Serological investigations of emerging viral zoonoses: Crimean-Congo haemorrhagic fever virus in Tanzania and severe acute respiratory syndrome coronavirus 2 in Scotland

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Abstract

Emerging viral zoonoses are a significant global health concern, brought into sharp focus in recent times by the emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the subsequent global pandemic. Other emerging and re-emerging zoonotic viruses also continue to present significant threats to public health, including Crimean-Congo haemorrhagic fever virus (CCHFV), a tick-borne virus that can cause severe disease and high case fatality rates in people, but with a complex ecology that remains poorly understood in many areas of the world, particularly in Africa. Serological tools are vital for understanding emerging zoonotic viruses both at a population- and individual-level. Population-level surveillance of animal and human populations can shed light on patterns of viral circulation and aid in identification of risk factors associated with infection, while individual-level serological investigations help to characterise the immune response to infection, providing insights into the nature of protective immunity, possible vaccine targets and improving methods of disease detection.

The initial focus of this thesis was the development of a diagnostic enzyme-linked immunosorbent assay (ELISA) for detection of CCHFV, and its subsequent use to explore the epidemiology of the virus in livestock and people in northern Tanzania. However, the start of the coronavirus disease 19 (COVID-19) pandemic part way through this research resulted in an additional set of research questions relating to SARS-CoV-2 diagnostic assay performance and epidemiology during the first wave of the pandemic in Glasgow, UK. This thesis therefore explores diagnostics assay development and epidemiology of CCHFV and SARS-CoV-2. Firstly, an indirect ELISA for detection of anti-CCHFV antibodies in livestock was developed and optimised. It showed good potential for use as an in-house assay for detection of CCHFV exposure in animals. Secondly, the performance of two indirect ELISA assays against the S1 subunit of the SARS-CoV-2 spike protein (S1) and the receptor binding domain (RBD) was investigated, establishing a cut-off value for interpretation of these assays, determining sensitivity and specificity, and exploring measures of assay precision. Both assays showed good ability to distinguish between positive or negative serum samples for anti-SARS-CoV-2 IgG

antibodies. These ELISAs were then used to investigate levels of SARS-CoV-2 exposure in a patient population in Glasgow during the first wave of the COVID-19 pandemic, demonstrating that overall seroprevalence remained low throughout this period. Additionally, ELISA responses were compared against levels of neutralising antibodies (NAbs), measured using HIV(SARS-CoV-2) pseudotype virus neutralisation assays (PVNAs), demonstrating heterogeneity in IgG and NAb responses, and highlighting an association between disease severity and higher levels of IgG and neutralising antibodies.

The epidemiology of CCHFV in northern Tanzania was explored through analysis of a large cross-sectional study of livestock and people in linked households, using a commercially produced species-independent ELISA. This study demonstrated for the first time that CCHFV is circulating in northern Tanzania. High levels of exposure were found in cattle (n = 1530/3098, 49.4%), goats (n = 823/2475, 33.3%), and sheep (n = 582/2124, 27.4%) across the region, and an overall seroprevalence of 15.1% (n = 53/351) was observed in people, despite an absence of confirmed clinical disease in the country (Temur et al., 2021). Substantial heterogeneities were observed in levels of exposure between study sites for both livestock and people, indicating that local context is important for determining exposure to CCHFV. However, patterns of village-level exposure varied between people and livestock, possibly suggesting different drivers of exposure. Risk factors associated with CCHFV seropositivity were also investigated in livestock, and demonstrated that increasing age, and extensive, pastoral agro-ecological settings were associated with higher levels of exposure. Additionally, a novel association was identified between pig keeping and higher exposure in cattle.

The work presented in this thesis demonstrates the application of serological methods for investigation of two important emerging and re-emerging zoonotic viruses, SARS-CoV-2 and CCHFV. This research adds substantially to our knowledge of CCHFV epidemiology in northern Tanzania, demonstrating high levels of exposure to the virus in livestock populations and highlighting its potential as a public health concern in the country, while the work on SARS-CoV-2 provided important information on population-level immunity and

the nature of the immune response during the early phases of the global pandemic.

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Acknowledgements

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During the COVID-19 pandemic I was fortunate enough to be able to join a fantastic team of scientists to work on the pandemic response. Particular thanks go to my co-author Julien Amat, for the ggplot wisdom and tea breaks, Jo Haney for all the ELISAs, and Pablo Murcia and Mafalda Viana for all they have taught me and for giving me the opportunity to lead part of the SARS-CoV-2 serology work. Thanks also go to Antonia Ho for her encouragement and patience, and for giving me the opportunity to continue working on SARS-CoV-2 in Malawi.

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constant love and support, immense generosity, and ability to always make me laugh, even at the toughest times.

Dedication

This thesis is dedicated to the memory of
Dr Patrick Michael Hughes
1923-2012
who would have enjoyed every word

Author's declaration

I declare that this thesis and the research contained within it is my own work unless otherwise stated, and no part of it has been submitted as part of any other degree or qualification.

Ellen Clare Hughes

April 2022

Abbreviations

ACE2	Angiotensin-converting-enzyme-2
AGDP	Agar-gel diffusion precipitin
AIC	Akaike Information Criterion
ASF	African swine fever
AUC	Area under the curve
BBSRC	Biotechnology and Biological Sciences Research Council
BSL	Biosafety level
CCHF	Crimean-Congo haemorrhagic fever
CCHFV	Crimean-Congo haemorrhagic fever virus
CDR	Complementarity determining regions
CFR	Case fatality rate
CI	Confidence interval
COVID-19	Coronavirus disease 2019
CV	Coefficient of variation
CVR	MRC-University of Glasgow Centre for Virus Research
DfID	Department for International Development (UK Government)
DMEM	Dulbecco's modified Eagle's medium
DSTL	Defence Science and Technology Laboratory (UK Government)
DUGV	Dugbe Virus
ELISA	Enzyme-linked immunosorbent assay
ESRC	Economic and Social Research Council
FAO	Food and Agriculture Organization of the United Nations
FOI	Force of infection
GCE	General contextual effect
GLMM	Generalised linear mixed model
HAZV	Hazara virus
HCA	Hierarchical cluster analysis
HCoV	Human coronavirus
HIV	Human immune deficiency virus
ICC	Intra-class or intra-cluster correlation coefficient
IFA	Immunofluorescent assay
IFR	Infection fatality rate
IgG	Immunoglobulin G

IgM	Immunoglobulin M
KCRI	Kilimanjaro Clinical Research Institute
MFA	Multiple factor analysis
MOD	Median Odds Ratio
MRC	Medical Research Council
MSD	Meso scale discovery immunoassay
NAb	Neutralising antibody
NERC	Natural Environment Research Council
NHS	National Health Service
NHSGGC	NHS Greater Glasgow and Clyde
NIBSC	National Institute for Biological Standards and Control
NP	Nucleoprotein
NSDV	Nairobi sheep disease virus
OD`	Optical density
OFAT	One factor at a time
OIE	World Organisation for Animal Health
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with 0.05% Tween-20
PHE	Public Health England
PHS	Public Health Scotland
PPRV	Peste de petit ruminants virus
R&D	Research and development
RBD	Receptor binding domain
rBV	Recombinant baculovirus vector
ROC	Receiver operating characteristic
RT-PCR	Reverse transcription polymerase chain reaction
RVFV	Rift Valley fever virus
S	Spike protein
S1	S1 subunit of the spike protein
SARS-CoV-1	Severe acute respiratory syndrome coronavirus 1
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SCE	Specific contextual effect
SCE	Specific contextual effects
Se	Sensitivity
SEEDZ	Social, economic and environmental drivers of zoonoses project

*Sf*21	*Spodoptera frugiperda* 21 (cell line)
SFTSV	Severe Fever with Thrombocytopenia syndrome virus
SNBTS	Scottish National Blood Transfusion Service
Sp	Specificity
Spp.	Species
SSA	Sub-Saharan Africa
TZG	Tripartite Zoonoses Guide
UFI	Undifferentiated febrile illness
WHO	World Health Organisation
ZELS	Zoonoses and Emerging Livestock Systems project

1 Introduction

1.1 Emerging viral zoonoses

Zoonotic pathogens are those which can be transmitted from animals to people, either directly or via a suitable vector species, resulting in disease in human hosts. Zoonoses are one of the most important causes of human infectious disease, with an estimated 60% of all human pathogens originating in animals (Taylor et al., 2001). These include ancient pathogens such as rabies virus or brucella species (Fooks et al., 2017, Moreno, 2014), which continue to cause substantial disease globally, but of increasing concern are newly emerging and re-emerging zoonotic pathogens, which have the potential to cause severe and widespread public health emergencies (Sikkema and Koopmans, 2021).

Spill-over events, whereby a virus is able to switch to a new host species are common in viral evolution but most of these events end in dead-end hosts or limited transmission chains (Longdon et al., 2014). However, recent decades have seen an increase in the incidence of novel disease outbreaks originating in animals that have the potential for onwards human-to-human transmission (Grubaugh et al., 2019, Jones et al., 2008). Viruses, especially RNA viruses, are particularly important sources of emerging zoonoses, due to their high genetic diversity and potential for rapid evolution, with up to 90% of RNA viruses capable of infecting humans being of zoonotic origin (Woolhouse et al., 2013). RNA viruses of zoonotic origin have been the cause of the most notable global health emergencies of the last decade including H1N1 influenza virus, Ebola virus, Zika virus, and the three novel coronavirus emergences, severe acute respiratory syndrome coronavirus 1 (SARS-CoV-1), Middle Eastern respiratory syndrome coronavirus (MERS-CoV), and most recently severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (CDC, 2019, Reperant et al., 2016, Morens and Fauci, 2020). The emergence of such viruses is a source of global concern for a several reasons, including the potential for rapid spread of novel pathogens through naïve populations without existing immunity, and the amplification of that spread by unprecedented local, regional and global interconnectedness, which can

facilitate rapid transmission of pathogens over large areas (Suk et al., 2014). Additionally, the public health consequences of emerging viruses may be increased by a lack of specific therapeutics and vaccines in the early phases of pathogen emergence, as well as by issues of poverty and poor health care infrastructure when emergence occurs in low-income settings.

In addition to novel viruses, re-emergence of known zoonotic pathogens into new populations and regions, or with increasing frequency, is also of considerable global concern. Significant Ebola epidemics in west and central Africa in the last decade demonstrated the huge public health impact of re-emergence events (Kaner and Schaack, 2016, Delamou et al., 2017), and other viral haemorrhagic fevers, such as Crimean-Congo haemorrhagic fever virus (CCHFV), Lassa fever and Marburg virus disease, remain a particular concern due to the potential severity of clinical disease, high case fatality rates, and diagnostic challenges of diagnosing febrile illness (Pigott et al., 2017). Zoonotic viruses that continue to circulate in animal and vector populations, such as Crimean-Congo haemorrhagic fever virus (CCHFV), may re-emerge into human populations at unpredictable intervals, with the potential for significant public health consequences (Leblebicioglu et al., 2016b). This may be of particular concern in sub-Saharan Africa and other tropical regions of the world. Changing climate, higher biodiversity, combined with increased human-wildlife interactions as well as habitat loss and land-use changes may make spill-over events more likely in these areas (Allen et al., 2017), while lack of surveillance infrastructure, under-resourced health services, and poverty may make the impact of such outbreaks more substantial in these communities.

1.2 Serological techniques for emerging viral zoonoses

Serological laboratory techniques are based on evaluation of components of blood serum, usually targeting antibodies against or antigens from infecting pathogens. By targeting different elements of the humoral immune system, they can be used to diagnose current infections, for example through detection of immunoglobulin M (IgM) antibodies, as well as to indicate past infection status, through detection of immunoglobulin G (IgG). This ability to

detect past infection constitutes a principal advantage of serological approaches over molecular detection techniques such as reverse transcription polymerase chain reaction (RT-PCR) or sequencing methods, which are typically limited to detection of current infections. In addition, many serological techniques require fewer resources and can produce results more rapidly than molecular methods, making them suitable for rapid diagnostics in clinical settings, as well as wider scale sero-surveillance, particularly in low-income settings (Arnold et al., 2018).

Serology is vital for population-level surveillance of pathogens, enabling investigation of trends and patterns in exposure, even where clinical disease has not been diagnosed on an individual-level. However, serological approaches are also essential for answering questions relating to individual immune responses, such as characterisation of immunoglobulin and neutralising antibody responses, duration and effectiveness of immunity, and identification of vaccine targets or novel therapeutics (Lu et al., 2020, Fernández-Barat et al., 2020, Krammer and Simon, 2020).

1.2.1 Enzyme linked immunosorbent assays (ELISA)

Enzyme-linked immunosorbent assays, more commonly referred to as ELISAs, are a type of solid phase immunoassay and are one of the most widely used methods for detecting antigens, antibodies or other analytes in biological samples, making use of the highly specific antigen-antibody binding reaction. Although simple in principle, the ELISA is one of the most long-standing assays available and has proven its worth in over four decades of serological research (Engvall, 2010, Lequin, 2005). ELISAs remain the backbone of much serological diagnosis for humans and animals, both in research and commercial laboratories, due to their high specificity, simple principles, and adaptability (Aydin, 2015).

Several types of ELISA are commonly used for serological assays, the principals of which are shown in Figure 1.2.1 . Each has advantages and disadvantages but all act on the principal of using a visually detectable enzyme linked to a bound antigen/antibody complex to elicit a colour change

when a suitable substrate is added. The indirect ELISA format, wherein immobilised antigen is bound by the target antibody in serum, which is then detected by a conjugated secondary antibody, is used in Chapters 2 and 3 of this thesis. A commercially produced double-antigen sandwich ELISA (IDvet, ID Screen® CCHF Double Antigen Multi-species ELISA, IDvet, Grabels, France), which uses a secondary conjugated antigen instead of an antibody, was used to test samples described in Chapter 4 and 5.

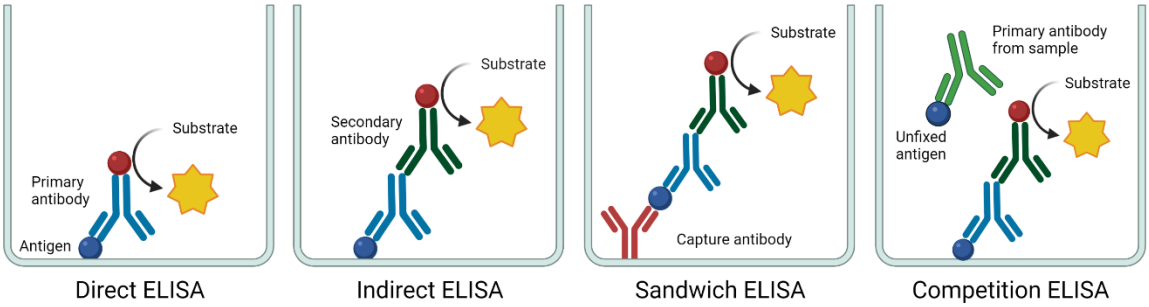

Figure 1.2.1 Principals of four common ELISA types.
Direct ELISA: antigen of interest is immobilised to the well and detected by addition of a conjugated primary antibody leading to a colour change. Indirect ELISA: Wells are coated with antigen of interest, which is then bound by the addition of a primary antibody, usually from a sample under investigation. This is then bound by addition of conjugated secondary antibody leading to a colour change. Sandwich ELISA: A capture antibody is immobilised to the well, which then binds the antigen of interest if present in the sample being tested. This antigen is then bound by an unconjugated primary antibody, which in turn is bound by a conjugated secondary antibody leading to a colour change. Competition ELISA: Sample of interest is incubated with free antigen which binds to antibody in the sample if present. This complex is then incubated with the bound antigen in the well and a primary antibody. After washing to remove unbound antigen-antibody complexes a conjugated secondary antibody is added. Only antibody not already bound to the free antigen can bind meaning the more antigen in the sample, the less antibody available to bind to the fixed antigen, resulting in a reduced or absent colour change. Can be direct or indirect (shown here) and adapted for antigen or antibody detection. Created with Biorender.com

1.2.2 Pseudotype virus neutralisation assays

Alternatives to live-virus assays, instead using replication-defective virus particles, are now widely used in virology to study mechanisms of viral entry, evaluate potential anti-viral compounds, and investigate neutralising antibody responses (King et al., 2016). Pseudotyped viruses (PVs) are composed of the viral core of one virus, surrounded by a cell-derived

membrane baring the external envelope proteins of a second virus. Genes encoding the envelope proteins of the core virus are deleted, preventing the PV from producing infectious virus. These genes can be replaced by marker gene such as firefly luciferase (*luc*) to allow detection of viral entry (Logan et al., 2016). Envelope proteins from the coating virus are not encoded by the core virus but instead are derived from the cell membrane of transfected cells during budding of the PV. The envelope proteins of the PV allow it to enter cells and be neutralised by antibodies in the same way the infectious virus would but without the risk of onwards replication. This allows pseudotyped versions of highly pathogenic RNA viruses such as SARS-CoV-2 and Ebola virus to be investigated under biosafety containment level 2 conditions, enabling more rapid and accessible research into these pathogens (Bentley et al., 2015, Steeds et al., 2020, Cantoni et al., 2021). In Chapter 3 of this thesis a neutralisation assay using a human immunodeficiency virus (HIV)(SARS-CoV-2) pseudotype virus is employed to investigate neutralising responses to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).

1.2.3 Exposure, infection and seropositive: a note on terminology

Exposure of an individual to a pathogen may result in infection, which typically leads to production of an antibody response, although the type, strength and duration of this response will vary depending on the type and load of the infecting pathogen, as well as host immune characteristics. Alternatively, exposure may occur but not result in infection, or its subsequent consequences. Additionally, an infection may occur following exposure but be rapidly cleared by physical barriers or the innate immune response and so result in a low or undetectable antibody response. Exposure, infection and seropositivity can therefore indicate subtly different interactions between host and pathogen. However, the presence of antibodies to a pathogen is usually evidence of exposure and infection with that pathogen, although special cases such as maternally derived immunity can complicate this picture. For the purposes of this thesis, ‘exposure’ and ‘infection’ will be treated as synonymous with ‘seropositive’, unless otherwise stated.

1.3 Thesis development: a note on changes of direction resulting from the SARS-CoV-2 pandemic

The original goal of this thesis research was to fill knowledge gaps regarding the presence and epidemiology of an important emerging tick-borne zoonosis, Crimean-Congo haemorrhagic fever virus (CCHFV), in Tanzania. This pathogen, discussed in detail below, is endemic in much of East Africa and is a priority pathogen in the World Health Organisation's research and development framework due to its high case fatality rate in humans (WHO, 2022a). Despite this, evidence of its presence in Tanzania was extremely limited, particularly in 2018 when research for this thesis commenced, and no studies had investigated the epidemiology of the virus in animals or people in the country. It was unclear whether the virus was actively circulating in Tanzania, and if it was, what the patterns of exposure were amongst animals, people and ticks. Additionally, at commencement of this research no commercially produced serological diagnostic tests were available for the detection of CCHFV antibodies in animals, although several assays were available for human sera.

To begin to answer these questions it was planned to utilise an existing, highly comprehensive set of serum samples from cattle, goats, sheep and people in linked households in northern Tanzania in 2016 as part of the Social, Economic and Environmental Drivers of Zoonoses project, hereafter referred to as SEEDZ (part of Zoonoses and Emerging Livestock Systems (ZELS) program, grant no: BB/L018926/1). The first challenge in pursuit of these research aims was to establish a method to detect antibodies to CCHFV in these samples and so initial laboratory work involved developing an ELISA to detect anti-CCHFV antibodies, with the plan to use this assay to screen all livestock samples to enable downstream epidemiological analysis of CCHFV exposure in the study population. During work into the development of this ELISA (described in Chapter 2) a commercially produced ELISA kit was released by IDvet (IDvet, Grabels, France) and funding for use of this kit to test all SEEDZ samples was provided through the Supporting Evidence Based Interventions (SEBI) project, (University of Edinburgh - grant number R83537).

This resulted in a change of direction away from using the in-house ELISA towards using the commercial kit, in order to better compare across species.

Part way through the research for this thesis, however, the emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) resulted in unpredicted but unavoidable changes to research priorities, as it did for infectious disease researchers around the world. The pandemic resulted in a cessation of most routine laboratory work, including work on CCHFV for this thesis, for several months, ultimately preventing some elements of the planned thesis being completed. However, the events of 2020 also presented an opportunity to use skills in diagnostic serology and epidemiology developed during the first half of this thesis research to contribute to the local pandemic response in Glasgow, UK, both in developing ELISAs to explore population- and individual-level immune responses to SARS-CoV-2 and in exploring the epidemiology of exposure to the virus in a local patient population. The resulting work now forms Chapter 3 of this thesis.

This thesis, therefore, brings together substantial work on the epidemiology of CCHFV in Tanzania, with a valuable study of SARS-CoV-2 immunology and epidemiology in Scotland. Although different pathogens, with different emergence histories and different research priorities, similar serological techniques were used here to answer key epidemiological questions for both viruses, highlighting the advantages of using serological techniques in understand emerging viral zoonoses at the population level.

1.4 Crimean-Congo haemorrhagic fever virus

CCHFV is often described as one of the most important and widespread arboviruses in the world. It can cause a rapidly fatal disease in people and has the potential for onwards human-to-human transmission. This epidemic potential, as well as its widespread distribution, severity of clinical disease, and lack of specific therapeutics or vaccine, have made CCHFV one of the World Health Organisation's priority pathogens for Research and Development (R&D) (WHO, 2022a). Despite this, the epidemiology and ecology of the virus in humans, asymptomatic animal hosts, and ticks is not fully understood, particularly in low-resource settings such as in sub-Saharan Africa (SSA), where the virus has the potential to pose a severe public health threat (Temur et al., 2021).

1.4.1 History

Cases of what is now known as Crimean-Congo haemorrhagic fever (CCHF) were first identified in the Crimean Peninsula during 1944, when around 200 Soviet soldiers and numerous local farm workers developed an acute febrile illness, typically accompanied by haemorrhage and shock, resulting in a fatality rate of around 10% (Bente et al., 2013, Hoogstraal, 1979, Grashchenkov, 1945). Cases were linked to tick bites and the syndrome was named 'Crimean haemorrhagic fever' (CHF). Attempts to isolate the causative agent of the infection were hampered by a lack of success in culturing, but the development of suckling mice models to cultivate the virus enabled it to be isolated for the first time in 1967 (Bente et al., 2013). This breakthrough also enabled production of associated antigens and antibodies, which led to an increase in research into the seroprevalence of the virus and transmission in nature (Bente et al., 2013, Whitehouse, 2004, Spengler et al., 2016b). In 1956, a virus causing similar symptoms was isolated from what is now the Democratic Republic of Congo (DRC) and named 'Congo virus' (Simpson et al., 1967, Woodall et al., 1967). In 1969 it was shown that this virus was identical to the Drosdov strain of CHF virus isolated through the early suckling mouse model work (Casals, 1969) and this recognition eventually led to a change in nomenclature resulting in its current name of Crimean-Congo haemorrhagic fever virus (CCHFV). Although only officially

recognised in the twentieth century, historical reports of disease and modern techniques investigating the most recent common ancestor of the virus suggest it has a much more ancient origin and has likely been circulating in central Eurasia for centuries, causing sporadic disease in humans (Carroll et al., 2010, Ergönül, 2006, Bente et al., 2013, Whitehouse, 2004).

1.4.2 Virology and classification

CCHFV is a negative sense single stranded RNA virus with a tri-segmented genome, of around 19kb, divided into small (S), medium (M) and large (L) segments (Spiropoulou and Bente, 2021). The genome is encapsidated in nucleoprotein (NP) (encoded by the S segment) and includes an RNA dependant RNA polymerase (encoded by the L segment) for initiation of transcription and replication (Bente et al., 2013). The virion's lipid envelope is coated in external glycoproteins, Gn and Gc, which are encoded by the M segment and enable virion binding to host cell receptors (Zivcec et al., 2016) (Figure 1.4.1).

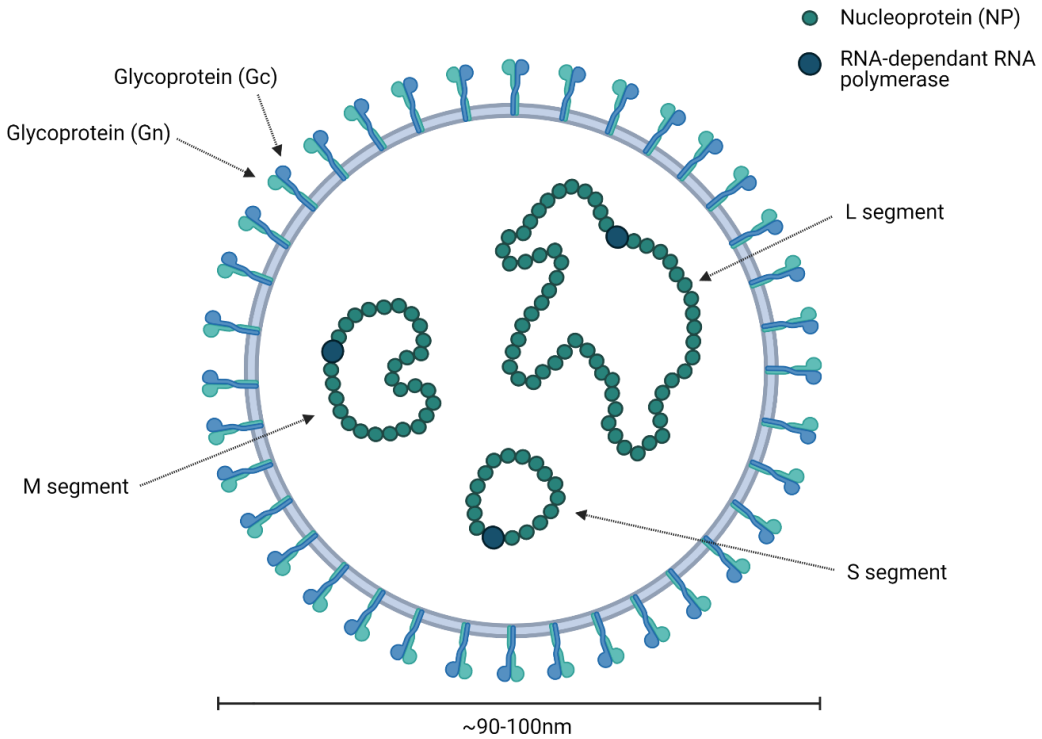

Figure 1.4.1 Schematic representation of CCHFV virion.
Adapted from Bente et al. 2013 and Zivcec et al. 2016. Created with BioRender.com.

CCHFV is a member of the genus *Orthonairovirus*, part of the *Nairovirus* family of the order *Bunyavirales*, which also encompasses other important

viral genera including *Orthobunyaviruses*, *Hantaviruses* and *Phleboviruses* (Adams et al., 2017). Like other nairoviruses it is distinguished from the rest of the Bunyavirus order by the notably long length of its L segment. Orthonarioviruses are further divided into at least seven serogroups including the CCHFV group, which includes CCHFV and Hazara virus (HAZV), and the Nairobi sheep disease virus group which includes Nairobi sheep disease virus (NSDV) and Dugbe virus (DUGV) (Whitehouse, 2004, Walker et al., 2016). HAZV is not known to cause disease in humans or animals and has been proposed as a model for studying CCHFV infection and antiviral development outside of biosafety level 4 laboratories (Monteil et al., 2020, Dowall et al., 2012a, Flusin et al., 2011, Begum et al., 1970). NSDV is primarily a pathogen of small ruminants but can also rarely infect humans, while DUGV can cause a mild febrile illness in people (Krasteva et al., 2020, Burt et al., 1996). Both are found in Eastern Africa, but their impact and epidemiology are poorly understood (Krasteva et al., 2020, David-West et al., 1975, Johnson et al., 1980).

Unusually for an arbovirus, CCHFV is one of the most genetically diverse viruses in the world (Deyde et al., 2006). Phylogenetic analysis has grouped the virus into at least six clades based on S segment sequence analysis: I West Africa; II Democratic Republic of Congo (DRC); III South Africa and West Africa; IV Asia and the Middle East; V Europe and Turkey; and VI Greece (Carroll et al., 2010, Deyde et al., 2006). These clades are further subdivided into different local variants. High genetic diversity likely reflects a long history of co-evolution with ticks and their hosts species (Honig et al., 2004, Xia et al., 2016), but geographic mixing of clades also contributes, with genetically and geographically diverse variants emerging in far-distant regions, possibly as a result of long-distance translocation of infected tick by migratory birds and transboundary livestock movements (Bente et al., 2013, Leblebicioglu et al., 2014, Hewson et al., 2004). Very recently, viruses in the Greece clade (clade VI), which consists of those closely related to the CCHFV isolate known as AP-92, have been reclassified as a distinct virus, now known as Aigai virus (AIGV). It has been proposed that this virus be defined as a separate species of Orthonairovirus, *Orthonairovirus parahaemorrhagiae*,

with all other clades of CCHFV being designated *Orthonairovirus haemorrhagiae* (Marklewitz et al., 2020, Papa et al., 2022).

1.4.3 Viral transmission and maintenance

In nature, CCHFV is maintained in tick-to-tick and tick-vertebrate-tick cycles, involving both small and large mammalian hosts (Figure 1.4.2). Ticks are both the vector and reservoir of the virus, with mammalian hosts likely acting principally as amplification hosts (Randolph and Rogers, 2007). The role of ticks as vectors for CCHFV, first identified in early research into viral outbreaks, has been confirmed by studies of both naturally infected ticks and in experimental studies (Gargili et al., 2017), while identification of vertebrate hosts has principally been through serological studies, due to challenges in identifying active infection in non-human hosts as a result of transient, typically asymptomatic, viraemia (Spengler et al., 2016a, Spengler et al., 2016b).

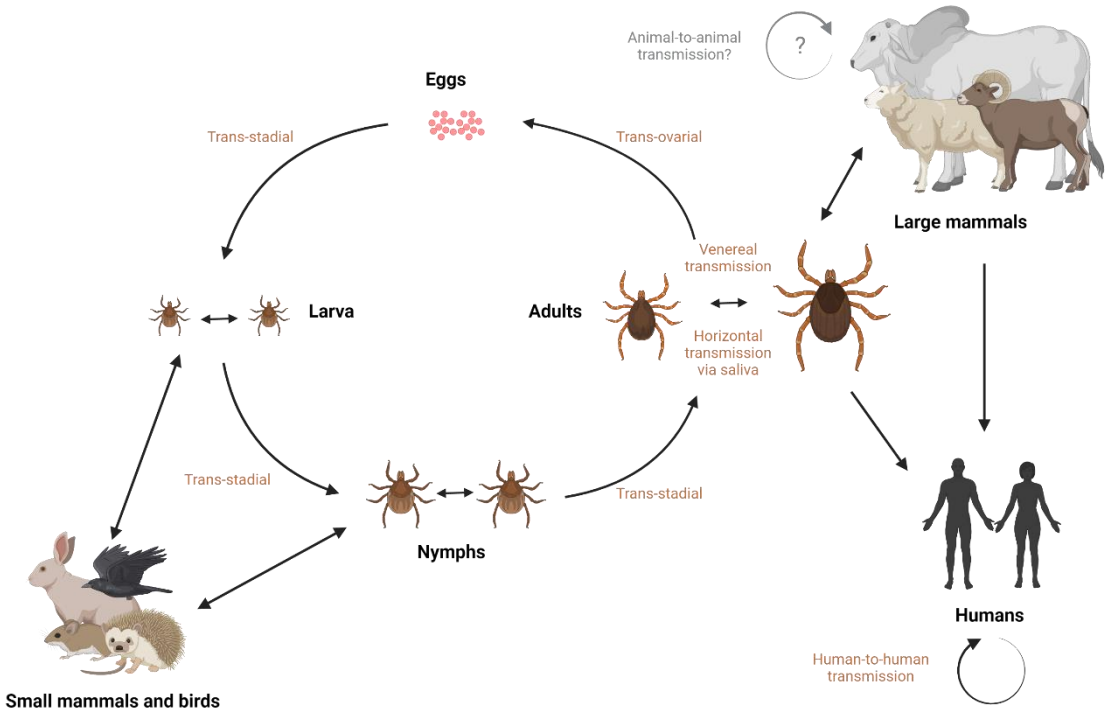

Figure 1.4.2 Transmission routes for CCHFV.

Routes of transmission of CCHFV between typical two-host *Hyalomma* spp., mammalian hosts and humans. Arrows indicate direction of viral transmission, major vertebrate hosts and tick life

stages are reported in black, known routes of transmission are reported in orange text, and a theoretical route of transmission is reported in grey text. Ticks. Created with BioRender.com.

Competent tick vectors are defined as those in which the virus can successfully replicate and pass by trans-stadial transmission from larval to nymph to adult stages, and via vertical trans-ovarial transmission from adult females to their eggs (Figure 1.4.2) (Shepherd et al., 1991, Dohm et al., 1996, Gordon et al., 1993, Bente et al., 2013, Spengler and Estrada-Pena, 2018). Sexual transmission can also occur between males and female ticks at mating (Gonzalez et al., 1992) and horizontal transmission can occur between ticks feeding in close proximity on the same host, via exchange of infected saliva (Shepherd et al., 1989a, Nuttall and Labuda, 2003, Nuttall and Labuda, 2004).

Unlike other nairoviruses, which tend to be specific to a single tick genus (Bente et al., 2013), CCHFV can be transmitted by more than one tick genus (Spengler and Estrada-Pena, 2018, Gargili et al., 2017). Global correlation between recorded CCHFV circulation and *Hyalomma* spp ranges, as well as vector competency studies indicate that *Hyalomma* species are the dominant vector globally, but experimental studies have demonstrated that certain *Rhipacephalus* and *Amblyomma* spp. can also be competent vectors (Gargili et al., 2017, Spengler and Estrada-Pena, 2018). The relative importance of different vector species is likely to vary by geographic region and local conditions. In areas such as sub-Saharan Africa (SSA) where the most common vector species, *Hyalomma marginatum*, is not present, it remains unclear which vector species are the most important (Spengler and Estrada-Pena, 2018, Gargili et al., 2017).

Infection of mammalian hosts occurs when an infected tick bites to feed, leading to viral replication in tissues and entry into the blood stream. The feeding tick may remain on the host for several days or weeks increasing the likelihood that transmission will occur. If infection results in a suitably high level of host viraemia naïve ticks may also become infected through feeding during this viraemic phase, demonstrating the amplifying role animal species can play in viral maintenance. Different mammalian hosts appear to have

different susceptibility to infection, and tick species may also differ in the level of viraemia required for a successful infection (Shepherd et al., 1991, Shepherd et al., 1989a), but the role played by different mammalian species in viral maintenance is poorly understood.

No studies have investigated whether direct animal-to-animal transmission is possible via exposure to viraemic tissues or blood, for example during fighting or parturition, but given that animal-to-human, and human-to-human transmission is known to occur it may be theoretically possible. This hypothesis is further supported by evidence from a recent paper using machine-learning to predict reservoir hosts of RNA virus. Machine learning models used in this work consistently identified CCHFV as falling outside the arthropod vector group, suggesting a possible under-recognised role for direct transmission of the virus (Babayan et al., 2018), although further biological work would be needed to investigate this possible transmission route.

Transmission to humans is usually via the bite of an infected tick or through contact with tissues or bodily fluids of a viraemic animal. Several cases of human disease have been directly linked to animal contact, such as butchering a goat or handling tissue from slaughtered cattle (Nabeth et al., 2004, Chinikar et al., 2010, Mustafa et al., 2011), and risk factor studies investigating associations between human cases and seroprevalence have found increased risk associated with animal contact, butchery and abattoir work (Adham et al., 2021, Mustafa et al., 2011, Kadanali et al., 2009, Lwande et al., 2012, Sargianou et al., 2013). Transmission to humans from animals and ticks is usually sporadic, resulting in isolated cases or small localised outbreaks.

Another important, though less common, route of human infection is via human-to-human transmission through exposure to blood or bodily fluids from a viraemic patient, needlestick injuries, or aerosol-generating procedures (Pshenichnaya and Nenadskaya, 2015, Leblebicioglu et al., 2016c). Most cases of human-to-human transmission occur in a nosocomial setting, with community transmission rare (Tsergouli et al., 2020, Nabeth et al., 2004). Health care workers are most at risk of contracting disease but do not appear

to develop symptomatic infections via this route if appropriate barrier techniques are used (Ergonul et al., 2007). Aerosol routes of transmission are of particular concern as aerosol-generating procedures such as intubation may be performed in the initial phase of the disease when symptoms appear like influenza or other respiratory infections and prior to the implementation of isolation of barrier nursing required to prevent CCHFV transmission (Pshenichnaya and Nenadskaya, 2015, Conger et al., 2015). This fact highlights the importance of patient and clinician awareness of CCHFV as a differential diagnosis and the need for readily available diagnostic tests for rapid diagnosis.

1.4.4 Disease in humans and animals

Clinical cases of CCHF in people can be severe and fatal, but it is estimated that up to 90% infections remain asymptomatic or sub-clinical (Bodur et al., 2012). Where infections do progress to clinical disease, symptom onset typically occurs between 1 and 13 days post-exposure, although this may vary with the route of transmission. The most rapid onset is likely to occur following tick bites, with slightly slower onset following exposure to blood or tissues from infected animals or people (Swanepoel et al., 1987). Clinical manifestation of CCHF typically falls into three phases. Firstly, a pre-haemorrhagic phase occurs in the first week after symptom onset, characterised by fever, malaise, myalgia, headaches and other non-specific signs (Ergönül, 2006, Spiropoulou and Bente, 2021). This may be followed by an acute haemorrhagic phase lasting 2-5 days, with bleeding from various sites and often including splenic and hepatic changes (Fillatre et al., 2019). In fatal cases, death typically occurs 5-14 days after the onset of symptoms (Çevik et al., 2008). If the patient recovers there follows a, sometimes prolonged, period of convalescence where symptoms such as generalised weakness, tachycardia and shortness of breath may continue for up to a year (Spiropoulou and Bente, 2021).

Infections in mammalian hosts, both wild and domestic, are typically considered to be asymptomatic, although research is limited (Spengler et al., 2016b). In livestock species, a small number of studies have been undertaken

to evaluate the outcome of CCHFV infection following experimental infection with the virus. In general, these studies support the idea that common livestock species develop a viraemia that lasts around 5-7 days, but no clinical signs following infection (Zarubinsky et al., 1976, Smirnova, 1979, Wilson et al., 1991). However, one study in cattle (Causey et al., 1970) and two studies in sheep (Gonzalez et al., 1998, Shepherd et al., 1989b) found evidence of mild clinical signs, including a transient fever, dullness and inappetence, in experimentally infected animals suggesting that mild clinical signs may sometimes result from infection.

1.4.5 Serological responses to CCHFV

The high levels of diversity observed in the CCHFV genome do not appear to be replicated in its antigenicity, with little apparent difference observed in serological responses to different clades of the virus (Whitehouse, 2004, Tignor et al., 1980). Neutralising antibodies (NAbs) are raised against the external glycoproteins Gn and Gc during infection (Fels et al., 2021) and are typically detectable around 10 days after the onset of clinical signs, while non-neutralising immunoglobulins are raised both against these proteins and principally against the abundant nucleoprotein (N protein) (Karaaslan et al., 2021, Ergunay et al., 2014). In people, both IgM and IgG antibodies are usually detectable from between 5 and 7 days after the onset of clinical signs, peaking at around 2-3 weeks (Spiropoulou and Bente, 2021). IgM levels remain detectable up to around 4 months, while IgG levels typically remain detectable for at least 3 to 5 years post infection (Spiropoulou and Bente, 2021, Shepherd et al., 1989c). In fatal cases antibody levels are often low or undetectable (Shepherd et al., 1989b).

In livestock, evidence of antibody kinetics and duration of immunity is limited. However, the small number of experimental studies available suggest that IgM levels become elevated 3-7 days following infection and remain detectable for up to three months (Gonzalez et al., 1998). IgG responses become detectable around 24 hours after IgM and may last for 15 months or more (Gonzalez et al., 1998, Wilson et al., 1991). Further evidence of duration of antibody response in livestock has not been investigated

experimentally, but epidemiological studies consistently show increasing seroprevalence with increasing age in livestock, a pattern which is suggestive of a fully immunising infection, producing livelong immunity (Adam et al., 2013b, Balinandi et al., 2021a, Schulz et al., 2021). Epidemiological evidence also supports the possibility of re-infection in livestock, with a longitudinal study undertaken in Senegal finding that seropositive animals infested with ticks had higher antibody levels compared to those without, a pattern that may suggest re-infection (Zeller et al., 1997).

Antibody responses raised against CCHFV have the potential to cross-react with other related orthonairoviruses, although the degree to which this occurs is not fully characterised. Evidence for substantial cross-reactivity between CCHFV and the most important related orthonairoviruses, Nairobi sheep disease virus (NSDV) and Dugbe virus (DUGV), is limited but some studies have found evidence of low levels of cross-reactivity between DUGV or NSDV and CCHFV (Davies et al., 1978, Casals and Tignor, 1980, Ward et al., 1992). However, detection of cross-reactivity is frequently dependant on the testing method, with techniques such as immunofluorescence and hemagglutination-inhibition assays identifying cross-reactivity where ELISAs or neutralisation assays have not (Hartlaub et al., 2021a, Hartlaub et al., 2021b, Davies et al., 1978). Large scale testing of serum samples against potentially cross-reactive orthonairoviruses using ELISA techniques have revealed very low levels of cross-reactivity between CCHFV and NSDV or DUGV (Burt et al., 1996, Grech-Angelini et al., 2020). This suggests that modern, widely used serological techniques such as ELISAs are unlikely to be hindered by substantial cross-reactivity, although further research is required to clarify these antigenic relationships.

1.4.6 Detection of CCHFV infection

Due to the severity of human infection, potential for onwards human-to-human transmission, and lack of effective prophylaxis and treatments, CCHFV is classified as an Advisory Committee on Dangerous Pathogens (ACDP)Hazard Group 4 agent, requiring all direct work with infectious virus to be carried out in containment level-4 (CL4) laboratory conditions (HSE, 2021). Although

live virus assays have generated much of the current knowledge of CCHFV virology, this biosafety level means that work with live virus is not practical in the majority of laboratories, so molecular and serological approaches are vital both for diagnostic and research purposes.

In the acute phase of viral infection, reverse transcription polymerase chain reaction (RT-PCR) can be used to detect viral RNA in serum and bodily fluids from patients (Mazzola and Kelly-Cirino, 2019). However, the high genetic variability observed between different clades of CCHFV can reduce the sensitivity of molecular detection methods (Gruber et al., 2019). This can be addressed through combined use of molecular and serological tests to improve diagnostic sensitivity (Mertens et al., 2013, Fernandez-García et al., 2014, Drosten et al., 2003), as the broad antigenic similarity between CCHFV clade means serological assays as less sensitive to genetic variation. Additionally, in recent years primers sets and multiplex RT-PCR assays have been developed that allow for detection of all viral clades (Atkinson et al., 2012a, Sas et al., 2018b). However, for clinical diagnostics, viral shedding declines around 7-10 days post-infection, often making molecular detection difficult after this (Fillatre et al., 2019). Clinical signs in this first phase of infection are typically those common to other febrile illnesses, so CCHF may not be suspected until it is too late to detect by molecular means.

Serological methods to detect antibodies against CCHFV, either in combination with RT-PCR or alone, are therefore highly important for individual-level diagnostics, as well as being vital for population-level sero-surveillance. Reliable and well-characterised tools for serological detection of CCHFV exist and have been used extensively in clinical and research settings (Vanhomwegen et al., 2012). Several ELISA and immunofluorescent assay (IFA) kits are available commercially to detect IgM and IgG in human patient samples (Emmerich et al., 2021) and some of these have been successfully adapted to detect animal immunoglobulins in research settings (Mertens et al., 2015, Schuster et al., 2016b). Additionally, many laboratories have developed in-house ELISAs for detection of anti-CCHFV antibodies in both human and animal sera (Burt et al., 1993, Dowall et al., 2012b, Mertens et al., 2015). Most serological assays target the abundant nuclear (N) protein,

which results in a strong immune response in humans and animals (Papa et al., 2015). For population surveillance and addressing questions around the ecology of CCHFV in natural hosts, species-independent ELISAs are needed, and several assays have been developed in recent years that allow antibody detection in multiple species through the use of competition or sandwich ELISA formats (Schuster et al., 2016a, Sas et al., 2018a, Shrivastava et al., 2021).

1.4.7 Global distribution

CCHFV has one of the widest geographical distributions of any arbovirus, and molecular or serological evidence of the virus has been detected in more than 30 countries to date (Mild et al., 2010). The virus appears to be endemic in much of south-eastern Europe, the Middle-East, central Asia, the Indian sub-continent and Sub-Saharan Africa, and only remains undetected in the Americas, Australia, and north-western Europe (Messina et al., 2015, Shahhosseini et al., 2021). It has not been detected further north than 47° North latitude, which is also the northern boundary of *Hyalomma* tick range (Esser et al., 2019, Fernandez-García et al., 2014), although several travel-associated cases have been reported in northern European countries beyond this boundary, including the UK (Atkinson et al., 2012b, Leblebicioglu et al., 2016a, Lumley et al., 2014). Most of the early-identified endemic regions were found around the Crimean and Black sea region of Eurasia, but in recent decades new foci of human infections have emerged, with multiple cases now reported annually in countries including Turkey, Iran and Pakistan (Shahhosseini et al., 2021, Spengler et al., 2019). Sporadic and small-scale outbreaks also occur outside these regions, including Uganda, Mauritania, India, and China (Balinandi et al., 2021b, Nabeth et al., 2004, Mourya et al., 2012, Yadav et al., 2014, Sun et al., 2009, Shahhosseini et al., 2021).

Increasing case numbers and instances of detection of CCHFV have contributed to concerns over emergence of the virus into new geographical areas and re-emergence of higher case number in endemic areas (Leblebicioglu et al., 2015, Spengler et al., 2019). The complex ecology of CCHFV, involving multiple vector and mammalian hosts, in combination with

changing climate and alterations in land-use patterns may result in changes to the range of CCHFV and potential emergence of new human disease foci in the coming decades (Gale et al., 2009, Estrada-Pena et al., 2012). Concerns over the potential for emergence in southwestern Europe in particular have elevated the profile of the virus on the international stage (Fanelli and Buonavoglia, 2021, Monsalve Arteaga et al., 2021, Negredo et al., 2021, Spengler and Bente, 2017) but re-emergence in resource poor settings such as many regions of SSA, should be considered as important, and may have greater public health impacts due to limited diagnostic capacity and less robust public health systems (Temur et al., 2021).

1.4.8 CCHFV in sub-Saharan Africa

Although considered endemic in much of SSA (WHO, 2017), knowledge of the epidemiology, disease burden, distribution, and ecology of the virus on the African continent is limited. However, as in Europe and Asia, case reports and seroprevalence studies have increased in the last two decades, and nine SSA countries have reported their first case of CCHF since 2000 (Temur et al., 2021). These include several countries where evidence of the virus in ticks, or serological evidence of exposure in animals had been previously reported. For example, in Kenya, serological evidence of exposure in humans was first observed in the 1980's but the first recognised case occurred in 2000 (Johnson et al., 1983, Dunster et al., 2002). Since then, further evidence of viral presence in ticks and serological exposure in people has been observed and five further sporadic cases reported in the country (Lwande et al., 2012, Sang et al., 2011, Nyataya et al., 2020). Similarly, evidence of serological exposure in Sudan was reported in animals and people in the 1980s and the virus found in ticks collected in the 1990's, but the first recorded human case in the country did not occur until 2008 (Hassanein et al., 1997, Aradaib et al., 2011, Morrill et al., 1990, McCarthy et al., 1996). These patterns are not dissimilar to those seen in other endemic regions, where virus circulation is recorded prior to human infections, but they highlight the potential for future outbreaks of CCHF across the continent. Several countries across SSA have recorded 20 or more cases of CCHF, including Namibia, Nigeria, South Africa, Sudan and Uganda, but ecological or epidemiological reasons for cases

in these countries specifically are not immediately apparent (Temur et al., 2021). Identification of cases in these countries may reflect better diagnostic capacity and awareness of the virus, rather than an intrinsically higher risk of disease in these areas.

1.4.9 CCHFV in Tanzania

Tanzania is considered endemic for CCHFV based on the known presence of competent vector species and a limited body of evidence supporting livestock and human exposure in the country (WHO, 2017). Evidence of livestock exposure is limited to a single study carried out at the East Africa Virus Research Institute (Entebbe) in 1974-5 and reported in Hoogstraal's comprehensive review of CCHFV in 1979 (Hoogstraal, 1979). This study tested 1048 cattle sera from four regions of central and northern Tanzania using an agar-gel diffusion precipitin (AGDP) test. Overall seroprevalence was 9.0%, ranging from 0.6% in the Northern province study sites (Longido, Monduli and Tengeru) to 16.3% in the Lake Victoria coastal region. In the decades since this study was reported no further investigations into animal exposure to CCHFV in Tanzania have been published.

Evidence of exposure in humans is even more limited, and no clinical cases of CCHF have been reported within Tanzania. The only evidence consistent with human transmission in the country was a clinical case reported in Zambia in January 1986. A 26-year-old male traveller became sick several days after leaving Tanzania where he reported being bitten by immature ticks while walking in the Kigoma area in the west of the country. He was treated in South Africa and recovered (Swanepoel et al., 1987). Serological evidence of exposure in humans is also limited, with no studies having investigated this at the start of this thesis research. Very recently, a small study was published which investigated seroprevalence and risk factors for a selection of zoonotic viral haemorrhagic fevers in Tanzania and found evidence of IgG antibodies to CCHFV in six of 500 healthy participants across eight districts of central and south-eastern Tanzania (Rugarabamu et al., 2021).

No evidence is available regarding infections in ticks from Tanzania but of the 28 species of tick found in the country six are confirmed to be competent vectors of CCHFV, as well as other species for which evidence of vector competency has not been demonstrated but that have been found to be exposed to the virus (Walker et al., 2003, Gargili et al., 2017). Competent vector species whose range include northern Tanzania are: *Amblyomma variegatum, Hyalomma rufipes, Hyalomma truncatum, Rhipicephalus apendiculatus, Rhipicephalus evertsi* and *Rhipicephalus pulchellus*.

The lack of research into CCHFV in Tanzania reflects the wider picture of lack of research into the virus across SSA (Temur et al., 2021) but does not reflect the potential true distribution of the virus. A predictive risk mapping study, using an ecological niche modelling approach, was carried out by Messina et al. (2015) and used environmental and vector distribution data from areas with good CCHF reporting to predict risk of CCHF cases across the globe. The resulting risk map predicted areas of high probability for human CCHF cases across the Sahel region of Africa, extending into East Africa, including substantial areas of northern Tanzania (Messina et al., 2015). The limited laboratory-confirmed evidence of CCHFV in Tanzania, in combination with risk-mapping studies, confirms that the virus is present in Tanzania and that more information on its distribution and circulation in animal hosts, humans and ticks is needed to better characterise the risk of this potential public health threat.

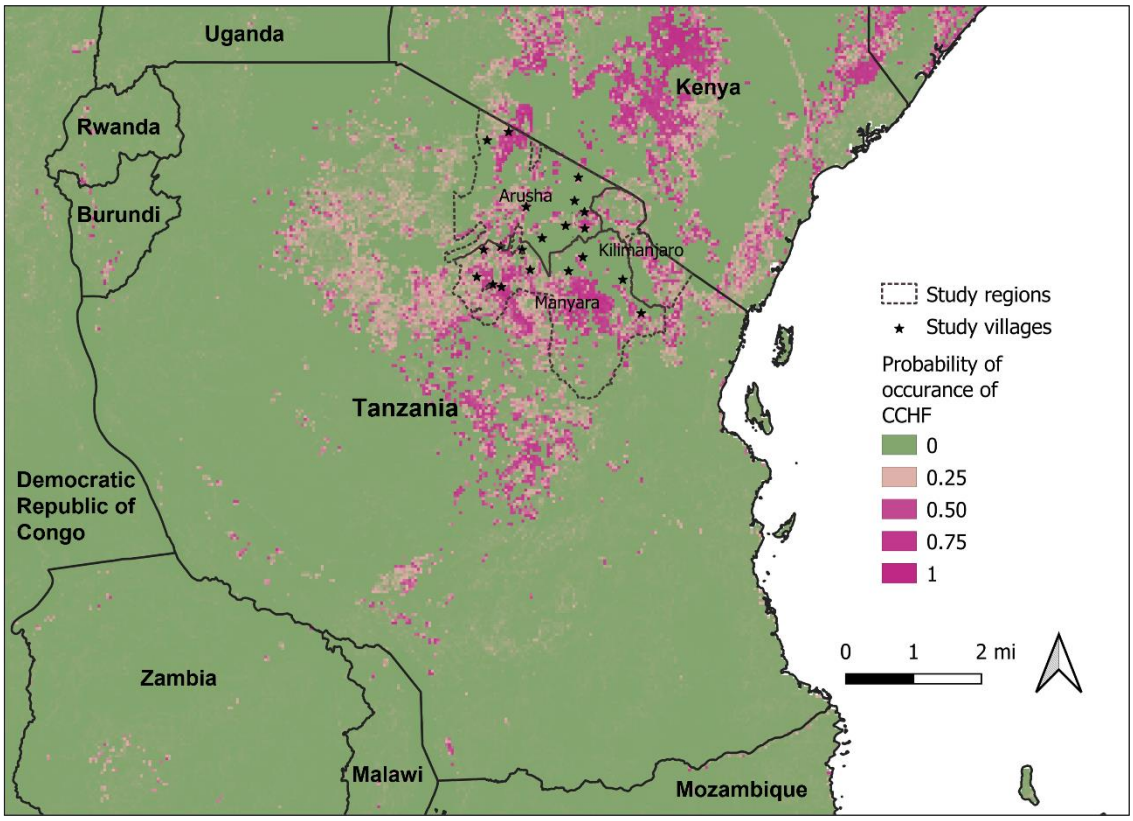

Figure 1.4.3 Probability of CCHF occurance in Tanzania.

Probability of CCHF occurance in people in Tanzania as calculated by Messina et al. (2015). Areas in purple are those most suitable for transmission and areas in green are those least suitable. Study regions and villages investigated in this thesis are highlighted.

1.5 Severe acute respiratory syndrome Coronavirus 2 (SARS-CoV-2)

Since its emergence in late 2019, COVID-19 and its causative agent SARS-CoV-2, have become the most significant public health challenge of our time. As of 3rd March 2022 more than 400 million confirmed infections have been recorded globally and more than 5.9 million deaths (WHO, 2022b). Given the rapid pace and extraordinary volume of research undertaken by the scientific community during this period a comprehensive review of the SARS-CoV-2 pandemic is beyond the scope of this thesis. Ongoing advances in knowledge of SARS-CoV-2 will be discussed as they relate to the work presented in Chapter 3, but research presented in this thesis will be framed in the context of the first pandemic wave in Scotland between March and May 2020.

1.5.1 Emergence

In December 2019 a cluster of pneumonia cases of unknown cause were identified by local health authorities in Wuhan, Hubei Province, China, and were reported to the World health Organisation (WHO) on 31st December (WHO, 2020a). In January 2020 the infectious agent was identified as a novel coronavirus that showed high genetic similarity to, but was distinct from, Sever acute respiratory syndrome coronavirus (now known as SARS-CoV-1) the cause of the SARS epidemic in 2002 (Zhou et al., 2020). Cases in Wuhan increased rapidly between December and January with sustained human-to-human transmission demonstrated (Li et al., 2020a), and the first case outside China was reported on 13th January (WHO, 2020a), although retrospective analysis suggests cases outside China may have occurred before this (Roberts et al., 2021). In February 2020 the novel coronavirus was officially renamed SARS-CoV-2 and the disease caused by it designated as Coronavirus disease 2019 (COVID-19) (WHO, 2020b). During February and March 2020 SARS-CoV-2 infections spread rapidly both within and beyond China, with community transmission established in much of Europe and north America. On 11th March 2020 the WHO declared a global pandemic (Cucinotta and Vanelli, 2020).

1.5.2 SARS-CoV-2 in Scotland and the United Kingdom during the first pandemic wave 2020

The first confirmed cases of COVID-19 in the UK were identified on 28th January 2020 in an individual who had recently returned from Hubei province, China to England and a close household contact who had not travelled (Lillie et al., 2020). In Scotland, the first case of confirmed COVID-19 was reported on 1st March 2020 in an individual who had recently travelled to northern Italy, an area with high case numbers at the time (Hill et al., 2020). Subsequent genomic evaluation of SARS-CoV-2 in Scotland during this period indicated that community transmission rapidly became established in early March, and that multiple introduction events contributed to the growing epidemic (da Silva Filipe et al., 2021). Cases increased rapidly in both Scotland and the rest of the UK during March, and a national lockdown was implemented on 23rd March 2020. Lockdown measures had the effect of dramatically reducing person-to-person transmission through enforced physical distancing and was effective at reducing case numbers, hospitalisations, and deaths (Talic et al., 2021). Restrictions were eased from the middle of May 2020 (IfG, 2021).

1.5.3 Virology and classification

Coronaviruses are a highly diverse family of viruses which infect a range of species and can cause mild to severe disease (Su et al., 2016). Four genera exist within the family - alpha, beta, gamma, and delta - with alpha and beta coronaviruses most significant for humans and animals (Cui et al., 2019). Coronaviruses have been recognised as a serious potential human health risk since the occurrence of two major zoonotic spill-over events in the twenty-first century, with SARS-CoV-1 emerging into human populations in 2002 (Zhong et al., 2003, Ksiazek et al., 2003) and Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012 (Coleman and Frieman, 2013). In recognition of this threat, SARS-CoV-1, MERS-CoV, and novel human coronaviruses were classified as priority pathogens for Research and Development by the WHO in 2018 (WHO, 2022a). Prior to these recent emergences, coronaviruses were not considered to be highly pathogenic in humans, with most causing mild respiratory illness (Cui et al., 2019),

although several alphacoronaviruses were the cause of significant disease in other mammalian species including cats (Feline coronavirus (FeCoV)) and pigs (swine acute diarrhoea syndrome coronavirus (SADS-CoV)) (Alluwaimi et al., 2020, Turlewicz-Podbielska and Pomorska-Mól, 2021).

SARS-CoV-2 is an enveloped positive-sense single-stranded RNA virus of the genus *Betacoronavirus*, family *Coronoviridae*, subfamily *Coronavirinae*, and order *Nidovirales* (ICTV, 2020). The virion is spherical, around 60-100nm in diameter and its external surface studded with spike proteins (S). Membrane proteins (M) and envelope proteins (E) are found between the S proteins and the RNA genome is encapsidated by nucleocapsid protein (N) (Jin et al., 2020b). The spike protein is homotrimeric, with the outward-facing bulbous S1 segment including the receptor binding domain (RBD), and the stalk-like S2 segment embedded in the envelope membrane (Figure 1.5.1). The RBD of the S1 subunit interacts with host Angiotensin-converting enzyme 2 (ACE2) receptors to facilitate cell entry (Huang et al., 2020, Lan et al., 2020).

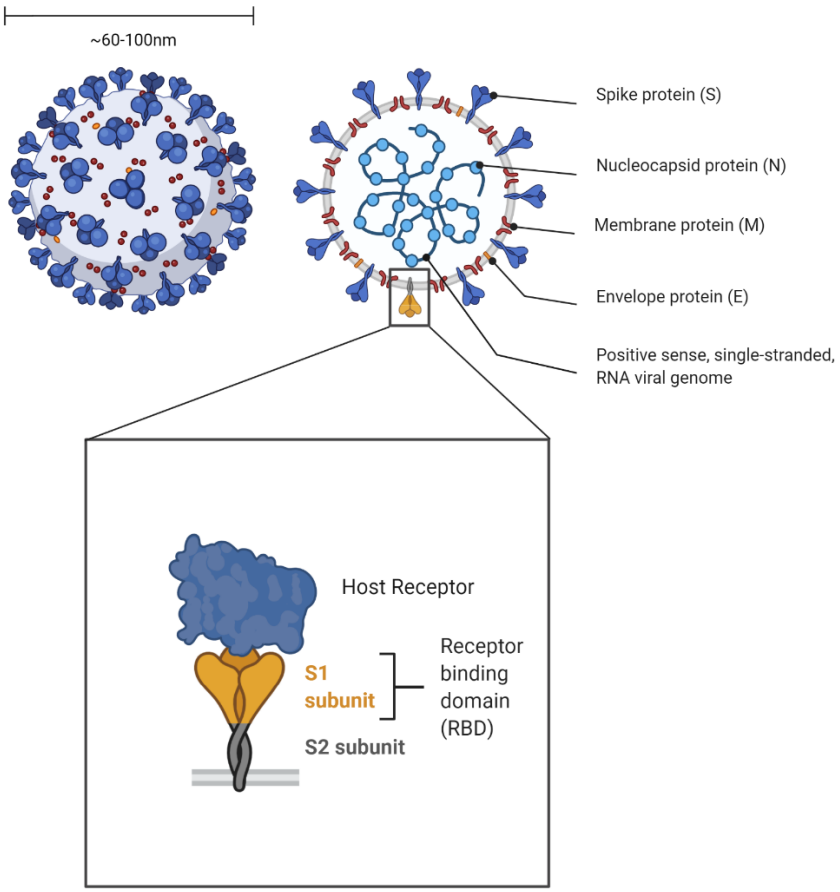

Figure 1.5.1 Schematic of SARS-CoV-2 virion, with inset of the spike protein.
Created with BioRender.com

1.5.4 Serological response to SARS-CoV-2

Antibody responses to SARS-CoV-2 are predominantly raised against the spike protein, principally against the S1 portion that includes the receptor binding domain (RBD), and the nucleoprotein (N) (Pang et al., 2021, Batra et al., 2021), but antibodies are also produced against other structural proteins M and E (Bates et al., 2021, Ahmed et al., 2020) (Figure 1.5.1). Neutralising responses appear to be raised principally against the spike protein but targets include numerous epitopes, including those outside the receptor binding domain (Chi et al., 2020, Wec et al., 2020, Voss et al., 2021). The kinetics of antibody development varies with different antigen targets (Chen et al., 2020b, Chvatal-Medina et al., 2021), as well as disease severity but patterns relating to the development and duration of antibody responses are broadly consistent across populations. Following infection with SARS-CoV-2, IgM antibodies are detectable from around 2 weeks post infection, typically

closely followed by IgG responses, which also become detectable around this time (Post et al., 2020, Zhao et al., 2020, Lou et al., 2020, Ma et al., 2020). IgM levels peak at 2 to 5 weeks post infection before declining to undetectable levels between 6 and 8 weeks (Hou et al., 2020, Jin et al., 2020a). IgG responses typically peak between 3 and 7 weeks post symptom onset and decline slowly over the course of several months (Yamayoshi et al., 2021). Neutralising antibody (NAb) responses also develop early in infection and peak at between 30 and 90 days post infection depending on the severity of disease (Lau et al., 2021). Neutralising responses typically also decline slowly over time (Seow et al., 2020, Crawford et al., 2021), although the duration of NAb responses varies across individuals, declining rapidly in some while persisting at high levels in others (Chia et al., 2021).

1.5.5 Serological diagnostics

During the early months of the pandemic knowledge of the humoral response to SARS-CoV-2 was limited and serological tests were urgently required to investigate this response at an individual and population level. Between January and May 2020 several commercial tests were developed to detect antibodies against SARS-CoV-2, as well as a multitude of in-house assays from different laboratories around the world (Amanat et al., 2020, Krammer and Simon, 2020, Stadlbauer et al., 2020, Zhong et al., 2020). Between January and May 2020 several commercial tests were released on the UK market and used by Public Health England (PHE), Public Health Scotland (PHS) and others to begin evaluating test performance and exploring populations antibody levels (PHE, 2020c, PHE, 2020a, PHE, 2020b). Commonly used tests were the EUROIMMUN-Anti-SARS-CoV-2 ELISA [IgG] (Euroimmun, London, UK), the Abbott Architect SARS-CoV-2 IgG (Abbott, Illinois, USA), and the DiaSorin LIAISON® SARS-CoV-2 S1/S2 IgG (DiaSorin, Saluggia, Italy), which all utilised solid phase immunoassay techniques to detect antibodies to SARS-CoV-2.

In addition to commercially produced assays, research groups and health authorities also developed and utilised in-house assays during this period. Development of these in-house assays was essential to enabling sero-surveillance and immunological investigations to progress during the early

pandemic, when commercial assays often had limited availability or were unsuited to flexible investigations of immune responses. Many in-house assays utilised an indirect ELISA format, which provided simple, specific, and cost-effective quantification of antibody levels. Additionally, pseudotype virus neutralisation assays were also rapidly developed which enabled characterisation and quantification of neutralising responses to SARS-CoV-2 infections. As the pandemic has progressed serological investigations have enabled characterisation of the level, type, and duration of immune response to SARS-CoV-2, providing vital information about natural and vaccine-induced antibody responses, including to the different variants (Chvatal-Medina et al., 2021, Willett et al., 2022).

1.6 Thesis objectives and structure

Emerging viral zoonoses present a global threat to public health both in the form of novel, newly emerged viruses such as SARS-CoV-2, and more established, widespread viruses such as CCHFV, which are re-emerging as a significant risk to human health in both endemic and novel regions but remain under-researched in many areas of the world. Serological methods such as ELISA and PVNAs provide methods to detect past exposure to these viruses, and to characterise the immune response to infection. With these facts in mind, the over-arching goal of this thesis was therefore to develop and then utilise serological methods to investigate the epidemiology of two important emerging viral pathogens, firstly CCHFV, and additionally SARS-CoV-2, in Tanzania and Scotland respectively.

Research carried out for this thesis was led by the author but also involved work undertaken by other colleague and collaborators. For chapters relating to CCHFV, the samples and metadata used were collected as part of the Social economic and envirnonmental drivers of zoonoses (SEEDZ) project in 2016. As these samples were not collected for the specific purpose of this PhD, several people in addition to the author were instrumental in this research. Roles and contributions are summarised in Table 1.6.1.

Table 1.6.1 Contributions of researchers to the SEEDZ study

SEEDZ study	
Activity	**Contributors**
Study design	W. de Glanville, S. Cleaveland, A. Davies
Sample collection and shipment to UK	W. de Glanville, K. Thomas, T. Kibona, SEEDZ field teams
Household questionnaire data collection	W. de Glanville, A. Davies, T. Kibona, SEEDZ field teams
CCHFV serological analyses	E. C. Hughes
CCHFV data analyses	E. C. Hughes

During the pandemic response, work on SARS-CoV-2 was undertaken as part of a team of virologists, immunologists, and epidemiological modellers. All work

reported in Chapter 3 was led by the author but was supported by the wider MRC-University of Glasgow Centre for Virus Research (CVR) serology team. Specific roles and contributions are set out in Table 1.6.2

Table 1.6.2 Contributions of researchers to concept development, implimentation and execution of serology research into SARS-CoV-2 during 2020

Activity	Concept development	Laboratory analysis	Data analysis
Study concept and sample collection	P. Murcia, B. Willett, NHSGGC team		
Screening of community sera by ELISA assay	B. Willett, P. Murcia, NIBSC team	E. Hughes, J. Haney, Y. Parr, B. Willett, I. Herbert	E. Hughes, J. Amat
Screening of community sera by PVNA	B. Willett, N. Logan	N. Logan, U. Arthur	E. Hughes, J. Amat
Evaluation of ELISA assay performance	E. Hughes, B. Willett	E. Hughes, M. Manali, D. Cretu	E. Hughes
Bayesian state space model	M. Viana		M. Viana

This thesis consists of four data chapters, overviews of which are outlined below.

1.6.1 Chapter 2

Work for this chapter reports the adaptation of an existing in-house, indirect ELISA against Rift Valley fever virus (RVFV) for the detection of antibodies against CCHFV, and the subsequent optimisation of this assay for use in sheep and goats. A set of 300 serum samples from livestock in northern Tanzania were tested on both the in-house ELISA and a newly available commercial ELISA kit for the detection of anti-CCHFV antibodies in animals, and results demonstrated the presence of CCHFV antibodies in the country for the first time in forty years.

1.6.2 Chapter 3

This chapter reports work carried out during the first wave of the global SARS-CoV-2 pandemic. The performance of two ELISAs, against the S1 subunit and receptor binding domain (RBD) of SARS-CoV-2, was evaluated and cut-off values determined using receiver operating characteristic (ROC) analyses. These ELISAs were then used to investigate patterns of immunoglobulin G (IgG) responses in a patient population in Glasgow, UK, including weekly seroprevalence throughout the first wave and investigation of heterogeneities in seroprevalence between different patient demographic groups. Additionally, pseudotype virus neutralisation assays (PVNAs) were used to evaluate the neutralising antibody response to SARS-CoV-2 exposure in the same patient population.

1.6.3 Chapter 4

This chapter explors seroprevalence and patterns of exposure to CCHFV in cattle, sheep, goats, and people in linked households in Arusha and Manyara districts of northern Tanzania. Comparison was made of exposure levels between species and mixed effects logistic regression models were used to undertake a general contextual analysis to evaluate the influence of village and household grouping on individual exposure risk, including patterns of spatial autocorrelation in village-level log odds of exposure.

1.6.4 Chapter 5

This chapter built on the work reported in Chapter 4 to investigate individual-, household- and village-level risk factors associated with CCHFV exposure in cattle, sheep, and goats in the study population, through the addition of fixed effects to mixed effects logistic regression models.

1.6.5 Summary

This thesis aimed to fill important gaps in our knowledge of CCHFV in Tanzania, which prior to this research was extremely limited. CCHFV is a serious public health concern given high potential fatality rates amongst people and despite fatalities occurring on the continent, is severely under-

researched across Africa. Changes to working practices and priorities as a result of the emergence of SARS-CoV-2 and subsequent pandemic led to the introduction of a second pathogen to this thesis research. As a result, an additional goal of this thesis was to use similar techniques to those used to explore CCHFV, to investigate diagnostic test performance and the epidemiology of SARS-CoV-2 in Glasgow, UK.

2 Development of an indirect ELISA for the detection of CCHFV antibodies in livestock

2.1 Introduction

2.1.1 Background to assay development

At the beginning of research for this thesis no assay for the detection of antibodies to Crimean-Congo haemorrhagic fever virus (CCHFV) in animal sera was commercially available. Previous studies into seroprevalence in animals had used a variety of techniques, either adapting versions of human ELISA tests or through development of in-house assays (Burt et al., 1993, Sas et al., 2018a, Schuster et al., 2016a). For this reason, in order to be able to investigate exposure to CCHFV in Tanzanian livestock, an in-house ELISA was developed to detect CCHFV antibodies in livestock sera. However, during the initial phases of this research a commercial assay became available from IDvet (ID Screen® CCHF Double Antigen Multi-species ELISA, IDvet, Grabels, France). This assay was initially used to compare and inform the results of the in-house assay and was not intended for further use due to financial constraints. However, funding for the IDvet ID Screen® test kit was later provided through the Supporting Evidence Based Interventions project (University of Edinburgh, grant number R83537 CH) enabling all samples to be tested using this commercial kit. To ensure continuity and facilitate meaningful comparisons in the analysis all samples for epidemiological investigations (see Chapters 4 and 5) were subsequently tested using this commercially produced ELISA. However, the in-house test continues to have value as an alternative method of identifying seropositive animals and so the work into its development and optimisation is presented below.

