Morbillivirus infections in animal hosts of the Serengeti District of Northern Tanzania: PPRV and CDV in multi-host livestock communities, and CDV in African wild dogs (*Lycaon pictus*).

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Abstract

Morbilliviruses are responsible for some of the most devastating outbreaks of disease in animals, accompanied by high morbidity and mortality rates. Canine distemper virus (CDV) and peste des petits ruminants virus (PPRV) are highly promiscuous morbilliviruses which have widely expanded their host range in recent decades. However, the extent of infection in atypical hosts and the genetic impact upon endangered, vulnerable species remain poorly understood, particularly in East Africa. The development of a highly accurate serological method capable of differentiating between circulating morbilliviruses is required. Further, the need for longitudinal and clinical monitoring of animals is essential to understand the infection dynamics occurring in susceptible and emerging hosts. Finally, there is a lack of research on the impact which decades of morbillivirus outbreaks have had on the critically endangered African wild dogs of Tanzania, which is paramount to understanding how this species can be further protected and preserved.

This thesis comprises two studies. The first study aims to confirm the presence of PPRV and CDV in cattle, sheep, and goats from ten households across the Serengeti District in Northern Tanzania. This work involves a longitudinal serological study underpinning active infection of animals, using a pseudotyped-virus neutralization assay (PVNA) to detect highly specific antibodies to PPRV and to CDV. This study also monitored clinical signs to investigate the disease manifestations of infections in livestock hosts, using a logistic regression model to test for associations between infection and signs of disease in each species.

The second study aims to clarify whether genetic change has occurred in the wild dog population of the Serengeti over recent decades, by investigating neutral

(microsatellite) and adaptive (MHC DRB1) genetic markers from published data (Marsden *et al*, 2012), and recently developed data following a fatal CDV outbreak in 2017. It also aims to determine whether there is evidence of natural selection on adaptive markers, and evidence of genetic variation attributable to factors beyond demographic change.

Results from these studies demonstrate that cattle in a mixed livestock setting become naturally infected with PPRV with no associated clinical disease. Data also show for the first time that cattle, sheep, and goats become naturally infected with CDV with no associated clinical disease. CDV seropositivity was detected predominantly in cattle. Infections occurred throughout the study with no patterns associated between household and infection, indicating widespread circulation of both viruses beyond the household level. The source of infection remains to be established, although livestock trade and sporadic outbreaks in domestic dogs are likely sources of PPRV and CDV infection, respectively. This work provides strong evidence that PPRV sub-clinically infects cattle in multi-host livestock communities, and that CDV sub-clinically infects livestock, predominantly cattle.

This thesis provides the first analysis of genetic changes in the Serengeti population of African wild dogs. This work utilized the pseudotype-based virus neutralization assay to detect CDV-specific neutralizing antibodies in sera of wild dogs which survived the 2017 CDV outbreak, while RT-qPCR was used to detect CDV in tissue samples from deceased wild dogs. Results showed an overwhelming proportion of PCR positives and lack of protective immune response in deceased and surviving animals, respectively. Allele frequency data showed fluctuations in DRB diversity over time coinciding with CDV outbreaks. Heterozygosity varied for both neutral and immune markers over time but showed no excess or evidence of population

bottleneck. Bayesian analysis of microsatellites found some genetic structuring over time (K = 2). The test for selection indicated balancing selection during at least two time points at the DRB, in keeping with CDV outbreaks. This study provides strong evidence of population structuring and the potential link between adaptive markers and disease outbreaks in wild dogs.

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Dedication

This is for the gardeners in my life who have cultivated me and helped me to bloom over the last five years. In particular:

For Ziggy – You are a daily reminder of everything that is joyful, innocent, sweet, and wholesome in this world. Thank you for adopting me.

For my parents – Thank you for reminding me of who I am when I felt lost. Thank you for your unconditional love and support and for always believing in me. I hope I have made you proud.

For my sister and best friend, Dr Catherine 'Babe Bennett' Arthur - Your compassion, strength, resilience, and humour were a huge source of motivation to keep going every day. Thank you for setting such a tremendous example of how to boss through life despite everything, with such determination and excellent patter.

For Uncle Paddy – Your support was invaluable, and I am forever grateful.

For Grandad – Although you could not be here to see it, you helped me to get through this PhD. I hope I would make you proud.

Most of all, this is for me.

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.

Ursula Pomeroy-Arthur, October 2022

Chapter 1: Introduction

Overview

Zoonotic pathogens with expanding host ranges pose one of the greatest challenges for disease control and prevention. Pathogens emerge into new host species when species interact within complex ecosystems. The *Morbillivirus* genus of viruses endemic to East Africa is an example of zoonotic pathogens with expanding host ranges. Peste des petits ruminants virus (PPRV) has plagued small ruminant populations globally for centuries, causing massive loss to small-holding farms across East Africa. PPRV is now known to cause disease in protected wildlife populations. Canine distemper virus (CDV) was traditionally considered a pathogen of dogs, though it now causes disease in many protected species across numerous taxa and is endemic to East Africa. In particular, outbreaks of CDV have devastated critically endangered populations of wildlife within the Serengeti National Park in Tanzania. Though these viruses are well studied, the parameters of their expanding host range into new species are not understood.

The aim of this thesis is to investigate the ecology of PPRV and CDV in a mixed-host livestock system in northern Tanzania. PPRV outbreaks have caused huge economic losses across farms in northern Tanzania in recent decades and is the target of a OIE eradication program by mass-vaccination by 2030. This virus has also caused outbreaks in endangered wildlife species. In recent years there has been a growing body of evidence that PPRV also infects cattle, though the dynamics of infection within mixed-livestock systems is not understood. Further, this thesis aims to

investigate whether livestock in the Serengeti District can become infected with CDV by detecting CDV antibodies and monitoring seroconversion and clinical signs of disease. This is based on some preliminary findings and the close contact between livestock and domestic dogs, which experience sporadic outbreaks in this endemic area. Finally, this thesis aims to investigate whether CDV outbreaks have affected genetic change in the critically endangered African wild dog (*Lycaon pictus*) within the Serengeti National Park, which has suffered severe decline due to outbreaks in the past and as recent as 2018.

This chapter will introduce general methods and frameworks used to study the emergence of morbilliviruses, and other pathogens, into new species. The current knowledge of the role cattle play in PPRV infection dynamics, CDV in atypical host species, and genetic impact of disease outbreaks on African wild dogs will be reviewed.

1.1 Morbillivirus Overview

The genus *Morbillivirus* comprises closely related single-strand, non-segmented, negative sense RNA viruses, and belongs to the *Paramyxoviridae* family (Conceicao & Bailey, 2021). Morbilliviruses are highly contagious, promiscuous, and cause severe respiratory and gastrointestinal disease during infection (Pfeffermann *et al*, 2018). As with other paramyxoviruses, morbilliviruses spread primarily via the respiratory and gastrointestinal tracts. Experimental infection of animal models has shown that most transmission occurs via airborne droplets from an infected animal to a naïve recipient animal (Dunkin & Laidlaw, 1926; Ludlow *et al*, 2014; de Vries *et al*, 2017). During

infection, these viruses cause immunosuppression in hosts, which can lead to susceptibility to secondary infections. The only member of this family which infects humans is Measles virus, which has provided a framework for much of the current knowledge on morbilliviruses. Other members of the genus include phocine distemper virus (PDV); cetacean morbillivirus (CeMV); feline morbillivirus (FeMV); bat morbillivirus (BMV); the extinct rinderpest virus (RPV); CDV, and PPRV.

1.1.1. Virion structure

The morbillivirus structure comprises the RNA genome, around which is a nucleocapsid and a complex of ribonucleoprotein components (Fig. 1.1.1).

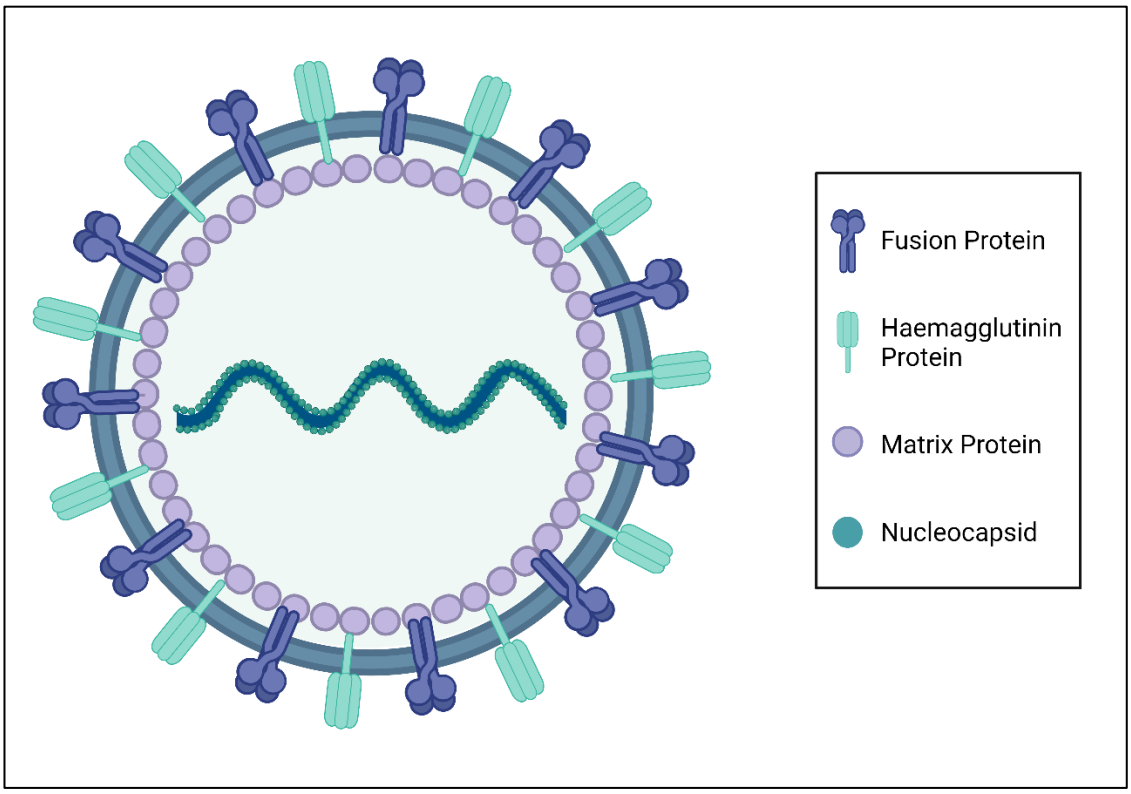


Figure 1.1.1. General structure of morbilliviruses. Created with Biorender.com.

Attached to the nucleocapsid is a phosphoprotein (P), which aids virus solubility in the cytoplasm upon cell entry. Further attached to this complex is the polymerase protein, which is essential for morbillivirus transcription once inside a host cell. This ribonucleoprotein complex (RNP) is surrounded by a protective core of matrix proteins. Matrix (M) proteins are essential to the virus structure by mediating RNA encapsidation and structural protein recruitment in progeny viruses. Beyond this inner protein complex is an envelope. On the surface of morbilliviruses are two key glycoproteins, the structure of which determine host cell specificity. First, the hemagglutinin (H) protein binding domains attach to the host cell receptor (CD150 or Nectin-4). The H protein then undergoes conformational changes which activate the receptor binding domain of the fusion (F) protein. The F protein is critical in binding the morbillivirus to host cells, and in binding host cells together, enabling virus entry and spread. These glycoproteins are key in morbillivirus' ability to recognize host cell receptors, and in subsequent infection.

1.1.2. Genome structure

The morbillivirus genome is between 15.7 – 16KB in length and is formed of hexamer units, whereby it is bound to the N protein every six nucleotides. The genome encodes six structural proteins which make up structural components of the mature virus (N; M; P; H; F, and two large polymerase (L)), and two non-structural proteins which are genome encoded but not part of the virion's physical structure (C and V)(Pfeffermann *et al*, 2018). The C and V accessory proteins are not essential to the infection of a host but act as inflammatory antagonists, thus play an important role in host immune

suppression (Barron & Barrett, 2000; Gotoh *et al*, 2001; Horvath *et al*, 2004; Bailey *et al*, 2005).

