IMMUNE RESPONSES TO CRIMEAN-CONGO HAEMORRHAGIC FEVER VIRUS AND MOLECULAR CHARACTERIZATION OF VIRAL ISOLATES

by

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DECLARATION

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Dominique Goedhals Date

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LIST OF ABBREVIATIONS

ABTS	azino di-ethyl-benzothiazoline-sulfonic acid
BLAST	Basic Local Alignment Search Tool
BSL	biosafety level
CCHF	Crimean-Congo haemorrhagic fever
CCHFV	Crimean-Congo haemorrhagic fever virus
CFR	case fatality rate
CHF	Crimean haemorrhagic fever
cRNA	complementary ribonucleic acid
DIC	disseminated intravascular coagulopathy
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
HIV	human immunodeficiency virus
HRPO	horse-radish peroxidase
IEDB	Immune Epitope Database
IFN-α	interferon alpha
IFN-γ	interferon gamma
IHRQL	impaired health related quality of life
ISG15	interferon-stimulated gene product 15
L	large
M	medium
MEGA	Molecular Evolutionary Genetics Analysis
MHC	major histocompatibility complex

NCR	non-coding region
NGS	next generation sequencing
NICD	National Institute for Communicable Diseases
NK cells	natural killer cells
OD	optical density
ORF	open reading frame
OTU	ovarian tumour
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PTSD	post-traumatic stress disorder
RdRp	RNA-dependent RNA-polymerase
RNA	ribonucleic acid
RT-LAMP	reverse transcription loop-mediated isothermal amplification
RT-PCR	reverse transcription polymerase chain reaction
RVF	Rift Valley fever
S	small
SFC/10^6	spot forming cells per million
STAT-1	signal transducer and activator of transcription-1
TNF-α	tumour necrosis factor alpha
vRNA	viral RNA

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SUMMARY

Crimean-Congo haemorrhagic fever virus (CCHFV) is a tick-borne virus belonging to the family *Bunyaviridae*, genus *Nairovirus*. The distribution of the virus correlates with that of the principal vector, ticks belonging to the genus *Hyalomma*. This includes areas in Africa, Asia, Eastern Europe and the Middle East, with recent emergence in Turkey, Greece and India. CCHFV is associated with haemorrhagic fever in humans, with a case fatality rate of up to 30%. Current patient management relies on supportive therapy and administration of ribavirin, but the efficacy of this antiviral drug is controversial. Although an inactivated vaccine has been used in Eastern Europe and the former Soviet Union, it has not been accepted for widespread use. An understanding of immune correlates is therefore needed to guide further development of therapeutic and preventative interventions. This study aimed to investigate immune responses in survivors of CCHF in South Africa, focusing on the presence of detectable memory T lymphocyte responses and the identification of epitopic regions within the nucleoprotein and glycoproteins. In order to ensure applicability of identified epitopes to geographically distinct isolates, viral sequence diversity was also investigated by means of next generation sequencing and phylogenetic studies.

A synthetic overlapping peptide library was used to screen for interferon gamma production by peripheral blood mononuclear cells from survivors of CCHFV infection in ELISPOT assays. Ten potential epitopic regions were identified, the majority of which were located on the nucleoprotein with only two regions identified on the glycoprotein G_C in a single patient. Long-lived memory CD8+ T cell responses were detected in survivors of CCHF up to 13 years after infection. These findings indicate the presence of effective long term cellular immune responses which could be modulated through vaccination and gives an indication of epitopic

regions that should be considered in candidate vaccines and testing vaccine immunogenicity. The presence of detectable memory responses in the absence of re-exposure or chronic infection will allow future studies to fully characterize T cell responses in survivors.

With an expanding area of CCHFV endemicity, safe, sensitive and specific serological assays are required for diagnostic and serosurveillance purposes. As the biosafety level 4 facilities required to culture the virus are lacking in many endemic areas, alternative means of producing reagents for diagnostic assays are needed which will not pose a safety risk to laboratory workers. The use of synthetic peptides in serological assays is one such alternative approach. In addition, identification of immunodominant epitopic regions may have application in vaccine development if they induce protective immunity. The peptide library was used to screen for antibodies recognizing human defined linear B cell epitopic regions in survivors of CCHFV infection by means of an enzyme-linked immunosorbent assay (ELISA). Two potential epitopic regions were identified on the G_C glycoprotein with reactivity in 13 – 14 of 15 patients tested. Further investigation will be required to determine whether these epitopic regions also correlate with immune protection and to identify non-contiguous B cell epitopes which are likely to play an important role in antibody induction during natural infection with CCHFV.

With new foci of CCHFV infections emerging in recent years, it is important to ascertain whether genomic variation will influence applicability of vaccine candidates and diagnostic assays in distinct geographic areas. Next generation sequencing techniques were used to obtain complete genome sequences for ten southern African CCHFV isolates. This is the first application of next generation sequencing technology to CCHFV isolates and proved to be a

rapid and cost effective alternative to standard Sanger sequencing which can be effectively applied to the approximately 20kb CCHFV genome. The phylogenetic results confirmed that although there is extensive variability among geographically distinct CCHFV isolates at a genomic level, conserved areas are present which could be targeted for vaccine development and diagnostic purposes. The genetic variability seen results from point mutations and segment reassortment, which was shown to occur commonly in southern African CCHFV isolates. Despite the extensive variation in primary sequence, at a protein level, the motifs involved in protein function are well conserved. Prediction software analysis confirmed the presence of conserved OTU-like cysteine protease and RNA dependent RNA polymerase (RdRp) domains in the L segment of diverse southern African CCHV isolates. The RdRp is essential for viral replication while the OTU-like protease likely plays a role in immune evasion and therefore affects viral pathogenicity. Analysis of the M segment showed conservation of the basic protein coding strategy, with two structural and three non-structural glycoproteins. However, amino acid variation was notable across all predicted proteins but particularly in the variable mucin-like domain which is thought to play a role in viral pathogenicity. This study identifies targets for further investigation of viral pathogenicity which may include *in vivo* studies in animal models and mutagenicity assays.

OPSOMMING

Krimeaanse-Kongo hemorragiese koors virus (KKHKV) is ‘n bosluisoorgedraagde virus wat tot die familie *Bunyaviridae* en genus *Nairovirus* behoort. Daar is ‘n positiewe korrelasie tussen die verspreiding van die virus en die voorkoms van die hoof vektor wat bosluise in die genus *Hyalomma* is. Endemiese areas sluit areas in Afrika, Asië, Oos-Europa en die Midde-Ooste in met die onlangse verskyning in Turkye, Griekeland en Indië. KKHKV word geassosieer met hemorragiese koors in mense met ‘n sterftekoers van tot 30%. Huidiglik bestaan die hantering van pasiënte uit ondersteuningsterapie en die toediening van ribavirin, maar die effektiwiteit van hierdie middel is nog kontroversieel. Daar is ‘n geïnaktiveerde entstof wat in Oos-Europa en in die voormalige Sowjet-Unie gebruik word, maar nog nie aanvaar is vir wydverspreide gebruik nie. Begrip van die immuun korrelate word benodig vir die verdere ontwikkeling van terapeutiese en voorkomende intervensies. Die doelstelling van hierdie studie was om die immuunrespons in KKHKV oorlewendes te ondersoek in Suid-Afrika met die fokus op die teenwoordigheid van ‘n opspoorbare geheue T-limfosiet reaksie en die identifikasie van epitopiese areas in die nukleoproteïen en glikoproteïene. Ten einde die toepaslikheid van die geïdentifiseerde epitope in geografies geskeide isolate te verseker was virale volgorde diversiteit ook ondersoek deur volgende generasie volgordebepaling en filogenetiese studies.

‘n Sintetiese oorvleuelende peptied versameling was gebruik om die periferale bloed mononukleêre selle van KKHKV te ondersoek vir die produksie van interferon gamma in ELISPOT toetse. Sodoende was tien potensiële epitopiese areas geïdentifiseer, waarvan die meerderheid op die nukleoproteïen geleë was. In ‘n enkele pasiënt was twee ander areas geïdentifiseer op die glikoproteïen G_C. ‘n Langdurige geheue CD8+ T-sel reaksie in KKHK

oorlewendes van tot 13 jaar na infeksie was geïdentifiseer. Die bevindinge dui op die teenwoordigheid van ‘n effektiewe langdurige sellulêre immuunrepons wat deur inenting gemoduleer kan word en bied ‘n goeie aanduiding vir die epitopiese areas wat oorweeg moet word vir kandidaat-entstowwe en toetsing vir entstof immunogenisiteit. Die teenwoordigheid van ‘n opspoorbare geheue reaksie in die afwesigheid van herblootstelling of chroniese infeksie sal toekomstige studies instaat stel om die volledige T-sel reaksie van oorlewendes te karakteriseer.

As gevolg van die groeiende area van KKHKV endemisiteit word veilige, sensitiewe en spesifieke serologiese toetse benodig vir diagnostiese en serum-waarnemende doeleindes. Aangesien die bioveiligheids vlak 4 fasiliteite wat benodig word vir die kweeking van die virus in baie endemiese areas ontbreek, moet daar alternatiewe maniere vir die voorbereiding van reagense vir diagnostiese toets gevind word, wat nie ‘n veiligheidsrisiko vir die laboratorium werkers inhou nie. Een van die alternatiewe is die gebruik van sintetiese peptiede in serologiese toetse. Daarbenewens kan die identifikasie van immunodominante epitopiese gebiede, indien ‘n beskermende immuunrespons ontlok word, van toepassing wees vir entstof ontwikkeling. Teenliggame gerig teen menslike gedefinieerde linieêre B-sel epitopiese gebiede was opgespoor deur die gebruik van ‘n peptied versameling in ‘n ensiem-gebonde immunosorbent toets. Twee potensiële epitopiese gebiede was geïdentifiseer op die G_C glikoproteïen met reaktiwiteit in 13 - 14 van die 15 getoetste pasiënte. Verdere ondersoek sal benodig word om te bepaal of die epitopiese gebiede ook korreleer met ‘n beskermende immuunrespons, asook vir die identifikasie van nie-aangrensende B-sel epitope wat moontlik ‘n belangrike rol in teenliggaam induksie in ‘n natuurlike infeksie met KKHKV speel.

Met die opkoms van nuwe foci van KKHKV infeksie meer onlangs is dit belangrik om te bepaal of die genomiese variasie die toepaslikheid van entstof kandidate en diagnostiese toetse in geografies geskeide areas sal beïnvloed. Volgende generasie volgordebepalings tegnieke was gebruik om die volledige genoom volgordes te bepaal vir tien suider-Afrikaanse KKHKV isolate. Hierdie is die eerste toepassing van volgende generasie volgordebepaling tegnologie vir KKHKV isolate en het bewys dat dit 'n vinnige en koste-effektiewe alternatief tot standard Sanger volgordebepaling is en wat effektief was vir die ongeveerd 20kb KKHKV genoom. Die filogenetiese resultate het bevestig dat daar groot variasie tussen geografies geskeie KKHKV isolate op 'n genomiese vlak is en dat gekonserveerde gebiede teenwoordig is en geteiken kan word vir entstof ontwikkeling en diagnostiese doeleindes. Die genetiese variasie was as gevolg van puntmutasies en segment herrangskikking wat algemeen in die suider-Afrikaanse KKHKV isolate voorkom. Ten spyte van die groot variasie in die primêre volgorde, by proteïen vlak, is die motiewe wat 'n rol in die funksie van proteïene speel gekonserveer. Voorspellings sagteware analise het die teenwoordigheid van OTU-agtige sisteïen protease en RNA-afhanklike-RNA-polimerase (RaRp) domeine in die L segment in die diverse suider-Afrikaanse KKHKV isolate bevestig. RaRp is noodsaaklik vir virale replikasie, terwyl die OTU-agtige protease waarskynlik 'n rol speel in immuunstelsel ontduiking en affekteer dus virale patogenese. Ontleding van die M segment dui die behoud van die basiese proteïen koderings strategie met twee strukturele en drie nie-stukturele glikoproteïene aan. Die aminosuur variasie was waarneembaar oor al die voorspelde proteïene, maar veral in die veranderlike mucin-agtige domein wat moontlik 'n rol in virale patogenisiteit speel. Hierdie study bied teikens vir verdere ondersoek in virale patogenisiteit wat *in vivo* studies in diere en mutageniese toetse kan insluit.

CHAPTER 1

Orientation, rationale and aims of the study

Introduction

Crimean-Congo haemorrhagic fever virus (CCHFV) is a tick-borne virus belonging to the family *Bunyaviridae* and genus *Nairovirus*. It occurs widely in Africa, Eastern Europe, Asia and the Balkans, and the geographic distribution correlates with the distribution range of ticks of the genus *Hyalomma*. Descriptions of a disease likely to be Crimean-Congo haemorrhagic fever (CCHF) date back to the twelfth century, however reports in which the disease was first given the name Crimean haemorrhagic fever (CHF) occurred in peasants harvesting crops on the Crimean peninsula in 1944. The causative agent was isolated in 1967 when suckling mice were used as laboratory hosts. In 1969, CHF was shown to be identical to Congo haemorrhagic fever isolated from a febrile child in the Belgian Congo (now Democratic Republic of the Congo) and the names were combined. The virus is now referred to as Crimean-Congo haemorrhagic fever virus.

Initial observations suggested that the African strain was less pathogenic than the Asian strain. However this assumption was based on a limited numbers of cases. Prior to 1979 the virus had only been identified in Africa in countries to the north of, and including Tanzania, and whereas fatality rates up to 40% had been noted in eastern Europe and Asia from numerous outbreaks and cases, only 1 fatality out of 15 cases had been reported in Africa. In February 1981, the first case of CCHF was confirmed in South Africa leading to debate regarding the recent introduction of the virus or previous existence of the virus that had been undetected. Serological surveys of stored livestock sera from South Africa, Zimbabwe

and Namibia were instrumental in establishing that the virus had pre-existed in the country and that it was present throughout the country with higher frequency in areas where the species of *Hyalomma* tick were present. Increased awareness of the virus and the establishment of a diagnostic facility for laboratory confirmation of CCHFV infections facilitated further recognition of the incidence of cases and it was established that on average there are about 1-20 cases occurring annually. Current figures indicate a fatality rate of approximately 30% and it is widely accepted that African strains are no less pathogenic than Asian. There is now debate regarding the presence of less pathogenic strains from Greece and Turkey, especially with regard to the Greek strain initially isolated from a tick in 1982, strain AP92. Further studies are required to confirm or refute the existence of less pathogenic strains.

Problem identification

Transmission of the virus to humans occurs through tick-bites, crushing of ticks with bare hands, contact with blood or tissues of infected animals, and contact with blood or tissues of infected patients. Infection in humans is characterized by fever, headache, myalgia, rash and often a haemorrhagic state, and as mentioned above, with a case fatality rate of approximately 30%. No vaccine is available and treatment consists of supportive therapy and administration of the antiviral drug ribavirin although the efficacy of antiviral treatment is still controversial. CCHFV has a single-stranded, segmented RNA genome. There is limited information available regarding immune responses in patients infected with CCHFV however cellular responses to date have not been elucidated. These studies are hindered by the

requirement of a biosafety level (BSL) 4 containment laboratory and until recently, the lack of an animal model.

It has not yet been determined what facilitates clearance of the virus. The kinetics of antibody responses have been determined and despite appearance of a detectable humoral antibody response, IgG and IgM, the antibody response does not always correlate with clearance of the virus. It has been shown that IgM antibody has no significant influence on outcome or decrease in viral load. Similarly although IgG antibody levels are inversely related to viral load, the viral titers in survivors appear to decrease independent of detectable antibodies. The observations indicate a role for innate or cellular immune responses in viral clearance. Investigation of cellular responses during the acute phase of illness will necessitate use of BSL 4 containment due to the biohazardous nature of the virus and investigation of T cell responses in survivors will be dependent on a detectable cellular immune response. As the frequencies of T cells in blood recognizing specific viral proteins or antigens is low, and has been noted to be less than 1:10 000 in peripheral blood mononuclear cells, the techniques that measure T cell immunity need to be highly sensitive. In addition there is debate regarding the ability to detect T cell responses against acute infections that are not boosted by re-exposure or persistence of the virus. Patients that have CCHF infections are seldom likely to be re-exposed to the virus and the virus does not persist. Hence it was deemed necessary to determine if survivors have a T cell memory response that could be detected irrespective of the time after illness. The subsequent identification of specific CCHFV T cell epitopes could therefore aid the development of effective protective or therapeutic vaccines by providing information as to which part of the viral genome should be targeted.

No effective vaccine is available at present. A formalin-inactivated, suckling mouse brain vaccine was used in parts of Eastern Europe and the former Soviet Union but has not been accepted for widespread use. More recently, a DNA vaccine which expresses the glycoproteins G_C and G_N was developed and was shown to elicit neutralizing antibodies in mice. A number of challenges exist in the development of an effective vaccine including the limited knowledge regarding what comprises a protective immune response.

The immune correlates of protection against CCHF are unknown and studies to date are limited probably due to the biohazardous nature of the virus and dependence on current infections that occur sporadically and unpredictably. Hence, we have selected to begin to determine the role of T lymphocytes in infection by investigating the memory T cell responses of survivors and to use the memory response to identify epitopic regions and to determine whether the T cell epitopes reside predominantly within the CCHFV nucleoprotein or glycoproteins. Current treatment of the disease consists of supportive and replacement therapy with blood products. Vaccines that promote cellular immunity may have a role in protection and/or treatment. The disease remains a significant public health concern hence it is important to investigate immune correlates that could contribute to development of novel candidate vaccines. In addition, the identification of human defined immunodominant epitopic regions that induce detectable antibody responses could play a role in development of safe recombinant reagents for diagnosis and for use as tools for surveillance studies. The recent emergence of CCHFV in previously non-endemic regions in Eastern Europe and the presence of the vector of this virus in Southern Europe emphasizes the need for improved diagnostic capacity and increased surveillance capacity. Currently diagnostic reagents are dependent on culturing the virus within BSL4 facilities which limit

the number of laboratories that can prepare their own reagents. In addition standardization and validation of reagents will play an important role in commercialization of assays. Identifying immunodominant epitopes may have application in development of recombinant or alternative reagents.

Antigenic and serological cross reactivity exists between geographically distinct isolates and to date there is no indication that there are antigenically distinct strains. However genetically distinct isolates do circulate within geographically distinct regions and within similar regions. Hence the development of vaccines and both serological and molecular tools for detection that are based on subunits, specific epitopic regions or targeting of specific regions of the genome respectively must take into consideration genetic diversity. To date only 31 complete genome sequences were available on GenBank for geographically distinct isolates of CCHFV. It was deemed necessary to determine additional sequence data for southern African isolates to determine if epitopic regions were conserved, to confirm reassortment events and to identify and determine if protein motifs were conserved with particular respect to southern African isolates.

Aims and objectives

The aims of the study were to :

1. identify cellular and humoral immune responses in survivors of CCHFV infection
2. investigate sequence diversity among geographically distinct CCHFV isolates
3. characterize sequence data for conservation of epitopic regions and protein motifs.

Specific objectives:

1. To ascertain if there were long lived memory T cell responses that can be used to further study the role of T lymphocytes in CCHFV infection. To identify if there were T cell epitopic regions on the nucleoprotein and/or structural glycoproteins of CCHFV by means of interferon gamma ELISPOT assays using a library of overlapping peptides and peripheral blood mononuclear cells collected from survivors of CCHFV infection.

2. To identify human defined linear B cell epitopes by means of enzyme linked immunosorbent assay (ELISA) screening of the overlapping peptide library.

3. To determine sequence diversity among CCHFV isolates with an emphasis on southern African isolates, and evaluate the effect of genome reassortment on sequence diversity and viral pathogenicity.

4. To identify conserved motifs at the amino acid level to determine targets for further studies into protein function and disease pathogenesis.

In recent years, CCHFV has emerged as an important tick borne pathogen with a growing number of cases and endemicity expanding into Europe and Asia. Current management relies chiefly on supportive therapy and an effective vaccine is lacking. Due to the significant public health concern and high mortality associated with CCHFV, the identification of immune correlates which may provide targets for novel vaccine candidates or therapeutic interventions are required. To ensure that diagnostic assays and therapeutic or preventative interventions will be broadly applicable against CCHFV isolates with diverse geographic origins, availability of complete genome sequence data will have application.

Structure of the thesis

This thesis is presented as a series of research articles which will be submitted for publication in various scientific journals. The first article, presented in Chapter 2, is a review article. As there are a plethora of review articles on CCHFV that have been published recently in the international literature, this article will be submitted to a South African journal to increase awareness of the disease locally and provide an update on current knowledge of the virus. The next two articles, presented as Chapters 3 and 4, investigate immune responses in survivors of CCHFV infection. Chapter 3 specifically investigates long-lived memory T cell responses in a cohort of South African CCHFV survivors and aims to identify if human defined T cell epitopes are present on the nucleoprotein or glycoproteins, while Chapter 4 focuses on the identification of human defined linear B cell epitopic regions using a peptide based ELISA. The next two chapters investigate sequence diversity among CCHFV isolates. In Chapter 5, next generation sequencing techniques are used to sequence the complete genomes of ten southern African CCHFV isolates. These are analysed along with available complete sequence data from the GenBank database. This manuscript has been submitted to Epidemiology and Infection and has been provisionally accepted pending minor revisions. In Chapter 6, the sequences are analysed at amino acid level using predictive software to identify conserved protein domains and potential protein functions. Finally, in Chapter 7, the overall conclusions of the study and future perspectives for further research are provided.

CHAPTER 2

Crimean-Congo haemorrhagic fever virus: review of a virus endemic in South Africa

Historical background

The clinical features of Crimean haemorrhagic fever (CHF) were first described following an outbreak in Crimea in 1944 (Hoogstraal, 1979). During this outbreak approximately 200 cases of the disease were reported in peasants involved in harvesting activities. The aetiological agent of CHF was shown to be a virus following experimental inoculation of psychiatric patients undergoing pyrogenic therapy with filtered blood collected from acutely ill CHF patients. The virus was also detected in *Hyalomma* ticks which were collected in the area and which were later shown to be the principal vector responsible for virus transmission (Chumakov, 1965; Chumakov *et al.*, 1968a). The virus was first propagated in the laboratory in 1967, following intracerebral inoculation of newborn white mice with the blood of CHF patients (Chumakov *et al.*, 1968a).

CHF was subsequently shown to be antigenically indistinguishable from isolates of Congo virus which had been recovered from patients in the Congo and Uganda with a similar clinical picture (Chumakov *et al*., 1968b). Casals *et al*. (1970) therefore suggested the name CHF-Congo virus, but finally the name Crimean-Congo haemorrhagic fever virus (CCHFV) was adopted (Hoogstraal, 1979).

The virus

CCHFV belongs to the family *Bunyaviridae*, genus *Nairovirus* (Calisher, 1991; Casals, 1969; Karabatsos, 1985). This family includes four other genera, namely *Orthobunyavirus, Hantavirus, Phlebovirus,* and *Tospovirus*. Each of these, with the exception of the *Tospovirus* genus, includes viruses of medical importance in humans. The *Nairovirus* genus is comprised of at least 34 viruses that have been grouped into seven serogroups (also referred to as species, http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/fs_index.htm). The serogroups were initially defined according to serological cross reactivity using complement fixation, haemagglutination, neutralization, immunoprecipitation and immunofluorescence techniques (Calisher and Karabatsos, 1989; Casals and Tignor, 1980). CCHFV belongs to the serogroup of the same name. Other members of the CCHFV serogroup include Hazara virus, the closest known antigenic relative, and Khasan virus (Nichol *et al*., 2006). CCHFV is the only member of the group known to be medically significant. Hazara virus was isolated from *Ixodes redikorzevi* ticks in Pakistan and Khasan virus was isolated from *Haemaphysalis longicornis* ticks from the former USSR (Begum, 1970; Smirnova, 1979).

CCHF virions are spherical and approximately 100 nm in diameter. The host cell derived lipid bilayered envelope contains surface projections of 5-10 nm in length which consist of the viral glycoproteins G_C and G_N. The virions contain three structural proteins, namely the glycoproteins (G_C and G_N) and a nucleocapsid protein, and one non-structural protein which is the viral RNA-dependent RNA-polymerase (Schmaljohn and Hooper, 2001; Whitehouse, 2004). The negative sense, single-stranded RNA genome consists of three segments designated large (L), medium (M) and small (S). The highly conserved complementary terminal nucleotide sequences of each segment result in loosely circular RNAs which

together with the nucleocapsid protein, make up the three helical nucleocapsids. These base-paired ends likely act as a functional promoter region which interacts with the viral RNA-dependent RNA-polymerase (RdRp) (Flick *et al.*, 2002). The L, M and S segments encode the viral RdRp, the viral glycoproteins and the viral nucleocapsid protein respectively (Schmaljohn and Hooper, 2001).