2.1.2 Enzyme-linked immunosorbent assays (ELISAs)

Enzyme-linked immunosorbent assays (ELISAs) are a type of solid phase immunoassay commonly used in infectious disease research to detect antibodies to a specific pathogen in serum samples from animals or people. ELISAs can take several forms but a simple and frequently used format is the

indirect ELISA, which was used in the work presented below to detect anti-CCHFV antibodies. The structure of an indirect ELISA is outlined here and visually represented in Figure 2.1.1**Error! Reference source not found.**. Initially, the antigen of interest is bound to the bottom of the wells of a 96-well plate manufactured to have a high binding affinity for proteins. On addition of serum, this fixed antigen is bound by its primary antibody, if antibodies against the antigen are present in the serum sample. At this stage a secondary antibody targeting the bound IgG and conjugated with an enzyme such as alkaline phosphatase (ALP) or horseradish peroxidase (HRP), is added. Finally, a suitable substrate is added which undergoes a colour change in the presence of the conjugated enzyme. The optical density (OD) or absorbance of this coloured product can then be read.

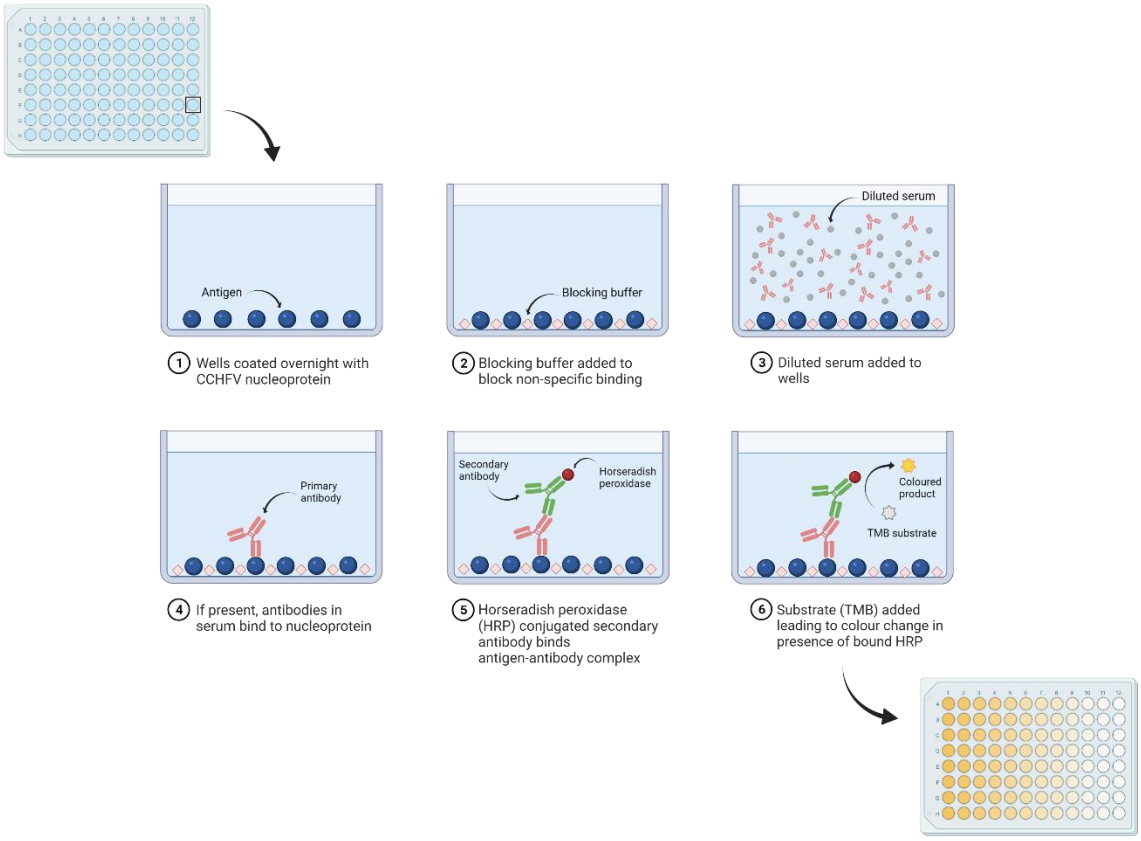

Figure 2.1.1 Principals of indirect ELISA used to detect antibodies against Crimean Congo haemorrhagic fever virus (CCHFV) antibodies.
Wells of a 96-well plate are first coated with antigen of interest (1), before being blocked with the blocking buffer (2). Diluted serum is then added to the coated well (3) and if antibody is present this binds to the fixed antigen (4). A secondary antibody conjugated with horseradish peroxidase is then added and binds to the bound antigen-antibody complex (5). Finally, a substrate (TMB) is added which undergoes a colour change in the presence of HRP. This signal is then read as optical density. Created with BioRender.com.

Although antigen-antibody binding is highly specific, excess “noise” or background OD can be detected in ELISA assays if non-specific binding is not adequately blocked. Following fixation of the antigen to the polystyrene base of the well, it is essential to perform a blocking step where-by areas of the well not coated in antigen become coated with the blocking buffer. This buffer is typically a solution of non-reactive protein which prevents non-specific binding to anything other than the bound antigen but does not obscure or alter the epitopes for antibody binding.

Optical density (OD) readings are usually interpreted either in relation to a defined cut-off value, above which samples are considered positive and

below negative, or in relation to a quantified control or series of controls with known quantities of the antibody of interest present. This enables the plotting of a standard curve from which tested sample OD values can be compared. Alternatively, samples can be tested in serial dilution to calculate an antibody titre for each sample.

2.1.3 ELISA optimisation

Factors most likely to affect ELISA performance include but are not limited to, volume of antigen, sample dilution factor, concentration and type of secondary antibody, blocking buffer, and duration and temperature of incubation steps. Optimisation of the in-house CCHFV ELISA was undertaken using an experimental design approach whereby factors likely to affect ELISA performance were identified and then systematically investigated (Shaw et al., 2015, Sitta Sittampalam et al., 1996). Key factors were identified and initially screened one-factor-at-a-time (OFAT), followed by a factorial approach whereby one or more factors were investigated together.

2.1.4 Chapter objectives

Work presented in this chapter had the following objectives:

1. Adapt an existing in-house ELISA against Rift Valley fever virus (RVFV) to detect IgG antibodies against CCHFV in livestock
2. Screen samples from a suspected endemic region to identify strongly and weakly reacting samples
3. Optimise the in-house CCHFV ELISA to maximise difference between negative and positive samples and to minimise laboratory time and resources

2.2 General methods

The work presented in this chapter was undertaken in an iterative manner, with each experiment building on the results of previous investigations. Specific methods and results for each experiment are therefore presented together to aid clarity. This general methods section outlines the steps taken in the research for this chapter, as well as the protocols for the initial ELISA assay, and the IDvet ID Screen® CCHFV ELISA assay.

2.2.1 Chapter workflow

The flow chart below shows the steps taken in the development and optimisation of the in-house indirect ELISA described in this chapter.

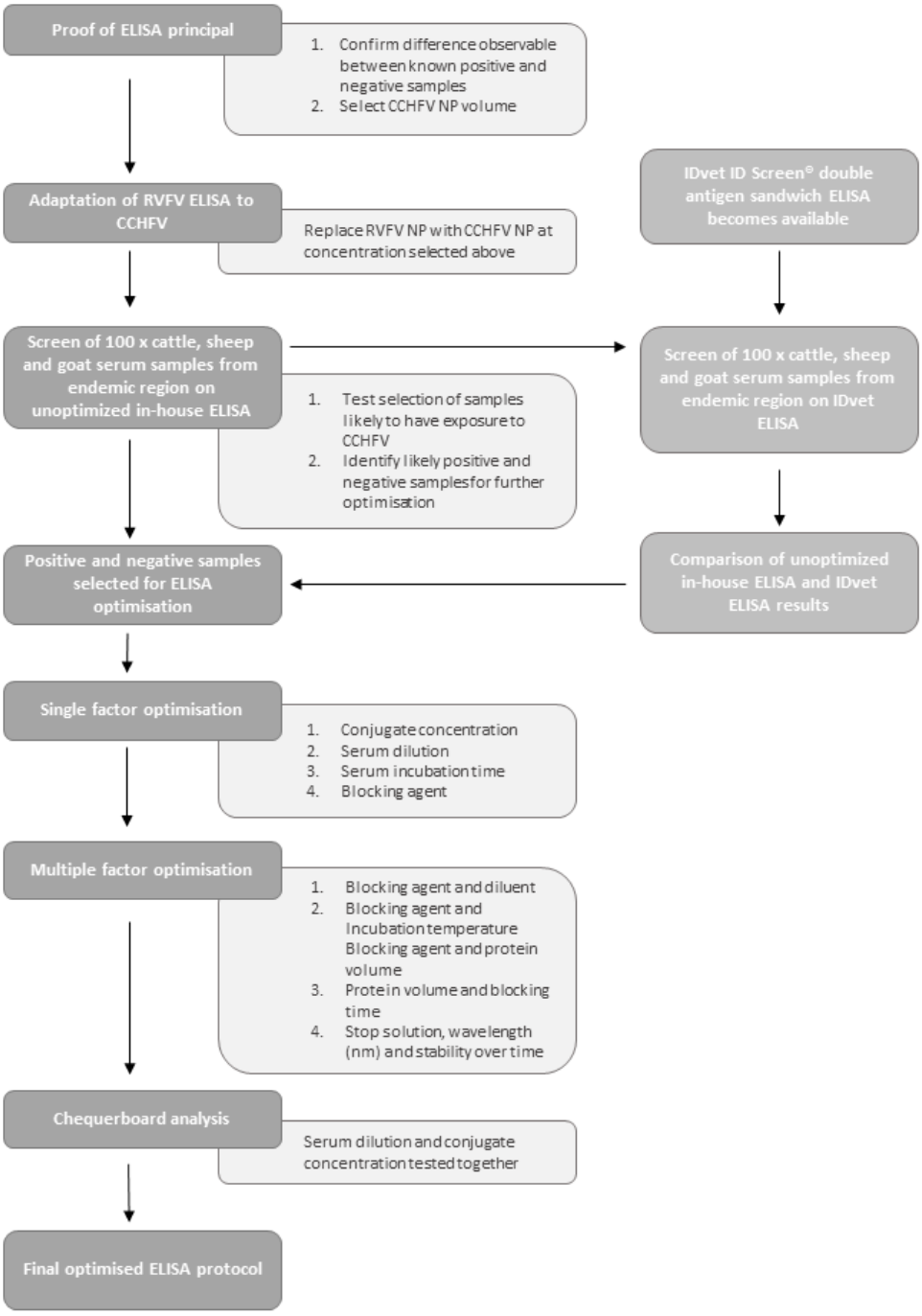

Figure 2.2.1 Flow diagram of steps taken in the research carried out for this chapter

2.2.2 Protein production

CCHFV nucleoprotein (NP) of CCHF-Baghdad-12 strain (GenBank accession, AJ538196) was obtained from Public Health England (PHE). Full production

methods can be found in Dowall et al. (2012b). Briefly, CCHFV NP of the above strain was expressed in a recombinant baculovirus vector (rBV). *Spodoptera frugiperda* 21 (*Sf*21) cells were infected and incubated with a this rBV and infected cells harvested 48h post-infection. Cells were lysed and 6x-histidine-tagged recombinant CCHF nucleoprotein purified by immobilized metal ion affinity chromatography (IMAC). Samples were also tested against RVFV and Severe Fever with Thrombocytopenia syndrome virus (SFTSV) nucleoproteins (NP) to confirm the specificity of the response. RVFV and SFTSV NP used for proof of principal testing were produced at the University of Glasgow by Dr. Ping Li using an *Escherichia coli* vector to express 6x-histidine-tagged RVFV or SFTSV NP, which was then purified using IMAC (Nyarobi, 2019).

2.2.3 Un-optimised indirect ELISA protocol

The initial ELISA protocol was based on a previous in-house ELISA developed to detect RVFV antibodies (Nyarobi, 2019). Firstly, 96-well 2HB Immulon® ELISA plates (ImmunoChemistry Technologies, LLC, Minnesota, USA) were coated with 100ng/well CCHFV NP in 100µl/well coating buffer (100mM sodium bicarbonate and 33mM sodium carbonate anhydrous pH 6.9) and incubated overnight at 4℃. Following incubation, coating buffer was removed, and plates washed five times with the wash buffer (Phosphate buffered saline (PBS) containing 0.1% Tween-20 (PBST)). Next, 200µl/well blocking buffer, made up of 1:10 casein (10x casein solution, Vector laboratories inc. Burlingame, CA 94010) in PBS was added and plates incubated at room temperature for one hour. Blocking buffer was removed and plates washed five times as previously described. Serum samples were diluted 1:400 in the dilution buffer (10% casein in PBST) and loaded into a 96-well PCR plate prior to 100µl/well being added to the ELISA plate using a multichannel pipette. Plates were incubated at room temperature for two hours. Samples were tested either in triplicate or duplicate within-plate repeats. Following washing, 100µl/well of the appropriate species secondary antigen conjugated with horseradish peroxidase (anti-human, anti-goat, anti-sheep and anti-cattle IgG (heavy and light chain) conjugate: Bethyl Laboratories, Montgomery, USA) was diluted 1:1000 with dilution buffer

before being added to the plates and incubated at room temperature for 1 hour. Following another washing step, 100µl/well tetramethylbenzidine (TMB) substrate (Sigma-Aldrich) was added before incubation in the dark for 15 minutes. Finally, 50µl/well H_2SO_4 was added to each well to stop the reaction and the OD value read at a wavelength of 420nm.

2.2.4 IDvet ID Screen® CCHF Double antigen multi-species ELISA

The ID Screen® is a novel double antigen sandwich ELISA released by IDvet (IDvet, Grabels, France) in 2018 and based on the assay developed by Sas et al. (2018a). 96-well plates were supplied ready-coated with recombinant purified CCHF NP antigen. All reagents were supplied as concentrated solutions for dilution with distilled water, freeze dried for reconstitution with a supplied buffer, or ready to use. All plates were run as per the test kit protocol. Samples were prepared, in singlicate, in an empty 96-well PCR plate. 30µl sample was diluted in 50µl dilution buffer. Supplied negative and positive controls were also prepared in the same way but in duplicate. After dilution, controls and samples were loaded onto the pre-coated ELISA plate using a multichannel pipette and the plate was incubated for 45 minutes at room temperature. Plates were next washed 5 times with a minimum of 300µl wash solution per well, before adding 50µl of appropriately diluted conjugate to each well. Plates were then covered and incubated for 30 minutes at room temperature. After this, plates were again washed and 100µl of substrate solution added to each well before incubation in the dark for 15 minutes at room temperature. Finally, 100µl of stop solution was added to each well and optical density read at 450nm.

Plates were validated if the mean of the positive control OD was greater than 0.350 and the ratio of the mean positive and negative control OD was greater than 3. For interpretation, raw OD values were used to calculate the S/P% (sample OD/positive control OD * 100), which was used to assign positive or negative status. Samples with S/P% ≤30% were considered negative, while those > 30% were positive.

2.2.5 Statistical analysis

Statistical analysis and data visualisation was undertaken in Graphpad Prism, version 8.4.0 (Prism). Most data were non-normally distributed so non-parametric tests, Mann-Whitney tests and Kruskal-Wallis tests, were used to compare two or more groups of OD values. The coefficient of variance (CV) was calculated to compare the variation between sample repeats (Standard deviation of OD/Mean OD*100). Comparison between IDvet ID Screen® OD results and in-house OD results was undertaken using Pearson's correlation coefficient. Statistical significance was set at p value ≤ 0.05.

2.3 Initial screen

2.3.1 Proof of principal

To confirm a response to CCHFV protein in an indirect ELISA format, six human serum samples with known serological status was tested against the nucleoprotein of three related bunyaviruses, CCHFV, RVFV and SFTSV, also known as Dabie bandavirus (Kuhn et al., 2020), using the protocol described above.

Serum from a confirmed case of CCHF, known to be positive for CCHFV antibodies, was obtained from Public Health England (PHE) and used as a positive control. Pooled serum from the measles UK negative reference sera (WHO International Standard, 3rd International Standard for Anti-Measles, NIBSC code: 97/648) was used as a negative control. It was assumed that the contributing individuals to these pooled sera had not been exposed to CCHFV, RVFV or SFTSV, as these are all absent from the UK. All serum samples were tested against 50ng, 100ng and 200ng CCHFV NP, as well as 100ng RVFV NP and 100ng SFTSV NP as controls. In the original RVFV ELISA 50ng/well of protein was found to be optimal but 200ng of protein was included for the initial attempt based on the use of 0.2µg of CCHFV protein used in previously described in-house indirect ELISA's (Maiga et al., 2017, Mertens et al., 2015). Anti-human IgG conjugate was used.

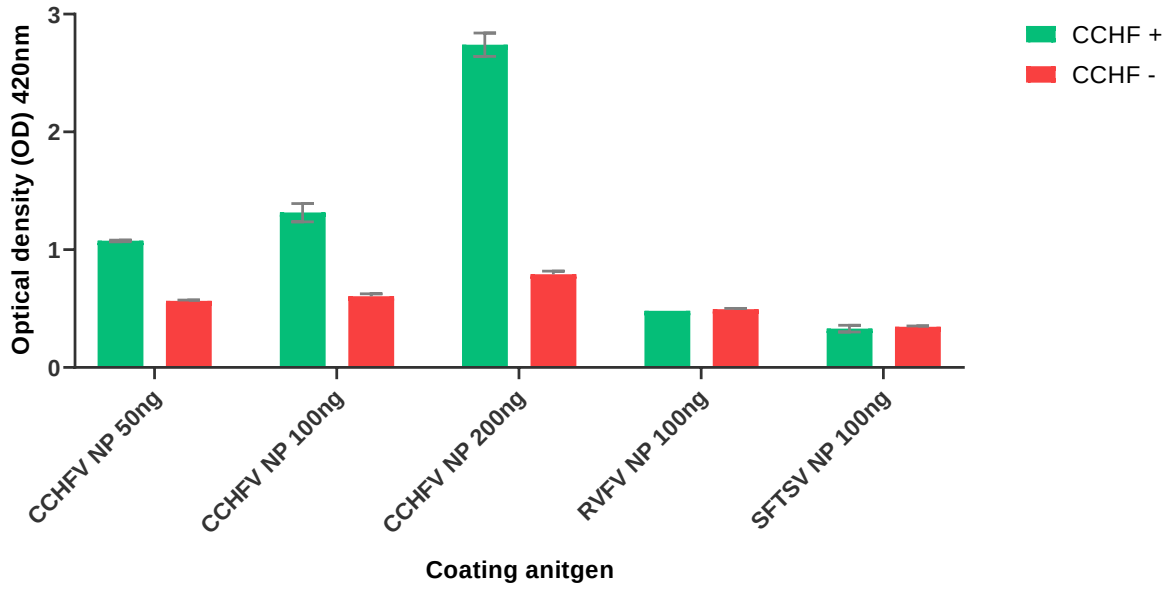

Figure 2.3.1 Proof of principal testing for an in-house indirect ELISA to detect antibodies against CCHFV.

Samples known to be positive or negative for antibodies to CCHFV were tested against three volumes of CCHFV nucleoprotein (50ng, 100ng and 200 ng) as well as 100ng RVFV nucleoprotein and100ng SFTSV nucleoprotein as controls.

Results showed that the positive CCHFV serum resulted in higher OD values against all concentrations of CCHFV N protein when compared to the negative control. This confirmed that the ELISA could differentiate between positive and negative samples. The relative difference in OD values between positive and negative samples at different volumes of protein demonstrated that altering assay parameters could enhance the differentiation ability of the assay. No difference was observed in OD values for the positive and negative CCHFV samples against the RVFV or SFTSV proteins, suggesting that antibodies to CCHFV are not cross-reacting with these viral antigens.

2.3.2 Sample selection

Following this confirmatory step, a subset of samples from cattle, sheep, and goats, were selected for use in exploring initial responses to CCHFV via the unoptimized ELISA and the IDvet ID Screen® described above. One hundred samples from each species were randomly selected from the Social, Economic and Environmental Drivers of Zoonoses project (part of Zoonoses and Emerging Livestock Systems program, funded through BBSRC, DfID, ESRC, MRC, NERC and DSTL - grant no: BB/L018926/1), a large cross-sectional study

of livestock conducted northern Tanzania in 2016. A full description of the study and dataset can be found in Chapter 4.

2.3.3 Un-optimised ELISA screen

Initial screen samples were tested using the basic protocol described above. All samples were first tested on the unoptimized in-house ELISA to identify likely positive and negative samples which could then be used for further optimisation. Wells were coated with 100ng/well CCHFV N protein, based on the results of the proof of principal tests. Cattle and goat sera of New Zealand (NZL) origin and sheep sera of UK origin were used as negative controls. A positive control was not available at the time of testing so was not included on these screening plates. All samples were tested in duplicate.

2.3.4 IDvet ID Screen® CCHF Double antigen multi-species ELISA

Following release of the ID Screen® CCHF Double Antigen Multi-species kit (IDVet, Grabels, France), in 2018, the initial screen samples were also tested using this kit for comparison with the in-house assay. Plates were run as per the test kit protocol, described above, with each sample run in singlicate. The true status of these samples could not be determined from the commercial ELISA alone as it was not a true gold standard test. However, in the absence of a gold standard diagnostic test, such as live virus neutralisation, and without access to samples of known status (for example through experimental infection), the ID Screen® was the best available test for determining antibody status and so was used as a proxy for a gold standard.

2.3.5 Results

2.3.5.1 Comparison of unoptimized in-house ELISA and ID Screen results

Screening of the initial cattle, sheep and goat samples using the un-optimised in-house ELISA demonstrated clear distinctions between samples with low and high OD values. Following testing of these samples using the ID Screen® ELISA it was possible to categorise these into likely-positives and likely-negatives. OD values of samples obtained on the in-house test were categorised as

positive or negative based on the results of the ID Screen®. Figure 2.3.2 shows that there was a significant difference between median OD values for all three species, showing good distinction between positive sample OD values and negative sample OD values for sheep and goats in particular, and to a lesser extent for cattle.

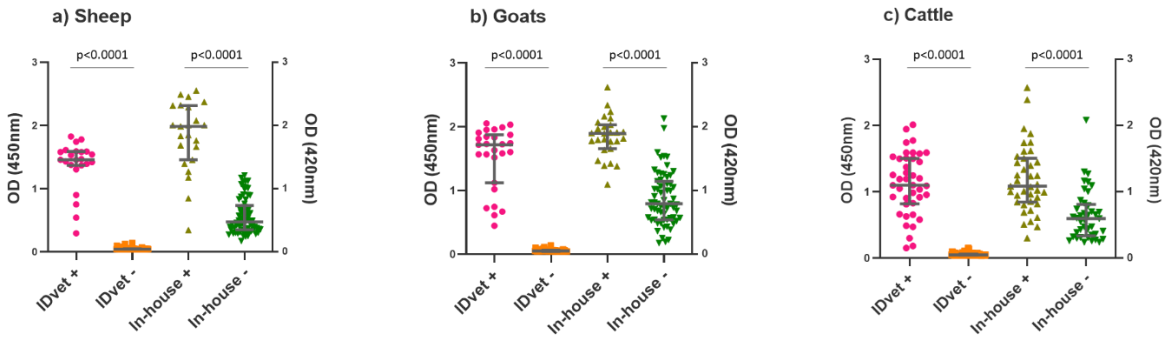

Figure 2.3.2 Comparison of IDvet ID Screen® CCHFV ELISA and an in-house CCHFV ELISA antibodies in a) sheep, b) goats and c) cattle.

Samples were categorised as positive or negative based on the results of the IDvet ELISA. Error bars show median and interquartile range.

Correlation between OD values from the in-house ELISA and ID Screen® were significant for all species but were particularly well correlated in sheep (r = 0.90, 95% CI 0.85-0.93, p=<0.0001) compared to goats (r=0.74, 95% CI 0.63-0.82, p=<0.0001), compared to cattle (r=0.69, 95% CI 0.55-0.79, p=<0.0001) Figure 2.3.3.

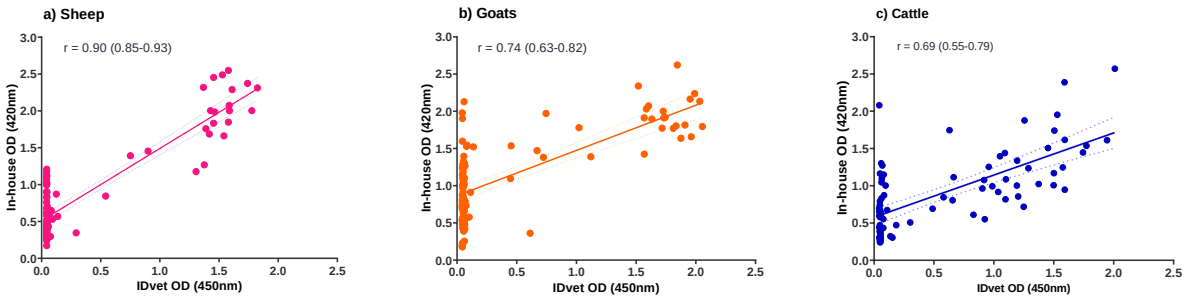

Figure 2.3.3 Correlation between IDvet ID Screen® CCHFV ELISA OD values and in-house CCHFV ELISA OD values for a) sheep, b) goats and c) cattle samples.

Pearson's correlation coefficient (r) and 95% confidence intervals are shown.

2.4 Optimisation of CCHFV Nucleoprotein ELISA for sheep and goats

2.4.1 Aims

Results of the initial screen using the unoptimized ELISA suggested that there was more overlap between negative and positive responses to CCHFV in cattle compared to sheep and goats (Figure 2.3.2 and Figure 2.3.3). For this reason, the latter two species were initially chosen as the focus of assay optimisation and steps were taken to optimise the performance of the in-house assay. A Design of Experiment (DOE) approach was taken in the optimisation of the assay (Sitta Sittampalam et al., 1996, Shaw et al., 2015).
The aims of the optimization process were:

1. Minimise the OD values of negative controls (i.e. to reduce background noise)
2. Maximise the ratio of positive OD to negative OD (PN ratio)
3. Minimise the use of resources and cost (e.g. the amount of CCHF protein used)
4. Minimise the time taken to complete the ELISA

2.4.2 Sample selection

Eight goat samples and eight sheep samples were selected for further optimisation of the assay. Three goat samples (GP1-GP3) and 4 sheep samples (SP1-SP4), shown to be positive on the ID Screen® and also to have high OD values on the unoptimized in-house ELISA, as well as four samples from each species (goats, GN1-GN4; sheep, SN1-SN4) that were negative on the ID Screen® with correspondingly low OD values on the in-house ELISA, were selected as reference samples for further optimisation of the in-house assay (

Table 2.4.1). As no cut-off values were available for the unoptimized ELISA, high and low values were characterised as those in the top or bottom 20% of initial screen samples tested, respectively. One inconclusive goat sample (GW1), which was negative on the ID Screen® but had a high OD on the in-house ELISA was also selected. No inconclusive sheep samples were identified from the two ELISAs. Between two and four positive and negative samples from each species were used in optimization experiments, depending on the plate set up for each step.

Table 2.4.1 Optical density values from unoptimized in-house ELISA , ID Screen® CCHF Double antigen multi-species ELISA and status of Tanzanian sheep and goat samples used for optimisation.

Sample mean OD and positive and negative control mean OD are shown for each assay.

Species	Sample code	In-house mean OD (420nm)	In-house negative control mean OD (420nm)	ID Screen OD (450nm)	ID Screen positive control OD (450nm)	ID Screen negative control OD (450nm)	Sample status
Sheep	SN1	0.28	0.79	0.0432	0.58055	0.0453	Negative
	SN2	0.25	0.79	0.0436	0.58055	0.0453	Negative
	SN3	0.29	0.82	0.0443	0.58055	0.0453	Negative
	SN4	0.24	0.82	0.0425	0.58055	0.0453	Negative
	SP1	2.37	0.79	1.7414	0.58055	0.0453	Positive
	SP2	2.29	0.82	1.61	0.58055	0.0453	Positive
	SP3	2.31	0.82	1.8251	0.58055	0.0453	Positive
	SP4	2.55	0.82	1.5793	0.58055	0.0453	Positive
Goats	GN1	0.18	1.14	0.0444	0.7507	0.04435	Negative
	GN2	0.52	1.05	0.0607	0.7507	0.04435	Negative
	GN3	0.43	1.14	0.048	0.7507	0.04435	Negative
	GP1	2.03	1.05	1.5851	0.7507	0.04435	Positive
	GP2	2.34	1.14	1.5186	0.7507	0.04435	Positive
	GP3	1.92	1.05	1.7434	0.7507	0.04435	Positive
	GP4	2.16	1.05	1.9521	0.7507	0.04435	Positive
	GW1	1.98	1.14	0.0436	0.7507	0.04435	Inconclusive

2.4.3 Screening tests

Factors were initially investigated one-factor-at-a-time (OFAT) and were typically tested without in-plate repeats in order to minimise the amount of protein used. Testing was carried out in an iterative fashion, altering the protocol based on the results of the previous experiment. At this stage, some factors, such as incubation temperature and protein volume, with two or three levels, were tested against each other. Where results showed a convincing preference for one combination of factors over another, no further investigations were carried out. If results were inconclusive, or where there was a plausible biological reason for interaction between factors, further investigations were undertaken. A fractional factorial approach, whereby the factors shown to be most influential on the outcome of the assay in the screening tests or those with large impacts of assay cost or usability were investigated further in combination with each other, was taken to maximise the useful outputs from limited resources. A summary of factors investigated and how they were tested can be found in Table 2.4.2
.

Table 2.4.2 Factors investigated during the optimisation of an in-house indirect ELISA against CCHFV nucleoprotein.

Levels of the factor investigated, the type of screening test used and a summary of the results are shown.

Factor	Levels	Screening tests	Results
Conjugate	8 levels - doubling dilutions from 1:1000 to 1:128000	Single factor screen; chequerboard analysis	1:8000 for goat sample and 1:4000 for sheep samples resulted in highest PN ratios
Serum	9 levels – doubling dilutions from 1:100 to 1:25600	Single factor screen; chequerboard analysis	1:400 (original protocol) resulted in the highest PN ratio in both species
Blocking agents	10% casein in PBS; 10% casein in PBST; 1%, 2%, and 4% Bovine serum albumin (BSA); Commercial tris-buffered gelatin block (Roche)	Single factor screen; also investigated against protein volume, incubation times and incubation temperature	10% casein in PBST performed consistently well across all blocking tests with low negative OD values and high PN ratios.

Diluent	PBS; 10% casein in PBST; 1%, 2%, and 4% Bovine serum albumin (BSA); Commercial tris-buffered gelatin block (Roche)	Investigated in conjunction with blocking agent	10% casein in PBST as both blocking agent and diluent for all stages of the assay consistently resulted in the lowest negative OD values and highest PN ratios.
Protein volume	100ng; 50ng	Tested against blocking agent (4% BSA and 10% casein) and block incubation time (15 minutes and 1 hour)	100ng protein gave consistently high PN ratios for both species and improved PN ratios compared to 50ng for sheep.
Stop solution	H2SO4; HCL 1N; HCL 2N	Single factor screen and compared against wavelength	No difference between stop solutions was observed at either wavelength (420nm or 450nm). HCL was more stable over time for both concentrations so was selected.
Wavelength	650nm (no stop solution); 420nm; 450nm	Investigated with stop solutions	450nm resulted in slightly higher negative OD values but much higher PN ratios. This wavelength will be used to read the optimised assay.

Serum incubation time	2 hours; 1 hour	Single factor screening and compared against original and updated conjugate concentrations	No difference was observed in mean OD values when serum was incubated for 1 hour rather than 2 hours. Serum will be incubated for 1 hour for the optimised protocol.
Block incubation time	1 hour; 15 minutes	Single factor screening and compared against protein volume	Blocking well for 15 minutes compared to 1 hour led to no difference in average OD values. Blocking will be carried out for 15 minutes in the optimised assay.
Incubation temperature	Room temperature; 37°C	Investigated in conjunction with blocking agent.	Room temperature for all incubation steps led to lower negative OD values and higher PN ratios. All steps will be carried out at room temperature in the optimised assay.

2.4.4 Assessment of results

Low background noise, as represented by low negative sample OD values, and the greatest distinction between positive and negative samples (signal to noise ratio), as represented by high positive to negative (PN) ratios, were prioritised when deciding which factor level or combination to take forward for further investigation. PN ratios were calculated as the mean of the positive samples divided by the mean of the negative samples for each species in each experiment. Sample GW1 typically behaved as a positive sample during optimisation but due to uncertainty surrounding its true status it was excluded from the mean value and PN ratio calculations. Where the difference between levels was qualitatively assessed as small, simplicity of assay protocol was favoured. Final conditions were selected to give the greatest distinction between positive and negative samples using minimal resources to ensure time and cost-effectiveness.

2.4.5 Single factor evaluation

2.4.5.1 Conjugate concentration

Doubling dilutions of anti-goat IgG conjugate and anti-sheep IgG conjugate (Bethyl Laboratories, Montgomery, USA) were prepared, ranging in concentration from 1:1000 to 1:128000. All selected samples were tested against each concentration on a single plate using the original protocol described above. OD values decreased with decreasing concentrations of conjugate in all samples in a sigmoidal manner (Figure 2.4.1). Mean values of positive and negative samples were used to calculate the PN ratio (Figure 2.4.2). PN ratios indicated that the conjugate concentration used in the original protocol (1:1000 for both species) was not optimal for maximising the distinction between positive and negative samples. The OD graphs (Figure 2.4.1) also showed high levels of background at this concentration. PN ratios suggest that a concentration of 1:8000 for goats and 1:4000 for sheep provide the greatest distinction between positive and negative samples. The large difference in outcome across the dilution levels demonstrates that conjugate

concentration is an important factor and so results from this factor were used to inform subsequent optimisation steps.

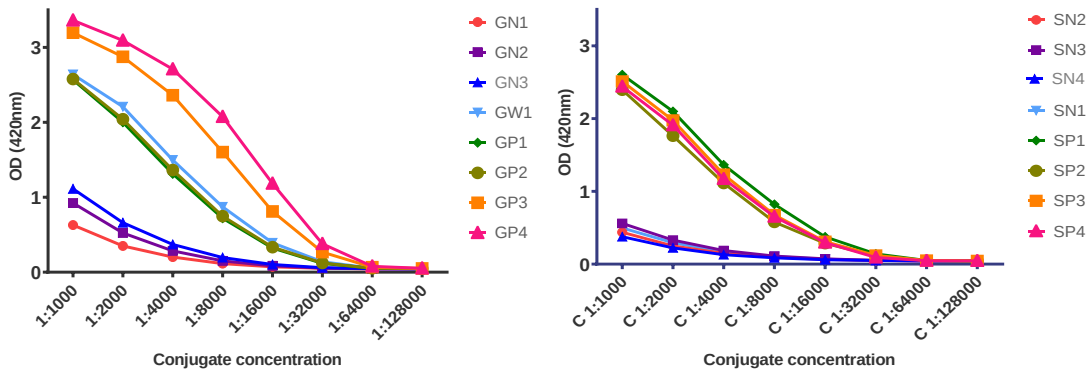

Figure 2.4.1 OD values of goat (left) and sheep (right) serum samples tested using doubling dilutions of conjugate

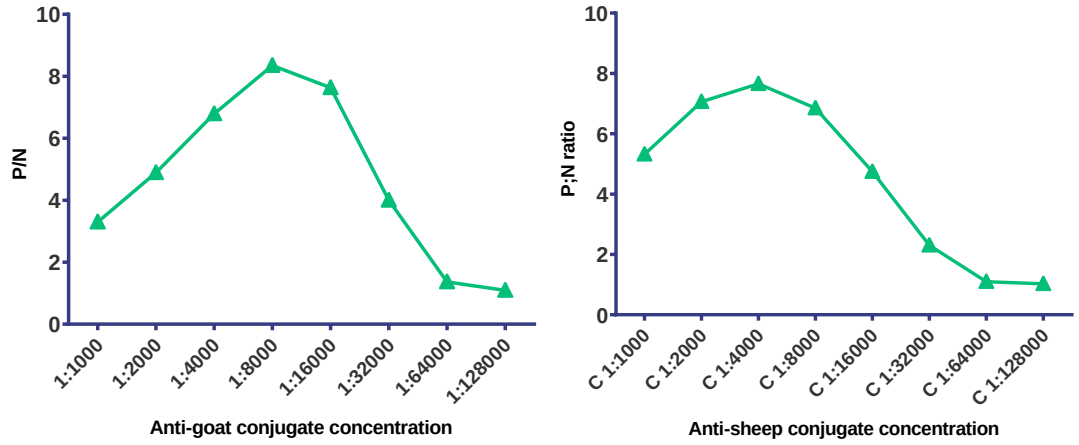

Figure 2.4.2 PN ratio of mean OD values of goat serum (left) and sheep serum (right) samples tested using doubling dilutions of conjugate.

2.4.5.2 Serum dilution

Doubling dilutions of serum were prepared using 10% casein PBST as the diluent in concentrations ranging from 1:100 to 1:25600. All parameters from the original protocol were used apart from the conjugate concentration which were updated based on the results of the conjugate plates (goat conjugate 1:8000; Sheep conjugate 1:4000).

OD values increased with decreasing serum dilutions in a sigmoidal manner in both species (Figure 2.4.3). PN ratios (Figure 2.4.4) indicated that the serum

dilution used in the original protocol (1:400) was optimal for maximising the PN ratio in both species.

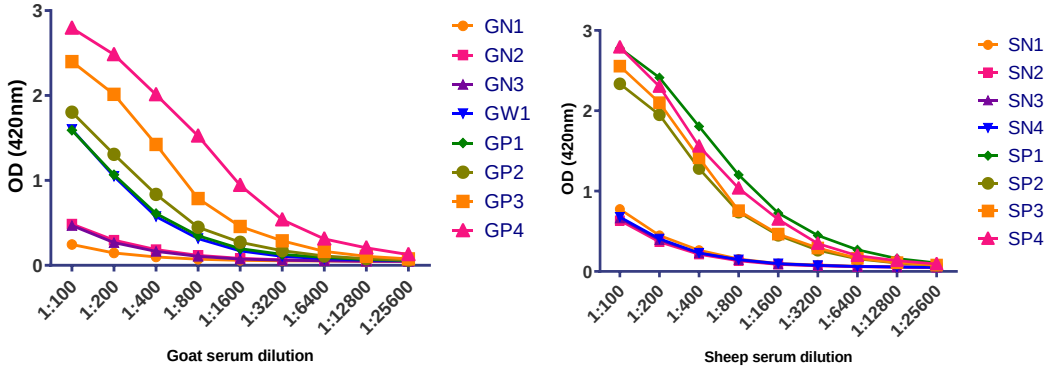

Figure 2.4.3 OD values of goat (left) and sheep (right) serum samples tested in doubling dilutions.

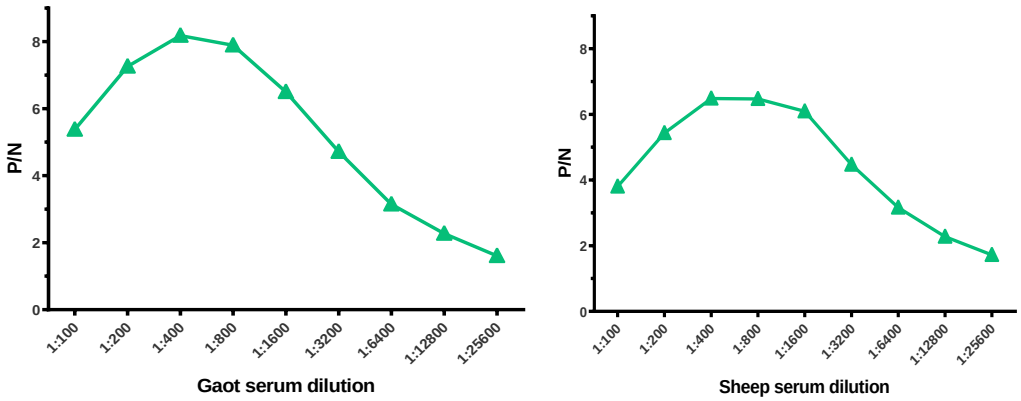

Figure 2.4.4 PN ratio of mean OD values of goat serum (left) and sheep serum (right) samples tested using doubling dilutions of conjugate.

2.4.5.3 Serum incubation time

A comparison was made of the OD values for serum incubated on the plate for 2 hours, as per the original protocol, verses 1 hour. Samples were tested using both the original (Goats 1:1000; Sheep 1:1000) and updated (goats 1:8000; sheep 1:4000) conjugate concentrations. Serum was diluted 1:400. OD values resulting from serum incubation times of 1 hour compared to 2 hours were highly consistent using either the original or the updated conjugate concentration (Figure 2.4.5). All samples had coefficients of variance (CV) (SD/Mean*100) under 10% and no significant difference was

found between the mean OD values in the different groups. Based on these plates, the incubation time for further screening tests was changed to 1 hour.

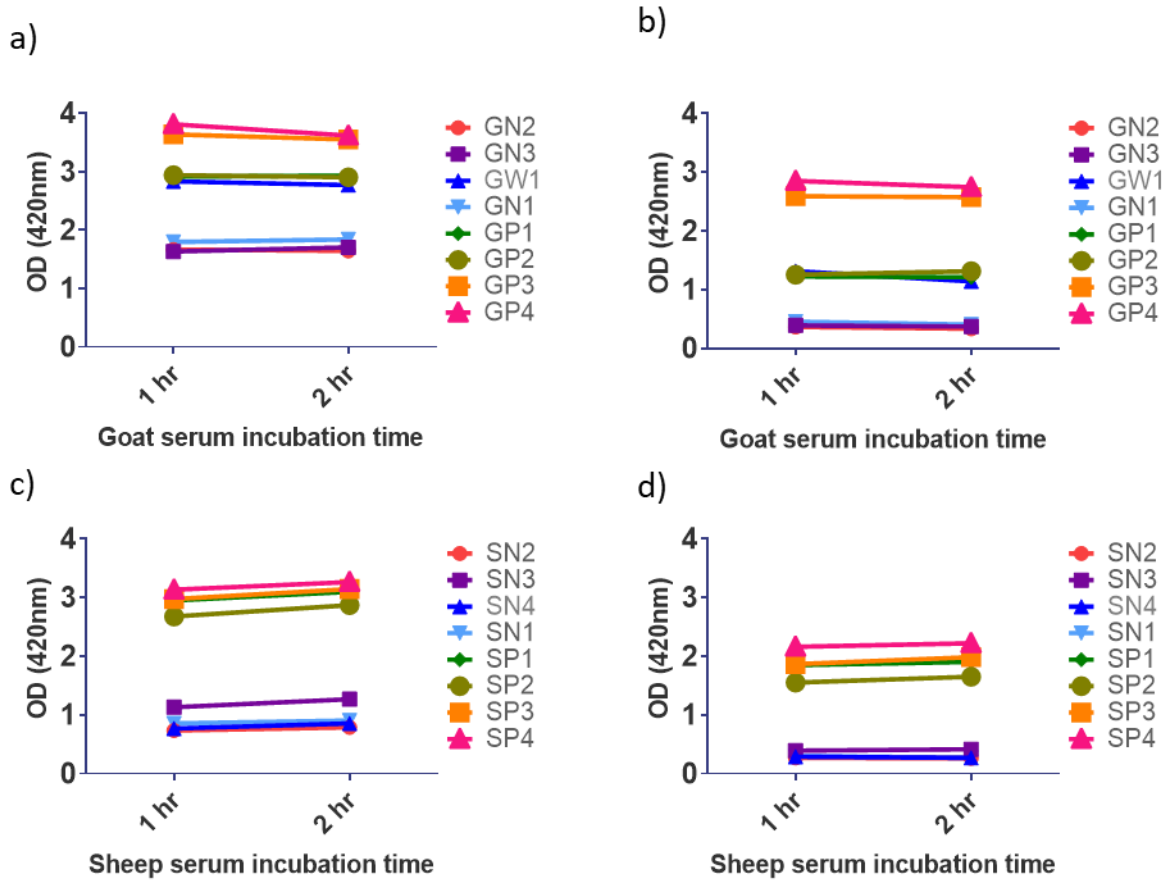

Figure 2.4.5 Comparison of serum incubation time and conjugate concentration. Comparisons of 1 hour verses 2 hour serum incubations for goat samples with a) 1:1000 conjugate and b) 1:8000 conjugate and sheep samples with c) 1:1000 conjugate and d) 1:8000 conjugate

2.4.5.4 Blocking agent

Six alternative blocking solutions were first tested using the original protocol, but with conjugate concentration and time of serum incubation updated according to the outcome of the previous steps (i.e. conjugate concentration: goats 1:8000; sheep 1:4000 and serum incubation time 1 hour). The different blocking solutions were used throughout the assays both as blocking solution and as the diluent for serum and conjugate dilution. The only exception to this was the 10% casein in PBS block, which was used as per the unoptimised protocol with serum and conjugate diluted using 10% casein and PBST. Blocking agents were chosen based on those used in previous in-house assays,

other reported ELISAs and those produced commercially for this purpose (Dowall et al., 2012b, Sas et al., 2018a). Blocking agents tested were: the original blocking agent of 10% casein in PBS; 10% casein in PBS with Tween 0.05% (PBST); solutions of 1%, 2% and 4% bovine serum albumin (BSA) in PBST, and a commercially produced Tris-buffered gelatin-based blocking agent (Roche diagnostics GmbH, Roche Applied Science, 68298, Mannheim, Germany). Background noise in OD values, which can be caused by poor blocking of non-specific binding, is seen most clearly in negative control OD values, so blocking effectiveness was assessed by comparison of negative sample OD values, as well as the PN ratio.

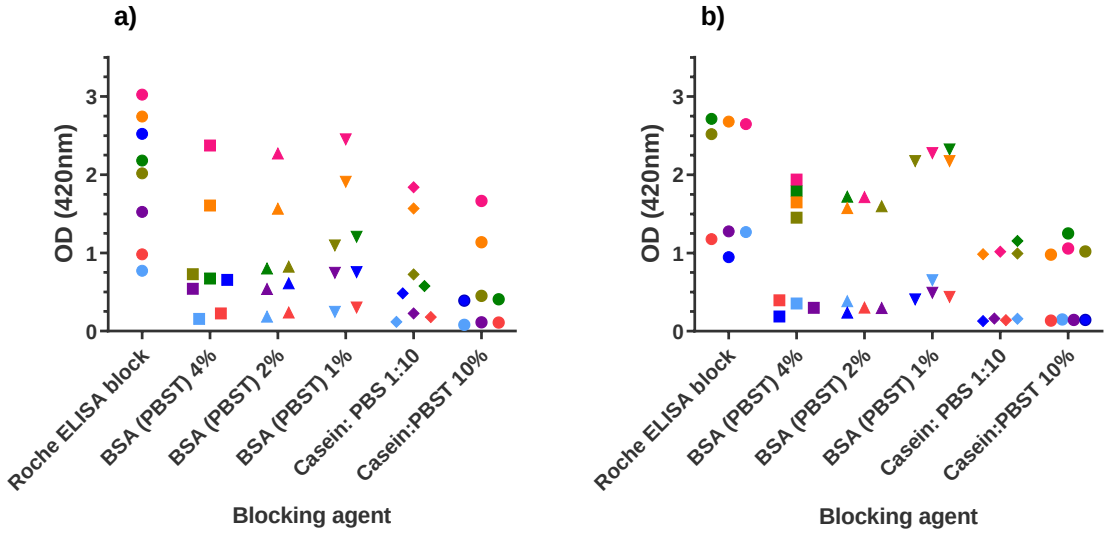

Figure 2.4.6 Comparison of different blocking agents in a) goat and b) sheep samples.
Six blocking solutions are compared: Roche ELISA block, bovine serum albumin (BSA) (PBST) 4%, BSA (PBST) 2%, BSA PBST) 1%, Casein: PBS 1:10, and Casein PBST 1:10.

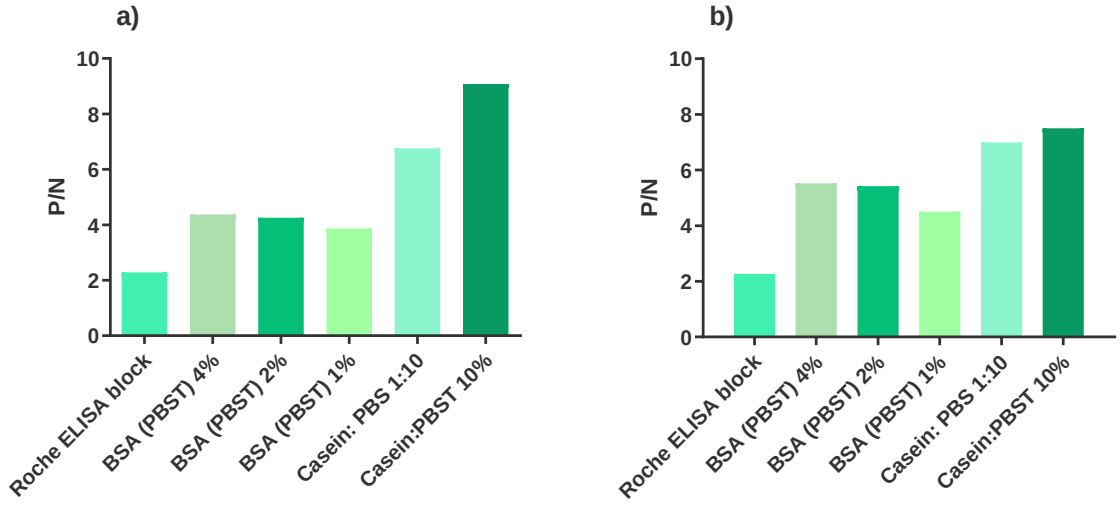

Figure 2.4.7 PN ratios for a) goat and b) sheep samples tested with different blocking agents.
Six blocking solutions are compared: Roche ELISA block, bovine serum albumin (BSA) (PBST) 4%, BSA (PBST) 2%, BSA PBST) 1%, Casein: PBS 1:10, and Casein PBST 1:10.

The best performing blocking agent was 10% casein in PBST, which had the lowest negative sample OD values and the highest PN ratio for both goats and sheep (Figure 2.4.6 and Figure 2.4.7).

To confirm this finding, this blocking agent as well as the best performing BSA concentration (4%) were repeat tested over several days using two negative and two positive samples from each species. The commercial block was also repeated due to observed inconsistencies in opacity between batches, which may have had an impact on the product's performance. A new batch was used for the repeat testing. Casein produced consistently lower mean negative OD values, and higher PN ratios compared to both alternative blocks, although the performance of the commercial blocking agent was substantially worse than either the casein or BSA based blocks (Figure 2.4.8 and Figure 2.4.9). The casein block also results in smaller coefficient of variation of repeated samples, suggesting that the use of this blocking agent may also lead to more consistent and repeatable results.

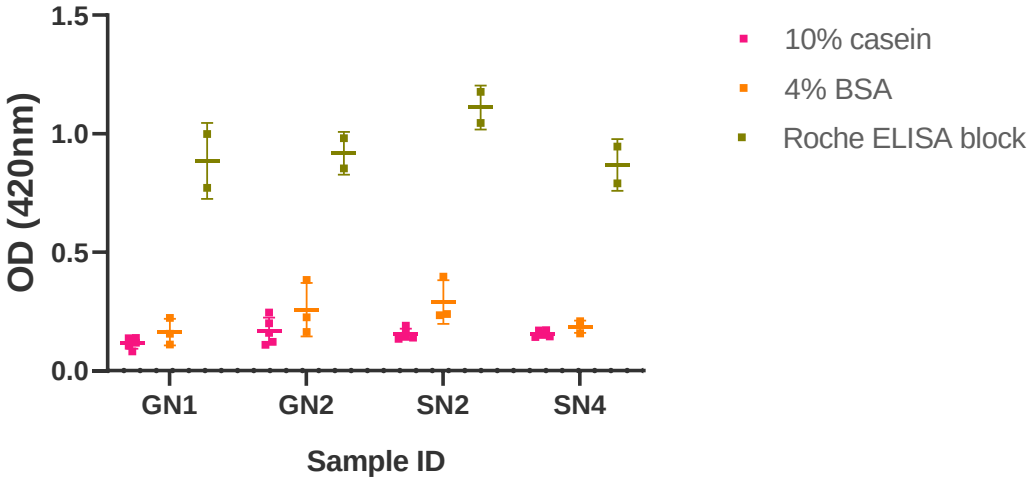

Figure 2.4.8 Comparison of three blocking agents.

Error bars show mean OD and standard deviation for negative goat and sheep samples.

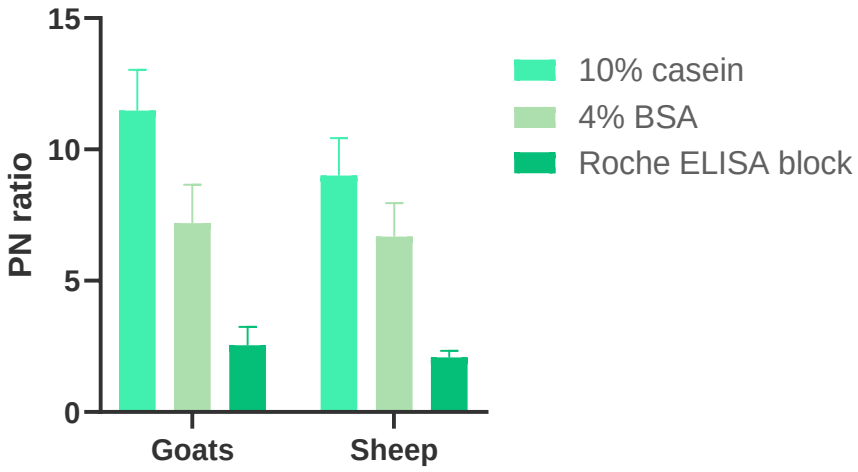

Figure 2.4.9 Comparison of PN ratios for three different blocking agents.

Error bars show SD of the mean.

Across all blocking experiments, casein-based blocks resulted in lower negative sample OD values, compared to both BSA-based blocking agents and the commercial gelatin-based block, as well as maximising PN ratios. In the unoptimized protocol the blocking solution was 10% casein in PBS while the diluent for serum and conjugate was 10% casein in PBST. Comparison of this protocol with 10% casein in PBST used for all blocking and dilution steps showed marginal improvement in both negative control OD and PN ratio. Ten percent casein in PBST was therefore used for all further experiments. This

combination also had the added practical advantage of increasing assay simplicity by using the same solution for both blocking and dilution steps.

2.4.5.5 Conclusions from single factor evaluation

Following investigation of the above factors the following adjustments or confirmations were made to the ELISA protocol:

1. Conjugate concentration was changed to a 1:8000 dilution for anti-goat IgG (heavy and light chain) conjugate (Bethyl Laboratories, Montgomery, USA) and to a 1:4000 dilution for anti-sheep IgG (heavy and light chain) conjugate (Bethyl Laboratories, Montgomery, USA)
2. Serum dilution was maintained at 1:400 for both goat and sheep ELISAs
3. Serum incubation time was reduced to 1 hour

2.4.6 Multiple factor evaluation

2.4.6.1 Blocking agent and serum/conjugate diluent

To further investigate the benefit of using the 10% casein in PBST blocking agent for all three stages (blocking, serum dilution and conjugate dilution), comparisons were made between different combination of blocking agent and PBST in the three stages. Both 10% casein in PBST and 4% BSA were investigated as blocking agents in the following combinations: 1) blocking agent used for all stages, 2) blocking agent used for block and serum dilution, 3) blocking agent used for blocking stage only, with PBST used for stages where the blocking agent was not used.

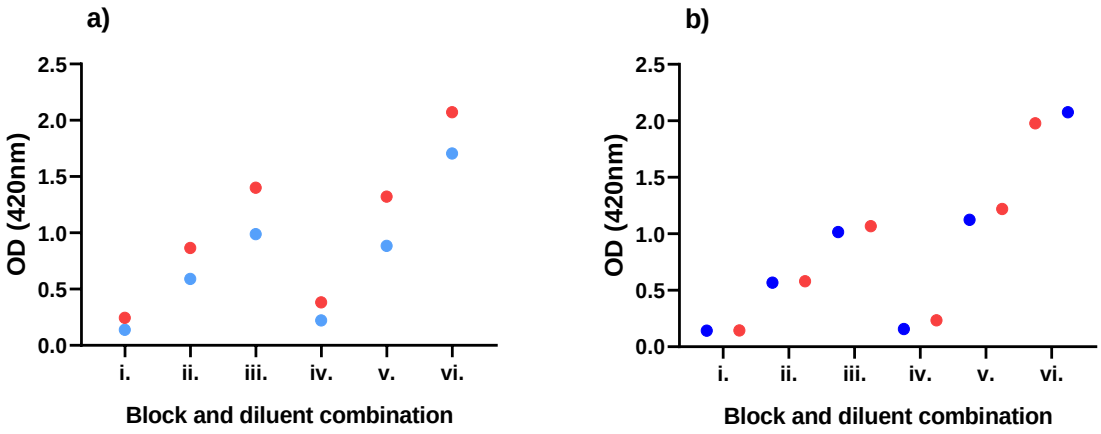

Figure 2.4.10 Comparison of the use of blocking agent or PBST alone in three different assay stages, blocking, serum dilution and conjugate dilution.

OD values of negative samples from a) goats and b) sheep resulting from the following combination of blocking agent and diluent: i. 10% casein for all stages; ii. 10% casein used as block and for serum diluent, PBST used as conjugate diluent; iii. 10% casein used as block, PBST used for serum and conjugate diluent; iv. 4% BSA used for all stages; v. 4% BSA used

as block and serum diluent, PBST used as conjugate diluent; vi. 4% BSA used as block, PBST used as serum and conjugate diluent.

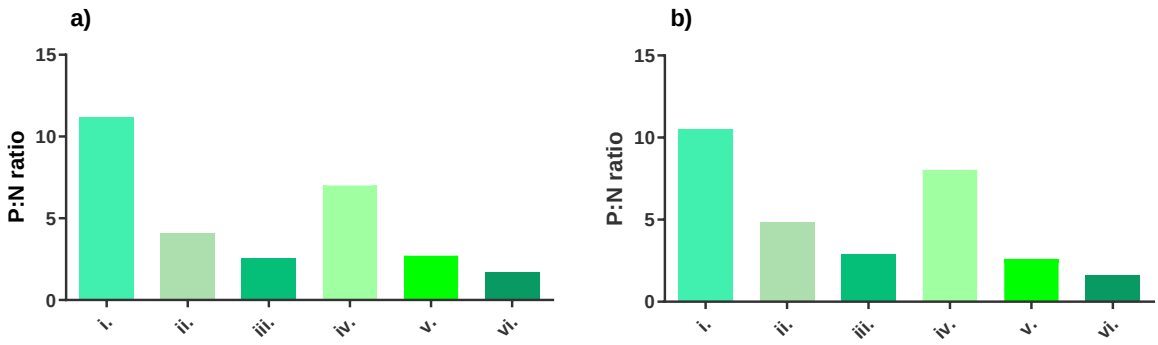

Figure 2.4.11 Comparison of PN ratios for the use of blocking agent or PBST alone in three different assay stages, blocking, serum dilution and conjugate dilution.
PN ratios for a) goat and b) sheep samples resulting from the following combination of blocking agent and diluent: i. 10% casein for all stages; ii. 10% casein used as block and for serum diluent, PBST used as conjugate diluent; iii. 10% casein used as block, PBST used for serum and conjugate diluent; iv. 4% BSA used for all stages; v. 4% BSA used as block and serum diluent, PBST used as conjugate diluent; vi. 4% BSA used as block, PBST used as serum and conjugate diluent

Use of either 10% casein or 4% BSA blocking solution for all stages of the assay resulted in significantly lower negative OD values and higher PN ratios compared to use of PBST for one or both dilution steps (Figure 2.4.10 and Figure 2.4.11). This demonstrated the benefit of using a blocking agent throughout the assay. These finding indicate that blocking action is beneficial during serum and conjugate incubation as well as for blocking background activity in the ELISA plate wells.

2.4.6.2 Incubation temperature and blocking agent

Comparisons were made to assess the impact of temperature during blocking, serum and conjugate incubation periods between room temperature (19.5°C) and 37°C. For room temperature (RT) steps the assay was undertaken as in the previous runs. For the 37°C level ELISA plates were placed in a humidified incubator at 37°C and 5% CO_2 for 1 hour during block, serum and conjugate

incubation steps. Comparisons of incubation temperature were carried out in conjunction with blocking agent comparisons described above (2.4.5.4).

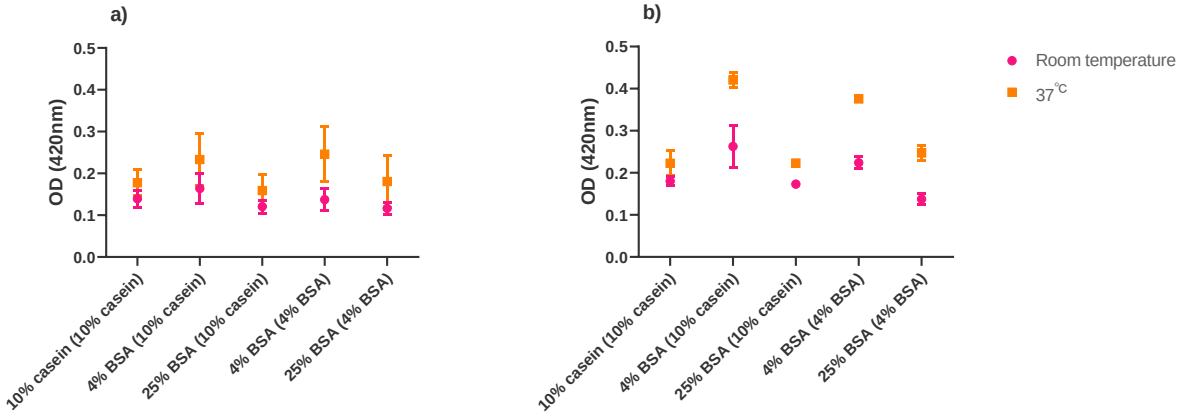

Figure 2.4.12 Comparison of assay temperature and blocking agent.
Mean OD values (error bars show range) of negative samples from a) goats (GN1 and GN2) and b) sheep (SN2 and SN4) resulting from incubation at RT or 37℃ of various combinations of blocking agents and diluents.

Negative OD values were consistently lower under RT conditions compared to 37℃ (Figure 2.4.12). PN ratios were higher under RT conditions across all blocking agent and serum diluent conditions (Figure 2.4.13). 10% casein in PBST resulted in the best combination of low OD and high PN ratio under both temperatures.

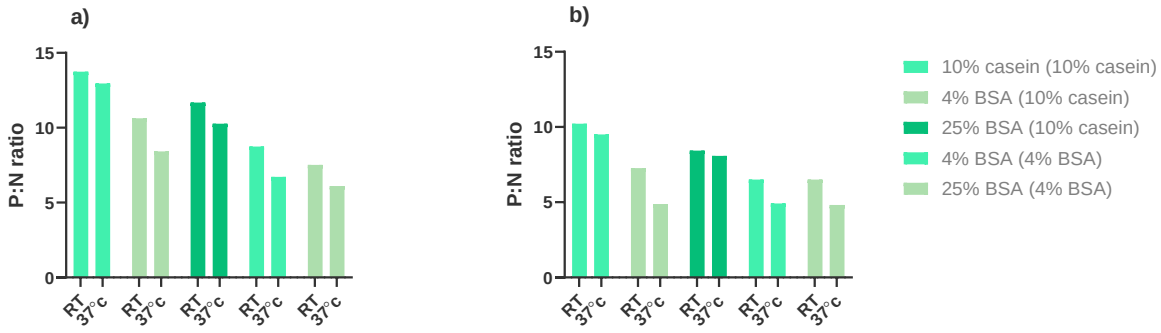

Figure 2.4.13 Comparison of PN ratios for assay temperature and blocking agent.
PN ratios resulting from a) goat and b) sheep samples tested under two different temperature and five different block and diluent conditions. Diluent solution is shown in brackets next to blocking agent.

25% BSA with casein as a diluent produced similar OD values to 10% casein in PBST used for both and would likely provide a similar assay performance. However, 25% BSA solution was challenging to prepare due to the high viscosity of BSA at high concentrations and the protocol required preparation of different solutions for block and diluent, reducing the efficiency of the protocol. Higher PN ratios for 25% BSA block with 10% casein in PBST as a diluent compared to 25% casein with 4% BSA as a diluent suggests that some of the improvement seen with 25% casein is attributable to the blocking action of the casein diluent.

2.4.6.3 Protein amount and blocking agent

The original protocol used 100ng of CCHFV N protein per well based on the preliminary confirmation plates (2.3.1). Plates were run to compare 10% casein and 4% BSA, used as block and diluent, when used with two different volumes of protein, 100ng and 50ng.

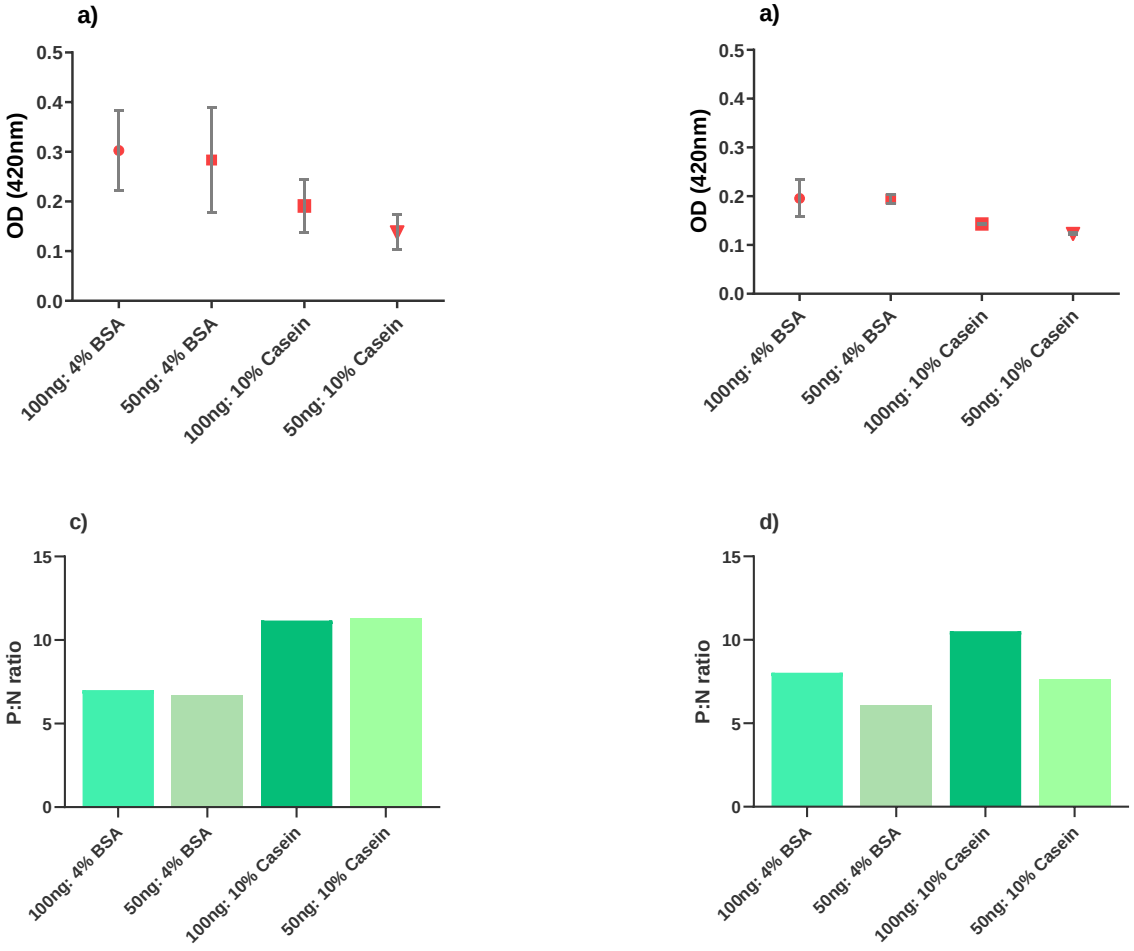

Figure 2.4.14 Comparison of protein amount and blocking agent.
a-b) Comparison of OD values of negative a) goat (GN1 and GN2) and b) sheep (SN2 and SN4) samples using 100ng of protein versus 50ng protein; c-d) Comparison of PN ratios for c) goat and d) sheep samples using 100ng of protein versus 50ng protein. Block and diluent agents were either 4% BSA or 10% casein for all graphs. Error bars show mean and range.

In confirmation of previous blocking agent and diluent comparisons, 10% casein produced the lowest negative OD values and highest PN ratios for both species at both protein volumes (Figure 2.4.14). In goats, 50ng protein resulted in slightly lower OD values for negative samples but similar PN ratios, suggesting 50ng of protein would be result in similar outcomes while using less resource. In sheep, OD values with 50ng protein were lower but the PN ratio was also reduced, suggesting 100ng protein would result in better distinction between positive and negative samples.

2.4.6.4 Protein amount and blocking time

To further investigate the effect of protein volume and the blocking step on assay outcome, further plates were run to compare protein volume against the time of block incubation. All goat and sheep samples were run with in-plate duplicates of 100ng and 50ng protein volumes. On one plate the blocking step lasted for 1 hour as per the previous protocol, while in the second plate the blocking agent was left on for 15 minutes prior to washing. 10% casein in PBST was used as blocking and diluent throughout.

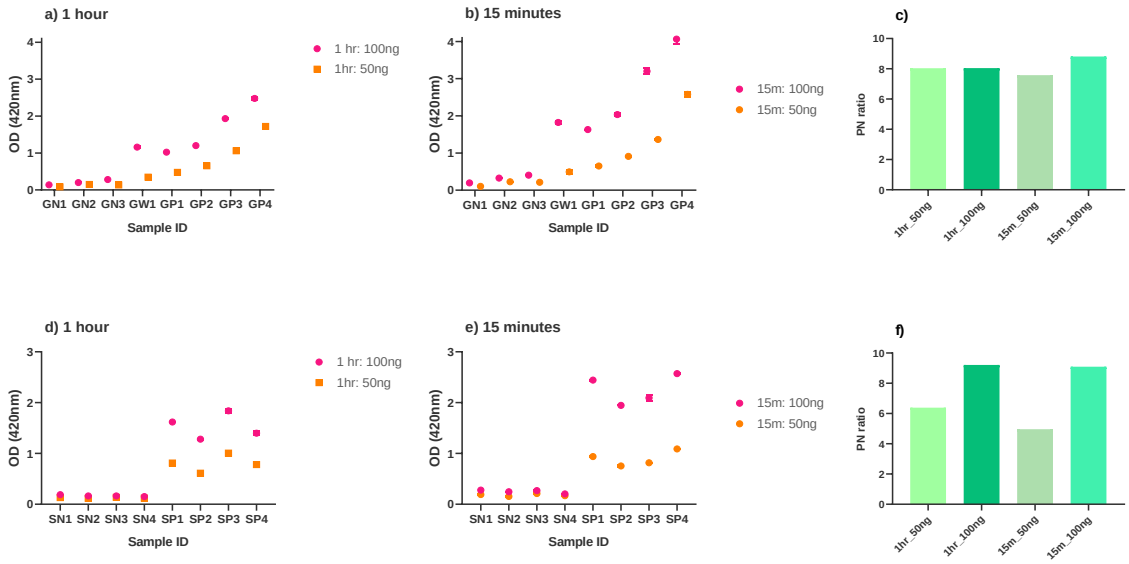

Figure 2.4.15 Comparison of protein amount and blocking time.
a-b) Mean OD values from in-plate duplicates of goat samples tested using 100ng or 50ng protein with blocking solution (10% casein) incubated for a) 1 hour or b) 15 minutes; c) shows PN ratios under the different conditions. d-e) Mean OD values from in-plate duplicates of sheep samples tested using 100ng or 50ng protein with blocking solution (10% casein) incubated for d) 1 hour or e) 15 minutes; f) shows PN ratios under the different conditions.

