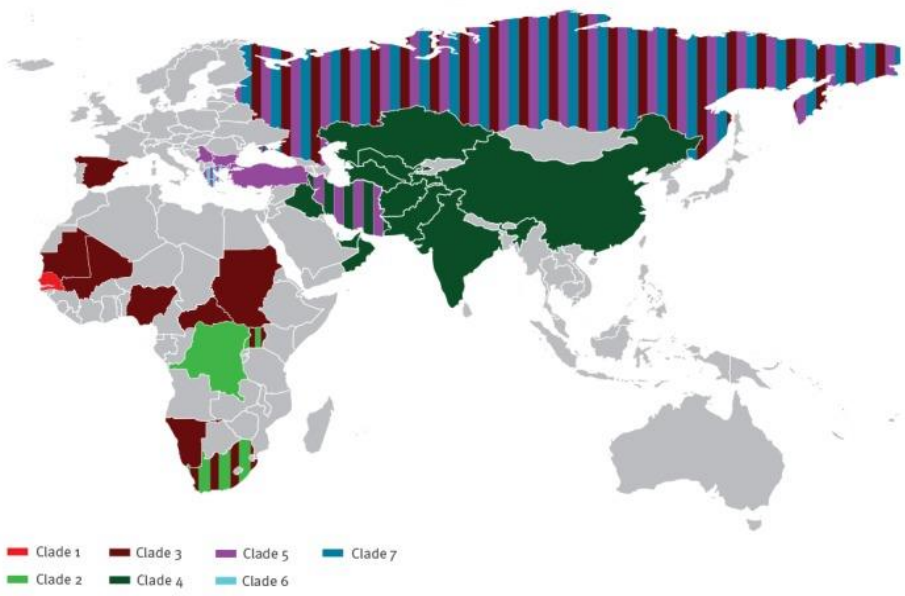

Figure 5. Geographical distribution of CCHFV clades, based on complete CCHFV S segment sequences. Clade I (red), clade II (light green) and clade III (brown) are found in Africa. Clade IV (dark green) is detected in Asia. Moreover, Clade V (purple), clade VI (light blue), and clade VII (blue) can be found in Europe. Reprinted from Bartolini et al. (2019).

2.6 Maintenance and transmission of CCHFV

2.6.1 Tick reservoir and vector

Ticks are arthropods from the suborder Ixodida in the class of Arachnida (Francischetti et al., 2009). The Ixodida include three families: the Argasidae, Ixodidae, and Nuttalliellidae. The Ixodidae are also known as hard ticks, characterized by a scutum (hard shield), while the Argasidae are called soft ticks according to their morphological characteristics, and lack a scutum. In the Nuttalliellidae, only one species is known so far, i.e. *Nuttalliella namaqua*, and its morphological characteristics include a dorsal pseudo-scutum, ball-and-socket joints, which articulate the leg segments, a small and a highly wrinkled cuticle with elevated rosettes and numerous pits (Nicholson et al., 2019). Ticks are ectoparasites of terrestrial mammals, birds, reptiles and amphibians. They bite these animals and feed on blood of vertebrates to obtain the nutrients needed for their further development characterized by the stages of egg, larva, nymph and adult tick. In particular, adult females need to feed on blood as a prerequisite to produce eggs (Francischetti et al., 2009).

Ticks are considered as a natural reservoir for CCHFV, as the virus is maintained in several species of the ixodid ticks through venereal, transovarial, transstadial and co-feeding transmission. Hence, the ticks remain infected and infectious throughout their lifetime (Logan et al., 1989; Gonzalez et al., 1992; Bente et al., 2013). CCHFV has been isolated from soft ticks (family Argasidae), hard ticks (family Ixodidae) and biting midges. However, the isolation of CCHFV does not necessarily lead to the recognition of these arthropods as vectors or reservoirs unless, been demonstrated that they maintain the virus throughout their life span and transmit it to vertebrates. Experimental studies on soft ticks shown that CCHFV fails to disperse in their tissues and is not transstadially transmitted to further developmental stages. CCHFV isolated from biting midges, which were believed to be recovered from midges that had recently fed on a viremic animal (Bente et al., 2013).

Ixodid ticks, in particular ticks of the genus *Hyalomma,* are considered to be the most important arthropods in the ecology and epidemiology of CCHFV (Whitehouse, 2004), which is evident from experimental studies performed by Logan et al. (1989) and Gonzalez et al. (1992). Furthermore, many human patients suffering from CCHF reported *Hyalomma* tick bites (Bente et al., 2013).

2.6.2 Vertical transmission of CCHFV

To become competent vectors of CCHFV, ticks need to support a sustainable replication of the virus in their tissues. Furthermore, transmission of virus from adult males to adult females, from females to their eggs, then sequentially through the developmental stages of the tick from the larva, via the nymph to the adult tick (Shepherd et al., 1991; Gonzalez et al., 1992; Gordon et al., 1993; Dohm et al., 1996). CCHFV replication occurs in the lining of the midgut of the tick after ingestion of the blood meal, then the virus spreads to distinct tissues, with higher virus titers in the salivary glands and reproductive tissues as compared to other tissues (Dickson and Turell, 1992). Female ticks generates thousands of eggs, which may be infected with virus since it is transovarially transmitted, and consequently, maintains the virus in the tick population, even in absence of a vertebrate host (Bente et al., 2013).

2.6.3 Horizontal transmission of CCHFV

The transmission of CCHFV between mammals and ticks takes primarily place during spring and summer. During this period, larvae and nymphs require blood meals for their developmental

growth, and the adult female ticks to produce eggs. When infected ticks bite vertebrates, the virus is transmitted, replicates in tissue cells of the vertebrate host resulting in viremia in the animal. The quantity of virus required for infection of ticks varies among tick species. Ticks may attach to the host for a couple of weeks, which may increase the probability of virus infusion into the host if the tick is infected with CCHFV, or the intake of virus from an infected host if the tick is naïve (Bente et al., 2013). CCHFV can be transmitted from an infected tick to uninfected ticks while they are co-feeding on the same host (Gordon et al., 1993). After feeding on a host, the larva stays on it for some time, plunges then off from the host and moults to the next developmental stage (nymph), which searches for another host (Bente et al., 2013).

Many species that are competent for transmitting CCHFV belong to the genus *Hyalomma*, especially to the species *Hyalomma marginatum*, which is a two-host tick, i.e. larvae and nymphs feed on birds, hares and rodents, whereas adults feeds on ungulates (cattle, sheep, goat, etc.) (Sonenshine and Roe, 2014). *Hyalomma* ticks, also known as, “hunting ticks”, because they can quest up to 400 meters to search for a host suitable for blood feeding (Bente et al., 2013).

Birds play an important role in CCHFV distribution, as they can transport infected ticks over long distances and thus introduce the virus into new areas, although they are not susceptible to infection with CCHFV (Hoogstraal, 1979). If ruminants are infected with CCHFV, the virus replicates in them and causes a viremia, which lasts approximately from four to 7 days. Infected ruminants fail to show clinical signs or symptoms upon infection with CCHFV (Gonzalez et al., 1998). *Hyalomma* ticks bite humans living in the rural areas especially in spring and summer, when these ticks are questing for a blood meal (Bente et al., 2013), and infected if bitten by an infected tick. In addition to transmission through the bite of an infected tick, humans can also become infected with CCHFV by contact with blood or tissues of viremic animals, e.g. at the slaughterhouse, or with tissue or body fluids, in particular blood, from infected humans, e.g. when treating or caring for infected patients (nosocomial infection) (Maltezou et al., 2009). Abattoir workers, veterinarians, farmers and people involved in health care are therefore high-risk professional groups for contracting CCHFV infections (Vawda et al., 2018).

The CCHFV-tick-vertebrate-tick life- cycle, is displayed in Figure 6. It also illustrates the possible vertical and horizontal transmission routes in the tick population.

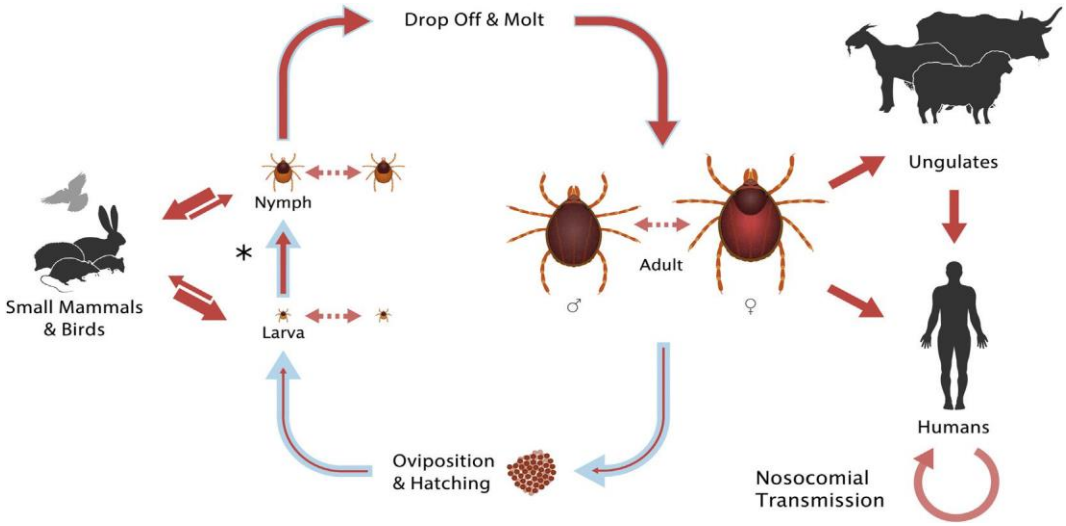

Figure 6. Scheme of CCHFV transmission. The blue arrow indicates the course of the infection in ticks (vertical, i.e. transovarial and transstadial transmission). After hatching, the larvae, which have developed from eggs, bite small mammals and take a blood meal, moult to nymphs or fall off and moult then. Subsequently, nymphs that have moult on the same animal take another blood meal or seek another animal to feed on. The nymphs engorge and drop off from the animal and moult into adult ticks. Finally, the adult ticks seek a larger animal (where they also mate), take a blood meal, engorge and then drop off for oviposition. It should be noted, that CCHFV also been transmitted between ticks of different stages; result of co-feeding on the same host (dashed brown arrows). Humans get infected with CCHFV by the bite of an infected tick or by contact with tissues or body fluid, in particular blood, of infected animals or humans. The efficiency of CCHFV transmission is demarcated by the thickness of the red arrow. Reprinted from Bente et al. (2013).

2.7 Epidemiology of CCHFV

CCHFV is distributed in a wide geographical area around the world including areas from western part of China over south Asia towards the Middle East, south-eastern Europe, and large parts of Africa (Bente et al., 2013). Areas other than known regions endemic for CCHFV may portray absence of viral foci, or lack of detection; however, absence of human cases in the presence of surveillance system usually reflects absence of the virus (Spengler et al., 2019). CCHFV may be introduced into new geographical areas as a consequence of climate change, tick transportation

by birds or due to anthropogenic factors (changes in the natural ecosystem or land-use by human influence) (Gargili et al., 2017). Warm summers and mild winters represent favourable conditions for CCHFV vectors (i.e. *Hyalomma* ticks) to survive in the environment. An increase in average annual temperature may thus allow them to spread into new habitats, especially towards northern latitudes of the globe, where CCHFV is not yet prevalent (Mertens et al., 2013).

2.7.1 CCHFV in Europe

CCHFV came into limelight after an outbreak in 1944-1945 in Crimea (Hoogstraal, 1979). Extensive research has since been done on this disease, in particular by the pioneering work of Chumakov. Diagnostic tools were developed that allowed identification of CCHF cases in humans. A few years after the detection of CCHF on the Crimean peninsula, CCHF was found in Bulgaria (Bente et al., 2013), where 1105 CCHF cases were reported between 1953 and 1974. CCHFV was isolated from *H. marginatum* ticks in Bulgaria (Papa et al., 2004). The first CCHF case in Kosovo was reported in 1954. Since 1990, the number of outbreaks increased and the seroprevalence has considerably risen from 5.2% in 1991 to 24.3% in 1995 (Mertens et al., 2013). In Albania, the first CCHF case was identified in 1986, and since then sporadic cases been reported every year (Papa et al., 2002a). Anti-CCHFV antibodies also have been detected in ruminants in Albania (Lugaj et al., 2014a). The CCHFV strains circulating in the Europe are Europe 1, Europe 2, and AP92 (Papa et al., 2010; Bente et al., 2013). In Greece, the CCHFV (AP92 strain) was first isolated in 1975 in ticks (*Rhipicephalus bursa*) from goats. This strain is considered mildly virulent, as no human cases with clinical symptoms have been reported, however, antibodies were detected among them. The first human case was reported in 2008, and the CCHFV strain identified was closely related to CCHFV strains from other Balkan states (Papa et al., 2010). In Hungary, CCHFV was first isolated in 1972, while the first and only human case was reported in 2004. Yet, *Hyalomma* ticks and anti-CCHFV antibodies have been detected in ruminants in Hungary (Hornok and Horváth, 2012). Although no human CCHF case has so far been reported from Romania, *H. marginatum* was found on cows, sheep and horses in the country. Moreover, a CCHFV-specific seroprevalence of 27.8% been detected among sheep (Ceianu et al., 2012). In 2010, CCHFV has first been detected in *H. lusitanicum* in deer in Spain. The virus strain was similar to African CCHFV strains. It has been suggested that migratory birds may have introduced it into the country (Estrada-Pena et al., 2012b). Subsequently, two CCHF cases in 2016 (Cajimat et al., 2017) and one case in 2018 were reported in Spain (ISID, 2018).

The first human CCHF case in Turkey was reported in 2002. From 2002 to 2005, 500 cases were observed with a case-fatality ratio of 5.2% (Ergönül, 2006). Among cattle, a seroprevalence of 79% has been reported (Gunes et al., 2009). *Hyalomma marginatum* ticks collected from cattle, sheep and human, have been found infected at percentages of 19%, 28% and 9%, respectively (Gunes et al., 2011). In Turkey, Europe 1, Europe 2 and strains that have similarity with AP92 were found (Midilli et al., 2009; Gargili et al., 2011).

2.7.2 CCHFV in Africa

CCHFV was first isolated in Africa in 1956 in the Belgian Congo (now Democratic Republic of Congo) (Simpson et al., 1967; Woodall et al., 1967). At that time, it was known as Congo virus, but later its similarity with the CHF virus isolated on the Crimean peninsula in the former USSR became obvious, so that the virus finally named CCHFV (Casal, 1969; Casals et al., 1970; Hoogstraal, 1979). In Nigeria, the virus was identified in ticks, domestic livestock and hedgehog in a study conducted from 1964-1968 (Causey et al., 1970). The first CCHF case in South Africa was identified in 1981 in a young boy with a *Hyalomma* tick attached on his scalp (Gear et al., 1982). Subsequently, from 1981 to 1987, up to 50 cases of CCHF occurred (Swanepoel et al., 1989). In a serological survey conducted from 1964 to 1985, anti-CCHFV antibodies were detected in large wild animals (giraffe, rhinoceros, eland, buffalo, zebra, gemsbok and Kudu), hares, rodents and domestic dogs (Shepherd et al., 1987). In another study, 28% of cattle were positive for CCHFV antibodies and 78% of cattle herds contained seropositive animals (Swanepoel et al., 1987). CCHF cases and anti-CCHFV antibodies in animals were also reported from Sudan (Aradaib et al., 2010). In Kenya, CCHFV has been detected in *Rhipicephalus* ticks feeding on sheep in 1970. In 2008, the virus was also found in *Hyalomma* ticks. The first human CCHF case was reported in 2000 (Sang et al., 2011; Lwande et al., 2012). The first human CCHF case in Mauritania was reported in 1983. In 2003, an outbreak occurred in the country affecting 38 people. Subsequently, animal sera and ticks collected from the area nearby the affected human population. Seropositive animals were found and CCHFV genome detected in ticks (Nabeth et al., 2004a). In Senegal, CCHFV infections have been reported among humans, animals and ticks (Nabeth et al., 2004b). CCHFV was isolated from *Rhipicephalus* ticks collected from cattle in 1985 in Madagascar. Putative human infections were reported in 1988 in two seropositive humans. Madagascar is considered to be low a risk area for CCHFV infection due to the absence of *Hyalomma* ticks (Andriamandimby et al., 2011). CCHFV was isolated from *Hyalomma* ticks in Mali in 2011, but no human CCHF case has been reported so far (Zivcec et

al., 2014). Previously, unrecognized CCHF infections were detected in a study conducted in Sierra Leone between 2007 and 2014, in which 13 human sera (2%) contained CCHFV-specific antibodies. Although the prevalence was low, it suggested the possible presence of a CCHFV reservoir and of a vector in the country, and therefore a potential risk for the human population (O'Hearn et al., 2016). So far, no human CCHF case been reported in Egypt, but CCHFV in *Hyalomma* ticks and anti-CCHFV antibodies have been detected in animals in the country. Egypt has imported animals from CCHFV-endemic countries, so that this and the movement of migratory birds from affected areas could be a possible reason for the influx of the virus into the country (Morrill et al., 1990; Mohamed et al., 2008; Chisholm et al., 2012).

2.7.3 CCHFV in Asia

CCHFV has been distributed in a wide geographical area in Asia including Turkey (already mentioned in the section on Europe) Tajikistan, Khazakhstan, Pakistan, China, Afghanistan, Iran, India, United Arab Emirates, Saudi Arabia, Oman, Iraq, and Kuwait. The CCHFV strains circulating in this region are Asia-1 and Asia-2. The first CCHF case in Tajikistan was recorded in 1943, and until 2010, 237 cases had been reported (Bente et al., 2013). In Kazakhstan, the first case was registered in 1948, and 108 cases have been reported until 2000 (Chinikar et al., 2010; Bente et al., 2013). In 1950, the first CCHF case in Afghanistan was reported. The number of outbreaks increased since 1998 with more than 100 cases observed within a few years (Chinikar et al., 2010). A serological survey conducted in 2009 identified a seroprevalence of 11.2% in humans, 79.1% in cattle and 75% in sheep (Mustafa et al., 2011). The first CCHF case in Iran was recorded in 1970. It seems that most of the country (23 out of 30 provinces) is endemic for CCHF (Chinikar et al., 2010). CCHFV also been detected in different tick species including *H. marginatum* in many regions of country (Telmadarraiy et al., 2015). Anti-CCHFV antibodies been detected in sheep, cattle, goats, and ostriches (Mostafavi et al., 2013a; Mostafavi et al., 2013c). In the United Arab Emirates, the first CCHF case reported in 1979. In 1994, an outbreak occurred among butchers and livestock workers, in which CCHFV antibodies were also detected among local and imported livestock (Khan AS et al., 1997). Moreover, in other Middle Eastern countries including Iraq, Kuwait, Oman, and Saudi Arabia, a lower frequency of CCHF cases has been reported (Chinikar et al., 2010). The first CCHF case in India was reported in 2011. A study conducted between 2010 and 2011 revealed that 43% of the tested sera from domestic animals were positive for CCHFV-specific antibodies. Moreover, in the same report it is mentioned that CCHFV was also detected in *Hyalomma* ticks (Mourya et al., 2012). CCHF has been endemic in

China, where the first outbreak was reported in 1965 and where CCHFV has also recently been isolated from *Hyalomma* ticks (Moming et al., 2018; Zhang et al., 2018). In livestock, a CCHFV seroprevalence of 12.7% was observed (Sun et al., 2009).

CCHFV was first reported in Pakistan in 1970 in *Hyalomma* ticks (Begum et al., 1970). The first known CCHF outbreak occurred in 1976, in which a shepherd in the northern part of the country was diagnosed with the disease and died later. The disease was also transmitted to members of his family as well as to health care workers in the hospital during treatment (Hoogstraal, 1979). In 1983, antibodies against CCHFV have been detected in rodents, cattle and buffaloes (Darwish et al., 1983). Multiple outbreaks occurred since the first report of CCHF in the country, in particular in the south-western part i.e. the province of Balochistan (Alam et al., 2013a; Ansari et al., 2018). From 2003 to 2008, 57 (67%) out of 85 CCHF human cases were reported in this province compared to other provinces in the country (Atif et al., 2017), and the case-fatality ratio recorded in the province of Balochistan was up to 20% (Khurshid et al., 2015).

2.8 Infection with CCHFV in humans and its zoonotic importance

2.8.1 Clinical manifestation and pathogenesis

The course of a CCHFV infection in humans consists of following phases: incubation period, pre-haemorrhagic phase, haemorrhagic phase and convalescence (Hoogstraal, 1979). The incubation period mainly depends on the route of exposure to the virus. It is around 3 days in case of a tick bite, 5 days after contact with tissue or blood of an infected animal, and around 6 days in case of contact with blood of an infected human (Whitehouse, 2004). The pre-haemorrhagic period is characterized by the onset of fever, headache, chills, nausea, lumbar, rheumatic or epigastric pain, vomiting, liquid stools or loss of appetite (Hoogstraal, 1979). An anti-viral drug (Ribavirin; trade name Virazole®) that disrupts viral replication is more likely to be effective during this stage (Ergonul, 2008). Then, petechiae and haemorrhages appear on the skin and on the mucous membranes of the intestine, the uterus and the respiratory organs. Bleeding of nose, buccal mucosa, gums, conjunctivae, and of the ears occurs. In more advanced cases, several organs get involved with a prominence of enlargement of spleen and liver. In case of multi-organ failure, the patient enters into shock and death occurs within 5-14 days (Bente et al. (2013). If the patient survives, the convalescence period begins with hair loss, asthenia, labile pulse and polyneuritis. CCHF patients are usually discharged from hospital after 3-6 weeks; depending on return to normal body function (Hoogstraal, 1979).

The case-fatality ratio in human CCHFV infection ranges from 5% to 80%. This variation depends mainly on the quality of the public health facilities, infected persons' access to the disease reporting system of the respective country (Yilmaz et al., 2009; Sas et al., 2018b) and the conditions of exposure (tick bite, or contact with body fluid or tissue of infected human or animal). The pathogenesis of human CCHF infection is poorly understood. CCHF cases are sporadic, they usually occur in rural areas, which lack autopsy facilities to establish the cause of death in fatal cases and obtain new insights into the pathogenesis of CCHF infections. In addition, a high containment biosafety laboratory (BSL-4) is required to perform research. Most importantly, animal model are lacking to understand the pathogenesis. Current knowledge is mainly based on the blood profile and biopsy results from CCHF patients (Whitehouse, 2004).

The course of CCHF infection in humans is illustrated in Figure 7. In the plasma of fatal cases of CCHF fatal patients, pro-inflammatory cytokines, including tumour necrosis factor (TNF)-α and interleukin (IL)-6, are present. A considerable decline of fibrinogen occurs and lymphocyte counts drop. Plasma concentrations of aspartate (AST) and alanine aminotransferases (ALT) increase, prothrombin and partial thromboplastin times prolongs, and white blood cells decrease or increase depends on the time of sample collection (Ergonul, 2008). Endothelial damage causes capillary fragility with haemostatic failure and consequently thrombocytopenia (low level of blood platelets) (Whitehouse, 2004). The viral load in fatal cases is $\geq 1 \times 10^9$ RNA copies per ml, compared to non-fatal cases with a considerably lower viral load (Cevik et al., 2007). As with the inception of haemorrhages and shock, viremia declines and IgM antibodies are detected, most probably in non-fatal cases, i.e. if an antiviral therapy has been successfully conducted along with supportive treatment and if there was no multi-organ failure. The virus can be detected in the blood by reverse transcriptase polymerase chain reaction (RT-PCR) after the inception of illness (Ergonul, 2008).