The L segment is approximately 12 000 bases in length and has a single open reading frame which contains two conserved protein motifs, namely the OTU cysteine protease-like domain and the RdRp domain (Duh *et al.*, 2008; Honig *et al.*, 2004; Kinsella *et al.*, 2004; Ozdarendeli *et al.*, 2010; Yadav *et al.*, 2013). The OTU cysteine protease domain facilitates immune evasion by hydrolysing ubiquitin and interferon-stimulated gene 15 (ISG15) (Frias-Staheli *et al.*, 2007), while the RdRp is responsible for mRNA synthesis and genome replication (Honig *et al.*, 2004). The M segment is approximately 5 400 bases in length and encodes a single precursor polypeptide including two structural glycoproteins, G_N and G_C, and a number of non-structural glycoproteins, namely the amino-terminal mucin-like domain, GP38, NS_M, GP85 and GP160 (Altamura *et al.*, 2007; Sanchez *et al.*, 2006). Only the structural glycoproteins have been identified as virion components, while the remaining CCHFV glycoproteins are likely soluble proteins (Sanchez *et al.*, 2006). The function of these non-structural glycoproteins is unclear although, by analogy with Ebola virus, the mucin-like variable domain may play an important role in viral pathogenesis (Yang *et al.*, 2000). G_N and G_C are responsible for attachment to cell surface receptors which allows entry into the host cell cytoplasm by endocytosis, where replication takes place. The viral envelope is acquired by budding through the endoplasmic reticulum into the Golgi cisternae. The virions are then transported in vesicles to the cell membrane and released by fusion of the vesicles and

plasma membranes (Schmaljohn and Hooper, 2001; Whitehouse, 2004). The S segment is approximately 1 600 bases in length and encodes the viral nucleoprotein which binds the viral RNA to form ribonucleoprotein complexes (Schmaljohn and Hooper, 2001).

Epidemiology and geographic distribution

CCHFV has been documented in more than 30 countries of Africa, Asia, Europe and the Middle East, with a distribution following that of its vectors, ticks belonging to the genus *Hyalomma* (Flick and Whitehouse, 2005). Cases of naturally acquired human infection have been documented in the former Soviet Union, China, Bulgaria, Yugoslavia, Albania, Kosovo, Greece, Pakistan, Iran, Iraq, United Arab Emirates, Saudi Arabia, Oman, Tanzania, Central African Republic, Democratic Republic of Congo, Uganda, Kenya, Mauritania, Burkina Faso, South Africa and Namibia (Al Tikriti *et al.*, 1981; Burney *et al.*, 1980; Dunster *et al.*, 2002; El Azazy and Scrimgeour, 1997; Gear *et al.,* 1982; Hassanein *et al.*, 1997; Hoogstraal, 1979; Msimang *et al.*, 2013; NICD Comm Dis Surveill Bull, 2011; Papa *et al*., 2002; Papa *et al.*, 2008; Saluzzo *et al.*, 1984; Saluzzo *et al.*, 1985; Schwarz *et al.*, 1995; Suleiman *et al.*, 1980; Tantawi *et al.*, 1980; Watts *et al.*, 1989). Virus has also been isolated from ticks or non-human mammals in Madagascar, Senegal, Nigeria, Central African Republic, Ethiopia, Afghanistan, Hungary, Morocco and Spain (Estrada-Pena et al., 2012; Németh et al., 2013; Palomar *et al.*, 2013; Watts et al., 1989). Serological evidence has been reported from Zimbabwe and Benin although no clinical cases have been documented, and limited serological observations have been reported from Portugal, France, Egypt and Kuwait (Watts et al., 1989).

The recent emergence of CCHFV in Turkey, with more than 7000 human cases reported since 2002, has highlighted the importance of active surveillance and vigilance for cases of CCHF even in areas not previously considered to be endemic for the virus (Maltezou *et al.*, 2010). Other areas of recent emergence include human cases in Greece and India (Mishra *et al.*, 2011; Papa *et al.*, 2008), serological evidence of CCHFV in sheep in Romania (Ceianu *et al.*, 2012), and detection of CCHFV in *Hyalomma* ticks in Spain (Estrada-Peña *et al.*, 2012).

CCHFV is transmitted to humans by tick bite or squashing of infected ticks with bare fingers, or by contact with blood or tissues of infected animals or humans. High risk occupations therefore include those with frequent contact with livestock, particularly those performing procedures such as castration or slaughtering such as farmers, farm workers, abattoir workers, and veterinarians (Hoogstraal, 1979; Swanepoel *et al.*, 1998). Nosocomial transmission has also been documented in a number of countries, including Turkey, Albania, Iran, United Arab Emirates, Pakistan, Sudan and South Africa, resulting in infection of health care workers and patients exposed to cases of CCHF (Elata *et al.*, 2011; Gürbüz *et al.*, 2009; Harxhi *et al.*, 2005; Hasan *et al.*, 2013; Naderi *et al.*, 2013; Van Eeden *et al*., 1985).

The virus has been isolated from numerous ixodid tick species, as well as two argasid species (Camicas *et al.*, 1991; Hoogstraal, 1979; Swanepoel *et al.*, 1983; Watts *et al.*, 1989; Zeller *et al.*, 1994). For the majority of these species, evidence that they could serve as vectors is lacking and it seems likely that the virus detected was present in a blood meal taken from a viraemic host. However, ixodid ticks of the genus *Hyalomma* appear to be competent vectors (Turrel, 2007). In addition, the distribution of CCHFV cases correlates with that of the distribution of ticks belonging to the genus *Hyalomma*. *Hyalomma* ticks are two-host ticks, with immature forms (larvae and nymphs) feeding on birds and small mammals, and

adult ticks feeding on large wild and domestic animals and sometimes large birds. Humans are incidental hosts and do not play a role in the maintenance of the virus in nature (Hoogstraal, 1979).

Transovarial transmission of CCHFV from infected female ticks to their progeny has been reported (Wilson, 1991), as well as transstadial transmission of the virus from tick larva to nymph to adult (Shepherd *et al*., 1989; Shepherd *et al*., 1991). Ticks have also been shown to become infected when co-feeding with infected ticks on a vertebrate host, even in the absence of a detectable viraemia in the vertebrate host (Gordon *et al*., 1993). Venereal transmission from infected male to uninfected female ticks also occurs (Gonzalez *et al*., 1992).

Viraemic infection of various small mammals has been documented, including hedgehogs, hares, susliks and rodents such as ground squirrels, veld rats and gerbils (Hoogstraal, 1979; Shepherd *et al*., 1989). Small mammals have previously been identified as reservoir hosts for CCHFV. Serological evidence of CCHFV infection has been detected in a wide range of larger wild animals including buffalo and various antelope species. Large domestic animals including cattle, horses, donkeys, sheep, goats and pigs have also been implicated as being capable of transmitting the virus (Hoogstraal, 1979; Shepherd *et al*., 1987). Although these mammals have detectable viraemias and show seroconversion following infection with CCHFV, they do not develop clinical disease. To serve as reservoirs or amplifying hosts of CCHFV, viremia must reach a threshold level that allows transmission of the virus, hence the exact role of many large mammals in the natural cycle remains undetermined.

Most birds have been shown to be refractory to CCHFV infection with no detectable viraemia and an absence of or short-lived seroconversion following exposure to the virus.

Ostriches appear to be the exception and have been implicated in more than one outbreak of CCHF in abattoir workers. This was supported by the demonstration of high-level viraemia and antibody responses in ostriches following experimental infection (Shepherd *et al*., 1987; Swanepoel *et al*., 1998). Although most birds do not act as amplifying hosts, ticks may become infected by cofeeding or venereal transmission while feeding on birds, and non-viremic transmission has been reported (Jones et al., 1987). In addition, migratory birds transport infected larval and nymphal ticks over large distances (Berezin *et al*., 1971; Zeller, 1994).

Genetic diversity

Genetic diversity in arboviruses with RNA genomes is a complex interaction between the need to maintain fitness in both vertebrate and arthropod hosts which favours genome conservation, and the RdRp which lack proof-reading mechanisms and therefore show high error frequencies during replication thus favouring genetic diversity (Coffey *et al.*, 2008; Steinhauer *et al.*, 1992). However, CCHFV shows a surprisingly high level of genetic diversity, especially in the M segment with nucleotide variation of 20%, 31% and 22%, for the S, M and L segments and amino acid variation of 8%, 27% and 10% for the respective proteins (Deyde *et al*., 2006). Phylogenetic analysis of complete genomes has identified groupings of CCHFV isolates into seven lineages which reflect the geographic origin of the viral isolates, namely group I (West Africa), group II (Democratic Republic of Congo), group III (South Africa and West Africa), group IV (Asia and the Middle East), group V (Europe and Turkey), group VI (Greece), and group VII (Mauritania). More recent isolates from China do not cluster within the existing groups (Zhou *et al.*, 2013).

Segment reassortment can occur when dual infection of a cell takes place. This phenomenon has been described for each of the CCHFV segments but appears to be more common for the M segment. The reason for the more frequent reassortment of the M segment is not clear (Burt *et al.*, 2009; Deyde *et al.*, 2006; Hewson *et al*., 2004; Morikawa *et al*., 2007). Segment reassortment between African and Asian isolates indicates that the virus circulates between the two continents, probably dispersed by movement of ticks on migrant birds and/or livestock trade. Evidence of recombination has also been found in the S RNA segment (Deyde *et al.*, 2006; Lukashev, 2005). The exchange of genetic material by reassortment or recombination is thought to occur within the tick vectors due to the longer duration of infection and potential exposure to multiple infected vertebrate hosts (Deyde *et al.*, 2006; Hewson *et al.*, 2004; Morikawa *et al*., 2007).

Molecular epidemiology studies have shown that genetically related isolates have been identified circulating within the same region as well as in geographically distinct regions and genetically distinct isolates have also been shown to circulate within the same geographic area. These studies support the movement of CCHFV within and between continents. It is likely that genetic diversity within regions has resulted from movement and trade in livestock and bird migration with consequent movement of infected ticks and introduction of genetically distinct isolates. Generation of genetic diversity could also be a consequence of reassortment and recombination events.

CCHFV in South Africa

The first case of CCHF was recognized in South Africa in 1981, when a 13 year old boy contracted the disease following a tick bite while attending "veldschool" in Bloemhof. This prompted a number of serological surveys which showed that the virus was widely distributed in South Africa and had been present for many years before the first clinical case was recognized (Swanepoel *et al*., 1983; Swanepoel *et al*., 1985). Since then, 194 cases of CCHFV have been recorded in South Africa, with a case fatality rate of 24% and more than half of the cases occurring in the Free State and Northern Cape provinces. The vast majority of infections have occurred in males (91%), particularly farmers and other agricultural workers, with patients reporting either tick bites or exposure to infected animals as likely routes of transmission (personal correspondence, Prof JT Paweska). Three species of *Hyalomma* ticks are found in South Africa, namely *H. marginatum rufipes, H. glabrum,* and *H. truncatum* and act as the local vectors. Immature ticks feed largely on hares and ground feeding birds, with adult ticks feeding on a variety of larger wild and domestic herbivores (Shepherd *et al*., 1987). These ticks are found most commonly in the central and western areas of South Africa and are rare in the eastern and southern coastal areas, corresponding to the occurrence of CCHFV cases in humans. Nosocomial transmission has been reported in an outbreak at Tygerberg Hospital in the Western Cape, involving an index case followed by seven secondary cases of CCHF (van Eeden *et al*., 1985). The infection was fatal in two of these patients.

Clinical features

The incubation period following a tick bite is approximately 1-3 days, while it is slightly longer following exposure to infected blood or tissues of animals or patients at

approximately 5-6 days (Swanepoel *et al.*, 1987; Whitehouse, 2004). The onset of symptoms is often sudden with non-specific symptoms including fever, rigors, chills, headache, sore throat, dizziness, malaise and myalgia. In addition, backache, nausea and vomiting, abdominal pain, neck stiffness and photophobia may occur early in the illness. Fever is a common feature, but may be intermittent. During the early stages of illness, lassitude, depression and somnolence may be apparent as well as neuropsychiatric changes such as confusion and aggression. Hyperaemia of the face, neck and chest, injected conjunctivae and chemosis may also be present. By day 3-6 of illness, a petechial rash may be seen and is often followed by larger ecchymoses and bruising (Hoogstraal, 1979; Swanepoel *et al.*, 1987). Less commonly, a macular or maculopapular rash may be present (Akyol *et al*., 2010; Ergonul *et al.*, 2004). When present, the onset of haemorrhagic manifestations occurs on day 4 to 5. The severity of the bleeding tendency varies from leakage or oozing of blood from injection or venipuncture sites to epistaxis, haematemesis, haematuria, melaena, gingival bleeding, and bleeding from other orifices. Hepatomegaly and right hypochondrial pain may be present early in the course of infection and may progress to jaundice during the second week of illness (Hoogstraal, 1979; Swanepoel *et al.*, 1987). Ocular findings are usually related to the haemorrhagic state with subconjunctival and retinal haemorrhages occurring (Engin *et al.*, 2009). Pulmonary parenchymal haemorrhage presenting with haemoptysis, chest pain and dyspnoea has been described (Dogan *et al.*, 2011). Infrequent presentations during the acute stage of illness include epididymo-orchitis, parotitis, peritoneal and pleural effusions, acalculous cholecystitis and intraabdominal abscesses (Aksoy *et al.*, 2010; Guner *et al.,* 2011; Kaya *et al.*, 2012; Şensoy *et al.*, 2011; Tanir et al., 2009). Cardiac involvement in the form of depressed cardiac function and pericardial effusion have also been documented (Engin *et al.*, 2009).

Fatalities occur mostly from days 5 to 14 of illness due to multi-organ failure or haemorrhagic complications such as intracranial haemorrhage. The reported fatality rate varies from 5-50% and is higher in nosocomial outbreaks than in sporadic cases (Gozalan *et al.*, 2007; Hoogstraal, 1979; Swanepoel *et al.*, 1989). Case fatality rates (CFR) vary significantly between distinct geographic regions, with an average fatality rate of 24% in South Africa and up to 5% in Turkey (Ergonul *et al.*, 2006a; Swanepoel *et al.*, 1987). The considerable differences in CFR have not yet been definitively linked to differences in CCHFV strain pathogenicity, but serosurveys in Turkey and Greece have shown that a large proportion of infections in these areas appear to be subclinical. In Greece, only a single case of CCHFV infection has been reported (Papa *et al.*, 2008) but serosurveys revealed approximately 4% seroprevalence of antibodies to CCHFV antibodies (Papa *et al.*, 2011; Papa *et al.*, 2013; Sidira *et al.*, 2012). These findings led Papa *et al.* (2013) to hypothesize that non-pathogenic or low-pathogenicity strains may be circulating in these areas. Similarly, a large serosurvey in Turkey revealed a seroprevalence of about 10% resulting in estimated subclinical infections in 88% of cases (Bodur *et al.*, 2012). AP92 and AP92-like strains may represent such a low-pathogenic strain of the virus. AP92 was originally isolated from a tick in Greece in 1972 (Deyde *et al.*, 2060) but the strain was not isolated in humans until 2007. Only a single case of documented clinical disease linked to an AP92-like strain was identified in a 6 year-old boy from Turkey who was infected with CCHFV following a tick bite. The child presented with fever, raised liver enzymes, and prolonged prothrombin time and activated partial thromboplastin time but made a full recovery. By day 10 of illness, the haemorrhagic manifestations had cleared and laboratory findings had returned to normal (Elevli *et al.*, 2009; Midilli *et al.*, 2009).

Patients who recover usually begin to improve by day 9 to 10 although the convalescent period may be prolonged. Residual symptoms during this time may include conjunctivitis, weakness, confusion, amnesia, polyneuritis, headache, dizziness, nausea, anorexia, alopecia, vision and hearing loss, and poor memory (Hoogstraal, 1979; Swanepoel *et al.*, 1987). Long term follow up of CCHF survivors have identified post-traumatic stress disorder (PTSD) and impaired health related quality of life (IHRQL) in nearly half of patients at 12 months post-infection. Rates of PTSD and IHRQL were significantly higher in patients requiring intensive care admission or the administration of blood products, and in those with bleeding (Gul *et al.*, 2012). Similar findings have been described in survivors of other acute, life-threatening conditions such as myocardial infarction and strokes and therefore likely relate to the severity of the illness and not CCHFV itself.

Although the clinical and laboratory features of CCHFV infection in children appear similar to those in adults, tonsillopharyngitis, rash and gastro-intestinal symptoms such as nausea, vomiting, and diarrhoea are more common. A tendency towards milder disease has also been noted, with a reported mortality rate below 5% (Dilber *et al.*, 2009; Tezer *et al.*, 2010). Maternal CCHFV infection during pregnancy may result in intrauterine or perinatal CCHFV infection in infants, with resultant abortion or haemorrhagic manifestations at birth (Ergonul *et al.*, 2010). However, delivery of a healthy infant with no evidence of transplacental transmission following maternal infection at 30 weeks gestation has also been documented (Aydemir *et al.*, 2010). There is currently insufficient data to determine the incidence of congenital infections following maternal infection or whether factors such as gestational age influence transmission and outcome, as has been described with other

viral infections. Only two cases of CCHFV infection have been described in breastfeeding women and transmission to their infants was not detected in either case (Erbay *et al.,* 2008).

Pathogenesis and immune responses

A clear understanding of the pathogenesis of CCHFV infection is lacking but it appears to involve a combination of viral and host immune-mediated mechanisms. Following inoculation, the virus is released from the basolateral membrane of epithelial cells facilitating haematogenous dissemination. Local amplification in tissue macrophages and dendritic cells may allow spread of the virus to lymph nodes and spleen further facilitating haematogenous dissemination (Akinci *et al.*, 2013). Replication in the blood, liver and spleen amplifies the viraemia allowing spread to other organs including lungs, kidneys, and brain, as demonstrated in a STAT-1 knockout mouse model (Bente *et al.*, 2010). Viral replication in the liver and adrenal glands may result in a decrease in coagulation and plasma protein synthesis as well as dysregulation of blood pressure homeostasis (Geisbert and Jahrling, 2004). Histopathological findings in CCHFV infected tissues of the liver, kidneys and adrenal glands include coagulative necrosis of these organs which supports this hypothesis (Burt *et al.*, 1997).

The haemorrhagic manifestations and increased vascular permeability with capillary leakage resulting in the clinical features of CCHF are primarily due to endothelial damage by direct viral replication and immune mediated mechanisms such as immune complex deposition and complement activation (Connolly-Andersen *et al.*, 2011; Joubert *et al.*, 1985). Proinflammatory cytokines interleukin (IL)-6, IL-8, IL-10 and tumour necrosis factor alpha

(TNF-α) are released during CCHFV infection and play a role in endothelial permeability. Increased levels of these cytokines have been linked with disease severity (Bente *et al.*, 2010; Ergonul *et al.*, 2006b; Papa *et al.*, 2006) potentially resulting in immune dysregulation and a so-called cytokine storm (Akinci *et al.*, 2013). TNF-α and IL-6 also stimulate monocyte activation which may result in haemophagocytosis. This phenomenon has been described both in adults and children with CCHFV infection and may contribute to the cytopaenias observed by phagocytosis of blood cells (Dilber *et al.,* 2009; Fisgin *et al.*, 2008; Karti *et al.*, 2004). A possible pathogenic role for TNF-α has been suggested in other viral haemorrhagic fevers (Kanerva *et al*, 1998; Linderholm *et al*, 1996). Known functions of TNF-α include activation of macrophages, stimulation of production of vasodilating substances, and antifibrinolytic activity. IL-6 is produced by a variety of cell types including Kupfer cells and is released following liver injury, which may account for the raised levels in CCHFV infected patients (Papa *et al*., 2006).

The haemorrhagic manifestations of CCHF also involve thrombocytopaenia, liver dysfunction, decreased levels of coagulation factors and disseminated intravascular coagulopathy (DIC) (Akinci *et al.*, 2013; Burt *et al.*, 1997; Swanepoel *et al.*, 1989).

Innate immune responses often play an important role in protection against and recovery from viral infections but limited information is available for their role in CCHFV infection. High natural killer (NK) cell counts have been identified as a possible prognostic marker linked to high fatality rates. It is thought that the high NK cell counts result from a strong response to a high viral load, or due to excessive cytokine release (Yilmaz *et al*., 2008). In the latter case, the immune response itself may play a role in the pathogenesis of the disease. Interferon alpha (IFN-α) has antiviral activity and inhibits CCHFV replication

through interferon-induced proteins such as MxA (Andersson *et al*., 2004; Andersson *et al*., 2006). Although CCHFV replication is inhibited by IFN-α, the virus is able to evade IFN by delaying induction of IFN synthesis and counteracting IFN signalling (Akinci *et al.*, 2013; Weber and Mirazimi, 2008).

Although the dynamics of IgG and IgM responses have been well defined, little information is available about the epitopes against which these antibodies are directed. Despite the genetic variation in the M segment of the genome and the associated antigenic variation, cell culture studies have demonstrated cross-reactive, neutralizing antibodies against a conserved G_C glycoprotein epitope (Ahmed *et al*., 2005). Although a decline in CCHF viral load has been correlated with the appearance of antibody in clinical infections, antibody production does not always correlate with viral clearance (Wölfel *et al.*, 2007). Data is lacking regarding the role of T cell responses in the protection or recovery from CCHFV infection. A study of Puumala virus, another member of the *Bunyaviridae* family, has shown long-lasting memory CD8+ cell responses following acute infection which greatly exceeds the responses previously documented following acute viral infections such as influenza (Van Epps *et al.*, 2002).

Laboratory diagnosis

Routine chemistry and haematology tests can assist in the identification of suspected CCHF cases. Either leukocytosis or leukopaenia may be present, while raised liver enzymes and thrombocytopaenia are consistently found and associated with decreased plasma fibrinogen, raised fibrin degradation products, and increased thrombin and partial

thromboplastin times (Swanepoel *et al*., 1989). Marked elevation in transaminases and early thrombocytopaenia as well as other signs of coagulation abnormalities are associated with an increased mortality (Ergonul *et al.*, 2006a; Swanepoel *et al.*, 1989). Although the clinical features, chemistry and haematology tests may alert clinicians to the possibility of CCHFV infection, laboratory confirmation is required to distinguish CCHF from clinically similar conditions.

Due to its biohazardous nature, CCHFV requires manipulation in a biosafely level 4 environment. Viraemia is present during the acute stage of illness and may be detectable up to day 13 of illness, during which time the virus can be detected in clinical samples of blood or tissues. The virus can be isolated in cell cultures or laboratory animals. Susceptible cell lines include a variety of mammalian cell cultures such as Vero (African Green monkey kidney), BHK-21 (Syrian hamster kidney), and SW-13 (human adrenal carcinoma) cells. Isolation in cell cultures takes 1-7 days and must be confirmed by immunofluorescence as cytopathic effects are usually not seen. Intracranially inoculated suckling mice are also susceptible to CCHFV infection and usually succumb to infection within 5 - 10 days (Hoogstraal 1979; Shepherd *et al*., 1986; Swanepoel *et al*., 1989). Mouse inoculation has been shown to be more sensitive than cell culture methods (Shepherd *et al*., 1986).

Reverse-transcription polymerase chain reaction (RT-PCR) is a rapid and sensitive means of diagnosis in the early stages of illness as well as in fatal cases where antibodies are not produced. Both conventional and real-time RT-PCR methods have been developed for the detection of CCHFV RNA targeting conserved regions of the S and L segments (Atkinson *et al*., 2012; Burt *et al*., 1998; Duh *et al.*, 2006). Another method for the detection of CCHFV RNA is reverse transcription loop-mediated isothermal amplification (RT-LAMP) which has

the advantage of isothermal amplification and the potential for naked eye turbidity read out of results which would be beneficial in resource-limited settings (Osman *et al.*, 2013).

Enzyme linked immunosorbent assay (ELISA) detection of viral antigen has also been investigated but has not been widely adopted for diagnostic use due to a lower sensitivity than viral isolation and PCR methods (Saijo *et al.*, 2005b; Shepherd *et al.*, 1988).

The serological diagnosis of CCHFV infection relies on demonstration of seroconversion or a four-fold increase in IgG on paired serum samples, or detection of IgM in a single serum sample. IgG and IgM become detectable from approximately day 7 and are detectable in almost all survivors by day 9. IgM generally remains detectable for up to 4 months, while IgG usually remains detectable for a number of years post-infection. Cases in which antibody remains undetectable beyond day 9 are invariably fatal (Shepherd *et al.*, 1989). Historically, methods such as complement fixation, immunodiffusion and haemagglutination inhibition were employed, but have now largely been replaced by indirect immunofluorescent assays and ELISA which can distinguish IgG and IgM. Both in-house and commercial ELISA assays are available using cell lysate and recombinant antigens (Burt *et al.*, 1994; Dowall *et al.*, 2012; Emmerich *et al.*, 2010; Marriott *et al.*, 1994; Saijo *et al.*, 2002; Saijo *et al.*, 2005a; Samudzi *et al.*, 2012; Tang *et al.*, 2003; Vanhomwegen *et al.*, 2012).