In goats (Figure 1.20: a-c), negative OD values were lower with 50ng protein compared to 100ng and all samples had lower ODs after 1 hour of block compared to 15 minutes. The PN ratios (excluding GW1) were broadly consistent across all combinations, with 100ng protein blocked for 15 minutes having a marginally higher value than the other combinations. The behaviour of GW1 in Figure 1.20 a) and b) is notable. When tested using 100ng, with either a 1 hour or 15 minute block, this inconclusive sample produces an OD

values that was well in the range of the positive samples. However, when tested using 50ng the sample OD is closer to the negative ODs. This sample tested negative on the ID Screen® test but produced an OD consistent with other positive samples on the first run of the in-house test now being optimized. The difference in OD value in this sample suggests that 50ng might produce a more sensitive result in goats.

In sheep (Figure 1.20: d-f), negative values were lower for both protein volumes after 1 hour of blocking compared to 15 minutes and the PN ratio was similar for both 1 hour and 15 minutes block with 100ng of protein. 100ng of protein produced notably higher PN ratios compared to 50ng of protein. From these plates, 100ng protein, used with either a 15 minute or a 1 hour block improved assay performance compared to 50ng.

Establishing a protocol that is the same for all cross-species aspects (i.e. all factors other than conjugate) will streamline assay performance as it would allow both sheep and goat samples to be run on the same plate if required and would allow plates to be pre-prepared. In sheep, the results indicate a clear improvement of PN ratio with the higher volume of protein. Although the above results suggest there may be some benefit to using a lower volume of protein in goats, this potential marginal improvement in PN ratio and negative OD values was not deemed sufficient to warrant a difference in protocol between species. For this reason, 100ng protein was maintained in the protocol.

OD values where not substantially different for samples blocked for 15 minutes compared to those blocked for one hour and in sheep the PN ratio was improved with a shorted block time. For this reason, in addition to the improved assay convenience of a shorter block time, block incubation was adjusted to 15 minutes in further experiments unless otherwise stated.

2.4.6.5 Stop solution, wavelength (nm) and stability over time

Slow TMB (3,3′,5,5′-Tetramethylbenzidine Liquid Substrate, Super Slow, for ELISA, Sigma Aldrich, UK) was used in the unoptimized protocol and for the optimised assay as per the manufacturer's recommendation. Comparisons were made between the original stop solution (H_2SO_4 1M) and two other stop solutions compatible with TMB (HCL 1M and HCL 2M), as well as using no stop solution but reading plates read at 650nm. Figure 2.4.16 shows that there was little difference in OD values between HCL and H_2SO_4 stop solutions but that reading plates at 650nm without stop solution led to a decrease in the OD of the positive samples.

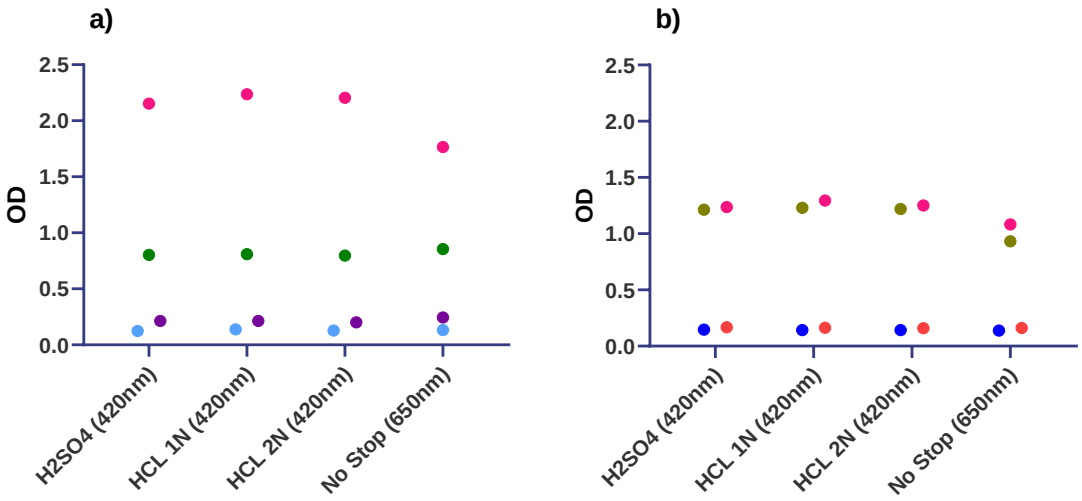

Figure 2.4.16 Comparison of stop solutions.

Comparison of OD values for four stop solutions: H_2SO_4, HCL 1M, HCL 2M (100µl volume read at 420nm) and no stop solution but plates read at 650nm. OD values for positive and negative samples for a) goats (GN1, GN3, GP1 and GP4) and b) sheep (SN2, SN4, SP2 and SP4) are shown by coloured points.

At this stage, reading the plate without stop solution was excluded from further testing. Further comparisons were next made of the three stop solutions, both over time and with different wavelengths. The same plates, with four in-plate repeats of each stop solution, were read at both 420nm and 450nm at 2 minutes following the addition of the stop solution (read 1) and then at five-minute intervals (reads 2 and 3) (Figure 2.4.17).

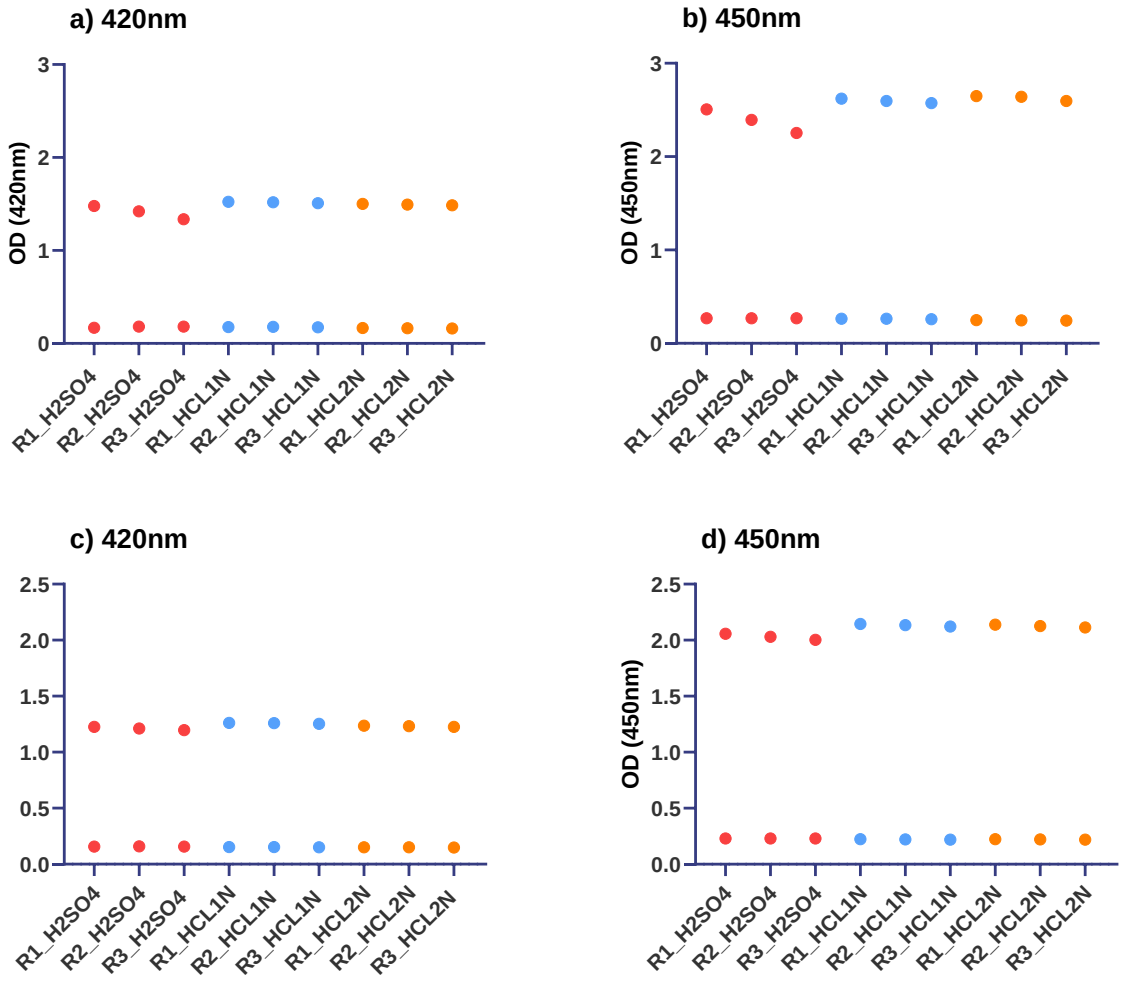

Figure 2.4.17 Comparison of stop solutions and read wavelengths over time.
a-b) Mean OD values of goat positive and negative samples (GN1, GN3, GP1 and GP4) read three times, five minutes apart (R1, R2, R3), for each different stop solution. A) shows OD values read at 420nm and b) shows those read at 450nm. C-d) Mean OD values of sheep positive and negative samples (SN2, SN4, SP2 and SP4) read three times, five minutes apart

(R1, R2, R3), for each different stop solution. c) shows OD values read at 420nm and d) shows those read at 450nm.

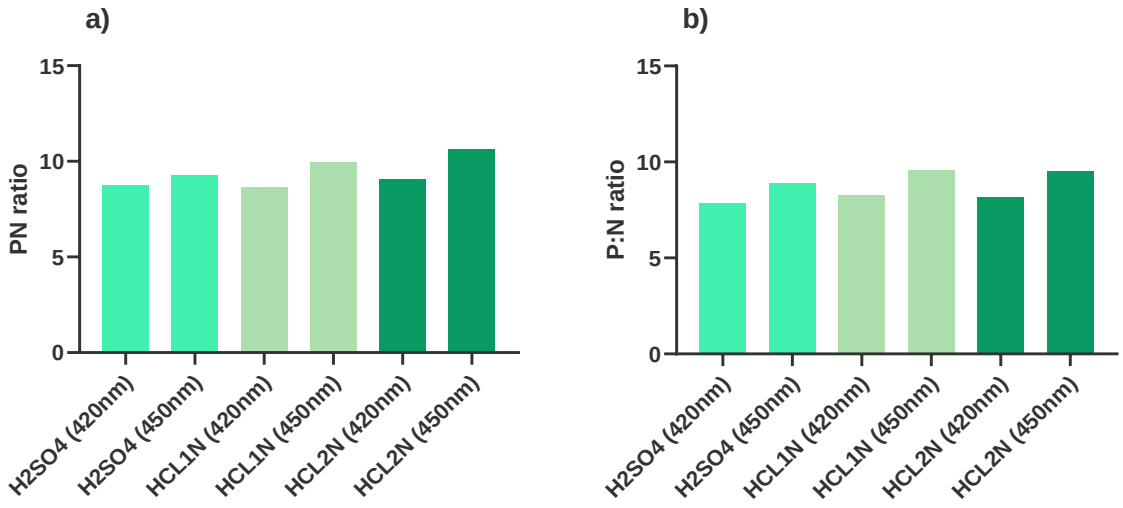

Figure 2.4.18 Comparison of PN ratios for stop solutions and wavelength.
a) goat (GN1, GN3, GP1 and GP4) and b) sheep samples (SN2, SN4, SP2 and SP4) read at either 420nm or 450nm after the addition of different stop solutions: H2SO4, HCL 1M, HCL 2M.

Both concentrations of HCL produced stable OD values over time. In goats, OD values for positive samples show a slight downwards trend when H2SO4 is used, particularly at 450nm (Figure 2.4.17; b). Although plates will typically be read at the same time for each assay, stability over time may be an advantage if assay reading is disrupted for any reason, so HCL was selected for future assays. No difference in OD values and only a marginal improvement in PN ratio was observed with using the higher concentration of HCL so a 1M solution was chosen for future use.

These experiments suggest that stop solution does not have a substantial effect on outcome, so no further factorial tests related to stop solution were needed. However, read wavelength (420nm verses 450nm) did result in a substantially different OD values as shown by comparison of Figure 2.4.17 graphs a) and b), and c) and d). Both positive and negative mean OD values were higher when read at 450nm but the increase in the positive OD is relatively greater than the negative. This greater distinction between positive and negative was confirmed by the PN ratios shown in Figure 2.4.18,

where PN ratios were consistently higher at 450nm than 420nm. The read wavelength was therefore changed to 450nm for future testing.

2.4.6.6 Conclusions from multiple factor evaluation

Following investigation of two or more factors simultaneously the following adjustments or confirmations were made to the ELISA protocol:

1. Solution of 10% casein in PBST used throughout assay for both blocking and dilution steps
   Incubation steps performed at room temperature
2. 100ng CCHFV N protein used to coat wells for both sheep and goat assays
3. Blocking step time reduced to 15 minutes
4. Stop solution was changed to 100µl HCL 1M
5. Wavelength at which plates were read changed to 450nm

2.4.7 Serum dilution and conjugate concentration chequerboard analysis

The greatest effect on OD values and PN ratios was seen in changes to the concentrations of serum and conjugate. For this reason, following the optimisation steps above, a final chequerboard analysis was undertaken to confirm that the concentrations selected for serum and conjugate dilutions remained optimal. One negative and one positive sample was selected for each species (goats: GN3 and GP2, sheep: SN3 and SP2).

For each sample a chequerboard of 6 conjugate concentrations, ranging from 1:1000 to 1:32000 was tested against 6 serum dilution ranging from 1:100 to 1:3200 (Figure 2.4.19). PN ratios were calculated for each conjugate, serum combination.

		Serum concentration											
		Negative sample						Positive sample					
		1:100	1:200	1:400	1:800	1:1600	1:3200	1:100	1:200	1:400	1:800	1:1600	1:3200
		1	2	3	4	5	6	7	8	9	10	11	12
Conjugate concentration	1:1000	A											
	1:2000	B											
	1:4000	C											
	1:8000	D											
	1:16000	E											
	1:32000	F											
	Blank	G											
	Blank	H											

Figure 2.4.19 Plate map for chequerboard comparison of conjugate and serum concentrations.

Layout of 96 well ELISA plate, using one negative and one positive sample. Wells are shaded according to the relative concentration of the two factors from most concentrated (dark yellow) to least concentrated (white).

PN ratios from the chequerboard analyses showed that several combinations of serum/conjugate dilutions resulted in similar performance. The concentrations chosen on single factor analysis did not result in the highest PN ratios but were only marginally lower than those with higher values, so they were deemed appropriate to be retained in the assay. If they had been substantially lower than other combinations following chequerboard analysis re-testing of other factors in combination with the improved serum/conjugate concentrations would be required.

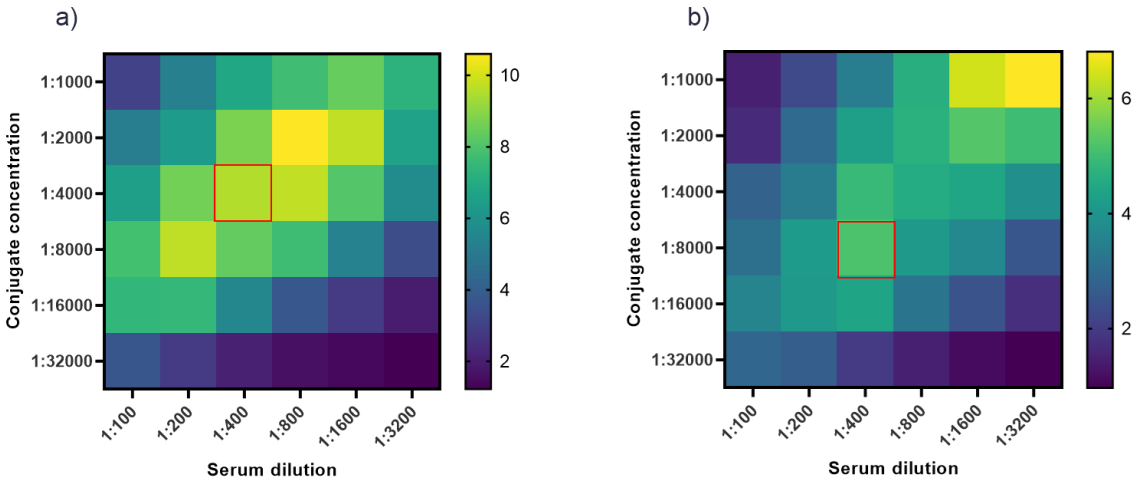

Figure 2.4.20 Heat maps of PN ratios for chequerboard analyses comparing conjugate concentration and serum dilution.

a) sheep and b) goats. Red squares indicate serum/conjugate concentration selected during the single factor analysis.

2.4.8 Optimised ELISA protocol

Following the optimisation steps described above the optimised ELISA protocol is given below, changes to the unoptimised protocol are shown in bold.

Coating buffer: 100mM sodium bicarbonate and 33mM sodium carbonate anhydrous pH 6.9

Washing buffer: Phosphate buffered saline (PBS) containing 0.1% Tween-20 (PBST)

Blocking buffer: 10x casein solution (Vector laboratories inc. Burlingame, CA 94010) diluted in PBST at a concentration of 1:10.

Dilution buffer: 10x casein solution (Vector laboratories inc. Burlingame, CA 94010) diluted in PBST at a concentration of 1:10

Stopping buffer: 1M solution of H_2SO_4

Protocol:

1. Coat each well of 96 well 2HB Immulon® ELISA plates (ImmunoChemistry Technologies, LLC, Minnesota, USA) with 100ng/well CCHFV nucleoprotein in 100µl/well coating buffer and incubate overnight at 4°C
2. Remove protein and coating buffer and wash plate 5 times using washing buffer
3. Add 200µl blocking buffer to each well, cover and incubate at room temp **for 15 minutes**
4. Remove blocking buffer and wash plate 5 times using washing buffer
5. Add 100µl/well of sera diluted 1:400 with dilution buffer in duplicate to plate, cover and incubate for **1 hour** at room temp
6. Remove serum and wash plates 5 times using washing buffer
7. Add 100µl/well diluted anti-goat IgG or anti-sheep IgG conjugate (Bethyl Laboratories, Montgomery, USA), cover and incubate at room temp for 1 hour
    a. Dilute anti-goat IgG conjugate at 1:8000 in dilution buffer
    b. Dilute anti-sheep IgG conjugate at 1:4000 in dilution buffer
8. Remove conjugate and wash 5 times using washing buffer

9. Add 100µl/well neat tetramethylbenzidine (TMB) substrate (3,3′,5,5′-Tetramethylbenzidine Liquid Substrate, Super Slow, for ELISA, cover and incubate in the dark for 15 minutes
10. Add 100µl/well of stop buffer **(HCL 1M)** to stop reaction
11. Read on plate reader at **450nm within 10 minutes** of adding stop buffer

2.5 Discussion

This chapter had three principal aims: to adapt an existing RVFV ELISA for use in the detection of antibodies against CCHFV in livestock, to use this ELISA to screen an initial subset of cattle, goat and sheep samples from Tanzania, and to optimise this ELISA to reduce the absolute OD values of the negative samples, improve distinction between low and high samples, and streamline the laboratory process. Proof of principal testing, using samples with confirmed IgG status to CCHFV, demonstrated that an existing in-house indirect RVFV ELISA could be successfully adapted to detect antibodies to CCHFV by substituting RVFV NP with CCHFV NP. The initial screen of samples collected in Tanzania confirmed that serum from all three species produced a high response to CCHFV NP, and use of a newly available commercially produced ELISA kit was able to categorise samples as positive or negative for use in further optimisation. Finally, the in-house ELISA was systematically optimised, leading to improved distinction between negative and positive samples and a more efficient laboratory procedure.

The in-house indirect ELISA platform utilised here offers a simple but effective means of measuring antibody responses and is highly flexible, enabling the same platform to be adapted to different viruses, viral proteins, species, and antibody types by changing the coating antigen or the secondary antibody. This flexibility, as well as its comparatively low cost, are the principal advantages of using an in-house system such as this compared to commercial kits, which are typically designed for a single purpose and have a high cost per plate. Adaptation of the unoptimised ELISA to detect antibodies against CCHFV rather than RVFV was straightforward and offered reasonable distinction between positive and negative samples even before optimisation. Adapted versions of the in-house assay could quickly and easily be developed to test for other antigenic components of the same virus (e.g. CCHFV external glycoproteins Gn and Gc) to investigate the breadth of immunological responses to infection, or to test for antibodies to closely related viruses to investigate cross-reactivity.

Cross reactions were not observed between CCHFV NP and RVFV antibodies in the proof of principal test, but these viruses are antigenically remote and therefore unlikely to show cross-reactivity. Previous studies have investigated cross-reactivity between related orthonairoviruses both in the CCHF serogroup, which includes CCHFV and Hazara virus (HAZV), and in the closely related Nairobi sheep disease virus (NSDV) serogroup which includes NSDV and Dugbe virus (DUGV). Several studies have investigated cross-reactivity through screening against multiple nairovirus antigens and have found no evidence of cross-reactivity between these viruses (Burt et al., 1996, Grech-Angelini et al., 2020). However, a recent study did identify some evidence of cross-reactivity between CCHFV and DUGV when samples were tested by immunofluorescence and immunoblot assays, although no cross-reactivity when tested by commercial CCHFV ELISA (Hartlaub et al., 2021a). This suggests that different assays may have different specificities for identifying antibodies to closely related viruses and highlights the need to fully explore the potential for cross-reactivity in different assays.

Without a commercially validated ELISA available at the time, the easy adaptability of the indirect ELISA platform was of huge advantage in the early stages of this investigation into CCHFV in Tanzania as it enabled a subset of samples from the study area (see Chapter 4 for a full description) to be tested rapidly and cheaply. This initial screen confirmed the presence of high antibody responses to CCHFV NP in some animals, and so confirmed that animals in this region were highly likely to have been exposed to the virus. This was the first time for more than 30 years that serological evidence of CCHFV presence in Tanzania was confirmed (Hoogstraal, 1979).

The release of the IDvet ID Screen® double antigen sandwich ELISA kit shortly after the testing of the initial samples, further confirmed exposure to the virus in Tanzanian livestock, as well as providing a comparison for the in-house ELISA and a method of categorising samples as positives or negative. Optimisation could have been performed without the confirmation of the IDvet assay by using samples with consistently high and low OD values as

controls, or using negative controls from non-endemic regions, but it provided a useful method of corroborating the status of the screened samples, as well as providing an early indication of prevalence in the sample set which was not possible from the unoptimised ELISA without an appropriate cut-off in place.

Correlation between unoptimised in-house ELISA OD values and those produced from the IDvet kit was good, particularly for sheep, with the main areas of difference seen in samples that were negative by the ID Screen® but that had mid-range or higher OD values on the in-house ELISA. Assuming these samples are “true negatives”, which is a reasonable assumption given the IDvet kit’s reported 100% specificity (IDvet, 2018, Sas et al., 2018a), the higher OD values of some samples with the in-house assay is likely due to high background noise or non-specific binding. These issues should be reduced through optimisation by selection for lower absolute negative control values, improved blocking, and optimal secondary antibody concentration. Unfortunately, given the unexpected restrictions to lab work imposed by the COVID-19 pandemic, it was not possible to retest these initial samples on the optimised ELISA to confirm this, but this would be a useful next step were the in-house ELISA to be used for sample testing in the future.

Differences in the degree of correlation between in-house and IDvet OD values for sheep, goats and cattle may suggest that the antigenic response to CCHFV varies between species or that antibodies of the different species react differently in the assay (Figure 2.3.3). The weaker correlation seen in cattle samples may be a result of increased non-specific binding in this species, as a subset of cattle antibodies have been shown to have an ultralong loop in the third complementarity determining region (CDR) of the heavy chain (Haakenson et al., 2018, Stanfield et al., 2018). This part of the antigen binding region is much longer in cattle compared to humans and other vertebrates and may allow binding to unusual antigenic targets (Wang et al., 2013) In an ELISA setting this could result in enhanced non-specific binding. The greater overlap between negative and positive samples in cattle

may reflect these differences in antibody structure and suggests that an in-house ELISA for cattle might be subject to lower sensitivity and specificity compared to one for small ruminant samples.

Potential differences in immune response between species also contributed to the decision to use samples of Tanzania origin to optimise the ELISA. For positive controls, no known-positive livestock sera were available for testing so other sources of samples was essential. For negative controls, samples from non-endemic regions could have been used, and goat, sheep and cattle serum from the UK and New Zealand were tested during the initial sample screens. However, as the assay was intended for use on Tanzanian samples it was felt that samples from this geographic origin would be more suitable as optimisation controls because Tanzanian livestock would be the population screened with the optimised assay. Livestock in non-endemic European countries are likely to be different from those in East Africa, both in terms of their genetics and their viral exposure history. In cattle, this may also extend to species differences, with principally *Bos taurus* cattle in Europe and a higher proportion of *Bos indicus* or Zebu cattle in Sub-Saharan Africa. Variation in immune responses to ticks and other parasites in *Bos taurus* and *Bos indicus* has been documented (Piper et al., 2009) and although differences in antibody and B cell responses are less well documented they may also occur and could influence the performance of tests developed using *Bos taurus* samples (Barroso et al., 2020). Optimisation using non-endemic animal sera therefore, particularly when originating from farmed populations with low genetic diversity (Doekes et al., 2018, Ablondi et al., 2022), may bias test performance in favour of European breeds, making an assay less useful when testing animals from endemic regions.

The final protocol following optimisation was shorter and more streamlined than the original protocol, making the assay quicker to run for many samples, which was the original purpose of this assay. For many of the factors investigated the difference in OD values and PN ratios between options was relatively small and so the choice of protocol to take forward was often

based on practicality more than quantitative improvement in assay performance. For example, the differences between 1:2000, 1:4000 and 1:8000 dilutions of anti-sheep conjugate were small so any of these concentrations would likely have been acceptable. The final chequerboard analysis of serum and conjugate dilution showed that there were several combinations that yielded similar PN ratios. Further chequerboard analyses may enable finer optimisation of this process. For example, reducing the amount of protein used would enable more plates to be completed, more cost effectively, so further investigation of the interaction between protein volume and serum/conjugate dilutions would be advisable if the assay was to be used on a large scale.

Choice of blocking buffer was a particularly important factor in the optimisation process, particularly considering the high OD values seen in some of the negative samples tested in the initial screen. Casein-based blocking buffers performed significantly better as a blocking agent, both in terms of minimising negative control OD values and maximising PN ratio, than either bovine serum albumin (BSA) which is commonly used in in-house ELISAs or the gelatin-based commercial ELISA blocking agent tested (Roche diagnostics, Mannheim, Germany). This is in keeping with other studies which found casein solutions to be a highly effective blocking agents (Pratt and Roser, 2014, Vogt et al., 1987). The poorer performance of gelatin is also consistent with reports from elsewhere (Huber et al., 2009). Optimisation experiments suggested that 15 minutes incubation with casein buffer was enough to provide adequate blocking. However, later use of both in-house and commercial ELISAs made it clear that, due to practical considerations, most plates would end up being blocked for longer than this. Laboratory workflow meant that plates were usually blocked while samples were diluted, a process that typically took between 30 minutes and one hour depending on the number of samples being tested. Although it was possible to put the block on for 15 minutes only, a longer blocking time would be more practical. As blocking should be saturating process, longer blocking times should not make substantial differences to OD values and so the 15 minutes should be

considered a minimum time. Blocking plates overnight, or bulk-blocking plates and freezing and then thawing them before use, would further improve assay usability, and should be explored for their effect on repeatability if this assay was to be used on a larger scale.

The optimisation process had some limitations. Samples were typically tested without within- or between plate-replicates due to limited antigen resources at this stage of the project. Duplicate or triplicate within-plate repeats, as well as repeated testing of key factor combinations on different plates would enable confirmation of conclusions. Were this ELISA to be used to test samples in the future, systematic analysis of repeatability and intermediate precision would be needed to ensure the results were reproducible. Evidence from factor investigations that did not affect assay performance, such as serum incubation time, do suggest that samples tested on this assay had good repeatability, but repeat testing of the same sample on the fully optimised assay would be required to confirm this.

Although the indirect ELISA system has several advantages, it also has a number of disadvantages over a commercially produced assays and other ELISA formats, which contributed to the decision not to take forward the in-house assay for the epidemiological components of this thesis. Although more expensive, commercial ELISA kits are typically highly repeatable within laboratories and reproducible between laboratories (i.e. have higher precision) and typically have extremely low background reactivity. Higher reproducibility across laboratories means that comparing results from different studies is more reliable. For CCHFV, where there is large geographical variation in animal seroprevalence (Spengler et al., 2016a), reducing this test-related variability by use of the same assay across many different studies would help to increase our understanding of global epidemiology of the virus. Additionally, for animal and emerging virus research, where multiple host species may be of interest, the in-house indirect ELISA has the disadvantage of being species independent. For common species of interest such as cattle, sheep and goats this is easily

remedied by use of secondary antibodies specific to that species, but it could pose a problem when investigating serological responses in less common animals, such as many wildlife species, where secondary antibodies are not readily available. For CCHFV, this may limit the usefulness of indirect ELISAs if testing of small mammal or avian hosts, who can be exposed to the virus through feeding of nymphal stages of infected ticks, were required. However, development of species-independent competition or sandwich ELISAs, could resolve this problem.

3 SARS-CoV-2 sero-surveillance and immunodynamics in a patient population in Glasgow, Scotland

3.1 Introduction

Severe acute respiratory syndrome Coronavirus 2 (SARS-CoV-2) infection in people was first reported in Wuhan, China in December 2019 and the virus has subsequently gone on to cause a global pandemic, constituting the most significant public health challenge of recent times. The first UK case of coronavirus disease 2019 (COVID-19) was confirmed in England on 28th January 2020, and the first case confirmed in Scotland on 28th February 2020 (Hill et al., 2020, Lillie et al., 2020). Following a rapid rise in cases and hospitalisations, the UK entered a national lockdown on 23rd March 2020 and remained under these guidelines until 28th May 2020 when restrictions began to ease.

Serological surveillance is a key tool in emerging and endemic disease epidemiology for determining rates of infection at a population scale, and such approaches are particularly useful for a pathogen like SARS-CoV-2 where asymptomatic and sub-clinical infections are common (Ma et al., 2021). Anti-SARS-CoV-2 immunoglobulin G (IgG) antibodies are typically detectable 7–21 days after infection, so assays which can detect the presence of these antibodies can be used to report past infection from this timescale and beyond (Zhao et al., 2020, Post et al., 2020). This presents an advantage of serological approaches over reverse transcription polymerase chain reaction (RT-PCR) identification of current infections, in being able to detect past infections. Additionally, serological testing can be readily used at a population-wide level, enabling more accurate determination of key epidemiological parameters such as case and infection fatality rates and the data with which to parameterise epidemiological models of transmission and interventions (Larremore et al., 2021, Metcalf et al., 2016).

Various serological tools, both for quantifying antibodies at an individual level and for population-level surveillance, were developed during the first pandemic wave and have since become vital tools to understanding the immune response and population spread of SARS-CoV-2, as well as developing novel antibody-based treatments (Chvatal-Medina et al., 2021, Galipeau et al., 2020). A commonly used assay for serosurveillance is the Enzyme-linked immunosorbent assay (ELISA) which have been widely utilised throughout the pandemic. Several commercial ELISA products were released during 2020 (Yassine et al., 2021, Meschi et al., 2020) but many studies also employed in-house ELISA assays due to the flexible and cost-effective nature of the assay format (Amanat et al., 2020, Zhong et al., 2020). An indirect enzyme-linked immunosorbent assay (ELISA) format was commonly used for in-house assays, typically utilising nucleoprotein, recombinant spike (S) protein, the S1 subunit of the S protein (S1), or the receptor-binding domain (RBD) as antigens. Pseudotype virus neutralisation assays were also rapidly developed in the early months of the pandemic and were used to provide insights into the nature of the neutralising immune response. HIV(SARS-CoV-2) pseudotype-based neutralisation assays were found to display a high correlation with live virus-based assays (Hyseni et al., 2020) and so could facilitate high-throughput screening outside of containment level 3.

Work for this chapter was undertaken during the first 9 months of the pandemic as part of a team of virologists, immunologists and epidemiological modellers. The paper resulting from this collaborative work can be found in Appendix 1. The work reported in this chapter was led by the E. Hughes and focuses on technical aspects of serological testing of human sera against SARS-CoV-2 IgG and neutralising antibodies, as well as the use of this data in characterising the progress of the pandemic and immune responses in a patient population in Glasgow, UK.

3.1.1 Chapter aims

Work reported in this chapter focussed on the following aims:

1. Establish appropriate cut-off values for ELISAs against the S1 subunit and RBD of SARS-CoV-2
2. Estimate the sensitivity and specificity of these ELISAs
3. Investigate precision and reliability of these ELISAs
4. Estimate levels of exposure and patterns of seroprevalence in a patient population in Glasgow during the first wave of the COVID-19 pandemic
5. Compare antibody (Ab) and neutralising antibody (NAb) responses in the same patient population

3.2 Methods

3.2.1 Serum sample origin and processing

Residual serum samples from patients undergoing routine biochemistry testing in both primary and secondary care settings were obtained from NHS Greater Glasgow and Clyde (NHSGGC). Primary care settings were principally general practitioner (GP) practices, while samples from secondary care were obtained from hospital settings. Between 500 and 1000 samples were randomly selected each week from all routine blood samples collected in NHSGGC and processed at the NHSGGC biorepository. Samples were anonymised and given a unique ID number which was associated with accompanying metadata including date of sampling, whether of primary or secondary care origin, patient age and sex, and previous patient PCR testing history. Approximately 500 samples collected per calendar week were randomly selected and residual serum from these transferred to the MRC-University of Glasgow Centre for Virus Research (CVR) where they were stored at -20°C until required. Samples were heat inactivated in a water bath at 56°C for 30 minutes before being stored at 4°c until testing.

3.2.2 Sample sets

3.2.2.1 Patient samples

For analysis of the first wave of SARS-CoV-2 infections in Glasgow, 7480 serum samples collected between 16th March 2020 and 24th May 2020 were randomly selected for testing. In some cases, multiple samples were obtained from the same patient, in which case only the first sample per calendar week was used for further analysis. Samples from patients under the age of 18, and those without a recorded care-type of origin, were excluded from further analysis. This sample set was used to undertake a serial cross-sectional study of SARS-CoV-2 seroprevalence in the NHSGGC patient population.

3.2.2.2 PVNA samples

A subset of these samples, those collected between 24th March and 24th April, were also tested using an HIV(Sars-CoV-2) pseudotype virus neutralisation assay (PVNA) to determine levels of neutralising antibodies (NAb). Sample dates and sample size were selected based on testing convenience.

3.2.2.3 Triplicate repeat samples

To assess assay precision, a random selection of 30 samples from the patient population described above was chosen for repeat testing. Seventeen samples had previously been identified as positive by either S1 or RBD ELISA and thirteen as negative. Sample size was opportunistically determined by the upper limit of samples that could be tested in triplicate on a 96-well ELISA plate.

3.2.3 Ethics

Ethical approval for use of the samples was obtained from NHSGGC Biorepository (application number 550).

3.2.4 Laboratory analyses

3.2.4.1 S1 and RBD protein production

S1 and RBD antigens were produced at The Francis Crick Institute, London, UK as described by Ng et al. (2020). Briefly, SARS-CoV-2 S1 (SARS-CoV-2 spike (UniProt ID: P0DTC2) residues 1-530 (MFVFL…GPKKS)) and RBD (SARS-CoV-2 spike (UniProt ID: P0DTC2) residues 319-541 (RVQPT…KCVNF)) constructs were produced with C-terminal Twin-Strep-tags. Secretion of the RBD construct was directed by a signal peptide from immunoglobulin kappa gene product (METDTLLLWVLLLWVPGSTGD). Proteins were transiently expressed in Expi293F cells grown in FreeStyle-293 medium (Thermo Fisher Scientific, UK). Conditioned media containing secreted proteins were harvested at 3-4, and 6-8 days post-transfection. Streptactin XT (IBA LifeSciences) was used to capture Twin-Strep-tagged proteins, which were then eluted and purified to homogeneity by size exclusion chromatography through Superdex 200 (GE

Healthcare). Purified SARS-CoV-2 antigens were concentrated to 1-5 mg/ml by ultrafiltration. Aliquots were snap-frozen in liquid nitrogen prior to storage at -80oC.

3.2.4.2 ELISA protocol

In-house indirect ELISA assays, based on a protocol received from the National Institute for Biological Standards and Control (NIBSC), were used to test all samples for IgG antibodies to S1 subunit (S1) and receptor binding domain (RBD) antigens of SARS-CoV-2 (Wuhan-Hu-1, GenBank: MN908947). Firstly, 96 well plates (Immulon 2HB, Fisher Scientific, Loughborough, UK) were coated over-night with purified S1 or RBD antigen at 50ng/well in phosphate buffered saline (PBS). The next day, plates were washed three times with the wash buffer made up of phosphate buffered saline (PBS) containing 0.05% Tween-20 (all subsequent wash steps followed this same protocol), before addition of 200µl per well of blocking buffer (PBS/0.05%tween20 supplemented with 10% casein (Vector laboratories, c/o 2BScientific, Upper Heyford, UK). Plates were then incubated at room temperature for one hour. Following a second wash step, 50µl sera diluted 1:100 in the blocking buffer, was added to each well. Sera was incubated for one hour at room temperature before another wash step. Next, 50µl anti-human IgG horseradish peroxidase-conjugated secondary antibody (Bethyl laboratories, c/o Cambridge Bioscience, Cambridge, UK) was diluted to 1:3000 in the blocking buffer and added to each well. Plates were then incubated for one hour before a final wash step. Following this, 50µl 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma-Aldrich/Merck, Dorset, UK) was added and plates incubated at room temperature in the dark for 10 minutes, after which the reaction was stopped by the addition of 50µl of 1M H_2SO_4. Absorbance was read immediately at 450 nm on a Labsystems Multiskan Ascent plate reader.

3.2.4.3 Control samples

Duplicates of pooled positive and negative controls were included on each plate. When this assay was first used, no sera of known status against SARS-

CoV-2 was available due to difficulties accessing such samples early in the pandemic response. To overcome this, one box of 100 samples was selected from the patient serum collected from NHSGGC and sacrificed to make controls. All samples were tested at least 3 times on the in-house S1 and RBD ELISAs, as well as once on the Euroimmun-Anti-SARS-CoV-2 ELISA [IgG] (Euroimmun, Lübeck, Germany). For negative controls, samples with the lowest OD values on repeat ELISA for both proteins, as well as low negative results on the Euroimmun ELISA were selected and pooled. For positive controls, samples with the highest in-house ELISA OD results as well as high positive results on the Euroimmun ELISA were selected and pooled.

3.2.4.4 Pseudotype virus neutralisation assay

3.2.4.4.1 *Cell lines and pseudotype production*

HEK293, HEK293T, and 293-ACE2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, UK) supplemented with 10% foetal bovine serum (FBS), 2mM L-glutamine, 100µg/ml streptomycin and 100 IU/ml penicillin (complete DMEM). HEK293T cells were transfected with the SARS-CoV-2 (Wuhan-Hu-1 strain, GenBank MN908947) S gene expression vector PcDNA6-S (N. Temperton, University of Kent, UK) in conjunction with pNL4-3-Luc-E−R−luc (Connor et al., 1995) using polyethylenimine (PEI, Polysciences, Warrington, USA). After 48 hours post-transfection HIV (SARS-CoV-2)-containing supernatants were filtered to harvest pseudotypes, aliquoted and frozen at -80°c for future use. Prior to use, each batch of pseudotype was tested at doubling dilutions in order identify the dilution which gave luciferase counts of $1x10^6$ counts per second per 50µl pseudotype added in each well. 293-ACE2 target cells were produced by stable transduction of HEK293 cells with pSCRPSY-human ACE2 (hACE2) (Matt Turnbull & Suzannah Rihn, MRC - University of Glasgow Centre for Virus Research). Selected 293-ACE2 cells were maintained in complete DMEM supplemented with 2µg/ml puromycin.

3.2.4.4.2 Pseudotype virus neutralisation assays

Serum samples were screened for neutralising antibodies at a fixed dilution. All samples were tested in duplicate, and a no-serum control included on every plate. Samples were diluted 1:25 in complete DMEM and 25µl per well plated onto white 96-well cell culture plates, before being incubated for 1 hour at 37°C and 5% CO_2 with an equal volume of HIV(SARS-CoV-2) pseudotype. Next, 50µl per well of $4x10^5$ cells/ml 293-ACE2 cells were added to make a final serum dilution of 1:50, and plates were incubated at 37°C and 5% CO_2 for a further 48-72 hours. Following this incubation, 75µl per well of Steadylite Plus chemiluminescence substrate (Perkin Elmer, Beaconsfield, UK) diluted 1:3 with distilled water was added. Luciferase activity was measured using a Perkin Elmer EnSight multimode plate reader (Perkin Elmer). Neutralising activity was assessed by calculating the percentage reduction in luciferase activity compared to the no serum control. A reduction in luciferase activity compared to the no-serum control of ≥90% was considered positive for demonstration of neutralising activity of the serum sample (Logan et al., 2016).

3.2.5 Statistical analyses

Data visualisation and statistical analyses were undertaken in R statistical environment, version 3.6.1 (R Core Team, 2021) and in Graphpad Prism, version 8.4.0 (Prism). Models were run using the lme4 package in R (Bates et al., 2015). Statistical significance was set at p=<0.05.

3.2.5.1 ELISA cut-off selection

Cut-off values for S1 and RBD ELISAs were selected using receiver operating characteristic (ROC) analysis. ROC analyses were performed using GraphPad Prism (version 8.4.0). Cut-off points were explored for raw OD values read at 450nm, as well as for corrected OD values where the raw OD was adjusted in relation to the negative control using the equation:

$$\frac{\text{(sample absorbance-negative control mean)}}{\text{negative control mean}}$$

All tested OD values were also plotted as distribution plots to visually assess the chosen cut-off values in relation to the two populations of samples, negative and positive.

Positive and negative control samples were tested as per the ELISA protocol above and used to produce ROC tables of sensitivity and specificity values for multiple potential cut-off values, from which a cut-off was selected. ROC curves were also produced by plotting the true positive rate (sensitivity) against the false positive rate (1-specificity) and the area under the curve (AUC) calculated. Negative control samples (n = 320), collected prior to the emergence of SARS-CoV-2 in December 2019, were obtained from the National Institute for Biological Standards and Control (NIBSC) and the Scottish National Blood Transfusion Service (SNBTS). Positive control samples (n = 128) were obtained from NIBSC and from a longitudinal study of health-care workers being undertaken at the MRC-University of Glasgow Centre for Virus Research. Positive samples were defined as those from patients with a positive result by reverse-transcription polymerase chain reaction (RT-PCR), or those who had recent clinical symptoms consistent with COVID-19, and whose serum sample tested positive on all other serological platforms on which it was tested (EUROIMMUN-Anti-SARS-CoV-2 ELISA [IgG] (Euroimmun, London, UK), Abbott Architect SARS-CoV-2 IgG (Abbott, Illinois, USA), or DiaSorin LIAISON® SARS-CoV-2 S1/S2 IgG (DiaSorin, Saluggia, Italy)). Testing of samples on other serological platforms was carried out by Public Health Scotland (PHS).

3.2.5.2 Sensitivity and specificity assessment

Control samples were tested against both S1 and RBD antigens and individual ROC analyses were undertaken for each. Cut-off values were chosen to optimise for the specificity of each individual antigen test, while maintaining a sensitivity above 90%. S1 and RBD results were then combined in parallel (i.e. if a sample tested positive for either one or both antigens it was

considered positive) and the overall sensitivity and specificity was recalculated using the following equations:

$$\text{Parallel sensitivity} = 1 - [(1-Se_{S1}) \times (1-Se_{RBD})]$$

$$\text{Parallel specificity} = Sp_{S1} \times Sp_{RBD}$$

where Se_{S1} is the sensitivity of the S1 ELISA, Se_{RBD} is the sensitivity of the RBD ELISA, Sp_{S1} is the specificity of the S1 ELISA and Sp_{RBD} is the specificity of the RBD ELISA (Thrusfield and Christley, 2018).

To further explore sensitivity and specificity, positive control samples were defined in different ways based on time since infection. Sensitivity (Se) and specificity (Sp) were recalculated based on positive samples being defined as those taken 14, 21, 28, 35 and 42 days post a positive RT-PCR test. Se and Sp were calculated using standard formulae:

$$\text{Sensitivity} = \frac{TP}{TP + FN}$$

$$\text{Specificity} = \frac{TN}{TN+FP}$$

where TP = number of true positive samples testing positive on the assay, FN = number of true positive samples testing negative on the assay, TN = True negative samples testing negative on the assay and FP = true negative samples testing positive on the assay.

3.2.5.3 Comparison of S1 and RBD responses

Comparison was made between antibody responses to S1 and RBD antigens using the patient samples. Pearson's correlation coefficient was used to calculate the degree of correlation between responses to each antigen, and paired t-tests were performed on log transformed data to compare the mean log OD value for each protein.

3.2.5.4 Assay precision and agreement

To assess markers of assay precision, samples from the triplicate repeat sample set were tested. Three different operators ran the same set of

samples three times on three different plates. All samples were tested on each plate in triplicate. All other parameters were kept as consistent as possible.

Assay precision can be measured as three distinct variables: repeatability, intermediate precision, and reproducibility (Andreasson et al., 2015). Repeatability is a measure of the variation observed between results for the same sample when all parameters are kept constant and the time between measurements is as short as possible. In this case, repeatability was represented by the within-plate variability between the same sample tested in triplicate wells on the same plate. Intermediate precision is a measure of the variability observed for the same sample, tested in the same laboratory but over a longer period (typically days or months) with other parameters allowed to change. In this case, intermediate precision was assessed in two ways: as the variability observed for the same sample tested by the same operator on separate plates (within-operator variability), and as the variability observed for the same sample tested by different operators across all plates (total variability). Repeatability can also be thought of as within-plate variability and intermediate precision as between-plate variability arising from different sources. Reproducibility is a measure of the between-laboratory variability, whereby the same sample is tested using the same assay in different laboratories, and so was not assessed here.

Repeatability and intermediate precision of OD values were assessed by calculating the mean and standard deviation of each set of replicates as well as the coefficient of variation (CV), where σ is the standard deviation and μ is the mean of the sample repeats:

$$CV = \frac{\sigma}{\mu}$$

An acceptable coefficient of variation for repeatability in ELISAs is often set at ≤15%, although this does not specifically relate to assay interpretation so may not be appropriate for all assays. CV values for intermediate precision and reproducibility are often slightly higher and so 20% may be considered

acceptable for these parameters. Here, within-plate and between-plate CV values were calculated for each sample. The proportion of those with CV values ≤15% for within-plate repeats, and the proportion with CV values ≤20% for between-plate repeats were calculated.

To assess how consistently samples were classified as positive or negative, Cohen's kappa coefficient was calculated to investigate the agreement in terms of assigned status (positive or negative based on the cut-off values) between sample repeats. Samples were initially classified as negative or positive based on the first time they were tested by ELISA. Following repeat testing, all replicates of each sample were assigned a new status based on the cut off values chosen in the ROC analysis. Number of samples which were negative or positive on both tests, number of samples which were negative and became positive, and number of samples which were positive and became negative, were calculated for S1 and RBD assays, as well as the two assays combined in parallel. The percentage agreement for positive and negative samples and the Cohen's Kappa coefficient of agreement were calculated using data. All measures of agreement were calculated for both OD values and corrected OD values. Cohen's Kappa statistic and 95% confidence intervals were calculated using the *DescTools* package in R (Signorell, 2021).

3.2.5.5 Comparison of raw OD and corrected OD values

All data from the triplicate repeat sample set, as well as the main patient samples, were analysed as raw OD values and as corrected OD values. Adjusting OD values using the formula outlined above resulted in some negative corrected OD values which meant that coefficient of variation was not an appropriate method of assessing the variance of this metric. Change in status was assessed through calculation of Cohen's Kappa coefficient to measure agreement between the status of the sample on repeated measurement compared to its original designation as positive or negative. Samples tested in triplicate were assigned a status based on the mean of each plate's triplicate repeat.

3.2.5.6 SARS-CoV-2 seroprevalence in a patient population in Glasgow

Unadjusted seroprevalence was calculated based on the number of samples positive by S1 or RBD ELISA divided by the number of samples tested from the patient population described above. Seroprevalence was calculated for each age group, patient sex, caretype and the week of sampling. Univariable logistic regression models were used to evaluate the associations between sero-status and patient sex, age group and care type using the unadjusted data. In the published paper relating to this research seroprevalence values were adjusted for test performance using a Bayesian State Space model which is not described here. Model-adjusted seroprevalence values are reported here but full methods can be found in Appendix 1 (Hughes et al., 2021).

3.2.5.7 Comparison of PVNA and ELISA

To determine if exposure to SARS-CoV-2 was likely to elicit a protective immune response, HIV(SARS-CoV-2) pseudotypes were used to measure levels of neutralising anti-SARS-CoV-2 antibodies. Comparison was made between IgG responses and percent neutralisation by plotting IgG antibody responses measured by the ELISAs against the results of the single screen pseudotype virus neutralisation assay (PVNA). To compare the magnitude of the two responses in different patient types, comparison was made between ELISA OD, and percent neutralisation, for patients from primary and secondary care settings. Statistical comparison between the two groups was undertaken using Mann-Whitney U tests.

3.2.5.8 Factors associated with neutralising antibody response

Multivariable logistic regression models were used to investigate associations between the presence of neutralisation at a single dilution, and ELISA OD values, care-type, age group and gender in ELISA positive samples (n=216). S1 and RBD responses were investigated separately because of high co-linearity between the responses.

3.3 Results

3.3.1 Cut-off selection

Cut-off values were chosen to maximise assay specificity whilst also retaining high sensitivity. Cut-off values with similar Se and Sp were selected for raw OD and corrected OD values. Final cut-off values and Se and Sp values for S1 and RBD are shown in Table 3.3.1.

Table 3.3.1 Final cut-off values for S1 and RBD ELISAs.

Cut-off values were selected by ROC analysis using either raw OD or corrected OD as the outcome.

OD cut-off					
		Sensitivity %	**95% CI**	**Specificity %**	**95% CI**
S1	> 1.090	92.97	87.18 - 96.26	98.44	96.40 - 99.33
RBD	> 0.7471	95.31	90.15 - 97.83	98.75	96.83 - 99.51
Corrected OD cut-off					
		Sensitivity %	**95% CI**	**Specificity %**	**95% CI**
S1	7.761	92.97	87.18 – 96.26	98.44	96.40 – 99.33
RBD	6.063	95.31	90.15 – 97.83	98.75	96.83 – 99.51

ROC curves were plotted and showed very good overall performance of the ELISA at distinguishing positive and negative samples, as demonstrated by the high area under the curve (AUC) for both S1 and RBD and for both the raw OD and corrected OD values (Figure 3.3.1).

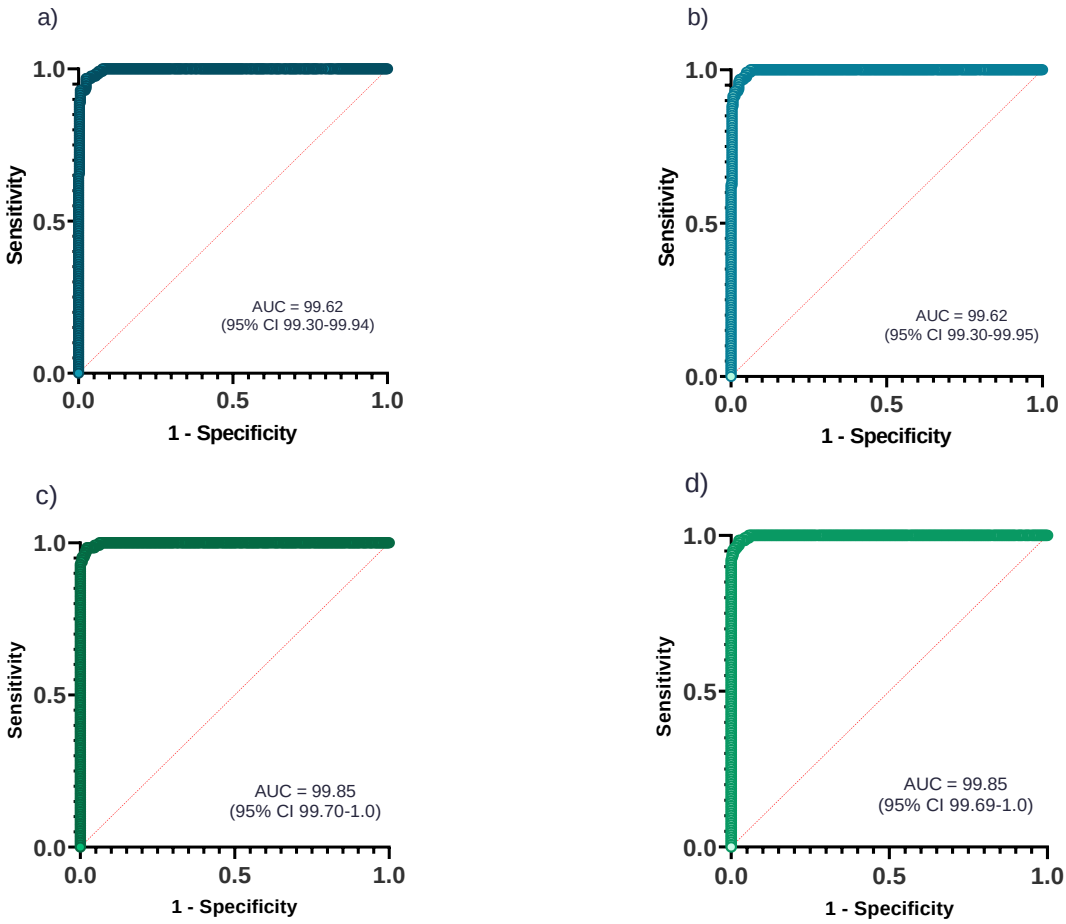

Figure 3.3.1 Receiver operator characteristic curves.

ROC curves with area under the curve (AUC) and 95% confidence intervals for a) S1 OD ELISA, b) S1 corrected OD ELISA, c) RBD OD ELISA and d) RBD corrected OD ELISA.

Control sample OD values were also plotted as density plots with the chosen cut-off marked to visualise the distribution of the two sample populations in relation to the cut-off value (Figure 3.3.2). Density plots show distinct

populations of negative and positive samples.

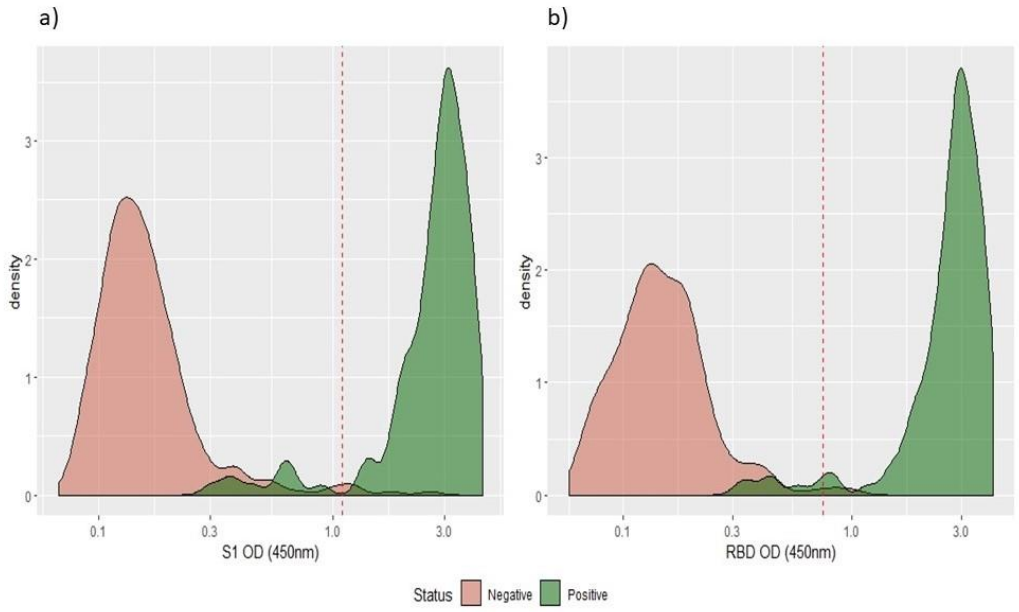

Figure 3.3.2 Density plot of OD values for all patient samples.
Plots show samples tested on the a) S1 and b) RBD ELISA. Cut off values for each ELISA are marked by the red dashed line. Sample densities are coloured by their status (red=negative, green=positive) according to the results of both ELISA test combined in parallel.

3.3.2 Sensitivity and specificity analysis

Final sensitivity (Se) and specificity (Sp) values for the two ELISA tests combined in parallel were Se = 95.31% (95% CI 90.08-98.26) and Sp = 97.19% (95% CI 94.73-98.71%). Assay specificity was consistent throughout, with 9 out of 320 pre-December 2019 samples testing positive for either S1 or RBD. However, assay sensitivity improved with time-since-infection, with 100% sensitivity found for samples tested at least 42 days post infection).

Table 3.3.2 Comparison of sensitivity and specificity for different times-since-infection.

Sensitivity, specificity and 95% confidence intervals for S1 and RBD ELISAs combined in parallel. Values are calculated according to different days-since-infection (confirmed by RT-PCR).

Time since infection	True status	ELISA positive	ELISA negative	Total tested	Sensitivity	95% CI	Specificity	95% CI
All	Positive	122	6	128	95.31	90.08-98.26	97.19	94.73-98.71
	Negative	9	311	320				
>14 days	Positive	88	4	92	95.65	89.24-98.80	97.19	94.73-98.71
	Negative	9	311	320				
>21 days	Positive	80	3	83	96.39	89.80-99.25	97.19	94.73-98.71
	Negative	9	311	320				
> 28 days	Positive	66	2	68	97.06	89.78-99.64	97.19	94.73-98.71
	Negative	9	311	320				
> 35 days	Positive	51	1	52	98.08	89.74-99.95	97.19	94.73-98.71
	Negative	9	311	320				
> 42 days	Positive	36	0	36	100.00	90.26-100.00	97.19	94.73-98.71
	Negative	9	311	320				

3.3.3 Comparison of S1 and RBD responses

Correlation between S1 and RBD responses was very high (r = 0.93, df=6633, p= <0.001). S1 OD values tended to be higher than RBD values, as reflected in the lower cut-off value for RBD as determined through the ROC analysis (Table 3.3.1). However, there was no significant difference between the log OD values for either protein (p = 0.209) (Figure 3.3.3.B). Although similar proportions of samples were deemed negative against one antigen and positive against the other, Figure 3.3.3.A shows that more samples negative for RBD had high OD values against S1 (values greater than 2) than vice versa.

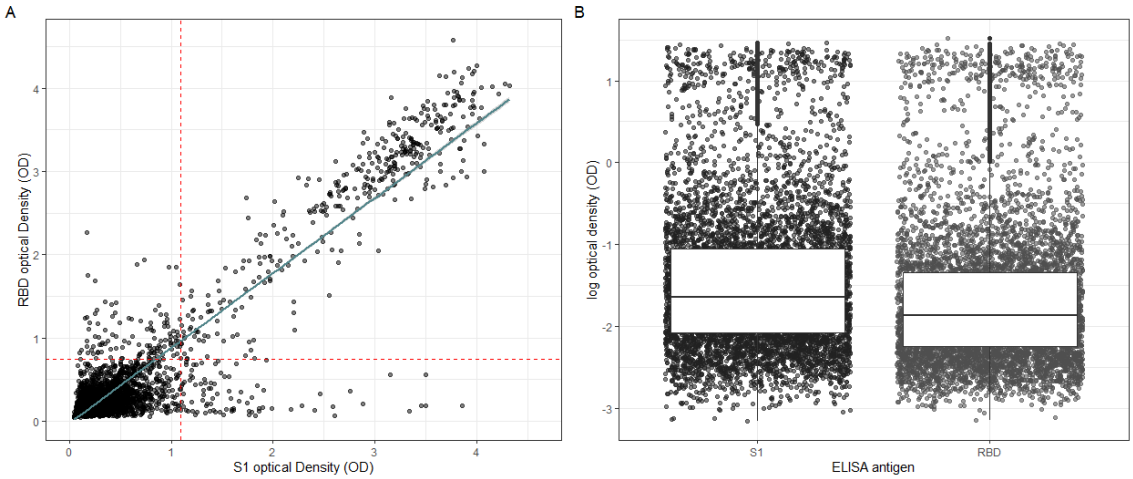

Figure 3.3.3 Comparison of ELISA optical density (OD) results against the S1 subunit (S1) and the receptor binding domain (RBD) of the SARS-CoV-2 spike protein.
A) correlation between S1 and RBD OD values, cut-off values for each antigen are shown in red-dashed lines; B) Box plot showing median and interquartile range of OD values for S1 and RBD ELISAs.

Number and proportion of samples positive or negative against both S1 and RBD antigens, and positive against only one antigen, as well as the proportion agreement are shown in (Table 3.3.3). Proportion agreement of negative samples was high for both S1 and RBD. The proportion agreement for positive samples was higher for S1 (79.24) than for RBD (74.34%).

Table 3.3.3 Comparison of S1 and RBD status.

Samples tested against S1 subunit (S1) and receptor binding domain (RBD) of the SARS-CoV-2 spike protein, showing proportion agreement.

	S1 negative (%)	**S1 positive (%)**	**Proportion agreement (%)**
RBD negative (%)	6065 (91.82)	88 (1.33)	98.57
RBD positive (%)	116 (1.75)	336 (5.09)	74.34
Proportion agreement (%)	98.12	79.24	

3.3.4 Assay precision

3.3.4.1 Repeatability

To assess assay repeatability, the variation between within-plate replicates was evaluated. Coefficient of variation of OD values for within-plate replicates were calculated for every plate. Overall, for the S1 ELISA 75.0% of samples had within-plate CV values of less than or equal to 15%. Of samples categorised as negative (by either S1 or RBD) this value was 67.25% and for positive samples 86.32%. The mean CV value for negative samples on the S1 ELISA was 15.6% and the median 10.4%, ranging from 5.62 to 110.0%, while the mean CV for positive samples was 8.2%, the median was 3.26% and the range was 3.14 to 58.7%.

For the RBD ELISA, 77.62% of all samples had within-plate CV values of ≤15%, with 69.75% of negatives and 87.90% positives. The mean CV value for negative samples was 14.5% (range 3.93-101.0%) and the median was 10.3, while for positive samples the mean was 7.11%, the median was 3.26% and the range was 6.48-46.9%. For both assays, relative variance, as represented by CV, was generally higher for samples with lower mean OD values (Figure 3.3.4)

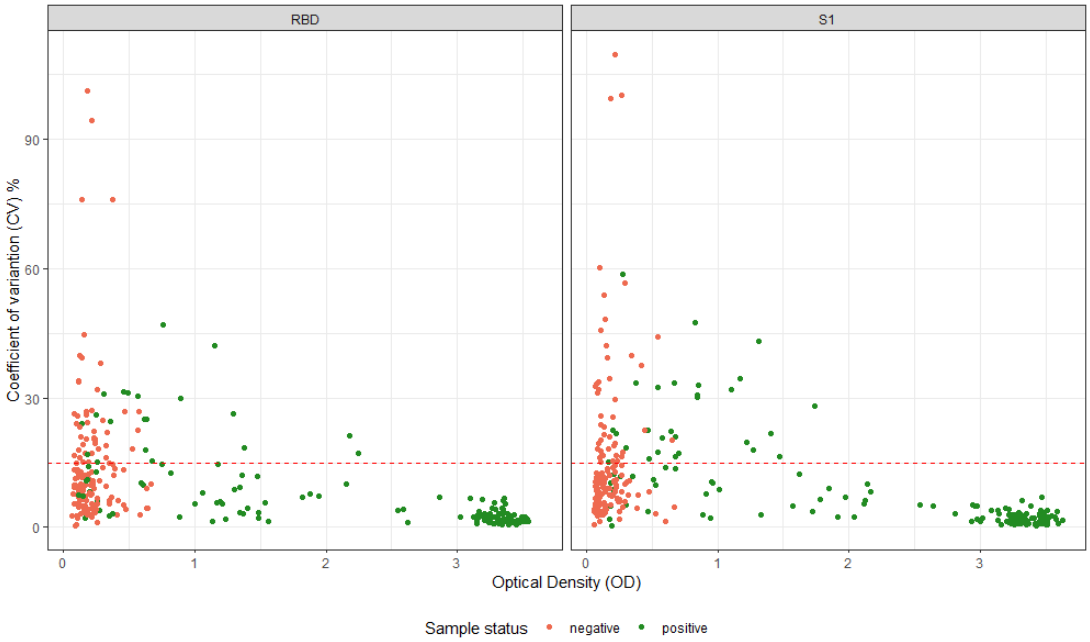

Figure 3.3.4 Coefficient of variation (CV) and optical density (OD) values for within-plate replicates tested on S1 and RBD ELISAs.

Samples originally classified as negative are shown in red and those originally classified as positive are shown in green. Red dashed line shows 15% CV value.

Figure 3.3.5 Optical density (OD) values for repeat tested samples on S1 and RBD ELISAs.

Red dashed lines indicate ELISA cut-off value. Samples originally classified as negative are shown in red and those originally classified as positive are shown in green. Samples are ordered according to the mean OD of all replicates.

3.3.4.2 Intermediate precision

Intermediate precision between plates was assessed by comparing the coefficient of variation of between-plate replicates (Table 3.3.4). The mean of the within-plate replicates was used as the sample OD value for each plate. Overall, for the S1 ELISA, 77.08% of samples had CV values ≤20%, with 70.18% of negative samples and 87.18% of positive samples. Overall, for the RBD ELISA, 70.83% of samples had CV values ≤20%, with 59.65% of negative samples and 87.18% of positive samples. Mean, median and range of between-plate CV values are shown in Table 3.3.4.

Table 3.3.4 Intermediate precision of between-plate replicates for S1 and RBD ELISAs. Table shows summary of number of replicates and coefficient of variation values for between-plate, within-operator intermediate precision of S1 and RBD ELISA optical density (OD) responses. Means of within-plate triplicate repeats were used to calculate CV values.

Between-plate replicates	Number of replicates	CV ≤ 20% (N)	CV ≤20% (%)	Mean CV	Median CV	Range
S1 ELISA						
Overall	96	74	77.08	15.8	9.72	0.83-121.0
Negative	57	40	70.18	18.5	12.1	3.21-67.2
Positive	39	34	87.18	13.2	5.38	0.83-121.0
RBD ELISA						
Overall	96	68	70.83	15.4	11.9	0.32-103.0
Negative	57	34	59.65	17.5	15.5	3.40-40.6
Positive	39	34	87.18	13.3	3.80	0.321-103

To evaluate intermediate precision across all repeats, CV values were calculated for each sample across all within-plate replicates, plates and operators. On the S1 ELISA 31.13% samples had an overall CV less than or equal to 20%. On the RBD ELISA 34.38% samples had an overall CV of less than or equal to 20%. Mean, median and range of overall CV values are shown in Table 3.3.5.

Table 3.3.5 Intermediate precision of across-plate replicates for S1 and RBD ELISAs. Summary of number of replicates and coefficient of variation values for between-plate and between operator intermediate precision of S1 and RBD ELISA optical density (OD) responses. Means of within-plate triplicate repeats across all plates were used to calculate CV values.

All replicates	Number of samples	CV ≤20% (N)	CV ≤20% (%)	Mean CV	Median CV	Range
S1 ELISA						
Overall	32	10	31.13	31.9	30.0	4.40 – 122.0
Negative	19	1	5.26	39.0	31.0	17.5 – 107.0
Positive	13	9	69.23	24.7	6.79	4.40 – 122.0
RBD ELISA						
Overall	32	11	34.38	28.6	28.5	2.66 – 132.0
Negative	19	2	10.53	32.8	32.1	16.7 – 68.7
Positive	13	9	69.23	24.3	5.83	2.66 - 132

3.3.4.3 Agreement by classification

Agreement in status (positive or negative) between samples repeats was investigated. Number of samples which were negative or positive on both tests, number of samples which were negative and became positive, and number of samples which were positive and became negative, as well as the proportion agreement and Cohen's Kappa coefficient of agreement, for raw OD values and corrected absorbance values, are shown in Table 3.3.6 and Table 3.3.7 respectively.

Table 3.3.6 Agreement characteristics of S1 and RBD ELISAs using OD as outcome variable.

Parameters reported are numbers of samples, percentage agreement and Cohen's Kappa coefficient (95% confidence intervals).

OD		**Repeats**			
		Negative	**Positive**	Agreement (%)	Cohen's Kappa coefficient (95% confidence interval)
Original S1	**Negative**	979	45	95.61	0.81 (0.78 – 0.84)
	Positive	110	586	84.20	
Original RBD	**Negative**	927	43	95.57	0.83 (0.80 – 0.85)
	Positive	102	648	86.40	
Original combined in parallel	**Negative**	860	2	99.77	0.80 (0.77 – 0.83)
	Positive	169	689	80.30	

Table 3.3.7 Agreement characteristics of S1 and RBD ELISAs using correct absorbance (SampleOD-negative controlOD/negative control OD) as outcome variable.
Parameters reported are numbers of samples, percentage agreement and Cohen's Kappa coefficient (95% confidence intervals).

Corrected absorbance		Repeat			
		Negative	Positive	Agreement (%)	Cohen's Kappa coefficient (95% confidence interval)
Original S1	Negative	1009	15	98.54	0.84 (0.81 – 0.86)
	Positive	117	579	83.19	
Original RBD	Negative	928	42	95.67	0.77 (0.73 – 0.80)
	Positive	153	597	79.60	
Original combined in parallel	Negative	861	1	99.88	0.74 (0.71 – 0.77)
	Positive	220	638	75.64	

All replicate OD values and corrected absorbance values for each sample were also plotted according to their original status on first testing (Figure 3.3.6). Negative samples rarely produced OD or corrected absorbance values above the cut-off value for each assay.

