Vaccine and assay development for Nairoviral Diseases

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Abstract

Emerging zoonotic diseases present a significant risk to global public health, as evidenced by the ongoing COVID-19 pandemic. Nairoviruses are a genus of viruses, which include Crimean Congo Haemorrhagic Fever Virus (CCHFV) and Nairobi Sheep Disease Virus (NSDV), and present a significant risk of epidemics in humans and animals. As such there is an urgent need to develop effective therapeutics and prophylactics to protect humans and livestock from nairovirus mediated infection and disease. Vaccines are a highly effective healthcare intervention, and ChAdOx vaccine platforms have been used extensively to develop vaccines against emerging diseases, including the ChAdOx1-nCoV19/AZD1222 vaccine. This thesis aims to characterise the development of ChAdOx vectored vaccines against a range of nairoviral antigens, and determine if nairoviral vaccines in ChAdOx vectors are immunogenic following vaccination in mice. As part of this thesis, new pseudotyping assays using CCHFV and NSDV glycoproteins were developed to test neutralising antibody responses induced following vaccination. This thesis demonstrated that CCHFV GPC (GlycoProtein Precursor), Gn, Gc, and NP (Nucleoprotein) antigens were all immunogenic, inducing cellular and humoral responses, and that ChAdOx vectored CCHFV GPC vaccination could induce neutralising antibodies. Further the immunogenicity of NSDV GPC, Gc, and NP antigens in ChAdOx1 vectors was demonstrated following vaccination. Development of multivalent ChAdOx1 CCHFV vaccines showed that a single vaccination could induce broad cellular and humoral immune responses. Anti-vector neutralisation responses induced by vaccination were also assayed in the ChAdOx1-nCoV19 vaccine clinical trial at the University of Oxford. The work in this thesis determined that no correlations were measured between anti-ChAdOx1 neutralisation titres and anti-SARS-CoV-2 spike immune responses following vaccination with ChAdOx1-nCoV19/AZD1222. Overall, this thesis highlights the development of immunogenic vaccines against the emerging pathogens CCHFV and NSDV, which will be crucial to managing future epidemics.

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Statement of Authorship

This thesis has been authored by Ciarán Gilbride and all work presented was performed primarily by Ciarán Gilbride unless otherwise stated. Design of CCHFV and NSDV vaccines was performed by Ciarán Gilbride, with input provided by Teresa Lambe, Sarah Gilbert and Dalan Bailey.

Designs of immunological assays, and mouse experiments were performed by Ciarán Gilbride. Design of molecular biology strategies and production of vaccines up to purification of ChAdOx bacterial artificial chromosomes were performed by Ciarán Gilbride. Mouse handling, processing of mouse blood and splenocytes, and IFN-γ ELISPOTs were performed primarily by Ciarán Gilbride, with manual assistance from Marta Ulaszewska, Hannah Sharpe, Jyothi Purushotham, Sandra Belij-Rammerstorfer, Elizabeth Allen, and Jack Saunders. ELISAs and CCHFV tec-VLP neutralisation assays were performed by Ciarán Gilbride with manual assistance from Jack Saunders. NSDV-VSV neutralisation assays were performed by Ciarán Gilbride. Clinical trials were designed by Teresa Lambe and Katie Ewer, and work conducted by the COVID-19 vaccine trial team. Anti-ChAdOx1 neutralisation were performed by Ciarán Gilbride, with assistance from Nick Edwards, Elizabeth Allen, Jack Saunders, Caitlin Blundell, and Susannah Blundell. All data analyses were performed by Ciarán Gilbride.

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List of abbreviations

ACDP – Advisory Committee on Dangerous Pathogens

ALP – Alkaline Phosphatase

AP – Alkaline Phosphatase

Ad – Adenovirus

BAC – Bacterial Artificial Chromosome

BCR – B-Cell Receptor

BGH – Bovine Growth Hormone

BoHV4 – Bovine Herpesvirus 4

BSL – Bio-Safety Level

CCHF – Crimean Congo Haemorrhagic Fever

CCHFV – Crimean Congo Haemorrhagic Fever Virus

CD – Cluster of Differentiation

ChAd – Chimpanzee Adenovirus

ChAdOx1 – Chimpanzee Adenovirus Oxford 1

ChAdOx2 – Chimpanzee Adenovirus Oxford 2

CL – Containment Level

CMV - Cytomegalovirus

CoV – Coronavirus

COVID-19 – Coronavirus Infectious Disease 2019

DMEM – Dulbecco's Minimum Essential Medium

DNA – Deoxyribonucleic acid

EBOV - Ebolavirus

ELISA – Enzyme Linked ImmunoSorbent Assay

ELISPOT – Enzyme Linked Immunospot

Gc – C-terminal surface Glycoprotein

GDP – Gross Domestic Product

GFP – Green Fluorescent Protein

GJMV – Ganjam Virus

GMP – Good Manufacturing Practice

Gn – N-terminal surface Glycoprotein

GP - Glycoprotein

GP38 – 38kdA Glycoprotein

GPC – Glycoprotein Precursor

HEK – Human Embryonic Kidney

IAV – Inactivated Vaccine

IFN – Interferon

IFN-γ – Interferon gamma

IgG – Immunoglobin G

IL – Interleukin

IQR – Interquartile Range

LAV – Live attenuated vaccine

LMIC – Low to Middle Income Countries

LNP – Lipid Nanoparticle

mAb – Monoclonal Antibody

MCL – Mucin like domain

MEME – Minimum Essential Eagle Medium

MERS – Middle East Respiratory Syndrome

MHC – Major Histocompatibility complex

mRNA – messenger Ribonucleic Acid

MVA – Modified Vaccinia Ankara

NHP – Non-human primate

NP - Nucleoprotein

NSD – Nairobi Sheep Disease

NSDV – Nairobi Sheep Disease Virus

NSm – Non-Structural Motif

ORF – Open Reading Frame

OTU – Ovarian Tumour De-ubiquitinase

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PEI – Polyethylimine

pNPP – para-Nitrophenyl Phosphate

PRF – Phenol Red Free

RdRP – RNA dependent RNA Polymerase

RLU - Relative Light Units

RNA – Ribonucleic acid

RT – Room Temperature

RT-PCR – Reverse Transcriptase Polymerase Chain Reaction

RVF – Rift Valley Fever

RVFV – Rift Valley Fever Virus

SAPO – Special Animal Pathogen Order

SARS – Severe Acute Respiratory Syndrome

SEAP – Secreted Alkaline Phosphatase

SFTS – Severe Fever with Thrombocytopenia Syndrome

SFTSV – Severe Fever with Thrombocytopenia Syndrome Virus

TAE – Tris-Acetate-EDTA

TCR – T-Cell Receptor

Tec-VLP – transcriptionally and entry competent Virus Like Particles

Th – T-helper

TNF – Tumour Necrosis Factor

UTR – Untranslated region

VLP – Virus-Like Particle

VNT – Virus Neutralisation Test

VSV – Vesicular Stomatitis Virus

VVV – Viral Vectored Vaccine

VZV – *Variacella Zoster* Virus

WHO – World Health Organisation

WT – Wild Type

Chapter 1 - Introduction

1.1 Emerging diseases

Emerging pathogens cause diseases of epidemic or pandemic potential which can periodically emerge into human or animal populations, causing outbreaks which can adversely affect human health or quality of life[1]. Emerging pathogens can be those which are recently identified within a population, or have previously been identified and either have emerged in a new geographical location, or have a rapidly increasing incidence in a previous or new location[1]. There are many causes for disease emergence, such as migration of infected animals or vectors into new geographical areas, changes in climate, or evolution of a pre-existing disease[2]. Emerging diseases which can sustain interhuman transmission can become established within the population causing pandemics[2]. The World Health Organisation (WHO) has established a list of priority emerging pathogens identified as being of epidemic or pandemic potential with no viable prophylactic or therapeutic measures[3]. The pathogens are presented in table 1.1, with their mammalian reservoirs, and their geographical ranges.

1.1.1 Risks of disease emergence

Zoonoses are a major source of emerging pathogens in human populations[4]. It is estimated that more than 60% of human pathogens originated in other vertebrate hosts, making zoonoses responsible for numerous epidemics and pandemics[5]. As demonstrated in table 1.1, all the WHO blueprint priority diseases have animal reservoirs where the pathogen circulates between spillover events. Figure 1.1 shows how zoonoses can be maintained within human or animal populations[6]. Each emerging pathogen will have different transmission dynamics, potentially being maintained by direct transmission between infected animals[7], or requiring an insect vector to spread between organisms[8]. Cycles of infection in vertebrate hosts maintain a reservoir of infectious virus which can

spillover into humans causing outbreaks[9]. Infections in humans can occur through direct contact with infected animals, or their tissues or bodily fluids, as shown during Ebola outbreaks[10] or in CCHFV transmission[11]. Outbreaks can also occur through insect vectors feeding on both the reservoir species and humans[8,12]. Infections of humans can spill over into other vertebrate species, such as with SARS-CoV-2, which has been identified in ferrets and white tailed deer, and infection is assumed to be from human to animal transmission, further increasing the complexity of trying to manage zoonotic transmission cycles[13,14].

For human health, the major risk occurs when pathogens can establish interhuman transmission after zoonotic spillover. With some outbreak pathogens such as avian influenza, interhuman transmission is negligible or non-existent, and outbreaks display high mortality but are controlled in their spread[15]. Epidemics, such as the 2014-2016 West African Ebola outbreak, occur when public health measures can bring interhuman transmission under control, and the outbreak can be slowed or stopped[16]. In the case of diseases such as COVID-19, interhuman transmission after the initial zoonotic spillover has been common and highly efficient[17], leading to a pandemic which has circumvented many public health measures designed to control interhuman infections, though public health measures remain essential for reducing the risks of infection and death during pandemics.

With an ever expanding scope to human activities, and the widespread ecological disruption caused by climate change, disruptive contact between humans and animals in their native habitats is increasing[18]. As such there are increasing opportunities for zoonotic transmission to occur, presenting global risks to public health.

WHO Blueprint disease	Mammalian reservoir(s)	Endemic geographical range
COVID-19	Fruit Bats (speculative)[19], pangolin (speculative)[20].	Worldwide[21]
Crimean Congo Haemorrhagic Fever (CCHF)	Cattle[22], goats[23], sheep[22], camels[24], horses[25], donkeys[26]	Eastern and Southern Europe[11], Middle East[27], Central Asia[28],China[29], Southern Asia[30], Africa[31]
Ebolavirus disease	Fruit bats[32]	Central and Western Africa[33]
Marburg Virus Disease	Fruit bats[34]	Central and Eastern Africa[35]
Lassa Fever	Multimammate mouse[36]	Western Africa[37]
MERS coronavirus disease	Bats, alpacas, camels[38]	Middle East and Northern Africa[39]
Nipah virus disease	Flying foxes[40]	Southern and South Eastern Asia[41]
Rift Valley Fever	Sheep, goats, cattle[42]	Eastern, North-Eastern, and Southern Africa[43] and Middle East[44]
SARS coronavirus disease	Horseshoe bats, palm civets[45]	China (speculative)[46]
Zika	Rhesus monkeys, sheep, goats, cows, horses, bats, carabaos, orangutans[47]	Central Africa, Southern Asia, Northen Oceania, Central and Southern America[48]
Disease X	Unknown	Unknown

Table 1.1: Table of the zoonotic WHO R&D blueprint diseases, their mammalian reservoir species, and the endemic geographical range of each species. Adapted from Gilbride et al 2021.

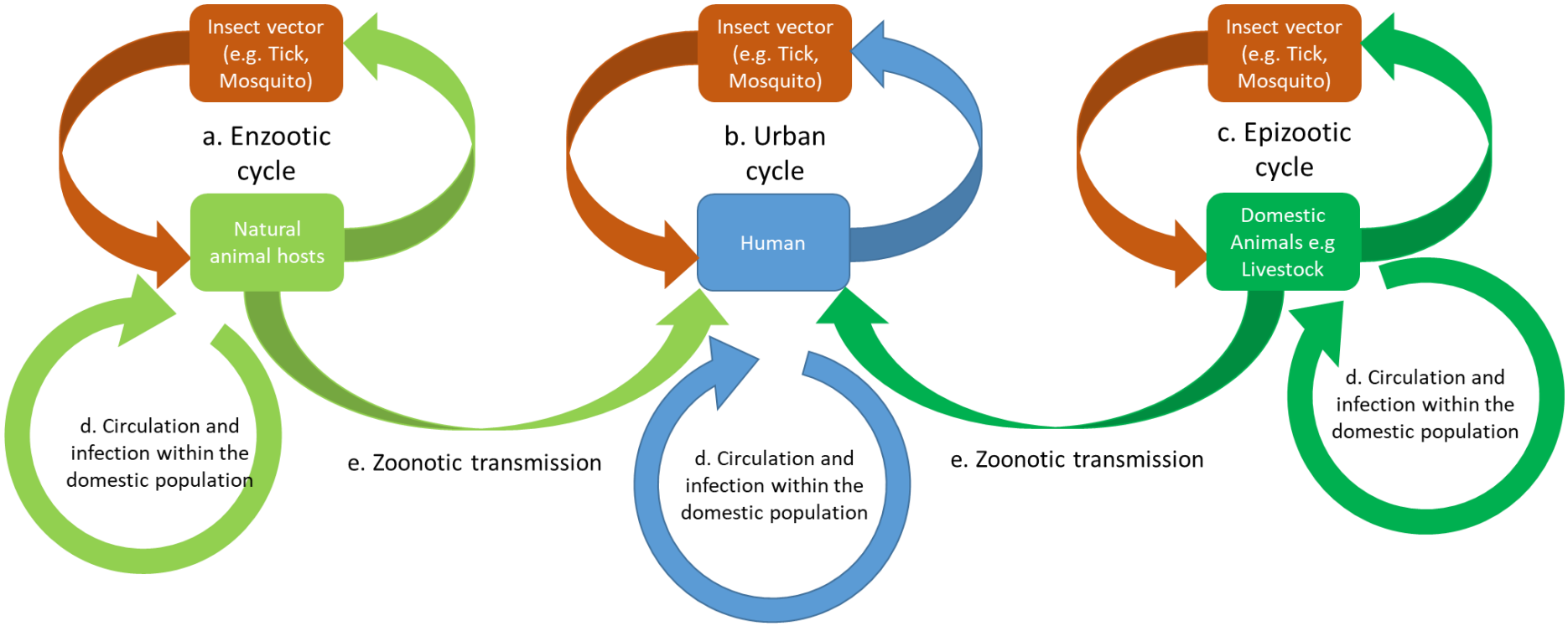


Figure 1.1: Cycles of infection for zoonotic transmission through insect vectors.
The figure shows cycles of infection for zoonoses with insect vectors and non-insect vector transmission. **A**) The enzootic cycle is a continuous cycle of pathogens transmitted in their natural hosts. Enzootic cycles generally occur in human uninhabited areas and are generally asymptomatic. **B**) The urban cycle occurs when diseases spillover into human populations and are maintained through insect vectors transmitting infections to uninfected humans. **C**) The epizootic cycle is a cycle of transmission in domestic animals and insect vectors. The epizootic cycle often includes transmission of disease into the domestic animal population and may cause animal disease. **D**) Pathogens may transmit between individuals within a population without the need for an insect vector. **E**) Zoonotic transmission occurs when a pathogen transmits from an animal host into a human host either through direct transmission or an insect vector. The zoonotic pathogen causes symptomatic disease in the human population.

1.1.2 Impacts of epidemics and pandemics

Zoonoses have been responsible for a rising number of epidemics and pandemics throughout the early 21st century. Epidemics are large outbreaks which can have high levels of mortality and devastate communities, but stay geographically contained. Throughout the first two decades of the 21st century zoonotic outbreaks caused epidemics including SARS-COV-1 in East Asia[49], Avian Influenza in China[50], MERS coronavirus in both the Arabian Peninsula[51] and Korea[52], and multiple Ebolavirus epidemics in Central[53] and West Africa[54].

The impacts of epidemics come from both loss of life and disruption of human activities in localised areas. The 2013-2016 West African Ebola Epidemic is estimated to have directly caused over 11,000 deaths[16] and the 2018 onwards epidemic in DRC was responsible for over 2,000 deaths[53]. The epidemics overwhelmed local healthcare systems, and the consequential breakdown of health infrastructure in Guinea, Sierra Leone, and Liberia is estimated to have caused similar numbers of death indirectly[55]. Epidemics can also cause significant economic burdens, whose impact extends far beyond those infected in the epidemic. The 2015 MERS outbreak is estimated to have reduced Korean GDP by 0.8%[56], while the 2003 SARS outbreak in Hong Kong is estimated to have cut 2.6% from Hong Kong's GDP[56]. The economic losses and disruption after epidemics continue to be felt for years after the end of outbreaks, and predominantly affect those worse off in society.

Pandemics are large outbreaks of disease which spread beyond a localised geographical area and affect a significant proportion of the population. Zoonoses have been responsible for the two most devastating pandemics in history, the bubonic plague[57] and the 1918 Influenza[58], along with more recent swine flu[59] and COVID-19 pandemics[60]. By definition the impact of pandemics are more widespread then epidemics and can even affect the climate, with the multiple pandemics caused by European settlers in the

Americas linked to drop in global temperature due to reforestation and carbon sequestration.[61]

The COVID-19 pandemic is ongoing at the time of this thesis, with the likely origin a zoonotic event from an unknown animal host, though a range of organisms have been speculated[19,20]. At present the pandemic has affected all seven continents and is officially confirmed to have caused over 5 million deaths[62], though estimates suggest poor recording of death in LMICs mean the actual death toll may be many times greater[63]. The COVID-19 pandemic has also devastated global commerce and freedom of movement, causing disruption to the global economy and reducing quality of life in a large number of communities, indirectly increasing morbidity and mortality[64]. Measures which can identify and control zoonoses can prevent the next outbreaks are therefore crucial to prevent epidemics or pandemics, and improve global health.

1.1.3 Bunyaviruses and Nairoviruses

Bunyavirales is an order of viruses containing pathogens of particular concern as emerging pathogens[65]. The taxonomy of *Bunyavirales*, regarding selected pathogens of interest can be seen in figure 1.2[66]. The order includes WHO blueprint diseases of concern CCHFV and RVFV[67], as well as further human outbreak pathogens such as Severe Fever with Thrombocytopenia Syndrome Virus (SFTSV)[68]. Bunyaviruses all share certain characteristics, as depicted in figure 1.3. All bunyaviruses are enveloped viruses with negative sense RNA genomes[65]. Their genetic material is tripartite, with small (S), medium (M) and large (L) segments[65]. The S segment expresses a nucleoprotein (NP) which binds the Bunyavirus RNA, the M segment expresses a glycoprotein precursor (GPC), and the L segment expresses an RNA dependent RNA polymerase (RdRP). The GPC is processed into a range of different peptide products in

different bunyaviruses, but all include transmembrane proteins termed Gn and Gc, which form a heterodimer on the surface of the Bunyavirus virion, and are responsible for viral tropism and cell entry[65].

Bunyaviruses are mostly arboviruses, meaning transmission mostly occurs through insect vectors[69]. Most bunyaviruses are spread by mosquito[70] or tick vectors[8], accounting for the wide geographic spread of bunyaviruses[69]. Climate change is altering the host range for a range of insect vectors, warming areas further from the equators, facilitating the spread of insect vectors which then become endemic[71], and extending the range into which they may be periodically introduced in summer months[72]. Expanding the geographical range of vectors increases the risk of Bunyavirus outbreaks in immunologically naïve populations of humans and animals.

1.1.3.1 Emerging diseases of concern among Bunyavirales

Bunyavirus outbreaks occur periodically throughout the world causing loss of life and livelihood. Beyond the WHO diseases of concern; RVFV, SFTSV, and CCHFV, further Bunyavirus families, such as Arenaviruses[73], Hantaviruses[74–76] and Nairoviruses, can cause disease in humans and should be closely monitored for their potential as emerging pathogens.

RVFV is mainly of concern in livestock, periodically emerging in East Africa, and is spread by infected mosquitos. Mortality rates are high in livestock, between 20-30% in adult animals, and approaching 100% in young animals[77], with rates of spontaneous abortion in infected mothers extremely high[77]. RVFV infections in humans mostly present as a self-limiting febrile illness[78], however in rare cases more severe haemorrhagic and neurological symptoms can present. For individuals presenting severe symptoms mortality rates are greater than 50%.[78]

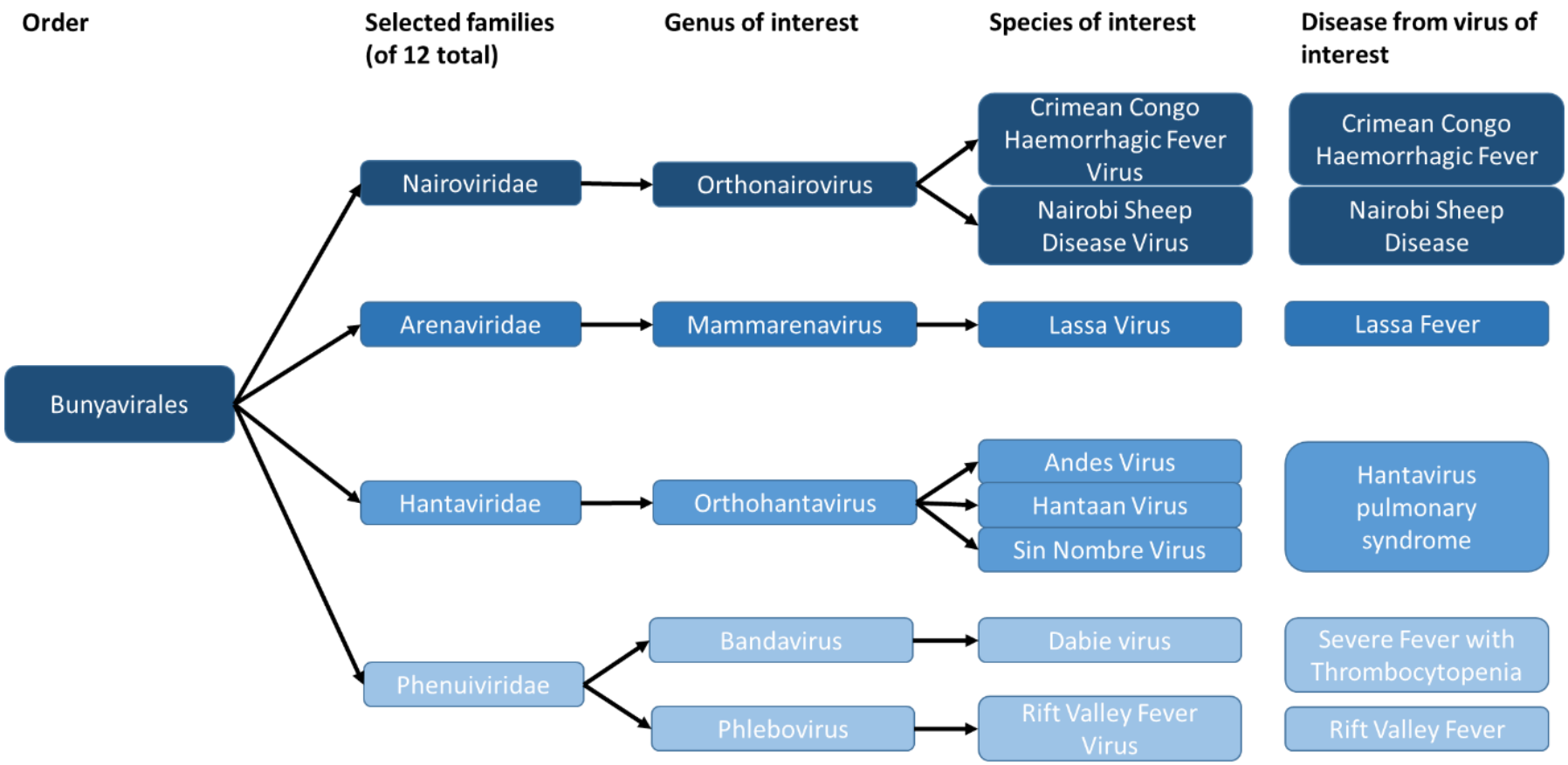


Figure 1.2: The taxonomy of the order *Bunyavirales*, with regards to specific disease of interest for veterinary and human health with the order
The figure shows the taxonomy of viruses causing selected disease of interest in veterinary and human health, specifically; Crimean-Congo Haemorrhagic Fever Virus, Nairobi Sheep Disease Virus, Lassa Virus, Andes Virus, Hantaan Virus, Sin Nombre Virus, Dabie Virus, Rift Valley Virus. Taxonomy from the International Committee on Taxonomy of Viruses[66].

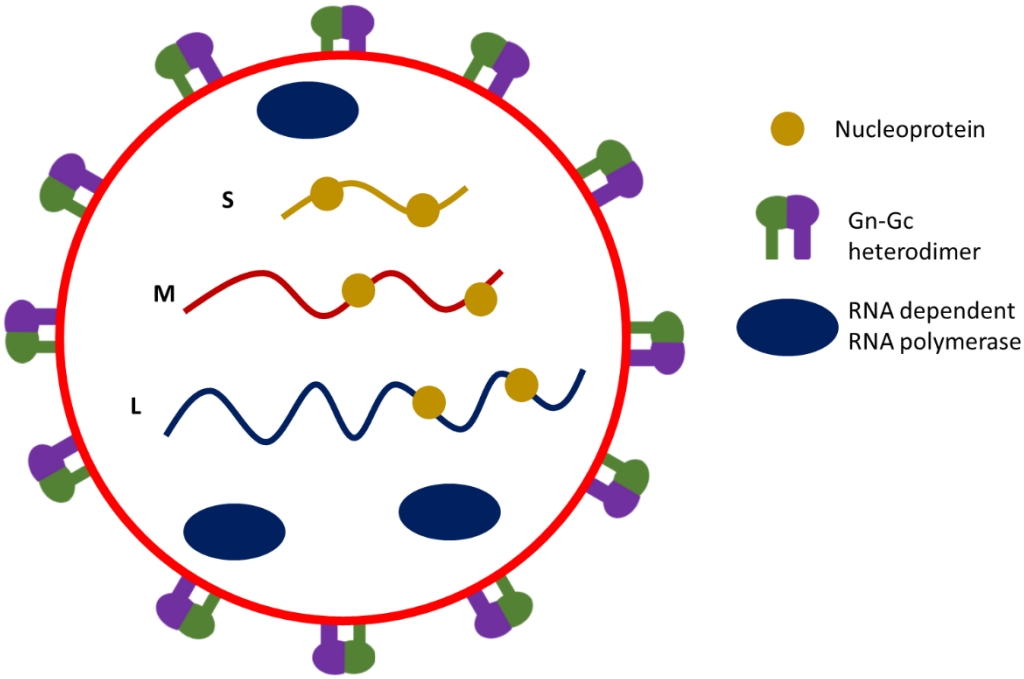


Figure 1.3: Schematic of the Bunyavirus virion
The Bunyavirus is an enveloped virus with the Gn and Gc heterodimer displayed on the surface of the virion. The Bunyavirus genome is segmented into S, M, and L segments. These genomic segments are bound by the viral nucleoprotein and replicated by the RNA dependent RNA polymerase.

SFTSV is a Bunyavirus which has emerged across the last decade in East Asia, spread by *H. longcornis* ticks[68]. Outbreaks have occurred in China, Japan and Korea, with particular foci in Henan province of China[79]. The main symptoms of infection are a high fever and thrombocytopenia, though gastrointestinal and haemorrhagic symptoms are often present in more severe cases. Estimates of mortality vary, with Japan and Korea reporting mortality of 27% and 23% respectively[80]. In China, where SFTSV surveillance is more widespread, mortality estimates are 6%, remaining an emergent threat[80].

1.1.3.2 Nairoviruses

In this thesis the pathogens most closely discussed will be from the genus *Orthonairovirus*, and family *Nairoviridae*, within the order of *Bunyavirales*[81]. The number of viruses in the genus nairovirus is rapidly expanding, due to continuous discovery within a range of different animal hosts, the present consensus separating the genus into nine distinct geno-groups[82]. Nairoviruses are found across the breadth of Europe, Asia and Africa, and are almost exclusively arboviruses, spread by tick vectors between vertebrate hosts[81]. Included within the genus *Orthonairovirus* is the WHO blueprint priority pathogen Crimean Congo Haemorrhagic Fever Virus (CCHFV), and Nairobi Sheep Disease Virus (NSDV), which can cause severe outbreaks in small ungulate herds[82].

Nairoviruses share several similar characteristics with other bunyaviruses, namely their tripartite negative sense RNA genome. Nairoviruses are also enveloped viruses, and express an NP, GPC, and RdRP protein from their genome segments. Where nairoviruses mainly differ is in their larger M and L segments, and consequently more complex GPC and RdRP[83]. The NP expressed from the S segment is thought to be responsible for binding and packaging RNA during viral replication[83]. The RdRP expressed from the L segment is important for synthesis of viral mRNA during viral replication[84,85], and is larger than in other bunyaviruses, containing a variety of additional protease domains. Particularly well studied is the N-terminal OTU protease domain, which is thought to modulate immune system signalling during infection by de-ubiquitination to supress innate immune activation[86,87]. Unique to nairoviruses among the order of bunyaviruses is the large GPC protein[83]. The GPC is processed into five separate proteins after expression, as shown in figure 1.4. At the N terminus the secreted proteins MCL and GP38 are cleaved[88]. MCL is poorly conserved across lineages, and its function is unknown. While the specific function of GP38 is still to be determined, its deletion prevents formation of infectious particles in CCHFV studies[89], and neutralisation of GP38 can prevent infection in CCHFV mouse models[90]. Together these suggest a crucial if unknown role for GP38 in virus formation and infection. Three transmembrane proteins are also produced, Gn, NSm and Gc[88,91]. Similarly to other bunyaviruses, Gn and Gc are thought to form a heterodimer on the surface of nairovirus virions[83], with homology studies against other bunyavirus proteins suggesting roles in receptor binding[92] and cell entry through fusion[93]. The function of the NSm subunit, cleaved between Gn and Gc, is currently unknown, though deletion does reduce the efficiency of virus production and release *in vitro*[89].

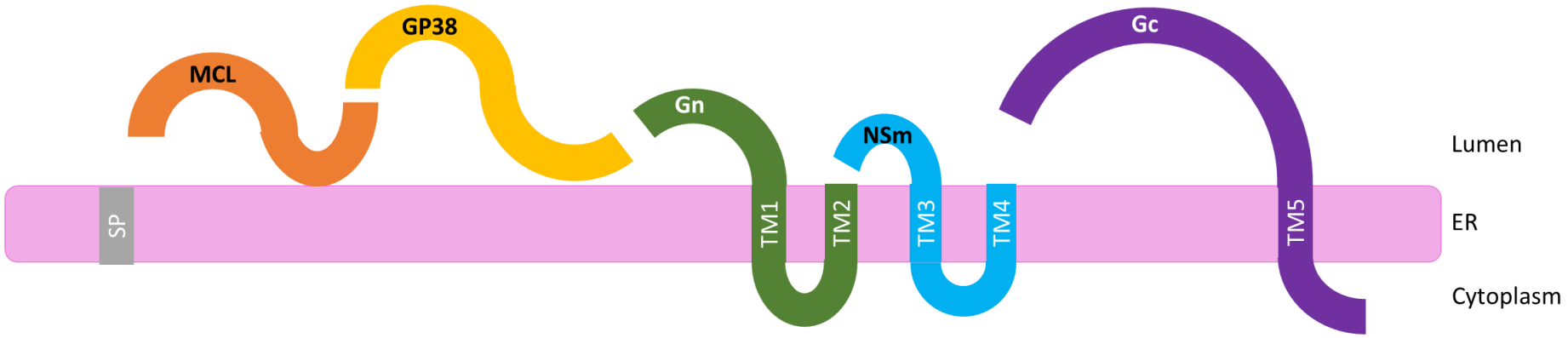


Figure 1.4: Diagram of the Nairovirus GPC and its proteolytic products.
The nairovirus GPC is expressed as single polyprotein and cleaved into 5 subunit glycoprotein products. The Nairovirus GPC is cleaved by a range of host proteases, producing two soluble proteins, MCL (orange) and GPC38 (yellow), and three transmembrane proteins, Gn (green), NSm (blue), and Gc (purple). SP – Signal Peptide, TM – transmembrane helix, ER – Endoplasmic Reticulum

1.2 CCHFV

1.2.1 Background to CCHFV

Crimean Congo Haemorrhagic Fever Virus (CCHFV) is an emerging nairovirus of concern, considered to have epidemic potential, and the causative agent of Crimean Congo Haemorrhagic Fever (CCHF)[94]. Evidence of CCHF-like disease in central Asia dates back to at least the 12th century[95]. CCHFV was initially reported in Crimea in 1944, as soldiers responsible for clearing overgrown land were infected and displayed symptoms of haemorrhagic fever following tick bites[95]. Subsequently similar haemorrhagic fever symptoms were identified throughout much of Eastern Europe, Central Asia and north and central Africa, before being realised as sharing an aetiological agent, and named CCHF[96]. CCHF is now recognised as the second most widespread arbovirus, after dengue fever, with a range that encompasses Southern and Eastern Europe, The Middle East, Central Asia, China, the sub-continent and all of Africa[97]. The large geographical area reflects the range of the primary vectors for CCHFV, ticks of the genus *Hyalomma*, [98]. The large endemic range of CCHFV leads to many opportunities for the disease to emerge into human populations causing outbreaks with mortality of 10-40%[99].

1.2.2 Pathogenesis of CCHFV

CCHFV is an arbovirus, maintained by tick-vertebrate-tick transmission in both epizootic and enzootic cycles[8]. Vertebrate hosts include a large range of domestic and wild mammal species (table 1.1). Infection of humans with CCHFV occurs through two major routes. The first route is through infected ticks carrying CCHFV. Exposure can occur through the bite of the infected tick[100], or through tick pulverisation exposing the individual to infected tick bodily fluids through open wounds[101]. Transmission can also occur through contact with the blood or bodily fluids of an infected animal, through an open wound or orifice[99,102]. Direct human to human transmission from symptomatic individuals has been demonstrated, with CCHFV considered a risk for nosocomial outbreaks[103]. Due to the methods of transmission, veterinarians, farmers and abattoir workers are considered high risk individuals for CCHFV infection due to their close proximity to livestock, alongside healthcare workers treating patients with CCHF[11,101,104].

After CCHFV infection, infection can cause disease, with a spectrum from mild to fatal cases. The length of disease incubation varies dependent on infection route with symptoms surfacing 3-5 days after tick bites, or 5-7 days after exposure to infected fluids or tissues[97]. Initial responses occur during the pre-haemorrhagic phase of illness[105], with symptoms encompassing sudden high fever, weakness, headaches, myalgia, and occasional diarrhoea and vomiting[97]. In mild cases of CCHF, disease can present as a non-specific febrile illness, which is difficult to distinguish from other similar diseases[106]. In more severe cases haemorrhagic symptoms present, initially developing petechial haemorrhages in mucosal membranes, and as rashes on the skin[105]. These progress into significant subcutaneous haemorrhages, with bleeding occurring from the gastrointestinal and urinary tracts[107]. Infections cause major inflammation in the liver and spleen[95], and further cerebral haemorrhages[107]. Death from CCHF can occur anywhere between 5-14

days after symptoms first present, with the cause of death being systemic haemorrhages, and multi-organ failure[99].

Working with the live CCHFV requires high containment facilities which limits the progress of research into CCHFV pathology[108]. Currently the receptors for cellular entry of CCHFV are unknown, but the virus is known to infect a wide range of cell types leading to pathology throughout the entire organism[106]. Upon virus entry initial replication appears to occur in the endothelial cells[109,110], and in dendritic cells recruited to the site of infection[111]. Infection of immune cells allows systematic infection through the lymph and circulatory systems, and causes leukopenia throughout disease[112]. As infection advances, foci of CCHFV infection are found in the liver, spleen and lungs[113,114]. CCHFV infection drives dysfunction in coagulation responses through a range of mechanisms in the circulatory system, with thrombocytopenia typical of severe infection[99]. CCHFV patients exhibit enhanced expression of pro-inflammatory cytokines[115]. Fatal outcomes are associated with further increased expression of TNFα, IL-6, IL-8, IL-10 and IFN-γ[115,116], however it has not been determined whether the association is causative of severe disease or merely correlative.

The mortality rates of CCHFV infection are unknown due to a lack of information on the true number of infections. Mortality rates during outbreaks are generally estimated to be between 10-40%[94,99], however mortality rates of up to 80% have been recorded[117]. Estimating mortality from CCHFV infection is difficult due to the non-specific symptoms presented in all stages of the disease, so CCHFV infection can only be confirmed by RT-PCR. Mortality during outbreaks may also be affected by the local strain of CCHFV, and the heterogeneity in healthcare provisions for treating symptomatic individuals in different regions, patient ethnicity, and co-morbidity. Sero-surveys in Turkey and Eastern Europe suggest that asymptomatic and mild CCHFV infections may be more common than previously thought[118,119]. Data suggests mild disease is common for CCHFV

infection in these areas and that mortality rates of infection may be lower than previously determined, with mortality rates since introduction to turkey estimated at approximately 5%[118]. Conversely, greater seropositivity rates suggest that CCHFV infection may be more widely spread than previously thought, and the disease burden larger than estimated, despite lower overall mortality.

1.2.3 Epidemiology of CCHFV

CCHFV circulation is vectored exclusively by hard bodied ticks of the *Ixodidae* family[120]. The predominant vectors are of the genus *Hyalomma* with *H. marginitum* being of particular interest, though many other members of the genus have been shown to transmit CCHFV[98]. Tick species of the *Rhipicephalus* and *Dermacentor* genuses have been shown to transmit CCHFV in laboratory conditions, and carry virus in the wild[121,122]. Ticks in the *Hyalomma* family are endemic to large areas of Africa, Asia and Eastern Europe[98], with their range closely reflecting the endemic range of CCHFV[123], with incidence of CCHFV closely correlating with the expansion of local *Hyalomma* populations[95]. Currently *H. marginitum* populations are expanding into and becoming endemic in southern Europe and autochthonous cases of CCHFV are occuring[124,125]. *H. marginitum* nymphs and larvae can be spread when feeding on flighted bird species, allowing seasonal introduction to non-endemic areas, but may in future present a periodic threat of CCHFV infection[126].

Infection of ticks with CCHFV occurs when a tick takes a blood meal from a host organism with CCHFV viremia[120], which then replicates in competent tick hosts, establishing a reservoir of CCHFV[127]. The highest viral titres of CCHFV are found in the salivary glands of the tick, facilitating CCHFV transmission during their next blood meal[127].

CCHFV transmission is maintained through a tick-vertebrate-tick transmission cycle. Ticks require a blood feed to progress through the different parts of their life cycle, and the vertebrate hosts vary throughout their life cycle[8], as shown in figure 1.5. The tick life cycle facilitates transmission and amplification of CCHFV in small animals during the larval and nymph stages[99], and transmission in ungulates and humans during their adult stages[8,99]. Tick based transmission of CCHFV can occur either from a direct transfer of an infected tick from a vertebrate host[120], or from horizontal transfer between co-feeding ticks[128]. In competent CCHFV hosts, vertical transmission can occur transovarially, with CCHFV passed from infected mothers into offspring[128].

Animal species act as reservoirs and amplifying hosts for CCHFV infection. CCHFV can infect a large number of different species from which *Hyalomma* ticks feed[129]. Most species are asymptomatic when infected with CCHFV despite many having detectable virus in their tissues[130], with humans as a notable exception who develop symptomatic disease. As hosts are asymptomatic, the risks of infection with CCHFV is unseen for those working closely with animals. Without active surveillance of animal populations, which is not widespread, CCHFV circulation will not be detected until outbreaks occur in humans.

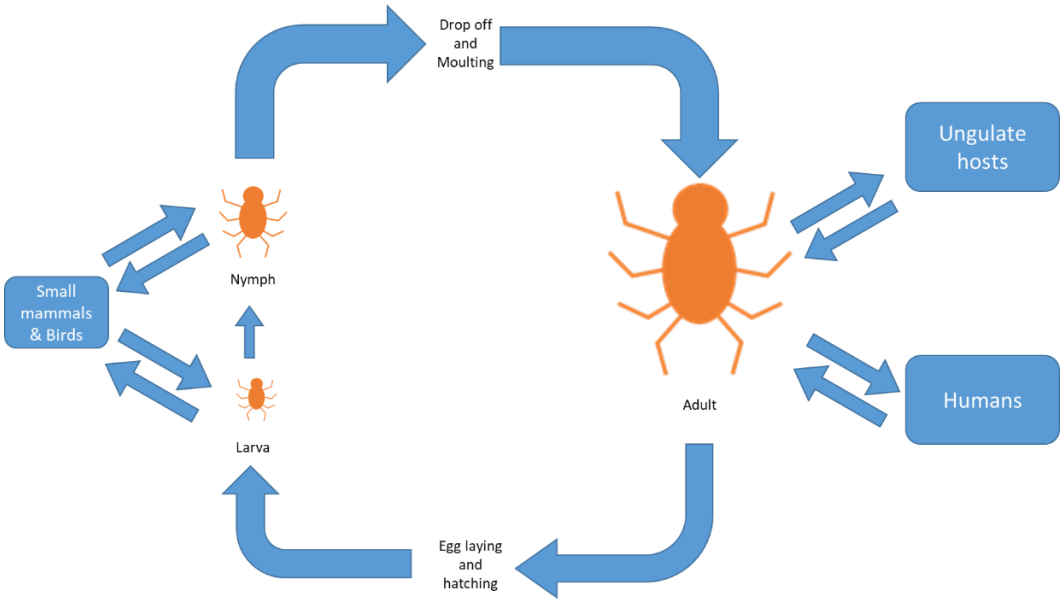


Figure 1.5: The life cycle and feeding patterns of Ixoxid ticks
After hatching, tick larvae's first blood meal is upon a small mammal or bird. In transitioning from larvae to nymph, ticks may continue to feed on the same host, or drop off then acquire a new small animal host for further blood meals. To transition from nymph to adults, following engorgement nymphs drop off and moult. Adult ticks then feed on large animals including ungulates and human hosts. Adult ticks mate while attached to a host and the engorged females drop off and undertake egg laying. These eggs hatch and seek out their first blood meal on small mammals or birds.

1.3 NSDV

1.3.1 Background to NSDV

Nairobi Sheep Disease Virus (NSDV), is the causative viral agent of Nairobi Sheep Disease (NSD), which is endemic to eastern sub-Saharan Africa[131]. Outbreaks of NSDV are characterised by a severe haemorrhagic gastroenteritis in sheep and NSDV mortality can approach 70% during an outbreak[132]. The mortality rate can be catastrophic for those whose livelihoods rely on sheep products. NSDV can also cause symptomatic disease in goats though mortality rates are significantly lower[133]. A strain of NSDV is also found in India and Sri Lanka, designated as Ganjam Virus (GJMV)[134]. NSD was first identified in 1917 during an outbreak among sheep which had been moved from an NSDV free region to an endemic region[133]. The first identification of GJMV in India occurred in tick samples from 1954[134]. Subsequent genetic analysis showed that NSDV and GJMV should be considered the same virus species[135]. Following discovery, infrequent outbreaks have been observed in sheep and goats throughout East Africa[132,136,137].

NSDV is an arbovirus spread by ticks of the *Rhipicephalus* genus in Africa[138] and *Haemaphysilis* ticks in India and Sri Lanka[139]. Outbreaks are low in endemic areas, as maternal antibodies are thought to protect newborn lambs until they develop their own adaptive immune response following pathogen exposure[131]. Outbreaks of NSDV occur when unprotected sheep or goats without pre-existing immunity are moved into endemic areas, or those incubating infections or infected ticks are moved into non-endemic areas[137]. NSDV is endemic to two geographically separate enclaves, and is not found in between the two, unlike CCHFV with its wide continuous geographical range. NSDV was likely seeded into India and Sri Lanka through movement of livestock and host transfer of ticks, where *Haemaphysilis* ticks were found as a competent vector allowing NSDV to replicate and continue a cycle of infection.

Research into NSDV has been severely limited due to a lack of resources. As such comparatively little is known about the pathology or epidemiology of NSDV. In addition, very little work has been performed on management, prophylactic or therapeutic interventions with NSDV, leading to a lack of understanding in how best to prevent outbreaks and control infections.

1.3.2 Pathogenesis of NSDV

NSDV infections occur through the bites of ticks infected with NSDV[133]. Entry through a break in the epithelium is likely needed for NSDV infection as evidenced by the lack of inter organism transmission without tick vectors[133], and the evidence of laboratory acquired infections in humans after needlestick injuries[140].

Following infection with NSDV, early symptoms of NSDV infection are febrile, with temperatures of up to 42ºC reported[132]. Fever can be constant or intermittent, but it is followed by gastrointestinal symptoms particularly bloody stools and diarrhoea[132,141]. Haemorrhages can also be observed in the nasal discharge[131], and subcutaneous haemorrhages can be observed in mucosal membranes and around the hooves, and are characteristic of NSDV infections[131]. Within a week of fevers presenting neurological symptoms are observed, typified by lethargy and a lack of response to stimuli, and are followed closely by a sharp drop in body temperature and death[132,136].

NSDV pathology impacts a wide range of organs within the body, suggesting infection is systemic[141]. In particular haemorrhages are observed throughout the gastrointestinal tract, resulting the bloody stools which are a characteristic symptom of NSDV infection[132,136,141]. NSDV has also been observed to cause respiratory discharge[132], and enlargement of the spleen and liver is characteristic of the infection[132,136,141], similar to symptoms presented in humans during CCHF. The immune system is also disrupted as evidenced by significant leukopenia starting from early infection[141]. Post mortem

observation also showed congestion within the brain of infected sheep[132,136], suggesting an explanation for late stage neurological symptoms. Following death from NSDV infection, viral antigens can be recovered from many organs and tissues within the body[132,141], further showing the systemic nature of NSDV infection.

NSDV infection in sheep induces a pro-inflammatory cytokine response[141], similar to the responses measured with the closely related nairovirus CCHFV. The pro-inflammatory responses can be associated with systemic shock and haemorrhagic symptoms observed during severe infection. Experimental infection with more pathogenic isolates induces greater TNFα, IL-6 and IL-12 expression and was associated with more severe infection[141]. However, it is unknown whether enhanced pro-inflammatory response is causative of severe disease, or merely correlative.

Infections in humans present as a mild febrile illness in confirmed cases, and infections can occur naturally[142], or through needlestick injuries in laboratory acquired infections[140]. In areas where NSDV circulates human populations display low levels of NSDV sero-positivity, suggesting that subclinical human infections do occur regularly but are not a public health threat[137].

1.3.3 Epidemiology of NSDV

As previously discussed NSDV is an arbovirus, spread through *Ixoxdae* ticks from a variety of genus'[137]. At present there is no known vertebrate reservoir of NSDV, though *Rhipicephalus* ticks have been shown to incubate NSDV for over two years[138], so a vertebrate may not be necessary for NSDV circulation. The range of NSDV is currently more limited than the geographical range of its host vectors[137]. Similarly to other arboviruses, the potential range in which NSDV can be endemic is changing and spreading due to climate change due to expansion of vector range. Recently *H. longcornis*

ticks carrying NSDV have been found in China, representing a new region in which NSDV may become endemic[143]. *H. longcornis* has also been observed in the USA[144], Australia[145], and New Zealand[146], representing a competent NSDV vector which could allow the pathogen to become endemic if introduced into North America, or Southern Oceania. Outbreaks of NSDV in non-endemic areas could devastate herds in developed Western countries and draw attention to neglected veterinary arboviruses and their impact. Across the next thirty years, models predict that NSDV may be able to spread from its East African and Indian enclaves, and expand further north in Africa, and into Eastern and Southern Asia[137].

1.4 Control measures for management of nairoviruses

For management of emerging diseases there are many methods to assess, understand, and control pathogens and their resultant diseases. For effective disease management and outbreak prevention, there is a need to understand the interdependent relationships of animals, humans, and their shared environments[147]. One Health approaches develop solutions to problems of zoonoses, acknowledging the entire ecosystem, and achieving better outcomes for both human and animal health[147].

Pathogen and disease outbreak can be controlled through managing vectors and the spread of infection, or by management through the use of therapeutics or prophylactics. These approaches can be used together to manage nairoviruses and prevent outbreaks in humans or animals.

1.4.1 Improved nairovirus diagnostics

Due to their sporadic nature, emerging pathogens are often far from public consciousness and awareness. This is further true of nairoviruses whose disease burden is diffuse and not concentrated in a single area for awareness. Studies have demonstrated that even in endemic areas, awareness of CCHFV among clinicians and veterinarians can be low[148]. Despite the impacts of CCHFV and NSDV, low levels of active surveillance lead to high levels of under-reporting of infections. Mild CCHFV infections can be subclinical or present clinical disease with non-specific febrile symptoms, which can be confused with other infections[118], and even severe CCHFV can present clinically with similar symptoms to other haemorrhagic fevers such as RVF or Ebola[94]. NSDV infections can often be conflated with those caused by peste de petits ruminants virus, due to similar outward pathologies[131]. The similarities in disease presentation only increase the need for increased awareness to ensure correct diagnosis of nairovirus infections, for assessing the burden of nairoviral infections more accurately, and for preventing and managing outbreaks.

To accompany raised awareness of nairoviral diseases in endemic areas, improved diagnostics are required to assess disease burden of nairoviruses. Rapid diagnostics are critical to controlling disease outbreaks and enabling of appropriate measures and treatments. The standard diagnostic test for CCHFV and NSDV is reverse transcriptase polymerase chain reaction (RT-PCR) to detect nairoviral RNA in infected individuals[131,149]. Though highly accurate, and rapid once the samples are received, most nairoviral infections occur in rural areas in LMICs where access to the laboratory facilities for RT-PCR may not be available on site, delaying diagnosis during outbreaks[150]. Diagnostic improvements can be made through the supply of equipment to more sites for diagnoses, and by developing diagnostic assays for CCHFV and NSDV which do not require extensive laboratory facilities[151,152].

1.4.2 Control measures for nairovirus insect vectors

Control of vector borne diseases can be achieved through controlling their vectors to prevent infections. Successes with vector management strategies are particularly evident with managing malaria infection from mosquitoes. These can range from strategies to reduce mosquito breeding sites, which helped eliminate malaria in Southern Italy[153], or use of mosquito nets to prevent infections, which has worked throughout Sub-Saharan Africa[154,155]. More technological vector management interventions include the release of sterile males to reduce mosquito populations in endemic areas[156]. All these measures have been used to reduce incidence of vector borne diseases.

CCHFV and NSDV are tick borne arboviruses, and as such vector control measures need to be specific to ticks. Due to the ecology of ticks and tick borne infections interventions such as nets and elimination of stagnant water would not be suitable for management of tick vectors[157]. Vector control for ticks has focused on preventing tick infestations in livestock to reduce circulation of vector borne nairoviruses. Targeting of ticks directly also has impacts on improving animal health regardless of tick infection status[158]. Acaricidal agents can be used to remove ticks from livestock, directly killing ticks[159]. However, many acaricidal agents are toxic organophosphates. These can contaminate animal products, and the ecosystem[160], and lead to more annual deaths than CCHFV does worldwide[159]. The environmental implications of preventative measures are vital to implementing a One Health approach to managing zoonoses. The ecological damage caused by acaricidal agents makes their uses non-feasible for livestock diseases[161].

Anti-tick vaccinations are an alternative measure to prevent arboviral infections, and allow an organism to mount an immune response stimulated by the tick feeding[162]. Anti-tick vaccinations do not carry the same risk of contamination in the environment that acaricidal agents do. Initial vaccines targeted antigens within the tick midgut, which does not prevent feeding but disrupts tick health and life cycle after drop off[163]. These vaccines

were shown to be highly effective at reducing tick transmission[163] and greatly reduce acaricide usage[164]. More recent vaccines have been developed targeting antigens in tick saliva, disrupting the tick's ability to feed on an organism and encouraging tick drop off[162,165]. Vaccines against tick saliva antigens has been shown to reduce pathogen transmission in a small animal model of infection[165].

Whilst effective at preventing infection, anti-tick vaccinations have limitations when applied to disease such as CCHFV and NSDV. Both diseases have multiple competent host vectors, and anti-tick vaccines are generally host specific[162]. To use anti-tick vaccines to prevent CCHFV and NSDV circulation would require a battery of different anti-tick vaccines to immunise livestock, or the targeting of conserved antigens within tick species, an endeavour which would require substantial time and financial investment.

1.5 Vaccination

Vaccination is one of the most effective healthcare interventions worldwide, responsible for protecting 2-3 million lives annually[166]. Worldwide vaccines have been responsible for saving millions if not billions of lives, and reducing the threat of circulating human diseases such as measles[167] or polio[168] across much of the world. Their impact has been shown during the ongoing COVID-19 pandemic, with the development of protective vaccines that have allowed societies to protect their most vulnerable against severe disease and death, saving hundreds of thousands of lives across the UK alone[169].

The purpose of vaccination is to induce an immune response against a pathogen which can prime and prepare a memory response within the immune system, in preparation of exposure to the pathogen of interest[166]. Immune memory through vaccination can be achieved through the use of a weakened or inactivated form of the pathogen[170], or through further engineered vaccines which expose the individual to a specific antigen from the

pathogen[171]. An antigen-specific immune-specific memory response is induced following vaccination and when the individual is subsequently exposed to the pathogen, the immune response induced through vaccination is stronger and more rapid than with a primary exposure[166]. An effective vaccine induced immune response is protective, reducing symptomatic infection and rates of hospitalisation and death[166]. The impact of reducing symptomatic infection may also reduce the rate at which vaccinated individuals can transmit pathogens[172].

1.5.1 The immunology of vaccination

The immune system can be largely divided into the innate and adaptive systems. Broadly speaking the innate immune system is responsible for the initial reaction to pathogenic threats and for activating the adaptive immune system[173]. The adaptive immune system is responsible for developing memory against specific pathogens after infection, and enacting effector responses against pathogens[173]. The aim of vaccination is to induce adaptive immune responses against antigens from a pathogen, responses which can be reactivated upon exposure to the pathogen by infection[166]. The re-activated immune response has multiple components which can be activated dependent on the specific pathogen, activating different effector functions which enable the immune system to identify, neutralise and eliminate pathogens.

1.5.1.1 Humoral immune responses

Humoral responses are mediated by antibodies which can bind to the external surface of pathogens[174]. Activation of humoral immunity is mediated by the B-cells which present the B-cell receptor (BCR) which can recognise accessible antigens on pathogens[175]. Once activated B-cells undergo a process of somatic hypermutation, affinity maturation and

class switching at germinal centres within the spleen[176]. The maturation process in the germinal centre mutates the antibodies sequence encoded in the germline to express a similar structure which allows the BCR to bind with higher affinity to the target antigen, and is reinforced through selective processes mediated by Tfh cells. B-cells can then differentiate into plasma cells which secrete antibodies into tissues, lymph and the circulatory system[176]. Antibodies have a variety of different functions within the humoral immune system[177]. Antibodies can bind to antigens on the surface of pathogens and perform a range of effector functions. Neutralising antibodies can bind and directly prevent the functionality of pathogen surface antigens, stopping pathogens gaining entry into cells and as such being highly prioritised in vaccine development. Antibodies can further interact with different components of the immune system when bound to a pathogen, through activation of the complement cascade, or recruitment of cytotoxic immune cells such as natural killer cells or monocytes in antibody dependent cellular cytotoxicity (ADCC)[178].

1.5.1.2 Cellular immune responses

Cellular based responses are immune effector functions based around the function of T-cells in response to infection. T-cells are effector cells within the immune system whose interactions are primarily mediated by the T-cell receptor (TCR)[179]. TCR molecules are highly divergent between T-cells[180] and can bind to and recognise a range of different epitopes presented on Major histocompatibility complex (MHC) proteins[179]. Small 9-11 amino acid epitopes are produced within cells by natural proteolytic processes[181]. When pathogens have entered a cell either by infection or by being consumed by a cell, the TCR molecules on T-cells are able to recognise the non-self epitopes presented by the MHC molecules, and initiate effector functions.

$CD4^+$ and $CD8^+$ T-cells have largely different functions within the immune system. $CD8^+$ effector T-cells recognise and kill cells infected by intracellular pathogens[182]. Once activated and expanded by antigen presenting cells $CD8^+$ T-cells respond to non-self antigens presented on MHC class I proteins, when a cell is infected by an intracellular pathogen[182]. CD8 effector T-cells bind to the infected cell and degranulate, releasing proteases and nucleases into the cytoplasm of the infected cell[183]. These enzymes induce controlled apoptosis in the infected cell, preventing necrotic processes following infection. The nucleases and proteases break down any pathogens within the cell, preventing release of pathogens from the infected cell and preventing the infection of further cells within a tissue[183].

$CD4^+$ T-cells instead recognise pathogen epitopes presented by the innate immune system on MHC class II proteins[184]. Once $CD4^+$ T-cells have recognised a non-self epitope, their effector functions guide the immune response, leading to their alternate name as T-helper cells[185]. $CD4^+$ T-cells are principally responsible for regulating the adaptive immune responses and guiding the activation of different cells to co-ordinate the immune response against the specific type of pathogen.

1.5.1.3 Memory immune responses

Once immune responses are induced by vaccination, with cellular and humoral effector functions, memory responses can be developed[166]. Following antigen exposure, the numbers of effector cells reduce once the antigen stimulation has been removed. Memory cells however are long lived cells within the immune system that primarily reside within the bone marrow long after initial exposure[186]. When the individual is re-exposed to an antigen or pathogen, memory cells are activated. They then rapidly replicate and differentiate into effector immune cells[187–189]. The rapid re-expansion of the memory cell

population produces a large number of antigen-specific B-cells and T-cells, much more quickly than during primary exposure to a pathogen or antigen. The high levels of effector cells are able to control the pathogen more rapidly than during primary exposure.

1.5.2 Vaccines

Vaccine platforms allow antigens from pathogens to be displayed within the immune system and generate immune responses, without the risks of infection and concomitant disease from the pathogen. There are a number of well-established vaccine methodologies including inactivated vaccines (IAVs) or Live attenuated vaccines (LAVs). Inactivated vaccines use pathogens which have been inactivated by chemical or temperature measures[170], whilst live attenuated vaccines (LAVs) are live pathogens which have been serially passaged or genetically modified to reduce the pathogenicity of the vaccine strain[170]. However, as live and actively replicating viruses, LAVs are generally not suitable for immunocompromised individuals nor for use with pathogens with high mortality rates[170]. Both IAVs and LAVs are optimal for producing humoral biased responses to pathogens, and though adjuvants can alter the induced immune response, their ability to induce effective CD8 T-cell responses is limited[190].

Subunit protein vaccines do not use whole pathogens, instead using independently expressed protein antigens[171]. Subunit protein vaccines therefore evade the need to handle high mortality pathogens, as with IAVs, or the risks of live virus vaccination, as with LAVs. Antigens for subunit vaccines are often soluble proteins from the surface of pathogens, as subunit protein vaccines generally generate a strong humoral biased response when combined with suitable adjuvants[171]. However, most need strong adjuvants to increase immunogenicity, which increases reactogenicity in vaccinees[171]. Though subunit protein vaccines have been successful in immunising against certain

pathogens, current research focuses on antigen development to induce more potent and more highly specific responses from protein vaccines[191].

Nucleic acid vaccines utilise RNA and DNA encoding the antigens of choice for the vaccine[192,193]. These platforms require a method of delivery to allow the nucleic acid to enter cells, as the immune system is effective at clearing uncoated nucleic acid, but once in cells, the nucleic acids are translated to express the antigen as a protein[193]. Nucleic acid vaccines express the target antigen within cells, and can induce potent cellular and humoral responses, dependent on the delivery mechanism[194–196]. Vaccines using mRNA, encapsidated in a lipid shell have become one of the leading vaccines used in developed countries during the COVID-19 pandemic[197,198].

1.5.3 Viral vectored vaccines

Viral vectored vaccines (VVVs) are recombinant viruses which act as delivery platforms for heterologous nucleic acid antigens[199]. VVVs infect host cells and express the heterologous antigen, generating immune responses against the encoded antigens[199]. VVVs allow vaccine antigens to be expressed and shed, generating humoral immunogenicity, and be processed by intracellular mechanisms generating potent cytotoxic cellular immunogenicity[199]. As live virus platforms VVVs also act as an adjuvant, guiding virus specific immune activation[199]. VVVs are an expanding field and three viral vectored vaccine platforms, using recombinant Vesicular Stomatitis Virus (rVSV)[200], Modified Vaccinia Ankara (MVA)[201], and adenovirus vectors[202,203], have been licenced as vaccines against Ebolavirus during the 2014-2016 West African Epidemic and the more recent outbreaks in DRC. In response to the COVID-19 pandemic multiple VVVs have been developed, mostly focused on the use of adenovirus as a vector[204–207].

Early viral vectored vaccines focused on the use of large poxvirus vectors[208]. Poxviruses are large double stranded DNA viruses, which had been well studied during the development of smallpox vaccines[209]. Poxvirus vectors offer a large antigen capacity due to their large genome size, however, this can make them more difficult to produce[209]. Instead VVVs based around smaller genomes have become more established in recent vaccine development.

1.5.3.1 Adenovirus as vaccine vectors

Adenovirus are a family of small double stranded DNA viruses, which can cause upper respiratory tract infections[210]. Adenovirus have a broad cell type tropism and have been used in genetic therapies, creating a breadth of research on the functionality of adenovirus genes and ability to express transgenes from their genome[211]. Adenovirus as vaccine vectors can be genetically modified to be replication incompetent making these viral vectors safe for use in immunocompromised individuals[212]. Despite being replication incompetent, adenovirus vectors have been shown to induce strong cellular and humoral immunogenicity against a range of different antigens[203,213–217].

Human adenoviruses have been utilised as vaccine vectors[218], however, human adenovirus can be limited as a vaccine platform due to pre-existing anti-vector immunity, generated by exposure to circulating adenoviruses within the human population[213,219]. Pre-existing immunity can reduce the immune responses induced against the vaccine antigens[205,213]. Therefore adenovirus vectors have been developed using adenovirus strains from a range of different host species[220], with Chimpanzee Adenoviruses (ChAds) being among the more common platforms[221]. Adenovirus vectors of both chimpanzee and human lineages have been studied extensively as vaccine vectors for a range of emerging and endemic pathogens.

ChAd vectors are continuously being developed and tested in preclinical studies, using antigens from malaria[222], TB[223], and filoviruses[224]. Chimpanzee adenoviruses have also been assessed clinically against diseases such as; influenza[225], malaria[226], TB[217], and ebolavirus[227], and licenced for use during the COVID-19 pandemic[228]. Adenovirus vectors are a flexible vaccine technology, and the speed, and low costs of production make adenovirus vectors ideal for responding to emerging pathogen outbreaks[221].

1.5.3.2 The ChAdOx1 vector

ChAdOx1 is a chimpanzee adenovirus vaccine platform developed at the Jenner Institute, University of Oxford. ChAdOx1 is derived from the replication competent ChAdY25 virus, selected due to a low rate of cross-reactive serum in diverse human populations, strong immunogenicity as a vaccine vector in preclinical studies[212]. ChAdOx1 was rendered replication incompetent by removal of the E1 and E3 regions of the ChAdY25 genome[212]. Heterologous vaccine antigens are then recombined into the ChAdOx1 genome at these sites[212].

Prior to the COVID-19 pandemic the ChAdOx1 vector had been used as a vaccine vector for a range of different antigens, and had entered into phase I clinical trials against Ebola[227], malaria[229], MERS[230], and influenza[225]. During the COVID-19 pandemic, ChAdOx1 was used as a vaccine vector encoding the SARS-CoV-2 spike protein in the vaccine ChAdOx1-nCoV19/AZD1222[204]. The vaccine was shown to be highly immunogenic[204], and protective in a two dose vaccination regime[228]. As of the time of writing over 2 billion doses of ChAdOx1-nCoV19 had been distributed[231].

Vaccination with ChAdOx1 induces strong humoral and cellular responses against the vaccine-encoded heterologous antigen. The profile of the immune response is Th1 biased, as evidenced by the cytokines secretion in vaccinated individuals[232]. A Th1 biased

response is optimal for inducing anti-viral protection with ChAdOx1 vaccination, but is also optimised for inducing immunogenicity against non-viral intracellular pathogens such as malaria and tuberculosis.

1.5.3.3 The ChAdOx2 Vector

ChAdOx2 is a second recombinant chimpanzee adenovirus vector generated by the Jenner Institute, University of Oxford. ChAdOx2 is a replication deficient chimpanzee adenovirus vector derived from ChAd68[233]. Similar to ChAdOx1, ChAdOx2 is replication incompetent due to the removal of the E1 and E3 genes, allowing genome space for recombination of heterologous antigens[233]. ChAdOx2 has been developed as an alternative ChAd vector to ChAdOx1[233]. Though currently it is unknown if ChAdOx1 vaccination would impact the efficacy of future ChAdOx1 vaccinations, it is important to have multiple vectors to allow for this possibility, and potentially allow for heterologous ChAdOx prime-boost regimens.