Histopathological features include hepatocellular necrosis, haemorrhage, fatty change, Kupffer cell hyperplasia and mononuclear portal inflammatory infiltrates, but are not pathognomonic for CCHFV infection. Immunohistochemistry for detection of CCHFV antigens can be used to confirm the diagnosis on tissue samples (Burt *et al.*, 1997).

Quantitative real time PCR has been used to determine CCHFV viral load in clinical samples as a predictor of disease severity and prognosis. Higher viral loads have been associated with a fatal outcome, although a definite cut-off for determining severe disease and poor prognosis have not yet been defined (Çevik *et al.*, 2007; Duh *et al.*, 2007; Papa *et al.*, 2007; Saksida *et al.,* 2010; Wölfel *et al.*, 2007; Yapar *et al.*, 2005).

Differential diagnosis

The differential diagnosis for CCHFV infection includes various bacterial, parasitic and viral infections as well as a number of non-infectious conditions. The geographic location, travel history and possible exposures, such as occupation and recreational activites, can assist in refining the differential diagnosis for individual patients. Infectious diseases which should be considered in the differential diagnosis include rickettsiosis (in South Africa, *Rickettsia conori* and *Rickettsia africae*), leptospirosis, borreliosis, malaria, Q fever (*Coxiella burnetti*), brucellosis, systemic anthrax, meningococcal infection, bacterial septicaemia, and causes of hepatitis including viral hepatitis and disseminated herpes simplex virus infection (Burt 2011; Flick and Whitehouse, 2005; Whitehouse, 2004). Other viral haemorrhagic fevers can be considered based on geographic location and travel history such as Ebola virus, Marburg virus, Lassa virus and Lujo virus in Africa, Rift Valley fever in Africa and the Middle East, yellow fever virus in Africa, South and Central America, and dengue virus in tropical and sub-tropical regions. Although CCHFV and Rift Valley fever virus are the only viral haemorrhagic fever viruses occurring endemically in South Africa, nosocomial transmission of Marburg virus (Gear *et al.*, 1975), Ebola virus (Richards *et al.*, 2000) and Lujo virus (Briese *et al.*, 2009) have been described via patients admitted for treatment to hospitals in Johannesburg. Non-

infectious conditions which should be considered include leukaemia, lymphoma, drug-induced hepatitis and auto-immune diseases (van Eeden *et al.*, 1985).

Treatment

Supportive therapy is essential to decrease mortality and should include maintenance of fluid and electrolyte balance, and aggressive administration of blood products including platelets, fresh frozen plasma and red cell preparations (Ergonul, 2008). Haemodialysis and respiratory support in the form of oxygen or mechanical ventilation should be administered as required (Leblebicioglu *et al.*, 2012). Although the administration of immune serum prepared from survivors of CCHFV infection has been attempted, the efficacy of this intervention is not clear and further studies are required to evaluate the use of specific immunoglobulin or monoclonal antibody preparations as a therapeutic intervention and for post-exposure prophylaxis (Keshtkar-Jahromi *et al.*, 2011; Kubar *et al.*, 2011). Additional difficulties with this approach include the limited availability of the product and lack of standardization. The use of corticosteroids may be beneficial in patients with severe disease and haemophagocytic syndrome (Dokuzoguz *et al.*, 2013; Erduran *et al.*, 2013).

Ribavirin is a broad spectrum antiviral agent which resulted in the inhibition of CCHFV replication in cell culture and reduced lethality in suckling mice (Paragas *et al.*, 2004; Tignor and Hanham, 1993). Although a number of case studies indicated improved outcome following the administration of oral ribavirin particularly within 4-5 days following onset of illness (Ergonul *et al.*, 2004; Izadi and Salehi, 2009; Mardani *et al.*, 2003; Ozkurt *et al.*, 2006), larger studies including two systematic reviews and meta-analyses evaluating a randomized

controlled trial and observational studies did not show a clear benefit to the use of ribavirin for the treatment of CCHFV infection (Ascioglu *et al.*, 2011; Ceylan *et al.*, 2013; Duygu *et al.*, 2012; Soares-Weiser *et al.*, 2010). Ribavirin therapy is reported to be well tolerated in patients with CCHFV infection. Adverse effects are reported only rarely, including mild anaemia, thrombocytosis and bradycardia (Ergonul *et al.*, 2004; Fisher-Hoch *et al.*, 1995; Ozkurt *et al.*, 2006; Uysal and Metan, 2012).

Prevention and control

Measures to minimize exposure to the virus may be effective in preventing CCHFV infection. General measures would include the use of acaricides for livestock, applying insect repellents to exposed skin, making use of clothing impregnated with permethrin, and avoiding direct exposure to potentially infected blood and tissues of animals for example, by wearing gloves. However, practical implementation of these measures is difficult.

As evidenced by a number of nosocomial outbreaks of CCHFV infection, infection of health care workers and patients in a hospital environment and laboratory workers can occur if appropriate precautions are not taken (Elata *et al.*, 2011; Gürbüz *et al.*, 2009; Harxhi *et al.*, 2005; Hasan *et al.*, 2013; Naderi *et al.*, 2013; Van Eeden *et al*., 1985). Treatment of CCHF patients requires the application of strict barrier-nursing techniques including isolation of the patient and the use of appropriate personal protective equipment. Although the efficacy of oral ribavirin for post-exposure prophylaxis has not been proven, it has been used for high-risk exposures such as needle-stick injuries and mucous membrane exposures in a nosocomial setting in South Africa.

An inactivated, suckling mouse brain vaccine has been in use in Bulgaria since 1974 but has not been accepted for widespread use (Papa *et al.*, 2011; Whitehouse, 2004). This vaccine elicited both T-cell responses to nucleoprotein and neutralizing antibodies in healthy volunteers, which were significantly increased by administering several doses of the vaccine (Mousavi-Jazi *et al.*, 2012). More recently, a recombinant DNA vaccine expressing the M segment coding region was developed and was shown to elicit neutralizing antibodies in approximately half of the immunized mice (Spik *et al*., 2006). A transgenic plant vaccine expressing G_N and G_C glycoproteins was administered both orally and by injection and resulted in IgG and IgA responses in immunized mice (Ghiasi *et al.*, 2011). Neutralizing antibodies were not investigated for this vaccine and challenge studies were not performed for either vaccine. A number of challenges exist in the development of an effective vaccine including the previous lack of a laboratory animal model, the limited target population for such a vaccine and the limited knowledge regarding what comprises a protective immune response. CCHFV has not been shown to cause disease in vertebrates other than humans, despite inducing a viraemia (Hoogstraal, 1979). Even non-human primates failed to show signs of clinical disease following CCHFV infection (Hoogstraal *et al.* 1979; Smirnova, 1979). Although newborn mice can be used for virus isolation using intracerebral or intraperitoneal inoculation (Bente *et al.*, 2013; Smirnova, 1979), they are not a suitable model for studies of pathogenesis, immunity or vaccine efficacy. The use of knockout mice lacking either the type 1 interferon receptor (Bereczky *et al.*, 2010) or the signal transducer and activator of transcription 1 (STAT-1) protein (Bente *et al.*, 2010) represent the first laboratory animal model which could be used for studies of pathogenesis and immunity and for development of effective antiviral and vaccine strategies.

The availability of an animal model will aid future studies on pathogenesis and vaccine development. Further studies on cellular immune responses in patients are required in order to better understand and possibly modulate the immune correlates of protection.

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CHAPTER 3

Identification of novel T cell epitopic regions on Crimean-Congo haemorrhagic fever virus and confirmation of long-lived memory T cell responses

Abstract

Crimean-Congo haemorrhagic fever virus (CCHFV) is a member of the *Nairovirus* genus of the *Bunyaviridae* family and is associated with haemorrhagic fever in humans. Although T lymphocyte responses are known to play a role in protection from and clearance of viral infections, specific T cell epitopes have yet to be identified for CCHFV. A panel of overlapping peptides covering the CCHFV nucleoprotein and the structural glycoproteins, G_N and G_C, were screened by ELISPOT assay to detect interferon gamma (IFN-γ) production *in vitro* by peripheral blood mononuclear cells from subjects with previous CCHFV infection. Sixteen peptides reacted and taking into account overlapping peptides this likely represented ten potential T cell epitopic regions. These regions were located predominantly on the nucleoprotein, with only a single subject reacting to two peptides from the glycoprotein G_C. No single epitope was immunodominant, but all subjects showed reactivity to at least one T cell epitope. These responses were present at high frequency and detectable several years after the acute infection despite the absence of continued antigenic stimulation. Depletion studies confirmed that IFN-γ production was mediated chiefly by CD8+ cytotoxic T cells. This is the first description of cytotoxic T cell epitopic regions for CCHFV and provides confirmation of long-lived memory T cell responses in survivors of CCHFV infection.

Introduction

Crimean-Congo haemorrhagic fever virus (CCHFV) is a member of the *Bunyaviridae* family and has a tripartite, single-stranded, negative sense RNA genome (Calisher, 1991; Casals, 1969; Karabatsos, 1985). The three segments are referred to as the large (L), medium (M) and small (S) segments (Clerx *et al.*, 1981). The L segment encodes the viral RNA dependant RNA polymerase which is responsible for mRNA synthesis and replication of the RNA genome (Honig *et al.*, 2004). The M segment encodes a number of non-structural glycoproteins and two structural glycoproteins, G_N and G_C (Altamura *et al.*, 2007; Sanchez *et al.*, 2006). The structural glycoproteins are responsible for attachment to host cell surface receptors and therefore determine the host range and cell tropism and are also the targets for neutralizing antibodies. The viral nucleoprotein, encoded by the S segment, binds the RNA segments for the formation of ribonucleoprotein complexes and shows endonuclease activity, although the role of this activity in CCHFV infection is not yet clear (Guo *et al.*, 2012; Schmaljohn and Hooper, 2001).

CCHFV is the only member of the CCHFV serogroup of medical importance as the other members of the group, Hazara virus and Khasan virus, have not been associated with disease in humans (Begum, 1970; Nichol *et al*., 2006; Smirnova, 1979). CCHFV infection in humans is associated with haemorrhagic fever and is fatal in up to 30% of cases (Bente *et al.*, 2013). The principal vectors of the virus are ticks belonging to the genus *Hyalomma* (Chumakov *et al.*, 1968). The distribution of disease follows that of the principal vector of the virus (Bente *et al*., 2013; Whitehouse, 2004). Clinical disease is well described in Africa, Asia, Eastern Europe and the Middle East and has recently emerged in Turkey, Greece and India (Maltezou *et al.*, 2010; Mishra *et al.*, 2011; Papa *et al.*, 2008). With over 7000 human

cases reported in Turkey since 2002, the development of effective preventative and therapeutic measures have now become a priority (Maltezou *et al.*, 2010). To this end, the correlates of protection against CCHFV need to be determined.

Antibody responses develop within 7-9 days of infection and include an IgM response which is present for up to 4 months following infection and has been detected in a small number of cases a year following onset of illness, and an IgG response which likely persists for life (Burt *et al.*, 2013; Shepherd *et al.*, 1989). The antibody response likely plays a role in clearance of CCHFV. In cases where an antibody response is not detectable by day 9, a fatal outcome is almost invariably seen (Shepherd *et al.*, 1989). However studies looking at viral load and antibody titre deduced that the detection of IgM had no influence on clearance of the virus or outcome, and viral load decreased independently of IgG. Hence, antibody production does not always correlate with viral clearance, implying that innate and T cell immunity likely also play an important role in viral clearance (Wölfel *et al.*, 2007). In addition, neutralizing antibodies identified in cell-culture assays do not always confer protection *in vivo*, while non-neutralizing antibodies may confer protection through mechanisms such as antibody-dependent cell-mediated cytotoxicity (Bertolotti-Ciarlet *et al.*, 2005). The nucleoprotein also induces antibody production and this region is widely used as the antigenic target in enzyme-linked immune-sorbent assays (ELISA) due to the robust nature of the antibody response to this protein following natural infection with CCHFV (Burt *et al.*, 1994; Dowall *et al.*, 2012; Emmerich *et al.*, 2010; Marriott *et al.*, 1994; Saijo *et al.*, 2002; Saijo *et al.*, 2005; Samudzi *et al.*, 2012; Tang *et al.*, 2003; Vanhomwegen *et al.*, 2012).

The Bulgarian CCHFV vaccine, which is an inactivated vaccine that was associated with a four-fold reduction in the number of CCHF cases following its introduction, has been shown

to induce both neutralizing antibody responses and T cell responses identified by interferon gamma (IFN-γ) enzyme-linked immunospot (ELISPOT) assays (Mousavi-Jazi *et al.*, 2012). Ideally, an effective vaccine will therefore likely require the induction of both B and T cell responses. The Bulgarian vaccine was shown to induce T cell responses to the nucleoprotein, but there are currently no data available evaluating T cell responses following natural infection with CCHFV in human cases.

To further understand the role of T lymphocytes in the immune response to CCHFV infection, we aimed to determine if it was possible to identify a memory T cell response in survivors of CCHFV infection and to identify T cell epitopes. An overlapping peptide library was used to screen for T cell responses by IFN-γ ELISPOT assay using peripheral blood mononuclear cells (PBMC) derived from subjects with previous CCHFV infection. The novel epitopic regions which were identified represent the first such T cell epitopes to be described following natural infection with CCHFV and may play an important role in vaccine development and evaluation of vaccine immunogenicity.

Methods

Study subjects

Ten patients with a history of laboratory confirmed CCHFV infection were included in the study. Laboratory confirmation of CCHFV infection was performed at the National Institute of Communicable Diseases, Johannesburg at the time of the acute illness by means of viral nucleic acid detection by reverse transcription polymerase chain reaction (RT-PCR) or virus isolation, or detection of CCHFV specific antibodies. Sodium heparin blood samples were

transported to the laboratory for processing within 4 to 6 hours after collection. Two additional subjects were included in the study as negative controls and were selected as they had no history of CCHFV infection, exposure to CCHFV or risk factors for such exposure. Informed consent was obtained from all subjects and approval was obtained from the institutional Ethics Committee (ECUFS NR 152/06, renewed to Dec 2014).

Synthetic peptides

An overlapping peptide library containing 156 peptides consisting of 19-mers with a 9-mer overlap and spanning the nucleoprotein (482 amino acids) and the mature glycoproteins, G_N (292 amino acids) and G_C (648 amino acids), were synthesized (Mimotopes) based on the deduced amino acid sequences of CCHFV isolate SPU103/87 (GenBank accession numbers DQ211647 and DQ211634). The peptide length was selected to reduce the cost of the library production and to decrease the number of cells required to perform the ELISPOT assays.

ELISPOT assays

Fresh PBMC were isolated from whole blood using Ficoll-Hypaque density gradient centrifugation. IFN-γ ELISPOT assays were performed using 96 well plates (MultiScreen-IP, Millipore) pre-coated with anti-IFN-γ antibody (clone 1-D1K; Mabtech) at 2μg/ml at 4°C overnight. After washing with sterile phosphate buffered saline (PBS), the plate was blocked with R10 (RPMI 1640 medium plus 1% penicillin-streptomycin, 1% L-glutamine and 10% foetal calf serum) for 2 hours at room temperature. Screening of the peptide library was performed by means of a matrix of 36 peptide pools containing 7 to 9 peptides each so that each peptide is included in two separate pools. Peptides were added at a final

concentration of 5µg/ml for each peptide. PBMC were then seeded at $1x10^5$ to $2x10^5$ cells per well in 100 µl final volume. For positive controls, PBMC were stimulated with phytohaemaglutinin (Sigma-Aldrich) and 0.1µg/ml monoclonal antibody to human CD3 (clone CD3-2, Mabtech) in separate wells. For negative controls, a well containing only media and duplicate wells of PBMC incubated with media only were included. Plates were incubated for 16 hours at 37°C and 5% CO_2. The plates were then washed 6 times with PBS and 2µg/ml of biotinylated anti-IFN-γ antibody (clone 7-B6-1, Mabtech) was added and incubated for 3 hours at room temperature. The plates were again washed with PBS and streptavidin-alkaline phosphatase conjugated antibody (Mabtech) was added and the plate incubated for 1 hour at room temperature in the dark. After a final wash, the alkaline phosphatase conjugate substrate kit (Bio-Rad) was used according to manufacturer's instructions to detect IFN-γ producing cells and the reaction was stopped using distilled water. After drying, the number of spots were counted manually and results were expressed as the number of spot forming cells per million cells (SFC/10^6 cells). A response was considered positive if it exceeded 50 SFC/10^6 cells while negative controls were consistently below 20 SFC/10^6. The peptides showing a positive response using the pool screening method were then tested individually using the same method and with a final peptide concentration of 5 µg/ml per well.

Subset depletion studies

To determine whether the positive responses obtained were predominantly due to CD4+ or CD8+ T cells, PBMC from patient VBD 02/13 were depleted of CD8+ T cells using Dynabeads® CD8 (Invitrogen) according to the manufacturer's instructions. The ELISPOT

assay was then performed using depleted and undepeleted PBMC with duplicate wells for each peptide previously shown to be reactive for this patient.

Epitope conservation

To determine whether the identified T cell epitopes are conserved amongst geographically distinct CCHFV isolates, available sequences were retrieved from the GenBank database.

Results

Ten subjects were included in the final analysis, all of whom were Caucasian males residing in the Free State, Northern Cape, and North West provinces of South Africa. The interval between CCHFV infection and sample collection for this study ranged from 10 months to 13 years. The details of the subjects are summarized in Table 1.

IFN-γ responses were detected against 16 peptides using the ELISPOT assays, with each subject responding to at least one peptide (Figure 1). As responses were detected to some overlapping peptides, a total of ten epitopic regions where identified and further studies using nonamers to determine the optimal epitopes are required. The details of the peptides showing positive responses are summarized in Table 2. The majority of the potential epitopes were located on the nucleoprotein, with only two located on the glycoprotein G_C. PBMC from two negative controls were tested against the 16 reactive peptides and showed no responses, confirming that the responses detected in the study subjects likely result from previous CCHFV exposure and were not due to non-specific reactivity.

Table 1

Summary of the subjects with previous CCHFV infection from whom peripheral blood mononuclear cells were extracted for interferon gamma ELISPOT assays.

Study number	Gender	Time since infection
VBD 07/13	Male	13 years
VBD 05/14	Male	12 years
VBD 05/13	Male	5 years
VBD 13/13	Male	5 years
VBD 04/13	Male	5 years
VBD 02/13	Male	4 years
VBD 10/13	Male	3 years
VBD 16/13	Male	3 years
VBD 04/14	Male	10 months
VBD 06/13	Male	13 years
VBD 14/13	Male	5 years
VBD 15/13	Male	8,5 years
VBD 19/13	Male	7 years

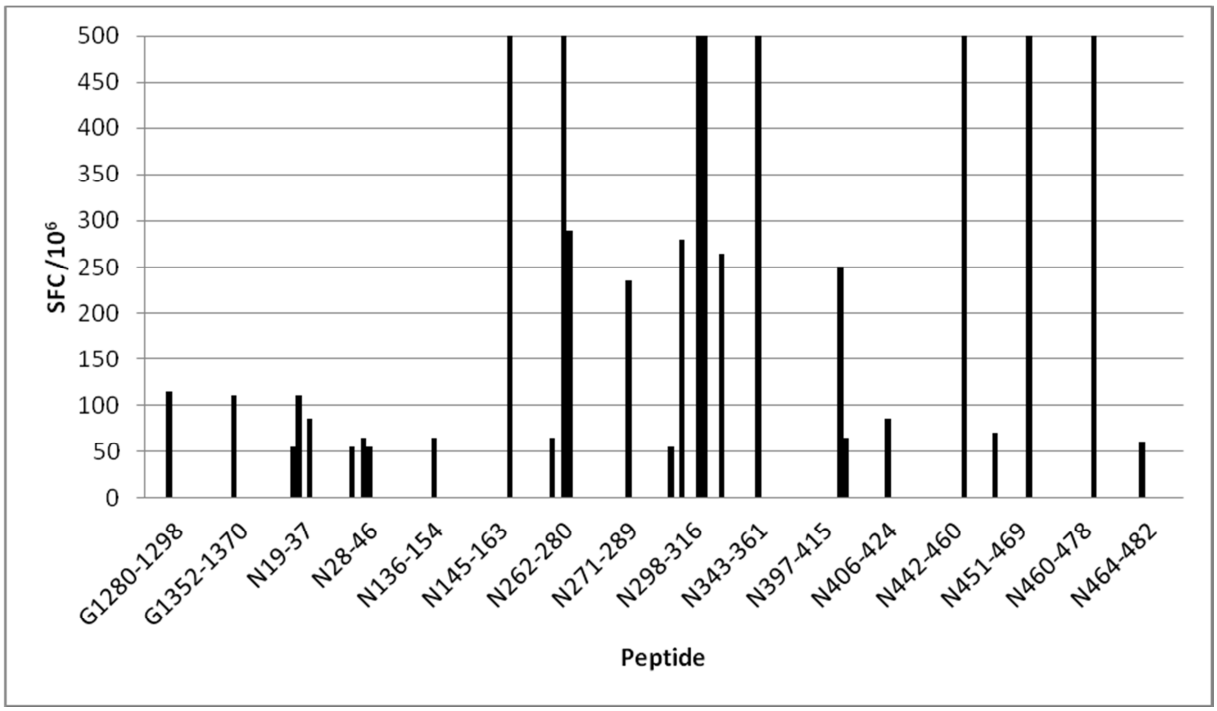

Figure 1

Detection of virus-specific T cell responses by interferon gamma ELISPOT assay. The magnitude of responses in spot forming cells per million (SFC/10^6) are indicated for each of the peptides to which study subjects showed a positive response.

Table 2

Details of the peptides representing potential epitopic regions identified by ELISPOT assay. Peptide names are derived from the relevant protein and the amino acid position relative to the coding regions of SPU 103/87 (DQ211647 and DQ211634). Six adjacent overlapping peptide pairs were reactive, with each pair likely representing a single epitopic region as indicated with a superscript numeric. Amino acid residues in the overlapping region are coloured red.

Peptide	Amino acid sequence	Number of positive responses	Range of magnitude of responses (SFC/10^6)
$G_{1280-1298}$	TLHPRIEEGFFDLMHVQKV	1	115
$G_{1352-1370}$	DGCDLDYYCNMGDWPSCTY	1	110
N_{19-37}^{1}	EFKKGNGLVDTFTNSYSFC	3	55 – 110
N_{28-46}^{1}	DTFTNSYSFCESVPNLDRF	3	55 – 65
$N_{136-154}^{2}$	DIGFRVNANTAALSNKVLA	1	65
$N_{145-163}^{2}$	TAALSNKVLAEYKVPGEIV	1	>500
$N_{262-280}^{3}$	DKHKDEVDRASADSMITNL	3	65 - >500
$N_{271-289}^{3}$	ASADSMITNLLHKIAKAQE	1	235
$N_{298-316}$	RAQGAQIDTAFSSYYWLYK	5	55 - >500
$N_{343-361}$	KMKKALLSTPMKWGKKLYE	1	>500
$N_{397-415}^{4}$	VANPDDAAQGSGHTKSILN	2	65 – 250
$N_{406-424}^{4}$	GSGHTKSILNLRTNTETNN	1	85
$N_{442-460}^{5}$	NIQDMDIVASEHLLHQSLV	1	>500
$N_{451-469}^{5}$	SEHLLHQSLVGKQSPFQNA	2	70 - >500
$N_{460-478}^{6}$	VGKQSPFQNAYNVKGNATS	1	>500
$N_{464-482}^{6}$	SPFQNAYNVKGNATSANII	1	60

No clear conclusion could be reached on the effect of the time interval between CCHFV infection and sampling with regard to the magnitude and range of T cell responses. Although the two subjects with intervals of 12 and 13 years respectively showed only low level responses (55 – 70 SFC/10^6) to one or two peptides, the subject with the most recent history of infection only 10 months prior to sampling also showed a low level response (65 SFC/10^6) to a single peptide. This variation likely is a consequence of individual patient responses rather than duration after infection.

Due to the number of patients showing IFN-γ responses and the magnitude of these responses (Figure 2), peptides $N_{262\text{-}280}$ and $N_{298\text{-}316}$ were selected for CD8+ depletion studies. Subject VBD 02/13 was selected due to strong responses to both of these peptides, with 290 and >500 SFC/10^6 detected respectively. Following CD8+ T cell depletion, the number of SFC/10^6 dropped to 5 and 25 respectively indicating a predominantly CD8+ T cell response.