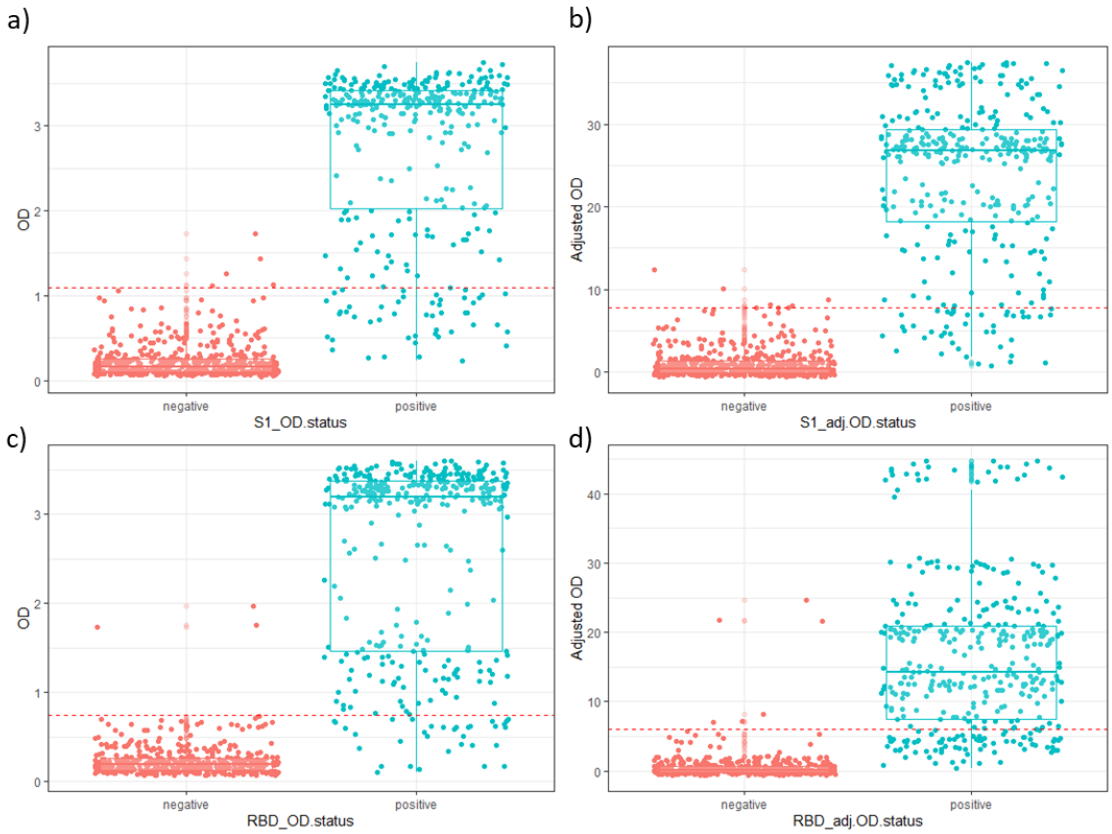

Figure 3.3.6 Agreement between replicates of positive and negative samples.
a) shows OD and b) corrected absorbance against S1, and c) OD and d) corrected absorbance against RBD. OD values of all replicates of 13 negative and 17 positive samples are shown as individual points with box plots. Samples and boxplots are coloured according to their status on first testing. ELISA cut-off values are shown by a red dashed line.

3.3.5 Comparison of OD and corrected OD values

While calculating weekly prevalence, primary care samples from weeks 18 and 20 showed unexpectedly high seroprevalence. Weekly seroprevalence estimates were calculated using raw OD values for primary care patients, who had on average lower ELISA responses, and secondary care patients, who had on average higher ELISA responses (see results for community study below). In primary care patients, prevalence spiked unexpectedly in week 20 and to a lesser extent in week 18 (Figure 3.3.7.b). As there was no such spike in secondary care patients (Figure 3.3.7.a) and no epidemiological reason to suspect these spikes were real reflections of SARS-CoV-2 seroprevalence, the original data was consulted to explore reasons for this pattern. Further investigation demonstrated that primary care samples from weeks 18 and 20

were tested on the same day and ELISA plates showed unusually high negative control values on this day.

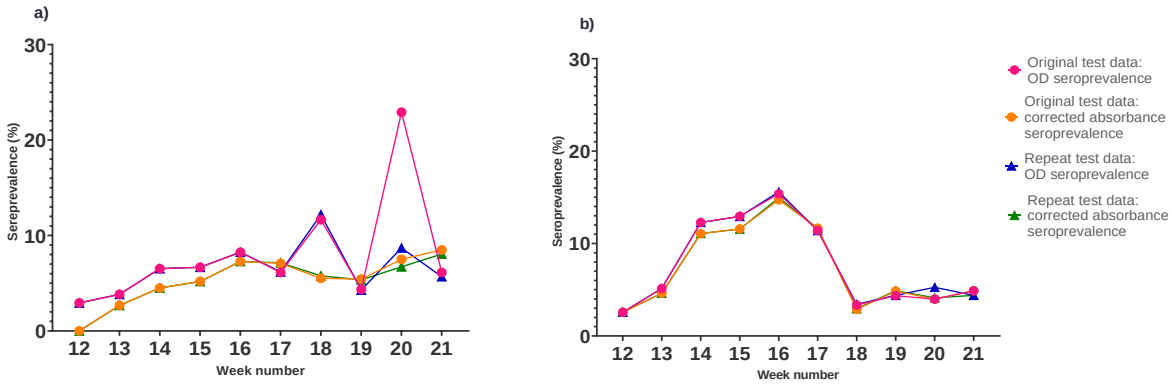

Figure 3.3.7 Weekly SARS-CoV-2 seroprevalence in a Glasgow patient population. Seroprevalence is shown for a) primary and b) secondary care patients calculated using original (circles) or repeated (triangles) testing data, and using either the raw OD (pink and blue) or corrected absorbance (orange and green) values

Plates on which these samples were tested had unusually high negative control values at the very upper limits of the distribution of the negative controls (Appendix 2) and this was reflected in higher OD values of the positive controls and across the plate, resulting in more samples having OD values above the cut-off point. To reassess these anomalous results, samples from these weeks were retested and results accepted if the control ODs were in the middle of the normal range. These repeated results were then used to recalculate the weekly seroprevalence, both using the raw OD and the corrected absorbance value. Negative control values for samples from week 20 were within the normal range but plates on which week 18 samples were re-tested also had very high negative control OD values. Following retesting, the size of the seroprevalence spike in week 20 was significantly reduced, but that of week 18 remained the same.

As an alternative to re-testing the samples from week 18 again, use of an adjustment method to account for these high OD plates was explored. Weekly seroprevalence was recalculated using the corrected absorbance (see 3.2.5.1) for the original as well as for the repeated samples. Seroprevalence calculated using the corrected absorbance eliminated the anomalous spikes in seroprevalence in weeks 18 and 20 (Figure 3.3.7). Corrected absorbance

seroprevalence from week 20 was similar to the seroprevalence calculated from the OD repeated samples for this week and was also consistent with the corrected absorbance seroprevalence calculated from the original test data. The corrected absorbance value for week 18 was equivalent when using the original or retested data. Corrected absorbance therefore appeared to account for laboratory variation more effectively than using raw OD so this was used for further analysis. Further investigations of agreement described above were undertaken after the analysis for publication had been completed and so were not taken into consideration when choosing which ELISA outcome variable to use.

3.3.6 SARS-CoV-2 seroprevalence in a patient population in Glasgow

A total of 7480 serum samples, collected during the ten-week period from March 16th 2020 (weeks 12 to 21 of 2020) were tested for IgG antibodies against the S1 subunit and RBD of SARS-CoV-2 using the in-house ELISAs described above. Of these samples, 6635 met the criteria for inclusion and so were used for further analysis. Samples were derived from primary (n = 2531, 38.15%) and secondary (n = 4104, 61.85%) care types and covered all age ranges except those under the age of 18 for whom few samples were available. Population demographics are shown in Figure 3.3.8.

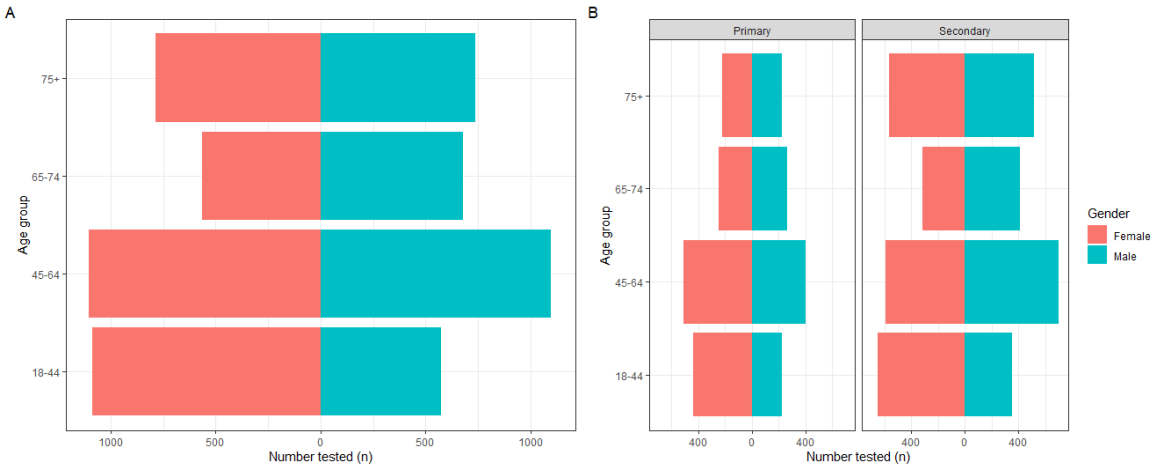

Figure 3.3.8 Demographics of sampled NHS Greater Glasgow and Clyde patient population.

A) Number of males (orange) and females (green) tested by age group; B) number of males (orange) and females (green) tested by age group in primary and secondary care settings.

Overall unadjusted seroprevalence was 7.81% (95% CI 7.17-8.48), but this varied week by week from a low of 2.68% in week beginning (w/b) 16th March to a high of 13.0% during w/b 13th April 2020. Seroprevalence increased to w/b 13th April then declined to w/b 27th April before stabilising (Figure 3.3.9, B). Seroprevalence in secondary care patients reached a higher peak than in primary care patients but decreased more steeply from this peak compared to primary care seroprevalence, which remained broadly consistent from the w/b 13th April peak to the end of the study period. Overall seroprevalence was significantly greater in patients sampled while in secondary care compared to primary care (OR 1.7, 95% CI 1.4-2.0, p= <0.0001), in males compared to females (OR 1.4 95% CI 1.2-1.7, p=0.0004) and in those aged 45-64 (OR 1.9 95% CI 1.5-2.5, p=<0.0001), 65-74 (OR 1.5 95% CI 1.1-2.0, p=0.007) and ≥75 (OR 1.8 95% CI 1.4-2.4, p=<0.0001) compared to those aged 18-44 (Figure 3.3.9, A).

Between age-groups, male patients aged 45-64y and 64-74y had higher seroprevalence compared to women. Across age groups, seroprevalence in primary care patients was similar in males and females but was higher in men in secondary care (10.73%, 95% CI 9.40-12.17) compared to women (7.60%, 95% CI 6.51-8.81) (Figure 3.3.9, C). Similarly, overall seroprevalence did not

vary greatly amongst primary care patients but older patients had higher prevalence in secondary care settings compared to those from primary care (Figure 3.3.9, C).

Table 3.3.8 Unadjusted and adjusted seroprevalence estimates in different demographic groups in the Glasgow patient study population.

Unadjusted values are those observed from the raw data, adjusted values are those adjusted for assay sensitivity and specificity using the Bayesian state space model described in Hughes et al. (2021)

		Seroprevalence (95% confidence interval)	
Demographic group	**Tested (N)**	**Unadjusted**	**Adjusted**
Sex			
Male	3092	9.06 (8.07–10.12)	6.49 (0.16–17.67)
Female	3543	6.72 (5.92–7.59)	4.23 (0.13–13.14)
Care type			
Primary	2531	5.69 (4.82–6.66)	2.95 (0.10–8.23)
Secondary	4104	9.11 (8.25–10.04)	6.73 (0.21–17.44)
Age			
18–44	1662	5.05 (4.05–6.22)	3.1 (0.10–9.05)
45–64	2202	9.36 (8.17–10.65)	6.67 (0.16–17.84)
65–74	1244	7.48 (6.08–9.08)	5.18 (0.15–13.98)
>75	1527	8.84 (7.46–10.38)	5.78 (0.17–14.96)
Overall	6635	7.81 (7.17–8.48)	5.29 (0.13–15.10)

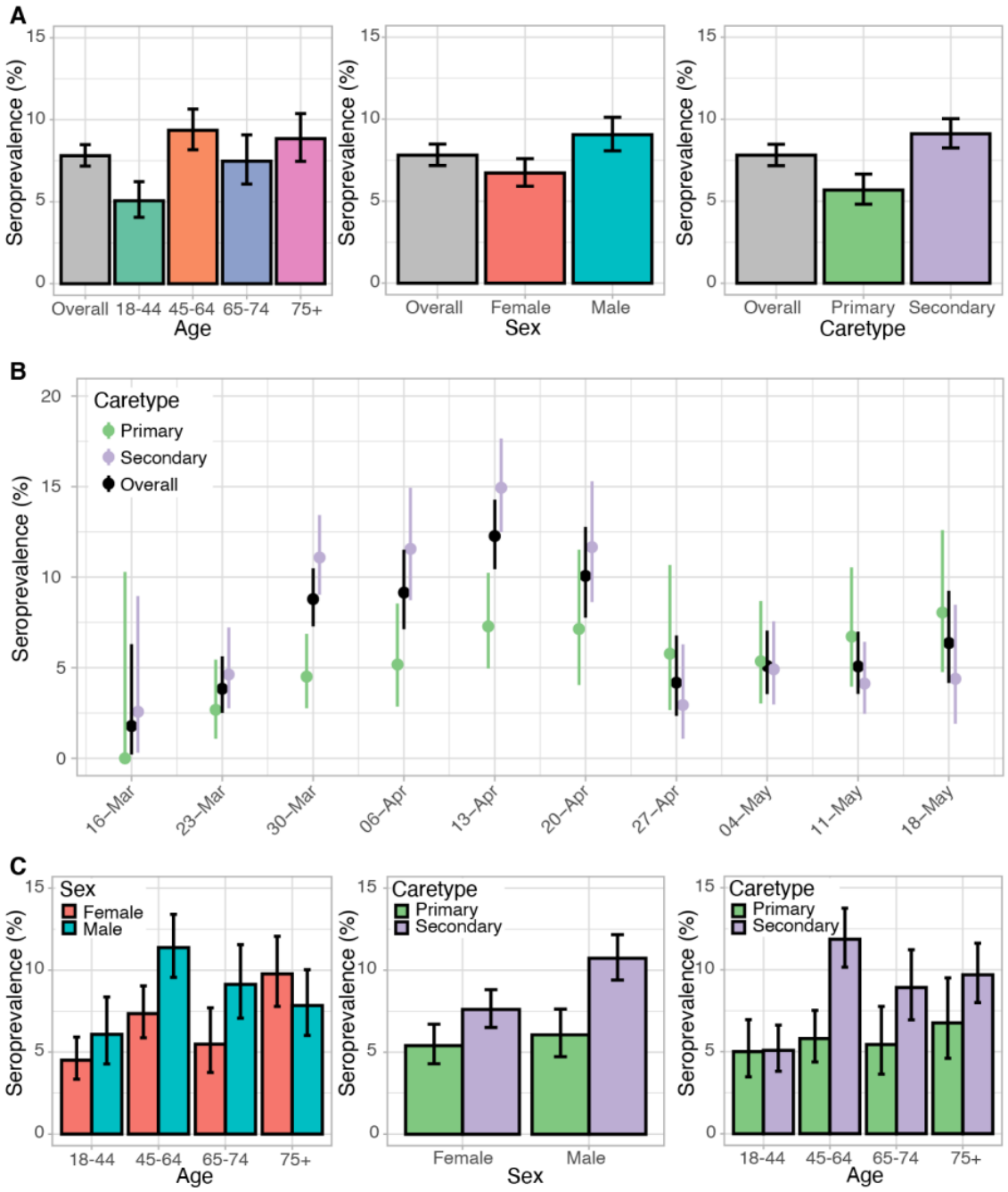

Figure 3.3.9 Unadjusted severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) seroprevalence in NHS Greater Glasgow and Clyde patient population.
Seroprevalence estimates and 95% confidence intervals are shown across age groups, sex, and healthcare setting (A), or date of sampling (B). Seroprevalence estimates and 95% confidence intervals investigated in sequential combinations of age group, sex, and healthcare setting are also shown (C).

3.3.7 Comparison of PVNA and S1 ELISA

Following ELISA testing, 1974 serum samples were also tested by pseudotype virus neutralisation assay (PVNA) as described above. Of these, 117 (54.17%)

S1 ELISA positive and 17 (0.97%) S1 ELISA negative samples were found to also have a neutralising antibody response (i.e. percentage neutralisation >90%) (Figure 3.3.10). S1 ELISA positive samples derived from patients in secondary care displayed significantly higher levels of neutralising activity compared to those from primary care settings (p = <0.0001) (Figure 3.3.10, C). IgG levels (as represented by ELISA corrected absorbance) were also higher in secondary care patients (p=0.016) (Figure 3.3.10, B). By comparison, S1 ELISA negative samples showed no difference in neutralising ability or IgG levels between care types.

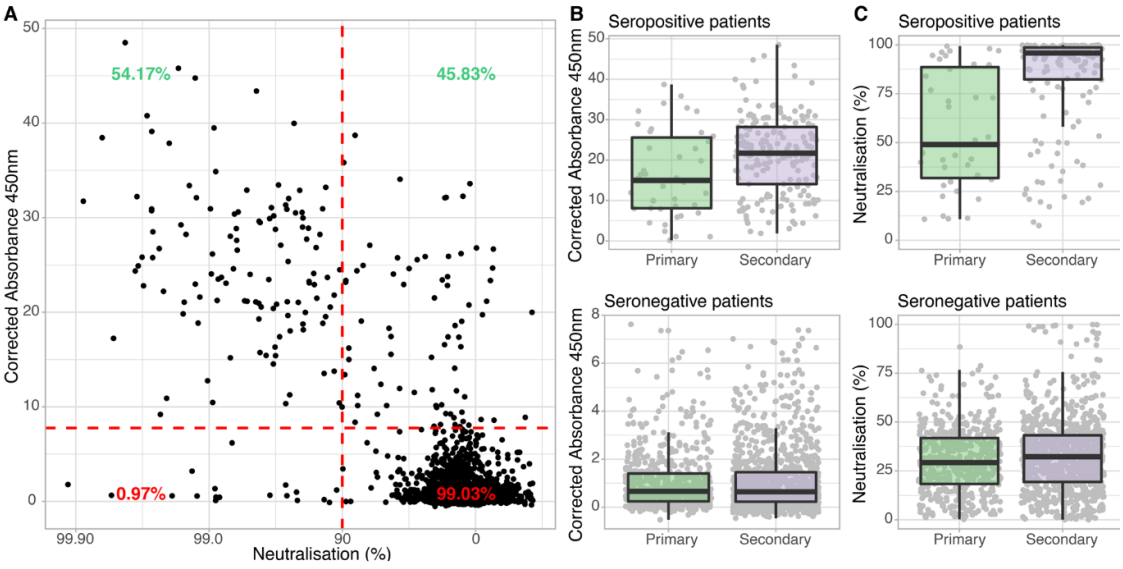

Figure 3.3.10 Antibody levels and subsequent virus neutralization activity.
Correlation between virus neutralization and antibody production is shown as a scatterplot (A), where each black dot represents one sample. Percentages reflect the sample distribution among seropositive patients (green numbers) and seronegative patients (red numbers), and between low (right) and high (left) virus neutralization. Boxplots of ELISA corrected-absorbance (B) and virus neutralisation (C) values are shown for samples seropositive or seronegative for SARS-CoV-2 in primary and secondary care patients.

3.3.8 Factors associated with neutralising antibody response

Multivariable logistic regression models confirmed that increasing absorbance values on ELISA were significantly associated with having a neutralising response greater than 90% (OR=1.15, 95% CI 1.10-1.21, p=<0.001). Multivariable models also confirmed that samples derived from secondary care had significantly higher odds of having neutralising ability compared to those derived from a primary care setting (S1 OR=6.77, 95% CI 2.68-18.75,

p=<0.001). No significant association was found between neutralising antibody response and age-group or sex.

Table 3.3.9 Multivariable logistic regression model for neutralising activity.
Estimated odds ratios, 95% confidence intervals and p values for explanatory variables, including corrected absorbance values against S1 antigen.

	Odds ratio (OR)	**95% confidence intervals**		**p value**
S1 corrected absorbance	1.15	1.10	1.21	< 0.001
Primary care	Reference			
Secondary care	6.77	2.68	18.75	<0.001
Female	Reference			
Male	1.18	0.59	2.34	0.642
18-44 years	Reference			
45-64 years	1.22	0.46	3.20	0.689
65-74 years	3.10	0.98	10.34	0.059
75+ years	0.95	0.32	2.78	0.919

Table 3.3.10 Multivariable logistic regression model for neutralising activity.
Estimated odds ratios, 95% confidence intervals and p values for explanatory variables, including corrected absorbance values against RBD antigen, in a logistic regression model for neutralising activity.

	Odds ratio (OR)	95% confidence intervals		p value
RBD corrected absorbance	1.16	1.12	1.21	<0.001
Primary care	Reference			
Secondary care	5.32	2.08	14.93	0.001
Female	Reference			
Male	1.12	0.52	2.40	0.766
18-44 years	Reference			
45-64 years	1.40	0.50	3.89	0.520
65-74 years	2.77	0.82	9.79	0.106
75+ years	1.39	0.44	4.41	0.576

3.4 Discussion

In response to an unprecedented public health emergency, two indirect ELISAs, against the S1 subunit (S1) and receptor binding domain (RBD) of SARS-CoV-2, were utilised for rapid sero-surveillance in a patient population in Glasgow, Scotland, and their performance evaluated. This study established cut-off values for these ELISAs, estimated sensitivity and specificity of the tests, and explored measures of assay precision. The ELISAs were used to estimate levels of exposure to SARS-CoV-2 in a patient population in Glasgow during the first wave of the COVID-19 pandemic, as well as to compare antibody (IgG) and neutralising antibody (NAb) responses in different patient groups.

This work was undertaken during a critical stage in the COVID-19 pandemic, before the establishment of large-scale sero-surveillance programmes and prior to the development of vaccines, when limited information existed around population-level exposure in Scotland or the wider UK and understanding of the humoral immune response to SARS-CoV-2 was still developing. The work reported in this study was shared throughout with Public Health Scotland (PHS) and NHS Greater Glasgow and Clyde (NHSGGC) and provided useful real-time information on seroprevalence levels, which helped to inform local and national responses to the pandemic.

The work reported in this chapter focussed on five aims, set out in the introduction, and discussed in turn below.

3.4.1 Establishment of cut-off values for S1 and RBD ELISAs

An essential first step in the use of the S1 and RBD ELISAs was to establish a cut-off value whereby the continuous output of the ELISA, in the form of optical density (OD) values, could be converted to a binary, assigning tested samples a status of either positive or negative. Numerous methods have been proposed for establishing appropriate cut-off points for ELISA and other continuous output assays (Frey et al., 1998, Xu et al., 1997, Lardeux et al., 2016, Nielsen

et al., 2004) but receiver operator characteristic (ROC) analysis is widely used in the medical literature when a gold standard test is available for comparison.

Here, ROC analyses were used to identify potential cut-off values, which were then selected based on sensitivity and specificity requirements. ROC analysis has an advantage over other methods of determining cut-off values in that plotting of ROC curves also provides a method of evaluating the discriminatory ability of a test to distinguish between truly positive and truly negative samples (Dohoo et al., 2009). The area under the curve (AUC) of the ROC curve provides a measure of the overall diagnostic accuracy of the test and can be interpreted as the probability that a truly positive individual will have a higher test value (in this case OD or corrected OD) compared to a random truly negative individual (Dohoo et al., 2009, Thrusfield and Christley, 2018, Kumar and Indrayan, 2011). The high AUC values obtained from the ROC analysis for both the S1 and RBD ELISAs demonstrated that they had very good power to distinguish between positive and negative samples, and that the probability of a truly positive sample having higher ELISA absorbance compared to a negative sample was greater than 99% for both S1 and RBD ELISAs (Figure 3.3.1)

3.4.2 ELISA sensitivity and specificity

ROC analysis enables a cut-off to be chosen based on the requirements of test interpretation. For example, if false positives were of particular concern a cut-off value with a higher specificity could be chosen at the expense of sensitivity. In this study, both false positive results and false negative results were of concern, but avoidance of false positive results was prioritised to avoid over-estimating seroprevalence. However, retaining a high sensitivity, and so minimising false negative results, was also important so cut-off values were chosen which maximised specificity without compromising sensitivity. This effectively meant the choosing a cut-off point close to the point at which both sensitivity and specificity were maximised.

The cut-off values chosen for S1 and RBD ELISAs resulted in an assay with good sensitivity and specificity for detecting IgG antibody responses to SARS-CoV-2 in patients from 2 weeks post-positive PCR test. Combining the two

ELISAs in parallel slightly reduced the overall sensitivity and specificity of the test but enabled individuals with atypical immune responses (i.e. only producing antibodies to S1 or to RBD) to be captured. The final sensitivity and specificity values determined for the combined ELISAs (Se = 95.31% (95% CI 90.08-98.26); Sp = 97.19% (95% CI 94.73-98.71%)) compared favourably with those reported for other in-house and commercial ELISAs against SARS-CoV-2 (Van Honacker et al., 2022, Vengesai et al., 2021, Ainsworth et al., 2020).

Test sensitivity could be improved to 100% by limiting the definition of "true positive" samples to those taken at least 42 days post a positive RT-PCR result (Table 3.3.2). The difference in the assay's ability to identify positive samples at different points post infection is consistent with individual differences in antibody production time. By 42 days post infection all positive samples in our study had measurable antibody response and this is consistent with other studies investigating the time-course of SARS-CoV-2 antibody production (Post et al., 2020).

Although the test performance was improved by limiting positive control samples to those with more than 42 days post infection, using the resulting sensitivity value for downstream analysis could be misleading in seroprevalence studies such as this one, where time-since-exposure of study participants was not known, and which aimed to provide a snap-shot of population-level exposure at a given point. False negative individuals are inevitable in a cross-sectional population due to individuals who have been exposed but not yet sero-converted, as well as those who have had mild infections resulting in lower resulting IgG responses that did not meet the ELISA threshold (Eyre et al., 2021). Using a sensitivity value that takes these individuals into account, therefore, will provide a more accurate estimation of population seroprevalence.

A small number of false positive results were identified in the sensitivity and specificity analysis. Nine pre-December 2019 samples tested positive by ELISA, 5 against S1 and 4 against RBD. These samples were all otherwise healthy individuals who could not have been exposed to SARS-CoV-2 and are unlikely

to have been exposed to SARS-CoV-1 given their UK origin. These positive results could have arisen for several reasons and similar patterns of reactivity to SARS-CoV-2 antigens prior to December 2019 have been found in other studies. The cause of these false positives may be cross-reactivity with seasonal human coronaviruses (HCoV) such as OC43 etc or SARS-CoV-1 (Zedan and Nasrallah, 2021, Woudenberg et al., 2021), or other idiosyncrasies of an individual's immune response.

3.4.3 ELISA precision and repeatability

The third aim of this study was to investigate the repeatability and precision of the ELISAs. This was undertaken by exploring the within-plate repeatability, between-plate intermediate precision, and the agreement between repeats of the same samples. Within-plate repeatability was generally good, with the majority of triplicate repeat samples having coefficient of variation (CV) values of 15% or lower. There was more relative variance (as represented by CV values) in samples with lower OD values compared to higher ODs (Figure 3.3.4) but even where high CV values between replicates of negative samples were observed, almost all OD values fell below the negative cut-off (Figure 3.3.6) demonstrating that status agreement for negative samples was high despite higher CV values.

Greater variation was observed between plates and between operators, as would be expected given the increased number of variables involved, but the majority of between-plate, within-operator CV values were below 20%. Across all within-plate replicates, plates and operators, CV values were higher and fewer samples had overall CV values below 20% (Table 3.3.5
Table 3.3.5). Variability in ELISA readings may be due to variations in the amount of IgG in each well, for example as a result of pipetting errors, or through variation in background "noise." Sources of variation can occur at many points in the protocol such as inefficient washing or blocking steps, or too low a concentration of the secondary antigen. In the assay described here these factors were either automated (washing) or kept constant between runs (blocking buffer concentration, volume and duration, and secondary antigen concentration) and so are unlikely to be the principal cause of plate-to-plate

or operator-to-operator variation. Inter-assay variability is more likely to arise from pipetting and dilution inconsistencies between users and other variables not captured in the data, including temperature fluctuations in the laboratory or inconsistencies in antigen binding effectiveness. Temperature was not recorded routinely for most of the assays used in this analysis but anecdotal reports suggest the laboratory temperature fluctuated within and between days during the testing period. Further research into the effect of ambient temperature on the assay, or of performing incubation steps in a temperature-controlled environment such as an incubator would be beneficial for future use of the assay. Uneven antigen coating of wells could also account for well-to-well and plate-to-plate variation and warrants further investigation. In this assay phosphate buffered saline (PBS) was used as a coating buffer, with a pH of around 7.4, but alternatives such as a carbonate/bicarbonate buffer with a higher pH may facilitate more consistent binding.

Despite the variation observed among OD values of repeat samples, agreement between repeats was generally high, indicating that the assay has good precision when interpreted as a binary (positive or negative) outcome. Cohen's kappa coefficient of agreement confirmed that most samples retained the same binary status on repeat testing even if the OD values varied. This was particularly apparent in samples with high and low mean OD values (i.e. the most obviously positive and negative samples) where variation was lower and status remained consistent between repeats. Mid-range samples, with mean OD values closer to the cut-off, showed the most variation and were more likely to change status on different test runs (Figure 3.3.5). The response of the samples observed here is typical of mid-range samples for any continuous ELISA output simplified to a binary response for two reasons. Firstly, the amount of antibody in these samples results in an optical density closer to the cut-off value and so small changes between replicates is more likely to result in a change of status. Secondly, ELISAs have a typically sigmoidal dose-response curve with mid-range samples lying around the upwards part of the curve. A small change in dose (antibody concentration) can therefore elicit a large change in the response (optical density). So small changes in the amount of

antibody present in the tested sample, for example due to pipetting or dilution errors, will result in proportionally greater differences in OD for these mid-range samples. Although reducing run-to-run variability would be beneficial, and if the assay were to be commercialised or used for individual-level diagnostics this would be essential, for the purposes of population-level surveillance, particularly in the time and resource-limited setting of the early pandemic, agreement was more important in practical terms than reducing variance.

Two ELISA outputs were considered in this study, i) raw OD values and ii) OD values corrected for the within-plate negative control, referred to as the "corrected absorbance". Corrected absorbance was chosen for use in the published analysis of these data because it was able to better adjust for non-specific laboratory variation observed on certain testing days (Figure 3.3.7), as well as being a common choice for ELISA interpretation. The cause of the variation on certain plates could not be determined with certainty but may have been due to high temperatures in the laboratory. By using corrected absorbance values the seroprevalence was adjusted to a level consistent with the repeated samples, which were assumed to being a more accurate representation of the true seroprevalence, and with estimates from the Bayesian state-space model (Appendix 1). Adjusting by the negative control had the effect of moving low positive values below the cut-off, thus making them negative, which eliminated the effect of the high overall plate values. For this reason, it was decided to use corrected seroprevalence for further analysis, although all models and analyses were investigated using raw OD as well as corrected seroprevalence and the patterns in the data remained the same. Corrected OD provided a more conservative estimate of prevalence and so was deemed the more appropriate choice of measure for publication.

Repeatability as represented by the coefficient of variation (CV) could not be assessed for corrected absorbance because the use of a biological negative control (i.e. not always the lowest value on the plate) resulted in negative corrected absorbance values, but agreement between replicates, as demonstrated by Cohen's kappa coefficient, was good for this corrected

measure. However, analysis of the triplicate repeat data, undertaken after the sero-surveillance study, showed that levels of agreement were higher for raw OD values compared to corrected absorbance, suggesting that OD without adjustment may be a better choice for assay interpretation in the future. However, it is important to note that raw OD values are still subject to laboratory variation which may result in spikes in the number of positives, as seen in the original results of samples tested from weeks 18 and 20. If OD was to be used for further analysis it would be sensible to further investigate the relationship between the value of the within-plate controls and the sample OD values and potentially to impose an upper and lower limit for these controls in order to avoid unusually high or low values across the plate. Alternatively, a cut-off value could be determined for each plate based on additional within-plate control samples as a means of mitigating against inter-plate variability. This would allow individual plates to have higher over-all values without affecting the outcome of the assay. Another alternative would be to set a zone of uncertainty around the cut-off, in which samples would be classified as of undetermined status rather than positive or negative.

Overall, the assay showed reasonable repeatability, although it was subject to day-to-day laboratory variation, the exact source of which was unclear. Despite this variation, measures of agreement between repeat tests runs were very good. This, in combination with the high AUC values on the RIC curves, confirmed that the assays were able to provide a good indication of antibody status.

3.4.4 Seroprevalence and patterns of exposure in a Glasgow patient population during the first wave of the COVID-19 pandemic

Use of the ELISA assays enabled sero-surveillance of a patient population in Glasgow during the first wave of the COVID-19 pandemic, undertaken in challenging circumstances with limited resources. Serological surveillance is a key element to understanding disease spread and impact and is vital for informing strategies around control or elimination of many infectious diseases (Arnold et al., 2018). This was, and remains, equally true in the context of

SARS-CoV-2 pandemic, particularly during the early phases when little was known about the epidemiology or immunology of the virus (Winter and Hegde, 2020). This study was able to track changes in exposure to SARS-CoV-2, as measured by seroprevalence, in the largest health board in Scotland in close to real time, and thus provided important insights into population level immunity during this period.

The study had several key findings: a) overall seroprevalence was under 10%, and remained broadly consistent, after the initial rise, in the most representative patient group; b) seroprevalence was higher in males, older age groups and those in secondary care; c) IgG responses to S1 and RBD were highly correlated; d) around half of patients with measurable IgG responses also had neutralising antibodies (NAbs); and e) IgG and NAbs were correlated with each other and were higher in secondary care patients.

Overall unadjusted seroprevalence during the study period from 16th March to 24th May 2020 was 7.81% (7.17–8.48%), with a steady increase in weekly seroprevalence observed to mid-April 2020, before a levelling off in the most representative patient group – those sampled in primary care settings. Secondary care weekly seroprevalence also rose steeply to w/b 13th April but then declined, whereas primary care seroprevalence remained broadly similar from mid-April onwards (Figure 3.3.9). The peak in secondary care seroprevalence occurred around two weeks after the peak of RT-PCR-confirmed cases in Glasgow (data not shown here, see Appendix 1 Figure 3D (Hughes et al., 2021)), and the decline from this point would be expected in a population heavily weighted towards COVID-19 patients. As infection rates declined, the proportion of patients hospitalised due to COVID-19 would be expected to decrease and so also the proportion of hospitalised patients seropositive for SARS-CoV-2. By contrast, patients attending primary care facilities would not have active symptomatic COVID-19, as seeing these patients in primary care was heavily restricted under NHS guidance at the time, so this population would be made up of patients who were asymptomatic, infected and recovered, or naïve to SARS-CoV-2 (Mulholland et al., 2020). After an initial rise in seroprevalence, consistent with the introduction of a novel

virus into the population, lockdown measures, imposed on 23rd March 2020, likely limited the continued rise in seroprevalence through reduction in viral transmission.

It is important to note that the population investigated here was that of individuals seeking and receiving healthcare, whether in primary or secondary care setting, and so generalisation to the wider community requires caution. Under usual circumstances residual biochemistry samples from primary care patients may be broadly representative of the wider population, and so provide a cost-effective and convenient method of population-level surveillance (Osborne et al., 2000). However, the unprecedented changes to routine healthcare access and health-seeking behaviours during the first wave of the pandemic are likely to have considerably altered the structure of this patient population (Mulholland et al., 2020). Patients who continued to be seen and have blood drawn in primary care settings included pregnant women and individuals with chronic conditions, both of whom were advised to shield at this stage of the pandemic (RCOG, 2020). This may mean these patients were less likely than the wider community to be exposed to SAR-CoV-2, thus leading to lower-than-expected seroprevalence. Conversely, changes in hospital admission and discharge policy during the first pandemic wave meant patients in secondary care were more likely to be in hospital as a direct result of COVID-19 disease (Mulholland et al., 2020) and so might be expected to have higher levels of exposure, and thus seroprevalence, compared to the wider population. Additionally, as time since infection was not known, patients in both care types may have been in the early stages of infection and so not yet have developed a measurable antibody response, leading to lower estimates of seroprevalence in both patient groups. However, even with these caveats, primary care patients were still likely to be a closer representation to the wider community compared to patients in secondary care and provided the most representative sample set available at the time.

Overall seroprevalence, after adjustment for test performance (for methods see Appendix 1), was 5.29% (0.13–15.10%) This was similar to that found in other European cities during an equivalent time period (Pollán et al., 2020,

Stringhini et al., 2020, Mattern et al., 2020), although the same caveats apply to uncertainty regarding how representative the study population was. From 20th April 2020 Public Health Scotland (PHS) also began collecting population level seroprevalence data, initially using primary care residual samples, before expanding to use blood donation samples from the Scottish National Blood transfusion service (SNBTS) and routine antenatal samples (Dickson et al., 2021, Mcauley et al., 2021, Palmateer et al., 2021). There is uncertainty around how representative of the wider population all these sample-sources are, but together they are likely to be broadly representative of patterns occurring at the wider population level (PHS, 2022). Seroprevalence data from PHS, adjusted for test performance using a Bayesian framework, was published from the week after our study ended (w/b 18th May 2020, week 21) and showed a countrywide seroprevalence for this week of 4.4% (95% confidence interval 3.4-5.4%), and a seroprevalence in NHS Greater Glasgow and Clyde of 5.0% (95% CI 2.9-6.8%) (PHS, 2022). These values support the seroprevalence found in our study and are also consistent with another study of Scottish blood donation samples which estimated seroprevalence in Glasgow at 5.4% (95% CI 3.2-7.9%) (Thompson et al., 2020). Ongoing serology work by PHS has since demonstrated that seroprevalence did not increase substantially until the roll-out of vaccinations from December 2020 (Mcauley et al., 2021, Palmateer et al., 2021), a finding also supported by serology studies in England and Wales (PHS, 2022, ONS, 2022). Demonstration of low proportion of individuals with antibodies to SARS-CoV-2 was particularly informative during the early stages of the pandemic when discussions around allowing the development of “herd immunity” through natural infections were occurring (Lourenço et al., 2020, Alwan et al., 2020). This work, which was regularly relayed to Public Health Scotland, clearly demonstrated low levels of population immunity after the first wave. These levels fell far short of the 43 – 70% coverage suggested at the time as potentially required to reach population-level protection, assuming a long lasting protective immune response (Britton et al., 2020, Fontanet and Cauchemez, 2020, Kadkhoda, 2021).

Heterogeneity was observed among seroprevalence levels in different parts of the Glasgow patient population, with males, those in older age groups, and

patients in secondary care settings having higher levels of seropositivity. Other community based seroprevalence studies covering similar stages of the pandemic did not find a difference in exposure by sex, so this finding may reflect a sex bias in COVID-19 presentation, particularly in secondary care settings, which could be related to sex differences in disease severity (Dudley and Lee, 2020, Qi et al., 2021, Fabião et al., 2022), or due to differences in social behaviours between the sexes, which led to increased exposure in certain sections of the male population (Bavel et al., 2020). Higher seroprevalence in secondary care patients was consistent with the bias towards COVID-19 patients in hospital admittance during this period. Higher seroprevalence in older age groups seen in the raw data may also reflect the bias towards older patients being sampled in secondary care settings. The patterns observed in the raw data presented here supported the conclusions of the modelling analysis, which found that probability of infection was significantly higher in males, secondary care patients and those in the 45-46 year old age groups (Appendix 1, (Hughes et al., 2021)).

ELISA results demonstrated that antibody responses to S1 and RBD were similar and correlated strongly, suggesting that S1 and RBD antibody responses develop at a similar time. This finding is consistent with other studies, which have found that RBD IgG responses may develop slightly earlier than S1 antibodies but both reach similar levels within days of each other (Brochot et al., 2020) and may suggest that a large portion of the S1 response is against the RBD. Although there was no significant difference in the mean OD values for all samples tested against S1 and RBD there were more samples with a strong response to S1 and a negative response to RBD than vice versa. This again may suggest that for many individuals, the antibody response is against the RBD portion of S1 but that for some, an IgG response is raised against a different portion of S1, not involved in receptor binding.

3.4.5 Comparison of IgG antibody (Ab) and neutralising antibody (NAb) responses

ELISA testing also enabled comparison between the levels of IgG antibodies with those of neutralising antibodies, detected using the HIV(SARS-CoV-2)

pseudotype neutralisation assay (PVNA) and providing insight into postexposure antibody-mediated immunity. Pseudotype neutralisation assays have previously been shown to display a high correlation with live virus neutralisation (Hyseni et al., 2020) and have the significant advantage over live-virus neutralisation assays in being able to be used in a biosafety containment level 2 laboratory, making rapid and extensive testing of samples straightforward. Approximately half of individuals with a positive IgG response also showed neutralising antibodies to SARS-CoV-2 (Figure 3.3.10) and there was a significant positive association between the presence of neutralising antibodies and increasing OD values, as well as with samples derived from secondary care (Table 3.3.9 and Table 3.3.10).

The heterogeneity observed here in antibody and NAb responses was in keeping with similar findings in other studies (Luchsinger et al., 2020). Heterologous responses are likely to reflect the make-up of the patient population, with individuals in different stages post-infection and with different disease severity, likely ranging from asymptomatic to being hospitalised with COVID-19. Neutralising antibodies may develop at a different rate to IgG responses, so samples that were positive by ELISA but negative by PVNA may not yet have developed a neutralising response that could still develop later (Post et al., 2020). Low NAb levels, which are present but do not reach the 90% neutralisation cut-off point, could also account for ELISA positive samples classified as neutralisation negative. Setting a lower cut off (for example 50% neutralisation at a single dilution) and calculating antibody titres for these samples may reveal low NAb titres for some of these individuals. A small proportion of samples (0.97%) showed neutralising activity but were negative by ELISA. These could be false negative results on the ELISA, or they may suggest the presence of epitopes outside the S1 subunit or RBD that are involved in neutralisation. Since the publication of this work several specific NAbs have been described that neutralise sites outside the S1 subunit or RBD (Chi et al., 2020, Wec et al., 2020).

Although half of ELISA positive sample did not have neutralising activity, amongst those which did, higher absorbance values obtained from the S1 ELISA

were significantly associated with a greater likelihood of being positive by PVNA, suggesting that higher ELISA responses are more likely to indicate a neutralising immune response. This research also identified significantly greater immune responses, both in terms of IgG and neutralisation, in patients sampled while in a secondary care setting. Hospital access to non-COVID patients was limited during the period of sampling so patients in secondary care at this time are likely a good proxy for severe COVID-19 infections. These findings therefore suggest that patients with more severe disease elicit an immune response of greater magnitude compared to those with mild or asymptomatic infections, which were more likely to be represented by the primary care patient group. Subsequent research elsewhere has confirmed this finding, with numerous other studies also observing higher IgG and NAb levels following more severe clinical infection (Crawford et al., 2021, Guthmiller et al., 2021, Röltgen et al., 2020, Zeng et al., 2020)

3.4.6 Conclusions

This chapter reports work undertaken during the first wave of the COVID-19 pandemic in Glasgow, Scotland. ELISAs against S1 and RBD were rapidly employed to monitor levels of antibodies against SARS-CoV-2 in a patient population using readily available residual clinical samples. A cut-off value was determined that enabled samples to be classified as positive or negative and resulted in excellent overall test performance and good sensitivity and specificity. The assays were subject to day-to-day laboratory variation but agreement between sample repeats in terms of status was good. ELISAs were used to test more than 7000 samples from primary and secondary care patients between March and May 2020, providing insight into patterns and demographic factors influencing SARS-CoV-2 exposure and immunity. The low prevalence found, in combination with heterogeneity in IgG and neutralising antibody-mediated responses, suggested that in the absence of vaccinations or non-pharmaceutical interventions, future waves of SARS-CoV-2 infections would likely cause significant disease burden – a conclusion that was confirmed by high morbidity and mortality since the date of this study.

4 Crimean-Congo haemorrhagic fever virus (CCHHFV) seroprevalence in livestock and humans in northern Tanzania

4.1 Introduction

Crimean-Congo haemorrhagic fever (CCHF) is a potentially fatal disease of people with a high case fatality rate. It is caused by the tick-borne orthonairovirus, Crimean-Congo haemorrhagic fever virus (CCHFV), which can infect humans and animals and has the potential for onwards human-to-human transmission (Bente et al., 2013). Due to its epidemic potential, it is categorised by the World Health Organisation (WHO) as a priority pathogen in its Research and Development (R&D) Blueprint (WHO, 2022a).

Transmission to humans occurs either through the bite of an infected tick or via contact with blood or tissues from viraemic animals (Whitehouse, 2004). People are most commonly dead-end hosts, but human-to-human transmission can occur and is most common in nosocomial settings where exposure to blood and tissues from viraemic patients is high (Aradaib et al., 2010, Naderi et al., 2011, Tsergouli et al., 2020). Outside human infections, the virus circulates in tick-vertebrate-tick cycles, and mammalian hosts, including livestock species, play an important role in viral maintenance (Bente et al., 2013, Whitehouse, 2004, Spengler et al., 2016a). A wide range of non-human hosts can become infected but typically remain asymptomatic during infection. In ruminant livestock species, asymptomatic infections result in a transient viraemia of around 7 days and exposure to blood and tissues from animals in this viraemic phase presents a direct route of transmission to humans (Spengler et al., 2016b, Nabeth et al., 2004, Hoogstraal, 1979). The asymptomatic nature of livestock infection makes identification of active infection difficult, either via clinical examination or molecular methods (Fanelli et al., 2021). However, livestock species produce a measurable humoral immune response following CCHFV infection which enables prior exposure to be identified through serological assays (Sas et al.,

2018a). This, combined with the more tractable nature of largescale livestock compared to human sampling, means that understanding patterns of exposure in livestock can provide important information both on the distribution and dynamics of CCHFV, as well as indicating potential areas of elevated risk for human disease emergence (Spengler et al., 2016a, Hoogstraal, 1979).

Although the global distribution of CCHFV is widespread, data from areas beyond the well-recognised foci of human disease is often limited. For example, the virus is considered endemic in much of Sub-Saharan Africa (SSA) (WHO, 2017), but molecular evidence of viral infection or serological evidence of exposure in both humans and animals is limited across the continent (Temur et al., 2021). In East Africa, a single case of CCHF was identified in Kenya in 2000 (Dunster et al., 2002) but the presence of the virus has become a greater concern since 2013 due to intermittent outbreaks of human disease in Uganda (Balinandi et al., 2021b, Mirembe et al., 2021). Despite these regional concerns, evidence of viral circulation in neighbouring Tanzania remains extremely limited. No information on CCHFV presence in livestock in the country has been recorded since the 1970s, when cattle were found to have antibodies against the virus (Hoogstraal, 1979). No cases of disease in people have been reported in the country and, until very recently, no studies had investigated serological evidence of exposure in people (Rugarabamu et al., 2021). Despite this lack of research, global risk mapping has identified much of northern Tanzania as at high probability of CCHF occurrence in people, including the area of northern Tanzania where research for this thesis was located (Messina et al., 2015). This limited evidence indicates that CCHFV is present in Tanzania but also highlights the need for more comprehensive exploration of the presence, levels, and distribution of the virus in the country. A key aim of this study was therefore to establish estimates of seroprevalence in livestock and healthy human populations as a first step towards understanding the circulation of the virus and its potential risk to human health in Tanzania.

Serological data are widely used in Africa to provide information on occurrence and distribution of pathogens, particularly livestock diseases. Seroprevalence is typically reported as an average of the study population

and although this is a simple and accessible way to describe population-level exposure, such estimates do not provide information on potential heterogeneities underlying them. This is particularly important in large cross-sectional studies sampled over multiple study sites such as that under investigation here, where differences within and between groups may be substantial (de Glanville et al., 2018b). To capture these heterogeneities, modelling approaches are required that account for hierarchically structured data where samples are clustered (Sommet and Morselli, 2017). Mixed effects models are suitable for analysing associations with pathogen exposure in hierarchically structured data and are frequently used in human and veterinary epidemiology (Victora et al., 1997, Stryhn and Christensen, 2014, Dohoo et al., 2009). The focus of such studies is often on identifying risk factors (fixed effects) associated with individual pathogen exposure such as intrinsic characteristics like age and sex, local management practices, or environmental conditions. Frequently, little attention is given to the random effects incorporated into these mixed-effects models (Merlo et al., 2018a). However, random effects can also provide useful information about patterns of disease in a study population (Merlo et al., 2018a). Model-derived random effects variance can be used to quantify the effect of the context or group in which an individual lives on their risk of exposure, through calculation of what are known as general contextual effects (GCEs), a selection of measures of variance and clustering including, but not limited to, the intra-cluster correlation coefficient (ICC) and the Median odds ratio (MOR) (Merlo et al., 2018a, Merlo, 2006). The general contextual effect can also be thought of as the group-level effect (Rodriguez and Goldman, 1995) or population-level effect (Merlo et al., 2004), and represents the overall influence of an individual's group or setting on the outcome of interest. Examples of such an approach in human health literature include exploration of the effects of neighbourhoods or hospitals on health outcomes (Glorioso et al., 2018, Merlo et al., 2016, Ghith et al., 2017), and also in neglected tropical diseases to evaluate the importance of social and environmental conditions on individual infection risk (de Glanville et al., 2018b, Kairu-Wanyoike et al., 2019, Oswald et al., 2019). An approach which considers patterns in the random effects as well as the fixed effects may be particularly useful for pathogens such as

CCHFV where many questions remain around routes of transmission and patterns of circulation.

A second key aim of this study, therefore, was to carry out a general contextual analysis to explore how the risk of exposure to CCHFV varied across different biologically relevant groupings of individuals, specifically addressing village- and household-level clustering of exposure risk for livestock and human hosts. Assessment of the variance within and between these groups was used to explore the importance of the household and village context on an individual's risk of CCHFV exposure.

4.1.1 Research questions

Work for this chapter focussed on the following research questions:

1. What is the estimated seroprevalence of CCHFV in cattle, goats, sheep, and humans in northern Tanzania?
2. How do patterns of exposure vary among species?
3. How is the risk of CCHFV exposure structured across different villages and households?

4.2 Materials and methods

4.2.1 Sample origin and justification for use

Serum samples and accompanying data were collected as part of a pilot and main study of the "Social, Economic and Environmental Drivers of Zoonoses," project, hereafter referred to as SEEDZ (part of "Zoonoses and Emerging Livestock Systems" (ZELS) program, funded through BBSRC, DfID, ESRC, MRC, NERC and DSTL - grant no: BB/L018926/1). The study, which is described in detail below, was undertaken in 2015 and 2016 with the aim of investigating the distribution, drivers and impacts of several zoonotic pathogens in humans and livestock in linked households across pastoral and agro-pastoral communities in northern Tanzania (de Glanville et al., 2020). Initial study design was focused on questions around *Brucella* spp., *Coxiella burnetii* and Rift Valley Fever Virus (RVFV), but the samples and linked meta-data have also been used to investigate other pathogens of importance for human or animal health, including Peste de Petit Ruminants Virus (PPRV) (Herzog et al., 2020a, Herzog et al., 2020b, Herzog et al., 2019) and *Neospora caninum* (Semango et al., 2019).

Although not specifically undertaken to investigate CCHFV, the rationale and design of the SEEDZ study allowed for samples and data to be used to determine seroprevalence and risk factors for a variety of livestock zoonoses including CCHFV. In addition, areas covered by the study have been identified as being at high risk of emergence of human cases of CCHF, and SEEDZ study villages provided locations in both high and low probability areas (Messina et al., 2015), making it a suitable dataset with which to investigate the epidemiology of the virus.

4.2.2 Ethics

The SEEDZ study was carried out in Tanzania with the permission of the Tanzania Commission for Science and Technology (2014-244-ER-2005-141). Local consent for study activities was also obtained from regional, district, ward, and village-level authorities. Ethical consent, including approval of the

study protocols, questionnaires, and consent documents, was obtained from the Kilimanjaro Christian Medical Centre (KCMC) (832) and National Institute of Medical Research (NIMR) (2028) ethics committees, the University of Otago Ethics Committee (H17/069), and University of Glasgow Medical, Veterinary and Life Sciences (MVLS) Ethics Committee (200140152). Consent for animal sampling was obtained from the University of Glasgow, School of Veterinary Medicine ethics committee (39a.15). Informed written consent was obtained from all study participants. Samples were imported into the UK under license TARP(S)2016/49.

4.2.3 Study area

The United Republic of Tanzania is situated on the East coast of Africa, bordering Kenya and Uganda to the north, Rwanda, Burundi and the Democratic Republic of Congo to the west, Zambia, Malawi and Mozambique to the south, and the Indian Ocean to the east. In 2016, the population was estimated at 50.9 million, and it is the largest country, by area and population, in the East African Community (East Africa Community Secretariat, 2019). The country is divided into a hierarchy of administrative units starting with 30 geographic regions, which are each sub-divided into districts, wards, villages, and sub-villages in rural settings, or Mtaa in urban settings. Wards typically comprise an average of three villages and are classified as rural, urban or mixed. In this study, a further division of sub-villages, into household or compound, was also included.

SEEDZ study sites were situated in two neighbouring regions, Arusha and Manyara, in northern Tanzania (Figure 4.2.1). Arusha region is divided into seven districts and had a population of around 1.7 million at the last census in 2012 (Tanzania National Bureau of Statistics, 2012). Samples were derived from six districts in Arusha (Arusha, Karatu, Longido, Meru, Monduli, and Ngorongoro) all of which were made up of >80% rural households (Tanzania National Bureau of Statistics, 2016a). Manyara region had a population of around 1.4 million in 2012 and is divided into 6 districts. Study sites were selected from four districts (Babati Rural, Babati Urban, Mbulu, and Simanjiro) all of which were predominantly rural apart from Babati urban

district where 65% of households were urban (Tanzania National Bureau of Statistics, 2016b). The environment of the study area contains a mixture of semi-arid and sub-tropical agro-ecological zones (Robinson, 2011), and farming practices include a mixture of pastoral, agro-pastoral and small-holder production systems (de Glanville et al., 2020). Pastoral production is characterised by extensive rearing of livestock as the principal means of household income, while agro-pastoral production combines livestock grazing on a smaller scale with increased crop production. Small-holder production is more common in urban and peri-urban settings and relies on crop production with small numbers of livestock (de Glanville et al., 2020).

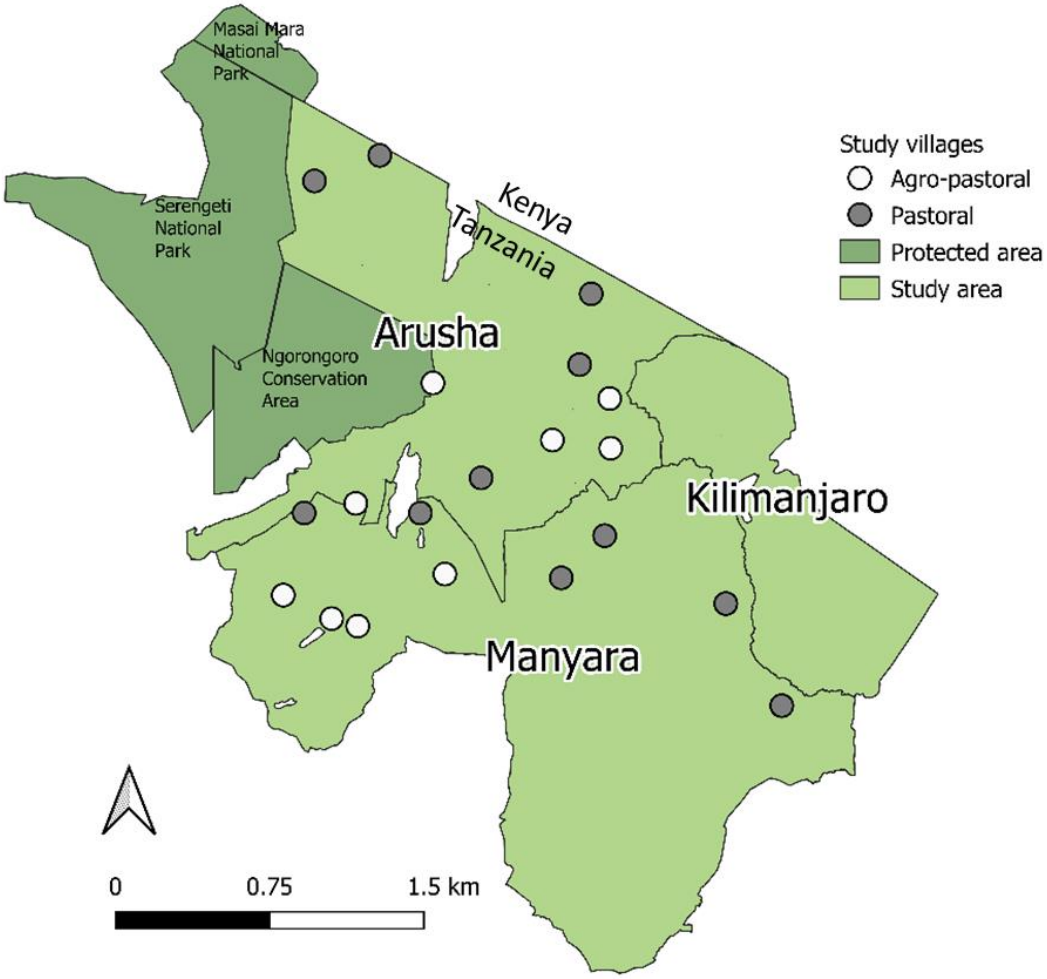

Figure 4.2.1 Study regions and villages.
Villages are coloured according to the dominant system of production, pastoral or agro-pastoral.

4.2.4 Village and household selection

4.2.4.1 SEEDZ study sites

A multi-level sampling frame was employed to select households and villages in the 10 study districts. A list of 1012 villages was compiled from the 2012 National Census data (Tanzania National Bureau of Statistics, 2012). Villages in wards classified as urban were excluded, as were those situated in the Ngorongoro Conservation Area (NCA), leaving 553 villages remaining. In consultation with District Veterinary Officers and District Livestock Officers, villages were then classified as predominantly ‘pastoral’ or ‘mixed’. Pastoral

villages were those in which livestock-rearing was considered to be the principal form of livelihood, while mixed villages included those where both livestock-rearing and crop-production were important sources of income. 20 villages, stratified to include 11 pastoral and 9 mixed locations, were then selected using a generalised random tessellation stratified sample approach in order to provide a spatially-balanced and probability-based sample (Stevens and Olsen, 2004).

Prior to commencement of the main study, serum samples were also collected from four pilot study villages during 2015 and 2016. Villages were purposely selected for sampling convenience and a balance of pastoral and mixed settings. Analysis was undertaken both including and excluding data from the pilot villages and was not found to have a significant impact on the results, so for the seroprevalence and general contextual analysis described in this chapter pilot villages were included.

A central point sampling approach was adopted whereby livestock keepers were invited to bring their animals to a pre-selected location for sampling and other disease control activities, organised through staff of the Tanzania Ministry of Livestock and Fisheries. Notification of sampling events was provided at least three days in advance and communicated through traditional village communication networks via village chairpersons and elders. At each sampling event a list of attending households was compiled and a random number generator was used to select a maximum of 10 households per sub-village for livestock and human sampling and follow-up questionnaire data collection. Human serum samples were collected from a random selection of households across 17 villages of the main SEEDZ villages, as well as the four pilot study sites. GPS co-ordinates of villages, central point locations and households were recorded using a handheld GPS (Garmin eTrex, Garmin Ltd, Olathe, Kansas, USA).

4.2.5 Livestock sampling

A maximum of 10 cattle, 10 sheep and 10 goats were randomly selected per household for sampling. Animals under 6 months of age were excluded from

the study. For cattle, up to five animals between 6 and 18 months and up to five over 18 months were selected. For sheep and goats up to five animals between 6 and 12 months, and up to five animals over 12 months were selected. The exact age of animals was frequently unavailable, so age was principally assessed by dentition, which provides an indication of age according to the number of adult incisor teeth present (Turton, 1999, Mitchell, 2003, Casburn, 2016). Female animals are more commonly kept than male animals so a minimum of two males per age group were selected. Animals were manually restrained, and samples were collected via jugular venepuncture into plain vacutainers.

4.2.6 Human sampling

Livestock keepers from households selected for sampling were visited within one week of the sampling event for follow-up for administration of a questionnaire and for human blood sampling. All members of each household were recorded and those eligible were approached for consent and participation. Individuals under 5 years of age, those who had been living in the household for less than 12 months, and those who could not provide informed consent were excluded from selection. Between 1 and 20 (minimum = 1, maximum = 20, mean = 2.4, median = 2) members of each household were subsequently selected for blood sampling after obtaining informed individual consent.

4.2.7 Sample processing and laboratory analyses

Animal and human blood samples were allowed to clot before having serum extracted on the day of sampling. Samples were divided into three aliquots and transferred to the Kilimanjaro Clinical Research Institute (KCRI) biotechnology laboratory in a mobile vehicle fridge for storage at minus 80°C. Samples were heat treated for a minimum of 120 minutes at 56°C prior to shipment to the University of Glasgow. Heat treatment of serum under these conditions was based on requirements for inactivation of any notifiable viral, bacterial or parasitic pathogens in these samples and exceeded the stated conditions of import stated by the Scottish Executive on the TARP(S) license (TARP(S)2016/49) as well as also being sufficient to inactivate CCHFV

(Weidmann et al., 2016). Two sets of aliquots were shipped to the University of Glasgow, in 2016 and 2019 respectively, while the remaining set of aliquots was stored at KCRI. All samples were tested at the MRC-Centre for Virus Research, Glasgow using the ID Screen® CCHF Double Antigen Multi-species ELISA (IDvet, Grabels, France) used according to the manufacturer's directions. The full protocol for this assay can be found in Chapter 2.

4.2.8 Statistical analyses

All statistical analyses were performed in R version 4.1.0 (R Core Team, 2021). Statistical significance was set at $p \leq 0.05$.

4.2.8.1 Apparent and adjusted seroprevalence

Livestock seroprevalence was estimated using three different methods: one without any adjustment (apparent prevalence), one to account for the complex study design (weighted prevalence), and one to account for the performance of the diagnostic test used (adjusted prevalence). Apparent seroprevalence was calculated as the proportion of positive samples divided by the total samples tested. Binomial 95% confidence intervals were calculated using the prop.test() function in base R (R Core Team, 2021). Weighted seroprevalence and 95% confidence intervals for livestock were calculated using the *Survey* package (Lumley, 2004), using village and household as cluster identifiers (village = primary sampling unit, household = secondary sampling unit), and sampling weights to account for the probability of a household being sampled from within a village and an individual animal being sampled from within the household. Sampling weights for each animal were calculated as,

$$\text{Sampling weight} = \frac{1}{(n/N*m/M)}$$

where n = the number of households sampled per village, N = the total number of households in each village, m = the number animals sampled per household and M = the total number of animals per household (Dohoo et al., 2009).

Seroprevalence and 95% confidence intervals for human samples were also calculated using the *Survey* package in R using village and household as cluster identifiers (village = primary sampling unit, household = secondary sampling unit) but due to the small sample size and more uniform number of individuals in each household no sampling weights were applied.
Adjusted seroprevalence values were calculated based on the test sensitivity and specificity values reported by the manufacturer of the ID Screen® CCHF Double Antigen Multi-species (IDvet, Grabels, France) (Table 4.2.1), using the formula set out by Rogen and Gladen (Rogan and Gladen, 1978):

$$\text{Adjusted prevalence} = \frac{\text{Apparent prevalence} + \text{Specificity} - 1}{\text{Sensitivity} + \text{Specificity} - 1}$$

Table 4.2.1 **Manufacturer-reported sensitivity and specificity values for cattle, sheep, goats and human sera tested on the ID Screen® CCHF Double Antigen Multi-species ELISA (IDvet, Grabels, France).**

	Specificity		Sensitivity	
Species	**Specificity (%)**	**95% Confidence intervals**	**Sensitivity (%)**	**95% confidence intervals**
Cattle	**100** (n = 402)	99.1 - 100 %	**97.9** (n = 95)	92.6 – 99.4 %
Sheep	**100** (n = 402)	99.1 - 100 %	**99.0** (n = 102)	94.7 – 99.8 %
Goats	**100** (n = 402)	99.1 - 100 %	**100** (n = 74)	95.1 – 100 %
Human	**100** (n = 257)	98.5 - 100 %	*Not assessed*	-

Specificity estimates were available from IDvet for human samples and were also explored in-house. Both in-house testing and the manufacturer's assessment estimated 100% specificity of the IDvet ID Screen® for human

samples. Sensitivity for human samples was not assessed by IDvet, or through in-house investigations, due to a lack of access to known-positive human samples. Given the high test-specificity and lack of sensitivity data, human seroprevalence estimates were not adjusted for test performance.

4.2.8.2 Mixed-effects logistic regression models

The hierarchical structure of the data, with individual animals clustered in households, which were in turn clustered in villages (*Figure 4.2.2*), means that the data are not independent, thus invalidating one of the assumptions required of logistic regression (Dohoo et al., 2009). To account for this multilevel structure, mixed-effects logistic regression models, a type of generalised linear mixed model (GLMM), were used. Random effects (village and household) were selected based on the hierarchical study-design, but model fit with and without random effects, as well as with only village or only household-level random effects, was also compared using the Akaike Information Criterion (AIC) to confirm that inclusion of these random effects improved model fit. Mixed effects models were run using the *lme4* package in R (Bates et al., 2015).

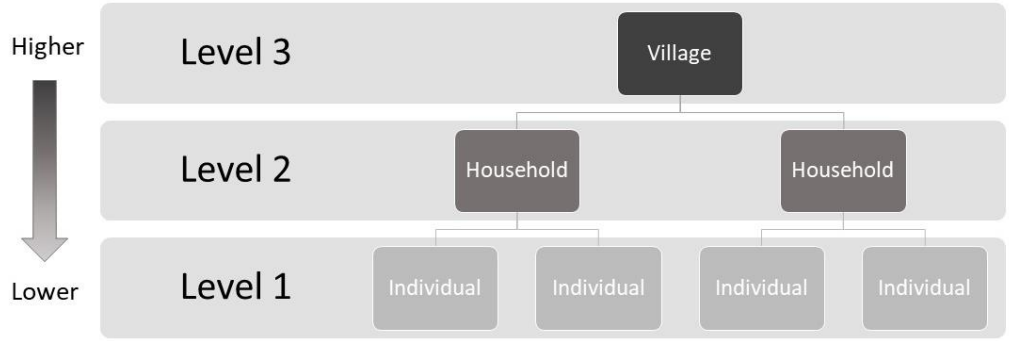

Figure 4.2.2 **Schematic of hierarchical or multi-level structure of the data. Village and household were considered as random effects.**

4.2.8.3 Differences in prevalence between species

Differences in prevalence between species were investigated by addition of species as a fixed effect to an all-livestock model with village and household as random effects. To further compare patterns of village-level seroprevalence between species, including humans, correlation of village-

level seroprevalence between species-pairs was assessed using Pearson's correlation coefficient using the cor.test() function in R (R Core Team, 2021).

4.2.8.4 General contextual analysis: ICC and MOR

To investigate how CCHFV risk was structured across the study area, and to quantify the degree of clustering of exposure risk at the level of different sampling units (village or household), a general contextual analysis was performed. The relative effect of village or household grouping was explored through calculation of the intraclass correlation coefficient (ICC) and the median odds ratio (MOR), referred to collectively here as general contextual effects (GCEs). Null logistic regression models were run with CCHFV positivity as the dependent variable, village and household as random effects and no fixed effects (Sommet and Morselli, 2017). The estimated variance of the random intercepts for household and village levels, as derived from these null models, were used to calculate the ICC and MOR. General contextual effects were explored for each species separately using species-specific null models. The intraclass correlation coefficient provides a measure of the proportion of the individual variance in exposure odds that is attributable to each cluster level, in this case, villages or households (Merlo, 2006, Killip et al., 2004). It was calculated using the latent variable method (Snijders and Bosker, 2012, Merlo, 2006):

$$\text{Village level ICC} = \frac{(\text{vilvar})}{(\text{hhvar} + \text{vilvar} + (\pi^2/3))}$$

$$\text{Household level ICC} = \frac{(\text{hhvar} + \text{vilvar})}{(\text{hhvar} + \text{vilvar} + (\pi^2)/3))}$$

Where, vilvar = the village-level random effects variance, and hhvar = the household-level random effects variance.

The ICC takes a value between 0 and 1 and can be thought of as the correlation between individuals in the same sampling unit. A value of zero would indicate that the odds of seropositivity are completely independent of the sampling unit, while an ICC of 1 would indicate that all observations

within sampling units were identical, i.e. perfect correlation between individuals, all seropositive or all seronegative (Sommet and Morselli, 2017). The MOR represents the median odds of seropositivity between the highest and lowest risk units at each level. The MOR can be conceptualised as the average difference in odds of exposure, all else being equal, for an individual in any village or household, compared to that of an equivalent individual in a village or household with lower prevalence. Unlike a normal odds ratio the MOR is always greater than or equal to 1. A MOR of 1 would indicate no difference in odds of seropositivity between individuals in the unit in question (village or household). For example, a village-level MOR of 4 would indicate that an animal moving from a low prevalence village to a higher prevalence village would have odds of infection 4 times higher in the higher prevalence village compared to when it was in the lower prevalence village. It was calculated using the equation below (Merlo, 2006):

$$\text{Village level MOR} = \exp(0.95 * \text{sqrt}(\text{vilvar}))$$

$$\text{Household-level MOR} = \exp(0.95 * \text{sqrt}(\text{hhvar} + \text{vilvar}))$$

Where, vilvar = the village-level random effects variance, and hhvar = the household-level random effects variance.

Confidence intervals for the random effects variance were calculated using the conf() function (R Core Team, 2021). Methods for calculating confidence intervals for ICC and MOR in frequentist framework have not been well described and so for this reason only the point estimate of these parameters was calculated (Austin and Leckie, 2020).

4.2.8.5 Spatial distribution

Patterns of spatial autocorrelation in the log-odds of seropositivity at the village-level were investigated using Moran's I statistic. Moran's I statistic provides a measure of spatial autocorrelation, or in this case, the tendency for close villages to have similar exposure levels. Moran's I takes a value between -1 and +1. A value of 1 indicates perfect positive spatial

autocorrelation, while a value of -1 indicates perfect negative autocorrelation, or perfect dispersal. A value of 0 indicates a random spatial distribution. Village-level log odds of exposure were extracted from the null models and linked to GPS coordinates. Moran's I was calculated using the Monte-Carlo simulation method in the *spdep* package in R (Bivand and Wong, 2018). The number of simulations was set to 9999 and the significance level was 0.05. Moran's I was assessed for the 20 main SEEDZ villages only as these had GPS co-ordinates, while the pilot study sites did not have recorded GPS. Maps showing village-level seroprevalence were plotted in QGIS version 3.16.0 (QGIS.org, 2022).