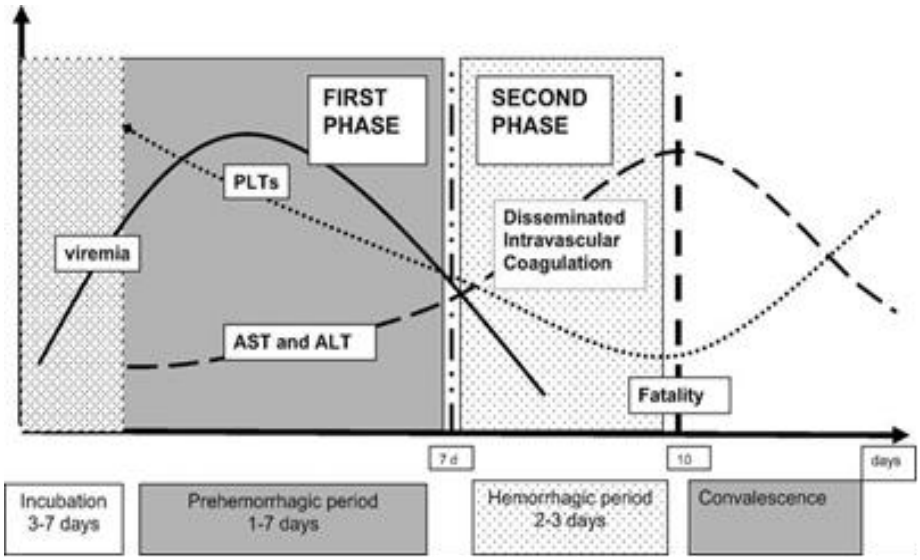

Figure 7. Course of CCHF infection in humans. The entry of the virus into the body is followed by the incubation period, the pre-haemorrhagic phase, the haemorrhagic phase and finally convalescence, if the patient survives. Reprinted from Ergonul (2008).

2.9 Prevention of CCHF

Personal protective measures are prerequisite for humans living in the CCHF endemic areas. It is pertinent to avoid areas with a high abundance of ticks, to wear proper clothing, to use repellents for avoiding tick attachment to the skin, and to wear gloves, if direct contact with animal blood or tissues is expected to occur, in particular on abattoirs and when bleeding animals (Ergönül, 2006; Atif et al., 2017). In health care facilities, proper safety measures are required as to avoid nosocomial infections from infected patient to health care workers (Ergönül, 2006). These measures include isolation of CCHF-suspected and confirmed patient in a special room, use of gowns, gloves, face shields or goggles by all healthcare workers, who may get in contact with the patient and any biomaterial from her or him. People visiting the patient must also be protected. Biosafety precautions must be taken in when handling samples, biopsy material, secretions and excretions of CCHF patients to avoid accidental transmission to healthcare workers or laboratory personnel. An awareness program is very important to educate people regarding the zoonotic importance of the disease, which is the responsibility of both health and veterinary officials (Vorou, 2009). Regular surveillance among livestock could be a useful early warning tool to protect the human population in areas at risk.

There is currently no vaccine available to protect humans from CCHF. A major constraint for developing effective vaccines against CCHF is the lack of an animal model. In 1970, a formalin-inactivated mouse brain CCHFV vaccine been developed in the former USSR, but it showed a low-level antibody response among recipients. A similar type of vaccine was developed in the Bulgaria, which induced a humoral and cellular response to CCHFV, but with low titers of neutralizing antibodies (Bente et al., 2013).

2.10 Treatment of CCHF

The treatment of a CCHF-infected human is currently based on supportive measures, checking of haematological parameters and coagulation status, taking appropriate symptomatic measures to stabilize the condition of the patient and the use of ribavirin as an antiviral drug (Ergonul, 2008). Ribavirin (trade name Virazole®) was first produced in 1972; it contains a purine nucleoside analogue, a modified base, and D-ribose sugar. *In vitro*, it prevents the replication of DNA and RNA of a wide range of viruses, and therefore, it has been considered to be a suitable broad spectrum antiviral drug (Ergonul, 2008). Ribavirin is licensed for the treatment of hepatitis C and respiratory syncytial virus infections (Bente et al., 2013). It is currently the only antiviral drug used for the treatment of CCHF. It showed antiviral efficacy *in vitro* when tested against CCHFV isolates from different countries (Ergonul, 2008). Ribavirin treatment of suckling mice reduced the replication of the virus in the liver, but viremia not completely prevented. Any further effect of the drug could not be demonstrated due to the lack of a suitable animal model for CCHF (Ergonul, 2008). The clinical use of ribavirin among humans in several countries in early phases of illness has proven beneficial (Bente et al., 2013).

2.11 Diagnosis of CCHF

A human patient is suspected for CCHFV infection when he or she shows clinical signs such as high fever, fatigue, myalgia, loss of appetite, headache (Mertens et al., 2013), coagulation defect, vascular leak (Bente et al., 2013), tick bite history, contact with animal blood or tissue, or contact with CCHF-positive patients (Mertens et al., 2013). Further initial laboratory diagnosis performed to check for thrombocytopenia, leukopenia and elevated serum ALT and AST levels (Bente et al., 2013). Patients with suspected CCHF are confirmed by detection of viral RNA in the blood by reverse transcriptase polymerase chain reaction (RT-PCR) or detection of anti-CCHFV IgM or IgG antibodies by immunological assays such as enzyme-linked Immunosorbent assay (ELISA)

or immunofluorescence assay (IFA) (Mertens et al., 2013). The “gold standard” for the diagnosis of CCHFV is virus isolation, but for this purpose, a Biosafety Level (BSL) 4 laboratory is required, which exists only in South Africa and China among the CCHF endemic countries (Bente et al., 2013; Han et al., 2019).

The virus can be detected in the blood for up to 14 days. IgM antibodies can be found from the 4th day of illness up to 4 months, while IgG antibodies are shown from day 6-post infection up to 5 years (Mertens et al., 2013). CCHFV can be isolated through cell culture, which is simple and fast (2-5 days), but less sensitive as compared to traditional intracranial inoculation into new born mice. The cell lines used for the virus culture are Vero, LLC-MK2, SW-13 and BHK-21. Successful isolation requires samples with relatively high virus concentration; blood obtained from patients during the initial 5 days of disease is usually suitable. The virus may have no or little cytopathic effect; infected cells therefore detected by immunofluorescent antibody tests using CCHFV-specific monoclonal antibodies. The reverse transcriptase PCR (RT-PCR) is a rapid, sensitive and specific method to detect virus genome in samples (Ergönül, 2006). It has also limitations as virus genome can only detected when a patient is in a viremic state. Secondly, strain variations may also reduce its sensitivity. Therefore, immunological assays could also be performed along with the RT-PCR to diagnosis CCHFV infections (Mertens et al., 2013).
In animals, the virus genome can be detected in the viremic phase, and, antibodies may be detected from 6-14 days of post-infection to several years (Shepherd et al., 1989a). Several in-house ELISAs have been developed for the detection of antibodies to CCHFV in animal sera (Burt et al., 1993; Qing et al., 2003; Garcia et al., 2006; Mertens et al., 2015; Schuster et al., 2016a; Schuster et al., 2016c). In a novel-in-house ELISA, recombinant N-protein antigen has been used instead of whole virus preparations. This test can therefore be performed outside BSL 4 laboratories (Mertens et al., 2015; Schuster et al., 2016a; Schuster et al., 2016c). A commercially available indirect CCHFV ELISA (Vector Best, Novosibirsk, Russia) and IFA (Euroimmun, Lubeck, Germany) for human sera have been adopted for animal sera to detect anti-CCHFV antibodies (Mertens et al., 2015; Schuster et al., 2016c). Most recently, a one-step multiplex real-time RT-qPCR has been developed by Sas et al. (2018b) with a specific primer sets for all of the known six CCHFV genotypes to detect CCHFV genome in animal sera, human sera and ticks.

3. Materials and methods

3.1 Study area

Balochistan is, with 347,190 km², the largest province of Pakistan (Majeed, 2015). It is located in the south-western part of the country (Figure 8). The climate is arid and semiarid. In total, 93% of Balochistan is covered by rangelands, of which 28% are suitable for animal husbandry. The sheep and goat population of the province forms 48% (12.8 million) and 22%, (11.8 million) respectively, to the total sheep and goat population of the country (Government of Balochistan, 2016). In Balochistan up to 47% of the provincial economy depends on animal husbandry (Government of Balochistan, 2016), therefore livestock plays an important role in the sociocultural and socioeconomic survival of the people (Raziq et al., 2010). The livestock husbandry system of sheep and goats in the province is divided into nomadic (30%), semi-nomadic (60%; trans-humane/agro pastoral) and sedentary (10%). Most of the livestock can be found in the north part of Balochistan (76%), while only around 24% of provincial livestock lives in the southern plains (Government of Balochistan, 2016). The northern zone includes Zhob, Loralai, Pishin, Quetta, Sibi, Nasirabad, Kohlu, Kalat, and northern part of Khuzdar districts, while the southern zone includes Kharan, south part of Khuzdar, Chagai, Lasbela, Turbat, Gwadar and Panjgur districts (Ahmad and Islam, 2011). The less arid and poor conditions in the ranges compel the nomadic and semi-nomadic graziers to migrate their livestock seasonally to feed them (Government of Balochistan, 2016).

3.2 Epidemiological investigations of CCHFV infection in sheep and goats in Balochistan

3.2.1 Study design

A cross-sectional study was conducted to determine the prevalence of CCHFV in sheep and goats in the province of Balochistan, Pakistan. Due to lack of information on the seroprevalence regarding CCHFV in the small ruminant population, the sample size was calculated for large sheep and goat populations with an expected prevalence of 50%, with 95% confidence level and 5% desired precision (Cannon and Roe, 1982). The required sample size was 384 for sheep and 384 for goats for the whole province of Balochistan. We rounded to 400 animals in case some samples could not be analysed. To assess the prevalence in livestock farms as well as in small holdings for sheep and goats, the sample size was set to 1600 animals (400 goats in small holdings, 400 sheep in small holdings, 400 goats in livestock farms, and 400 sheep in livestock farms). Livestock farms were defined as commercial animal holdings that reared sheep and goats

for breeding or producing milk to provide meat and milk product to the local market. Small holdings were defined as residential households that reared up to 15 livestock animals as a cheap protein source only for family members. Both types of farms have similar husbandry practices. Each livestock farm and each small holding was considered as an epidemiological unit. The study was conducted from July to September 2016. It was decided to sample 5 sheep and 5 goats within each farm. In total, 160 farms were sampled. Because of logistic issues, only farms in three randomly selected divisions (Quetta, Sibi and Zhob) were visited. To obtain spatial representativeness, the farms were stratified to the districts and divisions proportional to the number of animals in each division (Government of Balochistan, 2016). The animals were not tagged, therefore, they were selected systematically, so that each animal of the farm had the same chance to be selected, irrespective of age, sex and breed. Before sampling, the owner of the farm was informed regarding the purpose of the study and consent was obtained.

3.2.2 Sample collection

The blood samples were collected from sheep and goats with the help of trained veterinarians. From the jugular vein, 5 ml of blood were drawn using labelled BD Vacutainer®. The samples were transferred to the laboratory and centrifuged. The obtained serum was transferred to Eppendorf tubes, then sealed with paraffin foil, labelled and stored at -20 °C until shipment. The cold chain was intact through the whole shipment process to the Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany.

3.2.3 Questionnaire

A standard questionnaire was used to collect information related to farm management and the individual animals (Appendices). The questionnaire included both closed and open-ended questions. A moderated interview was conducted with the owner of the farm in the local language. All interviews were performed by the same investigator, who also entered the answers into the questionnaire form. The investigator also recorded the hygiene conditions on each farm as poor or satisfactory by assessing the disposal of dung, potential feed contamination with dung and the cleanliness of the water supply for animals.

3.2.4 Detection of antibodies directed against CCHFV

The sheep and goat sera were serologically tested as described by Schuster et al. (2016). We followed a hierarchical diagnostic decision tree, in which all samples were first tested in a species-specific indirect in-house CCHFV-IgG enzyme-linked immunosorbent assay (ELISA) and in an adapted commercial species-specific indirect CCHFV-IgG ELISA (Vector Best, Novosibirsk, Russia). In the indirect in-house CCHFV-IgG ELISA, the final result for each sample was calculated as fR = [R-sample/R-positive]*100, where fR is the final result, R-sample the final OD value of a sample and R-positive the final OD-value of the positive control sample. For the goat samples, an fR >16% was considered positive and for the sheep samples an fR >28%. In the adapted commercial species-specific indirect CCHFV-IgG ELISA, sheep and goat samples with OD value >0.7 were considered positive. In a second step, samples with divergent results in the two ELISAs were run in a commercial species-adapted indirect CCHFV-IgG immunofluorescence assay (IFA) (Euroimmun, Lübeck, Germany) to obtain the final result.

3.2.5 Detection of CCHFV RNA by real-time RT-qPCR

Serum samples were divided into pools of five to detect CCHFV RNA. RNA was prepared using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer`s instructions. Further, a one-step multiplex real-time RT-qPCR developed by Sas et al. (2018a) was used to detect viral S-segment of the CCHFV RNA. It detects all known CCHFV strains as it utilizes specific primer sets for each of the six known CCHFV genotypes. The RT-qPCR reaction was performed with 5 µl of sample RNA, 15 pmol of each CCHF-deg primer, 1 pmol of each genotype specific CCHF primer, 3 pmol of each CCHF probe (also CCHF-CoProbe) and the QuantiTect Probe RT-PCR kit (Qiagen). The total reaction volume was 30 µl. For the extraction control, IC2-RNA (*in vitro* transcript of enhanced green fluorescent protein) was employed, and for its detection EGFP-Mix 1 (5 pmol of each primer) and EGFP-HEX (3 pmol) were used. The cycling conditions included reverse transcription for 30 min at 50°C, followed by polymerase activation at 95°C for 15 min, denaturation 10 s at 95°C, annealing 25 s at 55°C and elongation 25 s at 72°C.

3.2.6 Statistical Analysis

Statistical analyses were performed using R (R Core Team, 2013, R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria; URL, http://www.R-project.org/) and R-Studio; an integrated development environment for R software

for statistical computing and graphics (RStudio, 2016). Maps were produced using the ArcGIS version 10.5.1 (Esri, 380 New York Street, Redlands, CA 92373, USA). The prevalence of CCHFV serology at species level was calculated as the number of seropositive animals divided by total number of animals sampled. For the herd serological prevalence, a farm was considered positive if at least one sheep or goat on the farm was seropositive for CCHFV. The exact 95%-Clopper & Pearson interval method was used to estimate binomial confidence intervals (CI) for proportions by using the binom package with the binom.test function in R (Dorai-Raj, 2014). The Mann-Whitney U-Test was applied using the wilcox.test function to determine potential associations between the continuous or discrete independent variables and categorical dependent variables. Potential risk factors associated with the prevalence of CCHFV were determined by constructing a multivariable logistic regression model (fixed effects only, no random effects). Separate models were built on farm level, animal level (including both sheep and goats), and also separately each for sheep and goat species. The presence or absence of CCHFV-specific antibodies was considered as the dependent variable and data obtained during the moderated interviews were used as independent or explanatory variables. In a univariable analysis, all, biologically plausible variables were analysed (Appendix A). Pearson`s Chi-squared test of association was conducted using the gmodels package with the CrossTable function in R (Warnes et al., 2015). Variables with $p \leq 0.2$ were included in the multivariable logistic regression model. Variance inflation factor (vif) and its inverse (tolerance) (1/vif) were measured to check for multi-collinearity among the independent variables. The glm() function was used to perform the multivariable logistic regression analysis in R. Finally, a manual backward stepwise selection strategy was used to exclude variables one by one, starting with the variable with the highest p-value, until the variables left had $p<0.05$. The confounding effect of a variable was evaluated by assessing the change in the remaining coefficient estimate of variables after removing the variable; if the change was greater than 20% as compared to the full model, the variable was considered to be a confounder and re-entered into the final model (Bursac et al., 2008; Hosmer et al., 2013). All variables with $p<0.05$ were considered statistically significant. The Scale-Location plot and normal Q-Q plot of the residuals of the final model were examined for homogeneity of variance and normal distribution of residuals, respectively using the plot (model) function. Additionally, Kolmogorov-Smirnov test for normality was also performed. The odds ratios (OR) for the regression coefficients and their 95% CIs were calculated using the exp() and exp(confint()) functions, respectively. Pseudo R^2, Akaike information criterion (AIC) values and the Pearson goodness-of-fit statistic were applied to assess the quality and model fit of the multiple logistic regression

model. Pseudo R^2 was estimated using pscl package with pR2 function (Jackman et al., 2017). Cohen's kappa was calculated using EpiTools (https://epitools.ausvet.io/comparetwotests).

3.3 CCHFV in ticks collected from livestock in Balochistan

3.3.1 Study design

A cross-sectional study was conducted from September to November 2017 in the province of Balochistan, Pakistan. The sample size was calculated for large populations with 50% expected prevalence to avoid potential loss of precision due to a higher or lower true prevalence, at the 95% confidence level and for 10% desired precision, resulting in 96 livestock farms to be sampled (Cannon and Roe, 1982). The details of the administrative units were obtained from the local municipality authority. A multi-stage cluster sampling approach was used to select the livestock farms in each division. Four out of six divisions in Balochistan were randomly selected. In each division, three districts, and in each district, two union councils were selected. In each union council, four livestock farms were randomly selected. In each farm, a minimum of three animals of each existing species (cattle, sheep or goats) was randomly selected irrespective of age, sex and breed. The animals on the farm were not tagged. Therefore, a systematic sampling approach was used, so that each animal at a farm had an equal chance to be selected. Before sampling, the farmer was informed about the purpose of sampling and his consent was obtained.

3.3.2 Tick collection

Ticks were collected from cattle, sheep and goats on the livestock farms. Adequate personal measures were adopted by wearing protective clothing to cover the whole body during the tick collection process to protect the tick collectors from tick-borne infections. The entire bodies of the animals were examined for ticks, in particular ears, neck, chest, scrotum, perineum and base of the tail. The ticks were eventually collected from the hosts with blunt forceps, transferred into appropriately labelled safety-lock Eppendorf tubes®. The ticks were stored at -20 °C until further processing. They were then transferred to the University of Veterinary and Animal Sciences, Lahore, Pakistan for morphological and molecular identification securing the cold chain. For further analysis, the ticks were shipped frozen on dry ice to maintain cold chain to the Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany.

3.3.3 Tick identification

3.3.3.1 Morphological identification

Ticks were identified based on their morphological features under the stereomicroscope using a multiple electronic key (Walker et al., 2005), and the re-description of tick species by Apanaskevich and Horak (2005); Apanaskevich et al. (2010). The ticks were identified up to the species level. After morphological identification, 10 specimens that belonged to different species were selected to confirm the morphological identification using a molecular method.

3.3.3.2 Molecular identification

DNA was extracted using Halos et al. (2004) protocol with the following modifications: Ticks were crushed separately using liquid nitrogen and 1.5 ml of lysis buffer, followed by addition of 0.125 µl of 20 mg/ml of proteinase K to each tube. The samples were incubated at 65°C for overnight. The concentrations of the extracted genomic DNA samples were quantified using a Nano drop ND-100 instrument. The extracted genomic DNA samples were stored at −20˚C until further use. The amplification of a partial fragment (750 nt) of the second internal transcribed spacer (ITS2) gene was performed using the primers as described earlier by Rehman et al. (2017). PCR was performed in a total reaction volume of 20 µl, containing 2 µl of each primer, 2 µl template DNA, 10 µl of 2X Green Master mix with Taq polymerase (Wiz Bio Solutions, South Korea), and 4 µl of DEPC water. The product was amplified in a thermocycler (G-storm, Surrey, UK) with initial denaturation at 95 ˚C for 5 min, followed by 40 cycles at 95 ˚C for 30s, 57 ˚C for 30 s and 72 ˚C for 50 s. A final extension at 72 ˚C for 5 min was also performed. The product was visualised on ethidium bromide-stained 1.5% agarose gels using a UV illuminator. The amplicons obtained were isolated and purified using a gel purification kit (GeneAll, Seoul, Korea) and the purified products were sent to 1st BASE, Singapore, for sequencing.

3.3.4 Molecular analysis of ticks for CCHFV genome

3.3.4.1 RNA extraction and real-time RT-qPCR

Each tick was homogenized in a 2 ml safety-lock Eppendorf tube® with 500 µl PBS, and a steel bead with 5 mm in diameter (Qiagen, Germany) by using TissueLyzer II (Qiagen, Germany) for 3 min at 30 Hz. The homogenate was centrifuged for 10 min at 10000 rpm. One-hundred- and-forty µl supernatant were added afterwards to 560 µl AVL buffer (Qiagen, Germany) in a 1.5 ml safety-

lock Eppendorf tube®. The tick homogenization procedure adopted in this part (2.5.1) and in the part 2.4.2 was performed according to two different protocols. RNA was extracted using the NucleoMag® VET kit (Macherey-Nagel, Germany) on a KingFisher Flex instrument (Thermo Fisher Scientific) according to the manufacturer`s instructions. Ten µl MS2-phage RNA were added to each well as an extraction control. In addition, 100 µl FKS P84 (RIC) was added to one well in each row of the plate as a negative control. The extracted RNA was stored at -80 °C until further processing.

To detect the RNA of the CCHFV S-segment, a one-step multiplex real-time RT-qPCR developed by Sas et al. (2018a) was used with the following modifications: It included primer sets for all known CCHFV genotypes. The AgPath-ID™ One-Step RT-PCR kit (Thermo Fisher Scientific) was used. The total reaction volume was 25 µl, consisting of 20 µl master mix (2 µl RNase free water, 12.5 µl 25X RT-PCR Buffer, 2 µl MS2-phage RNA Primer Probe mix, 2.5 µl genotypes-specific CCHF-Primer-Probe-CoProbe mix and 1 µl 25X µl RT-PCR Enzyme Mix) and 5 µl RNA template. As a positive control, CCHFV synthetic RNA was used as described by Sas et al. (2018a). The following cycling protocol was applied: 48 °C for 10 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 45 s. A CFX96 Real-Time PCR thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) was used for the real time RT-qPCR.

To confirm the results of the one-step multiplex real-time RT-qPCR for the S segment, a one-step multiplex real-time RT-qPCR was performed to detect CCHFV L-segment RNA, which again included primer sets for all known CCHFV genotypes. The AgPath-ID™ One-Step RT-PCR kit (Thermo Fisher Scientific) was used. The total reaction volume was 15 µl, consisting of 12 µl master mix (1.9 µl RNase free water, 7.5 µl 25X RT-PCR Buffer, 2 µl genotypes-specific CCHF-MS2-phage RNA Primer-Probe-CoProbe mix, and 0.6 µl 25X µl RT-PCR Enzyme Mix) and 3 µl RNA template. As a positive control, synthetic CCHFV RNA was used. The following cycling protocol was applied: 48 °C for 10 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 45 s. A CFX96 Real-Time PCR thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) was used for the real time RT-qPCR.

3.3.4.2 Sequencing

Samples that were positive in the one-step multiplex real-time RT-qPCR were further analysed to determine the CCHFV genotypes. The RNA template was amplified by using SuperScript™ III

One-Step RT-PCR System with Platinum™ *Taq* DNA Polymerase kit (Thermo Fisher Scientific) according to manufacturer`s recommendations using a Bio-Rad C1000™ thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). All genotype-specific CCHFV-primers were used as described by Sas et al. (2018a). The presence of the expected cDNA product obtained by RT-PCR was confirmed by electrophoresis in 2% agarose gels. The respective band was excised, cleaned using the Monarch® PCR & DNA Cleanup Kit (BioLabs) and sent to Eurofins Germany for sequencing. For phylogenetic analysis, CCHFV S segment reference strains of different genogroups were obtained from GenBank (www.ncbi.nlm.nih.gov). Multiple alignments of sequences were performed and a phylogenetic tree was constructed with the maximum-likelihood method using the Kimura-2 parameter model (Guindon et al., 2010) in the Geneious 11.1.5 software (Biomatters, Auckland, New Zealand).

3.3.5 Statistical analysis

For statistical analyses, R software (R Core Team, 2013, Vienna, Austria; http://www.R-project.org/) and R-Studio (an integrated development environment for R) (RStudio, 2016) were used. ArcGIS (version 10.5.1, Esri, 380 New York Street, Redlands, California, USA) was used to prepare maps. The CCHFV prevalence among ticks was calculated by dividing the number of CCHFV-positive ticks by the total number of ticks analysed. 95% confidence intervals (CI) for proportions were estimated by using the exact 95%-Clopper & Pearson interval method with the binom.test function in the binom package in R (Dorai-Raj, 2014).