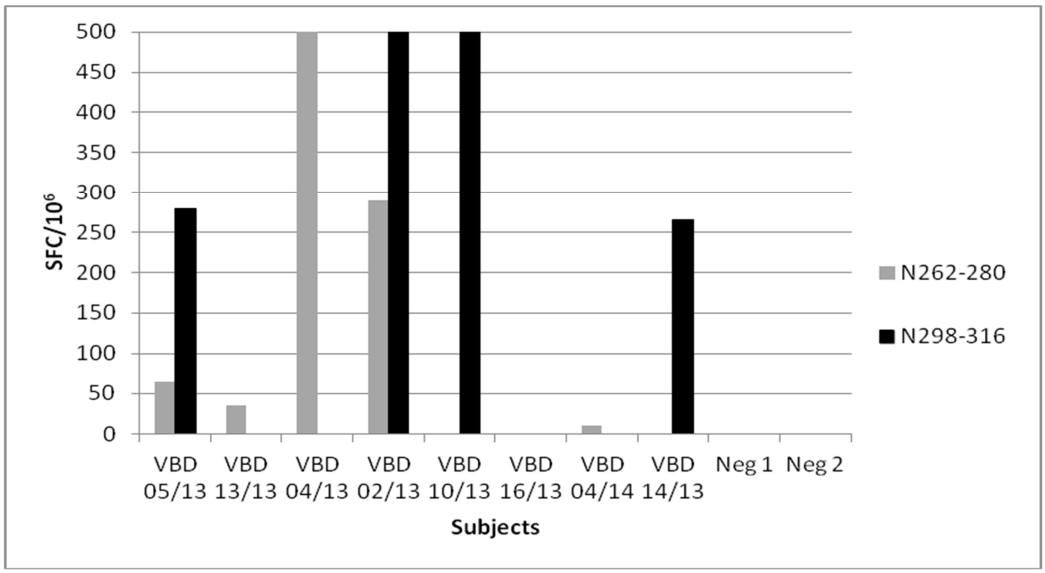

Figure 2

T cell responses to peptides $N_{262\text{-}280}$ and $N_{298\text{-}316}$ as determined by interferon gamma ELISPOT assay. The vertical axis is capped at 500 spot forming cells per million (SFC/10^6).

Comparison of the amino acid residues in the epitopic regions identified revealed some diversity among geographically distinct CCHFV isolates with a maximum of three amino acid differences compared to SPU103/87, with the exception of isolate AP92 which showed four amino acid differences for peptides $N_{262-280}$ and $N_{271-289}$. Amino acid sequence conservation was higher among the southern African isolates with a maximum of one amino acid difference across each 19mer.

Discussion

A library of overlapping peptides covering the nucleoprotein, G_N and G_C proteins of CCHFV were screened by ELISPOT assay to determine *in vitro* induction of IFN-γ production. The present study is the first description of T cell epitopes in survivors of CCHFV infection. Ten probable epitopic regions were identified in this way, residing predominantly on the nucleoprotein with only two epitopes on glycoprotein G_C. The predominance of nucleoprotein epitopes is similar to findings on Hantaan virus and Rift Valley fever (RVF) T cell epitopes (Wang *et al.*, 2011; Xu *et al.*, 2013). The reason for this is unclear but may result from the abundance of nucleoprotein production during CCHFV replication *in vivo*. RVF nucleoprotein derived subunit vaccines have been shown to protect against challenge with a lethal dose of virus using a mouse model. As antibodies directed against nucleoprotein are not neutralizing, it is likely that the protective mechanism is not dependent on induction of neutralizing antibodies but rather on a cellular immune response.

No single epitope was immunodominant, rather a variety of epitopes were identified in different patients. This likely represents the HLA diversity within the population studied and the differing HLA restrictions of the peptides, however HLA typing of patients and HLA restriction of epitopes was not determined in the study and will be included in future studies. A range of epitopes will most probably need to be included in any prospective vaccine candidates in order to induce protective T cell and antibody responses in all vaccine recipients. The high level of amino acid sequence conservation amongst the available southern African CCHFV isolates implies that variable responses to epitopes did not result from sequence variation which may result in an inability of T cells to recognise the synthetic peptides.

The strength of the T cell responses identified differed between peptides and between patients responding to the same peptide. The magnitude of T cell responses induced by longer peptides such as the 19mers used in this study, may be underestimated in comparison to the more effective responses induced by optimal nonamers. This increased efficiency of T cell response induction may result from direct binding of antigen presenting cells to the nonamers in the peripheral blood (Wang *et al.*, 2011). This phenomenon would not have influenced the outcomes of this study which aimed to identify the presence of T cell epitopes on viral proteins rather than to accurately quantify the magnitude of these responses. Further studies using nonamers of the epitopes identified will investigate this possibility and confirm the optimal epitopes.

Depletion studies confirmed that the T cell responses observed result from cytotoxic CD8+ cells rather than CD4+ helper cells. Further studies making use of flow cytometry are

planned to determine which lymphocyte subsets are responsible for the IFN-γ production observed and to evaluate functionality of the cells which respond to peptide stimulation.

CCHFV is transmitted to humans by tick bites, squashing of ticks with bare hands and contact with blood or tissues of infected humans and animals. Persons at high risk of exposure to CCHFV therefore include those likely to be exposed to ticks and those performing procedures resulting in exposure to blood or tissues of infected animals such as castration or slaughtering including farmers, farm workers, abattoir workers and veterinarians (Hoogstraal, 1979; Swanepoel *et al.*, 1998). Since the first recognised case of CCHFV infection in South Africa in 1981, a total of 194 cases have been recorded in this country. Of these cases, 91% have occurred in males and more than 50% originated in the Free State and Northern Cape Provinces (personal communication, Prof JT Paweska). The exclusively male cohort analyzed in this study is therefore representative of the gender distribution of cases in South Africa.

T cell responses to CCHFV peptides were detected in this study population up 13 years after acute CCHFV infection. The longevity of the responses points towards memory T cells playing an important role and support findings of the long-term presence of memory CD8+ T cells following infection with another bunyavirus, Puumala virus. This virus causes an acute infection with no known latent or chronic phase in humans, however Puumala virus specific memory T cell responses were present up to 15 years after infection despite the absence of continued antigenic stimulation or reexposure (van Epps *et al.*, 2002). These data imply that effective long term protection from infection may be achieved through vaccination if appropriate epitopes are targeted.

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CHAPTER 4

Identification of human linear B-cell epitope sites on the envelope glycoproteins of Crimean-Congo haemorrhagic fever virus

Abstract

A peptide library was used to screen for regions containing potential linear B-cell epitope sites in the glycoproteins and nucleoprotein of Crimean-Congo haemorrhagic fever virus (CCHFV) in an enzyme-linked immunosorbent assay (ELISA). The library consisted of 156 peptides, spanning the nucleoprotein and mature G_N and G_C proteins in a 19-mer with 9-mer overlap format. Using pooled serum samples from convalescent patients to screen the library, six peptides were identified as potential epitope sites. Further testing of these six peptides with individual patient sera identified two of these peptides as probable epitopic regions, with peptide $G_{1451\text{-}1469}$ reacting to 13/15 and peptide $G_{1613\text{-}1631}$ to 14/15 human sera. These peptides are situated on the G_C protein at amino acid positions 1451-1469 (relative to CCHFV isolate SPU103/97) (TCTGCYACSSGISCKVRIH) and 1613-1631 (FMFGWRILFCFKCCRRTRG). Identified peptides may have application in ELISA for diagnostic or serosurveillance purposes.

Introduction

Crimean-Congo haemorrhagic fever virus (CCHFV) is a tick-borne virus belonging to the family *Bunyaviridae* (Clerx *et al.*, 1981). The negative-stranded RNA genome consists of three segments (S, M and L) coding for the nucleoprotein, the envelope glycoproteins (G_N and G_C) and the viral polymerase respectively (Clerx *et al.*, 1981). The emergence of CCHFV in recent years in Southeast Europe and Southwest Asia highlighted the importance of developing serological assays for both diagnostic and serosurveillance purposes (Maltezou *et al.*, 2010). Although the preparation of CCHFV antigens from cell cultures or inoculation of suckling mice have been used, these techniques require the use of a biosafety level 4 laboratory which is not widely available and therefore limits the diagnostic and surveillance capabilities of many laboratories in endemic areas. Expression of recombinant CCHFV nucleoprotein antigens in baculovirus, mammalian and bacterial systems have also been utilized and have potential for application in detection assays (Samudzi *et al.*, 2012). Glycoproteins are more difficult to express, although by analogy with other bunyaviruses the glycoproteins should have application in detection assays.

Peptide libraries have been used to identify antigenic regions of CCHFV nucleoprotein, but epitopic regions of the glycoproteins have not previously been defined. In addition to their utility in diagnostic assays, the identification of antigenic regions or B-cell epitopes also play an important role in vaccine development, both in selecting appropriate peptides for subunit vaccines and in the evaluation of vaccine immunogenicity, as well as in elucidating virus-antibody interactions (Wu *et al.*, 2003). B-cell epitopes are classified as either conformational (discontinuous) or non-conformational (linear/continuous). Linear epitopes consist of short peptide fragments which are contiguous in the primary amino acid

sequence, while conformational epitopes are a set of amino acids that are brought into proximity by protein folding but are not contiguous in the primary sequence (Bottino *et al.*, 2013; Peters *et al.*, 2005). As antigen processing is not involved in immune recognition by B cells, these epitopes are often located on surface structures, are generally hydrophilic and are usually 4-8 amino acids in length (Bottino *et al.*, 2013). In the present study, we screened for linear B-cell epitope regions in the nucleoprotein and envelope glycoproteins of CCHFV using a synthetic peptide library and sera from CCHF survivors in an enzyme-linked immunosorbent assay (ELISA) format.

Methods

An overlapping peptide library consisting of 156 peptides (19-mers with a 9-mer overlap) spanning the 482 amino acids of the nucleoprotein, the 292 amino acids of the mature G_N and the 648 amino acids of the mature G_C were synthesized (Mimotopes) based on the deduced amino acid sequences of CCHFV isolate SPU103/87 (GenBank accession numbers DQ211647 and DQ211634). The peptide length was selected to reduce the cost of the library production while allowing coverage of the complete genes for the major structural proteins of CCHFV. A peptide ELISA was developed using the synthetic peptides to screen for linear B-cell epitope regions using the serum of survivors of CCHF infection. Polysorb plates (Nunc) were coated with 100µl per well of 20µg/ml peptide in 0.2M carbonate buffer (pH 9.6) overnight at 4^{o}C. The following day, plates were blocked with 10% skimmed milk in carbonate buffer for 2 hours at room temperature (22-24^{o}C). The plates were then washed using 0.05% Tween 20 in phosphate buffered saline (PBS, pH 7.2). Human sera diluted 1:100 in 10% skimmed milk in PBS were then added and incubated for 1 hour at 37^{o}C. After

washing, 100 µl of anti-human IgG horse radish peroxidase (HRPO) conjugate (Zymed) at a dilution of 1:1000 in 10% skimmed milk in PBS was added to each well and incubated for 1 hour at 37^{o}C. After further washing, plates were incubated with azino di-ethyl-benzothiazoline-sulfonic acid (ABTS®) peroxidase substrate (Kirkegaard and Perry Laboratories, KPL) for 40 minutes at room temperature in the dark and optical density (OD) values were read at 405nm/620nm. All assays were performed in duplicate.

For screening the full peptide library of 156 peptides, three pools of sera were generated to yield two positive and one negative pool each comprised of nine sera. The positive sera derived from survivors of previous infection with CCHFV as confirmed by ELISA or reverse transcription polymerase chain reaction testing performed at the National Institute for Communicable Diseases, Johannesburg, while the negative sera derived from volunteers with no history of CCHFV infection, exposure to CCHFV or risk factors for such exposure.

Results

The OD values obtained for each peptide of the envelope glycoproteins are illustrated in Figure 1. Six peptides derived from the envelope glycoproteins reacted to both positive pools but not the negative pool, however, no peptides in the nucleoprotein were found to be reactive to the pooled sera (data not shown). The details of the reactive peptides, as well as specific OD values obtained with each pool are shown in Table 1. Five peptides were located on the G_C protein, with one reactive peptide on the G_N protein.

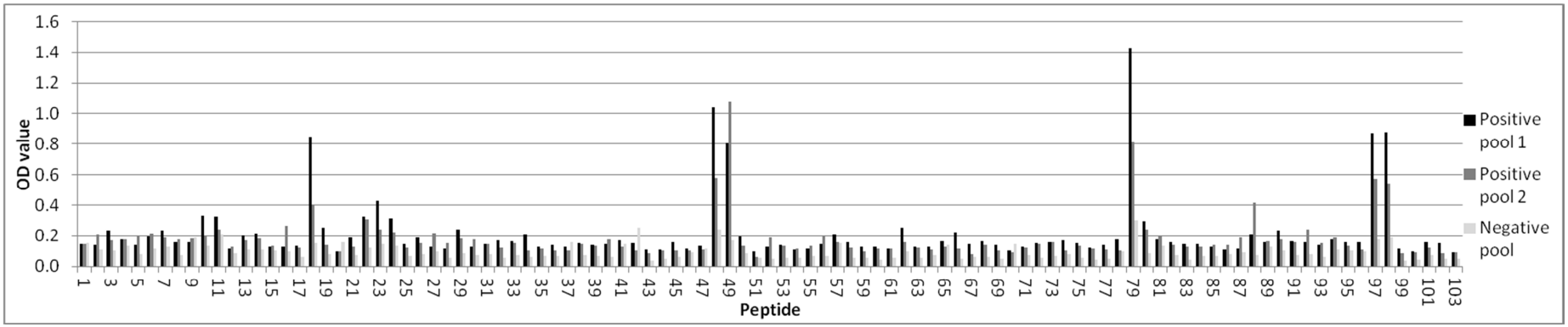

Figure 1

IgG-specific peptide ELISA illustrating OD values obtained for each peptide using pooled human sera. The horizontal axis denotes the peptide number and the vertical axis denotes the absorbance measured at 405nm/620nm. Peptides 1 – 32 represent the mature G_N protein and peptides 33 – 103 represent the mature G_C protein.

Table 1

Details of the six reactive peptides selected for further testing based on OD values obtained in the peptide ELISA using one negative and two positive serum pools. Two adjacent overlapping peptide pairs are noted with each pair likely representing a single epitopic region as indicated with a superscript numeric. Amino acid residues in the overlapping region are coloured red.

Peptide number	Peptide	Amino acid position relative to strain DQ211634		Protein	Positive pool 1 OD values	Positive pool 2 OD values	Negative pool OD values
		Start	End				
$G_{669-687}$	KIPLLGKMAIYICRMSNHP	669	687	G_N	0.845	0.407	0.152
$G_{1172-1190}^{1}$	RCGCTSSTCLHKEWPHSRN	1172	1190	G_C	1.038	0.583	0.241
$G_{1181-1199}^{1}$	LHKEWPHSRNWRCNPTWCW	1181	1199	G_C	0.803	1.073	0.170
$G_{1451-1469}$	TCTGCYACSSGISCKVRIH	1451	1469	G_C	1.434	0.811	0.305
$G_{1613-1631}^{2}$	FMFGWRILFCFKCCRRTRG	1613	1631	G_C	0.871	0.574	0.174
$G_{1622-1640}^{2}$	CFKCCRRTRGLFKYRHLKD	1622	1640	G_C	0.874	0.544	0.189

The six reactive peptides were then screened using individual sera from 15 survivors of previous CCHFV infection and three negative volunteers. The cut-off for each peptide was calculated using the mean of the negative sera plus two standard deviations. Peptides $G_{1451-1469}$ and $G_{1613-1631}$ were identified as including a probable linear B-cell epitope site with 13/15 and 14/15 sera showing positive OD results respectively (Supplementary data). Both of these peptides are located on the G_C envelope glycoprotein. The OD values of the positive sera obtained with peptide $G_{1451-1469}$ ranged from 0.385 – 0.931 (cut-off 0.352), while the OD values for peptide $G_{1613-1631}$ ranged from 0.408 – 1.257 (cut-off 0.317). Peptides $G_{1181-1199}$ and $G_{1622-1640}$ each reacted with 7/15 sera. These peptides showed lower reactivity in comparison to peptides $G_{1451-1469}$ and $G_{1613-1631}$, with OD values for positive sera obtained with peptide $G_{1181-1199}$ ranging from 0.318 – 0.576 (cut-off 0.317) and with peptide $G_{1622-1640}$ from 0.474 – 0.720 (cut-off 0.381). None of the sera reacted to peptide $G_{1172-1190}$ and only two sera had OD values above the cut-off determined for peptide $G_{669-687}$.

The two sera (VBD 49/10 and VBD10/09) which did not react to peptides $G_{1451-1469}$ and $G_{1613-1631}$ also failed to react to any of the other four peptides tested against the individual sera. As no further samples were available from these two patients at other time points, it could not be confirmed whether this reflects true lack of reactivity to all six peptides or whether sample integrity was compromised. However, sample VBD42/10 showed a low OD value on antibody ELISA testing in the laboratory, with an antibody titre of 1:100. The lack of reactivity to the peptides tested could likely be based on a low titred antibody rather than compromised sample integrity. Sample VBD10/09 was collected from a patient approximately three months after the onset of illness. The only other patient sampled at a similar time point showed relatively weak reactivity for peptides $G_{1451-1469}$ and $G_{1613-1631}$, and

negative results for the remaining peptides. The remainder of the samples in the panel were obtained between 11 months and 11 years post infection. It is known that epitope recognition varies with IgM and IgG class switching and with affinity maturation of the IgG response to viral infections (Gao *et al.*, 2012; Lenova *et al.*, 1998). As the majority of the serum samples included in the pools used to screen the peptide library were obtained at least one year post infection, it is possible that the six peptides identified in the initial ELISA screen are better recognized by antibodies in the later convalescent phase when extensive affinity maturation of IgG responses has occurred. Although acute phase and early convalescent phase serum panels were not available in the current study, it would be of interest to evaluate differences in peptide recognition at these time points after infection with CCHFV, particularly if the peptide ELISA were to be developed as a diagnostic assay for use in the acute phase of infection.

Sequence data for CCHFV isolates from patients sampled in this study were unavailable. Conservation of predicted epitopic regions was determined using complete genome sequence data for southern African isolates. Complete genome sequence data determined in our laboratory for 10 southern African isolates as well as four complete genomes from South African CCHFV isolates available on GenBank were used to determine the conservation of the epitopic regions identified (Figure 2). The deduced amino acid sequences for peptide $G_{1613\text{-}1631}$ show that this region is highly conserved with one isolate showing a single amino acid difference. Similarly, four isolates showed a single amino acid difference for peptide $G_{1451\text{-}1469}$ compared to isolate SPU103/87 which was used as the reference sequence for the construction of the peptide library. Peptides $G_{1172\text{-}1190}$ and $G_{1181\text{-}1199}$ showed 100% conservation in amino acid sequence, while peptides $G_{669\text{-}687}$ and $G_{1622\text{-}1640}$

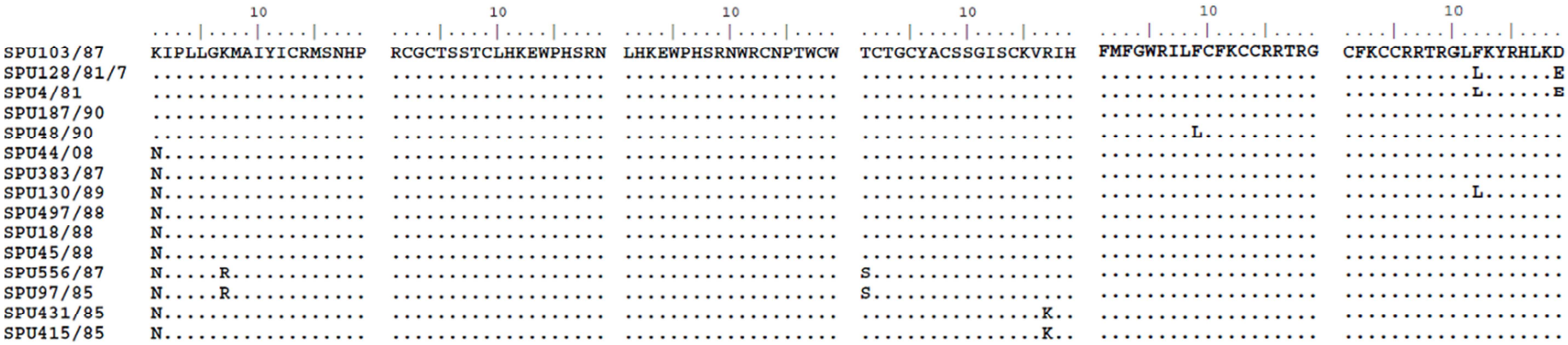

Figure 2

Alignment of amino acid sequences of reference SPU103/87 with 14 southern African CCHFV isolates illustrating conservation of peptides $G_{669-687}$, $G_{1172-1190}$, $G_{1181-1199}$, $G_{1451-1469}$, $G_{1613-1631}$ and $G_{1622-1640}$.

showed a maximum of two amino acid differences between isolates. All amino acid substitutions were conserved in polarity. The lack of reactivity to the individual patient sera by these peptides therefore cannot be explained by amino acid diversity, as all of the peptides found to be reactive in the screening ELISA were well conserved. In order to predict potential cross-reactivity of peptides $G_{1451\text{-}1469}$ and $G_{1613\text{-}1631}$ against other viruses, the peptide sequences were subjected to Basic Local Alignment Search Tool (BLAST) analysis using the protein-protein BLAST search (available at http://blast.ncbi.nlm.nih.gov/Blast.cgi). The BLAST search against peptide $G_{1451\text{-}1469}$ recognized only CCHFV glycoprotein with query coverage and identity greater than 80%, implying that the specificity of this peptide is likely to be high. The search against peptide $G_{1613\text{-}1631}$ returned hits for Hazara virus, Dugbe virus and Nairobi sheep disease virus glycoproteins although the coverage and identity were all below 80%. Further laboratory testing is required to determine whether this would lead to significant cross-reactivity and therefore lower the sensitivity of an assay based on this peptide.

The predicted amino acid sequences used to generate the peptide library were analysed using the Immune Epitope Database (IEDB) Analysis Resource B Cell Epitope Prediction Tools and compared to the epitopes identified by the screening ELISA. Although peptide $G_{669\text{-}687}$, located on the G_N protein, was identified by the Kolaskar and Tongaonkar Antigenicity software as a predicted protein antigen (threshold setting = 1.000) (Kolaskar and Tongaonkar, 1990), only four amino acids in the peptide were identified as a possible epitope by the Bepipred Linear Epitope Prediction software (threshold setting = 0.350) (Larsen *et al.*, 2006). Regions of peptides $G_{1172\text{-}1190}$, $G_{1181\text{-}1199}$, $G_{1451\text{-}1469}$, $G_{1613\text{-}1631}$ and $G_{1622\text{-}1640}$ on the G_C protein were all identified as potential antigens by the Kolaskar and

Tongaonkar Antigenicity software, while Bepipred Linear Epitope Prediction software identified only peptides $G_{1172\text{-}1190}$ and $G_{1181\text{-}1199}$. Parker Hydrophilicity Prediction was also performed and identified peptides $G_{1172\text{-}1190}$ and $G_{1451\text{-}1469}$ as hydrophilic peptides and therefore potentially epitopic with scores of 2.937 and 2.074 respectively (threshold setting 1.290) (Parker *et al.*, 1986). Peptides $G_{669\text{-}687}$, $G_{1181\text{-}1199}$, $G_{1613\text{-}1631}$ and $G_{1622\text{-}1640}$ were identified as hydrophobic, with scores of -0.426, 0.505, -1.311 and 0.179. As linear B-cell epitopes are mostly hydrophilic, the strongly hydrophobic score of peptide $G_{1613\text{-}1631}$ emphasizes the necessity of functional laboratory testing to support predictive software. As the precise epitopic region of peptide $G_{1613\text{-}1631}$ was not determined within the 19-mer, the true hydrophilicity score of this epitope could not be determined and may differ from the overall score for the peptide.

Discussion

As the aim of this study was to identify widely recognized epitopes for possible use in serological assays rather than the complete elucidation of linear B-cell epitope sites of CCHFV, the use of serum pools to screen the complete peptide library represented a practical approach given the size of the peptide library. Epitopes recognized at a low frequency in survivors of CCHFV infection may, however, not have been detected. The efficacy of synthetic peptides in immunoassays is influenced by their ability to bind to solid surfaces, the fact that antigenicity may be lost when binding to solid surfaces due to antigenic side chains not being accessible, and differing solubility (Tam, 1996). In a previous study, an epitopic site in the CCHFV nucleoprotein was identified using 8-mer overlapping peptide libraries (Burt *et al.*, 2013). The peptide used in the previous study was of higher

purity (95%) and was biotinylated and therefore bound to the surface via neutravidin. This technique significantly enhances binding properties and site accessibility which can be hidden when peptides are bound directly to a solid phase. As these characteristics cannot be reliably predicted, laboratory testing of peptide libraries or peptides based on prediction software is required to identify peptides with good binding ability which retain antigenicity in an immunoassay, such as the two peptides identified in this study. Unbiotinylated 19-mer peptides were used in the current study as they were primarily selected for ELISPOT assays for the study of T-cell responses in CCHFV survivors, for which biotinylated peptides are not used. The ease with which peptides are manufactured and stability are advantageous in a diagnostic setting. A combination of peptides may be required to ensure adequate sensitivity for the detection of IgM and IgG.