ChAdOx2 as vaccine vector has been tested for a smaller range of antigens than ChAdOx1, being a newer vaccine vector. ChAdOx2 HAV, expressing a four component fusion protein from *M. avium paratuberculosis*, has been shown to be tolerated and immunogenic in phase I human trials[234], and a ChAdOx2 vaccine expressing rabies G protein has been shown to be highly immunogenic in mice[235]. These results suggest ChAdOx2 is an immunogenic vector.

As a chimpanzee adenovirus it is likely that ChAdOx2 will present a Th1 biased immune phenotype, similar to that induced by vaccination with ChAdOx1. ChAdOx2 vaccination has been shown to produce T-cell based cellular immune responses, as well as inducing high titres of neutralising antibodies following vaccination, however the immunological profile remains to be demonstrated[234,235].

1.6 Nairovirus vaccination

1.6.1 Animal models of vaccination

To assess the immunogenicity and efficacy of vaccination, there is a need to use animal models to determine how a mammalian immune system will respond to the vaccines. Though there are ethical issues with the use of animals in scientific research, current in vitro organ models do not replicate the complexity of the adaptive immune system.

1.6.1.1 Mouse immunology models

Mice are a common model for assessing immunogenicity of vaccines. As small animals, mice are easy to house and feed and can be bred in large numbers rapidly, meaning experimentation can be performed at a greater rate than with larger animals. As they have mammalian adaptive immune systems, the types of immune responses generated can be a good guide for how a vaccine may behave in humans or livestock[236]. The mouse immune system is not identical to that of humans, but mice are a standardised model for assessing vaccine induced immunogenicity.

Throughout this thesis two different mouse strains have been commonly used for assessing vaccine immunogenicity. Balb/c are an inbred strain of *Mus musculus*[237]. As a mouse strain they are used for a range of different purposes, and are a standard strain for vaccinology and immunology research. As inbred mice they have little genetic diversity, with low divergence among MHC's[237]. CD-1 mice are an outbred stock of *Mus musculus*, also used extensively in immunology research. As an outbred strain of mice, CD-1 mice are more diverse and as such are expected to produce a wider range of immune responses after vaccination, with biological variation more akin to human immune responses[238].

1.6.1.2 Mouse challenge models of CCHFV

Humans are dead-end hosts for CCHFV and are the only organisms which develop symptomatic disease in response to natural infection[99]. Other immunocompetent animals present at most viremia with no clinical presentation of disease[130]. The lack of symptomatic disease from CCHFV infection in other mammals presents a difficulty in developing challenge models for testing CCHFV vaccines. Animal challenge models for infection with CCHFV with a measurable biological readout are needed for accurate assessment of vaccine efficacy. As CCHFV is a high containment pathogen, animals for challenge studies with CCHFV also need to be kept in high containment, further complicating development.

A solution to the lack of disease in mouse models is the use of immunocompromised mice. Models have focused on reducing the initial immune response to viral infection in these mice, disrupting their innate immune system, whilst allowing adaptive immune responses to develop in response to the vaccine[239,240]. STAT-1 knockout and IFN-I receptor knockout models in A129 mice have been developed which disrupt interferon responses to infection[239,240]. When infected with CCHFV, these mice show a pathology similar to human infection with haemorrhagic symptoms and severe disruption to immune cells, liver and spleen[239]. Immuno-compromised mice can be used to study CCHFV pathology but have also been used to assess vaccine efficacy[241]. CCHFV susceptibility can also be induced by antibody-induced blockade of interferon signalling, allowing CCHFV pathology to be studied in further mouse lines[242].

1.6.1.3 Non-human primate challenge of CCHFV

A non-human primate (NHP) model of infection is generally considered the gold standard for studying human diseases due to the close evolutionary distance. NHP species have

been tested as models for CCHFV infection, however, only cynomolgus macaques develop symptomatic infection[243]. In cynomolgus macaques, disease pathology is similar to humans, with a spectrum of mild to severe cases within the population challenged, with lethal infection in some cases[243]. The immunological profile is distinctly different to immunocompromised mouse models where CCHFV is lethal in all mice. Though an immunocompetent NHP model is superior from an immunological standpoint, NHP models are more expensive to maintain, and the use of primates presents the need for greater ethical considerations before undertaking challenge trials.

1.6.1.4 Ungulate challenge with NSDV

Unlike with CCHFV, NSDV challenge studies can be performed with sheep and goats. Challenge of sheep with NSDV has been performed over a long period of time, particularly following the initial discovery of NSDV[136,244]. Challenge models were also used in the 1970s to assess the efficacy on inactivated and live attenuated NSDV vaccines[244]. More recent challenge models have been more limited in number, but have offered insights into the pathology of NSDV infection[141]. These more recent uses are in immunocompetent sheep and could very easily be used as a challenge model for assessing NSDV vaccine efficacy.

1.6.2 CCHFV vaccine development

Since its recognition in 1944, there has been an acknowledgement of the need to protect individuals against CCHFV infection. As such there have been consistent attempt to develop an efficacious vaccine. However, despite some successes in animal challenge models, no vaccines have been widely licenced to protect against CCHFV.

Current trends in vaccine development require careful selection of antigenic targets. Nairoviruses have three major proteins, NP, GPC and RdRP, all of which could be used as antigens. RdRP is a poor choice as a vaccine antigen due to its large size and complexity, and the perceived risk of generating auto immune responses against human polymerases. Nucleoproteins have been selected as vaccine antigens because they are more highly conserved between pathogen strains due to a lack of external immunological selection pressure, and the same holds true with nairoviruses[245]. However, as nucleoproteins are often found only within the cell, humoral responses against nucleoproteins may not be useful for protection.

The Nairovirus GPC is a large polyprotein, with multiple proteins essential for viral tropism and cellular entry[88]. Surface glycoprotein are considered good antigenic targets for vaccines as both the cellular and humoral responses induced can be effective for protection. However, nairovirus GPCs are generally more diverse than NPs between species and strains[246]. Due to GPC heterogeneity some CCHFV vaccine development has focused on using more highly conserved GPC subunits as vaccines antigens.

1.6.2.1 Inactivated CCHFV vaccine

CCHF was a disease of concern within Eastern Europe and the USSR from the time of its discovery in the 1940s. An inactivated vaccine was developed from virus passaged in the brains of new-born mice, and then inactivated through heating to 58^{o}C and treatment with formaldehyde[247]. The vaccine was licenced for use throughout the USSR, with particularly use in Bulgaria, where widespread use of the vaccine in farm workers was proclaimed as responsible for a four-fold reduction in rates of CCHFV infection from 1975 to 1995[247].

Recent attempts to study the immunology of the inactivated vaccine have suggested the vaccine is not strongly immunogenic[248]. In human assessment, individuals needed four doses to induce low titres of neutralising antibodies, and T-cell immunogenicity was low[248]. Despite this the vaccine is still licenced for use in Russia and Eastern Europe, though has not found traction in other endemic regions.

1.6.2.2 Protein based CCHFV vaccines

Vaccine development against CCHFV moved on from the use of IAVs and LAVs, to develop vaccines based on specific antigen targets within CCHFV. Protein subunit vaccines have been developed with little success. Subunit-based CCHFV protein vaccines include a virus like particle (VLP) vaccine[249] and co-administration of CCHFV Gn and Gc protein antigens in the same vaccination[241]. The VLP was generated by co-expression of CCHFV N, GPC and RDRP in cell lines, without the capacity to package RNA expressing any of these proteins for further replication, producing an empty CCHFV virion[249].

Vaccination in triplicate with CCHFV VLPs generated neutralising antibodies in mouse models, though T-cell immunogenicity was poor, an immunological profile expected with VLP vaccination[249]. The immunogenicity profile was replicated in vaccination with CCHFV Gn and Gc, with strong neutralising antibodies induced[241]. However, in immunocompromised mouse models, neither immunisation with CCHFV VLPs nor with CCHFV Gn and Gc protected against lethal CCHFV challenge, despite the high titres of neutralising antibodies[241,249].

1.6.2.3 Nucleic acid CCHFV vaccine development

Nucleic acid vaccines have further been utilised in CCHFV vaccine development. Multiple DNA vaccines have been developed expressing CCHFV antigens, with common approaches utilising multiple antigens within a single vaccine. A DNA vaccine encoding CCHFV Gn, Gc and NP on separate plasmids was shown to be immunogenic for all three antigens[249]. Conversely a more recent approach used GPC and NP as antigens in a DNA vaccine, also inducing humoral and cellular immunogenicity[250]. Vaccines using mRNA lipid nanoparticle (LNP) platforms have been developed with NP, and a CCHFV GcGn fusion protein as antigens[251].

The efficacy of nucleic acid vaccines was tested by CCHFV challenge in immunocompromised mice. The Gn, Gc and NP vaccine induced a protective effect in mice, though not all vaccinated mice survived CCHFV challenge[249]. However, the DNA vaccine expressing GPC + NP was shown to be 100% effective at protecting mice from lethal CCHFV infections[250]. Whether the difference in protection is due to GPC being a more effective antigen, or due to advances in DNA vaccine technology between the two trials is unknown. With an mRNA-LNP vaccine, both NP and GcGn were shown to generate cellular and humoral immunogenicity[251]. Further, both antigens induced 100% protection in immunocompromised mouse models after a single vaccination, suggesting NP and GPC subunits are protective antigens in nucleic acid vaccines[251].

1.6.2.4 Viral vectored CCHFV vaccine development

A range of viral vectors have been developed using an NP antigen. A Modified Vaccinia Ankara (MVA) CCHFV NP vaccine was assessed in IFN-1 knockout A129 mice, given in a prime-boost regimen[252]. Despite inducing strong cellular responses, vaccination did not protect CCHFV challenged mice from lethal disease, though it did extend the time

course of infection. CCHFV NP has also been used as a vaccine in Ad5 and BoHV4 vectors[253]. In both vectors CCHFV as an antigen induced protection from lethal CCHFV challenge following two-dose vaccination regimens, suggesting anti-NP cellular responses can protect from CCHFV[253].

CCHFV NP as an antigen in VVVs does show mixed results however these results do suggest in the correct vector CCHFV NP could induce protection from CCHFV infection.

Viral vectored vaccines expressing CCHFV GPC have been shown to be immunogenic. An MVA GPC vaccine was shown to induce cellular and humoral immunogenicity in mice after two doses[254]. An rVSV vectored vaccine, using the same technology as the first licenced vaccine against ebolavirus[255], was shown to induce immunogenic responses after a single vaccination[256].

When assessed in mouse challenge models, both MVA and rVSV vectored vaccines were protective against lethal CCHFV challenge, suggesting immune responses induced against GPC are protective against infection[254,256]. Protection is is particularly notable with MVA CCHFV GPC as an MVA CCFHV NP vaccine was shown to not be protective against lethal CCHFV challenge.

1.6.2.5 Correlates of protection from CCHFV vaccine development

Correlates of protection are immune responses which are associated with survival during natural infection or associated with protective responses induced by vaccination at defined timepoints after immunisation and associated with particular regimens. For CCHFV the correlates of protection are unknown. Infections with CCHFV are sporadic and geographically diverse, often occurring away from well-resourced healthcare settings. However, studies of survivors of CCHFV infection have suggested that these individuals

have generated a stronger cytotoxic T-cell response during the course of infection than those who succumbed to the disease[257].

A lack of animal models for CCHFV infection can limit further the understanding of pathology of CCHFV. However, future research with a cynomolgus macaque model[243] may yield better understanding of CCHFV pathology and the correlates of protection in primates models of infection.

Analysis of protective responses induced by vaccination can also provide correlates of protection from lethal challenge, and vaccine induced immunogenicity comprises the biggest source of information on CCHFV correlates of protection. Work with CCHFV VLPs and CCHFV Gn and Gc protein antigens which induced strong neutralising antibody responses but poor T-cell immunogenicity[241,249] showed that cellular responses are necessary for protection and neutralising antibody responses alone are not sufficient for protection from CCHFV infection in this challenge model. The protective responses elicited by DNA and viral vector vaccines, which are predominantly cellular, also support the hypothesis, in particular the protection induced by Ad5, BoHV4 vectors and mRNA-LNPs containing CCHFV NP[253] with no efficacious neutralising antibody induction. However, other studies have suggested that cellular and humoral immunogenicity is necessary for protection from CCHFV challenge[258], and neutralising antibodies have been shown to be protective against CCHFV challenge in mice[90], suggesting that their induction by vaccination can contribute to protection from CCHFV.

1.6.3 NSDV vaccine development

NSDV vaccine development is significantly less advanced than CCHFV, with little investment and research on the immune response to NSDV and correlates of protection. The lack of research on NSDV vaccines is a direct result of the lack of funding on NSDV

research, likely because the disease is relatively controlled in endemic areas, and the threats of infection are not to major countries within the global north. The potential expanding range of NSDV may force a renewed course of study in NSDV, to develop greater understanding and encourage development of veterinary vaccines.

1.6.3.1 Inactivated NSDV vaccines

NSDV vaccine development did occur following pathogen discovery but progress has stalled across the last 50 years, and no vaccines have been utilised in field conditions. IAVs for NSDV were developed, and shown to induce high antibody titres in vaccinated animals[244]. LAVs were also produced by serial passage of NSDV within sheep cell lines, selecting for reduced pathogenicity[244], however, the reduced pathogenicity correlated with reduced immunogenicity[244]. In NSDV challenge trials, sheep immunised with an inactivated NSDV vaccine were protected from lethal challenge whilst those vaccinated with an attenuated vaccine showed clinical signs of disease, however, all survived exposure[244].

1.7 Pseudotyped Viruses

The use of, and experimentation with, highly pathogenic viruses carries risks for human and animal health. Pathogen outbreaks from laboratories in the UK have led to deaths in both humans and animals[259,260]. Due to the risks of laboratory escape, high containment facilities are needed for working with animal and human pathogens. These facilities can be extremely expensive to build run and maintain, as evidenced by the £145 million cost of the recently built containment building in the Pirbright Institute, UK for handling animal pathogens at a Special Animal Pathogen Order (SAPO) level 4 containment level[261]. Facilities needed for containing pathogens with threats to human health require

further complexity and expense[262]. The comparable lack of facilities to work with dangerous pathogens then creates a bottleneck of research, reducing the speed at which studies into virology, therapeutics and vaccines can be undertaken[263]. This is pertinent for emerging diseases which often have high mortality, and lack therapeutics or prophylactics to protect those working with the pathogen.

As such there is need to develop methods for studying pathogens which require high containment settings, in a lower containment safer setting. Pseudotyping is one such method for studying the proteins of dangerous pathogens, in replication deficient models, and BSL-2 containment settings. A pseudotype is a viral particle which presents a heterologous glycoprotein, supplied *in trans*, in a replication deficient virion[264]. The heterologous glycoprotein supplied can often be from an emerging pathogen, for which working with live wild-type viruses would require high containment facilities. The presentation of the heterologous glycoprotein on the surface of the virion alters the tropism and functionality of the virion to match that of the heterologous glycoprotein[265].

Pseudotype systems can be used to model a variety of different viral functions through the heterologous glycoproteins presented on the surface of the pseudotype virion. Functions include assessment of glycoprotein function in cell entry[266], identification of viral tropism and target cell receptors[267], and assessment of vaccine and infection induced neutralising antibody titres[268–270].

There are a range of different platforms which have been developed to produce pseudotyped viruses. Most pseudotyping systems are based on small RNA viruses such as influenza[271] VSV[265], or lentiviral based systems[272], or based on development of replication deficient virus like particles[273]. Different pseudotyping systems carry different benefits and drawbacks, for their ability to integrate virions with different heterologous glycoproteins and their capacity to study different viral functions.

1.8 Thesis outline

The aim of this thesis is; the development of new methods to assess neutralising antibodies induced against CCHFV and NSDV GPC, either through vaccination or natural infection; to design novel antigens against CCHFV and NSDV and produce immunogenic vaccines in ChAdOx viral vectors; and to assess the impact of a widespread ChAdOx1 vaccination campaign on anti-ChAdOx1 responses in the population, and their potential impact on immunogenicity induced by further ChAdOx1 vaccination.

Chapter 2 describes the experimental methods used to obtain the results throughout chapters 3-7. Chapter 3 describes the design and production of pseudotype particles for CCHFV and NSDV, and their assessment of suitability in a virus neutralisation test. Chapter 4 details the design of novel antigens and ChAdOx vectored vaccines against CCHFV, and the assessment of the immunogenicity induced by ChAdOx vectored monovalent CCHFV vaccines. Chapter 5 describes the development of ChAdOx1 vectored CCHFV vaccines, expressing multiple CCHFV antigens, and the immunogenicity induced by vaccination. Chapter 6 summarises the design of NSDV antigens, the development of ChAdOx1 vectored NSDV vaccines, and the immunogenicity induced by vaccination with ChAdOx1 vectored NSDV vaccines. Chapter 7 details the anti-ChAdOx1 neutralisation responses induced by vaccination with ChAdOx1-nCoV19 in human clinical trials, and assesses the impact of prior ChAdOx1 vaccination on CCHFV immunogenicity in a mouse immunisation model.

Chapter 2 - Materials and Methods

2.1 Materials

2.1.1 Reagents and suppliers

Material	Supplier
6 well flat bottom sterile cell culture plate	Costar, Corning Incorporated, USA
96F Nunclon Delta Microwell white plate	ThermoFisher Scientific, USA
96 well flat bottom sterile cell culture plate	Costar, Corning Incorporated, USA
96 well flat bottom black plate	Costar, Corning Incorporated, USA
Multiscreen Filter Plates	Merck Millipore, USA
Carbonate-Bicarbonate Buffer	Sigma, USA
Dulbecco's Phosphate Buffered Saline	Gibco, Thermofisher Scientific, USA
Phosphate Buffered Saline Tablets	Gibco, Thermofisher Scientific, USA
Minimum Essential Media Eagle	Sigma, USA
Dulbecco's Minimum Eagle Medium	Sigma, USA
Phenol Red Free Dulbecco's Minimum Eagle Medium	Sigma, USA
Foetal Bovine Serum	Sigma, USA
Penicillin/ Streptomycin	Sigma, USA
L-Glutamine	Sigma, USA
Genectin	Gibco, ThermoFisher Scientific, USA
100x Non Essential Amino Acids	Gibco, ThermoFisher Scientific, USA
CCHFV Glycoprotein Peptides	Mimotopes, Australia

CCHFV Nucleoprotein Peptides	Mimotopes, Australia
NSDV Glycoprotein Peptides	Mimotopes, Australia
NSDV Nucleoprotein Peptides	Mimotopes, Australia
Anti-Mouse IFNγ mAb AN18	Mabtech, Sweden
Anti-Mouse IFNγ mAb R4-6A2-Biotin	Mabtech, Sweden
Streptavidin-ALP	Mabtech, Sweden
F96 Maxisorb NUNC-Immuno Plate	ThermoFisher Scientific, USA
CCHFV Gn protein His-tag	Native Antigen Company, UK
CCHFV Gc protein Human-Fc tag	Native Antigen Company, UK
CCHFV NP protein His-tag	Native Antigen Company, UK
NSDV Gc protein Human-Fc tag	Native Antigen Company, UK
Tween-20	Sigma, USA
A1418 Goat Anti-Mouse IgG(Fc-specific)-AP	Sigma, USA
4-nitrophenyl phosphate disodium hexahydrate	Sigma, USA
25x AP color develop buffer	Biorad, USA
Diethanolamine Substrate Buffer 5x concentrate	Sigma, USA
AP Conjugate Substrate	Biorad, USA
PEI	Merck, USA
LT-1 tranfection reagent	Mirus Bio, USA
BrightGlo Luciferase Assay System	Promega, USA
Nano-Glo Substrate	Promega, USA
Nano-Glo Luciferase Assay Buffer	Promega, USA
Phospha Assay Buffer	Tropix Applied Biosystems, USA

Phospha Reaction Buffer Diluent	Tropix Applied Biosystems, USA
CSPD substrate	Tropix Applied Biosystems, USA
Restriction Enzymes	New England Biolabs, USA
CutSmart Buffer	New England Biolabs, USA
Gateway LR Clonase II enzyme mix	Invitrogen, USA
T4 DNA ligase	New England Biolabs, USA
T4 DNA ligase 10x buffer	New England Biolabs, USA
Antarctic Phosphatase	New England Biolabs, USA
Antarctic Phosphatase 10x buffer	New England Biolabs, USA
TAE 50x buffer	Fisher Scientific, USA
Sybr Safe DNA Gel Stain	Invitrogen, USA
Phusion Polymerase Master Mix	New England Biolabs, USA
Primers	GeneArt, Invitrogen, USA
DNA Sequences	GeneArt, Invitrogen, USA
DNA Molecular Weight Markers	New England Biolabs, USA
VSV-ΔG pseudoparticles	Kerafast, USA
DH5α cells	New England Biolabs, USA
Kanamycin	Sigma, USA
Chloramphenicol	Sigma, USA
Carbenicillin	Sigma, USA
In-Fusion 5x recombinase	Takara Biosciences, Japan
Blocker Caesin in PBS	ThermoFisher Scientific, USA

Table 2.1: Commercially available reagents

2.1.2 DNA sequences

DNA sequences for manufacture of vaccines and pseudotyped virus were ordered from GeneArt Synthesis (Invitrogen) as Genestrings.

Antigen DNA sequences can be found in appendix 1

2.1.2.1 CCHFV DNA sequences

The CCHFV antigens Gn, Gc, NP, and CCHFV del10 sequences were codon optimised by EBI EMBOSS (https://www.ebi.ac.uk/Tools/st/emboss_backtranseq/) for expression in *Homo sapiens*.

Sequences can be found in appendix 1.1

2.1.2.2 NSDV DNA sequences

All NSDV sequences were codon optimised by EBI EMBOSS (https://www.ebi.ac.uk/Tools/st/emboss_backtranseq/) for expression in *Ovis aries*.

Sequences can be found in appendix 1.2

2.1.2.3 CCHFV GPC sequence

Two different CCHFV GPC nucleotide sequences were used within this thesis.

The original sequence was used within the ChAdOx2-CCHFV GPC vaccine used in lethal Challenge assessment (chapter 4), and for ChAdOx1-CCHFV GPC in initial comparison with ChAdOx1-CCHFV Gn and ChAdOx1-CCHFV Gc (chapter 4).

All further uses of the CCHFV GPC used a recoded antigen for improved genetic stability. The amino acid sequence was unchanged.

Sequences can be found in appendix 1.3

2.1.3 Oligonucleotide sequences

Primer name	Sequence
CCHFGncPCRFw	GCGACGACTGCGTG
CCHFGncPCRRev	GGTCTTGGGGTGGTT
CCHFGccPCRFw	CGCCAAGGGCATGAA
CCHFGccPCRRev	GGCCGTAGAAGAAGCTC
CGseqCCHFGc1	GAGAAAGCCCCTGTTCC
CGseqCCHFGc2	CATCCAGCAGAAGCTGC
CGseqCCHFGc3	CCGACAGCACCTTCAAG
CGseqCCHFGcPromRev	CAGGGGCTTTCTCAGCA
CGseqCCHFGn1	GAAGGCTGCTGAGCGAG
CGseqCCHFGn2	CGAGATGCACGACCTGA
CGseqCCHFGnPromRev	CCAGGGCCGCCATAA
CGseqCCHFGn2ANP3	CGAGTGCGCCTGGGTGA
CGseqCCHFGn2ANP4	GAAGAAGGCCCTGCTGA
CGseqNSDGn1	CTGATGGCCGAGGAGGG
CGseqNSDGn2	GCTGCACGAGCTGAACT
CGseqNSDPromRev	CCAGTCGCCCTCCTCGG
VVCF5	ATCCACGCTGTTTTGACCTC
VVCF6	CAGCGAGCTCTAGCATTTAGG
J1172	GTCAATGGGGTGGAGACTTG
J3102	GAGCGTGCTGAGACAG
J3103	TGAACTGCAGCTACAA
J3110	TGGACAGCGTGGGCAA
J3614	GAACCTGCTGAGCGTGACAT

J3615	CTACAGCGCCAAGTGTTACA
J3616	AGCCAATATCGCCCTGAGCT
J3617	CTGCAGAGCTGTACACATGG

Table 2.2: Oligonucleotides sequences

2.1.4 Plasmids

2.1.4.1 Plasmids for viral vector production

Plasmid name	**Function**	**Source**
p2022	Protein expression plasmid and entry plasmid for ChAdOx1 and ChAdOx2 E1	Jenner Institute
p2563	ChAdOx1 Bacterial Artificial Chromosome	Jenner Institute
p3665	Entry plasmid for ChAdOx1 E4 entry	Jenner Institute
p4407	ChAdOx2 Bacterial Artificial Chromosome	Jenner Institute
p4771	ChAdOx1 Bivalent entry Bacterial Artificial Chromosome	Jenner Institute

Table 2.3: Plasmids used for viral vector production

2.1.4.2 Plasmids for pseudotype virus production

Plasmid name	Function	Source
p8.91	Expresses *gag-pol* of HIV-1	Jenner
pCSFLW	Expresses firefly luciferase for incorporation into pseudotyped lentivirus	Jenner
p4723	pCAGGs expressing VSV-G	Kerafast
p5666	PLCK expressing VL nanoluc	National Institute of Health, USA
p5667	pCAGGS expression codon optimised CCHFV GPC from IbAr10200	National Institute of Health, USA
p5670	pLCK expressing codon optimised CCHFV L protein from IbAr10200	National Institute of Health, USA
p5671	pCAGGS expressing T7 Polymerase	National Institute of Health, USA
p5672	pCAGGS expressing codon optimised CCHFV NP from IbAr10200	National Institute of Health, USA

Table 2.4: Plasmids for production of pseudotyped viruses

2.1.5 Solutions, Buffers and Cell Culture Media

TAE buffer had a final concentration of 40mM Tris, 40mM acetate, 1mM EDTA and was prepared by dilution of 50x concentrated TAE solution in dH_2O.

Phosphate buffered Saline had a final concentration of 2.6mM KCl, 1.47mM KH_2PO_4, 137mM NaCL, 8.10 mM Na_2HPO_4, and was prepared by dissolving dry powder in dH_2O.

10% MEME was prepared from Minimum Essential Medium Eagle, by addition of 50ml Foetal Bovine Serum, 5ml L-Glutamine, and 5ml Penicillin/Streptomycin.

0%DMEM was prepared from Dulbecco's Minimum Essential Medium, by addition of 5ml L-glutamine and 5ml Penicillin/Streptomycin.

2%DMEM was prepared from Dulbecco's Minimum Essential Medium, by addition of 10ml Foetal Bovine Serum, 5 ml L-glutamine and 5ml Penicillin/Streptomycin.

5%DMEM was prepared from Dulbecco's Minimum Essential Medium, by addition of 25ml Foetal Bovine Serum, 5ml L-glutamine and 5ml Penicillin/Streptomycin.

10%DMEM was prepared from Dulbecco's Minimum Essential Medium, by addition of 50ml Foetal Bovine Serum, 5ml L-glutamine and 5ml Penicillin Streptomycin.

10% PRF DMEM for Griptite 293 cells was prepared from Phenol Red Free Dulbecco's Minimum Essential Medium, by addition of 50ml Foetal Bovine Serum, 5ml L-Glutamine, 5ml Penicillin/Streptomycin, 6ml Genectin, and 5ml 100x Non-Essential Amino Acids.

2.1.6 Peptides

The peptides used for ELISPOT in this thesis were synthesised by Mimotopes Pty Ltd, Melbourne, Australia.

Sequences can be found in appendix 2.

2.1.7 Proteins for ELISA

Protein	Source
Crimean-Congo Hemorrhagic Fever Virus Gn Protein, His-Tag	Native Antigen Company, UK
Crimean-Congo Hemorrhagic Fever Virus Gc Protein, Human Fc-Tag	Native Antigen Company, UK
Crimean-Congo Hemorrhagic Fever Virus Nucleoprotein, His-Tag	Native Antigen Company, UK
Nairobi Sheep Disease Virus Glycoprotein Gc, Human Fc-Tag	Native Antigen Company, UK

Table 2.5: Proteins for ELISA

2.1.8 Cell Lines

HEK293T cells were a generous gift from the Wright Laboratory, University of Sussex

Huh-7 cells were a generous gift from the McKeating Laboratory, University of Oxford

A549 cells were obtained from the Jenner Institute, University of Oxford

Vero Cells were obtained from the Jenner Institute, University of Oxford

HEK293T Griptite cells were obtained from the Jenner Institute, University of Oxford

2.1.9 Pseudotyping Reagents

VSV-ΔG pseudoparticles were obtained from Kerafast, USA

2.2 Methods

2.2.1 Cloning & Molecular biology

Routine molecular biology protocols are outlined below. Specifics of these protocols are noted in the results chapters.

2.2.1.1 Polymerase Chain Reaction (PCR)

PCR reactions were performed with Phusion High-Fidelity DNA polymerase (New England Biolabs), with the following reaction mixture

50µl PCR mixture

25µl Phusion MasterMix

X µl for10-20ng of template DNA

1.5µl forward primer at 10mM

1.5µl reverse primer at 10mM

Y µl H_2O up to 50µl total volume

A Professional Thermocycler (Biometra) was used for all reactions according to the following cycle:

1. 2 minutes denaturation at 98°C
2. 20 seconds denaturation at 98°C
3. 20 second annealing at 54-65°C
4. 10 seconds per kb of PCR product extension at 72°C
5. Repeat steps 2-4 for 30 cycles
6. 5 minutes extension at 72°C

For colony PCR reactions from soluble bacterial culture 1µl of culture was used in place of template DNA. For bacterial culture from agar, the colony was swabbed using a clean 200µl tip and the colony suspended in the PCR reaction mixture.

2.2.1.2 Endonuclease Restriction Digests

Restriction digests were performed with a range of restriction enzymes according to the following generalised protocol:

20µl double restriction digest

2µl 10x Cutsmart buffer

1µl of each restriction enzyme

Xµl for 1µg target DNA

Yµl H_2O up to 20µl total volume

Restriction digests were incubated at 37°C for 1-2 hour. For vector restriction digests, add 1µl Antarctic phosphatase, and 2.5µl 10x Antarctic phosphatase buffer and further 15 minutes of digestion, to reduce the chances of re-ligation of complimentary ends. Fragments were then purified by gel electrophoresis.

2.2.1.3 In-fusion recombination

Recombination of PCR fragments was performed with In-Fusion HD Enzyme (Takara Bio).

10µl In-Fusion reaction

2µl 5x In-fusion mastermix

Xµl for 200ng linearised vector

Yµl for 200ng purified PCR fragments

Zµl H_2O for a total reaction volume of 10µl.

InFusion reactions were incubated at 50ºC for 15 minutes then placed on ice for a further 15 minutes, prior to separation by gel electrophoresis.

2.2.1.4 Agarose Gel Electrophoresis

DNA reaction products were separated by 1% agarose gel electrophoresis (100V, 30-50 minutes) in 1x Tris-acetate-EDTA (TAE), stained using Sybr Safe DNA stain, and visualised on a Dark Reader TransIlluminator (Clare Chemical Company). Size estimates were compared against 10kb ladder.

2.2.1.5 Agarose Gel Extraction

DNA digest fragment or PCR products were excised from agarose gels, and purified using a Qiaquick Gel Extraction Kit, as detailed below.

Gel excisions containing target DNA are dissolved in 300µl Buffer QG per gel excision in a clean 1.5ml tube and incubated at 50ºC for 10 minutes. Following agarose completely dissolving, add 100µl of isopropanol per gel excision and mix. Transfer the sample onto a Qiaquick spin column in a 2ml collection tube. Centrifuge at 16,000g for 1 minute, then empty buffer QG run through form collection tube. Wash DNA by addition of 0.75ml buffer PE to the Qiaquick column followed by centrifugation at 16,000g for 1 minute. Empty the collection tube and repeat centrifugation. Place the column onto a clean 1.5ml microcentrifuge tube. Add 50µl of buffer EB, and allow to incubate for 1 minute. Centrifuge for 1 minute at 16,000g, and collect the effluent.

2.2.1.6 DNA ligation

Ligations were performed with T4 DNA ligase (New England Biolabs).

15µl reaction mixture

1µl T4 DNA ligase

1.5µl 10x T4 DNA ligase buffer

Xµl for 50ng of vector DNA

Yµl for 50ng of insert DNA

Zµl of H_2O for a total reaction volume of 15µl

Reactions were either incubated overnight at 16ºC or at 25ºC for 15 minutes, then heat inactivated at 65ºC for 10 minutes, prior to further use or processing.

2.2.1.7 Bacterial Transformation

Competent DH5α *E.coli* cells (New England Biolabs) were transformed for plasmid and BAC growth, following ligation or recombination reactions.

50µl of DH5α E.*coli* cells were incubated on ice for 30minutes with 2-3µl of DNA reactions, followed by heat shock at 42ºC for 45 seconds. Cells were allowed to recover 1 hour at 37ºC, then plated onto 20ml LB agar containing selective antibiotics at 50µg/ml. Bacteria was then grown overnight at 37ºC before storage at 4ºC.

2.2.1.8 Plasmid DNA preparations

Single colonies of transformed bacteria were cultured individually in 1ml LB medium in selection antibiotics. Following overnight culture, plasmid constructs were purified by Qiagen Miniprep Kits, and screened using diagnostic restriction digests. Bacteria containing the correctly ligated or recombined plasmid were further amplified in 50ml of

LB culture medium with selection antibiotics. After overnight incubation, plasmid DNA was purified using Qiagen Plasmid Plus midiprep kits, and the DNA stored at -20ºC.

2.2.1.9 Production of Bacterial Artificial Chromosomes

Genomes for ChAdOx vectored vaccines were prepared by recombination between Bacterial Artificial chromosomes containing the ChAdOx genome, and shuttle vectors containing antigens.

For a total reaction volume of 10µl;

Xµl BAC containing 150ng DNA

Yµl Shuttle vector containing 150ng DNA

Zµl TE buffer, up to a total of 8µl

2µl of LR clonase II enzyme.

Reaction mixture was incubated at 25ºC for 1 hour. Following incubation, 1µl of proteinase K was added to the reaction mixture to terminate recombination. LR reactions were then immediately transformed into competent cells.

2.2.1.10 Preparation of BAC DNA

Single clones of BAC transformations were transferred into 5ml LB medium with 5µl of 50mg/ml chloramphenicol for antibiotic selection, overnight at 37ºC. 5ml cultures were then transferred into 50ml LB medium and incubated overnight at 37ºC. Cultures were also streaked out on LB agar plates with kanamycin and ampicillin. Successful single recombinants were selected by sensitivity to kanamycin, induced by loss of kanamycin resistance during recombination, whilst retaining chloramphenicol resistance. Successful double recombinants were selected kanamycin and ampicillin sensitivity, induced by a

double loss of resistance during recombination, whilst maintaining chloramphenicol resistance. 50ml cultures were further transferred into 500ml LB medium and incubated overnight at 37ºC. Cultures were pelleted at 6,000g for 15 minutes at 4ºC, and the pellet re-suspended in 15ml 4ºC P1 buffer (Qiagen), and transferred into a clean 50ml centrifuge tube (Corning). Bacteria were lysed by addition of 15ml P2 buffer (Qiagen) and incubation for 5 minutes. Lysis buffer was neutralised by addition of 15ml ice-cold P3 and incubated on ice for a further 20 minutes. BAC preparations were then centrifuged at 10,000g for 15 minutes at 4ºC to pellet precipitate, and the remaining supernatant removed into a clean 50ml centrifuge tube (Corning). A 100G column (Qiagen) was pre-equilibrated by application of 4ml QBT buffer (Qiagen). The supernatant was then run through the column and allowed to enter the resin. The column was washed twice with 10ml of QC buffer (Qiagen). BAC DNA was eluted with 3ml 65ºC QF buffer (Qiagen) and collected in a 15ml centrifuge tube (Corning). The elution was separated into 6x1.5ml micro-centrifuge tubes, containing 500µl each. Into each tube 350µl of 100% isopropanol was added, and the subsequent mixture centrifuged at 16,000g for 15 minutes at 4ºC to pellet DNA. Following centrifugation supernatant was aspirated and the pellet washed with 250µl 70% ethanol, followed by further centrifugation at 16,000g, for 15 minutes and 4ºC. The supernatant was then aspirated and the pellet allowed to air-dry for 10 minutes. DNA was re-dissolved in 10µl per tube TE buffer (Qiagen), and allowed to resuspend overnight at 4ºC to prevent shearing of DNA. DNA was combined into a single aliquot and stored at -20ºC until further use.

2.2.1.11 DNA concentration quantification

Quantification of DNA concentrations were performed by A260/A280 spectroscopy using Nanodrop One (ThermoFisher Scientific, USA).

2.2.1.12 Sequencing

DNA samples for sequencing were prepared at a concentration of 100ng/µl. A volume 5µl was prepared for each sequencing reaction. Primers for sequencing were prepared at a concentration of 3.2pmol/µl. A volume of 5µl was prepared for each sequencing reaction. Sequencing was performed by Source Biosciences (UK)

2.2.2 Immunology

2.2.2.1 Animals

6-7 week old female Balb/cOlaHsd or 21-25g ICR(CD-1) mice (both Envigo RMS Inc (Bicester, UK)) were used for all experiments. All procedures were carried out under the terms of the UK Animals (Scientific Procedures) Act 1986 Home Office Project Licence (P9804B4F1) and PIL (IDB5CB767).

2.2.2.2 Immunisations

Mice were anaesthetised by inhalation of 3% IsoFlo (Zoetis) prior to immunisation. All vaccines were diluted in sterile PBS to $2x10^9$ IU/ml (unless otherwise stated). 50µl of each vaccine was injected intramuscularly (i.m.) into the hind leg.

2.2.2.3 Tail vein bleed

Mice were restrained and a small nick made on the tail with a sterile blade. 40-60µl of blood was taken using serum separation tubes, and mice were restrained until the wound had clotted. Blood samples were centrifuged at 16,000g to separate blood components and serum was stored in 1.5ml micro-centrifuge tubes at -20^{o}C.

2.2.2.4 Mouse euthanasia

Mice were anaesthetised by inhalation of 3% IsoFlo (Zoetis) prior to all procedures. Mice were euthanised by exsanguination which was performed by cardiac puncture with a 25g syringe, and subsequent drawing of blood from the heart. Death was confirmed by cervical dislocation, followed by harvesting of spleens. Spleens were placed in 1ml of 10% MEME and stored on ice before use within 60-90 mins of harvesting. Blood was centrifuged at 16,000g to separate components and serum was stored in 1.5ml microcentrifuge tubes at -20^{o}C.

2.2.2.5 Ex-vivo IFN-γ Spleen Enzyme Linked Immunospot (ELISPOT)

MAIP plates were coated with mAB AN18 anti-mouse-IFNγ at a concentration of 5µg/ml in carbonate-bicarbonate buffer, then incubated overnight at 4^{o}C. After incubation plates were blocked with media for ~1h at 37^{o}C. Mouse spleens were harvested and crushed in PBS, with the resultant suspension passed through a sterile 70µm cell strainer and collected in 50ml tubes. The suspension was made up to a total of 15ml with sterile PBS then centrifuged (1350rpm x 5 minutes). The pellet was re-suspended in 5ml ACK buffer and incubated for 5 minutes at r.t. Subsequently, 25 ml of sterile PBS was added to each tube and centrifugation was repeated (1350 rpm x 5 minutes), followed by resuspension in 5ml of complete (10% HI FBS, 2mM L-glutamine, 1%pencillin/streptomycin) MEME. Splenocytes were counted using a CASY counter (Schärfe Systems, Reutlingen, Germany) and re-suspended to a concentration of $1x10^{7}$ cells/ml in complete MEME. A 2x serial dilution of cells was prepared in duplicate in coated wells by plating 100µl of cells then transferring 50µl into a coated well containing 50µl complete MEME. The process was repeated for a secondary serial dilution. Control wells were prepared by

plating in duplicate 50µl cells into coated wells. 50µl peptide was added to stimulated wells at 2x final concentration. To control wells 50µl complete MEME was added. Plates were then incubated for 18-20 hours at 37ºC, 5% CO_2. Plates were washed 6x with PBS and incubated for 2h at r.t. with biotiniylated anti-mouse IFNγ mAb R4-6A2-Biotin, at a concentration of 1µg/ml in PBS. Plates were washed 6x with PBS and incubated with Streptavidin-Alkaline Phosphatase at a concentration of 1µg/ml in PBS. Spots were developed by addition of 50µl colour development buffer (Deinoised water, 25x AP color development buffer, and the 2-component AP conjugate substrate at a ratio of 100:4:1:1 respectively) to each well. Spots were counted by ELISPOT counter (Autoimmun Diagnostika (AID), Straßberg, Germany). Results were expressed as spot forming units per million splenocytes. Responses in media only control wells were subtracted from those measured in peptide stimulated wells.

2.2.2.6 Enzyme Linked ImmunoSorbent Assay (ELISA)

Maxisorp 96 well plates were coated with 50µl per well of CCHFV protein. CCHFV Gn His-tag or CCHFV Gc human-Fc-tag were diluted to 1µg/ml in PBS, CCHFV NP His-tag was diluted to 2µg/ml. Following coating plates were incubated overnight at 4ºC. Plates were washed 3x with PBS with 0.05% Tween-20 (PBS-Tween) and blocked for 1 hour with 1% Caesin for 1h at r.t. Caesin was removed from each plate and 50µl mouse serum added in duplicate, diluted in PBS to be within the linear range of the standard curve. For anti-CCHFV Gn and anti-CCHFV NP responses a two-fold standard curve was established from a standard positive pool, spanning from 1:50 to 1:25600 in duplicate, to produce ten standard points which were assigned a value in ELISA Arbitrary Units (EAU). The lowest value of the standard curve was assigned a value of 390.625, which was set as the limit of detection for the ELISA. For anti-CCHFV Gc responses a three-fold standard curve was established from a standard positive pool, spanning from 1:200 to

1:3.94x10^6 in duplicate, to produce ten standard points that were assigned values in EAU. The lowest dilution was assigned a value of 13.72 EAU, and was set as the limit of detection for the ELISA. Internal controls were included at 1:400 dilution. Negative control wells contained casein alone. Plates were incubated for 2h at r.t. Plates were washed 6x with PBS-Tween and 50µl of A1418 alkaline phosphatase-conjugated goat anti-mouse IgG diluted 1:5000 in casein, was added to each well, and incubated for 1 hour at r.t. Plates were washed 6x in PBS-Tween and developed by addition 100µl per well pNPP substrate. Optical density (OD) values at 405nm were measured with a Bio-tek ELx800 Microplate Reader. Standard curve OD values were fitted to a 5-parameter logistic model (Graphpad Prism v9.0- 9.1.1) and test sera EAUs were calculated by interpolation to the standard curve.

2.2.3 Pseudotyped virus assays

2.2.3.1 Production of pseudotyped lentivirus

Producer cells were seeded in a 6-well dish at 1x10^6 cells per well in 2 ml 10% DMEM, and subsequently incubated at 37ºC 5%CO_2 for 24h. Following incubation producer cells were transfected with 1.5µg pCSFLW, 1µg p8.91, and 1µg of either negative control plasmid expressing no heterologous glycoprotein, positive control plasmid expressing EBOV GP, or nairovirus GPC plasmid expressing the del10 variant of either CCHFV or NSDV GPC. DNA was added to 200µl OptiMEM, and mixed with 10.5µg PEI transfection reagent and incubated for 20 minutes at RT. Transfection mixtures were transferred onto producer cells then incubated for 24h post transfection at 37ºC 5%CO_2. After 24h media on producer cells was exchanged for 3ml 10%DMEM followed by a further 48h incubation at 37ºC 5%CO_2. Following incubation supernatant containing lentiviral pseudotypes was harvested for either immediate usage or long term storage at -80ºC.

2.2.3.2 Production of CCHFV tec-VLPs

Producer cells were seeded in a 6-well dish at $5x10^5$ cells per well in 2ml 5%DMEM, and subsequently incubated at 37ºC 5%CO_2 for 24h. Following incubation each well of producer cells for tec-VLPs were transfected with 952ng pCAGGS T7 polymerase, 238ng pLCK-VL nanoluc, 952ng pCAGGS CCHFV NP, 476ng pLCK CCHFV RdRP, and 2381ng pCAGGS GPC for a total of 5µg of DNA. Each well of producer cells for negative control tec-VLPs were transfected with 952ng cCAGGS T7 polymerase, 238ng pLCK-VL nanoluc, and 3810ng negative control plasmid for a total of 5µg. DNA was added to 250µl OptiMEM and mixed with 15µg LT1 transfection reagent then incubated for 20 minutes at RT. Transfection mixtures were transferred onto producer cells then incubated for 24h at 37ºC 5%CO_2. After 24h media on producer cells was exchanged for 2ml 5%DMEM followed by a further 48h incubation at 37ºC 5%CO_2. Following incubation supernatant containing CCHFV tec-VLPs was harvested for either immediate use, or long term storage at -80ºC.

2.2.3.3 Production of pseudotyped VSV

Producer cells were seeded in a 6-well dish at $1x10^6$ cells per well in 2ml 10%DMEM, and subsequently incubated at 37ºC 5%CO_2 for 24h. Following incubation each well was transfected with 2µg of either a negative control plasmid, a positive control plasmid expressing VSV-G, or nairovirus GPC plasmid expressing the del10 variant of either CCHFV or NSDV GPC. DNA was added to 200µl OptiMEM and mixed with 6µg LT1 transfection reagent then incubated for 20 minutes at RT. Transfection mixtures were transferred onto producer cells then incubated for 24h at 37ºC 5%CO_2. After 24h media was removed and producer cells were infected with 10^8 RLU VSV-ΔG virions diluted in 500µl OptiMEM. Infections were incubated for 1h at 37ºC 5%CO_2. Subsequent to infection, producer cells were rinsed 4x with 500µl PBS, and 2x with 1ml PBS and 2ml

of 2%DMEM was added before incubation for 24h at 37ºC 5%CO_2. Following 24h incubation supernatant containing pseudotyped VSV was harvested and either used immediately or stored for later use at -80ºC.

2.2.3.4 Infection of target cells with pseudotyped lentivirus

Target cells were seeded in a 96-well sterile white plate at $2x10^4$ cells per well in 100µl 10%DMEM and subsequently incubated at 37ºC 5%CO_2 for 24h. For the infection 55µl supernatant containing pseudotyped lentivirus was mixed with 55ul of 0% DMEM in a sterile 96-well plate and incubated for 1h at 37ºC 5%CO_2. Media was removed from target cells and then 100µl of each supernatant media mix was transferred onto target cells for infection. Infections were incubated for 24h at 37ºC 5%CO_2 before media was exchanged for 100µl 10%DMEM, and incubated for a further 48h in identical conditions. Following incubation for 48h, media was removed from target cells, luminescence values were assayed using 100µl of 1:1 PBS:BrightGlo luminescent substrate. Cells were then incubated at RT for at least 5 minutes before luminescence values were measured using Skanit 4.0 software on a Varioskan Lux reader.

2.2.3.5 Infection of target cells with pseudotyped CCHFV tec-VLPs

Target cells were seeded in a 96-well sterile white plate at $1x10^4$ cells per well in 100µl 5%DMEM and subsequently incubated at 37ºC 5%CO_2 for 24h. For the infection 55µl supernatant containing pseudotyped lentivirus was mixed with 55ul of 5% DMEM in a sterile 96-well plate and incubated for 1h at 37ºC 5%CO_2. Infections were incubated for 24h at 37ºC 5%CO_2 before media was exchanged for 100µl 10%DMEM, and incubated for a further 24h in identical conditions. Following incubation, media was removed from target cells, luminescence values were assayed using 50µl of NanoGlo luminescent

substrate. Cells were then incubated at RT for at least 10 minutes before luminescence values were measured using Skanit 4.0 software on a Varioskan Lux reader.

2.2.3.6 Infection of target cells with pseudotyped VSV

Target cells were seeded in a 96-well sterile white plate at $2x10^4$ cells per well in 100µl 10%DMEM and subsequently incubated at 37ºC 5%CO_2 for 24h. For the infection 55µl supernatant containing pseudotyped lentivirus was mixed with 55ul of 2% DMEM in a sterile 96-well plate and incubated for 1h at 37ºC 5%CO_2. Media was removed from target cells and then 100µl of each supernatant media mix was transferred onto target cells for infection. Target cells were then incubated for 72h at 37ºC 5%CO_2. Following incubation, media was removed from target cells, luminescence values were assayed using 100µl of 1:1 PBS:BrightGlo luminescent substrate. Cells were then incubated at RT for at least 5 minutes before luminescence values were measured using Skanit 4.0 software on a Varioskan Lux reader.

2.2.3.7 Pseudotype Virus Neutralisation Test using CCHFV tec-VLPs

Target cells were seeded in a 96-well sterile white plate at $1x10^4$ cells per well in 100µl 10%DMEM and subsequently incubated at 37ºC 5%CO_2 for 24h. Serial dilutions were prepared in duplicate to a final volume of 55µl 5% DMEM in a 96-well plate. Dilutions began at 1/20, with 5 two fold serial dilutions being performed for each serum sample. To each assayed well 55µl of supernatant was added containing CCHFV tec-VLPs. Alongside 6 replicates of serum free positive and negative standards were prepared using CCHFV tec-VLPS and negative tec-VLPs respectively. Serum pseudotype mixtures were then incubated for 1h at 37ºC 5%CO_2. Following 1h incubation, media was removed from target cells and 100µl of each serum pseudotype mixture was transferred onto target cells

and then incubated for 24h at 37ºC 5%CO_2. Following incubation media was exchanged for 100µl 5%DMEM and cells were incubated for a further 24h in identical conditions. Following the second incubation, media was removed from target cells, luminescence values were assayed using 50µl of NanoGlo luminescent substrate. Cells were then incubated at RT for at least 10 minutes before luminescence values were measured using Skanit 4.0 software on a Varioskan Lux reader.

2.2.3.8 Pseudotype Virus Neutralisation Test using NSDV pseudotyped VSV

Target cells were seeded in a 96-well sterile white plate at $2x10^4$ cells per well in 100µl 10%DMEM and subsequently incubated at 37ºC 5%CO_2 for 24h. Serial dilutions were prepared in duplicate to a final volume of 55µl 2% DMEM in a 96-well plate. Dilutions began at 1/20, with 5 two fold serial dilutions being performed for each serum sample. To each assayed well 55µl of supernatant was added containing NSDV pseudotyped VSV. Alongside 6 replicates of serum free positive and negative standards were prepared using NSDV pseudotyped VSV and negative pseudotyped VSV respectively. Serum pseudotype mixtures were then incubated for 1h at 37ºC 5%CO_2. Following 1h incubation, media was removed from target cells and 100µl of each serum pseudotype mixture was transferred onto target cells and then incubated for 72h at 37ºC 5%CO_2. Following incubation, media was removed from target cells, luminescence values were assayed using 100µl of 1:1 PBS:BrightGlo luminescent substrate. Cells were then incubated at RT for at least 5 minutes before luminescence values were measured using Skanit 4.0 software on a Varioskan Lux reader.

2.2.4 Anti-ChAdOx1 neutralisation assay

2.2.4.1 Anti-ChAdOx1 neutralisation assay with human serum/plasma samples

Nab titres to the ChAdOx1 vector were measured using a SEAP reporter assay, measuring the reciprocal of serum/plasma dilution required to reduce expression of SEAP in ChAdOx1-SEAP infected cells by 50%, 24 hours post infection. ChAdOx1-SEAP was pre-incubated with and equal volume of serially diluted, heat inactivated (90 minutes at 56ºC) human plasma or serum for 1 hour at 37ºC. Dilutions were 1:18, 1:72, 1:288, 1:1152 and 1:4608. Serum or plasma with known neutralisation titres were used as positive and negative controls. Following incubation, 200µl of serum/plasma:virus dilutions were used to infect Griptite 293 cells (Invitrogen, USA), seeded in 96 well plates at $3x10^4$ 24 hours prior to infection. Serum/plasma: virus dilutions were exchanged for 200µl fresh media. Cell supernatants were collected 24 hours post transduction, and SEAP concentration was quantified using the Tropix PhosphaLite Chemiluminescent Assay Kit (Applied Biosystems, UK). Luminescence was measured using a Varioskan Flash Luminometer (ThermoScientific, UK). Neutralisation titres were expressed as the reciprocal of the serum/plasma dilution which reduced SEAP expression by 50%, by linear interpolation from adjacent values.

2.2.4.2 Anti-ChAdOx1 neutralisation assay with mouse serum samples

Nab titres to the ChAdOx1 vector were measured using a SEAP reporter assay, measuring the reciprocal of serum: virus dilution required to reduce expression of SEAP in ChAdOx1-SEAP infected cells by 50%, 24 hours post infection. ChAdOx1-SEAP was pre-incubated with an equal volume of serially diluted, heat inactivated (30 minutes at 56ºC) human plasma or serum for 1 hour at 37ºC. Dilutions were 1:72, 1:288, 1:1152, 1:4608, and 1:18432. Serum or plasma with known neutralisation titres were used as

positive and negative controls. Following incubation, 50µl of serum:virus dilutions were used to infect Griptite 293 cells (Invitrogen, USA), seeded in 96 well plates at $3x10^4$ 24 hours prior to infection. Serum/plasma: virus dilutions were exchanged for 200µl fresh media. Cell supernatants were collected 24 hours post transduction, and SEAP concentration was quantified using pNPP substrate. Optical density (OD) values at 405nm were measured with a Bio-tek ELx800 Microplate Reader. Neutralisation titres were expressed as the reciprocal of the serum/plasma dilution which reduced SEAP expression by 50%, by linear interpolation from adjacent values.

2.2.5 Analysis

Statistical analysis was performed using Graphpad Prism version 9.1.1. For data that was normally distributed, t-tests were used to compare differences between two groups, or Brown-Forsythe and Welch's ANOVA with Dunnett's T3 multiple comparisons test was used to compare differences between multiple groups. Non-parametric data were analysed by Mann-Whitney U test for differences between two groups or Kruskal-Wallis test was used to compare multiple groups of non-parametric data. $P<0.05$ was considered the threshold of significance.

Chapter 3 – Development of Pseudotype Neutralisation Tests for CCHFV and NSDV

3.1 Introduction

When developing effective therapeutics and vaccines against emerging diseases, there is a need to assess the efficacy of interventions against the disease of interest. Due to the inherent pathogenicity of many emerging diseases, and the high morbidity and mortality of these viruses, often high containment facilities are necessary to work with live virus[262]. High containment facilities are expensive to build and maintain, and require costly and highly specialised training for staff to operate safely in the facility, further increasing the cost[274].

In the UK CCHFV is designated as an Advisory Committee on Dangerous Pathogens (ACDP)-4 pathogen[275], due to high associated mortality of infection, and the lack of vaccines and therapeutics, to prevent or treat infection respectively. ACDP-4 pathogens need handling in containment level (CL) 4 facilities[275], requiring multiple levels of protection between the researcher and the pathogen of interest. Type and quantity of experiments possible under such conditions are limited and create a bottleneck in research on live CCHFV virus. It has been argued that CCHFV research should be downgraded to CL-3 facilities to increase the number of facilities able to participate in research and development of therapeutics and vaccines against CCHFV[108], though the risks of accidental release or infection would be increased by these measures.

Handling of live NSDV requires Specified Animal Pathogen Order (SAPO) level 4 facilities in UK[276], a different class of restriction, for pathogens that present a risk to wildlife and livestock but not to human health, and lack effective vaccines or therapeutics. While SAPO-4 facilities[277] are less restrictive than CL-4 facilities, due to the

lack of danger to human life, they are still expensive to maintain and perform experiments in. Again, expense and difficulty limit the breadth and pace of research into NSDV therapeutics and vaccines.

Due to the expense and difficulties of working with CCHFV and NSDV, the production of models and methodologies which can be used to replicate virus functionality in lower containment setting is essential to allow therapeutics and vaccines to be rapidly and accurately assessed.

As discussed in chapter 1, pseudotyping is a process of producing replication deficient viral particles, which incorporate heterologous envelope proteins within the viral particle, altering the tropism of the particle to reflect the targets of the heterologous protein[265,278]. As such, pseudoparticles can replicate wild type virus behaviour in specific assays, without exposing researchers or the environment to the same risks as live wild type virus.

The following chapter discusses the assessment of a range of pseudotyping systems for their use with CCHFV and NSDV glycoproteins, and their utility in developing virus neutralisation tests in BSL-2 level facilities.

3.1.1 Virus Neutralisation Tests

Virus neutralisation tests (VNTs) are *in vitro* assays that assess the ability of antibodies or inhibitors to neutralise a virus and prevent entry into target cells. A VNT needs a virus, a method of neutralisation and a reporter system to assay whether infection has taken place. Within these three requirements, there is a high level of variation between the different components leading to a large heterogeneity in described VNTs[279]. As such independent comparisons between assays can be difficult to make, though neutralisation responses often correlate across different assays[280].

The viral component can be either live virus or a pseudotype virus, with each offering advantages and disadvantages[281]. Use of live virus is considered optimal but as discussed previously, the need for containment of highly pathogenic viruses can be expensive and rate limiting. Work with replication deficient pseudotypes circumvents the issues of high containment levels, but may be considered more of an approximation of virus function. However, the perceived limitations of only using viral envelope proteins can prevent greater inference into their specific function in infection[281]. Correlations of neutralisation activity are often strong between live virus and pseudotype systems, and establishing a correlation is crucial to validating the accuracy of new pseudotyped assays[282].

VNTs help measure the ability of neutralising antibodies to bind to viral proteins and prevent entry into target cells. These antibodies can be purified monoclonal antibodies, to test their efficacy as therapeutics[283]. Alternatively, in assessing immunogenicity in response to vaccination or infection, serum, plasma, or other antibody containing fluids are used in the VNT, to measure the production of neutralising antibodies[284,285].

A VNT needs a reporter system to quantify the levels of infection by the viral component. With wild type live virus, the signal is often cytopathic effects on the cell layer, which lead to the formation of plaques[279]. Replication then allows the virus to spread from the initial infected cell and cause damage or death in the surrounding cells. In these cases, reductions in the number and size of plaques show evidence of neutralisation. With pseudotyping systems, which will not replicate and cause cell death, a reporter system is needed. Reporter systems often take the form of plasmids expressing fluorescent or luminescent proteins, which are packaged in the pseudotyped virus[286]. These plasmids are then expressed upon infection of the target cell and the fluorescent or luminescent signal used as a measurement of infection.

Figure 3.1 shows a generalised schematic of how the VNTs described throughout the rest of this chapter operate, using a pseudotyped virus, mouse serum containing antibodies,

and a luciferase based reporter system. In figure 3.1 the pseudotyped virus contains a luciferase reporter gene, which will be expressed upon entry into target cells. When incubated with neutralising antibodies as shown in path A, the antibodies block pseudotyped virus infection of the target cells and no luminescent signal is measured. In the right hand path, incubation of pseudotyped virus with non-neutralising antibodies does not block infection of the target cells and a luminescent signal is measured.

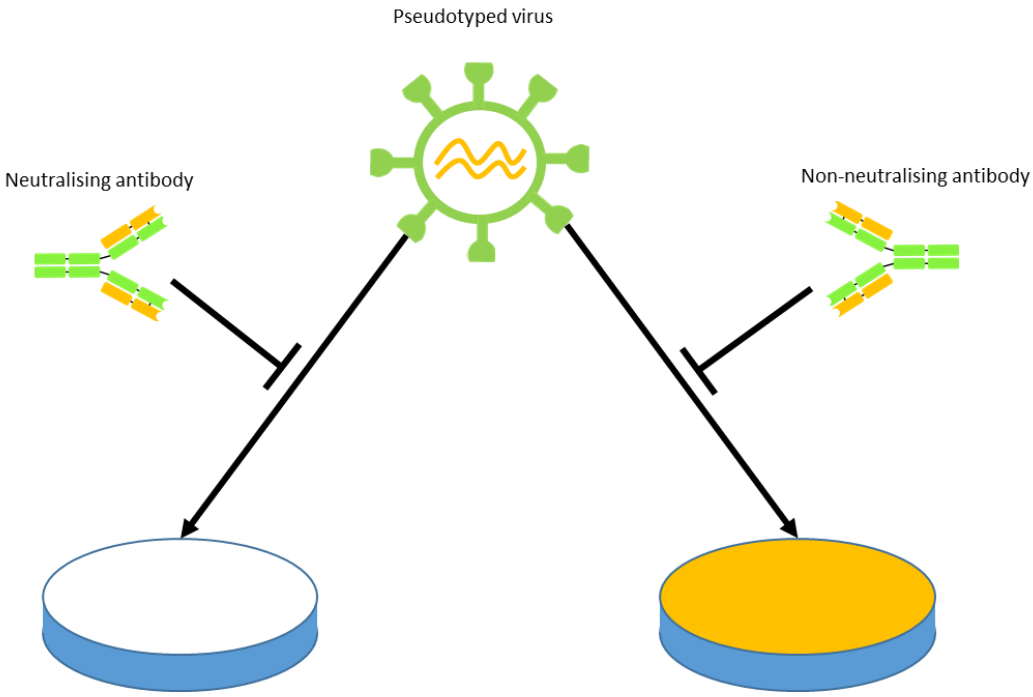


Figure 3.1: Virus neutralisation utilising luciferase expressing pseudotyped virus
Pseudotyped virus containing luciferase expressing genetic material is mixed with serum or plasma containing antibodies, and subsequently introduced to target cells for infection. The presence of neutralising antibodies in the serum or plasma blocks infection of target cells. 48-72 hours post infection, luminescence of target cells is assessed using a luminescent substrate.

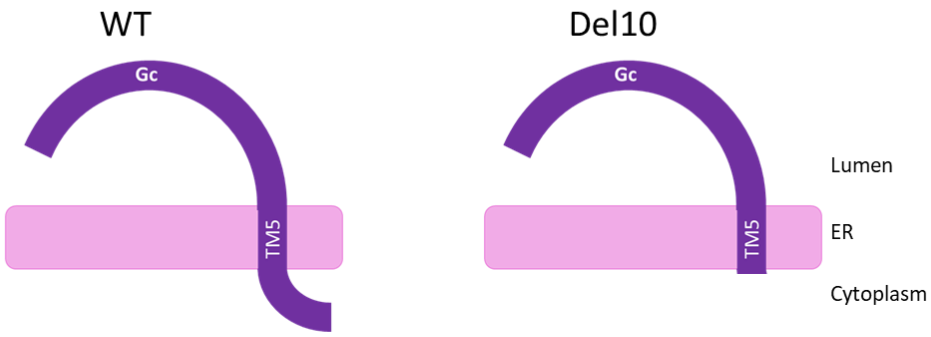
3.1.2 Prior use of truncated Nairovirus Glycoproteins

When developing a pseudotype virus, it is important to consider the heterologous envelope proteins. For nairoviruses, pseudotyping utilises the glycoprotein precursor (GPC), responsible for cell entry in live viruses, and the main antigen targeted by the vaccines developed in this thesis. The GPC is expressed from the M segment of nairovirus genomes, and is proteolytically processed into five glycoproteins, of which Gn and Gc are presented on the envelope surface[88].

Previous research into the pseudotyping of CCHFV has suggested that the GPC performs relatively poorly in producing infectious pseudoparticles[287,288]. Further research showed that C-terminal truncations of the CCHFV GPC increased expression in a VSV based pseudotyping system[288], with the most effective being a 50 amino acid truncation, which led to a 30 fold increase in pseudotype production (figure 6.2 B). The truncation occurs only a few amino acids beyond the predicted final TM helix of CCHFV Gc, cleaving the cytoplasmic tail of CCHFV Gc. The truncation does not reflect the natural cleavage of CCHFV Gc during proteolytic processing[289], and as such the actual function of cleaving the cytoplasmic tail has not been explored. The original authors speculated that the reduction in length of the cytoplasmic tail of CCHFV Gc, improved the ability of the CCHFV Gn and CCHFV Gc complex to package in the VSV virions of their pseudotyping system. Figure 3.2 A shows a topographical diagram comparing the wild type nairovirus Gc subunit to the truncated variant, referred to as Del10 in both the original literature and this DPhil thesis, due to the length of the remaining cytoplasmic tail.

At the time of beginning NSDV pseudotyping, there was no previous literature on the production of NSDV pseudotypes. As such, the design has been informed by pseudotyping CCHFV, and the work throughout this chapter uses nairovirus del10 mutants as the predominant glycoproteins, unless otherwise stated.

A



B

Variant	CCHFV VSV titre (RLU/ml)
Full length	200
Del10	6,250

Figure 3.2: Del10 variants of nairovirus GPC
The figure shows the effects of the truncation in the del10 mutation for nairovirus GPC. (**A**) Topographical diagram showing the effect of the 50 amino acid del10 truncation on the Gc subunit of nairovirus GPC. (**B**) Table adapted from Suda et al 2014, showing CCHFV VSV titres with full length glycoprotein, and Del10 truncation.

3.2 Results

For successful pseudotyping to occur several different conditions are needed to produce replication deficient virions which bear CCHFV or NSDV glycoproteins on their cell surface. The target cells used for production need to be able to correctly express the heterologous glycoprotein, and correctly present it. For large polyproteins, like nairovirus GPCs, multiple proteases are required to ensure correct cleavage into the requisite subunits. These subunits then need to be correctly presented to be incorporated into the virions of the pseudotype virus, and the efficacy of incorporation may change with the properties of different pseudotyping systems. If there are issues in the producer cells with processing the heterologous glycoproteins, or indeed the pseudotyping system, pseudotyped virions will not be produced effectively or in quantities necessary for future experimentation.

