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INNATE IMMUNE SIGNALLING INDUCED BY CRIMEAN-CONGO HAEMORRHAGIC FEVER VIRUS PROTEINS *IN VITRO*

Natalie Viljoen

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FAKULTEIT GESONDHEIDSWETENSKAPPE
FACULTY OF HEALTH SCIENCES

INNATE IMMUNE SIGNALLING INDUCED BY CRIMEAN-CONGO HAEMORRHAGIC FEVER VIRUS PROTEINS *IN VITRO*

Natalie Viljoen

PhD Medical Virology

Submitted in fulfilment of the requirements in respect of the PhD Medical Virology degree completed in the Division of Virology in the Faculty of Health Sciences at the University of the Free State

Promoter: Professor Felicity Jane Burt
Co-promoter: Professor Dominique Goedhals
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University of the Free State, Bloemfontein, South Africa
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Declaration

I, Natalie Viljoen, declare that the doctoral research interrelated, publishable manuscripts that I herewith submit at the University of the Free State is my independent effort and has not previously been submitted for a qualification at another institution of higher education. I furthermore waive copyright in favour of the University of the Free State and declare that all royalties with regard to intellectual property that was developed during the course of and/or in connection with the study at the University of the Free State, will accrue to the University.

X 

Natalie Viljoen

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"'Though the mountains be shaken and the hills be removed, yet my unfailing love for you will not be shaken nor my covenant of peace be removed,' says the Lord, who has compassion on you."
Isaiah 54:10 (New International Version)

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List of abbreviations

A549 – Adenocarcinoma human alveolar basal epithelial cells

Abs – absorbance

ACTB – actin, beta

ALT – alanine aminotransferase

AST – aspartate aminotransferase

ATCC – American Type Culture Collection

B2M – beta-2-microglobulin

BGH – bovine growth hormone

bp – base pair

BSL – biosafety level

BUNV – Bunyamwera virus

CCHF – Crimean-Congo haemorrhagic fever

CCHFV – Crimean-Congo haemorrhagic fever orthonairovirus

cDNA – complementary DNA

CHF – Crimean haemorrhagic fever

CHFV – Crimean haemorrhagic fever virus

CFU – colony forming units

CPE – cytopathic effect

Cq – cycle threshold

DAFF – Department of Agriculture, Forestry and Fisheries

DDX58 – DEAD (Asp-Glu-Ala-Asp) box polypeptide 58

DIC – disseminated intravascular coagulation

DMEM – Dulbecco's Modified Eagle's Medium

DNA – deoxyribonucleic acid

eIF4G – eukaryotic translation initiation factor 4 G

ELISA – Enzyme-linked immunosorbent assay

FBS – foetal bovine serum

GAPDH – glyceraldehyde-3-phosphate dehydrogenase

G_C – glycoprotein on the C-terminal

gDNA – genomic DNA

GFP – green fluorescent protein

G_N – glycoprotein on the N-terminal

GP – glycoproteins

HAZV – Hazara orthonairovirus

HEK – human embryonic kidney

HGDC – human genomic DNA contamination control

ICTV – Internal Committee on Taxonomy of Viruses

IFA – immunofluorescence assay

IFIH-1 – Interferon-induced with helicase C domain 1

IFN – interferon

IFNAR1 – Interferon (alpha, beta and omega) receptor 1

Ig – immunoglobulin

IL – interleukin

IP – interferon gamma-induced protein

IRF – Interferon regulatory factor

ISG – interferon-stimulated gene

ISGF – IFN-stimulated gene factor

JAK – Janus kinase

JCat – JAVA codon adaptation tool

L-segment – large segment

MAVS – Mitochondrial antiviral signalling protein

MCP – monocyte chemoattractant protein

MDA5 – melanoma differentiation-associated factor 5

MHC – major histocompatibility complex

mRNA – messenger RNA

M-segment – medium segment

MVA – Modified Vaccinia virus Ankara

MYD88 – Myeloid differentiation primary response gene (88)

NASBA – nucleic acid sequence-based amplification assay

NF – nuclear factor

NFKB1 – Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1

NP – nucleoprotein

NS_M – non-structural protein on the M-segment

NS_S – non-structural protein on the S-segment

OTU – ovarian tumour-like

PAMP – pathogen-associated molecular patterns

PCR – polymerase chain reaction

PRR – pattern recognition receptors

PPC – positive PCR control

qPCR – quantitative/real-time PCR

PVDF – polyvinylidene fluoride

RdRP – RNA-dependent RNA polymerase

RIG-I – retinoic acid-inducible

RLR – RIG-I-like receptors

RNA – ribonucleic acid

RTC – reverse transcription control

RT-LAMP – reverse transcriptase loop-mediated isothermal amplification assay

RT-PCR – reverse transcriptase polymerase chain reaction

RVFV – Rift Valley fever virus

SDS-PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis

siRNA – small interfering RNA

SPU – special pathogens unit

S-segment – small segment

STAT – signal transducer and activator of transcription

tcVLP – transcriptionally competent virus-like particle

tecVLP – transcription and entry competent virus-like particle

Th – T-helper

TLR – toll-like receptor

TNF – tumour necrosis factor

T_{reg} – regulatory T-lymphocytes

u/ml – units per millilitre

WHO – World Health Organisation

Presentations and publications

Oral presentations

- Non-structural Crimean-Congo haemorrhagic fever virus protein prevents immune activation. 50th University of the Free State Faculty of Health Sciences Research Forum, Bloemfontein, South Africa from 30-31 August 2018.
- Crimean-Congo haemorrhagic fever virus non-structural protein prevents immune activation. 7th Annual Free State Provincial Health Research Day, Bloemfontein, South Africa from 8-9 November 2018.
- Crimean-Congo haemorrhagic fever virus non-structural proteins modulate the innate immune response. Development of diagnostics and therapeutics for CCHFV workshop, Bloemfontein, South Africa from 6-7 December 2018.

Publication

Fritzen A, Risinger C, Korukluoglu G, Christova I, Corli Hitzeroth A, Viljoen N, Burt FJ, Mirazimi A, Blixt O. Epitope-mapping of the glycoprotein from Crimean-Congo haemorrhagic fever virus using a microarray approach. PLoS Negl Trop Dis 2018; 12:e0006598.

Viljoen N, Goedhals D, Burt FJ. A role for Crimean-Congo haemorrhagic fever virus NS_M protein in innate immune modulation. Preparation for submission to PLoS Pathogens.

Viljoen N, Goedhals D, Burt FJ. Modulation of innate immune signalling by Crimean-Congo haemorrhagic fever virus ovarian tumour-like protease. Preparation for submission to PLoS Pathogens.

Viljoen N, Goedhals D, Burt FJ. Characterisation of the innate immune transcriptional response to Hazara virus and Hazara viral RNA. Preparation for submission to Virus Research.

Ethics approval

Ethics approval for conducting this study was obtained from the Health Sciences Research Ethics Committee at the University of the Free State under the title “Innate immune signalling induced by Crimean-Congo haemorrhagic fever virus proteins *in vitro*” and allocated the ethics number ECUFS NR 34/2013C (refer to Appendix A for the letter of approval).

Department of Agriculture, Forestry and Fisheries approval

Permission to conduct research in terms of Section 20 of the Animal Diseases Act, 1984 (Act no. 35 of 1984) was obtained from the Department of Agriculture, Forestry and Fisheries (DAFF) and allocated the reference number 12/11/14 (refer to Appendix B for the original letter of permission and the letter of permission for requested amendment to the original approved methodology).

Note

All figures and tables herein have been self-constructed.

Summary

Crimean-Congo haemorrhagic fever orthonairovirus (CCHFV) is a tick-borne viral zoonosis associated with haemorrhagic fever in humans. The World Health Organisation identified CCHFV as a priority pathogen for research. The disease is widespread globally with regions of endemicity in Africa, Asia and eastern Europe; however, the emergence and re-emergence of disease in endemic and non-endemic regions is a cause for concern. Additionally, the lack of rapid point-of-care assays, a vaccine or therapeutic interventions approved for use in humans complicates the control and management of disease, which requires an improved understanding of the virus-host interactions.

Viruses have co-evolved with their host/s. Dysregulation of the immune response is a common strategy utilised to evade immune detection and clearance. The innate immune response is a robust non-specific response to infection with the aim of limiting virus replication and spread while activating the adaptive immune response that mediates virus clearance and protection. In this study, innate immune modulation by selected non-structural CCHFV proteins, including the NS_M protein and ovarian-tumour like (OTU) protease, and Hazara orthonairovirus (HAZV), a possible model for CCHFV, was investigated.

The CCHFV NS_M protein, encoded on the M-segment, was expressed *in vitro* to evaluate the ability of the protein to modulate innate immune signalling. In South Africa, isolates containing an M-segment that are genetically related to other South African isolates (non-reassortant) and isolates containing an M-segment that are genetically similar to Asian isolates (reassortant) have been identified. The CCHFV NS_M proteins from both reassortant and non-reassortant CCHFV isolates were evaluated. Despite 92,88% amino acid sequence similarity between the reassortant and non-reassortant NS_M protein, the non-reassortant NS_M protein downregulated key innate immune markers, including *DDX58* (RIG-1), *IFNB1*, *IFNAR1* and *STAT1*, whereas the reassortant NS_M protein upregulated *DDX58* (RIG-I) and *IFNB1* expression. The results indicate that the reassortant NS_M protein induces innate immune signalling, whereas the non-reassortant NS_M protein significantly downregulates innate immune signalling. The non-reassortant NS_M protein may

therefore potentially compromise virus recognition and the induction of an antiviral state.

The CCHFV OTU protease, encoded on the L-segment, has deubiquitinating and deISGylating activity that interferes with the regulation of cellular processes, including innate immune signalling. Modulation of innate immune signalling at transcriptional level by the CCHFV OTU protease was determined by gene expression analysis. Expression of the CCHFV OTU protease resulted in downregulation of *IFNB1*, *IFNAR1* and *STAT1* expression. The results suggest that, in addition to interfering with innate immune signalling by deubiquitination and deISGylation, the CCHFV OTU protease also downregulates innate immune signalling at transcriptional level. The CCHFV OTU protease may therefore significantly compromise the host's innate immune response to infection and represents a potential target for the development of therapeutic interventions.

HAZV, a closely related virus to CCHFV, has been proposed as a clinical surrogate for disease in type I IFN-receptor deficient mice. Innate immune signalling induced by HAZV and HAZV RNA was evaluated to determine whether HAZV modulates innate immune signalling. A significant downregulation in *IFNB1* expression during infection was observed and a lack of upregulation of several key innate immune markers. The results suggest that HAZV has the ability to downregulate innate immune signalling without completely abolishing innate immune activation. Further investigation to determine the mechanism utilised by HAZV may provide insights into whether HAZV would be suitable as a surrogate for the screening of therapeutic interventions to overcome innate immune signalling modulation post-infection. HAZV provides a safer alternative to CCHFV since HAZV can be cultured and handled in a biosafety level (BSL)-2 facility.

This study has identified targets for the development of therapeutic interventions and potential inclusion in vaccines. *In vivo* studies are required to determine the significance of the findings during infection.

Keywords

Crimean-Congo haemorrhagic fever virus; innate immune signalling; NS_M protein; OTU protease; Hazara virus; interferon

Chapter 1

Introduction, rationale and aims

Introduction

The human body is protected from foreign bodies by our immune system, which is comprised of the innate immune response and the adaptive immune response. The innate immune response is our body's first line of defence and will respond to threats in the same way on subsequent exposures without changes in the intensity or swiftness of the response. The innate immune response becomes activated almost immediately after initiation of infection and effector functions may be detected within hours of activation. The primary role of the innate immune response is to contain the infectious agent while activating the adaptive immune response. In the days leading up to full mobilisation and activation of the adaptive immune response through clonal expansion and differentiation into effector lymphocytes, the innate immune response attempts to retain control of the infectious agent. In a viral infection, the innate immune response attempts to limit virus replication and spread throughout the host until the adaptive immune response can respond to and eliminate the virus. However, viruses have evolved to alter the way in which our immune system responds by encoding one or more virulence factors that interfere with or delay immune activation. Interference with innate immune activation enables a virus to replicate uncontrollably and to spread.

In recent years, innate immune dysregulation has been demonstrated for many viruses. By improving our understanding of the virus-host interactions that result in innate immune dysregulation, insights into the mechanisms of viral pathogenesis may provide targets for the development of therapeutics and inclusion in vaccines. Crimean-Congo haemorrhagic fever orthonairovirus (CCHFV) causes a rapidly progressive disease and a delay or impairment of the innate immune response could have detrimental consequences for the host. CCHFV has been shown to delay innate immune activation by up to 48 hours *in vitro*. Considering that the incubation period can be as short as one day and fatalities are reported within five or more days after the onset of symptoms, a 48-hour delay in innate immune activation could significantly contribute to disease severity and/or outcome. To date, limited data suggest that strain-to-strain differences may exist in the way in which CCHFV strains interact with

the innate immune system. Different fatality rates are reported for different geographical areas and speculation over whether this may be related to differences in pathogenicity between geographically distinct strains or due to host-factors have not been substantiated.

The genomic diversity of CCHFV was shown to be generated almost solely within the tick host with no changes in the genomic composition associated with infection in a mammalian host. AP92 and AP92-like CCHFV strains have been isolated from ticks and have highly divergent genomes compared to all other CCHFV strains worldwide. AP92 or AP92-like strains are rarely associated with disease, which may suggest that cycling of the virus between hosts may impact virus fitness to infect and cause disease in mammals. In other words, if sufficient adaption to the tick host occurs, the ability of the virus to infect and cause disease in humans may be compromised or restricted. However, the mammalian host was represented by the Crimean-Congo haemorrhagic fever (CCHF) mouse model and the mouse model may not reflect the virus-host interactions in other mammalian hosts (such as humans) that may contribute to the accumulation of mutations within CCHFV. Within the tick host, the majority of mutations were found in the gene encoding the viral polymerase with fewer in the gene encoding the viral glycoproteins, which may indicate adaptation to infect and replicate within tick cells. The use of tick cell lines for isolating and studying CCHFV has also been proposed and was successfully achieved, not only in *Hyalomma* cell lines, but in several other tick cell lines. Additional information regarding the virus-host interactions are required, especially the contribution of host-factors to disease severity and outcome. The way in which the virus interacts with the host may, in part, be host-dependent and warrants investigation.

Problem identification

CCHFV is a tick-borne virus that is widespread globally and the distribution of disease correlates to the distribution of the primary vector, *Hyalomma* ticks. CCHFV infection causes disease in humans that varies in severity, which ranges from mild febrile illness to severe haemorrhagic disease with multiorgan failure, which may include liver and renal failure, and cardiac and/or pulmonary insufficiency (Swanepoel et al., 1987). The reported fatality rates differ significantly between geographical regions with Turkey

reporting a 5% fatality rate (Leblebicioglu et al., 2016) and smaller case series reporting higher fatality rates (Aradaib et al., 2011; Burney et al., 1980; Chinikar et al., 2010; Christova et al., 2009; Dunster et al., 2002; El-Azazy and Scrimgeour, 1997; Hoogstraal, 1979; Mishra et al., 2011; Mofleh and Ahmad, 2012; Mustafa et al., 2011; Nabeth et al., 2004b, 2004a; Papa et al., 2002a, 2002b, 2004, 2008; Saluzzo et al., 1985; Schwarz et al., 1997; Smego et al., 2004; Suleiman et al., 1980; Tantawi et al., 1980; Tikriti et al., 1981; Watts et al., 1989; Yadav et al., 2013; Yashina et al., 2003b, 2003a). The World Health Organisation (WHO) identified CCHFV as a priority pathogen for research due to the emergence and re-emergence of CCHFV in new and endemic regions, limited diagnostic capacity in endemic regions, and a lack of therapeutic agents and vaccines approved for human use (World Health Organization, 2018).

CCHFV has a widespread distribution globally and, since 2002, CCHFV has emerged in areas without previous recognition of CCHF cases, which is a cause for concern. The identification of CCHFV in regions previously considered to be naïve may indicate a new introduction of the virus or that the virus was previously unidentified within the region. Climate change has been suggested to play a role in tick abundance and the likelihood of ticks to colonise a new territory (Estrada-Peña et al., 2012a). In Turkey, habitat fragmentation was shown to be associated with an increase in CCHF cases and geographical disease spread (Estrada-Peña et al., 2010). However, modelling of climate change predicts that with time *Hyalomma marginatum* ticks may increase in population size and expand the northern limit of distribution (Estrada-Peña et al., 2012b). Concern over the spread of CCHFV to countries with an abundance of *Hyalomma* ticks and favourable environmental conditions exists (Al-Abri et al., 2017; Hoogstraal, 1979; Maltezou and Papa, 2010).

Limited diagnostic capacity in endemic areas presents a challenge to the identification, management and control of disease, which is partly due to the need for high containment facilities for the diagnosis of CCHF cases. Diagnostic tests suitable for implementation in peripheral laboratories and point-of-care tests are required for the early identification of cases to guide clinical management, and the implementation of infection prevention and control measures.

The lack of virus-specific therapy and a vaccine approved for human use contribute to difficulty in managing and controlling CCHF infections. Currently, treatment consists of supportive care and the replacement of blood products; however, in severe cases bleeding continues despite vigorous blood and blood product replacement therapy (Swanepoel et al., 1987). Despite a 4-fold reduction in CCHF cases in Bulgaria after the implementation of the Bulgarian CCHF vaccine in 1974, widespread acceptance of this vaccine is unlikely due to its crude preparation and preparation in the tissue of the central nervous system (Papa et al., 2011). Control of disease currently relies on physical measures of preventing contact with infectious material or tick-bite, which is difficult to sustain on a day-to-day basis in individuals with high-risk occupations. The availability of a CCHF vaccine may assist in controlling the disease in high-risk individuals. An improved understanding of the virus-host interactions is required to assist in the development of therapeutic strategies and vaccines and to improve knowledge of CCHFV pathogenesis.

Limited knowledge regarding the pathogenesis of CCHFV is available. A high viral load has been correlated to a fatal outcome, which in turn frequently occurs in the absence of a detectable antibody response (Shepherd et al., 1989). CCHFV has been shown to impair the innate immune response *in vitro*, which may delay activation of the adaptive immune response that is essential for viral clearance. The delay in innate immune activation has been attributed to the suppression of type I interferon (IFN) secretion, which resulted in the virus being insensitive to type I IFN at the time of secretion (Andersson et al., 2008). The delay in innate immune activation may impact virus replication and spread, which contributes to an unfavourable outcome of disease. The nucleoprotein (NP) was implicated in innate immune dysregulation after reduced activation of the IFN-β promoter by the highly virulent CCHFV Hoti strain NP, but not the less virulent IbAr10200 or AP92 strains, was demonstrated (Fajs et al., 2014). The results suggested that strain-to-strain differences may occur and that other viral components may contribute to innate immune dysregulation (Fajs et al., 2014). Additionally, the ovarian-tumour like (OTU) protease has been shown to have deubiquitinating and deISGylating activity, which interferes with innate immune activation (Frias-Staheli et al., 2007; Scholte et al., 2017). In this study, the involvement of the CCHFV NS_M protein and OTU protease in innate immune dysregulation was evaluated at a transcriptional level. Identification of the CCHFV

proteins that contribute to innate immune dysregulation and the innate immune components targeted may provide targets for therapeutic strategies and assist with rational vaccine design.

Currently, it is unclear whether geographically distinct strains of CCHFV display differences in pathogenicity. Additionally, it is unclear whether reassortment events may result in differences in pathogenicity. In South Africa, reassortant and non-reassortant CCHFV isolates have been identified with reassortant CCHFV isolates having acquired an M-segment that groups amongst Asian CCHFV isolates in group IV (Burt et al., 2009; Goedhals et al., 2014). In this study, the role of the CCHFV NS_M protein in innate immune modulation was investigated. The inclusion of reassortant and non-reassortant CCHFV NS_M proteins from South African CCHFV isolates may, in addition to profiling the innate immune response to the CCHFV NS_M protein, provide insight into strain-to-strain variation in the virus-host interactions with the immune response.

Currently, the mechanism utilised by CCHFV to impair the innate immune response and to cause disease is incompletely understood, which is partly due to the requirement of high containment biosafety level (BSL)-4 facilities for handling and culturing CCHFV. Hazara orthonairovirus (HAZV), a closely related virus to CCHFV, that can be cultured in a BSL-2 facility has been proposed as a model for CCHFV infection in adult type I IFN-receptor deficient mice (Dowall et al., 2012). In this study, HAZV innate immune modulation was evaluated to determine whether HAZV can be used as a surrogate for CCHFV innate immune modulation, which may assist with the screening of therapeutic agents that can overcome innate immune suppression. Additionally, it will represent a safer model to study innate immune modulation and may provide insights into mechanisms of viral pathogenesis.

Aims and objectives

The aims of this study were:

1. To investigate and compare innate immune modulation at a transcriptional level by CCHFV NS_M protein from a reassortant South African isolate and a non-reassortant South African isolate.

2. To investigate innate immune modulation at a transcriptional level by the CCHFV OTU protease from a South African isolate.
3. To evaluate HAZV, a closely related virus to CCHFV, as a model for innate immune modulation *in vitro*.

The objectives of this study were:

1. To express the CCHFV NS_M protein from a reassortant isolate and a non-reassortant isolate *in vitro* and, to determine and compare changes in innate immune signalling by analysing the transcriptional profile.

2. To express the CCHFV OTU protease from a South African isolate *in vitro* and to determine the changes in the transcriptional profile associated with innate immune modulation.

3. To determine the innate immune modulation by HAZV and HAZV RNA *in vitro*.

Structure of the thesis

This thesis is presented as an introduction and literature review followed by interrelated, publishable research articles according to the guidelines for submission of a thesis in article format from the University of the Free State. Chapter 1 contains an introduction to the thesis, the problem identification and aims and objectives. Chapter 2 is a literature review on CCHFV. Chapters 3 and 4 represent research describing the characterisation of innate immune modulation by the CCHFV NS_M protein and OTU protease, respectively. In Chapter 3, innate immune signalling induced in response to the expression of a reassortant and non-reassortant CCHFV NS_M protein from two South African isolates is described. Transcriptional modulation by a reassortant and non-reassortant CCHFV NS_M protein is compared to determine whether significant differences exist in the way in which two NS_M proteins from genetically diverse origins interact with host innate immune signalling. In Chapter 4, innate immune modulation by the OTU protease at a transcriptional level is described, which suggests that, in addition to physical interference, modulation of innate immune signalling may contribute to innate immune dysregulation. In Chapter 5,

characterisation of innate immune signalling in response to HAZV and HAZV RNA is described to determine innate immune modulation by HAZV and the immunostimulatory nature of HAZV RNA. In Chapter 6, the overall conclusions of the study and future research perspectives are provided.

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Chapter 2

Literature review: Crimean-Congo haemorrhagic fever virus

Introduction

In 1944, an outbreak of 200 cases of haemorrhagic fever, which was later referred to as Crimean haemorrhagic fever (CHF), in the Crimean Peninsula incited investigation into the aetiology of the disease (Chumakov, 1965). After exclusion of possible bacterial (including leptospiral and rickettsial) agents, a viral aetiology was demonstrated for CHF in 1945. The disease-causing agent was filterable from patient serum collected during the acute phase of disease and was identical to an agent isolated from ticks belonging to the genus *Hyalomma* (Chumakov, 1965). Study of the causative agent was complicated by unsuccessful isolation in animals and CHF virus (CHFV) was studied by passage in psychiatric patients as a form of pyrogenic therapy (Chumakov, 1965). In Africa, Dr Courtois first isolated Congo virus in 1956 from the serum of a febrile 13-year-old boy, by inoculation of newborn white mice (Simpson et al., 1967). Congo virus was not antigenically related to any of the arboviruses recognised in Africa at the time, or to herpes simplex virus, (Woodall et al., 1967) and was sent to the World Health Organisation International Reference Centre for Arboviruses, Yale Arbovirus Research Unit for identification.

Between 1946 and 1955, attempts to isolate the virus in selected primary and transplantable cell lines were unsuccessful; however, after the exploration of several more cell lines, virus isolation with the induction of cytopathic effects (CPE) was achieved in an unspecified cell line (Chumakov, 1965). The identification of cell lines susceptible to infection by CHFV assisted with the development of tests for the diagnosis of CHF infection (Chumakov, 1965). Between 1967 and 1968, isolation of CHF in newborn mice and newborn albino rats facilitated virological and serological investigation of CHFV. Virus isolation in newborn rodents proved to be an easy and effective method to allow confirmation of a suspected diagnosis and serological surveillance in humans and animals in CHF endemic areas (Chumakov et al., 1968).

Initially, CHFV was believed to be unrelated to known human or animal viruses; however, in 1968 Casals demonstrated that CHFV and Congo virus were serologically

indistinguishable (Casals, 1969). In addition to confirming Casals's findings, Chumakov and colleagues demonstrated cross-neutralisation between geographically distinct strains (Chumakov et al., 1969). The virus was subsequently designated Crimean-Congo haemorrhagic fever virus and was recently renamed Crimean-Congo haemorrhagic fever orthonairovirus (CCHFV) (Adams et al., 2017).

Virus classification and mode of development

CCHFV, a tri-segmented single-stranded ribonucleic acid (RNA) virus, was initially grouped within the *Bunyaviridae* family of viruses based on the morphology and mode of development of virus particles within infected host cells (Korolev et al., 1976). Within the family *Bunyaviridae*, CCHFV was identified as a member of the genus *Nairovirus* based on antigenic relatedness and structure and was shown to be antigenically different from representative members of the *Bunyavirus*, *Uukuvirus* and *Phlebovirus* genera (Casals and Tignor, 1980; Clerx et al., 1981). Viruses within the genus *Nairovirus* were further classified into seven antigenic groups, including the Crimean-Congo haemorrhagic fever (CCHF), Nairobi sheep disease, Dera Ghazi Khan, Qalyub, Hughes, Sakhalin and Thiafora groups (Casals and Tignor, 1980; Clerx et al., 1981; Zeller et al., 1989). Regrouping of the viruses was proposed based on the genomic characterisation of the genus *Nairovirus*. Phylogenetic analysis revealed that Hazara orthonairovirus (HAZV), which initially grouped within the CCHF group based on antigenic relatedness, was genetically more closely related to Tofla virus (Kuhn et al., 2016). The order, family and genus of CCHFV was reclassified by the International Committee on Taxonomy of Viruses (ICTV) into the order *Bunyavirale*s, family *Nairoviridae* and genus *Orthonairovirus* (Adams et al., 2017).

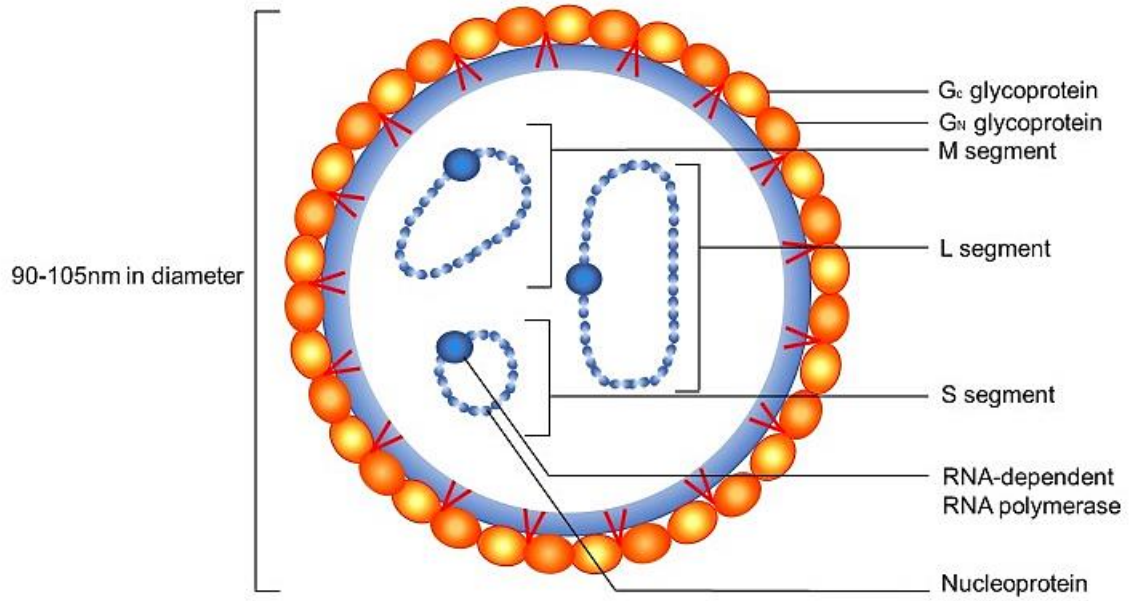

Figure 1: A schematic depiction of a Crimean-Congo haemorrhagic fever virus virion

Adapted from a schematic depiction of a CCHFV virion (Bente et al., 2013)

CCHFV virions are spherical particles of 90-105nm in diameter with surface projections of 8-10nm in length (Korolev et al., 1976). The CCHFV genome consists of three negative-sense single-stranded RNA segments, designated small (S), medium (M) and large (L) (Figure 1). The CCHFV S-segment encodes two proteins, the structural nucleoprotein (NP) and the non-structural protein on the S-segment (NS_S), using an ambisense coding strategy. The NP is involved in RNA binding and oligomerisation to form ribonucleoprotein complexes (Carter et al., 2012). Additionally, the CCHFV NP and eukaryotic translation initiation factor 4 G (eIF4G) have been demonstrated to facilitate the translation of capped and uncapped viral messenger (m)RNA (Jeeva et al., 2017). Whereas the highly conserved NS_S protein, encoded within the coding region of the NP, was recently shown to disrupt the mitochondrial membrane potential resulting in permeabilisation of the mitochondrial membrane, the activation of caspases and the induction of apoptosis (Barnwal et al., 2016). The M-segment encodes a single polypeptide, which after post-translational modification produces two structural glycoproteins (GPs), designated G_N and G_C, and a mucin-like domain, NS_M, GP38, GP85 and GP160 non-structural glycoproteins. The structural glycoproteins are embedded in the viral envelope and are essential for attachment to

host cells, whereas the non-structural proteins have not been shown to form part of the virion and are likely soluble proteins (Altamura et al., 2007; Sanchez et al., 2006). A fraction of the CCHFV NS_M protein has been shown to accumulate in the Golgi body with the structural glycoproteins and may play a role in virus assembly (Altamura et al., 2007). The L-segment encodes a viral RNA-dependent RNA polymerase (RdRP), which facilitates the transcription of virally encoded genes and viral replication. The polypeptide has been predicted to have other functions based on amino acid sequence homology with helicases, gyrases, topoisomerases, transcription factors and cytoskeleton-associated proteins. An ovarian tumour (OTU)-like protease has been identified at the N-terminal region of the polypeptide (Honig et al., 2004; Kinsella et al., 2004). The CCHFV OTU protease has the ability to hydrolyse ubiquitin and interferon (IFN)-stimulated gene (ISG)-15 product, which play an essential role in the innate immune response (Frias-Staheli et al., 2007; Scholte et al., 2017). The CCHFV OTU protease was shown to inhibit the nuclear factor (NF)-κβ dependent signalling pathway and antagonise the antiviral effects of ISG15 during Sindbis virus infection *in vivo*, which play key roles in the innate, adaptive and inflammatory responses to infection (Frias-Staheli et al., 2007).