4. Results

4.1 Epidemiological investigations of CCHFV infection in sheep and goats in Balochistan

4.1.1 Serology and detection of CCHFV genome in sheep sera

In the serological analysis, 149 (19%, CI: 16-21%) out of 800 sheep serum samples were positive for CCHFV-specific IgG antibodies, while 37 (5%, CI: 3-6%) out of 800 goat serum samples were positive for CCHFV-specific IgG antibodies. Among the sheep sera, 16 of 149 positive samples and among the goat sera, 11 out of 37 positive samples had yielded diverging results in the two ELISAs (Cohen's kappa for sheep sera 0.4980; 95% CI 0.4359- 0.5601; and for goat sera 0.3946; 95% CI 0.2814-0.5078). There were 147 sheep sera and 57 goat sera with diverging results in the ELISAs, which were tested by IFA to obtain the final result used for the prevalence estimates. Real-time RT-qPCR identified 8 (5%, CI: 2-10%) out of 160 sheep serum pools positive for CCHFV genome fragments. All goat serum pools were negative (0%, CI: 0-2%, 0 out 160).

4.1.2 Geographic distribution of CCHFV antibody positive farms

Out of 160 farms, 81 (51%, 95% CI: 43-59%) were seropositive for CCHFV-specific antibodies in the divisions of Zhob, Sibi and Quetta (Figure 8, Table 2). The prevalence estimates for the divisions and districts and the respective 95% confidence intervals are listed in Table 2.

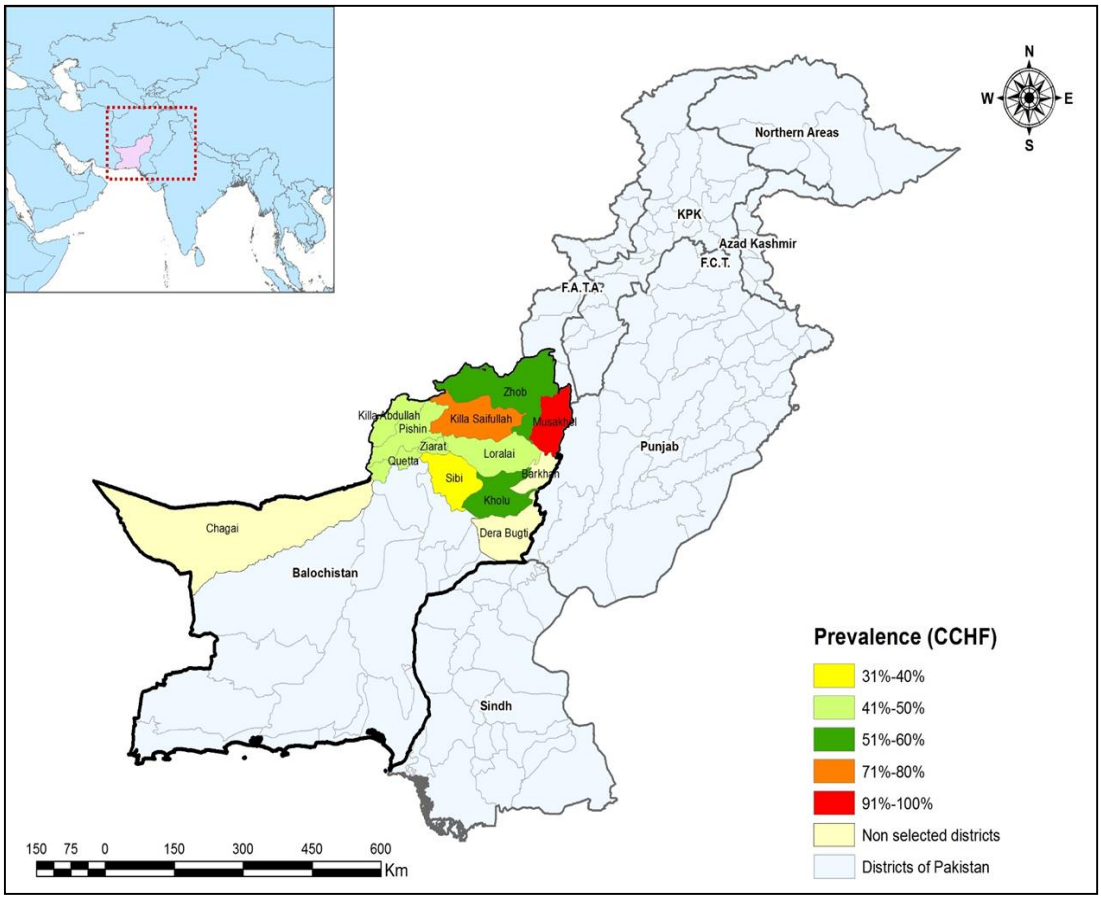

Figure 8. Geographical distribution of CCHFV infection seropositive livestock farms in Balochistan (district level), Pakistan.

Table 2. Seroprevalence of CCHFV in livestock farms (sheep and goats) in Balochistan (division level), Pakistan (n=160 farms).

	No. of positive farms	No. of farms	Prevalence in %	95% CI*
Zhob Division	**41**	**77**	**53**	**41-65**
Zhob	18	35	51	34-68
Loralai	15	32	47	29-65
Killa Saifullah	6	8	75	35-97
Musakhel	2	2	100	16-100
Sibi division	**27**	**56**	**48**	**35-62**
Kohlu	15	25	60	38-79
Sibi	10	27	37	19-57
Ziarat	2	4	50	7-93
Quetta division	**13**	**27**	**48**	**28-68**
Pishin	7	14	50	23-77
Killa Abdullah	5	11	45	17-76
Quetta	1	2	50	1-99

*95% CI for Prevalence.

4.1.3 Demographic characteristics of the farm structure

There were 126 farms with both sheep and goats, 13 farms with only sheep, and 21 farms with only goats. The median farm size was 78.5 (Q1-Q3: 15-265.5) animals with a range of 10-3510 animals. The median farm size of farms that were seropositive for CCHFV-specific antibodies was 133 (Q1-Q3: 17-300) animals with a range of 10-3510 animals, while the median farm size of farms seronegative for CCHFV-specific antibodies was 22 (Q1-Q3: 15-173) animals with a range of 10-2000 animals (p-value=0.015; U= 3909.5). The median age of sheep was 2.9 (Q1-Q3: 2.5-3.1) years with a range of 0.8-5 years. The median age of sheep seropositive for CCHFV-specific

antibodies was also 2.9 (Q1-Q3: 2.5-3.2) years with a range of 1.5-4.4 years and the median age of seronegative sheep was 2.9 (Q1-Q3: 2.5-3.1) years as well with a range of 0.8-5 years (p-value=0.236; U=51507). More sheep with an age ≥ 2 years (95%, 141 out of 149) were seropositive than sheep with aged less than 2 years (5%, 8 out of 149). In sheep, 19% (129 out of 664) male and 15% (20 out of 136) female animals were seropositive for CCHFV-specific antibodies (p=0.197; 1 df). The median age of goats was 2.9 (Q1-Q3: 2.4-3.1) years with a range of 1-4.5 years. The median age of goats seropositive for CCHFV-specific antibodies was 2.9 (Q1-Q3: 2.3-3) years with a range of 1.7-4.2 years, while the median age of goats seronegative for CCHFV-specific antibodies was also 2.9 (Q1-Q3: 2.4-3.1) years with a range of 1-4.5 years (p-value=0.589; U=13376). Moreover, more goats with an age ≥ 2 years (81%, 30 out of 37) were seropositive than goats less than 2 years (19%, 7 out of 37). In goats, 4% (28 out of 684) male and 8% (9 out of 116) female animals were seropositive for CCHFV-specific antibodies (p-value=0.082; 1 df).

4.1.4 Farmers' education level, knowledge on CCHFV, farm hygiene and treatment against tick infestation

This study was performed in a wide geographical area with scattered animal herds. We achieved a complete response rate (100%) and all questions were answered by the farmers. The education level among farmers was 45% (72 out 160) above and 55% (88 out of 160) below the intermediate level. There was no statistically significant association between the education level of the farmers and the seropositive status of farms (p-value=0.622) (Table 3). Among the farmers, 60% (96 out 160) had knowledge regarding CCHFV infection, while 40% (64 out 160) did not. There was no statistically significant association of the farmers knowledge on CCHFV and the seropositivity of the animals (p-value=0.605) (Table 3). On 90 out of 160 farms (56%) hygiene was assessed as poor, while in the remaining 70 farms (44%) the hygienic conditions were regarded as satisfactory. There was no statistically significant association of the hygiene status of the farms and the seropositivity of the animals (p-value=0.270). In total, 65% (104 out of 160) of the farmers used to adopt preventive measures when they were in contact with diseased or dead animals. The reasons for not treating animals against tick infestation using commercially available acaricides were expenses (64% farmers said that it was expensive), low effectiveness (21% had observed that the products they had used were not effective), or the use of alternatives (15% of farmers).

4.1.5 Farm management-related risk factors associated with CCHFV positivity

Flock size, presence of rural poultry, tick treatment, presence of vegetation in or around the farm, type of housing, other livestock farms nearby and feeding method had p-values ≦ 0.2 in the univariable analysis (Table 3) and were therefore included in the multivariable analysis. The diagnostic plots of the residuals of the final farm multivariable model showed normal distribution and equal variance of residuals. The type of housing had a significant effect on the seropositivity regarding CCHFV (Table 4). In addition, farms, on which the animals were allowed to graze had had a higher odds ratio of getting CCHFV infections as farms with trough-feeding within the farm perimeter as had farms with presence of vegetation in or around it and farms that did not practice tick treatment. Animals on farms without rural poultry had higher odds than animals on farms with rural poultry. The Akaike information criterion value (AIC) of the final multivariable logistic regression model was 187.47 (null deviance=221.78 with df=159, residual deviance=175.47 with df=154) as compared to 189.58 the full model. The pseudo R^2 values calculated for the final model was 0.208. The Pearson goodness-of-fit statistic showed that the model adequately fitted the data (p-value=0.113).

Table 3. Univariable analysis for associations between farm management-related risk factors and CCHFV infection (n=160).

Variable	OR	95% CI	df	X^2	p
Locality (Division)			2	0.4082	0.815
Quetta (n=27)	Ref				
Sibi (n=56)	1	0.4-2.51	1	0	0.995
Zhob (n=77)	1.22	0.51-2.95	1	0.21	0.648
Flock size			2	9.63	0.008
Small (n=78)	Ref				
Medium (n=35)	2.13	0.94-4.79	1	3.41	0.064
Large (n=47)	3.11	1.45-6.60	1	8.87	0.003
Presence of other animals species					
No (n=49)	Ref				
Yes (n=111)	0.86	0.41-1.79	1	0.16	0.682
Presence of rural poultry					
Yes (n=77)	Ref				
No (n=83)	2.24	1.13-4.44	1	6.38	0.011

Tick treatment					
Yes (n=25)	Ref				
No (n=135)	3.09	1.14-9.37	1	6.06	0.013
Animal Quarantine					
Yes (n=32)	Ref				
No (n=128)	0.75	0.34-1.64	1	0.51	0.476
Presence of Vegetation					
No (n=26)	Ref				
Yes (n=134)	4.2	1.50-13.61	1	9.42	0.002
Animal kept in diff age groups					
Yes (n=26)	Ref				
No (n=134)	1.23	0.53-2.87	1	0.25	0.618
Type of Housing					
Closed (n=40)	Ref				
Open (n=120)	4.31	1.84-10.82	1	14.01	<0.001
Presence of fences/boundary wall					
Yes (n=114)	Ref				
No (n=46)	1.4	0.7-2.77	1	0.89	0.343
Other livestock farms nearby					
≥200 m (n=85)	Ref				
<200 m (n=75)	0.67	0.34-1.31	1	1.58	0.208
Feeding method					
Trough (n=44)	Ref				
Grazing (n=116)	5.31	2.29-13.28	1	18.89	<0.001
Education level of farmer					
≥Inter level (n=72)	Ref				
<Inter level (n=88)	0.85	0.43-1.67	1	0.24	0.622
CCHF related knowledge of farmer					
Yes (n=96)	Ref				
No (n=64)	1.18	0.62-2.22	1	0.26	0.605

OR: odds ratio; CI: confidence interval; df: degree of freedom; X^2: Pearson`s Chi-squared test; Ref.: reference; m: meters

Table 4. Multivariable logistic regression model for statistically significant associations between farm management-related risk factors and CCHFV infection (p<0.05).

Variable	OR	95% CI	p
Type of housing			
Closed	Ref		
Open	3.76	1.57-9.56	0.003
Feeding method			
Trough	Ref		
Grazing	4.18	1.79-10.37	0.001
Presence of Vegetation			
No	Ref		
Yes	3.13	1.07-10.15	0.043
Tick treatment			
Yes	Ref		
No	3.31	1.16-10.21	0.029
Presence of Rural poultry			
Yes	Ref		
No	2.93	1.41-6.29	0.004

OR: odds ratio; CI: confidence interval; Ref: reference.

4.1.6 Animal-related risk factors associated with CCHFV positivity

In the univariable analysis, the animal level variables with p-values of ≤ 0.2 were age, tick infestation, and species (Table 5). Moreover, the variables for sheep with a p-value of ≤ 0.2 were age, sex and tick infestation, and for goats, the respective variables were sex and tick infestation. These variables were included in multivariable analyses separately for animal level, sheep and goats. The pseudo R^2 values for the final animal level (0.093), sheep (0.027), and goat (0.021) multivariable models were low. Also the diagnostic plots of the residuals of these respective models revealed deviations from a normal distribution. Therefore, we did not consider these multivariable models further in our results.

Table 5. Univariable analysis for the association between host species-related risk factors and CCHFV infection.

Variable	OR	95% CI	df	X^2	p
Age					
<2yrs (n=768)	Ref				
≥2yrs (n=832)	4.15	2.84-6.19	1	64.09	<0.001
Sex					
Female (n=252)	Ref				
Male (n=1348)	1.01	0.65-1.60	1	0.003	0.949
Breed					
Cross (n=166)	Ref				
Indigenous (n=1434)	1.26	0.73-2.32	1	0.71	0.398
Tick infestation					
No (n=558)	Ref				
Yes (n=1042)	2.35	1.59-3.52	1	20.80	<0.001
Species					
Goat (n=800)	Ref				
Sheep (n=800)	4.72	3.24-6.86	1	76.31	<0.001

OR: odds ratio; CI: confidence interval; df: degree of freedom; X^2: Pearson`s Chi-squared test; Ref: reference

4.2 CCHFV in ticks collected from livestock in Balochistan

4.2.1 Tick species identification and geographical distribution

Five-hundred-and-twenty-five of 529 ticks (99%, CI: 98-100%) were identified as *Hyalomma* spp. and four (1%, CI: 0.2-2%) ticks belonged to the genus *Rhipicephalus,* both genera belonging to the family Ixodidae (hard ticks) (Table 6). In the genus *Hyalomma,* the following species were identified: *H. marginatum* (28%, CI: 24-32%), *H. excavatum* (26%, CI: 22-30%), *H. dromedarii* (22%, CI: 19-26%), *H. anatolicum* (16%, CI: 13-19%), and *H. scupense* (8%, CI: 6-11%). In the genus *Rhipicephalus,* three ticks were *R. microplus* and one tick *R. turanicus.* Tick infestation on ruminants was detected in 58% (CI: 54-62%) of the examined sheep, 28% (CI: 24-32%) of the goats, and 14% (CI: 11-18%) of cattle. All collected ticks were identified as adults. Moreover, tick sequencing confirmed the morphological identification, and BLAST analysis showed 89-100%

similarity with the previously identified tick species. The geographical distribution of the ticks at the district level is shown in Figure 9.

Table 6. Tick species identified on host species in the districts of Balochistan, Pakistan (n=529).

District	Host species	*Hyalomma anatolicum*		*Hyalomma excavatum*		*Hyalomma marginatum*		*Hyalomma dromedarii*		*Hyalomma scupense*		*Rhipicephalus microplus*	*Rhipicephalus turanicus*
		M	F	M	F	M	F	M	F	M	F		
Quetta	Sheep	12	5	1	5	3	-	1	-	5	-	1	1
	Cattle	1	-	1	-	-	-	-	-	1	-	-	-
Killa Abdullah	Sheep	-	-	-	-	2	-	-	2	-	-	-	-
Pishin	Cattle	-	1	11	1	1	-	-	1	4	-	-	-
Kalat	Sheep	5	1	16	-	-	12	-	4	-	-	-	-
Khuzdar	Goat	1	-	-	3	1	22	59	-	-	-	-	-
Lasbela	Sheep	6	-	8	1	14	2	-	-	5	-	-	-
	Goat	11	1	14	1	4	11	6	-	-	6	-	-
Sibi	Cattle	2	-	2	3	9	-	33	3	-	-	2	-
Harnai	Sheep	4	12	35	12	17	1	2	2	4	-	-	-
Ziarat	Sheep	-	-	-	-	4	-	-	3	-	-	-	-
Zhob	Sheep	15	-	-	4	30	4	-	-	11	-	-	-
Loralai	Sheep	1	1	8	6	6	1	-	1	4	-	-	-
	Goat	-	-	-	-	-	4	-	-	2	-	-	-
Sherani	Sheep	3	-	-	3	1	-	-	-	-	-	-	-
Total		**61**	**21**	**96**	**39**	**92**	**57**	**101**	**16**	**36**	**6**	**3**	**1**

M: male; F: female

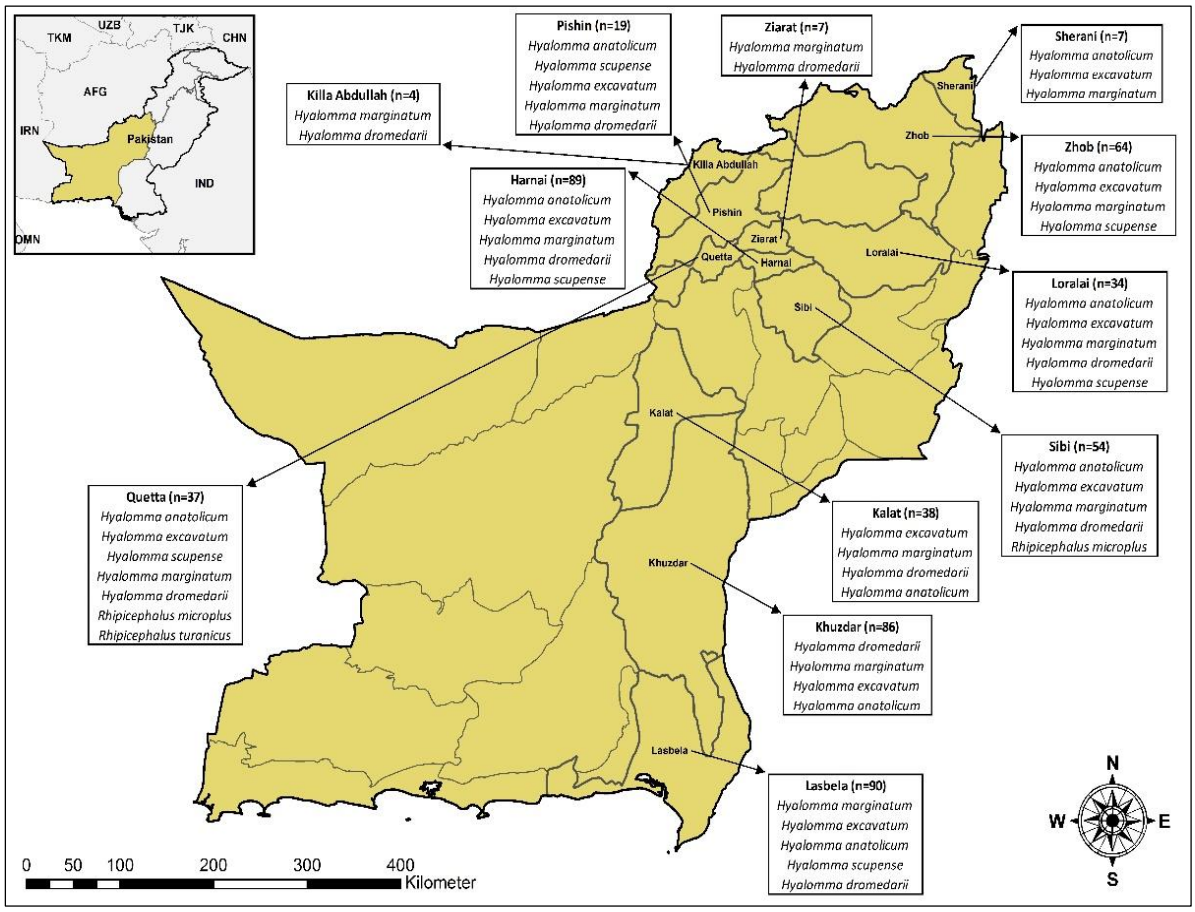

Figure 9. Geographical distribution of tick species in Balochistan (district level), Pakistan (n=529).

4.2.2 Prevalence of CCHFV in ticks

Five-hundred-and-twenty-five *Hyalomma* ticks were analysed, out of which 20 (4%, CI: 2-6%) ticks were positive for CCHFV S segment genome. All sequenced amplicons of the positive ticks clustered in the genotype Asia 1 (Figure 10). The Balochistan-42-2017-Pakistan sequence showed the closest proximity (99% nucleotide identity) with a CCHFV strain from Oman (DQ211645), followed by CCHFV strains from Iran (KJ566219, 97% nucleotide identity), and Pakistan (U88414, 97% nucleotide identity). Among the CCHFV positive ticks, 75% (15 out of 20) were female and 25% (5 out of 20) were male. CCHFV genomes were detected most frequently in *H. marginatum* (30%, 6 out of 20), followed by *H. dromedarii (*25%, 5 out of 20)*, H. excavatum* (20%, 4 out of 20), *H. anatolicum* (20%, 4 out of 20), and *H. scupense* (5%, 1 out of 20) (Figure 11). All positive ticks were found on sheep. The highest number of ticks were CCHFV-positive in the district of Kalat (60%, 12 out of 20), followed by the districts of Quetta (30%, 6 out

of 20) and Killa Abdullah (10%, 2 out of 20) (Figure 12). In Kalat, CCHFV genome was detected in *H. marginatum* (5 out of 12), *H. dromedarii* (4 out of 12), *H. anatolicum* (2 out of 12), and *H. excavatum* (1 out 12). In Quetta, *H. excavatum* (3 out of 6), *H. anatolicum* (2 out of 6), and *H. scupense* (1 out of 6) were positive for the CCHFV genome. In addition, in Killa Abdullah district, one *H. marginatum* and one *H. dromedarii* were positive for CCHFV genomes.