If pseudotypes are successfully produced, the target cells then need to be permissive to the heterologous glycoprotein used, to allow cell entry to the pseudotyped virions. The correct target cells are those which contain the receptor for the target virus, in these cases CCHFV and NSDV, or if receptors are unknown, cell lines derived from tissues permissive to CCHFV or NSDV. If pseudotypes are produced but cannot enter the target cells reporters will not be expressed and no signals will be observed. Cell lines which can be used to passage the WT virus are ideal as they are known to be both permissive to infection with the virus, and able to correctly process the heterologous glycoproteins.

3.2.1 Pseudotyping of Nairovirus Glycoproteins in a lentiviral system

Pseudotyping activity was first observed using lentiviruses[290,291], and their use continues for a range of different viruses including filoviruses and influenza[272,286]. The system used in this thesis utilises a replication deficient HIV genome but other lentiviruses are

effective for pseudotyping purposes[292–294]. Lentivirus based systems have several advantages for pseudotyping. Firstly, they are simple, requiring only the transfection of plasmids into producer cells, unlike other systems which may require separate infection and transfections. Secondly, lentivirus based systems can pseudotype a wide range of heterologous envelope proteins into the lentivirus virion, most recently being widely used in VNTs during the SARS-CoV-2 pandemic to assess vaccine immunogenicity[295] and the neutralisation capacity of convalescent serum[296].

Lentivirus pseudotypes can have a wide range of uses as shown by work with bunyaviruses, a virus family which includes RVFV, Hantavirus and SFTSV as wells as CCHFV and NSDV. Bunyavirus glycoproteins have been successfully pseudotyped in lentiviral vectors for a wide range of uses showcasing the potential of lentiviral systems. RVFV lentivirus pseudotypes have been used to develop neutralisation tests[297], SFTS lentivirus pseudotypes have been used to investigate virus entry into target cells[298], while Hantavirus pseudotypes have been used as vaccines to induce protection in mice[299], and to target gene therapies for vascular injury[300]. When the presented work was performed there was no literature on successful pseudotyping of nairoviruses in lentiviral systems, but subsequently successful CCHFV pseudotyped lentivirus has been demonstrated[301].

Figure 3.3 shows a schematic of the lentivirus production methods used throughout the following section. Target cells are co-transfected with three plasmids; p8.91, pCSFLW, and the heterologous glycoproteins. The p8.91 plasmid expresses the *gag* and *pol* of the HIV-1, on a single plasmid. The pCSFLW plasmid contains a firefly luciferase gene surrounded by packaging signals from HIV-1 which allow RNA encoding firefly luciferase to be packaged into the lentivirus pseudotypes. Plasmids containing heterologous glycoproteins are co-transfected to pseudotype the lentiviral particles. Expression of *gag-pol* allows formation of HIV virions from the ER of the infected cell, which can also incorporate the heterologous glycoproteins if properly expressed. These

pseudotyped virions will be secreted from cells into the supernatant, from which they can be harvested, and used to further infect cells matching the tropism of the heterologous glycoprotein.

3.2.1.1 Production of Nairovirus pseudotyped lentivirus in HEK293T cells

Development of a nairovirus pseudotype VNT requires identification of suitable producer cells and target cells. Producer cells are transfected with the plasmids necessary to assemble and express pseudotyped lentivirus. Target cells need to be permissive to the heterologous glycoproteins CCHFV or NSDV glycoproteins, to allow pseudotype infection. The specific receptors used by CCHFV and NSDV for cell entry are unknown. However, both diseases are known to infect a wide range of tissues within their target organisms[106,141], suggesting that they can either target multiple receptors, or the target receptor is found on a variety of cell types within the target organisms.

Initially the suitability of HEK293Ts cells was assessed as a producer cell for nairoviral pseudotypes. HEK293T cells are a secondary cell line, derived from human embryonic kidney (HEK) cells. HEK293T cells offered several advantages as a cell type, due to their rapid growth and resilience as a cell line, their permissiveness to transfected DNA, and high levels of protein production[302]. In addition, HEK293T cells have been used as producer cells extensively in bunyavirus pseudotyping, including production of RVFV and SFTS pseudotype lentivirus, and production of CCHFV pseudotyped VSV. As such HEK293T cells were used in initial transfections for production of CCHFV and NSDV pseudotyped lentivirus.

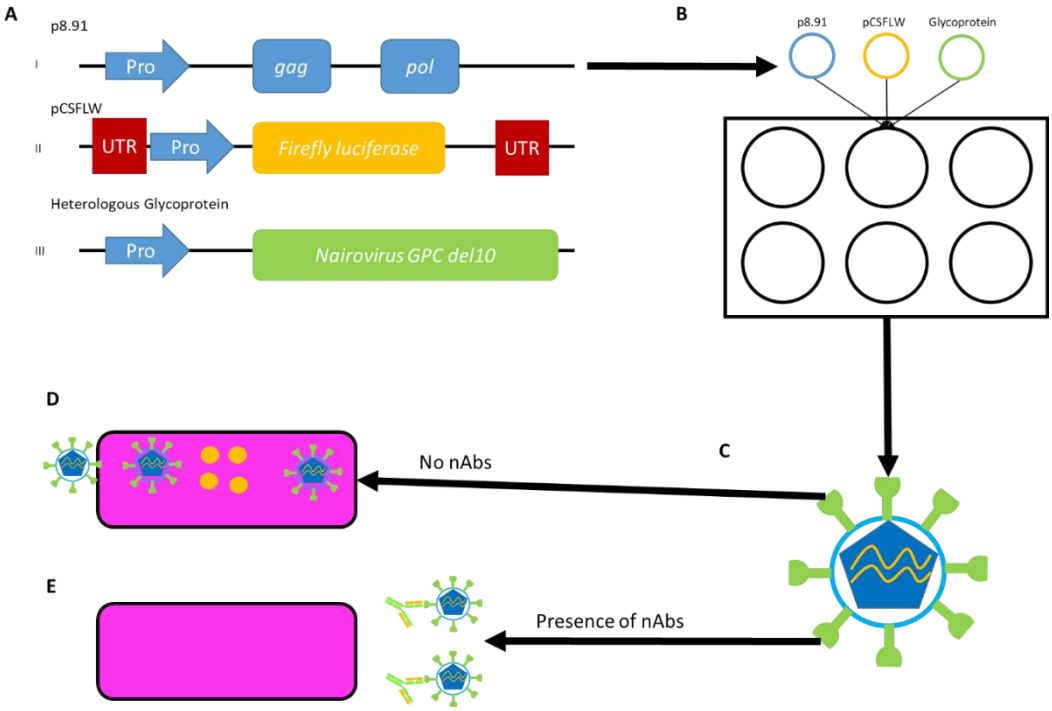


Figure 3.3: Production of nairovirus pseudotyped lentivirus
Figure shows the production and use of nairovirus pseudotypes in a lentivirus systems. (**A**) The plasmids used in production of lentivirus I) p8.91 expressing HIV *gag-pol* II) pCSFLW expressing firefly luciferase flanked by HIV UTR sequences for packaging into the lentivirus virion III) Heterologous glycoprotein plasmid expressing the del10 variant of a nairovirus GPC. (**B**) The three plasmids are transfected into producer cells to produce pseudotyped lentivirus. (**C**) Producer cells secrete pseudotyped lentivirus, displaying nairovirus GPC on the envelope surface of the HIV virion and carrying firefly luciferase genes. (**D**) Without neutralising antibodies (nAbs) pseudotyped lentivirus can infect target cells and firefly luciferase is expressed within the cells. (**E**) Neutralising antibodies can bind to and neutralise the pseudotyped lentivirus and prevent infection and luciferase expression.

Figure 3.4 shows the luminescence of target cells after infection with supernatant harvested from lentivirus producing cells. In the assay negative controls used supernatant harvested from cells transfected with an expression vector without any heterologous glycoprotein. The positive control was produced using supernatant harvested from cells transfected with a plasmid expressing EBOV glycoprotein, which pseudotypes well in a lentivirus system, representing a good positive control[303].

The pseudotypes were initially tested with two different target cells, in HEK293T cells and Vero cells, which are a cell line derived from green African monkey kidney cells. Both cell types had been previously shown to be permissive to CCHFV infection[304]. Vero cells have further been used as targets cells for CCHFV VSV pseudotypes[288], and are permissive to NSDV infection[305].

Figure 3.4A presents the results of using HEK293T cells as targets. Across 6 replicates, the luminescence in RLU from CCHFV and NSDV pseudotyped lentivirus showed no significant difference from the luminescence in the negative control. The strong signal of the EBOV pseudotyped positive control, approximately 3 orders of magnitude greater than negative control, suggests there are no issues with the production of lentivirus in HEK293T cells or the luciferase substrate. In figure 3.4B, the results of Vero cells as the target cells for pseudotyped lentivirus produced in HEK293T cells are presented. As target cells a difference was observed, with lower luminescence measured in both the negative and positive control pseudotypes in Vero target cells compared to HEK293T targets. However, a similar pattern is observed with CCHFV and NSDV pseudotypes with no significant difference in luminescent signal measured between the nairovirus pseudotyped lentivirus and the negative controls.

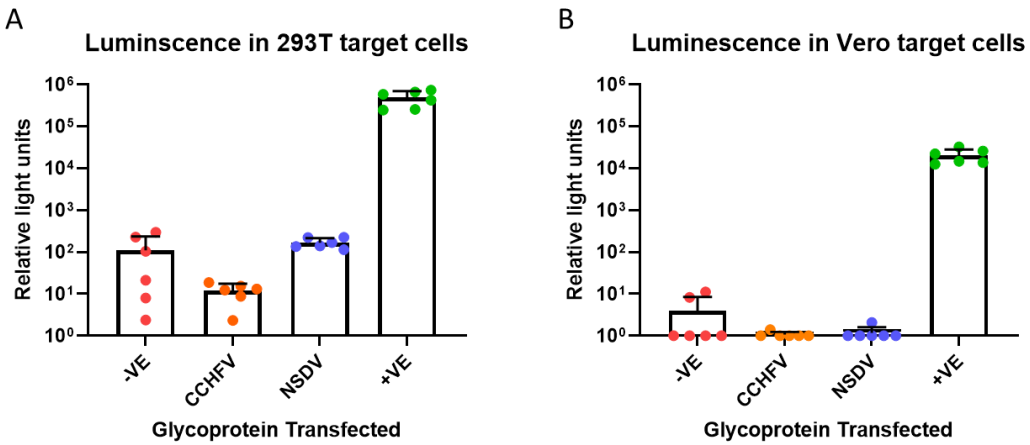


Figure 3.4: Luminescence in cells infected with CCHFV and NSDV pseudotyped lentivirus generated from 293T producer cells.
Infection performed by 100µl of 1:1 10%DMEM:Pseudoparticle supernatant added to target cells seeded at $2x10^4$ cells 24 hours prior to infection. Cells incubated at 37°C 5% CO_2 for 72h post infection. Cellular luminescence measured 5 minutes after lysis with 100µl 1:1 PBS:BrightGlo™ luciferase substrate. Group labels represent the heterologous glycoprotein used to produce pseudotyped lentivirus. The negative control (-VE) used non-pseudotyped lentivirus, without a heterologous glycoprotein. The positive control (+VE) uses lentivirus pseudotyped with EBOV glycoprotein. (**A**) Luciferase dependent luminescence in 293T target cells infected with pseudotyped lentivirus. All wells shown as individual data points (n=6) in Relative light units with mean and standard error of the mean also shown. (**B**) Luciferase dependent luminescence in Vero cells infected with pseudotyped lentivirus. All wells shown as individual data points (n=6) in Relative light units with mean and standard error of the mean also shown. All negative values relative to background have been plotted with a value of 1.

With HEK293T cells as producer cells, there was no observable signal from pseudotyping CCHFV and NSDV in lentivirus across multiple permissive target cells. There is however conclusive evidence that successful pseudotyping has taken place in the HEK293T cells, in the form of the positive control. This evidence, along with the previously referenced permissiveness of the target cells to CCHFV and NSDV, precludes issues with the lentiviral plasmids or luminescent reporter system as being behind the lack of signal messured from CCHFV and NSDV pseudotyped lentivirus. Therefore it would appear there is an issue with the incorporation of CCHFV and NSDV into lentivirus virions in HEK293T cells.

The aim of this work was to produce effective VNTs as low containment alternatives to use of live CCHFV and NSDV, however initial attempts to produce pseudotyped lentivirus were unsuccessful. Further research investigated the use of different cell types on pseudotyping success.

3.2.1.2 Production of Nairovirus pseudotyped lentivirus in Huh7 cells

Following unsuccessful attempts to produce CCHFV and NSDV pseudotyped lentivirus in HEK293T cells, other producer cells were used in an attempt to more successfully pseudotype lentivirus with nairovirus GPCs. Huh-7 cells were used as a variant producer cell line. Huh-7 cells are a hepatoma derived cell line[306], used for propagation of live CCHFV[114], showing that the cell line can both process CCHFV proteins, and is susceptible to infection from CCHFV. Huh-7 cells are also susceptible to EBOV infection[307], therefore EBOV pseudotyped lentivirus can continue to be used as a positive control.

Figure 3.5 shows the results of infecting 293T target cells with lentivirus pseudotypes produced in Huh-7 cells. No significant differences were measured between the

luminescence induced by NSDV pseudotyped lentivirus and the negative control. However, the luminescent signal from CCHFV pseudotyped lentivirus was significantly greater than the negative control, an effect maintained upon multiple repeats. As previously, EBOV pseudotyped lentivirus was used as the positive control and showed a signal approximately four orders of magnitude greater than the negative control.

Though the difference between the CCHFV signal and the negative control was statistically significant, the separation between the signals was relatively small, with the mean signal of CCHFV pseudotyped lentivirus being approximately 6 times greater than the mean of the negative control. The signal to background ratio is consistent with previous work which suggests CCHFV pseudotyping produces low titres, with CCHFV pseudotyped VSV production only showing an 8 fold increase against background previously[287]. Compared to the signal to background ratios utilised for pseudotype VNTs such as EBOV pseudotyped lentivirus[224] the ratios presented by CCHFV pseudotyped lentivirus are poor.

With little separation between CCHFV pseudotyped lentivirus signal and background noise, it would be difficult to develop a pseudotype VNT for CCHFV neutralising antibodies using CCHFV pseudotyped lentivirus. As such, despite production being observed from Huh-7 cells, it is unlikely nairovirus pseudotyped lentivirus can be used to within the scope of this thesis in a successful VNT for assessing antibodies neutralising CCHFV and NSDV. With a lack of success in lentiviral systems, research progressed onto investigating tec-VLP systems for CCHFV VNT development.

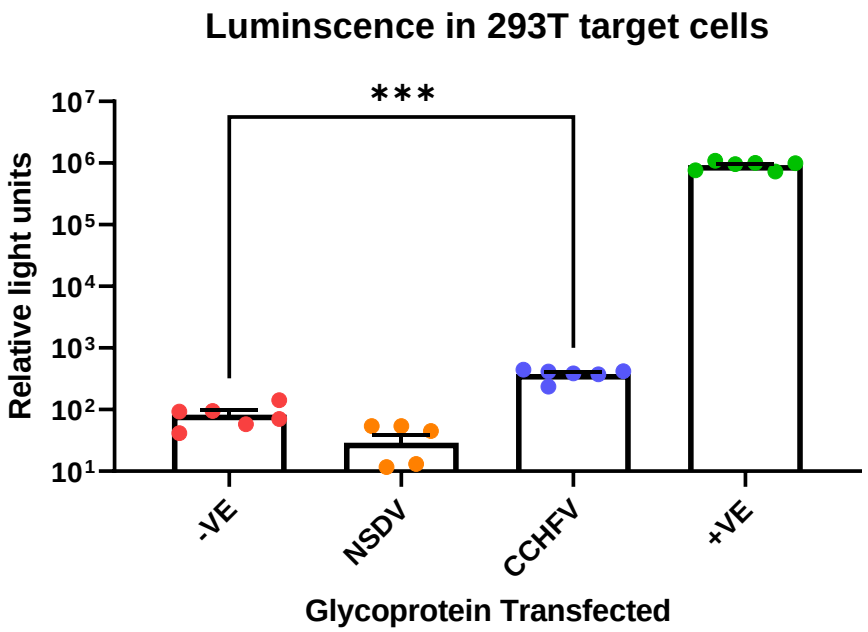


Figure 3.5: Luminescence in cells infected with NSDV and CCHFV pseudotyped lentivirus generated from Huh-7 producer cells.
Infection performed by 100µl of 1:1 10%DMEM: pseudoparticle supernatant added to target cells seeded at $2x10^4$ cells 24 hours prior to infection. Cells incubated at 37°C 5% CO_2 for 72h post infection. Cellular luminescence measured 5 minutes after lysis with 100µl 1:1 PBS:BrightGlo™ luciferase substrate. Group labels represent the heterologous glycoprotein used to produce pseudotyped lentivirus. The negative control (-VE) used non-pseudotyped lentivirus, without a heterologous glycoprotein. The positive control (+VE) uses lentivirus pseudotyped with EBOV glycoprotein. Luciferase dependent luminescence in 293T target cells infected with pseudotyped lentivirus. All wells shown as individual data points (n=6) in RLU with mean and standard error of the mean also shown. Significant differences were determined by Brown-Forsythe and Welch's ANOVA with Dunn's T3 multiple comparisons test (***p>0.001).

3.2.2 CCHFV tec-VLP Pseudotyping

With the previously discussed lentiviral system, pseudotyping efficiency and signal was not sufficient to produce a viable model for development of a pseudotype VNT. As such, work moved on to look at a further alternative system to develop a pseudotype VNT for CCHFV. Recently developed are transcript and entry competent virus like particles (tec-VLPs) for CCHFV[273,308]. These particles utilise a non-replicating CCHFV virion which carries a luminescent reporter gene. CCHFV tec-VLPs have an advantage over other pseudotyping methods in that they use all the proteins of the virus and as such can be used to assess the function of components beyond just the GPC. CCHFV tec-VLPs have been used to assess CCHFV inhibitor efficacy[273], RdRP function[308], and assess both vaccine induced[309] and naturally induced neutralising antibodies[310] against CCHFV.

Figure 3.6 shows the method of CCHFV tec-VLP production. The method shows similarities to production of pseudotyped lentivirus, requiring only a single transfection of plasmids for production. The producer cells are co-transfected with plasmids expressing the open reading frames of CCHFV S, M and L segments, expressing NP, GPC and RdRP respectively. All ORFS are expressed under the control of a T7 promoter in a pCAGGS plasmid backbone and are supplied in *trans*. Being expressed from the pCAGGs plasmid backbone, the CCHFV NP cannot package CCHFV expressing transcripts into the virions in the producer cells. As the virions do not contain the genetic material to express NP, GPC or RNARP, they cannot propagate beyond single replication cycle of production and infection of target cells. Also co-transfected is a T7 polymerase gene to facilitate expression of the CCHFV ORFs, and a luciferase reporter gene, with the 5’ and 3’ UTR of the CCHFV S segment, to facilitate packaging into the CCHFV tec-VLP and provide a reporter for tec-VLP infection. Following incubation after transfection, supernatant containing CCHFV tec-VLPs can be harvested and used to infect target cells.

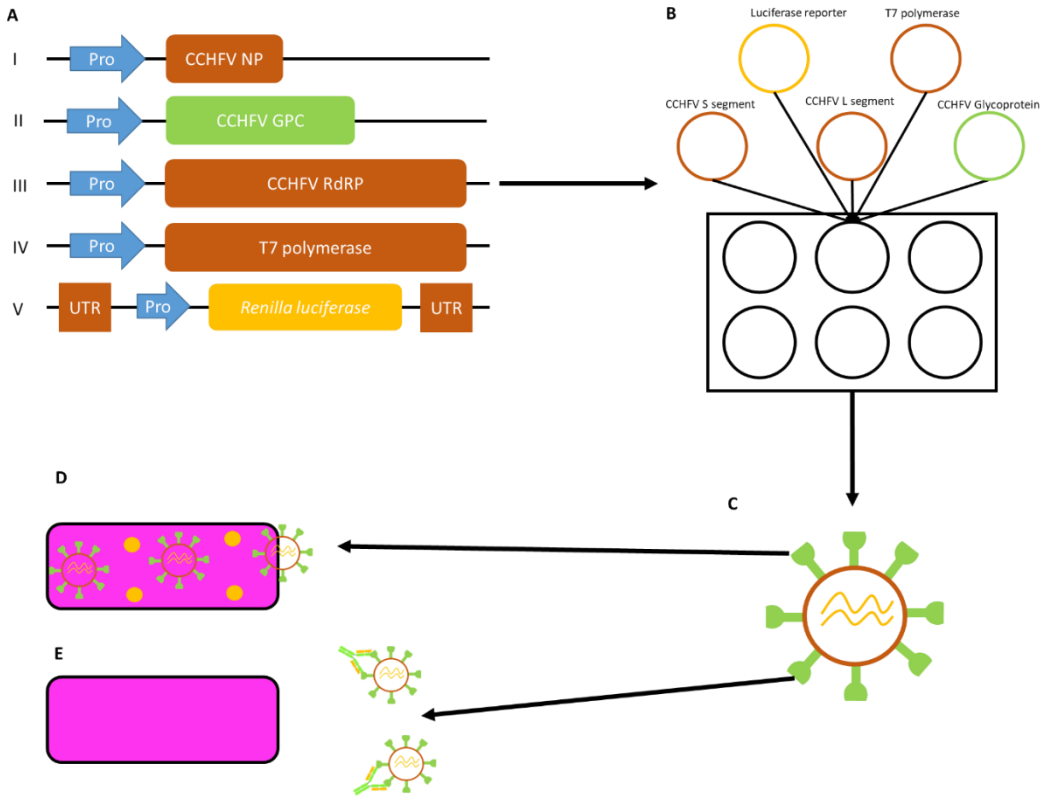


Figure 3.6: Production of CCHFV tec-VLP particles.
Figure shows the production and use of nairovirus pseudotypes in a lentivirus system. (**A**) The plasmids used in production of lentivirus I) pCAGGS expressing CCHFV NP under a T7 promoter II) pCAGGS expressing CCHFV GPC under a T7 promoter III) pLCK expressing a CCHFV RdRP under T7 promoter IV) pCAGGS expressing T7 polymerase V) pLCK expressing Renilla luciferase between the 5' and 3' UTR of CCHFV S-segment allowing packaging into the CCHFV virion. (**B**) The five plasmids are transfected into producer cells to produce CCHFV tecVLPs. (**C**) Producer cells secrete CCHFV tecVLPs, displaying CCHFV GPC on the envelope surface of the CCHFV virion and carrying renilla luciferase genes. (**D**) Without neutralising antibodies (nAbs) CCHFV tecVLPs can infect target cells and renilla luciferase is expressed within the cells. (**E**) Neutralising antibodies can bind to and neutralise the CCHFV tecVLPs preventing infection and luciferase expression.

3.2.2.1 Production of CCHFV tec-VLPs in Huh-7 cells

Unlike the previously assessed lentivirus pseudotyping system, the producer and target cells have been previously established for the CCHFV tec-VLP system, and thus have not been modified. Huh-7 cells are used as producer cells and A549, a lung carcinoma cell line, are used as targets for infection[273]. Initial work focused on establishing that the CCHFV tec-VLP production and infection previously performed in the literature could be replicated.

Figure 3.7 shows the results of infection of A549 cells with CCHFV tec-VLPs produced in Huh-7 cells. The negative control in the assay produced by transfection of all components of the T7 polymerase, luciferase reporter plasmid, and an empty pCAGGS plasmid to equal the quantity of DNA transfected. The luminescence results show clear evidence of successful tec-VLP production and infection with a significant difference measured between the negative control background and signal from CCHFV tec-VLP infection. The ratio between the signal and the background is greater than three orders of magnitude, a dynamic range sufficient to establish a pseudotype VNT for assessing CCHFV neutralisation. The del10 variant was also tested within this assay, but no evidence of packaging is observed via the luminescent reporter, likely because of the loss of the cytoplasmic tail from CCHFV GPC. The truncation may increase the expression and presentation of CCHFV Gc on the membrane as previously speculated, but these results suggest that the truncation of the CCHFV GPC tail may limit the specific packaging of the CCHFV Gc, limiting its usefulness in a CCHFV specific system rather than a generalised pseudotyping system.

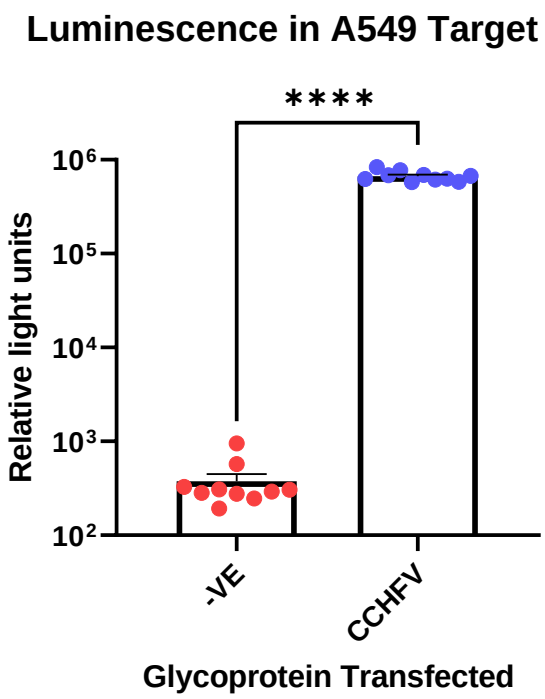


Figure 3.7: Luminescence in cells infected with CCHFV TecVLP or equivalent negative control

Infection performed by 100µl of 1:1 D5 media: TecVLP supernatant added to target cells seeded at $1x10^4$ cells 24h prior to infection. Cell incubated at 37°C 5% CO_2 for 48h post infection. Cellular luminescence measured 10 minutes after lysis with 50µl NanoGlo reagent. Group labels represent the heterologous glycoprotein used to produce pseudotyped lentivirus. The negative control (-VE) used transfection of an equal quantity of empty pCAGGS expression plasmid, with T7 polymerase and luciferase reporter. Luciferase dependent luminescence in A549 target cells infected with tecVLPs. All values shown as individual data points in Relative light units with mean and standard error of the mean also shown. Significant differences were determined by unpaired t test (****$p<0.0001$).

3.2.2.2 Establishment of pseudotype VNT using CCHFV tec-VLPs

Establishment of a pseudotype VNT with CCHFV tec-VLPs required assessing their sensitivity to antibodies against CCHFV GPC, to show their entry mechanism is CCHFV specific. As such neutralisation was assessed against a serial dilution of CCHFV specific mouse serum, to determine if the CCHFV tec-VLPs were sensitive to CCHFV antibodies, and if the response was specific compared to control serum. CCHFV specific serum was generated by vaccinating balb/c mice with a ChAdOx1-CCHFV GPC, and control serum was generated by vaccinating balb/c mice with a ChAdOx1 Control.

Figure 3.8 shows the results of luminescence signal of CCHFV tec-VLPs, neutralised by CCHFV specific mouse serum. Control serum was taken from mice vaccinated with an irrelevant ChAdOx1 control. Clear evidence of neutralisation was measured with CCHFV vaccinated serum, compared to serum from the control vaccination. Evidence of the serum specific effect is observed through the titration of the neutralisation effect as the CCHFV vaccinated serum was diluted. In combination with the lack of neutralisation measured following the use negative control serum, these results show a VNT can be established with CCHFV tec-VLPs, as infection with CCHFV tec-VLPs exhibit a large signal to background ratio, creating a large dynamic range for the assay, and show CCHFV specific inhibition, which can be used to assay vaccine induced neutralising antibodies against CCHFV.

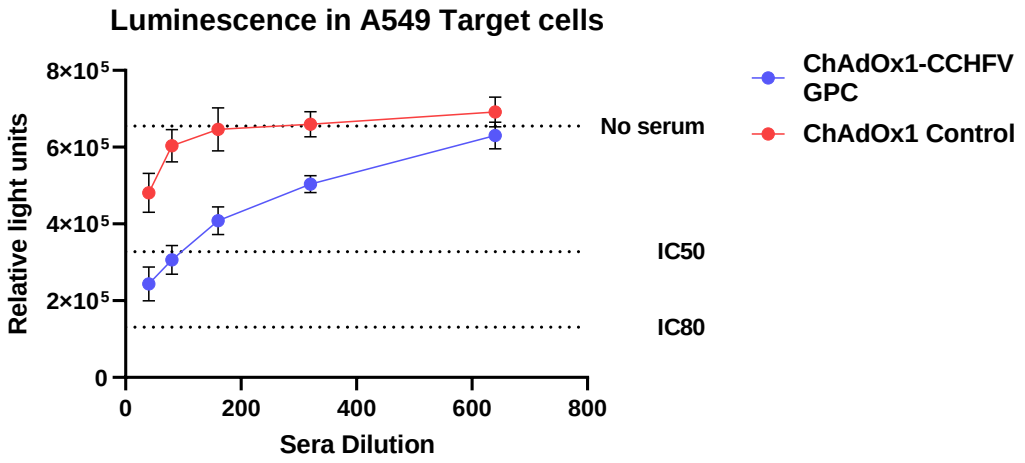


Figure 3.8: Neutralisation of CCHFV tecVLP by CCHFV GPC specific serum.
A serial dilution of vaccinated mouse serum was prepared in duplicate in D5 media to a total volume of 50µl. Fifty µl of supernatant containing CCHFV tecVLPs were incubated with the serum dilutions for 1h at 37°C 5% CO_2. CCHFV tecVLP-serum complexes were then transferred onto A549 target cells seeded at $1x10^4$ cells 24h prior to infection. Cells were incubated at 37°C 5% CO_2 for 48h post infection. Cellular luminescence was measured 10 minutes after lysis with 50µl NanoGlo luciferase substrate. Luciferase dependent luminescence in A549 target cells infected with tecVLP. The mean (n=6) is shown as a data point with SEM for each serum dilution assayed. CCHFV tecVLP only controls are shown as a line, with IC50 and IC80 thresholds.

3.2.3 Pseudotyping of Nairovirus Glycoproteins in a VSV system

Following the establishment of a VNT for assaying CCHFV neutralisation, there remained a need to establish a VNT for NSDV neutralisation. VSV pseudotyping systems are a more recent development than lentiviral systems[265], but have been used extensively for pseudotyping emerging disease for use in low containment settings. Use of VSV systems for transduction or gene therapies is less widespread, but pseudotyped VSV has been used in assessing virus glycoprotein functions and in pseudotype VNTs to assess neutralising antibodies.

VSV can be used to pseudotype virus function in both replication competent and deficient forms. VSV is often pseudotyped as a live virus, with the VSV glycoprotein (VSV-G) replaced in the VSV genome with a heterologous glycoprotein. The resulting virus is a VSV virion which presents the heterologous glycoprotein on its surface. Such live virus systems have been used with EBOV glycoproteins to produce protective vaccines[311], or with SARS-CoV-2 spike and GFP[312] to produce assay spike initiated cell entry and inhibition. Live CCHFV-VSV has been produced as a replication competent vaccine[256]. Though useful, these hybrid viruses remain replication competent and as such still require careful containment measures.

In contrast when pseudotyping replication deficient VSV, the heterologous glycoproteins are supplied in *trans*. The VSV-G gene is replaced with a reporter gene rather than a heterologous glycoprotein, generally fluorescent[313] or luminescent[265] creating a VSV-ΔG genome. Continued passage of the VSV-ΔG genome is only possible with glycoproteins supplied through transfection of infected cells. Replication deficient VSV systems have been used previously to pseudotype CCHFV GPC, for purposes of analysing GPC function in virus entry mechanisms[287,288]. CCHFV pseudotyped VSV has also been used to assess the neutralisation of antibodies from individuals previously infected with CCHFV[314].

Figure 3.9 shows the methods of producing pseudotyped VSV, a process which is more complex than the production of pseudotyped lentivirus previously described. Producer cells are initially transfected with the heterologous glycoprotein of interest. Following incubation, the transfected cells are then infected with a VSV-ΔG virions. These are VSV-G pseudotyped VSV, which infect the target cells and carry the VSV genome. Importantly, the VSV-G is supplied in *trans* during the production of these particles, and the VSV-ΔG genome cannot propagate independently. Following infection and transfection of the cells, supernatant is harvested which contains pseudotyped VSV particles, which can be used to infect further cells for pseudotype VNTs.

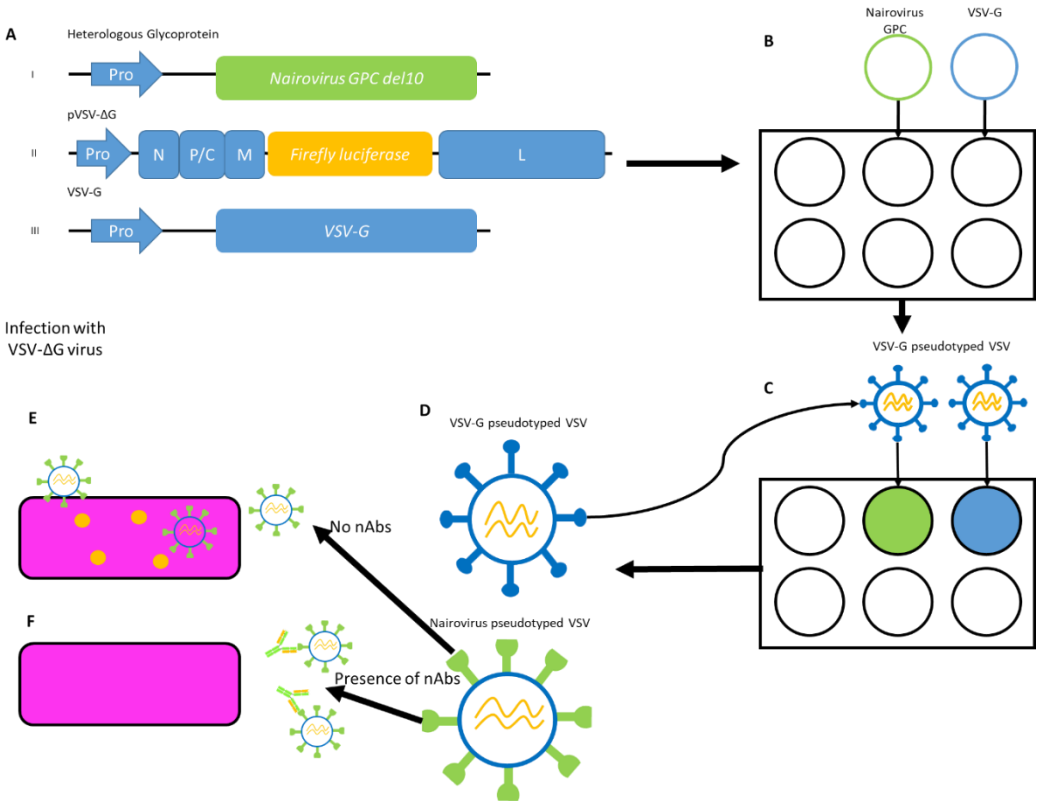


Figure 3.9: Production of nairovirus pseudotyped VSV
Figure shows the production and use of nairovirus pseudotypes in a VSV system. (**A**) The plasmids used in production of VSV. I) The plasmids expressing the heterologous glycoprotein Del10 variant nairovirus GPC. II) The VSV-ΔG genome, a VSV genome with *VSV-G* replaced with a firefly luciferase gene. III) A plasmid expressing VSV-G glycoprotein. (**B**) Target cells are transfected with heterologous glycoprotein (left) or VSV-G (right). (**C**) Producer cells are infected with VSV-G pseudotyped VSV to introduce the VSV-ΔG genome. (**D**) Producer cells secrete pseudotyped VSV. VSV-G pseudotyped VSV (top) is harvested and used to infect further cells in step C. Nairovirus pseudotyped VSV presents the Nairovirus GPC on the surface of the virion and can be used to assess neutralisation. (**E**) Without neutralising antibodies (nAbs) pseudotyped VSV can infect target cells and firefly luciferase is expressed within the cells. F) Neutralising antibodies can bind to and neutralise the pseudotyped VSV and prevent infection and luciferase expression.

3.2.3.1 Production of Nairovirus pseudotyped VSV in Huh-7 cells

Following successful establishment of a pseudotype VNT for CCHFV with CCHFV tec-VLPs, research continued to establish a pseudotype VNT for assessing NSDV neutralisation. As such, relevant producer and target cells were needed to produce NSDV pseudotypes, and a VSV system was investigated as the basis of an NSDV pseudotyping system. Initially, Huh-7 cells were investigated as the producer cells, informed by work with CCHFV. As discussed previously Huh-7 cells have been used to passage live CCHFV. In addition, Huh-7 showed minor success as producer cells for CCHFV lentivirus systems, and Huh-7 cells are used as the producers in the CCHFV tec-VLP system as previously discussed. As such, it is hoped Huh-7 can correctly process nairovirus GPCs to produce NSDV pseudotyped VSV.

Figure 3.10 shows the results of infection of HEK293T cells with nairovirus pseudotyped VSV produced in Huh-7 cells. The negative control in this assay was VSV-ΔG pseudotyped without a heterologous glycoprotein. The positive control in this assay is VSV-ΔG virions pseudotyped with VSV-G. The background luminescence of the VSV pseudotyping system is higher than lentiviral pseudotyping systems discussed in section 3.2.1, but with a comparatively higher positive control, and large dynamic range remaining, the pseudotyping system is still valid.

The luminescent signal from CCHFV pseudotyped VSV showed no significant difference from the negative control luminescence. The luminescent signal from NSDV pseudotyped VSV is significantly greater than the background of the negative control pseudotyped VSV. Presented here is first recorded evidence of pseudotyping with NSDV GPCs in any system. Importantly the separation between the signal of NSDV pseudotyped VSV and the background is almost three orders of magnitude, a sufficient dynamic range for a potential VNT to be established with NSDV pseudotyped VSV.

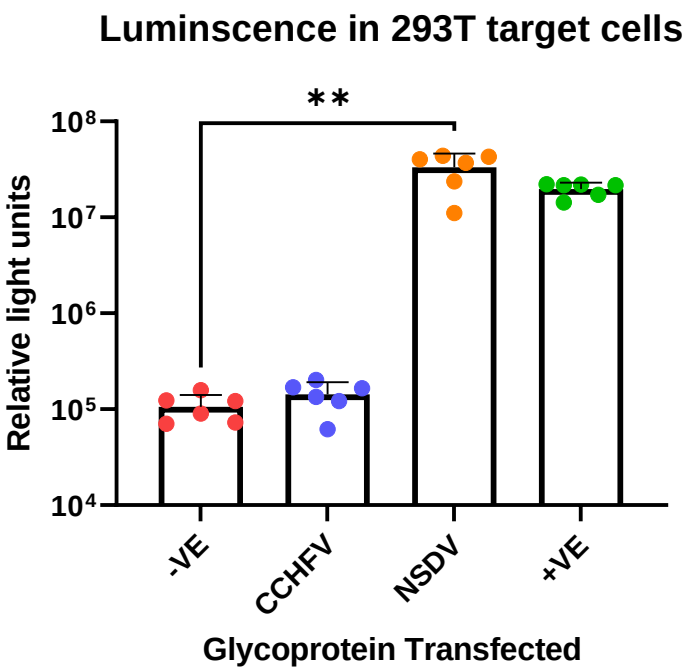


Figure 3.10: Luminescence in cells infected with NSDV or CCHFV pseudotyped VSV generated from Huh producer cells.
Infection performed by 100µl of 1:1 D2 media: pseudoparticle supernatant added to target cells seeded at $2x10^4$ cells 24h prior to infection. Cells incubated at 37°C 5% CO_2 for 72h post infection. Cellular luminescence measured 5 minutes after lysis with 100µl 1:1 PBS:Brightglo™ luciferase substrate. Group labels represent the heterologous glycoprotein used to produce pseudotyped lentivirus. The negative control (-VE) used non-pseudotyped lentivirus, without a heterologous glycoprotein. The positive control (+VE) uses lentivirus pseudotyped with VSV glycoprotein. Luciferase dependent luminescence in 293T target cells infected with pseudotyped VSV. All wells shown as individual data points in Relative light units with mean and standard error of the mean also shown. Significant differences were determined by Brown-Forsythe and Welch's ANOVA with Dunnetts T3 multiple comparisons test (**$p<0.01$).

3.2.3.2 Establishment of pseudotype VNT using NSDV pseudotyped VSV

Following on from the luminescent signal measured from infection with NSDV pseudotyped VSV, a pseudotype VNT was established using serum from vaccinated mice to assess neutralisation. Similar to work with CCHFV tec-VLPs, neutralisation was assessed against a serial dilution of mouse serum to determine if NSDV pseudotyped VSV was sensitive to NSDV antibodies, and if the response was specific compared to control serum. NSDV specific serum was generated by vaccination of balb/c mice with ChAdOx1-NSDV GPC. Control serum was generated by vaccination of balb/c mice with ChAdOx1-control.

Figure 3.11 shows the luminescence signal of NSDV pseudotyped VSV, and how entry has been neutralised by a serial dilution of mouse serum. Serum from mice vaccinated with the ChAdOx1 NSDV GPC vaccine, discussed in chapter 5, has been used to establish the sensitivity of the NSDV pseudotyped VSV to NSDV specific antibodies. Used as a control for serum specific effects was serum taken from mice vaccinated with an unrelated ChAdOx1 vaccines.

Clear evidence of neutralisation was measured with NSDV specific serum, an effect which showed a clear titration as the dilution of NSDV specific serum was reduced. With the control serum no evidence of neutralisation was measured at any dilution of serum. Taken together these results suggest that NSDV pseudotyped VSV is sensitive to NSDV antibodies, and the NSDV specific neutralisation can be separated from any background serum specific effects. Therefore we can establish that NSDV pseudotyped VSV can be used in a pseudotype VNT, and can be used to assess neutralisation induced by antibodies against NSDV pseudotyped VSV.

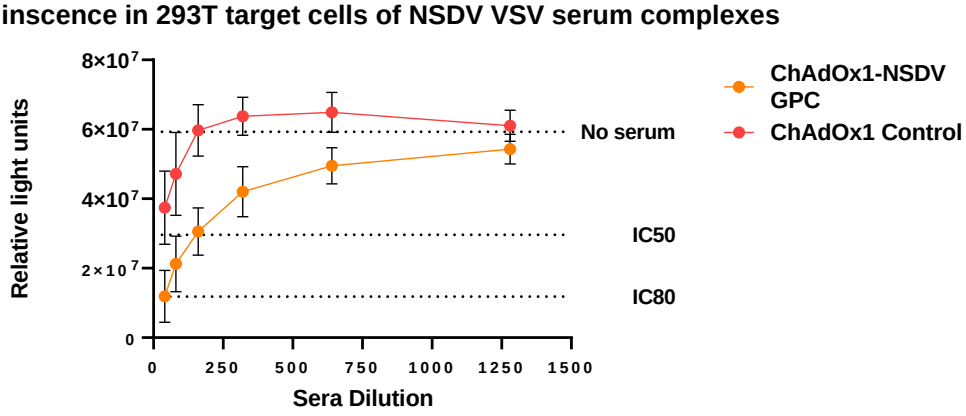


Figure 3.11: Neutralisation of NSDV pseudotyped VSV by NSDV GPC specific serum. A serial dilution, starting at 1/50, of vaccinated mouse serum was prepared in duplicate in D2 media to a total volume of 50µl. Fifty µl of supernatant containing NSDV-VSV pseudoparticles were incubated with the serum dilutions for 1h at 37°C 5%CO_2. NSDV VSV-serum complexes were then transferred onto 293T target cells seeded at $2x10^4$ cells 24h prior to infection. Cells were incubated at 37°C 5% CO_2 for 72h post infection. Cellular luminescence was measured 5 minutes after lysis with 100µl 1:1 PBS:Brightglo™ luciferase substrate. Luciferase dependent luminescence was observed in 293T target cells infected with pseudotyped VSV. The mean (n=6) is shown as a data point with SEM for each serum dilution assayed. Pseudotype only controls are displayed as a line of the graph, with IC50 and IC80 thresholds displayed.

3.3 Conclusions

The main aim of chapter 3 was to produce pseudotype VNTs for working with nairoviruses, a goal which has been successful, as the assays discussed in this chapter will be used throughout the remainder of the thesis to assess the neutralising antibodies elicited by vaccination with ChAdOx vectored vaccines.

Initial attempts at working with the lentivirus pseudotyping systems showed limited success. At the time of these experiments beginning there was no recorded success of nairovirus pseudotyping in a lentivirus system. The work discussed in this thesis reinforces previous literature, as no lentiviral production was demonstrated in 293T cells, the same cells used for production in previous CCHFV pseudotyping using a VSV system. Though Huh-7 producer cells produced a statistically significant luminescence signal for CCHFV pseudotyped lentivirus, the signal to background ratio was not sufficient to establish a pseudotype VNT.

Following the conclusion of the work, successful CCHFV pseudotyping has been reported in a lentivirus system[301], which uses a more complex two step method of production using both a modified HEK293T cell line and HeLa or Vero cells. Though pseudotyping was successful, the two step process and need for multiple cell lines negates the advantages of lentiviral systems in their simplicity. There is a chance that further experimentation with differing producer and target cell types may produce a pseudotype luminescent signal large enough to produce a pseudotype VNT, but such extensive work was outside the scope of this thesis.

The CCHFV tec-VLP system was well established, easy to use, and has been used previously for a wide range of assays. The specific system used and tec-VLP systems in general carry several advantages over more traditional pseudotyping systems, notably the inclusion of multiple external components of the viral genome, beyond just the envelope

or glycoproteins of the virus, with the capacity to study these proteins function. The use of multiple proteins of the virus in the VLP may limit their use to smaller genome RNA viruses, but the system still remains suitable for a wide range of emerging pathogens beyond just nairoviruses. For the purposes of this thesis CCHFV tec-VLPs have been an extremely useful system, producing a strong luminescent signal, and their use in pseudotype VNTs has been invaluable to interrogating the neutralisation responses induced by ChAdOx vectored CCHFV GPC based and CCHFV NP based vaccines.

There have been no instances in the literature of previous NSDV pseudotyping, which is not unexpected due to the lack of research performed on NSDV in both understanding function and in vaccines and therapeutics. Therefore, the success in producing NSDV pseudotyped VSV appears to be the first successful pseudotyping of NSDV, with the subsequently established pseudotype VNT being the first NSDV neutralisation assay suitable for lower containment settings. The assay established was robust and able to assess the neutralisation capacity of NSDV GPC based ChAdOx vectored vaccines. To further extend NSDV pseudotyping and to be able to more accurately assess any unlikely NSDV NP based neutralisation responses, there would be potential to develop an NSDV tec-VLP assay. The close homology on a nucleic and amino acid level of all NSDV proteins to their CCHFV counterparts may improve the likelihood of an NSDV tec-VLP system being viable. Such an assay would be able to assess wider levels of functionality than a pseudotyping system which only incorporates NSDV GPC.

An extension and advantage of pseudotype assays is the ability to easily substitute different strains of virus, comparative to live virus. Unlike with live virus, mutant pseudotypes can be developed with just a change of the DNA to be transfected during pseudotype production. In particular, experimentation with pseudotyped viruses allows for study of loss of function mutations which would not be viable in live replicating virus. The CCHFV tec-VLP system has previously been established with a range of different

CCHFV GPCs[315], showing the potential of the system to assess vaccine cross neutralisation between different strains of CCHFV.

With any pseudotyping assay comparison to live virus is extremely important. The assays established in this thesis at present are only a guide to the neutralisation elicited by ChAdOx vectored nairovirus vaccines. Due to the similarities of glycoprotein or envelope protein function between pseudotype systems and live virus, neutralisation often correlates strongly between pseudotype and live virus assays. As such, the results of the assays developed here, presented throughout chapter 4-6, should be looked at critically, and not considered definitive proof of induction of neutralising antibodies from ChAdOx vectored vaccines, until correlated with a live virus assay. However, they can be viewed optimistically as potential evidence of neutralisation.

In conclusion, the pseudotyping of nairoviruses offers an extremely valuable tool for assessing nairovirus function outside of high containment facilities, at lower complexity and expense. In this chapter a range of different pseudotyping approaches were investigated and two pseudotype VNTs were established for assessing neutralisation, a tec-VLP system for CCHFV and a VSV system for NSDV. These systems were used extensively to assess neutralising antibodies induced in mice by ChAdOx vectored nairovirus vaccines, and nairovirus pseudotypes have the potential to be expanded into a wider range functions beyond the work presented in this thesis.

Chapter 4 – Development of monovalent ChAdOx vectored vaccines against CCHFV

4.1 Introduction

CCHF is a potentially lethal disease of humans, caused by the pathogen CCHFV[99]. CCHFV is a pathogen of concern as part of the WHO R&D blueprint[3], due to its outbreak potential, and the current lack of therapeutics and vaccines to treat or protect against CCHFV infection. CCHFV infections can range from minor to severe, with mild symptoms displaying as a non-specific febrile illness making diagnosis difficult[99]. More severe cases of CCHFV cause gastrointestinal, neurological, and haemorrhagic symptoms, which can culminate in death. Mortality rates of CCHFV infections can vary from 10-40%[94], depending on CCHFV strain but the most severe outbreak recorded approached 80% mortality[117]. The true burden and case load of CCHFV is unknown in the population due to the non-specific symptoms of mild infections, and the unknown proclivity of asymptomatic infection.

CCHFV has the second largest geographical range of any arboviruses defined by its major vector, *Hyalomma* ticks[120], which are endemic to the majority of Africa and Asia, and Eastern Europe. With the impact of climate change[69,316], the range of *Hyalomma* ticks is expanding northwards into southern and western Europe, with sero-positivity being measured in humans and animals in new locations in the last few years[317–319]. CCHFV is maintained in a range of mammalian and bird species, both domesticated and wild[8]. Infections in most species are asymptomatic, acting only to amplify the virus, with humans considered dead end hosts of CCHFV[100]. The wide range of asymptomatic host species can create difficulties in eliminating or eradicating CCHFV, and maintaining control of CCHFV circulation[56]. Though vector control and better diagnostics may be

useful tools for protecting against CCHF, development of vaccines against CCHFV are a priority for protecting humans through both direct vaccination and livestock vaccinations.

A range of vaccines against CCHFV have been developed. Of these only one has been widely used, an inactivated vaccine based of the V42/81 strain[247]. Despite widespread use in the USSR and continued usage in Bulgaria, immunogenicity of the vaccine is low, with neutralising antibodies remaining low even after three booster doses[248], with the scalability of inactivated viral vaccine production also a concern. As such, vaccine development has continued and a range of vaccines in protein, nucleic acid and viral vectored platforms have been synthesised and shown to be immunogenic in mouse models[241,249,320,321]. However, lethal CCHFV challenges in immunocompromised mouse models have demonstrated that not all immunogenic vaccines induced a protective response in small animal models.

Viral vectored vaccines against CCHFV have performed well when assessed through CCHFV challenge in mouse models. An MVA-vectored CCHFV NP vaccine was immunogenic in mouse models but did not offer protection from lethal CCHFV infection[252]. In contrast, two doses of an MVA-vectored CCHFV GPC[254], a BoHV-4-vectored CCHFV NP[253], or an Ad-5-vectored NP[253] all induced protection against lethal challenge with CCHFV, and single dose protection has been observed with an rVSV vectored CCHFV GPC vaccine[256], the same vaccine platform as rVSV-EBOV, the first Ebolavirus vaccine licenced in humans[255].

Prior to the initiation of the work in this thesis, a ChAdOx2 vectored vaccine was developed expressing CCHFV GPC under a CMV promoter. Immunogenicity was assessed in balb/c mice and the CCHFV GPC antigen was shown to be immunogenic (unpublished data). A ChAdOx2-CCHFV GPC vaccine was then tested for vaccine efficacy in a mouse CCHFV challenge model. The model used immunocompromised A129 mice which had previously been used to assess protection against lethal CCHFV

challenge[252,254]. The presented CCHFV challenge was performed by a team at Public Health England in BSL-4 conditions, the results of which are shown in figure 4.1.

The immunogenicity of the ChAdOx2-CCHFV GPC vaccine was compared against an MVA-CCHFV GPC vaccine, two doses of which had previously demonstrated 100% protection against lethal CCHFV challenge. A single prime only vaccination of ChAdOx2-CCHFV GPC was assessed against a ChAdOx2 homologous prime-boost, MVA prime-boost, and a ChAdOx2-MVA heterologous prime boost. Cellular immunogenicity was demonstrated by IFN-γ ELISPOT, and no significant difference was measured between the responses post-vaccination of the ChAdOx2 prime only group and any of the prime-boost immunisation groups, presented in figure 4.1A. Humoral immunogenicity was assessed by total IgG ELISA against CCHFV Gc protein, and presented in figure 4.1B. Again, no significant differences were measured between the ChAdOx2 prime vaccinated group or any of the prime boost vaccinated groups.

Vaccinated mice were challenged with a lethal dose of CCHFV of the IbAr10200 strain, and survival curves are presented in figure 4.1C. All vaccinated groups showed 100% protection from disease and death from CCHFV infection. In contrast, all mice vaccinated with an irrelevant ChAdOx2 control vaccine showed clinical signs of CCHF and were culled having reached humane endpoints 5 days after challenge.

As all CCHFV vaccines induced 100% survival, it is difficult to draw conclusions related to correlates of protection against CCHFV infection. However, ChAdOx2-CCHFV GPC was protective in three different vaccination regimens, and showed protection against CCHFV challenge in mice, after a single dose. Such results supported the progression of ChAdOx2 CCHFV GPC into larger NHP challenge trials and subsequent human phase I trials.

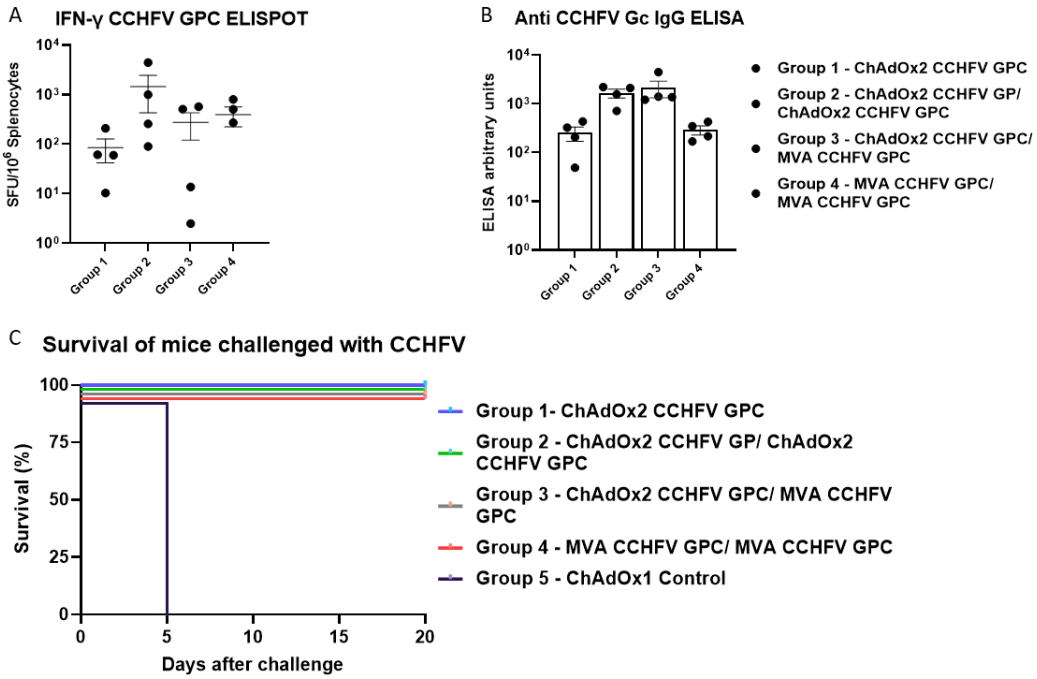


Fig. 4.1: CCHFV antigen specific antibody and cellular response in A129 mice immunized with ChAdOx2 CCHFV GPC and MVA CCHFV GPC vaccine homologous or heterologous prime-only or prime-boost regimes, and survival curves following lethal CCHFV challenge.

Mice in prime-boost regimes received prime vaccination on day 0 of experiment. Prime only regimes and prime-boost regimes received prime and boost vaccination respectively on day 14. Terminal bleed and spleen harvesting were performed on mice for immunogenicity time points on day 35. Mice in the challenge group were inoculated with a lethal dose of CCHFV on day 36 of the experiment. All surviving mice were culled on day 56, the experimental endpoint. CCHFV GPC specific IFNγ responses (**A**) were assayed in mouse splenocytes. Individual A129 mice (n=4) are shown here as individual data points measured in spot forming units per 10^6 splenocytes (SFU/10^6) with mean and standard error of the mean (SEM) also shown. No significant differences were determined by Brown-Forsythe and Welch ANOVA CCHFV Gc-specific IgG (**B**) responses in mouse sera were quantified by standardized ELISA. Individual A129 mice (n=4) are shown here as individual data points were expressed as ELISA arbitrary units and with mean and standard error of the mean also displayed. No significant differences were determined by Brown-Forsythe and Welch's ANOVA. Kaplan-Meier survival curve of mice challenged with CCHFV **(C)** shown to 20 days post challenge (N=6).

The Clinical Biomanufacturing Facility, University of Oxford aimed to generate a clonal product for GMP production of ChAdOx2-CCHFV GPC for use in phase I clinical trials. As part of the process, 19 separate clones of the ChAdOx2-CCHFV GPC were sequenced from the 5' and 3' ends of the antigen cassette, to assess the clonality and stability of the CCHFV GPC antigen cassette. The results of sequencing are shown in figure 4.2.

A high degree of instability was demonstrated within the ChAdOx2-CCHFV GPC antigen cassette. Of the samples sequenced, 47% showed the correct sequence at the 5' and 3' ends and the correct molecular weight (4.2A), suggesting the antigen sequence in ChAdOx2-CCHFV GPC is frequently modified when the virus is passaged. Of the errors discovered, 42% of the samples showed 4-5 kb deletions in the CCHFV antigen (4.2B&4.2C), a single clone showed an approximately 750bp deletion within the cassette, and the final clone showed deletion of the promoter and CCHFV GPC antigen in their entirety. These results demonstrate that ChAdOx2-CCHFV GPC is unstable, with a particular risk of the CCHFV GPC antigen being dropped out of the ChAdOx2 genome.

Previous research on other adenoviruses, for use in gene therapies, has suggested that genetically modified adenoviruses have a limited capacity to carry and express transgenes, at approximately 105% of the parent virus genome[322]. Above that limit, replication is slowed and transgenes are liable to be removed from the adenovirus genome. Work on the specific capacity limit in ChAdOx1 and ChAdOx2 has not been performed, and the ChAdOx2-CCHFV GPC is ~100% of the length of the size of the original chimpanzee adenovirus progenitor, suggesting the capacity limits of ChAdOx vectors may be lower than previously suggested in other adenoviruses.

Due to the instability of ChAdOx2-CCHFV GPC, despite demonstration of protection in mouse challenge models, further research is needed to produce alternative candidates for ChAdOx vectored vaccines against CCHFV.

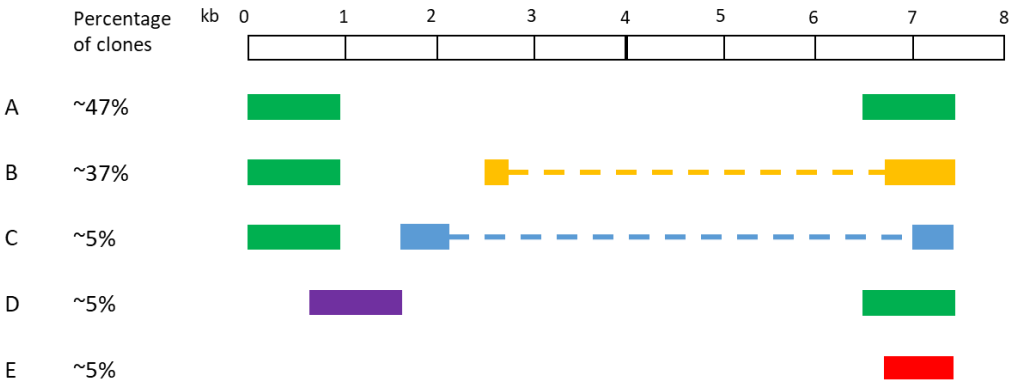


Figure 4.2: Representation of sequencing of single clones of ChAdOx2 CCHFV GPC genome.

The figure represents the 5' and 3' sequencing of the antigen cassette of the ChAdOx2-CCHFV GPC genome, following single virus infection. (**A**) represents samples of expected molecular weight, with no errors observed in 5' and 3' sequencing. (**B**) represents samples with a ~4kb deletion within the CCHFV antigen, with the deletion including a portion of the 3' sequence. (**C**) represents a sample with a ~5kb deletion of the CCHFV antigen, with the deletion including a portion of the 3' sequence. (**D**) represents a sample with a ~0.75kb deletion at an unknown location within the CCHFV antigen cassette, with no errors observed in 5' or 3' sequencing. (**E**) represents a sample with a ~7kb deletion of the majority of the CCHFV antigen cassette, with concatenation of the 5' and 3' sequencing.

4.2 Results

4.2.1 Small antigen CCHFV vaccine development

Investigations in reducing the size of the antigen cassette in ChAdOx vaccines focused in two directions, firstly investigating novel smaller CCHFV antigens, and secondly reducing the size of the promoter used to drive expression of CCHFV GPC. The CCHFV GPC of strain IbAr10200 is a large gene and protein, over 5kb and 1684 aa in length. Following expression of the GPC, it is processed by a range of known and unknown proteases into five glycoproteins; MCL, GP38, Gn, NSm, and Gc[88]. With the large geographical range of CCHFV a high level of variation is observed at both a DNA and protein level between different strains of CCHFV. To determine the variation in the full length GPC and the GPC subunits, a geographically diverse selection of CCHFV GPC protein sequences were obtained from Genbank and aligned with IbAr10200 sequence for GPC and the GPC subunits, using NCBI BLASTP. Included in the analysis were laboratory strains Hoti and Afg09, which have also been used to generate CCHFV vaccines. The results of these alignments are displayed in figure 4.3.

For the full length GPCs, percentage alignment against IbAr10200 GPC averaged ~80% amino acid identity. Unsurprisingly the highest identity was with the WT Nigerian strain, with Nigeria being the origin of the IbAr10200 strain prior to lab passage. The MCL subunit showed the lowest level of conservation of any GPC subunit, dropping below 40% identity in some cases, with only 4 of the 11 sequences analysed showing greater than 60% identity. Alignment of CCHFV GPC sequences to IbAr10200 GP38 and NSm were on average 80-85%, similar to the identity measured for full length GPC. The Gn and Gc subunits showed greater conservation across CCHFV strains, most strains showing ~90% conservation for Gn and ~95% conservation for Gc, a very high level of conservation across such a wide geographic range, especially compared to the rest of the GPC.

The Gn and Gc glycoproteins are the antigens found on the surface of CCHFV virus[323], thought to be responsible for viral tropism and cell entry, though the cell surface target for CCHFV is unknown. The mechanisms of virus entry, and roles of the secreted GPs, MCL and GP38, and the transmembrane NSm in CCHFV function are unknown. Gn and Gc antigen have been shown to induce protective responses in some mouse challenge trials[249,324], and antibodies targeting Gc particularly have been shown to neutralise CCHFV tec-VLPs[268]. However, other studies performed more recently have suggested Gn/Gc mediated protection may not be sufficient on its own creating a mixed picture of protection[90,320]. Due to their exposure on the virus surface, and high levels of conservation, CCHFV Gn and Gc were chosen as the initial subunit CCHFV GPC antigens.