Enveloped viruses gain entry to susceptible host cells by attaching to cell surface-associated receptors by means of the viral glycoproteins. The CCHFV G_C has been shown to interact with human cell surface nucleolin, which was proposed as an entry factor for CCHFV after cells that did not express cell surface nucleolin were shown to be resistant to infection by CCHFV (Xiao et al., 2011). Additionally, the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin, an innate immune receptor, was proposed as an entry factor for CCHFV (Suda et al., 2016). A study using transcription and entry competent virus-like particles (tecVLPs) demonstrated virus-dependent entry efficiencies depending on the glycoprotein of the virus with the IbAr10200 strain having a 100-fold lower entry efficiency compared to pathogenic CCHFV strains (Zivcec et al., 2015). CCHFV enters host cells by clathrin-mediated endocytosis (Simon et al., 2009a), is transported in a Rab5-dependent manner and delivered to multivesicular bodies, which are acidified compartments that allow virus-endosome fusion and are likely the last endosomal compartment the virus enters before being released into the cytoplasm (Shtanko et al., 2014). The 3' terminus of the S-, M- and L-segments of viruses that belong to the order *Bunyavirales* (previously

family *Bunyaviridae*) is conserved; however, the consensus sequence for viruses belonging to the *Nairoviridae* and *Peribunyaviridae* families (previously *Nairovirus* and *Bunyavirus* genera) differ from the 3' consensus sequence of the other members of the virus order (Clerex-Van Haaster et al., 1982). The inverted complementary sequences at the 3' and 5' termini of each segment have been shown to form stable non-covalently closed circular RNA molecules (Hewlett et al., 1977). After release into the cytoplasm, each segment associates with the virally encoded RdRP and transcription of each segment occurs as illustrated by a significant increase in complementary RNA, also known as mRNA or positive-sense RNA (Simon et al., 2009b). The viral and complementary RNA have been shown to co-localise with the NP in virus-infected cells (Andersson et al., 2012). The structural CCHFV glycoproteins (G_N and G_C) are synthesised in the endoplasmic reticulum, transported to the Golgi body to undergo post-translational modification (Bertolotti-Ciarlet et al., 2005) and associate with the ribonucleoprotein complexes. The complexes are comprised of circular RNA for each segment and is complexed with the viral NP. Translocation of the G_C protein to the Golgi body is dependent on the ectodomain of the G_N protein. Golgi targeting of the G_N protein, in turn, is dependent on the mucin-like and P35 domains at the N-terminus of the G_N protein (Bertolotti-Ciarlet et al., 2005). Virus particles mature by budding through the Golgi body into cytoplasmic vacuoles that are transported to the cell membrane and released by fusion with the cell membrane. Released virus particles contain an envelope derived from the host cell membrane with glycoprotein spikes protruding from the envelope (Ellis et al., 1981). The microtubule network has been shown to be essential for virus internalisation, assembly and release, but may also indirectly affect virus replication (Simon et al., 2009b).

Epidemiology

CCHFV is a tick-borne viral zoonosis that is widely distributed across the African, Asian and eastern European continents (Hoogstraal, 1979) and since 2002, cases have been reported in previously naïve areas including Turkey (Karti et al., 2004), Greece (Papa et al., 2008), India (Mishra et al., 2011; Patel et al., 2011) and Spain (Negredo et al., 2017) (Figure 2).

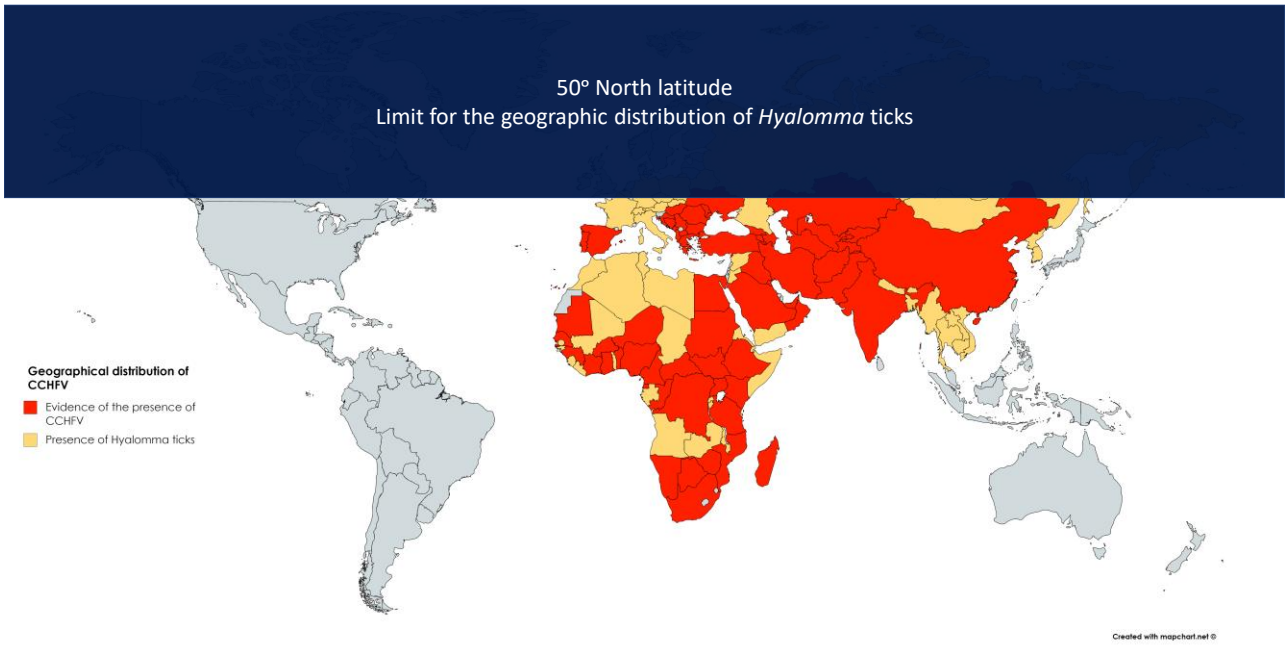

Figure 2: Geographical distribution of Crimean-Congo haemorrhagic fever virus

Countries coloured in red have evidence of the presence of CCHFV either by virus isolation or the detection of viral nucleic acid and/or CCHFV antibody in animals and/or humans, or virus isolation or the detection of viral nucleic acids from ticks. A complete list of the countries is included in Appendix C. The map was generated using MapChart.net (https://mapchart.net/).

CCHFV is transmitted by the bite of an infected tick or by contact with the blood and/or tissue from a viraemic host (Chumakov, 1965), including needlestick injury, which is an important route of transmission to healthcare workers (Celikbas et al., 2014; van Eeden et al., 1985a). Transmission may occur by squashing an infected tick without wearing gloves. CCHF cases have also been reported after handling meat that had been frozen and allowed to thaw (Metanat et al., 2017), which suggests that the process of freezing and thawing meat may not be sufficient to declare the contaminated meat safe for consumption. Ticks have been shown to develop high levels of viraemia ($6{,}8x10^7$ copies/ml in homogenate) (Papa et al., 2009), which may allow efficient transmission of the virus to animals and humans. Occupational and recreational activities may, therefore, predispose an individual to infection, especially individuals with frequent contact with animals or individuals involved in the castration or slaughtering of animals (Donchev et al., 1965; Hoogstraal, 1979; Swanepoel et al., 1998). Additionally, human-to-human transmission has been suggested to occur after

aerosol-generating medical procedures, including inhalation therapy, intubation and ventilation (Pshenichnaya and Nenadskaya, 2015) and sexual intercourse 1-2 days before the onset of symptoms and during a mild course of disease (Pshenichnaya et al., 2016). In nature, CCHFV is maintained between ticks, small mammals and ungulates. Although antibody detection and virus isolation have implicated a broad range of animals and ticks in maintaining CCHFV in nature, the significance of each of these animal or tick species as a reservoir or vector has not been determined (Hoogstraal, 1979). Antibody detection and virus isolation have been reported in animals and ticks in areas without any reported human CCHF cases (Hoogstraal, 1979). The emergence and re-emergence of CCHFV may be related to climate change favouring the reproductive rate of the vector population and anthropogenic factors, which include changes in hunting and agricultural activities (Maltezou and Papa, 2010; Spengler et al., 2019). CCHFV may further extend to new areas where *Hyalomma* ticks exist or have been introduced with favourable environmental conditions (Hoogstraal, 1979; Maltezou and Papa, 2010; Spengler et al., 2019).

The distribution of disease correlates with the distribution of the primary vector, which are ticks that belong to the genus *Hyalomma* (Hoogstraal, 1979). Although CCHFV has been isolated from several tick species, some tick species are rarely implicated in transmission to humans. *Hyalomma* ticks are two-host ticks and play a central role in the transmission of the virus to other animals and humans (Hoogstraal, 1979). The three species of *Hyalomma* ticks found in southern Africa are *Hyalomma rufipes*, *Hyalomma glabrum* and *Hyalomma truncatum*, which feed on hares and ground-feeding birds during the immature stage and large domestic and wild ungulates during the adult stage (Shepherd et al., 1987). The sporadic nature of cases reported in southern Africa may be explained by the host preference of the predominant *Hyalomma* species, which less frequently feed on humans (Shepherd et al., 1987). In more than 65% of CCHF cases in southern Africa, the tick responsible for transmission was identified as an adult *H. truncatum* tick (Swanepoel et al., 1987). Transstadial transmission allows CCHFV to persist through the different stages of the tick lifecycle from the larval stage to the adult stage and transovarial transmission has also been demonstrated for several *Dermacentor*, *Hyalomma* and *Rhicephalus* species. Shortly preceding CCHF epidemics, an increase in the number of ticks and hosts with the ability to act as amplifying hosts for CCHFV has been observed (Hoogstraal, 1979).

Additionally, severely cold weather conditions have been correlated to a decrease in the tick population and CCHF cases (Goldfarb et al., 1980).

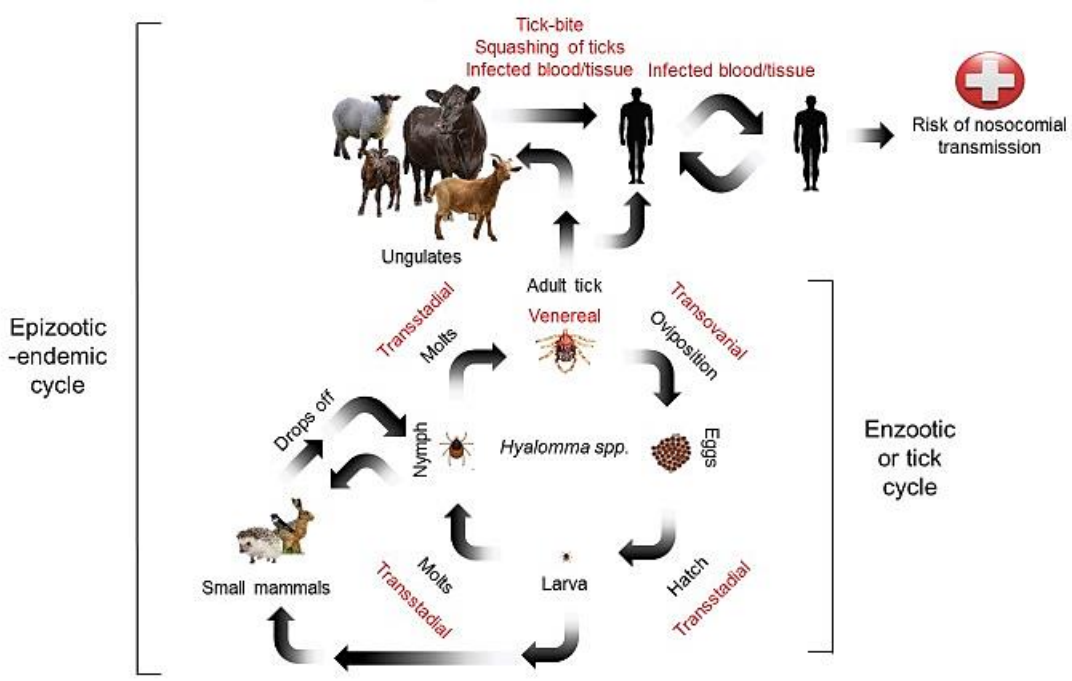

Figure 3: Transmission of Crimean-Congo haemorrhagic fever virus in southern Africa

Adapted from the transmission cycle of CCHFV (Bente et al., 2013)

The role of ground-feeding birds in the transmission of CCHFV is not clear; however, birds infested with ticks are thought to act as a vehicle of transport for infected ticks that facilitate intercontinental spread during seasonal migration (Causey et al., 1970; Hoogstraal, 1979). Except for ostriches, which develop viraemia with no signs of illness (Swanepoel et al., 1998), birds appear to be refractory to infection by CCHFV (Hoogstraal, 1979). More recently, serological evidence implicated bats, migratory flying mammals that are frequently infested with hard and soft ticks, as a putative host in the transmission cycle of CCHFV. Antibody that reacts with the CCHFV GP and has the ability to neutralise CCHFV virus-like particles and full CCHFV was detected in various bats species. Bats were hypothesised to contribute to the spatial distribution of CCHFV, similar to the suggested role of ground-feeding birds (Müller et al., 2016).

Additionally, animal trade and the movement of animals may contribute to the distribution of disease.

Antibody to CCHFV in animals and humans in endemic areas is widespread and the seropositivity rates differ from region to region, which may reflect the tick population and diversity. Antibody has been detected in a diverse group of animals despite the absence of apparent disease (Causey et al., 1970; Swanepoel et al., 1985a). Although the exact role of each animal in the maintenance and/or dissemination of CCHFV in nature is not clear, antibody detection has been correlated to the regions of virus isolation (Hoogstraal, 1979; Matevosyan et al., 1974). In South Africa, antibody to CCHFV in animals is widely distributed with lower seropositivity rates along the southern coast as opposed to the higher seropositivity rates in inland areas (Swanepoel et al., 1987), which is likely due to semi-arid regions that provide favourable conditions for the *Hyalomma* life cycle. Although CCHF cases have been reported in all nine provinces, the seropositivity rates correspond to the distribution of human cases with the majority of cases reported in the Northern Cape and Free State provinces (Msimang et al., 2013). The host preference of immature and adult *Hyalomma* ticks is reflected in the seropositivity rates in wild and domestic animals with the highest rates found in hares and large herbivores (Shepherd et al., 1987).

Smaller mammals, especially hares that belong to the genus *Lepus*, may act as a reservoir for CCHFV in southern Africa and ground-feeding birds are generally parasitised by immature ticks (Shepherd et al., 1987; Swanepoel et al., 1983). Antibody was detected in less than 0,02% of rodents tested in southern Africa and as rodents in South Africa are seldom parasitised by immature *Hyalomma* ticks, rodents are not considered to play an important role in the transmission of CCHFV in South Africa (Shepherd et al., 1987). Antibody was highly prevalent in wild and domestic animals in southern Africa, with high seropositivity rates in cattle and sheep suggesting that these animals play a significant role in the CCHFV ecology in South Africa (Shepherd et al., 1987; Swanepoel et al., 1983).

Genetic variation

RNA viruses have the potential to evolve rapidly, partly due to low fidelity of the viral RdRP, which introduces mutations during viral replication. However, the evolutionary rate of arboviruses is influenced by the need to maintain host competency in both the arthropod vector and vertebrate host/s (Coffey et al., 2008). Despite the need to maintain competency in both the arthropod and vertebrate hosts, a high level of diversity has been shown for CCHFV based on the nucleotide sequence and subsequent phylogenetic analysis. A high level of genetic diversity may reflect adaptation of CCHFV to hosts, including arthropod and vertebrate hosts that play a significant role in the CCHFV transmission cycle in different geographical regions. Although the nucleotide sequence variation between CCHFV strains is high with 20%, 31% and 22% for the S-, M- and L- segment, respectively, the deduced amino acid sequence shows a higher level of conservation with 8%, 27% and 10% variation for the S-, M- and L-segment, respectively. Divergence within the M-segment, involved in attachment to a susceptible host cell, is the highest and may be required to facilitate attachment to a wide range of host cells, including arthropod and vertebrate host cells (Deyde et al., 2006). Phylogenetic analysis initially revealed seven lineages, which coincided with the geographic origin and included group I (West Africa), group II (Democratic Republic of the 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). However, more recently the M- and L-segments of two isolates from China did not cluster within any of the identified groups and were proposed to constitute a new genotype (Zhou et al., 2013).

The genetic diversity may reflect adaptation of CCHFV to the arthropod and vertebrate species associated with maintaining the virus in geographically distinct regions (Deyde et al., 2006). Single nucleotide mutations were demonstrated after a single transstadial transmission in ticks, which became fixed with time allowing virus evolution. In contrast, no single nucleotide mutations were observed in a mammalian host, which was represented by a CCHF mouse model. The results suggested that ticks can act as a long-term reservoir for CCHFV, including overwintering of infected tick life stages, with adaptation to infect and replicate within the tick host (Xia et al., 2016). However, rodents are not thought to play a significant role in the CCHFV transmission cycle and may therefore not reflect changes in other mammals that play

a more significant role in the CCHFV life cycle. Further investigation is required to determine whether mutations accumulate within the CCHFV genome while adapting to other mammalian hosts. CCHFV has been successfully isolated in tick cell lines derived from *Hyalomma* species and other tick species, which may be an invaluable research tool to improve understanding of the virus-host interactions within ticks (Bell-Sakyi et al., 2012).

Recombination and reassortment events contribute to the genetic diversity observed within the CCHFV genome. Recombination events are rare and have been reported within the S-segment for African, Asian and southern European strains (Deyde et al., 2006; Lukashev, 2005) and possible recombination within the L-segment of group IV CCHFV strains (Deyde et al., 2006), whereas reassortment events have been reported more frequently (Burt et al., 2009; Deyde et al., 2006; Goedhals et al., 2014; Hewson et al., 2004). The S- and L-segments of a CCHFV isolate generally correlate with the geographic location based on phylogenetic grouping and have been hypothesised to be co-required to produce viable virus (Chamberlain et al., 2005), which may explain a higher frequency in M-segment reassortment (Burt et al., 2009; Deyde et al., 2006; Goedhals et al., 2014; Hewson et al., 2004; Kondiah et al., 2010). Interestingly, the S- and L-segments demonstrated different evolutionary rates, which may suggest that the S- and L-segments did not co-evolve (Carroll et al., 2010). Additionally, reassortment events between African and Asian isolates have confirmed dissemination of CCHFV across continents, possibly due to livestock trade and migrating birds carrying CCHFV infected ticks (Deyde et al., 2006). The significance of reassortment events in viral pathogenesis is not clear.

Clinical features

CCHF may be subdivided into four phases, which include the incubation, pre-haemorrhagic, haemorrhagic and convalescent phase; however, all four phases are not always present within a given patient. Disease may vary from mild non-specific febrile illness, which frequently goes undiagnosed leading to underestimation of the prevalence of CCHF, to severe haemorrhagic disease. A mathematical epidemiological model suggested that the ratio of inapparent to clinically overt CCHF infections in the Rostov region in the former Soviet Union is 5:1 (83,33% of infections

are subclinical) independent of the age of the infected individual (Goldfarb et al., 1980). In line with the predicted ratio of inapparent infections in the former Soviet Union, an estimated 88% of CCHF cases in Turkey were found to be subclinical after screening using a commercial enzyme-linked immunosorbent assay (ELISA) (Bodur et al., 2012). However, in South Africa, no evidence for a high rate of clinically inapparent infections exist. Field investigations undertaken in 1981 after the first reported CCHF case in South Africa, revealed a low rate of serological evidence in humans after screening using reversed passive haemagglutination inhibition assay and immunofluorescence assays (IFA) (5/74; 6,7%) (Swanepoel et al., 1983). A more recent seroepidemiologic survey that focussed on individuals who partake in high-risk occupational and recreational activities in the Northern Cape and Free State provinces revealed a low rate of serological evidence in humans (2/387; 0,5%) without reported clinically overt disease after screening using a commercial IFA (Vawda et al., 2018). A high rate of clinically inapparent infections may therefore not occur in all endemic regions and more extensive seroepidemiologic studies may be needed to confirm current evidence suggesting a low rate of clinically inapparent infections in South Africa with time.

The incubation period may vary depending on the route of transmission. Transmission by tick-bite generally has an incubation period of one to three days and transmission by contact with infected blood and/or tissue has an incubation period of five to six days (Swanepoel et al., 1987). On examination, patients frequently present with fever, which may be intermittent, tachycardia, lymphadenopathy, chills, rigor, dizziness, injected conjunctivae or chemosis with complaints of a severe headache, backache, neck stiffness and pain, myalgia, a sore throat, sore eyes and photophobia (Butenko and Chumakov, 1990; Swanepoel et al., 1987, 1989). Gastrointestinal symptoms frequently accompany disease and may include generalised abdominal tenderness or pain, nausea, vomiting and diarrhoea. Sharp mood swings, including confusion and aggression, may be present early in disease followed by depression, lassitude and somnolence. Generalised abdominal tenderness may localise to the upper right quadrant of the abdomen; however, hepatomegaly may not be discernible (Butenko and Chumakov, 1990; Swanepoel et al., 1987, 1989). In severe cases, haemorrhagic manifestations generally develop within three to five days of the onset of disease and start with petechiae of the throat, buccal mucosa and tonsils and a petechial rash on the trunk and limbs followed by the development of ecchymotic lesions and bruising

on the antecubital fossae, axillae and upper arms. Bleeding from injection and venepuncture sites are common with or without other haemorrhagic manifestations, which may include epistaxis, haematemesis, melaena, haematuria, haemoptysis, haematoma, as well as gingival and/or vaginal bleeding. Internal bleeding may be present in some patients, which include intracranial and retroperitoneal bleeding with abdominal distension (Butenko et al., 1971; Swanepoel et al., 1987, 1989). Disseminated intravascular coagulation (DIC) precedes the bleeding tendency and contributes to platelet depletion by thrombus formation resulting in severe thrombocytopaenia, subsequently exacerbating the haemorrhagic manifestations. Necrotic hepatitis of varying degrees may develop and jaundice may manifest in the second week of illness. In severe cases, haemorrhages may compromise the vascular system and patients become hypotensive and anaemic with or without pulmonary and cardiac involvement. Generally, despite aggressive volume replacement therapy and the administration of platelet and erythrocyte preparations, the bleeding tendency continues (Swanepoel et al., 1987, 1989). Patients become progressively lethargic, stuporous and comatose due to multiorgan failure, which may include hepatorenal failure and cardiac and/or pulmonary insufficiency. In non-fatal cases, substantial improvement is generally observed by day nine or ten; however, the convalescent phase is prolonged and patients may have conjunctivitis and experience asthenia, amnesia and slight confusion for a month or longer after infection (Swanepoel et al., 1987, 1989). Development of an autoimmune response during infection has been proposed to contribute to the long-term sequelae after recovery from viral haemorrhagic fevers (Bovendo and Kobinger, 2017); however, further investigation is required to confirm whether an autoimmune response occurs during CCHFV infection.

Clinical reports indicate that children present with milder disease compared to adults, which may be attributed to stronger immune activation in adults as indicated by higher levels of chemokine release (Arasli et al., 2015). Although cases of CCHF are rare during pregnancy, high rates of maternal and foetal or neonatal mortality have been reported. A systematic review revealed a 34% maternal mortality rate and a 58,5% foetal/neonatal mortality rate with the risk being highest when a bleeding tendency is present in the mother (Pshenichnaya et al., 2017). Additionally, high rates of nosocomial transmission were reported secondary to CCHF in pregnancy. Six reported CCHF cases during pregnancy resulted in 38 additional CCHF cases. The

high rate of secondary transmission is likely due to the absence of infection prevention and control measures required to prevent virus spread due to lack of suspicion of CCHF in these cases (Pshenichnaya et al., 2017).

Diagnosis of infection

Early diagnosis of CCHF is important, not only for patient care but also from an infection control perspective. CCHF is generally suspected based on a history of exposure to tick-bite or contact with infected serum or tissue, thrombocytopaenia and severe influenza-like disease. A clinical diagnosis is often difficult due to the clinical presentation being indistinguishable from disease caused by other potential bacterial, parasitic and viral pathogens, as well as non-infectious causes and is considered a preliminary diagnosis until laboratory confirmation. Special attention should be paid to the travel history, and occupational and/or recreational activities, which may predispose to infection (van Eeden et al., 1985b; Swanepoel et al., 1989). In a retrospective study, CCHF was initially misdiagnosed and attributed to other infectious causes, including upper or lower respiratory tract infection, gastroenteritis, pancytopaenia of unknown origin, urinary tract infection, malignancy, gastrointestinal haemorrhage, leptospirosis, vertigo or brucellosis in 68% (95/140) of cases admitted by the Department of Infectious Diseases and Clinical Microbiology of Ondokuz Mayis University in Turkey between March 2004 and August 2008. This study illustrated the importance of awareness and continuous medical education of medical staff to aid with early recognition of disease (Tasdelen Fisgin et al., 2010). In addition to a history of potential exposure, haematological abnormalities initially present include thrombocytopaenia, leukopaenia or leucocytosis and raised hepatic aminotransferases (alanine aminotransferase (ALT) and aspartate aminotransferase (AST)) (Swanepoel et al., 1989). Disproportionately higher levels of AST as compared to ALT have been suggested to be associated with a poor prognosis (Ergönül et al., 2004).

Suspected CCHF cases are diagnosed by a combination of virus detection and demonstration of a CCHFV-specific antibody response. During the viraemic phase, a diagnosis of CCHF may be confirmed by virus isolation in cell culture or suckling mice and/or viral nucleic acid detection by reverse transcriptase polymerase chain reaction

(RT-PCR). Viraemia generally persists for the first seven days after the onset of symptoms; however, the virus may be detected up to 12 days after the onset of disease (Butenko and Chumakov, 1990; Swanepoel et al., 1989). Virus isolation in cell culture is less sensitive compared to isolation in suckling mice but has been shown to reduce the time to diagnosis from 5-10 days to 1-6 days (Shepherd et al., 1986). Virus isolation is advantageous as it is highly sensitive and allows characterisation of the CCHFV strain; however, a BSL-4 facility is required and the time to diagnosis is not practical for a rapidly progressive disease such as CCHF. However, with the availability of newer sequencing technologies, genetic characterisation can be performed without the need for virus isolation. Currently, the most rapid method for CCHF diagnosis in the acute phase is the detection of viral nucleic acid in clinical samples by conventional or real-time RT-PCR. Detection by PCR is not only rapid but highly sensitive and suitable for early detection (Mazzola and Kelly-Cirino, 2019), which is imperative to establish a diagnosis of CCHF where a detectable antibody response is absent or delayed (Shepherd et al., 1989). However, high nucleic acid diversity within the CCHFV genome (Deyde et al., 2006) necessitates confirmation that assays are appropriate to detect all CCHFV strains circulating within the specific geographical area. Most viral nucleic acid amplification assays target highly conserved regions on the S-segment. A Simple-Probe® real-time PCR assay targeting the M-segment has been developed for differentiation of reassortant and non-reassortant CCHFV strains in southern Africa, since reassortant and non-reassortant viruses circulate in South Africa (Kondiah et al., 2010). The virus titre or viral load not only has diagnostic but prognostic implication with high viral loads being a poor prognostic indicator (Wölfel et al., 2007). A decrease in the viral load generally starts on day five after the onset of symptoms and has been shown to coincide with the appearance of detectable CCHFV-specific antibody (Butenko and Chumakov, 1990).

Diagnosis of recent or current CCHFV infections may be confirmed later in infection by the detection of CCHFV-specific immunoglobulin (Ig)M antibody or by demonstration of seroconversion indicated by a four-fold increase in the CCHFV-specific IgG antibody titre in paired sera or conversion from a negative to a positive result for CCHFV-specific IgG antibody (Shepherd et al., 1989). Antibody is generally detectable by day nine after the onset of symptoms in non-fatal cases but is

infrequently detectable in fatal cases. CCHFV-specific IgM titres peak at days 9-20 and may persist for up to four months, whereas CCHFV-specific IgG titres peak two to four months post-infection and may persist for years (Shepherd et al., 1989). Antibody may be detected in clinical samples using complement fixation, haemagglutination inhibition and immunodiffusion assays; however, ELISA and IFA have proven more useful for diagnosis due to the ability to differentiate CCHFV-specific IgM and IgG antibody facilitating differentiation between recent or current infections and past infections (Shepherd et al., 1989). Additionally, studies have identified immunodominant linear B-cell epitopes in CCHF survivors (Fritzen et al., 2018; Goedhals et al., 2015; Liu et al., 2014), which may have application in the development of new rapid point-of-care diagnostic assays.

Alternative assays for use in resource-poor areas that do not require sophisticated laboratory equipment have been explored for the diagnosis and surveillance of CCHF, which included the development of a low-density macroarray (Wölfel et al., 2009), reverse transcriptase loop-mediated isothermal amplification assay (RT-LAMP) (Osman et al., 2013) and nucleic acid sequence-based amplification assay (NASBA) (Pieters, 2015). These assays may prove useful for preliminary screening or diagnosis for CCHF cases occurring in remote areas where healthcare facilities have limited diagnostic capacity. One major challenge is the preparation of high-quality RNA in the field to use as input material. A device for in-field viral RNA extraction followed by PCR analysis has been described (Zhong et al., 2007) but is not commercially available.

Treatment, prevention and control

Treatment of CCHF consists mainly of supportive care by maintaining the electrolyte balance, volume replacement therapy and the administration of platelet and erythrocyte preparations. Additionally, all organ systems should be monitored and support provided as required. Currently, no virus-specific treatment has been approved for CCHF and the administration of immune serum prepared from CCHF survivors and ribavirin is highly controversial as the efficacy of these treatment interventions has not been determined in controlled clinical trials. Ribavirin was shown to effectively inhibit CCHFV replication *in vitro* (Watts et al., 1989) and, although not

approved, is frequently administered as a therapeutic agent in CCHF patients and post-exposure prophylaxis in close contacts. The administration of high-dose glucocorticoids has been suggested to reduce the required number of units of infused blood products (Dilber et al., 2010; Hashemi-Shahri et al., 2017). A novel antiviral recently showed excellent *in vitro* and *in vivo* activity against CCHFV in cell culture and in type I IFN-receptor deficient mice, respectively (Oestereich et al., 2014). Favipiravir (T-705), a nucleoside analogue, was shown to be more effective than ribavirin in a mouse challenge study with a 100% protection rate, no signs of disease or detectable virus in the blood and organs when administered up to 2 days post-infection (Oestereich et al., 2014). A compound, 2'-deoxy-2'-fluorocytidine, was demonstrated to be 200 times more potent than ribavirin and 17 times more potent than favipiravir *in vitro* using a reporter virus system. A synergistic effect was observed with co-administration of 2'-deoxy-2'-fluorocytidine and favipiravir (Welch et al., 2017). The reporter virus system may have application in high-throughput screening of potential therapeutic agents, which may assist in the rapid identification and assessment of potential therapeutic agents.

Currently, there is no approved vaccine against CCHFV for use in humans or animals. Therefore, prevention and control are reliant on minimising exposure to potential sources of infection, which may include the use of insect repellent on exposed skin and acaricides on livestock, to wear clothing impregnated with permethrin and to avoid contact with blood and/or tissue of animals. However, these preventative measures are difficult to implement on a day-to-day basis in high-risk groups, such as farmers, farm workers and abattoir workers. In the hospital and laboratory setting, secondary cases are effectively prevented by the isolation of an infected patient and the use of appropriate personal protective equipment to prevent percutaneous or mucosal exposure.

An unlicensed inactivated suckling mouse brain-derived vaccine, initially prepared in 1970 by inactivation with chloroform, heat treatment at 58˚C and adsorption to aluminium hydroxide, has been in use since 1974 in Bulgaria to vaccinate at-risk individuals and individuals who reside in CCHF endemic regions within the country. After implementation of the inactivated vaccine in Bulgaria, a significant decrease in the number of reported CCHF cases was evident, which decreased from 1105 cases between 1953-1974 to 279 cases between 1975-1996 (Papa et al., 2011). Factors

other than the vaccine, may have contributed to a decrease in CCHF cases but is unlikely to result in a 4-fold decrease suggesting that the Bulgarian vaccine did result in a decrease in CCHF cases reported in Bulgaria. Despite remaining unlicensed for use, the vaccine is still in use in Bulgaria. Genetic analysis of the CCHFV vaccine strain revealed that all three genomic segments cluster with the southeastern European and Turkish CCHFV strains (Papa et al., 2011). Evaluation of the immune response to the Bulgarian vaccine in healthy individuals revealed a robust cellular immune response post-immunisation with significant increases observed after the administration of booster doses. Despite high antibody titres after a single dose, low neutralising antibody titres were only detected after several vaccine doses were administered (Mousavi-Jazi et al., 2012).