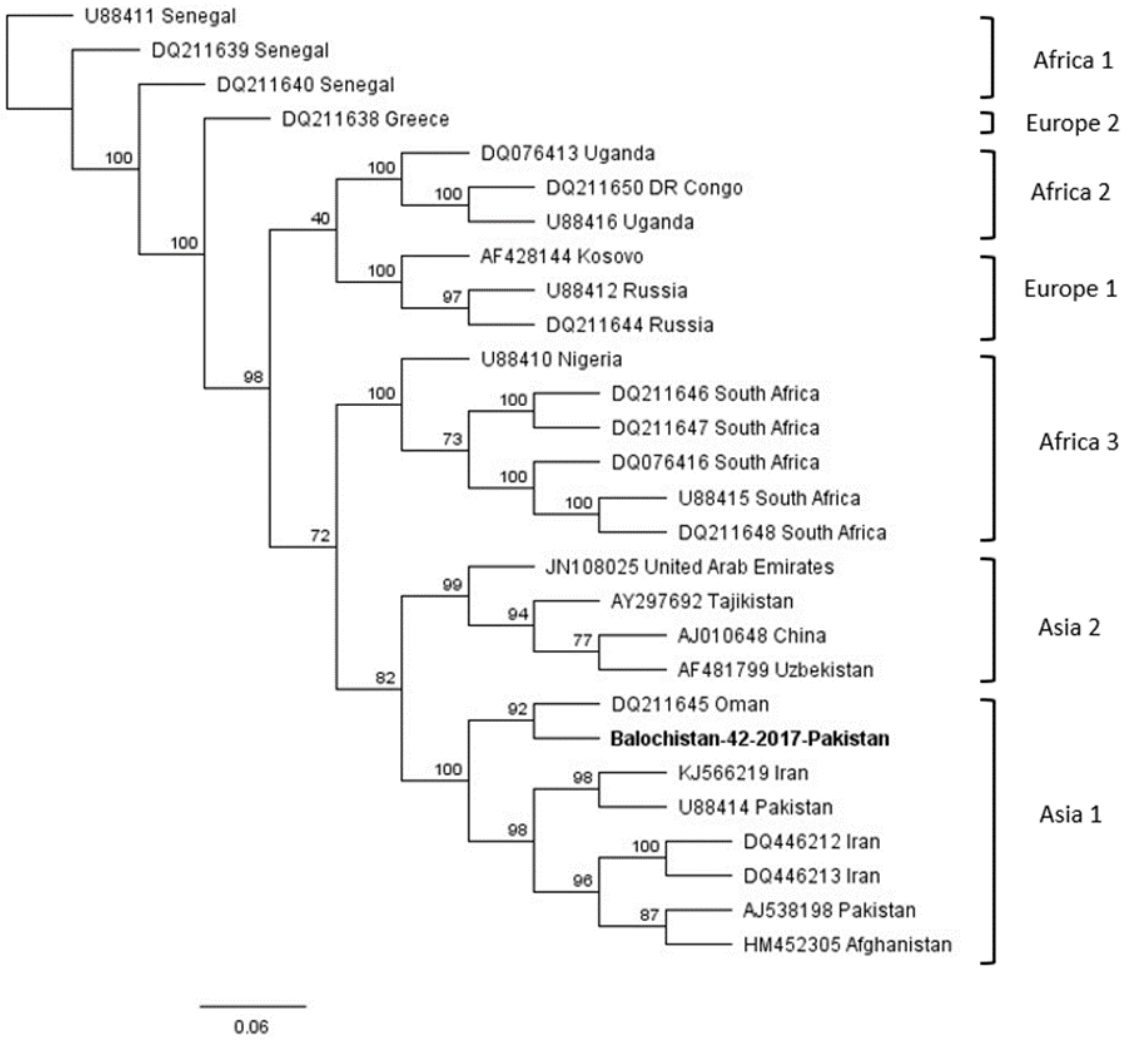

Figure 10. Phylogenetic tree of a partial S segment (180 nt) from the genome of CCHFV (isolate from this study in bold) with the maximum-likelihood method using the Kimura-2 parameter model. Bootstrap values at the nodes of above tree (percentage of replicate trees in which the interrelated taxa clustered together) obtained from the bootstrap test (1000 replicates).

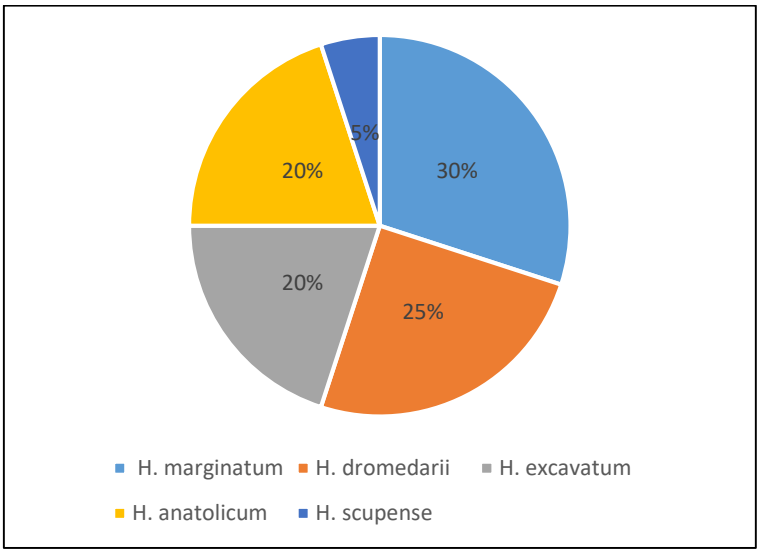

Figure 11. CCHFV genome-positive tick species found in Balochistan, Pakistan

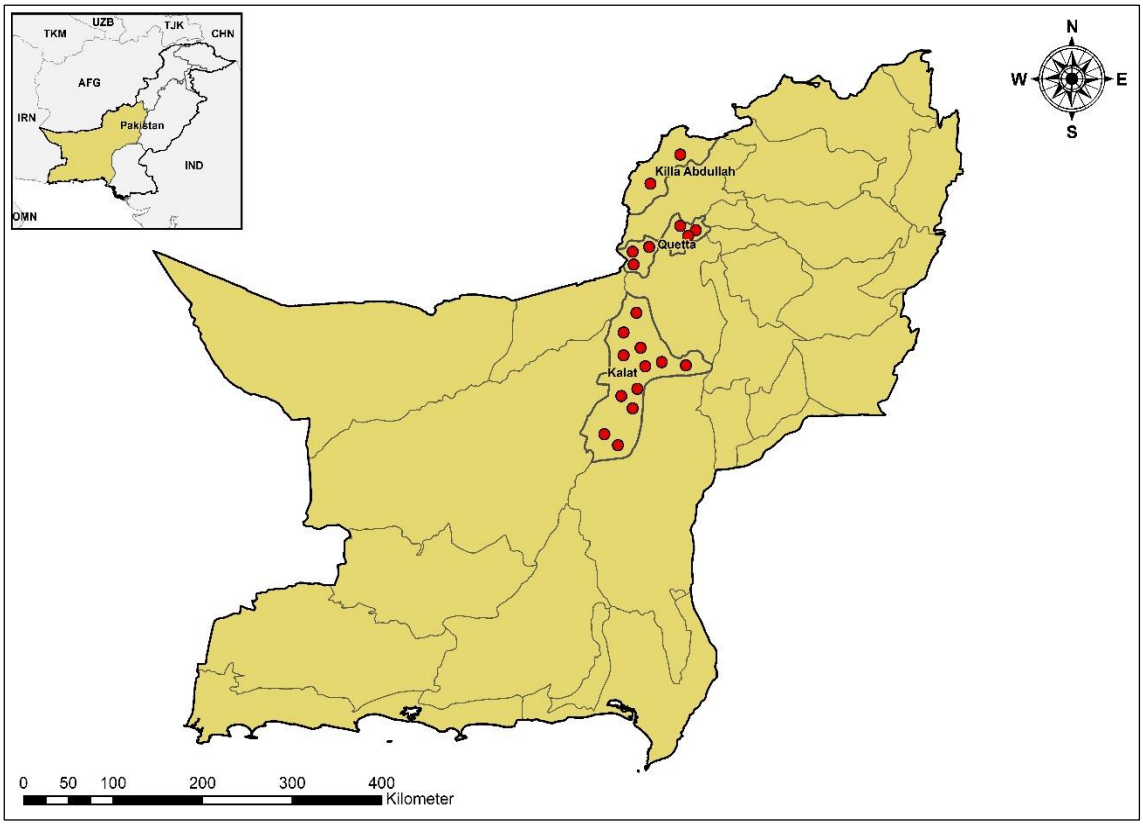

Figure 12. Geographic distribution of CCHFV-genome-positive ticks in Balochistan (district level), Pakistan.

5. Discussion

5.1 Epidemiological investigations of CCHFV infection in sheep and goats in Balochistan

This study is the first comprehensive epidemiological evaluation of CCHFV circulation in livestock in Balochistan, Pakistan. Since its first report in Pakistan in 1976, in humans multiple and sporadic cases have occurred (Hussain et al., 2016) and CCHFV is considered to be endemic in the country (Alam et al., 2013a). Pakistan has a vast agriculture sector with a large number of people involved in animal husbandry practices (Atif et al., 2017). The highest number of human cases in Pakistan were in recent years recorded in the province of Balochistan (Khurshid et al., 2015), where we conducted this study in sheep and goats. From 2003 to 2008, 57 (67%) out of 85 CCHF human cases were reported in this province compared to other provinces in the country (Atif et al., 2017). The case-fatality ratio recorded in this province was up to 20% (Khurshid et al., 2015).There is no surveillance system for CCHFV in Pakistan and only case-based data is available (Khurshid et al., 2015).

The presence of CCHFV-specific antibodies in domestic animals indicates the circulation of CCHFV in the area and an increased risk for the human population (Spengler et al., 2016a) and a seroprevalence estimate can help to quantify the proportion of the target species exposed to CCHFV. It has to be noted that livestock exposed to CCHFV form a reservoir for the virus and may therefore represent a risk for human infection. As to our knowledge, only two studies have reported CCHFV-specific antibodies in livestock in Pakistan in 1983 (Darwish et al., 1983) and in 1997 (Khan et al., 1997), but with limited epidemiological information. In our study, we detected not only CCHFV-specific antibodies in sheep and goat sera, but also CCFHV genome in samples obtained from sheep. In our findings, 8 (5%, CI: 2%-10%) out of 160 sheep serum pools were positive for CCHFV RNA. A previous study in Turkey reported 14% (6 out of 42) of sheep and 5% (3 out of 63) of goats positive for CCHFV in whole blood (Albayrak et al., 2012). Viraemia among small ruminants is usually transient. The animals do not show any clinical signs (Spengler et al., 2016b), but CCHFV-specific IgG-antibodies can be detected for several years after infection (Shepherd et al., 1989b). Therefore, antibody prevalence is a good indicator for the circulation of the CCHFV in a region (Mertens et al., 2016).

In our study, the seroprevalence of CCHFV-specific antibodies was significantly higher in sheep (19%) than in goats (5%) (p-value <0.001). A previous study conducted in Pakistan by Darwish et al. (1983) reported 2% seroprevalence in cattle (1 out of 45) and 4.5% in Buffalo (1 out of 22),

while 0% seroprevalence was found in sheep (n=46) and goats (n=48). A study conducted in the United Arab Emirates (UAE) reported one individual camel was seropositive, while one individual goat was seronegative for CCHFV specific antibodies. These animals had been imported from Pakistan into the UAE (Khan et al., 1997). We were not able to draw parallels to other study results from Pakistan, because there is only limited data with sero-epidemiological value. Consequently, we compared our seroprevalence results with studies from neighbouring countries, where the environmental conditions for the natural habitat of CCHFV and its tick vector were similar. Our findings showed a lower all over prevalence, but the relation in the prevalence between sheep and goats (sheep > goat) was similar to previous studies from India, which reported a seroprevalence of 41% in sheep and 34% in goats (Mourya et al., 2014). A second study from India reported 67% seropositivity in sheep and 30% in goats (Mourya et al., 2012). Moreover, a study performed in Iran reported seroprevalence of 59% in sheep and 25% in goats (Mostafavi et al., 2013b). Another study from Iran reported a seroprevalence of 42% in sheep and 33% in goats (Telmadarraiy et al., 2010). Furthermore, a higher seroprevalence among sheep was also reported in other studies from Iran by Bokaie et al. (2008) (sheep: 77%, goats: 46%) and Saidi et al. (1975) (sheep: 38%, goats: 36%). A higher seroprevalence in sheep was also reported in studies from Iraq (sheep: 58%, goats: 50%) (Tantawi et al., 1981), Egypt (sheep: 6%, goats: 1%) (Mohamed et al., 2008), Saudi Arabia (sheep: 4%, goats: 3%) (Hassanein et al., 1997), Turkey (sheep: 86%, goats: 67%) (Albayrak et al., 2012), and Bulgaria (sheep: 74%, goats: 60%) (Barthel et al., 2014). However, our findings differ from results from a study conducted in Turkey, which revealed a higher seroprevalence in goats (66%) as compared to sheep (31.8%) (Tuncer et al., 2014). Moreover, a higher seroprevalence among goats was also reported in studies from Kosovo (goat: 20%, sheep: 10%) (Fajs et al., 2014), Oman (goats: 27%, sheep: 23%) (Williams et al., 2000), United Arab Emirates (goats: 12%, sheep: 8%) (Khan et al., 1997), and Niger (goats: 4.9%, sheep: 2.95%) (Mariner et al., 1995). The variation of the seroprevalence in animals is often associated with the distribution of the tick vectors (Tuncer et al., 2014), the host preference of tick vectors, the tick load on a particular animal species (Spengler et al., 2016a) and the susceptibility of the animal species (Tuncer et al., 2014).

The seroprevalence among farms was slightly higher in the division of Zhob (53%) compared to Sibi (48%) and Quetta (48%). However, there was no significant association between localities and seropositivity to CCHFV (p-value=0.815). There were seropositive farms in all districts sampled in the study area. The seroprevalence was higher in the northern districts of the province, which are close to the border of Afghanistan. The farmers in Balochistan usually cannot afford to

purchase feed concentrates and mainly depend on traditional livestock feeding on rangelands. The rangelands in the northern part of the province have good quality ranges and are therefore grazed by large numbers of livestock animals. Moreover, the rangeland area in the border area between Balochistan and Afghanistan is jointly grazed by livestock from the two countries. The arid climate, low precipitation and livestock abundance provides a suitable environment for the CCHFV tick vector and makes these areas an epicentre for the circulation of CCHFV. Moreover, it has been evident that higher numbers of human outbreaks are reported every year in this area compared to other parts of the country (Alam et al., 2013a; Khurshid et al., 2015). Furthermore, nomadic flocks and some of transhumant flock owners usually migrate in winter from the north-uplands and the border of Afghanistan to the south-lowlands of the province and return back to the uplands in spring (Ahmad and Islam, 2011), and may subsequently introduce potentially infected ticks into new areas.

The pseudo R^2 values found in the final multivariable logistic regression model were limited, which means that the model explains only a small proportion of the total variation in seropositivity. Nevertheless, the factors identified in the model related to farm management variables showed that animals with an open type of housing had an increased chance of being seropositive than those with a closed type of housing. The closed type of housing investigated in this study included a well-organized farm structure with good constructed sheds, while the open type of housing had a poor housing structure, which usually consisted of sheds made of muddy material. The walls were made of mud bricks with cervices and had an open area without a window or mesh, through which wild birds easily entered the shed. The farmers usually depend on rangelands for livestock feeding and due to seasonal migration, they do not have permanent housing. This forces them to construct the open type of temporary housing for their livestock. Moreover, cracks and crevices in walls of the open type of housing provide a suitable environment for ticks to breed and hide (Muhammad et al., 2008).

Farms that fed the animals by grazing were four times as likely of being seropositive than farms that practiced only trough feeding. A previous study had shown that cattle grazing on an open system were significantly associated with CCHFV seropositivity (Ibrahim et al., 2015). The trough or stall feeding is only practiced up to some extent by sedentary farmers (Afzal and Naqvi, 2004), while most farmers depend on grazing for feeding animals in this area. The rangelands are widely used by different flocks in the area especially by nomads, who move often throughout the year for livestock feeding (Afzal and Naqvi, 2004) and may thus introduce infected tick vectors into

new areas (Alam et al., 2013a). The ticks attach themselves to grass and other vegetation and then approach animals passing nearby (Muhammad et al., 2008). *Hyalomma* ticks, known as "hunting ticks" have the ability to quest up to 400 m to find their hosts (Bente et al., 2013). Moreover, a previous study had shown that in a hyper-endemic region of Turkey, questing *Hyalomma* species ticks were found on low-lying vegetation (Gunes et al., 2011). In another study, *Hyalomma marginatum* was found on pastures in the CCHFV-endemic region of Kosovo (Sherifi et al., 2018). Furthermore, ticks of the genus *Hyalomma* collected from grazing sheep and goats were positive for CCHFV in Turkey (19%) (Albayrak et al., 2012), and *Hyalomma* spp. collected from grazing cattle in Albania were also positive for CCHFV (3%) (Papa et al., 2009). In addition, in our study, presence of vegetation in or around the farm posed three times as high chances of getting CCHFV infection than its absence. Presence of vegetation in or around the farm provides a safe habitat for ticks, which can easily approach animals, and consequently increases the risk of CCHFV infection to livestock.

Tick treatment is the most suitable method to control tick vectors and minimize CCHFV transmission among livestock. In our findings, farms that did not treat their sheep and goats against tick infestation were three times as likely to be seropositive for CCHFV then those that had treated their animals against ticks. A previous study in Pakistan reported that the use of acaricides as an anti-tick treatment was significantly associated with a low tick prevalence in livestock farms (Rehman et al., 2017). Furthermore, another study conducted in Pakistan showed that sheep and goats treated with acaricides had lower tick infestation compared to untreated group (Manan et al., 2007). The application of acaricides is widely used in livestock farming to control ticks (Muhammad et al., 2008), but farmers in Balochistan are poor, unskilled (Raziq et al., 2010) and often lack awareness for tick-related problems (Shafiq and Kakar, 2006). Another issue is that farms are scattered in many geographical areas (Kakar et al., 2008), and because of seasonal livestock migration and inaccessible rangelands, only 17% of the livestock in Balochistan has access to veterinary services (Shafiq et al., 2017). Moreover, Balochistan is rich in medicinal plants (Bibi et al., 2015), which are used for the treatment of various diseases among humans and to some extent also in veterinary practice (Sarangzai et al., 2013). It may be possible that such plants are used by some farmers as putative acaricides, but it is not known, to which extent this is practised, and there is no evidence for the success of these methods. Moreover, commercially available acaricides are expensive considering the economic status of local farmers as reported by our study participants.

Birds that pick ticks may play an important role in the biological control of the tick population. We found that farms without rural poultry were three times as likely of being seropositive regarding CCHFV then sheep and goat farms that kept rural poultry. Since CCHF is a tick-borne disease, tick control at farm level may ultimately reduce CCHFV infection among livestock. Birds pick ticks from animal bodies and from the ground, and may subsequently reduce the tick burden on the animals and in the environment (Muhammad et al., 2008). However, chickens can also support the tick population as they may act as intermediate host the immature tick stages (Bente et al., 2013). A previous study in Pakistan showed that absence of rural poultry at farms was significantly associated with a high tick prevalence (Rehman et al., 2017). Another study in the CCHFV-endemic areas of Kosovo reported that farms, which harboured chickens, had no or mild tick infestation compared to farms without poultry (Fajs et al., 2014). Therefore, chickens may be used in low-income countries to reduce the tick burden in ruminants, where acaricides are not affordable by farmers.

In the present investigation, in univariable analysis, animals infested with ticks had a significant association with being seropositive regarding CCHFV (p-value<0.001). Previous studies have also reported a significant association between ticks infested cattle and CCHFV infection (Adam et al., 2013). Ticks are both reservoirs and vectors for CCHFV and animals infested with ticks are more likely to be infected with CCHFV. Therefore, it is pertinent to adopt tick control measures at farm to minimize the chances of CCHFV transmission to animals.

5.2 CCHFV in ticks collected from livestock in Balochistan

CCHFV is endemic in Pakistan and causes a large number of human infections often with lethal outcomes. Livestock animals serve as feeding source and thus foster the increase of local tick populations. Moreover, they serve as amplification hosts for the virus. Both factors can lead to an increased exposure of the rural human population to this dangerous tick-borne disease by contact with blood or tissues from infected animals or tick bites, e.g. during activities in the field.

In this study, ticks collected from livestock (sheep, goats and cattle) in Balochistan, Pakistan, were analysed for CCHFV infections. The ticks belonged to the genera *Hyalomma* and *Rhipicephalus. Hyalomma* spp. infestation was higher as compared to *Rhipicephalus* spp. infestation, which is in accordance with previous studies from Pakistan (Sajid et al., 2011; Ali et al., 2013; Ahmad et al., 2014; Rehman et al., 2017). However, two other studies from Pakistan

reported a higher infestation of *Rhipicephalus* spp. as compared to *Hyalomma* spp. (Ahmed et al., 2012; Kakar et al., 2017). Moreover, among the genus *Hyalomma*, highest infestation was of *H. marginatum*, followed by *H. excavatum*, *H. dromedarii*, *H. anatolicum*, and *H. scupense.* This is in contrast to previous studies in Pakistan, which reported that *H. anatolicum* was most frequently found as compared to other *Hyalomma* species (Karim et al., 2017; Rehman et al., 2017).

We detected *Hyalomma* ticks in all districts in the study area. Previous studies in this area reported *Hyalomma* ticks (Iqbal and Nawaz, 2007; Rafique et al., 2015; Kakar et al., 2017; Karim et al., 2017), however, *H. marginatum* had not been found so far as to our knowledge. Moreover, there was hardly any information regarding the geographical distribution of the ticks in the area, in particular at the district level. The climate in the study area is mainly arid with low precipitation. It is mostly comprised of rangelands with grasses and shrubs (Sarfraz Ahmad and Islam, 2011), with abundant livestock (Government of Balochistan, 2016) which provides a favourable habitat for ticks. In addition, livestock farms in this area have mainly open type of housing with crevices and cracks in the walls where ticks can breed and hide (Muhammad et al., 2008). *Hyalomma* ticks have the ability to adapt, when introduced into new environments, especially in areas with dry climate. They also accustom easily in new animal housing facilities (wall crevices, under dried dungs, etc.)(ECDPC; Latif and Walker, 2004). Furthermore, livestock in this area is mainly fed by grazing on rangelands. This compels the farmers to move in search of pastures from one area to another, especially by nomadic and transhumant flock owners who migrate in winter from the northern part of province towards the southern part, and return back to the north in spring (Sarfraz Ahmad and Islam, 2011). *Hyalomma* ticks are known as “hunting ticks”, because they actively run towards their hosts (humans or animals) for distances of up to 400 m (Bente et al., 2013). Also, previous studies reported questing *Hyalomma* ticks on grazing pastures (Gunes et al., 2011; Sherifi et al., 2018). Livestock can support high infestation of up to 100 *Hyalomma* ticks on one single animal (ECDPC; Estrada-Pena et al., 2012a).

In this study, a prevalence of 4% CCHFV was detected in ticks in Balochistan. CCHFV was first diagnosed in ticks in Pakistan in 1970 in the north-eastern part of the country (Begum et al. (1970). We are not aware of any other published record of CCHFV detection in ticks from any other part of the country. We found that the CCHFV prevalence was higher in *H. marginatum* as compared to *H. dromedarii, H. excavatum*, *H. anatolicum*, and *H. scupense*. This result is in accord with previous reports from Turkey (Tonbak et al., 2006; Ozdarendeli et al., 2008; Gargili et al., 2011;

Gunes et al., 2011; Tekin et al., 2012), Iran (Zakkyeh et al., 2008; Fakoorziba et al., 2012) and Bulgaria (Gergova and Kamarinchev, 2013). *Hyalomma* ticks play a crucial role in the maintenance of CCHFV-endemic foci in nature. Moreover, it has been suggested that an increase in the population of *H. marginatum* is followed by an increase in CCHFV infections in humans in the affected area (Gargili et al., 2017). We found CCHFV-positive ticks in the districts of Kalat, Quetta, and Killa Abdullah, where CCHF cases among humans have previously been reported (Alam et al., 2013a; Khurshid et al., 2015). Also in a recent study, CCHFV genomes in sheep, and CCHFV-specific antibodies in sheep and goats were found in these areas (Kasi et al., 2019). This region is close to the border with Afghanistan, which is also endemic for CCHFV (Khurshid et al., 2015; Sahak et al., 2019). Balochistan is considered as a corridor for the trade of ruminant skins from Iran (also endemic for CCHFV) and Afghanistan for the leather industry, and also importation of livestock from Afghanistan to Pakistan is common (Raziq et al., 2010).

In the current study, all sequenced amplicons of the CCHFV-positive ticks clustered in the genotype Asia 1. The Balochistan-42-2017-Pakistan sequence shows high identity with CCHFV strains from Oman, Iran and Pakistan. The results are in accord with previous phylogenetic studies conducted with human CCHFV isolates from Pakistan, which also clustered in this genotype (Alam et al., 2013a; Khurshid et al., 2015). Genotype Asia 1 includes CCHFV strains from Pakistan, Iran, Afghanistan, Middle East and China (Alam et al., 2013a). However, Alam et al. (2013b) found a CCHFV isolate in Pakistan that clustered in the genotype Asia 2 with strains from Tajikistan and Dubai. This sequence was detected in a human CCHF patient from Balochistan. Genotype Asia 2 includes CCHFV strains from China, India, Kazakhstan, Tajikistan, Uzbekistan and Middle East (Alam et al., 2013b; Yadav et al., 2013).

Our phylogenetic analysis was conducted with a partial S segment sequence of the CCHFV genome. Similar (partial) sequences were also used in other studies for the classification of CCHFV into genotypes (Drosten et al., 2002; Papa et al., 2002b; Alam et al., 2013a; Alam et al., 2013b; Khurshid et al., 2015; Abdiyeva et al., 2019). Further research is needed to obtain the full-length CCHFV sequences of S, M, and L segments, to determine possible genetic re-assortment and recombination in the genome of CCHFV strains circulating in the area. Genetic re-assortment has been reported in European CCHFV strains (Lukashev et al., 2016). In Iran, which borders in the south-west with Balochistan, the circulation of genomic variants of CCHFV has been reported (Biglari et al., 2016).