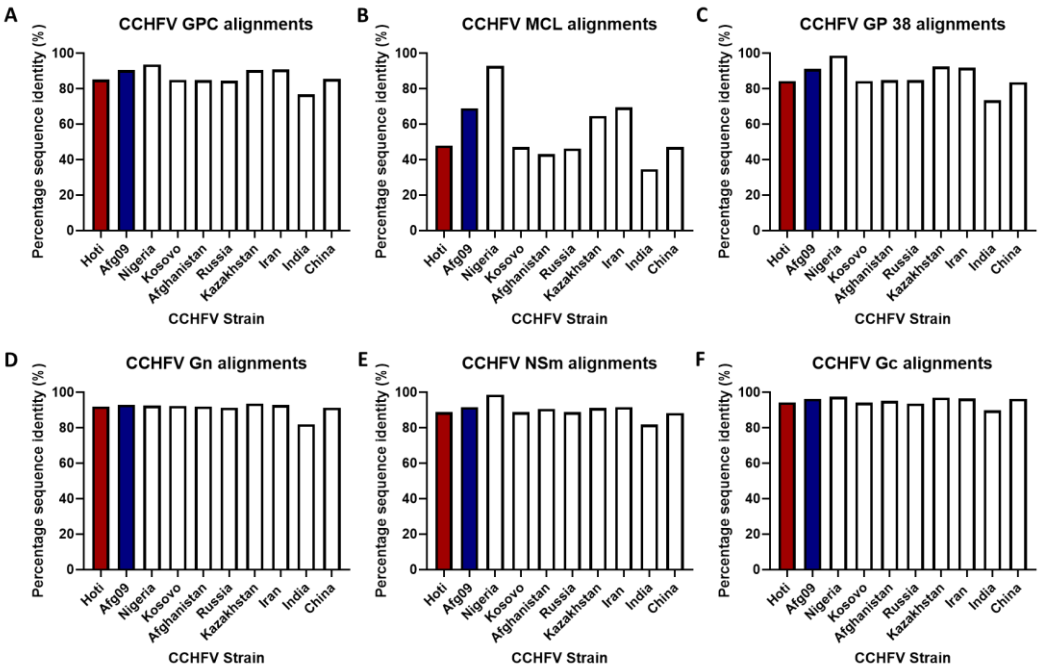


Figure 4.3: Protein sequence identity of selected full length CCHFV GPC and CCHFV GPC subunits against IbAr10200 GPC accession number NP_950235.1.
Accession numbers as follows; Hoti - ABW04159.1, Afg09 - HM452306.1, Nigeria - APB09910.1, Kosovo - AXH37970.1, Afghanistan - AGH10338.1, Russia - AQX83288.1, Kazakhstan - AOF43039.1, Iran - AZS19000.1, India - MN866218.1, China - AAK52742.1. (**A**) Percentage amino acid identity of CCHFV GPC against IbAr10200 CCHFV GPC, measured by alignment with BLASTP. (**B**) Percentage amino acid identity of CCHFV MCL against IbAr10200 MCL (amino acids 1-272), measured by alignment with BLASTP. (**C**) Percentage amino acid identity of CCHFV GP38 against IbAr10200 GP38 (amino acids 273-519), measured by alignment with BLASTP. (**D**) Percentage amino acid identity of CCHFV Gn against IbAr10200 CCHFV Gn (amino acids 520-769), measured by alignment with BLASTP. (**E**) Percentage amino acid identity of CCHFV NSm against IbAr10200 NSm (amino acids 770- 1040), measured by alignment with BLASTP. (**F**) Percentage amino acid identity of CCHFV Gc against IbAr10200 Gc (amino acids 1041-1684).

4.2.1.1 Design of antigen cassettes expressing CCHFV Gn and CCHFV Gc

Following selection of Gn and Gc as the antigens of interest, coding sequence from the IbAr10200 strain were selected. The designs of these antigens are displayed in figure 4.4. Both the Gn and Gc antigens use the CCHFV signal peptide, the first twenty amino acids of the GPC sequence, as both Gn and Gc have N-terminal extracellular domains. The signal peptide was followed by a ten amino acid linker region, which culminates in the Ski-1-like cleavage sites which mark the N-terminus of both Gn and Gc. Both Gn and Gc antigen expression constructs had unique linkers, which were the final 10 amino acids of their N-terminal subunits, GP38 and NSm respectively. The Gn subunit after the Ski-1-like cleavage site is 350 amino acids long, with a predicted molecular weight of 37kDa, as previously described[88]. The Gc subunit after the Ski-1-like cleavage site is a further 644 amino acids long, continuing to the C-terminus of the CCHFV GPC.

Both antigen sequences were cloned into shuttle vectors, for recombination into ChAdOx genomes. The antigen cassette in which the antigens were cloned is shown in figure 4.5. The antigens were expressed under the control of a short CMV promoter, to further reduce the size of the antigen cassette from ChAdOx2-CCHFV GPC. The short CMV promoter is ~800bp in length, compared to the ~2.3kb of the long CMV promoter which was used in ChAdOx2-CCHFV GPC. The polyA tail used in the antigen cassettes is from bovine growth hormone (BGH) of ~225bp.

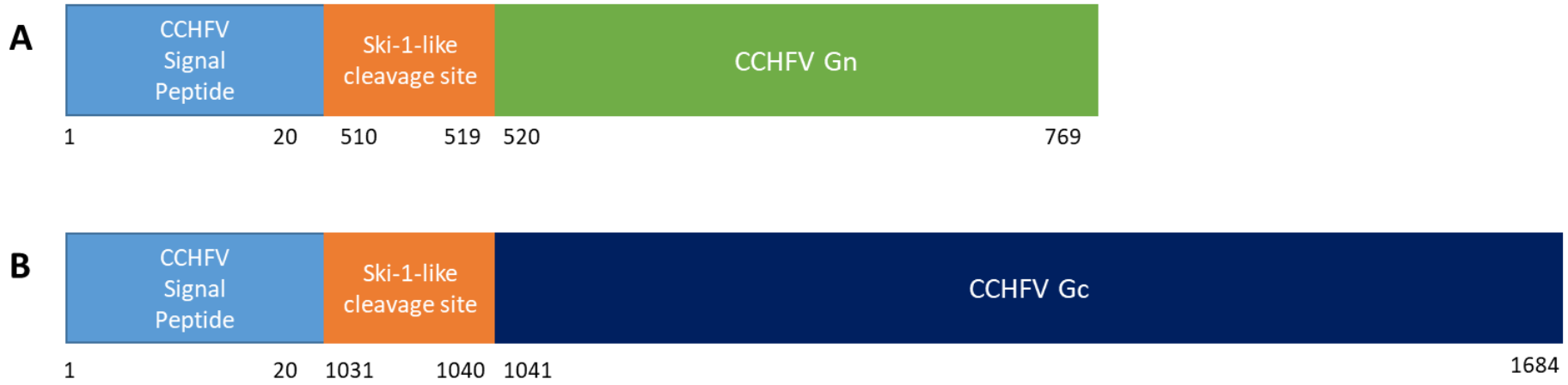


Figure 4.4: Design of CCHFV Gn and CCHFV Gc antigens.
All amino acid numbers given correspond to WT IbAr10200 GPC
A diagram of the construction of CCHFV Gn (**A**) consisting of the CCHFV signal peptide of 20 amino acid (1-20), a 10 amino acid linker with Ski-1-like cleavage site (510-519), and CCHFV Gn (520-769). A diagram of the construction of CCHFV Gc (**B**) consisting of the CCHFV signal peptide of 20 amino acids (1-20), a 10 amino acid linker with Ski-1-like cleavage site (1031-1040), and CCHFV Gc (1041-1684).

The CCHFV Gn and Gc antigens were ordered and cut from delivery plasmids by HindIII and KpnI. Sequences were ligated into a shuttle vector, linearised by HindII and KpnI. Ligation reactions were transformed into competent E.coli cells and successful plasmids selected after diagnostic digest with HindIII and KpnI. Shuttle vector containing CCHFV Gn or CCHFV Gc were recombined into the genome of the vector ChAdOx1 through the attL1 and attL2 sites, as shown in figure 4.5, and the ChAdOx1-CCHFV Gn and ChAdOx1-CCHFV Gc viruses were produced by the Viral Vector Core Facility, University of Oxford. ChAdOx1 was chosen for the initial experiments with CCHFV Gn and Gc antigens, as more is known about ChAdOx1 due to its usage in more trials for a wider variety of antigens.

Table 4.1 shows the size of the antigenic cassettes of ChAdOx1-CCHFV Gn and ChAdOx1-CCHFV Gc and their ChAdOx2 counterparts, compared against the antigen cassettes of ChAdOx vectored CCHFV GPC vaccines. The CCHFV Gn and CCHFV Gc antigen cassettes are significantly smaller than those used for CCHFV GPC, through a combination of using a shorter promoter and a smaller antigen.

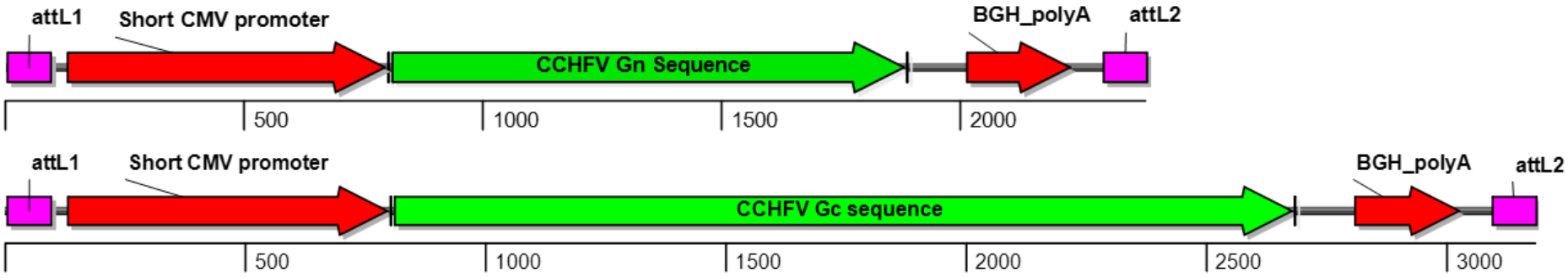


Figure 4.5: Antigen cassettes for ChAdOx CCHFV Gn and ChAdOx1 CCHFV Gc vaccines
Representation of (**A**) CCHFV Gn antigen cassette and (**B**) CCHFV Gc antigen cassette. AttL1 and AttL2 represent recombination sites for the antigen cassette into the ChAdOx1 genome. Short CMV promoter represents the 673 amino acid promoter sequence derived from CMV. BGH_polyA represents the polyA tail of bovine growth hormone for transcriptional termination.

Vaccine	Antigen Cassette	Size of antigen cassette (bp)	Size of ChAdOx1 Genome with antigen (bp)	Percentage of Original ChAdY25 genome size (%)	Size of ChAdOx2 Genome with antigen (bp)	Percentage of Original ChAd68 Genome size (%)
ChAdOx1/2-CCHFV GPC	CCHFV GPC long promoter	7571	36674	100	36556	100
ChAdOx1/2-CCHFV Gn	CCHFV Gn	2195	31298	85	31183	85
ChAdOx1/2-CCHFV Gc	CCHFV Gc	2990	32039	87	31975	87

Table 4.1: The size of ChAdOx vectored CCHFV genomes for CCHFV GPC, Gn and Gc antigens and their relationship to the size of their original ChAd genome.
The table shows the size of CCHFV GPC, CCHFV Gn and CCHFV Gc antigen cassettes, and their respective sizes when cloned into the ChAdOx1 and ChAdOx2 genome. ChAdOx1 genome sizes are then measured as a percentage of the progenitor ChAdY25 genome (36711 bp). ChAdOx2 genome sizes are then measured as a percentage of the progenitor ChAd68 genome (36521 bp).

4.2.1.2 Immunogenicity of ChAdOx1 vectored CCHFV Gn and Gc

With the reagents available at the time of vaccine development, Gn and Gc antigen expression from ChAdOx1 could not be directly assessed by western blot. Therefore research moved directly on to assessing the immunogenicity in mice of vaccination with CCHFV Gn and CCHFV Gc antigens in ChAdOx1, compared with immunogenicity after vaccination with ChAdOx1-CCHFV GPC vaccine. Balb/c and CD-1 mice were vaccinated with either ChAdOx1-CCHFV GPC, ChAdOx1-CCHFV Gn, ChAdOx1-CCHFV Gc, or a ChAdOx1 control vaccine, which expressed an irrelevant antigen.

Cellular immunogenicity was assessed by an IFN-γ ELISPOT assay. IFN-γ ELISPOT assesses the secretion of IFN-γ from T-cells stimulated by peptides specific to CCHFV. IFN-γ secretion is used as a proxy for overall cellular immune responses. Cellular responses from CD4+ and CD8+ T-cells are considered crucial for protection against, and clearing of, intracellular pathogens, including many viruses, and particularly CCHFV[249]. Correlates of protection have not been fully established for CCHFV, and therefore it is important to assess the cellular responses induced with ChAdOx vectored vaccines.

Figure 4.6 shows the cellular immunogenicity, in either balb/c or CD-1 mice, following a single immunisation of either ChAdOx1-CCHFV Gn or ChAdOx1-CCHFV Gc, compared with the responses following vaccination with ChAdOx1-CCHFV GPC.

Figures 4.6A and 4.6B show anti-Gn responses in Balb/c and CD-1 respectively. No anti-Gn responses were measured in control vaccinated mice, in either Balb/c or CD-1 strains. Anti-Gn responses were generally lower in CD-1 mice than Balb/c. No significant differences in ELISPOT responses were measured following vaccination with ChAdOx1-CCHFV Gn, compared with the response after vaccination with ChAdOx1-CCHFV GPC. These results suggest than CCHFV Gn is being expressed from ChAdOx1-CCHFV Gn,

and inducing an immune response that is equal to that induced by ChAdOx1-CCHFV GPC.

Figures 4.6C and 4.6D show anti-Gc IFN-γ responses from balb/c and CD-1 mice respectively. No non-specific responses were measured from the control vaccinated mice of either balb/c or CD-1 strains. Anti-Gc responses were broadly similar across the two mouse strains tested, and no significant differences in ELISPOT responses were observed following ChAdOx1-CCHFV Gc vaccination, compared with ELISPOT responses following ChAdOx1-CCHFV GPC vaccination. These results confirm expression of the CCHFV Gc antigen in the ChAdOx1 vector, and that immune responses generated by ChAdOx1-CCHFV Gc vaccination are equal to those induced by ChAdOx1-CCHFV GPC. The IFN-γ ELISPOT responses against the CCHFV Gc specific peptides appear to be larger than those against CCHFV Gn. This is not an unexpected result, with the CCHFV Gc pool containing more peptides, and analysis of peptides across the whole GPC showing Gc was more immunodominant than Gn, likely due to the larger extracellular regions represented in the Gc peptide pools.

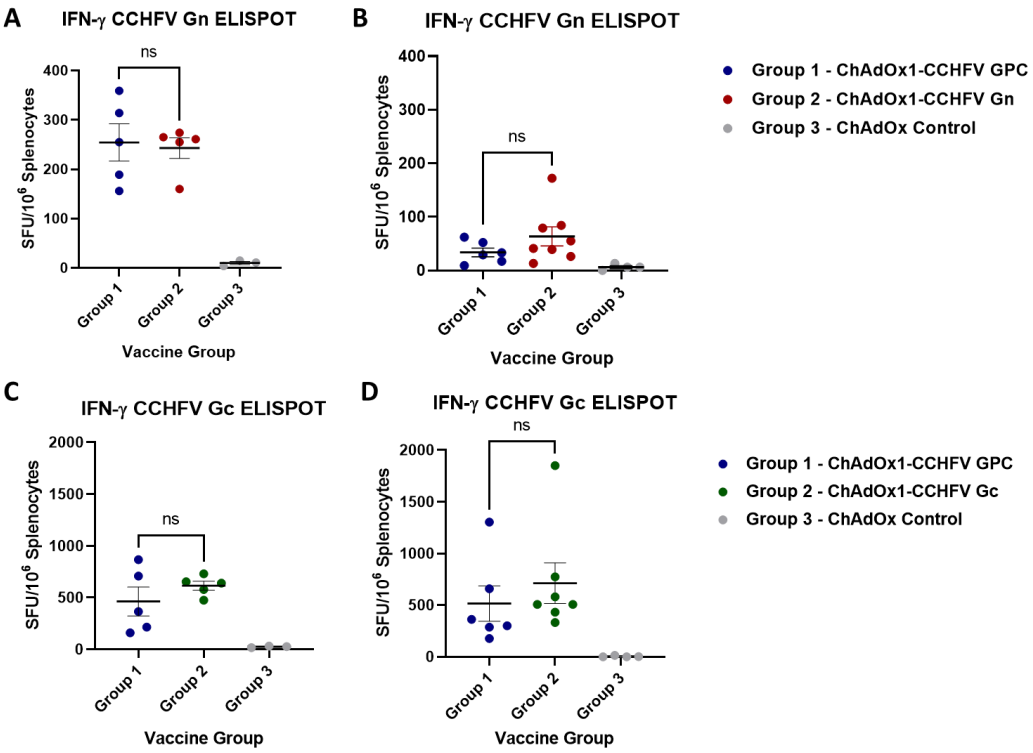


Figure 4.6: IFN-γ ELISPOTS against peptides specific to Gn or Gc of CCHFV IbAr10200 strain, following vaccination with ChAdOx1-CCHFV GPC or GPC subunit vaccines. 6 week old balb/c (**A/C**) or CD-1 Mice (**B/D**) were immunised with $1x10^8$ Infectious units (IU) of ChAdOx1 vaccine. 21 days after prime vaccination, mice were culled for immunological analysis. CCHFV Gn antigen specific IFN-γ responses in balb/c (**A**) and CD-1 (**B**) mice were assayed in mouse splenocytes by IFN-γ ELISPOT. A) Individual balb/c mice (groups 1/2 n=5 group 3 n=3) are shown here measured in spot forming units per 10^6 splenocytes (SFU/10^6) with mean and standard error of the mean (SEM) also shown. No significant differences were determined by Brown-Forsythe and Welch ANOVA. B) Individual CD-1 mice (group 1 n=7, groups 2 n=8, group 3 n=4) are shown here with mean and SEM. No significant differences were determined by Brown-Forsythe and Welch ANOVA. CCHFV Gc specific IFN-γ responses in balb/c (**C**) and CD-1 (**D**) mice were assayed in mouse splenocytes be IFN-γ ELISPOT. C) Individual B=balb/c (groups 1/2 n=5, group 3 n=3) are shown here with mean and SEM. No significant differences were determined by Brown-Forsythe and Welch ANOVA. D) Individual CD-1 mice (group 1 n=6, groups 2/3 n=7, group 4 n=4) are shown with mean and SEM. No significant differences were determined by Brown-Forsythe and Welch ANOVA.

Humoral responses can be crucial to protection against viral pathogens, and studies have shown their importance in protection against CCHFV infection[258]. In this thesis the humoral immunogenicity induced by vaccines was assessed by total IgG ELISA against CCHFV Gn and Gc proteins, and by assessing neutralisation titres with a CCHFV tec-VLP pseudotype VNT.

Separate ELISAs were performed for assessing both anti-CCHFV Gn and anti-CCHFV Gc total IgG. Unlike with the previously presented ELISPOT results, the anti-Gn and anti-Gc ELISA responses cannot be directly compared, as they are two separately standardised ELISAs.

Figure 4.7A and 4.7B show the anti-Gn total IgG in serum, induced following vaccination with ChAdOx1-CCHFV GPC or ChAdOx1-CCHFV Gn in Balb/c and CD-1 mice. Anti-Gn responses are poor in Balb/c mice with ChAdOx1-CCHFV GPC failing to induce any anti-Gn response, and the response induced by ChAdOx1-CCHFV Gn was inconsistent and low when present. Anti-Gn responses in the ELISA performed on serum from CD-1s showed greater antibody titres, both CCHFV GPC and CCHFV Gn antigens inducing a response, though within both groups some mice did not respond. Those that did produce measurable antibody titres, showed consistently higher responses than in balb/c mice. Due to the inconsistency in responses, there was no significant difference measured between mice vaccinated with ChAdOx1-CCHFV Gn and mice ChAdOx1-CCHFV GPC, and in some cases no significant differences were measured from animals vaccinated with a ChAdOx1 control vaccine.

Figure 4.7C and 4.7D show anti-CCHFV Gc total IgG responses in serum, induced after vaccination with ChAdOx1-CCHFV GPC or ChAdOx1-CCHFV Gc in balb/c and CD-1 mice. Anti-Gc antibody titres were not measured in control vaccinated mice of either strain, but antibody titres were induced consistently in all mice vaccinated with ChAdOx1 CCHFV GPC and ChAdOx1 CCHFV Gc. In balb/c mice all CCHFV antigen vaccinated

mice showed an anti-Gc response, however no significant difference was measured in anti-Gc antibody titres between mice vaccinated with ChAdOx1-CCHFV GPC and ChAdOx1-CCHFV Gc. In CD-1 mice, anti-Gc antibody titres were slightly higher, but similar to with balb/c mice, no significant difference was measured in anti-Gc ELISA antibody tires in CD-1 mice following vaccination with ChAdOx1-CCHFV Gc or ChAdOx1-CCHFV GPC.

The inconsistent anti-Gn humoral response is not unexpected as CCHFV Gn is a small antigen, with multiple transmembrane domains which would struggle to induce binding epitopes. The N-terminal extracellular region represented by the Gn protein used in the assay, is shorter with a limited number of potential binding epitopes, and responses observed to N-terminal regions of CCHFV GPC are generally focused on GP38 rather than Gn[90]. In contrast CCHFV Gc would be expected to produce a greater and more consistent response from both the CCHFV GPC and CCHFV Gc antigens, as previously demonstrated with vaccine induced responses[258,324].

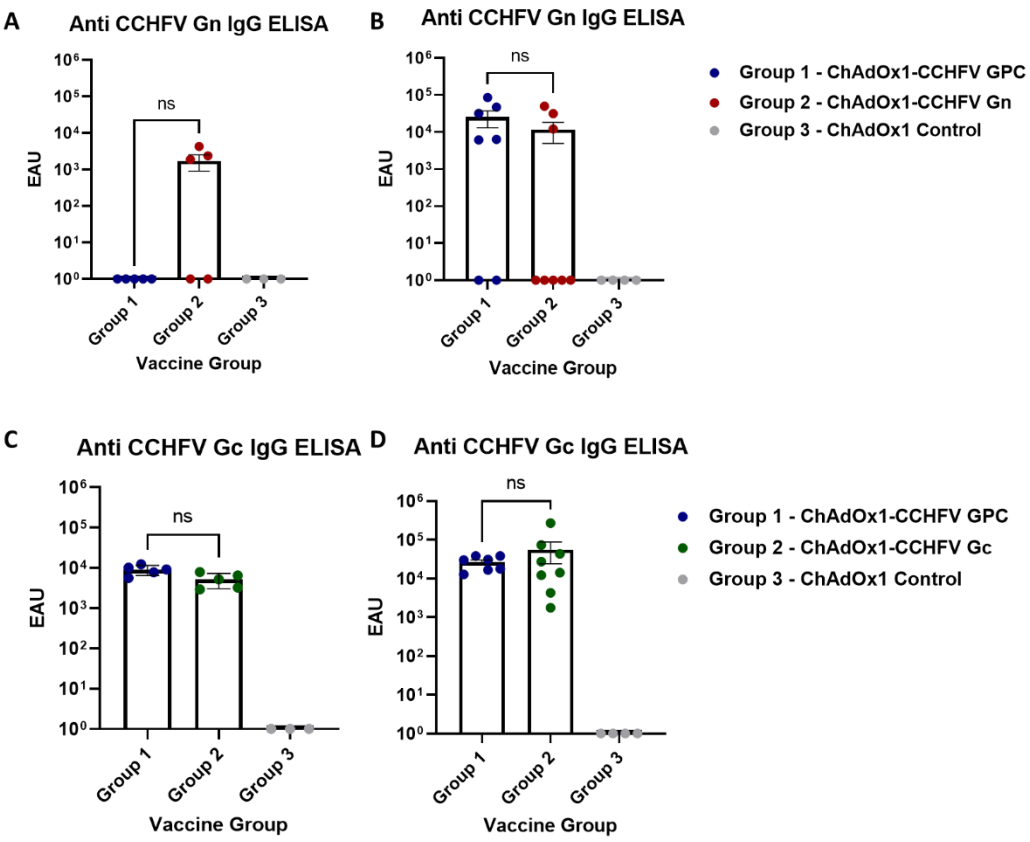


Figure 4.7: Total IgG ELISA against CCHFV Gn or Gc protein of the CCHFV IbAr10200 strain, following vaccination with ChAdOx1-CCHFV GPC or GPC subunit vaccines. 6 week old balb/c (A/C) or CD-1 Mice (B/D) were immunised with $1x10^8$ IU of ChAdOx1 vaccine. 21 days after prime vaccination, mice were culled for immunological analysis. CCHFV specific Gn responses were quantified in balb/c (**A**) and CD-1 (**B**) mice in a standardised ELISA. A) Individual mice (groups 1/2 n=5, groups 3/4 n=3) are shown as data points in ELISA arbitrary units (EAU) with mean and standard error of the mean (SEM) also shown. Responses below the limit of detection were assigned a value of 1EAU. No significant differences were determined by Kruskal-Wallis test. B) Individual mice (group 1 n=7, groups 2/3 n=8, group 4 n=4) are shown here as data points in EAU with mean and SEM also shown. Responses below the limit of detection were assigned a value of 1EAU. No significant differences were determined by Kruskal-Wallis test. CCHFV specific Gc responses were quantified in Balb/c (**C**) and CD-1 (**D**) mice in a standardised ELISA. C) Individual mice (groups 1/2 n=5, groups 3/4 n=3) are shown as data points in EAU, with mean and SEM also shown. Responses below the limit of detection were assigned a value of 1EAU. Significant differences were determined by Kruskal-Wallis test. D) Individual mice (group 1 n=7, groups 2/3 n=8, group 4 n=4) are shown here as data points in EAU with mean and SEM also shown. Responses below the limit of detection were assigned a value of 1EAU. No significant differences were determined by Kruskal-Wallis test.

The ability of vaccine induced antibodies to neutralise infection is important to assess. Existing studies are unsure of the role of neutralising antibodies to protect against CCHF or CCHFV infection, with some studies suggesting their importance[90,258], and others suggesting T-cell responses are more crucial for inducing protection[257].

The neutralising antibody response was assessed using a previously described CCHFV tec-VLP assay[273], from which a pseudotype VNT was developed as described in chapter 3. Figure 4.8 presents the neutralisation titres of balb/c and CD-1 mice vaccinated with ChAdOx1 CCHFV vectored vaccines. Figure 4.8A shows the results from vaccination in balb/c mice. The humoral response following vaccination with ChAdOx1-CCHFV GPC showed neutralisation responses above the limit of detection in all mice tested, and showed a significant difference from the responses measured after vaccination with a ChAdOx1 control vaccine. Conversely mice vaccinated with CCHFV Gn and Gc antigens showed a more variable response, with 3 and 4 mice respectively demonstrating responses above the limit of detection, and no significant differences were measured in neutralisation when compared to responses induced by vaccination with ChAdOx1 control. In CD-1 mice, presented in figure 4.8B, humoral responses induced following vaccination with ChAdOx1-CCHFV Gc and ChAdOx1-CCHFV GPC were all above the limit of detection, with a significant difference measured in both when compared to ChAdOx1 control vaccinated serum. In contrast, responses in mice vaccinated with ChAdOx1-CCHFV Gn showed poor neutralisation responses, with no significant differences measured compared to responses in CD-1 mice vaccinated with a ChAdOx1 control vaccine.

It was demonstrated in mice that vaccination with ChAdOx1-CCHFV Gn and ChAdOx1-CCHFV Gc induced lower neutralisation responses than vaccination with ChAdOx1-CCHFV GPC. The antigen CCHFV Gn showing no neutralisation in either balb/c or CD-1 models. CCHFV Gc did show greater evidence of neutralisation than CCHFV Gn, with

ChAdOx1-CCHFV Gc vaccination inducing a significant neutralisation response in CD-1 mice. The differences between neutralisation induced by CCHFV Gc and CCHFV GPC antigens were not significant, though in balb/c mice responses induced by ChAdOx1-CCHFV Gc vaccination were not significantly different from responses induced by ChAdOx1 control vaccination. An explanation for greater neutralisation responses measured after ChAdOx1-CCHFV GPC vaccination is the inclusion of the GP38 subunit, where neutralising responses have previously been shown to have a role in protection in mouse models[90].

The cellular and humoral immunogenicity responses after vaccination with ChAdOx1-CCHFV Gn of ChAdOx1-CCHFV Gc suggest these antigens are immunogenic. However, neutralisation responses were not induced following vaccination with ChAdOx1-CCHFV Gn, but neutralisation was detected in an outbred mouse strain following vaccination with ChAdOx1 CCHFV Gc, demonstrating the greater potential for CCHFV Gc as an antigenic target in ChAdOx vaccines.

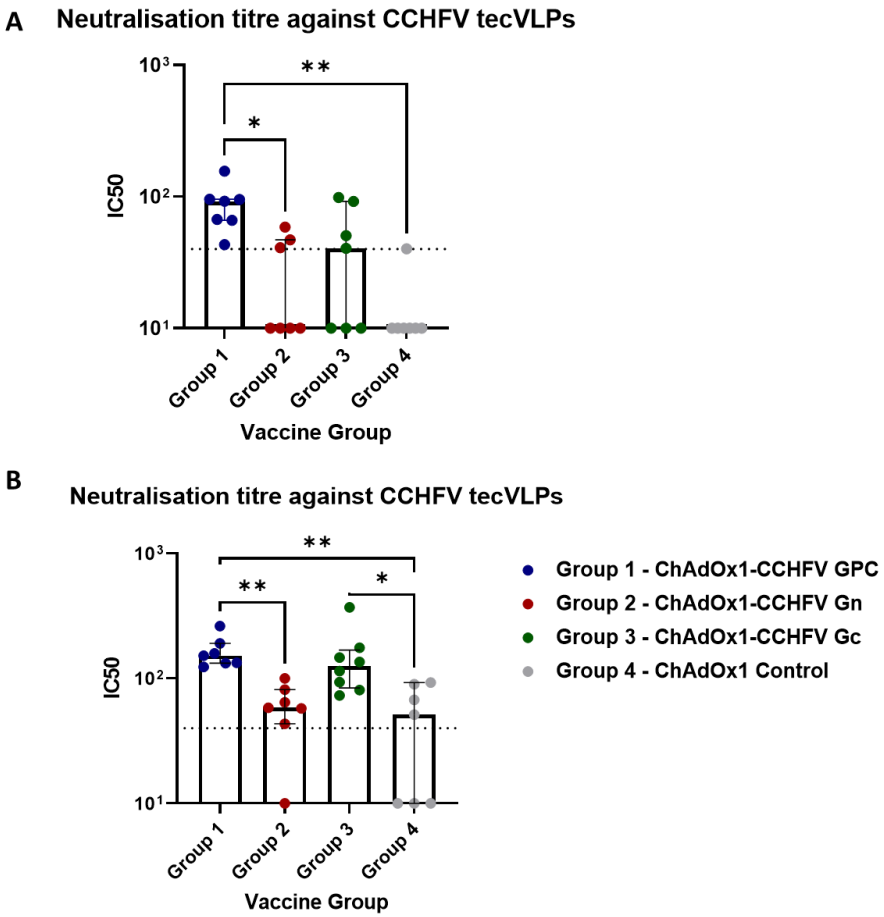


Figure 4.8: CCHFV tec-VLP IC50 neutralisation titres in Balb/c and CD-1 mice, following vaccination with ChAdOx1-CCHFV GPC or GPC subunit vaccines.
6 week old female balb/c (**A**) or CD-1 (**B**) mice were vaccinated with $1x10^8$ IU of ChAdOx1 Vaccine. 21 days after prime vaccination, mice were culled for immunological analysis. Neutralising antibody titres in balb/c mice (**A**) were determined by virus neutralisation test using CCHFV tec-VLPs. Individual data points (n=8) were expressed as IC50 values and shown here as a scatter dot plot with bars showing median and IQR. Values below the limit of detection were plotted with an IC50 of 10 for presentation purposes. Significant differences were determined by Kruskal-Wallis test with Dunn's multiple comparisons test (*p<0.05, **p<0.01). Neutralising antibody titres in CD-1 mice (**B**) were determined by virus neutralisation test using CCHFV tec-VLPs. Individual data points (group 1/2/4 n=7, group 3 n=8) were expressed as IC50 values and shown here as a scatter dot plot with bars showing median and IQR. Values below the limit of detection were plotted with an IC50 of 10 for presentation purposes. Significant differences were determined by Kruskal-Wallis test with Dunn's multiple comparison's test (*p<0.05, **p<0.01).

4.2.1.3 Design of antigen cassettes expressing CCHFV NP

Development of ChAdOx vectored vaccines carrying smaller CCHFV antigens continued with development of ChAdOx1-CCHFV NP, which includes the CCHFV NP sequence. CCHFV NP is expressed from the S segment of the CCHFV genome and is found within the CCHFV virion, coating the CCHFV genomic RNA. CCHFV NP is more highly conserved across CCHFV strains than GPC[245], making CCHFV NP a potential vaccine antigen for a broadly protective vaccine against multiple different CCHFV strains.

The structure of the antigen cassette for ChAdOx1-CCHFV NP is shown below in figure 4.9. The antigen cassette uses the short CMV promoter, as in ChAdOx1-CCHFV Gn and ChAdOx1-CCHFV Gc. The antigen cassette further utilised the same BGH polyA tail and was recombined into the ChAdOx1 genome. The CCHFV NP antigen itself was the full ORF from the S-segment of CCHFV IbAr10200. Unlike with CCHFV Gn and Gc, there was no need for modification as the entire NP antigen was comfortably within the size limits of the ChAdOx1 vector.

The CCHFV NP antigen was ordered and excised from delivery plasmid by digestion with HindIII and BamH1, then ligated into a shuttle vector linearised with HindIII and BamH1. Ligations were transformed into competent E.coli cells and analysed by diagnostic digest with HindIII and BamH1. Shuttle plasmids containing CCHFV NP antigen were recombined into the ChAdOx1 genome by the AttL1 and AttL2 sites. Once designed and cloned, the ChAdOx1-CCHFV NP virus was produced by the Viral Vector Core Facility, University of Oxford. Table 4.2 shows the size of the CCHFV NP antigen cassette compared to the previously discussed CCHFV GPC, CCHFV Gn and CCHFV Gc antigens. In size the CCHFV NP antigen cassette is between the sizes of CCHFV Gn and CCHFV Gc.

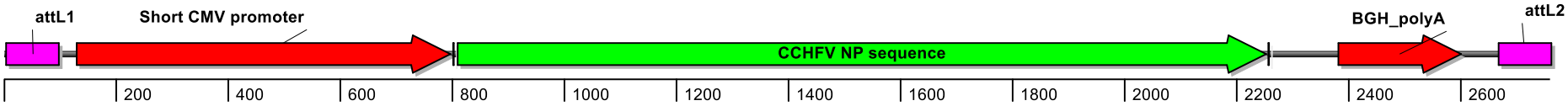


Figure 4.9: Antigen Cassettes for ChAdOx CCHFV NP vaccines.
Representation of CCHFV Gn antigen cassette. AttL1 and AttL2 represent recombination sites for the antigen cassette into the ChAdOx1 genome. Short CMV promoter represents the 673 amino acid promoter sequence derived from CMV. BGH_polyA represents the polyA tail of bovine growth hormone for transcriptional termination.

Vaccine	Antigen Cassette	Size of antigen cassette (bp)	Size of ChAdOx1 Genome with antigen (bp)	Percentage of Original ChAdY25 genome size (%)	Size of ChAdOx2 Genome with antigen (bp)	Percentage of Original ChAd68 Genome size (%)
ChAdOx1/2-CCHFV GPC	CCHFV GPC long promoter	7571	36674	100	36556	100
ChAdOx1/2-CCHFV Gn	CCHFV Gn	2195	31298	85	31183	85
ChAdOx1/2-CCHFV Gc	CCHFV Gc	2990	32039	87	31975	87
ChAdOx1/2 CCHFV NP	CCHFV NP	2564	31667	86	31549	86

Table 4.2: The size of ChAdOx vectored CCHFV genomes for CCHFV GPC, Gn, Gc, and NP, and their relationship to the size of their original ChAd genome
The table shows the size of the CCHFV GPC, CCHFV Gn, CCHFV Gc and CCHFV NP antigen cassettes, and their respective sizes when cloned into the ChAdOx1 and ChAdOx2 genome. ChAdOx1 genome sizes are then measured as a percentage of the progenitor ChAdY25 genome (36711 bp). ChAdOx2 genome sizes are then measured as a percentage of the progenitor ChAd68 genome (36521 bp)

4.2.1.4 Immunogenicity of ChAdOx1 vectored CCHFV NP

After production, immunogenicity of ChAdOx1-CCHFV NP was assessed by vaccination in Balb/c mice. Similarly to assessment of GPC and GPC subunits as antigens, immunogenicity was assessed by IFN-γ ELISPOT response to CCHFV NP specific peptides, total IgG ELISA against CCHFV NP protein, and a pseudotype VNT against CCHFV tec-VLPs.

The results of ChAdOx1 CCHFV NP immunogenicity are displayed in figure 4.10. Figure 4.10A shows the anti-CCHFV NP IFN-γ ELISPOT response. Responses against CCHFV NP are low but present, suggesting the vaccine has not induced a strong cellular response following immunisation. These results cannot however be compared directly to the IFN-γ ELISPOT responses induced by CCHFV GPC antigens, due to antigen size and the different properties of the peptides used. Anti-NP IgG responses, as presented in figure 4.10B, are also present in serum after vaccination with ChAdOx1-CCHFV NP. Whether sera from mice vaccinated with ChAdOx1-CCHFV NP could induce neutralisation responses was assessed but no evidence of neutralisation was observed despite total IgG ELISA responses.

The humoral responses are not unexpected as CCHFV NP would be expected to induce an antibody response, but due to the internal location of CCHFV NP on the CCHFV virion, these humoral responses may not be expected to be neutralising antibodies. As such, cellular responses against CCHFV NP may be expected to be the functional component in protection from CCHF, though they are unlikely to induce sterilising immunity.

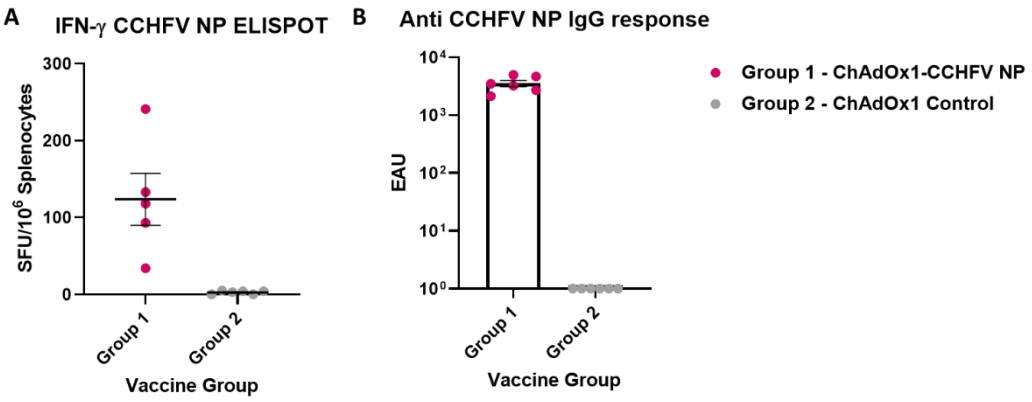


Figure 4.10: IFN-γ ELISPOT against peptides specific to CCHFV NP of strain IbAr10200 and anti IgG ELISA against CCHFV NP protein of strain IbAr10200, following vaccination with ChAdOx1-CCHFV NP.
6 week old balb/c were immunised with $1x10^8$ IU of ChAdOx1 vaccine. 21 days after prime vaccination, mice were culled for immunological analysis. CCHFV NP antigen specific IFN-γ responses (**A**) in balb/c mice were assayed in mouse splenocytes by IFN-γ ELISPOT. Individual balb/c mice (group 1 n=5, group 2 n=6) are shown here measured in spot forming units per 10^6 splenocytes (SFU/10^6) with mean and SEM also shown. Anti CCHFV NP IgG responses (**B**) in balb/c mice were measured by IgG ELISA. Individual balb/mice (n=6) are shown here in EAU with mean and SEM also shown.

CCHFV Gn, CCHFV Gc and CCHFV NP were all assessed as smaller CCHFV antigens, to improve stability and production in ChAdOx1 vectors for CCHFV, whilst maintaining immunogenic responses. All three antigens were shown to be immunogenic, producing both cellular and humoral immunogenicity. In particular, CCHFV Gn and CCHFV Gc antigens showed equivalent immunogenicity to CCHFV GPC, an antigen previously demonstrating protective efficacy in ChAdOx vectors.

4.2.2 Comparative ChAdOx2 vectored vaccine immunogenicity with CCHFV antigens

ChAdOx2 is an alternative ChAdOx vector, produced from modification of ChAd68 to make the vector replication deficient[233]. ChAdOx2 was used as the vector for CCHFV GPC, with vaccination producing an immunogenic response, and inducing protection against lethal CCHFV challenge in a mouse model. Despite ChAdOx2 CCHFV GPC inducing protection from lethal CCHFV challenge, the vaccine was genetically unstable and therefore unable to be produced in quantities necessary for larger trials.

4.2.2.1 Comparative immunogenicity of ChAdOx1 vectored vs ChAdOx2 vectored CCHFV GPC

The immunogenicity in the inbred balb/c mouse strain following vaccination with ChAdOx1-CCHFV GPC or ChAdOx2-CCHFV GPC is displayed in figure 4.11. The figure shows IFN-γ ELISPOT responses against CCHFV GPC peptides, anti CCHFV Gc total IgG ELISA, and neutralisation titres in a CCHFV tec-VLP pseudotype neutralisation assay following vaccination. Cellular responses, as measured by IFN-γ ELISPOT are presented in figure 4.11A, showed no significant difference following vaccination with ChAdOx1-CCHFV GPC or ChAdOx2-CCHFV GPC. Antibody titres were measured by

total IgG ELISA against CCHFV Gn or CCHFV Gc proteins. In balb/c mice no CCHFV Gn responses were measured above the limit of detection of the assay. Figure 4.11B presents the total IgG response against CCHFV Gc protein, where the humoral responses induced by ChAdOx2 CCHFV GPC vaccination were significantly greater than the response induced by vaccination with ChAdOx1 CCHFV GPC. Both vaccinations produced serum with a functional antibody response, displayed in figure 4.11C, with neutralising responses being observed against CCHFV tec-VLPs, with no significant difference in neutralisation measured between the ChAdOx1 and ChAdOx2 vectors.

These results show that vaccination with ChAdOx2-CCHFV induced significantly greater antibody anti-CCHFV Gc antibody titres in mice than vaccination with ChAdOx1-CCHFV GPC. However, no further significant differences were measured between vectors with regards to cellular or neutralisation responses induced by vaccination. ChAdOx2 has previously been shown to induce marginally greater cellular response with GFP as an antigen[233], but the antibody responses had not previously been published. These minor differences in humoral immunogenicity suggest that ChAdOx2 may be a more immunogenic vector than ChAdOx1, though whether the marginally greater immunogenicity will translate into a greater protective efficacy with ChAdOx2 vectored antigens is unknown.

With minor differences observed in immunogenicity between ChAdOx1 and ChAdOx2 as vectors for CCHFV GPC, the smaller CCHFV antigens previously assessed; CCHFV Gn, CCHFV Gc, and CCHFV NP, were cloned into the ChAdOx2 vector, to determine if immunogenicity would be different dependent on the ChAdOx vector.

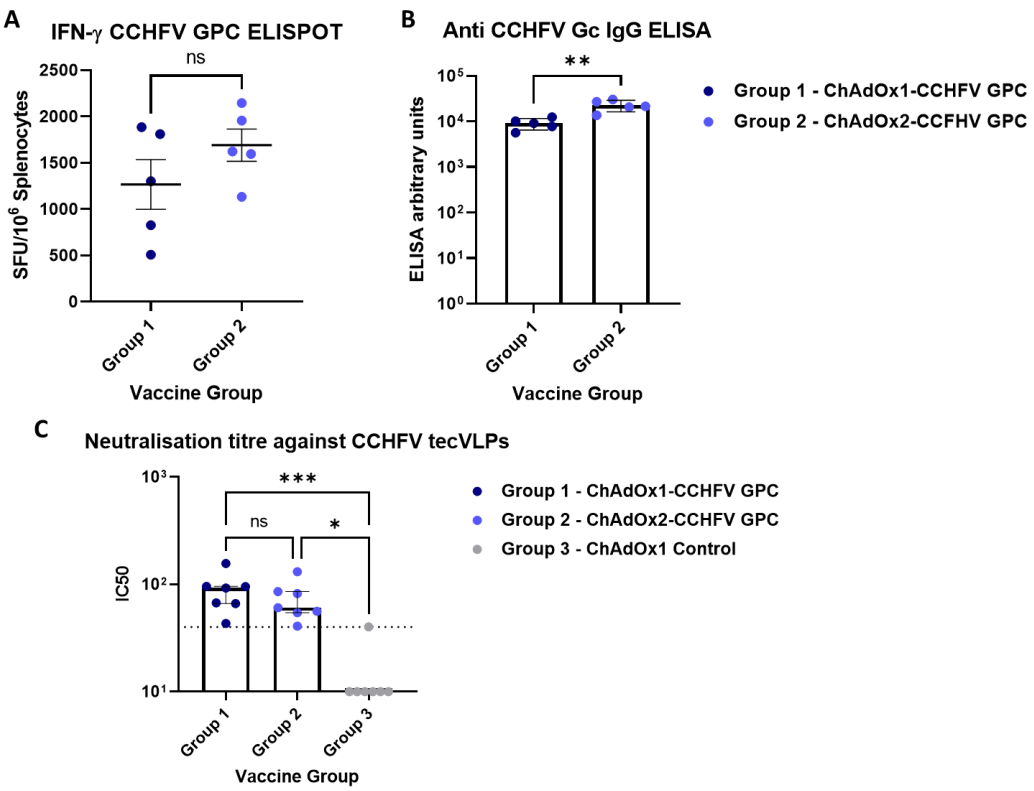


Figure 4.11: Comparison of IFN-γ ELISPOT against peptides specific to CCHFV GPC, anti IgG ELISA against CCHFV Gc protein, and neutralisation of CCHFV VLPs between ChAdOx1 and ChAdOx2 Vectored CCHFV GPC.

6 week old balb/c were immunised with 1x10^8 IU of ChAdOx vaccine. 21 days after prime vaccination, mice were culled for immunological analysis. CCHFV GPC antigen specific IFN-γ responses (**A**) in balb/c mice were assayed in mouse splenocytes by IFN-γ ELISPOT. Individual balb/c mice (n=5) are shown here measured in spot forming units per 10^6 splenocytes (SFU/10^6) with mean and SEM also shown. No significant differences were determined by Welch's t-test. Anti CCHFV Gc IgG responses (**B**) in balb/c mice were measured by IgG ELISA. Individual balb/mice (n=5) are shown here in EAU with mean and SEM also shown. Significant differences were determined by Mann-Whitney U test (**p<0.01). Neutralising antibody titres (**C**) were determined by virus neutralisation test using CCHFV tec-VLPs. Individual data points (n=7) were expressed as IC50 values and shown here as a scatter dot plot with bars showing median and IQR. Values below the limit of detection were plotted with an IC50 of 10 for presentation purposes. Significant differences were determined by Kruskal-Wallis test with Dunn's multiple comparisons test (*p<0.05, ***p<0.001).

4.2.2.2 Comparative immunogenicity of ChAdOx1 vs ChAdOx2 vectors with smaller CCHFV antigens

ChAdOx2 vectored vaccines were generated which incorporated the CCHFV Gn, CCHFV Gc, and CCHFV NP antigens. The antigen cassettes described previously in figures 4.5 and 4.9, were cloned into the ChAdOx2 genome with no further modification. Production of the ChAdOx2 viruses was performed by the Viral Vector Core Facility, and immunogenicity was compared between ChAdOx1 and ChAdOx2 vectored CCHFV antigens.

Immunogenicity in balb/c mice was compared between ChAdOx1-CCHFV Gn and ChAdOx2-CCHFV Gn, with assessment of cellular response by IFN-γ ELISPOT, antibody titres by anti-CCHFV Gn total IgG ELISA, and neutralisation titre by CCHFV tec-VLP pseudotype neutralisation assay, all presented in figure 4.12. CCHFV Gn specific IFN-γ ELISPOT responses following vaccination, displayed in figure 4.12A, were similar between ChAdOx1 and ChAdOx2 vectors with no significant differences determined. Anti-CCHFV Gn responses in a total IgG ELISA, displayed in figure 4.12B, showed an inconsistent response, with 42% of serum samples being below the limit of detection of the assay across both vaccine groups. With large variability measured in the ELISA, no significant differences were measured between the ChAdOx1 and ChAdOx2 vectors. No evidence of CCHFV tec-VLP neutralisation was measured after ChAdOx1-CCHFV Gn or ChAdOx2-CCHFV Gn vaccination. These results suggested very little difference in immunogenicity following vaccination with ChAdOx1-CCHFV Gn or ChAdOx2-CCHFV Gn.

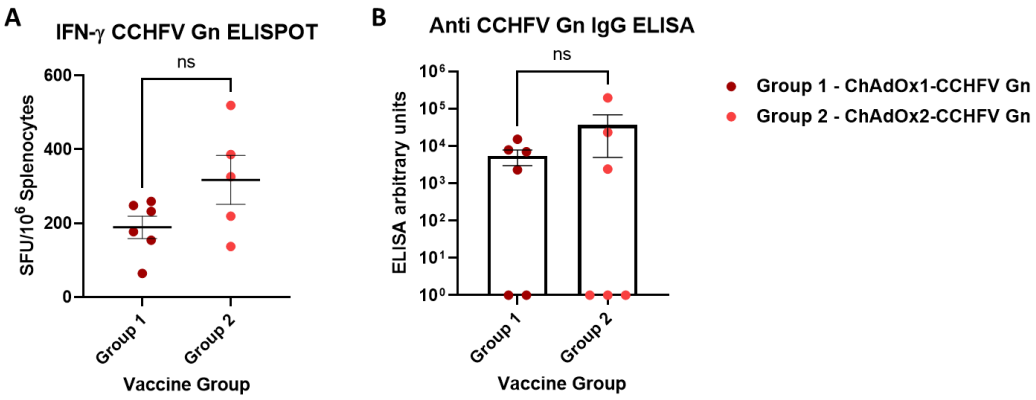


Figure 4.12: Comparison of IFN-γ ELISPOT against peptides specific to CCHFV Gn and total IgG ELISA against CCHFV Gn between ChAdOx1 and ChAdOx2 Vectored CCHFV Gn.

6 week old balb/c were immunised with $1x10^8$ IU of ChAdOx vaccine. 21 days after prime vaccination, mice were culled for immunological analysis. CCHFV Gn antigen specific IFN-γ responses (**A**) in balb/c mice were assayed in mouse splenocytes by IFN-γ ELISPOT. Individual balb/c mice (n=6) are shown here measured in spot forming units per 10^6 splenocytes (SFU/10^6) with mean and SEM also shown. No significant differences were determined by Welch's t-test. Anti CCHFV Gn IgG responses (**B**) in balb/c mice were measured by IgG ELISA. Individual balb/c mice (n=6) are shown here in EAU with mean and SEM also shown. Values below the limit of detection were assigned a value of 1EAU.

Responses against CCHFV Gc were assessed after vaccination with ChAdOx1-CCHFV Gc and ChAdOx2-CCHFV Gc. Immunogenicity was assessed by IFN-γ ELISPOT against CCHFV Gc peptides, anti-CCHFV Gc IgG ELISA, and CCHFV tec-VLP pseudotype VNT. Figure 4.13A shows IFN-γ ELISPOT responses to ChAdOx1 and ChAdOx2 vectored CCHFV Gc vaccination. No significant difference was measured after vaccination with ChAdOx1-CCHFV Gc or ChAdOx2-CCHFV Gc. Conversely, a significant difference was observed in the anti-CCHFV Gc responses following vaccination in total IgG ELISA, presented in figure 4.13B. The humoral response induced after ChAdOx2-CCHFV Gc vaccination was significantly greater than the response induced following vaccination with ChAdOx1-CCHFV Gc. The significant increase in humoral response did not translate to an increase in functional activity as neither ChAdOx1-CCHFV Gc nor ChAdOx2-CCHFV Gc vaccinated serum showed any evidence of neutralisation of CCHFV tec-VLPs.

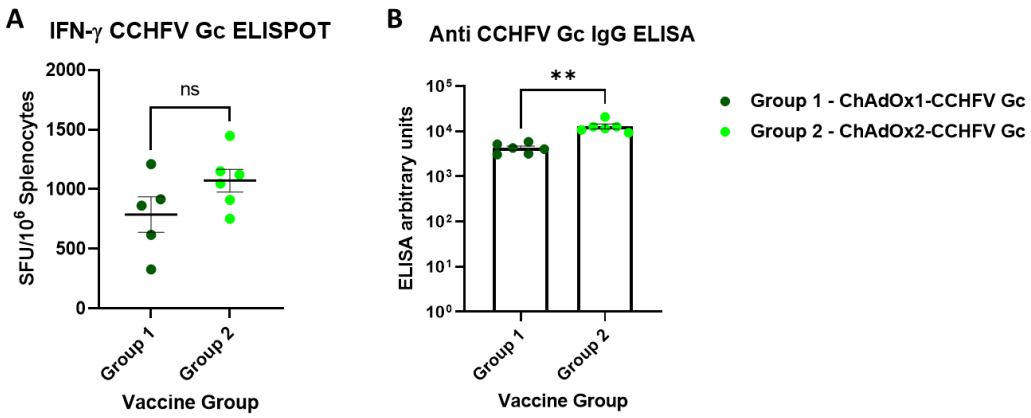


Figure 4.13: Comparison of IFN-γ ELISPOT against peptides specific to CCHFV Gc and total IgG ELISA against CCHFV Gc protein between ChAdOx1 and ChAdOx2 Vectored CCHFV Gc.

6 week old balb/c were immunised with $1x10^8$ IU of ChAdOx vaccine. 21 days after prime vaccination, mice were culled for immunological analysis. CCHFV GPC antigen specific IFN-γ responses (**A**) in balb/c mice were assayed in mouse splenocytes by IFN-γ ELISPOT. Individual balb/c mice (n=6) are shown here measured in spot forming units per 10^6 splenocytes (SFU/10^6) with mean and SEM also shown. No significant differences were determined by Welch's t-test. Anti CCHFV Gc IgG responses (**B**) in balb/c mice were measured by IgG ELISA. Individual balb/c mice (n=6) are shown here in EAU with mean and SEM also shown. Significant differences were determined by Mann-Whitney U test (**p<0.01).

Immune responses after vaccination with ChAdOx1 and ChAdOx2 vectored CCHFV NP were assessed through IFN-γ ELISPOT, anti-CCHFV total IgG ELISA, and CCHFV tec-VLP pseudotype VNT. Similar to CCHFV Gn and Gc as antigens, vaccination with ChAdOx2-CCHFV NP did not induce a significant difference in IFN-γ ELISPOT response, in figure 4.14A, compared to responses following vaccination with ChAdOx1-CCHFV NP. Anti-CCHFV NP humoral responses in total IgG ELISA, displayed in figure 4.14B showed no significant difference between the ChAdOx1 or ChAdOx2 vectors. Assessment of neutralisation showed no evidence of CCHFV tec-VLP neutralisation with serum vaccinated with either ChAdOx1-CCHFV NP or ChAdOx2-CCHFV NP.

The immunogenicity generated by CCHFV antigens after vaccination was compared in the ChAdOx1 and ChAdOx2 vectors. With the CCHFV Gn and CCHFV NP antigens, no significant differences were measured in immunogenicity between ChAdOx1 and the ChAdOx2 vector, in assays measuring either cellular, humoral, or neutralisation responses. With CCHFV GPC or CCHFV Gc as antigens, cellular and neutralisation responses induced following vaccination demonstrated no significant differences between ChAdOx1 and ChAdOx2 as vectors. For ChAdOx2-CCHFV Gc and ChAdOx2-CCHFV GPC significantly greater anti-CCHFV Gc responses were measured in total IgG ELISA, when compared with responses induced following vaccination with the corresponding ChAdOx1 vectored vaccines. These results suggest that ChAdOx2 may be improving the expression of correctly folded CCHFV Gc, though without a reliable test for protein expression, differences in expression cannot be confirmed. How these differential immune responses will impact efficacy against CCHF is still unclear.

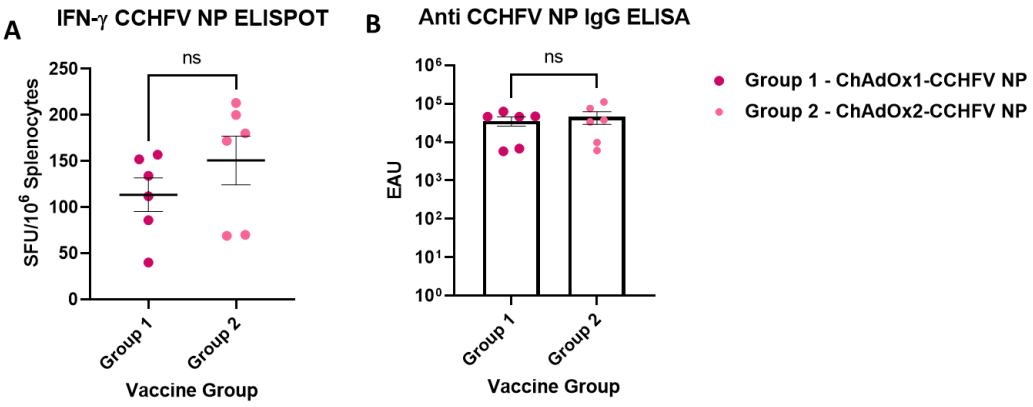


Figure 4.14: Comparison of IFN-γ ELISPOT against peptides specific to CCHFV NP and total IgG ELISA against CCHFV NP protein between ChAdOx1 and ChAdOx2 Vectored CCHFV NP.

6 week old balb/c were immunised with $1x10^8$ IU of ChAdOx vaccine. 21 days after prime vaccination, mice were culled for immunological analysis. CCHFV GPC antigen specific IFN-γ responses (**A**) in balb/c mice were assayed in mouse splenocytes by IFN-γ ELISPOT. Individual balb/c mice (group 1 n=5, group 2 n=6) are shown here measured in spot forming units per 10^6 splenocytes (SFU/10^6) with mean and SEM also shown. No significant differences were determined by Welch's t-test. Anti CCHFV NP IgG responses (**B**) in balb/c mice were measured by IgG ELISA. Individual balb/c mice (n=6) are shown here in EAU with mean and SEM also shown. No significant differences were determined by Mann-Whitney U test.

4.2.3 ChAdOx vectored vaccines expressing CCHFV GPC under different length promoters

Due to the cloning capacity of ChAdOx vectors, other avenues beyond smaller CCHFV antigens were explored, to reduce the size of the antigenic insert in the ChAdOx vectored vaccine. To that end ChAdOx1 and ChAdOx2 vectored CCHFV GPC vaccines were developed using a shortened promoter, with the antigen cassettes presented below in the figure 4.15. Cassette A utilises the short ~800 kB CMV promoter, and the same antigen cassette used to express the smaller CCHFV antigens previously described in this chapter. The cassette in figure 4.15B is the original cassette used in the protective ChAdOx2-CCHFV GPC vaccine, which uses a longer CMV promoter ~2.3kB in size. The only difference between the new ChAdOx2-sCMV-CCHFV GPC vaccine and the original ChAdOx2-CCHFV GPC vaccine is the size of the CMV promoter, with recombination site, polyA tail and antigen remaining the same. The shorter CMV promoter does not contain intron A, and when the efficacy of the two promoters was compared in an adenovirus vector, greater expression and immunogenicity was observed with the use of the longer promoter[325]. However, further internal work within the Jenner Institute suggests that not all antigens benefit from the longer promoter, suggesting the short CMV (sCMV) promoter is a viable alternative in vectors. More importantly, the size of the antigen cassette is reduced by ~1.4kB, reducing the size of the recombined ChAdOx genome below the accepted size limits of transgenic adenovirus[322]. The reduction in genome size would be expected to improve antigen stability and production of ChAdOx vectors.

The antigen cassette for production was produced by performing PCR on the long promoter antigen cassette, using primers which replicated the CCHFV GPC ORF with ends overlapping those of the short promoter shuttle plasmid, linearised with HindIII and BamH1. Recombination was performed by InFusion and recombination reactions cloned

into competent E.Coli cells. Successful recombinants were identified by diagnostic digest. Shuttle plasmids containing CCHFV GPC were recombined into the ChAdOx1 or ChAdOx2 genome by the attL1 and attL2 sites. Following production of the short CMV promoter CCHFV GPC (sCMV-CCHFV GPC) antigen cassette, ChAdOx1-sCMV-CCHFV GPC and ChAdOx2-sCMV-CCHFV GPC were produced by the Viral Vector Core Facility, University of Oxford.

Table 4.3 shows the size of the CCHFV GPC antigen cassette with the sCMV promoter compared to the original CCHFV GPC cassette with the full length CMV promoter. The antigen cassette with the sCMV promoter is smaller, though larger than the small CCHFV antigens presented in tables 4.1 and 4.2. When incorporated into the ChAdOx1 or ChAdOx2 genomes, the sCMV CCHFV GPC antigen cassette was ~96% of the original genome size, compared to 100% of progenitor genome size using the long promoter.

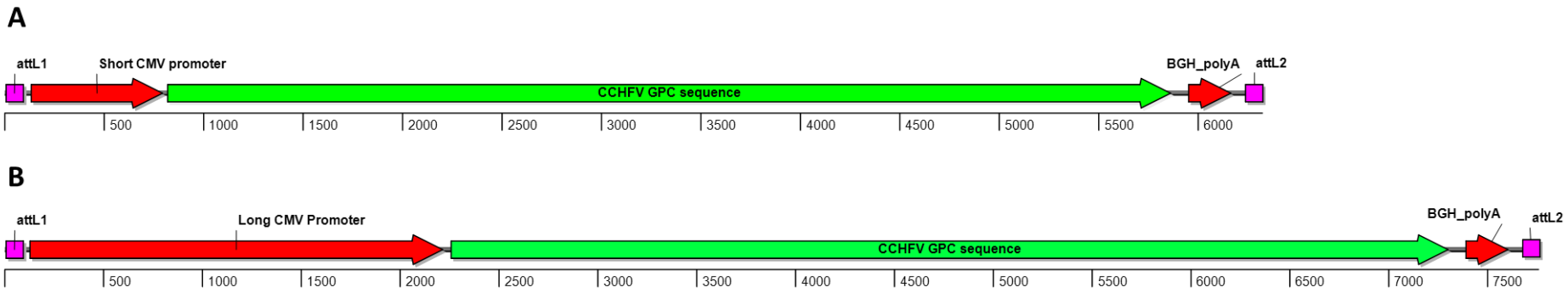


Figure 4.15: Antigen cassettes for CCHFV GPC with short and long promoters
Representation of CCHFV GPC antigen cassettes with (A) Short CMV promoter or (B) long CMV promoter. AttL1 and AttL2 represent recombination sites for the antigen cassette into the ChAdOx1 genome. Short CMV promoter represents the 673 amino acid promoter sequence derived from CMV. BGH_polyA represents the polyA tail of bovine growth hormone for transcriptional termination.

Vaccine	Antigen Cassette	Size of antigen cassette (bp)	Size of ChAdOx1 Genome with antigen (bp)	Percentage of Original ChAdY25 genome size (%)	Size of ChAdOx2 Genome with antigen (bp)	Percentage of Original ChAd68 Genome size (%)
ChAdOx1/2-CCHFV GPC	CCHFV GPC long promoter	7571	36674	100	36556	100
ChAdOx1/2-CCHFV-sCMV GPC	CCHFV GPC short promoter	6131	35234	96	35116	96

Table 4.3: The size of ChAdOx vectored CCHFV GPC genomes with long and short CMV promoters and their relationship to the size of their original ChAd genome
The table shows the size of the CCHFV GPC cassettes with long and short CMV promoters, and their respective sizes when cloned into the ChAdOx1 and ChAdOx2 genome. ChAdOx1 genome sizes are then measured as a percentage of the progenitor ChAdY25 genome (36711 bp). ChAdOx2 genome sizes are then measured as a percentage of the progenitor ChAd68 genome (36521 bp).

4.2.3.1 Immunogenicity of ChAdOx vectored CCHFV GPC vaccines with long and short CMV promoters

Following production of the ChAdOx1 and ChAdOx2-sCMV-CCHFV GPC vaccines, immunogenicity following vaccination was assessed in balb/c mice to determine if they could induce an anti-CCHFV GPC response. The results displayed in figure 4.16 show the IFN-γ ELISPOT responses against CCHFV GPC peptides, total IgG ELISA against CCHFV Gc, and CCHFV tec-VLP pseudotype VNT.

Figure 4.16A shows the IFN-γ ELISPOT responses to ChAdOx1 and ChAdOx2-sCMV-CCHFV GPC. Following vaccination, both vaccines produced a cellular response against CCHFV GPC specific peptides. Vaccination with ChAdOx2-sCMV-CCHFV GPC induced no significant differences in CCHFV GPC specific peptides in IFN-γ ELISPOT following vaccination, compared to post vaccination response induced by ChAdOx1-sCMV-CCHFV GPC vaccination. Total IgG response were measured by ELISA against CCHFV Gc protein, presented in figure 4.16B, and again both vaccines were shown to be immunogenic, inducing an anti-Gc IgG response in all mice vaccinated. No significant differences were measured in humoral responses following vaccination with either ChAdOx1-sCMV-CCHFV GPC or ChAdOx2-sCMV-CCHFV GPC. However, no functional neutralisation responses were measured with either vaccine.

As an antigen, CCHFV GPC would be expected to be immunogenic even under a different promoter. Surprisingly, CCHFV GPC under the short promoter did not present any evidence of significant neutralisation, unlike the long promoter counterparts. The ChAdOx1 against ChAdOx2 comparison was consistent with previous data presented for CCHFV antigens in this thesis, with no significant differences measured in immunogenicity.