Several different approaches to prepare a vaccine against CCHFV have been explored with varying levels of success. Evaluation of inactivated CCHFV vaccines prepared in mouse brain and cell culture demonstrated the ability to induce neutralising antibody responses but required booster doses to enhance the level of neutralising antibody activity (Canakoglu et al., 2015; Mousavi-Jazi et al., 2012). The cell-culture based inactivated demonstrated an 80% protection rate in type I IFN-receptor deficient mice (Canakoglu et al., 2015). Transgenic plant-based vaccines containing the viral GPs elicited an antibody response (Ghiasi et al., 2011; Kortekaas et al., 2015) but the G_C and G_N plant-based vaccines evaluated for protection failed to protect signal transducer and activator of transcription (STAT)1 knockout mice (Kortekaas et al., 2015). Two DNA vaccines encoding the CCHFV GPs expressed from the pWRG7077 plasmid under control of an early-immediate cytomegalovirus promoter demonstrated significant differences in immunogenicity. The DNA vaccine delivered by gene gun demonstrated poor immunogenicity (Spik et al., 2006), whereas the DNA vaccine delivered intramuscularly elicited an antibody and cell-mediated response and protected 75% of type I IFN-receptor deficient mice against viral challenge (Garrison et al., 2017). Two adenovirus-based vaccines expressing the GPs or NP were demonstrated to be immunogenic (Sahib, 2010; Zivcec et al., 2018). The adenovirus-based vaccine expressing the NP protected type I IFN-receptor deficient mice against viral challenge (Zivcec et al., 2018), whereas the adenovirus-based vaccine expressing the GPs failed to protect STAT1 knockout mice (Sahib, 2010). A transcriptionally competent virus-like particles (tcVLP) vaccine was

immunogenic but only protected 40% of type I IFN-receptor deficient mice against viral challenge (Hinkula et al., 2017). A Modified Vaccinia virus Ankara (MVA) based vaccine adapted to express the CCHFV NP failed to protect type I IFN-receptor deficient mice against CCHFV challenge (Dowall et al., 2016).

To date, the most promising candidate vaccines with a 100% protection rate include the MVA adapted to express the CCHFV GPs and the NP, G_N and G_C-encoding DNA vaccines with or without tcVLPs. The MVA-GP elicited a cell-mediated and humoral immune response and yielded a 100% survival rate in type I IFN-receptor deficient mice and 129Sv/Ev mice challenged with a lethal dose of CCHFV IbAr10200 (Buttigieg et al., 2014). Another study using DNA plasmids encoding the CCHFV NP, G_N and G_C with or without tcVLPs, showed a 100% survival rate in CCHFV IbAr10200 challenged type I IFN-receptor deficient mice with protection attributed to a predominantly T-helper (Th)1 response accompanied with a balanced Th2 response and the presence of neutralising antibody (Hinkula et al., 2017). DNA vaccines have demonstrated poor immunogenicity in larger mammals and the immunogenicity and protection in response to DNA vaccines will need to be verified in larger mammals.

Immune response to CCHFV

CCHFV has the ability to impair the immune response to infection, which affects the innate and adaptive immune response. Activation of the innate immune response is not only essential for limiting virus proliferation and dissemination early in infection by the induction of an antiviral state, but also for activation of the adaptive immune response that is essential for viral clearance and protection against disease. Type l IFNs, including IFN-α and –β, play a critical role in the induction of an antiviral state (Gerlier and Lyles, 2011) and CCHFV has been shown to be sensitive to IFN-α. A 100 to 1000 fold decrease in the virus titre was demonstrated in the presence of IFN-α (Andersson et al., 2006). Subsequently, replicating CCHFV was shown to delay the innate immune response by suppressing type l IFN secretion *in vitro* resulting in the virus being insensitive to type l IFN at the time of release. The delay was shown to last up to 48 hours post-infection allowing rapid virus proliferation, which may promote viral spread throughout the host (Andersson et al., 2008). The CCHFV Hoti strain NP was shown to suppress IFN-β promoter activity *in vitro* and other viral components

were suspected to be involved in the suppression of the innate immune response (Fajs et al., 2014). The delay in the innate immune response may be a contributing factor to the absence of a detectable antibody response in the majority of fatal CCHF cases due to delayed activation of the adaptive immune response. Other contributing factors may include interference by CCHFV with the maturation and functioning of antigen presenting cells, including dendritic cells and macrophages (Peyrefitte et al., 2010), no upregulation of major histocompatibility complex (MHC) class ll molecules on dendritic cells and macrophages in response to infection and the depletion of natural killer cells and lymphocytes despite activation (Bente et al., 2010). The depletion of lymphocytes may, in part, be attributed to a haemophagocytic syndrome described in up to 50% of CCHF patients (Cagatay et al., 2007; Karti et al., 2004).

The correlates of protection against CCHFV are unclear; however, recent vaccine studies and studies of the immune response in previously or currently infected individuals have significantly contributed to an improved understanding of the elements required to mediate protection against disease. A dysregulated immune response contributes to the severity of disease and significantly elevated levels of Th2 associated cytokines have been demonstrated in fatal CCHF cases as compared to CCHF survivors, which include interleukin (IL)-6, IL-8, monocyte chemoattractant protein (MCP)-1 and IFN gamma-induced protein (IP)-10 (Ergönül et al., 2017; Papa et al., 2016). Additionally, a weak Th1 response, which is important for viral clearance, is elicited by CCHFV (Papa et al., 2016). Long-lived $CD8^+$ T-lymphocyte responses have been demonstrated but were highly variable amongst CCHF survivors (Goedhals et al., 2017). Despite elevated levels of regulatory T-lymphocytes (T_{reg}) during the acute phase of disease, a proportion of the T_{reg} cells had impaired immunosuppressive capacity (Gazi et al., 2018). Regulation of immune activation is crucial to establish a balance between virus clearance and immunopathology, and impaired capacity to regulate immune activation may result in the induction of high cytokine and chemokine levels associated with increased disease severity.

Vaccine studies have illustrated the immunogenicity and/or protection associated with the different viral proteins. An MVA candidate vaccine prepared using the entire glycoprotein precursor coding region demonstrated complete protection after immunisation. Investigation of the cellular immune responses after vaccination revealed the highly immunogenic nature of the non-structural domains, including the

NS_M and GP38, and the structural G_C protein (Buttigieg et al., 2014). Whereas the NP, despite its immunogenicity, could not protect mice in a virus challenge study post-immunisation (Dowall et al., 2016). Interestingly, a recent study evaluating an adenovirus-vectored vaccine with CCHFV NP as the sole vaccine antigen demonstrated a survival rate of 78% after virus challenge (Zivcec et al., 2018), which indicates that the vaccine approach is crucial to the success of a vaccine not only the vaccine antigen/s included. Recently, a study by Hinkula and colleagues concluded that a Th1 immune response provides the most efficient protective immunity against CCHFV. The survival of mice with low neutralising antibody titres was attributed to a dominant Th1 and balanced Th2 response; however, the authors cautioned against undervaluing the role of neutralising antibody, because the majority of surviving mice had high neutralising antibody titres (Hinkula et al., 2017). CCHFV neutralisation may be context-dependent, since monoclonal antibodies targeting the G_N were shown to be non-neutralising in SW-13 cells, yet these antibodies provided more effective protection compared to monoclonal antibodies targeting the G_C protein in mice (Bertolotti-Ciarlet et al., 2005). The inability of several neutralising monoclonal antibodies that target the G_C protein to protect mice against a lethal CCHFV challenge suggested that a protective immune response against CCHFV may not be mediated solely by neutralising antibodies or that the neutralising capability of antibody may differ depending on the host cell/organism. Non-neutralising antibody-dependent mechanisms like antibody-dependent cellular cytotoxicity were suggested to play a role in protection *in vivo* (Bertolotti-Ciarlet et al., 2005). Protection is therefore likely mediated by an interplay between the innate, cellular and humoral immune responses with an unfavourable outcome if involvement from any one or more of the responses is absent or impaired.

Pathogenesis

CCHF pathogenesis is poorly understood due to the requirement of BSL-4 facilities for handling and culturing CCHFV and the limited availability of BSL-4 research facilities in endemic areas. Three mouse models that clinically replicate CCHF infection have been identified, two deficient in type l IFN-receptor and the other deficient in STAT1, which are essential components of the IFN-signalling pathway (Bente et al., 2010;

Bereczky et al., 2010; Zivcec et al., 2013). STAT1 has been demonstrated to be vital for early activation of type I IFN production and antiviral gene expression, and knockout of the *STAT1* gene resulted in a 1-day delay in upregulation of the innate immune markers (Bowick et al., 2012). In addition, the transcriptional innate immune signalling profile was more complex in wild-type mice compared to STAT1 knockout mice (Bowick et al., 2012). The identification and use of mouse models may provide insight into the mechanisms utilised by CCHFV for causing disease. The importance of type l IFN in viral suppression and protection against severe infection have been illustrated in the CCHF mouse models (Bente et al., 2010; Bereczky et al., 2010; Zivcec et al., 2013). Additionally, HAZV infection in type I IFN-receptor deficient mice presents with histopathological findings similar to the existing CCHFV mouse models (Dowall et al., 2012), which suggests the utility of HAZV as a surrogate model for testing of antivirals against CCHFV in the absence of high containment animal facilities.

More recently, an immunocompetent cynomolgus macaque disease model has been identified for CCHF. The non-human primate model did not display uniform lethality with infections ranging from asymptomatic to fatal (Haddock et al., 2018). Due to a low rate of severe infection in the non-human primate model, the costs associated with the use of the model to determine vaccine efficacy and protection against disease will be high, which may affect the utility of the model for use in vaccine development. However, since the model represents the full range of clinical presentations from asymptomatic to lethal infections, the model may prove more useful to study viral pathogenesis and the contribution of specific host-factors to the outcome of disease.

CCHFV preferentially enters and is released at the basolateral surface of epithelial cells, therefore requiring the virus first to traverse the epithelial lining or to be present in the bloodstream before being able to interact with the host cell receptors on the basolateral surface. Factors that may contribute to systemic spread include the release of virus at the basolateral surface into the bloodstream (Connolly-Andersen et al., 2007) and virus amplification in dendritic cells and macrophages, which may allow extravasation to the spleen and local lymph nodes (Connolly-Andersen, 2010). In the STAT1 knockout mouse model, initial virus replication was shown to occur in the blood with subsequent spread to the liver and spleen (Bente et al., 2010). CCHFV has the ability to replicate in various tissues after haematogenous spread, including the

adrenal gland, bone marrow, lymphatic gland (breast), hypothalamus, kidneys, liver, lungs, spleen and the wall of the large intestine (Butenko and Chumakov, 1990). CCHFV has been shown to mainly target endothelial cells, hepatocytes and macrophages with the liver as the main site for CCHFV virus replication (Burt et al., 1997), which may, in part, be due to the fenestrated liver sinusoids and lack of a basement membrane allowing access to the basolateral plasma membranes of endothelial cells and hepatocytes (Connolly-Andersen, 2010). Post-mortem examination of the liver has revealed extensive necrosis of hepatocytes and thrombi in the central and portal veins (Swanepoel et al., 1989). Viral infection results in damage to the host endothelium due to the direct and indirect effects of viral replication (Connolly-Andersen et al., 2009, 2011), which may contribute to activation of the intrinsic coagulation cascade (Swanepoel et al., 1989), increased vascular permeability and the initiation of an inflammatory immune response (Connolly-Andersen et al., 2011).

The intrinsic coagulation cascade is activated in response to dysregulated platelet aggregation, which is a consequence of endothelial damage and leads to a deficiency in clotting factors resulting in exacerbation of the bleeding tendency (Weber and Mirazimi, 2008). Additionally, the initiation of an inflammatory response may exacerbate damage to the host endothelium. Significantly elevated serum levels of IL-6 and tumour necrosis factor (TNF)-α have been detected in fatal CCHF cases as opposed to non-fatal cases (Ergonul et al., 2006; Kaya et al., 2014) and were positively correlated to the DIC score (Ergonul et al., 2006). In a more recent study, elevated levels of IL-6, IL-8 and MCP-1 were shown to predict a fatal outcome early in disease (first five days) (Ergönül et al., 2017). In addition to acting as an activator of endothelial cells in CCHF infection (Connolly-Andersen et al., 2009) and being correlated to the DIC score (Ergonul et al., 2006), TNF-α has also been correlated to disease severity indicating a significant role in the activation of endothelial cells and DIC in the pathogenesis of CCHFV (Papa et al., 2006).

Critical vascular functions regulated by endothelial cells are disrupted due to damage to the vascular endothelium resulting in increased vascular permeability, vasodilation and vascular leakage. The disruption of vascular homeostasis results in hypotension, multiple organ failure and shock, which frequently precedes death in CCHF patients (Swanepoel et al., 1989). The increase in vascular permeability may be mediated by

internalisation of the adherens junction protein that, in turn, perpetuates the inflammatory response (Connolly-Andersen et al., 2011) and/or the release of TNF-α resulting in the destabilisation of the microtubule network (Petrache et al., 2003). The causation of vascular leakage has been shown not to involve the tight junctions in the endothelium, which form the barrier between endothelial cells and are essential for vascular homeostasis (Connolly-Andersen et al., 2007).

Currently, there is no experimental data to support a difference in virulence between geographically distinct strains of CCHFV. The virus-host response was recently explored in CCHF infected individuals with regard to the expression of microRNAs that may regulate gene expression. A total of 106 microRNAs were differentially expressed, which have been proposed to be associated with cytokine expression, the secretion of adhesion molecules and escape from innate immunity (Demir et al., 2017). Disease is most likely attributable to the direct and indirect interaction of the virus with host cells. Currently, no specific host-factors have been identified that may predispose a host to more severe disease.

CCHF in South Africa

The first case of CCHF in South Africa was reported in 1981 in a 13-year old boy after attending a school camp at a nature reserve within the Bloemhof district, western Transvaal (today part of the North West Province). The boy initially presented with high fever, chills, severe headache and myalgia, and rapidly deteriorated to a severe haemorrhagic state within three days of the onset of symptoms (Gear et al., 1982). Despite aggressive volume replacement therapy and comprehensive support, the patient succumbed on day six of illness after cardiac arrest without successful resuscitation. A tick, retrospectively identified as a *Hyalomma* tick, was removed from the scalp of the boy on the first day of illness (Gear et al., 1982). Two subsequent studies found serological evidence that suggested the widespread distribution of antibody against CCHFV in South Africa and the presence of the virus before the first recognised case in 1981 (Swanepoel et al., 1983, 1985b).

In 1984, a nosocomial outbreak of CCHF was reported at Tygerberg Hospital in the Western Cape. After the admission of the index patient with evidence of a tick-bite,

seven healthcare workers were infected by direct and indirect contact with the index patient (van Eeden et al., 1985b). In the same year, an outbreak was reported on a dairy farm in the Free State after contact with the blood and/or tissue of an infected cow (Swanepoel et al., 1985a). In 1996, an outbreak at an ostrich abattoir near Oudtshoorn in the Western Cape led to a ban on the exportation of ostriches and ostrich meat to the European Union, which was later lifted based on the results obtained from the experimental infection of ostriches and implementation of the necessary precautions to prevent dissemination of CCHFV through export (Swanepoel et al., 1998).

In South Africa, the majority of cases have been reported in male agricultural workers with the most common routes of transmission being tick-bite or the squashing of a tick with bare hands. The fatality rate for CCHF in southern Africa based on laboratory-confirmed cases is around 24% (Msimang et al., 2013). In South Africa, suspected CCHF cases are referred to the National Institute for Communicable Diseases for confirmation of a CCHF diagnosis by RT-PCR or serological methods.

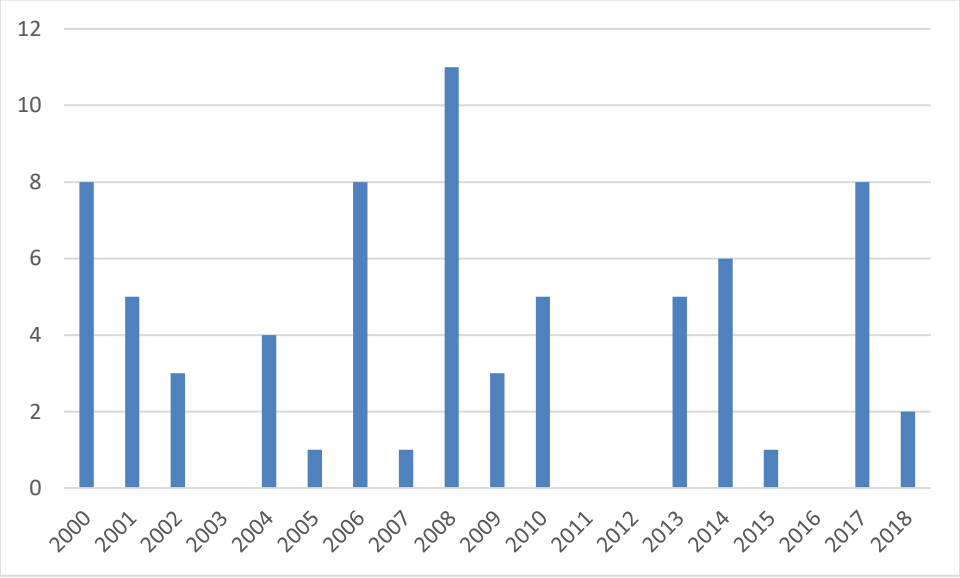

Figure 4: Confirmed Crimean-Congo haemorrhagic fever cases in South Africa

Constructed using data from the Communicable Diseases Communiqué from the National Institute for Communicable Diseases from 2000-2018.

The World Health Organisation (WHO) identified CCHFV as a priority pathogen for research due to the emergence of CCHFV in previously naïve regions, the need for rapid, point-of-care diagnostic tools and the lack of approved vaccines or therapeutic interventions (World Health Organization, 2018). Research that advances our knowledge on immune responses will contribute to our understanding of pathogenesis, immune correlates of protection and may contribute to the identification of novel therapeutic interventions and the development of vaccines.

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Chapter 3

A role for Crimean-Congo haemorrhagic fever virus NS_M protein in innate immune modulation

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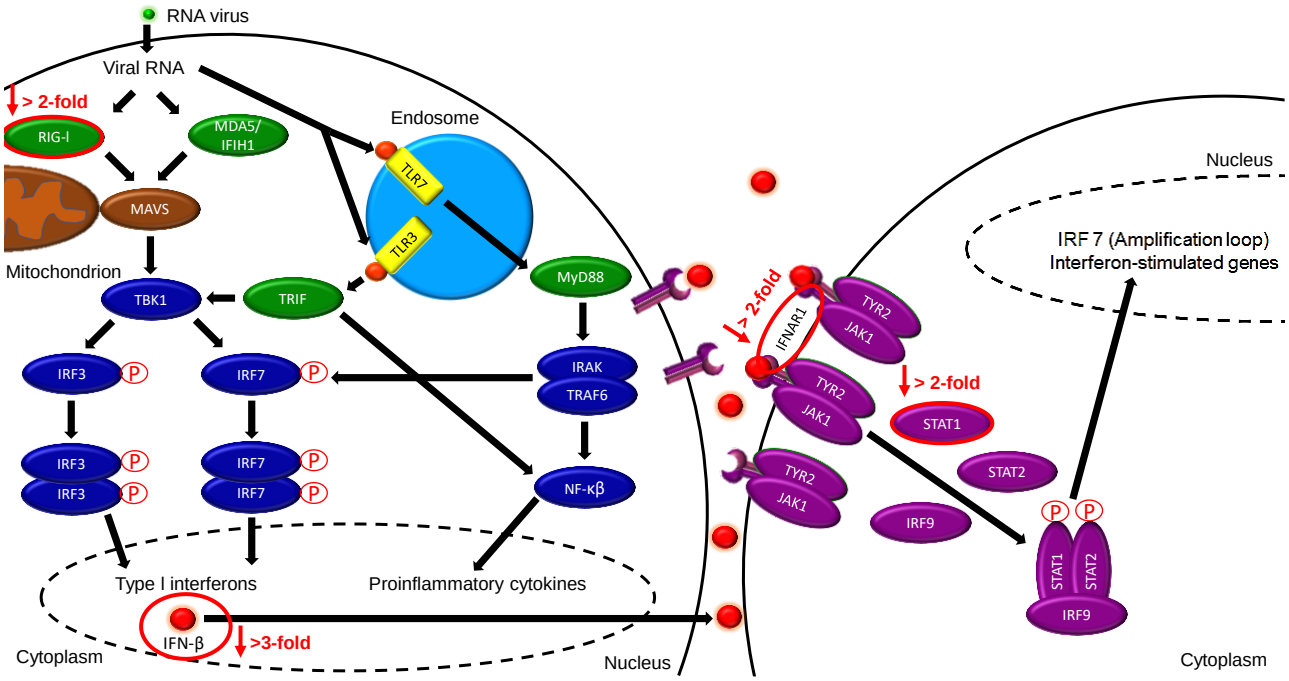
Summary

The innate immune response is crucial for virus recognition and limiting virus replication and spread during early infection. However, viruses have developed mechanisms to evade the immune system, which may render the immune response ineffective at protecting the host. Crimean-Congo haemorrhagic fever orthonairovirus (CCHFV) delays innate immune activation. To further explore the mechanism utilised, the CCHFV NS_M protein from reassortant and non-reassortant South African isolates were expressed *in vitro* and innate immune gene expression profiles determined using quantitative polymerase chain reaction (qPCR). The non-reassortant CCHFV NS_M protein induced transcriptional downregulation of *DDX58*, *IFNB1*, *IFNAR1* and *STAT1* gene expression, which may result in impaired virus recognition and subsequent innate immune activation. Interestingly, expression of the reassortant CCHFV NS_M protein resulted in transcriptional upregulation of all innate immune markers evaluated in this study. The most significant difference in gene expression between the CCHFV NS_M protein evaluated was a more than 6-fold difference in *IFNB1* gene expression. Improving our understanding of the virus-host innate immune interactions may assist in rational vaccine development and the identification of therapeutic interventions against CCHFV.

Keywords

Crimean-Congo haemorrhagic fever virus, NS_M protein, reassortant, non-reassortant, innate immune response, interferon

Graphical abstract



Introduction

Crimean-Congo haemorrhagic fever orthonairovirus (CCHFV) is a tripartite negative-sense single-stranded RNA virus that belongs to the family *Nairoviridae* and genus *Orthonairovirus*. CCHFV is widely distributed across the African, Asian and eastern European continents with the distribution of disease correlating to the distribution of the primary vector, which are ticks that belong to the genus *Hyalomma* (Hoogstraal, 1979). The potential for CCHFV to extend to new endemic foci where *Hyalomma* ticks are present and favourable environmental conditions exist is cause for concern. Of particular concern is the emergence and the high number of cases in Turkey since 2002 (Karti et al., 2004) and recent expansion to the southern European countries Greece (Papa et al., 2008) and Spain (Negredo et al., 2017). Disease presentation may vary from a mild non-specific febrile illness to severe haemorrhagic disease with

multiorgan failure (Swanepoel et al., 1987). Control of CCHFV is complicated by the lack of a vaccine, efficient long-term vector control strategies, the potential for nosocomial transmission, and the absence of virus-specific antiviral therapy. The development of vaccines and therapeutics require an improved understanding of the virus-host interactions to ensure an efficient, protective immune response.

CCHFV has been shown to interfere with the innate immune response *in vitro* resulting in a delay in interferon (IFN) secretion by up to 48 hours post-infection (Andersson et al., 2008). Additional mechanisms utilised for immune dysregulation include interfering with the maturation and functioning of antigen presenting cells (Peyrefitte et al., 2010), depleting natural killer cells despite activation, and inadequate upregulation of the major histocompatibility complex (MHC) class ll molecules on antigen presenting cells with concomitant lymphocyte depletion, despite activation (Bente et al., 2010). The interaction of CCHFV with the immune system may contribute to the lack of a detectable antibody response in the majority of fatal Crimean-Congo haemorrhagic fever (CCHF) cases. Determining the role of each CCHFV protein in innate immune modulation is crucial for rational vaccine design and the identification of potential therapeutic interventions. To investigate early virus-host interactions, innate immune modulation in the presence of the CCHFV non-structural protein encoded on the M-segment (NS_M) was evaluated *in vitro* using mammalian cell culture. The CCHFV NS_M protein was first described in 2007 (Altamura et al., 2007) and the role or function of the protein is yet to be determined. The function of the NS_M protein in other viruses belonging to the order *Bunyavirales* seems to be diverse. The Rift Valley fever virus (RVFV) NS_M protein has an anti-apoptotic function (Won et al., 2007) and is dispensable for virus maturation, replication, and infection (Gerrard et al., 2004). In contrast, Bunyamwera virus (BUNV) lacking the NS_M protein yielded no viable virus using a reverse genetics system indicating an essential role in virus maturation, replication and/or infection. The N-terminal region of the BUNV NS_M protein plays a role in virus assembly, and the C-terminal region may serve as an internal signal sequence for the G_C glycoprotein (Shi et al., 2006). Therefore, the function of the NS_M protein may not be conserved within the order *Bunyavirales* (previously *Bunyaviridae* family).

Currently, it is not clear if there are differences in pathogenicity of genetically distinct strains of CCHFV. Initially, it was postulated that Russian and Asian strains were more

pathogenic than African strains (Hoogstraal, 1979) and more recently it has been suggested that strains related to the Greek isolate AP92 may be less pathogenic than other strains (Ozkaya et al., 2010). Similarly, there is no clarity on differences in pathogenicity between reassortant and non-reassortant CCHFV strains. In South Africa, a high frequency of M-segment reassortment has been identified in clinical CCHFV isolates with reassortants acquiring an M-segment genetically identified as belonging to group IV with Asian CCHFV isolates (Burt et al., 2009; Goedhals et al., 2014). Studies investigating CCHFV genetic diversity in South Africa have been limited to partial sequence analysis with limited complete genome analysis, and no conclusive evidence exists to support a link between reassortment and the outcome of disease (Burt et al., 2009; Goedhals et al., 2014). Hence, in addition to profiling the innate immune responses to the CCHFV NS_M protein, by selecting M-segment gene sequences from a reassortant isolate belonging to lineage IV and a non-reassortant isolate belonging to lineage III this study may provide insight into possible strain-to-strain variation. The CCHFV NS_M coding regions are highly variable between genetically distinct lineages, and despite only 7,12% heterogeneity at amino acid level between the CCHFV NS_M proteins investigated in this study, variability within the encoded proteins may influence the role or functioning of the proteins.

In this study, modulation of innate immune signalling was evaluated at transcriptional level after transient expression of a South African reassortant and non-reassortant CCHFV NS_M protein *in vitro*. The innate immune activation profiles for the reassortant and non-reassortant CCHFV strains isolated from the same geographic area, but representing genetically diverse genes, were compared.

Methods

Cells

Human embryonic kidney (HEK)-293 cells (ATCC® CRL-1573™; RRID: CVCL_0045) were obtained from the American Type Culture Collection (ATCC®) and cultured in Dulbecco's Modified Eagle Medium (Lonza) supplemented with 10% foetal bovine serum (Gibco), 100 units per ml (U/ml) penicillin and streptomycin (Lonza), L-glutamine (Lonza) to a final concentration of 2mM and non-essential amino acids

(Lonza) at 37˚C in a humid 5% CO_2 atmosphere. Cells were passaged when 80-90% confluent and only used for electroporation within the first 15 passages after removal from cryopreservation.

Plasmids

Sequence data was retrieved from GenBank® for two CCHFV strains isolated from patients with a fatal outcome. The isolates selected as representatives of reassortant and non-reassortant strains were SPU45/88, with an M-segment that groups with Asian CCHFV isolates (group IV; reassortant), and SPU187/90, with an M-segment that groups with South African CCHFV isolates (group III; non-reassortant) (Goedhals et al., 2014). The nucleotide sequences encoding the CCHFV NS_M proteins (GenBank accession numbers: SPU187/90 M-segment KJ682814; SPU45/88 M-segment KJ682809) were codon-optimised, using the JAVA codon adaptation tool (JCat) (Grote et al., 2005; http://www.jcat.de/), for expression in human cells. A Kozak sequence was added to the 5' end to facilitate high-level expression in a mammalian cell. The 3' end was modified by the addition of a FLAG tag (amino acid sequence: Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) to facilitate detection and characterisation of the expressed proteins using mouse monoclonal anti-FLAG antibody (Thermo Scientific; RRID: AB_1957945). The CCHFV NS_M genes and the gene encoding a green fluorescent protein (GFP) were synthesised and supplied in pcDNA3.1(+) by GenScript®. Lyophilised plasmid DNA was reconstituted in nuclease-free water and chemically competent *Escherichia coli* JM109 cells (Zymo Research Mix&Go!) with a transformation efficiency of 10^8 CFU/µg were transformed using the heat-shock method. Positive transformants were selected using ampicillin to a final concentration of 100µg/ml, and plasmid construction was confirmed by polymerase chain reaction (PCR) and nucleotide sequence determination using the T7 forward (5'-TAA TAC GAC TCA CTA TAG GG-3') and bovine growth hormone (BGH) reverse (5'-TAG AAG GCA CAG TCG AGG-3') primers flanking the site of insertion. Confirmation of positive transformants was performed with GoTaq® G2 DNA polymerase (Promega) according to the manufacturer's instructions using 0,2µM of each primer. Cycling conditions included denaturation at 95˚C for two minutes followed by 40 cycles of denaturation at 95˚C for one minute, annealing at 45˚C for one minute and extension at 72˚C for one

minute and a final extension of 72˚C for five minutes. Sequence data were analysed using Chromas Pro version 1.6 (Technelysium), the open reading frames identified using ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/) and translated to amino acid sequence using the Expasy translational tool (Gasteiger et al., 2003; https://web.expasy.org/translate/). The nucleotide and amino acid sequences were aligned to the codon-optimised genes and translated amino acid sequences using Clustal Omega (Sievers et al., 2011; https://www.ebi.ac.uk/Tools/msa/clustalo/) to confirm that complete nucleotide and amino acid sequence identity was maintained. The use of double-stranded plasmid DNA for the introduction of the NS_M proteins may result in the upregulation of transcription of various innate immune markers due to overlap in the recognition and activation pathways for RNA and DNA viruses. To account for transcriptional activation due to the presence of double-stranded DNA, an empty vector construct for use as a negative control was prepared by excision of the gene encoding the GFP using *Hind*lll and *Bam*HI restriction endonucleases (Promega) from the pcDNA3.1(+)GFP plasmid, which was confirmed by nucleotide sequence analysis.