Humoral antibody responses to the nucleoprotein and glycoproteins can be detected following infection with CCHFV from approximately day 7 onwards after disease onset. While IgM responses generally decline to undetectable levels by 4-6 months post infection, IgG responses are detectable for at least 10 years (Burt *et al.*, 2013). In the current study, IgG responses to epitopes on the G_C glycoprotein were confirmed at least 11 years after infection. The envelope glycoproteins are responsible for binding of CCHFV to cell receptors and are targets for neutralizing antibodies which have been shown to protect mice in passive-immunization experiments (Bertolotti-Ciarlet *et al.,* 2005). Recombinant nucleoproteins are usually targeted for development of detection assays because they are more conserved and recombinant glycoproteins are difficult to express. However peptides mimicking epitopic or immunodominant regions from conserved regions of the glycoprotein may be useful for serological assay development particularly for serosurveillance which is

important for monitoring disease endemnicity. We identified two potential linear B-cell epitope sites on the G_C protein. Further investigation using shorter peptides would enable identification of specific epitopic sites within the peptides. Validation of the two peptides using larger numbers of serum samples would determine the usefulness of these peptides in serological assays.

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Supplementary data

Results of six reactive peptides tested with sera from 15 patients with previous CCHFV infection indicating OD values obtained, as well as the approximate duration from time of illness to sample collection. All OD values for CCHF survivors represent the mean of values obtained in duplicate testing. OD values above the relevant cut-off are indicated in red.

Laboratory number	Approximate duration since illness	Peptide 18 OD value	Peptide 48 OD value	Peptide 49 OD value	Peptide 79 OD value	Peptide 97 OD value	Peptide 98 OD value
52/10	10 years	0.271	0.227	0.315	0.846	1.257	0.625
10/09	3 months	0.083	0.100	0.112	0.092	0.115	0.088
41/10	8.5 years	0.324	0.261	0.279	0.717	0.868	0.654
06/11	11 months	0.242	0.204	0.318	0.632	0.970	0.474
38/11	8 months	0.180	0.151	0.332	0.499	0.733	0.422
51/10	2 years	0.277	0.391	0.373	0.714	1.013	0.503
30/10	10 years	0.502	0.338	0.455	0.931	1.210	0.720
42/10	2.5 years	0.154	0.156	0.231	0.200	0.324	0.156
39/11	7 years	0.201	0.192	0.576	0.636	1.173	0.270
40/11	8 months	0.215	0.167	0.287	0.478	0.720	0.394
43/11	11 years	0.160	0.180	0.412	0.525	1.100	0.277
49/11	3 years	0.182	0.157	0.354	0.428	0.812	0.258
51/11	5.5 years	0.183	0.162	0.232	0.411	0.591	0.343
54/11	2 years	0.209	0.208	0.266	0.457	0.671	0.347
20/13	3 months	0.170	0.199	0.168	0.385	0.408	0.338
Negative	N/A	0.169	0.214	0.133	0.184	0.189	0.218
Cut-off	N/A	0.290	0.508	0.317	0.352	0.317	0.381

CHAPTER 5

Next generation sequencing of southern African Crimean-Congo haemorrhagic fever virus isolates reveals a high frequency of M segment reassortment

Abstract

Crimean-Congo haemorrhagic fever virus (CCHFV) is a bunyavirus with a single-stranded RNA genome consisting of 3 segments (S, M and L), coding for the nucleocapsid protein, envelope glycoproteins and RNA polymerase respectively. To date only five complete genome sequences are available from southern African isolates. Complete genome sequences were generated for 10 southern African CCHFV isolates using next generation sequencing techniques. The maximum likelihood method was used to generate tree topologies for 15 southern African plus 26 geographically distinct complete sequences from GenBank. M segment reassortment was identified in 10 of 15 southern African isolates by incongruencies in grouping compared to the S and L segments. These reassortant M segments cluster with isolates from Asia/Middle East, while the S and L segments cluster with strains from South/West Africa. The CCHFV M segment shows a high level of genetic diversity, while the S and L segments appear to co-evolve. The reason why M segment reassortment occurs is not known. It has previously been suggested that M segment reassortment results in a virus with higher fitness but a clear role in increased pathogenicity has yet to be shown.

Introduction

Crimean-Congo haemorrhagic fever virus (CCHFV) is a member of the family *Bunyaviridae*, genus *Nairovirus* (Nichol *et al.*, 2006). This tick-borne zoonotic virus is associated with clinical disease ranging from a non-specific febrile illness to severe disease manifesting as haemorrhagic fever. CCHFV has a negative sense, single-stranded RNA genome consisting of three segments designated large (L), medium (M) and small (S). The highly conserved complementary terminal nucleotide sequences result in loosely circular RNAs which together with the nucleocapsid protein, make up the three helical nucleocapsids. The L segment encodes the viral RNA polymerase while the S segment encodes the viral nucleocapsid protein. The M segment encodes a polyprotein which undergoes proteolytic processing to yield the viral glycoproteins, G_C and G_N (Schmaljohn and Hooper, 2001).

CCHFV has been documented in more than 30 countries of Africa, Asia, Europe and the Middle East, with a distribution following that of *Hyalomma* ticks, the principal vector of the virus (Bente *et al.*, 2013; Whitehouse, 2004). Since the first description of clinical disease due to CCHFV infection in South Africa in 1981, 192 cases have been confirmed (Msimang *et al.*, 2013). However, complete S, M and L sequences are available for only four isolates collected from humans and one tick isolate from South Africa. These, as well as other published CCHFV sequences, were determined by Sanger sequencing using primer walking (Aradaib *et al.*, 2011; Chamberlain *et al.*, 2005; Deyde *et al.*, 2006; Duh *et al.*, 2008; Hewson *et al.*, 2004; Kinsella *et al.*, 2004; Meissner *et al.*, 2006; Olschläger *et al.*, 2011; Ozdarendeli *et al.*, 2010; Papa *et al.*, 2002; Seregin *et al.*, 2004; Yadav *et al.*, 2013; Zhou *et al.*2013). In recent years, a number of next generation sequencing (NGS) methods have been developed which yield large amounts of sequencing data at relatively low cost. Although the specifics

differ, these methods all make use of three steps, namely, library preparation, DNA capture and enrichment, and sequencing or detection. In the field of virology, these techniques have been employed for various purposes including the discovery of novel viruses, whole viral genome sequencing, and "deep" sequencing to determine viral quasispecies or genome variability (Capobianchi *et al.*, 2013). This study aimed to make use of NGS techniques to obtain whole genome sequences of southern African CCHFV isolates in order to perform genetic analysis including identification of reassortment events.

Methods

Viral isolates

Ten CCHFV isolates obtained from patients in southern Africa between 1985 and 2008 were sequenced retrospectively. RNA was extracted and supplied by Prof JT Paweska, Centre for Emerging and Zoonotic Pathogens, National Institute for Communicable Diseases (NICD), Johannesburg from cell culture preparations or mouse brain suspensions. Total RNA was extracted for isolate SPU44/08 using TRIzol® reagent according to the manufacturer's instructions. The remainder of the isolates were stored at -70°C as freeze-dried 10% suckling mouse brain suspensions at the level of mouse brain passage 2-3. The suspensions were inoculated into Vero cell cultures and total RNA extracted from the infected cells using the acid guanidium thiocyanate-phenol-chloroform method as described previously (Burt and Swanepoel, 2005). The extracted RNA was stored at -70°C until use.

Reverse transcription polymerase chain reaction and sequencing

The complete L segment was amplified using two previously described primer pairs that generated overlapping amplicons (Deyde *et al.*, 2006). The complete M segment was amplified using primers designed by Deyde *et al.* (2006). Existing sequences on GenBank were used to design primers SF1 and SR3 (Table 1) for amplification of the S segment. The respective forward primers were used to perform the reverse transcription step for each amplicon using SuperScript™ III Reverse Transcriptase (Invitrogen). PCR was performed with the Expand Long Template PCR System (Roche Diagnostics GmbH) using standard cycling conditions according to the manufacturer's instructions and an annealing temperature of 48°C. The PCR products were gel extracted and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega).

The complete genomes for 3 of 10 isolates (SPU431/85, SPU383/87 and SPU 130/89) were determined using the Ion Torrent PGM™ sequencer by the Central Analytical Facility, Stellenbosch University. For the remaining 7 isolates, the S segment data was previously determined in the laboratory using the Big Dye™ Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's instructions and three overlapping primer sets as described in Table 2. The L and M segments of these isolates were sequenced at the NICD, Johannesburg using the Roche 454 GS Junior™ sequencing system. Additional sequencing of all isolates for incomplete coverage and clarification of ambiguities including mixed bases and base insertions or deletions was performed as required using the Big Dye™ Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Details regarding the additional primers used are available from the corresponding author upon request.

Table 1

PCR and sequencing primers utilized. Approximate length of amplicons using primers LF1 and LR1 is 7800 bp, LF2 and LR2 is 6000 bp, MF and MR is 5350 bp and SF1 and SF3 1650 bp.

Primer name	**5'-3' position relative to SPU415/85**	**5'-3' sequence**	**Reference**
L1F	1 – 22	TCT CAA AGA TAT CAA TCC CCC C	Deyde 2006
L1R	7773 – 7752	TTG GCA CTA TCT TTC ATT TGA C	Deyde 2006
L2F	6137 - 6158	GAA GAG CTA TAT GAC ATA AGG C	Deyde 2006
L2R	12157 - 12134	TCT CAA AGA AAT CGT TCC CCC CAC	Deyde 2006
MF	1 – 18	TCT CAA AGA AAT ACT TGC	Deyde 2006
MR	5354 - 5337	TCT CAA AGA TAT AGT GGC	Deyde 2006
SF1	1 – 21	TCT CAA AGA AAC ACG TGC CGC	In house
SR1 *	590 – 569	GGT TCC TTC TCC TAA TCA TGT C	In house
SF2 *	467 – 487	GGT TTC CGT GTC AAT GCA AAC	In house
SR2 *	1114 – 1095	CAT TGG GGT GCT CAG CAG AG	In house
SF3 *	1029 - 1048	CGA CGG TGT CAC AGT TCC TC	In house
SR3	1673 - 1651	TCT CAA AGA TAT CGT TGC CGC AC	In house

*Primers designed for sequence determination

Table 2

Summary of data concerning the CCHFV sequences retrieved from the GenBank database and used in the study

Strain	Accession number			Year and location of isolation	Source of isolate
	L	M	S		
C-68031	DQ211616	DQ211629	DQ211641	1968 China	Sheep
Oman	DQ211619	DQ211632	DQ211645	1997 Oman	Human
ArD15786	DQ211614	DQ211627	DQ211640	1972 Senegal	Goat
ArD8194	DQ211613	DQ211626	DQ211639	1969 Senegal	Tick
ArD39554	DQ211615	DQ211628	DQ211641	1984 Mauritania	Tick
SPU415/85	DQ211622	DQ211635	DQ211648	1985 South Africa	Human
SPU97/85	DQ211620	DQ211633	DQ211646	1985 South Africa	Human
SPU103/87	DQ211621	DQ211634	DQ211647	1987 South Africa	Human
Drosdov	DQ211617	DQ211630	DQ211643	1967 Russia	Human
Kashmanov	DQ211618	DQ211631	DQ211644	1967 Russia	Human
Baghdad-12	AY947890	AJ538197	AJ538196	1979 Iraq	Human
Matin	AY422208	AF467769	AF527810	1976 Pakistan	Human
Kosova Hoti	EU044832	EU037902	DQ133507	2001 Kosovo	Human
Turkey 200310849	DQ211623	DQ211636	DQ211649	2003 Turkey	Human
UG3010	DQ211624	DQ211637	DQ211650	1956 DRC	Human
AP92	DQ211612	DQ211625	DQ211638	1975 Greece	Tick
TADJ/HU8966	AY720893	AY179962	AY049083	1990 Tajikistan	Human
ROS/HUVLV-100	AY995166	DQ206448	DQ206447	2003 Russian Federation	Human
Ibar10200	AY947891	AF467768	U88410	1966 Nigeria	Tick
Semunya	DQ076412	DQ094832	DQ076413	1958 Uganda	Human
Turkey-Kelkit06	GQ337055	GQ337054	GQ337053	2006 Turkey	Human
SPU128/81/7	DQ076414	DQ157174	DQ076415	1981 South Africa	Tick
SPU4/81	DQ076417	DQ157175	DQ076416	1981 South Africa	Human
Afg09-2990	HM452307	HM452306	HM452305	2009 Afghanistan	Human
NIVA 118594	JN572092	JN572084	JN572087	2011 India	Tick
NIV 112143	JN572091	JN572085	JN572089	2011 India	Human
NIVA 118595	JN572090	JN572083	JN572088	2011 India	Tick
YL04057	FJ562095	FJ562094	FJ562093	2004 China	Tick
Sudan AB1-2009	HQ378183	HQ378187	HQ378179	2009 Sudan	Human
Sudan Al-Fulah 3-2008	HQ378180	HQ378184	GQ862371	2008 Sudan	Human
79121M18	GU477492	GU477493	GU477494	1979 China	Rodent

Data analysis

Raw sequencing data was converted from SFF format to FASTQ files using the sff_extract script (available as part of seq_crumbs at http://bioinf.comav.upv.es/) and trimming and filtering of reads based on length and quality scores was then performed using PRINSEQ (Schmieder and Edwards, 2011). Sequences for the L, M and S segments available on Genbank were used to compile databases and separate the reads into L, M and S segment related data using filter_by_blast (available at http://bioinf.comav.upv.es/seq_crumbs/available_crumbs.html). De novo assembly of the blast-filtered and unfiltered reads was performed using MIRA (Chevreux *et al.*, 1999). The resulting contigs from both methods were assembled in Geneious (Geneious version 4.8.5 created by Biomatters, available from http://www.geneious.com/) and compared to known complete CCHFV sequences to identify areas of incomplete coverage or ambiguities which required further investigation. Ambiguities and homopolymers were investigated using Gap5 (Bonfield *et al.*, 1995) and the majority could be clarified by evaluation of the quality scores at each relevant position. The additional Sanger sequences were incorporated into the assembly using Geneious and ChromasPro version 1.42. Alignments were confirmed by visual inspection with reference sequences and comparison with previously published partial sequences.

Complete genome sequence data for 31 isolates were retrieved from GenBank as summarized in Table 2. The sequences were aligned using Clustal X version 2.0 (Larkin *et al.*, 2007) and analysed using Molecular Evolutionary Genetics Analysis (MEGA) version 5 (Tamura *et al.*, 2011) with the bootstrap maximum likelihood method with 1000 replicates. Sequence divergence was also determined using MEGA by calculating the average *P*

distances within and between sequence groups as well as pairwise distances for nucleotide and deduced amino acid sequences.

Results

Sequencing data

Full coverage of complete L, M and S segments was obtained for 10 southern African CCHFV isolates. The details of the 10 isolates are summarized in Table 3. Ion Torrent™ PGM sequencing yielded raw data of >80 000 reads including >13 million bases for each of the 3 isolates. Following stringent filtering and trimming of reads based on length as well as quality scores, >35 000 reads amounting to approximately 7 million bases were included in the final analysis of each of these isolates. The 454 GS Junior™ sequencing data were more variable and duplicate runs were performed for four of the isolates in order to obtain complete coverage. The raw data for these 7 isolates yielded between 2404 and 17 315 reads including 1-8 million bases per isolate. Following filtering and trimming, the final alignments were performed on between 1105 and 7042 reads with approximately 500 000 – 3.5 million bases per isolate. The alignments generated by MIRA de novo assembly of both blast-filtered and unfiltered data corresponded, although improved contiguous quality was achieved with some isolates following the application of filter_by_blast. A comprehensive sequence database and optimization of blast parameters were required to ensure effective filtering of reads without loss of coverage, particularly of the highly variable M segment.

Table 3

Summary of southern African CCHFV isolates for which complete genome sequences were obtained by next generation sequencing.

Strain	GenBank accession numbers			Year and location of isolation	Source of human infection	Outcome
	L	M	S			
SPU431/85	KJ682799	KJ682812	KJ682815	1985 Northern Cape	Nosocomial	Fatal
SPU383/87	KJ682801	KJ682806	KJ682816	1987 Free State	Tick	Survived
SPU556/87	KJ682798	KJ682811	KJ682817	1987 Northern Cape	Tick	Fatal
SPU18/88	KJ682803	KJ682810	KJ682818	1988 Northern Cape	Tick	Fatal
SPU45/88	KJ682796	KJ682809	KJ682819	1988 Free State	Tick	Fatal
SPU497/88	KJ682804	KJ682808	KJ682820	1988 Namibia	Livestock/ tick	Fatal
SPU130/89	KJ682802	KJ682807	KJ682821	1989 Northern Cape	Tick	Survived
SPU48/90	KJ682797	KJ682813	KJ682822	1990 North West Province	Unknown	Survived
SPU187/90	KJ682795	KJ682814	KJ682823	1990 North West Province	Abbatoir	Fatal
SPU44/08	KJ682800	KJ682805	KJ682824	2008 Free State	Livestock/ tick	Survived

Genetic analysis

The genetic relationship of isolates as determined by the maximum-likelihood method are shown for the S (Figure 1(a)), M (Figure 1(b)) and L (Figure 1(c)) segments. The phylogenetic groups were designated I-VII as defined previously (Deyde *et al.*, 2006; Carroll *et al.*, 2010).

The complete sequences confirm the phylogenetic grouping of southern African isolates SPU497/88, SPU130/89, SPU383/87, SPU18/88 and SPU45/88 and the occurrence of reassortment of the M segment of these isolates as suggested by partial sequencing (Burt *et al.*, 2009). The complete sequences also confirm that the incongruencies in M segment grouping were due to reassortment rather than recombination events. This pattern of M segment reassortment, showing clustering of the S and L segments with group III strains from South and West Africa while the M segments cluster with group IV isolates from Asia and the Middle East, was also noted for newly sequenced isolates SPU556/87, SPU44/08 and SPU431/85. Isolates SPU48/90 and SPU187/90 showed no evidence of reassortment, with all 3 segments falling within the group III South/West Africa lineage. Further evidence of M segment reassortment was seen in southern African isolates SPU97/85 and SPU415/85, as described previously (Deyde *et al.*, 2006). No evidence of S segment reassortment was seen in the available complete sequences, but L segment reassortment was seen in two CCHFV isolates from Senegal, ArD15786 and ArD8194 (Deyde *et al.*, 2006). The Chinese isolates YL04057 and 79121M18 appear to represent a new group as suggested by Zhou e*t al* (2013), with S segments related to group IV Asia and Middle East, while the M and L segments cluster separately from other known groups. Isolate SPU431/85 was obtained from the same patient but from a sample collected 3 days subsequent to previously published SPU415/85. The sequences correlated well with only a single non-synonymous mutation in the coding region of the L segment and 4 non-synonymous mutations in the coding region of the M segment, as confirmed by Sanger sequencing. RNA from both of these isolates was obtained from cell cultures and these additional passages may have contributed to the accumulated mutations noted.

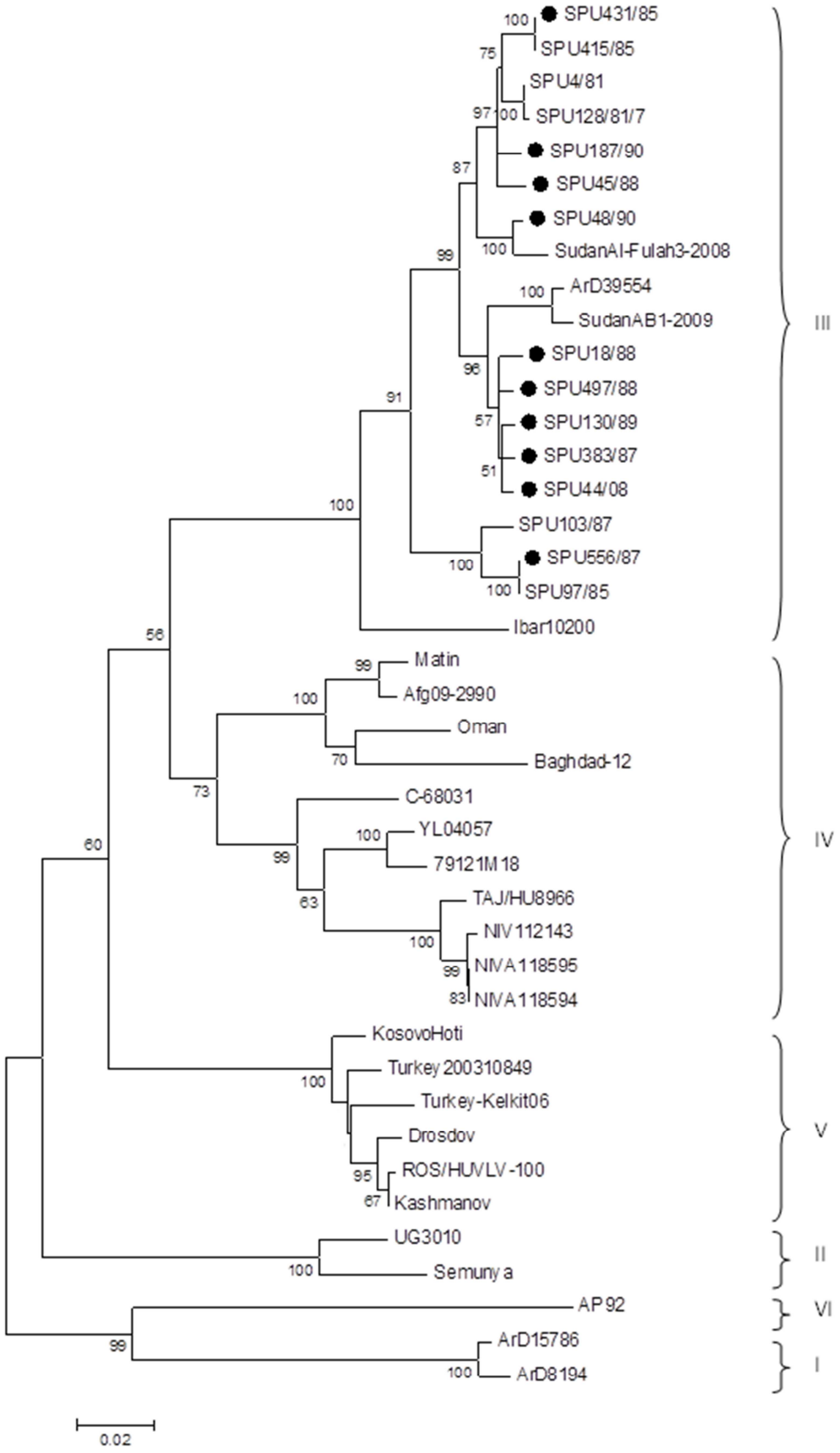
SPU431/85
SPU415/85
SPU4/81
SPU128/81/7
SPU187/90
SPU45/88
SPU48/90
SudanAl-Fulah3-2008
ArD39554
SudanAB1-2009
SPU18/88
SPU497/88
SPU130/89
SPU383/87
SPU44/08
SPU103/87
SPU556/87
SPU97/85
Ibar10200
Matin
Afg09-2990
Oman
Baghdad-12
C-68031
YL04057
79121M18
TAJ/HU8966
NIV112143
NIVA118595
NIVA118594
KosovoHoti
Turkey200310849
Turkey-Kelkit06
Drosdov
ROS/HUVLV-100
Kashmanov
UG3010
Semunya
AP92
ArD15786
ArD8194
III
IV
V
II
VI
I
0.02

Figure 1(a)