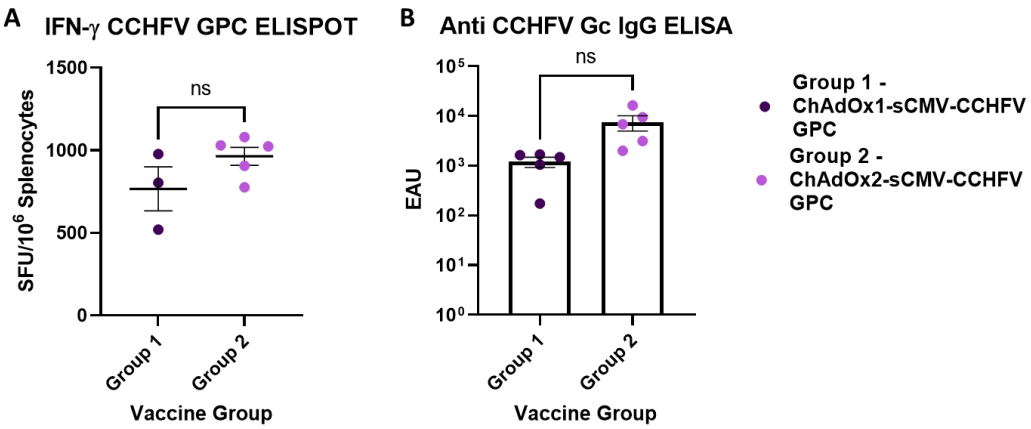


Figure 4.16: Comparison of IFN-γ ELISPOT against peptides specific to CCHFV GPC and total IgG ELISA against CCHFV Gc protein between ChAdOx1 and ChAdOx2 vectored CCHFV GPC expressed by the short CMV promoter.
6 week old balb/c were immunised with $1x10^8$ IU of ChAdOx1 vaccine. 21 days after prime vaccination, mice were culled for immunological analysis. CCHFV GPC antigen specific IFN-γ responses (**A**) in balb/c mice were assayed in mouse splenocytes by IFN-γ ELISPOT. Individual balb/c mice (group 1 n=3, group 2 n=5) are shown here measured in spot forming units per 10^6 splenocytes (SFU/10^6) with mean and SEM also shown. No significant differences were determined by Welch's t-test. Anti CCHFV Gc IgG responses (**B**) in balb/c mice were measured by IgG ELISA. Individual balb/mice (n=5) are shown here in EAU with mean and SEM also shown. No significant differences were determined by Welch's t-test.

It has not been established if the long and short CMV promoters used within ChAdOx vectors directly impact immunogenicity. Therefore, immunogenicity induced after vaccination with ChAdOx1-sCMV-CCHFV GPC and ChAdOx2-sCMV-CCHFV GPC was compared to the immunogenicity induced by vaccination with the original long promoter ChAdOx1 CCHFV GPC or ChAdOx2 CCHFV GPC vaccines.

Figure 4.17 shows the comparison of immunogenicity in balb/c mice following vaccination with ChAdOx1-CCHFV GPC and ChAdOx1-sCMV-CCHFV GPC vaccines. Immunogenicity was assessed through IFN-γ ELISPOT against CCHFV GPC peptides, anti-CCHFV Gc total IgG ELISA, and CCHFV tec-VLP pseudotype VNT. The IFN-γ ELISPOT results present in figure 4.17A showed no significant difference between the responses induced following vaccination by ChAdOx1-CCHFV GPC or ChAdOx1-sCMV-CCHFV GPC. The total IgG response against CCHFV Gc in figure 4.17B showed strong responses from both vaccines, but the response induced by ChAdOx1-CCHFV GPC vaccination was significantly greater than the response induced following vaccination with ChAdOx1-sCMV-CCFHV GPC. When assessing neutralisation activity, no neutralisation activity was detected following vaccination with ChAdOx1-CCHFV GPC or ChAdOx1-sCMV-CCHFV GPC. Whilst neutralisation data is consistent with previous results following ChAdOx1-sCMV-CCHFV GPC vaccination, previous experiments had demonstrated that vaccination with ChAdOx1-CCHFV GPC did induce significant neutralisation responses.

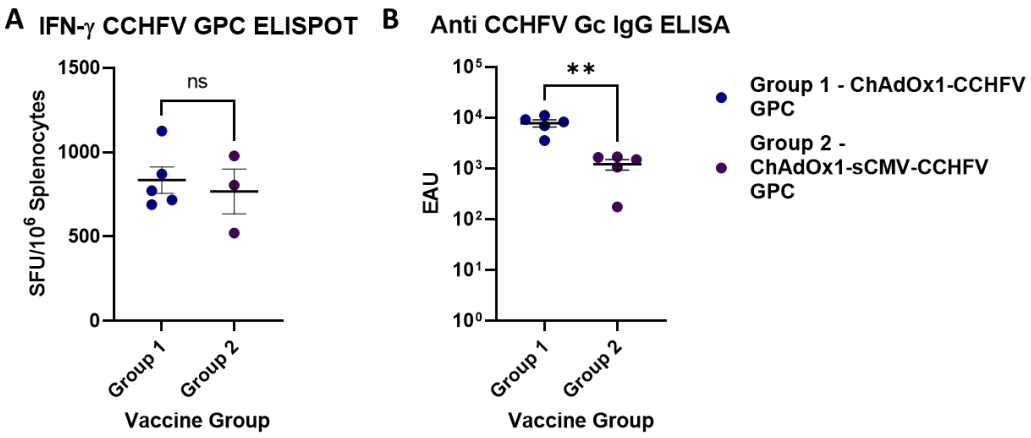


Figure 4.17: Comparison of IFN-γ ELISPOT against peptides specific to CCHFV GPC and total IgG ELISA against CCHFV Gc protein between ChAdOx1-CCHFV GPC vaccines using the CMV long or short promoter.

6 week old balb/c mice were immunised with $1x10^8$ IU of ChAdOx1 vaccine. 21 days after prime vaccination, mice were culled for immunological analysis. CCHFV GPC antigen specific responses (A) were quantified in balb/c mice by IFN-γ ELISPOT. Individual mice (group 1 n=5, group 2 n=3) are shown here as individual data points measured as spot forming units (SFU) per 1 million splenocytes, with mean and standard error of the mean (SEM) also shown. No significant differences were determined using Welch's t-test. CCHFV Gc antigen specific IgG responses (B) were quantified by total IgG ELISA. Individual mice (n=5) are shown as data points measured in EAU, with mean and SEM also shown. Significant differences were determined by Welch's t-test (**p>0.01).

The immunogenicity induced following vaccination with ChAdOx2-CCHFV GPC or ChAdOx2-sCMV-CCHFV GPC was assessed by IFN-γ ELISPOT against CCHFV GPC peptides, total IgG ELISA against both CCHFV Gn and CCHFV Gc proteins, and CCHFV tec-VLP pseudotype neutralisation, with the results displayed in figure 4.18. IFN-γ ELISPOT responses were shown in figure 4.18A, with no significant difference measured in cellular responses following vaccination. Unlike with previous responses from ChAdOx1 vaccines, anti-CCHFV Gn responses were measured by total IgG ELISA, displayed in figure 4.18B. However, these responses were inconsistent with one mouse vaccinated with ChAdOx2-CCHFV GPC, and three mice vaccinated with ChAdOx2-sCMV-CCHFV GPC showing no responses following vaccination. Figure 4.18C shows the anti-CCHFV Gc responses as assayed by total IgG ELISA. Responses induced by following ChAdOx2-sCMV-CCHFV GPC vaccination were significantly lower than the responses induced after vaccination with ChAdOx2-CCHFV GPC. Neutralisation of CCHFV tec-VLPs was assessed and presented in figure 4.18D. Evidence of significant neutralisation was measured following ChAdOx2-CCHFV GPC vaccination.

The results of comparing ChAdOx vaccine immunogenicity with long and short CMV promoters showed small differences between the two promoters. ChAdOx1-sCMV-CCHFV-GPC and ChAdOx2-sCMV-CCHFV GPC vaccines were both immunogenic producing both cellular and humoral responses, though no evidence of CCHFV neutralisation was measured following immunisation with either vaccine. When compared to their long CMV promoter counterparts, very little difference was measured in cellular responses, but anti-CCHFV Gc antibody titres were significantly greater for both long promoter vaccines when compared against their short promoter vaccine counterparts. However, without clear correlates of protection, it is unknown if these differences in humoral response would create a difference in protective efficacy of a ChAdOx vectored CCHFV vaccine.

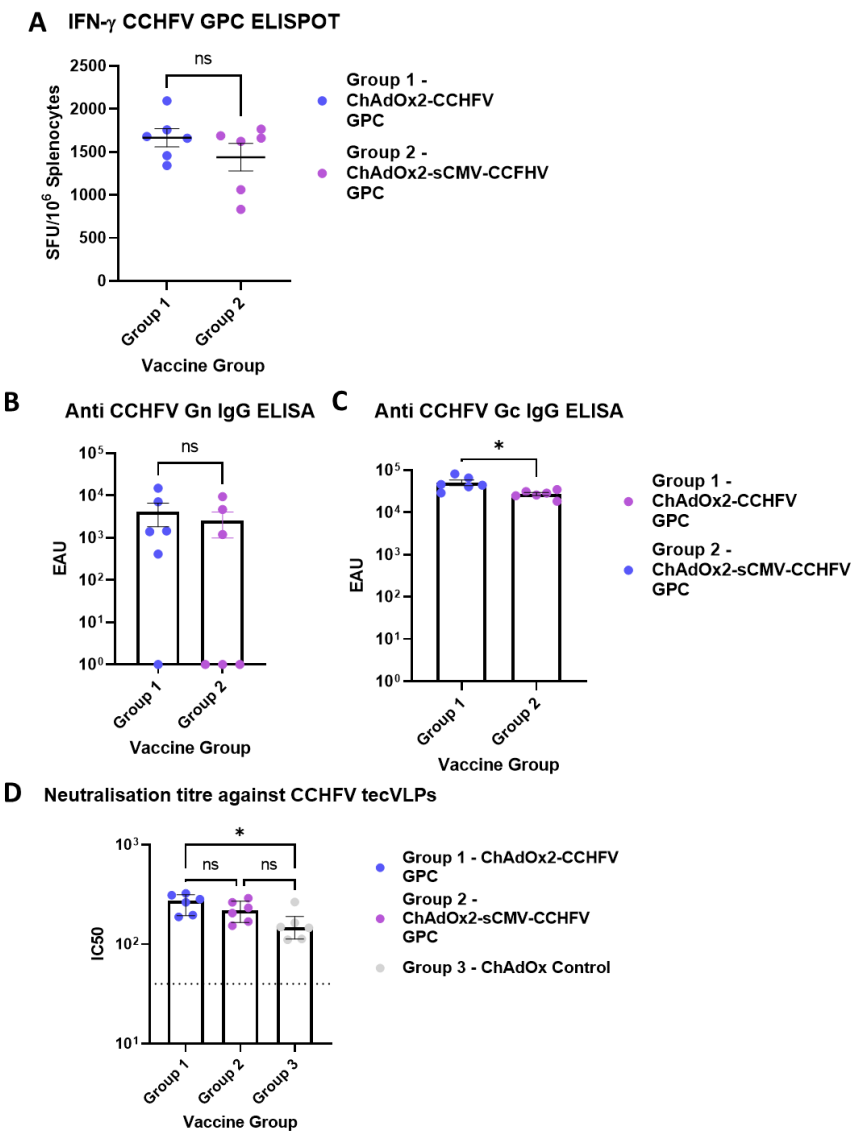


Figure 4.18: Comparison of IFN-γ ELISPOT against peptides specific to CCHFV GPC, total IgG ELISA against CCHFV Gn and Gc proteins, and CCHFV tec-VLP neutralisation between CHAdOx2-CCHFV GPC vaccine using the long or short promoter.

6 week old balb/c mice were immunised with 1x10^8 IU of ChAdOx1 vaccine. 21 days after prime vaccination, mice were culled for immunological analysis. CCHFV GPC antigen specific responses (**A**) were quantified in balb/c mice by IFN-γ ELISPOT. Individual mice (n=6) shown here as individual data points measured as spot forming units (SFU) per 1 million splenocytes, with mean and standard error of the mean (SEM) also shown. No significant differences were determined by Welch's t-test. CCHFV Gn antigen specific IgG responses (**B**) were quantified by total IgG ELISA. Individual mice (n=5) are shown as data points measure in EAU, with mean and SEM also shown. No significant differences were determined by Welch's t-test. CCHFV Gc antigen specific IgG responses (**C**) were quantified by total IgG ELISA. Individual mice (n=5) are shown as data points measure in EAU, with mean and SEM also shown. Significant differences were determined by Welch's t-test (*p<0.05). Neutralising antibody titres (**D**) were determined by virus neutralisation test using CCHFV tec-VLPs. Individual data points (n=6) were expressed as IC50 values and shown here as a scatter dot plot with bars showing median and IQR. Significant differences were determined by Ordinary One-way ANOVA with Tukey's multiple comparison's test (*p<0.05).

4.3 Discussion

CCHFV is a global disease of concern, and development of effective vaccines is an important step in controlling the virus and subsequent disease. To this end a ChAdOx2-CCHFV GPC vaccine was developed and tested in pre-clinical studies. Immunisation with ChAdOx2-CCHFV GPC was shown to be protective in a lethal CCHFV challenge mouse model. However, the vaccine was genetically unstable, likely due to the size of the antigenic cassette, and as such GMP production could not be scaled to the quantities needed for further assessment. The work described in this chapter has focused on developing vaccines which could be progressed into clinical trial through the reduction of the size of the antigen cassette and provide a more focused immune response for cross strain immunogenicity and protection.

The CCHFV GPC antigen in a ChAdOx vectored vaccine was protective in small animal challenge model, and therefore vaccine induced immunogenicity of two subunits antigens of GPC, Gn and Gc, was assessed and compared to immunogenicity induced by vaccination with the CCHFV GPC antigen in ChAdOx vectors. CCHFV Gn induced no significant difference in cellular responses compared to CCHFV GPC as a vaccine antigen, and induced humoral responses were too poor to draw any significant conclusions. The same pattern was repeated with the CCHFV Gc antigen, with no significant differences measured in cellular and humoral responses following vaccination with ChAdOx1 vectored CCHFV GPC and CCHFV Gc antigens. These immunogenicity results are particularly impressive as CCHFV Gn and Gc were expressed in ChAdOx vectors from a weaker promoter than the ChAdOx1-CCHFV GPC vaccine used as a comparator.

When neutralisation was assessed, results were less positive for smaller antigens. ChAdOx1 CCHFV GPC showed significant neutralisation in both inbred and outbred mouse strains. CCHFV Gn did not induce any neutralising response, which may be

expected due to the poor humoral responses measured in anti-CCHFV Gn ELISAs. In turn, CCHFV Gc did induce a significant neutralisation response in the outbred CD-1 strain but did not induce neutralisation in balb/c mice. It is unsurprising that CCHFV GPC, which includes the GP38 subunit shown to induce neutralising responses[90], induced a greater neutralisation response than CCHFV Gn and Gc. With the correlates of protection from CCHF unknown, but cellular responses likely more important for protection[257], the lack of neutralisation measured from vaccines encoding CCHFV Gn and Gc antigens may not affect their ability to induce protective responses against CCHFV. Further small animal models will be required to determine if the induced responses will be protective and if CCHFV Gn and CCHFV Gc antigens should be prioritised for clinical progression.

CCHFV NP was further assessed as a novel antigen in ChAdOx vectors. Assessment of immunogenicity showed a cellular and humoral response was induced against the antigen. The effectiveness of the humoral response is specifically unknown as it is unlikely CCHFV NP would be exposed to an antibody response. Furthermore, no evidence was measured of neutralisation activity in serum taken from mice vaccinated with ChAdOx vectored CCHFV NP, suggesting little functional activity for CCHFV NP antibodies, at least to the test presented within this thesis.

CCHFV antigens were assessed in two different vectors, ChAdOx1 and ChAdOx2. Initial evidence with CCHFV vaccines showed ChAdOx2 induced no significant difference in cellular responses with all the CCHFV antigens tested. Humoral responses following vaccination with ChAdOx1 and ChAdOx2 vectored CCHFV NP and CCHFV Gn antigens measured no significant differences. Research on the differences in immunogenicity and expression between ChAdOx vectors is limited, and without more knowledge on the differences between the two vectors, it is difficult to speculate on the reasons that ChAdOx2 vectored CCHFV GPC and CCHFV Gc vaccines induced greater

humoral responses, for which future studies examining expression levels of antigens are warranted.

Further attempts to reduce antigen cassette size were made by utilising the short CMV promoter with CCHFV GPC antigens. ChAdOx1 and ChAdOx2 vaccines expressing CCHFV GPC under the short CMV promoter were immunogenic, and when compared to their equivalent long promoter vaccines, showed little difference in cellular immunogenicity. Significantly greater anti-CCHFV Gc humoral responses were measured following vaccination with CCHFV GPC driven by the long CMV promoter in both ChAdOx1 and ChAdOx2 comparisons. Neither ChAdOx1-sCMV-CCHFV GPC nor ChAdOx2-sCMV-CCHFV GPC vaccination showed any evidence of induction of CCHFV neutralisation responses in the pseudotype neutralisation assay.

Across the work produced in this chapter a range of different ChAdOx vectored vaccines have been produced. These alternative vaccines have been effective in reducing the size of the antigen cassettes used in the vaccines to below the theoretical limits of the ChAdOx vector, to enhance stability and production of the viral vector. Cellular responses were broadly similar between following vaccination with ChAdOx vectors using either the long or short promoter to drive CCHFV GPC expression. Comparable cellular immunogenicity was also measured following vaccination with CCHFV GPC and small CCHFV antigens, Gn and Gc. Humoral responses varied to a greater degree, with anti-CCHFV Gn responses being highly variable, with not all vaccinated animal generating a humoral response, and the usefulness of the anti-CCHFV NP responses being debatable. Anti-CCHFV Gc humoral responses differed between the long and short promoter equivalents, and the antibody titres induced following vaccination with ChAdOx vectored CCHFV antigens were comparable to antibody titres induced by vaccination with CCHFV GPC in ChAdOx vectored vaccines. When assessing functional immunogenicity by neutralisation, only CCHFV GPC antigens expressed by the long

promoter showed evidence of neutralisation in a tec-VLP based neutralisation assay, when assessing in both balb/c mice and CD-1 mice, though vaccination with ChAdOx1-CCHFV GPC did not induce neutralising titres in all experiments. ChAdOx1 CCHFV Gc also exhibited a significant neutralising response when serum from vaccinated CD-1 mice was assessed, suggesting a possibility of neutralization responses being induced in other mouse lines or species.

There is still potential for further assessment of vaccine antigens in ChAdOx vectors. Since the design and choice of CCHFV Gn and Gc as antigens for vaccines, Gn and Gc have been shown to be protective antigens in small animal challenge models in mRNA vectored vaccines[324], reinforcing their selection as antigens in ChAdOx vectors. However, the role of the GP38 subunit in protective effects has also been further determined. GP38 is now thought to be an immuno-dominant epitope, producing a response from which neutralising monoclonal antibodies can be isolated, and have a role in the protection induced by some CCHFV GPC vaccines in mouse models[90,320]. The drawback of the antigen, as noted in figure 4.3, is the variation of the GP38 sequence across strains, but ChAdOx vectored CCHFV GP38 vaccines may be a relevant vaccine to protect against homologous challenge. In addition, further work could be performed trying to further utilise the long promoter and assess the expression and immunogenicity of CCHFV Gn, CCHFV Gc, and CCHFV NP in ChAdOx vectors under the long promoter.

Ultimately, when looking at the protection data from the ChAdOx2 CCHFV GPC vaccination, 100% survival was induced by all the vaccine regimens tested, and as such correlates of protection from cellular and humoral responses cannot be drawn. It is unknown if the immunogenicity induced by the alternative vaccines presented throughout this chapter will remain protective.

Smaller CCHFV antigens were assessed for multiple reasons and not only to reduce the size of the ChAdOx vaccine genome. CCHFV Gn, CCHFV Gc and CCHFV NP are more

highly conserved than the CCHFV GPC antigen often targeted, and as such provide a greater potential to protect against heterologous strains of CCHFV. Further work looked at the immunogenicity induced by co-administration of CCHFV vaccines and combination of multiple CCHFV antigens into a multivalent vaccine, with the purpose of providing immunity against multiple antigens for a broader range of protection and to lower the risk of infection and disease due to antigenic drift of CCHFV evading immune protections.

Chapter 5 – Development of multivalent ChAdOx vaccines against CCHFV

5.1 Introduction

A common concern for vaccine development is the ability for pathogens to mutate and evolve adaptions which evade the vaccine-induced immune response, without compromising pathogenicity[326]. Antigenic drift has been observed during the current COVID-19 pandemic, with the outbreaks of alpha, beta, and delta strain, which showed a measure of evasion to vaccine induced responses against the WT spike protein[327]. With CCHFV, infection levels are relatively low and poorly studied so the proclivity for antigenic drift is unknown. However, due to the large geographic range of CCHFV[8], and the existence of multiple clades of CCHFV[328], the same issues present themselves, with the potential for heterologous CCHFV strains to evade vaccine induced immunity. Challenge studies in small animal models have shown that vaccine induced protection can be reduced by challenge with a heterologous strain[329], showing concerns are not purely theoretical.

One method to address antigenic variation is to use a highly conserved antigen within the vaccine. As shown in Chapter 4, the CCHFV Gn, Gc and NP antigens were highly conserved in CCHFV, with the Gn and Gc subunit antigens showing greater amino acid conservation than the full length GPC. Greater conservation means that Gn and Gc are more similar between different strains, and fewer differences reduce the likelihood that immunogenicity will be evaded. The second approach is to combine highly conserved antigens with the delivery of multiple antigens within a vaccine platform, providing a greater breadth of cellular and humoral immunogenicity. Evasion of vaccine induced immunogenicity would therefore require multiple escape mutations. Vaccines targeting

multiple antigens are common in routinely administered protein based vaccines[330], and their use is expanding in further vaccine technologies[201,331].

Multivalent vaccines have been developed to induce immunogenicity and protective efficacy against CCHFV. DNA vaccines expressing CCHFV Gn, Gc and NP on separate, co-administered plasmids have shown 100% protection from lethal CCHFV challenge in small animal models[249]. Conversely in the same challenge, the administration of CCHFV tec-VLPs as a protein vaccine covering NP, GPC and the RdRP, did not induce protection despite the use of multiple antigens[332]. More recently, a DNA vaccine using co-immunisation of both GPC and NP showed 100% protection against lethal CCHFV challenge in a cynomolgus macaque model[250]. The evidence suggests that protection can induced by the use of multiple CCHFV antigens, though whether either antigen would be sufficient independently has not been tested in these challenge models.

Multivalent viral vectored vaccines, carrying multiple antigens have been developed for a range of emerging and pandemic potential pathogens. The commonly used MVA vector has been shown to have high carrying capacity, specifically in the licenced vaccine Mvabea[201], which carries three separate filovirus glycoprotein genes and a filovirus nucleoprotein gene as antigens[215]. ChAdOx vectored vaccines and other non-poxviral vectors, do not have the same capacity to carry antigens as MVA, but multivalent ChAdOx vectors have been developed through a variety of methods. ChAdOx1 NP+M1 is an experimental influenza vaccine[225], which targets the highly conserved nucleoprotein and matrix 1 protein of influenza A, with a single fusion protein expressed from ChAdOx1. Strong cellular responses were induced in phase 1 clinical trials which could be boosted with the NP+M1 antigen in an MVA vector.[225]

A different approach to multivalent ChAdOx1 vaccines was attempted in order to produce a cross-protective filovirus vaccine. A variety of different approaches were attempted to induce immunogenicity against Zaire Ebolavirus, Sudan Ebolavirus, and

Marburg virus[224]. Two of the vaccine candidates expressed a single transcript from the standard ChAdOx1 vector, expressing a single fusion polyprotein with glycine linkers, and three individually expressed glycoproteins, separated by 2A ribosomal self-cleaving linkers[224]. Further, a bivalent ChAdOx1 vector was developed with two separate entry sites for antigens at an E1 and E4 locus. Studies showed expression of glycoproteins could be induced from both antigen loci, and cellular and humoral immunogenicity was induced against both expressed glycoproteins[224].

With immunogenic multivalent ChAdOx1 vaccines being produced in multiple different approaches, CCHFV antigens were assessed in multivalent vaccines for the purpose of developing ChAdOx1 vaccines, with a wider breadth of immunogenicity, against CCHFV.

5.2 Results

5.2.1 Co-administration of monovalent CCHFV vaccines

Initial experimentation focused on co-administration of ChAdOx vectored CCHFV smaller antigen vaccines to determine if there was any evidence of antigenic interference between CCHFV Gn, CCHFV Gc and CCHFV NP.

5.2.1.1 Immunogenicity of co-administered ChAdOx1-CCHFV Gn and ChAdOx1-CCHFV Gc

CCHFV Gn and Gc are the surface glycoproteins of CCHFV[83] and are thought to form heterologous dimer on the surface of the CCHFV[333]. Immunisations were performed in balb/c mice, and the immunogenicity induced by immunisation with individual vaccines

was compared against the immunogenicity induced by vaccination with a 1:1 mix of ChAdOx1-CCHFV Gn and ChAdOx1-CCHFV Gc.

Figure 5.1 shows cellular responses induced by vaccination against CCHFV Gn and CCHFV Gc specific peptides, as assayed by IFN-γ ELISPOT. Figure 5.1A shows CCHFV Gn specific responses following vaccination with ChAdOx1-CCHFV Gn or the monovalent mix. Anti-CCHFV Gn responses were measured with both regimens, and no significant differences measured in cellular responses after vaccination with ChAdOx1-CCHFV Gn or the monovalent mixture. Figure 5.1B shows cellular responses against CCHFV Gc specific peptides. Vaccination with ChAdOx1-CCHFV Gc or the monovalent mixture induced anti-CCHFV Gc cellular responses, and no significant differences in cellular responses were measured between the two vaccinated groups.

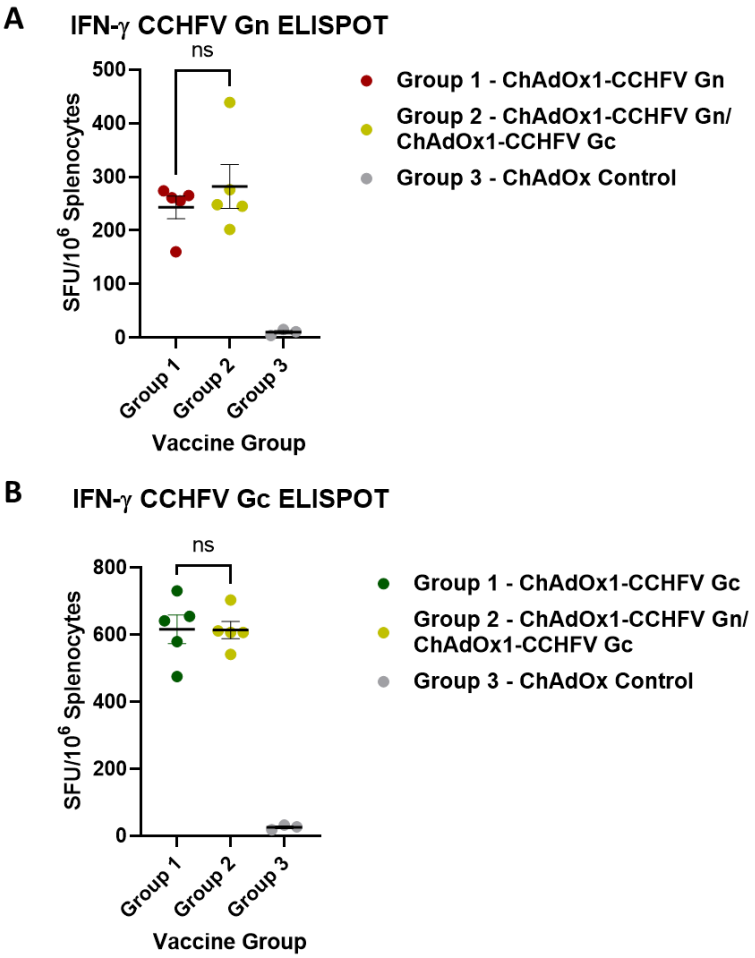


Figure 5.1: IFN-γ ELISPOT against peptide specific to Gn or Gc of CCHFV IbAr10200 strain, following monovalent or co-administered vaccination.
Six week old female balb/c mice were immunised with $1x10^8$ IU of ChAdOx1 vaccine. Twenty one days after prime vaccination, mice were culled for immunological analysis. CCHFV Gn antigen responses (**A**) in balb/c mice were determined by IFN-γ ELISPOT. Individual mice (groups 1/2 n=5, group 3 n=3) are shown here as individual data points measured as spot forming units per 10^6 ($SFU/10^6$) splenocytes, with mean and standard error of the mean (SEM) shown. No significant differences were determined by ordinary one-way ANOVA. CCHFV Gc antigen responses (**B**) in Balb/c mice were determined by IFN-γ ELISPOT. Individual mice (groups 1/2 n=5, group 3 n=3) are shown here as individual data points measured as spot forming units per 10^6 ($SFU/10^6$) splenocytes, with mean and standard error of the mean (SEM) shown. No significant differences were determined by Ordinary One-way ANOVA.

Humoral responses as measured by total IgG ELISA are shown in figure 5.2. Anti-CCHFV Gn humoral responses are shown in figure 5.2A. Vaccination with the monovalent mixture induced no detectable anti-Gn response, and responses following ChAdOx1-CCHFV Gn vaccination were inconsistent, with only three mice producing a detectable anti-Gn response. Consequently, no significant differences were measured by anti-Gn total IgG ELISA. Figure 5.2B displays anti-Gc total IgG ELISA responses. All mice vaccinated with ChAdOx1-CCHFV Gc showed a response along with the majority of the monovalent mixture group, however two mice vaccinated with the monovalent mixture did not show anti-Gc humoral responses. The mice that did respond showed similar levels to the monovalent vaccine, and no significant differences were measured between groups following vaccination, despite the non-responders. No evidence of neutralisation was measured following ChAdOx1-CCHFV Gn or ChAdOx1-CCHFV Gc vaccination, and consequently, no neutralisation was measured after vaccination with the monovalent mixture of CCHFV antigens.

In assessing immunogenicity induced by vaccination with ChAdOx1-CCHFV Gn and ChAdOx1-CCHFV Gc monovalent mix, no significant differences were measured from the respective responses to their monovalent equivalents. These results suggest no antigenic interference occurred following vaccination between CCHFV Gn and CCHFV Gc from ChAdOx1 vectors. Therefore assessment of co-administration progressed to vaccinations with CCHFV Gn, CCHFV Gc, and CCHFV NP.

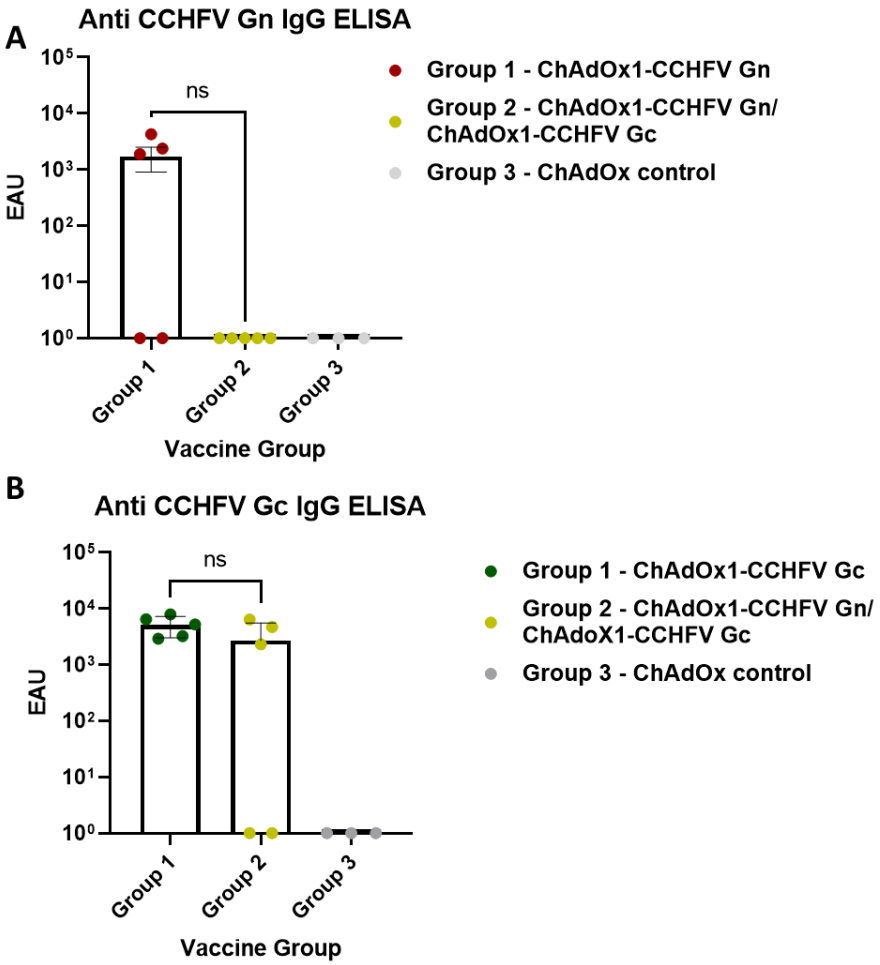


Figure 5.2: Total IgG ELISA against CCHFV Gn or Gc of the CCHFV IbAr10200 strain, following monovalent or co-administration vaccination.
Six week old balb/c mice were immunised with 1x10^8 IU of ChAdOx1 vaccine. Twenty one days after prime vaccination, mice were culled for immunological analysis. CCHFV specific Gn responses (**A**) were quantified in balb/c mice in a standardised ELISA. Individual mice (groups 1/2 n=5, group 3 n=3) are shown as data points in ELISA arbitrary units (EAU) with mean and standard error of the mean (SEM) also shown. Responses below the limit of detection were assigned a value of 1EAU for presentation purposes. No significant differences were determined by Kruskal-Wallis test. CCHFV specific Gc responses (**B**) were quantified in Balb/c mice in a standardised ELISA. Individual mice (groups 1/2 n=5, group 3 n=3) are shown as data points in EAU, with mean and SEM also shown. Responses below the limit of detection were assigned a value of 1EAU for presentation purposes. No significant differences were determined by Kruskal-Wallis test.

5.2.1.2 Immunogenicity of co-administered ChAdOx1-CCHFV Gn, ChAdOx1-CCHFV Gc, and ChAdOx1-CCHFV NP

As discussed in the introduction of the current chapter, co-administration of CCHFV Gn, Gc, and NP antigens in DNA vaccines has been shown to induce protection from lethal CCHFV challenge in animal models[249]. As such the co-administration of ChAdOx1 vectored CCHFV Gn, CCHFV Gc, and CCHFV NP was assessed for effects of antigenic interference on immunogenicity. Balb/c mice were vaccinated with either a monovalent vaccine, or a 1:1:1 mixture of ChAdOx1-CCHFV Gn, ChAdOx1-CCHFV Gc, and ChAdOx1-CCHFV NP.

Cellular responses were assessed by IFN-γ ELISPOT, and the results shown in figure 5.3. Figure 5.3A shows anti-CCHFV Gn responses, compared following vaccination with ChAdOx1-CCHFV Gn or the monovalent mixture. Cellular responses were measured with all vaccinated mice, however, no significant differences were measured in responses following vaccination, between the ChAdOx1-CCHFV Gn and the monovalent mixture groups. Anti-CCHFV Gc cellular responses were displayed in figure 5.3B. Both ChAdOx1-CCHFV Gc and the monovalent mixture induced an immune response following vaccination, and no significant differences were measured between the two groups. Figure 5.3C shows anti-CCHFV NP cellular responses by IFN-γ ELISPOT. Similar to responses to other CCHFV antigens, anti-CCHFV NP responses were observed following vaccination with ChAdOx1-CCHFV NP, and following vaccination with the monovalent mixture of vaccines. However, no significant differences in anti-CCHFV NP cellular responses were measured between the vaccine groups.

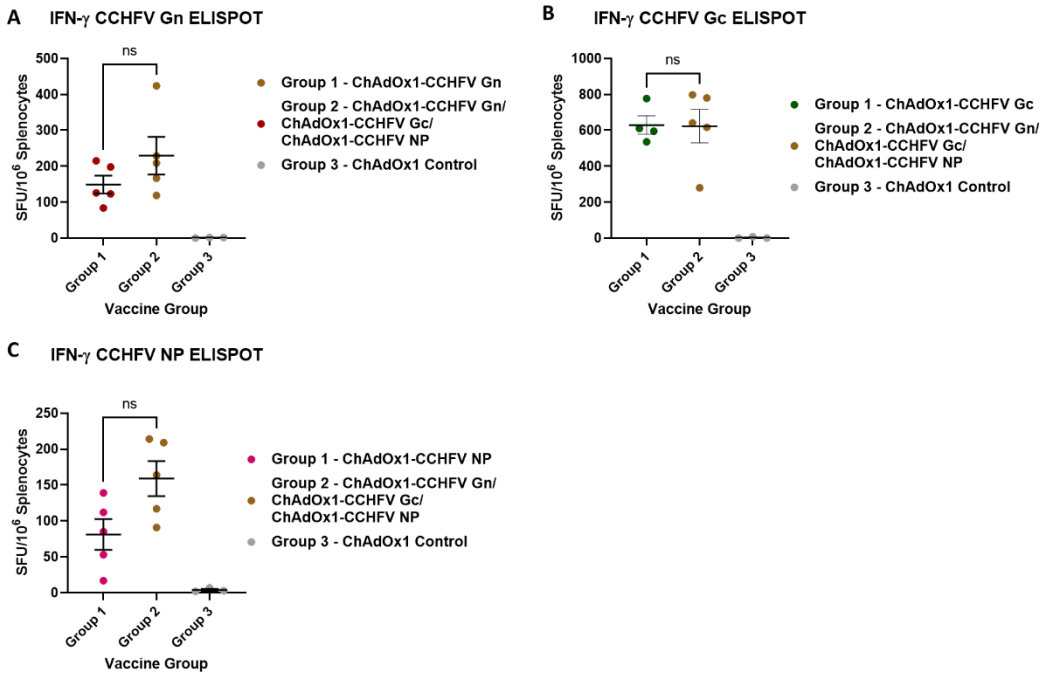


Figure 5.3: IFN-γ ELISPOT against peptide specific to Gn or Gc or NP of CCHFV IbAr10200 strain following monovalent or co-administration vaccination.
Six week old balb/c mice were immunised with $1x10^8$ IU of ChAdOx1 vaccine. Twenty one days after prime vaccination, mice were culled for immunological analysis. CCHFV Gn antigen responses (**A**) in balb/c mice were determined by IFN-γ ELISPOT. Individual mice (groups 1/2 n=5, group 3 n=3) are shown here as individual data points measured as spot forming units per 10^6 (SFU/10^6) splenocytes, with mean and standard error of the mean (SEM) shown. No significant differences were determined by Ordinary One-way ANOVA. CCHFV Gc antigen responses (**B**) in balb/c mice were determined by IFN-γ ELISPOT. Individual mice (groups 1/2 n=5, group 3 n=3) are shown here as individual data points measured as spot forming units per 10^6 (SFU/10^6) splenocytes, with mean and standard error of the mean (SEM) shown. No significant differences were determined by Ordinary One-way ANOVA. CCHFV NP antigen responses (**C**) in balb/c mice were determined by IFN-γ ELISPOT. Individual mice (groups 1/2 n=5, group 3 n=3) are shown here as individual data points measured as spot forming units per 10^6 (SFU/10^6) splenocytes, with mean and standard error of the mean (SEM) shown. No significant differences were determined by Ordinary One-way ANOVA.

Humoral responses were assessed and the results displayed in figure 5.4. Figure 5.4A shows anti-CCHFV Gn total IgG ELISA responses. Vaccination with the monovalent mixture and ChAdOx1-CCHFV Gn vaccines both induced inconsistent humoral responses, and no significant differences could be measured between the CCHFV vaccinated groups. Anti-CCHFV Gc humoral responses are presented in figure 5.4B. Both vaccination with ChAdOx1-CCHFV Gc and vaccination with monovalent mixture produced a strong IgG ELISA response. No significant differences were measured between the groups vaccinated with either ChAdOx1-CCHFV Gc or the monovalent mixture. Figure 5.4C shows humoral responses induced against CCHFV NP. IgG responses were induced in both vaccine groups, and no significant differences were measured following vaccination. No evidence of neutralisation was measured after vaccination with any of the monovalent vaccines assayed here, similar to the results presented in chapter 4. No significant neutralisation of CCHFV tec-VLPs was assayed following vaccination with the tri-vaccine monovalent mixture.

Through co-administration of ChAdOx1-CCHFV Gn, ChAdOx1-CCHFV Gc and ChAdOx1-CCHFV NP vaccines, there was no evidence of antigenic interference between CCHFV antigen, nor a subsequent reduction in immunogenicity. Cellular and humoral responses showed no significant difference between those induced by vaccination with an individual vaccine, and those induced by the mixtures of vaccines. With no evidence of antigenic interference between vaccine antigens, research could progress into developing multivalent ChAdOx vaccines expressing multiple CCHFV antigens.

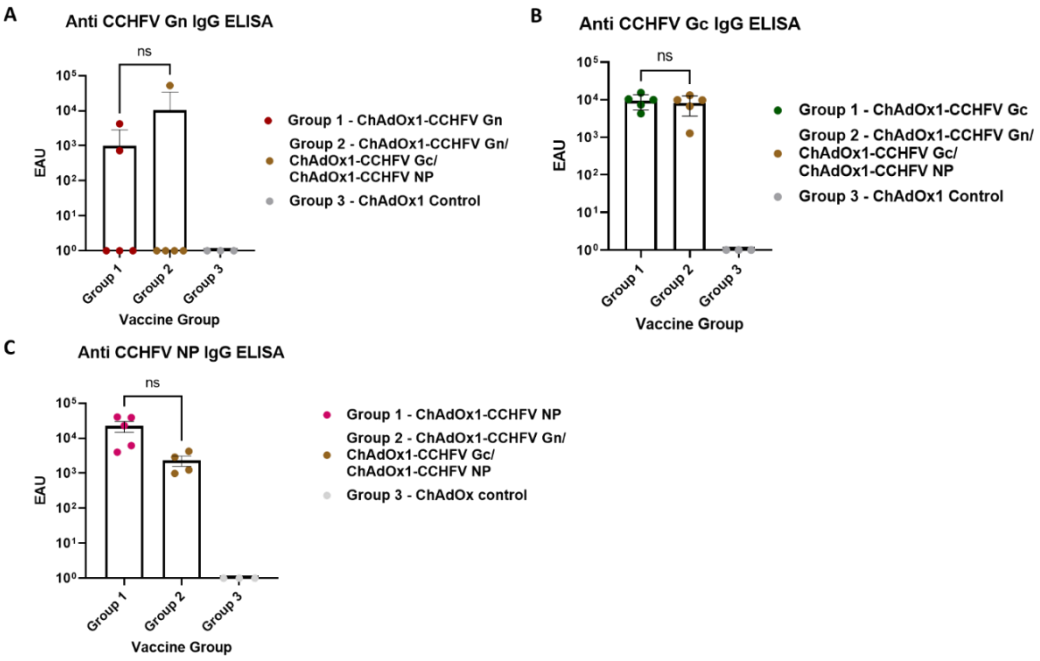


Figure 5.4: Total IgG ELISA against CCHFV Gn, Gc, or NP protein of the CCHFV IbAr10200 strain, following monovalent or co-administration vaccination.
Six week old balb/c mice were immunised with 1x10^8 IU of ChAdOx1 vaccine. Twenty one days after prime vaccination, mice were culled for immunological analysis. CCHFV specific Gn responses (**A**) were quantified in balb/c mice in a standardised ELISA. Individual mice (groups 1/2 n=5, group 3 n=3) are shown as data points in ELISA arbitrary units (EAU) with mean and standard error of the mean (SEM) also shown. Responses below the limit of detection were assigned a value of 1EAU. No significant differences were determined by Kruskal-Wallis test. CCHFV specific Gc responses (**B**) were quantified in balb/c mice in a standardised ELISA. Individual mice (groups 1/2 n=5, group 3 n=3) are shown as data points in EAU, with mean and SEM also shown. Responses below the limit of detection were assigned a value of 1EAU. No significant differences were determined by Kruskal-Wallis test. CCHFV specific NP responses (**C**) were quantified in Balb/c mice in a standardised ELISA. Individual mice (groups 1/2 n=5, group 3 n=3) are shown as data points in EAU, with mean and SEM also shown. Responses below the limit of detection were assigned a value of 1EAU. No significant differences were determined by Kruskal-Wallis test.

5.2.2 Design and production of multivalent ChAdOx vaccines

With no evidence of antigenic interference, design and development of multivalent vaccines could begin. The aim was to develop ChAdOx1 vaccines which contained multiple of the small CCHFV antigens; CCHFV Gn, CCHFV Gc, and CCHFV NP. Two multivalent vaccines were developed for this thesis, ChAdOx1 Bivalent CCHFV and ChAdOx1 Trivalent CCHFV.

The vector for vaccine development is the bivalent ChAdOx1 vector, described previously for production of a multivalent filovirus vaccine. The vector contains two separate antigen entry sites, the E1 and E4 loci. Figure 5.5 shows a schematic of the antigens included in the Bivalent CCHFV and Trivalent CCHFV vaccines. Both vaccines contained the CCHFV Gc antigen in the E1 locus. Due to the fact both vaccines use the CCHFV Gc antigen, CCHFV Gc was incorporated into the ChAdOx1 genome at the E1 locus, using the same shuttle vector as for the monovalent vaccine development. This meant only vectors expressing the Gn or Gn and NP needed to be redesigned and cloned, simplifying the production process for the multivalent vaccines. At the E4 locus, the Bivalent CCHFV Vaccine incorporated CCHFV Gn, and the Trivalent CCHFV vaccine incorporated the Gn-2A-NP expression construct.

The CCHFV Gn antigen was extracted from the antigen cassette used in production of ChAdOx1 and ChAdOx2 CCHFV Gn, by restriction digest with HindIII and KpnI restriction enzymes. CCHFV Gn DNA was purified and ligated into a linearised E4 entry shuttle plasmid. The antigen cassette for the E4 locus is shown in figure 5.6A. The antigen is expressed under the previously described short CMV promoter, and the polyA tail is obtained from the SV40 virus, unlike in the E1 entry cassette. Recombination into the ChAdOx1 vector is achieved through the flanking attL3 and attL4 sites.

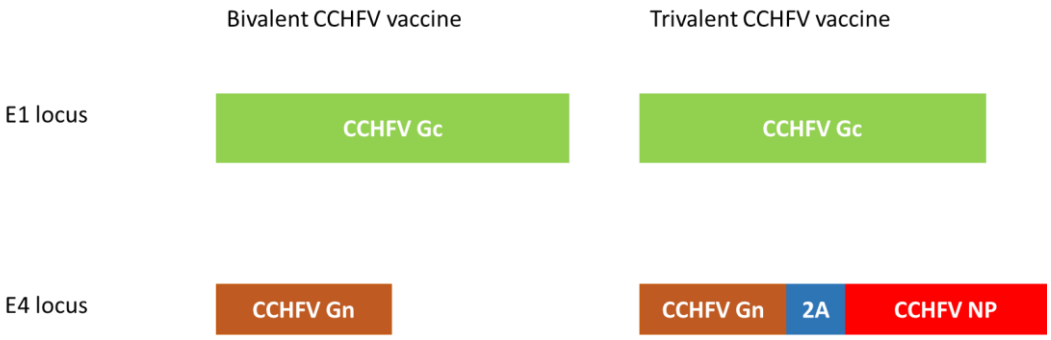


Figure 5.5: Schematic of antigens and locations in multivalent CCHFV vaccines.
A representation of the antigens expressed at both the E1 and E4 loci of the ChAdOx1 genome in both the Bivalent and Trivalent CCHFV vaccines. CCHFV Gc represents the CCHFV Gc sequence described in chapter 4. CCHFV Gn represents the CCHFV Gn sequence described in chapter 4. The 2A represents a ribosomal skipping peptide linker. CCHFV NP represents the native sequence of CCHFV NP from the IbAr10200 strain.

In the design of the trivalent CCHFV vaccine, the challenge was incorporating three CCHFV antigens into a two site vector. As previously discussed CCHFV Gc, the largest of these antigens, was incorporated into E1 locus as a single antigen, and therefore the issue became fitting both the Gn and NP antigens into E4 locus for expression. Design of antigen expression constructs was informed by previous research at the Jenner institute, expressing multiple antigens from a single site. The most effective method for inducing immunogenicity against two glycoproteins from the same site utilised antigen coding sequence connected with 2A domains[224]. The 2A domain is a naturally occurring motif which induces ribosomal skipping[334], which has been adopted for polyprotein expression in genetic engineering. The use of the 2A domain allows the antigen coding sequences to be translated as separate proteins from a single transcript.

As such the trivalent CCHFV vaccine was designed which expressed Gn-2A-NP from the E4 locus, displayed in figure 5.6B. The antigen consisted of the CCHFV Gn antigen sequence used in ChAdOx vectored CCHFV Gn vaccines, followed by a 2A linking domain, as previously used in the immunogenic ChAdOx1-triFilo(2A) vaccine. The 2A linker was followed by the CCHFV NP coding sequence as used in the monovalent CCHFV NP vaccines previously described. The CCHFV Gn-2A-NP was ordered as a gene string and recombined into a linearised E4 entry shuttle plasmid by In-Fusion recombination.

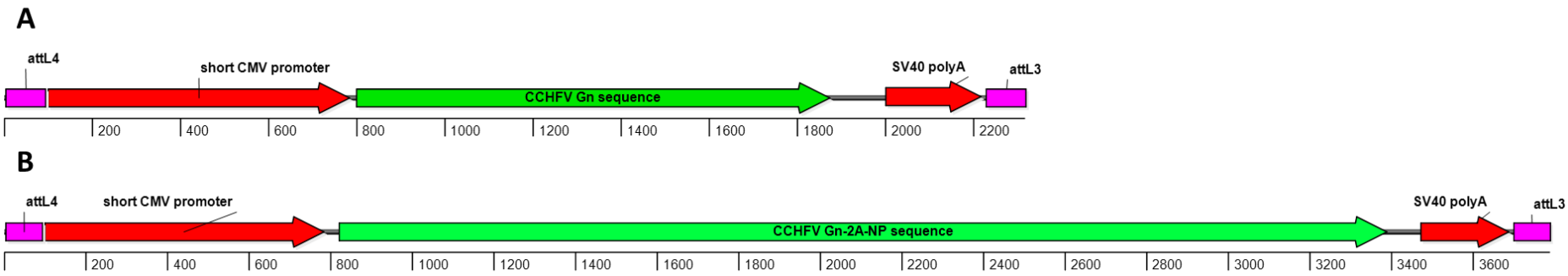


Figure 5.6: Antigen cassettes for E4 locus in ChAdOx1 Bivalent CCHFV and ChAdOx1 Trivalent CCHFV.
Representation of E4 entry antigen cassettes for expression of (**A**) CCHFV Gn and (**B**) CCHFV Gn-2A-NP. AttL3 and AttL4 represent recombination sites for the antigen cassette into the ChAdOx1 genome. Short CMV promoter represents the 673 amino acid promoter sequence derived from CMV. SV40 polyA represents the polyA tail of SV40 for transcriptional termination.

Following production of shuttle vectors, double recombination reactions were performed with the ChAdOx1 bi-entry genome. Once successful double recombinant had been confirmed by antibiotic selection and antigen sequencing, ChAdOx1 viruses were produced at the Viral Vector Core Facility, University of Oxford.

Both the ChAdOx1 Bivalent CCHFV and ChAdOx1 Trivalent CCHFV vaccines utilised the short CMV promoter at both antigen expression sites, to limit the size of the antigenic cassettes used in the vaccines. Table 5.1 shows the antigen cassettes and genome sizes of the multivalent CCHFV vaccines, and the ChAdOx vectored vaccines that they will be compared to throughout the next section. The genomes of ChAdOx1 Bivalent CCHFV and ChAdOx1 Trivalent CCHFV are smaller than ChAdOx2-CCHFV GPC, with ChAdOx1 Trivalent CCHFV being larger than the ChAdOx2-sCMV-CCHFV GPC vaccine.

Vaccine	Antigen Cassette	Size of antigen cassette (bp)	Size of ChAdOx Genome with antigen (bp)	Percentage of Original ChAdY25 genome size (%)	Size of ChAdOx2 Genome with antigen (bp)	Percentage of Original ChAd68 Genome size (%)
ChAdOx2-CCHFV GPC	CCHFV GPC long promoter	7571	N/A	N/A	36556	100
ChAdOx2-sCMV-CCHFV GPC	CCHFV GPC short promoter	6131	N/A	N/A	35116	96
ChAdOx1-CCHFV Gn	CCHFV Gn	2195	31298	85	N/A	N/A
ChAdOx1-CCHFV Gc	CCHFV Gc	2990	32039	87	N/A	N/A
ChAdOx1-CCHFV NP	CCHFV NP	2564	31667	86	N/A	N/A
ChAdOx1 Bivalent CCHFV	*CCHFV Gc – E1 entry*	*2990*	34289	93	N/A	N/A
	CCHFV Gn – E4 entry	*2129*			N/A	N/A
ChAdOx1 Trivalent CCHFV	*CCHFV Gc – E1 entry*	*2990*	35757	97	N/A	N/A
	CCHFV Gn-2A-NP entry	*3600*			N/A	N/A

Table 5.1: The size of multivalent ChAdOx vectored CCHFV genomes and their relationship to the size of their original ChAd genome
The table shows the size of Multivalent CCHFV vaccines and antigen cassettes, and selected others. Also displayed is their respective sizes when cloned into either the ChAdOx1 or ChAdOx2 genome. ChAdOx1 genome sizes are then measured as a percentage of the progenitor ChAdY25 genome (36711 bp). ChAdOx2 genome sizes are then measured as a percentage of the progenitor ChAd68 genome (36521 bp).

5.2.3 Immunogenicity of multivalent CCHFV vaccines

After production of the ChAdOx1 Bivalent CCHFV and ChAdOx1 Trivalent CCHFV, immunogenicity induced by vaccination was assessed. Immunogenicity assessment began with comparison between multivalent vaccines and their comparative monovalent mixes.

5.2.3.1 Comparative immunogenicity of multivalent CCHFV vaccines against monovalent vaccine mixtures

The immunogenicity induced by vaccination with ChAdOx1 Bivalent CCHFV or ChAdOx1 Trivalent CCHFV was assessed in balb/c mice. The immunogenicity induced by multivalent CCHFV vaccines, was compared against immunogenicity induced by two monovalent mixtures; a 1:1 co-administration of ChAdOx1-CCHFV Gn and ChAdOx1-CCHFV Gc, and a 1:1:1 co-administration of ChAdOx1-CCHFV Gn, ChAdOx1-CCHFV Gc, and ChAdOx1-CCHFV NP. The antigens in the two different monovalent mixtures corresponded to the Bivalent and Trivalent CCHFV vaccines respectively.

Figure 5.7 shows the cellular responses induced by vaccination, as measured by IFN-γ ELISPOT. Anti-CCHFV Gn responses remain low as displayed in figure 5.7A. All CCHFV Gn-containing vaccines induced an anti-Gn response following vaccination but the differences measured between groups were not significant. Contrastingly, significant differences were measured in figure 5.7B, showing anti-CCHFV Gc responses. All groups expressing CCHFV Gc were immunogenic. Cellular responses induced following vaccination with the bi-vaccine mixture and ChAdOx1 Bivalent CCHFV were both significantly greater than those induced following vaccination with the tri-vaccine mixture. Anti-Gc responses induced by vaccination with ChAdOx1 Trivalent CCHFV were also significantly greater than those induced by the tri-vaccine mixture. In figure 5.7C, anti-NP cellular responses are displayed. Vaccination with the tri-vaccine mixture

induced a cellular response against CCHFV NP specific peptides. ChAdOx1 Trivalent CCHFV vaccination induced a very low level response, which was almost indistinguishable from the background of the assay, and significantly lower than anti-CCHFV NP cellular responses induced after vaccination with the tri-vaccine mixture.

Immunogenicity was further assessed by total IgG ELISA with CCHFV proteins. Anti-Gc humoral responses are presented in figure 5.8A. Following vaccination, at least one mouse in each group did produce a detectable anti-Gn response, but the responses measured were inconsistent as previously observed, and most mice did not produce an anti-Gn humoral response. Figure 5.8B shows the anti-CCHFV Gc total IgG responses. Following vaccination all non-control groups showed anti-Gc humoral responses in all mice. However, no significant differences were measured in anti-Gc humoral responses following vaccination. Figure 5.8C displays the anti-NP response induced by vaccination. Immunisation with the tri-vaccine mixture induced a humoral response against CCHFV NP proteins, as shown previously following ChAdOx1-CCHFV NP vaccination. However, no anti-NP humoral responses were measured after vaccination with ChAdOx1 trivalent CCHFV. Neutralising responses induced by vaccination were assessed by a CCHFV tec-VLP pseudotype VNT, though no significant evidence of neutralisation was measured with any of the vaccinated groups.

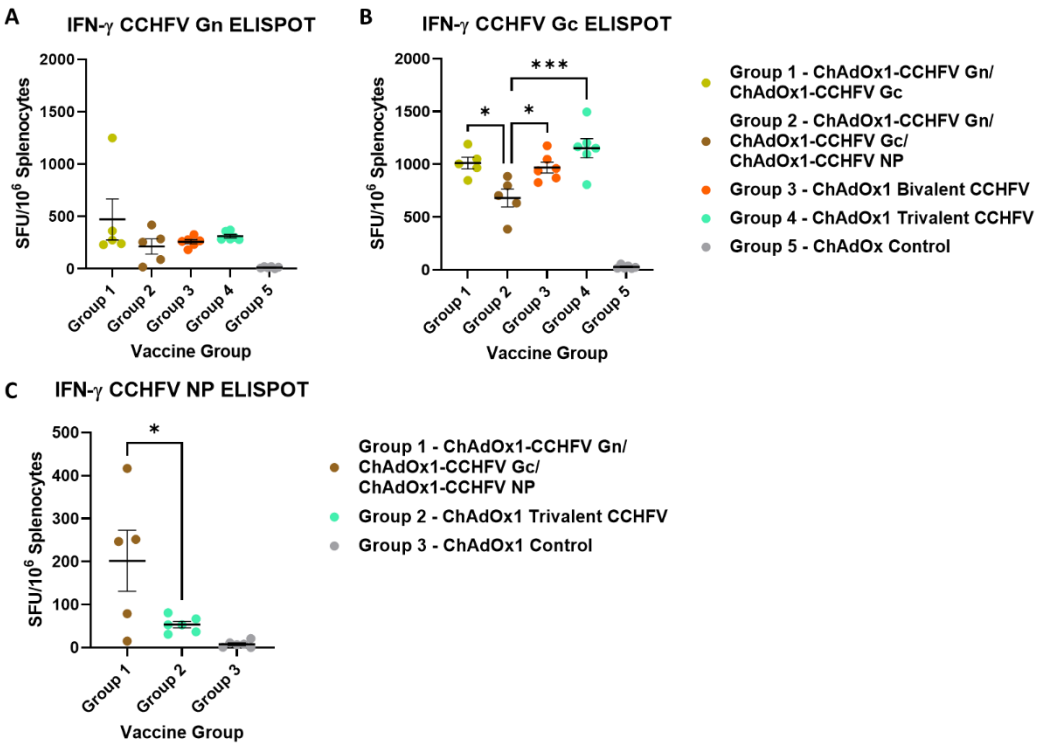


Figure 5.7: IFN-γ ELISPOT against peptide specific to Gn or Gc or NP of CCHFV IbAr10200 strain, following multivalent ChAdOx1 CCHFV or co-administration vaccination.

Six week old balb/c mice were immunised with $1x10^8$ IU of ChAdOx1 vaccine. Twenty one days after prime vaccination, mice were culled for immunological analysis. CCHFV Gn antigen responses (**A**) in balb/c mice were determined by IFN-γ ELISPOT. Individual mice (groups 1/2 n=5, groups 3/4/5 n=6) are shown here as individual data points measured as spot forming units per 10^6 (SFU/10^6) splenocytes, with mean and standard error of the mean (SEM) shown. No significant differences were determined by Ordinary one-way ANOVA. CCHFV Gc antigen responses (**B**) in Balb/c mice were determined by IFN-γ ELISPOT. Individual mice (groups 1/2 n=5, group 3/4/5 n=6) are shown here as individual data points measured as spot forming units per 10^6 (SFU/10^6) splenocytes, with mean and standard error of the mean (SEM) shown. Significant differences were determined by Ordinary One-way ANOVA, with Tukey's multiple comparisons test. CCHFV NP antigen responses (**C**) in Balb/c mice were determined by IFN-γ ELISPOT. Individual mice (group 1 n=5, group 2/3 n=6) are shown here as individual data points measured as spot forming units per 10^6 (SFU/10^6) splenocytes, with mean and standard error of the mean (SEM) shown. Significant differences were determined by Ordinary One-way ANOVA with Tukey's multiple comparisons test.

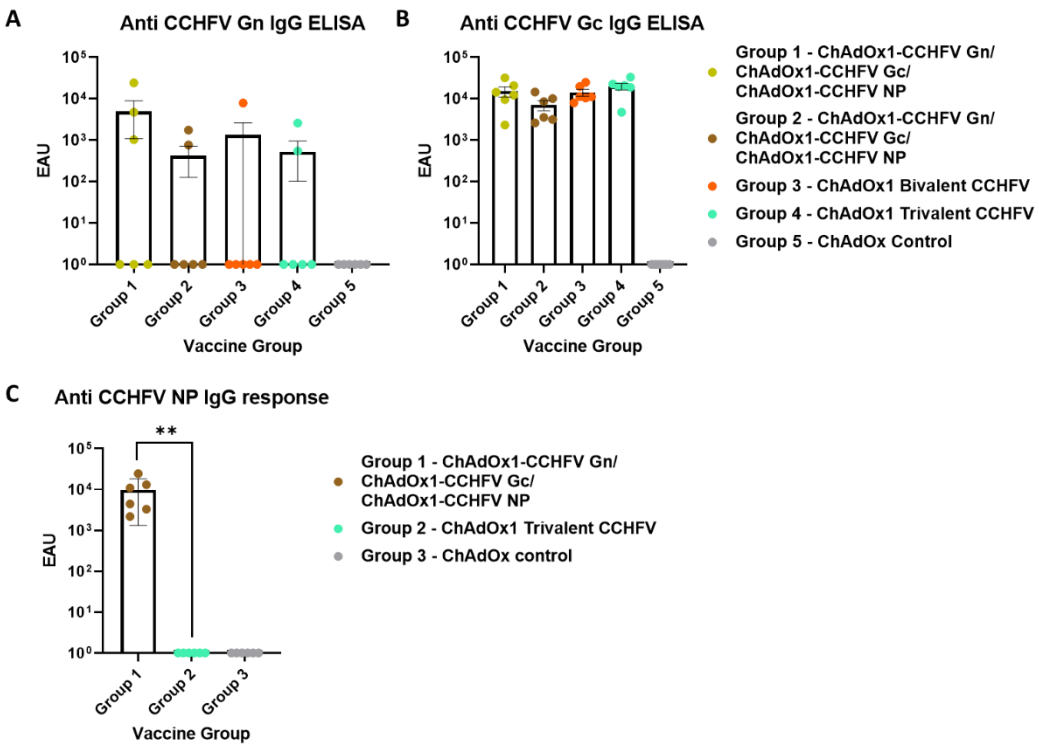


Figure 5.8: Total IgG ELISA against CCHFV Gn, Gc, or NP protein of the CCHFV IbAr10200 strain, following multivalent ChAdOx1 CCHFV or co-administration vaccination.

Six week old balb/c mice were immunised with 1x10^8 IU of ChAdOx1 vaccine. Twenty one days after prime vaccination, mice were culled for immunological analysis. CCHFV specific Gn responses (**A**) were quantified in balb/c mice in a standardised ELISA. Individual mice (n=6) are shown as data points in EAU with mean and SEM also shown. Responses below the limit of detection were assigned a value of 1EAU. No significant differences were determined by Kruskal-Wallis test. CCHFV specific Gc responses (**B**) were quantified in balb/c mice in a standardised ELISA. Individual mice (n=6) are shown as data points in EAU with mean and SEM also shown. Responses below the limit of detection were assigned a value of 1EAU. No significant differences were determined by Kruskal-Wallis test. CCHFV specific NP responses (**C**) were quantified in balb/c mice in a standardised ELISA. Individual mice (n=6) are shown as data points in EAU with mean and SEM also shown. Responses below the limit of detection were assigned a value of 1EAU. Significant differences were determined by Kruskal-Wallis test (**p<0.01).