1.1.3. Host cell recognition

Upon infection of a host, the primary method of attachment to host cells is via the CD150 receptor (Signaling Lymphocyte Activation Molecule, SLAM-1). This receptor is found on the surface of macrophages, dendritic cell subsets, and activated lymphocytes present on mucosal surfaces, for example in the upper respiratory tract. Binding of the virus H glycoprotein to the CD150 receptor on a host cell, conformational changes occur which cause the virus F glycoprotein to initiate fusion of the cell membrane with the virus membrane. This is a key process which allows for the viral RNP to enter the host cell cytoplasm, thereby starting infection. Once the virus disseminates and reaches lymphoid organs, it can be shed. To do this, the virus binds to the Nectin-4 (Polio Virus Receptor-Like Protein, PVRL-4) receptor present on epithelial cells, for example in the respiratory or gastrointestinal tracts. Despite being promiscuous viruses, morbilliviral glycoproteins have high host specificity, studies on which have mostly used MV and CDV as models (Shibahara *et al*, 1994; Bartz *et al*, 1996; Xie *et al*, 1999; Nielsen *et al*, 2001).

The receptor binding domain (RBD) of a host or pathogen receptor plays a critical role in the success of virus infection. It has been shown that changes in amino acid residues in this domain can either enable or prevent morbillivirus infection. Although morbilliviruses have high host-specificity, their high mutation rate has resulted in adaptation to different host species which share conserved portions of their SLAM-1 RBD (Prajapati *et al*, 2019). For example, the single amino acid change in the sequence

of human SLAM-1, which was shown to make human cells susceptible to CDV infection *in vitro* (Bieringer *et al*, 2013). Another example is the similarity between the SLAM-1 RBD sequences of cattle, sheep, and goats is likely to explain why they are all susceptible to PPRV and, previously, RPV (Ohishi *et al*, 2010; Abdullah *et al*, 2018). Antigenic similarity between morbillivirus glycoprotein sequences leads to cross-protection across species. For example, dogs vaccinated with MV were protected against clinical CDV disease following challenge infection (Strating *et al*, 1975; Appel *et al*, 1984; Nambulli *et al*, 2016). However, these virus similarities have raised concerns that spillover infection into atypical species is likely to occur (Nambulli *et al*, 2016). For example, it is unknown whether cattle, sheep, or goats are susceptible to CDV infection, though one study found that goat and sheep SLAM are able to bind to CDV H and F *in vitro* (Yadav *et al*, 2021; Yadav *et al*, 2019).

1.2. Morbillivirus detection: Serological and molecular methods

To date, most serological studies of PPRV have utilized monoclonal antibody-based enzyme-linked immunosorbent assays (ELISA). Due to its stability at room temperature and compact nature of available kits, this assay is a highly practical and inexpensive tool for field-based serological studies (Baron *et al*, 2017). Although there are various types of ELISA available, the basic premise of this method employs sera taken from an animal for a competition ELISA. The ELISA plate will have antigen coated on the bottom of wells containing a colour-changing molecule, to which sample and a competitive monoclonal antibody (MAb) is added with substrate. If antibodies are present in samples, they will compete with the MAb to bind to the antigen and initiate a colour change process. The more antibody is present in the sample, the more

the colour will change compared to the negative control. In theory, negative samples do not produce a signal. However, ELISAs have been associated with low sensitivity and specificity, producing 'false-negative' and 'false-positive' results. A low sensitivity means that PPRV antibodies are present in a sample but are not detected, whereas a low specificity means that the ELISA is detecting non-specific antibodies, including antibodies to antigenically similar morbilliviruses (Logan *et al*, 2016). These shortfalls have had implications for the accuracy of PPRV surveillance and prevalence estimates (Balamurugan *et al*, 2014; Libeau *et al*, 1992; Logan *et al*, 2015; Logan *et al*, 2016). Alternative serological methods have been developed and utilized to overcome this problem.

The live virus microneutralization assay (VNA) purports to have a higher sensitivity and specificity compared to the ELISA, and has been considered a 'gold-standard' for the purpose of PPRV sero-surveillance and detection (Rossiter *et al*, 1985; Kamel & El-Sayed, 2019). The basic mechanism of this immunoassay is to detect and quantify antibodies capable of inhibiting virus replication, by adding serially diluted sample to a fixed volume of prepared virus (Liu *et al*, 2015; Payne, 2017; Fakri *et al*, 2019; Yan *et al*, 2020). The reliance of the VNA on visual CPE means that virus-infected cells not showing CPE are not detected, thus results may not be accurate (Logan *et al*, 2016). A focal assay can be utilized in cases where CPE is not expected, for example with HIV, however, this is very time consuming. A further limitation of the VNA is that it cannot distinguish between PPRV-specific and cross-neutralizing antibodies, thus it has been found to provide inaccurate results (Baron *et al*, 2017; Logan *et al*, 2016). For example, PPRV and RPV seroprevalences could not be distinguished in sheep and goats before RPV was eradicated because antibodies to one virus could cross-neutralize the other (Osman *et al*, 2009; Obi & Patrick, 1984). The VNA has been

utilized for a variety of pathogens, however, the requirement for live virus means that this assay is limited to BSL3 laboratories for PPRV (Yan *et al*, 2020; Baron *et al*, 2017).

The development of the pseudotype-based virus neutralization assay has circumvented some of these challenges. This method involves a replication-defective vesicular stomatitis virus (VSVΔG) construct. In place of the VSV glycoprotein gene ('G'), the gene encoding the PPRV H or F glycoprotein is inserted. Due to its replication deficiency and very low infection risk, this construct can be engineered in CL2 laboratories and is now used for a variety of viruses (Bewly *et al*, 2021; Gilbert *et al*, 2020). A cell line expressing the appropriate SLAM receptor can then provide a model for infectivity. Serum samples from animals are then assayed with the model cell line and pseudotyped virus, which will be neutralized if there are antibodies present in the sample. By inserting the gene encoding the PPRV glycoprotein receptor binding domain into the VSV construct, only highly specific antibodies will bind and neutralize the pseudotyped virus, as the sequence is variable between morbilliviruses. A further advantage of this assay is the inclusion of a firefly luciferase gene within the VSV construct, which, upon target cell infection and addition of luciferase substrate, will emit light. This light is detectable and quantifiable using a luminescence plate reader, omitting the need to measure cytopathic effect with the naked eye. Previously, VSVΔG constructs were used bearing green fluorescent protein (GFP) to study virus-cell tropism (Seki *et al*, 2003). However, bioluminescent markers such as luciferase have been found to be more sensitive than fluorescent markers, thus offering optimal assay output (Fan & Wood, 2007; Chiem *et al*, 2019; Seki *et al*, 2003).

1.3. Ruminant morbilliviruses

RPV is considered the 'oldest' animal morbillivirus, recorded as 'cattle plague' before the first millennium BC (Pappas *et al*, 2008; Nambulli *et al*, 2016). The declaration of RPV eradication in 2011, achieved primarily through mass vaccination of cattle, has inspired current campaigns to eliminate other morbilliviruses. For example, in 2010 the World Health Assembly included the MV in the Global Vaccine Action Plan with the aim of eradicating it (WHO, 2012; Durrheim, 2020). However, the eradication of one ruminant morbillivirus has raised concerns that it created a vacated 'niche', wherein a closely related virus could take its place (Lloyd-Smith, 2013). This has been suggested in studies which have detected PPRV in large ruminants and other artiodactyls (such as deer, buffalo, and gazelle), which previously would have been hosts of RPV.

1.4. PPRV overview

PPRV is named the 'plague of small ruminants', causing devastating outbreaks in sheep and goat populations for decades. Morbidity and mortality rates during outbreaks can range from 80-100% (Mdetele *et al*, 2021; Lie *et al*, 2012). First discovered in Ivory Coast, West Africa in 1942, PPRV quickly became endemic to most of sub-Saharan Africa, spreading to the rest of the continent, to Asia, and to some of Europe (Gargadennec & Lalanne, 1942; Lembo *et al*, 2013; Dou *et al*, 2020). It is estimated that PPRV mortality in small ruminants reaches over 37.4 billion annually across 70 countries, with a global economic cost of up to $2.7 billion USD (Jones *et al*, 2016; Roger *et al*, 2021).

The virulence of PPRV reportedly varies between lineages (Couacy-Hymann *et al*, 2005). In East Africa, lineages II-IV are most prevalent and have caused recent outbreaks in Uganda (2007), Kenya (1999 and 2009), and Tanzania (2008, 2010, 2015) (Jones *et al*, 2020; Misinzo *et al*, 2014; Batten *et al*, 2011). PPRV was first reported in Tanzania in 2008, though retrospective serological studies suggest it was present as early as 1995 (Karimburibo *et al*, 2011; Dou *et al*, 2020). In 2016 the estimated small ruminant population of Tanzania was 21.7 million, all of which are deemed 'at risk' of PPRV outbreaks (Armson *et al*, 2021). These economic figure estimates are based on domestic sheep and goat populations, and there are currently no figures on atypical host species, including wildlife. However, as PPRV predominantly infects domestic sheep and goats, they are the target of a mass-vaccination strategy (PPR Global Control & Eradication Strategy, PPR-GCES) by the FAO-OIE. For the control and eradication of any pathogen to succeed, it is important to identify new and emerging sources of potential infection. In the case of PPRV, a high level of herd immunity required for eradication (80%) means that emerging hosts of the virus could threaten eradication efforts. It is therefore important to identify hosts of PPRV which are able to transmit and maintain the virus, and those which are considered 'dead-end' hosts, i.e., they become infected but cannot transmit the virus to another host. The role of atypical host species, such as cattle, in the epidemiology of PPRV is not well understood and remains a gap in current knowledge (Herzog *et al*, 2020; Mdetele *et al*, 2021).

1.4.1. PPRV transmission cycle

The predominant route of PPRV transmission is via respiratory droplets from an infected host to an uninfected recipient in close proximity. The virus has also been

found in feces from infected animals in shared grazing areas, providing a fecal-oral route of transmission (Ezeibe *et al*, 2008). Contaminated fomites, such as fencing, can also maintain the virus in droplets. Since farming practices greatly vary between households in areas with circulating PPRV, for example in their methods of housing, grazing, and livestock trade, virus transmission can be complex and is not fully understood (Herzog *et al*, 2020). Knowledge of transmission dynamics is particularly limited for households where cattle, sheep, and goats are kept in close proximity to one another (Herzog *et al*, 2020). Studies involving the role of cattle in PPRV transmission have concluded that they are 'dead-end' hosts, and that sheep and goats are the only hosts which transmit the virus (Couacy-Hymann *et al*, 2019). However, the role of cattle in virus transmission is still under experimental investigation (Schulz *et al*, 2018; Herzog *et al*, 2019; Herzog *et al*, 2020). The understanding of transmission dynamics and the associated risk factors is critical to the control of PPRV in susceptible hosts (Herzog *et al*, 2019).

1.4.2. PPRV clinical disease

Clinical manifestations in small ruminants include pyrexia; diarrhea; ulcerative lesions; mucopurulent oculo-nasal discharge; sneezing; pneumonia; anorexia, and death (Younus *et al*, 2020). Some atypical hosts of PPRV, such as Mongolian saiga, also succumb to the full range of clinical disease. However, some susceptible species can become infected without displaying any signs of disease, thus are sub-clinical hosts. For example, in some studies dromedary camels (*Camelus dromedarius*) were

found to have PPRV antibodies in endemic areas of Africa and Asia, with some reports of respiratory disease consistent with PPRV (Roger *et al*, 2000; Saeed *et al*, 2022). However, available evidence is conflicting as findings from other studies have found camels to be sub-clinically infected (Omani *et al*, 2019; Schulz *et al*, 2019). Knowledge on clinical PPRV disease in cattle is also not understood.