4.3 Results

4.3.1 Sample structure

A total of 7705 livestock samples were tested using the ID Screen® CCHF Double Antigen Multi-species ELISA. Of these, 7697 samples had accompanying metadata including species and were used for subsequent analysis. Two hundred and forty-one samples were collected under the pilot protocol and 7456 were collected during the main study period. A total of 3098 cattle, 2124 sheep and 2475 goats were tested from 434 households across 23 villages. A total of 351 human samples were also tested using the ID Screen® CCHF Double Antigen Multi-species ELISA. Human samples came from 145 households in 21 villages. One sampling location had only human samples (study site 24) and 3 study villages had only livestock samples (study sites 16, 19 and 20).

4.3.2 Seroprevalence estimates

Comparison was made between apparent livestock seroprevalence estimates, estimates adjusted for reported test performance, and weighted seroprevalence adjusted for the sampling framework (*Figure 4.2.2*).

Table 4.3.1 Seroprevalence estimates for cattle, sheep and goats in Arusha and Manyara regions, Tanzania.

Prevalence estimates calculated in four different ways, table shows sample size and 95% confidence intervals.

	Method of adjustment	Cattle		Sheep		Goats	
		N	% (95% CI)	N	% (95% CI)	N	% (95% CI)
Apparent prevalence	Apparent prevalence	3098	49.4 (47.6-51.2)	2124	27.4 (25.5-29.4)	2475	33.3 (31.4-35.2)
Adjusted prevalence	Adjusted for test performance as per Rogan and Gladen (1978)	3098	50.4 (48.7-52.2)	2124	27.7 (25.8-29.6)	2475	33.3 (31.4-35.2)
Weighted prevalence	Adjusted for sample structure and sampling weight using *Survey* package in R (Lumley, 2004)	2856	49.8 (40.4-59.2)	1954	28 (17.2-40.8)	2268	33.9 (21.8-47.6)

Adjusted for sample structure only	Adjusted for sample structure only using *Survey* package in R (Lumley, 2004)	3098	49.4 (40.1-58.6)	2124	27.4 (17.1-39.7)	2475	33.3 (21.7-46.3)

The high sensitivity and specificity reported for the ELISA, as well as the high proportion of seropositive animals, resulted in adjusted estimates of prevalence that did not vary substantially from the apparent estimates when adjusted for test performance. Given this small difference, the need to adjust for the complexity of the study design was considered to be of greater importance than adjusting for test performance. Using the *Survey* package ensured wider confidence intervals which better accounted for the clustered nature of the sample (Table 4.3.1). Addition of sample weights to the calculation reduced the sample size for calculating seroprevalence but did not substantially alter the estimate compared to adjusting for the sample frame alone (i.e. village and household clusters) (Table 4.3.1). Therefore, only adjustment for sample frame (village and household), and not for sample weights, was used going forward.

4.3.3 Overall seroprevalence

Overall seroprevalence by species as well as range of village-level seroprevalence is shown in Table 4.3.2.

Table 4.3.2 Seroprevalence for CCHFV exposure in cattle, goats, sheep, and humans in northern Tanzania.

95% confidence intervals and range of village-level seroprevalence are also shown.

Species	Overall seroprevalence %	95% confidence interval	Village-level seroprevalence range (95% CI)
Cattle	49.4%	40.1 – 58.6	5.3 (1.2-9.4) - 76.6 (70.3-82.8)
Goats	33.3%	21.7 – 46.3	0.0 (0.0-5.8) - 79.6 (68.3-90.8)
Sheep	27.4%	17.1-39.7	0.0 (0.0-3.8) - 71.0 (55.5-85.0)
Human	15.1%	8.5 - 24.0	0.0 (0.0-24.3) – 50.0 (30.8-69.2)

Across all study sites CCHFV seroprevalence in cattle was 49.4% (95% CI 40.1-58.6), sheep 27.4% (95% CI 17.1-39.7) and goats 33.3% (95% CI 21.7-46.3). Overall human seroprevalence was 15.1% (95% CI 8.5-24.0), and village-level

seroprevalence ranged widely between villages in all species (Table 4.3.2). Seroprevalence by species for each village can be found in Appendix 3.

4.3.4 Differences in seroprevalence between species

Both goats and sheep were found to have significantly lower odds of exposure compared to cattle when species was added as a fixed effect to an all-species model with village and household random effects (Table 4.3.3).

Table 4.3.3 Odds of exposure to CCHFV in sheep and goats compared to cattle. Coefficient, odds ratio (OD), 95% confidence intervals and p values from an all-species mixed effect logistic regression model with species as a fixed effect, and village and household as random effects are shown.

Species	ß	OR	95% confidence intervals	p value
Cattle	*Reference*			
Goats	-0.81	0.44	0.39 - 0.51	<0.0001
Sheep	-1.16	0.31	0.27 - 0.36	<0.0001

Correlation of village-level seroprevalence across livestock species-pairs are shown in Figure 4.3.1. There was a significant positive correlation between each pair of livestock species at the village level.

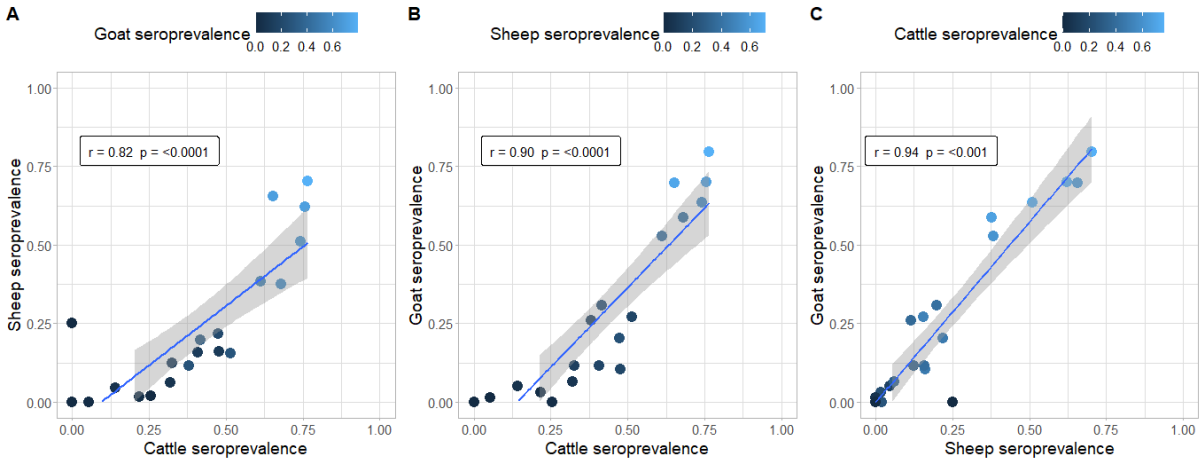

Figure 4.3.1 Correlation between village-level seroprevalence by species-pairs.
a) cattle and sheep, b) cattle and goats, c) sheep and goats. Points are coloured according to the village-level log odds of the third species.

Correlation of village-level human seroprevalence and each livestock species are shown in Figure 4.3.2. Correlation between village-level human and livestock seroprevalence was not significant for any species.

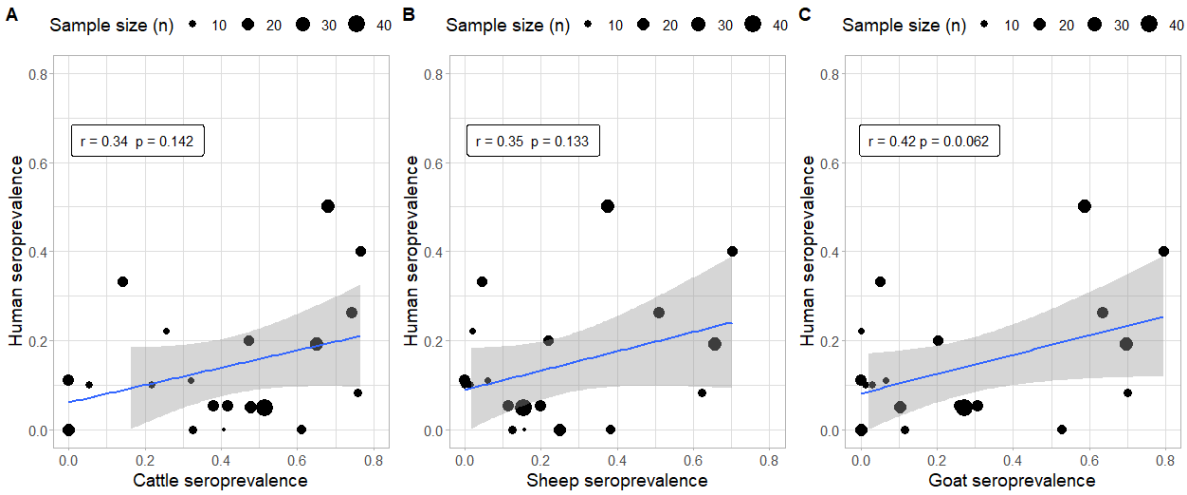

Figure 4.3.2 Correlation between village-level human and livestock seroprevalence.
a) human and cattle, b) human and sheep, c) human and goats. Points are sized according to the village-level sample size.

4.3.5 General contextual effects

Null models, without fixed effects, were used to calculate intraclass correlation coefficients (ICC) and median odds ratios (MOR) from species-specific models. Results are shown in Table 4.3.4.

Table 4.3.4 Random effects variance, intraclass correlation coefficient (ICC) and median odds ratios (MOR) derived from all livestock and individual species null models with only village and household as random effects and no fixed effects.

Confidence intervals for the random effects variance were calculated using the conf() function.

Species	Level	Random effect variance (95% CI)	ICC (%)	MOR
Livestock	Household	0.52 (0.40 - 0.68)	43.8	4.58
	Village	2.03 (1.12 - 4.15)	34.8	3.88
Cattle	Household	0.50 (0.34 - 0.78)	36.1	3.66
	Village	1.36 (0.71 - 2.91)	26.4	3.03
Sheep	Household	0.74 (0.45 - 1.15)	51.6	5.93
	Village	2.77 (1.44 - 5.95)	40.7	4.86
Goats	Household	0.70 (0.44 - 1.07)	57.4	7.40
	Village	3.73 (1.96 - 8.03)	48.3	6.27
Human	Household	0.02 (0.00 - 1.39)	20.5	2.40
	Village	0.83 (0.24 - 2.41)	20.0	2.37

4.3.6 Spatial distribution

Moran's I statistics for village-level log odds of infection are shown in Table 4.3.5. Maps showing the village-level seroprevalence for each species are shown in Figure 4.3.3.

Table 4.3.5 Moran's I statistics and p values for village level log odds of infection for all livestock and individual species.

Values are derived from the null model with village and household level random effects and no fixed effects.

Species	Moran's I	p value
Livestock	-0.08	0.558
Cattle	-0.09	0.598
Goats	-0.1	0.614
Sheep	-0.09	0.574
Human	0.43	<0.001

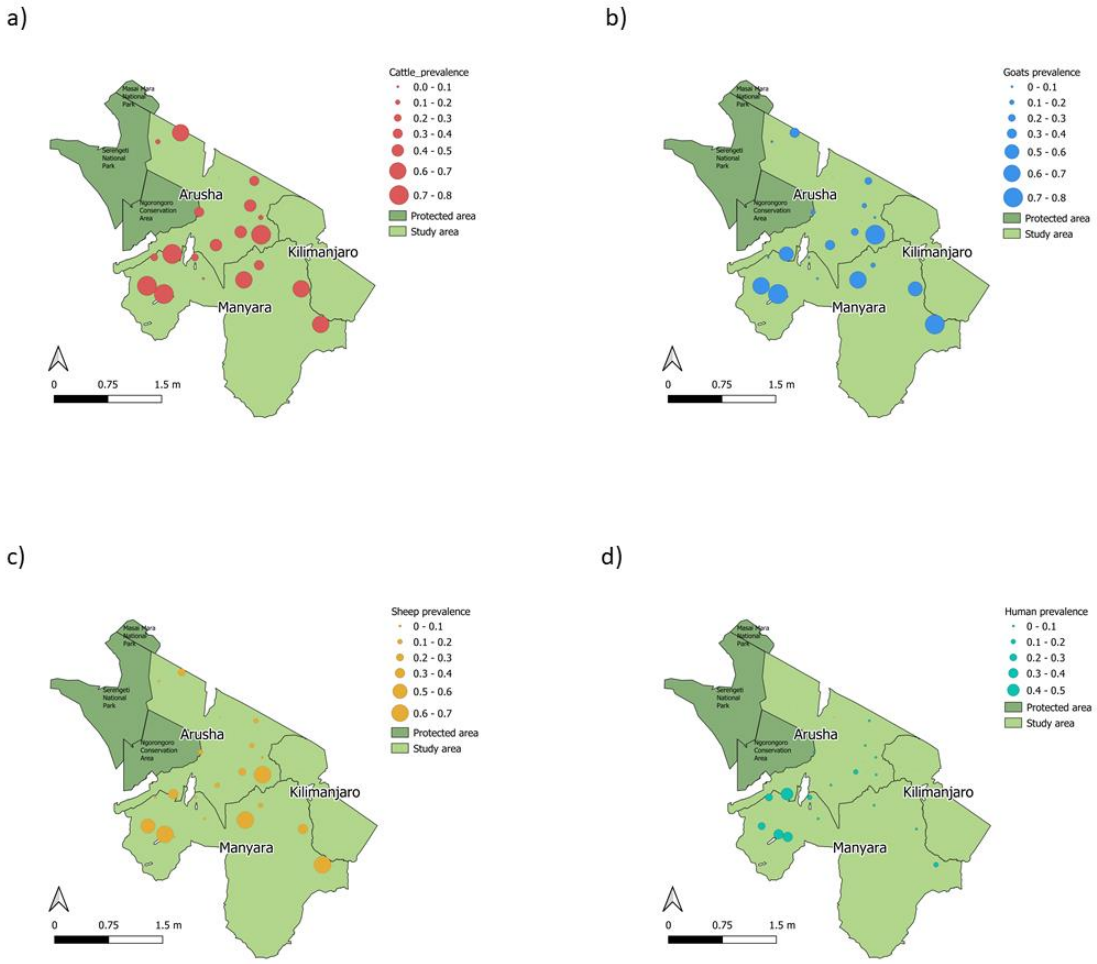

Figure 4.3.3 Village-level seroprevalence of Crimean-Congo haemorrhagic fever virus. a) cattle, b) goats, c) sheep and d) humans in study villages in Arusha and Manyara regions, Tanzania

4.4 Discussion

This study provides the first seroprevalence estimates for Crimean-Congo haemorrhagic fever virus (CCHFV) in livestock and humans in northern Tanzania. The study generated four key findings: a) exposure to CCHFV is widespread in all livestock species and in people in Arusha and Manyara regions; b) seropositivity in cattle (49.4%) was significantly higher than in goats (33.3%), sheep (27.4%) and people (15.1%); c) there was substantial variation in seroprevalence among villages and patterns of variation were different in livestock and people; and d) individual risk of exposure was strongly clustered within villages and household contexts particularly for livestock and to a lesser extent for people.

Seroprevalence estimates established here clearly demonstrate that CCHFV is present in northern Tanzania, confirming the findings of the only other report to investigate livestock exposure in the country. This single study, undertaken in 1974-5 and reported in Hoogstraal (1979) but not published independently, examined 1048 cattle from four regions of central and northern Tanzania. An overall seroprevalence of 9.0% was found, with a seroprevalence of 0.6% (19/256) in the districts of northern Tanzania closest to the SEEDZ study area (Longido, Monduli and Tengeru). This seroprevalence was substantially lower than that estimated in the current study and could suggest an increase in cattle CCHFV seroprevalence in this region over the last 4 decades. However, no details were provided of the study design or selection process for the tested animals and this, in combination with the different serological test used, as well as the large heterogeneity between sample sites observed in the current study, make it difficult to meaningfully compare these results.

Seroprevalence estimates from the current study are consistent, however, with similarly high livestock exposure found in neighbouring Uganda. A 1972 study reported cattle seroprevalence in Kenya/Uganda of 76.3% (Chumakov, 1972, Spengler et al., 2016a), while a more recent study in Ugandan cattle estimated seroprevalence between 48 and 90% in different districts using the

same commercially available assay employed here (Balinandi et al., 2021a). Elsewhere in SSA, seroprevalence estimates in cattle, sheep and goats reported in the literature have tended to be lower, although direct comparison is again challenging due to the small number of studies, typically small sample sizes, and variety of serological assays used (Spengler et al., 2016a).

Overall levels of livestock seroprevalence were significantly different between species. Exposure was greater in cattle than in small ruminants, a pattern that has also been seen elsewhere in SSA and other endemic regions (Body et al., 2016, Schulz et al., 2021). However, patterns of exposure between species are not consistent in the literature, with sheep and goats being found to have higher seroprevalence in certain endemic areas including India, the Middle East and Turkey (Mourya et al., 2014, Obaidat et al., 2021, Tantawi et al., 1981, Tuncer et al., 2014). In southern and south-eastern Europe, sheep have been identified as particularly important hosts and have been directly linked to human transmission events (Papa et al., 2013, Humolli et al., 2010). These inconsistencies may be due to regional differences in dominant tick vectors and host feeding preferences. In Tanzania, certain *Hyalomma* species, as well as *Amblyomma variagatum*, a competent vector species common in northern Tanzania are known to preferentially feed on cattle (Camicas, 1990, Lynen et al., 2007, Spengler and Estrada-Pena, 2018). However, if there was a very pronounced preference such a strong correlation between village-level species pairs might not be expected (Figure 4.3.1). Alternatively, higher prevalence in cattle may be a product of higher over-all tick burdens in this species, with a study of tick-borne pathogens in Kenya finding higher tick burdens on cattle compared to goats, sheep and pigs (Chiuya et al., 2020). Unfortunately, data on tick diversity and abundance in the study region is limited, so further research into which species are present, as well as their host preferences, is needed.

Seroprevalence estimates in the human study population confirm that people are also being exposed to CCHFV in northern Tanzania, despite an absence of reported clinical cases. The overall human seroprevalence found here is

similar to that found in other community studies in endemic regions such as Turkey (Cikman et al., 2016, Koksal et al., 2014) but higher than similar studies in other African countries including Mozambique (2.7%, n=300) and South Africa (3.9%, n=541) (Muianga et al., 2017, Msimang et al., 2021). Other than a case reported in Zambia in 1986, which may have originated in Tanzania (Swanepoel et al., 1987), there have been no reported human clinical cases of CCHF in the country, and until very recently no studies had investigated exposure to the virus through antibody detection. However, a small study published in 2021 recently identified IgG antibodies against CCHFV in six out of 500 (1.2%) community participants across 8 districts in central and south-eastern Tanzania (Rugarabamu et al., 2021). The overall seroprevalence found by Rugarabamu et al. (2021) was similar to that found in other studies in SSA but much lower than the overall seroprevalence identified in this thesis. A similar seroprevalence to that found here was observed in a study of health-seeking patients in northern Kenya, where 19% of individuals were positive for IgG antibodies to CCHFV. As these patients were actively seeking healthcare for febrile illness this estimate may be higher than that in the wider community. These comparisons with reported human seroprevalence elsewhere in SSA could suggest that community exposure in this area of Tanzania is particularly high. However, as in livestock, given the substantial between-village variation observed here, as well as the differences in study populations and design, limited conclusions can be drawn from these broader comparisons.

Overall seroprevalence in humans was lower compared to livestock and this is a typical finding across endemic regions (Nasirian, 2019, Spengler et al., 2016a). The high levels of seroprevalence observed in livestock, as well as evidence of human exposure, are of particular interest given the lack of reported clinical cases of CCHFV in people in Tanzania. As up to 90% of human CCHFV infections may be asymptomatic (Bodur et al., 2012), and clinical presentation can be highly variable, infections may be missed altogether or misdiagnosed as other febrile illnesses, particularly Malaria (Crump et al., 2013, Chandler et al., 2008). Awareness of the virus as a differential diagnosis for mild or non-haemorrhagic febrile symptoms may not

be well know and diagnostic tests for acute infection unlikely to be readily available (Petti et al., 2006). The relatively high exposure levels to CCHFV in people implies that clinical CCHF is a potentially serious, underdiagnosed health risk in this population and suggests that CCHF should be considered as a differential diagnosis for undifferentiated febrile illness in northern Tanzania.

Despite these valid concerns regarding human disease emergence in the country, evidence of livestock, human and tick exposure in areas without reported human clinical cases is not uncommon (Hoogstraal, 1979). In Turkey for example, CCHFV was known to circulate in animal populations long prior to the occurrence of clinical cases (Leblebicioglu et al., 2016b), and recent studies in regions of endemic countries without clinical cases have also confirmed the presence of the virus in such areas (Christova et al., 2018, Christova et al., 2017). An increasing number of seroprevalence studies, particularly in SSA, continue to highlight human and animal exposure in the absence of reported human clinical cases (Kuehnert et al., 2021, Phonera et al., 2021). The causes of disease emergence into such human populations are poorly understood but may in part be driven by land-use change or environmental disturbance leading to alterations in tick population dynamics, which in turn lead to increased human exposure to ticks (Estrada-Pena et al., 2012). Further research into regions such as northern Tanzania, where the virus is endemic but human disease has not been reported, is critical to understanding human disease emergence.

Although overall seroprevalence was high in all species, there was substantial heterogeneity in village-level seroprevalence, ranging from no seropositive individuals to >70% having anti-CCHFV antibodies. However, patterns of village-level exposure were different in livestock and people. In livestock there were significant differences in overall prevalence between species but the way in which this exposure was structured amongst villages was broadly similar. Village-level seroprevalence was significantly positively correlated between livestock species pairs, indicating that villages tended to have similar relative levels of exposure in the three different species. This may

suggest similar sources of infection for all species, exposure to which varies across different contexts, but which act similarly amongst species.

In addition, correlation between species exposure could be consistent with cross-species transmission as a source of exposure to CCHFV. Clustering of risk within local sampling units and the strong correlation between livestock species prevalence could support animal-to-animal transmission of the virus, whereby an introduction event in one cluster would likely lead to infection of closely kept animals. Although CCHFV is known to be predominantly tick-borne, a recent study using machine learning approaches to predict reservoir hosts and vectors for RNA viruses found the virus to be more similar to other directly transmitted livestock viruses than other tick-borne viruses, suggesting a potentially underappreciated role for direct transmission (Babayan et al., 2018). This raises a question of whether animal-to-animal transmission may play a role in CCHFV maintenance and evolution. Little investigation has been undertaken into livestock-to-livestock transmission of CCHFV but known human-to-human and livestock-to-human infection routes suggest it is possible. However, the lack of spatial autocorrelation observed in the livestock data may make this hypothesis less likely. For example, similar work has investigated the prevalence and structure of PPRV risk in the same livestock population studied here (Herzog et al., 2019). PPRV is highly contagious by direct animal-to-animal transmission and this work indicated strong spatial clustering of village level log odds of infection in cattle and goats, a pattern that is consistent with waves of epidemic viral spread moving through spatially correlated communities. Work on animal movements in this area of Tanzania has demonstrated that villages are highly interconnected through a network of livestock markets and private livestock transfers, enabling pathogens to spread quickly along these routes (Chaters et al., 2019). In contrast to the spatial autocorrelation observed in PPRV, CCHFV log odds of infection were not spatially correlated at the village level (Table 4.3.5). This makes epidemic transmission of the virus much less likely and strongly suggests endemic transmission among livestock species with exposure levels driven by local conditions, most likely equating to tick exposure and infection rates. Although this does not rule out animal-to-animal transmission

of CCHFV, no firm conclusions can be drawn from this data. Further work, ideally involving experimental infection studies, would be needed to fully explore this potential transmission route.

Human village-level seroprevalence was also highly varied, ranging from no positive individuals to half of those tested having antibodies against CCHFV. However, the pattern of this exposure was different from that seen in livestock, with some high prevalence human villages having low livestock seroprevalence and vice versa, and poor correlation between livestock and human village level seroprevalence (Figure 4.3.3). This heterogeneity, in combination with the differences in spatial distribution, may suggest different drivers of exposure in livestock and human populations, although the nature of this difference remains unclear. Discrepancies in sample size may also exaggerate differences so further linked investigations into human and livestock exposure and patterns of tick infection are required.

The general contextual analysis further confirmed the heterogeneity in CCHFV seropositivity across different study sites by demonstrating that risk of exposure was strongly clustered at the village-, and particularly at the household-level for all species. Intra-cluster correlation coefficients (ICCs) indicated that there were high levels of correlation, in terms of serostatus, between animals within the same village or household, implying that animals within these groups were more likely to be of similar status to each other than they were those of another sampling unit. This clustering was further confirmed by the high MORs which indicated the median change in odds of exposure between individuals in higher and lower prevalence villages. For example, in goats, the household ICC indicated that more than half (57.4%) of the variation in exposure risk was occurring at the level of the household (Table 4.3.4). The MOR for this species showed that a goat in a higher prevalence household had odds of exposure on average 7.4 times greater than those in lower prevalence households (Table 4.3.4). These findings indicate that the local context is extremely important in determining an individual’s risk of being exposed to CCHFV.

Higher household-level compared to village-level general contextual effects (GCEs) in all livestock species suggest that within-household drivers of exposure may be relatively more important than those between households, although direct comparison is difficult without confidence intervals, which are challenging to calculate for ICC and MOR in a frequentist framework (Austin and Leckie, 2020, de Glanville et al., 2018b). By comparison, in people, household and village-level GCEs were similar, suggesting that village and household context were similarly important in driving an individual's risk of being exposure to CCHFV. Both village- and household-level GCEs were lower in people compared to livestock implying that human exposure risk is comparatively less clustered by contextual grouping, which may suggest that local context is less important for humans than for livestock, although direct comparison is again challenging without measures of uncertainty. Although lower than for livestock, the ICC and MOR values for humans still suggest substantial levels of clustering of CCHFV exposure within villages and households (Otte and Gumm, 1997).

More contagious pathogens tend to have higher ICCs so the very high values observed here are notable in that they suggest high levels of transmissibility (Otte and Gumm, 1997). This would be consistent with direct transmission between animals in close contact i.e. within a household group or a village group where grazing was shared so animals from different households met frequently. However, previous studies demonstrate that ICC values for vector-borne diseases are highly variable, reflecting the distribution, abundance, and efficiency of local vectors (Otte and Gumm, 1997, Deem et al., 1993). The high degree of clustering observed here could therefore be driven by similarly high clustering in tick abundance and infection levels driven by local environmental, habitat and host-availability parameters.

No previous studies have reported GCEs or random effects variance for patterns of CCHFV exposure. However, substantial heterogeneity in seroprevalence between study sites has been observed in numerous other studies of CCHFV exposure both in livestock and humans (Lugaj, 2014, Tuncer et al., 2014, Balinandi et al., 2021a, Lotfollahzadeh et al., 2011). This is

likely to reflect differences in local vector dynamics as well as socioeconomic and environmental factors which may act and interact at different scales (Estrada-Peña et al., 2013, Morse, 1995, Colwell et al., 2011).

Although the investigation of GCEs demonstrated that an individual's risk of CCHFV exposure was strongly related to the village in which they lived, there was no observable spatial autocorrelation between village-level log odds of exposure. Non-significant Moran's I statistics in livestock indicated that village-level log odds of infection were randomly distributed across the study area. The distinction between these two different types of clustering, by spatial distribution and by village or household context, can be explained by thinking about an individual animal (individual A) in a randomly selected village: individual A's sero-status is more likely to be the same as another individual's in the same village than it is to those in another village, however, being in a village that is physically close to another village that has many positive animals does not make it any more likely that individual A will be seropositive. So, the status of animals in the first village is not associated with that of other near-by villages, although it is associated with the status of other animals within the sampling unit. The lack of spatial autocorrelation in livestock observed here does not rule out spatial autocorrelation in CCHFV exposure *per se*, but rather indicates that any spatial pattern in exposure is not observable at the broad landscape level captured by the village locations studied here. Study villages were typically more than 10km distant from one another so if spatial correlation of CCHFV risk does occur in this landscape, it is likely present at smaller scales than this. This finding points to the importance of local factors, likely reflective of tick populations, for driving CCHFV exposure. One challenge for the interpretation of these results is livestock movements, which were not considered in this analysis and may have an impact on spatial autocorrelation of CCHFV risk. Recent work into animal movement networks in this area of Tanzania has demonstrated dynamic networks of livestock movements through markets and private transactions (Chaters et al., 2019) and animals in pastoral systems may also travel long distances during seasonal grazing. These movements mean that exposure may not have occurred at the same location as the animal was

tested at, meaning that spatial patterns in log odds of antibody presence may not be equivalent to that of exposure events.

Patterns of spatial autocorrelation were different in humans compared to livestock species, showing evidence of positive spatial autocorrelation at the village level, with villages with higher human log odds of infection physically clustered in the western area of Manyara region (Figure 4.3.3). Observable spatial clustering at the level of villages may suggest that drivers of human exposure are more consistent between human communities at this broader landscape scale or that certain drivers of exposure are common to these locations. Although the human sample size was much smaller than livestock, and so may be a source of bias, the different spatial patterns further support differences observed in patterns of village-level exposure and GCEs, suggesting that drivers of exposure to CCHFV may be different in animals and humans. Further investigation into specific risk factors for CCHFV exposure in humans would be needed to more fully understand the patterns observed here.

In livestock, evidence of heterogeneity and clustering of risk implies that conditions specific to village and household context, whether vector, environmental or husbandry-related, are important drivers of exposure, although given the complexity of CCHFV transmission routes (Figure 1.4.2) these drivers are likely to be multiple and complex. Addition of specific contextual effects (SCEs), in the form of fixed effects variables, to the null models described above may help to explain some of this variation. Specific contextual effects of interest include variables which act both at the village and household levels as well as intrinsic factors related to individual animals (e.g. age, sex, breed). They may include environmental parameters such as temperature or elevation, husbandry-related variables such as grazing system or herd size, as well as over-arching categories such as agro-ecological system. Specific contextual effects are explored as fixed effects risk factors in Chapter five.

4.5 Conclusions

This study demonstrates substantial exposure to CCHFV in cattle, goats, sheep, and people in northern Tanzania for the first time. Overall levels of exposure were significantly different between species, with cattle having the highest seroprevalence levels. Although overall seroprevalence estimates were high, substantial variation was observed in seroprevalence among study villages. In livestock, levels of exposure were highly correlated among species between villages, but they did not correlate well with human seroprevalence. These findings suggest that the context in which an individual lives is highly important to their risk of exposure to CCHFV but that these patterns are different in livestock and humans, perhaps suggesting different drivers of exposure.

5 Risk factors associated with Crimean-Congo haemorrhagic fever virus seroprevalence in livestock in Tanzania

5.1 Introduction

Livestock species are common hosts of Crimean-Congo haemorrhagic fever virus (CCHFV) worldwide (Spengler et al., 2016a) and research reported in chapter 4 found high levels of exposure to the virus in cattle, goats and sheep in northern Tanzania. Viral transmission to livestock typically occurs via the bite of an infected tick, leading to a transient viraemia, during which period animals can infect naïve ticks consuming viraemic blood meals, resulting in amplification of the virus (Spengler et al., 2016b). Animal-to-animal transmission has not been demonstrated but could be possible given that infected animal blood and tissues are a source of infection for humans (Nabeth et al., 2004). Exposure of livestock to blood and tissues from injured, parturient, or slaughtered animals could represent an unexplored route of transmission.

A lack of overt clinical disease in livestock makes detection of active infection challenging, but serological responses can provide information on levels of viral circulation and transmission. Exploring risk factors associated with livestock exposure to CCHFV enables identification of systems and practices which may result in higher transmission levels in these species. Understanding these factors in livestock can shed light on routes of transmission and maintenance of the virus, furthering our understanding of the complex ecology of CCHFV. Additionally, exposure in livestock may provide indications of where there is increased risk to humans either through direct transmission from infected animals or via tick populations feeding on livestock, although patterns of human and livestock infection do not always correlate, as seen in the previous chapter.

Many studies have investigated risk factors associated with CCHFV seroprevalence in livestock across endemic regions including Sub-Saharan Africa (SSA), although the body of literature from the continent is smaller than that from Eurasia and elsewhere (Temur et al., 2021). This literature, as well as knowledge of the principals of CCHFV transmission, formed the basis from which to select variables for investigation in the current analysis. The analysis undertaken in Chapter 4 indicated that household-level drivers may be particularly important sources of CCHFV risk so variables acting at this level, including management practices and household-level environmental variables were investigated in this chapter, as well as individual- and village-level variables. Environmental and land-use parameters have been shown to be associated with increased CCHFV risk in a number of studies (Estrada-Peña et al., 2007, Wilson et al., 1990). Variation in environmental parameters such as temperature, precipitation, and elevation favour different vegetation types, resulting in differences in habitat and wildlife host availability for ticks, while use and cultivation of the land in crop and livestock agriculture also affects vegetation coverage and availability of domestic mammalian hosts. The complex interaction of these factors results in variation in tick abundance and host availability which in turn affect CCHFV transmission.

Given the complex nature of these potential ecological and agricultural drivers, this study also set out to investigate an aggregate variable in the form of agro-ecological production system, which grouped together associated environmental, ecological and agricultural variables common to different production systems. In much of East Africa, including Tanzania, three types of production system have been practised for centuries that can be broadly categorised as pastoral, agro-pastoral, and small-holder (de Glanville et al., 2020, Otte and Chilonda, 2003). Pastoral systems tend to be located in hotter, drier regions at higher elevation with lower human and livestock densities. Livelihoods predominately depend on extensive grazing of large herds of livestock, although some crops, such as beans and maize may also be grown. In northern Tanzania this production system is dominated by the Maasai tribe. Small-holder systems, in contrast, tend to be found in areas of comparatively higher rainfall and higher livestock and human population

densities. Average flock and herd sizes are typically smaller and household crop production is common, with sale of crops providing additional household income. Agro-pastoral systems typically fall between the pastoral and small-holder categories with herd and flock sizes smaller than pastoral but typically larger than small-holders. Crop production is common, and households tend to be located in areas of above average vegetation and forest cover (de Glanville et al., 2020).

Despite some inevitable overlap between these systems, as well as developments in production methods as livestock-keepers adapt to modern challenges such as climate change and changing patterns of land ownership, these traditional production systems remain representative of the environmental, production and socio-economic context in which much livestock farming in Tanzania takes place (de Glanville et al., 2020). Production system categories have been explored in detail for the study households used in the current research (de Glanville et al., 2020), with all households falling into one of the three traditional systems. This classification systems groups households according to environmental, agricultural, and socio-economic variables, including many that are hypothesised to be important drivers of CCHFV transmission. These include local environmental variables (e.g. precipitation, temperature, elevation), land-use characteristics (e.g. percentage cropland, grassland and forest cover, and type of crops grown) and livestock production variables (e.g. herd size and local livestock density).

Many measures of livestock production, including disease outcomes, productivity, and livelihood benefits vary between production systems (Robinson, 2011, Casey-Bryars et al., 2018, Herzog et al., 2019, Semango et al., 2019) and the pastoral, agro-pastoral and small-holder categories are familiar to animal and human health workers across the region. These agro-ecological systems therefore provide a convenient point of reference for understanding disease patterns and developing interventions. Understanding whether CCHFV exposure risk varies between agro-ecological system may provide a useful indication of where most CCHFV transmission is occurring

and which animals, and potentially people, are most at risk of infection, highlighting where interventions or further research might be most beneficial.

This chapter builds on the work reported in Chapter 4 which established that livestock exposure to CCHFV was widespread in Arusha and Manyara regions of northern Tanzania, but that there was substantial heterogeneity in levels of exposure across different villages and households. A general contextual analysis (Merlo, 2006) was undertaken whereby the effect of the general context in which an individual lived, in this case the village or household, on the odds of CCHFV exposure was characterised through investigation of the random effects in mixed-effects logistic regression models. This approach demonstrated that village, and particularly household, were highly important for understanding individual risk of exposure. The work reported in the current chapter builds on this exploration of the general context, to consider specific risk factors acting within these contexts, also defined as specific contextual effects (SCEs), through addition of fixed effects to the mixed-effects logistic regression models (Merlo et al., 2018a).

Investigation of the general contextual effect (GCE) provides an indication of the importance of an individual's immediate context to their risk of exposure, and are quantified using measures of clustering such as the intra-cluster correlation coefficient (ICC) and the Median Odds Ratio (MOR) (Chapter 4). However, measurement of the GCE does not provide information on specific factors that may be mediating these contextual effects. In order to explore this, investigation of associations between specific contextual effects (SCEs), more commonly known as fixed effects, and individual outcomes is required. By considering both GCEs and SCEs together it is possible to examine how much of the general contextual effect is explained by the addition of fixed effects. If the fixed effects are strongly related to the outcome and are context dependant (i.e. are acting as mediators) we could expect to see a decrease in the values of the GCE parameters after addition of fixed effects to the mixed effects models (Merlo et al., 2018a).

5.1.1 Research questions

This chapter aimed to investigate the following research questions:

1. What individual-level variables are associated with CCHFV seroprevalence in livestock?
2. How does CCHFV seroprevalence in livestock vary across agro-ecological setting?
3. What husbandry practices are associated with higher odds of CCHFV seropositivity in livestock?

5.2 Methods

5.2.1 Study design

Full details of the study design can be found in Chapter 4. Briefly, serum samples from cattle, goats and sheep in randomly selected households were collected during 2016 as part of the "Social, economic and environmental drivers of zoonoses," project, referred to as SEEDZ throughout this thesis. The study comprised 417 households in 20 villages, eleven pastoral and nine mixed, across 10 districts in Arusha and Manyara regions of northern Tanzania, which were selected using a multi-level sampling frame. Prior to the main study, a pilot study was also undertaken in three purposely selected villages.

5.2.2 Questionnaires

During collection of livestock blood samples, which took place at a central point location, questionnaires were conducted to gather information on individual animal characteristics. Following livestock sampling, livestock keepers from selected households were visited within one week of sampling for administration of a follow-up questionnaire. In total, questionnaires were administered at 397 households in the 20 main SEEDZ villages. Questionnaires were administered to the head of the household in the first language of the householder, usually Kiswahili or the local Masaai language, Maa. The questionnaire was designed to address research questions related to the social, economic and biological drivers of several zoonotic infections, principally *Brucella spp.*, *Coxiella burnettii* and Rift Valley fever virus (RVFV). Detailed information was gathered on demographic and socio-economic attributes of the households, livestock demographics, health and husbandry practices, as well livestock movements, crop production and other sources of income. Copies of both the individual animal and household questionnaires can be found in Appendix 4.

Most questions were asked at the household level, but some variables were collected at the level of the compound. In polygamous Maasai families, one

man may have several wives, with the wives and their children living in a separate family unit within a larger compound. These household units jointly use and manage resources, including livestock, within a 'boma' or single enclosure (Herzog et al., 2020b), which is defined as the compound. Variables collected at this level included herd size and counts of species other than cattle, goats, and sheep. Where households were grouped into compounds only one household from each compound was selected for collection of data for analysis of household-level exposures and this household was considered to be representative of all households in the compound (Herzog et al., 2020b). For this reason, household and compound-level variables have been used inter-changeably in this research.

Questionnaires were pre-tested during the pilot study. Some adaptations were made to the questionnaire between pilot and main study so that data gathered from the pilot study sites described in Chapter 4 took a slightly different form from the main SEEDZ study, with some questions not included and others in a different form. As a result, although data from the pilot study was included in calculations of prevalence and investigation of general contextual effects (described in Chapter 4) it was not used for risk factor analysis explored in this chapter.

5.2.3 Agro-ecological production system definitions

Agro-ecological settings were defined at either the village-level (as pastoral or mixed) or the household-level (as pastoral, agro-pastoral or small-holder). Village-level classification was defined prior to sampling in consultation with expert local opinion from District Veterinary Officers and District Livestock Officers in the area. The village-level classification reflected the predominant agricultural system in the village as perceived by these local experts. Villages classified as pastoral where those in which livestock-rearing was the dominant means of livelihood, while in villages classified as mixed both livestock-rearing and crop-production were important sources of income.

Household-level classification was assigned after sampling, using a data driven approach in which households were grouped according to similar production characteristics using the data generated from the SEEDZ questionnaire. A complete account of how the classifications were generated can be found in de Glanville et al. (2020). Briefly, dimension reduction was first performed on the full SEEDZ questionnaire and environmental data using a multiple factor analysis (MFA) to create a more parsimonious set of uncorrelated variables. Next, these variables were used to classify households into clusters using a hierarchical cluster analysis (HCA). This HCA produced three distinct clusters which correlated well with traditional agro-ecological classifications of pastoral, agro-pastoral and small-holder systems.

5.2.4 Laboratory analysis

Full details of laboratory analysis can be found in Chapters 2 and 4. All serum samples were tested for the presence of CCHFV antibodies using a commercially produced double antigen sandwich ELISA (ID Screen® CCHF Double Antigen Multi-species ELISA, IDvet, Grabels, France) used according to the manufacturer's directions.

5.2.5 Statistical analysis

5.2.5.1 Initial identification of variables

Questionnaire data collected during the SEEDZ study included more than 100 questions, resulting in multiple potential variables relating to household and village level characteristics, intrinsic livestock characteristics and livestock husbandry practices (Appendix 4). As the SEEDZ study was not designed to answer CCHFV-specific research questions not all information gathered through the questionnaire was relevant to this study. Investigation of all variables, without further refinement based on biological principals and targeted research questions, would likely result in spurious associations which are difficult to interpret meaningfully, as well as unstable coefficients due to collinearity between variables (Dohoo et al., 2009). For these reasons, potential variables were reviewed in relation to the literature on CCHFV risk factors for livestock and humans, knowledge of factors more broadly related

to livestock, zoonotic and vector-borne disease transmission, and biological principals of CCHFV transmission (Figure 1.4.2) before selection for inclusion in this study. Details of variables investigated can be found in Appendix 5.

5.2.5.2 Additional data cleaning and variable creation

Questionnaire data had been cleaned and some aggregate variables created prior to the start of this thesis research (initial data cleaning and organisation carried out by Will de Glanville) but some further data cleaning was undertaken, as well as the creation of new variables. Data cleaning and variable creation was undertaken in R version 3.6.1 (R Core Team, 2021). Some variables were complicated and were adapted to try to best represent the risk factor in question. Where low numbers of observations were observed in categorical variables, categories were combined where this made biological sense, or the variable was removed from further analysis. For example, “tethered” and “zero-grazed” were combined into a new variable, “tethered or zero grazed,” which represented households who practiced either of these management techniques for any part of the year. “Herding” and “free-roaming management” of animals were assumed to expose an animal to a similar risk profile so these were also combined into a new variable, “herded or free-roaming,” which represented households practicing either of these management techniques for any part of the year.

In some households, respondents reported not owning animals of a certain species, but samples from animals of that species were obtained and tested. For these households, the presence of sampled animals was used to define whether a household kept that species. These households were classified as NA for variables related to the number of each species kept and for variables relating to species interactions such as whether animals were confined or grazed together. Across the whole dataset most missingness could be classified as “Missing completely at random” and was predominantly due to unit non-response where livestock questionnaires were administered at sampling but household questionnaires were not followed up (Bhaskaran and Smeeth, 2014). Where NAs were introduced into the data through follow up questions to an initial negative response, subsequent responses were coded

as 0. For example, where a household did not own small ruminants, questions relating to co-confinement or grazing of small ruminants and cattle were coded as 0. Variables with greater than 90% of missing data were removed.

Variables were created for local livestock density. Cattle, goat, sheep and pig density data was downloaded from Gridded Livestock of the World (Gilbert et al., 2018a, Gilbert et al., 2018d, Gilbert et al., 2018c, Gilbert et al., 2018b, Gilbert et al., 2018e). Local density was extracted as the mean density in the $10km^2$ surrounding the village point GPS location using the *Raster* package in R (Hijmans, 2021). Village-level extraction of data was chosen to maximise the sample size for analysis, as not all households had GPS co-ordinates, but all villages did. The $10km^2$ scale was chosen to provide a village-level measure of density which included all household GPS points.

5.2.5.3 Hierarchical framework

Variables of interest were grouped according to the level at which they acted (individual, household or village), and their position in the hierarchical conceptual framework shown below (Figure 5.2.1) (Victora et al., 1997). Individual-level variables (sex, dentition, breed) were considered to be independent of the other variables. Agro-ecological classification system was considered distal to the variables which went into its construction (de Glanville et al., 2020).

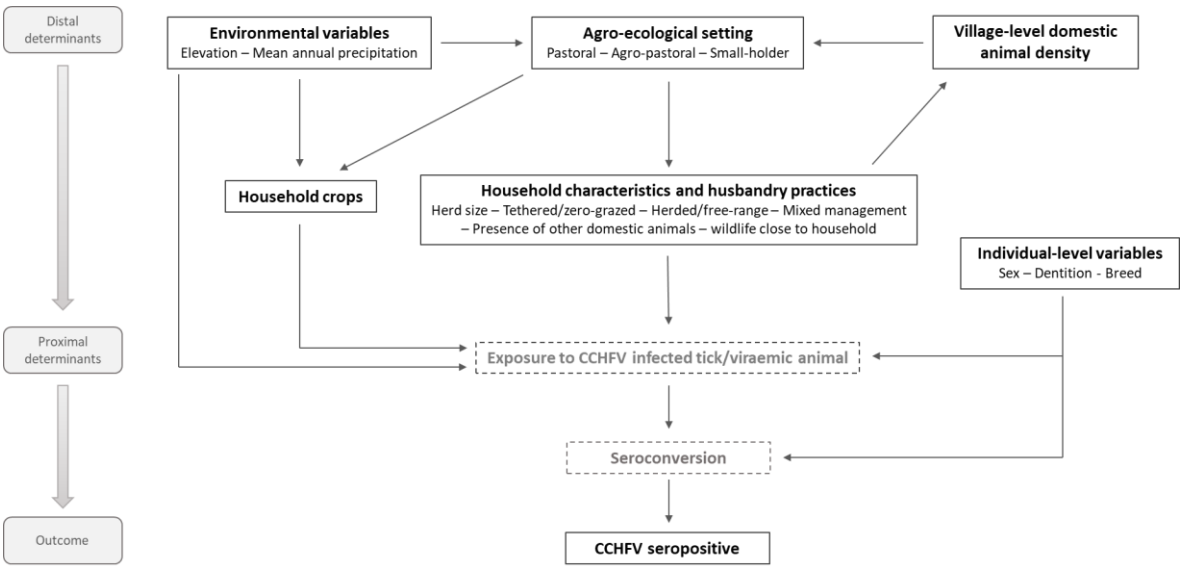

Figure 5.2.1 Hierarchical framework for variables investigated in relation to Crimean-Congo haemorrhagic fever virus seroprevalence.

Distal determinants are those further from the outcome. Dashed text boxes indicate variables that could not be measured.

5.2.5.4 Exploration of potential explanatory variables and descriptive statistics

All potential explanatory variables were explored using descriptive analyses. Mean, median, variance and percentiles were calculated for continuous variables, and frequency tabulations and plots for categorical variables. Given the complex and comprehensive nature of the questionnaire data, variables deemed likely to have high levels of collinearity were assessed using correlation plots and the variance inflation factor (VIF) from linear models, calculated using the vif() function from the *car* package in R (Fox and Weisberg, 2019). If the correlation was greater than 0.8 or the VIF was greater than 5, the variable deemed to be the least biologically relevant from evaluation of the literature was removed (Dohoo et al., 2009).

5.2.5.5 Univariable analyses

Following descriptive analysis and investigation of co-linearity, further exploration of variables of interest was undertaken through use of univariate mixed-effects logistic regression models to estimate crude odds ratios and 95% confidence intervals. Village and household were included as random effects in all models, based on the hierarchical study design, as well as

evidence of substantial clustering of CCHFV exposure risk at these levels described in Chapter 4. Models and summaries were produced using the *lme4* (Bates et al., 2015) and *FinalFit* packages in R (Harrison et al., 2020).

5.2.5.6 Multivariable models

Multivariable mixed-effects logistic regression models were used to investigate potential individual-level, household-level and environmental variables associated with CCHFV exposure in cattle, goats, and sheep. For multivariable models, only samples with full accompanying metadata were analysed to enable direct comparison of all models by building them on the same data set. Variables that had <20% but >10% missingness were investigated at the univariable level only so as not to limit the sample size in the multivariable models. Assessment of how missing data affected results was explored by running models with different data restrictions and any discrepancies in model conclusions investigated further.

Models were constructed in a hierarchical manner building on a null model, with only random effects (village and household), as described in Chapter 4. Variables were grouped according to the hierarchical framework set out in Figure 5.2.1 and explored one level at a time, starting with individual-level variables (age, sex, breed), followed by the most distal (agro-ecological setting) to most proximal (household-level husbandry variables). Further details of variables included at each level are set out below. Variables investigated in the univariable models were explored in the multivariable models except where large amounts of missingness (10-20%) limited the sample size. Due to the large number of potential risk factors investigated, backwards model selection was performed at each stage. All variables of interest at that level were included in the maximal models before removal of the least significant variable identified using likelihood ratio χ^2-tests using the drop1() function in R (R Core Team, 2021). Variables were removed in this way until all variables retained in the model were significant, to produce the most parsimonious model. Potential confounding was explored by adding and excluding potential confounding variables from the final model. Changes in coefficient values greater than 10% were considered potential confounders

and further exploration of these variables undertaken. Variables which were found to be significantly associated with CCHFV seropositivity on univariate analysis or at any stage of the multivariable model building but were not retained in the final models were investigated for potential confounding. Adjusted odds ratios and 95% confidence intervals were estimated at each level of the modelling process.

Statistical significance was set at a p value ≤ 0.05 for all models and pseudo-R^2 and AIC reported. Models and tables of univariable and multivariable models were made using the *lme4* and *FinalFit* packages in R (Bates et al., 2015, Harrison et al., 2020). Estimated marginal mean values were plotted using the *Effects* package (Fox and Weisberg, 2019). Final model fit was evaluated using adjusted R^2 for mixed effects model using the *rsq* package in R (Zhang, 2021). Marginal R^2 (explained by the fixed effects alone) and conditional R^2 (variance explained by both random and fixed effects) were both reported.

Analysis using the multivariable models aimed to address the three research questions outlined in the introduction and further described below.

5.2.5.6.1 Research question 1: What individual-level variables are associated with CCHFV seroprevalence in livestock?

Individual-level variables available and deemed to be of potential interest were: age, sex, breed and species. Previously, an all-species model, with village and household as random effects and species as a fixed effect, was used to investigate differences in prevalence between cattle, goats, and sheep (Chapter 4). This model indicated that cattle had significantly higher seroprevalence compared to goats and that sheep had significantly lower odds of exposure compared to both cattle and goats. For this reason, separate models were constructed for each species for risk factor analysis, so species was not included in the models. Records of dentition, recorded at the time of sampling, were used to estimate an animal's age (Dyce et al., 2010, Turton, 1999, Mitchell, 2003, Casburn, 2016). Dentition categories and implied age ranges are shown in Table 5.2.1. For young animals, the

minimum age was taken to be 6 months, as animals under this age were excluded from sampling.

Table 5.2.1 Dentition categories and implied age ranges for cattle and small ruminants

	Implied age range (Herzog et al., 2019, Dyce et al., 2010)	
Dentition category	**Cattle**	**Small ruminants**
Temporary (milk teeth)	6 months – 1.5 years	6 months – 1 year
One-pair (two-tooth)	> 1.5 – 2.5 years	> 1 – 1.5 years
Two-pair (four-tooth)	> 2.5 – 3.5 years	> 1.5 – 2 years
Three-pair (six-tooth)	> 3.5 – 4.5 years	> 2 – 3 years
Four-pair (full-mouth)	> 6.5 – 6 years	> 3 – 5 years
Worn	> 6 years	> 5 years

For all three species, breed was recorded as indigenous, exotic (typically European dairy breeds) or cross-bred. Cross-bred and exotic categories were combined to form a single category due to low numbers of animals in each group. All individual-level fixed effects, age (dentition), sex, and breed (indigenous and cross-bred or exotic), as well as an interaction term between age and sex, were added as fixed effects to the null model.

5.2.5.6.2 Research question 2: How does CCHFV seroprevalence in livestock vary across agro-ecological setting?

To address the second research objective, variables most distal to the outcome, describing agro-ecological production system at either the village or household level were investigated by addition of these variables to the final model from objective one. Village-level and household-level classification were investigated separately. Results from these models show the overall effect of these categories on CCHFV seropositivity unadjusted for mediating variables more proximal to the outcome. Household tribe was investigated as a confounding variable.

5.2.5.6.3 Research question 3: What husbandry practices are associated with higher odds of CCHFV seropositivity in livestock?

In order to address the third research objective, proximal variables potentially associated with both the production system and CCHFV exposure (i.e. mediators) were investigated. These were all household-level husbandry variables and included all variables deemed to be of potential biological significance that also went into the production of the household-level classification via the HCA analysis (de Glanville et al., 2020). Husbandry variables investigated at this stage were: tethering or zero-grazing of each species, herding or free-roaming of each species, presence and number of cattle, goats or sheep in the household, whether cattle were grazed with or confined with small ruminants, household ownership of pigs, donkeys, and chickens, observation of wildlife around the household, crop production at the household. Environmental factors investigated were household elevation and mean annual precipitation. Village cattle, sheep, goat, and pig densities were also investigated. For sheep and goat models, herding or free roaming was not included in the multivariable analysis because almost all small ruminants were managed in this way, leading to a very small group size in those that were not.

5.2.5.7 General Contextual Effects parameters

General contextual effects (GCEs), consisting of the intra-cluster correlation coefficient (ICC) and the median odds ratio (MOR) were calculated from the variance of the village and household random effects from the final models for each species using the methods described in Chapter 4. GCEs from the final models were compared to those from the null model containing only random effects. Null model parameters were re-run using the restricted dataset, so differ slightly from those reported in Chapter 4. The change in GCE was used to evaluate how much of the clustering in the data was explained by the addition of the specific contextual, or fixed, effects (Merlo et al., 2018b).

5.3 Results

5.3.1 Descriptive statistics

Serum samples from 7456 cattle, goats and sheep from 417 households in 20 villages were tested. All animals had accompanying individual-animal-level questionnaire data and 7078 animals from 397 households had accompanying questionnaire data. In all species, female animals were more frequently sampled than males (female n=5650 (73.5%), male n=2042 (26.5%)). Full-mouthed animals were the most common age group sampled (cattle n=1213 (40.3%); sheep n=897 (42.2%); goats n=1197 (50.3%)), followed by young animals with temporary teeth (cattle n=890 (29.6%); sheep n=514 (25.0%); goats n=440 (18.5%)). Older age groups were dominated by female animals while younger age groups had a more even distribution of males and females, reflecting the typical sex and age distribution of breeding herds and flocks (Figure 5.3.1).

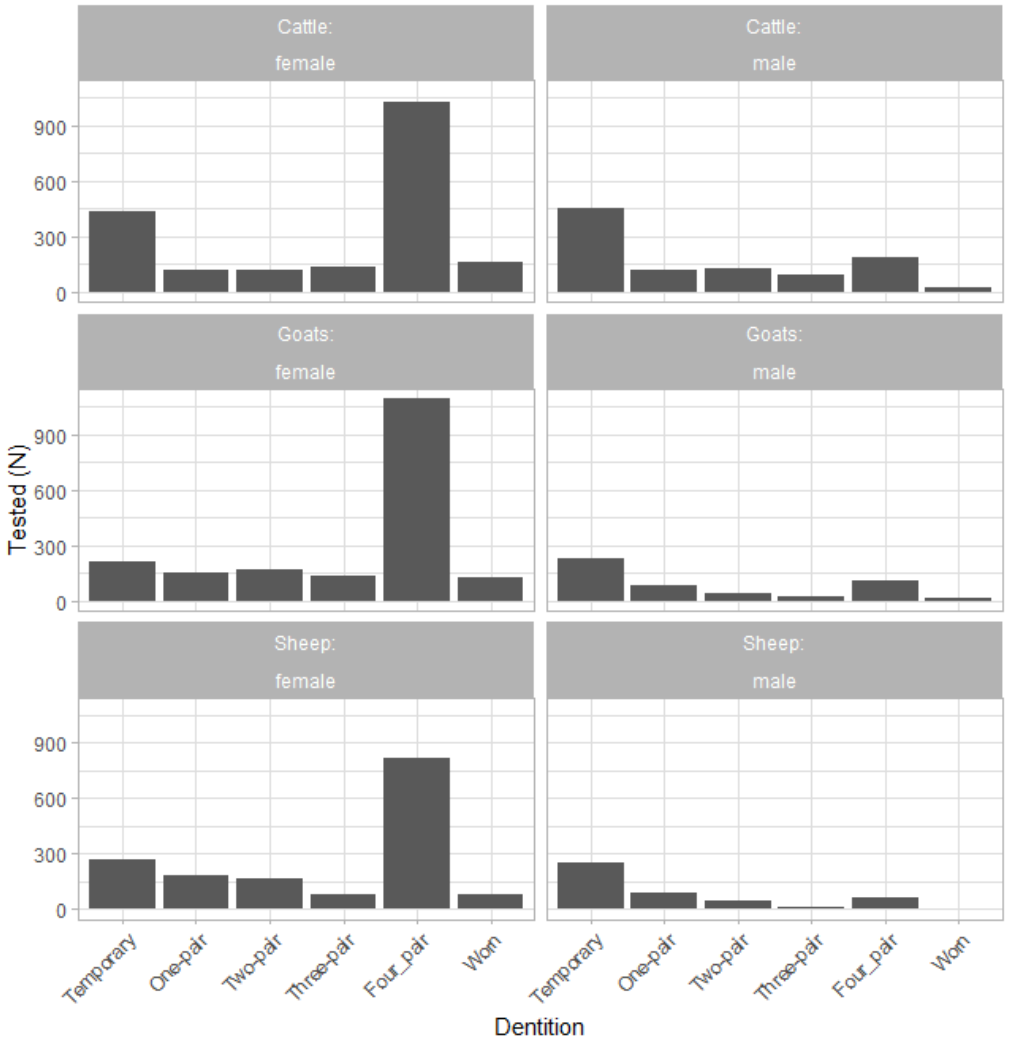

Figure 5.3.1 Age and sex characteristics of study population.
Numbers of male and female animals tested by age category for each species.

Despite consolidation of exotic or cross-bred animals into a single category this breed type accounted for only a small proportion of those sampled compared to indigenous animals, particularly in sheep where non-indigenous breeds were almost completely absent (cattle n=277 (9.2%); sheep n=9 (0.4%); goats n= 79 (3.3%).

Village-level classification was assigned prior to sampling, as previously described, with 62.5% of sampled animals from mixed and 37.5% animals from pastoral villages. Household-level classification identified 177 households as agro-pastoral, 171 as pastoral and 49 as small-holder, accounting for 2744, 3754 and 580 animals respectively. Household-level classification was broadly correlated with village-level classification, but 15.8% of households in

pastoral villages reported agricultural and livestock management practices that more accurately reflected agro-pastoral practices (de Glanville et al., 2020).

5.3.2 Final variables

Restriction of missing data in all potential variables resulted in datasets consisting of 2709 cattle from 345 households, 1822 sheep from 246 households and 2196 goats from 268 households across the 20 main SEEDZ villages, used for the multivariable models. Details of all variables investigated can be found in Appendix 5.

5.3.3 Seroprevalence and univariable analyses

Numbers tested, proportion positive or mean and standard deviation, crude odds ratios, 95% confidence intervals and p values for univariable cattle, goat and sheep analyses of categorical and continuous variable can be found in Appendix 6. Husbandry practices varied by household agro-ecological system. Seroprevalence for different management practices by household agro-ecological classification, as well as seroprevalence by sex and dentition, can be found in Appendix 7.

5.3.4 Multivariate models

The final multivariable models for cattle, goats and sheep are shown in Tables 5.3., 5.3.2 and 5.3.3 respectively.

Table 5.3.1 Risk-factors associated with Crimean-Congo haemorrhagic fever virus seroprevalence in cattle.

Estimates are derived from the final multivariable model.

Fixed effects						
Explanatory variables	**Levels**	**ß coefficient**	**Odds ratio (OR)**	**OR 95% confidence interval**		**p value**
				lower	**upper**	
Sex	female	Ref.				
	male	-0.27	0.76	0.51	1.14	0.183
Dentition	Temporary	Ref.				
	One-pair	0.10	1.10	0.60	2.04	0.753
	Two-pair	1.03	2.79	1.61	4.82	<0.0001
	Three-pair	1.47	4.36	2.54	7.48	<0.0001
	Four-pair	2.72	15.20	10.66	21.67	<0.0001
	Worn	3.33	28.01	15.46	50.74	<0.0001
Dentition*sex interaction	One-pair: male	1.03	2.80	1.21	6.48	0.016
	Two-pair: male	0.92	2.52	1.15	5.53	0.021
	Three-pair: male	0.78	2.19	0.97	4.97	0.061
	Four-pair: male	-0.37	0.69	0.37	1.28	0.241
	Worn: male	0.17	1.18	0.29	4.72	0.815

Agro-ecological setting (household-level)	Agro-pastoral	Ref.				
	Pastoral	0.62	1.86	1.09	3.17	0.023
	Small-holder	0.62	1.86	0.70	4.97	0.214
Own pigs	No	Ref.				
	Yes	0.78	2.19	1.14	4.20	0.019
Cattle tethered or zero-grazed	No	Ref.				
	Yes	-1.44	0.24	0.09	0.63	0.004
Confine cattle and small ruminants together	No	Ref.				
	Yes	0.79	2.21	1.24	3.95	0.007
Random effects						
Variables	**Variance**	**Std.Dev.**	**MOR**	**ICC (%)**		
Household	0.58	0.76	3.99	39.18		
Village	1.54	1.24	3.25	28.51		
Goodness of fit						
Adjusted R2						
Conditional	0.41					
Marginal	0.21					

AIC	
null model	3277.2
Full model	2707.5

Table 5.3.2 Risk-factors associated with Crimean-Congo haemorrhagic fever virus seroprevalence in goats.

Estimates are derived from the final multivariable model.

Fixed effects						
Explanatory variables	**Levels**	**ß coefficient**	**Odds ratio (OR)**	**OR 95% confidence interval**		**p value**
				lower	**upper**	
Sex	female	Ref.				
	male	-0.96	0.38	0.19	0.80	0.010
Dentition	Temporary	Ref.				
	One-pair	0.11	1.11	0.52	2.38	0.784
	Two-pair	0.36	1.44	0.73	2.84	0.294
	Three-pair	0.71	2.03	0.99	4.17	0.054
	four-pair	1.87	6.50	3.85	10.97	0.000
	Worn	2.21	9.10	4.35	19.05	0.000
Dentition*sex interaction	One-pair: male	1.37	3.95	1.23	12.73	0.021
	Two-pair: male	1.36	3.91	1.08	14.22	0.038
	Three-pair: male	3.05	21.20	4.94	90.99	0.000

	Four-pair: male	0.77	2.15	0.85	5.47	0.108
	Worn: male	2.03	7.61	1.39	41.58	0.019
Agro-ecological setting (household-level)	Agro-pastoral	Ref.				
	Pastoral	0.75	2.12	1.02	4.42	0.044
	Small-holder	-0.42	0.66	0.24	1.85	0.429
Graze cattle and small ruminants together	No	Ref.				
	Yes	0.67	1.95	1.23	3.07	0.004
Random effects						
Variables	Variance	Std.Dev.	MOR	ICC (%)		
Household	0.81	0.90	8.69	61.17		
Village	4.37	2.09	7.29	51.64		
Goodness of fit						
Adjusted R2						
Conditional	0.48					
Marginal	0.38					
AIC						

null model	2089.6
Full model	1881.2

Table 5.3.3 Risk-factors associated with Crimean-Congo haemorrhagic fever virus seroprevalence in sheep.

Estimates are derived from the final multivariable model.

Fixed effects						
Explanatory variables	**Levels**	**ß coefficient**	**Odds ratio (OR)**	**OR 95% confidence interval**		**p value**
				lower	**upper**	
Sex	female	Ref.				
	male	-1.19	0.30	0.15	0.63	0.001
Dentition	Temporary	Ref.				
	One-pair	0.54	1.72	0.92	3.22	0.089
	Two-pair	0.80	2.22	1.18	4.18	0.014
	Three-pair	1.32	3.76	1.76	8.03	0.001
	four-pair or worn	1.70	5.46	3.39	8.81	0.000
Dentition*sex interaction	One-pair: male	0.38	1.47	0.45	4.75	0.521
	Two-pair: male	2.08	8.01	2.16	29.78	0.002
	Three-pair: male	0.91	2.48	0.33	18.45	0.374
	Four-pair/worn: male	-0.01	0.99	0.34	2.82	0.980
Agro-ecological setting (household-level)	Agro-pastoral	Ref.				

	Pastoral	0.28	1.33	0.56	3.15	0.521
	Small-holder	-1.56	0.21	0.06	0.68	0.009
Random effects						
Variables	**Variance**	**Std.Dev.**	**MOR**	**ICC (%)**		
Household	0.77	0.88	7.61	58.12		
Village	3.80	1.95	6.37	48.35		
Goodness of fit						
Adjusted R2						
Conditional	0.43					
Marginal	0.37					
AIC						
null model	1655.1					
Final model	1517.2					

5.3.4.1 Individual-level variables

For all species, there was a trend of increasing odds of exposure with increasing age, apart from in male sheep where prevalence and odds of exposure decreased in the oldest age groups (three-pair teeth and full or worn mouth (Figure 5.3.2). All final models included a significant interaction term between sex and dentition. Odds of exposure were 28 times greater in the oldest age group for female cattle, 9.26 times for goats and 5.46 for sheep, compared to female animals with temporary teeth. For male cattle the oldest age group had 33.04 times odds of exposure, goats 68.65 times, and sheep 5.39 times compared to the youngest group (Table 5.3.4). Male cattle with one-pair dentition had significantly higher odds of exposure compared to females (OR=2.14, 1.02-4.48) and those with full-mouth dentition had lower odds compared to female (OR=0.53, 0.33-0.85). In goats, male animals with three-pair dentition had significantly higher odds of exposure compared to females, and in sheep males with full or worn dentition had lower odds of exposure compared to females (Table 5.3.2 and Table 5.3.3 and Figure 5.3.2).

Table 5.3.4 Odds ratios for sex*dentition interaction for cattle, goats and sheep, calculated from multivariable mixed effects logistic regression models.
The method used to calculate OR from the model coefficients is set out below the variable in the left-hand column.

		Cattle			**Goats**			**Sheep**		
	Level	OR	OR 95% CI		OR	OR 95% CI		OR	OR 95% CI	
Dentition (m)	Temporary (female)	Ref.			Ref.			Ref.		
Bdentition	One-pair (female)	1.10	0.60	2.04	1.11	0.60	2.06	1.72	0.92	3.22
	Two-pair (female)	2.79	1.61	4.82	1.44	0.83	2.49	2.22	1.18	4.18
	Three-pair (female)	4.36	2.54	7.48	2.03	1.18	3.48	3.76	1.76	8.03
	Four-pair (female)	15.20	10.66	21.67	6.50	4.56	9.27	NA		
	Worn (female)	28.01	15.46	50.74	9.10	5.02	16.49	NA		
	Four-pair or worn (female): sheep only	NA			NA			5.46	3.39	8.81
Dentition (m)	Temporary (male)				Ref.					
*Bsex + Bdentition + Bsex*dentition*	One-pair (male)	3.09	1.73	5.50	4.40	2.46	7.84	2.53	0.92	6.93
	Two-pair (male)	7.03	3.96	12.50	5.63	3.17	10.01	17.79	5.62	56.31

	Three-pair (male)	9.55	5.06	18.00	43.04	22.82	81.15	9.33	1.45	60.00
	Full-mouth (male)	10.53	6.20	17.86	13.98	8.24	23.72	NA		
	Worn (male)	33.04	9.16	119.16	69.21	19.19	249.58	NA		
	Full-mouth or worn (male): sheep only	NA			NA			5.39	2.09	13.88
Sex (temporary)	Female (temporary)				Ref.			Ref.		
Bsex	Male (temporary)	0.76	0.51	1.14	0.38	0.26	0.57	0.30	0.15	0.63
Male compared to female	One-pair (female)				Ref.			Ref.		
Bsex + *Bsex*dentition*	One-pair (male)	2.14	1.02	4.48	1.52	0.73	3.19	0.45	0.17	1.14
	Two-pair (female)				Ref.			Ref.		
	Two-pair (male)	1.93	0.98	3.79	1.51	0.76	2.96	2.44	0.82	7.25
	Three-pair (female)				Ref.			Ref.		
	Three-pair (male)	1.67	0.81	3.44	8.16	3.97	16.77	0.76	0.12	4.92

Full-mouth (female)				Ref.			NA		
Full-mouth (male)	0.53	0.33	0.85	0.83	0.51	1.33			
Worn (female)				Ref.			NA		
Worn (male)	0.90	0.24	3.41	2.93	0.77	11.06			
Full-mouth or worn (female): sheep only	NA			NA			Ref.		
Full-mouth or worn (male): sheep only							0.30	0.14	0.65

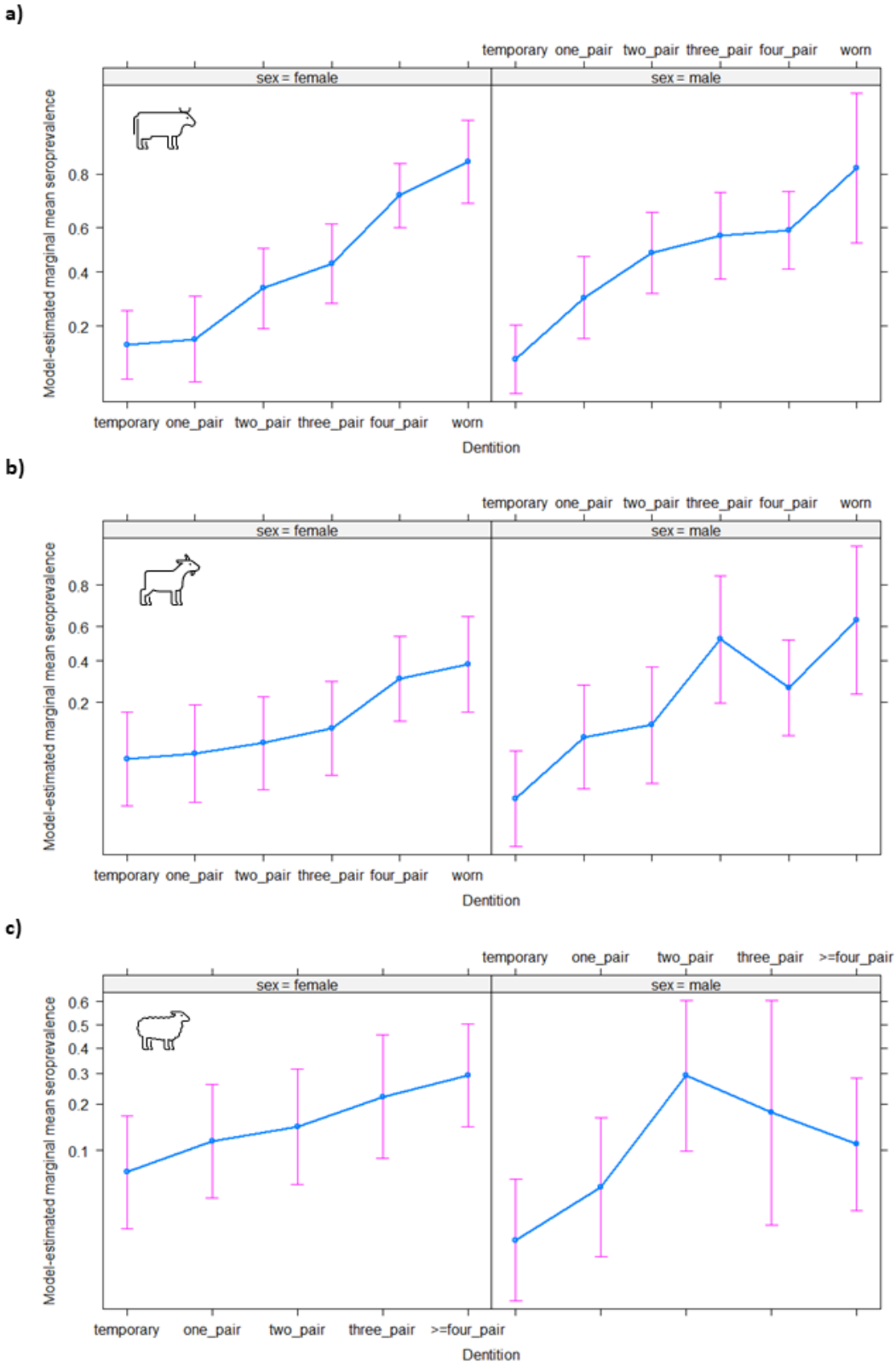

Figure 5.3.2 Estimated marginal mean seroprevalence for a) cattle, b) goats and c) sheep by sex and dentition.