The multivalent ChAdOx1 Bivalent CCHFV and ChAdOx1 Trivalent CCHFV vaccines were both assessed for immunogenicity induced by vaccination. Both vaccines were able to induce in mice anti-Gn cellular responses, and anti-Gc cellular and humoral responses. These results suggested expression of the Gc and Gn antigens, comparable to their respective monovalent ChAdOx1 vaccines. In contrast, anti-NP immunogenicity induced after ChAdOx1 Trivalent CCHFV vaccination was poor, with little cellular response and no humoral immunogenicity. These results combine to suggest that the Gn-2A-NP antigen is not expressing as expected, with Gn responses being measured, but no evidence of induced CCHFV NP immunogenicity. Despite the lack of NP responses induced, the anti-Gn and anti-Gc responses induced by vaccination with ChAdOx1 Bivalent CCHFV and Trivalent CCHFV were at least the equal of their respective monovalent mixtures, and specifically in anti-CCHFV Gc cellular responses, surpassed the equivalent monovalent CCHFV vaccine. In summary, the multivalent CCHFV ChAdOx vaccines are immunogenic and able to induce immunogenicity against multiple CCHFV antigens from a single immunisation.

5.2.3.2 Comparitive immunogenicity of multivalent CCHFV vaccines and ChAdOx2 CCHFV GPC vaccines

ChAdOx1 Bivalent CCHFV and ChAdOx1 Trivalent CCHFV vaccines both induced anti-CCHFV Gn and Gc responses when assessed. As they were immunogenic against multiple CCHFV antigens at a level comparable with monovalent CCHFV vaccines, the multivalent CCHFV vaccines were assessed against two leading vaccines described in Chapter 4. These were the ChAdOx2-CCHFV GPC vaccine, and the short promoter equivalent ChAdOx2-sCMV-CCHFV GPC. The ChAdOx2-CCHFV GPC vaccine induced protection from lethal CCHFV challenge in an A129 mouse model but was shown to be genetically unstable during GMP production. As such, comparable immunogenicity exhibited by a different vaccine would provide a positive signal for a protective vaccine.

Cellular immunogenicity induced by all the vaccines was assessed by IFN-γ ELISPOT. Figure 5.9A showed the responses induced against CCHFV Gn specific peptides. All the assessed non-control vaccines produced an anti-CCHFV Gn response. No significant differences were measured between anti-Gn cellular responses following vaccination with ChAdOx2-sCMV-CCHFV GPC, ChAdOx2-CCHFV GPC, or ChAdOx1 Bivalent CCHFV. However, the anti-Gn responses induced following vaccination with ChAdOx1 Trivalent CCHFV vaccination was significantly greater than cellular responses following vaccination with either of the ChAdOx2 vaccines expressing the CCHFV GPC antigen. Figure 5.9B displays the cellular responses against CCHFV Gc specific peptides. When assessing anti-Gc responses, all non-vaccines induced a cellular response following immunisation. The responses induced after ChAdOx1 Bivalent CCHFV vaccination were significantly greater than those induced following vaccination with either ChAdOx2 vectored vaccine expressing CCHFV GPC. The same pattern was measured following ChAdOx1 Trivalent CCHFV vaccination, with the anti-Gc cellular responses induced

following vaccination measured as significantly greater than cellular responses induced by vaccination with either ChAdOx2 vector CCHFV GPC vaccine. With anti-Gn and anti-Gc responses being greater in multivalent CCHFV vaccines, the total cellular responses induced against CCHFV GPC specific peptides were assessed, the results of which are presented in figure 5.9C. This analysis includes the cellular responses against the full length of CCHFV GPC induced by vaccination with ChAdOx2-CCHFV GPC or ChAdOx2-sCMV-CCHFV GPC, compared against the summed anti-Gn and anti-Gc responses induced by vaccination with ChAdOx1 Bivalent CCHFV or Trivalent CCHFV GPC. No significant differences were measured in total responses induced against CCHFV GPC peptides following vaccination with either ChAdOx2 vectored CCHFV GPC vaccine, or the multivalent ChAdOx1 CCHFV vaccines.

Humoral responses were assessed against CCHFV Gn and CCHFV Gc proteins for all vaccines. Figure 5.10A shows anti-CCHFV Gn total IgG responses, which were inconsistent with over half of mice vaccinated with CCHFV Gn or GPC antigens showing no measurable humoral response to CCHFV Gn. Anti-Gc total IgG ELISA results are displayed in figure 5.10B, and in contrast all non-control vaccinated mice displayed an anti-CCHFV Gc response. The anti-Gc response induced following ChAdOx2-CCHFV Gc vaccination was significantly greater than that induced after vaccination with any of the other assessed vaccines. No further significant differences were measured between anti-CCHFV Gc humoral responses following ChAdOx2-sCMV-CCHFV GPC, ChAdOx1 Bivalent CCHFV, or ChAdOx1 Trivalent CCHFV vaccination. Neutralisation responses were compared between vaccinated groups. In the analysis used, no significant differences could be measured between the vaccinated groups or the control serum, in contrast to the previous neutralising titres induced by vaccination with ChAdOx2-CCHFV GPC vaccination.

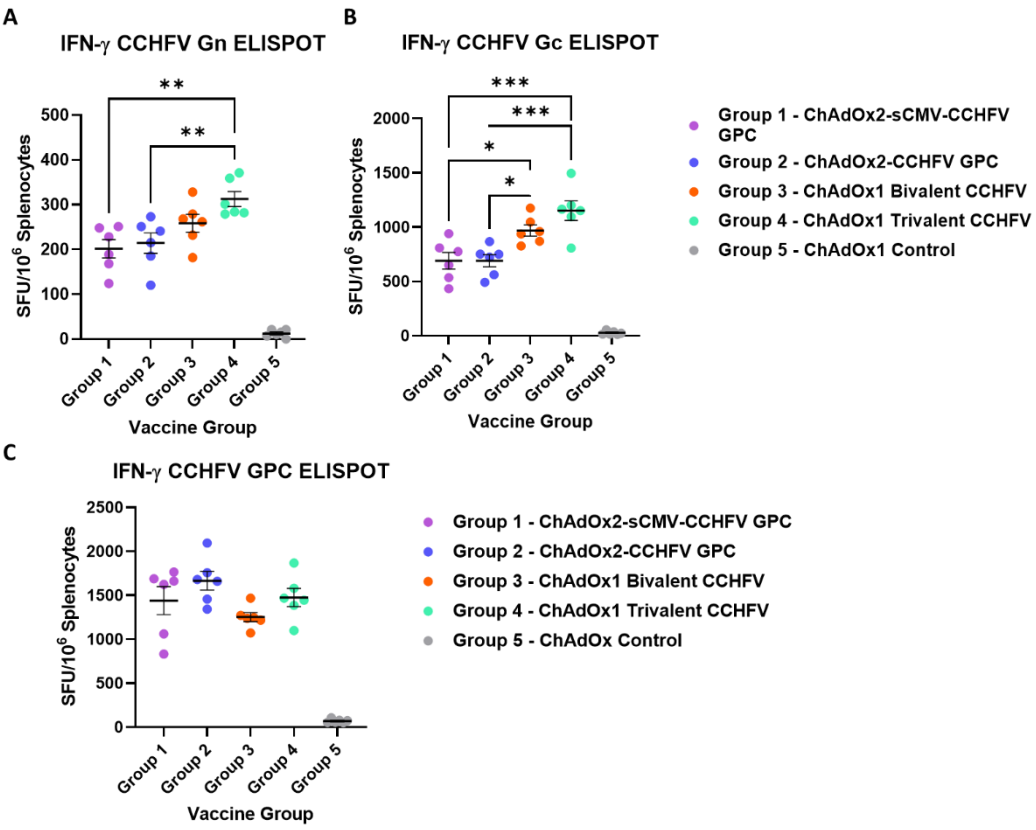


Figure 5.9: IFN-γ ELISPOT against peptides specific to Gn, Gc or GPC of CCHFV IbAr10200 strain, following multivalent ChAdOx1 CCHFV or ChAdOx2 vectored CCHFV GPC vaccination.

Six week old balb/c mice were immunised with $1x10^8$ IU of ChAdOx1 vaccine. Twenty one days after prime vaccination, mice were culled for immunological analysis. CCHFV Gn antigen responses (**A**) in balb/c mice were determined by IFN-γ ELISPOT. Individual mice (n=6) are shown here as individual data points measured as spot forming units per 10^6 (SFU/10^6) splenocytes, with mean and standard error of the mean (SEM) shown. Significant differences were determined by Ordinary one-way ANOVA with Tukey's multiple comparisons test (**p<0.01). CCHFV Gc antigen responses (**B**) in balb/c mice were determined by IFN-γ ELISPOT. Individual mice (n=6) are shown here as individual data points measured as spot forming units per 10^6 (SFU/10^6) splenocytes, with mean and standard error of the mean (SEM) shown. Significant differences were determined by Ordinary One-way ANOVA, with Tukey's multiple comparisons test (*p<0.05, ***p<0.001). CCHFV GPC antigen responses (**C**) in balb/c mice were determined by IFN-γ ELISPOT. Individual mice (n=6) are shown here as individual data points measured as spot forming units per 10^6 (SFU/10^6) splenocytes, with mean and standard error of the mean (SEM) shown. No significant differences were determined by Ordinary One-way ANOVA.

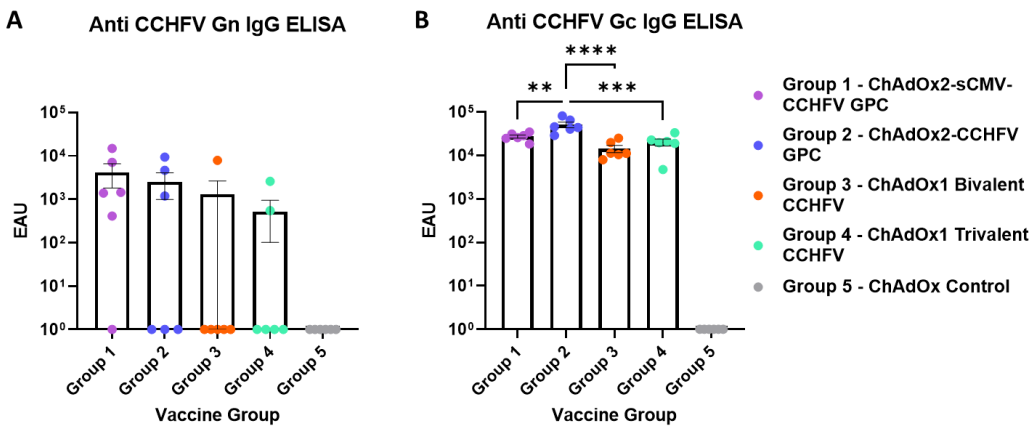


Figure 5.10: Total IgG ELISA against CCHFV Gn, Gc, or NP protein of the CCHFV IbAr10200 strain, following multivalent ChAdOx1 CCHFV or ChAdOx2 vectored CCHFV GPC vaccination.

Six week old balb/c mice were immunised with $1x10^8$ IU of ChAdOx1 vaccine. Twenty one days after prime vaccination, mice were culled for immunological analysis. CCHFV specific Gn responses (A) were quantified in balb/c mice in a standardised ELISA. Individual mice (n=6) are shown as data points in EAU with mean and SEM also shown. Responses below the limit of detection were assigned a value of 1EAU. No significant differences were determined by ordinary One-way ANOVA. CCHFV specific Gc responses (**B**) were quantified in balb/c mice in a standardised ELISA. Individual mice (n=6) are shown as data points in EAU with mean and SEM also shown. Responses below the limit of detection were assigned a value of 1EAU. Significant differences were determined by ordinary One-way ANOVA with Tukey's multiple comparisons test (**p<0.01, ***p<0.001, ****p<0.0001).

In comparison with ChAdOx2 vectored CCHFV GPC vaccines, both ChAdOx1 Bivalent CCHFV and ChAdOx1 Trivalent CCHFV induced comparable immunogenicity following vaccination. The cellular immunogenicity induced by vaccination against CCHFV Gn and Gc was greater than responses induced by vaccines using the CCHFV GPC antigen. Vaccination with ChAdOx2-CCHFV GPC did induce significantly greater anti-Gc responses than either multivalent CCHFV vaccine, but vaccination with both still produced a strong anti-Gc antibody response. In summary, ChAdOx1 multivalent CCHFV vaccines induced significant immunogenicity against CCHFV Gn and Gc, which correlated with the protective ChAdOx2-CCHFV GPC. The similar immunogenicity could translate into protection in small animal challenge trials and makes multivalent ChAdOx1 CCHFV vaccines an ideal candidate for further progression in preclinical and clinical assessment.

5.3 Conclusions

Multiple antigen vaccines have been researched extensively in the field of vaccination for the purposes of vaccination against multiple pathogens, or to broaden immune responses against a single pathogen and reduce the likelihood of immune evasion. For developing a ChAdOx vectored CCHFV vaccine, it was previously demonstrated that the CCHFV GPC antigen could be immunogenic in a ChAdOx2 vector, but the vaccine genome was unstable during clonal expansion. Following positive immunogenicity results from CCHFV Gn, CCHFV Gc and CCHFV NP antigens, multivalent vaccines were developed to broaden the immune response of a ChAdOx vectored CCHFV vaccine.

Antigenic interference between CCHFV Gn, CCHFV Gc, and CCHFV NP antigens following vaccination was assessed. No evidence was measured of antigenic interference affecting immunogenicity and as such ChAdOx1 Bivalent CCHFV and ChAdOx1 Trivalent CCHFV vaccines were developed, incorporating multiple CCHFV vaccines into a single ChAdOx1 vector.

Speculation has suggested the inclusion of multiple antigens would increase the magnitude immunogenicity against CCHFV induced by vaccination, particularly with regards to anti-Gn and Gc responses. In the native virus CCHFV Gn and Gc form a heterologous dimer on the cell surface, and it was thought that co-expression in a single cell may produce a complex which better reflected the native form of the dimer, potentially enhancing humoral responses. These speculations did not however play out in the data, with no significant difference measured in humoral or neutralisation responses measured following immunisation with multivalent CCHFV vaccines, and their equivalent individual monovalent vaccines. Enhancement was observed in cellular response, with significantly greater anti-Gc IFN-γ ELISPOT responses measured following ChAdOx1 Trivalent CCHFV vaccination, compared to responses induce by a mixture of monovalent ChAdOx1 vaccines. The observed anti-Gn and anti-Gc

immunogenicity was however offset by the lack of CCHFV NP immunogenicity induced after ChAdOx1 Trivalent CCHFV vaccination. CCHFV NP was the second part of the Gn-2A-NP expression construct, and cellular and humoral immunogenicity results suggest that expression was either extremely poor or non-existent beyond the 2A linker, an unexpected result given the previous successful expression from the 2A linker in ChAdOx1 vaccines[224].

Despite the poor CCHFV NP response, multivalent CCHFV vaccines were able to induce strong anti-Gn and anti-Gc responses following vaccination, and as such immunogenicity was compared against two leading previous ChAdOx candidates; ChAdOx2-CCHFV GPC, which is protective in small animal challenge models, and the short promoter equivalent ChAdOx2-sCMV-CCHFV GPC. Immunisation with both multivalent vaccines induced enhanced anti-Gn and anti-Gc IFN-γ responses when compared against responses induced by immunisation with ChAdOx2 vectored CCHFV GPC antigen vaccines When the cellular responses against the additional subunits MCL, GP38, NSm were included in the analysis, the summed anti CCHFV GPC responses showed no significant differences between multivalent ChAdOx1 CCHFV vaccines and ChAdOx2 vectored CCHFV GPC antigen vaccines. ChAdOx2-CCHFV GPC vaccination did however induce a significantly greater anti-Gc total IgG ELISA response following vaccination when compared against responses following vaccination with multivalent ChAdOx1 CCHFV vaccines, but the humoral responses induced following vaccination remained strong. Correlates of protection for CCHFV vaccines are unknown, with cellular and humoral responses both correlating with protection in different studies, though cellular responses CCHF is generally prioritised for protection. However, vaccination with both ChAdOx1 Bivalent CCHFV and ChAdOx1 Trivalent CCHFV induced cellular and humoral responses which were comparable to vaccination with ChAdOx2-CCHFV GPC. These correlates bode well for potential protection, but do not confirm it, as the protection induced by

ChAdOx2-CCHFV GPC vaccination may be from more specific cellular or non-neutralising antibody responses, against the MCL, GP38, or NSm regions which are not included in the multivalent CCHFV vaccines.

Disappointingly for the multivalent vaccines, neutralisation responses induced by a single vaccination were poor. Serum from balb/c mice vaccinated with CCHFV Gn, Gc and NP antigens showed no significant neutralisation in assays throughout chapter 4 and 5. The only significant neutralisation measured from balb/c serum was from mice vaccinated with CCHFV GPC antigens expressed under long promoters. Even following ChAdOx1/2-CCHFV GPC vaccination, neutralisation titres were inconsistent, and low, at least after a prime only. Inconsistencies within groups may suggest issues with the CCHFV tec-VLP neutralisation assays. However, responses within the assay were consistent between repeats with the same mouse serum and throughout assays reliability was measured with positive and negative controls. The more likely explanation is that the anti-CCHFV neutralisation titres induced following a single dose of ChAdOx vectored CCHFV vaccines are low, and even the greatest neutralising responses sit on the cusp of a significant response. Biological variation and limitations on group size reducing the statistical power of some responses following vaccination vary between significant and non-significant neutralisation titres.

Figure 5.11 shows the neutralisation responses measured from balb/c mice which have received a heterologous prime-boost of ChAdOx2-CCHFV GPC and an MVA-CCHFV GPC vaccine. A prime only of ChAdOx2-CCHFV GPC showed neutralisation consistent with that presented throughout the previous chapters, with a mean above that of the control serum but too large a biological variation to measure a significant difference. In contrast both heterologous vaccination regimes induced neutralising responses which were significantly greater than the control. These results clearly showed that the assay is CCHFV specific and can detect strongly neutralising responses, but may be less sensitive

to low level neutralisation responses induced by single immunisations with ChAdOx vectored CCHFV vaccines. If neutralisation responses are shown to be correlates of protection against CCHF or infection with CCHFV, there may be a need to use the vaccines assessed in chapters 4 and 5 in prime boost regimes to enhance neutralisation responses.

The aim across chapter 4 and 5 was to produce an immunogenic ChAdOx vectored CCHFV vaccine, in order to replicate the immunogenicity of the ChAdOx2 vaccine which was protective against lethal CCHFV challenge in small animal models. In addition, due to the instability of ChAdOx2-CCHFV GPC, there was a need to reduce the insert size of the ChAdOx vectored vaccines, aiming to increase the genomic stability of the virus. The genomes of ChAdOx1 Bivalent CCHFV and ChAdOx1 Trivalent CCHFV were significantly smaller than the ChAdOx2-CCHFV GPC genome and as such are predicted to be more stable for GMP manufacture.

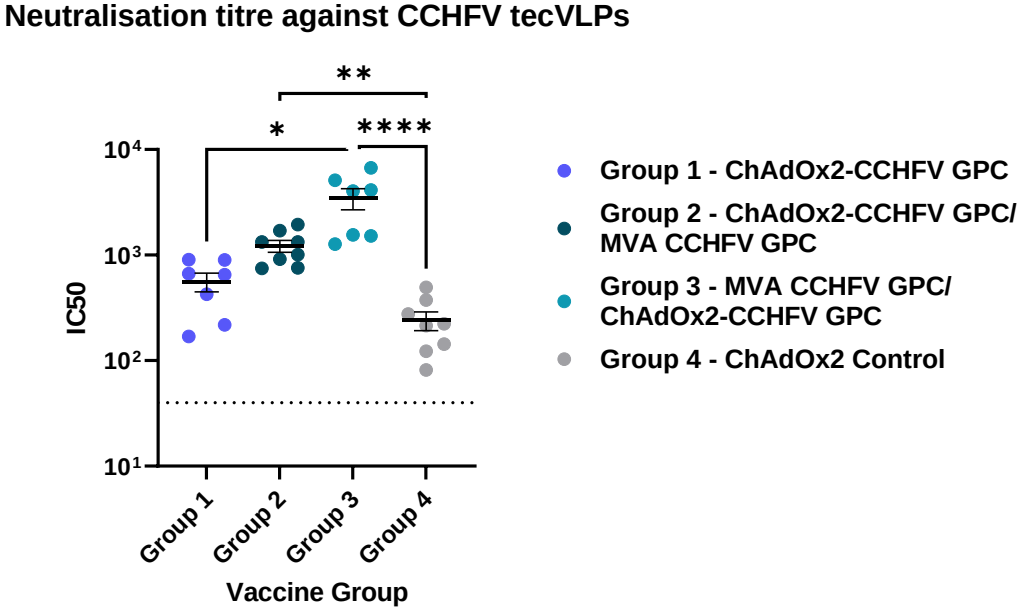


Figure 5.11: CCHFV tecVLP neutralisation titres following heterologous ChAdOx2-CCHFV GPC and MVA CCHFV GPC vaccination.
Eight week old balb/c mice were immunised in either prime or prime-boost vaccination regimes. On day 0 mice were vaccinated with either $1x10^7$IU of ChAdOx2-CCHFV GPC (Group2), or $5x10^6$PFU MVA-CCHFV GPC. On day 14 mice in groups 1, 3, and 4 were vaccinated with $1x10^7$IU of a relevant ChAdOx2 vaccine, and mice in group 2 were vaccinated with $5x10^6$PFU MVA CCHFV GPC. Twenty one days after final vaccination mice were culled for immunological analysis. Neutralising antibody titres were determined by virus neutralisation test using CCHFV tec-VLPs. Individual data points (group 1/3 n=7, groups 2/4 n=8) were expressed as IC50 values and shown here as a scatter dot plot with bars showing mean and SEM. Significant differences were determined by Kruskal-Wallis test (*p<0.05, **p<0.01, ****p<0.0001).

To assess genomic stability, testing was performed on a range of ChAdOx vectored CCHFV vaccines, discussed in both chapters 4 and 5, by the Viral Vector Core Facility, University of Oxford. The stability of ChAdOx2 vectored CCHFV GPC vaccines were compared against ChAdOx1 Bivalent CCHFV by serial passage, the results of which are presented in table 5.2. The ChAdOx1 Trivalent CCHFV vaccine was not selected to move forward into stability testing despite positive immunogenicity results, as there were concerns about the ancillary, likely unexpressed CCHFV NP sequence from the Gn-2A-NP antigen. The stability results showed that the ChAdOx2 CCHFV GPC was highly unstable as expected, with evidence of antigenic instability determined by PCR from passage 1 of the virus. ChAdOx2-sCMV-CCHFV GPC was less unstable, but additional bands were still observed in the PCR results, showing that genomic instability was likely still present. In contrast, ChAdOx1 Bivalent CCHFV showed no evidence of genetic instability, with expected PCR bands observed out to passage 10 of the virus.

Multivalent ChAdOx1 vectored vaccines are immunogenic, and immunogenicity following vaccination correlated well with the responses produced by ChAdOx2-CCHFV GPC. ChAdOx1 multivalent CCHFV vaccines are also more genetically stable, exhibited through repeated passage of the virus. As such these vaccines can be advanced into GMP production and expanded to quantities relevant for further challenge trials in mice or non-human primates, or for use in future phase I human trials. The lessons learned from design, production an immunogenicity analyses of ChAdOx vectored CCHFV vaccines can then be taken forward into the development of further nairovirus vaccines.

Vaccine	Passages	Stability comments	Pass/fail
ChAdOx2-CCHFV GPC	5	Extra bands found in flank-to-flank PCR	FAIL
ChAdOx2-sCMV-CCHFV GPC	5	Evidence of extra bands found in flank to flank PCR	FAIL
ChAdOx1 Bivalent CCHFV	10	No extra bands in flank-to-flank PCR. Stable	PASS

Table 5.2: Stability assessment of ChAdOx vectored CCHFV vaccines
The table shows the stability of CCHFV antigens in ChAdOx2-CCHFV GPC, ChAdOx2-sCMV-CCHFV GPC and ChAdOx1 Bivalent CCHFV. Stability was assessed by serial passage in Trex cells with antigen size assessed by flank-to-flank PCR, covering the antigen cassette, and the identification of PCR products with the expected size profiles for each antigen. Pass/Fail criteria were assessed by the Viral Vector Core Facility, University of Oxford.

Chapter 6 – Development of monovalent ChAdOx vaccines against NSDV

6.1 Introduction

Nairobi Sheep Disease Virus (NSDV) is the pathogen which causes Nairobi Sheep Disease (NSD), a severe disease of sheep and goats[131]. NSDV is a negative sense RNA virus with a tripartite genome in the genus *Nairovirus*, and family *Bunyaviridae*, the same genus and family as CCHFV[81]. NSDV is an arbovirus, with the major vector for infection being through the bites of infected ticks of the *Rhipicephalus* genus[131], or H*aemophysalis* genus in India[135]. NSDV infection in small ungulates causes a febrile illness, with distinctive symptoms being haemorrhagic gastroenteritis[141]. Outbreaks of NSDV are severe, with mortality of infection approaching 70%[335], an impact which can devastate herds of sheep or goats. NSDV is endemic to two specific and geographically distinct regions, sub-Saharan Africa, and India and Sri Lanka, where it is known as Ganjam virus[135]. Genomic studies have shown the NSDV and Ganjam virus are very closely related and are not considered distinct enough to be separate species[135].

No vaccines are currently available to protect against NSDV infection. However, inactivated and live attenuated vaccines were developed in the 1970s against CCHFV[244]. An inactivated NSDV vaccine induced protection against NSDV infection after a prime boost vaccination, whilst live attenuated vaccines were comparatively poorer at inducing protection[244]. Following the production of a protective inactivated vaccine over 45 years ago, development has stalled, and the inactivated vaccine has not been widely adopted, nor any side effects assessed, such as whether the vaccine may cause spontaneous abortions, as observed following inoculation with live attenuated RVFV vaccines[336].

NSDV antibodies in sheep are found extensively in endemic areas[337], and the biggest risks of NSDV outbreaks come from the movement of livestock from areas with a level

of herd immunity, to areas without[137]. In a continuously more globalised society, with a greater capacity to spread carriers of diseases, the threat is exacerbated[137]. The ability to immunise animals both prior to transport and in the area they will be introduced into, would greatly reduce the risk and impact of NSDV outbreak. In addition, similarly to CCHFV, the advance of climate change is expanding the range of tick vectors which can carry NSDV[316], further increasing the risk that if introduced to a region, NSDV could become endemic.

As NSDV can cause death rapidly after symptoms appear[338], there is an extremely small window to provide antivirals or therapeutic measures to prevent death. Furthermore, at present no such antiviral measures exist, and antiviral medications are often expensive treatments if available[339,340]. These factors emphasise the need for a cheap vaccine as a preventative measure, to be proactive rather than reactive to outbreaks. The research presented in this chapter has focused on developing NSDV vaccines in a ChAdOx1 vector.

Lessons learned from development of ChAdOx vectored CCHFV vaccines can be used to inform NSDV vaccine development. NSDV and CCHFV are closely related nairoviruses, with some debate as to whether they should be classified within the same serogroup[82,341]. Figure 6.1 shows the amino acid sequence identities between CCHFV IbAr10200 and NSDV Jilin strains for antigens tested in ChAdOx vectored vaccines. Though not as closely related as to be the same species of virus, NSDV and CCHFV are closely related enough that results with CCHFV will likely have some level of correlation with their NSDV counterpart antigens. Initial development of NSDV vaccines in this thesis looked to replicate previous CCHFV vaccine development by producing NSDV vaccines with NSDV GPC, NSDV Gn and NSDV Gc antigens.

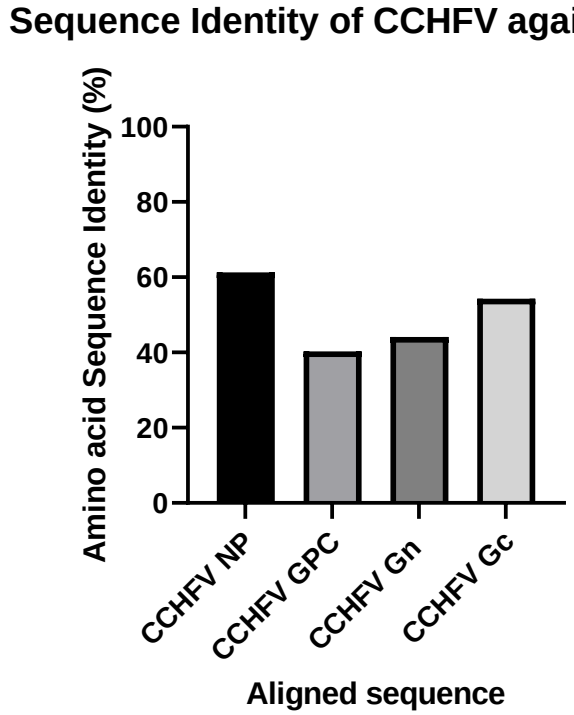


Figure 6.1: BLAST alignment of CCHFV IbAr10200 amino acid sequences against the corresponding NSDV Jilin amino acid sequence.
CCHFV NP sequences derived from NP_950237.1. CCHFV GPC sequences derived from AAA86616.2**.** NSDV NP sequences derived from YP_009361831.1**.** NSDV GPC sequences derived from YP_009361837.1

6.2 Results

6.2.1 ChAdOx1 NSDV GPC and GPC subunit vaccines

With research into NSDV correlates of protection and vaccine antigens being more limited than research into CCHFV, the experiences with CCHFV vaccines in this thesis were used to inform initial vaccine development against NSDV. The NSDV GPC is a polyprotein, also thought to be cleaved into five constituent proteins. Similar to other nairo- and bunyaviruses, the GPC subunits NSDV Gn and NSDV Gc are thought to form a heterodimer on the surface of the NSDV virion[342]. As such, vaccines were developed to target the full length GPC antigen, and the NSDV Gn and Gc antigens.

6.2.1.1 Design of ChAdOx1 NSDV GPC and GPC subunit vaccines

ChAdOx1 NSDV entry vectors were then designed for expression of the antigens in vaccines. The strain selected for use in the vaccines was the Jilin strain of NSDV. At the time of the project's inception, this was the only NSDV genome available from Genbank with complete sequences for all NSDV antigens. For design of the NSDV GPC antigen, the full open reading frame of the NSDV M segment was used, constituting the entire GPC sequence. The design of the NSDV Gn and Gc antigens followed the same processes as the design of the CCHFV Gn and Gc antigens, and the residues used were selected by BLASTP alignment with the previously designed CCHFV Gn and Gc antigens. Expression sequences were designed beginning with the signal peptide of NSDV GPC (aa1-20). For the NSDV Gn expression construct a 9 amino acid linker was used between the signal peptide and NSDV Gn sequence (aa 444-452) culminating in a Ski-1-like cleavage site. Following the linker was the NSDV Gn antigen which was a further 330 amino acids long (aa 453-782), equivalent in length to CCHFV Gn. The NSDV Gc antigen began with the same NSDV signal peptide (aa 1-20) followed by a

native 13 amino acid linker (aa 963-975) leading up to the NSDV Gc ski-1-like cleavage site. Following the cleavage site was the NSDV Gc antigen continued to the 50 amino acids C-terminus of NSDV GPC (aa 976-1577), to enhance expression similar to the previously discussed NSDV GPC del10, being 602 amino acids in length. The developed antigens were then cloned into antigen cassettes for expression in ChAdOx1 vectors. All NSDV antigens were expressed by the short CMV promoter to reduce the size of the antigen cassette, which is particularly pertinent for the NSDV GPC antigen, which is less than 150bp smaller than the equivalent CCHFV GPC.

NSDV GPC, Gn and Gc antigens were purchased as gene strings, and each ligated into an expression vector linearised by restriction digest with HindIII and BamH1 restriction enzymes. The ligation product was transformed into competent DH5α cells and transformed cells were selected by positive selection with Kanamycin. Plasmid DNA was extracted from transformed cells and plasmids containing NSDV antigens identified by diagnostic digest. Shuttle plasmids were recombined into a BAC containing the ChAdOx1 genome, and viruses were produced by the Viral Vector Core Facility, University of Oxford.

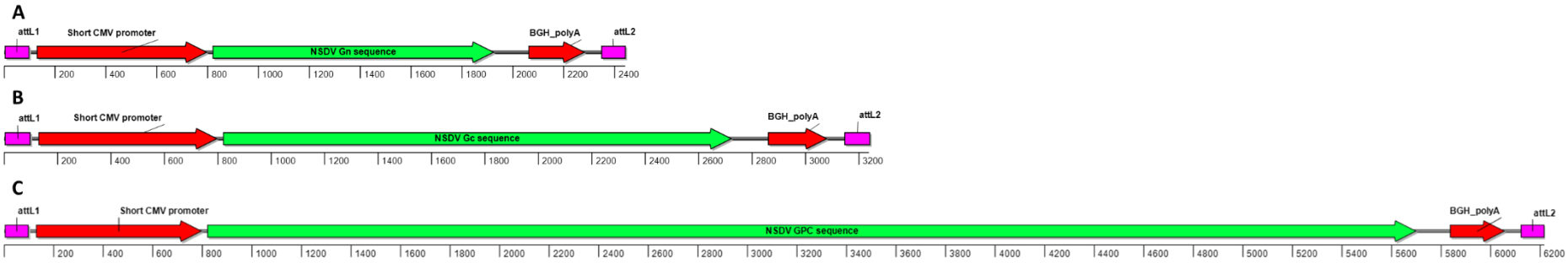


Figure 6.2: Antigen cassettes for ChAdOx1 NSDV Gn, ChAdOx1 NSDV Gc and ChAdOx1 NSDV GPC vaccines.
Representation of (**A**) NSDV Gn antigen cassette, (**B**) NSDV Gc antigen cassette, and (**C**) NSDV GPC. AttL1 and AttL2 represent recombination sites for the antigen cassette into the ChAdOx1 genome. Short CMV promoter represents the 673 amino acid promoter sequence derived from CMV. BGH_polyA represents the polyA tail of bovine growth hormone for transcriptional termination.

6.2.1.2 Immunogenicity of ChAdOx1 NSDV GP, Gn, and Gc vaccines

Similar to the situation for CCHFV vaccines, reagents were not available to assess expression of NSDV antigens directly by western blot. Instead, vaccine induced immunogenicity was assessed.

ChAdOx1 vaccines expressing NSDV GPC, NSDV Gn and NSDV Gc antigens were tested for immunogenicity induced in balb/c mice. In parallel, a 1:1 mixture of the monovalent vaccines ChAdOx1-NSDV Gn and ChAdOx1-NSDV Gc was assessed for immunogenicity. Immunogenicity was measured by IFN-γ ELISPOT against NSDV specific peptides, total IgG ELISA against NSDV Gc protein, and neutralisation by an NSDV-VSV pseudotype VNT.

IFN-γ ELISPOT responses are displayed in figure 6.3. Anti-Gn responses are shown in figure 6.3A. Following vaccination both ChAdOx1-NSDV GPC and ChAdOx1-NSDV Gn induced anti-Gn cellular responses. However, the response induced following ChAdOx1-NSDV Gn vaccination was significantly lower when compared with the anti-Gn cellular response induced by ChAdOx1-NSDV GPC vaccination. The anti-Gc responses contrasted and are displayed in figure 6.3B. ChAdOx1-NSDV GPC and ChAdOx1-NSDV Gc both induced anti-Gc cellular responses following vaccination. However, the responses induced after ChAdOx1-NSDV Gc vaccination were significantly greater than the anti-Gc cellular responses induced following vaccination with ChAdOx1-NSDV GPC. When responses from all GPC peptides were summed, shown in figure 6.3C, no significant difference was measured in total cellular responses induced following vaccination with ChAdOx1-NSDV GPC, ChAdOx1-NSDV Gc or the monovalent mixture of ChAdOx1-NSDV Gn and ChAdOx1 NSDV Gc.

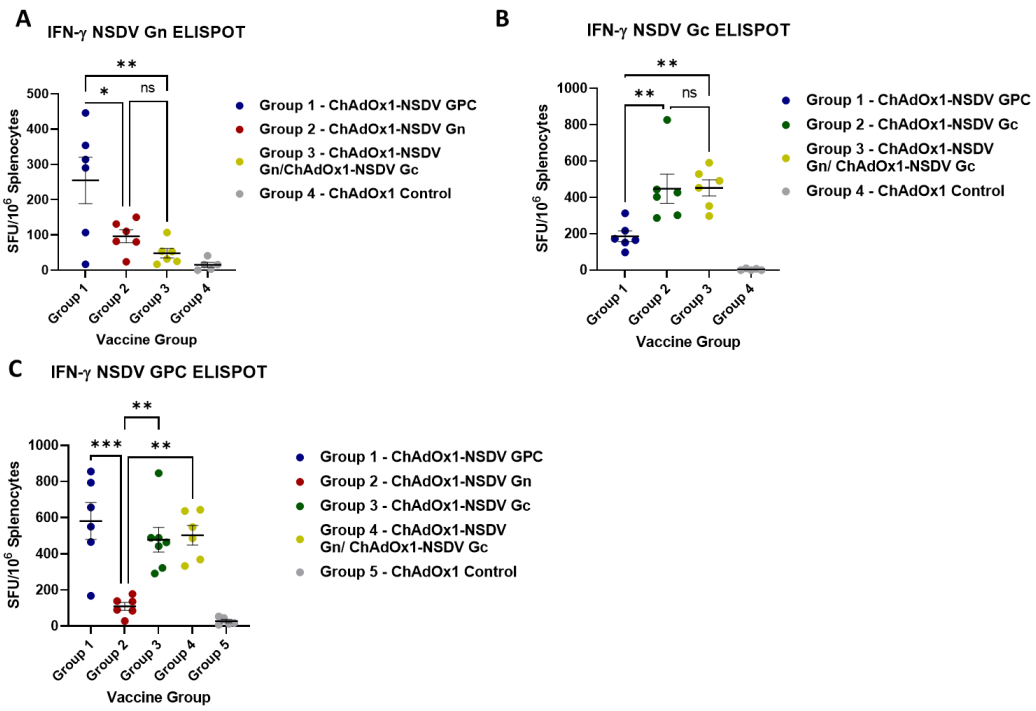


Figure 6.3: IFN-γ ELISPOT against peptides specific to Gn, Gc, or GPC of NSDV Jilin strain, following vaccination with ChAdOx1 NSDV GPC and GPC subunit vaccines. Six week old balb/c mice were immunised with $1x10^8$ IU of ChAdOx1 vaccine. Twenty one days after prime vaccination, mice were culled for immunological analysis. NSDV Gn antigen specific IFN-γ responses (**A**) were assayed in mouse splenocytes by IFN-γ ELISPOT. Individual balb/c mice (groups 1/2/3 n=6, group 4 n=5) are shown here measured in spot forming units per 10^6 splenocytes (SFU/10^6) with mean and standard error of the mean (SEM) also shown. Significant differences were determined by Ordinary One-Way ANOVA with Tukey's multiple comparison test (*p<0.05, **p<0.01). NSDV Gc antigen specific IFN-γ responses (**B**) were assayed in mouse splenocytes by IFN-γ ELISPOT. Individual balb/c mice (groups 1/3 n=6, group 2 n=7, group 4 n=5) are shown here measured in spot forming units per 10^6 splenocytes (SFU/10^6) with mean and standard error of the mean (SEM) also shown. Significant differences were determined by Ordinary One-Way ANOVA with Tukey's multiple comparisons test (**p<0.01). Total NSDV GPC antigen specific IFN-γ responses (**C**) were assayed in mouse splenocytes by IFN-γ ELISPOT. Individual balb/c mice (groups 1/2/4 n=6, group 3 n=7, group 5 n=5) are shown here measured in spot forming units per 10^6 splenocytes (SFU/10^6) with mean and standard error of the mean (SEM) also shown. Significant differences were determined by Ordinary One-way ANOVA with Tukey's multiple comparisons test (**p<0.01, ***p<0.01).

Total IgG ELISA was used to assess humoral responses induced by ChAdOx1 vaccination with NSDV GPC and NSDV Gc antigens. Attempts were made to produce a total IgG ELISA which could assess anti NSDV Gn response but the assay could not be standardised. The anti-Gc humoral responses measured by total IgG ELISA are displayed in figure 6.4. Vaccination with ChAdOx1-NSDV GPC and ChAdOx1-NSDV Gc induced a humoral response against NSDV Gc protein. However, no significant differences were measured in anti-Gc humoral responses following vaccination.

Neutralisation responses induced by vaccination were assessed using a pseudotype VNT with NSDV-VSV pseudoparticles, the results of which are presented in figure 6.5. Neutralisation responses were induced in all but one of the mice following vaccination with ChAdOx1-NSDV GPC or ChAdOx1-NSDV Gc. In contrast following vaccination with ChAdOx1-NSDV Gn, only two mice were measured with neutralisation titres above the lower limit of detection. No significant differences were measured in neutralisation titres between mice after vaccination with ChAdOx1-NSDV GPC, ChAdOx1-NSDV Gc, or the monovalent mixture.

NSDV GPC and GPC subunit antigens in ChAdOx1 vectors were assessed for their ability to induce immune responses following vaccination. NSDV GPC and Gc were immunogenic antigens in ChAdOx1 vectored vaccines, inducing cellular and humoral responses after vaccination, and with the induced humoral response producing neutralisation antibodies in a pseudotype VNT assay. In contrast the NSDV Gn antigen induced poor immunogenic responses following vaccination with ChAdOx1 vectors. ChAdOx1-NSDV Gn vaccination did not induce a significant neutralisation response unlike vaccination with ChAdOx1-NSDV GPC or ChAdOx1-NSDV Gc. By inducing cellular and functional humoral responses, ChAdOx1-NSDV GPC and ChAdOx1-NSDV Gc are both immunogenic vaccines and will be suitable for further progression in preclinical assessment.

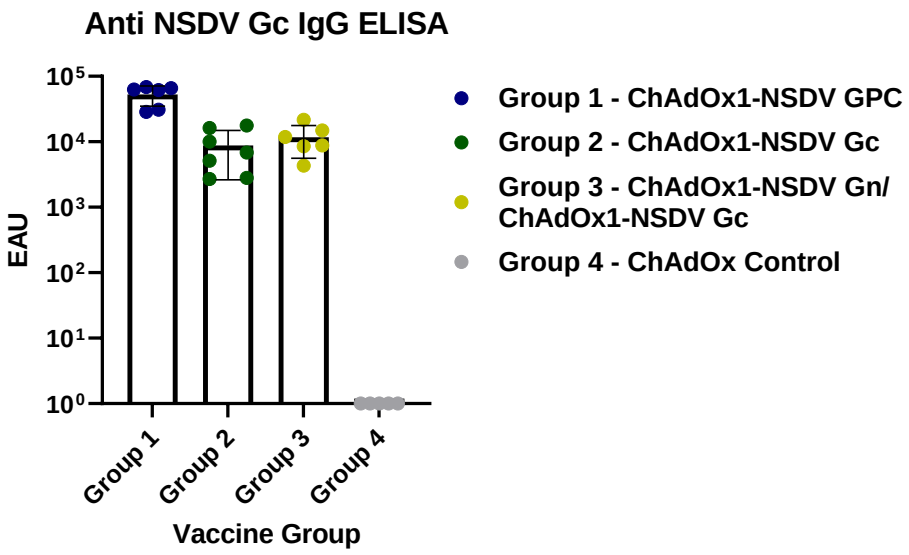


Figure 6.4: Total IgG ELISA against NSDV Gc of NSDV Jilin strain, following vaccination with ChAdOx1 NSDV GPC and GPC subunit vaccines.
Six week old balb/c mice were immunised with $1x10^8$ IU of ChAdOx1 vaccine. Twenty one days after prime vaccination, mice were culled for immunological analysis. CCHFV specific Gc responses were quantified in balb/c mice in a standardised ELISA. Individual mice (groups 1/3=6, group 2 n=7, group 4 n=5) are shown as data points in EAU, with mean and SEM also shown. Responses below the limit of detection were assigned a value of 1EAU. No significant differences were determined by Kruskal-Wallis test.

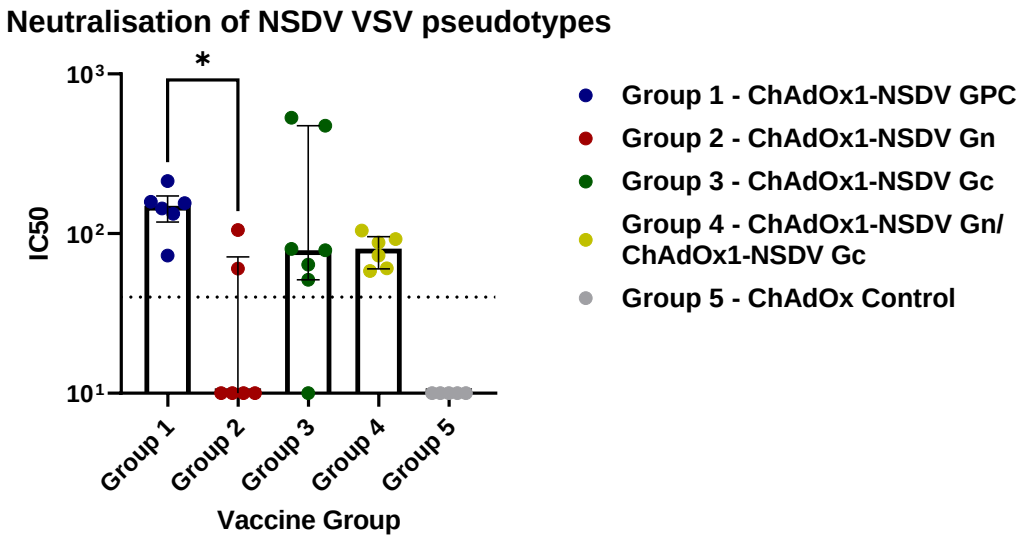


Figure 6.5: IC50 Neutralisation titre of vaccinated mouse serum against NSDV pseudotyped VSV, following vaccination with ChAdOx1 NSDV GPC and GPC subunit vaccines.

Six week old female Balb/c mice were vaccinated with $1x10^8$ IU of ChAdOx1 Vaccine. Twenty one days after prime vaccination, mice were culled for immunological analysis. Neutralising antibody titres were determined by virus neutralisation test using NSDV-VSV. Data points (group 1/2/4 n=6, group 3 n=7, group 4 n=5) were expressed as IC50 values and shown here as a scatter dot plot with bars showing median and IQR. Responses below the limit of protection were assigned an IC50 of 10 for presentation purposes. Significant differences were determined by Kruskal-Wallis test with Dunn's multiple comparison's test (*$p<0.05$).

6.2.2 ChAdOx1 vectored NSDV NP vaccines

Further informed by previous CCHFV vaccine development, NSDV NP was assessed as an antigen for ChAdOx vectored vaccines. NSDV NP is an internal antigen within the NSDV virion, and similar to many viral species, NP is thought to be more highly conserved than viral antigens under greater immunological selection pressures on the virion surface. As such, a ChAdOx1-NSDV NP vaccine was developed and assessed.

6.2.2.1 Design of ChAdOx1 vectored NSDV NP vaccine

A shuttle vector using NSDV NP as an antigen was designed for cloning into the ChAdOx1 genome. The NSDV antigen used the ORF from the NSDV S segment of the NSDV Jilin strain, the same isolate as used for development of NSDV GPC, NSDV Gn and NSDV Gc antigens. The NSDV NP antigen sequence was ordered as a Genestring and cloned into the shuttle vector, linearised by HindIII and BamH1. The antigen cassette for NSDV NP is presented in figure 6.6. The antigen is expressed by the short CMV promoter, the same promoter used to express NSDV GPC and GPC subunit antigens earlier in this chapter.

Following successful cloning of NSDV NP into a ChAdOx1 genome, ChAdOx1-NSDV NP was produced by the Viral Vector Core Facility, University of Oxford.

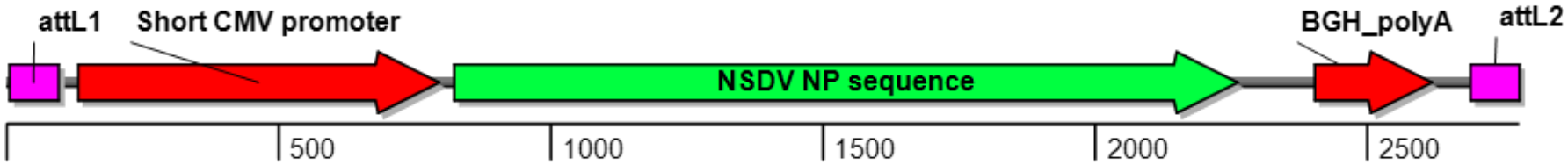


Figure 6.6: Antigen cassettes for ChAdOx1 NSDV NP
Representation of (**A**) NSDV NP antigen cassette. AttL1 and AttL2 represent recombination sites for the antigen cassette into the ChAdOx1 genome. Short CMV promoter represents the 673 amino acid promoter sequence derived from CMV. BGH_polyA represents the polyA tail of bovine growth hormone for transcriptional termination.

6.2.2.2 Immunogenicity of ChAdOx1 NSDV NP

Assessing immunogenicity of NSDV NP as an antigen presented difficulties, due to the lack of available reagents for working with NSDV and NSDV proteins. Cellular immunogenicity could however be assessed by IFN-γ ELISPOT against NSDV NP specific peptides. Balb/c mice were vaccinated with ChAdOx1-NSDV NP and the immunogenicity results following vaccination are presented in figure 6.7. ChAdOx1-NSDV NP vaccination induced a strong cellular response in balb/c as measured by IFN-γ ELISPOT. When compared to the immunogenicity induced by the ChAdOx1-CCHFV NP vaccine discussed in chapter 4, ChAdOx1-NSDV NP induces much stronger immunogenicity against NSDV NP specific peptides.

Unfortunately, humoral immunogenicity could not be assessed for the NSDV NP antigen. An ELISA assay could not be developed or standardised due to the lack of an NSDV NP protein for the ELISA, and the lack of facilities available to produce NSDV NP protein as it was not commercially available. Unlike for CCHFV pseudotyping, the NSDV pseudotype VNT developed included only the NSDV GPC, and as such neutralisation induced by NSDV NP could not be assessed, without development of an NSDV tec-VLP assay similar to the one used to assay CCHFV neutralisation.

However, humoral responses against NSDV NP would not be considered likely to induce any measure of protection against NSDV infection, as the NSDV NP specific antibodies would not be able to access the internal NSDV NP antigen and perform any functional role. Anti-NSDV NP cellular responses are more likely to be responsible for any potential anti-NP protective responses, and in this regard the ChAdOx1-NSDV NP was promising, inducing a strong anti NP cellular response as assayed by IFN-γ ELISPOT.

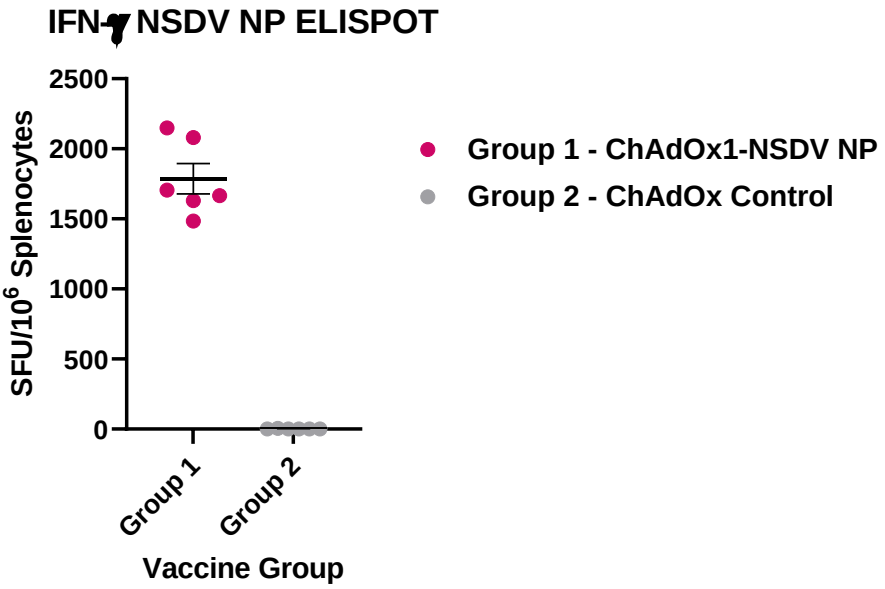


Figure 6.7: IFN-γ ELISPOT against peptides specific to NP of NSDV Jilin strain, following vaccination with ChAdOx1-NSDV NP.
Six week old balb/c mice were immunised with $1x10^8$ IU of ChAdOx1 vaccine. Twenty one days after prime vaccination, mice were culled for immunological analysis. NSDV NP antigen specific IFN-γ responses were assayed in mouse splenocytes by IFN-γ ELISPOT. Individual balb/c mice (n=6) are shown here measured in spot forming units per 10^6 splenocytes (SFU/10^6) with mean and standard error of the mean (SEM) also shown.

6.2.3 Co-administration of ChAdOx1 NSDV GPC and NSDV NP vaccines

The immunogenicity induced following vaccination with ChAdOx1 vectors expressing NSDV antigens followed different patterns to immunogenicity induced by vaccination with ChAdOx vectored CCHFV antigens, and as such different approaches were used to improve the breadth of immunogenicity. As the NSDV Gn antigen showed extremely poor immunogenicity, research did not investigate production of multivalent ChAdOx1 vectored NSDV vaccines. ChAdOx1-NSDV GPC immunisations induced cellular immunogenicity against all regions of GPC, and strong humoral responses against NSDV Gc. ChAdOx1 NSDV NP vaccination induced NP specific cellular responses. A bivalent ChAdOx1vaccine encoding NSDV GPC and NP antigens would likely exceed the capacity limits of the ChAdOx1 vector. As such, a co-administration of ChAdOx1-NSDV GPC and ChAdOx1-NSDV NP was assessed, in a regimen similar to a CCHFV DNA vaccine which was protective in small animal challenge models[250].

6.2.3.1 Immunogenicity of co-administered ChAdOx1-NSDV GPC and ChAdOx1-NSDV NP

Immunogenicity was assessed through IFN-γ ELISPOT against NSDV GPC and NP specific peptides, total IgG NSDV Gc ELISA, and neutralisation in an NSDV-VSV pseudotype VNT. IFN-γ ELISPOT responses are displayed in figure 6.8, with anti-GPC cellular responses displayed in figure 6.8A. Following vaccination with ChAdOx1 NSDV GPC a cellular immune response was induced, which was significantly greater than the anti-GPC cellular response induced following vaccination with a mixture of ChAdOx1-NSDV GPC and ChAdOx1-NSDV NP vaccines. Most mice vaccinated with ChAdOx1-NSDV NP did not show cellular responses against NSDV GPC, though one vaccinated mouse did show an anti-GPC response as measured by IFN-γ ELISPOT. Figure 6.8B shows IFN-γ ELISPOT responses against NSDV NP specific peptides. No significant differences were measured in responses following ChAdOx1-NSDV NP vaccination or following the co-administration of the ChAdOx1-NSDV GPC and ChAdOx1-NSDV NP. In addition, no responses against NP specific peptides were measured in mice vaccinated with ChAdOx1-NSDV GPC.

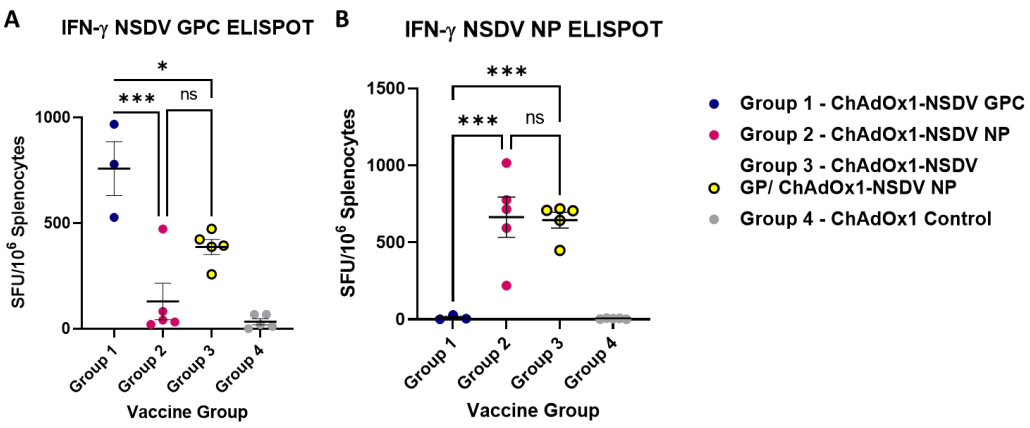


Figure 6.8: IFN-γ ELISPOT against peptides specific to NSDV GPC and NP, following vaccination with ChAdOx1-NSDV GPC, ChAdOx1-NSDV NP, or a monovalent mixture.

Six week old balb/c mice were immunised with $1x10^8$ of ChAdOx1 vaccine. Twenty one days after prime vaccination, mice were culled for immunological analysis. NSDV GPC antigen specific IFN-γ responses (**A**) were assayed in mouse splenocytes by IFN-γ ELISPOT. Individual balb/c mice (group 1 n=3, groups 2-4 n=5) are shown as data points measured in spot forming units per 10^6 splenocytes (SFU/10^6) with mean and standard error of the mean (SEM) also shown. Significant differences were determined by Ordinary One-Way ANOVA with Tukey's multiple comparison test (*p<0.05, ***p<0.001). CCHFV NP antigen specific IFN-γ (**B**) specific responses were determined by IFN-γ ELISPOT. Individual mice (group 1 n=3, groups 2-4 n=5) are shown here measured in SFU per 10^6 splenocytes with mean and SEM. Significant differences were determined by Ordinary One-Way ANOVA with Tukey's multiple comparisons test (***p<0.001).

Humoral responses against the NSDV Gc antigen were measured by a total IgG ELISA, and presented in figure 6.9. Following vaccination both ChAdOx1-NSDV GPC and the co-administration of ChAdOx1-NSDV GPC and ChAdOx1-NSDV NP produced a humoral response against NSDV Gc proteins, with no significant difference measured between the two different regimens. Humoral responses were furthered assessed by the capacity for serum from vaccinated mice to neutralise NSDV-VSV pseudoparticles, the results of the NSDV pseudotype VNT displayed in figure 6.10. Neutralisation responses were induced after vaccination in both the ChAdOx1-NSDV GPC and the co-administration groups, with no neutralisation measured following ChAdOx1-NSDV NP vaccination. No significant differences were measured in neutralisation titres between the group vaccinated with ChAdOx1-NSDV GPC, and the group receiving a co-administration of the ChAdOx1-NSDV GPC and ChAdOx1-NSDV NP vaccines.

In developing ChAdOx vectored NSDV vaccines, co-administration of multiple vaccines offers a different method of improving vaccine breadth that is in contrast to the methods discussed for CCHFV vaccine development. The co-administration regimen did show significantly lower anti-Gc cellular responses than a monovalent dose of ChAdOx1-NSDV GPC vaccine, but cellular responses were the only significant difference measured between vaccine regimens. Anti-NSDV NP cellular responses, anti-NSDV Gc total IgG ELISA responses, and neutralisation of NSDV-VSV bearing GPC on the surface all showed that the co-administration of ChAdOx1-NSDV GPC and ChAdOx1-NSDV NP were equally immunogenic as administration of a single dose of ChAdOx1-NSDV GPC. With co-administered GPC and NP antigens shown to be a protective measure in other nairovirus vaccines, vaccinating with ChAdOx1-NSDV GPC and ChAdOx1-NSDV NP may be a preferred method to induce a protective response against NSDV infection.

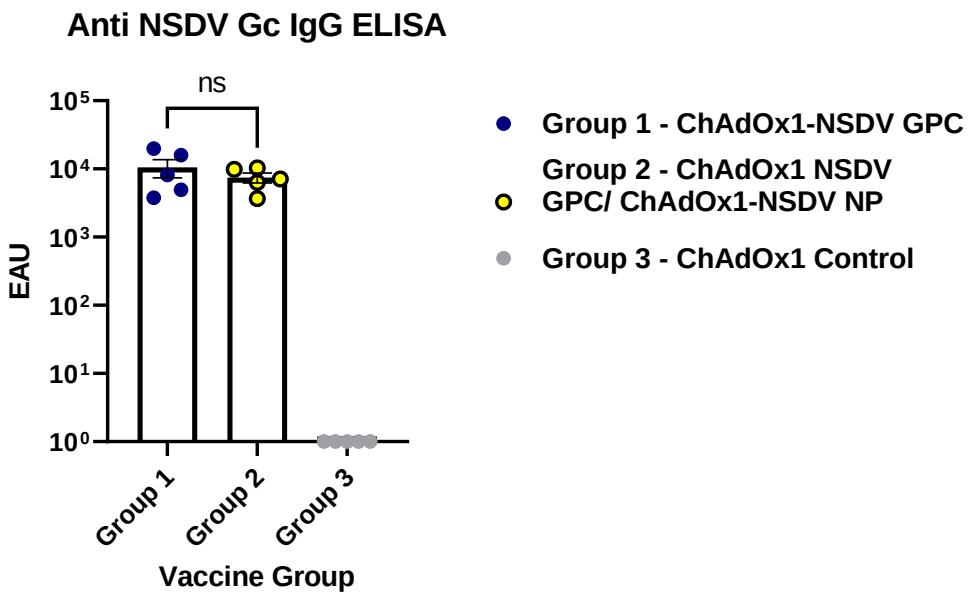


Figure 6.9: Total IgG ELISA against NSDV Gc of NSDV Jilin strain, following vaccination with ChAdOx1-NSDV GPC or a monovalent mixture.
Six week old balb/c mice were immunised with $1x10^8$ IU of ChAdOx1 vaccine. Twenty one days after prime vaccination, mice were culled for immunological analysis. CCHFV specific Gc responses were quantified in balb/c mice in a standardised ELISA. Individual mice (groups 1/3=6, group 2 n=7, group 4 n=5) are shown as data points in EAU, with mean and SEM also shown. Responses below the limit of detection were assigned a value of 1EAU. No significant differences were determined by Kruskal-Wallis test.

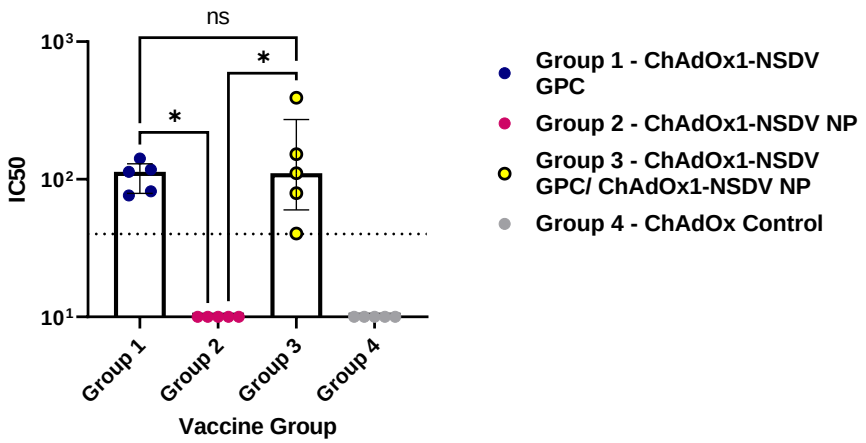


Figure 6.10: IC50 Neutralisation titre of vaccinated mouse serum against NSDV pseudotyped VSV, following vaccination with ChAdOx1-NSDV GPC, ChAdOx1-NSDV NP, or a monovalent mixture.

Six week old female balb/c mice were vaccinated with $1x10^8$ IU of ChAdOx1 Vaccine. Twenty one days after prime vaccination, mice were culled for immunological analysis. Neutralising antibody titres were determined by virus neutralisation test using NSDV pseudotyped VSV. Individual data points (n=5) were expressed as IC50 values and shown here as a scatter dot plot with bars showing median and IQR. Significant differences were determined by Kruskal-Wallis test with Dunn's multiple comparison's test (*$p<0.05$).

6.3 Conclusions

The aim in this chapter was to produce immunogenic NSDV vaccines, which could produce cellular and humoral responses against NSDV peptides, proteins and pseudotyped virus. It has been shown throughout this chapter that immunogenic vaccines have been developed using the ChAdOx1 vector. Use of NSDV GPC as the antigen was successful, with immunisation using ChAdOx1 vectors inducing cellular responses against NSDV Gn and Gc specific peptides, and the rest of the full length GPC antigen; MCL, GP38 and NSm. In addition, vaccination with ChAdOx1-NSDV GPC induced humoral responses against NSDV Gc, and induced neutralising antibody titres preventing NSDV GPC mediated cell entry in a pseudotype VNT. NSDV Gc as an antigen also induced a significant immune response from vaccination in ChAdOx1 vectors, with anti-Gc cellular responses induced following ChAdOx1-NSDV Gc vaccination significantly greater than those induced after ChAdOx1-NSDV GPC vaccination. ChAdOx1-NSDV Gc vaccination also induced a humoral response against NSDV Gc and induced significant neutralising antibody titres. NSDV Gc appears to be an immunogenic antigen in the ChAdOx1 platform and its use merits further investigation.