1.4.3. Expanding PPRV host range

Though it is mainly a pathogen of sheep and goats, PPRV has caused outbreaks in several host species and continues to expand its host range. Fatal outbreaks have also been reported in Sindh ibex (*Capra aegagrus blythi*), Himalayan bharal (*Pseudois nayaur*), and Dorcas gazelle (*Gazella Dorcas* (Pruvot *et al*, 2020; Bao *et al*, 2011; Asil *et al*, 2019)).

Wild boar and domestic pigs (*Sus scrofa*) have been reported to be infected with and able to transmit PPRV (Schulz *et al*, 2019). Large ruminants such as Indian buffalo (*Bubalis bubalis*) have suffered outbreaks with high morbidity (Govindarajan *et al*, 1997). Though the range of host species exhibiting severe disease is expanding, PPRV is also being detected in sub-clinical atypical species. For example, Chinese yak (*Bos grunniens*), water buffalo (*Bubalis bubalis*), and cattle (*Bos taurus*) were all found to have PPRV antibodies in multiple studies. Although there is evidence that cattle become infected with PPRV, there is little understanding on the clinical manifestations of infection or their epidemiological role within small ruminant populations.

1.4.4. PPRV in *Artiodactyla*

Within the order *Artyiodactyla* there are 9 families of mammals, some of which have become susceptible to outbreaks of and sub-clinical infections with PPRV. For example, PPRV antibodies have repeatedly been found in dromedary camels since 2002 (Haroun *et al*, 2002), and clinical disease from PPRV infection has been reported in recent years (Nafea *et al*, 2019; Fakri *et al*, 2019; Saeed *et al*, 2022). A further example of PPRV in camelid hosts is the alpaca (*Vicugna pacos*), recently shown to develop a neutralizing response to PPRV, though evidence of clinical disease was conflicted (Schulz *et al*, 2019; Liu *et al*, 2021). *Bovidae* is the largest family within *Artiodactyla*, comprising more than 100 species including large ruminants (buffalo, water buffalo, and cattle), and small ruminants (sheep and goats). Data on PPRV in large ruminants are limited and despite decades of research, evidence of PPRV infection is still conflicted. For example, an outbreak was reported and confirmed by PCR in domestic Asian water buffalo (*Bubalis bubalis*) in 1997 which had a 96% case fatality rate (Govindarajan *et al*, 1997). Serological studies have repeatedly found PPRV antibodies in buffalo (*Bubalis bubalis* and *Syncerus caffer*) (Khan *et al*, 2008; Balamurugan *et al*, 2012; Abubakr *et al*, 2015; Giridharan & Krishnamohan, 2021) although clinical disease was reportedly not observed. Despite sub-clinical infection, nasal swabs taken from buffalo have detected PPRV, indicating that they can shed the virus (Couacy-Hymann *et al*, 2005). However, the epidemiological role of these bovine species remains unclear. Thomson's gazelle (*Eudorcus thomsonii)*, Grant's gazelles (*Gazella granti*), wildebeest (*Connochaetes taurinus*), gerenuk (*Litocranius walleri*), yak (*Bos grunniens*), and hartebeest (*Alcelaphus buselaphus*) are further examples of bovine species found to become infected with PPRV in serological

surveys (Abubakr *et al*, 2014; Li *et al*, 2016; Asil *et al*, 2018; Pruvot *et al*, 2020; Jones *et al*, 2021).

Evidence of PPRV infection in cattle has been presented over recent decades in endemic areas with small ruminants (Anderson & McKay, 1994; Khan *et al*, 2008; Balamurugan *et al*, 2012; Lembo *et al*, 2013; Logan *et al*, 2016; Herzog *et al*, 2021). Serological studies have found seroprevalences as high as 42% (Khan *et al*, 2008) in some cattle populations. Experimental infections of cattle have been carried out to investigate whether they succumb to clinical disease when housed with infected sheep and goats (Sen *et al*, 2014; Schulz *et al*, 2019). With the exception of one calf, these studies were unable to find any clinical manifestation in cattle. However, research within a field setting is lacking, and such studies are cross-sectional thus only monitor infections at one period of time. Out of all bovine species not typically associated with PPRV infection, cattle are most likely to become the next susceptible host given their frequent and close proximity to sheep and goats within global farming communities.

1.4.5. PPRV in northern Tanzania

PPRV was first confirmed in Tanzania in June 2008, however, PPRV antibodies were found in sera collected in 2004, suggesting that the virus was present at least 4 years before it was officially confirmed (Karimuribo *et al*, 2011; Torsson *et al*, 2016). A serological survey was carried out in the Ngorongoro, Mara, and Arusha regions, finding PPRV seroprevalence of 45% overall (Swai *et al*, 2009; Torsson *et al*, 2016). It was believed that the spread into Tanzania came from cross-border livestock trade with Kenya, which had confirmed the presence of PPRV in 2007 (Dundon *et al*, 2015). Further serological investigation to assess the magnitude of outbreak found PPRV in

other districts, showing that it had spread southwards near the border with Mozambique (Swai *et al*, 2009; Dundon *et al*, 2015). PPRV is now endemic in sheep and goat populations of Tanzania. After the incursion of PPR outbreaks, livestock production in Tanzania dropped by 30% according to a socioeconomic survey (Kovarrubias *et al*, 2012). As the third largest livestock producers in Africa (after Ethiopia and Sudan) with 5 million sheep and 15 million goats, the threat of PPRV to livelihoods is huge (National Report, United Republic of Tanzania, 2013). In response to the 2008 outbreaks, emergency vaccinations with the attenuated Nigeria 75/1 strain were carried out in the districts worst affected by the disease. This vaccination was unsuccessful in containing the spread of the virus due to uncontrolled cross-border livestock movements, a lack of surveillance, and inadequate diagnostic protocols (Control Strategy, Southern African Development Community (SADC), 2012). In 2015 the SADC collaborated with the OIE and FAO to create the 'Global Strategy for the Control and Eradication of PPR', a mass-vaccination campaign modelled on the successful eradication of rinderpest (Britton *et al*, 2019).

Since it officially took hold in 2008, PPR has emerged into numerous species in Tanzania. Following the cessation of rinderpest vaccination, wild ungulates were serologically sampled in the Ngorongoro region to determine if PPRV had spread from neighbouring small ruminant communities. Results indeed showed spillover of PPRV infection from domestic ruminants into local wildlife species (Jones *et al*, 2021; Mahapatra *et al*, 2015). In the Serengeti ecosystem, serological surveys have found PPRV antibodies in multiple wildlife species, including buffalo, wildebeest, impala, and Thomson's and Grant's gazelle (Lembo *et al*, 2013; Mahapatra *et al*, 2015). The threat of PPRV to the Tanzanian (and East African) economy is heightened by the risk to wildlife species in this protected ecosystem. The extent of the spillover into new

host species is still under investigation. Given the spillover of PPRV into other bovines, it is not surprising that cattle have also been found to produce antibodies in areas where PPRV is circulating (Balamurugan *et al*, 2012; Lembo *et al*, 2013; Herzog *et al*, 2019; Herzog *et al*, 2020; Jones *et al*, 2021). Although multiple studies have concluded that cattle are susceptible to PPRV infection, their role in the epidemiology and transmission of the virus is still unclear. To achieve the goal of the FAO in eradicating PPRV by 2030, it is critical that the roles of emerging host species; particularly those in close contact with small ruminants; are fully understood.

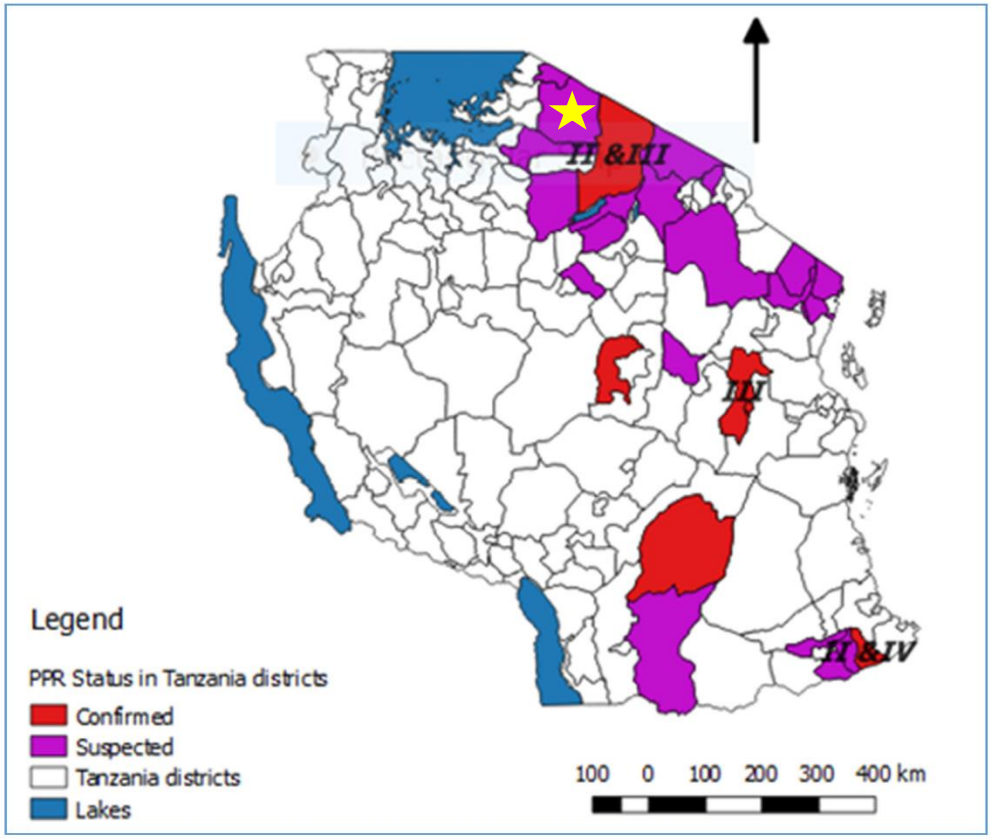


Figure 1.4.5. PPRV in Northern Tanzania (2008-2018), calculated by Mdetele *et al* (2021). The laboratory-confirmed presence of PPRV in Northern Tanzania between 2008-2018, as calculated by Mdetele *et al* (2021). PPRV lineages detected are noted in black. Areas in red are those with highest seroprevalence, and areas in purple are those which are suspected to have PPRV but have not yet been confirmed. The

Serengeti District where samples were collected for this study is marked with a yellow star.

1.5. CDV overview

Canine distemper is an often-fatal disease of canids and occurs globally (Beineke *et al*, 2015). CDV was first described in outbreaks of domestic dogs in the early 18th century (Stokholm *et al*, 2021). Despite mass vaccination of domestic carnivores over recent decades, new strains continue to evolve and cause outbreaks worldwide (Piewbang *et al*, 2019; Jo *et al*, 2019).

1.5.1. CDV transmission cycle

The primary route of CDV infection is via droplets from nasal, ocular, fecal and urinal secretions from clinically infected animals (Duque-Valenia *et al*, 2019). For example, infected domestic dogs can easily transfer the virus into the environment, the virus may then be shared with susceptible wildlife (Cleaveland, 1996; Viana *et al*, 2015). Given that approximately 50% of domestic dogs can shed CDV into the environment while infected sub-clinically, infection statistics attributed to this source of transmission are likely underestimated. Further, the extent of transmission from other sub-clinically infected reservoir species is not well understood. For example, between 2000-2010 morbidity in Washington sea otters (*Enhydra lutris*) was attributed to CDV infection confirmed with RT-PCR and sequencing, although it is not known how the virus could have been transmitted in their environment (Thomas *et al*, 2020).