Expression of the CCHFV NS_M protein

Plasmid DNA was purified from bacterial cultures using the QIAGEN® Plasmid *Plus* Midi Kit (Qiagen) according to the manufacturer's instructions and concentrated using the Eppendorf™ Vacufage™ Plus Concentrator (Eppendorf) to ensure DNA concentrations exceeding 1µg/µl. The concentration of plasmid DNA preparations was determined using a Nanodrop™ 2000 (Thermo Scientific) and cells were enumerated using the Countess™ II automated cell counter (Invitrogen). A single-celled suspension containing $5x10^6$ HEK-293 cells was electroporated with 10µg plasmid DNA using the Neon® 100 transfection kit (Life Technologies) with modifications to the manufacturer's instructions to ensure high-level, reproducible transfection efficiencies. Modifications applied after optimisation included pre- and post-electroporation incubations of at least 3 and 10 minutes, respectively. Each electroporation reaction was subdivided into two 6-well plates containing 45% of the reaction each and the residual cells plated in a 24-well plate containing a glass slide. Protein expression was confirmed using an indirect immunofluorescence assay (IFA)

targeting the 3' FLAG tag. Briefly, cells were fixed in a 1:1 solution of methanol (Merck) and acetone (Merck) at -20˚C for 20 minutes followed by incubation in blocking solution (phosphate buffered saline (PBS) at pH 7,4 containing 0,5% Triton X-100 and 10% sucrose) at room temperature for 20 minutes. Cells were reacted with mouse monoclonal anti-FLAG antibody (Thermo Scientific; RRID: AB_1957945) diluted 1:200 in blocking solution for 90 minutes at 37˚C in a humid atmosphere, washed thrice in PBS containing 1% Tween®20 (Promega) for one minute and reacted with goat anti-mouse IgG fluorescein isothiocyanate (FITC) antibody diluted 1:20 in 0,1% Evans blue (Merck) in PBS. Cells were washed as previously described and visualised by fluorescence microscopy. Untransfected cells were included as a negative control. An estimation of the transfection efficiency was determined using the number of fluorescing cells observed within two to three fields of vision. Cells expressing the CCHFV NS_M protein and cells transfected with an empty vector construct, pcDNA3.1(+), were harvested using a mammalian cell lysis buffer (150mM NaCl, 5mM EDTA, 50mM Tris at pH of 7,4 and bovine serum albumin to a final concentration of 1mg/ml with the addition of a protease inhibitor cocktail (Roche)) on ice and RNAprotect cell reagent (Qiagen) for protein and RNA analysis, respectively. All samples were stored at -20˚C until analysis.

Protein samples were separated on a 12,5% resolving and 4% stacking denaturing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel. A PageRuler™ pre-stained protein ladder (Thermo Scientific) and Magic Mark™ XP Western blot ladder (Thermo Scientific) were included in each run to control for protein transfer and allow size estimation of the blot, respectively. Separated proteins were transferred to a BioTrace™ polyvinylidene fluoride (PVDF) membrane (Pall Corporation) with a pore size of 0,2µm using a Trans-Blot® SD semi-dry electrophoretic transfer cell (Bio-rad). Transferred protein was analysed by Western blot analysis using the Pierce Fast Western blotting kit (Thermo Scientific) and mouse monoclonal anti-FLAG antibody (Thermo Scientific; RRID: AB_1957945) at a dilution of 1:1000 with modification to the manufacturer's instructions to include additional washes and supplemented with SuperSignal™ West Pico PLUS chemiluminescent substrate (Thermo Scientific). Western blot analysis was performed to confirm a similar level of protein expression for all prepared biological repeats and a similar level of reassortant and non-reassortant CCHFV NS_M protein expression.

Relative quantification

Cells collected in RNA stabilisation reagent, RNAprotect cell reagent (Qiagen), were thawed on ice, pelleted by centrifugation and RNA extracted according to the manufacturer's instructions with the addition of beta-mercaptoethanol (Merck) to the RLT buffer using the RNeasy Plus Mini kit (Qiagen). RNA integrity was determined using a 1,2% denaturing RNA gel (Aranda et al., 2012) and by evaluating the 18S and 28S rRNA bands after electrophoresis. The quality and quantity of the RNA were determined using a Nanodrop™ 2000 (Thermo Scientific) and Tris-buffered RNA. The 260/280 and 260/230 absorbance (Abs) ratios were used to confirm the quality of the RNA preparation with 2,0 and >2,0 respectively, reflecting a high-quality RNA preparation. Complementary DNA (cDNA) was prepared by reverse-transcribing 0,8µg high quality, intact RNA using the First Strand Synthesis kit (Qiagen) according to the manufacturer's instructions. The cDNA reaction was diluted using 91µl RNase-free water (Ambion) and relative quantification was performed using 0,9µl diluted cDNA in each reaction.

Relative quantification was performed using three reference and three quality control genes and ten innate immune signalling genes with a custom RT^2 profiler array (Table S1; SA Biosciences) using RT^2 SYBR Green ROX FAST Mastermix (Qiagen) according to the manufacturer's instructions on a Rotor-Gene Q cycler (Qiagen). Data were collected using the Rotor-Gene Q Series Software (RRID: SCR_015740). The threshold value was determined following the manufacturer's instructions and was determined to be 0,08303, which was applied for all subsequent runs. A dissociation curve was generated for each reaction to confirm qPCR specificity and no interference due to primer-dimer formation. The cycle threshold (Cq) values were exported and analysed.

Due to a decline in the *DDX58* amplification efficiency, *DDX58* expression levels were confirmed using primes designed to target a 158bp segment of *DDX58* mRNA (DDX58-FW 5'- GTGGGCAATGTCATCAAAATG-3' and DDX58-RV 5'-GTCTGAAGGCGTAAAATAGAGTC-3') and a 100bp segment of the *GAPDH* mRNA (GAPDH-FW 5'- TGACAACAGCCTCAAGAT-3' and GAPDH-RV 5'-TCCTTCCACGATACCAAAG-3') spanning exon-exon junctions. qPCR assays were developed and reactions prepared using RT2 SYBR® Green ROX FAST Mastermix

(Qiagen) and 0,5µM of each gene specific primer per reaction. Cycling conditions of the Rotor-Gene Q cycler (Qiagen), included denaturation at 95˚C for 10 minutes followed by 40 cycles of denaturation at 95˚C for 15 seconds and combined annealing and extension at 60˚C for 30 seconds. Fluorescence data acquisition was obtained at the end of each cycle and after the amplification cycles were completed melting curve analysis was performed using the default settings on the Rotor-Gene Q (Qiagen).

Analysis of qPCR data

The ΔΔCq method (Livak and Schmittgen, 2001) was used to calculate relative gene expression. Three reference genes (*ACTB*, *B2M* and *GAPDH*) were included for each sample and *GAPDH* was identified as the optimal gene for normalisation. Normalisation was performed by calculating the ΔCq value ($Cq_{\text{Gene of interest}} - Cq_{\text{GAPDH}}$) for each gene of interest. All Cq values > 33 were considered negative. The average ΔCq for biological repeats were used to calculate the ΔΔCq (Average $\Delta Cq_{\text{Experimental group}} - \Delta Cq_{\text{Control group}}$) between the control and each experimental group. The fold change ($2^{-\Delta\Delta Cq}$) and fold regulation (-1/fold change for fold change values < 1; fold change = fold regulation for fold change values > 1) were calculated to determine gene modulation. A change in gene modulation of ≥ 2-fold regulation is considered to be a biologically significant change in gene expression. To determine whether changes in gene expression are statistically significant, the p-value was determined using a Student's t-test with a two-tail distribution and equal variance between two groups using the normalised expression represented by the ΔCq value calculated for each biological repeat (Yuan et al., 2006). A p-value of < 0,05 was considered to be a statistically significant change in innate immune signalling.

Key resources table

Reagent/Resource	Source	Identifier
Antibodies		
Mouse monoclonal anti-FLAG antibody	Thermo Scientific	MA191878; RRID: AB_1957945
Bacterial strains		
Escherichia coli JM109 cells	Zymo Research Mix&Go!	T3003
Critical Commercial Assays		
Neon® 100 transfection kit	Life Technologies	MPK10025
RNeasy Protect Cell Mini Kit (Includes RNAprotect cell reagent)	Qiagen	74624
RT^2 First Strand Kit	Qiagen	330404
RT^2 qPCR FAST SYBR Green/Rox Mastermix	Qiagen	330622
Experimental Models: Cell Lines		
Human embryonic kidney (HEK)-293 cells	American Type Culture Collection	ATCC® CRL-1573™; RRID: CVCL_0045
Oligonucleotides		
Please refer to Table S1	SA Biosciences	Please refer to Table S1
Recombinant DNA		
pcDNA3.1(+) GFP	GenScript®	N/A
pcDNA3.1(+) NS_M187/90	GenScript®	N/A
pcDNA3.1(+) NS_M45/88	GenScript®	N/A
Software and Algorithms		
Chromas Pro	Technelysium	Version 1.6
Rotor-Gene Q Series Software	Qiagen	RRID: SCR_015740
Other		
Dulbecco's Modified Eagle Medium	Lonza	BE12-614F
Foetal bovine serum (Origin: South America)	Gibco	10493106
Penicillin and streptomycin	Lonza	DE17-602E
L-glutamine	Lonza	BE17-605E
Non-essential amino acids	Lonza	BE13-114E
HindIII	Promega	R6041
BamHI	Promega	R6021

Results

Downregulation of *DDX58* gene expression by a non-reassortant South African CCHFV NS_M protein

The recognition of double-stranded RNA replication intermediates by cytosolic receptors is crucial for activation of the innate immune response to RNA viruses. Retinoic acid-inducible (RIG)-I-like receptors (RLRs), including RIG-I and melanoma

differentiation-associated factor 5 (MDA5), are essential for the recognition of RNA viruses (Kato et al., 2006) and subsequent type I IFN-induction in a mitochondrial activator virus signalling (MAVS)-dependent manner. MAVS forms the critical link between virus recognition and innate immune signalling induction. IFN-induction is abolished in MAVS-deficient mice due to no IFN-regulatory factor (IRF) 3 or nuclear factor-κβ activation (Sun et al., 2006). Differential expression of *DDX58* (RIG-I), *IFIH1* (MDA5) and *MAVS* after CCHFV NS_M protein expression was investigated to determine whether the CCHFV NS_M protein can interfere with virus recognition by the innate immune response.

Innate immune signalling was determined using cells transfected with either the reassortant or non-reassortant CCHFV NS_M protein and compared to a control group. Transient expression of the reassortant NS_M protein induced low-level upregulation of *MAVS* and modest upregulation of *DDX58* (RIG-I) and *IFIH1* (MDA5) gene expression (Table 1; Figure 1a - c). In contrast, a 2-fold ($p = 0,0662$) downregulation in *DDX58* (RIG-I) gene expression was observed in response to the non-reassortant CCHFV NS_M protein, whereas low-level downregulation of *IFIH1* (MDA5) and *MAVS* gene expression were observed (Table 1; Figure 1a - c). The reduction in *DDX58* (RIG-I) gene expression may impact virus recognition and IFN-induction during early infection, especially considering recent characterisation of innate immune signalling associated with virus recognition and subsequent induction of an antiviral response using the CCHFV IbAr10200 strain. RIG-I has been identified as the most important receptor for virus recognition and subsequent activation of the innate immune response during infection. Knockout of RIG-I using small interfering (si)RNA transfection completely abrogated the type I IFN response to CCHFV IbAr10200, which in turn resulted in a reduction of IFN-stimulated gene (ISG) expression and increased virus replication, which could not be replicated by knockout of either MDA5 nor MAVS (Spengler et al., 2015). The non-reassortant CCHFV NS_M protein may significantly impact virus recognition by the innate immune response.

Transcriptional modulation of *IRF3* gene expression by a reassortant CCHFV NS_M protein and *IRF7* gene expression by a non-reassortant CCHFV NS_M protein

Type l IFN-signalling is primarily mediated by IRF3 and IRF7 in a two-phased induction mechanism (Sato et al., 2000). IFN-signalling is mediated by phosphorylation of IRF3 and IRF7 followed by nuclear translocation and the induction of IFN- and virus-inducible genes (Wathelet et al., 1998). IRF3 is crucial in early IFN-signalling and primarily induces IFN-β in an IFN-independent manner (Sato et al., 2000). The release of IFN-β induces the transcription of ISGs via IRF-9 (also known as IFN-stimulated gene factor (ISGF) 3), a transcriptional activator, after binding to the type l IFN-receptor on infected and uninfected cells (Levy et al., 1989). Activation of IRF-9 upregulates the transcription of IRF7, which is induced in an IFN-dependent manner and subsequently contributes to type l IFN expression (Sato et al., 1998). Gene knockout studies indicated that IRF3 is essential for efficient IFN-β expression but is unable to induce IFN-α expression in the absence of IRF7, whereas IRF7 contributed to IFN-α and IFN-β induction even in the absence of IRF3. However, the cooperation of IRF3 and IRF7 resulted in the most efficient IFN-α_4 and IFN-β induction compared to knockout models (Sato et al., 2000), which suggests that changes in the level of expression of either IRF3 or IRF7 could have a detrimental impact on type I lFN induction post-stimulation.

The transcriptional levels of IFN-signalling mediators *IRF3* and *IRF7* after NS_M protein expression revealed an important difference between the reassortant and non-reassortant NS_M protein. An almost 2-fold downregulation was observed in *IRF7* gene expression in response to the non-reassortant CCHFV NS_M protein, whereas an almost 2-fold upregulation in *IRF3* ($p < 0,05$) gene expression was observed in response to the reassortant CCHFV NS_M protein (Table 1; Figure 1d, e). Downregulation of *IRF7* gene expression may affect the type l IFN response, especially IFN-α, in virus-infected cells. CCHFV has been shown to be sensitive to IFN-α in pre-treated cells but becomes insensitive to IFN-α treatment at 6 hours post-infection (Andersson et al., 2008). The induction of type l IFN expression by IRF3 and IRF7 has been detected in IFN-primed cells at 6 hours post-stimulation and later in unprimed cells (Sato et al., 2000). Consequently, type l IFN induction mediated via IRF3 and IRF7 may not be effective at limiting CCHFV virus replication. Since the virus will be insensitive to IFNs at the time of induction, the expression of ISG products

may play a more significant role in limiting virus replication and spread than type l IFNs. Type l IFNs are essential signalling proteins that alert infected and uninfected cells to induce an antiviral state by stimulating the expression of ISGs. Downregulation of *IRF7* gene expression may result in a reduction in downstream type l IFN transcription, whereas upregulation of *IRF3* gene expression promotes the transcription of *IFNB1*.

Reassortant CCHFV NS_M protein upregulates *NFKB1* gene expression

Nuclear factor (NF)-κβ, an inducible transcription factor, functions by activating the transcription of an array of genes as part of the early response to infection and induces the transcription of pro-inflammatory cytokines, chemokines, cell adhesion molecules and immune receptors (Pahl, 1999). Additionally, during early infection the NF-κβ forms part of a transcriptional enhancer complex recruited to the IFN-β promoter to induce the transcription of *IFNB1* (Wathelet et al., 1998). Modest transcriptional upregulation of *MYD88* and *NFKB1* gene expression by the reassortant CCHFV NS_M protein in the absence of toll-like receptors (TLRs) indicates upregulation of *MYD88* and *NFKB1* transcription in a TLR-independent manner (Table 1; Figure 1f, g). Expression of the *NFKB1* gene remained unaltered in the presence of the non-reassortant CCHFV NS_M protein, despite low-level downregulation *MYD88* gene expression, which encodes an adaptor protein immediately upstream of NF-κβ in the signalling pathway (Table 1; Figure 1f, g). Despite the absence of TLRs in HEK-293 cells, modest upregulation of the transcription of NF-κβ pathway markers was induced in response to the reassortant CCHFV NS_M protein.

Transcriptional modulation of *IFNB1* gene expression and subsequent ISG-signalling

Transcription of the gene encoding IFN-β is significantly affected by IRF3 and IRF7 deficiency and is reduced by 90 and 50% in double-negative mutants, respectively, which indicates that IRF3 and IRF7 play essential roles in activating the human IFN-β response (Wathelet et al., 1998). IFN-β is responsible for inducing an antiviral state during early infection by activating the transcription of ISGs via interaction with type l

IFN-receptors, which depending on the specific interaction with type l IFN-receptors may be induced in a Janus kinase (JAK)/Signal transducer and activator of transcription (STAT)-dependent or -independent manner (de Weerd et al., 2013). IFN-β stimulation results in the upregulation of twice as many ISGs during early infection compared to IFN-α, indicating an essential role in innate immune signalling during early infection (Der et al., 1998). The transcriptional modulation of the gene encoding IFN-β may impact activation of the innate immune response and subsequently the adaptive immune response.

Expression of the non-reassortant CCHFV NS_M protein resulted in downregulation of *IFNB1* gene expression by more than 3-fold ($p = 0,0571$), whereas expression of the reassortant CCHFV NS_M protein resulted in upregulation of *IFNB1* gene expression by more than 3-fold resulting in a more than 6-fold ($p < 0,05$) difference in gene expression levels between the NS_M protein (Table 1; Figure 1h). The non-reassortant CCHFV NS_M protein may significantly downregulate the initial amplification component of the innate immune response. Additionally, *IFNAR1* and *STAT1* gene expression were downregulated by more than 2-fold ($p < 0,05$) in response to expression of the non-reassortant CCHFV NS_M protein (Table 1; Figure 1i, j). STAT1 is not only indispensable for an IFN response but also survival due to the critical role of the IFN response in the protection of the host from viral infections (Durbin et al., 1996). STAT1 is also an essential component of IRF9 (Müller et al., 1993), a positive transcription regulator, which rapidly becomes activated after IFN-α binds to the type l IFN-receptor translating an extracellular signal to a transcriptional response (Dale et al., 1989; Levy et al., 1989). Additionally, the expression of *IFNAR1* and *STAT1* play a significant role in protection against CCHF infection as illustrated in the CCHFV mouse models deficient in *IFNAR1* and *STAT1*. In the absence of innate immune control, uncontrolled virus replication and spread result in severe disease after infection with CCHFV (Bente et al., 2010; Bereczky et al., 2010; Zivcec et al., 2013).

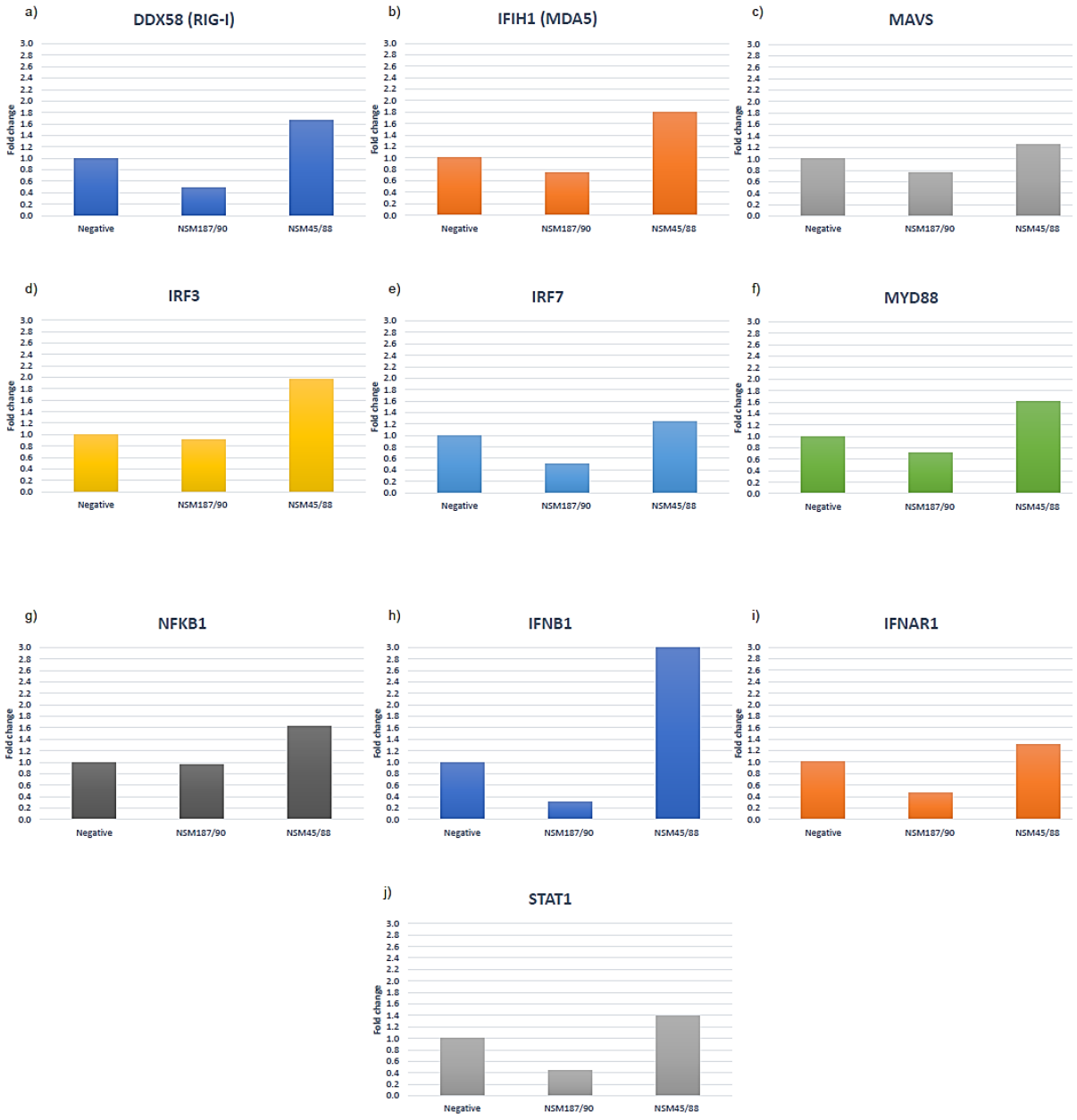


Figure 1: Modulation of gene expression by a reassortant and non-reassortant CCHFV NS_M protein

Modulation of gene expression determined in response to cells transfected with constructs expressing NS_M protein encoded by either a South African CCHFV non-reassortant isolate (NS_M187/90) or a reassortant isolate (NS_M45/88) and compared to a negative control in which cells were transfected with a pcDNA3.1(+) empty vector

Table 1: Innate immune modulation by the NS_M187/90 and NS_M45/88 protein

Protein:	Gene:	Fold regulation:	Fold change:	p-value:
Non-reassortant (NS_M187/90)	*DDX58*	-2,07	0,4830	0,0662
	IFIH1	-1,35	0,7388	0,3024
	MYD88	-1,42	0,7055	0,1757
	MAVS	-1,32	0,7596	0,1570
	IRF3	-1,09	0,9159	0,6330
	IRF7	-1,96	0,5093	0,0649
	NFKB1	-1,04	0,9593	0,8404
	IFNB1	-3,34	0,2994	0,0571
	IFNAR1	-2,17	0,4612	0,0109
	STAT1	-2,32	0,4313	0,0055
Reassortant (NS_M45/88)	*DDX58*	1,66	1,6586	0,2378
	IFIH1	1,79	1,7942	0,1714
	MYD88	1,62	1,6170	0,0842
	MAVS	1,25	1,2454	0,2890
	IRF3	1,96	1,9588	0,0203
	IRF7	1,23	1,2340	0,5207
	NFKB1	1,62	1,6245	0,0508
	IFNB1	3,04	3,0384	0,0897
	IFNAR1	1,31	1,3074	0,2961
	STAT1	1,39	1,3883	0,3120

*Genes modulated at biologically and statistically significant levels are indicated in red.

Comparison of innate immune transcriptional modulation by a reassortant and non-reassortant CCHFV NS_M protein

To date, a role for the CCHFV NS_M protein has not been established. In this study, transient expression of the NS_M protein from two South African CCHFV isolates resulted in modulation of the innate immune response at transcriptional level. The isolates were selected based on phylogenetic analysis of complete sequence data indicating reassortment and non-reassortment of the M-segment (Goedhals et al., 2014). Despite both isolates being associated with fatality in a human host, the transcriptional innate immune activation profiles for the reassortant and non-reassortant CCHFV NS_M protein revealed significant differences in gene expression modulation (Figure 2).

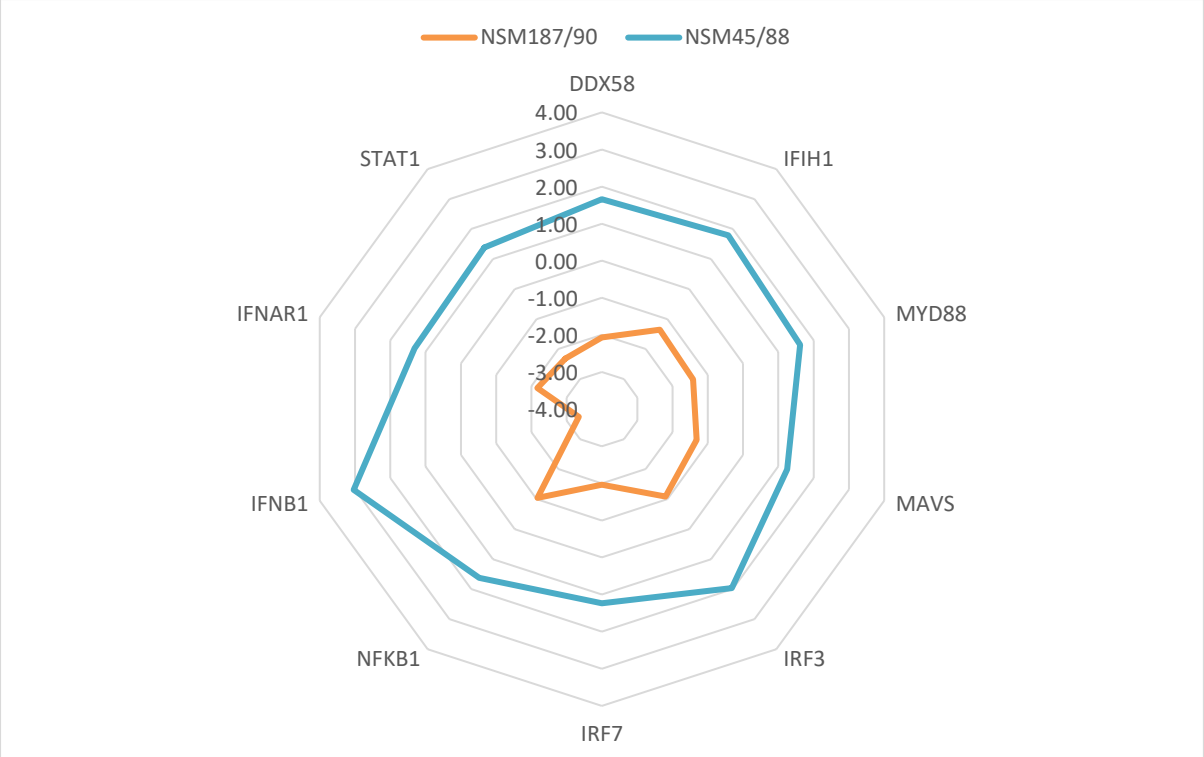


Figure 2: Comparison of innate immune gene expression profiles for a CCHFV non-reassortant and reassortant NS_M protein

Comparison of the modulation of gene expression determined in response to the NS_M protein encoded by either a South African CCHFV non-reassortant isolate (NS_M187/90) or a reassortant isolate (NS_M45/88)

Expression of the non-reassortant CCHFV NS_M protein resulted in transcriptional downregulation of *DDX58* (RIG-I), *IRF7*, *IFNB1*, *IFNAR1* and *STAT1* gene expression, which may have an impact on virus recognition, IFN-signalling and expression, and ISG-signalling. In contrast, expression of the reassortant CCHFV NS_M protein resulted in transcriptional upregulation of all selected innate immune genes evaluated with a significant increase in *IFNB1* gene expression. Moderate upregulation of gene expression suggests that the reassortant CCHFV NS_M protein upregulates innate immune signalling. The difference in fold regulation between the reassortant and non-reassortant CCHFV NS_M protein differs by more than 2-fold for *MAVS* ($p < 0,05$) and *NFKB1* ($p < 0,05$) gene expression and more than 3-fold for *DDX58* (RIG-I) ($p < 0,05$), *IFIH1*(MDA5) ($p < 0,05$), *MYD88* ($p < 0,05$), *IRF3* ($p < 0,05$), *IRF7* ($p = 0,0721$), *IFNAR1* ($p < 0,05$) and *STAT1* ($p < 0,05$) gene expression. The most significant

difference in gene expression was evident in *IFNB1* with more than 6-fold ($p < 0,05$) difference in expression between the reassortant and non-reassortant CCHFV NS_M protein (Table 2). The transcription profiles suggest a difference in the interaction of the CCHFV NS_M proteins with the innate immune response.

Table 2: Comparison between the innate immune modulation by the NS_M187/90 and NS_M45/88 proteins

Gene:	Difference in fold regulation:	p-value:
DDX58	3,73	0,0129
IFIH1	3,15	0,0248
MYD88	3,03	0,0000
MAVS	2,56	0,0108
IRF3	3,05	0,0100
IRF7	3,20	0,0721
NFKB1	2,67	0,0333
IFNB1	6,38	0,0022
IFNAR1	3,48	0,0059
STAT1	3,71	0,0164

*Genes modulated at biologically and statistically significant levels are indicated in red.

Discussion

CCHFV is one of the most widespread arboviruses globally with recent spread to previously non-endemic areas. Re-emergence of the virus in endemic areas and emergence in non-endemic areas is a cause for concern especially in light of the limited control measures against viral infection, challenges in the diagnosis of infection and the lack of approved, effective therapeutics and vaccines. The development of effective prevention and control strategies relies on an improved understanding of the virus-host interactions. In recent years, more information regarding the adaptive immune response to CCHF infection has become available by assessing the immune response in CCHF survivors and from the evaluation of candidate vaccines in the CCHFV mouse models (Bertolotti-Ciarlet et al., 2005; Buttigieg et al., 2014; Canakoglu et al., 2015; Dowall et al., 2016; Ergönül et al., 2017; Fritzen et al., 2018; Goedhals et al., 2015, 2017; Hinkula et al., 2017; Liu et al., 2014; Papa et al., 2016). However, an improved understanding of the interaction between viral components and the innate immune response is required.

The innate immune response is an ancient, conserved system that serves as the body's first line of defence against invading organisms. Many viruses have evolved to delay or impair the host immune response by encoding proteins that actively modify the immune response. The innate immune response consists of many interlinked pathways that become activated and subsequently activate the adaptive immune response. Interference with innate immune signalling could therefore not only result in a delay or impairment of the innate immune response but could also delay activation of the appropriate adaptive immune response. In limited studies, CCHFV has been shown to interfere with the innate immune response. CCHFV IbAr10200 was shown to delay activation of the innate immune response by 48 hours by delaying nuclear translocation of IRF3 (Andersson et al., 2008). Additionally, the ovarian-tumour like (OTU) protease encoded within the viral RNA-dependent RNA polymerase exhibited deconjugating activity that inhibited NF-κβ dependent signalling and antagonised ISG15, an ISG with antiviral effects (Frias-Staheli et al., 2007). In this study, the role of the CCHFV NS_M protein on transcriptional modulation involved in innate immune signalling was evaluated.

In South Africa, both reassortant and non-reassortant CCHFV isolates have been associated with severe disease and fatalities. Reassortant viruses appear to have acquired an M-segment from isolates circulating in Asia (Group IV) (Burt et al., 2009; Goedhals et al., 2014). The significance of M-segment reassortment on pathogenicity is unknown, hence the role of NS_M proteins encoded by representatives of naturally occurring reassortant and non-reassortant isolates were investigated *in vitro*. The transcription of key innate immune mediators was downregulated in the presence of the CCHFV NS_M protein from a non-reassortant South African isolate, including the gene encoding RIG-l cytosolic receptor, which is critical for sensing CCHFV infection and activating innate immune signalling (Spengler et al., 2015). Downregulation of the transcription of additional downstream genes involved in type l IFN-signalling and ISG-signalling further illustrates the ability of the CCHFV NS_M protein to impair the transcription of innate immune genes. The genes encoding IRF7, that is indispensable for IFN-α signalling (Sato et al., 2000), and IFN-β, that is critical for innate immune signalling during early infection to alert infected and uninfected cells to infection (Der et al., 1998), were downregulated. Additionally, the genes encoding the type l IFN-receptor that recognises type l IFNs and signals ISG induction (Dale et al., 1989; Levy

et al., 1989), and STAT1, a crucial component without which ISG transcription is abolished (Durbin et al., 1996), were also downregulated. The non-reassortant CCHFV NS_M protein significantly impaired the transcriptional response involved in virus recognition, IFN-signalling, and ISG-signalling.