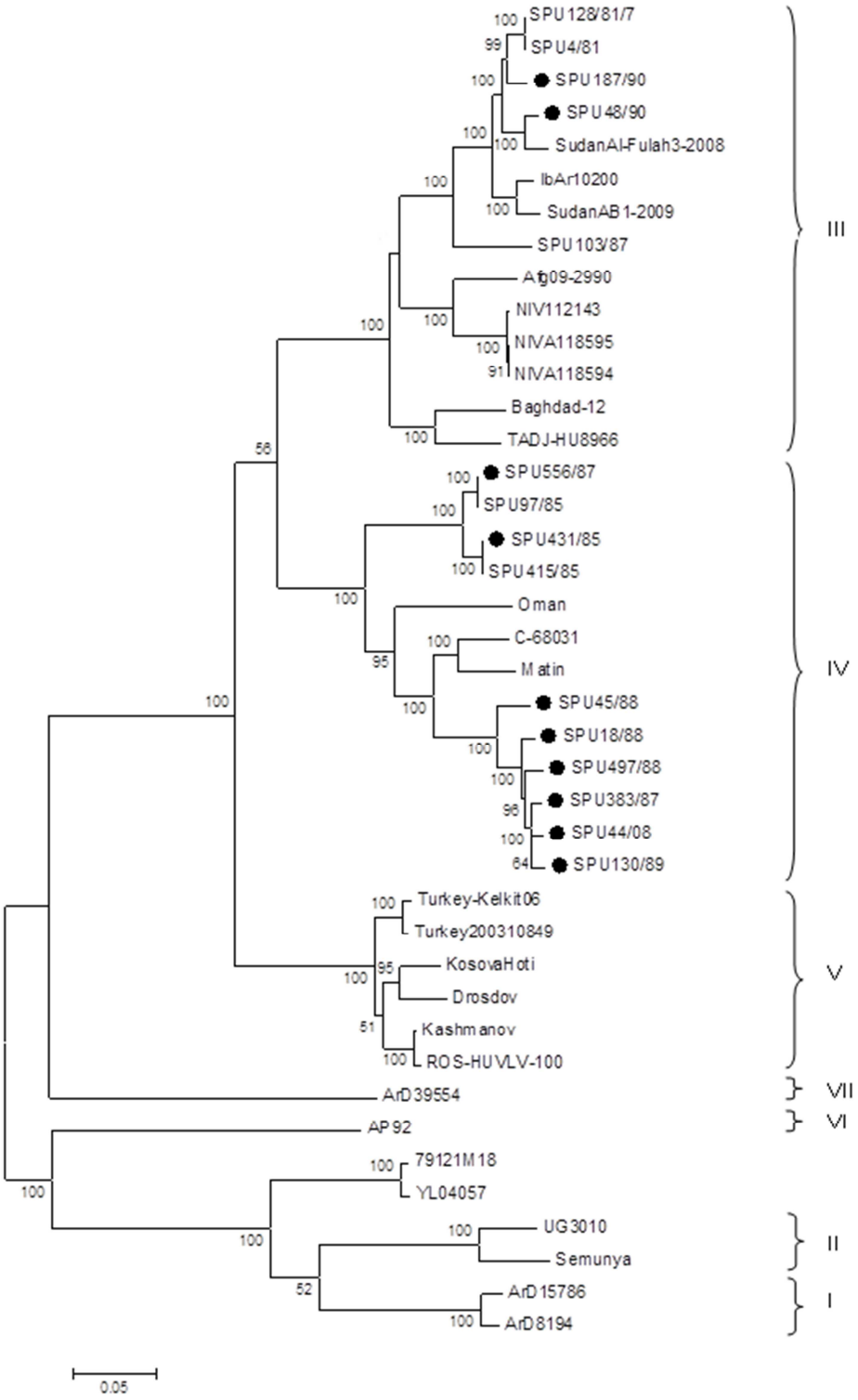
SPU128/81/7
SPU4/81
SPU187/90
SPU48/90
SudanAl-Fulah3-2008
IbAr10200
SudanAB1-2009
SPU103/87
Afg09-2990
NIV112143
NIVA118595
NIVA118594
Baghdad-12
TADJ-HU8966
SPU556/87
SPU97/85
SPU431/85
SPU415/85
Oman
C-68031
Matin
SPU45/88
SPU18/88
SPU497/88
SPU383/87
SPU44/08
SPU130/89
Turkey-Kelkit06
Turkey200310849
KosovaHoti
Drosdov
Kashmanov
ROS-HUVLV-100
ArD39554
AP92
79121M18
YL04057
UG3010
Semunya
ArD15786
ArD8194
III
IV
V
VII
VI
II
I
0.05

Figure 1(b)

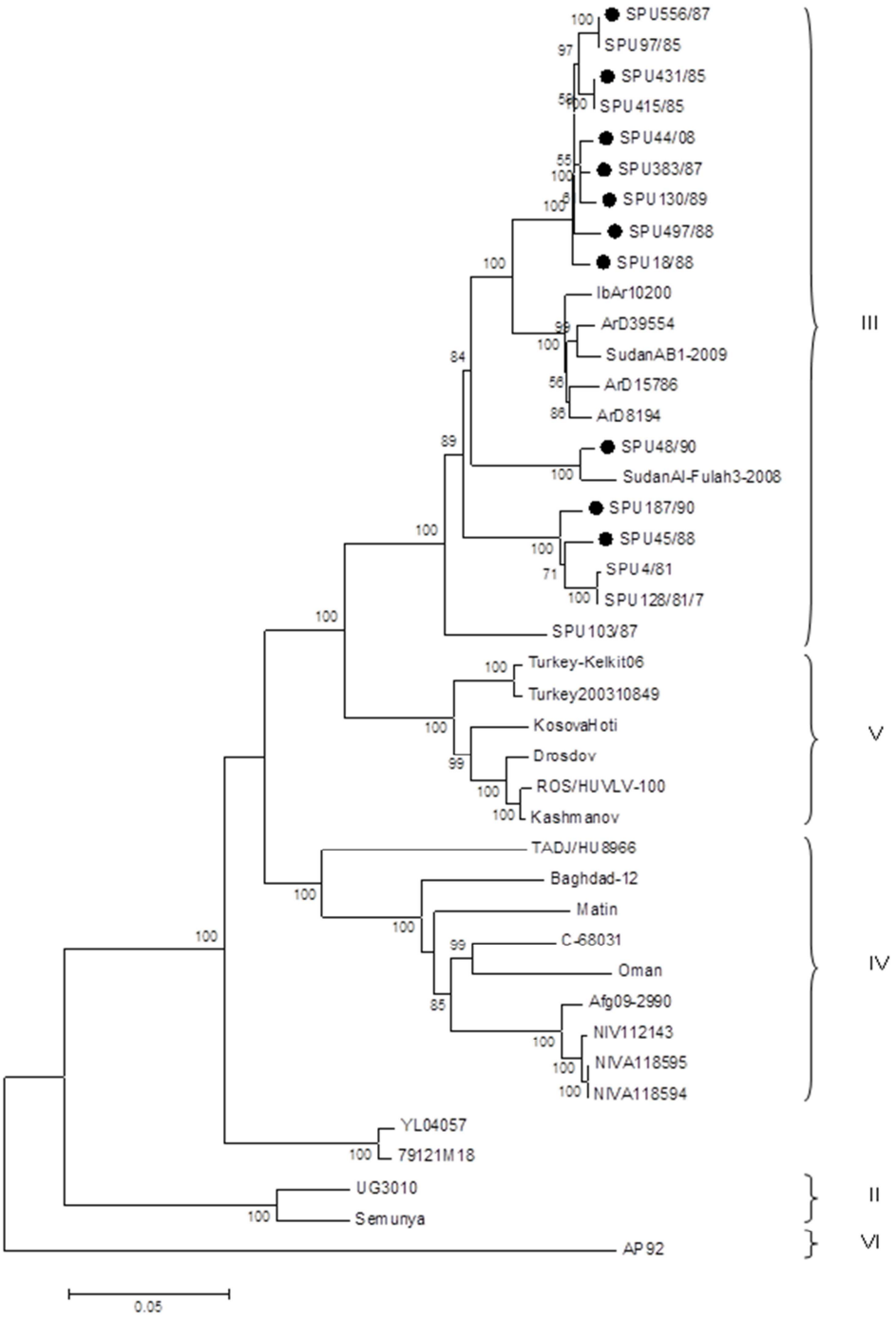

Figure 1 (c)

Phylogenetic analysis of complete coding regions of (a) S segments, (b) M segments, and (c) L segments of CCHFV using a bootstrap maximum-likelihood method with 1000 replicates, with bootstrap values greater than 50% indicated at the relevant nodes. Each sequence is designated by the isolate name and isolates sequenced in the current study are marked (•).

The geographical distribution of CCHFV groups is illustrated in Figure 2. The S and L segments show a strong correlation in the distinct geographical grouping. The map displaying M segment group distribution clearly illustrates the blending of Asian and African strains, while the remaining groups correlate with S and L segment distributions.

The mean *P* distances within groups and between groups as calculated with MEGA support the phylogenetic groupings. The S segment nucleotide distances within groups were generally low at between 0.1 – 1.4% and similar to the amino acid distances within groups which ranged from 0.1 - 1.8%. The nucleotide and amino acid distances between S segment groups were also similar at 2.5 – 6.8% and 2.8 – 8.4% respectively. The L segment showed a similar degree of diversity within groups at both nucleotide and amino acid levels, but increased diversity between groups. Nucleotide distances within groups ranged from 0.4 – 2.5 % and between groups from 3.8 – 11.3%, while amino acid distances within groups were between 0.5 – 1.8% and amino acid distances between groups were 2.9 – 10.1%. The M segment showed the greatest genetic diversity, particularly between groups, including numerous non-synonymous mutations. The nucleotide distances within groups ranged from 0.4 – 5.5%, while the nucleotide distances between groups were between 10.9 – 26.8%. At the amino acid level, the distances within groups were between 1.1 – 8.3% and the distances between groups were between 15.1 – 30.1%.

Among the southern African isolates, the S segment was highly conserved with both nucleotide and amino acid mean distances of <1% (range 0 - 1.3% and 0 - 1.7% respectively). The diversity was also low for the L group with mean distances of 1.7% at the nucleotide level (range 0 - 2.8%) and 1.2% at the amino acid level (range 0 - 2.1%).

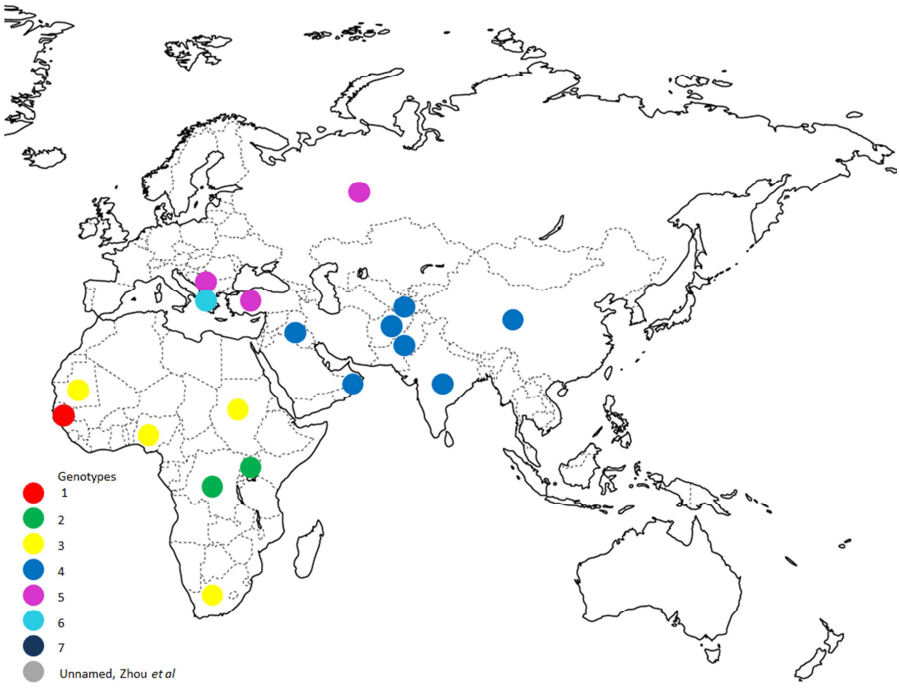

Figure 2(a)

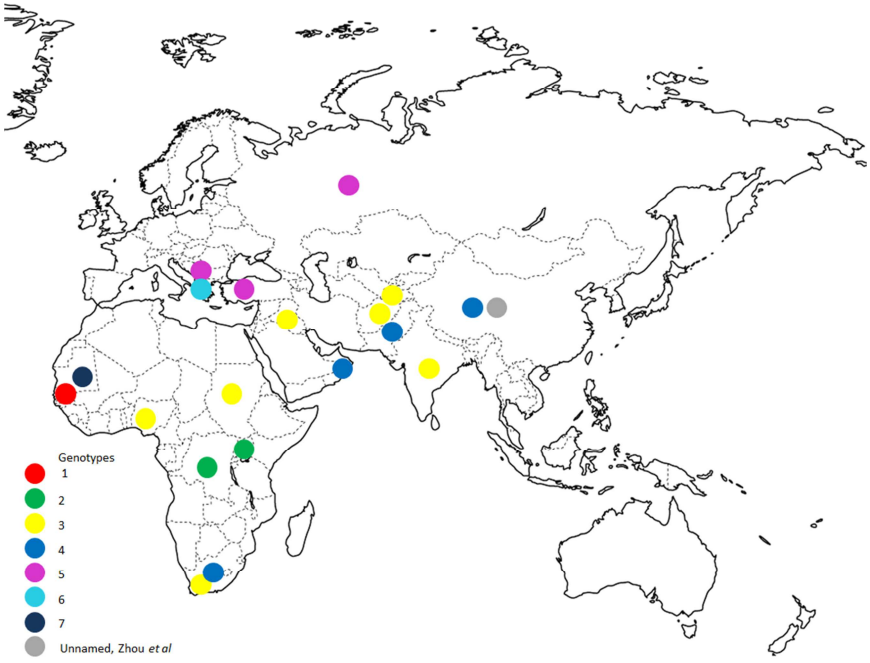

Figure 2(b)

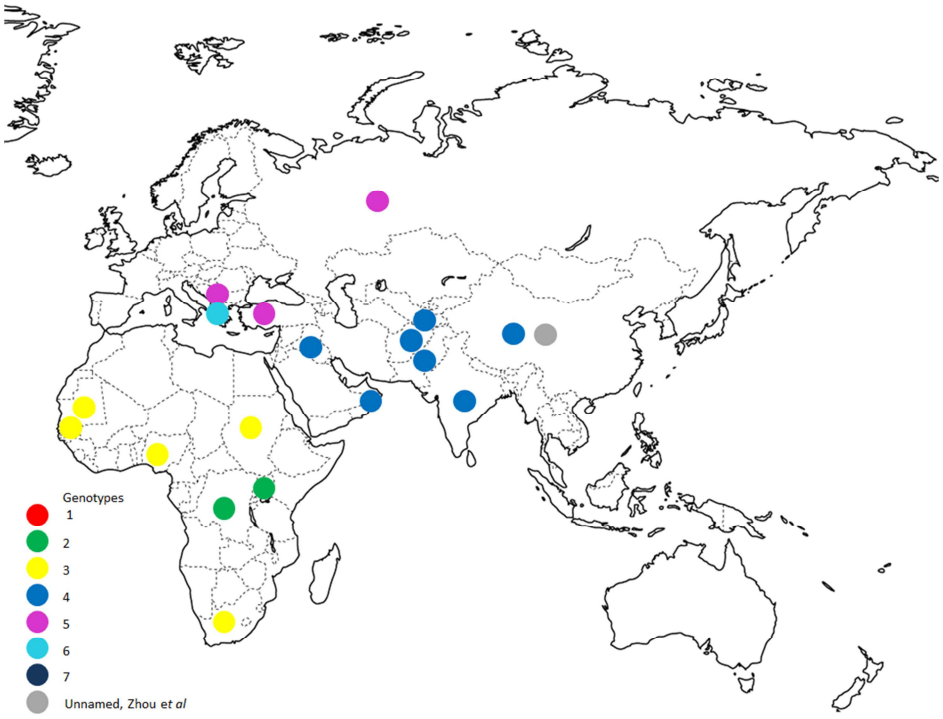

Figure 2(c)

Figure 2

Geographical distribution of CCHFV groups for the (a) S segment, (b) M segment and (c) L segment.

The diversity of M segments was higher among group IV isolates than group III, with mean nucleotide distances of 5% and 2.8% respectively and mean amino acid distances of 7.5% and 4.1% respectively. Overall, the southern African M segments showed distances of 0 - 12.4% at the nucleotide level and 0 - 15.6% at the amino acid level.

Although no association between M segment reassortment and pathogenicity was noted in the southern African isolates included in the current study, the number of both non-fatal

infections and non-reassortants included was small. Furthermore, no temporal association was seen with the isolation of reassortants.

Discussion

The S segment of CCHFV was previously investigated as a surrogate for complete sequencing due to its length and the fact that it is relatively conserved in comparison to the M and L segments thereby simplifying the sequencing process. However, the demonstration of both reassortment (Burt *et al.*, 2005; Burt *et al.*, 2009; Deyde *et al.*, 2006; Grard *et al.*, 2011) and, less commonly, recombination (Lukashev, 2005) among CCHFV genomes confirmed the necessity of at least partial sequencing of all three segments in order to perform accurate genetic analyses. The present study made use of two NGS platforms, namely the Ion Torrent PGM™ and the Roche 454 GS Junior™ sequencing systems, to obtain complete CCHFV sequences for ten southern African isolates. The largest collection of complete CCHFV genome sequences to date made use of 25 S, 40 M and 84 L primers in order to obtain complete sequence data for 13 geographically distinct isolates (Deyde *et al.*, 2006). NGS methods present a relatively simple alternative, requiring only appropriate primers for the generation of amplicons by RT-PCR and potentially a limited number of primers to verify isolated regions or bases. This process could be simplified further by performing NGS of CCHFV directly from clinical samples without prior amplification. This would not only negate the need for specific primers and therefore allow sequencing of diverse CCHFV isolates but would also remove bias introduced by PCR errors (Bracho *et al.*, 1998) as well as multiple passages sometimes required to generate adequate viral titres. This method has been

successfully used to sequence RNA viruses such as lyssaviruses directly from tissue samples and cell culture lysates (Marston *et al.*, 2013). The introduction of errors during PCR can largely be overcome by using high fidelity enzymes. Although the assembly of raw data generated by NGS platforms can be complex, the workflow described in the present study made use almost exclusively of open source software and could be applied to a range of datasets from various sources. Confirmation of the validity of the assemblies obtained was made possible by comparison with a number of partial sequences which were available from a previous study (Burt *et al.*, 2009), confirming the accuracy of the methods used.

Genetic evolution of arboviruses, including CCHFV, is a complex process influenced by multiple factors. As with other RNA viruses, the RNA-dependent RNA-polymerases lack proof-reading activity and show error frequencies of approximately 10^{-4} (Steinhauer *et al.*, 1992). This is offset by the effect of alternating infections of arthropods and vertebrates which constrains virus adaptation of arboviruses in comparison to other RNA viruses (Coffey *et al.*, 2008). Despite this, CCHFV shows a high level of genetic variability. Inclusion of diverse isolates in the current study, particularly from China and central and West Africa, led to nucleotide variation of up to 7, 27 and 11% for the S, M and L segments respectively and amino acid variation of up to 8, 30 and 10%. This is similar to previous studies with greater variability of the M and L segments in comparison to the S segment, which is contrary to expectation as the viral RNA polymerase is usually highly conserved (Anagnostou *et al.*, 2009; Deyde *et al.*, 2006).

Tree topologies for southern African isolates based on partial S, M and L sequence data correlated with topologies constructed using complete genome data (data available from corresponding author on request), providing further evidence that incongruencies in

grouping are likely reassortant events and not recombination (Burt *et al.*, 2009). Genetic relationships indicate movement of CCHFV isolates within and between continents. Isolates from the same group can be found in geographically distinct locations and isolates from different groups can be found to cocirculate in similar regions. Genetic diversity within regions supports movement of the virus by bird migration and livestock trade. Reassortment events have occurred between West African and southern African isolates and between southern African and Asian isolates. Interestingly, the reassortment events between West and southern Africa involved the L segment, whereas reassortment events between southern Africa and Asia involved the M segment. This may be related to the tick species found in these geographical areas. The mechanism of reassortment is unclear however, it is assumed to occur in the tick host where dual persistent infections are more likely. Few complete sequences are available from West Africa but as 3 of the 4 available isolates show either L or M segment reassortment, it appears that reassortant viruses may occur frequently in this area. The two novel Chinese isolates described by Zhou e*t al* (2013) cluster independently in an as yet unnamed group, suggesting the occurrence of further genetic groups. Additional complete genome sequence data from geographically distinct isolates are required to corroborate and expand these findings.

Reassortment is widely described for members of the *Bunyaviridae* family (Briese *et al.*, 2013). Both homologous and heterologous reassortment may occur and may result in altered viral phenotypes. Ngari virus is a reassortant of Bunyamwera and Batai viruses and is associated with haemorrhagic fever, in contrast to the mild disease associated with the parent viruses (Bowen *et al.*, 2001). Both increased neuroinvasiveness and enhanced transmission by insect vectors have been specifically linked to the M segment of La Crosse

virus, another member of the *Bunyaviridae* (Beaty *et al.*, 1981; Gonzalez-Scarano *et al.*, 1992). Burt *et al* (2009) suggested a possible association between M segment reassortment of CCHFV and pathogenicity although this finding was not statistically significant. The current study could not confirm this association but given the small number of isolates included, further investigation is warranted. As 10 of the 15 complete sequences of CCHFV from southern Africa show M segment reassortment, it would be of interest to determine whether these M segments provide a competitive advantage such as the increased transmissibility demonstrated in La Crosse virus reassortants. The postulated co-evolution of S and L segments, possibly related to functional interdependency (Chamberlain *et al.*, 2005), may account for the relative scarcity of reassortment of these segments.

It is likely that both genetic drift and shift of CCHFV genomes occur chiefly during infection of insect vectors rather than in mammalian hosts due to the longer period of infection and increased likelihood of superinfection with more than one strain (Hoogstraal, 1979; Kinsella *et al.*, 2004). In contrast to the short period of viraemia seen in humans (Shepherd *et al.*, 1986), ticks remain infected over much longer periods with transstadial and, more rarely, transovarial transmission playing an important role in virus perpetuation and therefore also virus evolution (Gonzalez *et al.*, 1992; Gordon *et al.*, 1993; Hoogstraal, 1979).

In conclusion, next generation sequencing methods provide a simple and effective alternative to Sanger sequencing for the determination of complete CCHFV genomes. The application of these methods to clinical samples without prior PCR amplification would provide further advantages and should be validated on CCHFV isolates. Our genetic analysis of complete genome sequences confirms the high frequency of M segment reassortment in southern African CCHFV isolates. Further studies are indicated to elucidate the possible

consequences of reassortment particularly relating to viral pathogenicity and transmissibility.

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CHAPTER 6

Comparative analysis of the L, M and S RNA segments of Crimean-Congo haemorrhagic fever virus isolates from southern Africa

Abstract

Crimean-Congo haemorrhagic fever virus (CCHFV) is a member of the *Bunyaviridae* family with a tripartite, negative sense RNA genome. This study used predictive software to analyse the L (large), M (medium) and S (small) segments of 14 southern African CCHFV isolates. The L segment's two domains, namely an OTU-like cysteine protease domain and the RdRp domain, are highly conserved among southern African CCHFV isolates. The M segment encodes the structural glycoproteins, G_N and $G_{C,}$ and the non-structural glycoproteins which are post-translationally cleaved at highly conserved furin and subtilase SKI-1 cleavage sites. All of the sites previously identified were shown to be conserved among southern African CCHFV isolates. The N-terminal variable mucin-like domain of the M segment is heavily O-glycosylated and shows the highest sequence variability of the CCHFV proteins. Five transmembrane domains are predicted in the M segment polyprotein resulting in three regions internal to and three regions external to the membrane across the G_N, NS_M and G_C glycoproteins. Eleven highly conserved N-glycosylation sites are predicted on the M protein. The corroboration of conserved genome domains and sequence identity among geographically diverse isolates may assist in the identification of protein function and pathogenic mechanisms, as well as the identification of potential targets for antiviral therapy and vaccine design.

Introduction

Crimean-Congo haemorrhagic fever virus (CCHFV) is a tick-borne virus belonging to the genus *Nairovirus* in the family *Bunyaviridae*. Other genera in this family are *Orthobunyavirus, Hantavirus, Phlebovirus* and *Tospovirus* (Nichol *et al*., 2006). The single-stranded, negative sense RNA genomes of the *Bunyaviridae* consist of three segments namely the small (S), medium (M) and large (L) segments (Clerx *et al.*, 1981). The CCHFV S segment encodes the nucleocapsid protein which encapsidates both viral RNA (vRNA) and complementary RNA (cRNA) to form ribonucleoprotein complexes. The vRNA serves as a template for both mRNA and cRNA, while the cRNA then serves as template for further vRNA (Bergeron *et al*., 2010). The nucleocapsid protein has a racket-shaped structure consisting of a head or globular domain and a stalk domain which are both composed predominantly of α-helices (Carter *et al*., 2012; Guo *et al*., 2012). In addition to binding RNA for the formation of the ribonucleoprotein complexes, the nucleocapsid protein also shows endonuclease activity attributed to the head domain (Guo *et al*., 2012). The polyprotein encoded by the M segment is co- and post-translationally cleaved into the two structural glycoproteins, G_N and G_C, and three non-structural proteins, NS_M, a mucin-like domain and GP38 (Altamura *et al*., 2007, Sanchez *et al.*, 2006). Two further non-structural glycoproteins, GP85 and GP160, contain GP38 and the mucin-like variable domain. These four non-structural glycoproteins are likely released from infected cells as secretory products (Sanchez *et al*., 2006). The L segment encodes the RNA-dependent RNA polymerase (RdRp) which is responsible for the synthesis of both mRNA for translation into proteins and cRNA for genome replication (Honig *et al.,* 2004).