Carnivores which consume infected meat can also be infected (Ludlow *et al*, 2014). For example, Marescot *et al* (2018) found that Serengeti spotted hyaenas with lower social status had a greater probability of contracting CDV, as they roam and hunt prey more frequently than higher status animals. Additionally, vertical transmission of CDV from mother to offspring is possible. For example, female tigers which consume infected prey may transmit the virus through infected milk to subsequent litters (Gilbert *et al*, 2014; Gupta *et al*, 2021). The transmission of CDV in known host species has been well researched and is a critical factor in the control of the virus. However, as the virus continues to emerge in unusual hosts and occurs sub-clinically in some species, the transmission dynamics become very unclear and require further understanding.

1.5.2. CDV clinical disease

Clinical manifestations include pyrexia; extreme lethargy; mucopurulent oculo-nasal discharge; diarrhea; neurological disturbances; hyperkeratosis of the foot pads and nose, and death (Duque-Valencia *et al*, 2019). Aside from measles, CDV is the only terrestrial morbillivirus which causes neurovirulence, whereby it targets glial cells of the central nervous system causing seizures, tremors, and ataxia (Alves *et al*, 2015). Due to its affinity for neuronal, lymphoid, and epithelial cells, the virus quickly becomes systemically distributed and infects all major organs of the body (Beineke *et al*, 2015). Since it is a multisystemic infection, the range of disease manifestations varies greatly between species and depends on the host's age, immune status, and virulence (Dik & Aslim, 2022). During the acute phase of infection, the virus infects

and depletes lymphoid tissue, causing mass syncytia and greatly reduces immune competency. This immunosuppression is a key factor in the death of many infected animals, as secondary infections with generally non-virulent pathogens become fatal. The neuronal manifestations of disease occur when the virus infects mononuclear cells which cross the blood-brain barrier, and via neurons in the olfactory mucosa (Lempp *et al*, 2014). Once inside neuronal tissue, the virus disseminates via the cerebrospinal fluid, where it infects further neuronal tissue. The extent of cytopathology varies for different cell types depending on the strain of CDV (Summers *et al*, 1984). The physical implications of the neuronal stage of CDV infection include apathy, ataxia, listlessness, confusion, and seizures. This stage of the disease causes mass cell death; however it is the immunosuppression during infection which most often leads to mortality (Origgi *et al*, 2012; Zhao & Ren, 2022). In less severe cases, animals may exhibit minimal pneumonia and lethargy, although many hosts do not display any signs of disease and are sub-clinically infected (Origgi *et al*, 2012). Animals which are able to launch an immune response to the virus may not produce any clinical disease and appear healthy while actively spreading the virus. The variety of disease manifestations of CDV infection means that differential diagnosis is difficult without laboratory confirmation, using either serological or molecular techniques. This is particularly challenging with sub-clinical infections in unusual hosts.

1.5.3. Expanding CDV host range

Previously, CDV was considered a virus of canids until a major outbreak occurred in the Serengeti lion (*Panthera leo*) population in 1994. Further outbreaks have occurred

in other endangered feline species such as the Amur tiger (*Panthera tigris altaica*) (Seimon *et al*, 2013), leopards (*Panthera pardus*), and jaguars (*Panthera onca*) (Terio & Craft, 2013; McAloose *et al*, 2013; Ohishi *et al*, 2014). In recent years, researchers have noted that CDV infects other carnivore species, such as black-footed ferrets (*Mustela nigripes*); bat-eared foxes (*Otocyon megalotis*); and spotted hyaenas (*Crocuta crocuta*)(Williams *et al*, 1988; Roelke-Parker *et al*, 1996; Ohishi *et al*, 2014; Kamath, 2020). Many of these susceptible species are within the suborders Feliforma and Caniforma (Visser *et al*, 1993; Kennedy *et al*, 2000; Ohishi *et al*, 2014). However, host range of the virus extends to include numerous other species including primates and ursids, for example Japanese monkeys (*Macaca fuscata*), rhesus macaques (*Macaca mulatta*), and giant pandas (*Ailuropoda melanoleuca*)(Yoshikawa *et al*, 1989; Kennedy *et al*, 2019; Feng *et al*, 2016). The impact upon populations of new host species has been devastating in many cases, for example in Santa Catalina Island foxes (*Urocyon littoralis catalinae*) (Timm *et al*, 2009), which lost 95% of the population as a result of CDV. Further, an outbreak in protected Chinese giant pandas was fatal to 5 out of 6 animals (Yoshikawa *et al*, 1989). Infection of new host species is therefore of great ecological concern, particularly in protected areas with endangered wildlife. Despite decades of research and the impact upon such a wide range of species, CDV is still being discovered in previously unknown hosts to differing degrees. It is estimated that up to 75% of CDV infections may be sub-clinical, for example in Sika deer and wild boar (Kameo *et al*, 2012; Ludlow *et al*, 2014; Bruyette, 2020; Weckworth *et al*, 2020). This poses a great challenge to the monitoring and control of CDV spread as many reservoir animals may be undetected.

1.5.4. CDV in *Artiodactyla*

Artiodactyla comprises a wide range of species including endangered wildlife and animals of economic importance. CDV outbreaks have been reported across multiple taxa within *Artiodactyla* in recent decades. For example, CDV was isolated from deceased collared peccaries (*Tayassu tajacu*) with acute encephalitis by Appel *et al* (1989), and a serological survey found that CDV antibodies were widespread in this species (Noon *et al*, 2003). Mustelids (including otters, ferrets) have also demonstrated susceptibility to acute CDV disease. For example, a raft of Asian clawless otters (*Aonyx cinereus*) suffered 100% mortality during a CDV outbreak in a Belgian zoo (Bosschere *et al*, 2005). However, the radiation of the CDV host range within *Artiodactyla* has not always been accompanied with obvious signs of disease. For example, during an outbreak in Japanese wildlife, a serological survey found CDV antibodies present in protected Sika deer (*Cervus nippon*)(Suzuki *et al*, 2015). Although no clinical disease was apparent, this was evidence that CDV could readily infect a protected ungulate species. In most of these examples, the source of infection was attributed to infected domestic dogs which had come into close contact with either the susceptible host, or with a human handler of the susceptible hosts. Much of the literature on the emergence of CDV into new hosts is limited to incidents of disease outbreak. However, if CDV can be directly or indirectly transmitted to new hosts from seemingly healthy animals, this poses a significant threat to many susceptible domestic and wild animals. The lack of current literature in this area also means that there is a need for more knowledge of new host species transmission dynamics. Further research and surveillance are required to understand which species are reservoirs for CDV, which are dead-end hosts, and which are likely to become new susceptible hosts for the virus.

1.5.5. CDV in northern Tanzania

Multiple outbreaks of CDV have affected wildlife in northern Tanzania, home to ecologically important national parks and reserves. These protected areas, namely the SNP and Ngorongoro Conservation Area, have become endemic with CDV (Viana *et al*, 2015). A serological survey by Packer *et al* (1999) found CDV antibodies in lions from the Ngorongoro Crater as early as 1984. Fatal outbreaks were thought to have killed bat-eared foxes (*Otocyon megalotis*) and black-backed jackals *(Canis mesomelas*) in the Serengeti ecosystem in 1978 (Banyard *et al*, 2012; Packer *et al*, 1999; Moehlman *et al*, 1983). This suggests that the virus may have been present in these species earlier than previously thought. The first major epidemic including non-canid species of the SNP occurred in 1993/1994, resulting in an estimated 1000 deaths of animals across the ecosystem (Cleaveland *et al*, 2007). It was thought that this outbreak originated from domestic dogs in the region, which roam freely across park boundaries where wildlife resides (Viana *et al*, 2015). The economic and ecological burden of this epidemic, in addition to outbreaks of rabies at the time, resulted in the dedication of research to the most effected species in the area. For example, lions, African wild dogs, and spotted hyaenas. However, little research has been done on other species which live closely to domestic dogs and could be the target of CDV host-switching. In particular, livestock in Northern Tanzania are kept close to domestic dogs for herding and guarding purposes, posing a daily risk of exposure to potential CDV infection.

1.5.6. CDV in African Wild Dogs

African wild dogs (AWD) (*Lycaon pictus*) are one of the most critically endangered carnivore species of sub-Saharan Africa, with approximately 6,000 individuals remaining in the wild (Cozzi *et al*, 2020). In northern Tanzania, approximately 120 individuals reside in the SNP across 10 packs, according to figures published by the Tanzanian Wildlife Research Institute (TAWIRI, 2019). Over recent decades population numbers have dwindled due to habitat loss, increased prey competition, and outbreaks of rabies and CDV. For example, in 2007 a fatal outbreak of CDV 12 Km north-east of the SNP killed approximately 60% of a free-ranging pack of wild dogs (Goller *et al*, 2010). Further, an outbreak in the Mkomazi Game Reserve in 2001 resulted in the deaths of 94% of AWD there (van der Bildt *et al*, 2002). The high fatality rate seen in small, fragmented populations such as these threatens their genetic fitness, and chance of survival as a species (Bucci *et al*, 2022). To revive the SNP population, the Tanzanian Wildlife Research Institute (TAWIRI) translocated a pack of wild dogs to the SNP's Nyasaori pack in 2015 (Wild Dog Report, TAWIRI, 2018). This was a success and gave hope for the re-establishment of the SNP AWD population, with reports of multiple litters observed from different adult pairs. However, in late 2017 a CDV outbreak killed at least 19 individuals from the Nyasaori pack. This devastating event again reduced the population to a low number. However, the extent of the damage to the population's stability is not currently known. Inbreeding and a loss of genetic variation arising from disease outbreaks are major concerns for this endangered population. Previously, Marsden *et al* (2012) investigated changes in genetic variation and structure in Serengeti wild dogs following earlier outbreaks of rabies, but this only utilized data up to and including 2009. Overall, this study aims to add to the existing body of knowledge to provide

information on the genetic status of the SNP population following more recent outbreaks of CDV to inform future conservation decisions.

1.6. Emerging infectious diseases

It is estimated that 75% of human emerging infectious diseases originate in animals (Cleaveland *et al*, 2001). The emergence of pathogens into new species has been accelerated in recent years by several factors, such as human activity in wildlife habitats, intensive farming of animals, illegal wildlife trade, and the consumption of wild animals (Tenorio, 2022). Further, the eradication of diseases can pave the way for new pathogens to take over. For example, the eradication of smallpox (Variola virus) by mass vaccination was a great success and saved millions of lives worldwide. However, in recent months the monkeypox virus has been increasingly observed in human hosts. Monkeypox was first diagnosed in a human in 1970 in the Democratic Republic of Congo, with sporadic outbreaks occurring solely in people in contact with wildlife reservoirs (Thornhill *et al*, 2022). This was evidence that monkeypox could infect humans opportunistically. However, secondary spread from person-person was limited and it was thought that the virus could not yet efficiently infect humans (Durski *et al*, 2018; Vaughan *et al*, 2018; Thornhill *et al*, 2022). In 1980 Smallpox was declared eradicated by the World Health Organization (WHO). The absence of Smallpox in the environment created a niche for a similar virus to take over and emerge into human hosts. With evidence that monkeypox could already opportunistically infect humans by undergoing a series of amino acid point mutations (microevolution), it makes sense that this virus adapted to infect more efficiently and rapidly between humans. Since

May 2022 there are approximately 300,000 reported cases globally, although this figure is likely to be underestimated (Thornhill *et al*, 2022).