Interestingly, the reassortant CCHFV NS_M protein upregulated the transcription of all genes investigated with a more than 3-fold increase in *IFNB1* gene expression, which suggests expression of the reassortant CCHFV NS_M protein stimulated innate immune signalling. The reassortant and non-reassortant CCHFV NS_M protein evaluated in this study share 92,88% amino acid sequence similarity; however, a significant difference can be observed in the innate immune transcriptional profiles induced. The ability of the non-reassortant CCHFV NS_M protein to impair the innate immune transcriptional response may impact disease severity and outcome. Since both isolates used in this study were obtained from fatal CCHF cases, it suggests that other viral factors may contribute to the pathogenicity of the reassortant virus or that host-factors may contribute to disease outcome. Additionally, the difference in transcriptional profiles between the non-reassortant and reassortant viruses can also be viewed as the difference between the roles of the NS_M proteins encoded by genetically diverse isolates of the virus. An important consideration is that *in vitro* overexpression of the CCHFV NS_M proteins may not reflect the relative abundance of this protein within an infected cell and may therefore not reflect the relative levels of transcriptional modulation within an infected cell. However, the level of suppression may be more pronounced in IFN-stimulated cells. It is also likely that other viral proteins have a role in immune modulation and that the response is multifactorial.

Poor innate immune control results in increased virus replication and spread throughout the host and may exacerbate disease. Dysregulation of the immune response is a common strategy employed by viruses to evade immune detection and elimination. The study of virus-host interactions is vital to rational vaccine design and the identification of therapeutic agents with the ability to overcome virus-induced immune dysregulation. Dysregulation of the innate immune response may explain two findings associated with severe disease or a fatal outcome, including the absence of a detectable antibody response in the majority of fatal CCHF cases and cytokine-mediated endothelial damage presenting with vascular leakage. The lack of a detectable antibody response may be the result of a delay in innate immune activation

or impairment of the innate immune response, subsequently delaying activation of the adaptive immune response. High viral titres disseminated throughout the host can, in part, be explained by poor innate immune control, which overwhelms the immune system at the time of activation resulting in high levels of cytokine release, which in turn promotes vascular leakage by compromising the host endothelium. Additionally, CCHFV has been shown to be insensitive to the effects of IFN-α at the time of release (Andersson et al., 2008), which may result in continued virus replication and spread even after immune activation. An alternative hypothesis may be that moderate downregulation of innate immune activation may modulate cytokine induction and as a result lower cytokine levels, which have been demonstrated to be associated with mild disease compared to high cytokine levels being correlated to severe disease (Ergönül et al., 2017; Papa et al., 2016). *In vivo* studies may provide clarity of the significance of innate immune signalling modulation by the CCHFV NS_M protein.

This study suggests a putative role for the CCHFV NS_M protein and suggests a difference in the mechanism of pathogenesis between reassortant and non-reassortant viruses circulating within the same geographical area. However, a role in transcriptional suppression of the innate immune response may be unique to non-reassortant South African CCHFV isolates due to the high amino acid sequence diversity within the CCHFV NS_M proteins amongst CCHFV isolates. All non-reassortant South African CCHFV strains with complete sequence data, except one, shows 98,3% amino acid sequence similarity within the NS_M coding region. On a global scale, CCHFV NS_M proteins only have 47,6% amino acid sequence similarity, which suggests that it is unlikely that the role of the NS_M protein is conserved among all CCHFV isolates but may reflect evolutionary changes to evade the immune response by certain CCHFV strains. In this study, key targets for innate immune modulation by the CCHFV NS_M protein were identified, which allows for more focused exploration of the underlying mechanisms of innate immune modulation. Other host cells may be explored in future studies to determine whether innate immune modulation occurs to a significant extent in cells targeted by CCHFV during infection. Determining the role of the CCHFV NS_M protein *in vivo* may provide insights into whether downregulation of innate immune signalling is beneficial or detrimental to the outcome of disease. Additionally, the role of microRNAs and siRNAs in the modulation of gene expression can be explored. Exploration to uncover any interaction of the

virus with the innate immune response in tick cells, the reservoir of CCHFV, may reveal the mechanism utilised for maintenance in ticks; however, the innate immune response in tick cells is not IFN-based and evaluation will require a different approach.

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Author contributions

Conceptualisation, F.J.B.; Methodology, formal analysis, investigation, validation, N.V.; Resources, F.J.B and D.G.; Writing – Original draft, N.V.; Writing – Review & Editing, F.J.B., D.G. and N.V.; Visualisation, N.V.; Supervision, F.J.B. and D.G.; Project administration, F.J.B.; Funding acquisition, F.J.B and N.V.

Declaration of interests

The authors declare no competing interests.

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Supplemental information

Table S1: Reference, quality control and innate immune signalling genes

Full name	Gene symbol	Reference sequence	RT2 catalogue number
Reference genes			
Actin, beta	*ACTB*	NM_001101	PPH00073
Beta-2-microglobulin	*B2M*	NM_004048	PPH01094
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	NM_002046	PPH00150
Quality control genes			
Reverse Transcription Control	*RTC*	SA_00104	PPX63340
Human Genomic DNA Contamination	*HGDC*	SA_00105	PPH65835
Positive PCR Control	PPC	SA_00103	PPX63339
Innate immune signalling genes			
DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	*DDX58*	NM_014314	PPH20774
Interferon-induced with helicase C domain 1	*IFIH1*	NM_022168	PPH18927
Myeloid differentiation primary response gene (88)	*MYD88*	NM_002468	PPH00911
Mitochondrial antiviral signalling protein	*MAVS*	NM_020746	PPH24180
Interferon regulatory factor 3	*IRF3*	NM_001571	PPH02025
Interferon regulatory factor 7	*IRF7*	NM_001572	PPH02014
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	*NFKB1*	NM_003998	PPH00204
Interferon, beta 1	*IFNB1*	NM_002176	PPH00384
Interferon (alpha, beta and omega) receptor 1	*IFNAR1*	NM_000629	PPH00869
Signal transducer and activator of transcription	*STAT1*	NM_007315	PPH00811

Table S2: Raw data for control and experimental conditions

Gene	**Control group (Empty vector)**	**NS_M187/90 (Non-reassortant)**	**NS_M45/88 (Reassortant)**
HGDC	N.D.	N.D.	N.D.
RTC	18,82	19,57	18,70
PPC	16,96	16,97	16,91
GAPDH	16,29	16,93	17,31
IFIH1 (MDA5)	22,93	24,00	23,11
MAVS	20,61	21,64	21,31
IRF3	19,30	20,06	19,35
IRF7	23,91	25,52	24,63
MYD88	22,75	23,89	23,08
NFKB1	23,07	23,77	23,93
IFNB1	28,00	30,38	27,43
IFNAR1	21,12	22,88	21,76
STAT1	20,19	22,04	20,74
Gene	**Control group (Empty vector)**	**NS_M187/90 (Non-reassortant)**	**NS_M45/88 (Reassortant)**
GAPDH	18,25	18,78	19,11
DDX58 (RIG-I)	21,34	22,92	21,47

The Cq value for each gene is the average Cq value for all biological repeats. N.D. – Not detected

Chapter 4

Modulation of innate immune signalling by Crimean-Congo haemorrhagic fever virus ovarian tumour-like protease

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Summary

The Crimean-Congo haemorrhagic fever orthonairovirus (CCHFV) ovarian tumour-like (OTU) protease has been shown to interfere with the innate immune response mainly by deubiquitinating and deISGylating cellular proteins. To explore additional mechanisms that the CCHFV OTU protease may utilise to modulate the innate immune response, modulation of innate immune genes was evaluated after expression of the OTU protease *in vitro*. Modulation of innate immune gene expression in response to the CCHFV OTU protease was determined using quantitative polymerase chain reaction (qPCR). The CCHFV OTU protease from a South African isolate downregulated *IFNB1*, *IFNAR1* and *STAT1* gene expression. A 3-fold decrease in *IFNB1* gene expression, despite not reaching statistical significance, was induced by the CCHFV OTU protease. Additionally, a greater than 2-fold decrease in *IFNAR1* and *STAT1* gene expression was induced by the CCHFV OTU protease, potentially compromising the induction of interferon-stimulated genes (ISGs). Further exploration to identify the mechanism of downregulation may provide potential target/s for the development of therapeutic intervention strategies.

Keywords

Crimean-Congo haemorrhagic fever virus, ovarian-tumour like protease, innate immune response, interferon

Introduction

Crimean-Congo haemorrhagic fever orthonairovirus (CCHFV) is a negative-sense single-stranded RNA virus maintained in nature by *Hyalomma* ticks, which are widely distributed globally. The virus was first identified as a cause of disease in the Crimean Peninsula in 1944 and later confirmed to be identical to a virus isolated from patient samples from the Belgian Congo (today Democratic Republic of the Congo) in 1956 (Casals, 1969). Fatality rates reported for CCHFV infections differ significantly between geographical areas with Turkey reporting a 5% fatality rate (Leblebicioglu et al., 2016) and smaller case series reporting higher fatality rates (Aradaib et al., 2011; Burney et al., 1980; Chinikar et al., 2010; Christova et al., 2009; Dunster et al., 2002; El-Azazy and Scrimgeour, 1997; Hoogstraal, 1979; Mishra et al., 2011a; Mofleh and Ahmad, 2012; Mustafa et al., 2011; Nabeth et al., 2004b, 2004a; Papa et al., 2002a, 2002b, 2004, 2008; Saluzzo et al., 1985; Schwarz et al., 1997; Smego et al., 2004; Suleiman et al., 1980; Tantawi et al., 1980; Tikriti et al., 1981; Watts et al., 1989; Yadav et al., 2013; Yashina et al., 2003b, 2003a). A fatal outcome has been shown to be correlated to a high viral load, which frequently occurs in the absence of detectable antibody levels (Shepherd et al., 1989) and has been correlated with high T-helper (Th) 2 associated cytokine levels (Ergönül et al., 2017; Papa et al., 2016). Currently, no approved vaccine or therapeutic interventions are available for use. The use of ribavirin and CCHFV immunoglobulins are highly controversial as consensus on the efficacy of these interventions has not been achieved. The mainstay for treatment of Crimean-Congo haemorrhagic fever (CCHF) is comprised of comprehensive supportive care with volume replacement therapy, the replacement of blood products, and monitoring and support for all organ systems. Improved understanding of the immune responses may contribute to developing novel therapeutic strategies.

Each virion contains three genomic segments designated the small (S), medium (M) and large (L) segments. The L-segment encodes the viral RNA-dependent RNA polymerase (RdRP), which facilitates viral replication within host cells. Within the RdRP coding region a viral ovarian tumour-like (OTU) protease is encoded (Honig et al., 2004; Kinsella et al., 2004) with 80% predicted amino acid sequence similarity between geographically distinct isolates. Virally-encoded OTU proteases have been considered to be virulence factors due to their deubiquitinating activity that can interfere with the regulation of cellular processes, including signalling processes and

subsequently the innate immune response. Despite sharing structural similarities with mammalian OTU proteases, additional motifs have been identified in virally-encoded OTU proteases, which were predicted to expand the range of substrates that can be targeted by virally-encoded OTU proteases (Bailey-Elkin et al., 2014). The CCHFV OTU, a cysteine protease, displays lower substrate specificity than mammalian OTU proteases and may, therefore, target a broader range of substrates (Frias-Staheli et al., 2007; van Kasteren et al., 2012). The CCHFV OTU does not appear to play a role in protein processing (Bergeron et al., 2010) but has been shown to possess deubiquitinating and deISG15ylating activity (Frias-Staheli et al., 2007; Scholte et al., 2017). Negative regulation of innate immune signalling by deubiquitination and deISGylation results in increased susceptibility to virus infection in mice (Frias-Staheli et al., 2007). Currently, the role of the CCHFV OTU protease in viral replication is not clear. The CCHFV OTU protease of IbAr10200 was previously shown to be dispensable for viral RNA replication (Bergeron et al., 2010). In contrast, the inactivation of the CCHFV OTU protease resulted in halting of viral replication independent of RdRP activity (Scholte et al., 2017). The deISGylating activity of the CCHFV OTU protease was implicated in protecting the L-segment from degradation and may be required for optimal viral replication in the presence of type I interferon (IFN) (Scholte et al., 2017). A role for the CCHFV OTU protease in directly antagonising innate immune activation by deubiquitination and deISGylation has been demonstrated (Frias-Staheli et al., 2007; Scholte et al., 2017). In this study, the aim was to determine whether the CCHFV OTU protease can modulate innate immune activation at a transcriptional level.

Since the CCHFV OTU protease has been identified as a potential target for developing antiviral therapeutic strategies against CCHFV, an improved understanding of the mechanisms utilised to interfere with innate immune activation is required to explore different therapeutic options.

Methods

Cells

Human embryonic kidney (HEK)-293 cells (ATCC® CRL-1573™; RRID: CVCL_0045), obtained from the American Type Culture Collection (ATCC®) were cultured in Dulbecco's Modified Eagle Medium (Lonza) supplemented with 10% foetal bovine serum (Gibco), 100 units per ml (U/ml) penicillin and streptomycin (Lonza), L-glutamine to a final concentration of 2mM and non-essential amino acids (Lonza) at 37˚C in a humid 5% CO_2 atmosphere and passaged when 80-90% confluent. Cells were used for electroporation within the first 15 passages after removal from cryopreservation. Cells were enumerated using the Countess™ II automated cell counter (Invitrogen).

Plasmids

The nucleotide sequence encoding the CCHFV OTU protease was retrieved from GenBank® (GenBank accession number: SPU187/90 L-segment KJ682795) and codon-optimised for expression in human cells using the JAVA codon adaptation tool (JCat) (Grote et al., 2005; http://www.jcat.de/). The gene was synthesised and supplied in pcDNA3.1(+) by GenScript® with modifications to include a Kozak sequence and a FLAG tag (DYKDDDDK) at the 5' and 3' ends, respectively. Similarly, a pcDNA3.1(+) plasmid containing the gene encoding a green fluorescent protein (GFP) was synthesised for use as a positive transfection control (GenScript®). An empty vector plasmid was prepared by excising the gene encoding the GFP from the pcDNA3.1(+)GFP plasmid using the *Hind*lll and *Bam*HI restriction sites flanking the GFP coding region. The empty vector was used to control for transcriptional changes due to the presence of double-stranded DNA used for the introduction of the gene encoding the CCHFV OTU protease. Plasmid DNA was introduced into chemically competent *Escherichia coli* JM109 cells (Zymo Research Mix&Go!) using the heat-shock method. After selection of positive transformants with ampicillin to a final concentration of 100µg/ml, polymerase chain reaction (PCR) and bidirectional nucleotide sequence analysis were performed on plasmid DNA purified with the PureYield™ Plasmid Miniprep kit (Promega). PCR and bidirectional nucleotide sequencing reactions were prepared using the T7 forward (5'-TAA TAC GAC TCA

CTA TAG GG-3') and bovine growth hormone (BGH) reverse (5'-TAG AAG GCA CAG TCG AGG-3') primers targeting sites flanking the CCHFV OTU protease coding region to confirm plasmid construction. PCR for the confirmation of positive transformants was performed using GoTaq® G2 DNA polymerase (Promega) according to the manufacturer's instructions using 0,2 µM of each primer. Amplification conditions included denaturation at 95˚C for one minute and 40 cycles of denaturation at 95˚C for one minute, annealing at 45˚C for one minute and extension at 72˚C for one minute and a final extension at 72˚C for five minutes. Chromas Pro version 1.6 (Technelysium) was used to analyse sequence data. The open reading frame was identified using ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/) and translated to amino acid sequence using the Expasy translation tool (Gasteiger et al., 2003; https://web.expasy.org/translate/). To confirm nucleotide and amino acid sequence identity, the nucleotide and translated amino acid sequences were aligned with the codon-optimised OTU nucleotide and translated amino acid sequences using Clustal Omega (Sievers et al., 2011; https://www.ebi.ac.uk/Tools/msa/clustalo/).

Expression of the CCHFV OTU protease

Transfection grade DNA was prepared using the QIAGEN® Plasmid Midi Kit (Qiagen) according to the manufacturer's instructions with lysate preparation by filtration using the QIAfilter cartridges (Qiagen) and the DNA concentration determined using a Nanodrop™ 2000 (Thermo Scientific). To ensure DNA concentrations exceeding 1µg/µl, plasmid DNA preparations were concentrated using the Eppendorf™ Vacufage™ Plus Concentrator (Eppendorf). A preparation of $5x10^6$ dissociated HEK-293 cells was electroporated with 10µg plasmid DNA using the Neon® 100 transfection kit (Life Technologies) with modifications to the manufacturer's instructions. Pre- and post-electroporation incubations were included in the electroporation protocol to ensure reproducible transfection efficiencies with a high-level of protein expression. Cells from a single electroporation were divided proportionally to allow confirmation of protein expression using an immunofluorescence assay (IFA) and Western blot, and RNA analysis. Cells attached to a glass slide in a 24-well plate were assayed for protein expression by an indirect IFA using mouse monoclonal anti-FLAG antibody (Thermo Scientific; RRID:

AB_1957945). Briefly, a 1:1 solution of acetone (Merck) and methanol (Merck) was used to fixed cells attached to a glass slide at -20˚C for 20 minutes followed by a 20 minute incubation in blocking solution (phosphate buffered saline (PBS) at pH 7,4 containing 0,5% Triton X-100 and 10% sucrose) at room temperature. Cells were reacted with mouse monoclonal anti-FLAG antibody (Thermo Scientific; RRID: AB_1957945) diluted 1:200 in blocking solution and incubated for 90 minutes at 37˚C in a humid environment followed by three washes for one minute with PBS containing 1% Tween®20 (Promega). Cells were reacted with goat anti-mouse IgG fluorescein isothiocyanate (FITC) antibody diluted 1:20 in 0,1% Evans blue (Merck) in PBS, washed as previously described and visualised by fluorescence microscopy. A glass slide containing untransfected cells was used as a negative staining control. The transfection efficiency was estimated based on the number of fluorescing cells within two to three fields of vision. For Western blot analysis, cells were harvested in lysis buffer (150mM NaCl, 5mM EDTA, 50mM Tris at pH of 7,4 containing 1mg/ml bovine serum albumin and protease inhibitor (Roche)). The supernatant from lysed cells was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a BioTrace™ polyvinylidene fluoride (PVDF) (Pall Corporation) membrane. A PageRuler™ pre-stained protein ladder (Thermo Scientific) was included in each run to allow confirmation of efficient protein transfer. In addition, a Magic Mark™ XP Western blot ladder (Thermo Scientific) containing immunoglobulin binding domains was included as a positive control for reactivity and to allow size estimation of the blot. Western blot analysis was performed using the Pierce® Fast Western Blotting kit (Thermo Scientific) with mouse monoclonal anti-FLAG antibody (Thermo Scientific; RRID: AB_1957945) according to the manufacturer's instructions with additional washes and supplemented with SuperSignal™ West Pico PLUS chemiluminescent substrate (Thermo Scientific). Western blot was used to confirm a similar level of protein expression in all biological repeats. For gene expression studies, cells expressing the CCHFV OTU protease were harvested in RNAprotect cell reagent (Qiagen) 24 hours post-transfection to stabilise the RNA profile within all cells.

Relative quantification of innate immune signalling markers

RNA was extracted from stabilised cells using the RNeasy Plus Mini kit (Qiagen) according to the manufacturer's instructions with the addition of beta-mercaptoethanol (Merck) to the lysis buffer. RNA integrity was confirmed by visualisation after electrophoresis on a 1,2% denaturing agarose gel (Aranda et al., 2012) and the quality and quantity of the RNA were determined using the Nanodrop™ 2000 (Thermo Scientific). High-quality RNA was reverse-transcribed to complementary DNA (cDNA) using the First Strand Synthesis kit (Qiagen) according to the manufacturer's instructions to ensure efficient removal of residual genomic DNA (gDNA). Additionally, during reverse transcription DNA and RNA controls were added to each reaction to allow determination of the efficiency of the real-time quantitative (qPCR) and reverse-transcription reactions, respectively. The determined reverse-transcription and qPCR efficiencies were used as quality indicators prior to gene expression analysis.

A custom RT^2 profiler array (SA BioSciences) was used to amplify three reference genes, three quality control genes and ten innate immune signalling genes (Table S1) using RT^2 SYBR Green ROX FAST Mastermix (Qiagen) according to the manufacturer's instructions on a Rotor-Gene Q cycler (Qiagen). Each cDNA synthesis reaction was diluted by the addition of 91µl RNase-free water (Ambion) according to the manufacturer's instructions and 0,9µl diluted cDNA was used in each reaction for relative quantification. The Rotor-Gene Q Series Software (RRID: SCR_015740) was used to collect data for each reaction. Data analysis was conducted according to the manufacturer's instructions, and the threshold value of 0,08303 was determined and used for all subsequent runs. The specificity of each qPCR reaction and absence of interference from primer-dimer were confirmed by analysis of the dissociation curve generated for each reaction. Gene expression analysis was performed on the exported cycle threshold (Cq) values.

qPCR assays were developed for confirmation of *DDX58* gene expression values due to a decline in the amplification efficiency of *DDX58* on the array. Primers spanning exon-exon junctions that target *DDX58* mRNA (DDX58-FW 5'-GTGGGCAATGTCATCAAAATG-3' and DDX58-RV 5'-GTCTGAAGGCGTAAAATAGAGTC-3') and *GAPDH* mRNA (GAPDH-FW 5'-TGACAACAGCCTCAAGAT-3' and GAPDH-RV 5'- TCCTTCCACGATACCAAAG-3')

were designed. Reactions were prepared using RT2 SYBR® Green ROX FAST Mastermix (Qiagen) according to the manufacturer's instructions with 0,5µM of each gene specific primer per reaction. Rotor-Gene Q (Qiagen) cycling conditions included denaturation at 95˚C for 10 minutes followed by 40 cycles of denaturation at 95˚C for 15 seconds and a combined annealing and extension cycle at 60˚C for 30 seconds with fluorescence data acquisition at the end of each cycle. Melting curve analysis was performed for each reaction to confirm the absence of primer-dimer or non-specific amplification.

Statistical analysis

The ΔΔCq method (Livak and Schmittgen, 2001) was used to perform relative gene expression analysis. Normalisation was performed relative to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene expression and modulation expressed relative to the negative control. The average ΔCq value for all biological repeats was calculated by subtracting the Cq value for the reference gene from each gene of interest and determining the average ΔCq value for each group. The average ΔCq value for the control group was subtracted from the average ΔCq value for the experimental group providing the ΔΔCq value, which was used to calculate the fold change ($2^{-\Delta\Delta Cq}$) and fold regulation (-1/fold change for fold change values < 1; fold change = fold regulation for fold change values > 1). A change was considered to be a biologically significant change if gene modulation resulted in a ≥ 2-fold regulation. The statistical significance of the changes in gene modulation was determined using the Student's t-test with a two-tail distribution and equal variance between two samples using the ΔCq values calculated for each biological repeat within a group (Yuan et al., 2006). Changes in gene modulation were considered to be statistically significant if a p-value of < 0,05 was obtained for the gene of interest.

Results

CCHFV OTU protease downregulates *DDX58* gene expression

The innate immune response is the body's first line of defence and early response to an invading pathogen is essential for limiting virus spread and replication. RNA viruses are frequently recognised by the retinoic acid-inducible (RIG)-I-like receptors (RLRs), which are essential for IFN-signalling via the mitochondrial activator virus signalling (MAVS) protein and nuclear factor κβ activation (Sun et al., 2006). Recently, RIG-I was identified as a critical mediator of CCHFV recognition within an infected cell and, without which, the type I IFN response was abolished entirely (Spengler et al., 2015). Activation of RIG-I after RNA binding and MAVS by RIG-I in response to viral infection requires K63 polyubiquitination, which is not only essential for virus recognition (Gack et al., 2007; Zeng et al., 2010), but also downstream activation of IFN-regulatory factor (IRF) 3 by MAVS to signal IFN expression (Zeng et al., 2010). The CCHFV OTU protease has been shown to prevent RIG-I activation by interfering with ubiquitination, which is imperative for regulating cellular processes by altering the activity, localisation or stability of the protein (Pickart and Eddins, 2004). The deubiquitinating activity of the virally-encoded OTU protease reverses activation of RIG-I preventing downstream IFN-signalling in response to the virus (van Kasteren et al., 2012; Scholte et al., 2017). To determine whether an additional mechanism of innate immune modulation exists for the CCHFV OTU protease, modulation of the transcriptional response after expression of the CCHFV OTU protease *in vitro* was assessed.

The transcriptional profile in cells expressing the CCHFV OTU protease was compared to that of cells stimulated with an empty vector (control group). An empty vector was used to determine the extent of activation by double-stranded DNA alone, which was accounted for when calculating transcriptional modulation by the CCHFV OTU protease. Expression of the CCHFV OTU protease resulted a 1,9-fold downregulation of *DDX58* (RIG-I) gene expression (Table 1; Figure 1a). Low-level downregulation of *IFIH1* (MDA5) and modest downregulation of *MAVS* gene expression was observed in CCHFV OTU protease expressing cells (Table 1; Figures 1b, c). In addition to directly reversing the activation of RIG-I within a host cell after innate immune activation by viral RNA, expression of the CCHFV OTU protease resulted in a reduction in *DDX58* (RIG-I) mRNA levels, which may compromise innate

immune activation downstream of the RIG-I cytosolic receptor, especially in light of the fact that MAVS activation that occurs immediately downstream of RIG-I activation may also be affected by the deubiquitinating activity of the CCHFV protease. Interference with either RIG-I or MAVS has been shown to result in impaired interferon-stimulated gene (ISG) expression, which ultimately mediates an antiviral response and positive feedback regulation in response to CCHFV infection (Spengler et al., 2015).

Transcriptional modulation of IFN-signalling innate immune genes by the CCHFV OTU protease

Signalling for IFN expression downstream of MAVS mainly involves signalling by IRF3 and IRF7 (Sato et al., 2000); however, signalling via the nuclear factor (NF)-κβ pathway strengthens type I IFN expression as NF-κβ forms part of the transcriptional enhancer complex for expression of type I IFNs and is specifically recruited to the IFN-β promoter (Wathelet et al., 1998). The CCHFV OTU protease has been shown to inhibit NF-κβ dependent-signalling by preventing nuclear translocation of endogenous p65 (Frias-Staheli et al., 2007). Additionally, activation of IRF3 and NF-κβ signalling was impaired by the CCHFV OTU protease, which resulted in impaired IFN-β activation (Scholte 2017).

Expression of the CCHFV OTU protease resulted in a 1,9-fold downregulation of *MYD88* ($p < 0,05$) and low-level downregulation of *NFKB1* gene expression (Table 1; Figures 1 d, e). Downregulation of gene expression in the absence of toll-like receptors may suggest that, in addition to interfering with NF-κβ dependent-signalling, the CCHFV OTU protease modulates transcription of the *MYD88* gene encoding the adaptor protein that activates NF-κβ. Disruption of NF-κβ activation may result in a downregulation in the transcription of the *IFNB1* gene. Modest downregulation of *IRF3* and *IRF7* gene expression (Table 1; Figures 1f, g) may affect downstream IFN-signalling.

IFN-β expression plays a critical role in activation of the innate immune response by binding to auto- and paracrine IFN-receptors and stimulating expression of the ISGs via the Janus kinase (JAK)/Signal transducer and activator of transcription (STAT) and alternative pathways (de Weerd et al., 2013). Expression of the gene encoding IFN-β, *IFNB1*, was downregulated by more than 3-fold ($p=0,1591$) (Table 1; Figure 1h) and

may impact the downstream expression of ISGs. IFN-β is crucial during early infection to mediate and coordinate the innate immune response to induce an antiviral state by inducing the expression of ISGs (Der et al., 1998).

Downregulation of *IFNAR1* and *STAT1* gene expression

In an attempt to limit the ability of a virus to replicate and spread throughout the host, expression of ISGs is induced to create an antiviral state within infected and uninfected cells. The importance of the expression of the type I IFN-receptor and STAT1 was demonstrated during the development of an animal model for CCHFV. Mice deficient in either the *IFNAR1* or *STAT1* gene developed clinical disease followed by death within days, whereas mice with an intact innate immune response did not develop disease after CCHFV challenge (Bente et al., 2010; Bereczky et al., 2010; Zivcec et al., 2013).

After recognition of an invading pathogen, IFN-β expression is induced and usually, despite innate immune suppression, not completely abolished. IFN-β forms the link between virus recognition and ISG-signalling via interaction with type I IFN-receptors (de Weerd et al., 2013). The ISG transcriptional response is mediated by IRF9 after associating with phosphorylated STAT1 and STAT2 (Dale et al., 1989; Levy et al., 1989). The CCHFV OTU protease downregulated *IFNAR1* gene expression by more than 2-fold ($p < 0,05$) (Table 1; Figure 1i), which may impact responsiveness to type I IFN and subsequent ISG induction. Additionally, the *STAT1* gene, essential for an early type I IFN response and ISG induction (Bowick et al., 2012), was downregulated by more than 2-fold ($p < 0,05$) (Table 1; Figure 1j). Downregulation of both the *IFNAR1* and *STAT1* genes may significantly impair innate immune activation to CCHFV, especially ISG-signalling.

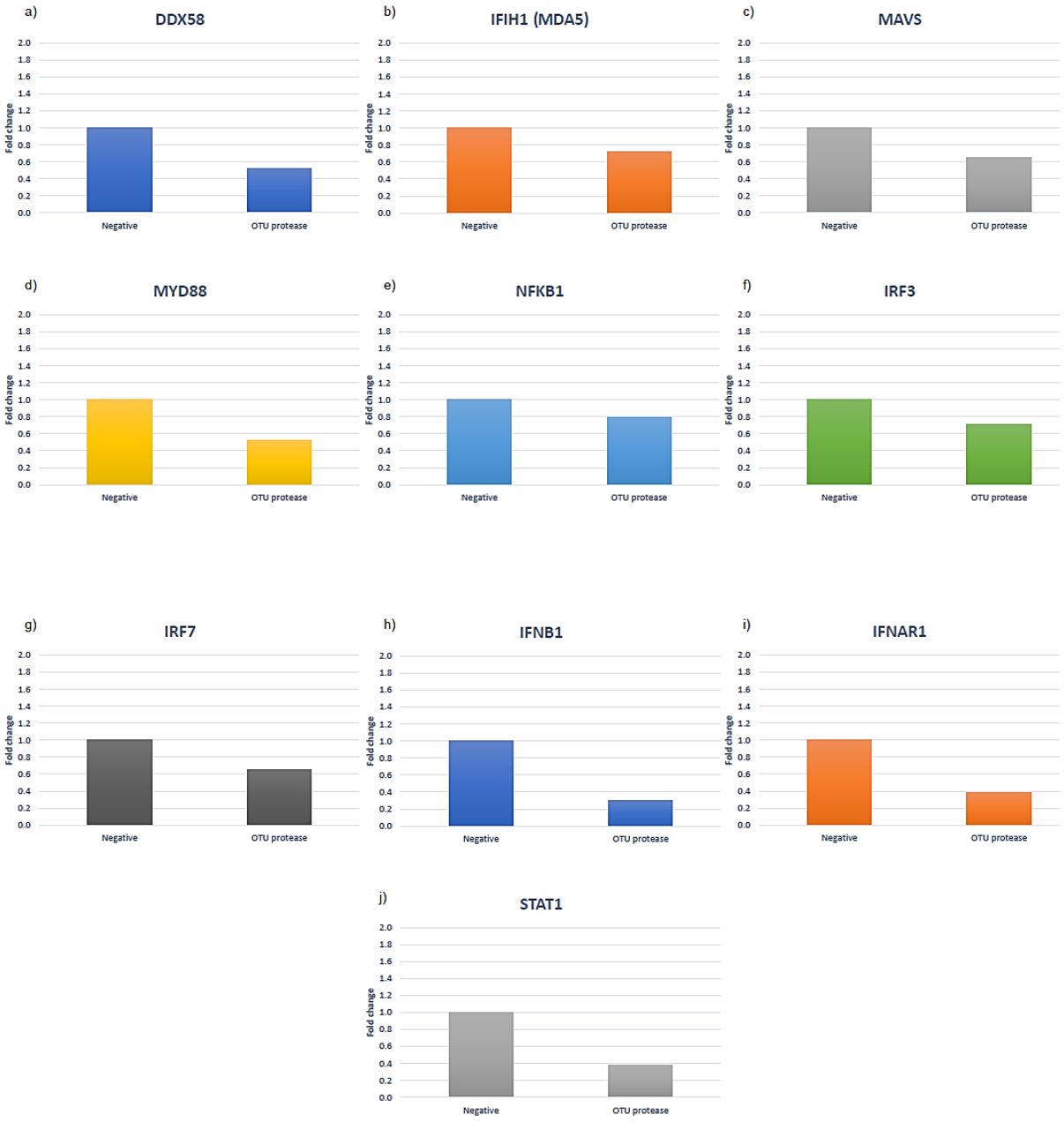

Figure 1: Modulation of gene expression by the CCHFV OTU protease

Modulation of gene expression determined in response to cells transfected with a construct expressing the CCHFV OTU protease and compared to a negative control in which cells were transfected with a pcDNA3.1(+) empty vector

Table 1: Innate immune modulation by the CCHFV OTU protease

Protein:	Gene:	Fold regulation:	Fold change:	p-value:
OTU protease	*DDX58*	-1,9053	0,52	0,1039
	IFIH1	-1,3883	0,72	0,3350
	MYD88	-1,9097	0,52	0,0411
	MAVS	-1,5476	0,65	0,0779
	IRF3	-1,4142	0,71	0,1127
	IRF7	-1,5192	0,66	0,1054
	NFKB1	-1,2541	0,80	0,2138
	IFNB1	-3,3481	0,30	0,1591
	IFNAR1	-2,6147	0,38	0,0122
	STAT1	-2,6329	0,38	0,0011

*Gene modulation with a biologically and statistically significant change is indicated in red.