The NSDV Gn antigen used in the ChAdOx1 platform was not strongly immunogenic, compared to NSDV GPC. Cellular responses induced by vaccination with ChAdOx1 NSDV Gn were poor as assayed by IFN-γ ELISPOT. Humoral responses could not be determined by a total IgG ELISA, but there was little evidence of any neutralisation response induced following ChAdOx1-NSDV Gn vaccination. With the receptors for NSDV and the methods of cell entry unknown, it is inconclusive if anti-Gn humoral responses would be expected to induce any neutralisation responses. The issue did not appear to be that NSDV Gn in itself was non immunogenic, as evidenced by the cellular responses against NSDV Gn specific peptides induced by ChAdOx1-NSDV GPC vaccination. Instead, the issue appears to stem from the expression of the designed NSDV

Gn construct from the ChAdOx1 vector. A redesign of the NSDV Gn antigen expression construct for ChAdOx1 vectors, as well as the development of assays to assess NSDV Gn protein expression, may allow the expression and immunogenicity of the NSDV Gn antigen to be more accurately assessed.

The ChAdOx1-NSDV NP vaccine was also assessed for induction of immunogenicity following vaccination. High levels of cellular immunogenicity were measured following ChAdOx1-NSDV NP vaccination. Humoral immunogenicity could not be assessed for NSDV NP, though the presumed effectiveness of humoral responses against an internal antigen in protection is low. As such, NSDV NP may also be considered an immunogenic NSDV antigen, meaning vaccines using NSDV NP are worthy of further investigation.

In contrast to approaches to deliver multiple CCHFV antigens, delivery of multiple NSDV antigens for vaccination looked initially at co-administration of ChAdOx1-NSDV GPC and ChAdOx1-NSDV NP in a single immunisation. In almost all immunological measures, co-administration induced immunogenicity following vaccination of similar magnitude to that induced by the individual vaccines. With the combination of GPC and NP antigens shown to induce protective immune responses in animal models of CCHFV challenge[250], the co-administration of CHAdOx1-NSDV GPC and ChAdOx1-NSDV NP provides an immune response that should be further investigated for protective efficacy. An alternative method of delivering multiple antigens with ChAdOx1 can use multiple antigen expression sites within the same vector. Both ChAdOx1-NSDV Gc and ChAdOx1-NSDV NP were immunogenic vaccines, inducing strong cellular responses following vaccination, and the inclusion of both antigen cassettes within a Bivalent ChAdOx1 genome would be below the theoretical capacity of adenovirus vectors.

The current issue with the development of nairovirus vaccines is the lack of correlates of protection from both infection and disease. The problem is exacerbated with NSDV compared to CCHFV with a dearth of research into the pathology of NSDV. Whilst

survival from NSDV infection is associated with the development of antibodies[337], there has been little research into whether humoral responses are the cause of protection or just a consequence of NSDV infection. With a lack of current vaccine development in NSDV and a comparative lack of challenge studies to further understand the pathology of infection, this lack of knowledge is unlikely to change in the near future. With a more thorough understanding of NSDV pathology, and the importance of various immune responses for survival from infection, vaccine induced immunogenicity can be analysed more accurately to determine if induced responses are likely to be protective.

Whilst challenge models have not been used in recent literature for assessing vaccine efficacy for NSDV, sheep have been used for assessing the pathology of infection[141], rather than assessing any correlates of protection in surviving individuals. A model using sheep to assess NSDV protection would be advantageous over the mouse or NHP models used in CCHFV research as pathogenic infection which does not directly replicate infection in the organism of interest, humans[239,243]. NSDV challenge in sheep is a more realistic model of infection, and it has been suggested that the pathology of NSDV infection could inform greater understanding of nairovirus pathology[56]. A sheep challenge model could therefore for be used to assess the protective effects of ChAdOx vectored NSDV vaccines.

Table 6.1 shows potential vaccine groups for a challenge experiment assessing protective efficacies of a single dose of ChAdOx1 vectored vaccines against NSDV. The challenge experiment would assess the protection induced by ChAdOx1 vectored NSDV GPC, NSDV Gc and NSDV NP antigens, all of which were shown to be immunogenic when used in mice. In addition, there would be three further groups assessing multiple NSDV antigens. Group 4 involves combining NSDV GPC and NSDV NP in ChAdOx1 vectors which has been shown in this thesis to be immunogenic in mice. Group 5 and 6 would assess the protection induced by co-administration of NSDV Gc and NSDV NP vaccines.

Both were immunogenic vaccines, and as previously discussed a combination of both in a ChAdOx1 vector would remain below the limits on ChAdOx1 genome size. Finally, a control group would be included as group 7, being vaccinated with a ChAdOx1 vector carrying an irrelevant antigen. Ideally a challenge experiment would be carried out in an outbred sheep population to show protection could be induced in individuals with a variety of MHC types. The use of up to 8 sheep per group in an outbred population has been used previously in RVFV challenge[343], but exact numbers needed for such a study would require consultation with a statistician to determine appropriate power, preventing wastage of sheep, and reducing costs from storage of excess sheep in SAPO-4 level facilities.

Table 6.1: Vaccination groups in a theoretical study to assess protection against lethal NSDV challenge.

Group	Vaccine received
1	ChAdOx1-NSDV GPC
2	ChAdOx1-NSDV Gc
3	ChAdOx1-NSDV NP
4	ChAdOx1-NSDV GPC/ ChAdOx1 NSDV NP
5	ChAdOx1-NSDV Gc/ ChAdOx1 NSDV NP
6	ChAdOx1 Bivalent NSDV
7	ChAdOx1 control

Chapter 7 – Anti-vector neutralisation responses against the ChAdOx1 vector

7.1 Introduction

Adenovirus vectors have been used extensively in medicine, as gene delivery vectors for genetic therapies[211] and as platforms for vaccine development[220,233]. Whilst human adenoviruses are a viable vaccine platform[213,215,344], pre-existing immunity to specific adenoviruses in the population can present an obstacle to widespread usage[213,345]. Adenoviruses are endemic in almost all populations across the world, with most infections causing mild respiratory symptoms, though other symptoms can present and infection can be severe in immunocompromised individuals[346]. Sero-reactivity against common vectors such as Ad5 and Ad26 vary across different population, with the greatest circulation in Africa, and seroprevalance in adult populations of up to 91% and 68% respectively[218].

The anti-vector responses induced by natural infection can produce detrimental effects on vaccine immunogenicity, a phenomenon most widely studied with the Ad5 vector. A previous Ad5-EBOV GP phase 1 trial assessed the effects of pre-existing Ad5 neutralising antibodies on anti-EBOV GP immunogenicity, and participants with anti-Ad5 titres greater than 1:200 showed significantly poorer anti-EBOV cellular and humoral immunogenicity than those with anti-Ad5 titres below 1:200[213]. Further research with Ad5 vector, centred on an Ad5-SARS-CoV-2 vaccine, showed that the negative impact on antigen immunogenicity in individuals with pre-existing exposure, could be overcome by increasing the vaccine dosage[345]. Though increasing vaccine dosage does present a possible avenue for circumventing anti-vector immune responses, such an approach would both decrease the number of doses of available vaccines, increase the cost of vaccines, and potentially increase the reactogenicity of the vaccine.

Pre-existing immunogenicity of human adenoviruses has led to the development of vaccine platforms using adenoviruses from non-human species to evade issues of pre-existing immunity. Adenoviruses from a variety of mammalian and bird species have been tested as gene expression vectors, including cows, pigs, dogs, and fowl[210]. However, the most well researched non-human adenovirus platforms have been isolated from simians. Many vaccines are in development using chimpanzee[214,235,347], gorilla[348,349], or monkey[350] derived adenoviruses.

More recently the ChAdOx1 platform has been used to produce the vaccine ChAdOx1-nCoV19 and is widely used throughout the world to vaccinate against SARS-CoV-2[204].. With the ChAdOx1 vector now being widely used to immunise a significant proportion of the world's population, there is a need to investigate the immune response directed against ChAdOx1 in vaccinated individuals. Furthermore, there is a need to assess how previous exposure to ChAdOx1 vaccines impacts immunogenicity against subsequent ChAdOx1 vectored vaccines.

Anti-ChAdOx1 neutralisation titres were determined by assaying infection in a cellular based neutralisation assay using a live ChAdOx1-SEAP virus[212]. Figure 7.1 shows a schematic of the reporter assay used to detect anti-ChAdOx1 neutralising antibodies. ChAdOx1-SEAP is a modified ChAdOx1 virus which carries a secreted alkaline phosphatase (SEAP) and is used to infect HEK-293T Griptite cells, a highly adherent HEK-293T. Upon infection of HEK 293T-Griptite cells, SEAP is expressed and secreted into the supernatant. Supernatant is harvested, and the amount of SEAP secreted can be assayed by luminescence when a luminescent alkaline phosphatase substrate is mixed with the supernatant. Anti-ChAdOx1 neutralising antibodies, present in serum or plasma, block ChAdOx1-SEAP infection, and in turn reduce SEAP expression and luminescence in supernatant.

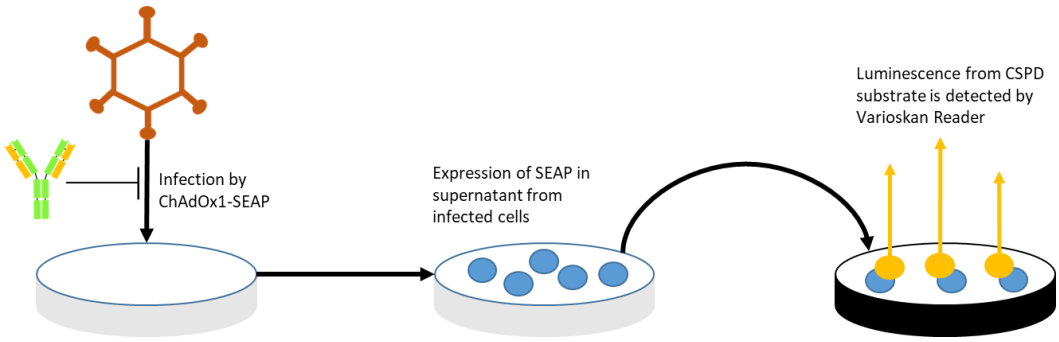


Figure 7.1: Schematic of the Secreted Alkaline Phosphatase (SEAP) reporter assay used in detection of anti-vector neutralisation.
HEK 293T-Griptite cells are plated at $3x10^4$ cells per well in a 96 well plate, then are incubated at 37°C 5%CO_2 for 24h. Cells are then infected with ChAdOx1-SEAP, mixed with the serum to be assayed for neutralisation, for 1h before being replaced with fresh 200µl 10% PRF Media and incubated for a further 24h in 37°C 5%CO_2. Following incubation 50µl supernatant containing SEAP is taken, and luminescence measured from reaction with a CSPD substrate.

7.2 Results

7.2.1 Anti ChAdOx1 neutralisation responses induced by vaccination with ChAdOx1-nCoV19

The clinical trials COV001, COV002, and COV006 assessed the immune responses to ChAdOx1-nCoV19, a ChAdOx1 vaccine which expressed the WT Spike protein of SARS-CoV-2[351–353]. As part of these trials, anti-vector neutralisation responses were assessed for a select group of participants, and Anti-ChAdOx1 responses were then analysed across a range of age groups and different vaccination regimens.

7.2.1.1 Anti-ChAdOx1 neutralisation in naïve individuals

The initial assessment of anti-ChAdOx1 responses was to determine pre-existing anti-ChAdOx1 neutralisation titres in a vaccine-naïve population prior to vaccination. All participants who were assessed in the studies described had not previously been exposed to ChAdOx1. If anti-vector neutralisation responses were detected they would likely reflect cross-reactivity from infection with other adenoviruses. The anti-ChAdOx1 responses in a ChAdOx1-nCoV19 naïve population are displayed in figure 7.2, stratified by age. The figure shows that most individuals showed no evidence of anti-vector neutralisation prior to vaccination. Further, in the small minority which did show evidence of anti-vector neutralisation, responses were low, falling below a threshold titre of 200, previously used in assessment of anti-Ad5 responses[213]. Less than 1% of participants showed anti-ChAdOx1 neutralisation responses above a threshold titre of 200, with no individuals in the 70+ age bracket showing responses of this magnitude. The greatest cross-reactivity against ChAdOx1 was measured in the 56-69 age range, with 22 % of individuals having a measurable anti-ChAdOx1 response. The reasons for the slight increase in the 56-69 age group are unknown and were not investigated further.

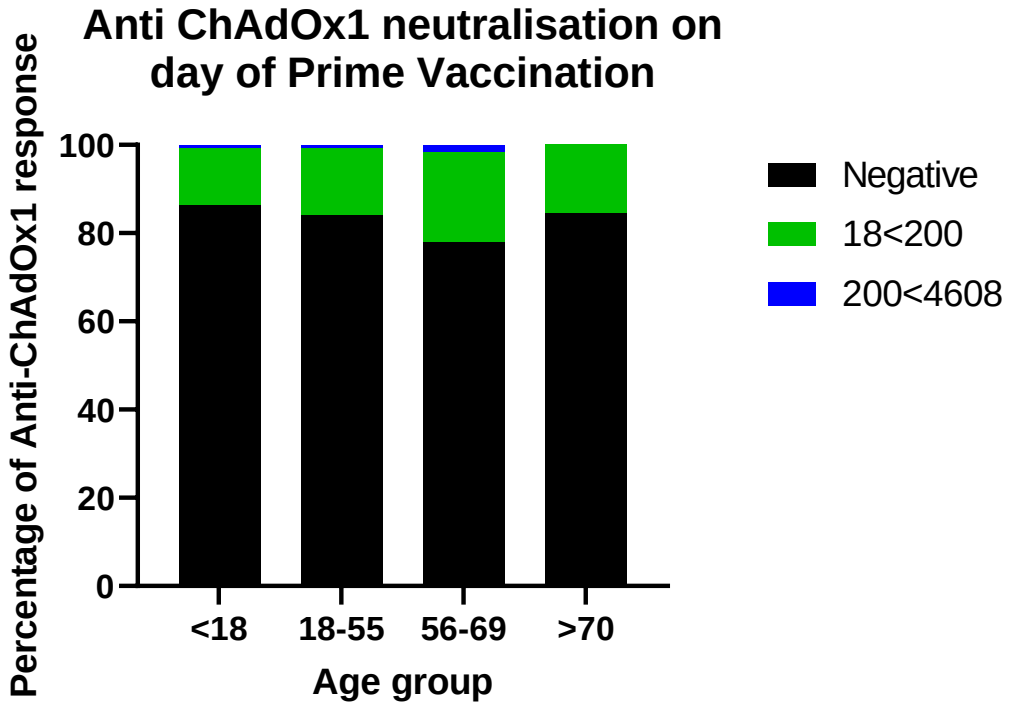


Figure 7.2: Anti-ChAdOx1 reactivity in naïve individuals prior to vaccination with ChAdOx1-nCoV19.

The figure shows the baseline anti-ChAdOx1 neutralisation IC50 titres in a naïve population prior to vaccination in the COV001, COV002 or COV006 clinical trials. The percentages of individuals in each age group were shown as one of three categories; those that were below the assays lower limit of detection (Negative), those who were between the limit of detection and a titre of 200 (18<200) and those with titres between 200 and the upper limit of detection in the assay (200<4608). For <18 N=144, for 18-55 N=150, for 56-69 N= 118, for 70+ N= 179.

7.2.1.2 Anti-ChAdOx1 neutralisation titres in response to ChAdOx1-nCOV19 vaccination

Following assessment of initial anti-ChAdOx1 responses in the population, anti-ChAdOx1 responses were assessed across a range of different vaccination regimens, encompassing differences in dosage and two dose interval, from the day of prime vaccination until 182 days after prime vaccination. The median results and IQR are displayed in figure 7.3 and show that differences appear to be minor between vaccinated groups. Anti-ChAdOx1 responses were induced in all groups by the initial prime vaccination. Little reduction in anti-ChAdOx1 neutralisation was measured following prime vaccination, and the receipt of a ChAdOx1-nCoV19 boost at an either 4 week or 8 week interval did not boost the anti-ChAdOx1 response. When observing responses out to 6 months post prime vaccination, again few differences were measured between the different groups, with anti-ChAdOx1 neutralisation titres showing long lived responses, with little reduction at the 6 month time point.

No significant differences were observed in anti-ChAdOx1 neutralisation time-courses between the different vaccine regimens presented. Anti-ChAdOx1 responses post first and second vaccination were stratified by a range of demographics, to determine if any differences were measured in responses between groups.

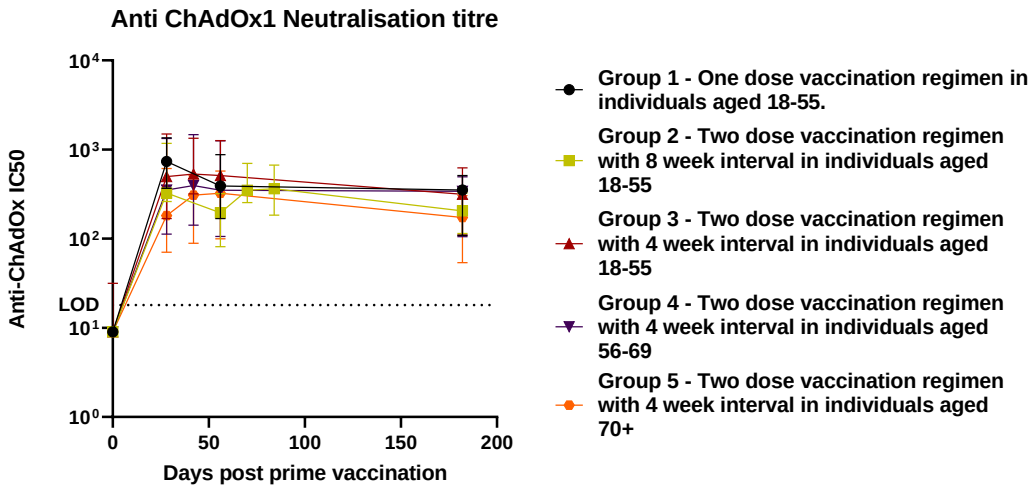


Figure 7.3: Time course of Anti-ChAdOx1 neutralisation titres across a range of vaccination regimes.

The figure shows anti-ChAdOx1 neutralisation IC50 titres both prior to and after receipt of the ChAdOx1-nCoV19 vaccine as part of the COV001 and COV002 clinical trials. For group 1 data was displayed for days 0, 28, 56 and 182. For group 2 data was displayed for days 0, 28, day of boost (D56), 14 days post-boost (D70), 28 days post-boost (D84) and day 182. For groups 3-5 data was displayed for days 0, day of boost (D28), 14 days post-boost (D42), 28 days post boost (D56) and day 182. Each data point displayed represents the median of the data set with the interquartile range. Values below the limit of detection (LOD) were assigned a value of 9 (1/2 LOD) for presentation.

The first analysis was assessing the impact of participant age on anti-vector neutralisation response, displayed in figure 7.4. Anti-ChAdOx1 neutralisation titres 28 days after a prime vaccination are shown in figure 7.4A. With each data point displayed it is evident that some individuals across all ages have not produced any anti-vector response post vaccination after prime vaccination. The correlation of anti-vector responses against age was assessed and a significant negative correlation was measured, suggesting that anti-ChAdOx1 neutralisation titres decreased in older participants which may be expected due to immunosenescence in older individuals. However, the slope of the line was shallow, and the R^2 value very low at 0.06, suggesting that though significant the correlation was very weak, with little relevance to predicting anti-vector neutralisation titres. Anti-ChAdOx1 responses post boost were correlated with age of participants, shown in figure 7.4B. Following boost, the previous correlation between anti-vector neutralisation and age was eliminated, with the relationship no longer being significant. In addition, fewer participants showed no detectable anti-vector response after a boost dose of ChAdOx1-nCoV19. With all future licenced ChAdOx1 vaccination regimens likely to utilise prime-boost vaccination due to the protective efficacy measured during clinical assessment of ChAdOx1-nCoV19, there is unlikely to be any significant age dependent correlations for anti-vector immunogenicity.

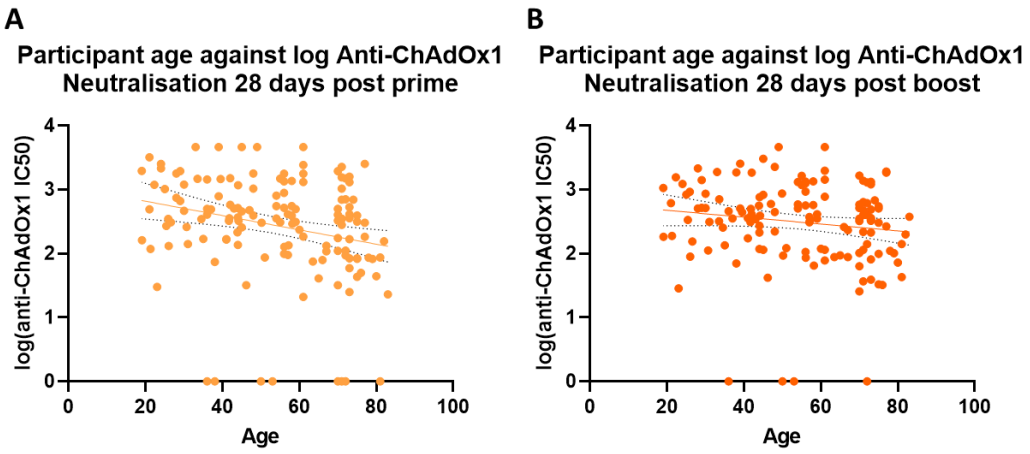


Figure 7.4: Anti-ChAdOx1 neutralisation titre against individual age in prime-boost vaccinated individuals.

The figure shows age of the individual plotted against anti-ChAdOx1 neutralisation IC50. Individuals were drawn from the COV001 and COV002 clinical trials. (**A**) Plot of age against anti-ChAdOx1 neutralisation IC50 28 days after prime vaccination. Each data point represents an individual. Anti-ChAdOx1 IC50 below the limit of detection (LOD) were assigned a value of log(9 (1/2LOD)) for presentation. A simple linear regression was performed producing a line of equation y=-0.0118.9x+3.042, with R^2=0.06122. The slope was significantly non-zero with a p-value of 0.0027. (**B**) Plot of age against anti-ChAdOx1 neutralisation IC50 28 days after boost vaccination. Each data point represents an individual. Anti-ChAdOx1 titres below the LOD were assigned a value of log(9 (1/2)LOD) for presentation. A simple linear regression was performed producing a line of equation y=-0.005342x+2.783, with R^2=0.02041. The slope was not significantly non zero with a p-value of 0.0910.

Within the clinical trials COV001 and COV002, two different doses of ChAdOx1-nCoV19 were assessed, standard dose (SD) and low dose (LD), which was approximately half the standard dose. Anti-ChAdOx1 neutralisation titres were measured for both regimes across a range of different age groups. Comparisons in 18-55 year olds are displayed in figure 7.5. After a single dose of either LD or SD, no significant differences could be measured in anti-ChAdOx1 neutralisation responses, as displayed in figure 7.5A, though the highest responders were part of the standard dose group. Following a boost vaccination with a homologous vaccine dose, anti-ChAdOx1 neutralisation responses were significantly greater in the SD/SD group, displayed in figure 7.5B. As would be expected, the greater dose of ChAdOx1 vaccine elicited a greater immunogenicity against ChAdOx1. However, at present it has not been possible to assess if these greater anti-ChAdOx1 responses affect the protective efficacy of ChAdOx1-nCoV19.

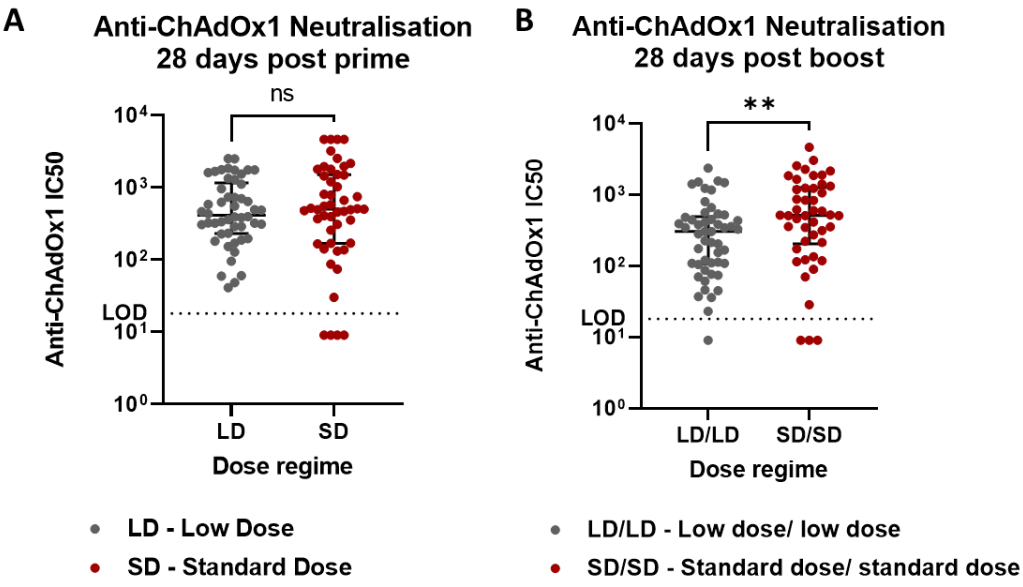


Figure 7.5: Comparison of anti ChAdOx1 neutralisation titres in 18-55 year olds receiving either a low or standard dose of ChAdOx1-nCoV19.
The figure shows the anti-ChAdOx1 neutralisation IC50 titres of individuals receiving a prime-boost of ChAdOx1-nCoV19 at a 4 week interval as part of the COV002 clinical trial. (**A**) Anti-ChAdOx1 IC50 titres are presented 28 days after receipt of a prime vaccination for groups receiving either a low dose (LD) or standard dose (SD) of ChAdOx1-nCoV19. Each data point represents an individual with median and inter quartile range of the group also shown. Values below the limit of detection (LOD) were assigned a value of 9 (1/2LOD) for presentation. No significant differences were determined by Mann-Whitney test. B) Anti-ChAdOx1 IC50 titres are presented 28 days after boost vaccination for groups receiving either a low dose (LD/LD) or standard dose (SD/SD) of ChAdOx1-nCoV19. Each data point represents an individual with median and interquartile range shown. Values below the LOD were assigned a value of 9 (1/2 LOD) for presentation. Significant differences were determined by Mann-Whitney test (**p<0.01).

Anti-vector responses stratified by SD or LD were also assessed in older age groups, and the results displayed in figure 7.6. Figure 7.6A and 7.6B show anti-vector neutralisation responses in 56-69 years old. Significant differences were measured following prime vaccination, with those receiving the SD showing significantly greater anti-ChAdOx1 neutralisation titres compared to individuals who had received the LD regimen. The significant difference measured after a prime vaccination was maintained after the boost in figure 7.6B, where those individuals in receipt of an SD/SD regimen showed significantly greater responses than those in the LD/LD group. The results for individuals over 70 contrasted, with no significant differences measured. As shown in figure 7.6C, SD induced anti-vector immunity trended greater than LD induced, but the difference measured was not significant. The trend continued after boosters, with individuals receiving the SD/SD regimen, as shown in figure 7.6D, showing a greater median anti-ChAdOx1 response, though the difference was not significant.

Despite the expectation that the SD would elicit greater anti-vector responses, this has not been borne out consistently across all vaccinated groups. Whilst the 18-55 needed a booster dose to show a significant difference, 56-69 year olds showed a significant difference after the prime alone, and contrastingly, the 70+ age group showed no significant differences in anti-ChAdOx1 neutralisation at any point. With little difference observed between groups, it will be difficult to assess if these minor differences will affect vaccine antigen immunogenicity efficacy in the different dosage groups.

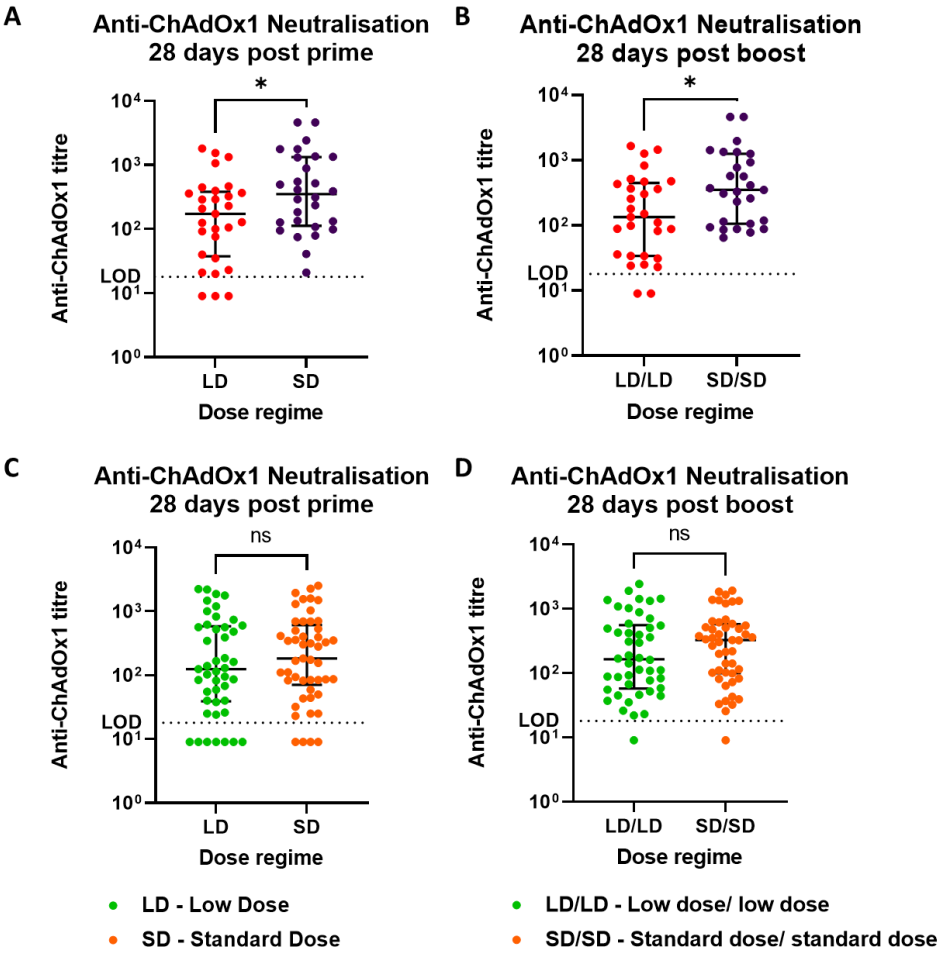


Figure 7.6: Comparison of anti ChAdOx1 neutralisation titres in 56-69 and 70+ year olds receiving either a low or standard dose of ChAdOx1-nCoV19.
The figure shows the anti-ChAdOx1 neutralisation IC50 titres of individuals receiving a prime-boost of ChAdOx1-nCoV19 at a 4 week interval as part of the COV002 clinical trial. (**A**) Anti-ChAdOx1 IC50 titres of the 56-69 age group are presented 28 days after receipt of a prime vaccination for groups receiving either a low dose (LD) or standard dose (SD) of ChAdOx1-nCoV19. Each data point represents an individual with median and inter quartile range of the group also shown. Values below the limit of detection (LOD) were assigned a value of 9 (1/2LOD) for presentation. Significant differences were determined by Mann-Whitney test (*p>0.05). (**B**) Anti-ChAdOx1 IC50 titres of the 56-69 age group are presented 28 days after boost vaccination for groups receiving either a low dose (LD/LD) or standard dose (SD/SD) of ChAdOx1-nCoV19. Each data point represents an individual with median and interquartile range shown. Values below the LOD were assigned a value of 9 (1/2LOD) for presentation. Significant differences were determined by Mann-Whitney test (*p<0.05). (**C**) Anti-ChAdOx1 IC50 titres of the 70+ age group are presented 28 days after receipt of a prime vaccination for groups receiving either a low dose (LD) or standard dose (SD) of ChAdOx1-nCoV19. Each data point represents an individual with median and inter quartile range of the group also shown. Values below the limit of detection were assigned a value of 9 (1/2 LOD) for presentation purposes. No significant differences were determined by Mann-Whitney test. (**D**) Anti-ChAdOx1 IC50 titres of the 70+ age group are presented 28 days after boost vaccination for groups receiving either a low dose (LD/LD) or standard dose (SD/SD) of ChAdOx1-nCoV19. Each data point represents an individual with median and interquartile range shown. Values below the limit of detection were assigned a value of 9 (1/2 LOD) for presentation purposes. No significant differences were determined by Mann-Whitney test.

The impact of biological sex on anti-vector immunogenicity was assessed, with the analysis displayed in figure 7.7. Anti-vector neutralisation titres after prime vaccination are shown in figures 7.7A, 7.7B, and 7.7C, representing the 18-55, 56-69, and 70+ age ranges respectively. There were no significant differences in anti-ChAdOx1 immunogenicity between male or female vaccinees in any of these groups. Anti-ChAdOx1 responses after a boost vaccination are shown in figures 7.7D, 7.7E, and 7.7F. Figures 7.7D and 7.7E represent the 18-55 and 56-69 age ranges, where no significant differences were measured in anti-vector neutralisation titres between the male and female groups. Figure 7.7F shows the male and female comparison in the 70+ age range after a prime-boost vaccination regime. A significant difference is measured in adults aged greater than 70, with anti-vector responses induced in female vaccinees being greater than those induced in male vaccinees. The relevance of the signficant difference in response between male and female participants in older adults is unknown, as the difference is only measured in one specific group.

In the 18-55 age group, different intervals between vaccinations were implemented and assessed for their impact on the induction of anti-vector immunity. Anti-ChAdOx1 responses in these groups are displayed in figure 7.8, where a 4 week and an 8 week interval between first and second dose vaccination were compared, with the anti-ChAdOx1 neutralisation titres 28 days after the boost dose displayed. Despite more individuals failing to mount an anti-ChAdOx1 neutralisation response in the 4 week interval group, no significant differences were measured between the groups. These results suggest that the vaccination interval did not have an effect on anti-vector neutralisation responses, though the results would be more conclusive with a larger sample size, covering a wider range of ages and vaccination intervals.

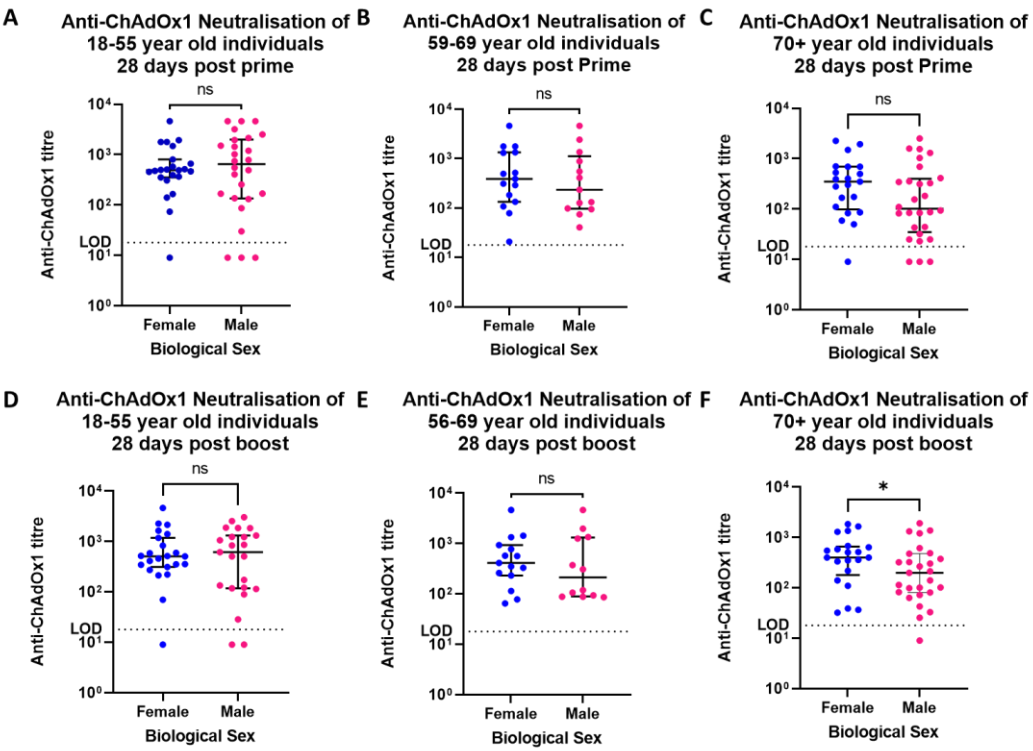


Figure 7.7: Female against Male comparison of anti-ChAdOx1 neutralisation after prime and boost vaccination.

The figure shows the comparison of anti-ChAdOx1 neutralisation IC50 between female and male individuals across a range of age groups. Individuals received a prime-boost of ChAdOx1-nCoV19 4 weeks apart as part of the COV002 clinical trial. (**A**) Anti-ChAdOx1 neutralisation IC50 in the 18-55 age group, separated by biological sex, 28 days post prime vaccination. Individuals are presented as data points, with median and IQR also shown. Values below the limit of detection (LOD) were assigned a value of 9 (1/2LOD) for presentation. No significant differences were determined by Mann-Whitney test. (**B**) Anti-ChAdOx1 neutralisation IC50 in the 56-69 age group, separated by biological sex, 28 days post prime vaccination. Individuals are presented as data points, with median and IQR also shown. No significant differences were determined by Mann-Whitney test. (**C**) Anti-ChAdOx1 neutralisation IC50 in the 70+ age group, separated by biological sex, 28 days post prime vaccination. Individuals are presented as data points, with median and IQR also shown. Values below the LOD were assigned a value of 9 (1/2LOD) for presentation. No significant differences were determined by Mann-Whitney test. (**D**) Anti-ChAdOx1 neutralisation IC50 in the 18-55 age group, separated by biological sex, 28 days post boost vaccination. Individuals are presented as data points, with median and IQR also shown. Values below the LOD were assigned a value of 9 (1/2LOD) for presentation. No significant differences were determined by Mann-Whitney test. (**E**) Anti-ChAdOx1 neutralisation IC50 in the 56-69 age group, separated by biological sex, 28 days post boost vaccination. Individuals are presented as data points, with median and IQR also shown. No significant differences were determined by Mann-Whitney test. (**F**) Anti-ChAdOx1 neutralisation IC50 in the 70+ age group, separated by biological sex, 28 days post boost vaccination. Individuals are presented as data points, with median and IQR also shown. Values below the LOD were assigned a value of 9 (1/2LOD) for presentation. Significant differences were determined by Mann-Whitney test (*p<0.05).

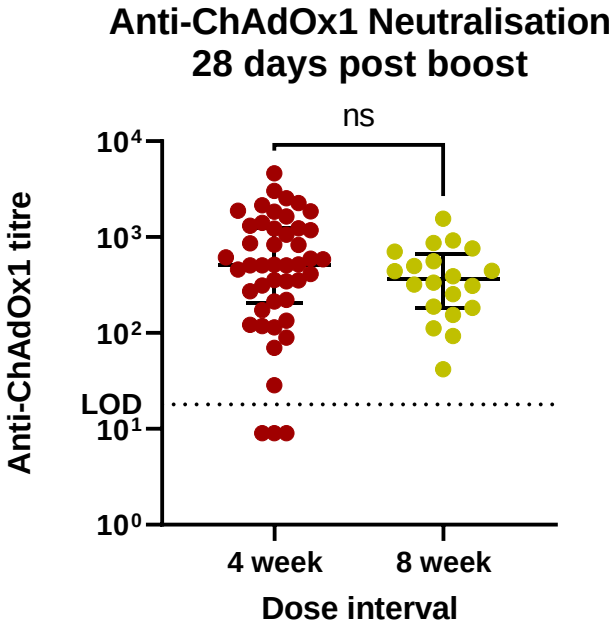


Figure 7.8: Comparison of Anti-ChAdOx1 neutralisation titres between a 4 week and 8 week prime-boost interval.
The anti-ChAdOx1 neutralisation IC50 28 days post boost in the 18-55 age group from the COV001 and COV002 clinical trials. Individuals received a prime-boost of ChAdOx1-nCoV19 either 4 or 8 weeks apart. Each data point represents an individual with median and IQR shown. Values below the limit of detection were assigned a value of 9 (1/2LOD) for presentation. No significant differences were determined by Mann-Whitney test.

7.2.1.3 Correlation of anti-ChAdOx1 neutralisation with anti-SARS-CoV-2 immunogenicity following vaccination with ChAdOx1-nCoV19

Analysis progressed to assessing if anti-vector responses at boost affected immunogenicity against SARS-CoV-2 spike protein after immunisation with ChAdOx1-nCoV19. Results of the aforementioned analysis are displayed in figure 7.9. Figure 7.9A shows IFN-γ ELISPOT responses against SAR-CoV-2 specific peptides. No significant correlations were observed between anti-SARS-CoV-2 cellular responses and anti-ChAdOx1 neutralisation titres. A similar pattern was presented in figure 7.9B, which showed total IgG ELISA responses against SARS-CoV-2 spike protein. Again, there was no significant correlation between the observed total IgG responses and anti-ChAdOx1 neutralisation titres. Finally, neutralisation titres from a SARS-CoV-2 pseudotype VNT were correlated with anti-ChAdOx1 neutralisation titres, the results of which are displayed in figure 7.9C. No significant correlations were observed between anti-SARS-CoV-2 neutralisation titres and anti-ChAdOx1 neutralisation titres.

The results presented throughout show that ChAdOx1 neutralisation titres were low within the tested population, and vaccination with ChAdOx1-nCoV19 induced a response against both SARS-CoV-2 spike and the ChAdOx1 vector. The use of the standard dose vaccine did induce a larger anti-vector response than the low dose, but after a prime-boost vaccination with ChAdox1-nCoV19, very few differences in immunogenicity were observed by age or biological sex. Immediate impacts of anti-vector neutralisation were not observed, with no differences measured in anti-SARS-CoV-2 immunogenicity with high or low neutralisation responses. However, anti-vector immunogenicity is known to effect vaccine immunogenicity, and as such it would be important to measure these effects in the long term. These may be important to the efficacy of booster doses of ChAdOx1-nCoV19 to maintain protective efficacy, or for the efficacy of future ChAdOx1 vectored vaccines.

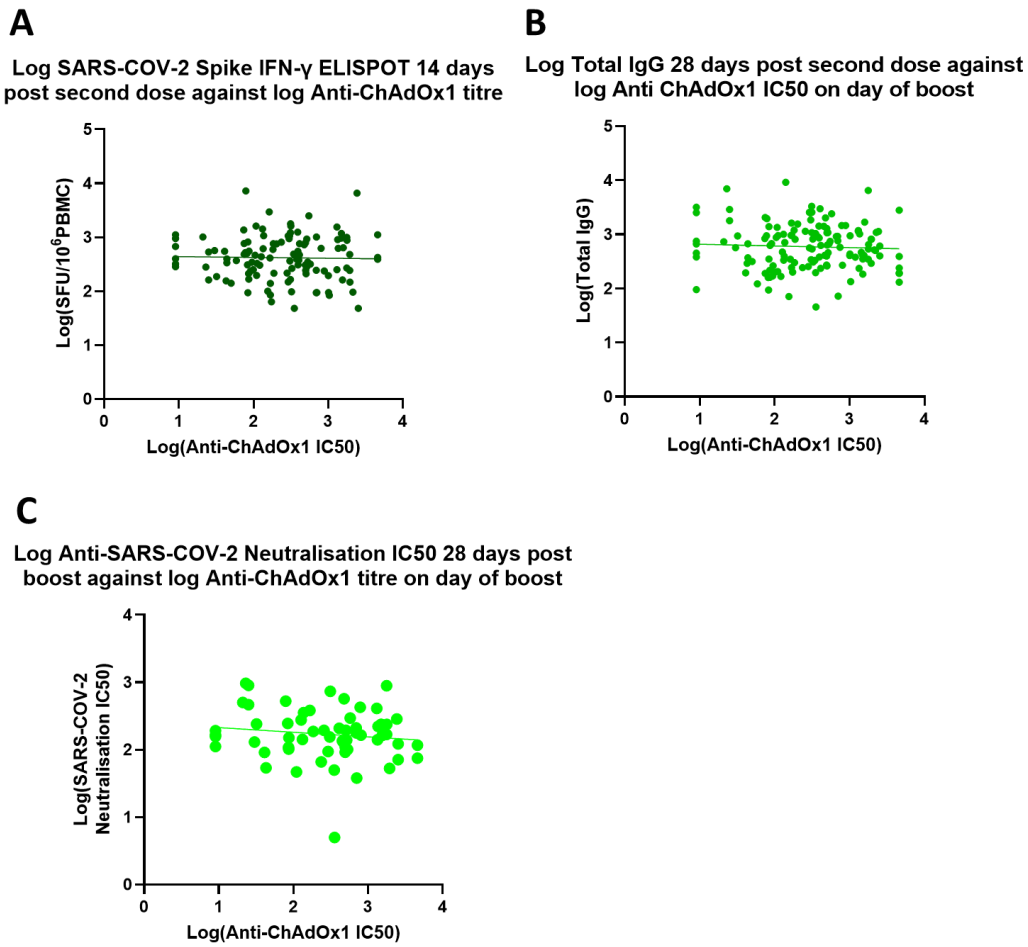


Figure 7.9: Correlations of anti SARS-CoV-2 immunogenicity with anti-ChAdOx1 neutralisation titre at day of second dose.

The figure shows anti SARS-CoV-2 immunogenicity post second dose of ChAdOx1-nCoV19 plotted against the anti-ChAdOx1 response on day of boost from individuals in the COV001 and COV002 trials. (**A**) Log Anti-ChAdOx1 neutralisation titres on day of second dose, plotted against Log anti-SARS-CoV-2 IFN-γ ELISPOT response 14 days post second dose. Each data point represents an individual. Individuals with anti-ChAdOx1 titres below the limit of detection were assigned a value of log(9 (1/2LOD)) for presentation purposes. A simple linear regression was performed producing a line of equation Y = -0.01553*X + 2.657, with R^2=0.00059. The slope was not significantly non-zero with a p-value of 0.7972. (**B**) Log Anti-ChAdOx1 neutralisation titres on day of second dose, against log anti-SARS-CoV-2 spike total IgG ELISA response 28 days post second dose. Each data point represents an individual. Individuals with anti-ChAdOx1 titres below the limit of detection were assigned a value of log(9 (1/2LOD)) for presentation purposes. A simple linear regression was performed producing a line of equation Y = -0.03099*X + 2.847, with R^2=0.002391. The slope was not significantly non-zero with a p-value of 0.5690. (**C**) Log Anti-ChAdOx1 neutralisation titres on day of second dose, against log anti-SARS-CoV-2 spike neutralisation titre in pseudotype VNT 28 days post second dose. Each data point represents an individual. Individuals with anti-ChAdOx1 titres below the limit of detection were assigned a value of log(9 (1/2LOD)) for presentation purposes. A simple linear regression was performed producing a line of equation Y = -0.06846*X + 2.396, with R^2=0.01745. The slope was not significantly non-zero with a p-value of 0.3102.

7.2.2 Assessing the impact of prior ChAdOx1 vaccination on antigen immunogenicity in mouse models

With ChAdOx1-nCoV19 being used across the world as a vaccine to protect against SARS-CoV-2, a proportion of the world's population will no longer be naïve to ChAdOx1. Anti-vector immunity may impact the next generation of ChAdOx vaccines for different antigens, if anti-ChAdOx responses do affect immunogenicity. As such, a series of experiments were set up in mouse models to assess if prior vaccination with a ChAdOx1 vectored vaccine could affect the immunogenicity induced against an antigen in a different ChAdOx1 vectored vaccine.

The first round of experiments measured the effects of a 4 week interval between first and second doses of a ChAdOx1 vectored vaccine, in both balb/c and CD-1 mouse lines. The vaccine used for assessment of immunogenicity was ChAdOx1-sCMV-CCHFV GPC, assessed in chapter 4. First dose vaccinations were performed with ChAdOx1-Zoster, a ChAdOx1 vaccine carrying GPe from *Variacella Zoster (*VZV). GPe is a highly expressed envelope glycoprotein form VZV, used as an antigen in the licenced VZV vaccine Shingrix[354]. ChAdOx1-Zoster has been used as a negative control for ChAdOx1 vaccination in previous assessments of ChAdOx vectored CCHFV vaccines, with no induction of anti-CCHFV cellular or humoral responses, as shown throughout chapters 4 and 5.

Balb/c or CD-1 mice were vaccinated with ChAdOx1-Zoster and following a 4 week interval were vaccinated with ChAdOx1-sCMV-CCHFV GPC. At the same time as the second vaccination, a control group also received ChAdOx1-sCMV-CCHFV GPC, without prior ChAdOx1 vaccination.

The immunogenicity in balb/c mice against the CCHFV GPC antigen following 4 week interval between an irrelevant ChAdOx1 prime vaccination and a boost ChAdOX1-

sCMV-CCHFV GPC vaccination are shown in figure 7.10. Figure 7.10B shows the CCHFV GPC specific IFN-γ ELISPOT responses, where no significant difference was measured. There was an impact on the anti-CCHFV total IgG ELISA responses, displayed in figure 7.10C. In mice primed with ChAdOx1-Zoster the anti-CCHFV Gc humoral responses were significantly lower in mice who had not received a prime dose of ChAdOx1-Zoster.

Following the experiment in balb/c mice, the same experimental regimen was repeated in the outbred CD-1 strain of mice, to determine if the same effects of ChAdOx1 prime vaccination could be measured in a more heterogeneous population. Figure 7.11 presents the immunological results of a 4 week interval between a prime ChAdOx1-Zoster vaccination and booster ChAdOx1-sCMV-CCHFV GPC vaccination, against a single ChAdOx1-sCMV-CCHFV GPC vaccination. IFN-γ ELISPOT results, shown in figure 7.11B, measured no significant difference between the groups, however, the primed groups showed an extremely unexpected distribution of results based on prior responses to vaccination with CCHFV GPC antigens in CD-1, with 6 of the group displaying a low ELISPOT response, with a further two displaying extremely high cellular responses. Anti-CCHFV Gc total IgG ELISA responses, displayed in figure 7.11C, measured no significant difference between groups who had received a ChAdOx1-Zoster prime vaccination, and the group which only received ChAdOx1-sCMV-CCHFV-GPC.

An anti-ChAdOx1 neutralisation assay was developed for use with small quantities of mouse serum, to determine if anti-ChAdOx1 neutralising antibodies may be responsible for the difference measured at the 4 week interval in balb/c mice. However, a single vaccination of ChAdOx1-Zoster did not elicit detectable anti-vector neutralisation responses in balb/c mice.

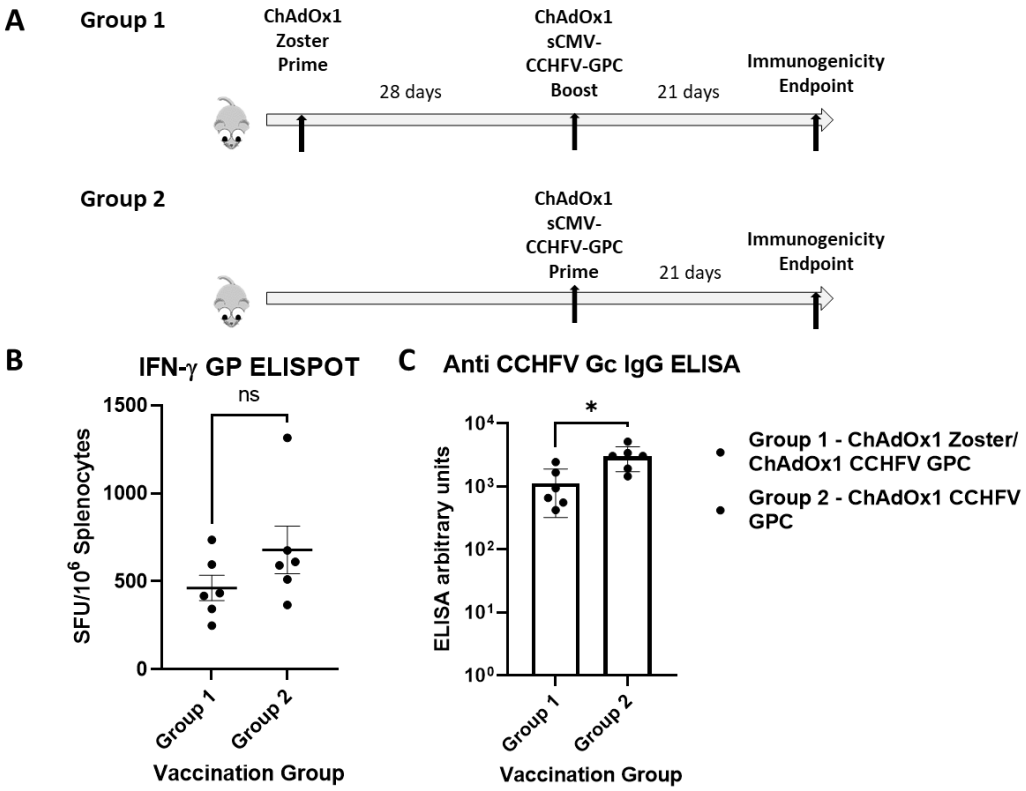


Figure 7.10: Comparison of IFN-γ ELISPOT against peptides specific to CCHFV GP, total IgG ELISA against CCHFV Gc protein between balb/c mice primed with ChAdOx1 4 weeks prior to vaccination or not primed prior to vaccination with ChAdOx1-sCMV-CCHFV GPC.

Vaccination regimens for groups 1 and 2 (**A**). Six week old Balb/c in group 1 were immunised with $1x10^8$ IU of ChAdOx1-Zoster vaccine. Twenty eight days after prime vaccination, mice in both group 1 and group 2 were immunised with $1x10^8$ IU ChAdOx1 CCHFV sCMV GPC. Twenty one days later, mice were culled for immunological analysis. CCHFV GPC antigen specific IFN-γ responses (**B**) in balb/c mice were assayed in mouse splenocytes by IFN-γ ELISPOT. Individual balb/c mice (n=6) are shown here measured in spot forming units per 10^6 splenocytes (SFU/10^6) with mean and SEM also shown. No significant differences were determined by Welch's t-test. Anti CCHFV Gc IgG responses (**C**) in Balb/c mice were measured by IgG ELISA. Individual Balb/c mice (n=6) are shown here in EAU with mean and SEM also shown. Significant differences were determined by Mann-Whitney U test (*$p<0.05$).

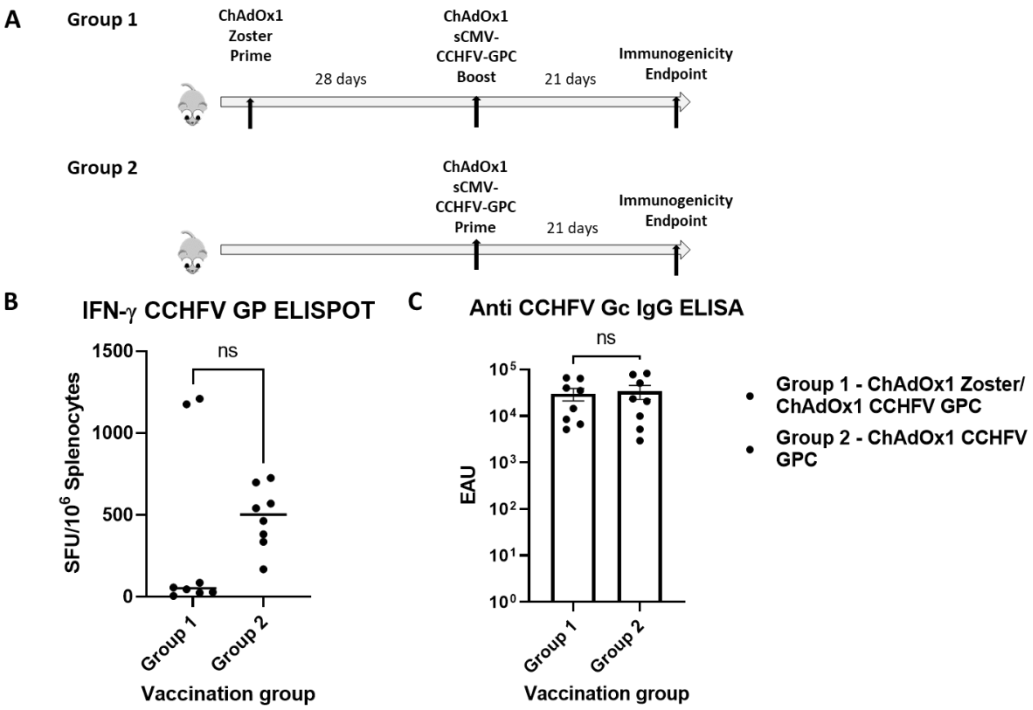


Figure 7.11: Comparison of IFN-γ ELISPOT against peptides specific to CCHFV GP, total IgG ELISA against CCHFV Gc protein between CD-1 mice primed with ChAdOx1 4 weeks prior to vaccination or not primed prior to vaccination with ChAdOx1-sCMV-CCHFV GPC.

Vaccine regimens for groups 1 and 2 (**A**). Nine to eleven week old CD-1 mice in group 1 were immunised with $1x10^8$ IU of ChAdOx1-Zoster vaccine. Twenty eight days after prime vaccination, mice in both group 1 and group 2 were immunised with $1x10^8$ IU ChAdOx1-sCMV-CCHFV GPC. Twenty one days later, mice were culled for immunological analysis. CCHFV GPC antigen specific IFN-γ responses (**B**) in CD-1 mice were assayed in mouse splenocytes by IFN-γ ELISPOT. Individual CD-1 mice (n=8) are shown here measured in spot forming units per 10^6 splenocytes (SFU/10^6) with mean and SEM also shown. No significant differences were determined by Welch's t-test. Anti CCHFV Gc IgG responses (**C**) in CD-1 mice were measured by IgG ELISA. Individual CD-1 mice (n=8) are shown here in EAU with mean and SEM also shown. No significant differences were determined by Mann-Whitney U test.

The effects on vaccine induced immunogenicity were assessed with a 12 week interval between a first dose vaccination with ChAdOx1-Zoster and a second vaccine dose of ChAdOx1-sCMV-CCHFV GPC, in both balb/c and CD-1 mice. CCHFV GPC specific IFN-γ ELISPOT responses are displayed in figure 7.12B. No significant differences were measured between the group primed with ChAdOx1-Zoster and the cellular responses after vaccination with only ChAdOx1-sCMV-CCHFV GPC. Figure 7.12C showed the total IgG ELISA response against CCHFV Gc. No significant differences were measured in anti-CCHFV Gc humoral responses, following vaccination with single ChAdOx1-sCMV-CCHFV GPC, or with a prior prime vaccination of ChAdOx1-Zoster.

Immunogenicity in CD-1 mice was also assayed with a 12 week interval between the ChAdOx1-Zoster prime and ChAdOx1-sCMV-CCHFV GPC immunisation. The cellular and humoral immunogenicity induced by vaccination are displayed in figure 7.13. Figure 7.13B shows anti-CCHFV GPC IFN-γ ELISPOT results, where no significant differences were measured between the groups who received a ChAdOx1-Zoster prime, and the group which only received a ChAdOx1-sCMV-CCHFV-GPC vaccination. Anti-CCHFV Gc total IgG ELISA results are displayed in figure 7.13C, with no significant differences measured, between the primed and non-primed groups. Neutralising responses were detected in the CCHFV tec-VLP pseudotype VNT and are presented in figure 7.13D. Both the primed and non-primed groups produced significant neutralisation, but no significant difference was measured between the two regimens.

Again, an anti-ChAdOx1 neutralisation assay was performed using serum quantities suitable for mouse samples. No anti-ChAdOx1 neutralisation was detected in naïve mice, or those who had received a ChAdOx1-Zoster prime vaccination, 1 day before receipt of ChAdOx1-sCMV-CCHFV GPC.

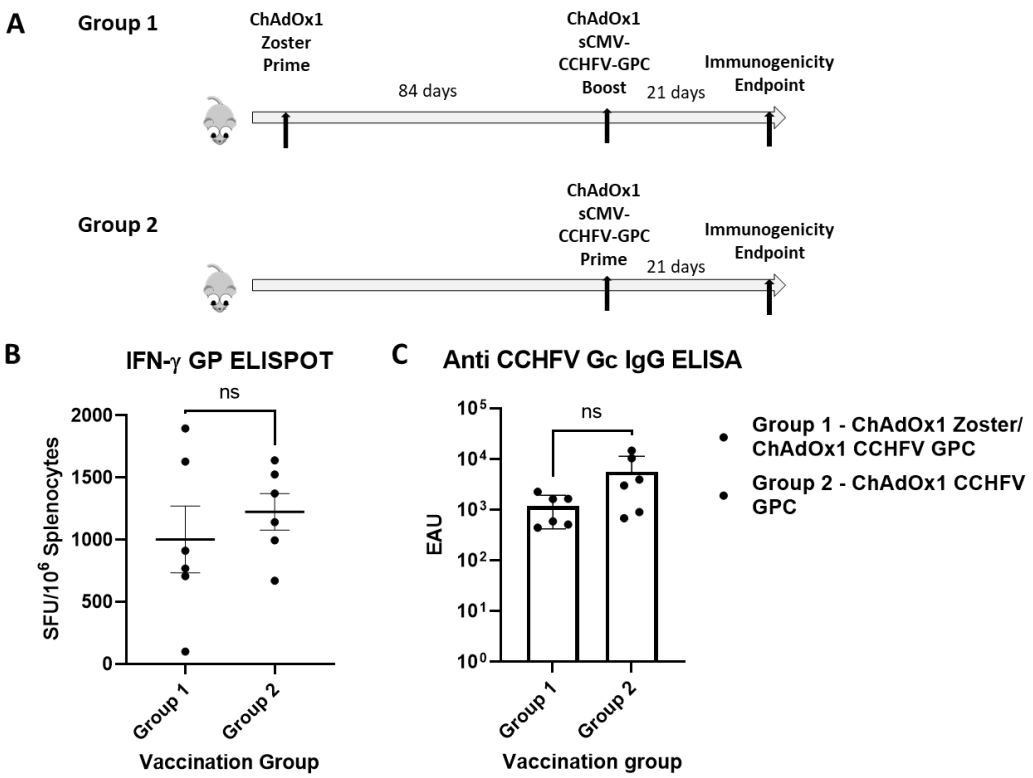


Figure 7.12: Comparison of IFN-γ ELISPOT against peptides specific to CCHFV GP, total IgG ELISA against CCHFV Gc protein between balb/c mice primed with ChAdOx1 12 weeks prior to vaccination or not primed prior to vaccination with ChAdOx1-sCMV-CCHFV GPC.

Vaccine regimens for groups 1 and 2 (**A**). Six week old balb/c in group 1 were immunised with $1x10^8$ IU of ChAdOx1-Zoster vaccine. Eighty four days after prime vaccination, mice in both group 1 and group 2 were immunised with $1x10^8$ IU ChAdOx1-sCMV-CCHFV GPC. Twenty one days later, mice were culled for immunological analysis. CCHFV GPC antigen specific IFN-γ responses (**B**) in balb/c mice were assayed in mouse splenocytes by IFN-γ ELISPOT. Individual balb/c mice (n=6) are shown here measured in spot forming units per 10^6 splenocytes (SFU/10^6) with mean and SEM also shown. No significant differences were determined by Welch's t-test. Anti CCHFV Gc IgG responses (**C**) in balb/c mice were measured by IgG ELISA. Individual balb/c mice (n=6) are shown here in EAU with mean and SEM also shown. No significant differences were determined by Mann-Whitney U test.