In recent months the direct impact of emerging disease has become very apparent for humans. However, disease emergence in animals is also increasing. The rinderpest virus of cattle ('cattle plague') devastated livelihoods of people across Europe, Asia, Africa, and the Middle East for thousands of years (Roeder *et al*, 2002; Pastoret *et al*, 2006). The mortality rate of cattle was 80-90% and spread around the globe with the development of international trade (Barrett & Rossiter, 1999). Following the success of the Global Rinderpest Eradication Program, rinderpest was declared eradicated in 2011 (Morens *et al*, 2011). This, however, created a niche for a similar virus, such as PPRV, to infect animals previously susceptible to rinderpest. For example, in Tanzania, wildlife sampled between 2008-2012 during rinderpest surveillance were negative for PPRV. However, in the years following rinderpest eradication, the wildlife sampled were rinderpest negative and PPRV positive (de Swart *et al*, 2012; Logan *et al*, 2016). It is thought that the rinderpest vaccine may have offered cross-protection against PPRV due to the closely related viral proteins (Logan *et al*, 2016; Taylor, 1979). Since the cessation of the vaccine, all recipient animals have since died leaving no rinderpest antibodies in subsequent generations. This means that any protection from PPRV offered by the rinderpest vaccine is no longer there, leaving animals more susceptible to PPRV infection (Taylor, 1979; Mariner *et al*, 1993; Jones *et al*, 1993).

Going forward, the WHO aims to eradicate the measles virus (MV), although this is not without potential cost to human health. It has long been established that vaccination against MV also protects against CDV in many cases (Taylor *et al*, 2001; de Vries *et al*, 2014). For example, following a fatal outbreak of CDV in non-human primates, de

Vries *et al* (2014) showed that the macaques were able to rapidly clear the infection if they were vaccinated with MV vaccine. Although CDV does not infect humans, it can readily infect non-human primates in addition to many other taxa. If MV were to be eradicated and vaccination eventually ended, a niche would be created for a related virus to jump the species barrier into humans, as demonstrated with other viruses such as monkeypox (Rendon-Marin *et al*, 2019). Considering the genetic similarities between humans and primates, including cell receptor sequences, it is entirely possible that CDV could eventually move into humans (Quintero-Gil *et al,* 2019; Rendon-Marin *et al*, 2019; Cosby *et al*, 2018).

1.7. Rationale for the study

1.7.1. Problem statement

Morbilliviruses are highly promiscuous in their host range and present a great threat to livestock owners and protected wildlife species globally, although the dynamics of infection in new host species are not well understood, particularly in mixed-livestock settings where other pathogens and morbilliviruses co-circulate. PPRV has been detected in cattle in northern Tanzania using ELISA techniques, although true serological estimates may be skewed by cross-reactivity from related morbilliviruses circulating in the environment. It is possible that other morbilliviruses, such as CDV, can now infect ruminants in a natural setting. Clinical manifestations of PPRV and CDV in cattle are unknown in the context of natural infection over time, therefore a robust serological and clinical monitoring are required to map infection of cattle to clarify the extent of host expansion.

Further, following decades of fatal CDV outbreaks in Serengeti African wild dogs, changes in neutral and adaptive genetic variation are completely unknown. Previous work by Marsden *et al* (2012) investigated these changes in samples up to and including 2009, however the extensive demographic changes since then may have impacted the genetic viability of remaining packs. An assessment of the changes in population structure and immune markers is required to elucidate the impact of population decline and identify a possible link between outbreaks and the diversity of adaptive markers.

Throughout this PhD several aspects of livestock data collection and generation were produced by a team of people. The roles and people involved are summarized in table A 1.7.1. Additionally, as African wild dog samples were not collected for this PhD, several people aside from the author contributed to this research. Contributions are summarized in table B 1.7.1.

1.7.1. Table A. Study contributions.

Livestock Samples	
Role	**Contributors**
Study design	S. Cleaveland, B. Willett, H. Auty, T. Lembo, U. Pomeroy-Arthur.
Sample collection	U. Pomeroy-Arthur, E Sindoya, L. James.
Sample processing and shipment	U. Pomeroy-Arthur, G. Shirima, I. Tesha, J. Mshanga, H. Auty.
Serological testing and analyses	U. Pomeroy-Arthur, N. Logan.
Clinical data analyses	U. Pomeroy-Arthur.

1.7.1. Table B. Contributions to the African wild dog study.

African Wild Dog Samples	
Role	**Contributors**
<1991, 2001-2009 sample collection	E. Eblate, E. Masenga, S. Cleaveland, S. Lelo, K. Laurenson.
2011-2017 sample collection	E. Eblate, E. Masenga.
2017 outbreak tissue analysis	U. Pomeroy-Arthur.
2017 outbreak sera genotyping and sequencing	E. Kilbride, B.K. Mable, B. Willett.
2017 outbreak data analyses	U. Pomeroy-Arthur, B.K. Mable.

1.7.2. Study justification

Understanding specific morbillivirus infection patterns in cattle and clinical manifestations will aid current research on their role in PPRV and CDV epidemiology in the Serengeti District of Tanzania, within mixed-livestock environments. Assessing changes in genetic structure and diversity of African wild dogs over time will inform research on their genetic progress and future decisions on their conservation.

1.8. Thesis objectives

This thesis comprises three data chapters, overviews, and objectives for which are described below.

1.8.1. Chapter 3

The first data chapter reports PPRV-specific neutralizing antibodies found over a one-year period in cattle across 10 participant households in the Serengeti District of Tanzania (see figure 1.8.1), using the PVNA. The tests conducted on samples in the first time period (September 2018) included: 347 cattle samples, 82 sheep samples, 76 goat samples, and 14 dog samples. The tests conducted on samples in the second time period (April 2019) included 365 cattle samples, 77 sheep samples, 75 goat samples, and 14 dog samples. The tests conducted on samples in the third time period (October 2019) included 292 cattle samples, 59 sheep samples, 57 goat samples, and 6 dog samples. The households from which samples were collected are shown in figure 1.6.1. Results found PPRV-specific antibodies and seroconversion occurring in cattle throughout the study. Patterns of infection were investigated using likelihood ratio tests (LRT). The chapter also reports findings from the clinical monitoring of cattle using logistic regression models for the first time in a longitudinal study of this kind.

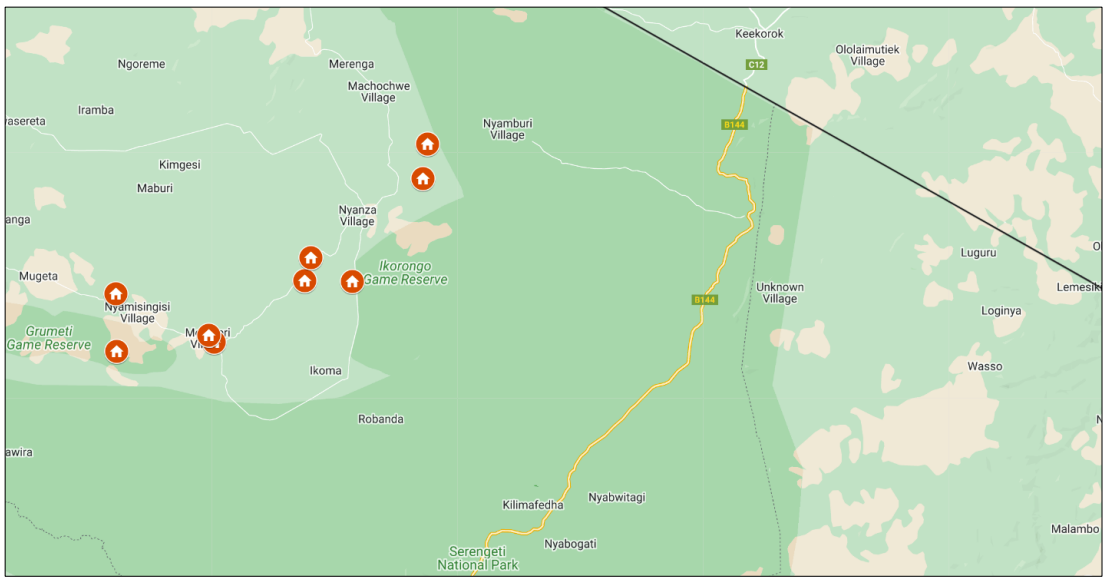


Figure 1.8.1. Map of the Serengeti District of Northern Tanzania showing approximate locations of 10 participant households in this study. (Google Inc.)

1.8.2. Chapter four

This chapter reports the serological and clinical findings of CDV in livestock using the same samples referred to in chapter 3. CDV-specific antibodies were detected in all species using the PVNA and seroconversions were observed in cattle throughout the study. Patterns of infection were assessed using LRT. The association of infection with clinical manifestations was investigated using logistic regression models and results showed for the first time that CDV is ubiquitous in livestock.

1.8.3. Chapter five

Work in this chapter explores the recent and fatal 2017 CDV outbreak in African wild dogs of the Serengeti, assessing the detection of CDV and immune response in wild dog samples. Using sample sets from pre-1991, 2001-2009, 2011-2016, 2017 outbreak tissue (deceased animals), and 2017 outbreak sera (unconfirmed surviving animals), this chapter investigates whether diversity at the MHC DRB1 locus has changed over time using allele frequency analyses. Changes in diversity of neutral microsatellite markers and genetic structuring was also investigated using Bayesian STRUCTURE and principal component analyses. A comparison of changes in adaptive and neutral markers was made to determine if change has occurred due to factors beyond demographic change, using a TPM model for excess heterozygosity. Finally, this chapter assesses the evidence of selection at the MHC DRB1 locus using Tajima's test for selection, and whether there is any association with CDV outbreaks in wild dogs.

Chapter 2 General Materials & Methods

2.1 Longitudinal field study

2.1.1 Study area

The study sites are located within the Serengeti District in the Mara region of northern Tanzania, between the Grumeti and Ikorongo Game Reserves bordering east of the Serengeti National Park (SNP) (Fig. 2). Both game reserves are protected areas for wildlife and the SNP is a UNESCO world heritage site, home to critically endangered wildlife species. The Serengeti District spans approximately 48-104Km from the Kenyan border. The Serengeti district, one of seven districts in the Mara region, spans approximately 10,373Km^2 with a population of 249,420 (NBS, 2012; Shirima & Kunda, 2016). Most households in this region are permanently settled and agropastoralist; they practice a mixture of livestock farming and crop-based agriculture (Kerario *et al*, 2017). Livestock herds in this area vary in size, ranging from one to more than 200 animals (Katale *et al*, 2013).

Geographic mapping of village and household locations was done using Google Map software. Coordinates taken during sampling periods was done using Google location feature and coordinates were given in Universal Transverse Mercator format (Google Inc, California USA). Coordinates initially plotted on the map were enlarged and zoomed out to ensure participants could not be identified from either map

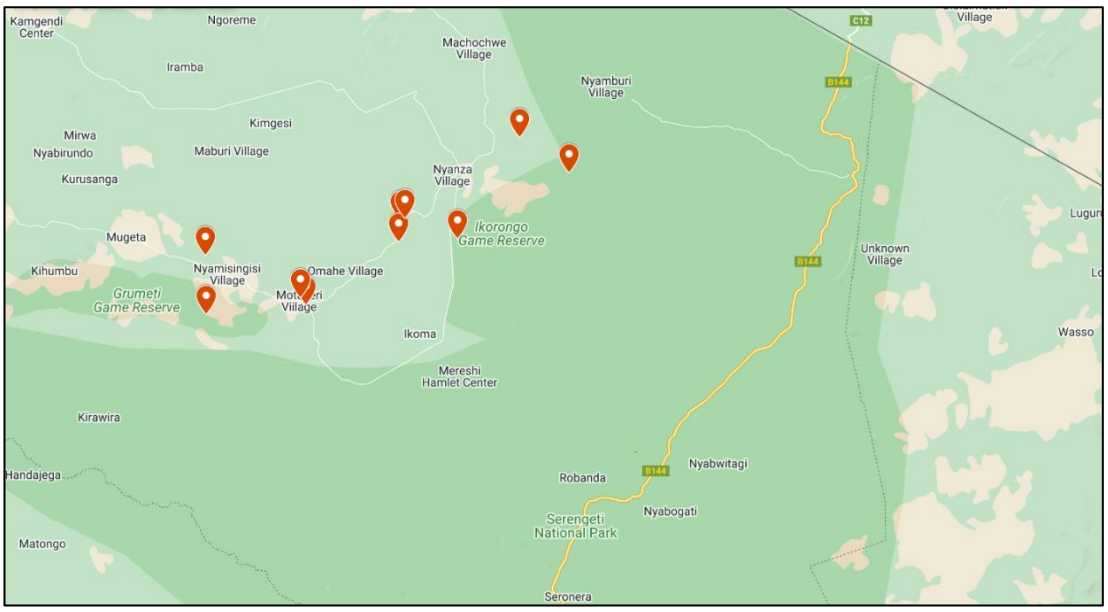


Figure 2.1.1 Map of the study area. Zoomed in map showing the distribution of participant households in relation to the Serengeti National Park, Grumeti and Ikorongo Game Reserves. (Google Inc.)