5.3 General discussion

This study is the first comprehensive epidemiological evaluation of CCHFV circulation in livestock and ticks in province of Balochistan, Pakistan. Since its first report in Pakistan in 1976, multiple outbreaks have occurred in humans (Hussain et al., 2016), and CCHFV is considered to be endemic in the country (Alam et al., 2013a).

Pakistan has a vast agriculture sector with a large number of people involved in animal husbandry practices (Atif et al., 2017). The highest number of human cases in Pakistan is reported from Balochistan province, with a case-fatality ratio of up to 20% (Khurshid et al., 2015). The presence of CCHFV-specific antibodies in domestic animals indicates the circulation of CCHFV in the area and an increased risk for the human population (Spengler et al., 2016a). A seroprevalence estimate can help to quantify the proportion of the target species exposed to CCHFV. Furthermore, identification of tick vectors for CCHFV and circulation of CCHFV genotypes among them in a particular geographical area gives an insight in understanding the zoonotic CCHFV tick-vertebrate-tick cycle infection.

In Pakistan, the epidemiological aspects of CCHFV infections in livestock, wild animals, and ticks have not been studied extensively so far (Atif et al., 2017). Therefore, considering the importance of the disease, a comprehensive sero-epidemiological investigation of CCHFV infections among sheep and goats was conducted in 2016 in the province of Balochistan, Pakistan. The resultant seroprevalence in sheep and goats and potential risk factors identified associated with it in this geographical area, gave rise to further investigate the distribution of CCHFV tick vectors and circulation of CCHFV genotypes among them in the same study area. Therefore, in 2017 a second study was conducted, in which ticks were collected from infested livestock. The ticks were characterised by determining their species and tested for CCHFV. Moreover, an additional administrative division was included to extend the scope of study to a wider geographical area.

5.3.1 Epidemiological investigations of CCHFV infection in sheep and goats

In the study, 8 (5%, CI: 2-10%) out of 160 sheep serum pools were positive for CCHFV genome fragments. CCHFV in whole blood was also found in whole blood of sheep and goats in a previous study in Turkey (Albayrak et al., 2012). In small ruminants, CCHFV viremia is usually transient and the animals do not show any clinical signs (Spengler et al., 2016b). The antibody prevalence

is a good indicator for the circulation of CCHFV in a region (Mertens et al., 2016), as CCHFV-specific IgG-antibodies can be detected for several years after infection (Shepherd et al., 1989b). The seroprevalence of CCHFV-specific antibodies in this study was significantly higher in sheep (19%) than in goats (5%) (p-value <0.001). This was in line with the studies reported in India (Mourya et al., 2012; Mourya et al., 2014), Iran (Saidi et al., 1975; Bokaie et al., 2008; Telmadarraiy et al., 2010; Mostafavi et al., 2013b), Iraq (Tantawi et al., 1981), Egypt (Mohamed et al., 2008), Saudi Arabia (Hassanein et al., 1997), Turkey (Albayrak et al., 2012), and Bulgaria (Barthel et al., 2014). However, our findings were in contrast to results from studies conducted in Turkey (Tuncer et al., 2014), Kosovo (Fajs et al., 2014), Oman (Williams et al., 2000), United Arab Emirates (Khan et al., 1997), and Niger (Mariner et al., 1995), which reported a higher seroprevalence in goats compared to sheep. The variation in seroprevalence in animals is often associated with the susceptibility of the animal species (Tuncer et al., 2014), the distribution of the tick vectors (Tuncer et al., 2014), the host preference of competent tick vectors, and the tick load on a particular animal species (Spengler et al., 2016a). Moreover, in the current investigation, the seroprevalence was higher in the northern districts of the province, which are close to the border with Afghanistan. An increased number of human cases is also reported in this part of the country (Alam et al., 2013a; Khurshid et al., 2015). The rangelands in the northern part of the province are of good quality and are therefore grazed by large numbers of livestock animals. The arid climate, low precipitation and livestock abundance provides a suitable environment for the CCHFV tick vector and makes these areas an epicentre for the circulation of CCHFV.

In the multivariable analysis, farm management variables showed that animals with an open type of housing had an increased chance of being seropositive as compared to those with a closed type of housing. Farmers usually depend on rangelands for livestock feeding and due to seasonal migration, they do not have permanent housing. This forces them to construct an open type of temporary housing for their livestock. Moreover, cracks and crevices in walls of the open type of housing provide a suitable environment for ticks to breed and hide (Muhammad et al., 2008). Furthermore, in the current study, farms that fed animals by grazing were four times as likely of being seropositive than farms that practiced only trough feeding. This was in accordance with a previous study, in which livestock grazing was significantly associated with CCHFV seropositivity (Ibrahim et al., 2015). Trough or stall feeding is only practiced up to some extent by sedentary farmers (Afzal and Naqvi, 2004), while most farmers depend on grazing for feeding animals in this area. The ticks attach themselves to grass and other vegetation and then approach animals passing nearby (Muhammad et al., 2008). Moreover, previous studies have reported presence of

Hyalomma ticks on grazing pastures in CCHF endemic areas (Papa et al., 2009; Gunes et al., 2011; Albayrak et al., 2012; Sherifi et al., 2018). In addition, in the current study, the presence of vegetation in or around the farm posed three times as high chances of getting CCHFV infection than absence of vegetation. Vegetation usually provides a safe habitat for ticks to hide. Moreover, in this study, farms that did not treat their sheep and goats against tick infestation were three times as likely to be seropositive for CCHFV then those that had treated their animals against ticks. Previous studies in Pakistan showed a low tick prevalence in livestock farms, where tick treatment was practiced (Manan et al., 2007; Rehman et al., 2017). Furthermore, in multivariable analysis in the current study, farms without rural poultry were three times as likely of being seropositive regarding CCHFV then sheep and goat farms that kept rural poultry. Birds that pick ticks may play an important role in the biological control of the tick population, and may ultimately reduce CCHFV infection among livestock. It has been reported in previous studies that farms with chickens had low tick prevalence (Fajs et al., 2014; Rehman et al., 2017). Therefore, chickens may be used in low-income countries to reduce the tick burden in ruminants, where acaricides are not affordable by farmers, and may thus reduce the risk of CCHFV infections in livestock and ultimately in the rural human population. Moreover, in current investigation, animals infested with ticks had a significant association with being seropositive regarding CCHFV. This is in accordance with the previous studies which have also reported a significant association between ticks infested livestock and CCHFV infection (Adam et al., 2013; Ibrahim et al., 2015). Animals infested with ticks are more likely to be infected with CCHFV (Ibrahim et al., 2015), as ticks are considered as reservoirs and vectors for CCHFV infection (Spengler and Estrada-Peña, 2018).

5.3.1.1 Limitations of the study

Some of the farmers were reluctant to allow sampling of live animals, as they were afraid of any harm to them (mostly farms where sheep or goats were grazing). In some cases, it was also difficult to convince the farmers to answer the questionnaire. However, we achieved a complete response rate (100%) and all questions were answered by the farmers. Yet, it cannot be completely ruled out that farmers did not fully understand questions and that they might have given incorrect answers. However, if this happened, it is unlikely that it had any major impact, because most farmers realised that they might benefit from the study and had therefore no reason to give deliberately wrong answers. If a few answers were mistakenly wrong, this cannot have had a major impact on the outcomes of the study.

A complete sampling frame was not available, therefore we carried out multistage cluster sampling proportional to the population size of livestock in each division to avoid selection bias. Interviewer bias was avoided as all interviews were performed by the same investigator, who also entered the answers into the questionnaire form. However, recall bias might have occurred by the respondents, especially regarding information related to individual animals in larger flocks. There was no valid serological assay for the CCHFV strains from Pakistan. Therefore, we had followed the hierarchical diagnostic decision tree adopted by Schuster et al. (2016b) to determine the anti-CCHFV antibodies in the sheep and goats serum samples. Both, the in-house and the adapted commercial ELISA were based on different CCHFV genotypes antigens. Divergent results in these assays were confirmed by an adapted commercial IFA, therefore, minimizing the chance of false-positive results, which could have led to an overestimation of the true prevalence. False-negative results cannot be ruled out, which may have led to an underestimation of the true seroprevalence. It is unlikely, however, that this had a major impact on the accuracy of the prevalence estimates, although an underestimation of the true seroprevalence seems more likely than an overestimation under the specific circumstances of this study.

5.3.2 CCHFV in ticks collected from livestock

In this study, ticks belonging to the genera *Hyalomma* and *Rhipicephalus* were identified in the province of Balochistan, Pakistan. Overall, the prevalence of *Hyalomma* ticks was higher compared to *Rhipicephalus* ticks, which is in accordance with previous studies reported in Pakistan (Sajid et al., 2011; Ali et al., 2013; Ahmad et al., 2014; Rehman et al., 2017). Furthermore, the prevalence *of H. marginatum* and *R. microplus* was highest in the genera *Hyalomma* and *Rhipicephalus,* respectively. *Hyalomma* ticks were detected in all of the districts in the study area. *Hyalomma* ticks can easily adopt themselves to a new environment (ECDPC; Latif and Walker, 2004). Moreover, the climate in the study area is favourable for these ticks to survive. Most importantly, livestock animals are frequently moved throughout the year in search of pastures, and consequently introduce the ticks they are infested with into new geographical areas (Sarfraz Ahmad and Islam, 2011).

In the current investigation, a CCHFV prevalence of 4% was detected in ticks. The CCHFV prevalence was higher in *H. marginatum* compared to *H. dromedarii, H. excavatum*, *H. anatolicum*, and *H. scupense*. This is in accordance with previous studies reported from Iran (Zakkyeh et al., 2008; Fakoorziba et al., 2012), Bulgaria (Gergova and Kamarinchev, 2013), and

Turkey (Tonbak et al., 2006; Ozdarendeli et al., 2008; Gargili et al., 2011; Gunes et al., 2011; Tekin et al., 2012). *H. marginatum* ticks are usually considered as the main reservoir and vector for CCHFV (Sherifi et al., 2014; Sherifi et al., 2018; Spengler and Estrada-Peña, 2018).

All sequenced CCHFV amplicons from ticks sampled in this study clustered in the Asia 1 genotype. This is in line with previous phylogenetic analysis studies in Pakistan on human samples, which reported the clustering of CCHFV strains in the Asia 1 genotype (Alam et al., 2013a; Khurshid et al., 2015). However, one study conducted in the country reported a CCHFV strain that clustered in the Asia 2 genotype. The sample was obtained from a patient in Balochistan (Alam et al., 2013b). However, genomic variants of CCHFV strains have been previously reported in CCHFV endemic countries (Biglari et al., 2016).

5.3.2.1 Limitations of the study

Also for this part of the study, a complete sampling frame was not available, therefore we carried out multistage cluster sampling to select livestock farms in each division to avoid selection bias. The phylogenetic analysis in this study was limited to partial sequences from the S segment of the CCHFV genome, which may have led to a loss of information as compared to full S segment or full CCHFV genome sequences. Nevertheless, partial S segment sequences were also used in previous studies for the classification of CCHFV into genotypes (Drosten et al., 2002; Papa et al., 2002b; Alam et al., 2013a; Alam et al., 2013b; Khurshid et al., 2015; Abdiyeva et al., 2019).

5.3.3 Conclusions

The sero-epidemiological study (part I; 5.3.1) was conducted in Quetta, Sibi and Zhob divisions in the province of Balochistan, Pakistan in 2016. While the geographical distribution of CCHFV tick vectors study (part II; 5.3.2) was conducted in Quetta, Sibi, Zhob, and Kalat division of Balochistan in 2017. Both studies confirms the circulation of CCHFV in the livestock and ticks in this geographical area. In sero-epidemiological investigation (part I), CCHFV genome was detected only in sheep serum, while CCHFV-specific IgG antibodies were detected in both sheep and goats. The CCHFV sero-prevalence among small ruminants was detected higher in the northern part of the province. Furthermore, potential risk factors identified in this study associated with the seropositivity of CCHFV were open type of housing, grazing, presence of vegetation in or around the farm, no tick treatment, absence of rural poultry, animals with age $\geq$ 2 years, animals

infested with ticks, and sheep species. In the second study (part II), ticks belonging to the genera *Hyalomma* and *Rhipicephalus* were found on sheep, goats, and cattle. CCHFV genome was detected mainly in *Hyalomma* ticks. The CCHFV strain detected in this study belonged to the Asia 1 genotype. The most dominant tick species infected with CCHFV was *H. marginatum*, followed by, *H. dromedarii, H. excavatum*, *H. anatolicum*, and *H. scupense*.

Altogether, it is pertinent to consider the risk factors identified in this research project to prevent the CCHFV infection among livestock, which may subsequently help to prevent CCHF outbreaks among humans who are in close contact with animals. An inexpensive method to reduce the exposure of livestock to potentially CCHFV-infected ticks may be keeping rural poultry together with sheep, goats and cattle. Moreover, it is also pertinent to develop a one health approach with an integrated surveillance system amongst humans and livestock.

6. Summary

Epidemiology of Crimean-Congo haemorrhagic fever virus in ticks and livestock in Balochistan, Pakistan

Crimean-Congo haemorrhagic fever (CCHF) is a tick-borne zoonotic disease caused by the arbovirus Crimean-Congo haemorrhagic fever virus (CCHFV). It causes fatal haemorrhagic disease in human. Ticks considered as reservoir and vector for CCHFV. Livestock serve as a transient reservoir for this virus, but do not show clinical signs.

In part I (3.2) of this thesis, a cross-sectional study has been conducted from July to September 2016, in which sheep and goats in Balochistan, Pakistan, were examined to determine the CCHFV seroprevalence, spatial distribution of seropositive sheep and goats, and to identify potential risk factors for seropositivity to CCHFV in these animals. To this end, farms and animals selected by systematic sampling, blood samples from 800 sheep and 800 goats collected and information regarding farm management and the kept animals were retrieved using a standard questionnaire. Sera tested for antibodies against CCHFV in two independent ELISA formats and an immunofluorescence assay (IFA) following a hierarchical diagnostic decision tree. By these assays 149 (19%, 95%-CI: 16%-21%) out of 800 sheep serum samples and 37 (5%, 95%-CI: 3%-6%) out of 800 goat serum samples were positive for CCHFV-specific IgG antibodies. Interestingly, at least 8 (5%, 95%-CI: 2%-10%) out of 160 sera pools were from CCHFV viremic sheep, as sera (in pools of 5) tested positive for CCHFV genome by real time PCR (RT-qPCR). Risk factor analysis revealed that the open type of housing (OR=3.76, 95%-CI:1.57-9.56, p-value=0.003), grazing (OR=4.18, 95%-CI:1.79-10.37, p-value=0.001), presence of vegetation in or around the farm (OR= 3.13, 95%-CI: 1.07-10.15, p-value=0.043), lack of treatment against ticks (OR=3.31, 95%-CI: 1.16-10.21, p-value=0.029), absence of rural poultry (OR=2.93, 95%-CI: 1.41-6.29, p-value=0.004), animals with age $\geq$ 2 years (OR=4.15, 95%-CI: 2.84-6.19, p-value<0.001), animals infested with ticks (OR=2.35, 95%-CI: 1.59-3.52, p-value<0.001), and sheep species (OR=4.72, 95%-CI:3.24-6.86, p-value<0.001) represented statistically significant risk factors associated with seropositivity to CCHFV. Taken together this part of study confirms the circulation of CCHFV in livestock in Balochistan, Pakistan. The identification of risk factors might help to reduce the risk of infection in sheep and goats, which may also mitigate the risk for human infection. An interesting option for reducing the risk of CCHFV infection in small ruminants is keeping also chickens, since they pick ticks that transmit CCHFV.

In part II (3.3), a cross-sectional study has been conducted from September to November 2017, in the province of Balochistan, Pakistan. Ticks were collected from cattle, sheep and goats in the livestock farms. The ticks were identified morphologically and the result confirmed by genotyping. Further, ticks were analysed to detect CCHFV genome by one-step multiplex real-time RT-qPCR, and positive ticks were sequenced to determine the CCHFV genotype. In 529 livestock infested ticks, 525 (99%) ticks belonged to the genus *Hyalomma*, and four (1%) ticks were from the genus *Rhipicephalus*. Within the genus *Hyalomma, H. marginatum* (n=149; male=92, female=57), *H. excavatum* (n=135; male=96, female=39), *H. dromedarii* (n=117; male=101, female=16), *H. anatolicum* (n=82; male=61, female=21), and *H. scupense* (n=42; male= 36, female=6) were identified. In the genus *Rhipicephalus*, *R. microplus* (n=3), and *R. turanicus* (n=1) were found. The tick infestations on ruminants were 58 % in sheep (n=307), 28 % in goats (n=146), and 14 % in cattle (n=76). All collected ticks were adults. Four percent (20 out of 525, 95%-CI: 2%-6%) ticks were positive for CCHFV genome (S segment). All CCHFV sequences obtained from the ticks clustered in the Asia-1 genotype. Among the CCHFV-positive ticks, 75% (15 out of 20) were female and 25% (5 out of 20) were male. CCHFV genome was detected most frequently in *H. marginatum* (30%, 6 out of 20), followed by, *H. dromedarii (*25%, 5 out of 20)*, H. excavatum* (20%, 4 out of 20), *H. anatolicum* (20%, 4 out of 20), *and H. scupense* (5%, 1 out of 20). All CCHFV-positive ticks were found on sheep. The highest number of CCHFV-positive ticks was detected in the Kalat district (60%, 12 out of 20), followed by Quetta (30%, 6 out of 20) and Killa Abdullah (10%, 2 out of 20) districts. This part of the study confirms the circulation of CCHFV in ticks in the south-western part (Balochistan) of Pakistan. It is imperative to take effective tick-control measures in this area, especially to control livestock infestation with ticks, to reduce the risk of CCHF outbreaks in the human population.

7. Zusammenfassung

Epidemiologie des hämorrhagischen Krim-Kongo-Fiebervirus bei Zecken und Nutztieren in Belutschistan, Pakistan

Krim-Kongo-Hämorrhagisches Fieber (CCHF) ist eine durch Zecken übertragene Zoonose, die durch das Krim-Kongo-Hämorrhagisches Fieber Virus (CCHFV), ein Arbovirus, verursacht wird. Es verursacht tödliche hämorrhagische Erkrankungen beim Menschen. Zecken gelten als Reservoir und Vektor für CCHFV. Vieh dient als vorübergehendes Reservoir für dieses Virus, zeigt jedoch keine klinischen Anzeichen.

In Teil I (3.2) wurde von Juli bis September 2016 eine Querschnittsstudie durchgeführt, in der Schafe und Ziegen in Belutschistan, Pakistan, untersucht wurden, um die CCHFV-Seroprävalenz, die räumliche Verteilung von seropositiven Schafen und Ziegen sowie mögliche Risikofaktoren für Seropositivität gegenüber CCHFV bei diesen Tieren zu bestimmen. Zu diesem Zweck wurden Betriebe und Tiere durch systematische Probenahme ausgewählt, Blutproben von 800 Schafen und 800 Ziegen entnommen und Informationen zur Betriebsführung und den gehaltenen Tieren unter Verwendung eines Standardfragebogens abgerufen. Die Seren wurden in zwei unabhängigen ELISA-Formaten und einem Immunfluoreszenz-Assay (IFA) nach einem hierarchischen diagnostischen Entscheidungsbaum auf Antikörper gegen CCHFV getestet. Mit diesen Tests waren 149 (19%, 95%-CI: 16%-21%) von 800 Schafserumproben und 37 (5%, 95%-CI: 3%-6%) von 800 Ziegenserumproben positiv für CCHFV-spezifische IgG-Antikörper. Interessanterweise stammten mindestens 8 (5%, 95%-CI: 2%-10%) von 160 Serumpools von virämischen CCHFV-Schafen, da Seren (in Pools von 5) in der realtime-PCR positiv auf das CCHFV-Genom getestet wurden. Die Risikofaktoranalyse ergab, dass Offenställe (OR = 3,76, 95%-CI: 1,57-9,56, p-Wert = 0,003), Weidegang (OR = 4,18, 95%-CI: 1,79-10,37, p-Wert = 0,001), Vorhandensein von Vegetation in oder um den Betrieb (OR = 3,13, 95%-CI: 1,07-10,15, p-Wert = 0,043), fehlende Behandlung gegen Zecken (OR = 3,31, 95%-CI: 1,16-10,21, p-Wert = 0,029), Abwesenheit von Geflügel (OR = 2,93, 95%-CI: 1,41-6,29, p-Wert = 0,004), Tiere mit einem Alter von > 2 Jahren (OR = 4,15, 95%-CI: 2,84-6,19, p-Wert <0,001), mit Zecken befallene Tiere (OR = 2,35, 95%-CI: 1,59-3,52, p-Wert <0,001) und Schafarten (OR = 4,72, 95%-CI: 3,24-6,86, p-Wert <0,001) statistisch signifikante Risikofaktoren darstellten, die mit der Seropositivität gegenüber CCHFV assoziiert waren. Somit bestätigt dieser Teil der Studie die Verbreitung von CCHFV bei Nutztieren in Belutschistan, Pakistan. Die Identifizierung von Risikofaktoren könnte dazu beitragen, das Infektionsrisiko bei Schafen und Ziegen zu verringern, was auch das Risiko

einer Infektion des Menschen verringern kann. Eine interessante Option zur Verringerung des Risikos einer CCHFV-Infektion bei kleinen Wiederkäuern ist die Haltung von Hühnern, da sie Zeckenfressen, die CCHFV übertragen.

In Teil II (3.3) wurde von September bis November 2017 eine Querschnittsstudie in der pakistanischen Provinz Belutschistan durchgeführt. In den Viehbetrieben wurden Zecken von Rindern, Schafen und Ziegen gesammelt. Die Zecken wurden morphologisch identifiziert und der Befund durch Genom-Typisierung bestätigt. Weiterhin wurden Zecken analysiert, um das CCHFV-Genom durch Multiplex-realtime-RT-qPCR nachzuweisen. Positive Proben wurden sequenziert, um den CCHFV-Genotyp zu bestimmen. Von insgesamt 529 Zecken, die bei Schafen, Ziegen oder Rindern abgesammelt wurden, gehörten 525 (99%) zur Gattung Hyalomma, und 4 (1%) zur Gattung Rhipicephalus 4 (1%). In der Gattung Hyalomma waren *H. marginatum* (n = 149; männlich = 92, weiblich = 57), *H. excavatum* (n = 135; männlich = 96, weiblich = 39), *H. dromedarii* (n = 117; männlich = 101) weiblich = 16), *H. anatolicum* (n = 82; männlich = 61, weiblich = 21) und *H. scupense* (n = 42; männlich = 36, weiblich = 6). In der Gattung Rhipicephalus wurden *R. microplus* (n = 3) und *R. turanicus* (n = 1) identifiziert. Insgesamt waren 307 Schafe (58%) 146 Ziegen (28%) und 76 Rinder (14%) mit den Zecken befallen. Alle abgesammelten Zecken waren Adulte. 4% (20 von 525, 95%-CI: 2%-6%) Zecken waren positiv für das Genom des CCHFV-S-Segments. Alle CCHFV-Sequenzen aus den Zecken clusterten im Asia-1-Genotyp. Unter den CCHFV-positiven Zecken waren 75% (15 von 20) weiblich und 25% (5 von 20) männlich. Das CCHFV-Genom wurde in *H. marginatum* (30%, 6 von 20) am häufigsten nachgewiesen, gefolgt von *H. dromedarii* (25%, 5 von 20), *H. excavatum* (20%, 4 von 20), *H. anatolicum* (20%, 4 von 20) und *H. scupense* (5%, 1 von 20). Alle CCHFV-positiven Zecken wurden an Schafen gefunden. Die höchste Anzahl von CCHFV-positiven Zecken war im Bezirk Kalat zu verzeichnen (60%, 12 von 20), gefolgt von den Bezirken Quetta (30%, 6 von 20) und Killa Abdullah (10%, 2 von 20). Dieser Teil der Studie bestätigt die Verbreitung von CCHFV in Zecken im südwestlichen Teil Pakistans (Belutschistan). In diesem Bereich sollten dringend wirksame Maßnahmen zur Zeckenbekämpfung ergriffen werden, insbesondere um den Zeckenbefall bei Nutztieren zu bekämpfen, und so das Risiko von Ausbrüchen von CCHF in der menschlichen Bevölkerung zu verringern.