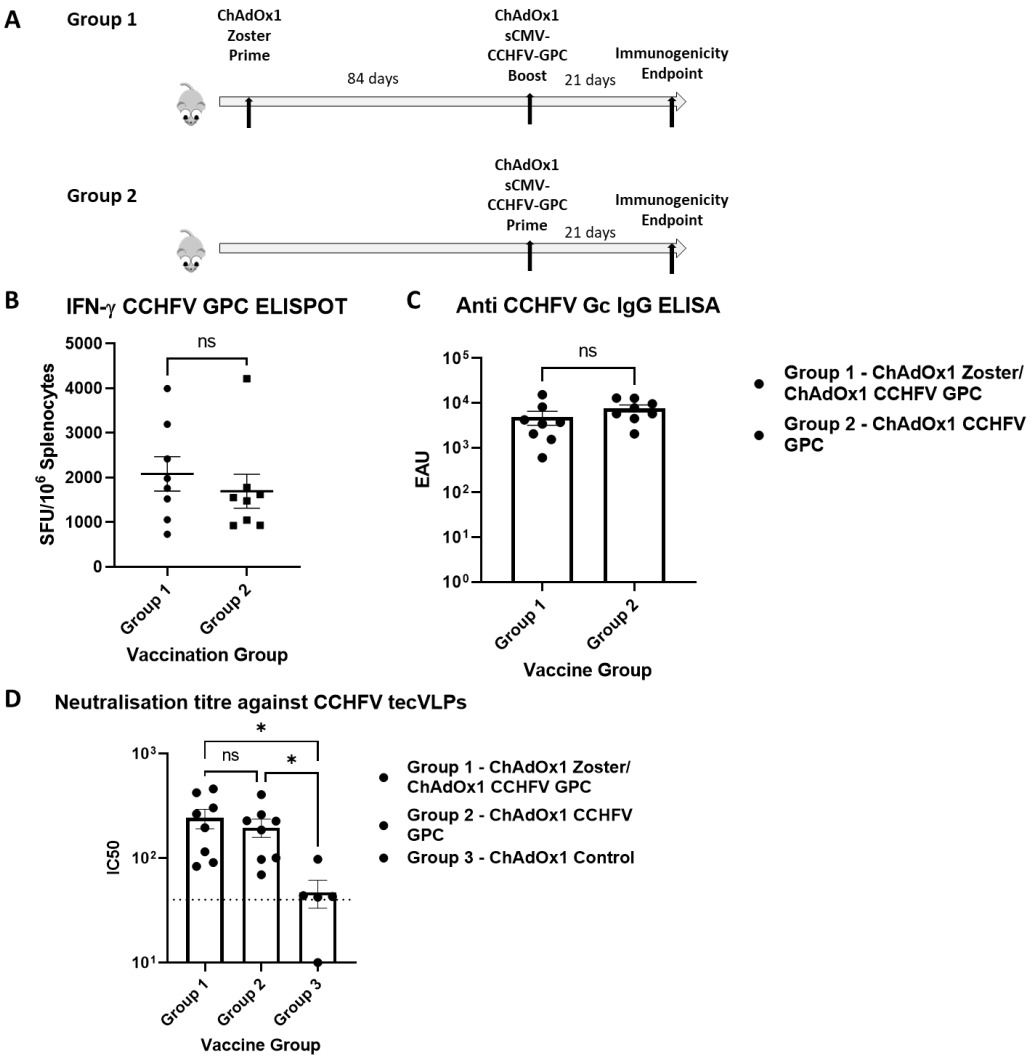


Figure 7.13: Comparison of IFN-γ ELISPOT against peptides specific to CCHFV GP, total IgG ELISA against CCHFV Gc protein, and neutralisation of CCHFV VLPs between CD-1 mice primed with ChAdOx1 4 weeks prior to vaccination or not primed prior to vaccination with ChAdOx1-sCMV-CCHFV GPC.

Vaccine regimens for groups 1 and 2 (**A**). Nine to eleven week old CD-1 mice in group 1 were immunised with $1x10^8$ IU of ChAdOx1-Zoster vaccine. Eighty four days after prime vaccination, mice in both group 1 and group 2 were immunised with $1x10^8$ IU ChAdOx1-sCMV-CCHFV GPC. Twenty one days later, mice were culled for immunological analysis. CCHFV GPC antigen specific IFN-γ responses (**B**) in CD-1 mice were assayed in mouse splenocytes by IFN-γ ELISPOT. Individual CD-1 mice (n=8) are shown here measured in spot forming units per 10^6 splenocytes (SFU/10^6) with mean and SEM also shown. No significant differences were determined by Welch's t-test. Anti CCHFV Gc IgG responses (**C**) in CD-1 mice were measured by IgG ELISA. Individual CD-1 mice (n=8) are shown here in EAU with mean and SEM also shown. No significant differences were determined by Mann-Whitney U test. Neutralising antibody titres (**D**) were determined by virus neutralisation test using CCHFV tec-VLPs. Individual data points (group 1/2 n=8, group 3 n=5) were expressed as IC50 values and shown here as a scatter dot plot with bars showing median and IQR. Values below the limit of detection were assigned an IC50 of 10 for presentation purposes. No significant differences were determined by Kruskal-Wallis test.

7.3 Conclusions

Anti-vector responses from prior exposure through infection can adversely affect the immunogenicity of viral vectored vaccines[213]. With the increased number of individuals exposed to ChAdOx1 following the receipt of ChAdOx1-nCoV19 during COVID-19 pandemic, there is need to assess the anti-ChAdOx1 responses induced by vaccination with a ChAdOx1 vector. As the current pre-existing level of anti-vector responses in the population are low across all age groups, the impacts of anti-ChAdOx1 immunogenicity on future mass vaccination campaigns is unknown.

Pre-existing immunity to adenoviruses can occur through infection with circulating adenoviruses or through vaccination with an adenoviral vectored vaccine. Regardless of the source of pre-existing immunity, anti-vector responses can impact immunogenicity induced against antigens in adenoviral vectored vaccines. It is however important to question whether different magnitudes of anti-vector immunity may be induced by replicating wild-type adenovirus, and replication deficient adenoviral vectors. It is likely that immune responses induced after infection with a replication competent live virus, such as wild-type Ad5, will be greater than those induced by vaccination with a self-limiting replication incompetent adenoviral vector. Therefore, pre-existing immunity induced by infection with circulating adenoviruses would likely have a greater impact on immunogenicity against antigens in viral vectored vaccines, than prior vaccination with an adenoviral vector.

The generation of anti-ChAdOx1 responses after vaccination with ChAdOx1-nCoV19 was assessed with individuals vaccinated as part of the COVID-19 vaccine trials. Almost all individuals vaccinated with ChAdOx1-nCoV19 developed anti-ChAdOx1 neutralising antibodies, and the responses are long lived, with very little reduction six months after prime vaccination. Responses across a range of demographics were assessed and after a two dose regimen, very few differences were measured across age and biological sex of

vaccinees. However, unsurprisingly significantly lower anti-ChAdOx1 neutralising responses were measured in individuals who received a low dose of ChAdOx1-nCoV19.

The relationship between anti-ChAdOx1 neutralising antibodies and anti SARS-CoV-2 spike immunogenicity induced by ChAdOx1-nCoV19 vaccination was investigated. No differences in immunogenicity were measured between individuals across a range of anti-ChAdOx1 neutralisation titres. Whether the same lack of correlation would hold for a larger sample size is unknown, and if there are titres of neutralising antibodies that may affect future vaccination regimens requires further investigation.

Work with clinical samples from the COVID-19 vaccine trials focused on the impact of homologous two dose vaccination with ChAdOx1-nCoV19 on anti-ChAdOx1 neutralisation titres. Experiments were designed in mouse immunology models to instead assess the impact of a prior vaccination with a ChAdOx1 vectored vaccine on immunogenicity induced by further heterologous ChAdOx1 vaccinations. In balb/c mice a ChAdOx1 vaccination 4 weeks prior to a further vaccination induced reduced anti-CCHFV Gc humoral responses, but no further significant differences were measured with a longer interval in balb/c mice, or with CD-1 mice across two interval regimens. The immunogenicity results determined in balb/c mice do correlate with efficacy results measured in humans who received two doses of ChAdOx1-nCoV19 at different intervals.

No significant differences in anti-ChAdOx1 neutralisation titres were detected between interval lengths either in mice or in human samples, but differences were measured in immunogenicity against the vaccine antigen or efficacy of vaccine protection against hospitalisation. With no differences in measured anti-ChAdOx1 neutralisation, differences in vaccine efficacy are therefore likely to be mediated by other anti-vector effects, likely cellular based anti-ChAdOx1 responses which were not measured. As such it may be important in future assessment of ChAdOx1 vectors to consider the underlying cellular responses in the population, and their response to vaccine. Through the suggested

experiments a correlative or causative relationship could be determined between anti-vector cellular responses, and immunogenicity against the target antigen.

It will be important to determine if the effects measured on immunogenicity will have an impact on future vaccination regimes using the ChAdOx vectored vaccines against CCHFV and NSDV, developed and presented in chapter 4-6 of this thesis. ChAdOx1 NSDV vaccines, if used, are likely to be deployed only in sheep and goats. At present no ChAdOx1 vaccines are licenced as veterinary vaccines and as such the effect of ChAdOx1 responses across multiple heterologous vaccinations warrants further investigation.

Through vaccination campaigns a significant proportion of the global population have anti-ChAdOx1 immune responses due to vaccination with ChAdOx1-nCoV19, which must be taken into account with future vaccination regimes. The induction of immunogenicity with ChAdOx1 vaccination, may instead guide what pathogens ChAdOx1 is used for. While it is likely based on current evidence that the initial annual SARS-CoV-2 booster doses will not have an effect on immunogenicity, the effects of regular boosting of anti-ChAdOx1 responses over multiple years will need to be further elucidated, though improvements in antigen design and work towards universal influenza coronavirus vaccines may reduce the need for repeated boosters.

Where ChAdOx1 vaccines may be most effective is in the circumstances presented during the COVID-19 pandemic, in responding to outbreak pathogens, due to the rapid production timelines and relatively cheap manufacture of ChAdOx1 vectored vaccines. The need for outbreak pathogen vaccines will likely be irregular and widely spaced both geographically and temporally, and which would therefore lead to little concern for the impact of anti-vector responses on vaccine efficacy during these outbreaks. The capacity to develop ChAdOx vaccines quickly and effectively, and to produce doses on a large scale makes them ideal for protection against epidemics or pandemics.

8. Conclusions and future directions

Development of vaccine against emerging diseases is a priority for global health, to prevent epidemics and pandemics. The impact of nairoviruses on human and animal health has been neglected, despite their widespread impact. Due to the ability of vaccines to protect against disease, development of human and veterinary vaccines for CCHFV and NSDV is a priority for a One Health approach to protecting human and animal health.

There is currently a human vaccine against CCHFV, though it has limited licensure, availability, and scalability, and a lack of evidence as to its protective efficacy[248]. There is no clear leading candidate for a worldwide CCHFV vaccine, though DNA, mRNA and viral vectored vaccines have all been demonstrated to be immunogenic and protective in animal challenge models with limited vaccines progressing into clinical trials. Crucially, mRNA and rVSV platforms have both previously been shown to be protective in mouse models after a single vaccination dose[256,324]. This thesis starts from the completed challenge study establishing that ChAdOx2 vectored CCHFV GPC can also induce protection from lethal CCHFV challenge in immunocompromised mice after a single vaccination.

However, ChAdOx2-CCHFV GPC was genetically unstable, encoding the large CCHFV GPC antigen. The focus was therefore on the development of second generation vaccines encoding smaller nairovirus antigens for use in ChAdOx vectored vaccines. These antigens were designed to express the CCHFV GPC subunits CCHFV Gn and CCHFV Gc, and CCHFV NP. It was demonstrated that these CCHFV antigens could induce both cellular and humoral immune responses in ChAdOx vectors, equivalent to the immunogenicity induced by CCHFV GPC. It was further demonstrated that multiple small novel CCHFV antigens could be incorporated into the same ChAdOx1 vector, without observing interference. Multivalent ChAdOx1 CCHFV vaccines were

demonstrated to induce robust cellular and humoral immunogenicity after vaccination in mice. The vaccine-induced immunogenicity was equivalent to that induced by the protective ChAdOx2 CCHFV GPC vaccine, suggesting that multivalent ChAdOx1 vectored CCHFV vaccines may induce protection from lethal CCHFV challenge in mice.

Informed by ChAdOx vectored CCHFV vaccine design, vaccines were designed to immunise against NSDV. This thesis demonstrated that the NSDV antigen NSDV GPC, NSDV Gc, and NSDV NP, were immunogenic, with NSDV GPC and NSDV Gc determined to have induced both cellular and humoral immunogenicity.

An issue in the field of nairovirus virology is the need for high-containment facilities to work with live virus. In this thesis, the utility of two pseudotyping systems for use with CCHFV and NSDV was demonstrated. These assays were used to assess neutralising antibodies induced by ChAdOx vectored vaccines, and their use could be expanded to further interrogate the function of CCHFV and NSDV GPCs.

Anti-vector immunity is a concern for the use of adenoviruses as repeat vaccines in vaccination schemes. Due to the now widespread usage of ChAdOx1 vectors globally it is important to determine how anti-vector immunity will impact immune response to vaccine encoded antigens. This thesis demonstrates that anti-ChAdOx1 neutralisation responses are low in individuals not previously vaccinated, but increased in those vaccinated with ChAdOx1-nCoV19. Anti-ChAdOx1 neutralisation titres were not correlated with anti-SARS-CoV-2 immunogenicity induced by ChAdOx1-nCoV19 vaccination, and a few significant differences in anti-ChAdOx1 neutralisation titres when stratified by a range of demographics. The lack of correlation measured with vaccine antigen immunogenicity will have a broad impact on the further use of ChAdOx vectors for vaccines beyond SARS-CoV-2. Further experimentation in mouse models demonstrated prior ChAdOx1 vaccination had no long term effects on immunogenicity induced by ChAdOx1 mouse models.

8.1 Conclusions

8.1.1 Pseudotyping of nairovirus glycoproteins

The purpose of nairovirus pseudotype experimentation was to develop assays to assess nairovirus functionality in BSL-2 containment facilities, a goal which was achieved. A VSV based system was used to pseudotype NSDV GPC. This is the first recorded example of NSDV being successfully pseudotyped and such a system could be used to interrogate NSDV GPC functionality, in a cheaper assay and lower containment facility than with live NSDV virus.

Pseudotyping of CCHFV GPC was successful in tec-VLP particle, used on multiple occasions throughout the literature, for determination of CCHFV GPC function and pharmaceutical or vaccine efficacy. Though previously used and established, it was important to determine that such assays were reproducible and could be used throughout the field.

Work within this thesis could not replicate the previously established work by Suda et al, on producing a VSV pseudotyped CCHFV[288], the reasons for which were unclear. However, this thesis was able to re-inforce Suda et al's work on truncation of nairovirus glycoproteins, as development of an NSDV pseudotyping assay showed that NSDV GPC with the del10 truncation incorporated more efficiently into VSV pseudoparticles than WT NSDV-VSV.

8.1.2 Nairovirus based virus neutralisation test development

Following the development of pseudotype systems with could incorporate CCHFV and GPC into replication deficient virions, virus neutralisation tests were established for both glycoproteins. An NSDV-VSV based VNT was demonstrated, and was able to assess the induction of neutralising antibodies by vaccination with ChAdOx vectored NSDV

antigens. The insight into vaccine induced immunogenicity will inform and improve the further development of ChAdOx vectored NSDV vaccines, and future vaccination regimens using ChAdOx vectored NSDV antigens.

The use of CCHFV tec-VLPs was further demonstrated in a VNT for detecting neutralising antibodies induced by ChAdOx vectored vaccination with CCHFV antigens. The assay was shown to detect neutralising antibodies from ChAdOx vectored GPC vaccines, though the assay was not sensitive enough to detect antibodies in all mice after a single immunisation. The assay was able to detect consistent neutralising titres from mice receiving two doses of a ChAdOx vectored CCHFV vaccine.

8.1.3 Immunogenicity of monovalent ChAdOx vectored CCHFV vaccines

From earlier immunogenicity and challenge experiments, it was demonstrated that CCHFV GPC was immunogenic in ChAdOx vectors and protective against lethal CCHFV challenge in mice in the ChAdOx2 vector. Due to the established instability of ChAdOx2-CCHFV GPC likely due to exceeding the capacity of ChAdOx vectors, vaccines expressing small CCHFV antigens Gn, Gc and NP were developed. In ChAdOx vectors, CCHFV Gn and Gc antigens it was established that equivalent cellular and humoral immunogenicity was induced following vaccination with CCHFV GPC as an antigen. ChAdOx vectors encoding CCHFV Gn and Gc antigens were not able to generate equivalent neutralising antibody titres, in a balb/c mouse immunogenicity model. ChAdOx vectored CCHFV NP induced humoral and cellular responses after vaccination, though it is unknown if the responses would generate protection from CCHFV.

ChAdOx vectors were further developed with CCHFV GPC antigens, under the control of different CMV derived promoters, to reduce the size of the antigen expression cassette. It was demonstrated that CCHFV GPC expressed with the short CMV promoter induced significantly lower humoral responses, though it is unknown if the reduced humoral responses would reduce protective efficacy against CCHFV challenge.

8.1.4 Immunogenicity of multivalent ChAdOx vectored CCHFV vaccines

To improve breadth of anti-CCHFV responses in a vaccine, CCHFV Gn, CCHFV Gc and CCHFV NP antigens were combined in a single ChAdOx1 vector. The ChAdOx1 Bivalent CCHFV and ChAdOx1 Trivalent CCHFV vaccines showed no evidence of antigenic interference, or reduction in anti CCHFV Gn and Gc immunogenicity compared to their monovalent counterparts. Further multivalent ChAdOx vectored vaccines were demonstrated to induce greater cellular responses than ChAdOx2 vectored CCHFV GPC antigens, and robust humoral responses. However, the ChAdOx1 trivalent CCHFV vaccine was partially unsuccessful, failing to induce anti-CCHFV NP cellular or humoral immunogenicity.

The immunogenicity results raise the potential that multivalent CCHFV vaccines may be genetically stable and can induce a protective immune response against CCHFV, though protection remains to be confirmed by CCHFV challenge.

8.1.5 Immunogenicity of ChAdOx vectored NSDV vaccines

With very little published research on NSDV vaccines the work presented in this thesis represents a significant step forward in vaccine development against NSDV. Vaccines

against NSDV were developed in ChAdOx1 vectors with four NSDV antigens; NSDV GPC, NSDV Gn, NSDV Gc, and NSDV NP. NSDV Gn failed to induce cellular or humoral responses, however, NSDV GPC and NSDV Gc both induced robust cellular and humoral responses, and were able to neutralise pseudoparticles bearing NSDV GPC. ChAdOx1 NSDV NP was demonstrated to induce extremely high cellular responses.

The correlates of protection for NSDV are unknown, and it is therefore unknown whether the responses induced by ChAdOx1 vectored NSDV GPC, Gc or NP, will be protective against lethal disease. Further work will require confirmation with NSDV challenge, however, the induction of both cellular and humoral immunogenicity after vaccination is encouraging.

8.1.6 Anti-vector Immunity to ChAdOx1-nCoV19

Non-human adenovirus vaccine platforms were developed to evade the effects of anti-vector immunogenicity, which reduced immunogenicity induced by vaccine antigens encoded in human adenovirus vectors. The widespread usage of ChAdOx1 vaccines as part of global vaccination schemes against SARS-CoV-2, prompts the need to investigate if anti-ChAdox1 responses induced during human vaccination will impact vaccine antigen immunogenicity. In this thesis it was demonstrated that ChAdOx1-nCoV19 vaccination induced anti-ChAdOx1 neutralising antibodies. The titres of these antibodies were not significantly boosted by a second dose of ChAdOx1-nCoV19, and were maintained out to 6 months post prime vaccination. No correlations were demonstrated between age of vaccinees and their anti-ChAdOx1 titres after two doses of ChAdOx1-nCoV19. Significant differences in anti-ChAdOx1 neutralisation titres were measured dependent on biological sex in only vaccinees 70 years of age or older. Vaccine dose dependent effects were demonstrated after two doses of ChAdOx1-nCoV19 in 18-55 year

olds and 56-69 year olds. However, no correlations were observed between anti-ChAdOx1 titres and the anti-SARS-CoV-2 immunogenicity induced by two doses of ChAdOx1-nCoV19.

Further limited mouse experiments attempted to determine the impact of a prior ChAdOx1 vaccination on immunogenicity using a ChAdOx1-sCMV-CCHFV GPC vaccine. A significant reduction in humoral immunogenicity was observed in inbred mice with a ChAdOx1 vaccination 4 weeks prior to ChAdOx1-sCMV-CCHFV GPC vaccination, however, these effects were not measured with a 12 week vaccination interval, and no significant differences were measured in outbred mice. Combined these results suggest the impacts of anti-ChAdOx1 immunogenicity will not affect future immunogenicity to ChAdOx1 vectored vaccines, however, the long term impact will require further investigation.

8.1.7 Conclusions on ChAdOx vectored nairovirus vaccines

To positively impact on human and animal health, the ChAdOx vectored nairovirus vaccines developed as part of this thesis will need to be shown to be effective at protecting against nairovirus induced disease. The results within this thesis suggest that CCHFV Gc, and CCHFV Gn antigens will be able to replicate immunogenicity induced by the CCHFV GPC antigen, and the vaccine ChAdOx1 Bivalent CCHFV induced superior cellular responses, and robust anti-Gc humoral responses, though significantly lower than those induced following vaccination with the known protective vaccine ChAdOx2-CCHFV GPC. Whilst no correlates of protection are known for NSDV, the induction of NSDV-specific cellular and humoral immunogenicity is a positive sign, prior to any challenge studies being established.

Having produced immunogenic vaccines, it is also important to discuss how they may be used in practice. Veterinary vaccines can be used to improve both animal and human health. Many vaccines have been used successfully in domestic animals and livestock to improve animal health, with examples such as rinderpest. Upon introduction into Africa through movement of infected animals from an endemic area to a non-endemic one, rinderpest devastated livestock and wild ungulate populations for approximately 70 years, but through vaccination rinderpest was eliminated in the wild. ChAdOx1 vectored NSDV vaccines may be optimally applied to vaccinate animals moving between endemic and non-endemic areas or vice versa, and to reduce circulation within endemic areas.

Veterinary vaccines have been used to manage zoonotic pathogens such as rabies, preventing infections in both humans and domestic animals. The recently assessed ChAdOx1 RVFV vaccine also provides the same role, as a vaccine designed to protect both humans and livestock from RVFV. These examples have relevance to the use of CCHFV vaccines, as vaccination in livestock will likely reduce the risk to humans, through reducing circulation in tick vectors, and the risk of transmission through infected bodily fluids. Such a regimen would likely be more effective at preventing outbreaks than reactive vaccinations to control CCHFV outbreaks. However, vaccination against CCHFV does not benefit animal health, as CCFHV does not cause symptomatic disease within livestock populations. The lack of direct protection is in contrast to administration of vaccinations such as ChAdOx1 RVF, in which vaccination would directly protect livestock from a high mortality pathogen. A closer parallel is the recently assessed ChAdOx1 MERS vaccine. MERS has a camel animal reservoir and can cause severe disease in humans. Camel vaccination with ChAdOx1 MERS has been shown to reduce shedding of virus and reduce transmission of virus to uninfected animals[355], which would likely protect human health and decrease transmission of MERS from animals to humans. However, without direct benefit to animal health, the case for veterinary vaccination to

reduce CCHFV and MERS transmission may require further engagement with stakeholders, economic incentives, and larger campaign to improve pathogen awareness, reduce circulation and transmission, and protect human lives.

8.2 Future Directions

8.2.1 Improving pseudotype virus neutralisation tests for nairovirus glycoproteins

The development of successful pseudotyping systems for CCHFV and NSDV GPCs provide the capacity to extend the understanding of the function of these proteins, both inside and outside vaccinology. The ability to pseudotype nairovirus GPCs allows the assessment neutralising antibodies induced by vaccination. The CCHFV tec-VLP system used to pseudotype CCHFV GPC uses a lab passaged strain of CCHFV from Nigeria. However, the system has been shown to be able to pseudotype a range of different CCHFV GPCs derived across CCHFV's endemic range[273]. With CCHFV having divergent GPCs, the ability for neutralising antibodies to neutralise different strains of the disease will be important in vaccine development and the standardisation of a number of VNTs to assess cross-strain neutralisation will be important to select further vaccine candidates.

NSDV has two divergent enclaves in NSDV and GJNV[135]. The GPCs show ~85% homology on an amino acid level[356]. As such it will be important to assess whether GJNV GPCs can be pseudotyped into a VSV based pseudotyping system, and if cross-reactive neutralising antibodies are generated by ChAdOx vectored NSDV vaccines.

Due to the dangers of working with live CCHFV and NSDV viruses, very little is known about the function of their glycoproteins. With CCHFV some functions have been elucidated through genetic knockout of GPC subunits[89], and recently structure of CCHFV

Gc[333], findings which taken together can help improve understanding of CCHFV GPC functionality. However, almost nothing is known of NSDV GPC function, and there are many further discoveries needed to fully understand CCHFV GPC structure and function. In particular, the cellular receptors for CCHFV and NSDV are unknown at present. Pseudotyped viruses, such as those discussed in this thesis could be used in future, in low containment facilities, to elucidate cellular receptors for nairovirus, improving understanding on the variable tropism of these closely related pathogens.

8.2.2 Future Nairovirus antigen development for ChAdOx vectors

The design of novel small antigens for CCHFV and NSDV vaccines was generally successful. For CCHFV, Gn, Gc, and NP antigens encoded in ChAdOx vectors induced both cellular and humoral immunogenicity following vaccination. For NSDV, ChAdOx1-NSDV Gc vaccination induced cellular and humoral immunogenicity, and vaccines expressing NSDV NP could only be assayed for cellular immunogenicity, but showed a robust response. In addition, multivalent CCHFV vaccines were developed with no evidence of antigenic interference between antigens and a greater breadth of response.

The first vaccine improvement in future work would involve redesign of the ChAdOx1-NSDV Gn and ChAdOx1 trivalent CCHFV vaccines. Immunogenicity experiments failed to detect any evidence of Gn expression from ChAdOx1-NSDV Gn, and further redesign and investigation may be needed to produce a vaccine which can express NSDV Gn as an immunogenic antigen. The ChAdOx1 Trivalent CCHFV vaccine strongly expressed two antigens which generated cellular and humoral immunogenicity against CCHFV Gn and CCHFV Gc antigens. However, the antigen Gn2ANP antigen construct did not induce immunogenic responses against CCHFV NP. Antigens separated by 2A linker sequences have been shown to be immunogenic in previous ChAdOx1 vectors[224], and as such the

approach is likely to work again. Determining expression of the CCHFV NP sequence from a new Gn2ANP construct will be crucial to producing a fully immunogenic ChAdOx1 trivalent CCHFV vaccine.

During the work in this thesis establishing which antigens to select for ChAdOx vectored vaccines, CCHFV Gn and Gc were selected, due to their predicted function as a heterodimer on the envelope surface, and their comparatively greater levels of conservation compared to other parts of the GPC. Nairovirus Gn and Gc can be immunogenic antigens in ChAdOx1, and CCHFV Gn and Gc have been established as protective antigens in other vaccine platforms following challenge studies. However, recently the GP38 subunit of nairovirus GPCs has been shown to play a larger role in function than was previously thought. Importantly it has been shown that neutralising antibodies targeting CCHFV GP38 are protective against CCHFV challenge[90], and GP38 was necessary for GPC induced protection against lethal CCHFV challenge in a DNA vaccine vector[320]. These data taken together suggest that future research should assess GP38 as a suitable nairovirus antigen in ChAdOx vectored vaccines.

8.2.3 Further understanding immune responses induced by ChAdOx vectored CCHFV vaccines

There is a need to further understand the correlates of protection required to protect organisms from lethal CCHFV infection, to aid in vaccine design. Correlates of protection induced by vaccination are poorly described and data is principally available from challenge studies in immunocompromised mouse models, and limited NHP models. As many vaccines tested in immunocompromised mouse models either show 100% protection or no evidence of protection, it is difficult to delineate which immune

responses are necessary for protection particularly with GPC based vaccines which often induce both cellular and humoral immunogenicity.

Within the scope and timelines of this thesis it was not possible to fully interrogate the immune responses induced by ChAdOx vectored CCHFV vaccinations. In particular, it would be important to investigate the generation of tissues resident memory cells and the ability to reside or enter the liver. CCHFV is a liver centric disease, and previous CCHFV vaccination regimes in mice have measured liver-specific immune responses[324]. ChAdOx1 is known to be a liver-tropic vaccine, and vaccination regimens have been designed using ChAdOx1 to specifically enhance the development of tissue resident memory cells in the liver, for use in malaria vaccines[357]. Therefore, it will be important to assess if ChAdOx vectored CCHFV vaccines can induce liver-specific immune responses after vaccination, and in whether these responses result in the development of tissues resident memory cells. It will also be important to assess if liver specific memory responses induced by vaccination can enhance protection from CCHFV infection and from severe CCHF.

8.2.4 Prime-Boost vaccination regimens for enhancing nairovirus immunogenicity

At the commencement of this thesis, consensus towards the use of ChAdOx vectored vaccines focused on their use as single dose vaccinations. Clinical trial data has shown that ChAdOx vectored vaccines can induce protection from severe disease and hospitalisation after a single dose[204], but also show that the use of two doses induces greater immunogenicity and protection against disease[228], including disease caused by variant viruses. As such, it will be important to consider ChAdOx vectored nairovirus

vaccines in future as two dose vaccines, something not considered within this thesis, but which could enhance the immunogenicity of ChAdOx vectored nairovirus vaccines.

The use of prime boost regimens for ChAdOx vectored nairovirus vaccines raises questions related to design of regimens, specifically related to the preference of inducing a broad antigenic response or to specifically boost responses to a single specific antigen. Regimens with ChAdOx vectored vaccines could look to continuously boost GPC responses, an antigen known to induce protective responses. However, alternative approaches to generating GPC specific responses with a boost vaccine could include CCHFV antigens from heterologous strains, improving the breadth of protection against different CCHFV strains. Alternatively, prime vaccination with a ChAdOx vectored GPC antigen would induce a broad cellular and humoral response against GPC, which could be boosted with a vaccine such as ChAdOx1 Bivalent CCHFV, focusing the immune response to vaccination onto the Gn and Gc antigens found on the virion surface. Such an approach would need to be studied to determine if targeted or focused immunogenicity would be an improvement from a prime-boost regimen with a homologous antigen.

In early work with ChAd vaccines, heterologous vaccinations using multiple viral vectors were common, such as the licensed ChAd-MVA boost regime for protection against Ebola haemorrhagic fever[203]. However, in response to outbreaks with a novel pathogen, the need to assess safety and immunogenicity for two separate vaccines can represent a bottle neck for the use of heterologous regimens during outbreaks settings. Concordant to this, most vaccine regimens tested in clinical trials during the COVID-19 pandemic have utilised homologous boosts[198,204,358], a trend which has changed with the administration of mRNA third doses after initial adenovirus vaccination in the UK and US. Recent research in mice has suggested that the use of ChAdOx vectored vaccines in heterologous vaccination regimens can generate greater immunogenicity than either vaccine given as a homologous prime-boost[359]. The use of heterologous doses for vaccination against SARS-

CoV-2 had been measured in human clinical trials and showed heterologous vaccination with ChAdOx1 and mRNA vaccine dose can increase vaccine immunogenicity[360,361]. For known emerging pathogens, such as CCHFV, prime-boost vaccine regimens can be tested prior to outbreaks and the immunogenicity determined. CCHFV antigens have been incorporated into both the ChAdOx1 and ChAdOx2 vectors. In developing prime-boost regimens for nairovirus vaccination, it will be important to assess if heterologous prime-boosts with two different ChAdOx vectors can enhance immunogenicity, compared to homologous ChAdOx prime-boost regimens. An additional consideration for heterologous prime-boost regimens requiring multiple different vaccines with different vaccine antigens, is the subsequent increase in manufacturing costs, due to the need to generate multiple vaccines for the vaccination regimen.

8.2.5 The future of ChAdOx vectors in vaccinations

ChAdOx vectors have proven to be an invaluable vaccine platform for managing the COVID-19 pandemic, with the production of over 2 billion doses, delivered as a low cost vaccine globally available, with the majority of doses being used in LMICs[231]. However, the future of ChAdOx vaccines as vectors for human and veterinary vaccines is far from certain.

ChAdOx vectors have received comparatively negative publicity throughout the pandemic, against the received response to mRNA vaccines. Partially negative publicity stems from confusing initial results which showed markedly lower efficacy than mRNA SARS-CoV-2 vaccines. Despite the issues with initial reporting, in population level studies following licensure ChAdOx1-nCoV19/AZD1222 was shown to be either more or equally effective as the BNT162b vaccine, at preventing symptomatic disease and hospitalisation, in a range of demographics[362].

Of greater concern for the future use of ChAdOx vectors was the extremely rare blood clot related serious adverse events correlated with the receipt of ChAdOx1-nCoV19/AZD1222[363]. Though the rates of blood clot events were significantly lower than some other nominative medicines, such as hormone based contraceptive pills, such reactions are still of concern. Rates of abnormal blot clots were extremely low within the population, with an additional 66 events of venous thromboembolisms recorded per 10 million people receiving ChAdOX1-nCoV19 in the UK population[363]. Importantly thrombotic events were many times more likely in unvaccinated individuals who were infected with SARS-CoV-2. These occurrence of extremely rare events would have been impossible to spot in the small sizes of animal research and even with late stage clinical trials. More recent data has suggested that very rare thrombotic events have not been detected in all geographical areas, with lower rates of thrombotic events observed in LMICs following vaccination with ChAdOx1-nCoV19[364]. This encouraging data suggests ChAdOx1 should continue to be developed as a platform to protect against emerging pathogens, and that the extremely low risks associated with vaccination appear to be even lower in the communities who could most benefit from emerging pathogen vaccines.

Promisingly for use of ChAdOx1 in future vaccination regimens, mechanistic reasons that may facilitate the development of very rare thrombotic events following vaccination have been suggested. Namely, ChAdOx1 capsid hexons appear to interact with clotting cascade protein PF4, which may potentially disrupt the blood clotting cascade, and in very rare cases initiate thrombosis and thrombocytopenia[365]. With the potential causative factor being determined, ChAdOx capsids can be modified to alter the electrostatic environment on the capsid surface, abolishing interaction between PF4 and the capsid. In addition, medical treatment can be issued following vaccination to prevent the development of thrombotic events, or to rapidly manage extremely rare cases if they occur.

By reducing the very low risks of ChAdOx vaccines as a platform, development of ChAdOx vaccines can continue as a safer biological technology. An improved safety profile, along with the high efficacy levels shown by ChAdOx1-nCoV19/AZD1222 during the COVID-19 pandemic make ChAdOx vectored vaccines a positive future technology for vaccination schemes against epidemic and pandemic level emerging pathogens.

8.3 Final remarks

The work compiled in this thesis describes the development of ChAdOx vectored vaccines against CCHFV and NSDV, using novel nairovirus antigens. The thesis also includes the development of new immunological assays for assessing vaccine immunogenicity, and assessment of the impact of widespread ChAdOx1 use on anti-vector neutralisation within the population. The research presented shows that ChAdOx vectored nairovirus antigens can induce cellular and humoral responses in mice after a single vaccination. CCHFV antigens Gn and Gc could be administered in a single ChAdOx1 Bivalent CCHFV vaccine, which was able to induce comparable immunogenicity following immunisation to ChAdOx2-CCHFV GPC, which was demonstrated to be protective from CCHFV in small animal challenge models. This work has produced new vaccines which can be pursued first into animal challenge trials to assess protective efficacy, and further into phase I clinical trials to protect human health.

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Appendices

Appendix 1 – DNA Sequences

Appendix 1.1 – CCHFV DNA sequences

CCHFV Gn

ACAGCCTCCGGTTAAGCTTATGCACATCAGCCTGATGTACGCCATCCTGTGCCTGCAGCTGTGCG

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CCHFV Gn-2A-NP

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Appendix 1.2 – NSDV DNA sequences

NSDV GPC

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NSDV Gc

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NSDV NP

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NSDV GPC Del10

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Appendix 1.3 – CCHFV GPC DNA sequences

Original CCHFV GPC sequence

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GGATGAGCTGGGACGGCTGCGACCTGGACTACTACTGCAACATGGGCGACTGGCCCAGCTGCA

CCTACACCGGCGTGACCCAGCACAACCACGCCAGCTTCGTGAACCTGCTGAACATCGAGACCGA

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A

Recoded CCHFV GPC

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ACTGAGCCAGGGCCTGAAGAAGTACTACGGCAAGATCCTGAGACTGCTGCAGCTGACCCTGGA

AGAGGATACAGAGGGACTGCTGGAATGGTGCAAGAGAAACCTGGGCCTCGACTGCGACGACA

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GAGCCTTTCAAGTCTTACTTCGCCAAGGGCTTCCTGTCCATCGACAGCGGCTACTACAGCGCCAA

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CCAGCATCTTCAAAGAGCACCGCGAGGTCGAGATCAACGTGCTGCTGCCTCAAGTGGCCGTGA

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GCCACGTCAAGCTGAGCAGAGGATCTGAAGTGGTGCTGGACGCCTGTGATACCTCTTGCGAGA

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CGTGATCACCTGTATCCTGTGCAAGGCCATCTTCTACCTGCTGATCATCGTGGGCACACTCGGCA

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CTATCGAAGCTCCCTGGGGCGCTATCAATGTGCAGAGCACCTACAAGCCAACCGTGTCCACAGC

CAATATCGCCCTGAGCTGGTCCTCTGTGGAACACAGAGGCAACAAGATTCTGGTGTCTGGCCGC

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ACGTGAAGGACCTCTTCACCGATTACATGTTCGTGAAGTGGAAGGTTGAGTACATCAAGACCGA

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CCTCCAGAGATCATCACACTGCACCCTCGGATCGAAGAGGGCTTCTTCGACCTGATGCATGTGC

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ATCTGCAGGTCTACCACATCGGAAATCTGCTGAAGGGCGACAAAGTGAACGGCCACCTGATCCA

CAAGATCGAGCCTCACTTCAATACCAGCTGGATGAGCTGGGACGGCTGCGACCTGGACTACTAC

TGCAATATGGGCGACTGGCCCAGCTGCACATACACCGGCGTGACACAGCATAACCACGCCAGTT

TCGTGAACCTCCTGAACATCGAGACAGACTACACCAAGAATTTCCACTTCCACAGCAAGAGAGT

GACAGCCCACGGCGACACACCTCAGCTGGATCTGAAAGCCAGACCAACATACGGCGCAGGCGA

GATCACAGTGCTGGTTGAGGTGGCCGATATGGAACTGCACACAAAGAAGATCGAGATCAGCGG

CCTGAAGTTCGCCAGCCTGGCCTGTACCGGATGTTACGCCTGTTCCAGCTCCATCAGCTGCAAA

GTGCGGATTCACGTGGACGAGCCCGATGAGCTGACAGTGCACGTGAAGTCCGACGATCCTGAT

GTGGTGGCCGCCTCTTCTAGCCTGATGGCCAGAAAGCTGGAATTCGGCACCGACTCCACCTTCA

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GAGCTGCAGCGAAGAGGACACTAAGAAATGCGTGAACACCAAGCTCGAGCAGCCTCAGTCTAT

CCTGATCGAGCACAAGGGCACCATCATCGGCAAGCAGAATAGCACCTGTACCGCCAAGGCCTCT

TGCTGGCTGGAAAGCGTCAAGAGCTTCTTCTACGGACTGAAGAACATGCTGTCCGGCATCTTCG

GCAATGTGTTTATGGGCATCTTCCTGTTTCTGGCTCCCTTCATCCTGCTGATTCTGTTCTTCATGTT

CGGCTGGCGGATCCTGTTCTGTTTCAAGTGCTGCAGACGGACCAGAGGCCTGTTCAAGTACAGA

CACCTGAAGGACGACGAGGAAACAGGCTACCGGCGGATCATCGAGAAACTGAACAACAAGAA

GGGCAAGAACAAACTGCTGGACGGCGAGAGACTGGCCGATGGAAGAATTGCCGAGCTGTTCA

GCACCAAGACACACATCGGCTGA

Appendix 2 – Peptides for ELISPOT

CCHFV GPC peptides

Peptide Number	Peptide Pool	Sequence	Peptide Number	Peptide Pool	Sequence
1	1	MHISLMYAILCLQLCGLGET	82	4	ESTGVALKRSSWLIVLLVLF
2	1	CLQLCGLGETHGSHNETRHN	83	4	SWLIVLLVLFTVSLSPVQSA
3	1	HGSHNETRHNKTDTMTTHGD	84	4	TVSLSPVQSAPIGQGKTIEA
4	1	KTDTMTTHGDNPSSEPPVST	85	4	PIGQGKTIEAYRAREGYTSI
5	1	NPSSEPPVSTALSITLDPST	86	4	YRAREGYTSICLFVLGSILF
6	1	ALSITLDPSTVTPTTPASGL	87	4	CLFVLGSILFIVSCLMKGLV
7	1	VTPTTPASGLEGSGEVYTSP	88	4	IVSCLMKGLVDSVGNSFFPG
8	1	EGSGEVYTSPPITTGSLPLS	89	4	DSVGNSFFPGLSICKTCSIS
9	1	PITTGSLPLSETTPELPVTT	90	4	LSICKTCSISSINGFEIESH
10	1	ETTPELPVTTGTDTLSAGDV	91	4	SINGFEIESHKCYCSLFCCP
11	1	GTDTLSAGDVDPSTQTAGGT	92	4	KCYCSLFCCPYCRHCSTDKE
12	1	DPSTQTAGGTSAPTVRTSLP	93	4	YCRHCSTDKEIHKLHLSICK
13	1	SAPTVRTSLPNSPSTPSTPQ	94	4	IHKLHLSICKKRKTGSNVML
14	1	NSPSTPSTPQDTHHPVRNLL	95	4	KRKTGSNVMLAVCKLMCFRA
15	1	DTHHPVRNLLSVTSPGPDET	96	4	AVCKLMCFRATMEVSNRALF
16	1	SVTSPGPDETSTPSGTGKES	97	4	TMEVSNRALFIRSIINTTFV
17	1	STPSGTGKESSATSSPHPVS	98	4	IRSIINTTFVLCILILAVCV
18	1	SATSSPHPVSNRPPTPPATA	99	4	LCILILAVCVVSTSAVEMEN
19	1	NRPPTPPATAQGPTENDSHN	100	4	VSTSAVEMENLPAGTWEREE
20	1	QGPTENDSHNATEHPESLTQ	101	4	LPAGTWEREEDLTNFCHQEC
21	1	ATEHPESLTQSATPGLMTSP	102	4	DLTNFCHQECQVTETECLCP
22	1	SATPGLMTSPTQIVHPQSAT	103	4	QVTETECLCPYEALVLRKPL
23	1	TQIVHPQSATPITVQDTHPS	104	4	YEALVLRKPLFLDSTAKGMK
24	1	PITVQDTHPSPTNRSKRNLK	105	5	FLDSTAKGMKNLLNSTSLET
25	2	PTNRSKRNLKMEIILTLSQG	106	5	NLLNSTSLETSLSIEAPWGA
26	2	MEIILTLSQGLKKYYGKILR	107	5	SLSIEAPWGAINVQSTYKPT
27	2	LKKYYGKILRLLQLTLEEDT	108	5	INVQSTYKPTVSTANIALSW
28	2	LLQLTLEEDTEGLLEWCKRN	109	5	VSTANIALSWSSVEHRGNKI

29	2	EGLLEWCKRNLGLDCDDTFF	110	5	SSVEHRGNKILVSGRSESIM
30	2	LGLDCDDTFFQKRIEEFFIT	111	5	LVSGRSESIMKLEERTGISW
31	2	QKRIEEFFITGEGHFNEVLQ	112	5	KLEERTGISWDLGVEDASES
32	2	GEGHFNEVLQFRTPGTLSTT	113	5	DLGVEDASESKLLTVSVMDL
33	2	FRTPGTLSTTESTPAGLPTA	114	5	KLLTVSVMDLSQMYSPVFEY
34	2	ESTPAGLPTAEPFKSYFAKG	115	5	SQMYSPVFEYLSGDRQVEEW
35	2	EPFKSYFAKGFLSIDSGYYS	116	5	LSGDRQVEEWPKATCTGDCP
36	2	FLSIDSGYYSAKCYSGTSNS	117	5	PKATCTGDCPERCGCTSSTC
37	2	AKCYSGTSNSGLQLINITRH	118	5	ERCGCTSSTCLHKEWPHSRN
38	2	GLQLINITRHSTRIVDTPGP	119	5	LHKEWPHSRNWRCNPTWCWG
39	2	STRIVDTPGPKITNLKTINC	120	5	WRCNPTWCWGVGTGCTCCGL
40	2	KITNLKTINCINLKASIFKE	121	5	VGTGCTCCGLDVKDLFTDYM
41	2	INLKASIFKEHREVEINVLL	122	5	DVKDLFTDYMFVKWKVEYIK
42	2	HREVEINVLLPQVAVNLSNC	123	5	FVKWKVEYIKTEAIVCVELT
43	2	PQVAVNLSNCHVVIKSHVCD	124	5	TEAIVCVELTSQERQCSLIE
44	2	HVVIKSHVCDYSLDIDGAVR	125	5	SQERQCSLIEAGTRFNLGPV
45	2	YSLDIDGAVRLPHIYHEGVF	126	5	AGTRFNLGPVTITLSEPRNI
46	2	LPHIYHEGVFIPGTYKIVID	127	5	TITLSEPRNIQQKLPPEIIT
47	2	IPGTYKIVIDKKNKLNDRCT	128	5	QQKLPPEIITLHPRIEEGFF
48	2	KKNKLNDRCTLFTDCVIKGR	129	5	LHPRIEEGFFDLMHVQKVLS
49	2	LFTDCVIKGREVRKGQSVLR	130	5	DLMHVQKVLSASTVCKLQSC
50	2	EVRKGQSVLRQYKTEIRIGK	131	5	ASTVCKLQSCTHGVPGDLQV
51	2	QYKTEIRIGKASTGFRRLLS	132	5	THGVPGDLQVYHIGNLLKGD
52	2	ASTGFRRLLSEEPSDDCVSR	133	5	YHIGNLLKGDKVNGHLIHKI
53	3	EEPSDDCVSRTQLLRTETAE	134	5	KVNGHLIHKIEPHFNTSWMS
54	3	TQLLRTETAEIHGDNYGGPG	135	5	EPHFNTSWMSWDGCDLDYYC
55	3	IHGDNYGGPGDKITICNGST	136	5	WDGCDLDYYCNMGDWPSCTY
56	3	DKITICNGSTIVDQRLGSEL	137	6	NMGDWPSCTYTGVTQHNHAS
57	3	IVDQRLGSELGCYTINRVRS	138	6	TGVTQHNHASFVNLLNIETD
58	3	GCYTINRVRSFKLCENSATG	139	6	FVNLLNIETDYTKNFHFHSK
59	3	FKLCENSATGKNCEIDSVPV	140	6	YTKNFHFHSKRVTAHGDTPQ
60	3	KNCEIDSVPVKCRQGYCLRI	141	6	RVTAHGDTPQLDLKARPTYG
61	3	KCRQGYCLRITQEGRGHVKL	142	6	LDLKARPTYGAGEITVLVEV

62	3	TQEGRGHVKLSRGSEVVLDA
63	3	SRGSEVVLDACDTSCEIMIP
64	3	CDTSCEIMIPKGTGDILVDC
65	3	KGTGDILVDCSGGQQHFLKD
66	3	SGGQQHFLKDNLIDLGCPKI
67	3	NLIDLGCPKIPLLGKMAIYI
68	3	PLLGKMAIYICRMSNHPKTT
69	3	CRMSNHPKTTMAFLFWFSFG
70	3	MAFLFWFSFGYVITCILCKA
71	3	YVITCILCKAIFYLLIIVGT
72	3	IFYLLIIVGTLGRRLKQYRE
73	3	LGRRLKQYRELKPQTCTICE
74	3	LKPQTCTICETTPVNAIDAE
75	3	TTPVNAIDAEMHDLNCSYNI
76	3	MHDLNCSYNICPYCASRLTS
77	3	CPYCASRLTSDGLARHVIQC
78	3	DGLARHVIQCPKRKEKVEET
79	3	PKRKEKVEETELYLNLERIP
80	3	ELYLNLERIPWVVRKLLQVS
81	3	WVVRKLLQVSESTGVALKRS
143	6	AGEITVLVEVADMELHTKKI
144	6	ADMELHTKKIEISGLKFASL
145	6	EISGLKFASLACTGCYACSS
146	6	ACTGCYACSSSISCKVRIHV
147	6	SISCKVRIHVDEPDELTVHV
148	6	DEPDELTVHVKSDDPDVVAA
149	6	KSDDPDVVAASSSLMARKLE
150	6	SSSLMARKLEFGTDSTFKAF
151	6	FGTDSTFKAFSAMPKTSLCF
152	6	SAMPKTSLCFYIVEREHCKS
153	6	YIVEREHCKSCSEEDTKKCV
154	6	CSEEDTKKCVNTKLEQPQSI
155	6	NTKLEQPQSILIEHKGTIIG
156	6	LIEHKGTIIGKQNSTCTAKA
157	6	KQNSTCTAKASCWLESVKSF
158	6	SCWLESVKSFFYGLKNMLSG
159	6	FYGLKNMLSGIFGNVFMGIF
160	6	IFGNVFMGIFLFLAPFILLI
161	6	LFLAPFILLILFFMFGWRIL
162	6	LFFMFGWRILFCFKCCRRTR
163	6	FCFKCCRRTRGLFKYRHLKD
164	6	GLFKYRHLKDDEETGYRRII
165	6	DEETGYRRIIEKLNNKKGKN
166	6	EKLNNKKGKNKLLDGERLAD
167	6	KLLDGERLADGRIAELFSTK
168	6	GERLADGRIAELFSTKTHIG

CCHFV NP peptides

Peptide Number	Peptide Pool	Sequence
1	NP	MQNQIVADNKDAILAWHKTY

2	NP	DAILAWHKTYSEKHRLKSVL
3	NP	SEKHRLKSVLTNSASFCETI
4	NP	TNSASFCETIPDLSGYEVSM
5	NP	PDLSGYEVSMRLVSSESEKD
6	NP	RLVSSESEKDSVYASALVAA
7	NP	SVYASALVAATKFCAPILEC
8	NP	TKFCAPILECAWTSCTGMVE
9	NP	AWTSCTGMVERGLDWFENNK
10	NP	RGLDWFENNKETVKIWDAEY
11	NP	ETVKIWDAEYGKLRTEAPSP
12	NP	GKLRTEAPSPEQLLGYQRAA
13	NP	EQLLGYQRAALKWRKDTKYG
14	NP	LKWRKDTKYGINRNTAALAA
15	NP	INRNTAALAAAIATEYRVPG
16	NP	AIATEYRVPGSIVVNVKDML
17	NP	SIVVNVKDMLSDMIRRRNKI
18	NP	SDMIRRRNKILNRDGSEDVP
19	NP	LNRDGSEDVPKRGPVSKEHI
20	NP	KRGPVSKEHIDWARDLAQGK
21	NP	DWARDLAQGKFLVVFNPPWG
22	NP	FLVVFNPPWGDINKAGKSGI
23	NP	DINKAGKSGIALAATGMAKL
24	NP	ALAATGMAKLIELDGPKIAE
25	NP	IELDGPKIAEDLRESLKGLV
26	NP	DLRESLKGLVAWINAHKDEV
27	NP	AWINAHKDEVENGKEVVDGL
28	NP	ENGKEVVDGLTKHLQKALEL
29	NP	TKHLQKALELAKQSSAMRAQ
30	NP	AKQSSAMRAQGAQIDTVFSS
31	NP	GAQIDTVFSSYYWLWKAGVT
32	NP	YYWLWKAGVTAEMFPTVSQF
33	NP	AEMFPTVSQFLFELGKVPRG
34	NP	LFELGKVPRGNKKMKKALSS

35	NP	NKKMKKALSSMPLKWGKKLL
36	NP	MPLKWGKKLLALFADDSFTA
37	NP	ALFADDSFTANRIYMHPGVL
38	NP	NRIYMHPGVLTAGRMSELGV
39	NP	TAGRMSELGVCFGAIPVANP
40	NP	CFGAIPVANPDDAAEGSGHI
41	NP	DDAAEGSGHIKNILAQTTDT
42	NP	KNILAQTTDTQAGNPCAQNI
43	NP	QAGNPCAQNIVALFNIQKAG
44	NP	VALFNIQKAGFDIESMDIVA
45	NP	FDIESMDIVALEHLLHQSLV
46	NP	LEHLLHQSLVGKRSPFQNAY
47	NP	GKRSPFQNAYNIRGNATSIQ
48	NP	NIRGNATSIQII

NSDV GPC peptides

Peptide Number	Peptide Pool	Sequence	Peptide Number	Peptide Pool	Sequence
1	1	MVSLEKGLILLLLMHIAIAS	76	4	VKRLSWFSVLVTLFILTISP
2	1	LLLMHIAIASTGGSLGLPGL	77	4	VTLFILTISPVQGFPLESPP
3	1	TGGSLGLPGLNGEDHTDDPE	78	4	VQGFPLESPPIGAATDSRIM
4	1	NGEDHTDDPEVGGDGPTEEY	79	4	IGAATDSRIMYGVVGFMFAL
5	1	VGGDGPTEEYTEDDPESTTV	80	4	YGVVGFMFALIMMLNLKKSH
6	1	TEDDPESTTVSIPTTPPVLG	81	4	IMMLNLKKSHYGSIFHVIDG
7	1	SIPTTPPVLGQGTEEVPATS	82	4	YGSIFHVIDGFGRCNLCDTR
8	1	QGTEEVPATSGEAEAGYSTT	83	4	FGRCNLCDTRIDSLMEETAH
9	1	GEAEAGYSTTDVPDTVSEKV	84	4	IDSLMEETAHDLCCMFDCCP
10	1	DVPDTVSEKVEDEQDTTPST	85	4	DLCCMFDCCPYCHRVSESNG
11	1	EDEQDTTPSTQVTPDQPEQS	86	4	YCHRVSESNGSHCFHVRICV
12	1	QVTPDQPEQSTTSLPDPWTA	87	4	SHCFHVRICVAKEISKKMLK
13	1	TTSLPDPWTAISTRIGRELL	88	4	AKEISKKMLKAGGRLFTNKL

14	1	ISTRIGRELLSVESDTTKSS	89	4	AGGRLFTNKLVSREGTFANR
15	1	SVESDTTKSSLVTVGHPQQP	90	4	VSREGTFANRIQLLYRRSKV
16	1	LVTVGHPQQPIFLDGQPVEE	91	4	IQLLYRRSKVVSTLVVTVLL
17	1	IFLDGQPVEEVTHPVNERDV	92	4	VSTLVVTVLLLLVLLKPTTA
18	1	VTHPVNERDVTEALRKYDKM	93	4	LLVLLKPTTAFDSGPLPDGT
19	2	TEALRKYDKMKKLLSQRILS	94	4	FDSGPLPDGTWEEEEKLVRN
20	2	KKLLSQRILSKMGMARDFTD	95	4	WEEEEKLVRNCGQECILDQG
21	2	KMGMARDFTDEELDVWCHER	96	4	CGQECILDQGECLCPGEHRA
22	2	EELDVWCHERFDNCSSNDIE	97	4	ECLCPGEHRAGRKLLFLNGL
23	2	FDNCSSNDIENRIRDFFLIS	98	4	GRKLLFLNGLQDAARRMTES
24	2	NRIRDFFLISDQTECFDEVL	99	5	QDAARRMTESHRLLTSVSID
25	2	DQTECFDEVLVKRLCESTNP	100	5	HRLLTSVSIDAPWGAINIES
26	2	VKRLCESTNPIVGEPWQVAG	101	5	APWGAINIESTFKPVLAASN
27	2	IVGEPWQVAGIKEEIVRREM	102	5	TFKPVLAASNIDLSWNSAEE
28	2	IKEEIVRREMGRRIFRFFTP	103	5	IDLSWNSAEEQGDKIILSGR
29	2	GRRIFRFFTPTLKVTCVSGS	104	5	QGDKIILSGRSTGIIKLQER
30	2	TLKVTCVSGSLNPSDQFVRF	105	5	STGIIKLQERTGLMWKMSSE
31	2	LNPSDQFVRFYDPVIERTSG	106	5	TGLMWKMSSEKASESKNLLV
32	2	YDPVIERTSGPTIQNYDSMH	107	5	KASESKNLLVSVMDFSQLYN
33	2	PTIQNYDSMHCLNIENGLAK	108	5	SVMDFSQLYNSIFQYITGDR
34	2	CLNIENGLAKPSKMVVLNVL	109	5	SIFQYITGDRSLSEWPKAVC
35	2	PSKMVVLNVLMTTVEVRLES	110	5	SLSEWPKAVCTGDCPDRCGC
36	2	MTTVEVRLESCRAFINAHQC	111	5	TGDCPDRCGCQTSTCYHKEW
37	2	CRAFINAHQCIYTQHADGQV	112	5	QTSTCYHKEWPHTRNWRCNP
38	2	IYTQHADGQVMVPTFVDHSG	113	5	PHTRNWRCNPTWCWGIGTGC
39	2	MVPTFVDHSGTRRTIGAYTM	114	5	TWCWGIGTGCTCCGMDVERY
40	2	TRRTIGAYTMSFNLTDERNR	115	5	TCCGMDVERYFNKYFGVKWA
41	2	SFNLTDERNRACIIRTTCVV	116	5	FNKYFGVKWALEYVRTDVVV
42	2	ACIIRTTCVVKGKEVKKGQS	117	5	LEYVRTDVVVCVELTNEERH
43	2	KGKEVKKGQSQLRGFPTSIR	118	5	CVELTNEERHCDLVQAGSRF
44	2	QLRGFPTSIRLFKSVTGKRR	119	5	CDLVQAGSRFSIGPVSVTLS
45	2	LFKSVTGKRRLMAEEGDWTD	120	5	SIGPVSVTLSDPQNVVNRLS

46	2	LMAEEGDWTDCGSGTQLDMT	121	5	DPQNVVNRLSSNIMTIQEIA
47	3	CGSGTQLDMTMAVSVHNDKK	122	5	SNIMTIQEIADNGILDLMHV
48	3	MAVSVHNDKKGGPGKKLTIC	123	5	DNGILDLMHVSKVISAENAC
49	3	GGPGKKLTICNGTTVSDSTL	124	5	SKVISAENACKLQSCTHGSP
50	3	NGTTVSDSTLNEGLGCYTIN	125	5	KLQSCTHGSPGDLQILHTDN
51	3	NEGLGCYTINKVITGKACKV	126	5	GDLQILHTDNLIKGDMSGGI
52	3	KVITGKACKVGNSTGSCEVQ	127	5	LIKGDMSGGINLAHLDPQVN
53	3	GNSTGSCEVQTELQKCETGK	128	5	NLAHLDPQVNTSWMSWEGCD
54	3	TELQKCETGKCILVKQKSKG	129	5	TSWMSWEGCDLDYYCTVGDW
55	3	CILVKQKSKGVVKLKRGKTV	130	5	LDYYCTVGDWPSCTYTGVNS
56	3	VVKLKRGKTVIITECQGSCL	131	6	PSCTYTGVNSINTDSFTNLI
57	3	IITECQGSCLFAIPQDTGDI	132	6	INTDSFTNLINTETDYTGKF
58	3	FAIPQDTGDITIDCSGGRQH	133	6	NTETDYTGKFHFHSKRISAR
59	3	TIDCSGGRQHYLEINIVDIH	134	6	HFHSKRISARDDTLQMDLKA
60	3	YLEINIVDIHCPGKDKWKGF	135	6	DDTLQMDLKARPNSGGGEMT
61	3	CPGKDKWKGFMLYICRVSSR	136	6	RPNSGGGEMTVLVEVNGLEL
62	3	MLYICRVSSRPLIALTFGLW	137	6	VLVEVNGLELHSKRISLKGL
63	3	PLIALTFGLWLAAGYLITCL	138	6	HSKRISLKGLRLTNLKCDGC
64	3	LAAGYLITCLVSFIIYNAVL	139	6	RLTNLKCDGCFACSQGLTCT
65	3	VSFIIYNAVLLLSIAIKKVR	140	6	FACSQGLTCTITAKLESPDE
66	3	LLSIAIKKVRQGREKKGDLC	141	6	ITAKLESPDEFTIHLRSTSR
67	3	QGREKKGDLCIKCEQHCMNL	142	6	FTIHLRSTSRDVVVAETSVT
68	3	IKCEQHCMNLYDQELHELNC	143	6	DVVVAETSVTARKVETGARS
69	3	YDQELHELNCSFNLCPYCAN	144	6	ARKVETGARSGFRAFAVRDV
70	3	SFNLCPYCANRLSDEGLPRH	145	6	GFRAFAVRDVKKLCLEVVEK
71	3	RLSDEGLPRHVPRCPKKSER	146	6	KKLCLEVVEKDYCPSCTVED
72	3	VPRCPKKSERLEEIELYINY	147	6	DYCPSCTVEDLKICVDVSLE
73	3	LEEIELYINYTRVPCILRWI	148	6	LKICVDVSLEPPKDILIEHK
74	3	TRVPCILRWILSTSVQVGTA	149	6	PPKDILIEHKGTIIQHYNKS
75	3	LSTSVQVGTAVKRLSWFSVL	150	6	GTIIQHYNKSCDEGYNCWVG
			151	6	CDEGYNCWVGSVSGFFVGVK
			152	6	SVSGFFVGVKDFFEKNLGGV

153	6	DFFEKNLGGVLIGLVSTILP
154	6	LIGLVSTILPLVLTVLFFIY
155	6	LVLTVLFFIYGKKLFCLCRL
156	6	GKKLFCLCRLCHKKCCRGSG
157	6	CHKKCCRGSGRGRDGYSRLS
158	6	RGRDGYSRLSQEEEIKEIIK
159	6	QEEEIKEIIKKFSRNGELLG
160	6	KFSRNGELLGKGENDKRTVA
161	6	KGENDKRTVARMFMDGQSTK
162	6	RMFMDGQSTKKAIKEVA

NSDV NP peptides

Peptide Number	Peptide Pool	Sequence
1	NP	MQNQIVADNKDAILAWHKTY
2	NP	DAILAWHKTYSEKHRLKSVL
3	NP	SEKHRLKSVLTNSASFCETI
4	NP	TNSASFCETIPDLSGYEVSM
5	NP	PDLSGYEVSMRLVSSESEKD
6	NP	RLVSSESEKDSVYASALVAA
7	NP	SVYASALVAATKFCAPILEC
8	NP	TKFCAPILECAWTSCTGMVE
9	NP	AWTSCTGMVERGLDWFENNK
10	NP	RGLDWFENNKETVKIWDAEY
11	NP	ETVKIWDAEYGKLRTEAPSP
12	NP	GKLRTEAPSPEQLLGYQRAA
13	NP	EQLLGYQRAALKWRKDTKYG
14	NP	LKWRKDTKYGINRNTAALAA
15	NP	INRNTAALAAAIATEYRVPG

16	NP	AIATEYRVPGSIVVNVKDML
17	NP	SIVVNVKDMLSDMIRRRNKI
18	NP	SDMIRRRNKILNRDGSEDVP
19	NP	LNRDGSEDVPKRGPVSKEHI
20	NP	KRGPVSKEHIDWARDLAQGK
21	NP	DWARDLAQGKFLVVFNPPWG
22	NP	FLVVFNPPWGDINKAGKSGI
23	NP	DINKAGKSGIALAATGMAKL
24	NP	ALAATGMAKLIELDGPKIAE
25	NP	IELDGPKIAEDLRESLKGLV
26	NP	DLRESLKGLVAWINAHKDEV
27	NP	AWINAHKDEVENGKEVVDGL
28	NP	ENGKEVVDGLTKHLQKALEL
29	NP	TKHLQKALELAKQSSAMRAQ
30	NP	AKQSSAMRAQGAQIDTVFSS
31	NP	GAQIDTVFSSYYWLWKAGVT
32	NP	YYWLWKAGVTAEMFPTVSQF
33	NP	AEMFPTVSQFLFELGKVPRG
34	NP	LFELGKVPRGNKKMKKALSS
35	NP	NKKMKKALSSMPLKWGKKLL
36	NP	MPLKWGKKLLALFADDSFTA
37	NP	ALFADDSFTANRIYMHPGVL
38	NP	NRIYMHPGVLTAGRMSELGV
39	NP	TAGRMSELGVCFGAIPVANP
40	NP	CFGAIPVANPDDAAEGSGHI
41	NP	DDAAEGSGHIKNILAQTTDT
42	NP	KNILAQTTDTQAGNPCAQNI
43	NP	QAGNPCAQNIVALFNIQKAG
44	NP	VALFNIQKAGFDIESMDIVA
45	NP	FDIESMDIVALEHLLHQSLV
46	NP	LEHLLHQSLVGKRSPFQNAY
47	NP	GKRSPFQNAYNIRGNATSIQ
48	NP	NIRGNATSIQII

Appendix 3 – ChAdOx Vaccines produced and their genome sizes

Vaccine	Antigen Cassette	Size of antigen cassette (bp)	Size of antigen in ChAdOx1 Genome (bp)	Percentage of Original ChAdY25 genome size (%)	Size of antigen in ChAdOx2 Genome (bp)	Percentage of Original ChAd68 Genome size (%)
ChAdOx1/2-CCHFV GPC	CCHFV GPC long promoter	7571	36674	100	36556	100
ChAdOx1/2-CCHFV-sCMV GPC	CCHFV GPC short promoter	6131	35234	96	35116	96
ChAdOx1/2-CCHFV Gn	CCHFV Gn	2195	31298	85	31183	85
ChAdOx1/2-CCHFV Gc	CCHFV Gc	2990	32039	87	31975	87
ChAdOx1/2 CCHFV NP	CCHFV NP	2564	31667	86	31549	86
ChAdOx1 Bivalent CCHFV	*CCHFV Gc – E1 entry*	*2990*	34289	93	N/A	N/A
	CCHFV Gn – E4 entry	*2129*			N/A	N/A
ChAdOx1 Trivalent CCHFV	*CCHFV Gc – E1 entry*	*2990*	35757	97	N/A	N/A
	CCHFV Gn-2A-NP entry	*3600*			N/A	N/A
ChAdOx1-NSDV GPC	*NSDV GPC*	*6019*	35,268	96	N/A	N/A
ChAdOx1-NSDV Gn	*NSDV Gn*	*2195*	31298	85	N/A	N/A
ChAdOx1-NSDV Gc	*NSDV Gc*	*3043*	32,292	88	N/A	N/A
ChAdOx1-NSDV NP	*NSDV NP*	*2584*	31,833	87	N/A	N/A