2.1.2. Household and Animal Recruitment

All households selected were within the Serengeti district boundaries. The cohort comprised one group of ten households that were non-randomly selected to represent households with a high probability of exposure to morbilliviruses. These households comprised: (a) five households that had experienced a disease outbreak in sheep and goats with signs consistent with PPR ('clinical households' and (b) five households involved in a parallel on-going study of foot-and-mouth disease (FMD) which were selected for this study based on previous serological detection of morbilliviruses ('non-clinical households') (Lembo *et al*, 2013)). For each household, approximately 40 cattle, 10 sheep, 10 goats and any domestic dogs available were recruited into the study.

The clinical households were recruited during a suspected outbreak of PPR in July 2018 and included livestock owners who had reported clinical disease consistent with a PPR outbreak in their livestock, to the District Veterinary Officer. These villages comprised Iharara, Nyichoka, Mbilikili, Natta, and Nyamburi. Animals displaying clinical signs of disease (diarrhoea, nasal lacrimation, ocular discharge, sneezing, anorexia, fever) within these households were tagged with unique numbers for later identification when being revisited for the subsequent sampling periods of the study. To ensure enough animals were recruited, once all clinically ill animals were ear-tagged remaining individuals were then recruited in the same way until there were 40 cattle, 10 sheep, and 10 goats with ear tags. The only exception to this method was in the case of small households with fewer animals, in which case all available animals were recruited.

The five non-clinical households were selected from a total of 22 FMD study herds on the basis of results of previous detection of morbillivirus-specific antibodies (PPRV, CDV and RLV) using a pseudotype-based neutralisation assay carried out on samples collected in 2017 and 2018.. There was no clinical basis for selection of these households. Individual cattle, sheep and goats were selected. Animals were identified through ear-tags previously fitted as part of the FMD study.

2.1.3. Sample Strategy

Each of the ten households was visited three times for sampling during the study: September 2018, April 2019 and October 2019. The sampling cohort comprised 40 cattle, 10 sheep, 10 goats and all available domestic dogs for each of ten households

(see figure 2.1.3). To compensate for animal losses after the first sampling period, new animals were recruited and ear-tagged in April 2019. A breakdown of animals sampled at each time point is given in the table below.

Table 2.1.3. Breakdown of animals sampled at each time period within the study.

	Oct-18		Apr-19		Oct-19		
Species	No. Sampled	No. Tested	No. Sampled	No. Tested	No. Sampled	No. Tested	**No. Repeat Sampled (at least twice)**
Cattle	347	141	361	257	292	193	311
Sheep	82	4	77	14	59	15	17
Goats	76	14	75	14	57	12	14
Dogs	14	11	14	13	6	6	6

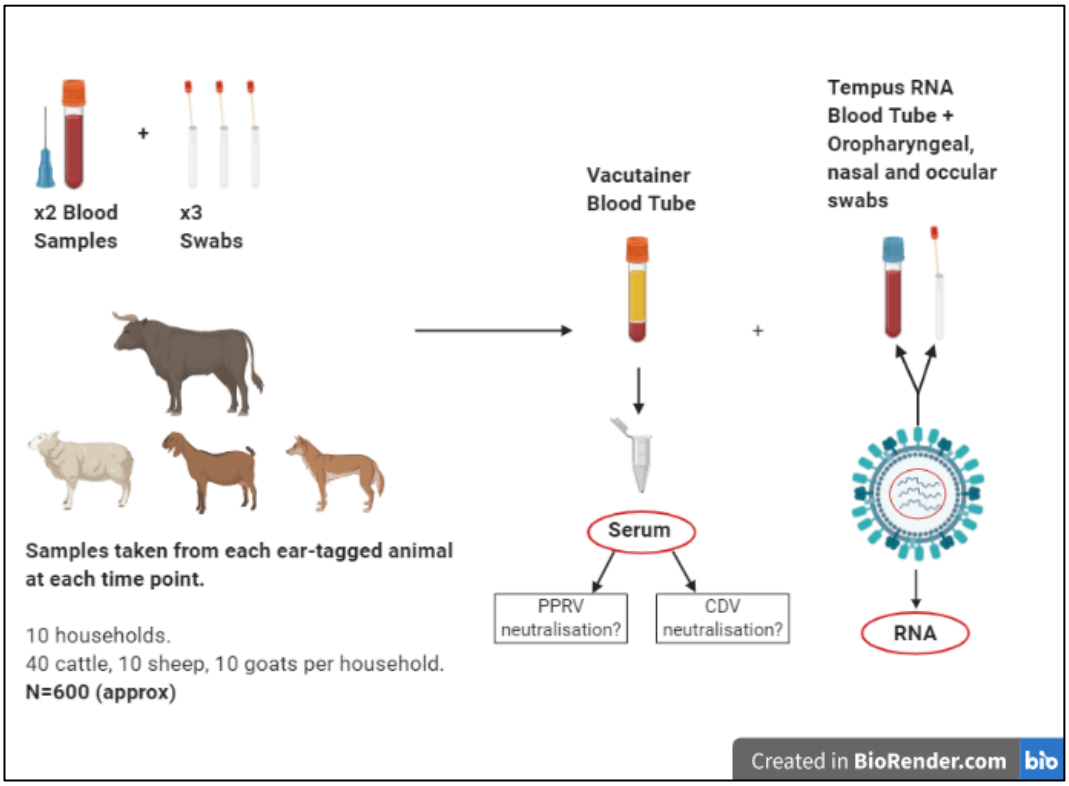


Figure 2.1.3. Breakdown of samples and processes. Samples taken from each

species (left), and the purpose for each sample (right). The RNA samples (right) were unable to be tested due to COVID-19 restrictions, thus no data are available, but this remains a part of the methods used in this study.

2.1.4. Sample Size

The initial sample size calculation for this study was based on the proportion of animals required to detect viral RNA from blood and swab samples over the course of a year, as described in figure 2.1.3. Previous diagnostic studies on natural outbreaks have found PPRV RNA in as few as 1-4% of clinically sick animals during peak infection (Omani *et al*, 2019; Rahman *et al*, 2019). The ideal sample size for this study also aimed to be large enough to detect seroconverting cattle at each time point, given that duration of morbillivirus infection lasts at least one year. Preliminary serological data from bovine morbillivirus research suggests a PPRV seroprevalence of 28%, and CDV seroprevalence of 9% (Sen *et al*, 2014; Balamurugan *et al*, 2012). Therefore, the chosen sample size of 400 cattle would statistically be able to provide positive samples by RT-qPCR and would be expected to result in 112 PPRV and 36 CDV seroconversions.

Due to the change in the scope of the study questions, a retrospective power analysis was done to determine whether the sample size could give enough resolution to answer the serological questions outlined in the aims. The sample size (as shown below) was enough to estimate seroprevalence in cattle at each time point and carry out descriptive analyses. However, a larger sample size may be needed to investigate more complex interactions and patterns of seropositivity.

Given the changing dynamics of herd sizes due to deaths and livestock trade, the initial sample size may decrease with a dropout rate of up to 25%. Further, due to the redirection of the study, the key research questions changed to expand the serology aspect and thus the sample size also accounts for this. Assuming these factors, the serological-based sample size was calculated to detect the number of animals seroconverting and the seroprevalence:

Where: SS = Sample size

P = Percentage seropositive in average population

Z = Standard error given 95%CI (**1.96**)

$SS = (Z\text{-score})^2 * p*(1-p) / (\text{margin of error})^2$

Where: SS = Sample size

P = Percentage seropositive in average population

Z = Standard error given 95%CI (**1.96**)

$SS = (Z\text{-score})^2 * p*(1-p) / (\text{margin of error})^2$

$SS = (1.96)^2 * 0.3*(1-0.3) / (0.05)^2$

$SS = 3.8416 * 0.21 / 0.0025$

$SS = 322$

2.1.5. Ageing of Animals

All livestock being sampled were assessed at each sampling period for their age by dentition, as described by Pace & Wakeman (1983). To avoid language

miscommunication for the local livestock team, diagrams were provided with dental descriptions in the SOP. These diagrams are given below.

Table 2.1.5. A breakdown of the major dental developmental stages in cattle. Adapted from Ensminger (1983).

	Age	**Description**
	Less than one year	Some or all temporary incisors are present.
	One to two years	Central pair of temporary incisors are replaced by permanent incisors.
	Three to four years	First and second intermediates either side of the pinchers are replaced with permanent teeth. They begin to wear at four years.
	Five to six years	Permanent pinchers and incisors are levelled, and corner teeth begin to wear.
	Seven or more years	Pinchers, middle pairs, and corner teeth show increased wear. Teeth begin to separate and appear triangular in shape. Mouth arch begins to straighten out.

2.1.6. Protocol for blood sampling (data not included due to COVID-19 impact)

Individual animals were restrained and carefully handled by trained livestock officers. Ear tagged cattle, sheep and goats were sampled in the same way once restrained. A sterile 18G (cattle) or 21G (sheep and goats) 1.5-inch needle was inserted into the animal's jugular vein at a 45° angle through a vacutainer collection cup. Once blood appears in the cup, a vacutainer tube is inserted into the cup and is pierced by the needle, allowing the flow of blood into the tube. Once filled, the tube is disconnected and thoroughly wiped with methylated spirits before being labelled and placed in a cool box. The vacutainer tubes were centrifuged once returned to the Mugumu research base in Serengeti District after the household sampling was complete. The 9mL Tempus RNA tube is then placed in the collection cup in the same way as the vacutainer. Once filled to capacity (3mL blood) the tube is removed and vigorously shaken for 10 seconds to allow complete lysis of blood cells and stabilization of any RNA products. The tube is then cleaned with methylated spirits and labelled before being placed on ice packs in the cool box. The needle is then carefully removed from the vein and placed into a biohazard sharp bin, and the cup is washed with water and methylated spirits. The area of needle insertion is cleaned with methylated spirits before swab sampling commences. The protocol for blood sampling was taken from Qiagen Tempus RNA reagent kit (ThermoFisher Scientific, Warrington, UK).

2.1.7 Protocol for swab sampling (data not included)

Swabs were targeted to the ocular, nasal and oropharyngeal cavities to maximise the likelihood of detecting excreted virus, as demonstrated in experimental infection

studies (Couacy-Hymann *et al*, 2007). Following blood sampling, a sterile cotton swab was taken and inserted gently into the corner of the eye and under the upper eyelid. To reduce the number of consumables and increase chance of virus detection, the same swab was then used in the nasal cavity before being clipped and placed in a 2mL cryovial tube containing RNA Later preservative. A second swab was then taken and inserted to the back of the mouth before being placed into the same tube as the first swab, with extra care taken to ensure cotton tips were in the preservative. This tube was then cleaned with methylated spirits and placed in a secure bag on the ice packs in the cool box. The swab protocol is a standard procedure carried out routinely by the livestock officer.

2.1.8 Clinical signs scoring

All ear-tagged animals in each household was assessed for clinical signs consistent with PPRV and CDV in known hosts as clinical signs of PPRV and CDV in cattle have not been documented. These included nasal and ocular lacrimation, pneumonia, anorexia, fever, diarrhoea, lethargy, and neurological problems based on predominant clinical signs of PPRV in sheep and goats (Parida *et al.* 2015) and CDV in dogs (Pope *et al*. 2016). During sampling this was achieved using a tick-box protocol with a list of clinical signs. An example of this layout is shown below. During monthly clinical signs monitoring the information collected was restricted to the ear-tag number and list of clinical signs of disease specific to PPRV and CDV infection in known hosts.