Discussion

CCHFV is an arboviral infection that is widely distributed and primarily transmitted by the bite of *Hyalomma* ticks or by crushing ticks with bare hands. Since 2002, the virus has emerged in areas where CCHFV was previously not reported, including Turkey (Karti et al., 2004), Greece (Papa et al., 2008), India (Mishra et al., 2011b; Patel et al., 2011) and Spain (Negredo et al., 2017). CCHFV has been identified as a priority pathogen for research by the World Health Organisation due to limited diagnostic capacity, emergence and re-emergence in endemic and previously non-endemic areas and the lack of approved vaccines or therapeutics for use in humans (World Health Organization, 2018). CCHFV causes disease by direct and indirect interaction with various host tissues and can replicate in the blood, liver, spleen, kidneys, the adrenal gland, bone marrow, lymphatic gland, hypothalamus and the wall of the large intestine (Butenko and Chumakov, 1990). The virus preferentially replicates in endothelial cells, hepatocytes and macrophages (Burt et al., 1997). An improved understanding of the virus-host interactions is required to improve prevention and treatment strategies.

The innate immune response is an immediate non-specific, robust response elicited in response to the detection of a foreign body, which does not have a memory component. Detection of RNA viruses induces type I IFNs, which in turn induces the transcription of ISGs that mediate an antiviral response in infected and uninfected cells. The innate immune response aims to contain the virus while activating the

adaptive immune response. Consequently, impairment of the innate immune response may impact the activation and coordination of the adaptive immune response and subsequently compromise protection against disease and the outcome of disease. Currently, the way in which CCHFV interacts with the innate immune response is not completely understood and unravelling the mechanisms and responses may assist in vaccine development and the identification or development of novel therapeutic interventions. The nucleoprotein (Fajs et al., 2014), OTU protease (Frias-Staheli et al., 2007; Scholte et al., 2017) and the NS_M protein (unpublished data), have been shown to interfere with the innate immune response suggesting that modulation of the innate immune response is multifactorial. Strain-to-strain differences in innate immune modulation have been observed for the nucleoprotein and the NS_M protein, which suggest that innate immune modulation by these proteins may differ from isolate-to-isolate or amongst geographically distinct strains. Expression of the CCHFV nucleoprotein from the IbAr10200 and AP92 strains activated IFN-β promoter activity, whereas the CCHFV nucleoprotein from the Hoti strain, a virulent strain, resulted in a decrease in IFN-β promoter activity (Fajs et al., 2014). The results suggest that a difference in the interaction between highly virulent and less virulent strains with the host cell may exist. Interestingly, two NS_M proteins, one that groups with South African CCHFV strains and the other with Asian CCHFV strains, associated with fatality significantly differed in their interaction with the host cell. The CCHFV NS_M protein that grouped with South African isolates significantly downregulated innate immune activation at transcriptional level, while the NS_M protein that grouped with Asian isolates induced innate immune activation (unpublished data). The results suggest that, despite both isolates being associated with fatality, significant differences could be observed, which indicate that innate immune modulation is a multifactorial process and/or host-factors may significantly contribute to the outcome of disease.

Alignment of the predicted amino acid sequence of the CCHFV OTU protease coding region from genetically and geographically distinct isolates suggests that the function of the CCHFV OTU protease may be conserved in many strains with identified critical residues being well conserved. Mutations at specific positions have been shown to affect enzyme activity and specificity. An E128G mutation within the OTU protease encoded by the CCHFV isolate Ug 3010 resulted in a significant decrease in

deubiquitinating and deISGylating activity (Capodagli et al., 2011). An A129R mutant OTU protease was shown to induce robust ISGylation with reduced expression of the nucleoprotein and viral loads indicating that deISGylation is essential for viral replication in the presence of type I IFN (Scholte et al., 2017).

Dysregulation of the innate immune response by the CCHFV OTU protease has been shown to be mediated by deubiquitination during early infection reversing activation of various innate immune components and deISGylation later in infection mainly targeting ISG15 (Scholte et al., 2017). The significant role of the deubiquitinating function was demonstrated by disruption of the function by mutagenesis, which resulted in increased immune activation and a lower viral load (Scholte et al., 2017). In this study, transcriptional modulation of selected innate immune genes was demonstrated. In addition to directly targeting innate immune components, the transcriptional response for key innate immune components was downregulated (Table 1; Figure 2). Based on the results from this study, the innate immune gene regulation has been illustrated in Figure 2.

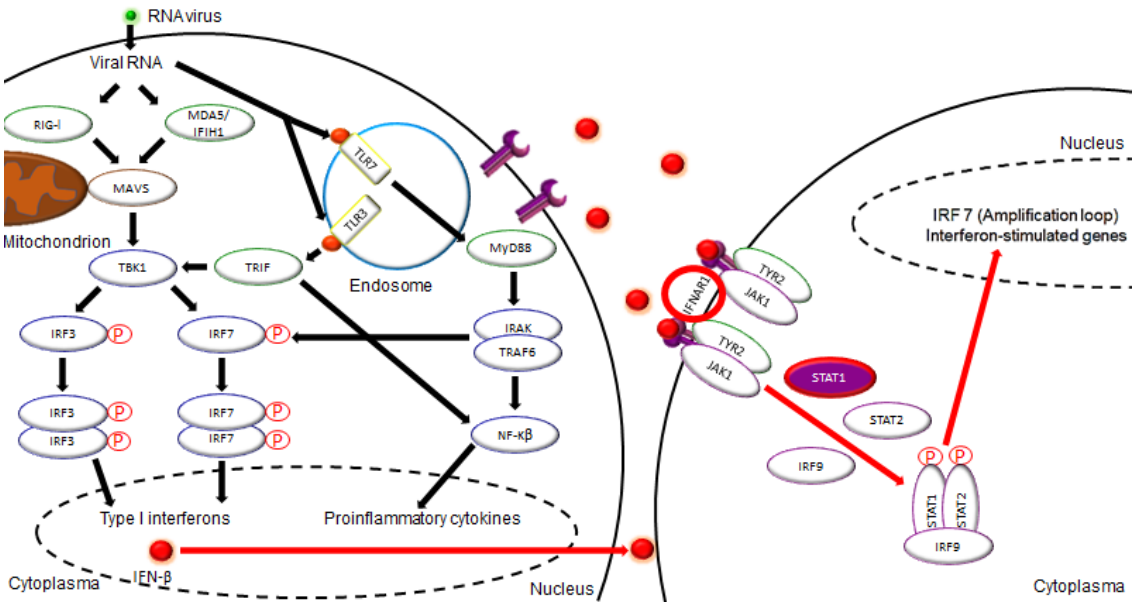

Figure 2: Graphical representation of innate immune gene modulation by the CCHFV OTU protease

The innate immune proteins with downregulated gene expression are encircled in red and the processes likely affected are indicated with a red arrow.

Briefly, the CCHFV OTU protease from a South African isolate demonstrated the ability to downregulate the transcription of the *DDX58* (RIG-I) gene by 1,9-fold, which may affect virus recognition and subsequent activation of the innate- and adaptive immune response. RIG-I was recently shown to be indispensable for CCHFV recognition and subsequent immune activation (Spengler et al., 2015) and is not only targeted directly by the deubiquitinating activity of the CCHFV OTU protease, but also by downregulation of *DDX58* gene expression. The CCHFV OTU protease also downregulated *IFNB1*, *IFNAR1* and *STAT1* gene expression, which are activated downstream of RIG-I and are crucial mediators of innate immune activation to induce ISG expression. Downregulation of *IFNB1*, *IFNAR1* and *STAT1* expression may affect the level of IFN-β released from an infected cell and the ability of the cell to bind IFN-β and induce ISG expression to create an antiviral state. Additional work is required to determine whether CCHFV OTU-induced innate immune modulation also occurs in primary macrophages, which are key target cells for CCHFV after entry (Burt et al., 1997).

The CCHFV OTU protease, not only directly inactivates innate immune proteins, but also downregulates the transcription of key innate immune components. Expression of the CCHFV OTU does not completely inhibit the induction of ISGs or inactivate all ISG15, but a significant decrease has been demonstrated (Frias-Staheli et al., 2007; Scholte et al., 2017). Similarly, transcriptional downregulation will unlikely abolish the innate immune response to CCHFV, but may contribute to a significant extent to impair or delay the innate immune response and may compromise the adaptive immune response and viral clearance. The CCHFV OTU protease, therefore, remains a potential target for the development of therapeutic agents, which may target the OTU protease activity or target the changes mediated by the OTU protease. Additionally, study of the immunogenicity of the OTU protease, especially a cell-mediated response, should be undertaken to determine whether inclusion in candidate vaccines may induce a response that will counteract the OTU protease activity when exposed to the virus. Targeting the OTU protease may not only counteract innate immune modulation but may potentially affect viral replication. The deISGylating function of the CCHFV OTU protease seems to either stabilise the L-segment or prevent the L-segment from being targeted for degradation (Scholte et al., 2017). Blocking of the deubiquitinating and deISGylating function by inducing a C40A mutation resulted in

the inability to recover CCHFV, whereas blocking of the deISGylating function by the introduction of a A129R mutation resulted in a decrease in the expression of the protein encoded on the L-segment but still allowed virus replication in selected cell lines (Scholte et al., 2017). A therapeutic intervention with the ability to counteract the deubiquitinating and deISGylating activity of the CCHFV OTU protease may therefore prevent virus replication, which will likely reduce the severity of disease and improve the outcome of disease. Additionally, the role of micro and small-interfering RNAs may be explored and can be targeted as a therapeutic strategy.

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Author contributions

Conceptualisation, F.J.B.; Methodology, formal analysis, investigation, validation, N.V.; Resources, F.J.B and D.G.; Writing – Original draft, N.V.; Writing – Review & Editing, F.J.B., D.G. and N.V.; Visualisation, N.V.; Supervision, F.J.B. and D.G.; Project administration, F.J.B.; Funding acquisition, F.J.B and N.V.

Declaration of interests

The authors declare no competing interests.

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Supplemental information

Table S1: Reference, quality control and innate immune signalling genes

Full name	Gene symbol	Reference sequence	RT2 catalogue number
Reference genes			
Actin, beta	*ACTB*	NM_001101	PPH00073
Beta-2-microglobulin	*B2M*	NM_004048	PPH01094
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	NM_002046	PPH00150
Quality control genes			
Reverse Transcription Control	*RTC*	SA_00104	PPX63340
Human Genomic DNA Contamination	*HGDC*	SA_00105	PPH65835
Positive PCR Control	PPC	SA_00103	PPX63339
Innate immune signalling genes			
DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	*DDX58*	NM_014314	PPH20774
Interferon-induced with helicase C domain 1	*IFIH1*	NM_022168	PPH18927
Myeloid differentiation primary response gene (88)	*MYD88*	NM_002468	PPH00911
Mitochondrial antiviral signalling protein	*MAVS*	NM_020746	PPH24180
Interferon regulatory factor 3	*IRF3*	NM_001571	PPH02025
Interferon regulatory factor 7	*IRF7*	NM_001572	PPH02014
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	*NFKB1*	NM_003998	PPH00204
Interferon, beta 1	*IFNB1*	NM_002176	PPH00384
Interferon (alpha, beta and omega) receptor 1	*IFNAR1*	NM_000629	PPH00869
Signal transducer and activator of transcription	*STAT1*	NM_007315	PPH00811

Table S2: Raw data for control and experimental conditions

Gene	**Control group (Empty vector)**	**CCHFV OTU protease**
HGDC	N.D.	N.D.
RTC	18,82	19,57
PPC	16,96	16,96
GAPDH	16,29	17,07
IFIH1 (MDA5)	22,93	24,18
MAVS	20,61	22,01
IRF3	19,30	20,57
IRF7	23,91	25,29
MYD88	22,75	24,46
NFKB1	23,07	24,17
IFNB1	28,00	30,52
IFNAR1	21,12	23,28
STAT1	20,19	22,36
Gene	**Control group (Empty vector)**	**CCHFV OTU protease**
GAPDH	18,25	19,26
DDX58 (RIG-I)	21,34	23,28

The Cq value for each gene is the average Cq value for all biological repeats. N.D. – Not detected

Chapter 5

Characterisation of the innate immune transcriptional response to Hazara virus infection and Hazara viral RNA

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Summary

Hazara orthonairovirus (HAZV), a closely related virus to Crimean-Congo haemorrhagic fever orthonairovirus (CCHFV), has been shown to be a disease model for CCHFV in type I interferon (IFN)-receptor knockout mice. In this study, innate immune modulation in response to HAZV infection and HAZV RNA was determined using gene expression analysis targeting selected innate immune response genes by quantitative polymerase chain reaction (qPCR). Low-level upregulation of innate immune gene expression was demonstrated after stimulation with HAZV RNA, whereas HAZV infection resulted in significant downregulation of *IFNB1* gene expression. The results suggest that one or more HAZV proteins may possess the ability to antagonise the IFN response. Further investigation is required to determine whether HAZV may act as a surrogate for CCHFV innate immune modulation, which would allow work in biosafety level (BSL)-2 facilities and testing of potential novel therapeutics to overcome innate immune suppression.

Keywords

Hazara virus; Hazara virus RNA; innate immune response; interferon

Introduction

Hazara orthonairovirus (HAZV), a closely related virus to Crimean-Congo haemorrhagic fever orthonairovirus (CCHFV), belongs to the order *Bunyavirales*, family *Nairoviridae* and genus *Orthonairovirus* (Adams et al., 2017). HAZV was first isolated from *Ixodes redikorzevi* hard ticks removed from Royle's Mountain voles (*Alticola roylei*) caught in the Kaghan Valley within the Hazara district in West Pakistan (Begum et al., 1970). At that time HAZV was shown to be serologically related, but distinct, from two other viruses (designated JD206 and JD213), which had been isolated from *Hyalomma anatolicum anatolicum* hard ticks collected from cattle and were subsequently identified as Congo virus (now CCHFV) (Begum et al., 1970). Contradictory findings have been reported for serological cross-reactivity between HAZV and CCHFV, which seems to depend on the serological test, virus strains and directionality of cross-reactivity. One way serological cross-reactivity has been demonstrated between CCHFV and HAZV antiserum using hemagglutination inhibition assays (Casals and Tignor, 1974) and limited cross-reactivity using complement fixation (Begum et al., 1970; Smirnova, 1979). Convalescent sera from individuals that recovered from Crimean haemorrhagic fever (now Crimean-Congo haemorrhagic fever (CCHF)) have been shown to react with the HAZV nucleoprotein (NP), but not against the glycoprotein (Clerx et al., 1981). Despite marked cross-neutralization of CCHFV Ug 3010 strain by anti-HAZV hyperimmune serum *in vitro* (Buckley, 1974), no cross-neutralization (Casals and Tignor, 1974; Smirnova, 1979) or cross-protection could be demonstrated *in vivo* (Smirnova, 1979). Interestingly, observations of cross-protection against CCHFV infection after exposure to HAZV have been reported as unpublished data (Foulke et al., 1981), which warrants further investigation and characterisation of HAZV for use in the development of a vaccine and therapeutics against CCHFV. Currently, there are no studies investigating similarities in the innate immune response induced by CCHFV and HAZV.

HAZV can be handled and cultured within a biosafety level (BSL)-2 facility, whereas BSL-4 facilities, which are limited, are required for handling and culturing CCHFV. Due to the relatedness of HAZV and CCHFV, HAZV has been investigated as a disease model for CCHFV. Type I interferon (IFN)-receptor knockout mice had similar histopathological findings after inoculation with HAVZ as the existing CCHFV mouse

models, suggesting the use of the HAZV mouse model as a surrogate model for testing the efficacy of antivirals against CCHFV *in vivo* (Dowall et al., 2012). RNA interference studies in cell culture using small interfering RNA (siRNA) illustrated that targeting the HAZV NP can inhibit HAZV replication (Flusin et al., 2011; Garcia et al., 2005), but siRNAs that target the CCHFV NP failed to inhibit HAZV replication (Garcia et al., 2005). Therefore, cross-protection by siRNA is unlikely, but well-designed siRNAs targeting CCHFV may prove more successful at inhibiting CCHFV replication. A synergistic effect after co-administration of HAZV NP siRNA and ribavirin was demonstrated *in vitro* (Flusin et al., 2011). *In vivo* studies are required to confirm efficient inhibition of virus replication using siRNAs with or without ribavirin.

In this study, modulation of innate immune signalling during HAZV infection and in the presence of HAZV RNA was investigated to characterise the innate immune signalling by HAZV. CCHFV has been shown to suppress innate immune signalling *in vitro* by up to 48 hours (Andersson et al., 2008), which is crucially important in a rapid, progressive infection and may affect the severity and outcome of disease, especially since the innate immune response activates the adaptive immune response, which mediates virus clearance. Similar modulation by HAZV would justify further investigation into the role of HAZV as a potential model for CCHFV innate immune modulation.

Methods

Cells

Vero cells (ATCC® CCL-81™; RRID:CVCL_0059) and human embryonic kidney (HEK)-293 cells (ATCC® CRL-1573™; RRID:CVCL_0045) were obtained from the American Type Culture Collection (ATCC®) and were initially cultured in Dulbecco's Modified Eagle Medium (DMEM; Lonza) supplemented with 10% foetal bovine serum (FBS; Gibco) supplemented with 100 units per ml (U/ml) penicillin and streptomycin (Lonza), L-glutamine to a final concentration of 2mM (Lonza) and non-essential amino acids (Lonza). After removal from cryopreservation, Vero and HEK-293 cells were maintained in advanced DMEM (Gibco) supplemented with 5% and 10% FBS (Gibco),

respectively, and 100 U/ml penicillin and streptomycin (Lonza), L-glutamine to a final concentration of 2mM (Lonza) and non-essential amino acids (Lonza) at 37˚C in a humid 5% CO_2 atmosphere. Cells were sub-cultured when 80-90% confluent.

Adenocarcinoma human alveolar basal epithelial (A549) cells (ATCC® CCL-185™; RRID:CVCL_0023) were obtained from Cellonex. Cells were initially cultured in DMEM supplemented with 20% FBS (Cellonex). Cells were cryopreserved and maintained in advanced DMEM (Gibco) supplemented with 5% FBS (Gibco), 100 U/ml penicillin and streptomycin (Gibco), L-glutamine to a final concentration of 2mM (Gibco) and non-essential amino acids (Lonza) at 37˚C in a humid 5% CO_2 atmosphere after removal from liquid nitrogen storage and were sub-cultured when 80-90% confluent.

Culture and quantification of Hazara virus titres

HAZV JC280 was kindly provided by Prof Robert B. Tesh from the University of Texas Medical Branch. A 90% confluent T25 cell culture flask of Vero cells was inoculated with 50µl seed virus reconstituted in 0,5ml sterile water (Ambion). Cells were cultured at 37˚C in a humid 5% CO_2 atmosphere in advanced DMEM (Gibco) supplemented with 2% FBS (Gibco), 100 U/ml penicillin/streptomycin (Lonza), L-glutamine to a final concentration of 2mM (Lonza) and non-essential amino acids. Virus stocks were prepared by sub-culturing virus-infected cells. Infected cells were assayed for HAZV using an indirect immunofluorescent assay with convalescent CCHF serum; however, CCHF antibody was poorly cross-reactive. During serial passage of virus, cell culture supernatant was harvested and RNA extracted using the Viral RNA Isolation kit (Qiagen) according to the manufacturer's instructions. Infection of cells was confirmed by reverse-transcription polymerase chain reaction (RT-PCR) using the SuperScript™ III One-Step RT-PCR System with Platinum™ *Taq* DNA polymerase (Invitrogen) and 0,2µM of each HAZV-specific primer (HAZV iFW 5'-CGACCAAGACAAGGCAGAAGACC-3' and HAZV iRV 5'-CCTGCCTTCCAAAGCCAGTAGTA-3') according to the manufacturer's instructions. Cycling conditions included a complementary DNA (cDNA) synthesis cycle at 55˚C for

30 minutes followed by pre-denaturation at 94˚C for two minutes and 40 cycles of denaturation at 94˚C for 15 seconds, annealing at 55˚C for 30 seconds and extension at 68˚C for 30 seconds followed by final extension at 68˚C for five minutes. Primers were designed using sequence data retrieved from GenBank® for HAZV JC280 strain (GenBank accession number: JC280 S-segment KP406725). The primers target a 150bp region of the gene encoding the NP. Virus was passaged in Vero cells five times and adapted to A549 cells by passaging the virus-infected cells six times.

HAZV RNA in the virus stock was quantified by real-time quantitative PCR (qPCR) analysis to determine the viral load. Briefly, a 5µl aliquot of RNA isolated from the supernatant collected from virus-infected cells was reverse-transcribed using the SuperScript™ III reverse transcriptase kit (Invitrogen) according to the manufacturer's instructions. Quantification was performed using 1µl cDNA as template. Standards were prepared by 10-fold serial dilution of the 150bp HAZV DNA amplicon and the DNA quantity confirmed using the Qubit™ dsDNA BR Assay kit (Invitrogen), which were included in triplicate within each run. qPCR was performed using the RT^2 SYBR Green ROX FAST Mastermix (Qiagen) according to the manufacturer's instructions on a Rotor-Gene Q cycler (Qiagen) and the optimal primer concentration was determined to be 0,5µM of each HAZV-specific primer per reaction. A standard curve was generated using the average cycle threshold (Cq) value obtained for each standard, which was used to calculate the number of viral copies within the qPCR reaction and the viral copies per millilitre within the prepared virus stocks. Inclusion criteria for a valid standard curve, included a qPCR efficiency of 100±5%, which correlates to a slope of -3,3 and an R^2 value of ≥ 0,99 illustrating an excellent fit of curve.

HAZV infection

A549 cells were enumerated using the Countess™ II automated cell counter (Invitrogen) and seeded at $7{,}5x10^5$ cells per well in triplicate in a 6-well plate and infected 24 hours after seeding with ten viral genome equivalents per cell used for seeding. Virus adsorption was allowed for one hour with gentle agitation every 10

minutes prior to removing the inoculum and incubating cells in maintenance media at 37˚C in a humid 5% CO_2 atmosphere for 24 hours. The cell culture supernatant was collected and cells harvested in RNAprotect Cell Reagent (Qiagen). Uninfected A549 cells were used as a negative or unstimulated control. Total RNA was isolated from stabilised cells using the RNeasy Protect Cell Mini kit (Qiagen) according to the manufacturer's instructions, including the addition of beta-mercaptoethanol (Merck) to the lysis buffer. HAZV infection within A549 cells was confirmed with the HAZV qPCR as described previously using total RNA extracted from cells harvested at 24 hours post-infection.

Electroporation of HAZV RNA

HAZV RNA isolated from supernatant of HAZV cultures was quantified using a HAZV-specific qPCR. The Viral RNA Isolation kit (Qiagen) co-purifies poly-A RNA added to prevent RNA degradation and improve the RNA isolation efficiency. Hence to control for the contribution of poly-A RNA on innate immune signalling, control RNA was prepared by isolation of RNA from uninfected A549 cultures. HAZV RNA was introduced into HEK-293 cells by electroporation using the Neon® transfection kit (Life Technologies) with modifications to the manufacturer's instructions, which included pre- and post-electroporation incubations of 3 and 10 minutes, respectively, resulting in improved viability and electroporation efficiency. Aliquots containing $5x10^6$ HEK-293 cells were electroporated with $4{,}5x10^7$ HAZV copies, which equated to 9 genome equivalents per cell, in triplicate. Control reactions containing RNA isolated from uninfected A549 cells were volume matched to account for poly-A RNA content and prepared in triplicate. A pcDNA3.1(+) plasmid encoding a green fluorescent protein (GFP) was included within each run and prepared in parallel to assess electroporation efficiency and cell survival. The run was only accepted if the control reaction, pcDNA3.1(+)GFP electroporated cells, had high electroporation efficiencies (>80%) and cell survival rates. Cells were harvested at 24 hours post-electroporation in RNAprotect cell reagent (Qiagen) to ensure immediate stabilisation of the gene expression profile within harvested cells.

RNA isolation and qRT-PCR

Total RNA, for gene expression analysis, was isolated using the RNeasy Plus Mini kit (Qiagen) according to the manufacturer's instructions, including the addition of beta-mercaptoethanol (Merck) to the RLT buffer. RNA integrity was confirmed using a 1,2% denaturing RNA gel (Aranda et al., 2012). RNA quality and quantity were determined using the Nanodrop™ 2000 (Thermo Scientific). cDNA synthesis was performed on 0,8µg total RNA with a combination of random hexamer and poly-dT primers using the First Strand Synthesis kit (Qiagen) according to the manufacturer's instructions. cDNA was diluted in 91µl RNase-free water (Ambion) and 0,9µl cDNA template added per reaction. RNA and cDNA were stored at -80˚C until analysis. A custom RT^2 profiler array (SA Biosciences) was used targeting selected innate immune genes for relative quantification (Table 1).

Table 1: Genes selected for characterisation of HAZV innate immune signalling

Full name	Gene symbol	Reference sequence	RT2 catalogue number
Reference genes			
Actin, beta	*ACTB*	NM_001101	PPH00073
Beta-2-microglobulin	*B2M*	NM_004048	PPH01094
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	NM_002046	PPH00150
Quality control genes			
Reverse Transcription Control	*RTC*	SA_00104	PPX63340
Human Genomic DNA Contamination	*HGDC*	SA_00105	PPH65835
Positive PCR Control	PPC	SA_00103	PPX63339
Innate immune signalling genes			
DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	*DDX58*	NM_014314	PPH20774
Interferon-induced with helicase C domain 1	*IFIH1*	NM_022168	PPH18927
Myeloid differentiation primary response gene (88)	*MYD88*	NM_002468	PPH00911
Mitochondrial antiviral signalling protein	*MAVS*	NM_020746	PPH24180
Interferon regulatory factor 3	*IRF3*	NM_001571	PPH02025
Interferon regulatory factor 7	*IRF7*	NM_001572	PPH02014
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	*NFKB1*	NM_003998	PPH00204
Interferon, beta 1	*IFNB1*	NM_002176	PPH00384
Interferon (alpha, beta and omega) receptor 1	*IFNAR1*	NM_000629	PPH00869
Signal transducer and activator of transcription	*STAT1*	NM_007315	PPH00811

qPCR reactions were prepared using RT^2 SYBR Green ROX FAST Mastermix (Qiagen) according to the manufacturer's instructions and runs were performed on a Rotor-Gene Q cycler (Qiagen). Due to a decline in the amplification efficiency of *DDX58* mRNA on the array, qPCR assays targeting a 158bp segment of *DDX58* mRNA and a 100bp segment of *GAPDH* mRNA were developed for confirmation of relative *DDX58* gene expression on a Rotor-Gene Q (Qiagen). qPCR reactions were prepared using RT2 SYBR® Green ROX FAST Mastermix (Qiagen) according to the manufacturer's instructions and 0,5µM pf each gene specific primer (DDX58-FW 5'-GTGGGCAATGTCATCAAAATG-3', DDX58-RV 5'-GTCTGAAGGCGTAAAATAGAGTC-3', GAPDH-FW 5'- TGACAACAGCCTCAAGAT-3' and GAPDH-RV 5'- TCCTTCCACGATACCAAAG-3'). Cycling conditions included denaturation at 95˚C for 10 minutes followed by 40 cycles of denaturation at 95˚C for 15 seconds and a combined annealing and extension cycle at 60˚C for 30 seconds with fluorescence data acquisition at the end of each cycle followed by melting curve analysis. Data acquisition was captured by the Rotor-Gene Q Series Software (RRID: SCR_015740), and subsequently, the threshold value was determined according to the manufacturer's instructions and set at 0,08303 for all subsequent runs. qPCR amplification specificity was confirmed using a dissociation curve generated for each reaction. Data was exported and the Cq values were analysed.

qPCR data analysis

The ΔΔCq method (Livak and Schmittgen, 2001) was utilised to determine modulation of gene expression. *GAPDH* was selected as the reference gene used for normalisation and modulation of expression was expressed relative to a negative or unstimulated control. Cq values > 33 were considered negative. The ΔCq value was calculated to allow normalisation of gene expression by subtracting the Cq value of *GAPDH* from the gene of interest for each preparation. The average value for each condition tested in triplicate was used to calculate the ΔΔCq, fold change and fold regulation values in response to HAZV infection or HAZV RNA. The ΔΔCq value was calculated by subtracting the average ΔCq for the negative control from the ΔCq for each experimental condition. To determine the fold change, $2^{-\Delta\Delta Cq}$ was calculated for

each experimental condition. The fold change was used to calculate the fold regulation by determining -1/fold change for fold change values < 1 and fold change = fold regulation for fold change values > 1. A fold regulation value of ≥ 2 was considered to be a significant biological change in the level of gene expression. Statistical analysis using a Student's t-test on the ΔCq value for each sample within the control and experimental groups with two-tailed distribution and equal variance between two samples were used to determine whether changes in gene expression were statistically significant ($p < 0,05$).

Results

Innate immune signalling associated with the recognition of HAZV or HAZV RNA

Activation of the innate immune response relies on the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). Antiviral immunity is induced in response to the recognition of viral nucleic acids by the host cell via two major types of PRRs, including retinoic acid-inducible (RIG)-I-like receptors (RLRs) and Toll-like receptors (TLRs). RIG-I cytosolic receptors have been demonstrated to be required for recognition and innate immune activation in response to CCHFV (Spengler et al., 2015). After RIG-I or melanoma differentiation-associated factor 5 (MDA5) bind viral RNA or replication intermediates, downstream signalling is mediated in a mitochondrial activator virus signalling (MAVS)-dependent manner. MAVS induces an IFN response by activation of IFN-regulatory factor (IRF) 3 or nuclear factor (NF)-κβ activation (Sun et al., 2006).