CCHFV infection in humans can result in haemorrhagic fever with a fatality rate of up to 30%. In recent years, the distribution of disease has expanded to numerous countries in Africa, Asia, the Middle East and Europe (Bente *et al*., 2013). The expanding endemicity in the absence of an effective vaccine or antiviral therapy has prioritized CCHFV research to identify possible conserved targets for such interventions. Antiviral drugs targeting enzymes involved in viral replication are now available for viral infections such as human immunodeficiency virus (HIV), the herpes viruses and hepatitis C. Molecules inhibiting viral adsorption and entry into host cells, such as the CCR-5 inhibitors used for the treatment of HIV, and immunotherapy for pre- and post-exposure prophylaxis are other approaches which have been shown to be effective for managing diverse viral infections. If similar approaches are to be followed for the management of CCHFV infection, then detailed information regarding viral proteins and their functions in geographically distinct isolates will be required. A thorough understanding of the pathogenesis of CCHFV disease is also paramount and this may, in part, be elucidated by the comparison of gene products and protein functions of other viral haemorrhagic fevers such as Ebola and Lassa viruses. In this study, nucleotide and deduced amino acid sequences were analysed using a range of predictive software to identify conserved domains, O- and N-linked glycosylation sites, transmembrane helices, cleavage sites and genetic distances among southern African CCHFV isolates.

Methods

Sequence data set

Complete genome sequences for 14 southern African CCHFV isolates were included in the analysis. The sequence data for ten of the CCHFV isolates were determined in a previous study (Goedhals et al., submitted for review), namely SPU431/85, SPU383/87, SPU556/87, SPU18/88, SPU45/88, SPU497/88, SPU130/89, SPU48/90, SPU187/90 and SPU44/08. A further four complete sequences were retrieved from GenBank, namely SPU415/85, SPU97/85, SPU103/87 and SPU4/81. All of the CCHFV isolates were from humans, with one isolate originating in Namibia while the remaining isolates were from South Africa including the Free State, Northern Cape and North West Provinces. Sequences were aligned using ClustalX version 2.0 (Larkin *et al*., 2007) and manually edited using BioEdit version 7.2.3 (available at http://www.mbio.ncsu.edu/bioedit/bioedit.html).

Sequence analysis

Genome cyclization due to interactions between the complementary 5' and 3' non-coding regions (NCR) was investigated by joining the 5' NCR with an approximately equivalent number of bases from the relevant 3' NCR using a 50 base poly-A spacer (Khromykh *et al*., 2001). The poly-A linked construct for each segment was analyzed using the Mfold Web Server (available at http://mfold.rna.albany.edu/?q=mfold) with default parameters and folding predictions at 37^{o}C (Zuker, 2003). Sequence diversity within specific motif regions was calculated with Molecular Evolutionary Genetics Analysis v5 (MEGA5) using the p-distance option (Tamura *et al*., 2011).

Protein analysis

The deduced amino acid sequences of the L segment were submitted to InterProScan 4 (Zdobnov and Apweiler, 2001) and PSI-BLAST (Altschul *et al*., 1997) to identify functional

sites and conserved protein domains. Prediction of transmembrane helices was performed on the M segment amino acid sequences using TMHMM 2.0 (Krogh *et al.,* 2001) and signal sequence cleavage sites were predicted using SignalP version 4.1 (Petersen *et al*., 2011). Potential sites of N-linked and O-linked glycosylation sites were determined using N-Glycosite (available at www.hiv.lanl.gov/sequence/GLYCOSITE/glycosite.html) (Zhang *et al*., 2004) and NetOGlyc 4.0 (Steentoft *et al*., 2013) respectively. Visual inspection of sequences was performed using BioEdit.

Results

Complete genome sequences

The characteristics of the southern African CCHFV complete genome sequences are summarized in Table 1. The complete L segment, including the 5' and 3' NCR, ranged from 12157 – 12170 nucleotides in length for the southern African CCHFV isolates. The open reading frame (ORF) was 11838 nucleotides in length therefore encoding a protein of 3945 amino acid residues (approximate nucleotide position 77 – 11911). The variability in length of the L segment was chiefly due to variations in size of the 3' NCR (243 – 256 nucleotides) while the 5' NCR (76 nucleotides) and ORF were conserved in length. The 3945 amino acid ORF of the L segment had a GC content of 41%.

Table 1

Southern African CCHFV genome characteristics.

Characteristic	**S segment**	**M segment**	**L segment**
Length (nucleotides)	1671 - 1673	5344 – 5364	12155 – 12170
% GC content	45.9 – 47.7	43.4 – 45.0	41.2 – 41.4
5’ NCR length – nucleotides	54 – 55	77 – 93	76
ORF length – nucleotides (amino acids)	1449 (482)	5055 - 5070 (1684 – 1689)	11832 - 11838 (3943 - 3945)
3’ NCR length – nucleotides	168 – 169	198 – 218	243 - 256

The complete M segment, including the 5’ and 3’ NCR, ranged from 5344 – 5365 nucleotides in length. The M segment 5’ and 3’ NCR were more variable than the L segment, at 77 – 91 nucleotides and 198 – 218 nucleotides in length respectively. The M segment coding region was between 5055 – 5070 nucleotides in length, encoding a polyprotein of 1684 – 1689 amino acid residues. The GC content of the M segments ranged from 43.4 – 45.0%.

The S segment ORF encoded a single nucleoprotein of 482 amino acid residues (1449 nucleotides) in length for all available southern African CCHFV isolates. The complete S segment was 1671 nucleotides long, including a 5’NCR of 55 nucleotides and a 3’NCR of 169 nucleotides and with a GC content of 45.9 – 47.7%.

5’ and 3’ NCR cyclization analysis

Mfold analysis confirmed that the complementary 5' and 3' UTR of each segment allow for cyclization to form a panhandle RNA structure. Figure 1 shows representative examples of the three most stable structures according to the estimated change in Gibbs free energy predicted by Mfold and ranged from -37.17 to -51.91 using SPU97/85 as an example.

Molecular characterization of the L segment ORF

Conserved domains identified by InterProScan and PSI-BLAST analysis included an N-terminal cysteine-protease motif of the ovarian tumour (OTU) protein superfamily and the RNA-dependent RNA polymerase (RdRp) motif. The OTU domain was located at approximate amino acid residues 29 – 158 of the L segment ORF and showed little sequence diversity, with a maximum of two amino acid differences between isolates over the 118 amino acid region and calculated p-distances of 0 – 1.7%. The catalytic triad of the CCHFV OTU-like cysteine protease is predicted to contain cysteine, histidine and aspartic acid residues, initially identified potentially as G**D**GN**C**FYHSIAX$_{100}$**H**FD with the position of the catalytic triad indicated in bold (Duh *et al*., 2008; Kinsella *et al*., 2004). A recent study, however, has identified the catalytic triad as GDGN**C**FYHSIAX$_{100}$**H**F**D** by means of kinetic studies using OTU mutated at the candidate residues (Capodagli *et al*., 2011). The amino acid sequence of the OTU-like cysteine protease catalytic triad was conserved among the southern African CCHFV isolates. The approximate position of the catalytic domain of the RdRp was predicted to occur at amino acid residue positions 2043 – 2776 using PFAM and 2342 – 2551 using PROFILE relative to SPU97/85. The exact position of the RdRp catalytic domain has yet to be experimentally determined. Sequence diversity within the catalytic site ranged from 0 – 1.6% between isolates.

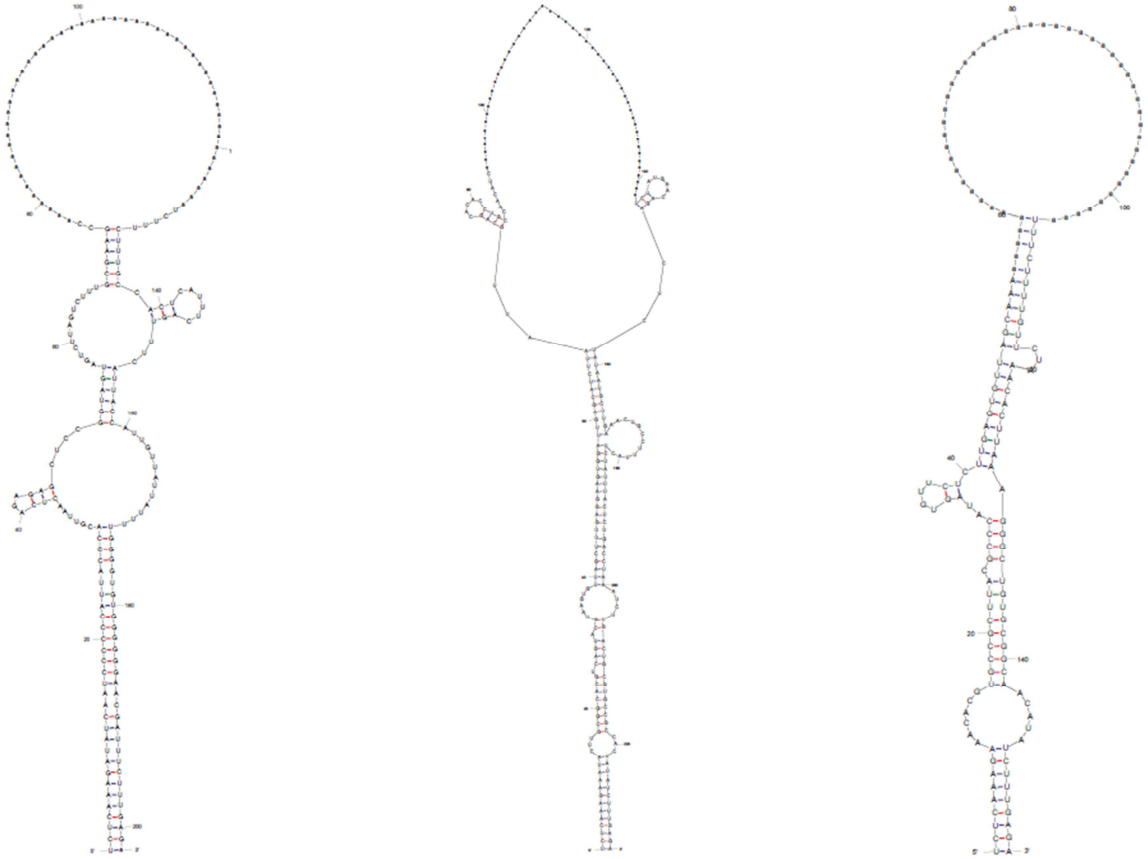

Figure 1

5’ and 3’ NCR complementary regions forming panhandle structures as modelled using Mfold Web Server. A) L segment, dG = -40.48. B) M segment, dG = -51.91. C) S segment, dG = -37.17.

The C2H2-type zinc finger motif (amino acid residue positions 609 – 632) identified previously (Duh *et al*., 2008, Ozdarendeli *et al*., 2010, Yadav *et al*., 2013) was not predicted using either InterProScan or PSI-BLAST although the amino acid sequence in this region was highly conserved. In comparison to Turkey-Kelkit06, Kosova Hoti, NIVA118594, NIVA118595 and NIV112143 CCHFV isolates, a single, semi-conserved amino acid residue substitution of arginine to lysine at position 611 was present in 2/14 southern African isolates and the C2H2 motif was identified visually in all isolates. Similarly, the leucine zipper motif (amino acid residue positions 1386 – 1407) identified in strain IbAr10200 was not predicted in the southern African isolates although a maximum of two amino acid substitutions were present in the region (Honig *et al*., 2004, Kinsella *et al*., 2004). Isoleucine was substituted for leucine at position 1386 in 4/14 isolates and serine for glycine at position 1389 in 13/14 isolates in comparison with IbAr10200. The conserved protein domains in the L ORF are illustrated in Figure 2(a).

Molecular characterization of the M segment

Analysis of the southern African M segments using SignalP identified a signal peptide cleavage site at the N terminus for all isolates with the exception of SPU415/85. The exact position of the cleavage site differed between isolates, occurring variably between amino acid residues 20/21 (SPU187/90 and SPU48/90), 22/23 (SPU4/81 and SPU103/87), 24/25 (SPU383/87, SPU130/89, SPU497/88, SPU431/85, SPU97/85, SPU556/87, SPU45/88 and SPU18/88) or 27/28 (SPU44/08). Signal peptides are commonly found in proteins which are targeted to organelles such as the endoplasmic reticulum or Golgi, and in many membrane-bound proteins. The mucin-like variable region stretches from the signal peptide cleavage site to a furin cleavage site motif, RSKR, which was conserved among all southern African

isolates at either amino acid residue position 244 - 247 or 249 - 252. The calculated p-distance for the mucin-like domain of southern African isolates showed nucleotide sequence diversity of 0 – 40.7% (amino acid diversity 0 – 60.1%) for this region. The furin cleavage site is followed by GP38 which was 804 nucleotides (268 amino acid residues) in length for all southern African CCHFV isolates studied. The nucleotide and amino acid sequences in this region were more conserved than the mucin-like region with p-distances calculated at 0 – 12.7% at the nucleotide level and 0 – 16.8% at the amino acid level. The N terminus of G_N is cleaved from GP38 at a protease SKI-1 cleavage site motif, RRLL, which was conserved among southern African isolates at amino acid residue position 516 – 519 or 521 – 524. The smaller of the two envelope glycoproteins, G_N, was 852 nucleotides (268 amino acid residues) in length and showed nucleotide variation of 0 – 8.1% (amino acid 0 – 8.5%). The C terminus of the G_N protein is cleaved at a further SKI-1 cleavage site motif, RRLL or RKLL, at amino acid residue position 804 – 807 or 809 – 812. Calculated p-distances for the 687 nucleotide (229 amino acid) long NS_M protein were 0 – 10.5% at the nucleotide level and 0 – 11.5% at the amino acid level. A final cleavage site, RRPL or RKPL, was situated at amino acid residue position 1037 - 1040 or 1042 - 1045, at the G_C N terminus. The 1935 nucleotide long G_C protein showed the highest sequence conservation of the M segment polyprotein with amino acid divergence of 0 – 4.8% (nucleotide divergence 0 – 6.2%). The genome organization of the M segment is illustrated in Figure 2(b).

Five transmembrane helices were predicted for all southern African CCHFV isolates analyzed, resulting in three regions internal to and three regions external to the membrane. Using isolate SPU97/85 as an example, the transmembrane helices were located at approximate amino acid residue positions 700 – 722, 825 – 847, 862 – 884, 974 – 996, and

Figure 2(a)

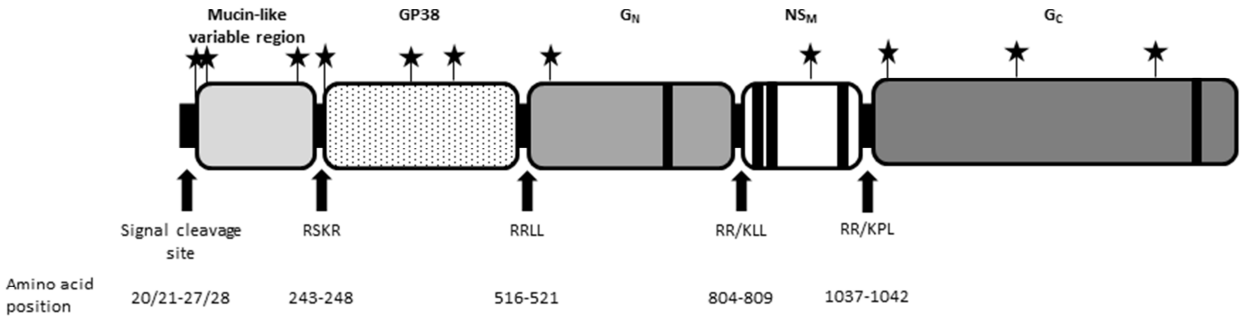

Figure 2(b)

Figure 2

Schematic representation of the protein analysis of the complete genome of southern African CCHFV isolates (not drawn to scale). (a) L segment. (b) M segment. Cleavage sites are indicated by arrows with the amino acid residues and positions indicated below. Transmembrane helices are indicated as black lines in the relevant ORFs. N-glycosylation sites are indicated by stars.

1600 – 1622. These regions were located within the G_N, NS_M, and G_C proteins. Predicted mucin-type GalNAc O-glycosylation sites were identified in 5.4 – 6.5% of the M segment polyprotein, predominantly in the mucin-like variable region where 29.4 – 34.0% of amino acid residues were predicted to be O-glycosylated. In contrast, only 1.3 – 1.9% of the rest of the M segment was predicted to have O-linked glycosylation. O-linked glycosylation involves the addition of N-acetyl-galactosamine to serine or threonine residues. The serine and threonine content in the M segment ORF averaged 9.1% (range 8.9 – 9.3%) and 8.6% (8.3 – 9.1%), respectively. In comparison, the mucin-like variable region showed increased serine and threonine content at 14.6% (range 12.1 – 16.9%) and 17.4% (16.1 – 19.0%), correlating with the distribution of predicted O-linked glycosylation sites. The positions of predicted N-glycosylation sites for all the aligned M segment sequences are summarized in Table 2. Eleven of these predicted sites were conserved in 13 - 14 of the southern African isolates as indicated in Figure 2(b), excluding position 760 which was within a predicted transmembrane helix region. The M segment of the southern African CCHFV isolates contained 79 cysteine residues which were highly conserved. This may suggest the presence of a large number of disulphide linkages, however the G_N cytoplasmic tail has been shown to contain two ββα-type zinc fingers with a **C**X_2**C**$X_{11\text{-}12}$**H**X_3**C** motif of cysteine and histidine residues (Estrada and De Guzman, 2011). These motifs were conserved in the southern African isolates (Figure 3) beginning at amino acid residue positions 736/741 and 761/766 respectively and separated by a 4 amino acid linker, specifically SYNI.

Table 2

N-linked glycosylation sites on the M segment ORF as predicted by N-Glycosite. Amino acid residue positions are based on the aligned ORF sequences to allow uniformity of numbering. Position 760 forms part of a predicted transmembrane helix and is therefore unlikely to be N-glycosylated.

	30	35	37	46	82	108	116	186	201	205	229	248	381	431	562	760	981	1059	1350	1568
SPU4/81	N	N		N					N	N		N	N	N	N	N	N	N	N	N
SPU187/90		N		N					N	N		N	N	N	N	N	N	N	N	N
SPU48/90	N	N		N				N		N		N	N	N	N	N	N	N	N	N
SPU103/87	N	N								N		N	N	N	N	N	N	N	N	N
SPU44/08	N	N								N		N	N	N	N	N	N	N	N	N
SPU383/87	N	N								N		N	N	N	N	N	N	N	N	N
SPU130/89	N		N							N		N	N	N	N	N	N	N	N	N
SPU497/88	N	N								N		N	N	N	N	N	N	N	N	N
SPU18/88	N	N								N		N	N	N	N	N	N	N	N	N
SPU45/88	N	N				N				N		N	N	N	N	N	N	N	N	N
SPU556/87	N	N			N		N			N		N	N	N	N	N	N	N	N	N
SPU97/85	N	N			N		N			N		N	N	N	N	N	N	N	N	N
SPU431/85	N	N			N					N	N	N	N	N	N	N	N	N	N	N
SPU415/85	N	N			N					N	N	N	N	N	N	N	N	N	N	N

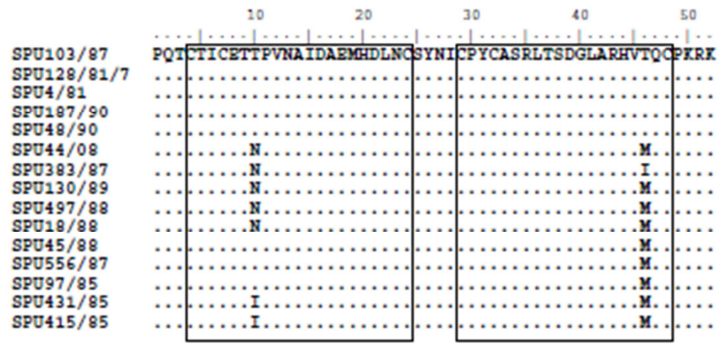

Figure 3

The amino acid sequences of the dual CCHC-type zinc finger motifs in the G_N tail. Conserved residues are indicated by a dot, while substitutions are indicated by the relevant amino acid. The conserved **C**-X_2-**C**-$X_{11\text{-}12}$-**H**-X3-**C** motifs of each zinc finger are outlined.

Discussion

Characterization of the deduced amino acid sequences for the L segment of 14 southern African CCHFV isolates confirmed two conserved functional domains as previously identified, namely the OTU cysteine protease domain and the RdRp catalytic domain (Duh *et al*., 2008, Honig *et al*., 2004, Kinsella *et al*., 2004, Ozdarendeli *et al*., 2010, Yadav *et al*., 2013). Analysis of domains in southern African isolates was consistent with geographically distinct isolates. It has been shown that the OTU cysteine protease domain situated in the N terminus of the CCHFV L protein is not required for viral replication or RdRp function (Bergeron *et al.*, 2010). Rather, these proteases hydrolyze ubiquitin and interferon-stimulated gene product 15 (ISG15) thereby allowing the virus to evade the type 1 interferon and tumour necrosis factor alpha cytokine pathways (Frias-Staheli *et al*., 2007). This domain is highly conserved, showing 98.3 - 100% sequence identity among southern

African CCHFV isolates. Similarly, the predicted catalytic domain of the RdRp is highly conserved among isolates as would be expected of the functional site of an enzyme which is essential for viral replication. Despite the sequence conservation of the previously identified C2H2-type zinc finger motif and leucine zipper among geographically diverse isolates (Duh *et al*., 2008; Honig *et al.,* 2004; Kinsella *et al.,* 2004; Ozdarendeli *et al*., 2010; Yadav *et al*., 2013), these motifs were not identified by the predictive software used in this analysis. To confirm that sequence differences were not the reason for this discrepancy, the published isolates were analysed and the zinc finger and leucine zipper were not predicted. This may be due to the dynamic nature of the hidden Markov model based databases such as Pfam (Finn *et al*., 2010) and the extensive variation in the primary protein structure between nucleotide polymerases and RdRp. The L segment translational product is poorly defined with regards to posttranslational modifications including proteolytic cleavage and maturation and experimental studies are required to confirm such motifs.

The genome organization of CCHFV M segment follows the sequence: 5'NCR-mucin like region-GP38-G_N-NS_M-G_C-3'NCR. Cleavage of the M polyprotein is accomplished at four cleavage sites including one furin cleavage site (RSKR) and two subtilase SKI-1 cleavage sites (RRLL, RR/KLL) (Vincent *et al*., 2003; Sanchez *et al.,* 2006). The final cleavage site (RR/KPL), which separates G_C from NS_M, has been shown to be poorly cleaved by SKI-1 although the protease involved in this cleavage event has not been identified (Vincent *et al*., 2003). The four cleavage sites are conserved among all CCHFV isolates including those from southern Africa. The structural glycoproteins, G_N and G_C, were the most conserved of the M segment proteins with up to 91.9% nucleotide (91.5% amino acid) and 95.2% nucleotide (93.8% amino acid) sequence identity between southern African isolates respectively. The role of

the non-structural or secretory glycoproteins (mucin-like variable domain, GP38, NS_M, GP85 and GP160) in CCHFV infection is currently unclear, but a similar mucin-like domain glycoprotein of Ebola virus plays a role in viral pathogenesis by causing endothelial cell disruption resulting in increased vascular permeability (Yang *et al*., 2000). Sequence diversity was highest in the mucin-like variable region with divergence of up to 40.7% at the nucleotide level and 60.1% at the amino acid level. CCHFV utilizes various vertebrate and tick hosts in distinct geographical regions. This ability may relate to the variation within the mucin-like region (Ozdarendeli *et al*., 2010). This is supported by grouping of isolates based on phylogenetic analysis of the mucin-like region which correlates with the complete M segment (data not shown) showing geographic linkage of isolates within groups. The mechanism underlying the variablility of mucin-like regions of negative stranded RNA viruses including CCHFV has been explained chiefly by a relaxation of purifying selection in this region. This implies that the amino acid composition of the region is not important for its function as long as the O-glycosylation is maintained (Wertheim and Worobey, 2009).

Although the high cysteine content of the M protein which is conserved in all available CCHFV isolates may point to extensive disulphide bonds and a complex secondary structure, the presence of two CCHC-type zinc finger motifs in the G_N cytoplasmic tail also contribute to this cysteine content. The G_N tail has been shown to bind RNA and likely plays a role in RNA packaging and viral assembly by associating with the viral ribonucleoprotein complexes, as do the glycoprotein zinc fingers of hantaviruses, also belonging to the *Bunyaviridae* family (Estrada and De Guzman, 2011).