Table 2.1.8. Table showing the layout of sampling information collected for each animal in the study.

Cattle 1	**Clinical Signs**	**Samples collected**
Age: Sex: Ear Tag Number: Is this animal a calf kept with sheep and goats? Yes / No	Diarrhoea □ Nasal Discharge □ Ocular Discharge □ Oral Lesions □ Anorexia □ Other?	Ocular/nasal/oropharyngeal swabs □ Whole blood for serum □ Whole blood for Tempus Tube □
Has this animal received any treatment in the last 6 months?		

2.1.9. Additional collected data

Following sample collection at the boma, livestock owners were asked questions as part of the data collection surrounding PPRV and CDV infection. Acute PPRV and CDV infections can run their course in host populations within fourteen days, thus the questions aimed to gather information on any signs of clinical disease between the sampling periods of six months (Hammouchi *et al*, 2012). This was done by asking the following main questions:

- Have your cattle, sheep or goats suffered any disease in the last six months? If so, how?

- Have you observed any signs of PPR in your cattle, sheep, or goats in the last six months? Please give details.
- Have your animals been ill in the last six months? If so, how?
- Have your animals been vaccinated? Please give details.

An example of the questionnaire can be found in the appendix. The additional questions provided herd-level information which included relevant information for animals not ear-tagged. Vaccination information was collected to ensure any antibodies detected were due to natural infection. All questions were asked in English with translation into Kiswahili by the Veterinary Livestock Officer, and vice versa with answers.

2.1.10. Clinical Monitoring

Between April and October 2019 all households were visited each month by the Livestock Field Officer to assess ear-tagged animals for any clinical signs of disease. This was done using a list of ear-tag numbers in numerical order, and a column for each clinical sign of PPRV/CDV infection. If any clinical signs were observed in an animal, it was ticked on the sheet.

2.1.11 Ethical Clearance and Consent

Work carried out for this study in Tanzania was permitted by the Tanzania Commission for Science and Technology (COSTECH, permit number 2019-495-NA-2018-329) and SRUC Animal Experiments Committee. All study participant

households were provided with a participant information document written in English, which was verbally translated to Kiswahili by the Veterinary Livestock Officer. Separate consent forms were provided at the beginning of each sampling period before any sampling or participant questionnaires were carried out. Signatures of the livestock owner, Veterinary Livestock Officer and lead researcher were recorded on all consent forms before commencing.

To access the field sites and base in Mugumu, permits were obtained from the Tanzanian Wildlife Research Institute due to the close proximity of the study sites and protected areas.

2.1.12 Data Protection

In accordance with the General Data Protection Regulation (2018) and Data Protection Act (1998), all participant information was pseudo-anonymised by assigning codes to all study households. During sample analysis and statistical analyses households were referred to by codes only. No person other than the lead researchers had access to the identities of these households. All data collected (with no personal identifiers) on paper were filed and stored in a secure location. Data was transferred manually to a Microsoft Excel spreadsheet in a secured folder on OneDrive (University of Glasgow) and Dropbox with password-protected access only. Visualisation of participant households on a map was limited to village-level identification.

2.2 Laboratory Methods

2.2.1 Sample processing and storage

At NM-AIST plastic sealable bags containing the sera collected in 2mL cryovial tubes were sprayed and wiped with 10% Virkon^{TM} to eliminate contamination with pathogens including FMDV, as shown by Hole *et al* (2016). Bags containing sera tubes were then placed in a water bath at 56°c for two hours to eliminate active virus within tubes. Following this heat-treatment, bags were removed from the water bath. Enclosed inside each bag were three bags of serum aliquots. These bags of aliquots were each emptied onto a tray filled with 10% Virkon where they were thoroughly wiped, dried, and sprayed with 70% IMS before being placed into new sterile, labelled bags. All new sterile bags of sera were packaged on a separate laboratory bench to minimise risk of cross-contamination. Samples were then stored at -80°c.

2.2.2 Sample shipment

Sera and RNA samples (see table 2.2.2) were packaged at NM-AIST in TC-20 thermal control units (AirSea, Merseyside, UK) containing 20Kg dry ice and ice packs per unit. The shipment port of exit was Kilimanjaro International Airport. Export of samples from Tanzania was authorised by the Tanzanian Ministry of Livestock and Fisheries (permit number **VIC/AR/ZIS/1595**). Importation of samples to the UK was authorised through a **TARP(s) 2019/15** permit issued by the Scottish Government. Delivery of the shipment was completed by courier to the University of Glasgow, where samples were removed from thermal control units and stored at -20°c for testing.

Table 2.2.2. Samples obtained and processed.

Sample Type	**Specification**	**Processing**	**Storage**
Blood 10mL	Vacutainer Blood Tube, Qiagen	Centrifugation for 10 minutes at 1300G. All sera were extracted and divided into three aliquots.	-20°c
Blood 3mL (Tube contains 6mL stabilizing reagent)	Tempus RNA Blood Tube, Qiagen	Tubes vigorously shaken for 10 seconds before storing.	-20°c initially before transfer to -80°c.
Swabs (Nasal, ocular, oropharyngeal)	Cotton tip plastic swabs, Medical Wire	Swabs placed in 2mL screwcap tubes with 300uL RNA Later (Qiagen) before storing.	-20°c initially before transfer to -80°c.

2.2.3. Production of target cells for measuring virus neutralising antibodies

A cDNA encoding the canine SLAMF1 (signalling lymphocyte activation molecule) from a domestic dog (*Canis familiaris*) was amplified from RNA previously extracted from mitogen-stimulated canine peripheral blood mononuclear cells (Logan *et al*, 2016) using the primers dogSLAM Bgl: 5 -GCTCAGATCTGAGAGCTTGATGAATTGCCCAG-3 and dogSLAM Sal: 5 -GCTCGTCGACGCTCTCTGGGAACGTCAC-3. The dogSLAM cDNA was

digested with BglII and SalI and ligated into mammalian expression vector pDisplay (Thermofisher Scientific, Warrington, UK) as described previously Logan *et al* (2016). HEK293 cells were then transfected with the pDisplay-dogSLAM construct utilising linear polyethyleneimine, MW 25,000 (Polysciences Inc, Northampton, UK). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (Merck Life Science UK Ltd, Gillingham, UK), 2mM L-glutamine, and 100IU/ml penicillin and 100µg/ml streptomycin (complete DMEM). Cells were then resuspended in complete DMEM and selected with 800µg/ml G418 (Thermofisher Scientific, Warrington, UK). Cells were maintained in T150 flasks (Merck Life Science UK Ltd, Gillingham, UK) and passaged approximately every two days once cells reached confluency.

2.2.4. Pseudotype production

A recombinant vesicular stomatitis virus (VSV) lacking the glycoprotein (G) which was replaced with a firefly luciferase gene (*luc*) was previously produced (VSVΔG*luc*) (Whitt, 2010). The haemagglutinin (H) and fusion (F) protein sequences of the vaccine strain of PPRV (Nigeria 1975/1) cDNA were amplified using the following primers:

- PPRV-H-NotWtF: 5 -CCGGCGGCCGCACCATGTCCGCACAAAG-3,
- PPRV-H-BamHIR: 5 -GGGGGATCCTCAGACTGGATTACATGTT-3,
- PPRV-F-Wt-NotF: 5 -GGGGCGGCCGCACCATGCATGCGCCGA-3,
- PPRV-F-BamHI-WtR: 5 -GGGGGATCCGCCTACAGTGATCTCACGT-3,
- PPRV-F-BamHI- D633R: 5 -GGGGGATCCTGGTTATCTCCCCTTACAG-3.

Restriction sites within the PPRV amplicons were Bam*HI* and *NotI*. The H and F gene sequences of the vaccine strain of CDV (Onderstepoort) cDNA were amplified using the following primers:

- CDV H Sal: 5 -GTCGAC-ACC-ATGCTCCCCTACCAAGACAAGGT-3,
- CDV H Not: 5 -GGGCGGCCGC-TTAACGGTTACATGAGAATCTTA-3,
- CDV F Sal: 5 -GGGTCGAC-ACC-ATGCACAGGGGAATCCCCAAAAG3,
- CDV F D633 Not: 5 -GGGCGGCCGC-TTGCTAGCGTCTTTTACAACAGTAAATCAGCA-3.

Restriction sites within the CDV amplicons were *SalI* and *NotII*. Both PPRV and CDV amplicons were amplified using the following thermocycling parameters:

Table 2.2.4. Thermocycling conditions for PPRV H/F and CDV H/F.

Step	**Temperature**	**Time**	**Cycles**
Denaturation	94◦C	5 mins	1
Denaturation	94◦C	30 sec	35
Annealing	50◦C	60 sec	1
Extension	72◦C	120 sec	1
Extension	72◦C	10 mins	1

PCR products were digested with *SalI* and *NotI* and ligated into the expression vector VR1012 (Vical Inc.). The transformed VR1012 vectors containing PPRV H and F, and CDV H and F proteins were transfected into HEK293T cells before superinfection of cells with VSVΔG*luc* (Whitt, 2010) in 10cm culture dishes (Merck

Life Science UK Ltd, Gillingham, UK). After 48 hours incubation at 37◦C, pseudotypes were harvested and split into 3ml aliquots before being frozen at -80◦C. The titre of each batch of pseudotypes was determined as described previously by Logan *et al* (2016), whereby the titre is the point at which 90% neutralization occurs.

2.2.5 Neutralisation assays

Sera collected during the study were diluted 1:32 with complete DMEM and added to 96-well white flat-bottomed plates (Greiner Bio-One, UK) in triplicates of 25µL. Pseudotypes were diluted in complete DMEM at a concentration of 2.5 x 10^3 $TCID_{50}$, and 25µL was added to each well. Plates were incubated for 45 minutes at 37◦C. Confluent HEK293T cells expressing dogSLAM were added to wells in volumes of 25µl at a concentration of 8 x 10^5 cells per ml. At 48 hours post-infection, luciferase activity was quantified by the addition of 75µL of Steady Lite substrate solution (Perkin Elmer Ltd) each well. Luciferase activity was quantified using an EnSight multimode plate reader (Perkin Elmer, Coventry, UK) to quantify luciferase counts per minute per well. Lower counts per minute indicated reduced signal from expressed *luc* gene as a consequence of antibody-specific neutralisation. Higher counts indicated less signal inhibition suggesting an absence of specific neutralising antibodies. A negative, no-serum control was included on each plate.

2.2.6 Definition of seropositivity

The antibody titre was defined as the serum dilution at which neutralising activity was estimated to reduce luciferase activity by 90% (see figure 2.2.6). Hence, as the initial serum dilution was 1:32, sera were considered positive for neutralising activity

if at a dilution of 1:32 or greater, luciferase activity was reduced by 90% (Logan *et al*, 2016). Each serum sample was titrated by serial dilution. Antibody titres were calculated by estimating the point at which the luciferase counts (CPM) were reduced to 90% of the negative control (no serum) wells on the same plate. The 90% points were predicted using the INDEX and MATCH functions in Excel. For example, the 90% point would be calculated by taking the mean value of the three replicates for the no serum control wells and multiplying by 0.1. This value would then be MATCHed with the mean values in the 7 serum dilutions and the predicted 90% titre calculated using an interpolation formula using INDEX.

Given the complexity of the ecosystem, animals may be exposed to more than one morbillivirus. Similarly, some epitopes may be shared between morbilliviruses, hence individuals may possess neutralising antibodies that either a) recognise epitopes conserved between morbilliviruses or b) have been elicited by exposure to a related morbillivirus. Previous work shows that an animal generates a higher titre of neutralising antibody against the virus to which it was exposed (Logan *et al*, 2016). However, if an animal is exposed to more than one virus it is possible that it could elicit an immune response specific to each pathogen. Hence, for the purpose of this study, if an animal is seropositive for antibodies neutralising more than one morbillivirus, it is assumed that it was exposed to more than one morbillivirus, with the caveat that the animal may be cross neutralising the virus against which it elicited the lower titre.