Marginal mean values were estimated from final multivariable models.

5.3.4.2 Agro-ecological setting

Agro-ecological setting was investigated at both the village- and household-level in multivariable models but village-level classification was not significantly associated with CCHFV seroprevalence, so only household-level classification was included in subsequent investigations. Household-level agro-ecological classification was significantly associated with CCHFV seropositivity. For cattle and goats, animals in pastoral households had higher odds of exposure compared to those in agropastoral households (cattle OR=1.86,1.09-3.17, p=0.023; goats OR 2.18, 1.05-4.53, p=0.037). In sheep, animals in small-holder households had significantly lower odds of exposure compared to those in agro-pastoral households (OR=0.21, 0.06-0.68, p=0.01) (Table 5.3.1, Table 5.3.2, Table 5.3.3).

5.3.4.3 Household-level husbandry variables

For cattle, the household-level husbandry practices of owning pigs (OR 2.19, 1.14-4.02) and confining cattle and small ruminants together (OR 2.21, 1.24-3.95), were found to be significantly associated with higher odds of CCHFV exposure. The practice of tethering or zero-grazing cattle was associated with significantly lower odds of exposure (OR 0.24, 0.09-0.63). For goats, only one household-level practice, that of grazing cattle and small ruminants together, was associated with a significantly higher odds of CCHFV exposure (OR 1.95, 1.23-3.07) in the final multivariable model. For sheep, none of the household-level husbandry variables investigated were significant in the final model. The final sheep model included only the dentition*sex interaction and household agro-ecological classification as fixed effects.

5.3.4.4 Potential confounding variables

In the final cattle model, a positive association was observed for small-holder households (OR 1.86, 0.70-4.97, p=0.214) compared to agro-pastoral households, although the confidence intervals were wide and the relationship was not significant. Removal of household-level classification from the final model led to a 33% decrease in the coefficient value of the zero-grazed or tethered variable. To further investigate this relationship the final model was

run again with classification included but zero-grazed or tethered excluded. Exclusion of the zero-grazed or tethered variable led to a 93.7% decrease in coefficient for small-holder classification and changed the direction of the effect from positive to negative, although small-holder odds ratios were not significantly different from the reference level (agro-pastoral) in either model.

Cattle kept in Iraqw households had higher apparent seroprevalence (64.3%, univariable OR 1.36, 0.60-3.12) than those kept in other tribal households, although this was not significant on univariate or multivariable analysis. Addition of household tribe to the final multivariable model led to a 4.3% decrease in coefficient, not considered sufficient to be considered confounding. However, when pig keeping was removed from this model, while retaining tribe, the coefficient of the Iraqw tribe increased by 13.2%, suggesting that pig keeping is confounding the effect of the Iraqw tribe variable. Amongst Iraqw households, cattle seroprevalence for those that kept pigs was 74.9 (95% CI 70.7-79.1), while among those which did not keep pigs it was 50.2 (95% CI 44.6-55.8).

5.3.5 General contextual effects

The amount of within-group (household or village) correlation, represented by the intra-cluster correlation coefficient (ICC), and between-group heterogeneity, represented by the median odd ratio (MOR) were compared for the null model, with only random effects, and the final models after adjustment for fixed effects. ICC and MOR values were similar in both the null and final models for all species (Table 5.3.5).

Table 5.3.5 General contextual effects for cattle, goats and sheep. Intra-cluster correlation coefficient (ICC) and Median odds ratio (MOR) calculated from the random effects variance from the null and final models.

General contextual effects		Cattle		Goats		Sheep	
	Level	**null model**	**final model**	**null model**	**final model**	**null model**	**final model**
Random effect variance	Household	0.55	0.58	0.79	0.81	0.82	0.77
	Village	1.21	1.54	3.77	4.37	3.04	3.80
ICC (%)	Household	34.90	39.18	58.09	61.17	54.02	58.12
	Village	24.02	28.51	48.02	51.64	42.52	48.35
MOR	Household	3.53	3.99	7.60	8.69	6.47	7.61
	Village	2.85	3.25	6.32	7.29	5.24	6.37

5.4 Discussion

This study aimed to address three research questions relating to Crimean-Congo haemorrhagic fever virus (CCHFV) exposure in livestock: 1) what individual-level variables are associated with CCHFV seroprevalence; 2) how does CCHFV seroprevalence vary across agro-ecological setting; and 3) what husbandry practices are associated with CCHFV seropositivity? Several key findings were identified. Increasing age was associated with a significant increase in odds of seropositivity in all species and a significant interaction between sex and age categories was also identified in all three species. Household-level agro-ecological system was significantly associated with CCHFV exposure, with higher odds of exposure in pastoral households for cattle and goats and lower odds of exposure in small-holder households in sheep. Keeping pigs in the household was associated with increased odds of exposure in cattle, as was confining cattle with small ruminants, while the practice of tethering or zero-grazing cattle was associated with lower odds of exposure. Grazing cattle and small ruminants together was associated with increased odds of exposure in goats. Several of these findings (positive associations with increasing age and pastoral practices and negative association with zero- or restricted grazing practices) are consistent with risk factors previously identified in the literature, while others (positive associations with pig keeping and mixed-management practices), are novel.

5.4.1 Research question 1: Individual-level variables

Increasing age was associated with a significant increase in odds of seropositivity in all three species and this trend was consistent in both male and female animals, although a significant interaction between sex and age categories was also identified in all species. Greater odds of exposure with increasing age, here represented by dentition categories, is consistent with CCHFV being endemic in livestock in northern Tanzania and is consistent with studies of livestock CCHFV seroprevalence elsewhere (Adam et al., 2013b, Balinandi et al., 2021a, Schulz et al., 2021). This pattern of seropositivity is typical of circulation of a fully immunising viral pathogen that elicits long-lived antibody responses. Data on the duration of antibody response to CCHFV in livestock are limited but similar age-seroprevalence patterns

observed elsewhere imply antibodies are long lasting (Schulz et al., 2021), and evidence of reinfection suggests antibody levels may be boosted by repeat infection through life (Spengler et al., 2016b). Increasing odds of seropositivity with age is therefore consistent with an interpretation of endemic CCHFV infection, whereby individuals are increasingly likely to have been exposed to CCHFV with time.

The lack of a significant difference between male and females in the multivariable analysis is consistent with most other studies (Mostafavi et al., 2012, Ibrahim et al., 2015, Wilson et al., 1990). A small number of studies that have found female animals were at higher odds of exposure compared to males (Balinandi et al., 2021a, Phonera et al., 2021) but they either did not control for age or had small sample sizes, and it is possible that the higher exposure in females may be a result of female animals being retained in the herd for longer for breeding purposes. Results of the current study emphasise the importance of accounting for age in any analysis of individual-level risk factors.

This study is the first to report a significant interaction between age and sex, which was consistent for all species (Figure 5.3.2 and Table 5.3.4). It is unknown whether the absence of this finding in other studies is due to a lack of investigation or a true absence of this interaction. There is no immediate explanation for the association seen in this study in terms of exposure risk or transmission dynamics, and interpretation is constrained by the small size of the effect (Table 5.3.4). Differences in odds of exposure at different ages could suggest differences in the rate at which exposure occurs, also known as the force of infection (FOI). Age-specific force of infection can be generated through analysis of cross-sectional serological data infection (Dohoo et al., 2009, Herzog et al., 2020a) and may be useful to determine whether this interaction indicates a true difference in age-related exposure patterns between males and females. Further exploration of these patterns by household class may also be warranted to explore whether different settings have an impact on the rate of exposure.

5.4.2 Research question 2: Agro-ecological classification

Household-level agro-ecological classification was significantly associated with CCHFV exposure in livestock. All species displayed a similar relationship between odds of exposure in the different household types, with odds highest in pastoral and lowest in small-holder households. Sheep in small-holder households had significantly lower odds of exposure (OR=0.21, p=0.009), while cattle and goats in pastoralist households had significantly higher odds of exposure (cattle OR=1.86, p=0.023 ; goats OD=2.18, p=0.037), both compared to animals in agro-pastoral households. This was despite the raw overall seroprevalence being higher in agro-pastoral households, particularly for cattle. This discrepancy is partly a result of the highly clustered nature of the study population, and partly a result of an unusually high seroprevalence in pig-keeping households, which were almost exclusively found in agro-pastoral settings. Risk associated with pig keeping households is further discussed below.

In this study, agro-ecological system was a focus for investigation as it encompassed land-use, environmental characteristics, and management practices, all of which may affect transmission of CCHFV from ticks to livestock. In comparison with agropastoral and smallholder settings, pastoral households tend to be located in flatter, drier areas, with lower vegetation and crop cover, but higher average annual temperatures and a higher proportion of grassland cover. These conditions are consistent with the preferred habitats of the seven known competent vector species present in Tanzania, particularly the three Hyalomma species: *Hyalomma Impeltatum*, *Hy. Rufipes*, and *Hy. Truncatum* (Walker et al., 2003). However, detailed information on distribution and preferences of these tick species, particularly in relation to agro-ecological settings, is limited and what evidence does exist suggests several of the competent vector species have broad and often overlapping habitat ranges. A study conducted across Tanzania in 2007 evaluated the distribution and habitat preferences of certain *Amblyomma* and *Rhipecephalus* species including two competent CCHFV vectors, *A. variagatum* and *R. appendiculatus* (Lynen et al., 2007). Both species were

found to be widespread across the SEEDZ study area and tended to be generalists in terms of their habitat preferences, although *A. variegatum* was positively associated with cultivated land and *R. appendiculatus* distribution was negatively associated with bushland. These subtle differences in habitat preference are likely to be similar in other competent vector species and suggest that, although in general terms the typical environmental characteristics of pastoral households may favour greater tick populations, the association with CCHFV exposure in this system is likely to be multi-factorial and not a simple reflection of tick habitat preference.

No previous studies have specifically investigated how the different agro-ecological systems described here are associated with CCHFV risk, although many risk factor studies in both animals and humans, have investigated environmental variables and husbandry practices typical of these systems, or investigated association with similar livestock production systems. For example, although not significantly associated with CCHFV seroprevalence on multivariable analysis in this study, environmental variables such as elevation, temperature and rainfall are associated with agro-ecological system (de Glanville et al., 2020) and have been found to be associated with CCHFV elsewhere (Esser et al., 2019, Vescio et al., 2012). Studies in Senegal found seroprevalence in sheep was greatest in the northern part of the country where the climate was arid and vegetation sparse and decreased consistently toward the damper more forested areas in the south (Wilson et al., 1990, Chapman et al., 1991, Zeller et al., 1997), while a recent study in Malawi found that animals grazed in drier and more elevated upland areas were >4 times more likely to be exposed to CCHFV compared to those grazed in seasonally waterlogged dambo wetlands. Extensive grazing practices characteristic of pastoral livestock management are also associated with higher tick burdens compared to more intensive management practices and so may contribute to the increased risk in pastoral settings (Swai et al., 2005b, Chepkwony et al., 2021).

Where other studies have considered associations between CCHFV exposure and different livestock production systems, grazing appears to be related to

increased CCHFV exposure. Two studies in Sudan, where CCHF is an emerging concern following outbreaks in 2008, 2009 and 2010 (Aradaib et al., 2011, Aradaib et al., 2010), considered the risk associated with different grazing systems: nomadic, stationary or a combination of the two systems. Ibrahim et al. (2015) found that animals managed under a nomadic grazing system had odds of exposure 27 time higher than those managed under a stationary system. However, this association was not consistent, as another study undertaken elsewhere in the country found no significant difference in risk between nomadic, semi-nomadic and stationary grazing systems (Adam et al., 2013a). Nomadic herders in Sudan practice extensive grazing of livestock and this pastoral system may share similarities with pastoral livestock production in northern Tanzania. Grazing was also identified as a risk factor for CCHFV infections in Pakistan although little further information on what these variables was provided (Kasi et al., 2019).

Higher odds of exposure in pastoral households are therefore consistent with findings from elsewhere. Animals in pastoral households spend more time grazing and travel further from the household, both of which are likely to place them at higher exposure to ticks. In addition, pastoral households tend to be located in higher, drier and hotter areas of grassland which are the preferred habitat of the *Hyalomma spp*. of ticks which are found in Tanzania. Tick burdens have been found to be higher in extensive systems in Tanzania compared to small-holder systems, further supporting the hypothesis of higher tick exposure in these settings may underlay higher CCHFV exposure risk (Swai et al., 2005a, Swai et al., 2008).

No association was found between village-level agro-ecological system and CCHFV seroprevalence in livestock. This village-level classification is representative of that used by veterinary officials and workers in the local area, so understanding its limitations as an indication of CCHFV exposure may be important for local risk assessments. From the more detailed household classification system constructed by de Glanville et al. (2020) it is clear that the village-level classification was not always representative of the reported agro-ecological practices of households within the village, particularly as

households adapt their practices to changing climatic and economic conditions (Mccabe et al., 2010, Pretty et al., 2011). This variation in production practices between households in the same village may account for the lack of association with CCHFV seroprevalence at the village-level.

Despite inconsistencies between village- and household-level classifications observed here, other investigations of the SEEDZ data have found strong associations between village-level classification and other pathogens, notably peste des petit ruminants virus PPRV (Herzog et al., 2019). It is not unexpected that pathogens with different transmission routes and dynamics would behave differently, but it is important to note that village-level classification can be a useful predictor for some pathogens, further highlighting the lack of association at this level for CCHFV. This may suggest that some drivers of CCHFV exposure in livestock are acting at a smaller scale than the village level, an hypothesis consistent with the greater degree of clustering of CCHFV risk at the household compared to the village level for livestock observed in Chapter 4.

5.4.3 Research question 3: Household-level husbandry risk factors

Investigation of variables below the level of household classification was able to further explain some of the specific risk factors associated with CCHFV seroprevalence in our study population. Unlike the similar patterns observed amongst species in age, sex and household classification variables, specific risk factors were not the same between species. In cattle, an association was found between CCHFV odds of exposure and three variables: tethering or zero-grazing of cattle (OR = 0.24), confining cattle and small ruminants together (OR = 2.21) and keeping pigs in the household (OR = 2.19). In goats, a positive association was found between CCHFV exposure and co-grazing of cattle and small ruminants (OR = 1.95); and in sheep no additional risk factors were identified at this level.

A significant protective effect was observed for cattle in households where tethering or zero-grazing was practiced. Both practices result in the

confinement of the individual to a single space, whether outside in the case of tethering, or typically inside a shed or hut in the case of zero-grazing. Animals are thus unable to roam and so remain in the same environment, which is likely to lead to reduced tick exposure. Few other studies have considered associations between confinement or tethering and CCHFV seroprevalence, although the effect of this practice has been implied from other associations and is in direct contrast to the increased risk associated with extensive grazing practices described above. For example, low frequency of pasture usage amongst dairy cattle was hypothesised as the reason for their relatively lower exposure levels compared to small ruminants in Turkey (Tuncer et al., 2014), and although related to a different species, confinement was found to be a highly effective method of preventing hard tick infestation in pigs in south eastern Tanzania (Braae et al., 2013, Kabululu et al., 2018). Similarly, the large difference in CCHFV seroprevalence found between camels (57.3%) and sheep (6.7%) in Inner Mongolia by Li et al. (2020b) was hypothesised to be in part due to differences in time spent outside, with camels spending more time outside of stock confinement and moving further than sheep, thus exposing them to greater tick burdens. Although the species and environments were different, the strong protective effect of confinement against hard tick infestation provides support for the hypothesis that CCHFV exposure is lower in confined or tethered cattle due to reduced tick exposure.

This association was not observed in small ruminant species, although seroprevalence was lower in tethered or zero-grazed sheep and goats but this difference was not significant on either univariable or multivariable analysis. In cattle there was evidence of confounding between small-holder classification and tethering or zero-grazing, with much of the protective effect of small-holder production coming from this practice, as demonstrated by the increase in small-holder coefficient when tethering was added into the model and the higher seroprevalence in small-holder households where tethering or zero-grazing was not practiced (84.0%, 73.8-94.2%) compared to those who did (33.2%, 26.5-39.8%) (Appendix 7).

Increased odds of CCHFV exposure in cattle were also observed in households where cattle were confined with small ruminants (OR=2.20, 1.24-3.89). This practice most commonly refers to confinement of animals at night, and as such is distinct from full confinement (zero-grazing) or tethering practices which were associated with lower risk of exposure. Co-grazing of cattle and small ruminants was not a significant risk factor for cattle but this practice was significantly associated with increased exposure odds in goats (OR=1.8, 1.24-2.94). No association with either co-management strategy was observed in sheep. No significant association was found with the presence of other livestock species in the household when included as a binary variable (e.g. sheep present or absent from the household), only with these specific practices. However, almost all households keep a mix of livestock, so comparison was hampered by the small number of households keeping only one or two species. A lack of association between CCHFV exposure and the presence of different livestock species was also observed in Sudan and Malawi (Adam et al 2013; Phonera et al 2021) but no other studies have found a positive association with co-management practices as identified here. Similar drivers may be reflected in both the confinement and grazing associations, although the cause of this relationship is not clear. This evidence may further support the hypothesis that livestock-to-livestock transmission could be occurring, with increased contact between species leading to increased transmission events and so increased seroprevalence. However, co-management may also result in as yet undefined variation in tick populations which could explain higher levels of CCHFV. Co-management of different livestock species has been found to increase tick burdens in the UK (Lihou et al., 2020) but further research is needed to better understand these relationships in African ticks.

Keeping pigs in the household was found to be associated with 2.12 times increase in odds of exposure in cattle compared to households that did not keep pigs. In our study, pigs were only kept by Iraqw households, with most pig-keeping households classified as agro-pastoral and a minority as small-holder. As pig-keeping was restricted to a single tribal group and to just three village locations, careful investigation of potential confounding was

undertaken. However, even after adjustment for potential confounding, keeping pigs remained associated with higher odds of CCHFV exposure. When the data was stratified to only consider Iraqw households, seroprevalence was higher in households that kept pigs (74.9% 95% CI 70.7-79.1) than those that did not (50.2%, 95% CI 44.6-55.8).

The causes of this association are unclear. Data on CCHFV infection in pigs, either domestic or wild, is extremely limited. Four studies have tested porcine sera but none identified antibodies to CCHFV (Darwish et al., 1978, Mourya et al., 2012, Chiuya et al., 2020, Spengler et al., 2016a). Chiuya et al. (2020) also tested ticks recovered from pigs at slaughter slabs in Kanya for several pathogens, including CCHFV, but none were positive for the virus. Given the wide range of mammalian hosts CCHFV is known to infect it seems likely that exposure in pigs could lead to generation of an antibody response, despite the current lack of serological evidence to support this. More extensive testing of pig sera, particularly in areas of high CCHFV seroprevalence in livestock would be an important first step in furthering our understanding of what role, if any, pigs may play. Unfortunately, pig serum samples were not available from the study area for serological testing during the current investigations.

Pig production has increased rapidly over the last two decades, both in Tanzania and elsewhere in sub-Saharan Africa. In 2015 around 2.4 million pigs were kept in the country, with greater than 90% of these kept by small-holder farmers (Kabululu et al., 2018, Kimbi, 2015, Wilson and Swai, 2014). Production in this setting tends to be dominated by traditional practices, with herd sizes under 10 and low productivity, with pigs being kept as an additional source of food security and income to crops and other livestock (Wilson and Swai, 2014, Karimuribo et al., 2011, Kimbi, 2015). There is little literature available on pig husbandry practices within Iraqw households but studies elsewhere in Tanzania found pigs were typically kept in one of three management systems: fully confined year-round, partially confined, or tethered, dependant on time of day and season, or free-roaming year-round. In the same way that confinement was found to be associated with lower

CCHFV exposure risk in cattle in this study, it is likely that different pig management systems will result in different tick exposure patterns in pigs. Further understanding of pig production in villages and households with high CCHFV risk in our study area is therefore likely to be important to unravelling the relationship between pigs and cattle CCHFV exposure.

One hypothesis for the cause of this association is that tick populations carrying CCHFV are amplified in the presence of pigs. Pigs are commonly associated with soft ticks of the genus *Ornithodorus*, the vector of African Swine Fever Virus (ASFV), although they can also host various hard-tick species (Walker et al., 2003, Braae et al., 2013). Ticks of the *Ornithodoros moubata* complex, including *Ornithodoros porcinus* the main vector of ASFV, are present in Tanzania (Sanchez Vizcaino et al., 2015, Walker et al., 2003), and CCHFV has been found in ornithodoros ticks feeding on hosts elsewhere (Sureau et al., 1980, Tahmasebi et al., 2010). However, no evidence has so far been found to suggest that any species of soft tick (family: *Argasidae*) are competent vectors for CCHFV, although experimental studies are limited (Durden et al., 1993). Shepherd et al. (1989a) inoculated various soft ticks including *Or. savignyi* and *Or. porcinus*, both species present in Tanzania (Walker et al., 2003), but could not detect virus more than one day post inoculation, suggesting these species are not competent vectors.

However, pigs may also be hosts for various hard-tick species, which are more likely to carry and transmit CCHFV. A study of ectoparasites on pigs in Mbaya region in south-western Tanzania investigated prevalence of hard ticks on pigs kept under three different management systems: full confinement, free-roaming and mixed (Braae et al., 2013). Free-roaming pigs had significantly higher prevalence of hard tick exposure (50%, 95% CI 32-68) compared to those who were confined (1%, 0-3) or managed in a mixed system (13%, 7-19). Ticks were only identified to the genus level, but four genera were found: *Amblyomma spp.*, *Rhipicephalus spp*. *Haemaphysalis spp* and *Boophilus spp.*. Domestic pigs are not considered typical hosts for most species in these genera so the presence of substantial numbers of hard ticks on these pigs may suggest that hard tick infestation of pigs is more common than previously

recognised in the region and could have implications for transmission of tick-borne pathogens, particularly where pigs are free-roaming. Free-roaming pigs may be more likely to come into contact with other livestock species, and if they are also more likely to act as hosts for hard-tick species, this might lead to transmission of ticks between species. However, the lack of previous identification of pigs as a potential viral reservoir or of any known link between pigs and CCHFV exposure in any species, makes direct transmission less likely. Despite this, the evidence of association identified here, as well as evidence of hard tick presence on pigs in Tanzania indicates that further investigation of this potential link should be pursued.

Potential confounding by other available variables was investigated to rule this out as a cause of the association between higher CCHFV odds in cattle and pig keeping, and no obvious source of confounding was identified. However, it may be that this association is masking the effect of another unobserved variable. For example, pig keeping may result from or in differences in vegetation or environmental setting of the household, which might in turn lead to an increased tick abundance in these areas, even if these ticks are not directly related to the presence of the pigs. For example, pig keeping households tend to be at higher altitude, both in our study and across the country (Kimbi, 2015), and although this variable was not found to be a confounding factor in this analysis, similar environmental variables not considered here might be associated with both pig-keeping and increased CCHFV exposure. Unrecorded differences in Iraqw tribe husbandry or management practices could also act as confounding factors. Further investigation into pig husbandry practices therefore, as well as pig seroprevalence and tick burdens may help to explain this association.

Findings discussed in Chapter 4 demonstrated that patterns of exposure in cattle, goats and sheep varied in magnitude but retained a similar relative relationship across sampling units. It is therefore notable that no association was found between pig-keeping and small ruminant CCHFV exposure. If pigs were leading to amplification of tick populations in general, the increase in CCHFV exposure might be expected to be seen across species. However,

host-feeding preferences are poorly understood for many of the potential tick species involved in CCHFV transmission in this area. If pigs were leading to amplification of tick populations which preferentially fed on cattle, this could explain the lack of association with small ruminants. Transmission routes in these settings are likely to be complex and multifaceted with the potential for subtle differences in tick species abundance and host preference to result in variations in levels of exposure.

5.4.4 General contextual effects

Recalculation of general contextual effect (GCE) parameters demonstrated that there is still a high degree of clustering at village and household level even after adjustment for fixed effects, with similar intra-cluster correlation coefficients (ICCs) and median odds ratios (MORs) in both the null and final models. If the fixed effects were responsible for a large part of the variance observed in the null models, the random effects (RE) variance would be expected to decrease when the fixed effects were added. As the RE variance, and so the GCEs calculated from them, do not vary greatly between null and final models for any species, we can conclude that the addition of the fixed effects do not explain a large proportion of the variance and do not explain the group-level effects observed in Chapter 4. Marginal r^2 values show that much of the variation in the data is explained by the random effects rather than the fixed effects, suggesting that the unexplained variation between closely grouped sets of animals (within a village or household context) has a greater influence on CCHFV exposure than the individual and specific fixed effects considered in this risk factor analysis (Merlo et al., 2005). This unexplained variance suggests that important explanatory variables are not captured in these models.

5.4.5 Limitations

Although extensive and wide ranging, the questionnaire administered during the SEEDZ study was not designed with CCHFV investigation in mind and thus misses some potentially important information. The most obvious gap in the potential explanatory variables is the absence of tick-associated parameters,

including accaracide use, individual-level and local environmental tick prevalences, and these should be a priority for further research.

5.4.6 Conclusion

Overall trends appear to support the hypothesis that CCHFV exposure risk for livestock is higher in extensive pastoral systems, perhaps in particular where animals are co-managed. Higher risk in pastoral systems is consistent with higher tick exposures found in similar systems in other studies, and is also consistent with the protective effect observed in small-holder systems, and in animals that were tethered or zero-grazed. An exception to this appears to be households in which pigs are also kept, none of whom employed extensive grazing of livestock. Further work is needed to understand the drivers behind this association between pig keeping and CCHFV.

6 Discussion

6.1 Overview

This thesis aimed to use serological methods to investigate the epidemiology of two emerging viral zoonoses: Crimean-Congo haemorrhagic fever virus (CCHFV) and severe acute respiratory syndrome virus 2 (SARS-CoV-2). CCHFV is a likely ancient, but now emerging and re-emerging, tick-borne viral concern in much of Eurasia and sub-Saharan Africa, due to its high case fatality rates in people, potential for onwards human-to-human transmission, and lack of effective therapeutics and vaccines (Bente et al., 2013). Although it causes substantial morbidity and mortality across endemic regions each year (Leblebicioglu et al., 2017), outbreaks tend to be sporadic, and where human-to-human transmission does occur it is usually containable with appropriate barrier nursing and standard personal protective measures (Tsergouli et al., 2020). In contrast, SARS-CoV-2 emerged as a novel virus in December 2019, with high transmissibility, and has since gone on to cause a global pandemic, accounting for more than 400 million cases and nearly 6 million deaths to date (WHO, 2022b).

Research questions relating to these two viruses, and the research environment in which they might be answered, are therefore very different. CCHFV has a complex and incompletely understood ecology involving ticks, wild and domestic mammalian hosts, and people, while SARS-CoV-2, although of animal origin (Holmes et al., 2021) and with potential to spillback to animal hosts (Bashor et al., 2021), is now predominantly of concern due to its impacts on and transmission between humans. Despite these differences, serological approaches are applicable to the investigation of both viruses and are vital tools in understanding both population-level exposure and patterns of individual-level immunological response.

This thesis reported aspects of the development, optimisation, use, and downstream analysis of results obtained from several enzyme linked immunosorbent assays (ELISAs): three in-house indirect ELISAs, one against

Crimean-Congo haemorrhagic fever virus (CCHFV) and two against severe acute respiratory coronavirus 2 (SARS-CoV-2), as well as a commercially produced ELISA against CCHFV (ID Screen® CCHF Double Antigen Multi-species ELISA, IDvet, Grabels, France). These tools were highly similar, sharing the same basic principles with some adaptations, but were used to address different questions related to population-level surveillance and immunological responses in two very different viruses, under very different circumstances.

6.2 Serology for a re-emerging zoonosis: CCHFV in Tanzania

6.2.1 CCHFV in livestock in northern Tanzania

Research undertaken for this thesis demonstrated the presence and circulation of Crimean-Congo haemorrhagic fever virus (CCHFV) in livestock and humans in northern Tanzania, substantially increasing knowledge of the virus in this area, which was previously poorly characterised. Evidence of wide-spread anti-CCHFV antibody responses in cattle, goats and sheep, and patterns of increasing seropositivity with age, confirmed that CCHFV is endemic in livestock in Arusha and Manyara regions in northern Tanzania (Chapters 4 and 5). This was previously suspected due to the presence of competent vector species and some serological evidence from cattle but had not been confirmed prior to this research (Messina et al., 2015, Temur et al., 2021). Viral exposure, as measured by seroprevalence, was high overall, but odds of exposure varied substantially between different village and household settings, demonstrating that local environments were strongly associated with differences in risk, and offering opportunities for exploring the causes of these differences.

Some of the variation in individual odds of exposure was associated with the agro-ecological setting in which livestock were kept, with pastoral settings, where animals are extensively grazed in typically higher, dried areas with high grassland coverage and low crop production, associated with greater odds of exposure. Increased risk of exposure in pastoral settings is likely at

least partly related to higher tick abundance in these environments and higher individual-animal tick burdens, although these variables were not measured in this study. In addition, co-management of cattle and small-ruminants, typical of pastoral management, was also associated with increased odds of CCHFV exposure. The reasons for this association are less obvious but may also be due to impacts on vector populations caused by mixed grazing, or might suggest routes of between species transmission. Small-holder settings, where fewer animals are kept per household, in typically lower, more humid areas with higher crop coverage, were associated with lower seroprevalence. At least some of this protective effect is likely due to practices of confining or zero-grazing of livestock, leading to lower tick exposure during roaming or grazing. This trend towards higher risk in pastoral and lower risk in small-holder setting has also been observed in several other pathogens in this same data set including leptospirosis and brucellosis in cattle, *Neospora* in cattle, and Peste des petits ruminants virus (PPRV) in cattle and small ruminants (de Glanville et al., 2018a, Herzog et al., 2019, Semango et al., 2019). Pastoral livestock are likely to be at higher general risk of infectious disease spread for several reasons including high levels of animal mobility, mixing of herds, and limited access to veterinary care (Vanderwaal et al., 2017, Megersa et al., 2009). For CCHFV these factors are also likely to contribute to elevated exposure odds, along with environmental features of pastoral landscapes that may help to sustain suitable vector populations. Limited access to veterinary resources may affect CCHFV exposure patterns in pastoral communities despite typically asymptomatic infection livestock as, although not subject to direct veterinary intervention, lack of access to acaracides or their incorrect use may contribute to higher tick burdens (Swai et al., 2005b).

An unexpected relationship was also identified between increased odds of CCHFV exposure in cattle and the presence of pigs in the household, a finding not previously seen in the literature. The mechanism behind this association is not immediately obvious but two hypotheses are proposed: firstly, that the presence of pigs leads to amplification of tick populations around the households, resulting in higher burdens and higher CCHFV exposure in cattle,

and secondly that pig presence is masking another unmeasured variable which is the direct cause of increased CCHFV exposure. Research related to pigs and ticks in Africa is dominated by questions around African swine fever (ASF) so information on pig-tick interactions for non-ASF vectors is limited. Additionally, the body of literature on pig keeping practices in Tanzania, although a growing field, remains small and no published studies involve the Iraqw tribal group, who dominated pig keeping in this study. Further research into both these areas, as well as investigation of serological responses to CCHFV in pigs, are needed to test these hypotheses.

6.2.2 CCHFV in humans in northern Tanzania

Research presented in Chapter 4 outlines the first comprehensive study of CCHFV seroprevalence in people in Tanzania, revealing evidence of high levels of anti-CCHFV antibodies in otherwise healthy people in the country for the first time. This confirms that viral exposure is occurring despite no clinical cases of Crimean-Congo haemorrhagic fever (CCHF) being reported in Tanzania. Across endemic regions it is not unusual to find human and animal exposure in the apparent absence of clinical disease, but this finding indicates that CCHF should be considered a differential diagnosis for undifferentiated febrile illness (UFI) in Tanzania.

Febrile illness is a major cause of morbidity and mortality in SSA (Maze et al., 2018) but multiple potential aetiologies make characterisation of this burden challenging and often impedes individual diagnostic success (Chappuis et al., 2013, Maze et al., 2018, Elven et al., 2020). Presenting signs for many fever-causing pathogens are non-specific and this, combined with limited diagnostic capacity and an over-reliance on Malaria as a presumptive diagnosis (Stoler and Awandare, 2016), means that the true cause of febrile illness is often missed. This is compounded by difficulties in attributing clinical presentation to a single cause when multiple co-infections are identified (D'Acremont et al., 2014). Studies into causes of undifferentiated febrile illness (UFI) in Africa have become more common in recent years but there remains a lack of research in this area (Prasad et al., 2015). A lack of available rapid diagnostic tests, and focus only on a limited number of pathogens, may result in bias in

these studies towards known pathogens of concern. For evaluation of CCHFV as a cause of UFI limited knowledge of where the virus circulates may lead to its exclusion from investigations of febrile illness, even in areas where it may be causing disease. Identification of sub-clinical exposure through investigation of community seroprevalence is therefore vital for informing studies that are better able to characterise causes of UFI. In Tanzania, several studies have investigated the epidemiology of febrile illness but none of these have included CCHFV in diagnostic panels (Crump et al., 2013, D'Acremont et al., 2014). In contrast, in studies elsewhere in eastern and southern Africa, where CCHFV has been included for investigation, acute cases of CCHF have been identified in patients with febrile illness (Bower et al., 2019, Nyataya et al., 2020, Muianga et al., 2017, Elven et al., 2020). Robust and well-characterised diagnostics for CCHF are available (Vanhomwegen et al., 2012) but access in low-income health care settings may be limited (Mcnerney, 2015). Improved access to, and awareness of diagnostic tests for acute CCHF (typically IgM detection, if molecular methods are not available), are therefore needed both in hospital settings and specific febrile illness studies to further understand the true burden of CCHF in Tanzania.

This thesis also identified substantial heterogeneities in human seroprevalence across different study sites, ranging from villages where no study participants were seropositive to those in which half (50.0%, 95% CI 30.8-69.2%) were. If seroprevalence can be assumed to be related to the risk of clinical disease, this variation may enable identification of risk factors for disease emergence. Investigation of such specific risk factors was beyond the scope of this thesis but investigations into patterns of exposure in Chapter 4 suggested that human and livestock exposure was not structured in the same way, with poor correlation between village-level livestock and human seroprevalence. This finding may suggest that different drivers of exposure are acting on livestock and human populations, and cautions against over-interpreting livestock seroprevalence as an indication of human risk. Extrapolation of animal serology studies to human disease risk, both in

Tanzania and elsewhere, remains challenging and additional studies are needed to further explore this relationship.

6.2.3 Findings highlight complexity of CCHFV transmission

Variation in livestock and human exposure patterns (Chapter 4), unexplained variation in the livestock models, and unexpected findings such as the association with pig keeping (Chapter 5), all serve to highlight the complexities of CCHFV transmission dynamics. This complexity is also demonstrated in the literature by the wide range of reported mammalian hosts, multiple competent tick vectors, and reported heterogeneities in human disease emergence, and remains poorly understood. Across endemic areas there is a lack of understanding of the complex interrelationships that govern viral circulation in ticks and wild and domestic animal hosts, as well as the emergence of the virus as human disease. Interactions between tick species, mammalian hosts, climate, social-change, land use and vegetation changes are likely to determine how, when, and where human cases emerge, but there continues to be a distinct lack of understanding of how these factors interact, particularly in sub-Saharan Africa (SSA). Hoogstraal, in his foundational review of 1979 lamented the dearth of knowledge and lack of studies designed to investigate these complex interactions, and nearly forty years later, as Estrada-Pena et al. (2012) highlight in their more recent review, this situation remains largely unaltered, despite many published studies on seroprevalence and clinical aspects of the virus (Pigott et al., 2017, Temur et al., 2021).

The complex web of interactions between multiple host and vector species and multiple factors makes predicting and understanding CCHFV emergence extremely challenging. However, some common themes do emerge. Climatic and environmental variables have been associated with cases of human CCHF and livestock seroprevalence in many studies (Vescio et al., 2012, Estrada-Peña et al., 2013). Although household elevation and annual mean precipitation were not found to be significantly associated with CCHFV seroprevalence in livestock in this thesis, the relationship observed between higher risk pastoral settings and lower risk in small-holder settings may be

partly driven by their different climatic settings. The impact of climatic conditions on CCHFV transmission is likely to be driven by local context and inter-related to local vector populations, hosts, and farming systems, so the effects of changing climate will also vary from region to region, and from year to year as patterns change, and may be difficult to predict (Rohr et al., 2011, Gilbert, 2021). In southern Europe changes towards a hotter drier climate may result in permanent establishment of *Hyalomma marginatum* populations beyond its current range, providing competent vectors for CCHFV circulation (Nuttall, 2021, Gale et al., 2009). In East Africa, increasing temperatures and increased variability in precipitation are predicted to occur as a result of climate change (Ongoma et al., 2018, Shongwe et al., 2011) but exactly how these changes will impact CCHFV is difficult to predict due to the multifaceted nature of transmission and maintenance.

Changes in land-use patterns, whether due to climate change, conflict, or socio-economic drivers, are associated with increased human cases of CCHF as well as changes in tick-host dynamics and are likely to be a significant factor in the emergence of CCHF cases in the future (Estrada-Peña et al., 2010). Observations of an association with land use change go back to the first identification of the disease in Crimea in the 1940's when farmers and soldiers returned to land previously abandoned during the second world war. More recently, similar patterns were seen in Central Turkey with an increase in cases seen as residents returned to farms following civil disturbances (Bente et al., 2013). These outbreaks were likely driven by an increase in small mammal hosts during the period of abandonment, with a corresponding reduction in livestock hosts leading in an abundance of *Hyalomma* tick nymphs searching for a large mammalian hosts for their adult feed. Land use change in Africa, due to changing climate and socio-economic pressures, and resulting in increased fragmentation (Estrada-Peña et al., 2010) may lead to similar increases in human CCHFV exposure, although, as with climatic variation, the exact nature of how these changes will affect the complex dynamics of CCHFV transmission will likely be heavily dependent on local conditions. Studies that address these complexities are therefore needed. However, it is also important to note that in SSA in particular, understanding of CCHFV risk is hampered by a lack of basic data on seroprevalence in

animals and humans, as well as viral prevalence in different tick species. Studies such as this one therefore, which investigate the presence and distribution of the virus in space and in different hosts, remain important. The research reported in this thesis goes some way to filling gaps in knowledge around seroprevalence and patterns of risk in livestock and humans in a previously data-poor area of SSA.

The complexity of CCHFV dynamics, as well as the many unknowns in terms of factors that influence transmission, should be carefully considered when designing interventions for CCHFV. Without a clear understanding of how tick, vertebrate host, and human transmission cycles interact, poorly planned interventions could result in at best negligible benefits, and at worst an increase in virus circulation, again emphasising the need for improved understanding of the complex relationships described above. Studies that consider a single aspect of CCHFV risk in isolation may result in similarly narrowly focussed intervention strategies, with unpredictable consequences when applied in a real-world complex and interrelated system (Şekercioğlu, 2013). Ultimately, interventions targeting livestock may not be necessary for CCHFV given the lack of clinical or production impact as well as the questions that remain about the nature of the relationship between animal exposure levels and cases of human disease. Interventions which target better characterised aspects of CCHFV transmission, namely improved diagnostic capacity and knowledge amongst clinicians treating febrile illness, implementation of barrier nursing, and increased knowledge around removal of ticks in people may be as effective as more complex interventions.

6.2.4 Is CCHFV really emerging?

Reported cases of CCHF and the known-geographic distribution of the virus have both increased over the last two decades (Nasirian, 2019, Nasirian, 2020) leading to CCHFV being characterised as an emerging pathogen (Serretiello et al., 2020). However, the true character of this emergence is complicated by the concurrent increase in available diagnostic tests, surveillance and prioritisation at a global policy level by the World Health Organisation (WHO, 2022a), resulting in improved diagnostic capability and an

upsurge in published research into all aspects of the virus (Dereli and Kayser, 2018). These factors alone could account for increases in reported case numbers and serological evidence for the virus, which in many regions is likely to have been endemic in ticks and animals for decades or longer (Bente et al., 2013, Whitehouse, 2004). In these areas CCHFV can be considered a re-emerging pathogen, increasingly important in terms of public health awareness, but not necessarily increasing in levels of circulation. An example of this is seen in the re-emergence of human CCHF cases in regions following prolonged periods without detection such as south-western Russia and Central Africa (Grard et al., 2011, Maltezou et al., 2010). However, there is also evidence that CCHFV may be truly emerging, in the sense of an expansion of viral range, for example into western Europe (Espunyes et al., 2021, Monsalve Arteaga et al., 2021), or in sustained outbreaks in areas of the world where previously only serological evidence was reported such as Turkey (Leblebicioglu et al., 2016b). Climate change and land-use change in the coming decades, potentially resulting in changes or expansion of the geographic ranges of competent vector species, may increase this true emergence into new regions contiguous with endemic areas. Additionally, the virus may be imported into new areas further distant from endemic foci through transboundary animal movements, translocation of ticks via migratory birds or movement of people (Spengler et al., 2019), although it is important to highlight that importation of human cases to non-endemic regions when transmission occurred elsewhere is unlikely to constitute expansion, being typically isolated cases without onwards chains of transmission (Lumley et al., 2014, Atkinson et al., 2012b). Infected vector species can be introduced into new areas though livestock movements or via migratory birds (Capek et al., 2014, Gale et al., 2012) and may be able to establish permanent populations if there are available host species and suitable climatic conditions (Spengler et al., 2019, Estrada-Peña et al., 2012). In addition, the high genetic diversity of CCHFV, along with its wide host range, may make emergence into new vector species, which are suited to conditions beyond the current geographic range of the virus a possibility.

6.2.5 Further work: CCHFV

The work presented in this thesis provides the first step towards understanding CCHFV risk in northern Tanzania, but it raises many further questions and highlights areas for future research. Three initial research areas could be explored using existing resources without additional field studies, and so should be prioritised for future work:

1. **What specific risk factors are associated with CCHFV seroprevalence in people in Tanzania?**

   Heterogeneities in odds of exposure for people across different village sites in this study presents an opportunity to further explore risk factors that may help explain these differences. Although the sample size for people is small compared to livestock, it is large enough to explore individual-level variables and agro-ecological setting. Investigation of these risk factors and comparison of how they relate to patterns in livestock observed here should be the next step in this research.

2. **Is CCHFV seroprevalence in livestock and people associated with further environmental variables?**

   Research in Chapter 4 highlighted specific risk factors associated with CCHFV risk in livestock but also identified a large amount of variation in the models that remained unexplained. Some of this variation may be associated with local environmental, land-use and habitat variables, which may act of proxies for suitable vector habitats, in the absence of tick species and abundance data (Zannou et al., 2021, Da Re et al., 2019). These include the Normalized Difference Vegetation Index (NDVI), land-use indices and other remote sensing data (Barrios et al., 2012, Randolph and Rogers, 2007). It will also be important to explore these variables at different scales, from the immediate household environment to larger spatial scales, particularly when considering the differences in risk between agro-ecological settings, where animals may experience environmental variables over varied scales due to differences in management practices.

3. Molecular detection of CCHFV in northern Tanzania

Gaining molecular insight into which clades of CCHFV are circulating in Tanzania is an important next step towards better understanding of how the virus circulates in the region. Additionally, identification of strain type could assist in understanding the risk of human disease from CCHFV in Tanzania as some strains may be less virulent than others (Hua et al., 2020). An obvious place to begin would be in ticks also collected during the SEEDZ project. Ticks were collected opportunistically from sampled animals but no data on individual-level or environmental tick burdens were recorded at the time. The non-systematic collection methods make them unsuitable for estimating prevalence, but they are an excellent resource for identifying tick species and potentially detecting CCHFV genomes. Unfortunately, this could not be carried out within the scope of this PhD thesis, but work on this has now begun. Additionally, serum samples used for serological analysis in this review could also be tested for viral RNA. Viraemia in livestock species lasts around 7 days and as it is not accompanied by clinical signs typically goes undetected. However, with such large numbers of samples, some individuals may have been viraemic at the time of sampling. A cross-sectional study undertaken in Pakistan tested 1600 serum samples from sheep and goats for CCHFV antibodies, as well as pooling samples to test them by RT-qPCR for viral RNA. They found 19% of sheep and 37% of goats were seropositive and 5% (95% CI 2-10%) of sheep pools showed evidence of viral RNA. Another study found 6.6% animals were viraemic during cross-sectional sampling (Tuncer et al., 2014). It is therefore possible that a proportion of the samples collected as part of the SEEDZ study would be positive by RT-qPCR. Positive samples could then be followed up with sequencing to identify clades circulating in this area of Tanzania. Pooling samples for RT-qPCR or utilising newer sequencing technologies such the minion (Oxford Nanopore Technologies, Oxford, UK) could make such an approach practical.

As well as these questions that relate to data and samples already available, the work of this thesis indicates three other areas in which further field or

laboratory studies would help to expand our understanding of CCHFV in northern Tanzania.

1. **Human hospital-based surveillance studies**

The finding of substantial seroprevalence in human communities to CCHFV in northern Tanzania suggests that CCHF may be an unrecognised public health risk in the country. Lack of awareness among clinicians and poor diagnostic resources may mean that cases of CCHF are going unreported in the country, so health care-based surveillance of patients presenting with mild to severe febrile illness of unknown aetiology may help to identify such cases. Investigation of CCHF as a differential diagnosis for febrile illness would not only increase our understanding of the burden of CCHFV but would also contribute to improvements in the management of febrile patients more generally by increasing knowledge of the epidemiology of febrile illness in Tanzania (Maze et al., 2018).

2. **Investigation of pigs and CCHFV**

The identification in this thesis of an association between pig presence and CCHFV exposure in cattle is an entirely novel finding and raises questions around the role of vertebrate hosts in CCHFV transmission and maintenance. Beyond serological investigations of exposure there is a paucity of research into the ability of different vertebrate hosts to support the circulation of CCHFV and to transmit the virus to humans, ticks, or other animals. The association with pigs identified here further highlights the need to rectify this lack of knowledge relating to vertebrate hosts and suggests that exploration of species beyond the common livestock host species, cattle, sheep, and goats, is warranted. Field studies investigating exposure in pigs, as well as further characterisation of pig, tick and cattle interactions may help to explain these findings.

3. **Cross-reactivity studies**

Few recent studies have addressed questions around antigenic cross-reactivity between related nairoviruses using modern laboratory techniques, and the limited evidence that is available is not conclusive (Ward et al., 1992, Grech-Angelini et al., 2020). Dugbe virus (DUGV) and Nairobi sheep disease virus (NSDV) are known to circulate in East Africa,

and so confirming the specificity of antibody responses to CCHFV would enhance confidence in the conclusions drawn from serological studies of the virus. A small number of recent studies have compared antibody responses to related orthonairoviruses using ELISAs and have not found evidence of widespread cross-reactivity (Grech-Angelini et al., 2020, Hartlaub et al., 2021a). However, these relationships remain poorly characterised and so further investigation is needed to confirm that the commonly used assays are in fact measuring what they purport to, and to distinguish between cross-reactivity and co-infection. This is particularly important given the restrictions to working with CCHFV outside high containment laboratories, which makes confirmation of serological response using live virus techniques unfeasible in most cases. Ideally, samples of known exposure status to the different viruses would be used to explore cross-reactivity against different viral proteins but obtaining specifically infected sera typically requires experimentally infected animals, which are expensive and labour intensive. An alternative would be to test samples of known CCHFV status against viral proteins from potentially cross-reactive viruses and to compare patterns of exposure. In the absence of a pseudotype virus system for nairoviruses, indirect ELISA assays provide a starting point for such investigations.

Beyond these specific areas of further research there is a need, both in Tanzania and in many endemic but data-poor countries, for more detailed research in a number of broad areas, in order to better understand the complex ecology of CCHFV in different settings and species.

The paucity of data on tick abundance and fine-scale species distribution, particularly as they relate to different environmental and habitat parameters, presents a limitation for understanding many tick-borne pathogens, including CCHFV, across Africa. Tick studies should aim to address questions around tick-host interactions and host-species preferences through collection of ticks directly from livestock species. In addition, collection of questing ticks, which have not yet fed, and molecular detection of CCHFV in these ticks, would aid in the identification of competent vector species in the

region, and would complement further laboratory-based tick competency studies, particularly in non-*Hyalomma* spp., which are also needed. In Tanzania, studies designed specifically to characterise which species are present, in what abundance and in what environments, could be focussed around the three agro-ecological settings examined in this thesis (pastoral, agro-pastoral and small-holder) and these data used to further understand the patterns observed in the serological data.

As well as pathogen-specific research, the findings of this thesis have also emphasised the need for improved collection of household and livestock-level metadata. Household-level classification, as determined by de Glanville et al. (2020), was associated with CCHFV exposure odds in livestock and provides a more nuanced representation of an individual animal's agro-ecological environment than the broader village-level classification system, being based on multiple reported household behaviours and practices. As such, it is more likely to represent truly similar groups and provide a more realistic insight into risk. However, identification of these household classifications required substantial data collection and analysis. Livestock production data is rarely collected through national surveys, and when it is, is frequently limited. This is despite livestock production being of huge importance to individuals and the wider economy in Tanzania (Covarrubias, 2012). The association identified here between this detailed household classification and CCHFV seroprevalence reinforces the need for improved collection of comprehensive livestock data which can inform risk management and aid in the development of policy (de Glanville2020, Pica-Ciamarra2014).

6.3 Serology in a pandemic response: SARS-CoV-2

6.3.1 SARS-CoV-2 in Glasgow, Scotland

The early phases of the covid-19 pandemic in 2020 brought unprecedented changes to routine scientific work and the need for an all-hands-on-deck approach to research into SARS-CoV-2. This resulted in a change of direction for part of this thesis and the introduction of additional serology work investigating a second emerging viral pathogen. However, the skills and techniques developed through working on an in-house ELISA for CCHFV (Chapter 2) provided a foundation on which to build when using similar indirect ELISAs to investigate patterns of SARS-CoV-2 seroprevalence in a patient population in Glasgow, UK.

In the early phases of the COVID-19 pandemic several commercial solid-phase immunoassays to detect anti-SARS-CoV-2 antibodies were developed, but they were difficult to obtain in large quantities due to the intense global demand, and the high costs involved made them unsuitable for large-scale sero-surveillance unless significant funding was available. For these reasons, as well as the speed with which in-house assays could be developed compared to long wait times for commercial products, in-house assays were developed and utilised by many academic and government studies. At the MRC-University of Glasgow Centre for Virus Research (CVR), previous experience of ELISA development for other viruses (Parr et al., 2021), extensive experience with pseudotype virus neutralisation assays (Logan et al., 2016), and the large number of samples to be tested without specific research funding initially, made in-house assays the most appropriate choice.

The basic protocol for indirect ELISAs against the S1 subunit (S1) and receptor binding domain (RBD) of SARS-CoV-2 was obtained from the National Institute for Biological Standards and Control (NIBSC) but assay interpretation and assessment of performance was carried out in Glasgow and is reported in Chapter 3. Receiver operating characteristic (ROC) analysis demonstrated that over-all the assays performed well at distinguishing between positive and negative samples and had a high sensitivity and specificity (Se = 95.31% (95%

CI 90.08-98.26); Sp = 97.19% (95% CI 94.73-98.71%)). A cut-off value that presented a balance between high specificity (true negative rate) without compromising sensitivity (true positive rate) was chosen for interpretation, as both false positives and false negative results were considered a concern in terms of population-level sero-surveillance. Both ELISAs showed a higher than anticipated degree of day-to-day laboratory variation in raw optical density (OD) values between repeated samples, but the causes of much of this variation were difficult to identify. However, assessment of agreement between repeated samples by comparing their status based on the selected cut-off, demonstrated that the ELISAs had good precision when interpreted as a binary (positive or negative). Thus, even without extensive validation, which was not possible in the time and resource-limited setting of the early pandemic, the ELISAs reported here provided an efficient, cost-effective, and reliable method of monitoring population level exposure to SARS-CoV-2.

Residual samples from routine biochemistry testing undertaken in the National Health Service (NHS) Greater Glasgow and Clyde (NHSGGC) provided an excellent sample set with which to monitor levels of antibody responses to SARS-CoV-2 in the weeks following its introduction to Scotland, between 16th March and 24th May 2020. Under normal circumstances, such samples, particularly those collected in primary care settings, could be expected to provide a reasonably representative sample of the wider community as blood samples are collected for a wide variety of reasons from both healthy and sick individuals (Osborne et al., 2000). During the first pandemic wave this changed considerably, with severe restrictions placed on routine NHS care limiting the number and type of blood tests taken (Thorlby et al., 2020). However, even with these changes, the samples tested in this study, from primary and secondary care, provided an important insight into antibody levels in the Glasgow patient population, at a time when establishing a new study with a more representative population would have been impossible. Patients in primary care settings, although subject to the biases discussed in Chapter 3, were likely the closest representation of the wider community available at the time. Although extrapolation to the general population should be considered with caution, one of the key findings of this study – that

mean seroprevalence remained low in primary care patients during the first pandemic wave – is likely to be representative of the Glasgow population at large and is consistent with low community seroprevalence estimates after the first wave from elsewhere in Europe (Vaselli et al., 2021). Secondary-care samples collected during this time likely reflected the bias towards COVID-19 patients with disease that could not be managed at home, who were prioritised for hospital care. As such, they are unlikely to be representative of the wider community but do provide a useful proxy for more severe COVID-19 cases. Comparison of ELISA positive individuals from primary and secondary care types demonstrated that those in secondary care had both higher levels of IgG and neutralising antibody (NAb) responses. This association between disease severity and antibody levels was not well-characterised at the time but has since been supported by other studies (Chen et al., 2020a, Maciola et al., 2022, Crawford et al., 2021).

Comparison was also made between IgG responses to the S1 subunit and neutralising antibody responses. This revealed heterogeneities in immune responses, with around half (54.17%) of ELISA positive samples also showing neutralising activity. This variation in IgG and NAb responses has since been supported by other studies (Maciola et al., 2022, Luchsinger et al., 2020) and demonstrates the broad and varied humoral immune response to SARS-CoV-2 infection (Wang et al., 2022). Although the presence of neutralising antibodies is often associated with improved protective immunity, lower levels of neutralising antibodies in primary care patients, who are likely to have had milder or asymptomatic infections, suggests that non-neutralising antibody responses can be sufficient to clear infection, at least in mild cases (Chvatal-Medina et al., 2021).

The work presented in Chapter 3 was led by the author, but an expanded version of this study, including a Bayesian state-space model developed and implemented by M. Viana, has been published and is available in the appendix of this thesis (Appendix 1). The model was used to adjust the seroprevalence estimates for test sensitivity and specificity, estimate the weekly probability of infection, and evaluate the impact of age, sex and care

type on the probability of an individual being positive for anti-SARS-CoV-2 antibodies. The Bayesian model showed that even though test sensitivity and specificity was high, overall seroprevalence following adjustment (5.29%, 0.13%–15.10%) was lower than the crude estimates (7.81%, 7.17%-8.48%). This difference is to be expected but demonstrates the importance of considering test performance in conjunction with population parameters for assays where sensitivity and specificity are not close to 100%. The model was also used to estimate the weekly probability of infection, which remained largely consistent throughout the study period following an early peak in probability the same week that RT-PCR confirmed cases peaked, and two weeks prior to the peak in seropositivity. This consistent and low probability of infection was likely due to lock-down restrictions imposed during this period, emphasising the overall low seroprevalence observed in the population, and supporting the conclusion that lockdown and social distancing measures reduced the probability of transmission (Talic et al., 2021). The published study also used the seroprevalence observed to estimate the infection fatality rate (IFR) in different age groups. Although the same caveats for extrapolating these seroprevalence estimates to the wider population apply, the pattern of IFRs observed amongst age groups reflected those reported in other studies from this time, with individuals older than 65 years having a much higher IFR than the younger age groups (COVID-19 Forecasting Team, 2022, Brazeau et al., 2020).

6.3.2 Findings highlight the importance of serological data in pandemic response

The scientific response to the SARS-CoV-2 pandemic has been unprecedented in terms of research outputs, and has fundamentally changed the research and publishing landscape (Aviv-Reuven and Rosenfeld, 2021, Else, 2020). The exceptional volume of research into all aspects of SARS-CoV-2 can make it difficult to place studies such as this one, which took place early in the pandemic, in their contemporary research context. However, it is important to remember that when the sero-surveillance study discussed here was undertaken, knowledge of SARS-CoV-2 epidemiology and immunology was far less complete than it is now.

The findings of this study fit into a number of key areas that were incompletely understood at the time and help to highlight the benefits gained from using serological approaches during the early pandemic. The first of these relates to evidence of the levels of anti-SARS-CoV-2 antibodies in the population following the first wave, as derived from real-world data rather than mathematical models. Mathematical modelling studies were particularly common at the start of the pandemic when real-world data was sparse (James et al., 2021, Panovska-Griffiths, 2020), so studies such as this one, which provided evidence of seroprevalence levels in a large UK city, albeit in a patient rather than community population, provided an important indication of real-world exposure and antibody levels. During the first wave, and much of 2020 prior to the release of vaccinations, discussions were taking place both in the media and in scientific circles around whether natural infections could provide levels of population immunity sufficient to reduce viral transmission, and how policy should reflect this (Abbasi, 2020, Wise, 2020, Greenhalgh et al., 2020). Evidence from seroprevalence studies, including this one, established that population levels of anti-SARS-CoV-2 antibodies were low after the first wave and remained so until the widespread rollout of vaccination, providing evidence that naturally acquired infection would be unlikely to result in high level of population immunity required to limit transmission without significant morbidity and mortality.

Discussions around population immunity relied on the assumption that immunity from natural infection would be highly protective and long lasting, but the degree of protective immunity was not well characterised in 2020. The first confirmed case of re-infection occurred in August 2020 (To et al., 2021) and many questions remained around the constituents of protection into 2021 and beyond. With the emergence of new variants and evidence of vaccine escape (Willett et al., 2022, Harvey et al., 2021), these questions remain pertinent today. In 2020, diagnostic assays which enabled characterisation of the humoral immune response to SARS-CoV-2 infection were urgently needed to complement RT-PCR tests used in diagnosis, and to address questions around correlates of immunity. ELISAs and pseudotype virus

neutralisation assays (PVNAs) described here enabled exploration of the immune response in a large sample of patients with different clinical and demographic histories. Although this study investigated a series of cross-sectional population samples and so could not provide information on longevity of immunity or the relationship between antibody response and protection from reinfection, the findings here, of variation in IgG and NAb responses as well as lower immune responses associated with milder infections, suggested heterogeneities in individual immune responses that could have implications for the degree of protection elicited by natural infection. The link between disease severity and humoral immune response was incompletely characterised at this stage of the pandemic so the evidence provided in this study of a link between disease severity and increased IgG and NAb responses was an important indication of the nature of this relationship.

Serological studies continue to play a vital role in our ability to understand and tackle COVID-19. The widespread roll-out of vaccinations in Europe now means that a large majority of the population have antibodies against SARS-CoV-2 (ONS, 2022) but sero-surveillance can still be used to monitor antibody persistence, and assays which target the N protein, not involved in many vaccine responses, can distinguish between vaccinated and naturally induced immunity, meaning serology can still offer insights into the levels of viral exposure in the population (Duarte et al., 2022). Beyond Europe, vaccine roll-outs have been limited in many low and middle income countries, particularly in sub-Saharan Africa (Lawal et al., 2022). RT-PCR surveillance and diagnostics are also often limited in these settings, so sero-surveillance continues to have an important role to play in monitoring population levels of exposure (Chisale et al., 2022).

6.3.3 Further work SARS-CoV-2

In such a rapidly developing field, many of the questions that arose from the work presented here have since been addressed by research groups both in the UK and around the world, but as the pandemic progresses and new variants emerge, there remain many unanswered questions around SARS-CoV-

2 immunology which serological approaches can help to answer. In the UK, where vaccine coverage is high and there is national, ongoing population-level surveillance of infections and seropositivity facilitated by the government (ONS, 2022), the use of serological techniques to explore immune responses to SARS-CoV-2 infection, reinfection and vaccination is now more pertinent than smaller scale seroprevalence studies. Investigation of differential humoral responses to SARS-CoV-2 variants of concern including how antibody levels differ between vaccinated individuals and those naturally infected with different variants will help to characterise immunity generated by different variants as well as increase our understanding of potential vaccine escape. Pseudotype virus neutralisation assays, made using the spike proteins from different variants, are ideally suited to these comparative studies.

6.4 Serology and sero-surveillance for emerging and re-emerging infectious diseases

The research presented in this thesis demonstrated the benefits of using serological approaches to address questions around emerging and re-emerging viral pathogens. Serological approaches, both to evaluate population-level patterns of exposure and individual-level immunological responses, should be key tools in the emerging infectious disease tool-box. Such conclusions are not new, with use of serological methods widespread across infectious disease research, but in the face of newer, often higher-profile molecular technologies, the advantages of serological investigation are worth emphasising.

Novelty is increasingly prioritised when considering studies for selection by many of the major funding bodies (Cohen, 2017) and there can be no doubt that innovative approaches are important for furthering scientific knowledge. In CCHFV for example, despite an increase in CCHFV research in the last decades there are still large gaps in our understanding of complex interactions between vectors and hosts, and novel and innovative studies are likely to be needed to address these questions. However, novelty can sometimes be prized over established approaches, to the detriment of more

basic but still vital research. In CCHFV research, the need for novel approaches to better understand a complex system does not negate the continuing need, particularly in data-poor regions such as SSA, for simple epidemiological studies that make use of well-established serological techniques and study designs to fill gaps in our basic knowledge of viral distribution and prevalence.

One of the most important applications of serology is in population-level surveillance, a vital tool in understanding and controlling emerging, endemic, and neglected pathogens. Sero-surveillance provides means of detecting re-emerging viruses in novel regions or species, as well as estimating the true burden of endemic and neglected pathogens. Serology offers simple, cost-effective methods for large-scale and wide-ranging surveillance, the knowledge gained from which can then be built upon using more detailed molecular techniques. During the SARS-CoV-2 pandemic, molecular surveillance and diagnostics have been vital, but they could provide only limited information on population exposure and immunity levels without concurrent employment of serological approaches (Galipeau et al., 2020, Winter and Hegde, 2020). For CCHFV, serological surveillance, in both animals and people, is an obvious choice to help fill the gaps in knowledge of distribution and extent of virus, which will form a foundation for further targeted ecological, molecular, and patient diagnostic work.

The SARS-CoV-2 pandemic has brought into sharp focus the threat of emerging novel viruses and has highlighted the need for flexible and dynamic surveillance of both newly emerging and re-emerging zoonotic viruses. Although molecular detection of novel viruses with the potential to spill-over to humans is important for identifying future threats (Mollentze et al., 2021, Holmes et al., 2018), serological monitoring of emerging and re-emerging viruses in animal hosts is also important for understanding potential risk to people. Serological surveillance is particularly important in low-income settings, where data are often limited but climatic conditions and increased human-wildlife contact make spill-over events more likely (Allen et al., 2017) and endemic zoonoses are highly prevalent (Maudlin et al., 2009).

Implementation of routine surveillance is particularly important for zoonoses, as these pathogens often fall between human and animal reporting systems and may go under-reported as a result (Maudlin et al., 2009, Halliday et al., 2012). However, novelty is also often prized in surveillance systems, particularly when focussed on novel pathogen discovery. Moves to develop novel surveillance strategies to detect newly emerging pathogens can neglect existing systems and overshadow the need for routine surveillance of endemic or re-emerging and neglected pathogens, which cause heavy disease burdens in resource poor settings, potentially diverting funding and awareness from the places they are most needed (Halliday et al., 2007). Investing in surveillance infrastructure in low-income settings is vital both for early detection of novel pathogens and for characterising and limiting the burden of endemic zoonoses (Worsley-Tonks et al., 2022). Systems which incorporate surveillance of both emerging and endemic zoonoses, as well as building on and strengthening existing systems and using local expertise, will lead to improved efficiency and ultimately better data collection, with benefits for both local communities, health, and veterinary professionals in understanding endemic disease, and the global community in monitoring emerging pathogens (Halliday et al., 2012, WHO, 2019).

Gathering large scale surveillance data is challenging however, particularly long-term, routine reporting in the absence of a specific and pressing health threat such as SARS-CoV-2. Research programmes such as the “Social, economic and environmental drivers of zoonoses” (SEEDZ) study used in this thesis can help fill surveillance gaps where routine or centrally organised surveillance systems are not in place. The broad scope of the SEEDZ study design meant that the samples and data collected have been suitable for use in the investigation of multiple zoonotic pathogens, providing insights beyond its initial focus. Use of existing samples and metadata in this way should be applied whenever possible to increase the cost-effectiveness of, and to maximise data obtained from, intensive field studies (Metcalf et al., 2016).

Effective surveillance in low-income settings can also be limited by lack of available laboratory and diagnostic resources (Halliday et al., 2012,

Onyebujoh et al., 2016). The in-house ELISA techniques described in this thesis offer relatively cost-effective diagnostics for population-level surveillance, which can be used under biosafety containment level 2 laboratory conditions without the need for tissue culture or higher containment facilities that are often unavailable in low-income settings. The use of in-house assays to facilitate surveillance in low-income settings may be particularly effective when supported in collaboration with laboratories in middle- or high-income countries with capacity to confirm and validate findings with more infrastructurally demanding and costly techniques such as PVNAs. For example, the SARS-CoV-2 ELISAs described in Chapter 3 have since been adapted for use in a large, ongoing community study into SARS-CoV-2 seroprevalence and immunodynamics in Malawi, with samples also shipped to Glasgow for further testing using PVNAs.

6.5 Future directions in serology

This thesis has demonstrated that readily available, cost-effective serological methods can be used to explore important population- and individual-level questions about emerging and re-emerging zoonoses. The ELISA and PVNA techniques used here remain important and present opportunities to broaden surveillance of zoonoses in resource poor settings. However, serological methods continue to develop, and new technologies present exciting opportunities for higher throughput and more detailed analysis of serological samples. Recent developments in ELISA platforms include meso scale discovery (MSD) immunoassays which utilise multi-spot technology to analyse a single, small volume samples against multiple analytes simultaneously (MSD, 2022). This technology allows for rapid, high throughput screening against multiple viral antigens and typically has higher sensitivity and dynamic range compared to traditional ELISAs. Current antigen panels are available for various SARS-CoV-2 antigens, seasonal human coronaviruses, and other respiratory pathogens, but this technology has the potential to be applied to a wide range of emerging viral pathogens. Multiplex assays such as this are particularly beneficial for integrated surveillance where multiple pathogens are of concern, for example in the diagnosis of febrile illness, in

exploring questions of cross-reactivity between related viruses, or in identifying patterns of co-infections (Arnold et al., 2018).

6.6 The need for One Health approaches

The need for surveillance of emerging, re-emerging, and endemic pathogens is clear, and serological approaches, both well-established and more recently developed, play an important role in this. Both pathogens examined in this thesis also highlight the utility of a 'One Health' approach to zoonoses research and the potential benefits to be gained from framing research in this context. The global pandemic of the last two years has demonstrated the need for joined up approaches to pathogen surveillance, reporting, responses, and interventions (Thoradeniya and Jayasinghe, 2021, Lambert et al., 2020), which also apply to other emerging zoonotic viruses including CCHFV (Greene et al., 2022). The One Health paradigm, which emphasises the inter-relatedness of human, animal, and environmental health (Gibbs, 2014, Zinsstag et al., 2011) is ideally suited to facilitate the joined-up approach required to tackle the challenge of zoonotic viral emergence (EClinicalMedicine, 2020). This approach has been embraced over the last two decades by the World Health Organisation (WHO), the Food and Agriculture Organization of the United Nations (FAO) and the World Organisation for Animal Health (OIE), particularly in response to zoonoses, with the publication in 2019 of a joint One Health framework, the Tripartite Zoonoses Guide (TZG) (FAO et al., 2019) which aimed to encourage and facilitate multisectoral and interdisciplinary approaches to zoonotic diseases surveillance, reporting, and control. Zoonoses such as rabies and brucellosis, are often considered classic One Health pathogens but it is important to note that the benefits of a One Health approach are not limited to directly transmitted or neglected zoonoses. Vector-borne pathogens, as well as those that cause disease only in livestock, or only in humans following an initial spill-over event, can also benefit from framing research questions in the context of animal, human and environmental health. The complexity of CCHFV ecology particularly lends itself to a One Health approach, with more integrated studies that consider linked human, animal and vector

transmission and surveillance, needed (Sorvillo et al., 2020, Gilbride et al., 2021).

Since its emergence into human populations, global concerns around SARS-CoV-2 have rightly been dominated by health concerns specific to human populations, but the virus's likely animal origins (Holmes et al., 2021), as well as outbreaks in other species also demonstrate the importance of a One Health approach for this virus. Several spill-back events have now been reported whereby the virus has been transmitted from humans to wild or domestic animals resulting in circulation in these species (Pickering et al., 2022, Hammer et al., 2021, Molenaar et al., 2020). As well as having the potential to cause disease in these new host species (Fenollar et al., 2021), such events may also result in evolution of new variants which could then spill-back into human populations, possibly with greater severity than the original strains (OIE, 2022, Bashor et al., 2021, Colson et al., 2022).