Innate immune signalling in response to RNA isolated from uninfected cells containing poly-A RNA was compared to unstimulated cells and no significant modulation of innate immune signalling was observed for any of the genes of interest. Although the presence of poly-A RNA within RNA preparations is unlikely to influence gene modulation of innate immune markers confirmation was deemed necessary. HAZV RNA induced a low-level upregulation of *DDX58* (RIG-I) gene expression, whereas modest downregulation of *DDX58* (RIG-I) gene expression during HAZV infection was demonstrated (Table 2; Figure 1a). In contrast, HAZV infection resulted in modest

upregulation of *IFIH1* (MDA5) ($p < 0,05$) gene expression with minimal upregulation in response to HAZV RNA (Table 2; Figure 1b). Downregulation of *DDX58* (RIG-I) gene expression during HAZV infection, in contrast to low-level upregulation in response to HAZV RNA, may suggest that the virus has the ability to prevent upregulation of *DDX58* (RIG-I) expression in response to infection. Further investigation is required to determine whether HAZV is preferentially recognised by the RIG-I or MDA5 cytosolic receptor, but the results in this study suggest that MDA5 may play a role in recognition of HAZV *in vitro*. Alternatively, additional investigation to determine whether a HAZV component antagonises *DDX58* (RIG-I) gene expression may reveal a virulence mechanism to prevent virus recognition. *MAVS* gene expression remained unaltered in response to virus infection and viral RNA (Table 2; Figure 1c). The NF-κβ pathway represents an alternative recognition pathway responsible for the induction of cytokines, chemokines, immune receptors and cell adhesion molecules, which is mainly activated after recognition by TLRs but alternative stimuli exist (Pahl, 1999). HEK-293 cells lack TLRs, whereas A549 cells have TLRs yet the level of upregulation of *MYD88* gene expression, encoding an adaptor protein responsible for NF-κβ activation, was similar during HAZV infection and after stimulation with HAZV RNA. Low-level upregulation of *MYD88* gene expression in HEK-293 cells in response to HAZV and HAZV RNA suggest that some upregulation can occur in a TLR-independent manner (Table 2; Figure 1d). Interestingly, low-level upregulation of *NFKB1* gene expression was demonstrated in response to viral RNA in HEK-293 cells that lack TLRs, which primarily activate the NF-κβ pathway, but unaltered *NFKB1* gene expression was demonstrated during infection in A549 cells that contain TLRs (Table 2; Figure 1e).

IFN-β signalling in response to HAZV infection and HAZV RNA

IFN-β plays a key role in early innate immune activation and binds to autocrine and paracrine IFN-receptors on infected and uninfected cells to induce an antiviral state by the induction of interferon-stimulated genes (ISGs) via IRF9 (Levy et al., 1989). The two most important mediators of type I IFN-signalling are IRF3 and IRF7, which follow a two-phased induction mechanism with IRF3 being crucial to the early induction of

IFN-β expression (Sato et al., 2000). IRF7 expression is induced via positive feedback regulation in an IFN-dependent manner and plays a significant role later in the innate immune response (Sato et al., 1998). Additionally, knockout studies have provided evidence that IRF7 is the sole inducer of IFN-α expression and that IRF3 and IRF7 cooperation is required for efficient IFN-α_4 and IFN-β induction (Sato et al., 2000).

Stimulation with HAZV RNA resulted in low-level upregulation of *IRF3* and *IRF7* gene expression, whereas low-level downregulation of *IRF7* gene expression was demonstrated in HAZV infected cells (Table 2; Figures 1f, g). Despite the changes not reaching the threshold for significance, upregulation in the presence of HAZV RNA suggests that some innate immune signalling is induced in response to viral RNA only. However, when the intact virus is present, despite the presence of viral RNA and replication intermediates, some downregulation occurs, which may indicate that one or more viral components restrict IRF7 induction or that HAZV infection affects ISG-signalling and subsequently *IRF7* gene expression.

The expression of IFN-β has been shown to induce a broad array of ISGs compared to IFN-α, which are crucial to induce an antiviral state (Der et al., 1998). Stimulation with HAZV RNA upregulated *IFNB1* gene expression, whereas HAZV infection resulted in significant downregulation of *IFNB1* gene expression by more than 3-fold ($p < 0,05$) at 24 hours post-infection (Table 2; Figure 1h). The results suggest that one or more HAZV components antagonise *IFNB1* gene expression. Further investigation is required to determine the mechanism utilised by HAZV to modulate *IFNB1* gene expression. The CCHFV nucleoprotein of the Hoti strain has been shown to antagonise the IFN-β promoter (Fajs et al., 2014). Additionally, the CCHFV NS_M protein and OTU protease from a South African strain have been shown to downregulate *IFNB1* expression (unpublished data).

ISG-signalling downstream of IFN-β expression

IFN-β is released from infected cells after activation of innate immune signalling and, after specific interaction with type I IFN-receptors, induces transcription of ISGs in a JAK/STAT-dependent or -independent manner (de Weerd et al., 2013). STAT1 has

been implicated in protection against viral infection and is essential for the induction of an IFN response (Durbin et al., 1996). In CCHFV infection, the type I IFN-receptor and STAT1 has been demonstrated to be critical for control of virus replication and spread (Bente et al., 2010; Bereczky et al., 2010; Zivcec et al., 2013). Additionally, HAZV has been identified as a clinical model for CCHFV infection in type I IFN-receptor deficient mice (Dowall et al., 2012).

Upregulation of *IFNAR1* and *STAT1* gene expression in response to HAZV infection and HAZV RNA (Table 2; Figure 1i, j) suggests that, despite significant downregulation of *IFNB1* gene expression at 24 hours post-infection with HAZV, upregulation downstream of IFN-β expression could still occur. HAZV may, therefore, antagonise the IFN-β response but does not completely abolish innate immune activation. However, IFN-β suppression by HAZV may explain the low-level upregulation of other innate immune response genes evaluated, since positive feedback regulation may be significantly affected by a significant reduction in IFN-β expression.

Table 2: Innate immune modulation by HAZV infection and HAZV RNA

Condition:	**Gene:**	**Fold regulation:**	**Fold change:**	**p-value:**
HAZV infection	*DDX58*	-1,50	0,6659	0,1607
	IFIH1	1,61	1,6096	0,0212
	MYD88	1,19	1,1920	0,2081
	MAVS	1,01	1,0140	0,8375
	IRF3	1,12	1,1173	0,2456
	IRF7	-1,18	0,8448	0,0610
	NFKB1	-1,03	0,9749	0,6406
	IFNB1	-3,74	0,2673	0,0008
	IFNAR1	1,23	1,2283	0,1751
	STAT1	1,34	1,3410	0,0584
HAZV RNA	*DDX58*	1,12	1,1199	0,2862
	IFIH1	1,27	1,2716	0,1966
	MYD88	1,19	1,1920	0,1913
	MAVS	1,09	1,0867	0,3702
	IRF3	1,33	1,3318	0,0317
	IRF7	1,11	1,1070	0,4303
	NFKB1	1,15	1,1540	0,1140
	IFNB1	1,16	1,1620	0,8038
	IFNAR1	1,05	1,0521	0,7647
	STAT1	1,13	1,1303	0,3975

*Gene regulation at a biologically and statistically significant level is indicated in red.

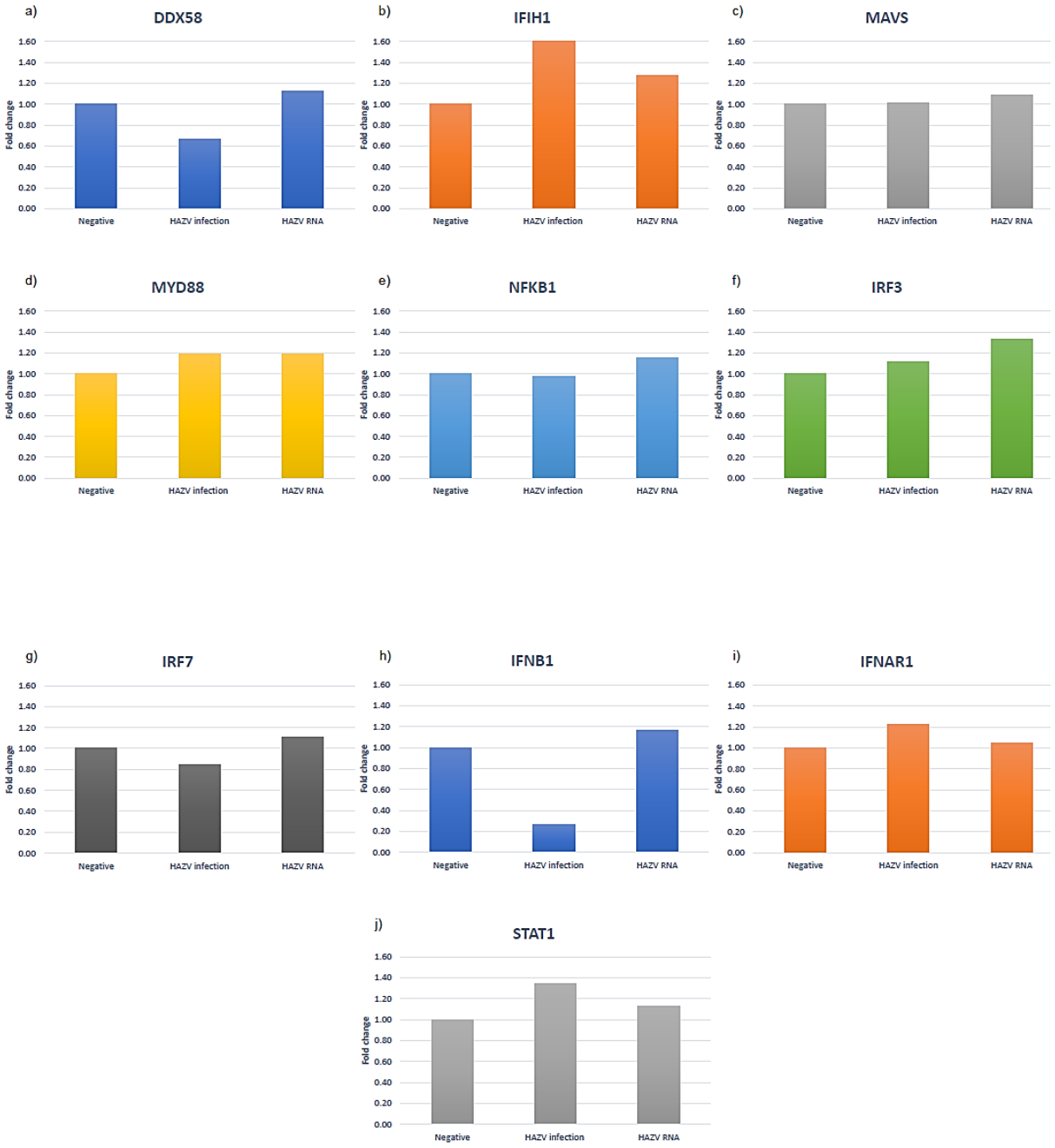

Figure 1: Modulation of innate immune signalling in response to HAZV infection and HAZV RNA *in vitro*

Modulation of gene expression determined in response to HAZV infection and stimulation with HAZV RNA, which were compared to unstimulated cells or cells stimulated with RNA extracted from uninfected cultures, respectively.

Discussion

HAZV, a closely related virus to CCHFV, is not considered to be a medically significant virus since human infection is not known to occur. In contrast to CCHFV with a broad host range, HAZV seems to mainly infect ticks, which may in part be due to the remote terrain where the host from which the ticks containing HAZV was first isolated, is found. HAZV was isolated from adult *Ixodes redikorzevi* hard ticks removed from Royle's Mountain voles caught at a high altitude (12 000 ft approximately 3650m) in subarctic terrain (Begum et al., 1970), which is therefore unlikely to be transmitted to humans. HAZV has been demonstrated to be antigenically closely related to CCHFV, a medically significant pathogen that causes sporadic human cases and was initially grouped within the CCHF antigenic group with CCHFV (Casals and Tignor, 1980; Clerx et al., 1981; Zeller et al., 1989). However, phylogenetic analysis has subsequently revealed significant genetic differences between HAZV and CCHFV, which resulted in HAZV grouping with Tofla virus, rather than CCHFV (Kuhn et al., 2016). HAZV has been suggested to be a disease model in type I IFN-receptor deficient mice, because histopathological findings were similar to the histopathological findings demonstrated in the CCHFV mouse models (Dowall et al., 2012). In this study, innate immune modulation by HAZV and HAZV RNA was investigated to determine whether HAZV have potential as a surrogate to allow investigation of CCHFV innate immune modulation in a BSL-2 facility.

HAZV RNA induces low-level upregulation of innate immune signalling and a high quantity of viral RNA may be required to induce significant innate immune modulation. Alternatively, the presence of poly-A RNA in viral RNA preparation may influence the innate immune modulation, despite no significant changes having been observed between unstimulated and poly-A stimulated cells. Despite low-level upregulation in response to HAZV RNA, trends could be identified, including downregulation or a lack of upregulation in the presence of intact HAZV compared to HAZV RNA only, which may provide targets that require further investigation. Identified targets include RIG-I, IRF7 and IFN-β. Interestingly, despite upregulation of *DDX58* (RIG-I) expression in response to HAZV RNA, downregulation of gene expression was observed during HAZV infection, which may indicate a virus-induced mechanism that contributes to downregulation of *DDX58* (RIG-I) gene expression. Alternatively, HAZV may be

recognised by the MDA5 cytosolic receptor, not RIG-I, as identified for CCHFV. Further investigation is required to determine whether HAZV is recognised by RIG-I or MDA5. In addition, investigation of the role of NF-κβ during HAZV infection may provide insights into the mechanism utilised to downregulate the expression of *IFNB1*. Minimal upregulation of *NFKB1* expression in HEK-293 cells that lack TLRs, the main activators of the NF-κβ pathway, with unaltered gene expression in A549 cells that contain TLRs may suggest a mechanism to prevent NF-κβ activation, which may subsequently contribute to downregulation of IFN-β expression. NF-κβ is part of the transcriptional enhancer complex that is recruited to the IFN-β promoter to induce type I IFN expression (Wathelet et al., 1998). *IRF7* expression is downregulated in response to HAZV infection but upregulated in response to HAZV RNA, which may indicate a mechanism to interfere with *IRF7* transcription. Lastly, significant downregulation in *IFNB1* gene expression was demonstrated at 24 hours post-infection with HAZV. HAZV, therefore, likely has one or more virulence factors that interfere with *IFNB1* transcription, which may directly target *IFNB1* or an innate immune component upstream of *IFNB1* gene expression. Further investigation is required to determine the mechanism utilised to downregulate *IFNB1* gene expression.

Future investigation is required to confirm whether HAZV can act as a surrogate for CCHFV innate immune modulation. Additional work may include side-by-side comparison of infectious CCHFV and HAZV. Additionally, *in vitro* findings should be confirmed *in vivo*. Despite demonstrating virus replication, virus adaptation to host cells for more extended periods may be required to achieve virus replication at higher levels, which may result in more significant changes in innate immune modulation. Study of virus evolution during adaptation to a host cell, including tick and mammalian cells may provide insights into changes required to adapt to a specific host cell.

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Author contributions

Conceptualisation, F.J.B.; Methodology, formal analysis, investigation, validation, N.V.; Resources, F.J.B and D.G.; Writing – Original draft, N.V.; Writing – Review & Editing, F.J.B., D.G. and N.V.; Visualisation, N.V.; Supervision, F.J.B. and D.G.; Project administration, F.J.B.; Funding acquisition, F.J.B and N.V.

Declaration of interests

The authors declare no competing interests.

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Supplemental information

Table S1: Raw data for control and experimental conditions

Gene	Un-infected A549 cells	HAZV infection	Volume matched control RNA	HAZV RNA
HGDC	N.D.	N.D.	N.D.	N.D.
RTC	17,95	17,98	18,12	18,13
PPC	17,11	17,08	16,95	16,93
GAPDH	15,70	16,03	17,55	17,66
IFIH1	25,43	25,08	24,09	23,86
MYD88	20,49	21,02	22,91	22,77
MAVS	21,41	21,72	21,83	21,82
IRF3	19,64	19,81	20,47	20,17
IRF7	27,31	27,89	26,14	26,11
NFKB1	22,90	23,27	24,69	24,60
IFNB1	28,69	30,92	29,73	29,63
IFNAR1	23,30	23,33	22,43	22,47
STAT1	20,05	19,96	21,70	21,64
Gene	**Un-infected A549 cells**	**HAZV infection**	**Volume matched control RNA**	**HAZV RNA**
GAPDH	18,98	18,77	19,64	19,64
DDX58	22,01	22,39	22,75	22,59

The Cq value for each gene is the average Cq value for all biological repeats. N.D. – Not detected

Chapter 6

Conclusions and future perspectives

Viruses co-evolve with their respective host/s, which allows adaptation to infect and replicate within a specific host or host range. Crimean-Congo haemorrhagic fever orthonairovirus (CCHFV) has a broad host range, including ticks and small and large vertebrates, which play a role in maintaining the virus in nature. Humans are referred to as dead-end hosts. CCHFV, an RNA virus, has been shown to have a slow evolutionary rate (Carroll et al., 2010), which likely reflects the need to retain fitness in this broad range of hosts. The M-segment has been reported to have the highest evolutionary rate (Carroll et al., 2010), which likely indicates adaptation of the virus to infect different host cells via the encoded structural glycoproteins that facilitate binding to and entering host cells. Interestingly, a high evolutionary rate has been demonstrated in ticks (Xia et al., 2016). Co-evolution with a host may result in the development of strategies to evade the host immune response, which may target the innate and/or adaptive immune response.

The innate immune system developed prior to the separation of the plant and animal kingdoms and therefore represents an ancient branch of the immune system, whereas the adaptive immune response developed later allowing for a pathogen-specific response. The innate immune response plays a critical role in activating and coordinating the adaptive immune response by guiding antigen selection by B- and T-lymphocytes (Fearon and Locksley, 1996). Dysregulation of the innate immune response may, therefore, compromise the innate and adaptive immune responses. Subsequently, protection against disease may be compromised and potentially the longevity of protection in survivors of infection. The immune response to CCHFV infection is a multifaceted process and an improved understanding of the virus-host interactions is imperative to develop novel therapeutic strategies and vaccines. Research aimed at understanding the virus-host interactions with regard to the immune response has been primarily focused on understanding the adaptive immune response to CCHFV post-infection and in response to different vaccine strategies. Considering the critical role of the innate immune response during early infection, an improved understanding of the virus-host innate immune interactions may provide

insights into strategies that aid in controlling the virus during early infection, allowing the adaptive immune response to mediate virus clearance. In this study, modulation of innate immune signalling in response to selected non-structural CCHFV proteins was investigated.

The CCHFV NS_M protein encoded on the M-segment is produced by post-translational processing of the glycoprotein precursor polypeptide. The NS_M protein was detected in cell lysates from CCHFV-infected cells but not in virion lysates, which suggested that it is a non-structural protein produced within infected cells (Altamura et al., 2007). To date, the role or function of the CCHFV NS_M protein in the virus life cycle is undetermined. Within the order *Bunyavirales* (previously family *Bunyaviridae*), the function of NS_M proteins is not conserved. In this study, the CCHFV NS_M protein from two South African isolates, a reassortant and non-reassortant isolate, were transiently expressed *in vitro* in human embryonic kidney (HEK)-293 cells to determine whether the NS_M protein plays a role in innate immune modulation. The M-segment of the reassortant isolate grouped within the Asian lineage of CCHFV isolates, whereas the non-reassortant isolate grouped within the predominantly South African lineage of CCHFV isolates. Therefore, the NS_M proteins evaluated in this study, not only represented the NS_M protein from reassortant and non-reassortant South African isolates but may also be viewed as representing different lineages, namely Asian and African lineage NS_M proteins, respectively. The non-reassortant or South African NS_M protein demonstrated the ability to suppress transcriptional upregulation of key innate immune markers required in CCHF infection for virus recognition and the induction of an antiviral state. In contrast, the reassortant or Asian NS_M protein induced transcriptional upregulation of innate immune markers. Analysis of the amino acid composition of the CCHFV NS_M proteins evaluated in this study revealed 92,88% amino acid sequence similarity (refer to Appendix D). To determine whether NS_M proteins from other CCHFV isolates may act as innate immune antagonists, global amino acid sequence analysis was performed. Substantial amino acid heterogeneity between geographically distinct isolates was revealed with only 47,6% amino acid sequence similarity amongst CCHFV isolates (refer to Appendix D). In contrast, phylogenetic analysis revealed grouping of South African NS_M proteins from non-reassortant isolates with 98,3% amino acid sequence similarity and one non-reassortant South African isolate with 92,8% amino acid sequence similarity.

Therefore, the function of the South African NS_M protein as innate immune antagonist may be conserved amongst non-reassortant South African isolates, which may reflect evolutionary changes to evade the host's immune response.

The role as innate immune antagonist is the first putative role proposed for a CCHFV NS_M protein. Investigation to determine the role or function of CCHFV NS_M proteins from genetically diverse CCHFV isolates may be explored. In this study, higher rates of cell death were consistently observed in cells expressing the reassortant or Asian CCHFV NS_M protein as compared to all other conditions applied. To ensure accurate results, the level of protein expression was matched with the level of expression of the non-reassortant NS_M protein; however, higher rates of cell death may suggest a role in inducing apoptosis. Study of the evolution of the NS_M protein may not only provide insights into the evolution of CCHFV but may reveal potential function/s.

A recent study revealed an immunodominant linear B-cell epitope located on the CCHFV NS_M protein. The identified peptide reacted significantly with antibody in the sera of CCHF survivors from Turkey and vaccinated individuals from Bulgaria but not CCHF survivors from South Africa (Fritzen et al., 2018), which suggests that the CCHFV NS_M protein is immunogenic and that the level of expression during infection may be sufficient to induce long-term memory B-cell responses. The lack of reactivity with sera from South African CCHF survivors may reflect a difference in immunogenicity, host response and/or a significant difference in amino acid composition. Phylogenetic analysis of the NS_M protein at amino acid level revealed that Bulgarian and Turkish isolates group within a single clade, which may indicate that sera from geographically distinct regions with circulating virus that have a significantly different amino acid composition may lack reactivity or poorly react with the identified peptide. Within the immunodominant region consisting of 20 amino acids ($_{951}$NVMLAVCKRMCFRATIEASR$_{970}$), amino acid differences could be identified at seven positions when assessing CCHFV isolates representing all CCHFV lineages. The Bulgarian and Turkish isolates (identical within the identified peptide) differ from South African isolates by two to three amino acids within the identified peptide, which may explain the lack of reactivity or poor reactivity. Further investigation is required to determine whether poor reactivity is attributed to amino acid differences, poor immunogenicity of the South African NS_M protein possibly due to innate immune dysregulation, due to the test setup used in the study or host responses to the NS_M

protein. Investigation may provide clarity on the use of the CCHFV NS_M protein for the development of diagnostic tools that can be applied worldwide, especially in light of the significant differences amongst CCHFV NS_M proteins. Alternatively, the use of different variants of the peptide may be required to be applied worldwide, which will significantly influence the cost of the assay.

Virus ovarian-tumour like (OTU) proteases are virulence factors that have been demonstrated to interfere with ubiquitination, which plays a key role in regulation of cellular processes including immune activation. Structural differences between the nairoviral and eukaryotic OTU proteases suggest divergent evolution (Bailey-Elkin et al., 2014). Within the family *Nairoviridae*, OTU protease motifs have been identified within the coding region of the RNA-dependent RNA polymerase (RdRP) on the L-segment. Despite relative variability within the OTU coding region, specific amino acid residues associated with deubiquitinating and deISGylating function are highly conserved amongst all nairoviruses (Kuhn et al., 2016). The CCHFV OTU protease has been demonstrated to have strong deubiquitinating and deISGylating activity (Frias-Staheli et al., 2007; Scholte et al., 2017), which facilitate dysregulation of cellular processes and the innate immune response. In this study, the CCHFV OTU protease demonstrated the ability to inhibit the upregulation of innate immune signalling, which may impact virus recognition and the induction of an antiviral state. The CCHFV OTU protease remains an attractive target for the development of novel therapeutic strategies, because of its role in directly interfering with innate immune signalling. In this study, an additional mechanism was identified for innate immune dysregulation by the CCHFV OTU protease. In a recent study, inactivation of the CCHFV OTU protease resulted in no recoverable virus and blocking of the deISGylating function resulted in a significant reduction in the expression of the L-segment (Scholte et al., 2017), which suggests that targeting the CCHFV OTU protease may not only counter innate immune dysregulation, but potentially affect the ability of the virus to replicate and/or infect host cells. Therefore, investigation to identify therapeutic interventions to counteract the deubiquitinating and deISGylating activity of the CCHFV OTU protease is warranted. Further investigation is required to determine whether inclusion in candidate vaccines provide a strategy for countering the CCHFV OTU protease activity.

Interestingly, the CCHFV OTU protease has been shown to target RIG-I by employing its deubiquitinating function directly, which prevents RIG-I induced interferon (IFN)-β transcription (Scholte et al., 2017). In this study, transcriptional downregulation of *DDX58* (RIG-I) gene expression was demonstrated in response to the CCHFV NS_M protein and OTU protease. Recent findings demonstrated that knockout of *DDX58* (RIG-I) gene expression, not only impaired IFN-stimulated genes (ISG) induction but allowed uncontrolled virus replication (Spengler et al., 2015), which suggests that RIG-I is key to the induction of an antiviral response and intervention strategies that can overcome RIG-I dysregulation may improve the outcome of disease. However, studies investigating the role of selected proteins in innate immune modulation are limited to *in vitro* study and a limitation is the need to overexpress the protein/s in order to detect the effects the proteins may exert on innate immune signalling. The levels of expression therefore may not replicate the level of expression during infection. *In vivo* studies are required to provide clarity on the significance of each of the identified innate immune antagonist in infection. However, the study of innate immune signalling *in vivo* has been hampered by the need for an immunocompetent CCHFV model, which until recently was not available (Haddock et al., 2018).

In vivo studies may reveal the effect of innate immune dysregulation on the severity and outcome of disease. Currently, two theories exist with regard to the effect on the outcome of disease. One theory is that innate immune dysregulation may result in poor innate immune control followed by uncontrolled virus replication and spread throughout the host, which in turn overwhelms the host at the time infection is recognised. Overstimulation of the immune system may then result in high levels of cytokine and chemokine release in an attempt to control the virus infection, which in turn results in immunopathology and contributes to disease severity and the outcome of disease. However, an alternative theory is that innate immune dysregulation without complete or high-level suppression may assist the host by preventing high-levels of cytokine and chemokine release associated with more severe infections. The first theory may explain why high viral loads and the absence of detectable antibody levels are frequently demonstrated in fatal CCHF cases.

Furthermore, investigating the effect of CCHFV proteins with the ability to modulate the innate immune response on different cell types may provide clarity on whether specific cells are more conducive to CCHFV replication due to the interaction of the

viral proteins with the cell defensive mechanisms. Involvement of micro and small interfering RNAs in gene expression modulation may be explored and provides a target to counter gene modulation. In-depth transcriptomic analysis may reveal additional mechanisms and/or targets for therapeutic interventions.

Hazara orthonairovirus (HAZV), a closesly related virus to CCHFV, has been proposed as a disease model for CCHF in type I IFN-receptor deficient mice (Dowall et al., 2012). In this study, innate immune modulation during HAZV infection and in response to HAZV RNA was investigated to determine whether HAZV has potential as a surrogate for CCHFV innate immune modulation. The response to HAZV RNA and intact HAZV was compared. Since the innate immune system responds to viral RNA, differences in the response to HAZV RNA and intact HAZV may reveal mechanisms of innate immune modulation, which may not necessarily result in downregulation but prevent upregulation. A significant downregulation in the transcription of *IFNB1*, the gene that encodes IFN-β, suggests that HAZV does modulate innate immune signalling. Further investigation is required to determine the mechanism underlying downregulation of *IFNB1* gene expression. Additionally, it may be warranted to investigate the ability of the HAZV OTU protease to deubiquitinate and deISGylate proteins. If the HAZV OTU protease demonstrates a similar level of deubiquitinating and/or deISGylating activity to the CCHFV OTU protease, then HAZV may be a useful model for therapeutic interventions to overcome the effects of the OTU protease.

Despite not being able to determine whether HAZV is a suitable surrogate for CCHFV innate immune modulation, this study revealed targets for further exploration to investigate the potential of HAZV as a surrogate for CCHFV innate immune signalling. Virus adaptation to a specific cell line may be required to achieve higher rates of virus replication and more robust changes in innate immune modulation. Study of HAZV evolution while adapting to a host cell, including tick and mammalian cells may suggest specific changes required to infect and replicate to high-levels within a specific host cell. Further investigation may reveal whether HAZV can act as a surrogate for innate immune modulation. HAZV may potentially be an invaluable tool at research institutions where high-level biosafety facilities are lacking and may provide a safer alternative for screening therapeutic interventions against CCHFV.

Despite the fact that type I IFN may be ineffective at controlling CCHFV, which can be attributed to the virus becoming insensitive to the effects of type I IFN during early infection (Andersson et al., 2008), the role of type I IFNs should not be undervalued. Type I IFNs play a key role in signalling ISGs, which are responsible for inducing an antiviral state. ISG expression may be more effective at limiting virus replication but is compromised due to virus-host interactions. The use of ISG products may, therefore, provide a strategy to limit virus replication and reduce the severity of disease and/or improve the outcome of disease.

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Appendix A: Letter of ethics approval

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

IRB nr 00006240
REC Reference nr 230408-011
IORG0005187
FWA00012784

26 February 2016

MS N VILJOEN
C/O PROF FJ BURT
DEPT OF MEDICAL MICROBIOLOGY (VIROLOGY)
FACULTY OF HEALTH SCIENCES
UFS

Dear Ms Viljoen

ECUFS NR 34/2013 C (SUB STUDY)
PROJECT TITLE: INNATE IMMUNE SIGNALLING INDUCED BY CRIMEAN-CONGO HAEMORRHAGIC FEVER VIRUS PROTEINS IN VITRO.

1. You are hereby kindly informed that, at the meeting held on 23 February 2016, the Health Sciences Research Ethics Committee (HSREC) approved the above project.
2. The Committee must be informed of any serious adverse event and/or termination of the study.
3. Any amendment, extension or other modifications to the protocol must be submitted to the HSREC for approval.
4. A progress report should be submitted within one year of approval and annually for long term studies.
5. A final report should be submitted at the completion of the study.
6. Kindly use the **HSREC NR** as reference in correspondence to the HSREC Secretariat.
7. The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; The International Conference on Harmonization and Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Tripartite), Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

Yours faithfully

DR SM LE GRANGE
CHAIR: HEALTH SCIENCES RESEARCH ETHICS COMMITTEE

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
T: +27 (0)51 401 7795/7794 | F: +27 (0)51 444 4359 | E: ethicsfhs@ufs.ac.za
Block D, Dean's Division, Room D104 | P.O. Box/Posbus 339 (Internal Post Box G40) | Bloemfontein 9300 | South Africa
www.ufs.ac.za

Appendix B: Letter of Department of Agriculture, Forestry and Fisheries approval and approval of amendment

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/4

Felicity Jane Burt
Room 410, Block C, 2nd Floor, Francois Retief Building
Department of Medical Microbiology and Virology
Faculty of Health Sciences
University of the Free State
Bloemfontein

Email: burtfj@ufs.ac.za

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

Dear Felicity Jane Burt

Your application requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions :

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. Approval may be required in terms of the Genetically Modified Organisms Act, 1997 (Act No 15 of 1997) prior to the start of the study;

4. A veterinary import permit may be required prior to the importation of synthesized CCHFV and Hazara virus genes from Genscript, China;
5. Only a registered waste disposal company may be utilised for the removal of waste generated during the study;
6. The study may only be conducted under BSL 2 conditions at the Department of Medical Microbiology and Virology, University of the Free State.

Title of research/study: Innate immune signalling induced by Crimean-Congo haemorrhagic fever virus proteins *in vitro*
Researcher: FJ Burt
Your Ref./ Project Number: ECUFS NR 34/2013C
Institution: University of the Free State
Our ref Number: 12/11/1/4
Expiry: 31 December 2019

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH

Date: 2018 -01- 2 9

SUBJECT: *PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)*

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/4

Felicity Jane Burt
Room 410, Block C, 2nd Floor, Francois Retief Building
Department of Medical Microbiology and Virology
Faculty of Health Sciences
University of the Free State
Bloemfontein

Email: burtfj@ufs.ac.za

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Dear Felicity Jane Burt

Your Email dated 29 August 2018, requesting an amendment to permission granted on 29 January 2018 under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform research projects/studies, refers.