8. Literature

Abdiyeva, K., Turebekov, N., Dmitrovsky, A., Tukhanova, N., Shin, A., Yeraliyeva, L., Heinrich, N., Hoelscher, M., Yegemberdiyeva, R., Shapiyeva, Z., Kachiyeva, Z., Zhalmagambetova, A., Montag, J., Dobler, G., Zinner, J., Wagner, E., Frey, S., Essbauer, S., 2019. Seroepidemiological and molecular investigations of infections with Crimean–Congo haemorrhagic fever virus in Kazakhstan. International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases 78, 121-127. https://doi.org/10.1016/j.ijid.2018.10.015.

Adam, I.A., Mahmoud, M.A., Aradaib, I.E., 2013. A seroepidemiological survey of Crimean Congo hemorrhagic fever among cattle in North Kordufan State, Sudan. Virol J 10, 178. https://doi.org/10.1186/1743-422X-10-178.

Afzal, M., Naqvi, A.N., 2004. Livestock Resources of Pakistan: Present status and future trends. Quarterly Science Vision. http://www.sciencevision.org.pk/BackIssues/Vol9/22.livestock.pdf (accessed 11 April 2018).

Agricultural Census Organization, S.D., Government of Pakistan, 2006. Pakistan Livestock Census 2006. http://www.pbs.gov.pk/sites/default/files/aco/publications/pakistan-livestock-cencus2006/All%20Pakistan%20Report%20%28Introduction%29.pdf (accessed 11 April 2018).

Ahmad, I., Khawja, A., Shams, S., Ayaz, S., Khan, S., Akbar, N.u., Waqar, M., Alam, S., Khan, M.A., Rehman, A., Zakir, M., 2014. Detection of babesiosis and identification of associated ticks in cattle. Int J Bioassays 3, 3195-3199.

Ahmad, S., Islam, M., 2011. Rangeland Productivity and Improvement Potential in Highlands of Balochistan, Pakistan. *https://www.researchgate.net/publication/221916004*_Rangeland_Productivity_and_Improvement_Potential_in_Highlands_of_Balochistan_Pakistan (accessed 10 April 2018).

Ahmed, S., Numan, M., Manzoor, A.W., Ali, F.A., 2012. Investigations into Ixodidae ticks in cattle in Lahore, Pakistan. Veterinaria italiana 48, 185-191.

Alam, M.M., Khurshid, A., Sharif, S., Shaukat, S., Rana, M.S., Angez, M., Zaidi, S.S.Z., 2013a. Genetic analysis and epidemiology of Crimean Congo Hemorrhagic fever viruses in Baluchistan

province of Pakistan. BMC infectious diseases 13, 201. https://doi.org/10.1186/1471-2334-13-201.

Alam, M.M., Khurshid, A., Sharif, S., Shaukat, S., Suleman, R.M., Angez, M., Zaidi, S.S., 2013b. Crimean-Congo hemorrhagic fever Asia-2 genotype, Pakistan. J Emerg Infect Dis 19, 1017-1019. https://doi.org/10.3201/eid1906.120771.

Albayrak, H., Ozan, E., Kurt, M., 2012. Serosurvey and molecular detection of Crimean-Congo hemorrhagic fever virus (CCHFV) in northern Turkey. Tropical animal health and production 44, 1667-1671. https://doi.org/10.1007/s11250-012-0122-4.

Ali, Z., Maqbool, A., Muhammad, K., Khan, M.S., Younis, M., 2013. Prevalence of theileria annulata infected hard ticks of cattle and buffalo in punjab, Pakistan. J Anim Plant Sci 23, 20-26.
Altamura, L.A., Bertolotti-Ciarlet, A., Teigler, J., Paragas, J., Schmaljohn, C.S., Doms, R.W., 2007. Identification of a novel C-terminal cleavage of Crimean-Congo hemorrhagic fever virus PreGN that leads to generation of an NSM protein. Journal of virology 81, 6632-6642. https://doi.org/10.1128/JVI.02730-06.

Andriamandimby, S.F., Marianneau, P., Rafisandratantsoa, J.T., Rollin, P.E., Heraud, J.M., Tordo, N., Reynes, J.M., 2011. Crimean-Congo hemorrhagic fever serosurvey in at-risk professionals, Madagascar, 2008 and 2009. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology 52, 370-372. https://doi.org/10.1016/j.jcv.2011.08.008.

Ansari, J.A., Khan, M.A., A, R.M., R, R.T., G, K., M, S., Hassan, N., Qammar, A., Ikram, A., 2018. Crimean Congo hemorrhagic fever in Pakistan: Case control study, 2012-2015. Pak J Public Health 8. https://doi.org/10.32413/pjph.v8i1.98.

Apanaskevich, D., Horak, I., 2005. The genus Hyalomma Koch, 1844. II. Taxonomic status of *H.anatolicum Koch*, 1844 and *H.excavatum Koch*, 1844(Acari, Ixodidae) with redescriptions of all stages. Acarina 13, 181-197.

Apanaskevich, D.A., Filippova, N.A., Horak, I.G., 2010. The genus Hyalomma Koch, 1844. X. Redescription of all parasitic stages of *H.(Euhyalomma) scupense Schulze*, 1919 (= *H. detritum*

Schulze)(Acari: Ixodidae) and notes on its biology. Folia Parasitologica 57, 69-78. https://doi.org/10.14411/fp.2010.009.

Aradaib, I.E., Erickson, B.R., Mustafa, M.E., Khristova, M.L., Saeed, N.S., Elageb, R.M., Nichol, S.T., 2010. Nosocomial outbreak of Crimean-Congo hemorrhagic fever, Sudan. Emerging infectious diseases 16, 837-839. https://doi.org/10.3201/eid1605.091815.

Atif, M., Saqib, A., Ikram, R., Sarwar, M.R., Scahill, S., 2017. The reasons why Pakistan might be at high risk of Crimean Congo haemorrhagic fever epidemic; a scoping review of the literature. Virol J 14, 63. https://doi.org/10.1186/s12985-017-0726-4.

Atkinson, B., Chamberlain, J., Logue, C.H., Cook, N., Bruce, C., Dowall, S.D., Hewson, R., 2012a. Development of a Real-Time RT-PCR Assay for the Detection of Crimean-Congo Hemorrhagic Fever Virus. Vector borne and zoonotic diseases 12, 786-793. https://doi.org/10.1089/vbz.2011.0770.

Atkinson, B., Latham, J., Chamberlain, J., Logue, C., apos, Donoghue, L., Osborne, J., Carson, G., Brooks, T., Carroll, M., Jacobs, M., Hopkins, S., Hewson, R., 2012b. Sequencing and phylogenetic characterisation of a fatal Crimean – Congo haemorrhagic fever case imported into the United Kingdom, October 2012. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin 17, 20327. https://doi.org/10.2807/ese.17.48.20327-en.

Barthel, R., Mohareb, E., Younan, R., Gladnishka, T., Kalvatchev, N., Moemen, A., Mansour, S.S., Rossi, C., Schoepp, R., Christova, I., 2014. Seroprevalance of Crimean-Congo haemorrhagic fever in Bulgarian livestock. Biotechnology, biotechnological equipment 28, 540-542. https://doi.org/10.1080/13102818.2014.931685.

Bartolini, B., Gruber, C.E., Koopmans, M., Avšič, T., Bino, S., Christova, I., Grunow, R., Hewson, R., Korukluoglu, G., Lemos, C.M., Mirazimi, A., Papa, A., Sanchez-Seco, M.P., Sauer, A.V., Zeller, H., Nisii, C., Capobianchi, M.R., Ippolito, G., Reusken, C.B., Di Caro, A., 2019. Laboratory management of Crimean-Congo haemorrhagic fever virus infections: perspectives from two European networks. Euro surveillance : bulletin Europeen sur les maladies transmissibles =

European communicable disease bulletin 24, 1800093. https://10.2807/1560-7917.ES.2019.24.5.1800093.

Begum, F., Wisseman, C.L., Jr., Casals, J., 1970. Tick-borne viruses of West Pakistan. IV. Viruses similar to or identical with, Crimean hemorrhagic fever (Congo-Semunya), Wad Medani and Pak Argas 461 isolated from ticks of the Changa Manga Forest, Lahore District, and of Hunza, Gilgit Agency, W. Pakistan. American journal of epidemiology 92, 197-202. https://dx.doi.org/10.1093/oxfordjournals.aje.a121199.

Bente, D.A., Forrester, N.L., Watts, D.M., McAuley, A.J., Whitehouse, C.A., Bray, M., 2013. Crimean-Congo hemorrhagic fever: history, epidemiology, pathogenesis, clinical syndrome and genetic diversity. Antiviral research 100, 159-189. https://doi.org/10.1016/j.antiviral.2013.07.006.

Bergeron, E., Vincent, M.J., Nichol, S.T., 2007. Crimean-Congo hemorrhagic fever virus glycoprotein processing by the endoprotease SKI-1/S1P is critical for virus infectivity. Journal of virology 81, 13271-13276. https://doi.org/10.1128/JVI.01647-07.

Bertolotti-Ciarlet, A., Smith, J., Strecker, K., Paragas, J., Altamura, L.A., McFalls, J.M., Frias-Staheli, N., Garcia-Sastre, A., Schmaljohn, C.S., Doms, R.W., 2005. Cellular localization and antigenic characterization of crimean-congo hemorrhagic fever virus glycoproteins. Journal of virology 79, 6152-6161. https://doi.org/10.1128/JVI.79.10.6152-6161.2005.

Bibi, T., Ahmad, M., Tareen, N.M., Jabeen, R., Sultana, S., Zafar, M., Zain-ul-Abidin, S., 2015. The endemic medicinal plants of Northern Balochistan, Pakistan and their uses in traditional medicine. J Ethnopharmacol 173, 1-10. https://doi.org/10.1016/j.jep.2015.06.050.

Biglari, P., Chinikar, S., Belqeiszadeh, H., Telmadarraiy, Z., Mostafavi, E., Ghaffari, M., Javaherizadeh, S., Nowotny, N., Fooks, A.R., Shahhosseini, N., 2016. Phylogeny of tick-derived Crimean-Congo hemorrhagic fever virus strains in Iran. Ticks and tick-borne diseases 7, 1216-1221. https://doi.org/10.1016/j.ttbdis.2016.07.012.

Bokaie, S., Mostafavi, E., Haghdoost, A.A., Keyvanfar, H., Gooya, M.M., Meshkat, M., Davari, A., Chinikar, S., 2008. Crimean Congo Hemorrhagic Fever in Northeast of Iran. J Anim Vet Adv 7, 343-350.

Bursac, Z., Gauss, C.H., Williams, D.K., Hosmer, D.W., 2008. Purposeful selection of variables in logistic regression. Source Code Biol Med 3. https://doi.org/10.1186/1751-0473-3-17.

Burt, F.J., Swanepoel, R., Braack, L.E., 1993. Enzyme-linked immunosorbent assays for the detection of antibody to Crimean-Congo haemorrhagic fever virus in the sera of livestock and wild vertebrates. Epidemiology and infection 111, 547-557. https://doi.org/10.1017/s0950268800057277.

Cajimat, M.N.B., Rodriguez, S.E., Schuster, I.U.E., Swetnam, D.M., Ksiazek, T.G., Habela, M.A., Negredo, A.I., Estrada-Peña, A., Barrett, A.D.T., Bente, D.A., 2017. Genomic Characterization of Crimean–Congo Hemorrhagic Fever Virus in Hyalomma Tick from Spain, 2014. Vector borne and zoonotic diseases 17, 714-719. https://doi.org/10.1089/vbz.2017.2190.

Cannon, R.M., Roe, R.T., 1982. Livestock disease surveys : A field manual for Veterinarians, first ed. Australian Government Pub. Service, Canberra, Australia.

Carroll, S.A., Bird, B.H., Rollin, P.E., Nichol, S.T., 2010. Ancient common ancestry of Crimean-Congo hemorrhagic fever virus. Molecular phylogenetics and evolution 55, 1103-1110. https://doi.org/10.1016/j.ympev.2010.01.006.

Carter, S.D., Surtees, R., Walter, C.T., Ariza, A., Bergeron, É., Nichol, S.T., Hiscox, J.A., Edwards, T.A., Barr, J.N., 2012. Structure, Function, and Evolution of the Crimean-Congo Hemorrhagic Fever Virus Nucleocapsid Protein. Journal of virology 86, 10914-10923. https://doi.org/10.1128/jvi.01555-12.

Casal, J., 1969. Antigenic Similarity between the Virus Causing Crimean Hemorrhagic Fever and Congo Virus. Exp Biol Med 131, 233- 236. https://doi.org/10.3181/00379727-131-33847.

Casals, J., Henderson, B.E., Hoogstraal, H., Johnson, K.M., Shelokov, A., 1970. A Review of Soviet Viral Hemorrhagic Fevers, 1969. The Journal of infectious diseases 122, 437-453. https://dx.doi.org/10.1093/infdis/122.5.437.

Causey, O.R., Kemp, G.E., Madbouly, M.H., David-West, T.S., 1970. Congo Virus from Domestic Livestock, African Hedgehog, and Arthropods in Nigeria. The American journal of tropical medicine and hygiene 19, 846-850. https://doi.org/10.4269/ajtmh.1970.19.846.

Ceianu, C.S., Panculescu-Gatej, R.I., Coudrier, D., Bouloy, M., 2012. First serologic evidence for the circulation of Crimean-Congo hemorrhagic fever virus in Romania. Vector borne and zoonotic diseases 12, 718-721. https://doi.org/10.1089/vbz.2011.0768.

Cevik, M.A., Erbay, A., Bodur, H., Eren, S.S., Akinci, E., Sener, K., Onguru, P., Kubar, A., 2007. Viral load as a predictor of outcome in Crimean-Congo hemorrhagic fever. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 45, e96-100. https://doi.org/10.1086/521244.

Chamberlain, J., Cook, N., Lloyd, G., Mioulet, V., Tolley, H., Hewson, R., 2005. Co-evolutionary patterns of variation in small and large RNA segments of Crimean-Congo hemorrhagic fever virus. The Journal of general virology 86, 3337-3341. https://doi.org/10.1099/vir.0.81213-0.

Chinikar, S., Ghiasi, S.M., Hewson, R., Moradi, M., Haeri, A., 2010. Crimean-Congo hemorrhagic fever in Iran and neighboring countries. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology 47, 110-114. https://doi.org/10.1016/j.jcv.2009.10.014.

Chisholm, K., Dueger, E., Fahmy, N.T., Samaha, H.A., Zayed, A., Abdel-Dayem, M., Villinski, J.T., 2012. Crimean-congo hemorrhagic fever virus in ticks from imported livestock, Egypt. Emerging infectious diseases 18, 181-182. https://doi.org/10.3201/eid1801.111071.

Darwish, M.A., Hoogstraal, H., Roberts, T.J., Ghazi, R., Amer, T., 1983. A sero-epidemiological survey for Bunyaviridae and certain other arboviruses in Pakistan. Transactions of the Royal Society of Tropical Medicine and Hygiene 77, 446-450. https://doi.org/10.1016/0035-9203(83)90108-6.

Deyde, V.M., Khristova, M.L., Rollin, P.E., Ksiazek, T.G., Nichol, S.T., 2006. Crimean-Congo hemorrhagic fever virus genomics and global diversity. Journal of virology 80, 8834-8842. https://doi.org/10.1128/JVI.00752-06.

Dickson, D.L., Turell, M.J., 1992. Replication and tissue tropisms of Crimean-Congo hemorrhagic fever virus in experimentally infected adult Hyalomma truncatum (Acari: Ixodidae). Journal of medical entomology 29, 767-773. https://doi.org/10.1093/jmedent/29.5.767.

Dohm, D.J., Logan, T.M., Linthicum, K.J., Rossi, C.A., Turell, M.J., 1996. Transmission of Crimean-Congo hemorrhagic fever virus by Hyalomma impeltatum (Acari:Ixodidae) after experimental infection. Journal of medical entomology 33, 848-851.

Dorai-Raj, S., 2014. Binom Package: Binomial Confidence Intervals For Several Parameterizations. https://CRAN.R-project.org/package=binom (accessed 5 March 2018).

Dowall, S.D., Buttigieg, K.R., Findlay-Wilson, S.J., Rayner, E., Pearson, G., Miloszewska, A., Graham, V.A., Carroll, M.W., Hewson, R., 2016. A Crimean-Congo hemorrhagic fever (CCHF) viral vaccine expressing nucleoprotein is immunogenic but fails to confer protection against lethal disease. Human vaccines & immunotherapeutics 12, 519-527. https://doi.org/10.1080/21645515.2015.1078045.

Drosten, C., Minnak, D., Emmerich, P., Schmitz, H., Reinicke, T., 2002. Crimean-Congo hemorrhagic fever in Kosovo. Journal of clinical microbiology 40, 1122-1123.

ECDPC, E.C.f.D.P.a.C., An agency of the European Union, Hyalomma marginatum - Factsheet for experts. https://ecdc.europa.eu/en/disease-vectors/facts/tick-factsheets/hyalomma-marginatum (acessed 18 September 2019).

Ergonul, O., 2008. Treatment of Crimean-Congo hemorrhagic fever. Antiviral research 78, 125-131. https://doi.org/10.1016/j.antiviral.2007.11.002.

Ergönül, Ö., 2006. Crimean-Congo haemorrhagic fever. The Lancet. Infectious diseases 6, 203-214. https://doi.org/10.1016/S1473-3099(06)70435-2.

Estrada-Pena, A., Jameson, L., Medlock, J., Vatansever, Z., Tishkova, F., 2012a. Unraveling the ecological complexities of tick-associated Crimean-Congo hemorrhagic fever virus transmission: a gap analysis for the western Palearctic. Vector borne and zoonotic diseases 12, 743-752. https://doi.org/10.1089/vbz.2011.0767.

Estrada-Pena, A., Palomar, A.M., Santibanez, P., Sanchez, N., Habela, M.A., Portillo, A., Romero, L., Oteo, J.A., 2012b. Crimean-Congo hemorrhagic fever virus in ticks, Southwestern Europe, 2010. Emerging infectious diseases 18, 179-180. https://doi.org/10.3201/eid1801.111040.

Fajs, L., Humolli, I., Saksida, A., Knap, N., Jelovsek, M., Korva, M., Dedushaj, I., Avsic-Zupanc, T., 2014. Prevalence of Crimean-Congo hemorrhagic fever virus in healthy population, livestock and ticks in Kosovo. PloS one 9, e110982. https://doi.org/10.1371/journal.pone.0110982.

Fakoorziba, M.R., Golmohammadi, P., Moradzadeh, R., Moemenbellah-Fard, M.D., Azizi, K., Davari, B., Alipour, H., Ahmadnia, S., Chinikar, S., 2012. Reverse transcription PCR-based detection of Crimean-Congo hemorrhagic fever virus isolated from ticks of domestic ruminants in Kurdistan province of Iran. Vector borne and zoonotic diseases 12, 794-799. https://doi.org/10.1089/vbz.2011.0743.

Flick, R., Whitehouse, C.A., 2005. Crimean-Congo hemorrhagic fever virus. Current molecular medicine 5, 753-760.

Francischetti, I.M.B., Sa-Nunes, A., Mans, B.J., Santos, I.M., Ribeiro, J.M.C., 2009. The role of saliva in tick feeding. Front Biosci (Landmark Ed) 14, 2051-2088. https://doi.org/10.2741/3363.

Garcia, S., Chinikar, S., Coudrier, D., Billecocq, A., Hooshmand, B., Crance, J.M., Garin, D., Bouloy, M., 2006. Evaluation of a Crimean-Congo hemorrhagic fever virus recombinant antigen expressed by Semliki Forest suicide virus for IgM and IgG antibody detection in human and animal sera collected in Iran. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology 35, 154-159. https://doi.org/10.1016/j.jcv.2005.02.016.

Gargili, A., Estrada-Pena, A., Spengler, J.R., Lukashev, A., Nuttall, P.A., Bente, D.A., 2017. The role of ticks in the maintenance and transmission of Crimean-Congo hemorrhagic fever virus: A review of published field and laboratory studies. Antiviral research 144, 93-119. https://doi.org/10.1016/j.antiviral.2017.05.010.

Gargili, A., Midilli, K., Ergonul, O., Ergin, S., Alp, H.G., Vatansever, Z., Iyisan, S., Cerit, C., Yilmaz, G., Altas, K., Estrada-Pena, A., 2011. Crimean-Congo hemorrhagic fever in European part of

Turkey: genetic analysis of the virus strains from ticks and a seroepidemiological study in humans. Vector borne and zoonotic diseases 11, 747-752. https://doi.org/10.1089/vbz.2010.0030.

Gear, J.H.S., Thomson, P.D., Hopp, M., Andronikou, S., Cohn, R.J., Ledger, J., Berkowitz, F.E., 1982. Congo-Crimean haemorrhagic fever in South Africa - report of a fatal case in the Transvaal. South African medical journal = Suid-Afrikaanse tydskrif vir geneeskunde 62, 576-580.

Gergova, I., Kamarinchev, B., 2013. Comparison of the prevalence of Crimean-Congo hemorrhagic fever virus in endemic and non-endemic Bulgarian locations. Journal of vector borne diseases 50, 265-270.

Gonzalez, J.P., Camicas, J.L., Cornet, J.P., Faye, O., Wilson, M.L., 1992. Sexual and transovarian transmission of Crimean-Congo haemorrhagic fever virus in Hyalomma truncatum ticks. Res Virol 143, 23-28. https://doi.org/10.1016/S0923-2516(06)80073-7.

Gonzalez, J.P., Camicas, J.L., Cornet, J.P., Wilson, M.L., 1998. Biological and clinical responses of West African sheep to Crimean-Congo haemorrhagic fever virus experimental infection. Res Virol 149, 445-455. https://doi.org/10.1016/S0923-2516(99)80013-2.

Gordon, S.W., Linthicum, K.J., Moulton, J.R., 1993. Transmission of Crimean-Congo hemorrhagic fever virus in two species of Hyalomma ticks from infected adults to cofeeding immature forms. The American journal of tropical medicine and hygiene 48, 576-580.

Government of Balochistan, P., 2016. Livestock & Dairy Development Department. http://balochistan.gov.pk/index.php?option=com_docman&task=cat_view&gid=1368&Itemid=677 (accessed 2 April 2018).

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Systematic biology 59, 307-321. https://doi.org/10.1093/sysbio/syq010.

Gunes, T., Engin, A., Poyraz, O., Elaldi, N., Kaya, S., Dokmetas, I., Bakir, M., Cinar, Z., 2009. Crimean-Congo hemorrhagic fever virus in high-risk population, Turkey. Emerging infectious diseases 15, 461-464. https://doi.org/10.3201/eid1503.080687.

Gunes, T., Poyraz, O., Vatansever, Z., 2011. Crimean-Congo hemorrhagic fever virus in ticks collected from humans, livestock, and picnic sites in the hyperendemic region of Turkey. Vector borne and zoonotic diseases 11, 1411-1416. https://doi.org/10.1089/vbz.2011.0651.

Halos, L., Jamal, T., Vial, L., Maillard, R., Suau, A., Menach, A.L., Boulouis, H.-J., Vayssier-Taussat, M., 2004. Determination of an efficient and reliable method for DNA extraction from ticks. Journal of veterinary research 35, 709-713. https://doi.org/10.1051/vetres:2004038.

Han, X., Yi, H., Haixia, M., Bobo, L., Weiwei, X., Donglin, S., Zhiming, Y., 2019. Biosafety Level 4 Laboratory User Training Program, China. Emerging Infectious Disease journal 25. https://doi.org/10.3201/eid2505.180220.

Hassanein, K.M., El-Azazy, M.E., Yousef, H.M., 1997. Detection of Crimean-Congo haemorrhagic fever virus antibodies in humans and imported livestock in Saudi Arabia. Trans. R. Soc. Trop. Med. Hyg. 91, 536-537. https://doi.org/10.1016/s0035-9203(97)90014-6.