7 Appendices

7.1 Appendix 1: Severe Acute Respiratory Syndrome Coronavirus 2 Serosurveillance in a Patient Population Reveals Differences in Virus Exposure and Antibody-Mediated Immunity According to Host Demography and Healthcare Setting

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MAJOR ARTICLE

Severe Acute Respiratory Syndrome Coronavirus 2 Serosurveillance in a Patient Population Reveals Differences in Virus Exposure and Antibody-Mediated Immunity According to Host Demography and Healthcare Setting

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Identifying drivers of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) exposure and quantifying population immunity is crucial to prepare for future epidemics. We performed a serial cross-sectional serosurvey throughout the first pandemic wave among patients from the largest health board in Scotland. Screening of 7480 patient serum samples showed a weekly seroprevalence ranging from 0.10% to 8.23% in primary and 0.21% to 17.44% in secondary care, respectively. Neutralization assays showed that highly neutralizing antibodies developed in about half of individuals who tested positive with enzyme-linked immunosorbent assay, mainly among secondary care patients. We estimated the individual probability of SARS-CoV-2 exposure and quantified associated risk factors. We show that secondary care patients, male patients, and 45–64-year-olds exhibit a higher probability of being seropositive. The identification of risk factors and the differences in virus neutralization activity between patient populations provided insights into the patterns of virus exposure during the first pandemic wave and shed light on what to expect in future waves.

Keywords. SARS-CoV-2; COVID-19; virus exposure, serology; virus neutralization; modelling; risk factors; seroprevalence.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first reported in China in December 2019 and spread rapidly across multiple countries. The first coronavirus disease 2019 (COVID-19) case in Scotland was confirmed on 28 February 2020, the country entered lockdown on 23 March, and restrictions were eased on 28 May [1]. Serological surveys are instrumental in determining infection rates at the population scale [2]. Assays based on the detection of anti-SARS-CoV-2 immunoglobulin (Ig) G antibodies, which are typically detectable 7–21 days after infection [3], can identify past viral exposure even in asymptomatic individuals. In-house assays commonly use an indirect enzyme-linked immunosorbent assay (ELISA) format, with recombinant S protein, S1 subunit of the S protein, or the receptor-binding domain (RBD) used as antigens. Virus neutralization assays provide insights into the effectiveness of the humoral immune response. Neutralization titers obtained with pseudotype-based tests are similar to those obtained with live virus [4], and 2 pseudotype-based methods are commonly used: human immunodeficiency virus (HIV)–based and vesicular stomatitis virus–based pseudotypes. Both methods produce similar results [5].

Models that link patient information (eg, age, sex, and time of sampling) with exposure and immunity enable the identification of factors associated with SARS-CoV-2 infection [6]. NHS Greater Glasgow and Clyde (NHSGGC) is the largest health board in Scotland and reported the most COVID-19 cases (n = 3876) and deaths (n = 1280) in the country between 1 March and 24 May [7]. We performed a serial cross-sectional study among primary and secondary care patients in NHSGGC to estimate levels of exposure to SARS-CoV-2

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since the introduction of the virus in Scotland and up to calendar week 21 (starting on 18 May 2020). Using a bayesian framework, we combined serological and patient information to estimate an individual's probability of testing positive for SARS-CoV-2 across various age groups, time and healthcare settings. We also performed neutralization assays to estimate the fraction of exposed individuals who developed an effective antibody response. Finally, we combined serological data with publicly available information on deaths to estimate the case-fatality ratio.

METHODS

Serum Samples

Ethical approval was provided by NHSGGC Biorepository (application 550). Random residual biochemistry serum samples (n = 7480) from primary (general practices) and secondary (hospitals) healthcare settings were collected by the NHSGGC Biorepository between 16 March and 24 May 2020. Associated metadata included date of collection, patient sex and age, partial postal code of the patient, and sample origin (primary or secondary care). All serum samples were inactivated at 56°C for 30 minutes before being tested.

ELISA Testing

S1 and RBD antigens were prepared as described elsewhere [8]. The SARS-CoV-2 RBD and S1 constructs, spanning SARS-CoV-2 S (UniProt ID P0DTC2) residues 319–541 (RVQPT…KCVNF) and 1–530 (MFVFL…GPKKS), respectively, were produced with C-terminal twin Strep tags. Proteins were produced by transient expression in Expi293F cells grown in FreeStyle-293 medium (Thermo Fisher Scientific). Proteins were harvested at 2 time points, 3–4 and 6–8 days after transfection. Twin Strep-tagged proteins were captured on Streptactin XT (IBA LifeSciences) and purified by size exclusion chromatography through Superdex 200 (GE Healthcare). Purified SARS-CoV-2 antigens, concentrated to 1–5 mg/mL by ultrafiltration were aliquoted and snap-frozen in liquid nitrogen before storage at −80°C.

Assays to detect IgG antibodies against recombinant S1 and RBD antigens of SARS-CoV-2 were performed as described elsewhere [9]. First, 96-well plates (Immulon 2HB, Fisher Scientific) were coated overnight with S1 or RBD antigen (50 ng per well). After being washed 3 times with phosphate-buffered saline (PBS)/0.05% Tween 20 (all subsequent wash steps followed the same protocol), serum samples were diluted 1:100 in PBS/0.05% Tween 20 (vol/vol) supplemented with 10% (vol/vol) casein (Vector Laboratories; 2BScientific) and incubated for 1 hour at room temperature before a second wash. Anti-human IgG horseradish peroxidase–conjugated secondary antibody (Bethyl Laboratories) diluted 1:3000 in PBS/0.05% Tween 20/casein was then added and incubated for 1 hour before a third wash. Next, 3,3′,5,5′-tetramethylbenzidine (Sigma-Aldrich/Merck) was added and incubated for 10 minutes in the dark.

The reaction was stopped by adding an equal volume of 1-mol/L sulfuric acid. Absorbance was read immediately at 450 nm on a Labsystems Multiskan Ascent plate reader. Duplicates of pooled known-positive and known-negative controls were included on each plate. Raw absorbance values were corrected using the following equation: (sample absorbance − negative control mean)/negative control mean. This value was used for downstream analysis. The cutoff between positive and negative values was selected using receiver operating characteristic (ROC) analysis undertaken with the corrected absorbance values of positive and negative control samples tested on the assay. A total of 320 serum samples collected before December 2019, obtained from the National Institute for Biological Standards and Control and the Scottish National Blood Transfusion Service, were used as negative controls.

Positive controls were defined as samples from patients with a positive reverse-transcription polymerase chain reaction result, or those who had recent clinical symptoms consistent with COVID-19 and whose serum sample tested positive on all other serological platforms (EUROIMMUN-Anti-SARS-CoV-2 ELISA [IgG], Abbott Architect SARS-CoV-2 IgG, or DiaSorin LIAISON SARS-CoV-2 S1/S2 IgG). A total of 128 samples were used as positive controls. Cutoff values for individual antigens were chosen to optimize for the specificity of each individual test, while maintaining a sensitivity >90%. All samples were tested against both S1 and RBD antigens, and separate ROC analyses were undertaken for each antigen. ROC analyses were performed using GraphPad Prism software (v9.0.0) (GraphPad) (Supplementary Figure 1). Final sensitivity and specificity values, and 95% confidence intervals (CIs), were calculated by applying the individual cutoff values for S1 and RBD, derived from the ROC analysis, to the control samples in parallel (ie, if a sample tested positive for either or both antigens, it was considered positive). The resulting numbers of true-positives and true-negatives, and false-positives and false-negatives, were then used to calculate the final sensitivity and specificity of the combined assays.

Neutralization Assays

HEK293, HEK293T, and 293–angiotensin-converting enzyme 2 (ACE2) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2-mmol/L L-glutamine, 100-µg/mL streptomycin and 100-IU/mL penicillin. HEK293T cells were transfected with the SARS-CoV-2 S (corresponding to Wuhan-Hu-1 strain; GenBank MN908947) gene expression vector pCDNA6-S (from N. Temperton, University of Kent), together with pNL4-3-Luc-E⁻R⁻luc [10] using polyethylenimine (Polysciences). HIV (SARS-CoV-2)–containing supernatants were harvested 48 hours after

transfection, aliquoted and frozen at −80°C before use. 293-ACE2 target cells were generated by stable transduction of HEK293 cells with pSCRPSY-human ACE2 (hACE2). Selected 293-ACE2 cells were maintained in complete DMEM supplemented with 2-μg/mL puromycin.

Neutralizing antibodies were measured using a fixed dilution screening. Duplicate serum samples were diluted 1:50 in complete DMEM and incubated for 1 hour with an equal volume of HIV (SARS-CoV-2) pseudotypes. The serum-virus mix was plated onto 293-ACE2 cells in 96-well white cell culture plates. After 48–72 hours, luciferase activity was quantified by adding Steadylite Plus chemiluminescence substrate (Perkin Elmer) and analyzed on a Perkin Elmer EnSight multimode plate reader (Perkin Elmer). Serum samples were considered to have high neutralizing activity if at a 1:50 dilution they reduced infection by HIV (SARS-CoV-2) pseudotypes by ≥90% [11].

COVID-19 Data

The number of laboratory-confirmed cases was obtained from the Scottish government Web site (https://www.gov.scot/coronavirus-covid-19/) and the West of Scotland Specialist Virology Centre. The number of COVID-19–associated deaths was obtained from the National Records of Scotland Web site (https://www.nrscotland.gov.uk/covid19stats).

Statistical Analysis

Multivariable logistic regression models were used to investigate associations between neutralization at a 1:50 dilution and corrected optical density values, care type, age group, and sex in ELISA positive samples (n = 216). Separate models were run for samples positive to S1 and RBD (Supplementary Tables 2 and 3). Univariate analyses comparing the mean corrected optical density, or percentage neutralization, between ELISA-positive samples from primary and secondary care types were undertaken using Mann-Whitney *U* tests. To determine a sample size for estimating the prevalence of partial postal code districts, we used a simple calculation, assuming a random sample from a large population. An assumed prevalence (p) of 10%, and a confidence of 95%, substituted into the equation $n = 1.96^2 p(1-p)/d^2$ (where d = precision = 0.05), resulted in a sample size of 138. Statistical analyses and data visualization were undertaken using R software [12], version 3.6.1. Models were run using lme4 package [13].

Bayesian State-Space Model

A state-space model was developed to estimate the weekly probability of infection of the patient population and to evaluate the impact of the different demographic factors affecting the probability of an individual being seropositive for SARS-CoV-2. The model followed methods published elsewhere [14] and comprised 2 coupled parts: a population-level process and an observation or individual-level process. The population process captured the weekly exposure dynamics through a linear predictor comprising a temporal trend and autocovariates (ie, first- and second-order autoregressive components capable of reconstructing potential exposure cycles). This results in a weekly probability of infection that reflects the average chance of being infected in a given week after adjustment for individual covariates in the observation process.

The observation process confronted the population probabilities by using individual-level data (ie, binary observed serological data from each patient) in a Bernoulli trial that adjusted seropositivity according to the sensitivity and specificity of the test and estimated an individual's probability of infection based on the population-level dynamics but also through a series of individual covariates such as sex, age, care type and week of sample collection. We noted that since further adjustment for population size resulted in differences of approximately 0.1% in group-based seroprevalence estimates, for simplicity this was omitted from the final state-space model. We ran the model in JAGS for 100 000 iterations and 50 000–iteration burn-in to achieve full convergence. Priors and the model code are provided in the Supplementary Material.

Infection Fatality Ratio

An infection fatality rate was calculated for each age group by estimating the fraction of SARS-CoV-2–confirmed deaths relative to the number of people exposed. The latter variable was approximated using the adjusted seroprevalence, multiplied by the corresponding group population size (455 739, 310 813, 106 435, and 80 745 for the 18–44-, 45–64-, 65–74-, and ≥75-year age groups, respectively). Mid-2019 population estimates were obtained from the National Records of Scotland (https://www.nrscotland.gov.uk).

RESULTS

A total of 7480 residual biochemistry serum samples from patients living in NHSGGC were tested for the presence of IgG antibodies against the S1 subunit of the SARS-CoV-2 spike protein and its RBD using 2 ELISA assays [9]. Of these, 6635 met the inclusion criteria and were used for further analysis. Samples spanned a 10-week period, starting on 16 March 2020 and covered all NHSGGC districts and all age groups, except for children and young adults <18 years of age for whom insufficient samples were available (Figure 1 describes the sample inclusion criteria and sample sizes). The underrepresentation of samples from pediatric patients reflected the reduction in general practitioner appointments, the prioritization of suspected COVID-19 cases during this period, parents' avoidance of attending medical facilities to protect children from the virus, and likely reduced risk of non–COVID-19 infections and injuries (the most common reason for emergency attendances in children) owing to physical distancing as well as the lower incidence of clinical signs in children [15, 16].

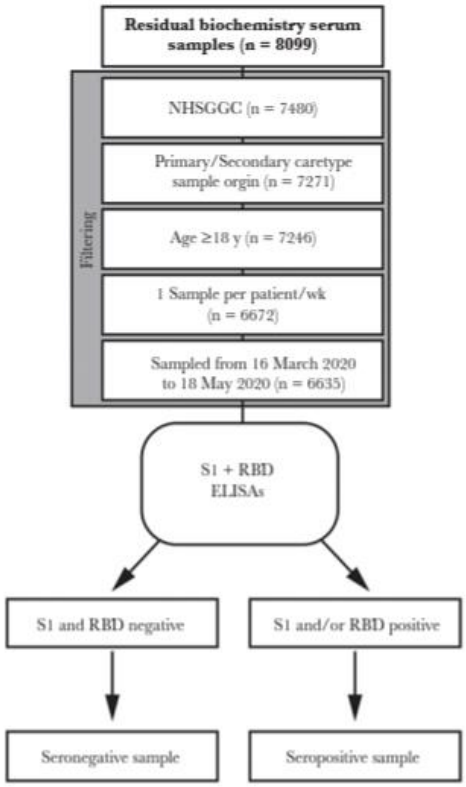

Figure 1. Diagram summarizing the flow of samples used in this study. Abbreviations: ELISAs, enzyme-linked immunosorbent assays; NHSGGC, NHS Greater Glasgow and Clyde; RBD, receptor-binding domain; S1, spike glycoprotein.

The overall unadjusted seroprevalence in our patient population was 7.81% (95% CI, 7.17%–8.48%) (Figure 2A). Seroprevalence was higher in 45–64-year-olds, in male patients, and in patients attending secondary care services (Figure 2A). A steady increase in seroprevalence was observed from the week beginning 16 March up to the week beginning 13 April in both primary and secondary care settings. However, while seroprevalence in the secondary care subpopulation was higher, and started to decrease from the week beginning 13 April, seroprevalence in primary care remained at a similar level after the week beginning 13 April to the end of our study period (Figure 2B). For some age groups (45–64 and 64–74 years) seroprevalence was higher in men (Figure 2C), perhaps driven by a sex bias in SARS-CoV-2–associated hospitalization [17], since men admitted to secondary care services had a higher seroprevalence (10.73%; 95% CI, 9.40%–12.17%) than women (7.60%; 6.51%–8.81%) (Figure 2C). This difference between sexes was not observed among primary care patients (6.06% [95% CI, 4.73%–7.63%] for men and 5.40% [4.29%–6.71%] for women) Figure 2C).

Patient seroprevalence was also calculated in a subset of districts (20 of 61) in which sample numbers provided sufficient power to estimate prevalence. Estimated seroprevalences ranged from 3.83% (95% CI, 1.67%–7.40%) to 12.94% (8.29%–18.94%) (Supplementary Table 1) suggesting that there may be geographically driven differences in infection risk. However, sample size limitations prevented more detailed analysis. Our bayesian state-space model [14] was used to adjust the crude patient seroprevalence rates for the sensitivity and specificity of the assays and to determine the factors associated with seropositivity in the study population. The model converged well and provided a good fit to the data (Figure 3A and Supplementary Figure 2). Although the test had high sensitivity (95.31%; 95% CI, 90.08%–98.26%) and specificity (97.20%; 94.76%–98.71%), the adjusted overall seroprevalence (5.29%; .13%–15.10%) was approximately half the crude estimates (Figure 3A and Table 1). The analysis indicated that patients receiving secondary care were twice as likely (odds ratio, 2.2; 95% CI, 1.6–3.1) to be seropositive as those in primary care (Figure 3B).

Male patients were 1.39 (95% CI, 1.1–1.8) times more likely to be seropositive, and individuals belonging to the 45–64-year age group were 2.2 (1.5–3.3) times more likely to be seropositive than those in the 18–44-year age group. However, belonging to the older age groups (≥65 years) did not significantly increase the probability of being seropositive (Figure 3B). Nonetheless, considering the adjusted seroprevalences per age group, and their associated population size and SARS-CoV-2–related deaths, we estimated a higher infection fatality ratio in older age groups (Table 1), consistent with findings from a previous United Kingdom–based study [18]. The probability of infection at the population level (Figure 3C) peaked once during the week beginning 30 March, 2 weeks before the week with highest seroprevalence and coincided with the peak of polymerase chain reaction–confirmed cases (Figure 3D). After this peak, there was a low and constant weekly probability of infection (median 10.2%; 95% CI, 3.1%–20.6%) (Figure 3C), likely reflecting the strict lockdown conditions of the study period. At the end of the study period, before lockdown was eased, we observed a slight increase in the probability of infection (Figure 3C), but further data would be required for confirmation. Together, these results suggest that while levels of infection by SARS-CoV-2 remained broadly constant from the introduction of the virus, they were higher among men, 45–64-year-old patients, and those who attended secondary care.

To determine whether exposure might elicit a protective immune response, HIV (SARS-CoV-2) pseudotypes were used to measure levels of neutralizing anti-SARS-CoV-2 antibodies in samples collected between 24 March and 24 April (n = 1974; 10.94% positive by ELISA). A total of 117 (54.17%) ELISA-positive

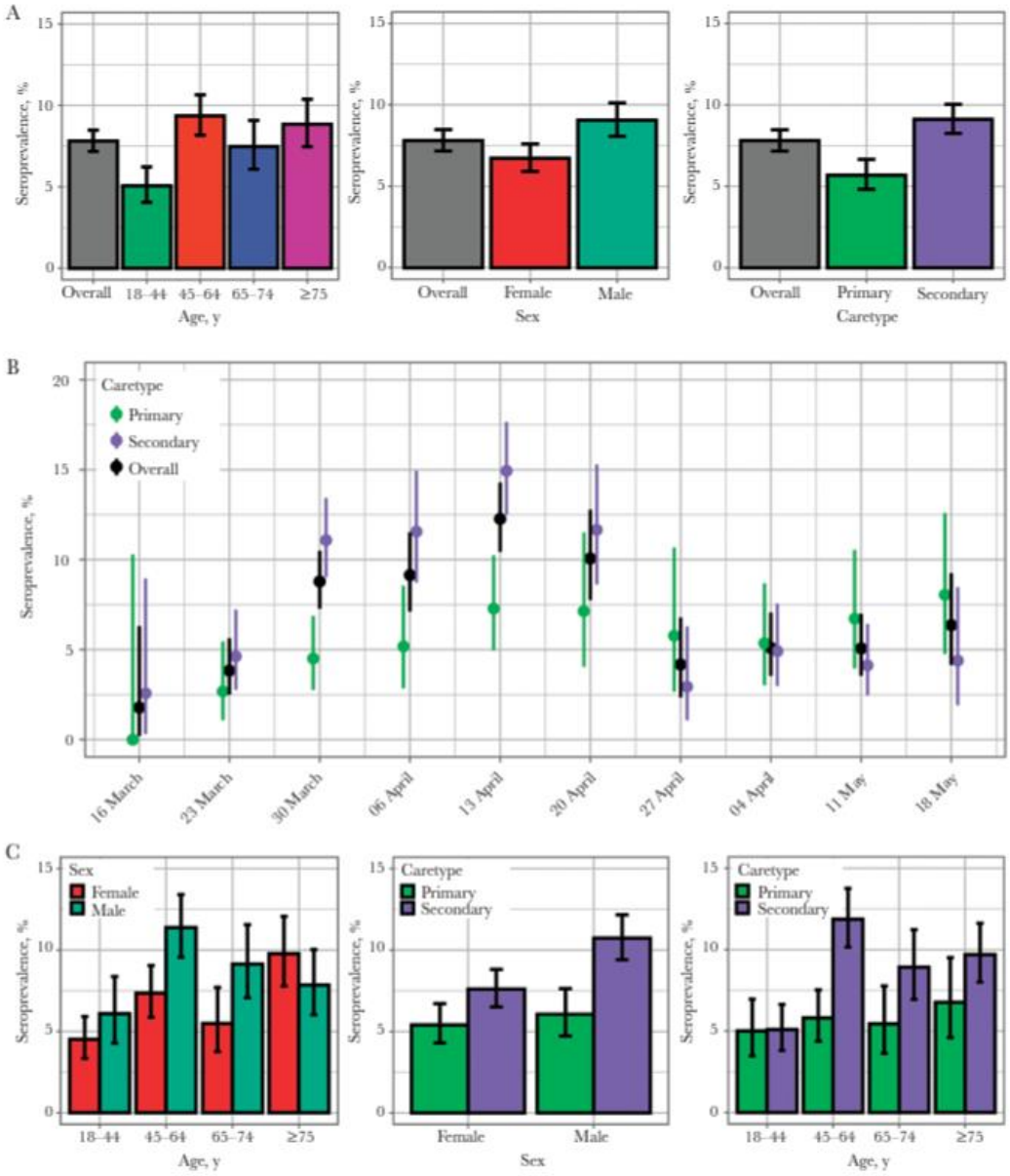

Figure 2. Unadjusted severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) seroprevalence in NHS Greater Glasgow and Clyde, Scotland, United Kingdom, patient population. *A, B*, Seroprevalence estimates and 95% confidence intervals are shown across age groups, sex, and healthcare setting (*A*), or date of sampling (*B*). *C*, Seroprevalence estimates and 95% confidence intervals investigated in sequential combinations of age group, sex, and healthcare setting.

and 17 (0.97%) ELISA-negative samples exhibited high neutralizing activity (Figure 4A). Serum samples were considered to have high neutralizing activity if they reduced infection by >90% at a 1:50 dilution. Overall, our results suggest that approximately half of those individuals who seroconverted elicited a highly neutralizing response. Serum samples with higher absorbance levels in

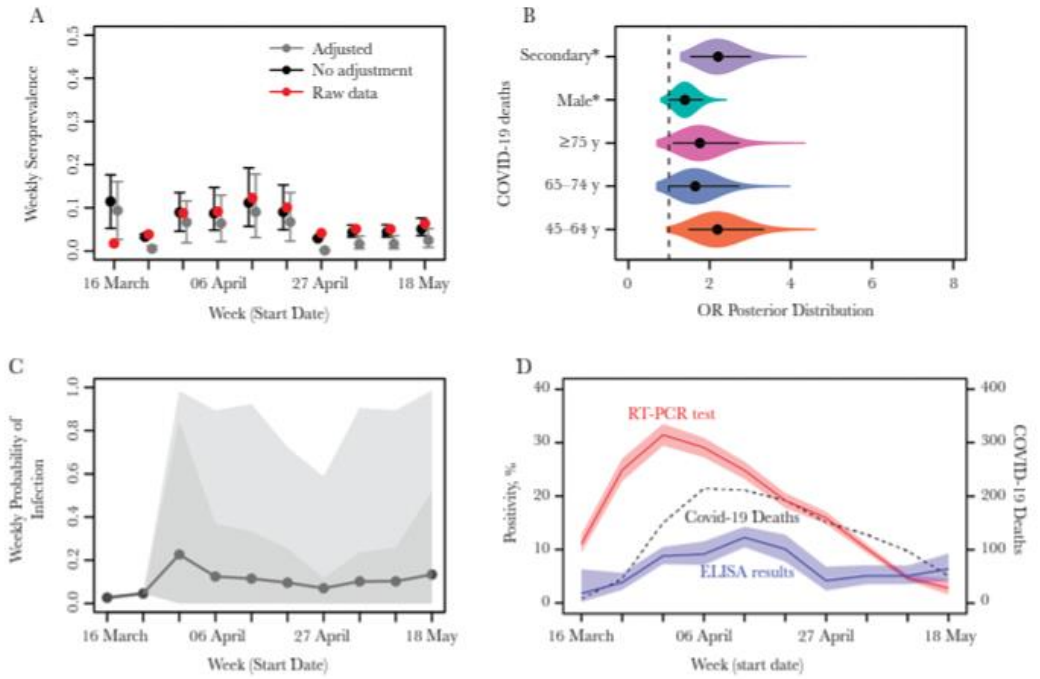

Figure 3. Posterior estimates obtained from the bayesian state-space model. *A*, Model fit (observed data in red vs estimated unadjusted seroprevalence in black) and estimated adjusted seroprevalence (*gray*). *B*, Odds ratios (ORs) of the effect sizes of age, sex and healthcare setting on the probability of a patient being seropositive for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) antibodies (95% confidence interval [CI] lines within violin). *C*, Estimated mean weekly probability of infection of the studied population, and associated 75% and 95% CIs. *D*, Unadjusted SARS-CoV-2 seroprevalence (blue), reverse-transcription polymerase chain reaction (RT-PCR)–confirmed coronavirus disease 2019 (COVID-19) cases (red), and COVID-19–related deaths (black) are shown. Abbreviation: ELISA, enzyme-linked immunosorbent assay.

ELISAs exhibited higher levels of virus neutralization (Figure 4A and Supplementary Tables 2 and 3).

In addition, serum samples derived from ELISA-positive patients in secondary care displayed significantly higher mean absorbance values ($P = .004$) (Figure 4B) and mean percentage neutralization than samples from antibody-positive patients in primary care (Figure 4C), implying that disease severity is associated with a stronger and more effective antibody-mediated response. Multivariable logistic regression models confirmed that increasing absorbance values on ELISA were significantly associated with neutralization (odds ratio, 1.15; 95% CI, 1.10–1.21; $P \leq .001$), and that samples derived from secondary care had significantly higher odds of neutralizing ability than those from primary care (6.77; 2.68–18.75; $P \leq .001$) (Supplementary Tables 2 and 3).

DISCUSSION

Serological surveys are key to informing strategies aimed at controlling the spread of disease. Our study showed that SARS-CoV-2 exposure during the first wave of the pandemic remained broadly consistent over time (likely due to lockdown conditions), but heterogeneous among different groups of the Glasgow patient population. After adjustment for test sensitivity and specificity, the overall seroprevalence in the patient population of NHSGGC (5.29%) was similar to reports from community-based cross-sectional studies carried out during an equivalent period in other European cities, such as Geneva [19] and Madrid [20]. However, because our study relied on analyses of residual biochemistry samples from a population of individuals seeking healthcare including—but not exclusively—people who are more likely to be symptomatic with SARS-CoV-2 infection than the general population, generalization beyond the study population requires caution. For example, male patients had a significantly higher risk of being seropositive in our study, although this was not a feature of the previous community-based studies, likely reflecting a sex bias in COVID-19 presentation [21] or differences in social behavior that led to increased exposure [22].

Table 1. Observed and Adjusted Seroprevalences in the Different Demographic Groups of the Study Population

Demographic Group	Population Size	Samples, No	Seroprevalence, Mean (95% CI), %		COVID-19–Related Deaths, No.	IFR, %
			Unadjusted	Adjusted		
Sex						
Male	459 189	3092	9.06 (8.07–10.12)	6.49 (.16–17.67)	606	NA
Female	494 556	3543	6.72 (5.92–7.59)	4.23 (.13–13.14)	627	NA
Care type						
Primary	NA	2531	5.69 (4.82–6.66)	2.95 (.10–8.23)	NA	NA
Secondary	NA	4104	9.11 (8.25–10.04)	6.73 (.21–17.44)	NA	NA
Age group, y						
18–44	455 739	1662	5.05 (4.05–6.22)	3.10 (.10–9.05)	8	0.06
45–64	310 813	2202	9.36 (8.17–10.65)	6.67 (.16–17.84)	103	0.50
65–74	106 435	1244	7.48 (6.08–9.08)	5.18 (.15–13.98)	164	2.97
>75	80 758	1527	8.84 (7.46–10.38)	5.78 (.17–14.96)	958	20.52
Overall	953 745	6635	7.81 (7.17–8.48)	5.29 (.13–15.10)	1233	NA

Abbreviations: CI, confidence interval; COVID-19, coronavirus disease 2019; IFR, infection fatality rate; NA, not available.

It is important to note that 38% of samples were derived from patients attending primary care, and this proportion remained stable during the studied period. Under normal circumstances, such samples would provide a cost-effective method of obtaining samples for serosurveillance that are broadly representative of the wider community [23]. However, the unprecedented changes to routine healthcare guidelines and health-seeking behavior [16] during the first wave of the pandemic are likely to have altered the structure of this population considerably. Patients in primary care were well enough to be managed in the community and so might be subject to similar exposure conditions as the general population.

At the same time, groups that continued to be seen in primary care for blood sampling, including pregnant women and those with chronic conditions, may have shielded during this period and thus have had lower exposure than the general population. The prevalence in this group may therefore be lower than the expected community prevalence. Conversely, the probability of exposure for individuals from secondary care might be higher than expected in the general population owing to the prioritization of severe COVID-19 cases in hospital settings during this period. In addition, some patients may have been in the early stages of infection and may not have seroconverted at the time of sampling, resulting in an underestimation of seroprevalence in both healthcare settings. Overall, and with the aforementioned caveats, the seroprevalence observed in the primary care subpopulation may be a better representation of the general population than that observed in secondary care.

Neutralization assays provided insight into postexposure antibody-mediated immunity. HIV (SARS-CoV-2) pseudotype-based neutralization assays display a high correlation with live virus-based assays [4]. Although we found a significant correlation between antibody levels and neutralizing activity, we also found, in agreement with other studies [24], that exposure to SARS-CoV-2 resulted in heterogenous responses. As samples from secondary care patients showed both significantly higher antibody levels and odds of neutralization capacity, our results suggest that disease severity may be associated with more effective immune responses. However, antibody levels change over time and our results should be considered within this context. Given the time frame of our study, our results are likely to represent the serological profiles of recent infections. Although our data set did not include clinical information on individual patients, the emphasis on reduction of routine procedures and prioritization of patients with COVID-19 during lockdown makes the secondary care population a suitable proxy for severe SARS-CoV-2 infections. Lower IgG and neutralizing responses in primary care patients could also reflect sampling at earlier points after infection. However, similar results linking disease severity and immune response were reported [25–27].

Neutralizing ability observed in a small number of ELISA-negative serum samples suggests that the presence of epitopes outside the SARS-CoV-2 S1 or RBDs may contribute to the neutralizing response. We note that while there is evidence linking the presence of neutralizing antibodies with protection [28], any inferences between antibody levels and protective immunity should be interpreted with caution. The determinants of a protective immune response to SARS-CoV-2 are unknown and recent studies have suggested that T-cell responses play an important role in SARS-CoV-2 immunity [29]. It has been postulated that between 43% and 70% of the population needs to be immune to SARS-CoV-2 to reach herd immunity [30, 31]. Achieving such levels without vaccination is unlikely in the short term, given that seroprevalence, even among secondary care patients who showed the highest seroprevalence, reached only 6.73% (95% CI, .21%–17.44%). The absence of a strong neutralizing response in a large proportion of seropositive patients raises questions regarding the protective nature of the humoral immune response, highlighting the urgent need for further studies into the duration of neutralizing responses and

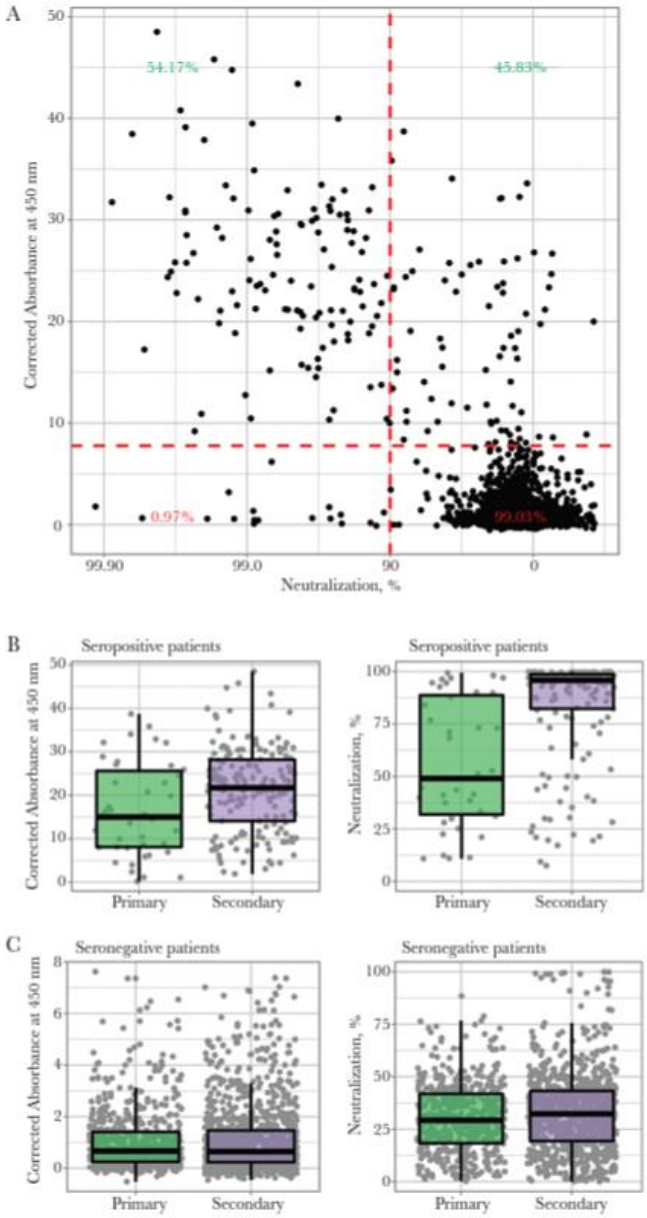

Figure 4. Antibody levels and subsequent virus neutralization activity suggest an association with disease severity. *A*, Correlation between virus neutralization and antibody production is shown as a scatterplot, where every sample is represented by a black dot. Percentages reflect the sample distribution among seropositive patients (*green numbers*) and seronegative patients (*red numbers*), and between low (*right*) and high (*left*) virus neutralization. Enzyme-linked immunosorbent assay corrected-absorbance (left) and virus neutralisation (right) values are shown in patients seropositive *B* or seronegative *C* for acute respiratory syndrome coronavirus 2.

the relationship between IgG response, neutralizing antibody levels, and protection from reinfection.

Our study provides an insight into the demographic factors that influence SARS-CoV-2 exposure and immunity. The low prevalence observed, combined with the heterogeneity of antibody-mediated neutralizing responses, suggests that in the absence of measures such as vaccination or nonpharmaceutical interventions, future waves of SARS-CoV-2 infection are likely to cause significant burden. Future developments in real-time community serological surveillance systems linked with robust correlations of virus immunity are necessary to design interventions and to prioritize those measures that safeguard public health at a minimal societal and economic cost.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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7.2 Appendix 2: Density plots of optical density (OD) values of within-plate controls for the S1 and RBD ELISAs

Optical density (OD) values for within-plate negative and positive controls, tested on every ELISA plate were plotted as density plots.

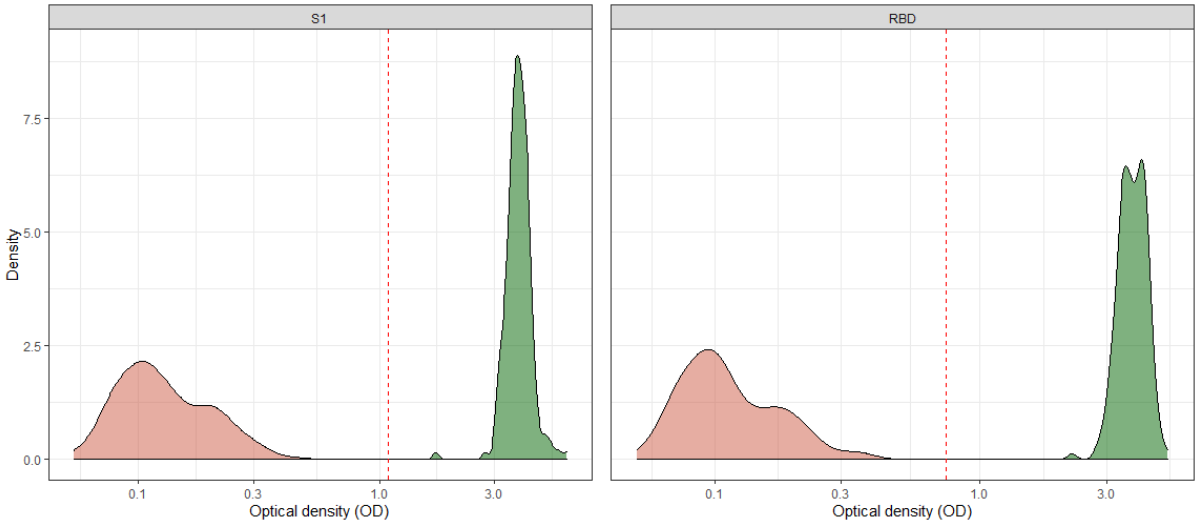

Figure 7.2.1 Density plots of optical density (OD) values of within-plate controls for the S1 ELISA (left) and RBD ELISA (right).

Negative control OD values are in red, positive OD values are in green and red dashed lines indicate ELISA cut-off values.

7.3 Appendix 3: Village-level CCHFV seroprevalence in livestock and humans

Figure 7.3.1 Village-level CCHFV seroprevalence by species

Village number	Village name	Village-level agro-ecological classification	Species	Tested (N)	Seroprevalence (%)	95% confidence interval (%)	
						Lower	Upper
1	Kimokowa	Pastoral	Cattle	176	38.1	30.9	45.2
			Goats	158	25.9	19.1	32.8
			Sheep	148	11.5	6.3	16.6
			Human	19	5.3	0.0	15.3
2	Engikaret	Pastoral	Cattle	197	47.7	40.7	54.7
			Goats	163	10.4	5.7	15.1
			Sheep	175	16.0	10.6	21.4
			Human	20	5.0	0.0	14.6
3	Naiti	Pastoral	Cattle	197	41.6	34.7	48.5
			Goats	254	30.7	25.0	36.4
			Sheep	136	19.9	13.1	26.6
			Human	19	5.3	0.0	15.3
4	Ruvu remitii	Pastoral	Cattle	164	65.2	58.0	72.5

			Goats	185	69.7	63.1	76.4
			Sheep	160	65.6	58.3	73.0
			Human	26	19.2	4.1	34.4
5	Kansay	Agro-pastoral	Cattle	225	68.0	61.9	74.1
			Goats	148	58.8	50.9	66.7
			Sheep	85	37.6	27.3	47.9
			Human	26	50.0	30.8	69.2
6	Maheri	Agro-pastoral	Cattle	241	74.3	68.8	79.8
			Goats	167	63.5	56.2	70.8
			Sheep	96	51.0	41.0	61.0
			Human	19	26.3	6.5	46.1
7	Endanyawish	Pastoral	Cattle	102	25.5	17.0	33.9
			Goats	62	0.0	0.0	0.0
			Sheep	49	2.0	0.0	6.0
			Human	9	22.2	0.0	49.4
8	Sarame	Agro-pastoral	Cattle	113	5.3	1.2	9.4
			Goats	152	1.3	0.0	3.1
			Sheep	95	0.0	0.0	0.0
			Human	10	10.0	0.0	28.6

9	Long	Agro-pastoral	Cattle	175	76.6	70.3	82.8
			Goats	49	79.6	68.3	90.9
			Sheep	37	70.3	55.5	85.0
			Human	15	40.0	15.2	64.8
10	Ngage	Pastoral	Cattle	170	61.2	53.9	68.5
			Goats	89	52.8	42.4	63.2
			Sheep	86	38.4	28.1	48.6
			Human	13	0.0	0.0	0.0
11	Komolo	Pastoral	Cattle	126	32.5	24.4	40.7
			Goats	157	11.5	6.5	16.4
			Sheep	129	12.4	6.7	18.1
			Human	12	0.0	0.0	0.0
12	Lositete	Agro-pastoral	Cattle	135	40.7	32.5	49.0
			Goats	26	11.5	0.0	23.8
			Sheep	57	15.8	6.3	25.3
			Human	7	0.0	0.0	0.0
13	Ilkerin	Agro-pastoral	Cattle	93	47.3	37.2	57.5
			Goats	109	20.2	12.6	27.7
			Sheep	133	21.8	14.8	28.8

			Human	15	20.0	0.0	40.2
14	Nambala	Agro-pastoral	Cattle	136	75.7	68.5	82.9
			Goats	113	69.9	61.5	78.4
			Sheep	103	62.1	52.8	71.5
			Human	12	8.3	0.0	24.0
15	Kisimiri	Agro-pastoral	Cattle	101	21.8	13.7	29.8
			Goats	63	3.2	0.0	7.5
			Sheep	64	1.6	0.0	4.6
			Human	10	10.0	0.0	28.6
16	Sukuro	Pastoral	Cattle	160	63.8	56.3	71.2
			Goats	138	63.8	55.7	71.8
			Sheep	135	67.4	59.5	75.3
17	Arri	Agro-pastoral	Cattle	64	14.1	5.5	22.6
			Goats	40	5.0	0.0	11.8
			Sheep	22	4.5	0.0	13.2
			Human	15	33.3	9.5	57.2
18	Mnjingu	Pastoral	Cattle	144	31.9	24.3	39.6
			Goats	107	6.5	1.9	11.2
			Sheep	99	6.1	1.4	10.8

			Human	9	11.1	0.0	31.6
19	Oloipiri	Pastoral	Cattle	144	21.5	14.8	28.2
			Goats	114	3.5	0.1	6.9
			Sheep	139	2.9	0.1	5.7
20	Engusero sambu	Pastoral	Cattle	152	59.9	52.1	67.7
			Goats	88	37.5	27.4	47.6
			Sheep	111	29.7	21.2	38.2
21	Sorenyi	Agro-pastoral	Cattle	10	0.0	0.0	0.0
			Goats	13	0.0	0.0	0.0
			Sheep	3	0.0	0.0	0.0
			Human	18	11.1	0.0	25.6
22	Engoshowazi	Agro-pastoral	Cattle	7	0.0	0.0	0.0
			Goats	10	0.0	0.0	0.0
			Sheep	4	25.0	0.0	67.4
			Human	20	0.0	0.0	0.0
23	Lengoolwa	Pastoral	Cattle	66	51.5	39.5	63.6
			Goats	70	27.1	16.7	37.6
			Sheep	58	15.5	6.2	24.8
			Human	41	4.9	0.0	11.5

24	RVF trial (Ngorongoro)	Pastoral	Human	16	18.8	0.0	37.9

7.4 Appendix 4: Social, economic and environmental driver of zoonoses (SEEDZ) project questionnaires

Individual animal questionnaire

3193525165

ZELS - CS ANIMAL QUESTIONNAIRE

Household Unique Identifier (HHID) Z L H - -

Form ID from HH Q

Language O Kiswahili O English O Maa

Individual ID (IndID) Z L A -

Interviewer ID

Date of Review (dd/mm/yyyy) / /

Date of Visit (dd/mm/yyyy) / /

Visit Type O CS HH O Parturition O Abattoir

Reviewer ID

SECTION 1: ANIMAL DESCRIPTION & OBSERVATIONS

1.1 Species O Cattle O Sheep O Goat

1.2 Breed O Exotic O Indigenous O Cross

1.3 Reported age *(Miaka)*
O Days O Months O Years O DK

1.4 Reported age class

O Adult (breeding age +)
O Juvenile (weaning to breeding age)
O Young (birth to weaning)

1.5 Dentition age class

O Temporary teeth
O 2 tooth
O 4 tooth
O 6 tooth
O Full mouth
O Full mouth + worn

1.6 Body condition score

O 1 - Emaciated
O 2 - Thin
O 3 - Average
O 4 - Heavy
O 5 - Fat

1.7 Ectoparasite presence:

Lice (Chawa) O Yes O No
Fleas (Viroboto) O Yes O No
Ticks (Kupe) O Yes O No

1.8 Tick infestation score

O No ticks
O 1-20 ticks
O 21-60 ticks
O >60 ticks

1.9 Animal origin

O Born at household (amezaliwa kwenye kaya)
O Purchased (amenunuliwa)
O Other (nyinginezo)
O DK

Comments:

1.10 How long ago was this animal born/acquired and moved to this household?
Ni muda gani tangu mnyama huyu kuzaliwa/kupatikana na kuhamia kwenye eneo hili?

O Days O Months O Years O DK

SECTION 2: REPRODUCTIVE CONDITION & HISTORY

2.1 Sex O Male O Female

Complete only the section below that is appropriate for the sex of the animal (for kiume, go to 2.2, then skip 2.5-2.14; for kike, skip 2.2-2.4 and go to 2.5)

Male animals *(Wanyama wa kiume)*

2.2 Has this animal been castrated?
Je mnyama huyu amehasiwa?

O Yes O No

If yes, skip to 4.1. If no, go to 2.3.

2.3 Do you use this animal to service females in your herd?
Unamtumia mnyama huyu kuwapandisha wanyama wa kike kwenye kundi lako?

O Yes O No

2.4 Do you lend this animal out to service females in other herds?
Je, unamuazimisha mnyama huyu nje kupandisha majike kwenye makundi mengine?

O Yes O No

Female animals *(Wanyama wakike)*

2.5 What is the current reproductive status of this animal?
Je, ni ipi hali ya uzazi kwa sasa ya mnyama huyu?

O Heifer (hajawahi kuzaa)
O Pregnant (ana mimba)
O Lactating (ananyonyesha)
O Dry (mkavu)

If heifer - skip to 4.1
If lactating, please ask if it is possible to collect a milk sample from this animal before moving on to 2.6

Milk Collection Permitted? O Yes O No

1075525162

Within its lifetime at this compound, has this animal:
Katika maisha yake katika eneo hili, mnyama huyu:

2.6 Given birth to any healthy live offspring?
Amezaa mtoto hai yeyote mwenye afya?
O Yes O No O DK

2.7 If yes, how many in total? *(enter 99 if unknown)*
Kama ndio, wangapi kwa ujumla? ☐☐

2.8 Given birth to any weak live offspring?
Amezaa watoto wowote dhaifu?
O Yes O No O DK

2.9 If yes, how many in total? *(enter 99 if unknown)*
Kama ndio, wangapi kwa ujumla? ☐☐

If no to 2.6 and 2.8, go to 2.11.

2.10 If yes, date of last live birth (mm-yyyy)? *(99-9999 if unknown)*
Mwaka na mwezi wa kuzaa mtoto hai mara ya mwisho (awe mwenye afya au dhaifu)? ☐☐ - ☐☐☐☐

2.11 Had an abortion or given birth to dead/stillborn offspring?
Mimba imeharibika au kuzaa mtoto mfu?
O Yes O No O DK

If no to 2.11, go to 2.14.

2.12 If yes, how many? *(enter 99 if unknown)*
Kama ndio, ngapi? ☐☐

2.13 If yes, date of last stillbirth/abortion (mm-yyyy)?
(enter 99-9999 if unknown)
Mwaka na mwezi wa kuzaa mtoto mfu mara ya mwisho
☐☐ - ☐☐☐☐

2.14 Had retained placenta/foetal membranes?
Kondo la nyuma halikutoka/utando za kichanga?
O Yes O No O DK

If no to 2.14, go to 3.1.

2.15 If yes, how many times? *(enter 99 if unknown)*
Kama ndio, mara ngapi? ☐☐

2.16 If yes, date of last retained placenta (mm-yyyy)?
(enter 99-9999 if unknown)
Mwaka na mwezi kwa mara ya mwisho kondo la yuma kubaki ndani?
☐☐ - ☐☐☐☐

SECTION 3: MILK

3.1 Is a calf/kid/lamb currently suckling from this animal?
Kwa wakati huu ndama/mtoto ananyonya kwa mnyama huyu?
O Yes O No

If no to 3.1, go to 3.2.

3.2 If yes, how old is the calf/kid/lamb that is suckling from this animal?
Kama ndio, ndama/mtoto anayemnyonya mnyama huyu ana umri gani?
O Days O Months O Years ☐☐

3.3 Have you ever collected milk from this animal?
Umewahi kukamua maziwa kwa mnyama huyu?
O Yes O No O DK

If no to 3.3, go to 4.1.

3.4 Have you sold milk collected from this animal within:
Je, umeuza maziwa yaliyokamuliwa kutoka kwa mnyama huyu katika kipindi cha:

the past 30 days (*katika siku 30 zilizopita*) O Yes O No O DK
the past 12 months (*katika miezi 12 iliyopita*) O Yes O No O DK

3.5 Are you currently milking this animal?
Kwa wakati huu unakamua maziwa kwa mnyama huyu?
O Yes O No

If no to 3.5, go to 4.1

3.6 How often do you milk this animal? *(e.g. x times per unit)*
Ni mara ngapi unakamua maziwa kwa myama huyu?
O Days O Weeks O DK ☐☐

3.7 How much milk did you collect from this animal last time it was milked?
Ulikamua maziwa kiasi gani kutoka kwa mnyama huyu mara ya mwisho?
O Cups O Litres O DK ☐☐.☐

SECTION 4: CLINICAL HISTORY

4.1 Does this animal currently have, or has it ever had (whilst owned by this household), any of the following clinical signs?
Je, mnyama huyu kwasasa ana, au amewahi kupata (jinsi unavyofahamu), yeyote ya dalili zifuatazo za kitabibu?

Indicate NA if the sign is not appropriate given the sex or reproductive condition of the animal

Sign	Current/*Kisasa*	Ever/ *Au huko nyuma*
swollen testicles *kende kuvimba*	O Y O N O NA	O Y O N O DK O NA
complete infertility *tasa kabisa*		O Y O N O DK O NA
breeding (conception and gestation) problems *matatizo ya kuzaa (kutopata mimba au mimba kutokukua)*		O Y O N O DK O NA
mastitis (including changes in the milk e.g. blood, clots of pus etc.) *ugonjwa wa kiwele (pamoja na mabadiliko ya maziwa mfano damu, usaha n.k.)*	O Y O N O NA	O Y O N O DK O NA
unexplained drop in milk production (i.e. not attributed to e.g. reduced diet) *kupungua utoaji maziwa kusikoelezeka (mfano, hakuhusiani na kupungua chakula)*	O Y O N O NA *NA if not lactating*	O Y O N O DK O NA
mouth lesions *vidonda mdomoni*	O Y O N	O Y O N O DK
joint swelling *kuvimba viungo*	O Y O N	O Y O N O DK
lameness *udhaifu wa miguu*	O Y O N	O Y O N O DK
incoordination or paralysis *kupooza au matatizo ya viungo kuwasiliana*	O Y O N	O Y O N O DK
bloody urine *mkojo wa damu*	O Y O N	O Y O N O DK
anaemia (e.g. pallor and pale membranes) *kupungua damu (weupe na weupe kwenye ngozi laini)*	O Y O N	O Y O N O DK
jaundice (e.g. yellow eyes) *manjano (mfano, macho kuwa manjano)*	O Y O N	O Y O N O DK

4.2 Has this animal ever been vaccinated against any diseases?
Je, mnyama huyu amewahi kupata chanjo dhidi ya magonjwa yoyote?
O Yes O No O DK

If yes, tick all that apply:

O Rabies (*Kichaa cha mbwa*)
O Anthrax (*Kimeta*)
O CBPP
O PPR
O Foot and Mouth Disease
O Other

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Household questionnaire

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ZELS - CS HOUSEHOLD QUESTIONNAIRE

Household Unique Identifier (HHID)

Z L H - ☐☐ - ☐☐☐

Date of Interview (dd/mm/yyyy) ☐☐ / ☐☐ / ☐☐☐☐

Interviewer ID ☐☐☐

Language

○ Kiswahili ○ English ○ Masai

Date of Review (dd/mm/yyyy) ☐☐ / ☐☐ / ☐☐☐☐

Reviewer ID ☐☐☐

Visit Type

○ CS HH ○ Parturition

SECTION 1: RESPONDENT DETAILS

1.1 Sex ○ Male ○ Female

1.2 Date of Birth (dd/mm/yyyy)

☐☐ / ☐☐ / ☐☐☐☐

If only the year of birth is known, record 01 for dd and 07 for mm.
If year of birth is known, skip to 2.4.

1.3 Age Class

○ 13-18 years ○ 19-34 years ○ 35-54 years ○ ≥ 55 years

1.4 What is your tribe? ***(Kabila lako?)***

○ Chagga ○ Pare ○ Maasai ○ Sambaa ○ Other

☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

1.5 How long have you lived in this village? ***(Ni muda gani umeishi katika kijiji hiki?)*** ○ Years ○ Months ○ Days ☐☐☐

1.6 Relationship to head of household? ***(Hali ya mahusiano wa mkuu wa kaya?)*** ○ Spouse ○ Sibling ○ Parent ○ Self (= H of H) ○ Child ○ Other

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SECTION 2: HOUSEHOLD LOCATION

2.1 Region ○ Kilimanjaro ○ Arusha

2.2 District

○ Arumeru ○ Mwanga
○ Arusha ○ Rombo
○ Longido ○ Hai
○ Moshi rural ○ Same
○ Moshi urban ○ Siha
○ Monduli

2.3 Ward

☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

2.4 Village/Street name

☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

2.5 Sub-village name *(leave blank if none)*

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2.6 How long does it take to walk to the nearest stream/river from your house?
Inachukua muda gani kutembea mpaka kwenye mto/mfereji wa karibu zaidi na nyumba yako?

☐☐ ○ Hours ○ Minutes ○ DK

2.7 How long does it take to walk to the nearest tarmac road from your house?
Inachukua muda gani kutembea mpaka barabara ya lami iliyo karibu zaidi na nyumba yako?

☐☐ ○ Hours ○ Minutes

☐☐☐☐☐

2.8 GPS E/W Coordinate ☐☐.☐☐☐☐☐

2.9 GPS N/S Coordinate ☐☐.☐☐☐☐☐

2.10 Altitude (metres) ☐☐☐☐

2.11 Waypoint ID ☐☐☐

2.12 GPS Accuracy <=10m ○ Yes ○ No *(If No, record the accuracy in 2.13)*

2.13 Accuracy (metres) ☐☐

SECTION 3: COMPOUND/HOUSEHOLD DESCRIPTION

3.1 How many inhabited houses/buildings within this compound are used by members of this household?
Kuna nyumba au majengo mangapi katika eneo/boma zinazotumiwa/kaliwa na wanakaya? ☐☐

3.2 How many adults live in this household (>/=18 years)?
Watu wangapi (>= 18 miaka) wanaishi kaya hii? ☐☐

3.3 How many children live in this household (< 18 years)?
Watu wangapi (<18 miaka) wanaisha kaya hii? ☐☐

3.4 How many sleeping rooms are used by the members of your household?
Kuna vyumba vingapi vya kulala katika nyumba yako? ☐☐

3.5 Head of household age (years)?
Umri wa mkuu wa kaya (miaka)? ☐☐☐

3.6 Head of household gender?
Jinsia ya mkuu wa kaya? ○ Male ○ Female

3.7 Head of household marital status?
Hali ya mahusiano ya ndoa ya mkuu wa kaya?

○ Married (nimeolewa/oa)
○ Single (sijaolewa/oa)
○ Divorced/Separated (nimeachika/acha)
○ Widow (mjane)

3.8 Head of household highest level of education?
Mkuu wa kaya kiwango cha juu kabisa cha elimu yako?

○ No education (sijasoma)
○ Primary (1-7 years) (msingi)
○ Secondary (8-11 years) (sekondari)
○ High School (12-13 years) (sekondari ya juu)
○ University/College (chuo kikuu/chuo)

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3.9 Is this land where this compound is:(choose only one)
Ardhi uliyojenga nyumba hii (chagua moja tu):

- O Common land (ardhi ya wote = kijiji)
- O Rented from others (umeikodi kwa wengine)
- O Owned by the members of the compound (Inamilikiwana mkaaji wa eneo la kaya hii)
- O Other

3.10 In the past 12 months, has there been standing water within this compound?
Katika miezi 12 iliyopita, kumekuwa na maji yaliyosimama/ mafuriko katika eneo la kaya hii?

O Yes O No

3.11 What is the primary source of drinking water for members of this household in the dry & wet seasons?
Chanzo kikuu cha maji ya kunywa kwa wanakaya katika kipindi cha ukame na masika ni kipi?

	Dry season primary source **Kiangazi chanzo kikuu (chagua moja tu)**	Wet season primary source **Masika chanzo kikuu (chagua moja tu)**	Other sources dry and/or wet **Vianzo vingine kiangazi na/au masika (chagua yote yanayohusika)**
Piped water into the home **Maji ya bomba nyumbani**	O	O	O
Public/communal well or standpipe **Kisima cha umma, bomba ya umma**	O	O	O
River or creek (moving water) directly **Moja kwa moja kutoka katika mto au mfereji (maji yanayotembea)**	O	O	O
Lake, pond, dam (standing water) directly **Moja kwa moja kutoka katika ziwa, dimbwi, au bwawa (maji yaliyosimama)**	O	O	O
Private well or pump **Kisima au pampu binafsi**	O	O	O
From a spring **Moja kwa moja kutoka katika chemchem**	O	O	O
Rainwater **Maji ya mvua**	O	O	O
Tanker truck **Gari la kubebea maji (boza)**	O	O	O
Cart or wheelbarrow with small tank or drum **Toroli/mkokoteni wa kubebea maji**	O	O	O
Bottled water **Maji ya chupa**	O	O	O
Other (specify) **Vinginevyo (ainisha)**	O	O	O

3.12 Is your drinking water treated (by filtering, boiling, chlorinating, straining, etc.)?
Huwa una tibu maji ya kunywa (kwa kuchuja, kuchemsha, kuweka dawa ya klorine, kuchuja kwa nguo)?

O Yes O No

If Yes, go to 3.13. If No, go to 3.15

3.13 If yes, how frequently do you treat your household drinking water?
Kama ndio, mara ngapi una tibu maji ya kunywa katika kaya yako ?

O Always O Sometimes

3.14 How do you treat it? (choose all that apply)
Kama ndio, unatibu vipi ? (chagua yote yanayohusika)

- O Boiling (Kuchemsha)
- O Strain it through a cloth (Kuchuja kwa nguo)
- O Adding disinfectant, such as chlorine or bleach (Kuweka dawa klorine/bleach/water guard)
- O Sedimentation and decant (Kuacha kwa muda yatwae/uchafu uende chini)
- O Filtering (Kuchuja)
- O Solar disinfection (Kuweka juani)
- O Other, specify (Nyinginezo, ainisha)

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3.15 What is the primary source of bathing water for members of this household in the dry & wet seasons?
Chanzo kikuu cha maji ya kuoga kwa wanakaya katika kipindi cha ukame na masika ni kipi?

	Dry season primary source **Kiangazi chanzo kikuu (chagua moja tu)**	Wet season primary source **Masika chanzo kikuu (chagua moja tu)**	Other sources dry and/or wet **Vianzo vingine kiangazi na/au masika (chagua yote yanayohusika)**
Piped water into the home **Maji ya bomba nyumbani**	○	○	○
Public/communal well or standpipe **Kisima cha umma, bomba ya umma**	○	○	○
River or creek (moving water) directly **Moja kwa moja kutoka katika mto au mfereji (maji yanayotembea)**	○	○	○
Lake, pond, dam (standing water) directly **Moja kwa moja kutoka katika ziwa, dimbwi, au bwawa (maji yaliyosimama)**	○	○	○
Private well or pump **Kisima au pampu ya binafsi**	○	○	○
From a spring **Moja kwa moja kutoka katika chemchem**	○	○	○
Rainwater **Maji ya mvua**	○	○	○
Tanker truck **Gari la kubebea maji (boza)**	○	○	○
Cart or wheelbarrow with small tank or drum **Toroli/mkokoteni wa kubebea maji**	○	○	○
Other (specify) **Vinginevyo, ainisha**	○	○	○

3.16 What is the primary source of washing water (clothes and dishes) for members of this household in the dry & wet seasons?
Chanzo kikuu cha maji ya kufulia nguo na kuoshea vyombo kwa wanakaya katika kipindi cha ukame na masika ni kipi?

	Dry season primary source **Kiangazi chanzo kikuu (chagua moja tu)**	Wet season primary source **Masika chanzo kikuu (chagua moja tu)**	Other sources dry and/or wet **Vianzo vingine kiangazi na/au masika (chagua yote yanayohusika)**
Piped water into the home **Maji ya bomba nyumbani**	○	○	○
Public/communal well or standpipe **Kisima cha umma, bomba ya umma**	○	○	○
River or creek (moving water) directly **Moja kwa moja kutoka katika mto au mfereji (maji yanayotembea)**	○	○	○
Lake, pond, dam (standing water) directly **Moja kwa moja kutoka katika ziwa, dimbwi, au bwawa (maji yaliyosimama)**	○	○	○
Private well or pump **Kisima au pampu ya binafsi**	○	○	○
From a spring **Moja kwa moja kutoka katika chemchem**	○	○	○
Rainwater **Maji ya mvua**	○	○	○
Tanker truck **Gari la kubebea maji (boza)**	○	○	○
Cart or wheelbarrow with small tank or drum **Toroli/mkokoteni wa kubebea maji**	○	○	○
Other (specify) **Vinginevyo, ainisha**	○	○	○

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3.17 What type of toilet system do members of your household normally use? (choose only one)
Ni aina gani ya mfumo wa choo ambao unatumika kawaida na wakazi wa kaya yako? (chagua moja tu)

- ○ Flush or pour toilet with septic tank, including squat toilet
  Choo cha kuchuchumaa, cha kumwaga maji na mfumo wa shimo la maji taka
- ○ Flush or pour toilet connected to sewer pipe
  Choo cha maji kilichounganishwa na bomba la maji taka
- ○ Pit latrine with covering slab
  Choo cha shimo kilichosakafiwa
- ○ Pit latrine without covering slab
  Choo cha shimo ambacho hakijasakafiwa (kisichosakafiwa)
- ○ Ventilated improved pit latrine (VIP)
  Choo cha shimo bora chenye bomba la kutoa hewa chafu (VIP)
- ○ Bucket or plastic bags
  Ndoo au mfuko
- ○ No facilites or field or bush
  Hakuna choo, kwenda porini

3.18 Do you have electricity at your house? ○ Yes ○ No
Una umeme wowote katika nyumba yako?

If yes, go to 3.19. If no, skip to 3.20

3.19 What kind of electricity do you have? *(Aina gani?)*

- ○ Grid (Gridi)
- ○ Solar (Solar)
- ○ Generator (Jenerata)
- ○ Other (Nyinginezo)

3.20 What are the energy sources used for cooking at this household? (primary and secondary)
Aina gani kuu (ya msingi) ya nishati inatumika kwa kupikia katika kaya hii?

	Primary source (choose one) **Chanzo cha kudumu**	Other source (choose all that apply) **Vyanzo vingine (chagua yote yanoyohusikaa)**
Electricity **Umeme**	○	○
Gas **Gesi**	○	○
Kerosene **Mafuta taa**	○	○
Cow dung **Kinyesi cha ng'ombe**	○	○
Firewood **Kuni**	○	○
Charcoal **Mkaa**	○	○
Other **Nyinginezo**	○	○

3.21 What is the roof of your house made of? (choose only one)
Je paa la nyumba yako limeezekwa na nini? (chagua moja tu)

- ○ Metal (Bati)
- ○ Thatch (Nyasi)
- ○ Wood (Mbao)
- ○ Tiles (Vigae)
- ○ Cement (Sementi)
- ○ Dirt or mud (Vumbi au tope)
- ○ Dung (Kinyesi cha mifugo)
- ○ Other (Nyinginezo)

3.22 What is the floor of your house made of? (choose all that apply)
Sakafu ya nyumba yako imetengenezwa na nini? (chagua yote yanayohusika)

- ○ Brick (Matofali)
- ○ Cement (Sementi)
- ○ Wood (Mbao)
- ○ Other (Nyinginezo)
- ○ Dirt or mud (Vumbi au tope)
- ○ Dung (Kinyesi cha mifugo)
- ○ Tile or linoleum (Vigae au sakafu ya mpira)

3.23 What are the walls of your house made of? (choose all that apply)
Ukuta wa nyumba yako umetengenezwa na nini? (chagua yote yanayohusika)

- ○ Mud or manure (Tope au kinyesi cha mifugo)
- ○ Burnt bricks (Matofali yaliyochomwa)
- ○ Mud bricks, uncooked (Matofali ya udongo)
- ○ Cement bricks (Matofali ya sementi)
- ○ Wood or planks (Mbao au fito)
- ○ Stone (Mawe)
- ○ Thatch (Nyasi)
- ○ Other (Nyinginezo ainisha)

3.24 Do you have any of the following items in your household (choose all that apply)?
Kuna chochote kati ya hivi katika nyumba yako (chagua yote yanayohusika)?

Ox plough *(Jembe la ng'ombe)*	○ Yes	○ No
Ox cart *(Mkokoteni wa ng'ombe)*	○ Yes	○ No
Bicycle *(Baiskeli)*	○ Yes	○ No
Motorbike *(Pikipiki)*	○ Yes	○ No
Car *(Gari)*	○ Yes	○ No
Tractor *(Trekta)*	○ Yes	○ No
Mobile phone *(Simu ya mkononi)*	○ Yes	○ No
Radio *(Redio)*	○ Yes	○ No
Television *(Luninga)*	○ Yes	○ No
Sofa *(Makochi)*	○ Yes	○ No
Bed net *(Chandarua)*	○ Yes	○ No
Refrigerator *(Jokofu au friji)*	○ Yes	○ No

3.25 What area of land was planted for crops by the members of your household during the last growing season?
Eneo gani la shamba lilioteshwa mazao na wanakaya wa kaya hii wakati wa msimu wa kilimo uliopita?

○ Acre ○ Hectare .

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3.26 What crops were grown at this compound in the past 12 months? (choose all that apply)
Katika kaya yako, mimea gani ililimwa katika miezi 12 iliyopita? (chagua yote yanayohusika)

- ○ Maize (Mahindi)
- ○ Beans (Maharage)
- ○ Bananas (Ndizi)
- ○ Coffee (Kahawa)
- ○ Avocado (Parachichi)
- ○ Sugarcane (Miwa)
- ○ Rice (Mpunga)
- ○ Other (Nyinginezo)

SECTION 4: Animals & Livestock

4.1 For the following species record if each is present:
Kwa aina zifuatazo andika iwapo kila aina ya mnyama inafugwa eneo hili:
If yes for a given species, please record the total number of adult and juvenile animals that are currently kept at this compound.
Record 000 if the species is present but no animals of the age group are present.
For mammals:Adult >/= 1 yr, Juvenile 0-1 yr
For birds:Adult = adult plumage, Juvenile = non-adult plumage

	Kept/**Kutunzwa**	Adults/ **wakubwa**	Juveniles/ **wadogo**
Pigs/**nguruwe**	○ Yes ○ No		
Chickens/**kuku**	○ Yes ○ No		
Dogs/**mbwa**	○ Yes ○ No		
Donkeys/**punda**	○ Yes ○ No		
Horses/**farasi**	○ Yes ○ No		
Cats/**paka**	○ Yes ○ No		
Ducks/**bata**	○ Yes ○ No		
Rabbits/**sungura**	○ Yes ○ No		
Doves or pigeons/**njiwa**	○ Yes ○ No		
Camels/**ngamia**	○ Yes ○ No		
Other/**wengineo specify**	○ Yes ○ No		

4.2 Are any animals of the following species kept at this compound?
Je kuna yeyote wa wanyama wafuatao wanatunzwa/fugwa katika eneo hili?

- Cattle *(Ng'ombe)* ○ Yes ○ No
- Sheep *(Kondoo)* ○ Yes ○ No
- Goats *(Mbuzi)* ○ Yes ○ No

If no for a given species, skip to the next species.
If a species is kept record the number of animals in each group.
Record 000 if no animals of a given age/sex group are kept.

4.3 Number of cattle kept at the household?
Namba ya ng'ombe wanatunzwa katika kaya?
(Juvenile = 0-1 year, Adult >/= 1 year)

Adult Males/ **Wakubwa wakiume**	Juvenile Males/ **Ndama dume**	Adult Females/ **Wakubwa wakike**	Juvenile Females/ **Ndama jike**	Total/**Jumla**

4.4 Number of sheep kept at the household?
Namba ya kondoo wanatunzwa katika kaya?
(Juvenile = 0-1 year, Adult >/= 1 year)

Adult Males/ **Wakubwa wakiume**	Juvenile Males/ **Wadogo wakiume**	Adult Females/ **Wakubwa wakike**	Juvenile Fem./ **Wadogo wakike**	Total/**Jumla**

4.5 Number of goats kept at the household?
Namba ya mbuzi wanatunzwa katika kaya?
(Juvenile = 0-1 year, Adult >/= 1 year)

Adult Males/ **Wakubwa wakiume**	Juvenile Males/ **Wadogo wakiume**	Adult Females/ **Wakubwa wakike**	Juvenile Fem./ **Wadogo wakike**	Total/**Jumla**

4.6 Have any animals of the following species been born or brought into the herd in the past 12 months (incl. animals that have been brought in but are no longer here and all juvenile animals present)?
Kuna yeyote wa wanyama wa aina zifuatazo amezaliwa hapa au kuletwa katika kundi katika miezi 12 iliyopita (ikihusisha wanyama walioletwa kwenye kundi lakini hawapo kwa sasa na wadogo wote)?

- Cattle *(Ng'ombe)* ○ Yes ○ No
- Sheep *(Kondoo)* ○ Yes ○ No
- Goats *(Mbuzi)* ○ Yes ○ No

If no for a given species, skip to the next species.
If animals of a species were brought in or born record the number of animals in each group. Record 00 if no animals of a given age/sex group were brought in or born.

Cattle *(Ng'ombe)* (Juvenile = 0-1 year, Adult >/= 1 year)

	Adult Males/ **Wakubwa wakiume**	Juv. Males/ **Ndama dume**	Adult Fem./ **Wakubwa wakike**	Juv. Fem. /**Ndama jike**	Total/ **Jumla**
Born at the household/ **Waliozaliwa katika kaya**					
Moved into the herd **Wamehamia katika kundi**					

Sheep *(Kondoo)* (Juvenile = 0-1 year, Adult >/= 1 year)

	Adult Males/ **Wakubwa wakiume**	Juv. Males/ **Ndama dume**	Adult Fem./ **Wakubwa wakike**	Juv. Fem. /**Ndama jike**	Total/ **Jumla**
Born at the household/ **Waliozaliwa katika kaya**					
Moved into the herd **Wamehamia katika kundi**					

Goats *(Mbuzi)* (Juvenile = 0-1 year, Adult >/= 1 year)

	Adult Males/ **Wakubwa wakiume**	Juv. Males/ **Ndama dume**	Adult Fem./ **Wakubwa wakike**	Juv. Fem. /**Ndama jike**	Total/ **Jumla**
Born at the household/ **Waliozaliwa katika kaya**					
Moved into the herd **Wamehamia katika kundi**					

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4.7 Have any of the following species died or left the herd in the past 12 months (including animals that left but have been brought back in)?
Kuna wanyama wowote katika aina zifuatazo amekufa au kuondoka katika kundi katika miezi 12 iliyopita (kihusisha wanyama waliokuwa wameondoka lakini wamerudishwa tena kwenye kundi)

Cattle *(Ng'ombe)* ○ Yes ○ No
Sheep *(Kondoo)* ○ Yes ○ No
Goats *(Mbuzi)* ○ Yes ○ No

If no for a given species, skip to the next species.
If animals of a species died/left record the number of animals in each group.
Record 00 if no animals of a given age/sex group died/left.