Of the 11 conserved N-linked glycosylation sites predicted for the M segments of southern African CCHFV isolates, three have thus far been confirmed experimentally namely one site

located on G_N (amino acid residue position 557) and two sites located on G_C (amino acid residue positions 1054 and 1563). The N-linked glycosylation site on G_N was shown to be important for glycoprotein localization and for transport of both G_N and G_C proteins from the endoplasmic reticulum to the Golgi apparatus (Erickson *et al*., 2007).

The conserved nucleotide complementarity of the 5' and 3' NCR of the L, M and S segments of CCHFV were modelled using Mfold and showed cyclization to form panhandle structures. This can be compared to the complementary genomic ends of another member of the *Bunyaviridae* family, Bunyamwera virus. In the case of Bunyamwera virus, the cooperation of the complementary 5' and 3' NCR were required for RNA synthesis to generate both mRNA and cRNA (Barr and Wertz, 2004). A similar role in CCHFV replication seems likely. The first nine nucleotides of the L, M and S segments are identical in CCHF, as in other nairoviruses such as Dugbe virus and Hazara virus, and may function as an RdRp recognition site for the initiation of viral mRNA transcription and replication (Lasecka and Baron, 2013; Marriott *et al*., 1992).

Genetic characterization of CCHFV isolates can help to identify protein functions and to suggest pathogenic mechanisms which require further examination. The development of diagnostic assays, vaccine design and identification of potential targets for antiviral interventions may also benefit, particularly when information from geographically distinct areas is available. It is clear that further experimental studies are required to investigate the significance and function of many of the CCHFV genome properties identified in this way. However, this examination of southern African CCHFV isolates provides corroboration of conserved genome domains and sequence identity which may direct further studies.

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CHAPTER 7

Conclusions and future perspectives

CCHFV is endemic in South Africa and is emerging as an important pathogen in Europe and Asia. In the absence of specific treatment or a vaccine, determination of immune correlates and a better understanding of pathogenic mechanisms are essential to guide investigation of therapeutic or preventative interventions. This study was aimed at investigating immune responses in survivors of CCHF in South Africa, with particular emphasis on investigating the presence of detectable memory T lymphocyte responses and epitopic regions within the nucleoprotein/or and glycoproteins. The human immune response to viral infections is a complex process comprised of innate and adaptive responses. The innate immune response includes factors such as interferon production and natural killer cells (Campbell and Hasegawa, 2013; Yan and Chen, 2012). In the case of CCHFV, the virus has been shown to delay interferon production, in part mediated by the OTU-like cysteine protease domain of the L segment, thereby allowing viral replication in the early stages of infection (Akinci *et al.*, 2013; Weber and Mirazimi, 2008). The adaptive immune response is comprised of humoral and cellular components. The humoral response consists primarily of virus-specific antibody production by B cell lymphocytes. The antibodies produced can both protect from infection and assist in clearance of established infection. This is accomplished not only through direct virus neutralization but also through antibody dependent cell-mediated cytotoxicity and complement activation (Dörner and Radbruch, 2007). Infection with CCHFV is known to induce antibody production and this attribute is widely used to aid diagnosis by means of

serological assays. The majority of antibody responses that have been described previously are based on ELISA and immunofluorescent techniques rather than virus neutralization assays. Using monoclonal antibodies raised in mice, cross-reactive neutralizing antibodies have been demonstrated to a conserved epitope on the G_C glycoprotein (Ahmed *et al.*, 2005). However, antibody production does not directly correlate with viral clearance and the immune response to CCHFV is likely to also involve both innate and cellular components (Wölfel *et al.*, 2007). Both humoral and cell mediated immune responses result in the production of long-lived memory cells which can recognize and respond rapidly following re-exposure to an antigen which has previously been encountered.

Although related bunyaviruses such as Hantaan virus and Puumala virus have been shown to induce long-lived memory T cell responses (Van Epps *et al.*, 2002; Wang *et al.*, 2011), it was unknown whether acute CCHFV infection would induce memory T cells present at high enough frequencies in the peripheral blood to be detectable using ELISPOT assays in the absence of continued antigenic exposure or reinfection. No data is currently available regarding T cell responses following CCHFV infection. Using a synthetic overlapping peptide library to screen for interferon gamma production by PBMC from survivors of CCHFV infection, ten potential epitopic regions were identified. The majority of these were located on the nucleoprotein with only two regions identified on the glycoprotein G_C in a single patient. T cell responses to the nucleoprotein of Rift Valley fever virus are detected in patients with a history of RVF infection and can be induced by vaccination. These vaccines can induce protective immunity in the absence of neutralizing antibody, likely through cell mediated immunity (Xu *et al.*, 2103). This study provides the first report of long-lived memory CD8+ T cell responses in survivors of CCHF up to 13 years after infection. For

vaccine development, it is necessary to differentiate between the mechanisms of protection and mechanisms of recovery. Cellular responses are frequently more significant for recovery from viral infection or viral clearance (Plotkin, 2010). An effective vaccine will likely require induction of both cellular and humoral responses. If the nucleoprotein induces memory T cells then one can theorize that a vaccine without a nucleoprotein component may not induce memory T cell responses. The significance of this will depend on the role that it plays in protection against disease or in recovery. The frequency of responses detected in some CCHFV survivors is similar to those detected in Hantaan and Puumala viruses and correlate with responses induced by other acute infections such as influenza viruses (Van Epps *et al.*, 2002; Wang *et al.*, 2011). These data indicates that induction of effective long term cellular immune responses should be possible through vaccination and gives an indication of epitopic regions that should be included in the vaccine and applied to assays testing vaccine immunogenicity. Although the magnitude and frequency of spot forming cells in the ELISPOT assays were similar to those detected previously for Hantaan and Puumala viruses, the levels were far lower than those detected in Rift Valley fever survivors (Xu *et al.*, 2103). It may be significant to determine whether there is a threshold level of memory response required to confer full protection or full clearance of the virus on re-exposure. Further studies are planned to confirm the optimal epitopes using peptide nonamers in an ELISPOT format and to determine the HLA restriction of these optimal epitopes. Depletion studies confirmed that CD8+ cells were likely the dominant phenotype secreting IFN-γ in the memory response. More detailed information regarding the subsets of memory T cells involved in the long-lived responses and the role of CD8+ and CD4+ cells in secretion of additional cytokines will be determined using flow cytometry. The reasons for severity of illness are not clear however cytokine activation may

play a role in pathogenicity during the acute phase of infection. A comparison of cytokine levels in serum samples has shown high levels of TNF-α associated with severe disease and IL-6 levels were high in both severe and mild disease (Papa *et al.*, 2006). A comparison of cytokine secretion from PBMC collected from fatal and non-fatal cases during acute illness would be useful for further defining the role of T cell responses. In addition, functional assays including the CD107a degranulation assay will be used to confirm that the cytokine secreting cells are functional and able to induce cytolysis. As correlates of protection may be dependent on antigen presentation on MHC and therefore HLA type, it will be important to determine HLA restriction of these epitopes before making significant decisions regarding vaccine development.

Rapid, sensitive and specific assays are required for increasing diagnostic and surveillance capacity. The use of synthetic peptides in ELISA provides an alternative approach for safe production of diagnostic reagents. Identification of epitopic regions on viral proteins would greatly aid the development of serological assays. If these epitopes induce protective antibody responses, then they could also find relevance in the production of subunit vaccines. Linear epitopes are short peptide fragments contiguous in the primary amino acid sequence which comprise approximately 10% of B cell epitopes. Although less frequent than discontinuous epitopes, they are potentially involved in inducing neutralizing antibodies and/or antibodies allowing detection and diagnosis. The peptide library was therefore used to screen for linear B cell epitopes in survivors of CCHFV infection. Two potential epitopic regions were identified on the G_C glycoprotein with reactivity in 13 – 14 of 15 patients tested. These two epitopic regions are located within a conserved region of the genome and within regions of hydrophilicity suggesting that they are exposed and therefore

increasing the likelihood that they have antigenic significance. One of the reactive peptides identified, peptide $G_{1613\text{-}1631}$, overlaps peptide $G_{1622\text{-}1640}$ which also showed reactivity although to a lesser extent and in only 7 of 15 patients tested. This lack of reactivity against $G_{1622\text{-}1640}$ in some sera could be as a result of a lower antibody titre or due to the position of the epitope which may partially overlap peptide $G_{1613\text{-}1631}$. In contrast the optimal epitope of the other reactive peptide, peptide $G_{1451\text{-}1469}$, is likely situated within the centre of the 19mer as no reactivity was observed against overlapping peptides. This region is also highly conserved within geographically distinct isolates. Further investigation will be required to determine whether these epitopic regions also correlate with immune protection and to identify non-contiguous B cell epitopes which are likely to play an important role in antibody induction during natural infection with CCHFV.

Expanding CCHFV complete genome sequence data for isolates from geographically distinct regions will contribute to studies investigating immune correlates of protection and development of diagnostic reagents for rapid detection and identification. Complete sequence data will also have application in identifying conserved epitopic regions that play a role in inducing memory T cell responses. Development of serological and molecular diagnostic assays that can be applied to isolates distributed worldwide will depend on knowledge of cross reactivity and conservation of targeted regions of the genome. To date there were 31 complete genome sequences available on GenBank hence it was deemed necessary to determine sequence data for additional isolates from our Southern African cohort. To investigate CCHFV sequence diversity among southern African isolates and isolates from other endemic countries, ten complete CCHFV genomes were sequenced using next generation sequencing techniques. With new foci of CCHFV infection emerging over

recent years, it is important to ascertain whether genomic variation is likely to have an effect on vaccine efficacy and ensure that vaccine candidates and diagnostic assays will be applicable in distinct geographic areas. This paper reports the first application of next generation sequencing technology to CCHFV isolates. These techniques are rapid and cost effective alternatives to standard Sanger sequencing which can be effectively applied to the approximately 20kb CCHFV genome. The results confirmed that, at a genomic level, there is extensive variability among geographically distinct CCHFV isolates. While point mutations are common and result in increased genetic diversity over time, whole segment reassortment is also common among CCHFV isolates and contributes significantly to genetic variability. The role of reassortment in viral pathogenicity in humans is as yet unclear, but animal studies using one of the recently described mouse models may shed some light on this. Despite the extensive variability noted, at a protein level, the motifs involved in protein function are well conserved. Prediction software confirmed the presence of conserved OTU-like cysteine protease and RNA dependent RNA polymerase (RdRp) domains in the L segment of diverse southern African CCHFV isolates. The RdRp is essential for viral replication while the OTU-like protease likely plays a role in immune evasion and therefore affects viral pathogenicity. Analysis of the proteins encoded by the M gene showed conservation of the basic protein coding strategy, with two structural and three non-structural glycoproteins. However, amino acid variation was notable across all predicted proteins but particularly in the variable mucin-like domain which is thought to play a role in viral pathogenicity. This study provides targets for further investigation into viral pathogenicity which may include *in vivo* studies in animal models and mutagenicity assays. The identification of conserved protein motifs such as the OTU-like cysteine protease has

application in the development of novel anti-viral therapies targeting these proteins such as cysteine-modifying agents (Casini *et al.*, 2002).

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APPENDIX A

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2013-11-06

REC Reference nr 230408-011
IRB nr 00006240

PROF F BURT
DEPT OF MEDICAL MICROBIOLOGY AND VIROLOGY
FACULTY OF HEALTH SCIENCES
UFS

Dear Prof Burt

ETOVS NR 152/06
PROF F BURT DEPT OF MEDICAL MICROBIOLOGY AND VIROLOGY
PROJECT TITLE: IMMUNE RESPONSES IN SURVIVORS OF CRIMEAN-CONGO HAEMORRHAGIC FEVER AND EVALUATION OF CANDIDATE VACCINES.

- You are hereby kindly informed that the Ethics Committee approved the following and it will be condoned at the meeting scheduled for 26 November 2013:
  - ***Amendment to the protocol***
  - ***Extension of the study period to December 2014***
  - ***Two additional collaborators: Dr A Meyers and Mr R Atkinson from the University of Cape Town***

- Committee guidance documents: Declaration of Helsinki, ICH, GCP and MRC Guidelines on Bio Medical Research. Clinical Trial Guidelines 2000 Department of Health RSA; Ethics in Health Research: Principles Structure and Processes Department of Health RSA 2004; Guidelines for Good Practice in the Conduct of Clinical Trials with Human Participants in South Africa, Second Edition (2006); the Constitution of the Ethics Committee of the Faculty of Health Sciences and the Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines.

- Any amendment, extension or other modifications to the protocol must be submitted to the Ethics Committee for approval.

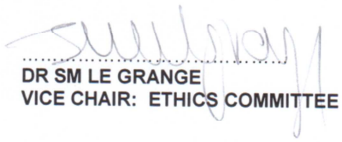
- The Committee must be informed of any serious adverse event and/or termination of the study.

- All relevant documents e.g. signed permission letters from the authorities, institutions, changes to the protocol, questionnaires etc. have to be submitted to the Ethics Committee before the study may be conducted (if applicable).

- A progress report should be submitted within one year of approval of long term studies and a final report at completion of both short term and long term studies.

- Kindly refer to the ECUFS reference number in correspondence to the Ethics Committee secretariat.

Yours faithfully



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DR SM LE GRANGE
VICE CHAIR: ETHICS COMMITTEE

APPENDIX B

UNIVERSITEIT VAN DIE VRYSTAAT
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Ms H Strauss

2008-03-13

DR F BURT
DEPT OF VIROLOGY
FACULTY OF HEALTH SCIENCES
UFS

Dear Dr Burt

ETOVS NR 152/06
PROJECT TITLE: IDENTIFICATION OF CTL EPITOPES IN SURVIVORS OF CRIMEAN-CONGO HAEMORRHAGIC FEVER INFECTION AND EVALUATION OF CANDIDATE VACCINES.

- You are hereby informed that at the meeting on 11 March 2008 The Ethics Committee approved the following:

- *Title changed to: "Immune responses in survivors of Crimean-Congo haemorrhagic fever and evaluation of candidate vaccines".*
- *Addition of students: Dr D Goedhals and Ms R Samudzi*
- *Addition of collaborators: Prof M Heise, University of North Carolina; Dr T Ross, Centre for Vaccine Research, Pittsburgh.*

- Committee guidance documents: Declaration of Helsinki, ICH, GCP and MRC Guidelines on Bio Medical Research. Clinical Trial Guidelines 2000 Department of Health RSA; Ethics in Health Research: Principles Structure and Processes Department of Health RSA 2004; the Constitution of the Ethics Committee of the Faculty of Health Sciences and the Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines.

- Any amendment, extension or other modifications to the protocol must be submitted to the Ethics Committee for approval.

- The Committee must be informed of any serious adverse event and/or termination of the study.

- A progress report should be submitted within one year of approval of long-term studies and a final report at completion of both short term and long term studies.

- Kindly refer to the ETOVS reference number in correspondence to the Ethics Committee secretariat.

Yours faithfully

for **PROF BB HOEK**
CHAIR: ETHICS COMMITTEE

339, Bloemfontein 9300,RSA ☎ (051) 405 2812 gndkhs.md@ufs.ac.za
Republiek van Suid-Afrika / Republic of South Africa

APPENDIX C ***CONSENT DOCUMENT 1*** ***ETOVS no***

CONSENT TO PARTICIPATE IN RESEARCH

You have been asked to participate in a research study. You have been informed about the study by your general practitioner or other healthcare worker.

You may contact Prof F J Burt at 051 405 3173 at any time if you have questions about the research. You may contact the Secretariat of the Ethics Committee of the Faculty of Health Sciences, UFS at telephone number (051) 4052812 if you have questions about your rights as a research subject.

Your participation in this research is voluntary, and you will not be penalized or lose benefits if you refuse to participate or decide to terminate participation. If you agree to participate, you will be given a signed copy of this document as well as the participant information sheet, which is a written summary of the research.

The research study, including the above information has been verbally described to me. I understand what my involvement in the study means and I voluntarily agree to participate. I consent that

1. I will donate blood on 3 separate occasions as described in the information document
2. my donated blood maybe stored and analyzed as part of future research
3. research data obtained from the study may be published anonymously in a scientific journal

Name of participant Telephone no

Email address

Approximate date of CCHF infection

_____ _____

Signature of Participant Date

_____ _____

Signature of Witness Date
(Where applicable)

_____ _____

Signature of Translator Date
(Where applicable)

INFORMATION DOCUMENT 1

Study title: Immune responses in survivors of Crimean-Congo haemorrhagic fever infection and evaluation of candidate vaccines.

Good morning
We, the Department of Virology, are doing research to identify how the human body responds to infections of Crimean-Congo haemorrhagic fever (CCHF). In this project we want to study which cells in your immune response contributed to your recovery from the infection

Invitation to participate: We are inviting you to participate in this research study

What is involved in the study – We are inviting patients with a confirmed history of infection with CCHF to participate in the study. We will request blood from you on 3 occasions. We require initially request 1x 10 ml blood tube and then 4 x 10 ml blood tubes on two separate occasions. Different cells in the body are activated when we have a viral infection and these cells all play a role in clearing the virus from our body. We will isolate blood cells from your sample and try to identify which cells were activated by the virus. We will use DNA from your blood to identify immune cells specific to you and we will use the blood to look at your immune response. Patients who survive infection with CCHF virus have antibodies against the virus. It is possible to isolate and immortalize (keep) the cells that are responsible for making these antibodies. We request permission to store these cells indefinitely and use them for related projects to study the CCHF virus.

Risks There are no foreseeable risks of being involved in the study:

Benefits There is very little information available regarding the mechanisms by which the body responds to infection against CCHF virus and we are hoping this study will lead to results that will contribute to development of a vaccine or specific treatment.

Participation is voluntary, and refusal to participate will involve no penalty or loss of benefits to which the subject is otherwise entitled; the subject may discontinue participation at any time without penalty or loss of benefits to which the subject is otherwise entitled.

Reimbursements We can reimburse you for any cost incurred at the pathology laboratory for collection of blood for this study.

Confidentiality: Efforts will be made to keep personal information confidential. Absolute confidentiality cannot be guaranteed. Personal information may be disclosed if required by law. Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as the Ethics Committee for Medical Research.

Contact details of researcher– Prof Felicity Burt, Department of Virology, University of the Free State, Bloemfontein. Tel 051 4053173, burtfj.md@ufs.ac.za

Contact details of REC Secretariat and Chair – For reporting complaints or problems please contact

Ms H Strauss
Research Division
Faculty if Health Sciences
University of the Free State
E-mail address: gndkhs.md@mail.uovs.ac.za
☎(051) 4052812

APPENDIX D ***CONSENT DOCUMENT 2*** ***ETOVS no***

CONSENT TO PARTICIPATE IN RESEARCH

You have been asked to participate in a research study. You have been informed about the study by your general practitioner or healthcare worker.

You may contact Prof F J Burt at 051 405 3173 any time if you have questions about the research. You may contact the Secretariat of the Ethics Committee of the Faculty of Health Sciences, UFS at telephone number (051) 4052812 if you have questions about your rights as a research subject.

Your participation in this research is voluntary, and you will not be penalized or lose benefits if you refuse to participate or decide to terminate participation. If you agree to participate, you will be given a signed copy of this document as well as the participant information sheet, which is a written summary of the research.

The research study, including the above information has been verbally described to me. I understand what my involvement in the study means and I voluntarily agree to participate. I consent that:
I will donate blood as described in the information document
my donated blood may be stored and analyzed as part of future research
research data obtained from the study may be published anonymously in a scientific journal

Name of participant Telephone no

Email address

Approximate date of CCHF infection

_____ _____
Signature of Participant Date

_____ _____
Signature of Witness Date
(Where applicable)

_____ _____
Signature of Translator Date
(Where applicable)

INFORMATION DOCUMENT 2

Study title: Immune responses in survivors of Crimean-Congo haemorrhagic fever infection and evaluation of candidate vaccines.

Good morning
We, the Department of Virology, are doing research to identify how the human body responds to infections of Crimean-Congo haemorrhagic fever (CCHF). In this project we want to study which cells in your immune response contributed to your recovery from the infection

Invitation to participate: We are inviting you to participate in this research study

What is involved in the study – We are inviting patients with a confirmed history of infection with CCHF to participate in the study. We will request blood 2x 10 ml blood tubes. Patients who survive infection with CCHF virus have antibodies against the virus. We want to use these antibodies to study how the body reacts to different proteins in the virus. We request permission to store your blood indefinitely for related projects to study the CCHF virus.

Risks There are no foreseeable risks of being involved in the study:

Benefits There is very little information available regarding the mechanisms by which the body responds to infection against CCHF virus and we are hoping this study will lead to results that will contribute to development of a vaccine or specific treatment.

Participation is voluntary, and refusal to participate will involve no penalty or loss of benefits to which the subject is otherwise entitled; the subject may discontinue participation at any time without penalty or loss of benefits to which the subject is otherwise entitled.

Reimbursements We can reimburse you for any cost incurred at the pathology laboratory for collection of blood for this study.

Confidentiality: Efforts will be made to keep personal information confidential. Absolute confidentiality cannot be guaranteed. Personal information may be disclosed if required by law. Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as the Ethics Committee for Medical Research.

Contact details of researcher– Prof Felicity Burt, Department of Virology, University of the Free State, Bloemfontein. Tel 051 4053173, burtfj.md@ufs.ac.za

Contact details of REC Secretariat and Chair – For reporting complaints or problems please contact
Ms H Strauss
Research Division
Faculty if Health Sciences
University of the Free State
E-mail address: gndkhs.md@mail.uovs.ac.za

APPENDIX E ***CONSENT DOCUMENT 3*** ***ETOVS no***

CONSENT TO PARTICIPATE IN RESEARCH

You have been asked to participate in a research study. You have been informed about the study by your general practitioner or healthcare worker.

You may contact Prof F J Burt at 051 405 3173 any time if you have questions about the research. You may contact the Secretariat of the Ethics Committee of the Faculty of Health Sciences, UFS at telephone number (051) 4052812 if you have questions about your rights as a research subject.

Your participation in this research is voluntary, and you will not be penalized or lose benefits if you refuse to participate or decide to terminate participation. If you agree to participate, you will be given a signed copy of this document as well as the participant information sheet, which is a written summary of the research.

The research study, including the above information has been verbally described to me. I understand what my involvement in the study means and I voluntarily agree to participate. I consent that:
I will donate blood as described in the information document
my donated blood may be stored and analyzed as part of future research
research data obtained from the study may be published anonymously in a scientific journal

Name of participant Telephone no

Email address

_____ _____
Signature of Participant Date

_____ _____
Signature of Witness Date
(Where applicable)

_____ _____
Signature of Translator Date
(Where applicable)

INFORMATION DOCUMENT 3

Study title: Immune responses in survivors of Crimean-Congo haemorrhagic fever infection and evaluation of candidate vaccines.

Good morning
We, the Department of Virology, are doing research to develop new laboratory tests for detecting Crimean-Congo haemorrhagic fever (CCHF) infections. In this project we want to use blood from volunteers that have not previously had CCHF virus. The blood will be used as negative controls for development of laboratory tests.

Invitation to participate: We are inviting you to participate in this research study

What is involved in the study – We are inviting patients with NO history of infection with CCHF virus to participate in the study. We will request blood 2x 10 ml blood tubes. We request permission to store your blood indefinitely for related projects to study the CCHF virus.

Risks There are no foreseeable risks of being involved in the study:

Benefits We are developing rapid and reliable tests for detecting CCHF virus infections and request blood from healthy donors with no history of infection with the virus that will be used to determine the specificity of the assays.

Participation is voluntary, and refusal to participate will involve no penalty or loss of benefits to which the subject is otherwise entitled; the subject may discontinue participation at any time without penalty or loss of benefits to which the subject is otherwise entitled.

Reimbursements We can reimburse you for any cost incurred at the pathology laboratory for collection of blood for this study.

Confidentiality: Efforts will be made to keep personal information confidential. Absolute confidentiality cannot be guaranteed. Personal information may be disclosed if required by law. Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as the Ethics Committee for Medical Research.

Contact details of researcher– Prof Felicity Burt, Department of Virology, University of the Free State, Bloemfontein. Tel 051 4053162, burtfj@ufs.ac.za
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Contact details of REC Secretariat and Chair – For reporting complaints or problems please contact
Ms H Strauss
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APPENDIX F

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Dominique Goedhals

Letter sent as PDF via email:
gnvrdg@ufs.ac.za

April 30, 2014

Dear Dominique Goedhals

D. Goedhals, P. A. Bester, J. T. Paweska, R. Swanepoel and F. J. Burt, "Next-generation sequencing of southern African Crimean-Congo haemorrhagic fever virus isolates reveals a high frequency of M segment reassortment", Epidemiology and Infection, (forthcoming).

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Yours sincerely

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Publishing Assistant
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