Honig, J.E., Osborne, J.C., Nichol, S.T., 2004. Crimean-Congo hemorrhagic fever virus genome L RNA segment and encoded protein. Virology 321, 29-35. https://doi.org/10.1016/j.virol.2003.09.042.

Hoogstraal, H., 1979. The Epidemiology of Tick-Borne Crimean-Congo Hemorrhagic Fever in Asia, Europe, and Africa. Journal of medical entomology 15, 307–417. https://doi.org/10.1093/jmedent/15.4.307.

Hornok, S., Horváth, G., 2012. First report of adult *Hyalomma marginatum rufipes* (vector of Crimean-Congo haemorrhagic fever virus) on cattle under a continental climate in Hungary. Parasites & vectors 5, 170. https://doi.org/10.1186/1756-3305-5-170.

Hosmer, D.W., Lemeshow, S., Sturdivant, R.X., 2013. Applied Logistic Regression,third ed. Wiley, New Jersey.

Hussain, B., Iqbal, A., Abubakar, M., 2016. Crimean-Congo Hemorrhagic Fever (CCHF): An Emerging Disease in Pakistan. Vet Sci: Res Reviews 2, 11-12. http://dx.doi.org/10.17582/journal.vsrr/2016.2.1.11.22.

Ibrahim, A.M., Adam, I.A., Osman, B.T., Aradaib, I.E., 2015. Epidemiological survey of Crimean Congo hemorrhagic fever virus in cattle in East Darfur State, Sudan. Ticks and tick-borne diseases 6, 439-444. http://dx.doi.org/10.1016/j.ttbdis.2015.03.002.

ICTV, 2018. International Committee on Taxonomy of Viruses. https://talk.ictvonline.org/taxonomy/ (accessed 31 January 2018).

Iqbal, A., Nawaz, M., 2007. Taxonomic studies of Haemaphysalis flava (Neumann), its seasonal prevalence and role in parasitic diseases of sheep/goat in Balochistan. Pak. Entomol. 29, 1-4.

ISID, P.I.S.f.I.D., 2018. Crimean-Congo Hemorrhagic Fever-Spain: (Castile and Leon) Fatal. www.promedmail.org (accessed 25 July 2019).

Jackman, S., Tahk, A., Zeileis, A., Maimone, C., Fearon, J., Meers, Z., 2017. Pscl: Political Science Computational Laboratory. https://CRAN.R-project.org/package=pscl (accessed 5 March 2018).

Kakar, M., Khan, M., Khan, M., Ashraf, K., Kakar, M., Hamdullah, Jan, S., Razzaq, A., 2017. Prevalence of tick infestation in different breeds of cattle in Balochistan. J Anim Plant Sci 27, 797-802.

Kakar, M.A., Raziq, A., Haq, K.M.U., Faqir, M., 2008. Trends and Potential in Dairy Production of Balochistan Province. Pak J Agri Sci 45.

Karim, S., Budachetri, K., Mukherjee, N., Williams, J., Kausar, A., Hassan, M.J., Adamson, S., Dowd, S.E., Apanskevich, D., Arijo, A., Sindhu, Z.U., Kakar, M.A., Khan, R.M.D., Ullah, S., Sajid, M.S., Ali, A., Iqbal, Z., 2017. A study of ticks and tick-borne livestock pathogens in Pakistan. PLoS neglected tropical diseases 11, e0005681. https://doi.org/10.1371/journal.pntd.0005681.

Kasi, K.K., Andrada Sas, M., Sauter-Louis, C., von Arnim, F., Gethmann, J.M., Schulz, A., Wernike, K., Groschup, M.H., Conraths, F.J., 2019. Epidemiological investigations of Crimean-Congo haemorrhagic fever virus infection in sheep and goats in Balochistan, Pakistan. Ticks and tick-borne diseases, 101324. https://doi.org/10.1016/j.ttbdis.2019.101324.

Khan AS, Maupin GO, Rollin PE, Noor AM, Shurie HH, Shalabi AG, e.a., 1997. An outbreak of Crimean-Congo hemorrhagic fever in the United Arab Emirates. The American journal of tropical medicine and hygiene 57, 519–525. https://doi.org/10.4269/ajtmh.1997.57.519.

Khan, A.S., GO, M., PE, R., AM, N., HH, S., AG, S., Wasef, S., Haddad, Y.M.A., Sadek, R., Ijaz, K., Peters, C.J., Ksiazek, T.G., 1997. An outbreak of Crimean-Congo hemorrhagic fever in the United Arab Emirates. Am. J. Trop. Med. Hyg. 57, 519–525. https://doi.org/10.4269/ajtmh.1997.57.519.

Khurshid, A., Hassan, M., Alam, M.M., Aamir, U.B., Rehman, L., Sharif, S., Shaukat, S., Rana, M.S., Angez, M., Zaidi, S.S.Z., 2015. CCHF virus variants in Pakistan and Afghanistan: Emerging diversity and epidemiology. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology 67, 25-30. https://doi.org/10.1016/j.jcv.2015.03.021.

Korolev, M.B., Donets, M.A., Rubin, S.G., Chumakov, M.P., 1976. Morphology and morphogenesis of Crimean Hemorrhagic Fever Virus. Archives of virology 50, 169–172. https://doi.org/10.1007/BF01318011.

Latif, A.A., Walker, A.R., 2004. An introduction to the biology and control of ticks in Africa. http://www.alanrwalker.com/assets/PDF/tick-biology-africa.pdf (accessed 19 September 2019).

Logan, T.M., Linthicum, K.J., Bailey, C.L., Watts, D.M., Moulton, J.R., 1989. Experimental transmission of Crimean-Congo hemorrhagic fever virus by Hyalomma truncatum Koch. The American journal of tropical medicine and hygiene 40, 207-212. https://doi.org/10.4269/ajtmh.1989.40.207.

Lugaj, A., Koni, M., Mertens, M., Groschup, M., Berxholi, K., 2014a. Serological survey of Crimean-Congo hemorrhagic fever virus in cattle in Berat and Kolonje, Albania. Albanian J Agri Sci, 325–328.

Lugaj, A., Koni, M., Mertens, M., Groschup, M., Berxholi, K., 2014b. Serological survey of Crimean-Congo hemorrhagic fever virus in cattle in Berat and Kolonje, Albania. Albanian J Agri Sci Special edition, 325-328.

Lukashev, A.N., Klimentov, A.S., Smirnova, S.E., Dzagurova, T.K., Drexler, J.F., Gmyl, A.P., 2016. Phylogeography of Crimean Congo Hemorrhagic Fever Virus. PloS one 11, e0166744. https://doi.org/10.1371/journal.pone.0166744.

Lwande, O.W., Irura, Z., Tigoi, C., Chepkorir, E., Orindi, B., Musila, L., Venter, M., Fischer, A., Sang, R., 2012. Seroprevalence of Crimean Congo hemorrhagic fever virus in Ijara District, Kenya. Vector borne and zoonotic diseases 12, 727-732. https://doi.org/10.1089/vbz.2011.0914.

Macleod, J.M.L., Marmor, H., Garcia-Sastre, A., Frias-Staheli, N., 2015. Mapping of the interaction domains of the Crimean-Congo hemorrhagic fever virus nucleocapsid protein. The Journal of general virology 96, 524-537. https://doi.org/10.1099/vir.0.071332-0.

Majeed, G., 2015. Land of Balochistan: A Historical Perspective. J Political Studies 22, 153-166.
Maltezou, H.C., Papa, A., Tsiodras, S., Dalla, V., Maltezos, E., Antoniadis, A., 2009. Crimean-Congo hemorrhagic fever in Greece: a public health perspective. International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases 13, 713-716. https://doi.org/10.1016/j.ijid.2008.11.011.

Manan, A., Khan, Z., Ahmad, B., Abdullah, 2007. Prevalence and identification of Ixodid tick genera in Frontier region Peshawar. J Agric Biol Sci 2.

Mariner, J.C., Morrill, J., Ksiazek, T.G., 1995. Antibodies to hemorrhagic fever viruses in domestic livestock in Niger: Rift Valley fever and Crimean-Congo hemorrhagic fever. The American journal of tropical medicine and hygiene 53, 217-221. https://doi.org/10.4269/ajtmh.1995.53.217.

Mertens, M., Schmidt, K., Ozkul, A., Groschup, M.H., 2013. The impact of Crimean-Congo hemorrhagic fever virus on public health. Antiviral research 98, 248-260. https://doi.org/10.1016/j.antiviral.2013.02.007.

Mertens, M., Schuster, I., Sas, M.A., Vatansever, Z., Hubalek, Z., Güven, E., Deniz, A., Georgiev, G., Peshev, R., Groschup, M.H., 2016. Crimean-Congo Hemorrhagic Fever Virus in Bulgaria and Turkey. Vector borne and zoonotic diseases 16, 619-623. https://doi.org/10.1089/vbz.2016.1944.

Mertens, M., Vatansever, Z., Mrenoshki, S., Krstevski, K., Stefanovska, J., Djadjovski, I., Cvetkovikj, I., Farkas, R., Schuster, I., Donnet, F., Comtet, L., Tordo, N., Ben Mechlia, M., Balkema-Buschmann, A., Mitrov, D., Groschup, M.H., 2015. Circulation of Crimean-Congo Hemorrhagic Fever Virus in the former Yugoslav Republic of Macedonia revealed by screening of cattle sera using a novel enzyme-linked immunosorbent assay. PLoS neglected tropical diseases 9, e0003519. https://doi.org/10.1371/journal.pntd.0003519.

Messina, J.P., Pigott, D.M., Golding, N., Duda, K.A., Brownstein, J.S., Weiss, D.J., Gibson, H., Robinson, T.P., Gilbert, M., William Wint, G.R., Nuttall, P.A., Gething, P.W., Myers, M.F., George, D.B., Hay, S.I., 2015. The global distribution of Crimean-Congo hemorrhagic fever. Transactions of the Royal Society of Tropical Medicine and Hygiene 109, 503-513. https://doi.org/10.1093/trstmh/trv050.

Midilli, K., Gargili, A., Ergonul, O., Elevli, M., Ergin, S., Turan, N., Sengoz, G., Ozturk, R., Bakar, M., 2009. The first clinical case due to AP92 like strain of Crimean-Congo Hemorrhagic Fever virus and a field survey. BMC infectious diseases 9, 90. https://doi.org/10.1186/1471-2334-9-90.

Mild, M., Simon, M., Albert, J., Mirazimi, A., 2010. Towards an understanding of the migration of Crimean-Congo hemorrhagic fever virus. The Journal of general virology 91, 199-207. https://doi.org/10.1099/vir.0.014878-0.

Mohamed, M., Said, A.R., Murad, A., Graham, R., 2008. A serological survey of Crimean-Congo haemorrhagic fever in animals in the Sharkia Governorate of Egypt. Veterinaria italiana 44, 513-517.

Moming, A., Yue, X., Shen, S., Chang, C., Wang, C., Luo, T., Zhang, Y., Guo, R., Hu, Z., Zhang, Y., Deng, F., Sun, S., 2018. Prevalence and Phylogenetic Analysis of Crimean-Congo Hemorrhagic Fever Virus in Ticks from Different Ecosystems in Xinjiang, China. Virologica Sinica 33, 67-73. https://doi.org/10.1007/s12250-018-0016-3.

Morikawa, S., Saijo, M., Kurane, I., 2007. Recent progress in molecular biology of Crimean-Congo hemorrhagic fever. Comparative immunology, microbiology and infectious diseases 30, 375-389. https://doi.org/10.1016/j.cimid.2007.07.001.

Morrill, J.C., Soliman, A.K., Imam, I.Z., Botros, B.A., Moussa, M.I., Watts, D.M., 1990. Serological evidence of Crimean-Congo haemorrhagic fever viral infection among camels imported into Egypt. The Journal of tropical medicine and hygiene 93, 201-204.

Mostafavi, E., Chinikar, S., Esmaeili, S., Amiri, F.B., Tabrizi, A.M.A., KhakiFirouz, S., 2012. Seroepidemiological survey of Crimean-Congo hemorrhagic fever among sheep in Mazandaran province, northern Iran. Vector borne and zoonotic diseases 12, 739-742. https://doi.org/10.1089/vbz.2011.0958.

Mostafavi, E., Chinikar, S., Moradi, M., Bayat, N., Meshkat, M., Fard, M.K., Ghiasi, S.M., 2013a. A case report of crimean congo hemorrhagic Fever in ostriches in iran. The open virology journal 7, 81-83. https://doi.org/10.2174/1874357901307010081.

Mostafavi, E., Haghdoost, A., Khakifirouz, S., Chinikar, S., 2013b. Spatial analysis of Crimean Congo hemorrhagic fever in Iran. Am. J. Trop. Med. Hyg. 89, 1135-1141. https://doi.org/10.4269/ajtmh.12-0509.

Mostafavi, E., Haghdoost, A., Khakifirouz, S., Chinikar, S., 2013c. Spatial analysis of Crimean Congo hemorrhagic fever in Iran. The American journal of tropical medicine and hygiene 89, 1135-1141. https://doi.org/10.4269/ajtmh.12-0509.

Mourya, D.T., Yadav, P.D., Shete, A., Majumdar, T.D., Kanani, A., Kapadia, D., Chandra, V., Kachhiapatel, A.J., Joshi, P.T., Upadhyay, K.J., Dave, P., Raval, D., 2014. Serosurvey of Crimean-Congo hemorrhagic fever virus in domestic animals, Gujarat, India, 2013. Vector borne and zoonotic diseases 14, 690-692. https://doi.org/10.1089/vbz.2014.1586.

Mourya, D.T., Yadav, P.D., Shete, A.M., Gurav, Y.K., Raut, C.G., Jadi, R.S., Pawar, S.D., Nichol, S.T., Mishra, A.C., 2012. Detection, isolation and confirmation of Crimean-Congo hemorrhagic fever virus in human, ticks and animals in Ahmadabad, India, 2010-2011. PLoS neglected tropical diseases 6, e1653. https://doi.org/10.1371/journal.pntd.0001653.

Mourya, D.T., Yadav, P.D., Shete, A.M., Sathe, P.S., Sarkale, P.C., Pattnaik, B., Sharma, G., Upadhyay, K.J., Gosavi, S., Patil, D.Y., Chaubal, G.Y., Majumdar, T.D., Katoch, V.M., 2015.

Cross-sectional Serosurvey of Crimean-Congo Hemorrhagic Fever Virus IgG in Livestock, India, 2013-2014. J Emerg Infect Dis 21, 1837-1839. http://dx.doi.org/10.3201/eid2110.141961.

Muhammad, G., Naureen, A., Firyal, S., Saqib, M., 2008. Tick control strategies in dairy production medicine. Pak Vet J 28, 43-50.

Mustafa, M.L., Ayazi, E., Mohareb, E., Yingst, S., Zayed, A., Rossi, C.A., Schoepp, R.J., Mofleh, J., Fiekert, K., Akhbarian, Z., Sadat, H., Leslie, T., 2011. Crimean-Congo hemorrhagic fever, Afghanistan, 2009. J Emerg Infect Dis 17, 1940-1941. https://doi.org/10.3201/eid1710.110061.

Nabeth, P., Cheikh, D.O., Lo, B., Faye, O., Vall, I.O., Niang, M., Wague, B., Diop, D., Diallo, M., Diallo, B., Diop, O.M., Simon, F., 2004a. Crimean-Congo hemorrhagic fever, Mauritania. Emerging infectious diseases 10, 2143-2149. https://doi.org/10.3201/eid1012.040535.

Nabeth, P., Thior, M., Faye, O., Simon, F., 2004b. Human Crimean-Congo hemorrhagic fever, Senegal. Emerging infectious diseases 10, 1881-1882. https://doi.org/10.3201/eid1010.040586.

Nicholson, W.L., Sonenshine, D.E., Noden, B.H., Brown, R.N., 2019. Chapter 27 - Ticks (Ixodida), in: Mullen, G.R., Durden, L.A. (Eds.), Medical and Veterinary Entomology (Third Edition), Academic Press, pp. 603-672.

O'Hearn, A.E., Voorhees, M.A., Fetterer, D.P., Wauquier, N., Coomber, M.R., Bangura, J., Fair, J.N., Gonzalez, J.-P., Schoepp, R.J., 2016. Serosurveillance of viral pathogens circulating in West Africa. Virol J 13, 163. https://doi.org/10.1186/s12985-016-0621-4.

Ozdarendeli, A., Aydin, K., Tonbak, S., Aktas, M., Altay, K., Koksal, I., Bolat, Y., Dumanli, N., Kalkan, A., 2008. Genetic analysis of the M RNA segment of Crimean-Congo hemorrhagic fever virus strains in Turkey. Archives of virology 153, 37-44. https://doi.org/10.1007/s00705-007-1056-4.

Papa, A., Bino, S., Llagami, A., Brahimaj, B., Papadimitriou, E., Pavlidou, V., Velo, E., Cahani, G., Hajdini, M., Pilaca, A., Harxhi, A., Antoniadis, A., 2002a. Crimean-Congo hemorrhagic fever in Albania, 2001. European journal of clinical microbiology & infectious diseases: official

publication of the European Society of Clinical Microbiology 21, 603-606. https://doi.org/10.1007/s10096-002-0770-9.

Papa, A., Bozovi, B., Pavlidou, V., Papadimitriou, E., Pelemis, M., Antoniadis, A., 2002b. Genetic detection and isolation of crimean-congo hemorrhagic fever virus, Kosovo, Yugoslavia. J Emerg Infect Dis 8, 852-854. https://doi.org/10.3201/eid0805.010448.

Papa, A., Christova, I., Papadimitriou, E., Antoniadis, A., 2004. Crimean-Congo hemorrhagic fever in Bulgaria. Emerging infectious diseases 10, 1465-1467. https://doi.org/10.3201/eid1008.040162.

Papa, A., Dalla, V., Papadimitriou, E., Kartalis, G.N., Antoniadis, A., 2010. Emergence of Crimean–Congo haemorrhagic fever in Greece. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases 16, 843–847.

Papa, A., Mirazimi, A., Koksal, I., Estrada-Pena, A., Feldmann, H., 2015. Recent advances in research on Crimean-Congo hemorrhagic fever. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology 64, 137-143. https://doi.org/10.1016/j.jcv.2014.08.029.

Papa, A., Papadopoulou, E., Tsioka, K., Kontana, A., Pappa, S., Melidou, A., Giadinis, N.D., 2018. Isolation and whole-genome sequencing of a Crimean-Congo hemorrhagic fever virus strain, Greece. Ticks and tick-borne diseases 9, 788-791. https://doi.org/10.1016/j.ttbdis.2018.02.024.

Papa, A., Velo, E., Papadimitriou, E., Cahani, G., Kota, M., Bino, S., 2009. Ecology of the Crimean-Congo hemorrhagic fever endemic area in Albania. Vector borne and zoonotic diseases 9, 713-716. https://doi.org/10.1089/vbz.2008.0141.

Porterfield, J.S., Casals, J., Chumakov, M.P., Gaidamovich, S.Y., Hannoun, C., Holmes, I.H., Horzinek, M.C., Mussgay, M., Oker-Blom, N., Russell, P.K., 1975. Bunyaviruses and Bunyaviridae. Intervirology 6, 13-24. https://doi.org/10.1159/000149449.

Qing, T., Saijo, M., Lei, H., Niikura, M., Maeda, A., Ikegami, T., Xinjung, W., Kurane, I., Morikawa, S., 2003. Detection of immunoglobulin G to Crimean-Congo hemorrhagic fever virus in sheep sera by recombinant nucleoprotein-based enzyme-linked immunosorbent and immunofluorescence assays. Journal of virological methods 108, 111-116. https://doi.org/10.1016/S0166-0934(02)00267-7.

Rafique, N., Kakar, A., Iqbal, A., Masood, Z., Razzaq, W., 2015. Identification of Three Species of Ticks *Hyalomma anatolicum Anatolicum*, *Hyalomma aegyptium* and *Dermacenter andersoni* in Quetta City of Balochistan, Pakistan. Glob Vet 14, 842-847.

Raziq, A., Younas, M., Rehman, Z., 2010. Prospects of Livestock Production in Balochistan. Pak Vet J 30, 181-186.

Rehman, A., Nijhof, A.M., Sauter-Louis, C., Schauer, B., Staubach, C., Conraths, F.J., 2017. Distribution of ticks infesting ruminants and risk factors associated with high tick prevalence in livestock farms in the semi-arid and arid agro-ecological zones of Pakistan. Parasites & vectors 10. https://doi.org/10.1186/s13071-017-2138-0.

RStudio, 2016. An Integrated development environment for R software environment for statistical computing and graphic, Version 1.0.44 – © 2009-2016 RStudio, Inc. 250 Northern Ave, Boston, MA 02210, USA. https://www.rstudio.com/ (accessed 2 November 2016).

Sahak, M.N., Arifi, F., Saeedzai, S.A., 2019. Descriptive epidemiology of Crimean-Congo Hemorrhagic Fever (CCHF) in Afghanistan: Reported cases to National Surveillance System, 2016-2018. International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases 88, 135-140. https://doi.org/10.1016/j.ijid.2019.08.016.

Saidi, S., Casals, J., Faghih, M.A., 1975. Crimean hemorrhagic fever-Congo (CHF-C) virus antibodies in man, and in domestic and small mammals, in Iran. The American journal of tropical medicine and hygiene 24, 353-357. https://doi.org/10.4269/ajtmh.1975.24.353.

Sajid, M.S., Iqbal, Z., Khan, M.N., Muhammad, G., Needham, G., Khan, M.K., 2011. Prevalence, associated determinants, and in vivo chemotherapeutic control of hard ticks (Acari: Ixodidae)

infesting domestic goats (Capra hircus) of lower Punjab, Pakistan. Parasitology research 108, 601–609. https://doi.org/10.1007/s00436-010-2103-8.

Sang, R., Lutomiah, J., Koka, H., Makio, A., Chepkorir, E., Ochieng, C., Yalwala, S., Mutisya, J., Musila, L., Richardson, J.H., Miller, B.R., Schnabel, D., 2011. Crimean-Congo hemorrhagic fever virus in Hyalommid ticks, northeastern Kenya. Emerging infectious diseases 17, 1502-1505. https://doi.org/10.3201/eid1708.102064.

Sarangzai, A.M., Ahmed, A., Laghari, S.K., 2013. Traditional uses of some useful medicinal plants of Ziarat district Balochistan, Pakistan. Fuuast J Biol 3, 101-107.

Sarfraz Ahmad, Islam, M., 2011. Rangeland Productivity and Improvement Potential in Highlands of Balochistan, Pakistan. *https://www.researchgate.net/publication/221916004* Rangeland Productivity and Improvement Potential in Highlands of Balochistan Pakistan (accessed 10 April 2018).

Sas, M.A., Vina-Rodriguez, A., Mertens, M., Eiden, M., Emmerich, P., Chaintoutis, S.C., Mirazimi, A., Groschup, M.H., 2018a. A one-step multiplex real-time RT-PCR for the universal detection of all currently known CCHFV genotypes. Journal of virological methods 255, 38–43. https://doi.org/10.1016/j.jviromet.2018.01.013.

Sas, M.A., Vina-Rodriguez, A., Mertens, M., Eiden, M., Emmerich, P., Chaintoutis, S.C., Mirazimi, A., Groschup, M.H., 2018b. A one-step multiplex real-time RT-PCR for the universal detection of all currently known CCHFV genotypes. Journal of virological methods 255, 38-43. https://doi.org/10.1016/j.jviromet.2018.01.013.

Schuster, I., Mertens, M., Kollner, B., Korytar, T., Keller, M., Hammerschmidt, B., Muller, T., Tordo, N., Marianneau, P., Mroz, C., Rissmann, M., Stroh, E., Dahnert, L., Hammerschmidt, F., Ulrich, R.G., Groschup, M.H., 2016a. A competitive ELISA for species-independent detection of Crimean-Congo hemorrhagic fever virus specific antibodies. Antiviral research 134, 161-166. https://doi.org/10.1016/j.antiviral.2016.09.004.

Schuster, I., Mertens, M., Mrenoshki, S., Staubach, C., Mertens, C., Bruning, F., Wernike, K., Hechinger, S., Berxholi, K., Mitrov, D., Groschup, M.H., 2016b. Sheep and goats as indicator

animals for the circulation of CCHFV in the environment. Experimental & applied acarology 68, 337–346. https://doi.org/10.1007/s10493-015-9996-y.