I am pleased to inform you that permission is hereby granted to perform the following amended research/study, with the following conditions :

Conditions:

1. All conditions contained in the original Section 20 approval, dated 2018-01-29, for the below mentioned studies must be complied with;
2. The only allowed deviation from the Section 20 approval is the following:
    - Approval is extended to include work with Vero cells (African green monkey kidney cell line) and A549 (human epithelial cell line);
    - Approval is granted to store cell culture stocks of Hazara virus in Vero and A549 cells in the Division of Virology, Faculty of Health Sciences, University of the Free State.

Title of research/study: Innate immune signalling induced by Crimean-Congo haemorrhagic fever virus proteins *in vitro*
Researcher (s): Felicity Jane Burt
Institution: University of the Free State
Our ref Number: 12/11/1/4

Kind regards

DR MPHO MAJA
DIRECTOR OF ANIMAL HEALTH

Date: 2018 -09- 17

Appendix C: Geographical distribution of CCHFV

Afghanistan (Mustafa et al., 2011)	Mali (Zivcec et al., 2014)
Albania (Papa et al., 2002a)	Macedonia (Mertens et al., 2015)
Armenia (Matevosyan et al., 1974)	Mauritania (Saluzzo et al., 1985)
Azerbaijan (Shafei et al., 2016)	Montenegro (Gligić et al., 1977)
Benin (Hoogstraal, 1979)	Morocco (Palomar et al., 2013)
Bosnia and Herzegovina (Gligić et al., 1977)	Mozambique (Muianga et al., 2017)
Bulgaria (Donchev et al., 1965)	Namibia (Swanepoel et al., 1989)
Burkina Faso (Saluzzo et al., 1984)	Niger (Mariner et al., 1995)
Cameroon (Gonzalez et al., 1989; Sadeuh-Mba et al., 2018)	Nigeria (Causey et al., 1970)
Central African Republic (Gonzalez et al., 1983)	Oman (Schwarz et al., 1995)
China (Papa et al., 2002b)	Pakistan (Burney et al., 1980)
Congo (Gonzalez et al., 1989)	Portugal (Filipe et al., 1985)
Democratic Republic of the Congo (Swanepoel et al., 1989)	Republic of Moldova (Aristova et al., 2001)
Egypt (Darwish et al., 1978)	Romania (Ceianu et al., 2012)
Equatorial Guinea (Gonzalez et al., 1989)	Saudi Arabia (El-Azazy and Scrimgeour, 1997; Hassanein et al., 1997)
Ethiopia (Wood et al., 1978)	Senegal (Nabeth et al., 2004)
Georgia (Zakhashvili et al., 2010)	Serbia (Gligić et al., 1977)
Ghana (Akuffo et al., 2016)	South Africa (Gear et al., 1982)
Greece (Papa et al., 2008)	South Sudan (Suliman et al., 2017)
Guinea (Butenko, 1996)	Spain (Negredo et al., 2017)
Hungary (Horváth, 1976)	Sudan (Aradaib et al., 2011)
India (Mishra et al., 2011; Patel et al., 2011)	Tajikistan (Seregin et al., 2004)
Iran (Alavi-Naini et al., 2006)	Tanzania (Swanepoel et al., 1989)
Iraq (Tantawi et al., 1980; Tikriti et al., 1981)	Tunisia (Wasfi et al., 2016)
Kazakhstan (Yashina et al., 2003)	Turkey (Karti et al., 2004)
Kenya (Dunster et al., 2002)	Turkmenistan (Kuhn et al., 2004)
Kosovo (Drosten et al., 2002)	Uganda (Simpson et al., 1967)
Kuwait (Al-Nakib et al., 1984)	Ukraine (Kovalenko et al., 2006)
Kyrgyzstan (Risaliev, 1979)	United Arab Emirates (Suleiman et al., 1980)
Macedonia (Mertens et al., 2015)	Uzbekistan (Yashina et al., 2003)
Madagascar (Mathiot et al., 1988)	Zimbabwe (Blackburn et al., 1982)

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Appendix D: Amino acid sequence alignments for the CCHFV NS_M proteins

Amino acid sequence alignment of the CCHV NS_M protein representing CCHFV isolates from all lineages

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CLUSTAL O(1.2.4) multiple sequence alignment


ABB30025.1        RKLLQVSESTGVALKRSSWMIVLLILLIVSMSPVQSAPISQERTVALYQSREDYMGICLV   60
APG38067.1        RRLLQVSESTGMALKRSCWMTTLLILLVVSLSPVQSAPVGQEKAIEVYQVRESYTSMCLF   60
AAW84282.1        RRLLQVSESTGMALKRSCWMTTLLILLVVSLSPVQSAPVGQGKAVEVYRVRESYTSICLF   60
ARB51467.1        RRLLQVSESTGMALKRSCWMTTLLILLVVSLSPVQSAPVGQGKAVEVYRVRESYTSICLF   60
ACM78471.1        RKLLQVSESTGMALKRSCWMITLLILLLVSISPVQSAPVGKERAIETYQARETYTGICLF   60
ABB30027.1        RKLLQVSESTGVALKRSCWIITLLILLTVSMSPVQSAPVGHKRAVEVYQIRESYTGICLF   60
ABB30026.1        RKLLQVSESTGVALKRSCWMITLLILLTVSMSPVQSAPVGHKRAVEVYQMRESYTGICLF   60
ARB51446.1        RKLLQVSESTGVALKRSCWMITLLILLTVSMSPVQSAPVGHKRAVEVYQMRESYTGICLF   60
ARB51458.1        RKLLQVSESTGVALKRSSWLLILVILLTVSMSPVQSAPINQQRAVEAYQAREGYTGICLF   60
ABB30028.1        RKLLQVSESTGVALKRSSWLVTLLVLLTVSLSPVQSAPIGQERTVVTYQVRESYTGMCLF   60
ADV31331.1        RKLLQVSESTGVVLKRSSWLIVLLVLLTVSLSPVQSAPVGHGKTIETYQTREGFTSICLF   60
ABB30030.1        RKLLQVSESTGVALKRSSWLIVLLVLLTVSLSPVQSAPVGHGKTIETYQTREGFTSICLF   60
APG38058.1        RKLLQVSESTGVALKRSSWLIVLLVLLTVSLSPVQSAPVGHGKTIETYQTREGFTSICLF   60
ALM24059.1        RKLLQVSESTGVALKRSSWLIVLLVLLPVSLSPVQSAPVGHGKTIETYQTREGFTSICLF   60
ACT88367.1        RKLLQVSESTGVALKRSSWLIVLLVLLTVSLSPVQSAPVGHGKTIEIYQTREGFASICLF   60
AOT86852.1        RKLLQVSESTGVALKRSSWLIVLLVLLTVSLSPVQSAPVGHGKTIETYQTREGFTSICLF   60
ABB72473.1        RKLLQVSESTGVALKRSSWLIVLLVLLTVSLSPVQSAPVGHGKTIETYQTREGFTSICLF   60
AAO62015.1        RKLLQVSESTGVALKRSSWLIVLLVLLTVSLSPVQSAPVGHGKTIETYQTREGFTSICLF   60
ABW04159.1        RKLLQVSESTGVALKRSSWLIVLLVLLTVSLSPVQSAPVGHGKTIETYQTREGFTSICLF   60
AWX63617.1        RKLLQVSESTGVALKRSSWLIVLLVLLTVSLSPVQSAPVGHGKTIETYQTREGFTSICLF   60
ARB18229.1        RKLLQVSESTGVALKRSSWLIVLLVLLTVSLSPVQSAPIGHGKTIETYQTREGYTSICLF   60
AIE16132.1        RKLLQVSESTGVALKRSSWLVVLLVLLTVSLSPVQSAPIGHGKTVEVYQTREGYTSICLF   60
NSm45.88          RKLLQVSESTGVALKRSSWLVVLLVLLTVSLSPVQSAPIGHGKTVEVYQTREGYTSICLF   60
AAM48107.1        RKLLQVSESTGVALKRSSWLVVLLVLLTVSLSPVQSAPIGHGKTVETYQIREGYTSVCLF   60
BAB84578.1        RKLLQVSESTGVALKRSSWLVVLLVLLTVSLSPVQSAPIGHGKTVETYQTREGYTSICLF   60
AAW84284.1        RKLLQVSESTGVALKRSSWLVVLLVLLTVSLSPVQSAPIGHGKTVETYQTREGYTSICLF   60
ABB30029.1        RKLLQVSESTGVALKRSSWLVVLLVLLTVSLSPVQSAPIGHGKTVETYQTREGYTSICLF   60
AAW84283.1        RKLLQVSESTGVALKRSSWLIVLLVLFAVSLSPVQSAPIGHGKTIETYQVREEYTSICLF   60
AHL45281.1        RKLLQVSESTGTVLKRSSWLIVLLVLFTVSLSPVQSAPIGHGKTIETYRVREEYTSICLF   60
ADQ57289.1        RKLLQVSESTGTVLKRSSWLIVLLVLFTVSLSPVQSAPIGHGKTIETYRVREEYTSICLF   60
ASW20660.1        RKLLQVSESTGTVLKRSSWLIVLLVLFTVSLSPVQSAPIGHGKTIETYRVREEYTSICLF   60
APG38028.1        RKLLQVSESTGVALKRSSWLIVLLVLLTVSLSPVQSAPIGHGKTIETYQTREGYTSICLF   60
SPU103/87         RRLLQVSESTGVALKRSSWLIVLLVLFTVSLSPVQSAPISHGKTVEAYQAREGYTGMCLF   60
AEO72051.1        RKLLQVSESTGIALKRSSWLIVLLVLFTVSITPVQSAPIGHGKTIEAYRAREEYTSICLF   60
ASV45881.1        RKLLQVSESTGIALKRSSWLIVLLVLFTVSLSPVQSAPIGHGKTIEAYRAREGYTSICLF   60
ATG31911.1        RKLLQVSESTGVTLKRSSWLIVLLVLFTVSLSPVQSAPIGQGKTIEAYRAREGYTSICLF   60
AIE16137.1        RKLLQVSESTGVALKRSSWLIVLLVLFTVSLSPVQSAPIGHGKTIEAYRAREGYTSICLF   60
NSm187.90         RKLLQVSESTGVALKRSSWLIVLLVLFTVSLSPVQSAPIGHGKTIEAYRAREGYTSICLF   60
ARB51465.1        RKLLQVSESTGVALKRSSWLIVLLVLFTVSLSPVQSAPIGHGKTIEAYRAREGYTSICLF   60
NP_950235.1       RKLLQVSESTGVALKRSSWLIVLLVLFTVSLSPVQSAPIGQGKTIEAYRAREGYTSICLF   60
AAM48106.1        RKLLQVSESTGVALKRSSWLIVLLVLFTVSLSPVQSAPIGQGKTIEAYRAREGYTSICLF   60
ARB51455.1        RKLLQVSESTGVALKRSSWLIVLLVLFTVSLSPVQSAPIGQGKTIEAYRAREGYTSICLF   60
AWX63620.1        RKLLQVSESTGVALKRSSWLIVLLVLFTVSLSPVQSAPIGQGKTIEAYRAREGYTSICLF   60
APG38034.1        RKLLQVSESTGVALKRSSWLIVLLVLFTVSLSPVQSAPIGQGKTTEAYRAREGYTSICLF   60
AEI70589.1        RKLLQVSESTGVALKRSSWLIVLLVLFTVSLSPVQSAPIGQGKTIEAYRAREGYTSICLF   60
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ABB30025.1        IMGSVLLAVSLLVRGLIDSISSTFFPGLSVCRTCHIGSINGFEIESHKCYCGLLCCPYCR   120
APG38067.1        VLGSVLFAVSWLVKGLIDGIGNSFFPGLSVCKTCSIGSINGFEIESHKCYCSLFCCPYCR   120
AAW84282.1        VLGSVLFAVSWLIKGLIDGIGNSFFPGLSVCKTCSIGSINGFEIESHKCYCSLFCCPYCR   120
ARB51467.1        VLGSVLFAVSWLIKGLIDGIGNSFFPGLSVCKTCSIGSINGFEIESHKCYCSLFCCPYCR   120
ACM78471.1        VLGSVLFAVSWLTKALIDGIGNSFFPGLSVCKTCSIGSINGFEIESHKCYCSLFCCPYCR   120
ABB30027.1        VLGGVLFAVSWLVKALIDSIGNSFFPGLSICKTCSIGSINGFEIESHKCYCSLLCCPYCR   120
ABB30026.1        VLGSVLFAVSWLVKALIDSIGNSFFPGLSICKTCSIGSINGFEIESHKCYCSLFCCPYCR   120
ARB51446.1        VLGSVLFAVSWLVKALIDSIGNSFFPGLSICKTCSIGSINGFEIESHKCYCSLFCCPYCR   120
ARB51458.1        ILGSVLFMTSWLTKGLIDSIGDSFFPGLFVCKTCSIGSINGFEIESHRCYCSLLCCPYCR   120
ABB30028.1        ILGSVLFAISWLTKGLVDSVGNIFFPGLSVCKTCSIGSINGFEIESHKCYCSLFCCPYCR   120
ADV31331.1        MLGSILFIVSCIVKGLVDSVSDSFFPGLSVCKTCSISSINGFEIESHKCYCSLFCCPYCR   120
ABB30030.1        MLGSILFIVSCLVKGLVDSVSDSFFPGLSVCKTCSIGSINGFEIESHKCYCSLFCCPYCR   120
APG38058.1        MLGSILFIVSCLVKGLVDSVSDSFFPGLSVCKTCSIGSINGFEIESHKCYCSLFCCPYCR   120
ALM24059.1        MLGSILFIVSCLVKGLVDSVGDSFFPGLSVCKTCSIGSINGFEIESHKCYCSLFCCPYCR   120
ACT88367.1        MLGSILFIVSCLVKGLVDSVSESFFPGLSVCKTCSIGSVNGFEIESHKCYCSLFCCPYCR   120
AOT86852.1        MLGSILFIVSCLVKGLVESVSDSFFPGLSVCRTCSIGSINGFEIESHKCYCSLFCCPYCR   120
ABB72473.1        MLGSILFLVSCLVKGLVDSVSDSFFPGLSVCKTCSIGSINGFEIESHKCYCSLFCCPYCR   120
AAO62015.1        MLGSILFLVSCLVKGLVDSVSDSFFPGLSVCKTCSIGSINGFEIESHKCYCSLFCCPYCR   120
ABW04159.1        MLGSILFIVSCLVKGLVDSVSDSFFPGLSVCKTCSIGSINGFEIESHKCYCSLFCCPYCR   120
AWX63617.1        MLGSILFIVSCLVKGLVDSVSDSFFPGLSVCKTCSIGSINXFEIESHKCYCSLFCCPYCR   120
ARB18229.1        VLGSILFIVSHLMKGLVDSVGNSFFPGLSICKTCSIGSINGFEIESHKCYCSLFCCPYCR   120
AIE16132.1        VLGSILFVVSCLMRGLVDSVGNSFSPGLSICKTCSIGSINGFEIESHKCYCSLFCCPYCR   120
NSm45.88          VLGSILFVVSCLMRGLVDSVGNSFSPGLSICKTCSIGSINGFEIESHKCYCSLFCCPYCR   120
AAM48107.1        VLGSILFIVSCLMKGLVDSDGNSFFPGLSICKTCSIGSINGFEIESHKCYCSLFCCPYCR   120
BAB84578.1        VLGSILFIVSCLMKGLVDSVGNSFFPGLSICKTCSIGSVNGFEIESHKCYCSLFCCPYCR   120
AAW84284.1        VLGSILFIVSCLMKGLVDSVGNSFFPGSSICKTCSIGSINGFEIESHKCYCSLFCCPYCR   120
ABB30029.1        VLGSILFIVSCLMKGLVDSVGNSFFPGLSICKTCSIGSINGFETESHKCYCSLFCCPYCR   120
AAW84283.1        VLGSILFIVSCLTKGLVNSVGNIFFPGLSICKTCSIGSINGFEIESHKCYCSLLCCPYCR   120
AHL45281.1        VLGSILFIVSFLMKGLVDGVGNIFFPGLSVCKTCSIGSINGFEIESHRCYCSLFCCPYCR   120
ADQ57289.1        VLGSILFIVSFLMKGLVDGVGNIFFPGLSVCKTCSIGSINGFEIESHKCYCSLFCCPYCR   120
ASW20660.1        VLGSILFIVSFLMKGLVDGVGNIFFPGLSVCKTCSIGSINGFEIESHKCYCSLFCCPYCR   120
APG38028.1        VLGGILFIVSCLMKGLVDSVGNNFFPGLTICKTCSISSINGFEIESHKCYCSLLCCPYCR   120
SPU103/87         VLGSILFLVSCLMKGLVDSVGNTFFPGLSICKTCSISSINGFEIESHRCYCSLFCCPYCR   120
AEO72051.1        VLGSVLFVVSCLMKGLVDSVGNIFFPGLFICKTCSIGSINGFEIESHKCYCSLFCCPYCR   120
ASV45881.1        VLGSILFIVSRLMKGMVDSVGNSFFPGLSICKTCSTSSINGFEIESHKCYCSLFCCPYCR   120
ATG31911.1        VLGSILFIVSCLMKGLVDSVGNSFFPGLFICKTCSISSINGFEIESHKCYCSLFCCPYCR   120
AIE16137.1        VLGSILFVVSCLMKGLVDSVGNSFFPGLSICKTCSIGSINGFEIESHKCYCSLFCCPYCR   120
NSm187.90         VLGSILFVVSCLMKGLVDSVGNSFFPGLSICKTCSIGSINGFEIESHKCYCSLFCCPYCR   120
ARB51465.1        VLGSILFIVSCLMKGLVDSVGNSFFPGLSICKTCSISSINGFEIESHKCYCSLFCCPYCR   120
NP_950235.1       VLGSILFIVSCLMKGLVDSVGNSFFPGLSICKTCSISSINGFEIESHKCYCSLFCCPYCR   120
AAM48106.1        VLGSILFIVSCLMKGLVDSVGNSFFPGLSICKTCSISSINGFEIESHKCYCSLFCCPYCR   120
ARB51455.1        VLGSILFIVSCLMKGLVDSVGNSFFPGLSICKTCSISSINGFEIESHKCYCSLFCCPYCR   120
AWX63620.1        VLGSILFIVSCLMKGLVDSVGNSFFPGLSICKTCSISSINGFEIESHKCYCSLFCCPYCR   120
APG38034.1        VLGSILFIVSCLMKGLVDSVGNSFFPGLSICKTCSISSINGFEIESHKCYCSLFCCPYCR   120
AEI70589.1        VLGSILFIVSCLMKGLVDSVGNSFFPGLSICKTCSISSINGFEIESHKCYCSLFCCPYCR   120
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ABB30025.1        ACSTDQDAHQFHLSVCKKRKAGSNVMLAVCKRMCFKAIIEASNKVLFIRNVINTTFVVCI   180
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AAW84282.1        ACSSDKITHRMHLNVCKKRKAGSNVMLAVCKRMCFKATVEASNTALLIRGIINSTFVICV   180
ARB51467.1        ACSSDKITHRMHLNVCKKRKAGSNVMLAVCKRMCFKATVEASNTALLIRGIINSTFVICV   180
ACM78471.1        ACSSDKNTHRMHLNVCKKRKMGSNVMLAVCKRMCFRATIEASNKALLIRSIINSTFVICI   180
ABB30027.1        ACSSDKITHRMHLNVCKKRKVGSNVMLAVCKRMCFKATIEANNRATFIRNIINSTFVICI   180
ABB30026.1        ACSSDKITHRMHLNVCKKRKVGSNVMLAVCKRMCFKATIEASNRATFIRNIINSTFVICI   180
ARB51446.1        ACSSDKITHRMHLNVCKKRKVGSNVMLAVCKRMCFKATIEASNRATFIRNIINSTFVICI   180
ARB51458.1        TCSADKDSHQLHLSICKKKKAGSNVMLAVCKRMCFKATIEASNRVLLIRSIINTTFIICM   180
ABB30028.1        YCSADRGAHQSHLSVCKKRKTGSNVMLAVCKRMCFRATLKVSSEALIIRSIINTTFVVCI   180
ADV31331.1        HCSADREIHQLHLSICRKRRTGSNVMLAVCKRMCFRATIEVSRRALLIRSIINTTFVICI   180
ABB30030.1        HYSADREIHQLHLSICKKRKTGSNVMLAVCKRMCFKATIEASRRALLIRSIINTTFVICI   180
APG38058.1        HCSADREIHQLHLSICKKRKTGSNVMLAVCKRMCFKATIEASRRALLIRSIINTTFVMCI   180
ALM24059.1        HCSADREIHQLHLSICKKRKTGSNVMLAVCKRMCFRATIEASRRALLIRSIINTTFAMCI   180
ACT88367.1        HCSADREIHQLHLSICKKRKTGSNVMLAVCKRMCFRATIEASRRALLIRSIINTTFVICI   180
AOT86852.1        HCSADREIHQLHLSICKKRKTGSNVMLAVCKRMCFRATMEASRRALLIRSIINTTFVICI   180
ABB72473.1        HCSADREIHQLHLSICKKRKTGSNVMLAVCKRMCFRATIEASRRALLIRSIINTTFVICI   180
AAO62015.1        HCSADREIHQLHLSICKKRKMGSNVMLAVCKRMCFRATIEASRRALLIRSIINTTFVICI   180
ABW04159.1        HCSADREIHQLHLNICKKRKTGSNVMLAVCKRMCFRATIEASRRALLIRSIINTTFVICI   180
AWX63617.1        HCSADREIHQLHLNICKKRKTGSNVMLAVCKRMCFRATIEASRRALLIRSIINTTFVICI   180
ARB18229.1        HCSADKEIHKLHLSICKKRKAGSNVMLAVCKRMCFRATMEVSNKALLIRSVINTTFVVCI   180
AIE16132.1        HCSADIEIHKLHLNICKKRKTGSNVMLAVCKRMCFRATMEVSSKALLIRNIINTTFVVCI   180
NSm45.88          HCSADIEIHKLHLNICKKRKTGSNVMLAVCKRMCFRATMEVSSKALLIRNIINTTFVVCI   180
AAM48107.1        HCSTDREIHKLHLSICKKRKTGSNVMLAVCKRMCFRATVEVSNKALLIRSIINTTFVVCI   180
BAB84578.1        HCSADREIHRLHLSICKKRKTWSNVMLAVCKRMCFRATVEVSNKALLIRNIINTTFVLCI   180
AAW84284.1        HCSADREIHKLHLSICKKRKTGSNVMLAVCKRMCFRATVEVSNKALLIRNIINTTFVVCI   180
ABB30029.1        HCSADREIHKLHLSICKKRKTGSNVMLAVCKRMCFRATVEVSNKALLIRNIINTTFVVCI   180
AAW84283.1        HCSADREIHQLHLSICKKRKTGSNVMLAVCKRMCFRATMEVSNKALFIHSIINTTFVVCI   180
AHL45281.1        HCSADGEIHQLHLSICKKRKTGSNVMLAVCKRMCFRATMEVSNKVLFIRSIINTTFVVCI   180
ADQ57289.1        HCSADGEIHQLHLSICKKRKTGSNVMLAVCKRMCFRATMEVSNKALFIRSIINTTFVVCI   180
ASW20660.1        HCSADGEIHQLHLSICKKRKTGSNVMLAVCKRMCFRATMEVSNKALFIRSIINTTFVVCI   180
APG38028.1        HCSADREIHQLHLSICKKRKTGSNVMLAVCKRMCLRVTTEVSNRALFIRSIINTTFVICI   180
SPU103/87         HCSANKEIHQLHLSICKKRKTGSNVMLAVCKRMCFRATMEVSNRALFIRSIINTTFVLCI   180
AEO72051.1        HCSADREIHQLHLSICKKRKTGSNVMLAVCKRMCFRTTMEVSNKALFVRSIINTTFVVCI   180
ASV45881.1        HCSTDKEIHKLHLSICKKRKTGSNVMLAVCKRMCFRATMEVSNKALFIRSIINTTFVLCI   180
ATG31911.1        HCSADKEIHKLHLSICKKRKTGSNVMLAVCKRMCFRATMEVSNRALFIRSIINTTFVLCI   180
AIE16137.1        HCSADKEIHKLHLSICKKRKTGSNVMLAVCKRMCFRATMEVSNRALFIRSIINTTFVLCI   180
NSm187.90         HCSADKEIHKLHLSICKKRKTGSNVMLAVCKRMCFRATMEVSNRALFIRSIINTTFVLCI   180
ARB51465.1        HCSADKEIHKLHLSICKKRKTGSNVMLAVCKRMCFRATMEVSNRALFIRSIINTTFVLCI   180
NP_950235.1       HCSTDKEIHKLHLSICKKRKKGSNVMLAVCKLMCFRATMEVSNRALFIRSIINTTFVLCI   180
AAM48106.1        HCSTDKEIHKLHLSICKKRKKGSNVMLAVCKLMCFRATMEVSNRALFIRSIINTTFVLCI   180
ARB51455.1        HCSTDKEIHKLHLSICKKRKTGSNVMLAVCKLMCFRATMEVSNRALFIRSIINTTFVLCI   180
AWX63620.1        HCSTDKEIHKLHLSICKKRKXGSNVMLAVCKLMCFRATMEVSNRALFIRSIINTTFVLCI   180
APG38034.1        HCSTDKEIHKLHLSICKKRKTGSNVMLAVCKRMCFRATMEVSNRALFIRSIINTTFVLCI   180
AEI70589.1        HCSTDKEIHKLHLSICKKRKTGSNVMLAVCKRMCFRATMEVSNRALFIRSIINTTFVLCI   180
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ABB30025.1        LVMVACIASSAAVEMENLPAGTWEREEELTNFCHQECQVTETECLCPYESFILRKPL      237
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AAW84282.1        LILVICVVSTSAVDMENLPAGIWEREEDLTNFCHQECQVTETECLCPYEALILRKPL      237
ARB51467.1        LILVICVVSTSAVDMENLPAGIWEREEDLTNFCHQECQVTETECLCPYEALILRKPL      237
ACM78471.1        LILTICVVSTSAVDMENLPAGIWEKEEDLTNFCHQECQVTETECLCPYEAMVLRKPL      237
ABB30027.1        LILVICVVSTSAVDMENLPAGIWEKEEDLTNFCHQECQVTETECLCPYEALMLRKPL      237
ABB30026.1        LILVICVVSTSAVDMENLPAGIWEKEEDLTNFCHQECQVTETECLCPYEALMLRKPL      237
ARB51446.1        LILVICVVSTSAVDMENLPAGIWEKEEDLTNFCHQECQVTETECLCPYEALMLRKPL      237
ARB51458.1        LILVVCVISTSAVEMEDLPAGTWEREEDLTNFCHQECQVTETECLCPYEAFVLRKPL      237
ABB30028.1        LILVVCVVSTSAVEMENLPAGTWEKEEDLTNFCHQECQVTETECLCPYEAFVLRKPL      237
ADV31331.1        LTLTICVVSTSAVEMENLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRKPL      237
ABB30030.1        LILAICVVSTSAVEMENLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRKPL      237
APG38058.1        LTLAICVVSTSAVEMEDLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRKPL      237
ALM24059.1        LTLTICVVSTSAVEMENLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRKPL      237
ACT88367.1        LTLTICVVSTSAVEMENLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRKPL      237
AOT86852.1        LTLTICVVSTSAVEMENLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRKPL      237
ABB72473.1        LTLTICVVSTSAVEMENLPSGTWEREEDLTNFCHQECQVTETECLCPYEALVLRKPL      237
AAO62015.1        LTLTICVVSTSAVEMENLPSGTWEREEDLTNFCHQECQVTETECLCPYEALVLRKPL      237
ABW04159.1        LTLTICVVSTSAVEMENLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRKPL      237
AWX63617.1        LTLTICVVSTSAVEMENLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRKPL      237
ARB18229.1        LILAVCVVSTSAVEMESLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRKPL      237
AIE16132.1        LILAVCVVSTSAVEMESLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRKPL      237
NSm45.88          LILAVCVVSTSAVEMESLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRKPL      237
AAM48107.1        LILAVCVVSTSAVEMESLPAGTWEREEDLINFCHQECQVTETECLCPYEALVLRKPL      237
BAB84578.1        LILAVCVVSTSAVEMESLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRKPL      237
AAW84284.1        LILAVCVVSTSAVEMESLPAGTWEREEDLTNFCHQECQATETECLCPYEALVLRKPL      237
ABB30029.1        LILAVCVVSTSAVEMESLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRKPL      237
AAW84283.1        LILAVCVVSTSAVEMESLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRRPL      237
AHL45281.1        LILAVCVVSTSAVEMESLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRRPL      237
ADQ57289.1        LILAVCVVSTSAVEMESLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRRPL      237
ASW20660.1        LILAVCVVSTSAVEMESLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRRPL      237
APG38028.1        LILAVCVISTSAVEMENLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRKPL      237
SPU103/87         LILAVCVVSTSAVEMESLPAGTWEREEDLTNFCHQECQVTDTECLCPYEALVLRRPL      237
AEO72051.1        LILAVCVVSTSAVEMESLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRRPL      237
ASV45881.1        LILAVCVVSTSAVEMENLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRKPL      237
ATG31911.1        LILAVCVVSTSAVEMENLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRKPL      237
AIE16137.1        LILAVCVVSTSAVEMENLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRRPL      237
NSm187.90         LILAVCVVSTSAVEMENLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRRPL      237
ARB51465.1        LILAVCVVSTSAVEMENLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRKPL      237
NP_950235.1       LILAVCVVSTSAVEMENLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRKPL      237
AAM48106.1        LILAVCVVSTSAVEMENLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRKPL      237
ARB51455.1        LILAVCVVSTSAVEMENLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRKPL      237
AWX63620.1        LILAVCVVSTSAVEMENLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRKPL      237
APG38034.1        LILAVCVVSTSAVEMENLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRKPL      237
AEI70589.1        LILAVCVVSTSAVEMENLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRKPL      237
                  * :. *: *::**:**.**:* **:**:* ********.*:********:::**:**
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113/237 = 47,67% amino acid sequence similarity

Amino acid sequence alignment of the CCHV NS_M 187/90 (non-reassortant) and CCHFV NS_M 45/88 (reassortant) proteins

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CLUSTAL O(1.2.4) multiple sequence alignment


NSm187.90       RKLLQVSESTGVALKRSSWLIVLLVLFTVSLSPVQSAPIGHGKTIEAYRAREGYTSICLF 60
NSm45.88        RKLLQVSESTGVALKRSSWLVVLLVLLTVSLSPVQSAPIGHGKTVEVYQTREGYTSICLF 60
                ********************:******:*****************:*.*::********** 

NSm187.90       VLGSILFVVSCLMKGLVDSVGNSFFPGLSICKTCSIGSINGFEIESHKCYCSLFCCPYCR 120
NSm45.88        VLGSILFVVSCLMRGLVDSVGNSFSPGLSICKTCSIGSINGFEIESHKCYCSLFCCPYCR 120
                *************:********** ********************************** 

NSm187.90       HCSADKEIHKLHLSICKKRKTGSNVMLAVCKRMCFRATMEVSNRALFIRSIINTTFVLCI 180
NSm45.88        HCSADIEIHKLHLNICKKRKTGSNVMLAVCKRMCFRATMEVSSKALLIRNIINTTFVVCI 180
                ***** *******.****************************.:**:**.*******:** 

NSm187.90       LILAVCVVSTSAVEMENLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRRPL    237
NSm45.88        LILAVCVVSTSAVEMESLPAGTWEREEDLTNFCHQECQVTETECLCPYEALVLRKPL    237
                ****************.*************************************:** 
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220/237 = 92,8% amino acid sequence similarity