Cattle *(Ng'ombe)* (Juvenile = 0-1 year, Adult >/= 1 year)

	Adult Males/ **Wakubwa wakiume**	Adult Fem./ **Wakubwa wakike**	Juveniles/ **Ndama**	Total/ **Jumla**
Slaughtered/ **Waliochinjwa**				
Died/ **Waliokufa**				
Sold/lost/stolen/moved/ **Waliouzwa/wa liopotea/au kukiwa/waliondoka kwenye kundi**				
Other/ **Mengine**				

Sheep *(Kondoo)* (Juvenile = 0-1 year, Adult >/= 1 year)

	Adult Males/ **Wakubwa wakiume**	Adult Fem./ **Wakubwa wakike**	Juveniles/ **Ndama**	Total/ **Jumla**
Slaughtered/ **Waliochinjwa**				
Died/ **Waliokufa**				
Sold/lost/stolen/moved/ **Waliouzwa/wa liopotea/au kukiwa/waliondoka kwenye kundi**				
Other/ **Mengine**				

Goats *(Mbuzi)* (Juvenile = 0-1 year, Adult >/= 1 year)

	Adult Males/ **Wakubwa wakiume**	Adult Fem./ **Wakubwa wakike**	Juveniles/ **Ndama**	Total/ **Jumla**
Slaughtered/ **Waliochinjwa**				
Died/ **Waliokufa**				
Sold/lost/stolen/moved/ **Waliouzwa/wa liopotea/au kukiwa/waliondoka kwenye kundi**				
Other/ **Mengine**				

4.8 Have any other animals kept at this compound died in the past 12 months (not including animals sent for slaughter)?
Kuna mnyama yeyote aliyewekwa katika eneo hili alikufa katika miezi 12 iliyopita (bila kuhusisha wanyama walioenda kuchinjwa)?

○ Yes ○ No

If no for all species, go to 4.9.
If yes for any species, please fill in table below. Include newborns that were born alive but died later
Record NA if species is not kept.

	Deaths/**Vifo**	Adults/ **Wanyama wakubwa**	Juveniles/ **Wanyama watoto**
Pigs/**nguruwe**	○ Yes ○ No ○ NA		
Chickens/**kuku**	○ Yes ○ No ○ NA		
Dogs/**mbwa**	○ Yes ○ No ○ NA		
Donkeys/**punda**	○ Yes ○ No ○ NA		
Horses/**farasi**	○ Yes ○ No ○ NA		
Cats/**paka**	○ Yes ○ No ○ NA		
Ducks/**bata**	○ Yes ○ No ○ NA		
Rabbits/**sungura**	○ Yes ○ No ○ NA		
Doves or pigeons/**njiwa**	○ Yes ○ No ○ NA		
Camels/**ngamia**	○ Yes ○ No ○ NA		
Other/**wengineo specify**	○ Yes ○ No		

4.9 Have any of the cattle, sheep or goats kept at this compound had joint problems in the past 12 months?
Je, yeyote wa ng'ombe, kondoo au mbuzi wako alikuwa na matatizo ya viungo katika miezi 12 iliyopita?

Record NA if the species is not owned.

Cattle *(Ng'ombe)* ○ Yes ○ No ○ NA
Sheep *(Kondoo)* ○ Yes ○ No ○ NA
Goats *(Mbuzi)* ○ Yes ○ No ○ NA

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SECTION 5: Herd Management

5.1 Do your animals ever use the same source of water as you use for household drinking water during the dry or wet season?
Je, wanyama wako huwa wanatumia chanzo kimoja cha maji na maji unayotumia kwa kunywa kwa kaya wakati wa msimu wa kiangazi/ukame au masika/mvua?

Record NA if species is not kept.

	Cattle/ **ng'ombe**	Sheep/ **kondoo**	Goats/ **Mbuzi**
Dry/**kiangazi**	○ Yes ○ NA ○ No	○ Yes ○ NA ○ No	○ Yes ○ NA ○ No
Wet/**masika**	○ Yes ○ NA ○ No	○ Yes ○ NA ○ No	○ Yes ○ NA ○ No

5.2 How do you graze your livestock during the day in the dry and wet seasons (choose one option for each species and season)?
Una chunga/lisha namna gani mifugo yako mchana wakati wa kiangazi/ukame na wakati wa mvua (chagua jibu moja kwa kila aina ya msimu)?

Record NA if species is not kept.

	Cattle/ **ng'ombe**		Sheep/ **kondoo**		Goats/ **mbuzi**	
	Dry	Wet	Dry	Wet	Dry	Wet
NA	○	○	○	○	○	○
Free-ranging (no herdsman)/**wanajichunga wenyewe (hakuna mchungi)**	○	○	○	○	○	○
Herded/**wanachungwa**	○	○	○	○	○	○
Tethered/ **Wanaofungwa**	○	○	○	○	○	○
Not grazed or confined/ **Wanakatiwa majani**	○	○	○	○	○	○
Other/ **Mengine**	○	○	○	○	○	○

5.3 Do you graze your cattle, sheep and goats together (choose only one)?
Je, unachunga ng'ombe, kondoo na mbuzi wako kwa pamoja (chagua moja tu)?

- ○ Cattle, sheep, goats together (ng'ombe, kondoo na mbuzi pamoja)
- ○ Cattle and sheep together (ng'ombe na kondoo pamoja)
- ○ Cattle and goats together (ng'ombe na mbuzi pamoja)
- ○ Sheep and goats together (kondoo na mbuzi pamoja)
- ○ Species herded separately (kila aina inatenganishwa)
- ○ NA (only 1 species owned, only 1 grazed, or none grazed)

5.4 Where are your adult livestock normally kept during the night in the dry/wet seasons (choose one for each species and season)?
Wanyama wako wakubwa kwa kawaida wanawekwa wapi wakati wa usiku wakati wa msimu wa mvua na ukame (chagua jibu moja tu kwa kila aina na msimu)?

	Cattle/ **ng'ombe**		Sheep/ **kondoo**		Goats/ **Mbuzi**	
	Dry	Wet	Dry	Wet	Dry	Wet
NA	○	○	○	○	○	○
Confined at the compound >10m of household location **Hufungwa zaidi ya mita 10 kutoka kwenye eneo la kaya ya eneo la kaya**	○	○	○	○	○	○
Confined at the compound within 10m of household location **Hufungwa mita 10 ndani ya eneo la kaya**	○	○	○	○	○	○
Not confined **Hawafungwi**	○	○	○	○	○	○
Confined elsewhere (outside the compound) **Hufungwa sehemu nyingine (nje ya maeneo ya nyumba)**	○	○	○	○	○	○
Other **Mengine**	○	○	○	○	○	○

If any species are confined at night:

5.5 Do you confine your cattle, sheep and goats together (choose only one)?
Je, unawaweka sehemu moja ng'ombe, kondoo na mbuzi wako (chagua moja tu)?

- ○ Cattle, sheep, goats together (ng'ombe, kondoo, na mbuzi pamoja)
- ○ Cattle and sheep together (ng'ombe na kondoo pamoja)
- ○ Cattle and goats together (ng'ombe na mbuzi pamoja)
- ○ Sheep and goats together (kondoo na mbuzi pamoja)
- ○ Species confined separately (kila aina inatenganishwa)
- ○ NA (only one species owned/confined)

5.6 Do any of your livestock ever come into contact with livestock from other herds and/or wildlife while grazing or watering?
Je, kuna yeyote kati ya mifugo yako huwa wanakutana na wanyama kutoka katika makundi ya watu wengine/au wanyama pori wakati wa kuchunga au kunywesha maji?

If respondent does not know about contacts while grazing, mark DK and skip to 6.1
If no animals graze, mark NA and skip to 6.1.
If animals do graze but do not come into contact with other animals, mark No and skip to 6.1.
If any animals graze and come into contact with other animals, mark Yes and fill in table.

○ DK ○ No ○ NA ○ Yes

If yes, indicate how frequently 1=daily, 2=weekly, 3=monthly, 4=less than monthly, 0=never.

	Ng'ombe ○ NA		Kondoo ○ NA		Mbuzi ○ NA	
	Dry	Wet	Dry	Wet	Dry	Wet
Cattle/**ng'ombe**						
Sheep/**kondoo**						
Goats/**mbuzi**						
Buffalo/**nyati**						
Wildebeest/**nyumba**						
Zebra/**punda mulia**						
Other/**wengineo**						

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SECTION 6: BREEDING, PARTURITION & ABORTION

6.1 How was service done in your herd in the past 12 months (indicate all that apply)?
Huduma ya kupandisha majike ilitolewa namna gani katika kundi lako kwa miezi 12 iliyopita (chagua yoyote yanayohusika)?
Choose NA if the species is not owned

	Cattle/ ng'ombe ○ NA	Sheep/ kondoo ○ NA	Goats/ mbuzi ○ NA
Male(s) from own herd/**madume kutoka kundi lako**	○ Yes ○ No	○ Yes ○ No	○ Yes ○ No
Males(s) hired/**dume au madume wakukodisha**	○ Yes ○ No	○ Yes ○ No	○ Yes ○ No
Males(s) borrowed/ **madume ya kuazima**	○ Yes ○ No	○ Yes ○ No	○ Yes ○ No
Artificial insemination/ **uzalishaji kwa mrija**	○ Yes ○ No	○ Yes ○ No	○ Yes ○ No
Females taken elsewhere/**jike lilipelekwa pengine**	○ Yes ○ No	○ Yes ○ No	○ Yes ○ No
Other/ **Mengine**	○ Yes ○ No	○ Yes ○ No	○ Yes ○ No

6.2 What normally happens to the placenta and foetal membranes after one of your cattle/sheep/goats give birth?
Nini huwa kwa kawaida kinafanyika kwa kondo la nyuma na utandu za kichanga baada ya mmojawapo ya ng'ombe/kondoo/mbuzi wako akizaa?
Ask respondent to volunteer a normal action (V) then proceed through the list of actions (Y/N). Record NA if the species is not owned.

	Cattle/ ng'ombe ○ NA	Sheep/ kondoo ○ NA	Goats/ mbuzi ○ NA
Nothing, material left/ **hakuna, vilachwa**	○ V ○ Y ○ N	○ V ○ Y ○ N	○ V ○ Y ○ N
Eaten by family members/ **vililiwa na wakazi katika familia**	○ V ○ Y ○ N	○ V ○ Y ○ N	○ V ○ Y ○ N
Buried/**vilifukiwa (zikwa)**	○ V ○ Y ○ N	○ V ○ Y ○ N	○ V ○ Y ○ N
Burned/**vilichomwa**	○ V ○ Y ○ N	○ V ○ Y ○ N	○ V ○ Y ○ N
Given raw to dogs/**vilipewa mbwa vikiwa vibichi**	○ V ○ Y ○ N	○ V ○ Y ○ N	○ V ○ Y ○ N
Given to dogs after cooking/ **vilipewa mbwa baada ya kupikwa**	○ V ○ Y ○ N	○ V ○ Y ○ N	○ V ○ Y ○ N
Thrown into the bush/ **vilitupwa porini**	○ V ○ Y ○ N	○ V ○ Y ○ N	○ V ○ Y ○ N
Other/ **Mengine**	○ V ○ Y ○ N	○ V ○ Y ○ N	○ V ○ Y ○ N

6.3 Have any of the youngstock (cattle, sheep and goats) born in the last 12 months been born weak with any of the following signs: difficulty breathing, difficulty suckling, difficulty standing or staggering while walking?
Kuna yeyote wa mifugo wadogo (ng'ombe, kondoo na mbuzi) amezaliwa katika miezi 12 iliyopita, alizaliwa mdhaifu na yeyote ya dalili zifuatazo: kupumua kwa shida, kunyonya kwa shida, kusimama kwa shida au kutetemeka akitembea?
If yes, record the number of weak youngstock born in the last 12 months.
NA if that species is not kept at this compound.

Cattle *(ng'ombe)*	○ Yes	○ No	○ NA	
Sheep *(kondoo)*	○ Yes	○ No	○ NA	
Goats *(mbuzi)*	○ Yes	○ No	○ NA	

6.4 Have any of the livestock (cattle/sheep/goats) at this compo aborted or delivered still-born offspring in the last 12 months?
Kuna mifugo yeyote (ng'ombe, kondoo na mbuzi) anayefuwa katika eneo hili ametupa/imeharibu mimba au amezaa mtoto mfu katika miezi 12 iliyopita?
If yes, indicate the number of abortions/stillbirths in the last12 months.
Record NA if that species is not kept at this compound.

Cattle *(ng'ombe)*	○ Yes	○ No	○ NA
Sheep *(kondoo)*	○ Yes	○ No	○ NA
Goats *(mbuzi)*	○ Yes	○ No	○ NA

6.5 What normally happens to the foetus, placenta and membranes after one of your cattle/sheep/goats has an abortion or stillbirth?
Nini huwa kwa kawaida kinafanyika kwa kichanga, kondo la nyuma na utandu baada ya mmojawapo ya ng'ombe/kondoo/mbuzi wako mimba ameharibika au kuzaa mtoto mfu?
Ask respondent to volunteer a normal action (V) then proceed through the list of actions (Y/N). Record NA if the species is not owned or no abortions have occurred.

	Cattle/ ng'ombe ○ NA	Sheep/ kondoo ○ NA	Goats/ mbuzi ○ NA
Nothing, material left/ **ahakuna, viliachwa**	○ V ○ Y ○ N	○ V ○ Y ○ N	○ V ○ Y ○ N
Eaten by family members/ **vililiwa na wakazi katika familia**	○ V ○ Y ○ N	○ V ○ Y ○ N	○ V ○ Y ○ N
Buried/**vilifukiwa (zikwa)**	○ V ○ Y ○ N	○ V ○ Y ○ N	○ V ○ Y ○ N
Burned/**vilichomwa**	○ V ○ Y ○ N	○ V ○ Y ○ N	○ V ○ Y ○ N
Given raw to dogs/**vilipewa mbwa vikiwa vibichi**	○ V ○ Y ○ N	○ V ○ Y ○ N	○ V ○ Y ○ N
Given to dogs after cooking/ **vilipewa mbwa baada ya kupikwa**	○ V ○ Y ○ N	○ V ○ Y ○ N	○ V ○ Y ○ N
Thrown into the bush/ **vilitupwa porini**	○ V ○ Y ○ N	○ V ○ Y ○ N	○ V ○ Y ○ N
Other/ **Mengine**	○ V ○ Y ○ N	○ V ○ Y ○ N	○ V ○ Y ○ N

6.6 Have any of the livestock (cattle, sheep and goats) at this compound had a case of retained placenta in the past 12 months?
Je, yeyote wa mifugo (ng'ombe, kondoo na mbuzi) katika eneo hili imetokea kondo la nyuma likaacha kutoka katika miezi 12 iliyopita?
If yes, record the number of cases (affected adult females) in the last 12 months.
Choose NA if that species is not kept at this compound.

Cattle *(ng'ombe)*	○ Yes	○ No	○ NA
Sheep *(kondoo)*	○ Yes	○ No	○ NA
Goats *(mbuzi)*	○ Yes	○ No	○ NA

6.7 Have you identified any infertile adult cattle, sheep or goats in the herd in the past 12 months?
Umemgundua ng'ombe, kondoo na mbuzi (wakike au wakiume) yeyote tasa (asiyeshika mimba) katika miezi 12 iliyopita?
○ Yes ○ No
If no, go to 6.8.
If yes, complete the table below, recording NA if species is not owned and 00 if species/sex is owned but none are infertile.

	Cattle/ ng'ombe ○ NA	Sheep/ kondoo ○ NA	Goats/ Mbuzi ○ NA
Males/ **wakiume**			
Females/ **wakike**			

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6.8 Have any of the livestock (cattle, sheep and goats) at this compound had reduced milk yield in the past 12 months that could not be explained by other factors (e.g. reduction in feed, drought)?
Je, kuna yeyote wa mifugo (ng'ombe, kondoo na mbuzi) katika eneo hili amekuwa na upungufu wa kutoa maziwa katika miezi 12 iliyopita ambao haukuweza kuelezeka kwa sababu zingine (mfano, kupungua malisho, ukame)?
If yes, record the number of cases (affected adult females) in the last 12 months NA if that species is not kept at this compound or if no animals of this species are milked.

Cattle *(ng'ombe)*	O Yes	O No	O NA	
Sheep *(kondoo)*	O Yes	O No	O NA	
Goats *(mbuzi)*	O Yes	O No	O NA	

SECTION 7: RODENTS

7.1 Have you seen rodents in your house in the past 30 days?
Je umewahi kuwaona panya katika nyumba yako siku thelathini zilizopita?

O Every day (kila siku)
O More than once a week (zaidi ya mara moja kwa wiki)
O Less than once a week (pungufu ya mara moja kwa wiki)
O Never (haijatokea)

7.2 Have you seen evidence of rodents (such as faeces, urine, rodent tracks, heard noises) in your house in the past 30 days?
Je umewahi kuona ushahidi wa panya (kama choo, mkojo, sauti, njia yake) katika nyumba yako katika siku 30 zilizopita?

O Every day (kila siku)
O More than once a week (zaidi ya mara moja kwa wiki)
O Less than once a week (pungufu ya mara moja kwa wiki)
O Never (haijatokea)

7.3 Have you seen evidence of rodents (such as faeces, urine, tracks, heard noises) in your kitchen or stored food in the last 30 days?
Je umewahi kuona ushahidi wa panya (kama choo, mkojo, sauti, njia yake, vitu vilivyoharibiwa na panya) katika jiko lako au chakula kilichohifadhiwa katika siku 30 zilizopita?

O Every day (kila siku)
O More than once a week (zaidi ya mara moja kwa wiki)
O Less than once a week (pungufu ya mara moja kwa wiki)
O Never (haijatokea)

7.4 Have you seen evidence of rodents (such as faeces, urine, tracks, heard noises) in your compound in the last 30 days?
Je umewahi kuona ushahidi wa panya (kama choo, mkojo, sauti, njia yake, vitu vilivyoharibiwa na panya) katika eneo lakokatika siku 30 zilizopita?

O Every day (kila siku)
O More than once a week (zaidi ya mara moja kwa wiki)
O Less than once a week (pungufu ya mara moja kwa wiki)
O Never (haijatokea)

7.5 Have you seen evidence of rodents (such as faeces, urine, tracks, heard noises) in your animal feed stores in the last 30 days?
Je umewahi kuona ushahidi wa panya (kama choo, mkojo, sauti, njia yake, vitu vilivyoharibiwa na panya) katika chakula cha mifugo kwenye stoo katika siku 30 zilizopita?

O Every day (kila siku)
O More than once a week (zaidi ya mara moja kwa wiki)
O Less than once a week (pungufu ya mara moja kwa wiki)
O Never (haijatokea)

7.6 Have you seen evidence of rodents (such as faeces, urine, tracks, heard noises) in the fields around your compound in the last 30 days?
Je umewahi kuona ushahidi wa panya (kama choo, mkojo, sauti, njia yake, vitu vilivyoharibiwa na panya) katika shamba lako katika siku 30 zilizopita?

O Every day (kila siku)
O More than once a week (zaidi ya mara moja kwa wiki)
O Less than once a week (pungufu ya mara moja kwa wiki)
O Never (haijatokea)

7.7 Do any members of your household do anything to control rodents?
Je yeyote wa wakazi wa kaya hii anafanya chochote kuzuia hawa panya? O Yes O No

If no to 7.7, skip to 7.10. If yes, go to 7.8.
7.8 If yes, what type of rodent control do you use (choose all that apply)?
Kama ndio, njia gani huwa unatumia (chagua yote yanayohusika)?

Mechanical *(kuwatega = mitego)*	O Yes	O No
Chemical *(kutumia dawa/kemikali)*	O Yes	O No
Biological *(kutumia njia za kibiologia = mfano kufuga paka)*	O Yes	O No
Other *(nyinginezo)*	O Yes	O No

If yes to chemical control, go to 7.9. If yes to other control methods, go to 7.10. If no to all, go to 7.11
7.9 What is the name or brand of the poison that you use?
Kama ndio kwa dawa/kemikali, nini jina au aina ya sumu/dawa/kemikali?

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7.10 How do you dispose of the carcasses of the rodents that you kill (choose all that apply)?
Kama ndio kwa kemikali/dawa au kuwatega au nyinginezo, unafanya nin mizoga ya panya ambao unawaua (chagua yoyote yanayohusika)?

Leave them where they die *(nawaacha walipokufa)*	O Yes	O No
Throw them into the bush *(kuwatupa porini)*	O Yes	O No
Burn *(choma moto)*	O Yes	O No
Bury *(kuwafukia)*	O Yes	O No
Feed to other animals *(kulishia wanyama wengine)*	O Yes	O No
Consume *(kuwala)*	O Yes	O No
Other *(nyinginezo)*	O Yes	O No

7.11 How many rodents do you see in the different seasons of the year?
Unawaona panya wangapi katika misimu tofauti ya mwaka?

	Long rain/ **mvua za muda mrefu**	Short rain/ **mvua za muda mfupi**	Dry/ **kiangazi**
Many/**wengi**	O	O	O
Few/**wachache**	O	O	O
None/**hakuna**	O	O	O
DK	O	O	O

SECTION 8: ZOONOTIC DISEASE AWARENESS

8.1 Do you know of any diseases that people can catch from livestock?
Je, unayajua magonjwa yoyote ambayo watu wanaweza kupata kutoka kwa mifugo?
O Yes O No

If yes, record the name of the 1st disease mentioned.

8.2 Do you know of any diseases that people can catch from rodents?
Je, unayajua magonjwa yoyote ambayo watu wanaweza kupata kutoka kwa panya?
O Yes O No

If yes, record the name of the 1st disease mentioned.

8.3 Do you know of any diseases that people can catch from dogs?
Je, unayajua magonjwa yoyote ambayo watu wanaweza kupata kutoka kwa mbwa?
O Yes O No

If yes, record the name of the 1st disease mentioned.

8.4 Do you know of any diseases that can cause abortions in your livestock?
Unafahamu magonjwa yoyote ambayo yanaweza kusababisha mimba kuharibika kwa mifugo yako? mifugo?
O Yes O No

If yes, record the name of the up to 3 diseases mentioned.

8.5 Have you heard of a disease called brucellosis?
Je, umewahi kusikia ugonjwa unaoitwa brucellosis/brusela?
O Yes O No

If no, go to 8.6

Do you know if this disease (brucellosis) affects only animals, only people or both)?
Je unafahamu kuwa ugonjwa huu (brusela) unaathiri wanyama peke yake, watu tu, au wote?
O Only animals (wanyama tu)
O Only humans (binadamu tu)
O Both (wote)

8.6 Have you heard of a disease called leptospirosis?
Je, umewahi kusikia ugonjwa unaoitwa leptospirosis?
O Yes O No

If no, go to 8.7

Do you know if this disease (leptospirosis) affects only animals, only people or both)?
Je unafahamu kuwa ugonjwa huu (leptospirosis) unaathiri wanyama peke yake, watu tu, au wote?
O Only animals (wanyama tu)
O Only humans (binadamu tu)
O Both (wote)

8.7 Have you heard of a disease called Q fever?
Je, umewahi kusikia ugonjwa unaoitwa Q fever?
O Yes O No

If no, go to 9.1

Do you know if this disease (Q fever) affects only animals, only people or both)?
Je unafahamu kuwa ugonjwa huu (Q fever) unaathiri wanyama peke yake, watu tu, au wote?
O Only animals (wanyama tu)
O Only humans (binadamu tu)
O Both (wote)

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SECTION 9: Household diet

The following questions are about the types of foods that you or anyone else in your household ate in the past 4 days. This includes all food prepared within the household that is consumed outside of the home (for example, food eaten while working in the fields). It does not include food that is bought or prepared outside your household.
Maswali yafuatayo yanahusu aina ya vyakula ambavyo wewe au mtu yeyote katika kaya yenu alikula katika siku nne zilizopita. Hi itajumuisha vyakula, vyote vilivyotayarishwa nyumbani na vimeliwa nje ya nyuma (kwa mfano, vyakula vinavyoliwa wakati wa kazi za shamba). Hii haihusishi chakula ambacho kilinunuliwa au kutengenezwa nje ya kaya yako.

9.1 In the past 4 days have you or anyone else in the household eaten the following foods?
Katika siku nne zilizopita wewe au mtu yeyote katika kaya yako alikula vyakula vifuatavyo?

Any bread, plantains, rice, noodles, biscuits, millet, maize, ugali, chapati or any other food made from rice, wheat, millet or sorghum? **Mkate, ndizi, wali, tambi, biskuti, ulezi, mahindi, ugali, chapati au chakula chochote kingine kilichotengezwa kutokana na mchele, ngano, ulezi au mtama?**	○ Yes ○ No
Any potatoes, yams, cassava or any other foods made from roots or tubers? **Viazi, magimbi, mihogo, au chakula chochote kinachotokana na mizizi?**	○ Yes ○ No
Any vegetables? **Mboga za majani?**	○ Yes ○ No
Any fruits? **Matunda?**	○ Yes ○ No
Any chicken, duck or other birds (including liver, kidney, heart or other organ meats from such animals)? **Nyama ya nkuku, nyama ya bata au ndege wegine (hata maini, figo, moyo au nyama nyingine ya ndani)?**	○ Yes ○ No
Any beef, pork, lamb, goat, rabbit, wild game or liver, kidney, heart or other organ meats from these animals? **Nyama ya ngombe, nyama ya nguruwe, nyama ya kondoo, nyama ya mbuzi, nyama ya sungura, wayama wa porini, maini, figo, moyo au nyama nyingine ya ndani?**	○ Yes ○ No
Any eggs? **Mayai?**	○ Yes ○ No
Any fresh or dried fish or shellfish? **Samaki wabichi au wakukausha au makaya?**	○ Yes ○ No
Any foods made from beans, peas, lentils or nuts? **Vyakula vyovyote vilivotengenezwa kutokana na maharage, njegere, dengu au karanga, korosho?**	○ Yes ○ No
Any cheese, yogurt, milk or other milk products? **Siagi, jibini, mtindi, maziwa au mazao mengine ya yatokanayo na maziwa?**	○ Yes ○ No
Any foods made with oild, fat, or butter? **Vyakula vinavyotokana na mafuta ya mimea, wanyama au siagi?**	○ Yes ○ No
Any sugar or honey? **Sukari au asali?**	○ Yes ○ No
Any other foods such as condiments, coffee, tea? **Vyakula vyingine kama viungo, kahawa, majani ya chai?**	○ Yes ○ No

The following question is about how many times household members ate in the past 24 hours.
Maswali yafuatayo yanahusu ni mara ngapi wanakaya walikula masaa ishirini na nne yalioyopita.

9.2 Were any houshehold members absent from the home in the past 24 hours?
Je kuna wanakaya wowote hawakuwepo nyumbani maasaa ishirini na nne yaliyopita?
○ Yes ○ No

9.3 If yes, how many household members were absent from the home in the past 24 hours?
Kama ndiyo, ni wanakaya wangapi hawakuwepo nyumbani masaa ishirini na nne yaliyopita?
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9.4 Have yesterday or today been usual days for your household (for example,not a funeral, feast or fast for religious/ceremonial purposes?
Siku ya jana au leo zimekuwa siku ya kawaida kwa kaya yako (kwa mfano hamna mazishi, karamu, au kufunga kwa ajili ya dini au sherehe)?
○ Yes ○ No

If yes, proceed to 9.5. If no (meaning yesterday was not a normal day for the household), the interviwer should return another day this week to ask 9.5, if possible.

9.5 During the past 24-hour period, did you or anyone in your household consume food prepared at the household at the following times (note that chai with milk should be considered a meal).
Katika kipindi cha masaa ishirini na nne yaliyopita wewe au yeyote kwenye kaya yako alikula (au kunywa maziwa au chai ya maziwa) katika mida iuatayo:
Mark 'yes' if anyone in the household consumed food at one of these times.

Any food before a morning meal? **Chakula chochote kabla ya kifungua kinywa?**	○ Yes ○ No
A morning meal? **Chakula cha asubuhi/kifungua kinywa?**	○ Yes ○ No
Any food between morning and midday meals? **Chakula chochote katikati ya kifungua kinywa na chakula cha mchana?**	○ Yes ○ No
A mid-day meal? **Chakula cha mchana?**	○ Yes ○ No
Any food between midday and evening meals? **Chakula chochote katikati ya chakula cha mchana na chakula cha jioni?**	○ Yes ○ No
An evening meal? **Chakula cha jioni?**	○ Yes ○ No
Any food after the evening meal? **Chakula chochote baada ya chakula cha jioni?**	○ Yes ○ No

Please thank the respondent for their time and for their participation in this research.
Comments:

☐☐☐☐☐

7.5 Appendix 5: Variables of interest as CCHFV risk factors

Table 7.5.1 Variables of interest used in univariable and multivariable analysis of CCHFV exposure in livestock.

Hierarchical level	Variable name	Levels	Data cleaning and comments	Rationale for investigation	Univariable analysis	Multivariable analysis
Individual	Species	Cattle; goats; sheep	Significant difference between species found in chapter 4 so each species modelled separately in chapter 5.	Species differences observed in seroprevalence literature. Cattle > small ruminants (Grech-Angelini et al., 2020, Schulz et al., 2021) Small ruminants > cattle (Obaidat et al., 2021, Tuncer et al., 2014, Mourya et al., 2014)	Yes, chapter 4	No
	Sex	Male; female		Sex differences sometimes observed in	Yes - cattle (C), goats	Yes - C, G, S

				seroprevalence literature. Females > males (Balinandi et al., 2021a, Phonera et al., 2021)	(G) and sheep (S)	
	Dentition	Temporary; two tooth; four-tooth; sex-tooth; full mouth;	Oldest two age groups combined for sheep due to 0 male animals in final category (worn).	Age often associated with increasing seroprevalence. (Adam et al., 2013b, Balinandi et al., 2021a, Lotfollahzadeh et al., 2011, Mohamed et al., 2008, Mostafavi et al., 2012)	Yes - C, G, S	Yes - C, G, S
	Breed	Cross-bred or exotic; indigenous breeds	Cross-bred and exotic categories from original questionnaires	Cross-breed animals associated with higher seroprevalence (Adam et al., 2013b)	Yes - C, G	

			combined due to low numbers. No exotic/cross-breed sheep so breed excluded from sheep models			
Agro-eco setting	Village class	Pastoral; mixed		Village-level agro-ecological system found to be significanlty associated with other pathogens in this study population (Herzog et al., 2019)	Yes - C, G, S	Yes - C, G, S
	Household class	Pastoral; agro-pastoral; small-holder		Household-level agro-ecological system offers aggregate variable encompassing many	Yes - C, G, S	Yes - C, G, S

				variables potentially associated with CCHFV exposure.		
Household characteristics and husbandry practices	Number cattle owned (log)	Numeric		Group size may be associated with frequency dependant transmission.	Yes - C, G, S	Yes - C, G, S
	Number goats owned (log)	Numeric		As above	Yes - C, G, S	Yes - C, G, S
	Number sheep owned (log)	Numeric		As above	Yes - C, G, S	Yes - C, G, S
	Household ownership of cattle	Binary		Presence of other species may be associated with alterations in tick abundance and species dynamics.	Yes - C, G, S	Yes - G, S

	Household ownership of goats	Binary		As above	Yes - C, G, S	Yes - C, S
	Household ownership of sheep	Binary		As above	Yes - C, G, S	Yes - C, G
	Household ownership of pigs	Binary		As above	Yes - C, G, S	Yes - C, G, S
	Household ownership of donkeys	Binary		Presence of other species may be associated with alterations in tick abundance and species dynamics. Contact with donkeys identified as human risk factor (Lwande et al., 2012). High seroprevalence in	Yes - C, G, S	Yes - C, G, S

				donkeys found in Bulgaria (Barthel et al., 2014)		
	Household ownership of chickens	Binary		Absence of chickens around household associated with higher odds of CCHFV in Pakistan (Kasi et al., 2019)	Yes - C, G, S	Yes - C, G, S
	Tethering or zero-grazing	Binary, for each species	Variable created from combination of two variables	Confinement/restricted grazing associated with decreased odds of CCHFV in sheep and camels (Li et al., 2020b). Hypothesise fewer ticks in these settings.	Yes - C, G, S	Yes - C, G, S
	Herded or free roaming	Binary, for each species	Variable created from	Grazing associated with higher CCHFV exposure	Yes - C, G, S	Yes - C, G

			combination of two variables	(Kasi et al., 2019, Ibrahim et al., 2015, Sargianou et al., 2013)		
	Cattle confined with small ruminants	Binary		Presence of other species may be associated with alterations in tick abundance and species dynamics.	Yes – C, G, S	Yes – C, G, S
	Cattle grazed with small ruminants	Binary		Presence of other species may be associated with alterations in tick abundance and species dynamics.	Yes – C, G, S	Yes – C, G, S
	Transhumance	Binary, for each species	Higher missingness in these variables so excluded	Extensive grazing practices associated with higher odds of CCHFV (Ibrahim et al.,	Yes - C, G, S	No (sample size restricted dataset due to

			from multivariable analysis to prevent restriction of sample size	2015, Adam et al., 2013b, Kasi et al., 2019)		>20% missingness)
	Household tribe	Arusha; Iraqw; Maasai; other	Tribal identity of head of household	Cultural differences between tribal groups may result in variation in livestock management practices e.g. pigs are only kept by the Iraqw tribe. Tribal practices could be potentially confounding.	Yes - C, G, S	Yes - C, G, S
	See wildlife around household	Binary	Aggregate variable capturing all	Numerous wildlife species are known to be hosts for CCHFV	Yes - C, G, S	Yes - C, G, S

			wildlife sightings from the household	(Spengler et al., 2016a). Wildlife may affect tick abundance (Keesing et al., 2013). Exploratory analyses did not suggest a link between CCHFV seroprevalence and sighting of particular wildlife species so an aggregate variable was used in the multivariable analysis.		
Environmental variables	Household elevation	Meters above sea-level		Positive correlation with CCHFV seropositivity identified in literature (Sargianou et al., 2013, Sisman, 2013, Hakan Mete Dogan, 2009).	Yes - C, G, S	Yes - C, G, S

	Household mean annual precipitation	mm per year		Relative levels of precipitation associated with higher or lower CCHFV seroprevalence dependent on location (Aker et al., 2015, Wilson et al., 1990, Esser et al., 2019). Rainfall levels may affect tick abundance (Keesing et al., 2018).	Yes - C, G, S	Yes - C, G, S
	Mean annual temperature	Celcius	Highly co-linear with elevation so excluded from further models in favour of elevation, as elevation is a	Temperature associated with increased CCHFV levels (Esser et al., 2019, Vescio et al., 2012, Wilson et al., 1990)	Yes - C, G, S	No

			consistent variable and not subject to climatic change.			
	Household crop production	Binary		Presence of vegetation in or around the household may affect tick populations (Kasi et al., 2019, Vescio et al., 2012)	Yes - C, G, S	Yes - C, G, S
Village-level	Cattle density	Animals per $1km^2$		Livestock density has been associated with CCHFV exposure (Estrada-Peña et al., 2013, Aker et al., 2015).	Yes - C, G, S	Yes - C, G, S
	Goat density	Animals per $1km^2$		As above.	Yes - C, G, S	Yes - C, G, S

	Sheep density	Animals per $1km^2$		As above.	Yes - C, G, S	Yes - C, G, S
	Pig density	Animals per $1km^2$		As above.	Yes - C, G, S	Yes - C, G, S

7.6 Appendix 6: Univariable analysis of potential risk factors for CCHFV exposure in livestock

Table 7.6.1 Univariable analysis of categorical variables for CCHFV exposure in cattle.

Showing seroprevalence, odds ratio (OR), 95% confidence intervals and p values for categorical variables.

Categorical variables	levels	Total tested (N)	Missing (N)	Total tested per level (%)	N positive (seroprevalence %)	OR (univariable with random effects)	95% confidence intervals	P value
Individual-level								
Breed	Cross or exotic	3015	0	277 (9.2)	144 (52.0)	Ref.		
	Indigenous			2738 (90.8)	1352 (49.4)	1.07	0.62 - 1.85	0.811
Sex	Female	3012	3	2003 (66.5)	1093 (54.6)	Ref.		
	Male			1009 (33.5)	402 (39.8)	0.44	0.36 - 0.53	<0.001
Dentition	Temporary	3007	8	890 (29.6)	203 (22.8)	Ref.		
	One pair			242 (8.0)	72 (29.8)	1.79	1.22 - 2.63	0.003
	Two pair			245 (8.1)	122 (49.8)	3.84	2.65 - 5.56	<0.001
	Three pair			228 (7.6)	116 (50.9)	5.11	3.51 - 7.46	<0.001

	Four pair			1213 (40.3)	820 (67.6)	14.75	11.29 - 19.27	<0.001
	Worn			189 (6.3)	160 (84.7)	29.95	17.91 - 50.08	<0.001
Agro-ecological setting								
Village-level	Agro-pastoral	3015	0	1283 (42.6)	705 (54.9)	Ref.		
	Pastoral			1732 (57.4)	791 (45.7)	0.96	0.36 - 2.57	0.939
Household-level	Agro-pastoral	2856	159	1204 (42.2)	638 (53.0)	Ref.		
	Pastoral			1409 (49.3)	682 (48.4)	1.84	1.15 - 2.93	0.01
	Small-holder			243 (8.5)	106 (43.6)	0.8	0.44 - 1.47	0.48
Potential confounders								
Household tribe	Arusha	2856	159	495 (17.3)	190 (38.4)	Ref.		
	Iraqw			711 (24.9)	457 (64.3)	1.36	0.60 - 3.12	0.462
	Maasai			1343 (47.0)	661 (49.2)	1.46	0.83 - 2.54	0.188
	Other			307 (10.7)	118 (38.4)	1.15	0.54 - 2.45	0.721
Livestock husbandry								
Own goats	No	2975	40	248 (8.3)	104 (41.9)	Ref.		

	Yes			2727 (91.7)	1369 (50.2)	1.57	1.04 - 2.37	0.033
Own sheep	No	2964	51	441 (14.9)	228 (51.7)	Ref.		
	Yes			2523 (85.1)	1244 (49.3)	1.14	0.81 - 1.60	0.452
Own pigs	No	2856	159	2450 (85.8)	1122 (45.8)	Ref.		
	Yes			406 (14.2)	304 (74.9)	1.95	1.11 - 3.44	0.021
Own donkeys	No	2856	159	1009 (35.3)	526 (52.1)	Ref.		
	Yes			1847 (64.7)	900 (48.7)	1.43	1.04 - 1.95	0.026
Own chickens	No	2801	214	419 (15.0)	189 (45.1)	Ref.		
	Yes			2382 (85.0)	1211 (50.8)	0.88	0.61 - 1.28	0.51
Cattle herded or free-range	No	2843	172	35 (1.2)	10 (28.6)	Ref.		
	Yes			2808 (98.8)	1409 (50.2)	1.88	0.69 - 5.09	0.215
Cattle tethered or zero-grazed	No	2843	172	2619 (92.1)	1346 (51.4)	Ref.		
	Yes			224 (7.9)	73 (32.6)	0.35	0.19 - 0.65	0.001
Graze cattle and small ruminants together	No	2834	181	2106 (74.3)	1039 (49.3)	Ref.		
	Yes			728 (25.7)	379 (52.1)	1.04	0.77 - 1.42	0.787

Confine cattle and small ruminants together	No	2807	208	2604 (92.8)	1268 (48.7)	Ref.		
	Yes			203 (7.2)	121 (59.6)	1.73	1.04 - 2.88	0.033
Cattle ronjo	No	2722	293	1566 (57.5)	831 (53.1)	Ref.		
	Yes			1156 (42.5)	546 (47.2)	1.18	0.84 - 1.65	0.338
Does not see wildlife	No	2856	159	2453 (85.9)	1204 (49.1)	Ref.		
	Yes			403 (14.1)	222 (55.1)	0.84	0.56 - 1.26	0.4
Crops grown in the compound	No	2856	159	415 (14.5)	191 (46.0)	Ref.		
	Yes			2441 (85.5)	1235 (50.6)	1.26	0.85 - 1.87	0.255

Table 7.6.2 Univariable analysis of continuous variables for CCHFV exposure in cattle.

Showing mean and standard deviation (SD), odds ratio (OR), 95% confidence intervals and p value for continuous variables.

Continuous variables	Total tested (N)	Missing (N)	Mean (SD)			OR (univariable with random effects)	95% confidence intervals	p value
			Total	CCHFV negative	CCHFV positive			
Number cattle kept	2856	159	61.5 (126.4)	59.0 (116.6)	64.1 (135.5)	1.00	1.00 - 1.00	0.95
Number sheep kept	2856	159	59.1 (166.9)	53.7 (139.6)	64.5 (190.2)	1.00	1.00 - 1.00	0.244
Number goats kept	2856	159	64.6 (126.4)	57.5 (107.6)	71.8 (142.5)	1.00	1.00 - 1.00	0.146
Cattle density ($10km^2$)	3015	0	2999.4 (1413.5)	2952.3 (1346.5)	3047.3 (1477.4)	1.00	1.00 - 1.00	0.986
Sheep density ($10km^2$)	3015	0	1056.8 (595.5)	1078.6 (565.9)	1034.7 (623.6)	1.00	1.00 - 1.00	0.58
Goat density ($10km^2$)	3015	0	1613.3 (1835.4)	1457.9 (1733.8)	1771.0 (1920.8)	1.00	1.00 - 1.00	0.717
Pig density ($10km^2$)	3015	0	38.4 (15.8)	37.5 (15.3)	39.3 (16.3)	1.00	0.97 - 1.03	0.957

Household elevation (m)	2824	191	1470.9 (449.1)	1445.3 (404.5)	1496.3 (488.3)	1.00	1.00 - 1.00	0.575
Household annual mean precipitation (mm)	2834	181	812.6 (129.5)	814.0 (137.8)	811.2 (120.5)	1.00	0.99 - 1.00	0.152
Household annual mean temperature (C°)	2834	181	19.3 (2.3)	19.4 (2.1)	19.2 (2.5)	1.12	0.90 - 1.40	0.313

Table 7.6.3 Univariable analysis of categorical variables for CCHFV exposure in goats.

Showing Seroprevalence, odds ratio (OR), 95% confidence intervals and p values for categorical variables.

Categorical variables	Levels	Total tested (N)	Missing (N)	Total tested per level (%)	N positive (seroprevalence %)	OR (univariable with random effects)	95% confidence intervals	p value
Individual-level								
Breed	Cross or exotic	2382	0	79 (3.3)	39 (49.4)	Ref.		
	Indigenous			2303 (96.7)	765 (33.2)	1.12	0.50 - 2.51	0.791
Sex	Female	2382	0	1886 (79.2)	674 (35.7)	Ref.		
	Male			496 (20.8)	130 (26.2)	0.48	0.36 - 0.65	<0.001
Dentition	Temporary	2379	3	440 (18.5)	55 (12.5)	Ref.		
	One pair			230 (9.7)	44 (19.1)	2.04	1.18 - 3.53	0.011
	Two pair			213 (9.0)	55 (25.8)	2.74	1.60 - 4.69	<0.001
	Three pair			160 (6.7)	46 (28.8)	4.23	2.39 - 7.48	<0.001
	Four pair			1197 (50.3)	519 (43.4)	9.57	6.42 - 14.26	<0.001
	Worn			139 (5.8)	84 (60.4)	18.84	10.23 - 34.69	<0.001

Agro-ecological setting								
Village-level	Agro-pastoral	2382	0	867 (36.4)	342 (39.4)	Ref.		
	Pastoral			1515 (63.6)	462 (30.5)	0.77	0.14 - 4.36	0.772
Household-level	Agro-pastoral	2268	114	897 (39.6)	320 (35.7)	Ref.		
	Pastoral			1194 (52.6)	398 (33.3)	2.17	1.12 - 4.18	0.021
	Small-holder			177 (7.8)	53 (29.9)	0.64	0.26 - 1.57	0.328
Potential confounders								
Household tribe	Arusha	2268	114	481 (21.2)	111 (23.1)	Ref.		
	Iraqw			446 (19.7)	228 (51.1)	1.5	0.38 - 5.90	0.561
	Maasai			1110 (48.9)	377 (34.0)	1.51	0.73 - 3.14	0.266
	Other			231 (10.2)	55 (23.8)	0.64	0.21 - 1.94	0.432
Livestock husbandry								
Own cattle	No	2372	10	125 (5.3)	21 (16.8)	Ref.		
	Yes			2247 (94.7)	782 (34.8)	1.26	0.57 - 2.79	0.575
Own sheep	No	2378	4	168 (7.1)	51 (30.4)	Ref.		
	Yes			2210 (92.9)	753 (34.1)	1.7	0.91 - 3.16	0.096

Own pigs	No	2268	114	1996 (88.0)	599 (30.0)	Ref.		
	Yes			272 (12.0)	172 (63.2)	1.34	0.58 - 3.11	0.492
Own donkeys	No	2268	114	782 (34.5)	280 (35.8)	Ref.		
	Yes			1486 (65.5)	491 (33.0)	1.61	1.06 - 2.45	0.026
Own chickens	No	2242	140	354 (15.8)	106 (29.9)	Ref.		
	Yes			1888 (84.2)	658 (34.9)	0.89	0.54 - 1.47	0.643
Goats herded or free-range	No	2250	132	16 (0.7)	3 (18.8)	Ref.		
	Yes			2234 (99.3)	762 (34.1)	0.47	0.08 - 2.90	0.414
Goats tethered or zero-grazed	No	2250	132	2113 (93.9)	742 (35.1)	Ref.		
	Yes			137 (6.1)	23 (16.8)	0.48	0.19 - 1.25	0.134
Graze cattle and small ruminants together	No	2262	120	1640 (72.5)	513 (31.3)	Ref.		
	Yes			622 (27.5)	257 (41.3)	1.81	1.20 - 2.73	0.005
Confine cattle and small	No	2251	131	2090 (92.8)	706 (33.8)	Ref.		

ruminants together								
	Yes			161 (7.2)	60 (37.3)	1.52	0.77 - 2.98	0.229
Goat ronjo	No	2145	237	1443 (67.3)	490 (34.0)	Ref.		
	Yes			702 (32.7)	246 (35.0)	1.81	1.16 - 2.82	0.009
Does not see wildlife	No	2268	114	1947 (85.8)	639 (32.8)	Ref.		
	Yes			321 (14.2)	132 (41.1)	0.94	0.56 - 1.59	0.82
Crops grown in the compound	No	2268	114	346 (15.3)	112 (32.4)	Ref.		
	Yes			1922 (84.7)	659 (34.3)	0.99	0.58 - 1.69	0.967

Table 7.6.4 Univariable analysis of continuous variables for CCHFV exposure in goats.

Showing mean and standard deviation (SD), odds ratio (OR), 95% confidence intervals and p value for continuous variables.

Continuous variables	Total tested (N)	Missing (N)	Mean (SD)			OR (univariable with random effects)	95% confidence intervals	p value
			Total	CCHFV negative	CCHFV positive			
Number cattle kept	2268	114	57.9 (125.2)	52.7 (120.2)	68.1 (134.0)	1.00	1.00 - 1.00	0.896
Number sheep kept	2268	114	54.1 (126.7)	53.9 (132.1)	54.4 (115.7)	1.00	1.00 - 1.00	0.106
Number goats kept	2268	114	69.9 (123.4)	64.4 (120.0)	80.5 (129.2)	1.00	1.00 - 1.00	0.473
Cattle density ($10km^2$)	2382	0	2933.1 (1434.1)	2898.8 (1333.1)	3000.4 (1612.8)	1.00	1.00 - 1.00	0.578
Sheep density ($10km^2$)	2382	0	975.0 (593.7)	1027.1 (553.2)	872.8 (654.5)	1.00	1.00 - 1.00	0.173
Goat density ($10km^2$)	2382	0	1515.3 (1792.1)	1382.9 (1679.8)	1775.1 (1969.7)	1.00	1.00 - 1.00	0.644
Pig density ($10km^2$)	2382	0	39.0 (15.3)	38.0 (14.9)	40.9 (15.8)	1.02	0.97 - 1.08	0.403
Household elevation (m)	2252	130	1381.9 (410.3)	1387.1 (369.5)	1371.9 (479.6)	1.00	1.00 - 1.00	0.306

Household annual mean precipitation (mm)	2262	120	790.8 (126.3)	795.0 (127.8)	782.7 (123.2)	1.00	0.99 - 1.00	0.213
Household annual mean temperature (C°)	2262	120	19.7 (2.1)	19.7 (1.9)	19.8 (2.4)	1.18	0.85 - 1.65	0.318

Table 7.6.5 Univariable analysis of categorical variables for CCHFV exposure in sheep.

Showing Seroprevalence, odds ratio (OR), 95% confidence intervals and p values for categorical variables.

Categorical variables	levels	Total tested (N)	Missing (N)	Total tested per level (%)	N positive (seroprevalence %)	OR (univariable with random effects)	95% confidence intervals	P value
Individual-level								
Breed	Cross or exotic	2059	0	9 (0.4)		Excluded		
	Indigenous			2050 (99.6)				
Sex	Female	2058	1	1595 (77.5)	505 (31.7)	Ref.		
	Male			463 (22.5)	67 (14.5)	0.3	0.21 - 0.42	<0.001
Dentition	Temporary	2056	3	514 (25.0)	63 (12.3)	Ref.		
	One pair			275 (13.4)	54 (19.6)	2.14	1.31 - 3.49	0.002
	Two pair			209 (10.2)	56 (26.8)	3.78	2.28 - 6.28	<0.001
	Three pair			93 (4.5)	32 (34.4)	6.37	3.37 - 12.03	<0.001
	Four pair or worn			965 (46.9)	367 (38.0)	7.36	5.06 - 10.70	<0.001

Agro-ecological setting								
Village-level	Agro-pastoral	2059	0	692 (33.6)	211 (30.5)	Ref.		
	Pastoral			1367 (66.4)	361 (26.4)	0.96	0.19 - 4.72	0.956
Household-level	Agro-pastoral	1954	105	643 (32.9)	174 (27.1)	Ref.		
	Pastoral			1151 (58.9)	330 (28.7)	1.38	0.64 - 2.98	0.409
	Small-holder			160 (8.2)	41 (25.6)	0.33	0.13 - 0.84	0.02
Potential confounders								
Household tribe	Arusha	1954	105	395 (20.2)	78 (19.7)	Ref.		
	Iraqw			280 (14.3)	104 (37.1)	0.83	0.17 - 3.90	0.809
	Maasai			1102 (56.4)	324 (29.4)	1.69	0.78 - 3.68	0.187
	Other			177 (9.1)	39 (22.0)	0.44	0.13 - 1.48	0.186
Livestock husbandry								
Own goats	No	2059	0	75 (3.6)	31 (41.3)	Ref.		
	Yes			1984 (96.4)	541 (27.3)	0.65	0.27 - 1.53	0.321
Own cattle	No	2055	4	53 (2.6)	19 (35.8)	Ref.		

	Yes			2002 (97.4)	553 (27.6)	0.4	0.15 - 1.07	0.067
Own pigs	No	1954	105	1784 (91.3)	471 (26.4)	Ref.		
	Yes			170 (8.7)	74 (43.5)	0.79	0.27 - 2.30	0.661
Own donkeys	No	1954	105	639 (32.7)	205 (32.1)	Ref.		
	Yes			1315 (67.3)	340 (25.9)	1.11	0.69 - 1.78	0.661
Own chickens	No	1928	131	326 (16.9)	74 (22.7)	Ref.		
	Yes			1602 (83.1)	463 (28.9)	0.93	0.54 - 1.58	0.781
Sheep herded or free-range	No	1876	183	28 (1.5)	2 (7.1)	Ref.		
	Yes			1848 (98.5)	519 (28.1)	4.57	0.66 - 31.51	0.123
Sheep tethered or zero-grazed	No	1876	183	1745 (93.0)	496 (28.4)	Ref.		
	Yes			131 (7.0)	25 (19.1)	0.64	0.24 - 1.68	0.362
Graze cattle and small ruminants together	No	1945	114	1390 (71.5)	376 (27.1)	Ref.		
	Yes			555 (28.5)	167 (30.1)	1.13	0.70 - 1.85	0.614
Confine cattle and small	No	1937	122	1818 (93.9)	519 (28.5)	Ref.		

ruminants together								
	Yes			119 (6.1)	21 (17.6)	0.74	0.33 - 1.70	0.483
Sheep ronjo	No	1850	209	1215 (65.7)	317 (26.1)	Ref.		
	Yes			635 (34.3)	216 (34.0)	2.15	1.37 - 3.37	0.001
Does not see wildlife	No	1954	105	1673 (85.6)	459 (27.4)	Ref.		
	Yes			281 (14.4)	86 (30.6)	0.85	0.48 - 1.51	0.589
Crops grown in the compound	No	1954	105	334 (17.1)	97 (29.0)	Ref.		
	Yes			1620 (82.9)	448 (27.7)	0.86	0.49 - 1.49	0.581

Table 7.6.6 Univariable analysis of continuous variables for CCHFV exposure in sheep.

Showing mean and standard deviation (SD), odds ratio (OR), 95% confidence intervals and p value for continuous variables.

Continuous variables	Total tested (N)	Missing (N)	Mean (SD)			OR (univariable with random effects)	95% confidence intervals	p value
			Total	CCHFV negative	CCHFV positive			
Number cattle kept	1954	105	61.4 (127.7)	55.2 (116.8)	77.4 (151.2)	1.00	1.00 - 1.00	0.536
Number sheep kept	1954	105	65.5 (144.2)	64.4 (149.7)	68.2 (128.9)	1.00	1.00 - 1.00	0.239
Number goats kept	1954	105	75.2 (134.6)	67.6 (127.0)	94.9 (150.9)	1.00	1.00 - 1.00	0.353
Cattle density ($10km^2$)	2059	0	2892.5 (1487.7)	2892.3 (1394.2)	2892.9 (1708.2)	1.00	1.00 - 1.00	0.499
Sheep density ($10km^2$)	2059	0	1001.7 (631.6)	1076.1 (584.8)	808.2 (704.2)	1.00	1.00 - 1.00	0.141
Goat density ($10km^2$)	2059	0	1416.9 (1752.3)	1342.1 (1688.4)	1611.2 (1896.3)	1.00	1.00 - 1.00	0.662
Pig density ($10km^2$)	2059	0	37.6 (15.0)	36.1 (14.6)	41.4 (15.1)	1.02	0.97 - 1.07	0.446
Household elevation (m)	1938	121	1399.5 (429.3)	1422.8 (401.4)	1339.5 (489.3)	1.00	1.00 - 1.00	0.901

Household annual mean precipitation (mm)	1945	114	793.6 (130.7)	801.5 (133.4)	773.2 (121.5)	1.00	0.99 - 1.00	0.651
Household annual mean temperature (C°)	1945	114	19.6 (2.2)	19.6 (2.1)	19.9 (2.4)	0.96	0.71 - 1.30	0.786

7.7 Appendix 7: CCHFV seroprevalence by sex and dentition, and household-level husbandry variables in different agro-ecological settings

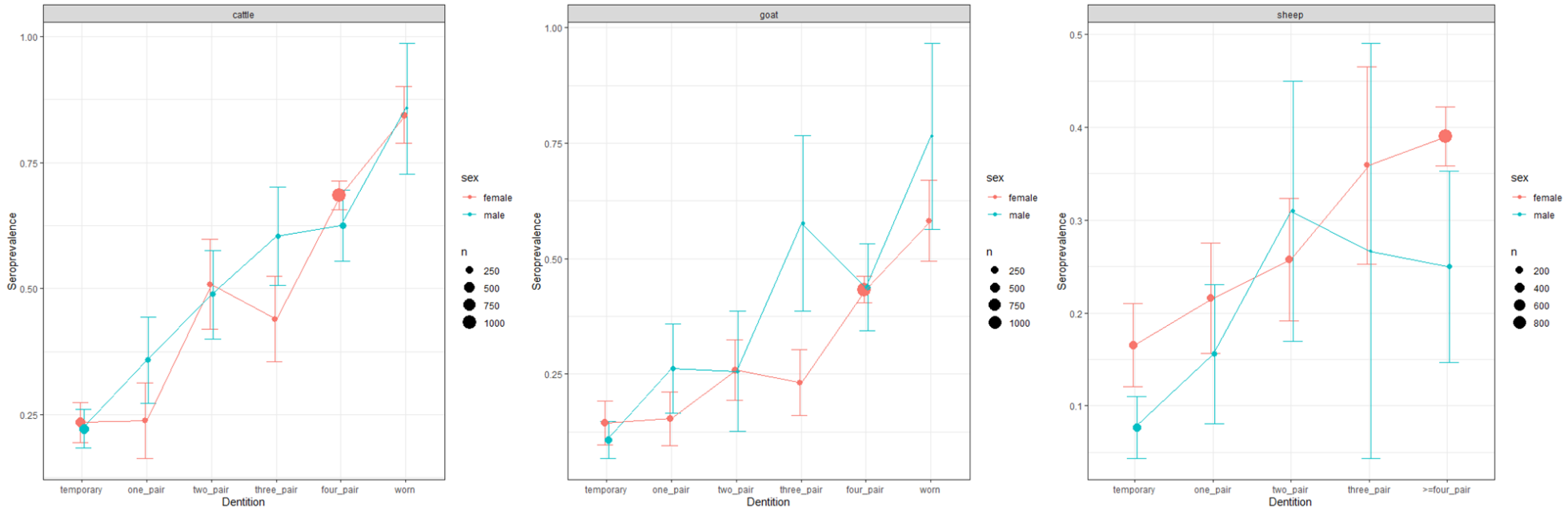

Figure 7.7.1 Seroprevalence estimates by sex and dentition category for cattle, goats and sheep.

Error bars show 95% confidence intervals

Table 7.7.1 Cattle seroprevalence for husbandry variables in different household agro-ecological settings

Husbandry variables	Levels	Agro-pastoral				Pastoral				Small-holder			
		Tested (N)	Prevalence (%)	95% confidence interval		Tested (N)	Prevalence (%)	95% confidence interval		Tested (N)	Prevalence (%)	95% confidence interval	
Own goats	No	132	48.48	39.96	57.01	80	37.50	26.89	48.11	36	27.78	13.15	42.41
	Yes	1072	53.54	50.56	56.53	1329	49.06	46.37	51.75	207	46.38	39.58	53.17
Own sheep	No	288	57.29	51.58	63.00	100	43.00	33.30	52.70	53	37.74	24.69	50.79
	Yes	916	51.64	48.40	54.87	1309	48.82	46.11	51.52	190	45.26	38.19	52.34
Own cattle	No	NA				NA				NA			
	Yes	1204	52.99	50.17	55.81	1409	48.40	45.79	51.01	243	43.62	37.39	49.86
Own pigs	No	808	42.33	38.92	45.73	1409	48.40	45.79	51.01	233	42.06	35.72	48.40
	Yes	396	74.75	70.47	79.03					10	80.00	55.21	104.79
Own donkeys	No	664	56.02	52.25	59.80	182	38.46	31.39	45.53	163	51.53	43.86	59.21
	Yes	540	49.26	45.04	53.48	1227	49.88	47.08	52.68	80	27.50	17.72	37.28
Own chickens	No	69	53.62	41.86	65.39	330	44.85	39.48	50.21	20	20.00	2.47	37.53
	Yes	1115	52.91	49.98	55.84	1044	49.71	46.68	52.75	223	45.74	39.20	52.28

Cattle tethered or zero-grazed	No	1170	53.59	50.73	56.45	1399	48.39	45.77	51.01	50	84.00	73.84	94.16
	Yes	31	29.03	13.05	45.01	NA				193	33.16	26.52	39.80
Goats tethered or zero-grazed	No	1024	53.13	50.07	56.18	1309	48.97	46.26	51.68	54	81.48	71.12	91.84
	Yes	27	44.44	25.70	63.19		0.00	0.00	0.00	153	33.99	26.48	41.49
Sheep tethered or zero grazed	No	838	51.07	47.69	54.46	1259	49.40	46.64	52.17	48	79.17	67.68	90.66
	Yes	28	28.57	11.84	45.30		0.00	0.00	0.00	129	32.56	24.47	40.64
Cattle herded or free-range	No	4	0.00	0.00	0.00		0.00	0.00	0.00	31	32.26	15.80	48.71
	Yes	1197	53.13	50.31	55.96	1399	48.39	45.77	51.01	212	45.28	38.58	51.98
Sheep herded or free-range	No		0.00	0.00	0.00		0.00	0.00	0.00		0.00	0.00	0.00
	Yes	866	50.35	47.02	53.68	1259	49.40	46.64	52.17	143	46.15	37.98	54.32

Goats herded or free-range	No		0.00	0.00	0.00		0.00	0.00	0.00		0.00	0.00	0.00
	Yes	1051	52.90	49.88	55.92	1309	48.97	46.26	51.68	171	46.78	39.30	54.26
Graze cattle and small ruminants together	No	643	51.79	47.93	55.65	1310	48.63	45.92	51.33	153	45.10	37.21	52.98
	Yes	561	54.37	50.25	58.49	87	45.98	35.50	56.45	80	42.50	31.67	53.33
Confine cattle and small ruminants together	No	1005	50.35	47.26	53.44	1371	48.21	45.57	50.86	228	44.30	37.85	50.75
	Yes	170	63.53	56.29	70.77	18	44.44	21.49	67.40	15	33.33	9.48	57.19
Do not see wildlife	No	954	51.47	48.30	54.64	1364	48.17	45.52	50.82	135	41.48	33.17	49.79
	Yes	250	58.80	52.70	64.90	45	55.56	41.04	70.07	108	46.30	36.89	55.70

Crops grown by household	No	10	50.00	19.01	80.99	386	47.67	42.69	52.65	19	10.53	-3.27	24.33
	Yes	1194	53.02	50.18	55.85	1023	48.68	45.62	51.74	224	46.43	39.90	52.96
Household tribe	Arusha	400	37.75	33.00	42.50	94	40.43	30.50	50.35	1	100.00	100.00	100.00
	Iraqw	660	65.76	62.14	69.38	7	0.00	0.00	0.00	44	52.27	37.51	67.03
	Maasai	76	35.53	24.77	46.29	1257	50.20	47.43	52.96	10	30.00	1.60	58.40
	Other	68	38.24	26.68	49.79	51	25.49	13.53	37.45	188	42.02	34.97	49.08

Table 7.7.2 Goat seroprevalence for husbandry variables in different household agro-ecological settings

Husbandry variables	Levels	Agro-pastoral				Pastoral				Small-holder			
		Tested (N)	Prevalence (%)	95% confidence interval		Tested (N)	Prevalence (%)	95% confidence interval		Tested (N)	Prevalence (%)	95% confidence interval	
Own goats	No	132	48.48	39.96	57.01	80	37.50	26.89	48.11	36	27.78	13.15	42.41
	Yes	1072	53.54	50.56	56.53	1329	49.06	46.37	51.75	207	46.38	39.58	53.17
Own sheep	No	288	57.29	51.58	63.00	100	43.00	33.30	52.70	53	37.74	24.69	50.79
	Yes	916	51.64	48.40	54.87	1309	48.82	46.11	51.52	190	45.26	38.19	52.34
Own cattle	No	NA				NA				NA			
	Yes	1204	52.99	50.17	55.81	1409	48.40	45.79	51.01	243	43.62	37.39	49.86
Own pigs	No	808	42.33	38.92	45.73	1409	48.40	45.79	51.01	233	42.06	35.72	48.40
	Yes	396	74.75	70.47	79.03					10	80.00	55.21	104.79
Own donkeys	No	664	56.02	52.25	59.80	182	38.46	31.39	45.53	163	51.53	43.86	59.21
	Yes	540	49.26	45.04	53.48	1227	49.88	47.08	52.68	80	27.50	17.72	37.28
Own chickens	No	69	53.62	41.86	65.39	330	44.85	39.48	50.21	20	20.00	2.47	37.53
	Yes	1115	52.91	49.98	55.84	1044	49.71	46.68	52.75	223	45.74	39.20	52.28

Cattle tethered or zero-grazed	No	1170	53.59	50.73	56.45	1399	48.39	45.77	51.01	50	84.00	73.84	94.16
	Yes	31	29.03	13.05	45.01	NA				193	33.16	26.52	39.80
Goats tethered or zero-grazed	No	1024	53.13	50.07	56.18	1309	48.97	46.26	51.68	54	81.48	71.12	91.84
	Yes	27	44.44	25.70	63.19		0.00	0.00	0.00	153	33.99	26.48	41.49
Sheep tethered or zero grazed	No	838	51.07	47.69	54.46	1259	49.40	46.64	52.17	48	79.17	67.68	90.66
	Yes	28	28.57	11.84	45.30		0.00	0.00	0.00	129	32.56	24.47	40.64
Cattle herded or free-range	No	4	0.00	0.00	0.00		0.00	0.00	0.00	31	32.26	15.80	48.71
	Yes	1197	53.13	50.31	55.96	1399	48.39	45.77	51.01	212	45.28	38.58	51.98
Sheep herded or free-range	No		0.00	0.00	0.00		0.00	0.00	0.00		0.00	0.00	0.00
	Yes	866	50.35	47.02	53.68	1259	49.40	46.64	52.17	143	46.15	37.98	54.32

Goats herded or free-range	No		0.00	0.00	0.00		0.00	0.00	0.00		0.00	0.00	0.00
	Yes	1051	52.90	49.88	55.92	1309	48.97	46.26	51.68	171	46.78	39.30	54.26
Graze cattle and small ruminants together	No	643	51.79	47.93	55.65	1310	48.63	45.92	51.33	153	45.10	37.21	52.98
	Yes	561	54.37	50.25	58.49	87	45.98	35.50	56.45	80	42.50	31.67	53.33
Confine cattle and small ruminants together	No	1005	50.35	47.26	53.44	1371	48.21	45.57	50.86	228	44.30	37.85	50.75
	Yes	170	63.53	56.29	70.77	18	44.44	21.49	67.40	15	33.33	9.48	57.19
Do not see wildlife	No	954	51.47	48.30	54.64	1364	48.17	45.52	50.82	135	41.48	33.17	49.79
	Yes	250	58.80	52.70	64.90	45	55.56	41.04	70.07	108	46.30	36.89	55.70

Crops grown by household	No	10	50.00	19.01	80.99	386	47.67	42.69	52.65	19	10.53	-3.27	24.33
	Yes	1194	53.02	50.18	55.85	1023	48.68	45.62	51.74	224	46.43	39.90	52.96
Household tribe	Arusha	400	37.75	33.00	42.50	94	40.43	30.50	50.35	1	100.00	100.00	100.00
	Iraqw	660	65.76	62.14	69.38	7	0.00	0.00	0.00	44	52.27	37.51	67.03
	Maasai	76	35.53	24.77	46.29	1257	50.20	47.43	52.96	10	30.00	1.60	58.40
	Other	68	38.24	26.68	49.79	51	25.49	13.53	37.45	188	42.02	34.97	49.08

Table 7.7.3 Sheep seroprevalence for husbandry variables in different household agro-ecological settings

Husbandry variables	Levels	Agro-pastoral				Pastoral				Small-holder			
		Tested (N)	Prevalence (%)	95% confidence interval		Tested (N)	Prevalence (%)	95% confidence interval		Tested (N)	Prevalence (%)	95% confidence interval	
Own goats	No	54	46.30	33.00	59.60	10	50.00	19.01	80.99	11	9.09	0.00	26.08
	Yes	589	25.30	21.79	28.81	1141	28.48	25.86	31.10	149	26.85	19.73	33.96
Own sheep	No	NA				NA				NA			
	Yes	643	27.06	23.63	30.49	1151	28.67	26.06	31.28	160	25.63	18.86	32.39
Own cattle	No	27	22.22	6.54	37.90	25	52.00	32.42	71.58	1	0.00	0.00	0.00
	Yes	616	27.27	23.76	30.79	1126	28.15	25.53	30.78	159	25.79	18.99	32.59
Own pigs	No	483	22.15	18.45	25.86	1151	28.67	26.06	31.28	150	22.67	15.97	29.37
	Yes	160	41.88	34.23	49.52	NA				10	70.00	41.60	98.40
Own donkeys	No	361	32.41	27.58	37.24	175	28.00	21.35	34.65	103	37.86	28.50	47.23
	Yes	282	20.21	15.53	24.90	976	28.79	25.95	31.63	57	3.51	0.00	8.29
Own chickens	No	33	48.48	31.43	65.54	281	20.64	15.91	25.37	12	0.00	0.00	0.00
	Yes	608	25.66	22.19	29.13	846	31.44	28.31	34.57	148	27.70	20.49	34.91

Cattle tethered or zero-grazed	No	594	27.78	24.18	31.38	1116	28.32	25.67	30.96	50	42.00	28.32	55.68
	Yes	18	11.11	0.00	25.63	NA				109	18.35	11.08	25.62
Goats tethered or zero-grazed	No	575	25.57	22.00	29.13	1131	28.56	25.93	31.19	50	42.00	28.32	55.68
	Yes	7	28.57	0.00	62.04	NA				99	19.19	11.43	26.95
Sheep tethered or zero grazed	No	583	26.42	22.84	29.99	1111	28.89	26.23	31.56	51	41.18	27.67	54.68
	Yes	30	20.00	5.69	34.31	NA				101	18.81	11.19	26.43
Cattle herded or free-range	No	NA				NA				26	7.69	0.00	17.94
	Yes	612	27.29	23.76	30.82	1116	28.32	25.67	30.96	133	29.32	21.59	37.06

Sheep herded or free-range	No	NA				NA				28	7.14	0.00	16.68
	Yes	613	26.10	22.62	29.58	1111	28.89	26.23	31.56	124	30.65	22.53	38.76
Goats herded or free-range	No	NA				NA				23	8.70	0.00	20.21
	Yes	582	25.60	22.06	29.15	1131	28.56	25.93	31.19	126	30.16	22.15	38.17
Graze cattle and small ruminants together	No	250	19.20	14.32	24.08	1073	29.26	26.54	31.99	67	20.90	11.16	30.63
	Yes	393	32.06	27.45	36.68	78	20.51	11.55	29.47	84	29.76	19.98	39.54
Confine cattle and small ruminants together	No	534	28.28	24.46	32.10	1130	29.03	26.38	31.67	154	25.97	19.05	32.90
	Yes	102	18.63	11.07	26.18	11	9.09	0.00	26.08	6	16.67	0.00	46.49

Do not see wildlife	No	478	25.31	21.42	29.21	1111	29.07	26.40	31.74	84	17.86	9.67	26.05
	Yes	165	32.12	25.00	39.25	40	17.50	5.72	29.28	76	34.21	23.54	44.88
Crops grown by household	No	15	20.00	0.00	40.24	312	30.13	25.04	35.22	7	0.00	0.00	0.00
	Yes	628	27.23	23.75	30.71	839	28.13	25.09	31.17	153	26.80	19.78	33.82
Household tribe	Arusha	328	20.43	16.06	24.79	62	17.74	8.23	27.25	5	0.00	0.00	0.00
	Iraqw	261	37.16	31.30	43.03	1	0.00	0.00	0.00	18	38.89	16.37	61.41
	Maasai	37	8.11	0.00	16.90	1056	30.21	27.44	32.98	9	22.22	0.00	49.38
	Other	17	41.18	17.78	64.57	32	0.00	0.00	0.00	128	25.00	17.50	32.50

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