Schuster, I., Mertens, M., Mrenoshki, S., Staubach, C., Mertens, C., Brüning, F., Wernike, K., Hechinger, S., Berxholi, K., Mitrov, D., Groschup, M.H., 2016c. Sheep and goats as indicator animals for the circulation of CCHFV in the environment. Experimental & applied acarology 68, 337-346. https://doi.org/10.1007/s10493-015-9996-y.

Shafiq, M., Kakar, M.A., 2006. Current Livestock Marketing and its Future Prospects for the Economic Development of Balochistan–Pakistan. Int J Agric Biol 8.

Shafiq, M., Khan, J., Azhar, A., Rafiq, N., 2017. Constraints in Livestock Health System in Balochistan-Pakistan and its Impact on the Economy. Biol Forum 9, 243-247.

Shepherd, A.J., Leman, P.A., Swanepoel, R., 1989a. Viremia and antibody response of small African and laboratory animals to Crimean-Congo hemorrhagic fever virus infection. The American journal of tropical medicine and hygiene 40, 541-547.

Shepherd, A.J., Swanepoel, R., Leman, P.A., 1989b. Antibody response in Crimean-Congo hemorrhagic fever. Reviews of infectious diseases 11 Suppl 4, S801-806.

Shepherd, A.J., Swanepoel, R., Shepherd, S.P., Leman, P.A., Mathee, O., 1991. Viraemic transmission of Crimean-Congo haemorrhagic fever virus to ticks. Epidemiology and infection 106, 373-382. https://doi.org/10.1017/S0950268800048524.

Shepherd, A.J., Swanepoel, R., Shepherd, S.P., McGillivray, G.M., Searle, L.A., 1987. Antibody to Crimean-Congo hemorrhagic fever virus in wild mammals from southern Africa. The American journal of tropical medicine and hygiene 36, 133-142. https://doi.org/10.4269/ajtmh.1987.36.133.

Sherifi, K., Cadar, D., Muji, S., Robaj, A., Ahmeti, S., Jakupi, X., Emmerich, P., Kruger, A., 2014. Crimean-Congo hemorrhagic fever virus clades V and VI (Europe 1 and 2) in ticks in Kosovo, 2012. PLoS neglected tropical diseases 8, e3168. https://doi.org/10.1371/journal.pntd.0003168.

Sherifi, K., Rexhepi, A., Berxholi, K., Mehmedi, B., Gecaj, R.M., Hoxha, Z., Joachim, A., Duscher, G.G., 2018. Crimean-Congo Hemorrhagic Fever Virus and Borrelia burgdorferi sensu lato in Ticks from Kosovo and Albania. Front Vet Sci 5, 38. https://doi.org/10.3389/fvets.2018.00038.

Simon, M., Johansson, C., Lundkvist, A., Mirazimi, A., 2009a. Microtubule-dependent and microtubule-independent steps in Crimean-Congo hemorrhagic fever virus replication cycle. Virology 385, 313-322. https://doi.org/10.1016/j.virol.2008.11.020.

Simon, M., Johansson, C., Mirazimi, A., 2009b. Crimean-Congo hemorrhagic fever virus entry and replication is clathrin-, pH- and cholesterol-dependent. The Journal of general virology 90, 210-215. https://doi.org/10.1099/vir.0.006387-0.

Simpson, D.I.H., Knight, E.M., Courtois, G., Williams, M.C., Weinbren, M.P., Kibukamusoke, J.W., 1967. Congo virus: a hitherto undescribed virus occurring in Africa. Part 1. Human isolations-clinical notes. East African medical journal 44, 87-92.

Sonenshine, D.E., Roe, R.M., 2014. Biology of ticks, Second ed. Oxford University Press.

Spengler, J.R., Bente, D.A., Bray, M., Burt, F., Hewson, R., Korukluoglu, G., Mirazimi, A., Weber, F., Papa, A., 2018. Second International Conference on Crimean-Congo Hemorrhagic Fever. Antiviral research 150, 137–147. https://doi.org/10.1016/j.antiviral.2017.11.019.

Spengler, J.R., Bergeron, É., Rollin, P.E., 2016a. Seroepidemiological Studies of Crimean-Congo Hemorrhagic Fever Virus in Domestic and Wild Animals. PLoS neglected tropical diseases 10, e0004210. https://doi.org/10.1371/journal.pntd.0004210.

Spengler, J.R., Bergeron, É., Spiropoulou, C.F., 2019. Crimean-Congo hemorrhagic fever and expansion from endemic regions. Current opinion in virology 34, 70-78. https://doi.org/10.1016/j.coviro.2018.12.002.

Spengler, J.R., Estrada-Peña, A., 2018. Host preferences support the prominent role of Hyalomma ticks in the ecology of Crimean-Congo hemorrhagic fever. PLoS neglected tropical diseases 12, e0006248. https://doi.org/10.1371/journal.pntd.0006248.

Spengler, J.R., Estrada-Pena, A., Garrison, A.R., Schmaljohn, C., Spiropoulou, C.F., Bergeron, E., Bente, D.A., 2016b. A chronological review of experimental infection studies of the role of wild animals and livestock in the maintenance and transmission of Crimean-Congo hemorrhagic fever virus. Antiviral research 135, 31-47. https://doi.org/10.1016/j.antiviral.2016.09.013.

Sun, S., Dai, X., Aishan, M., Wang, X., Meng, W., Feng, C., Zhang, F., Hang, C., Hu, Z., Zhang, Y., 2009. Epidemiology and phylogenetic analysis of crimean-congo hemorrhagic fever viruses in xinjiang, china. Journal of clinical microbiology 47, 2536–2543. https://doi.org/10.1128/JCM.00265-09.

Swanepoel, R., Gill, D.E., Shepherd, A.J., Leman, P.A., Mynhardt, J.H., Harvey, S., 1989. The clinical pathology of Crimean-Congo hemorrhagic fever. Reviews of infectious diseases 11 Suppl 4, S794-800. https://doi.org/10.1093/clinids/11.supplement_4.s794.

Swanepoel, R., Shepherd, A.J., Leman, P.A., Shepherd, S.P., McGillivray, G.M., Erasmus, M.J., Searle, L.A., Gill, D.E., 1987. Epidemiologic and clinical features of Crimean-Congo hemorrhagic fever in southern Africa. The American journal of tropical medicine and hygiene 36, 120-132. https://doi.org/10.4269/ajtmh.1987.36.120.

Tantawi, MO, S., SK, A.-T., 1981. Antibodies to Crimean-Congo haemorrhagic fever virus in domestic animals in Iraq: a seroepidemiological survey. International journal of zoonoses 8, 115-120.

Tekin, S., Bursali, A., Mutluay, N., Keskin, A., Dundar, E., 2012. Crimean-Congo hemorrhagic fever virus in various ixodid tick species from a highly endemic area. Veterinary parasitology 186, 546-552. https://doi.org/10.1016/j.vetpar.2011.11.010.

Telmadarraiy, Z., Chinikar, S., Vatandoost, H., Faghihi, F., Hosseini-Chegeni, A., 2015. Vectors of Crimean Congo Hemorrhagic Fever Virus in Iran. Journal of arthropod-borne diseases 9, 137-147.

Telmadarraiy, Z., Ghiasi, S.M., Moradi, M., Vatandoost, H., Eshraghian, M.R., Faghihi, F., Zarei, Z., Haeri, A., Chinikar, S., 2010. A survey of Crimean-Congo haemorrhagic fever in livestock and

ticks in Ardabil Province, Iran during 2004–2005. Scandinavian journal of infectious diseases 42, 137–141. https://doi.org/10.3109/00365540903362501.

Tonbak, S., Aktas, M., Altay, K., Azkur, A.K., Kalkan, A., Bolat, Y., Dumanli, N., Ozdarendeli, A., 2006. Crimean-Congo hemorrhagic fever virus: genetic analysis and tick survey in Turkey. Journal of clinical microbiology 44, 4120-4124. https://doi.org/10.1128/JCM.00644-06.

Tuncer, P., Yesilbag, K., Alpay, G., Dincer, E., Girisgin, O., Aydin, L., Uyar, Y., Ozkul, A., 2014. Crimean-Congo Hemorrhagic Fever infection in domestic animals in Marmara region, Western Turkey. Ankara Üniv Vet Fak Derg. 61, 49-53. https://doi.org/10.1501/vetfak_0000002604.

Vawda, S., Goedhals, D., Bester, P.A., Burt, F., 2018. Seroepidemiologic Survey of Crimean-Congo Hemorrhagic Fever Virus in Selected Risk Groups, South Africa. Emerging infectious diseases 24, 1360-1363. https://doi.org/10.3201/eid2407.172096.

Vorou, R.M., 2009. Crimean-Congo hemorrhagic fever in southeastern Europe. International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases 13, 659-662. https://doi.org/10.1016/j.ijid.2009.03.028.

Walker, A.R., Matthews, J., Preston, P.M., 2005. The development of electronic keys for the identification of ticks. Int J Trop Insect Sci 25, 2-5. https://doi.org/10.1079/IJT200546.

Warnes, G.R., Bolker, B., Lumley, T., Johnson, R.C., 2015. Gmodels package: Various R programming tools for model fitting. https://CRAN.R-project.org/package=gmodels (accessed 5 March 2018).

Whitehouse, C.A., 2004. Crimean-Congo hemorrhagic fever. Antiviral research 64, 145-160. https://doi.org/10.1016/j.antiviral.2004.08.001.

Williams, R.J., Al-Busaidy, S., Mehta, F.R., Maupin, G.O., Wagoner, K.D., Al-Awaidy, S., Suleiman, A.J., Khan, A.S., Peters, C.J., Ksiazek, T.G., 2000. Crimean-congo haemorrhagic fever: a seroepidemiological and tick survey in the Sultanate of Oman. Tropical medicine & international health : TM & IH 5, 99-106. https://doi.org/10.1046/j.1365-3156.2000.00524.x.

Woodall, J.P., Williams, M.C., Simpson, D.I.H., 1967. Congo virus: a hitherto undescribed virus occurring in Africa. Part 2. Identification studies. East African medical journal 44, 93-98.

Xiao, X., Feng, Y., Zhu, Z., Dimitrov, D.S., 2011. Identification of a putative Crimean-Congo hemorrhagic fever virus entry factor. Biochemical and biophysical research communications 411, 253-258. https://doi.org/10.1016/j.bbrc.2011.06.109.

Yadav, P.D., Cherian, S.S., Zawar, D., Kokate, P., Gunjikar, R., Jadhav, S., Mishra, A.C., Mourya, D.T., 2013. Genetic characterization and molecular clock analyses of the Crimean-Congo hemorrhagic fever virus from human and ticks in India, 2010-2011. Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases 14, 223-231. https://doi.org/10.1016/j.meegid.2012.10.005.

Yilmaz, G.R., Buzgan, T., Irmak, H., Safran, A., Uzun, R., Cevik, M.A., Torunoglu, M.A., 2009. The epidemiology of Crimean-Congo hemorrhagic fever in Turkey, 2002-2007. International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases 13, 380-386. https://doi.org/10.1016/j.ijid.2008.07.021.

Zakkyeh, T., Moradi, A.R., Vatandoost, H., Mostafavi, E., Oshaghi, M.A., Zahirnia, A.H., Haeri, A., Chinikar, S., 2008. Crimean-Congo hemorrhagic fever: a seroepidemiological and Molecular survey in Bahar, Hamadan province of Iran. Asian J Anim Vet Adv 3, 321-327. https://doi.org/10.3923/ajava.2008.321.327.

Zhang, Y., Shen, S., Fang, Y., Liu, J., Su, Z., Liang, J., Zhang, Z., Wu, Q., Wang, C., Abudurexiti, A., Hu, Z., Zhang, Y., Deng, F., 2018. Isolation, Characterization, and Phylogenetic Analysis of Two New Crimean-Congo Hemorrhagic Fever Virus Strains from the Northern Region of Xinjiang Province, China. Virologica Sinica 33, 74-86. https://doi.org/10.1007/s12250-018-0020-7.

Zivcec, M., Maiga, O., Kelly, A., Feldmann, F., Sogoba, N., Schwan, T.G., Feldmann, H., Safronetz, D., 2014. Unique strain of Crimean-Congo hemorrhagic fever virus, Mali. Emerging infectious diseases 20, 911-913. https://doi.org/10.3201/eid2005.131641.

9. Appendices

9.1. Appendix A (Questionnaire-I)

Farm ID: _____ Date: ____/____/_____

Personnel information

Name of farm: _____-

_____

Address:

_____

_

Division:

_____

_

Contact No. _____ Age:

_____

Gender: □ Male □ Female

Occupation:

_____

Location: X-Coordinate _____ Y-Coordinate

_____

Farm level information

1. Size of the flock:

   _____

2. How many people live on your farm?

Category	Number
No. of men over 18 years	
No. of women over 18 years	
No. of children over 5 years	
No. of children up to 5 years	

3. Presence of other animal species:

Animal species	Number
Cattle	
Buffalo	
Sheep	
Goat	
Dogs	
Cats	
Chickens	
Turkey	
Other poultry: Please specify	
Horses	
Donkeys	
Other animals: Please specify:	

4. When you observe the tick infestation at your farm?

S.No.	Month	Tick occur	Highest infestation
1	January		
2	February		
3	March		
4	April		
5	May		
6	June		
7	July		
8	August		
9	September		
10	October		
11	November		
12	December		

5. Tick control:
   □ Acaricides (compound name) _____ □ Local plants/traditional medicine _____ □ Collect ticks □ No □ Do not know
6. Tick treatment frequency:
   □ Within 6 months □ Within 6-12 months □ After 12 months

7. Do you treat the animals in different groups according to their age?
   □ Yes □ No □ Do not know
8. Please indicate any reasons, why you do not use acaricides: (If not in No. 5)
   □ They are too expensive □ I do not believe in their effect
   □ I use alternative treatment methods □ I do not have access to acaridices
   □ Ticks do not occur □ It is normal that animals have ticks
   □ Animals do not seem to suffer from the ticks
   □ Other reasons, please specify:
   _____
   □ Do not know
9. Did you observe any change in animal health/ behavior infested by ticks?
   □ Yes, please specify what type of changes
   _____
   _____
   □ No □ Do not know
10. Did you observe loss of the weight in animals infested with ticks?
    □ Yes □ No □ Do not know
11. Quarantine of purchased animals/return from sale market:

Species/Breed	**Sheep**	**Goat**	**Other**
Time Period (Days/months)			

12. Vaccination or tick treatment of purchase animals/return from sale market:

□ Yes □ No

13. Dispose of animal dung:

□ Daily □ Monthly □ Yearly

14. How you dispose of dead animals/wastes:

□ Bury □ Burn □ No □ Do not know

15. Presence of the vegetation in or around the farm:

□ Trees □ Shrubs/ bushes

□ Other

_____

□ No

16. How often did veterinary doctor or para-veterinary staff visit your farm?

□ Regular (monthly or once in year)

_____

□ Only when there is morbidity/mortality □ Only for vaccination of animals □ No visits

17. Sheep/goat are kept separately or jointly with other species of animals:

□ Separately

□ Jointly, please specify the specie

_____

18. Different age groups of sheep/goat are kept separately or together at the farm:

□ Separately, please specify on which basis they are divided

_____

_____

_____

□ Jointly

19. Type of the housing for the animals:

S G

□ □ Open houses

□ □ Closed sheds

□ □ Other, please specify

_____

_____

20. Type of material used for the housing of animals:

□ Concrete bricks □ Mud bricks □ Wood □ Straw

21. Type of floor used for the housing of animals:

□ Hard floor (concrete or other hard material) □ Soil floor □ Straw bedding

22. Presence of fences/boundary wall around the farm periphery:

□ Yes

□ Not around the entire farm, but the area where the ruminants are kept

□ No

23. Presence of other livestock farm near your farm:

□ Yes, please specify the distance

_____

□ No

24. Contact of your farm animals with other farm animals:

□ Yes □ No

25. Visit of other farm worker to your farm:

□ Yes, for what purpose?

_____

_____

_____

□ No

26. Feeding method used for feeding of the animals:

S G

□ □ Grazing

□ □ Floor feeding

□ □ Trough feeding

27. Where did you store feed at the farm:

□ Open air on the ground □ Closed building

28. The supplier of the feed to your farm is same who supplies to other farm in the area:

□ Yes □ No

29. Repeat grazing on same area:

□ Yes □ No

30. Grazing area used by animals of other farms:

□ Yes □ No

31. How many sheep were ill in the farm since last 6 months?

_____

32. What were the signs and symptoms?

□ Number of animals

_____

□ Increased temperature □ Weight loss □ Off feed

□ Low water intake □ Abortion □ Skin infection

□ Other,

_____

□ Do not know

33. How many goats were ill in the farm since last 6 months?

_____

34. What were the signs and symptoms?

□ Number of animals

_____

□ Increased temperature □ Weight loss □ Off feed

□ Low water intake □ Abortion □ Skin infection

□ Other,

_____

□ Do not know

35. Level of morbidity at farm?

□ Low □ Medium □ High

36. How many sheep died since last 6 months?

_____

_____

37. What were the signs and symptoms?

□ Increased temperature □ Weight loss □ Off feed

□ Low water intake □ Abortion □ Skin infection

□ Other,

_____

□ Do not know

38. How many goats died since last 6 months?

_____

_____

39. What were the signs and symptoms?

□ Increased temperature □ Weight loss □ Off feed

□ Low water intake □ Abortion □ Skin infection

□ Other,

_____

□ Do not know

40. Is there proper drainage system for the farm?

□ Yes □ No □ Do not know

41. Farm hygiene:

□ Good □ Bad

42. Do you know about zoonotic disease?

□ Yes, can you name it

_____

_____

_____

□ No

43. Do you know about the CCHF disease?

□ Yes □ No

44. Did you, your farm worker or any of your family member had suffered from CCHF disease?

□ Yes, when and what were the sign and symptoms?

_____

_____

_____

□ No

45. Did you know how to handle the live ticks?

□ Yes, can you tell the procedure?

_____

_____

_____

□ No

46. Did you use any of following equipment while in contact with diseased or dead animal?

□ Gloves □ Protective clothing □ Mask □ Gum boots

□ No

47. Did you wash your hand with soap or any detergent after in contact with animals?

□ Yes □ No

48. What is your level of education?

□ Intermediate or above level □ Elementary level □ No schooling

□ Other, please specify,

_____

9.2. Appendix B (Questionnaire-II)

Animal level information

Animal ID	Date	Farm ID	Age	Sex (M/F)	Species	Breed	Tick infestation (Y/ N)	Body condition (weak / fatty)	Previous history of disease (If yes, please specify the signs and symptoms)

10. Research articles published/submitted, and conferences and workshops attended

10.1. Research article published

Kasi, K.K., Andrada Sas, M., Sauter-Louis, C., von Arnim, F., Gethmann, J.M., Schulz, A., Wernike, K., Groschup, M.H., Conraths, F.J., 2019. **Epidemiological investigations of Crimean-Congo haemorrhagic fever virus infection in sheep and goats in Balochistan, Pakistan**. *Ticks and Tick-borne Diseases*, 101324. https://doi.org/10.1016/j.ttbdis.2019.101324.

Khushal Khan Kasi, Felicitas von Arnim, Ansgar Schulz, Abdul Rehman, Amna Chudhary, Muhammad Oneeb, Miriam Andrada Sas, Tariq Jamil, Pavlo Maksimov, Carola Sauter-Louis, Franz J. Conraths, Martin H. Groschup. **Crimean-Congo haemorrhagic fever virus in ticks collected from livestock in Balochistan, Pakistan**. *Transboundary and Emerging Diseases*. https://doi: 10.1111/tbed.13488.

10.2. Conferences/symposiums/workshops attended

a. Zoonoses 2019- International Symposium on Zoonoses Research, 16-18, October, 2019, Berlin, Germany.

b. Workshop on “Inside Tick”, 12-13, September, 2019, University of Greifswald, Germany.

c. Poster presentation at 13th International symposium on Ticks and Tick-borne Diseases, 28-30 March, 2019, Weimar, Germany. “Epidemiological investigation of Crimean-Congo Hemorrhagic fever virus foci among livestock in the endemic region of Pakistan”.

d. Oral presentation at 13th International Veterinary Congress, May 02-03, 2019, London, UK. “Epidemiological distribution of the Bovine Viral Diarrhea Virus (BVDV) among small ruminants in Pakistan”.

e. Poster presentation at International Meeting on Emerging Diseases and Surveillance, November 9-12, 2018, Vienna, Austria. “Risk factors for Crimean-Congo Hemorrhagic fever virus infection in livestock in Pakistan”.

f. National Symposium on Zoonoses Research 2018, October 17-19, 2018 Berlin, Germany.

g. Poster presentation at Junior Scientist Symposium, 24-26 September 2018, Friedrich-Loeffler-Institute Greifswald-Insel Riems, Germany. “Khushal Khan Kasi, Miriam Andrada Sas, Carola Sauter-Louis, Jörn Martin Gethmann, Martin H. Groschup, Franz J. Conraths. Seroprevalence of Crimean-Congo Hemorrhagic Fever in small ruminants in Balochistan, Pakistan”.

h. VECMAP Lite workshop, Avia-GIS, 24th March 2018, Institute of Parasitology and Tropical Veterinary Medicine, Freie University Berlin, Germany.

i. Poster presentation at 28th Annual Meeting of the German Society for Parasitology, 21-24 March 2018, Freie University Berlin, Germany. **“**Khushal Khan Kasi, Miriam Andrada Sas, Jörn Martin Gethmann, Martin H. Groschup, Franz J. Conraths. Epidemiology of Crimean-Congo Hemorrhagic Fever in Livestock animals of Balochistan, Pakistan: A risk indicator for the human population.

j. “Presenting (in) Science- How to own the stage on (international) conferences”, May 11-13, 2017, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany.

k. “Epi Days 2017- Epidemiologie in der praktischen Anwendung” on 23-24 March, 2017, Greifswald, Germany organized by Institute of Epidemiology, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany.

11. Acknowledgments

I am very grateful to Professor Dr. Franz J. Conraths for supervising my PhD thesis, and giving me a great opportunity to carry out research work in the Friedrich-Loeffler-Institut (FLI) Greifswald-Insel Riems, Germany. He always guided me during my research project with his expert suggestions. His supervision helped me to improve my epidemiological and scientific writing skills, which resulted in accomplishment of this PhD thesis.

I am also thankful to Professor Dr. Martin H. Groschup for his guidance during my research project. He gave me the opportunity to analyse the samples in his laboratory. He was always willing to assist me whenever I needed any help during my research project.

Furthermore, I would like to express my gratitude to Dr. Miriam Andrada Sas for her supervision in the laboratory. She helped me to learn the laboratory techniques to analyse the samples, and to improve my scientific writing skills. She was very kind, friendly, and always there to help me.

Moreover, I also extend my special gratitude to Dr. Carola Sauter-Louis, Dr. Jörn Martin Gethmann, Dr. Kerstin Wernike, Dr. Carolina Probst, Dr. Felicitas von Arnim, Dr. Katja Schulz, Dr. Pavlo Maksimov, Sonja Schell, Patrick Wysocki, Nicole Reimer, Suman Bhowmick, Rene Schottner, Martina Abs, Maria Justiniano Suarez, Ansgar Schulz, Arian Kohler, Ibrahim Moharam, and all other colleagues in the FLI, for their heartiest guidance and support during my stay at FLI.

I would like to thank my parents, brother, sisters, and other family members for their motivation and support in accomplishment of this doctoral degree.

I am also grateful to Professor Dr. Tariq Abbas for his guidance in this research project. Moreover, I am thankful to all local veterinary officials for their support to carry out sampling in the field. In addition, the farm owners, for their extended cooperation by allowing us to collect samples from their animals, and farm and animal related information.

It is pertinent to mention and thank the Higher Education Commission of Pakistan (HEC) and German Academic Exchange Service (DAAD) for awarding me the PhD fellowship to accomplish doctoral thesis in Germany.

Khushal Khan Kasi

12. Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen Anspruch genommen habe.

Berlin, 16.03.2020 Khushal Khan Kasi

mbvberlin mensch und buch verlag

49,90 Euro | ISBN: 978-3-96729-043-1