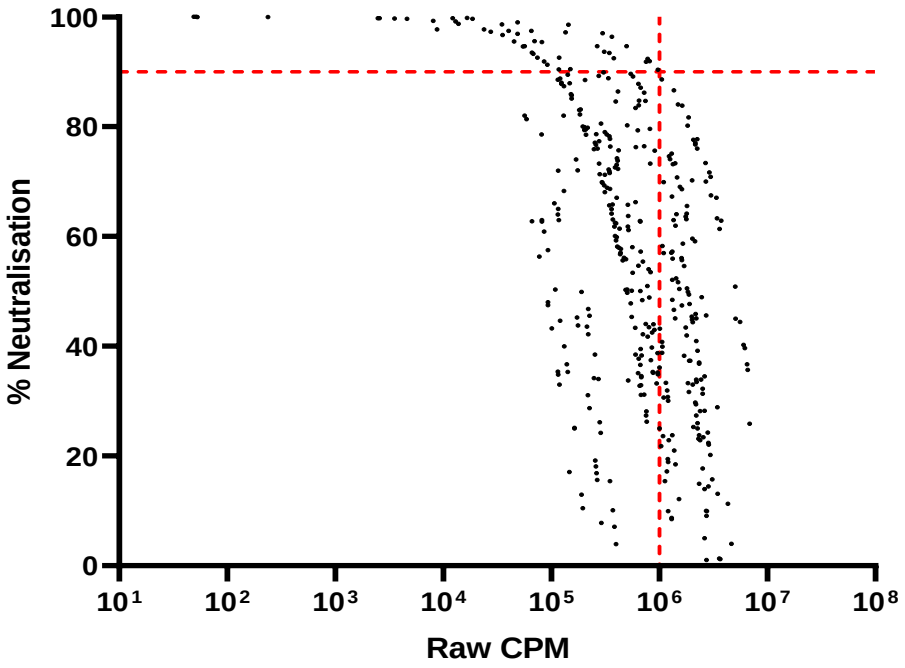


Figure 2.2.6 Scatter plot showing the percentage neutralisation (y axis) against the raw number of luciferase CPM. The y-axis cut-off is marked at 90% neutralisation, above which are samples considered seropositive. The cut-off point on the axes mark the lowest CPM value of the negative controls and the highest CPM value of samples with at least 90% neutralisation relative to those negative controls ($y = 1.1 \times 10^6$). The area above the line at y and to the left of the line at x contains the samples considered seropositive.

2.2.7 Calculation of Titre

Serum samples which demonstrated neutralisation at IC90 compared to the negative control were considered to be positive, as shown by Logan *et al* (2016). To determine if this was a true result, these samples were further tested using a serial dilution of sera ranging in concentration from 1:8 to 1:32,768, whereby the dilution of serum is doubled each time. If a serum sample exhibits 90% neutralisation of *luc*-

expressing pseudotyped virus at a dilution of 1:32,768 compared to the negative control then it was considered truly positive. The titre of pseudotyped virus was calculated using the Spearman-Karber method as demonstrated by Nicholson & Prestage (1982), whereby the titre (intercept) is 2.5 standard deviations from the mean counts per minute of the negative control:

Intercept x = 2 + $\frac{1}{B}$ [E - $\sqrt{E^2}$ - 2B (Y_2 - D)],

and the end-point titre of the test sample is X = d * nx^{-1}

The titre of positive sera was calculated by plotting data on a simple XY graph with a fitted line, estimating the point at which there was 90% reduction of luciferase activity, using the equation:

Y = a * X + b, IC90 = (0.9 – b)/a.

2.2.8 Data Management and cleaning

All serological data for this study were analysed and figures created using GraphPad Prism Software version 8 (GraphPad Software, San Diego, USA).

Data for this study contained sensitive information including evidence of PPRV and CDV infection in cattle within various participant households. Data was kept in a secured folder on OneDrive (University of Glasgow) and a secure Dropbox account where all data files and versions were uploaded. Cleaning of data was done using Microsoft Excel 365 (Microsoft Inc, Washington, USA) and R Studio software version 1.3.1073 (RStudio, Boston, USA). Data cleaning involved removal of errors

and unnecessary characters in the data set, and formatting values (binary, categorical, continuous) to ensure consistency in the data within variables. In RStudio the 'recode' and 'na.strings' functions were used to reorganise variables (see appendix for full script).

2.2.9 Study Limitations

There were several limitations to this study. Firstly, a widespread outbreak of FMDV occurred at the beginning of the study, thus transport of heat-treated sera from households experiencing active FMD infection was impossible. As this study was building on earlier longitudinal studies that had involved repeat herd sampling (see 2.1.3), this resulted in gaps in the longitudinal data for investigating patterns of seroconversion in cattle for some households.

A further limitation of this study was the aging of animals by dentition. This was carried out by a trained livestock officer; however errors can still be made particularly if the animal is not well-restrained. Estimations of age using this method can also be affected by other factors, such as breed and level of inbreeding (Mwacharo *et al*, 2006). In instances where an animal could not be assessed by dentition, the recorded age was entered as the age reported by the livestock owner, based on their recall with prompts from the enumerator, such as the number of offspring produced Finally, swab and Tempus blood RNA samples were not analysed using RT-qPCR as initially planned. This was a consequence of the SARS-CoV-2 outbreak causing the COVID-19 pandemic and restrictions in laboratory access. Initial objectives relating to cattle viraemia, and shedding could therefore not be addressed in this study.

3.1. Summary

Peste des petits ruminants virus (PPRV) infection has previously been documented in cattle in Africa, including northern Tanzania. However, given that other morbilliviruses also circulate in the environment, questions remain about the specificity and interpretation of cattle seropositivity. Limited data are available on the clinical manifestation of natural PPRV infection in cattle. Further, the relationship between PPR infection in cattle, sheep, and goats in not understood in mixed livestock systems. The strength of the antibody response using the pseudotyped virus-based neutralisation assay (PVNA) provides a case definition for seropositivity in cattle which accounts for potential cross-reactivity with other morbilliviruses. Samples (n=400) collected at each of three time points from 10 households were tested using the PVNA. Findings confirmed specific seroreactivity to PPRV in cattle from all households. There was no association between cattle seropositivity and clinical signs in either cattle, sheep, or goats, consistent with previous findings of sub-clinical infection in cattle. Seroconversion in cattle was also detected when there were no reported clinical signs of infection in cattle, sheep, or goats. These findings suggest that PPRV can circulate below current thresholds of disease detection in mixed livestock environments.

3.2. Introduction

PPRV poses one of the greatest threats to the global livestock trade and livelihoods of subsistence farmers worldwide (Baron *et al*, 2017). In recent years there has been a growing body of evidence showing that the host-range of PPRV is expanding to infect new hosts, including bovine species (Pruvot *et al*, 2020; Schulz *et al*, 2019). The virus has rapidly spread in recent years to include much of Asia (China, Mongolia, Tibet), over 60% of the African continent, and was first reported in Europe in 2016. Since global demand for livestock trade is expected to at least double in the next decade, this virus is subject to prioritised eradication efforts by the OIE and FAO by 2030 (OIE, 2015; Kumar *et al*, 2014). This follows the example of the closely related rinderpest virus of large ruminants, successfully eradicated in 2011 (FAO, 2015; ul-Rahman *et al*, 2018).

Several studies show that cattle are able to produce antibodies to PPRV when exposed in a natural setting (Anderson & McKay, 1994; Abraham *et al*, 2005; Khan *et al*, 2008; Prajapati *et al*, 2021). The studies span a range of geographic locations, including serological surveys across Pakistan (Khan *et al*, 2008), Ethiopia (Abraham *et al*, 2005), Nepal (Prajapati *et al*, 2021), and Tanzania (Lembo *et al*, 2013). Recently, Aguilar *et al* (2020) found an overall seroprevalence of 19% in cattle residing near PPRV infected wildlife in Kasese District, Uganda. Analysis of bovine sera collected from the Serengeti ecosystem between 2005 and 2012 (Logan *et al*, 2016) demonstrated neutralising antibody titres against PPRV. These findings suggest that cattle are capable of hosting the virus and launching a humoral response.

Previous serological studies of mixed livestock populations suggest that PPRV seroconversion in cattle results from spill over from small ruminants in endemic areas (Herzog *et al*, 2020; Balamurugan *et al*, 2011). This has been a recurring finding from epidemiological studies on integrated farming systems (Balamurugan *et al*, 2011). However, the role of cattle in PPRV circulation within livestock herds remains unclear and undefined. To date, no longitudinal studies have been conducted to monitor seroconversion of cattle in a natural, multi-species livestock setting. Clinical data from PPRV endemic locations indicate that while cattle do seroconvert, it is limited to sub-clinical infection (Schulz *et al*, 2018; Couacy-Hymann *et al*, 2019). Despite this observation, a natural outbreak of PPRV was reported in wild buffalo (*Bubalus bubalis*) from southern India in 1997 (Govindarajan *et al*, 1997). PPRV was isolated from infected buffalo and inoculated into naïve buffalo calves, which subsequently developed clinical disease and died (Govindarajan *et al*, 1997). More recently, other bovine species have been reported to have suffered clinical outbreaks of PPRV for the first time, such as water deer (*Hydropotes inermis*), Mongolian Saiga (*Saiga tatarica mongolica*), ibex (*capra sibirica*), and gazelle (*Gazella subgutterosa*) (Zhou *et al* 2018; Pruvot *et al*, 2020). This expansion of the PPRV host range to include more subfamilies of *bovinae* raises the question of whether cattle could be susceptible to clinical disease in a natural setting (Baron *et al*, 2016). It is important to establish the parameters of PPRV infection in cattle to determine their epidemiological role in livestock communities. This will be essential for successful disease elimination.

It has been suggested that the eradication of rinderpest created a ‘niche’ in bovine host species which could potentially be occupied by a closely related, emerging virus such as PPRV (de Swart *et al*, 2012). This ‘niche’ could be filled by PPRV if the receptor-binding domain of the H surface glycoprotein adapts to recognise bovine cell receptors. Sequence similarities between surface antigens have previously been shown to elicit cross-reactive antibodies (Logan *et al*, 2015; Logan *et al*, 2016). This occurs if antibodies are raised against a conserved domain on the glycoprotein and is thought to offer some cross-protection for the host (Holzer *et al*, 2016; Dardiri *et al*, 1977). An example of this was observed when rinderpest (before eradication) was found to produce antibodies which cross-neutralised PPRV (Abubakar *et al*, 2017; Nambulli *et al*, 2016; Holzer *et al*, 2016). However, after a decade following the elimination of rinderpest, there are very few, if any, animals remaining which received the rinderpest vaccine. Rinderpest antibodies are no longer circulating in livestock, thus any cross-protection previously offered is no longer available. For this reason, it is thought that cattle may be susceptible to PPRV infection, the degree to which requires more clarification.

Nearly all studies on cattle seropositivity utilise the cELISA assay which has important limitations addressed in this study. The cELISA has been found to have lower specificity than live virus neutralisation assays (VNA) in several studies on PPRV (de Swart *et al*, 2005; Anderson *et al*, 1991; Diallo *et al*, 2003; Couacy-Hymann *et al*, 2007). The VNA has previously been used as the ‘gold standard’. However, VNA assays are labour-intensive and restricted to containment level 3 laboratories, thus the ELISA is commonly utilised for PPRV antibody screening (Baron *et al*, 2014). It is not uncommon for a PPRV ELISA to be positive when

tested with a related morbillivirus such as CDV (Logan *et al*, 2016). This diagnostic limitation, combined with the general lack of antibody surveillance in cattle, means that PPRV seroprevalence estimates for cattle are likely to be inaccurate.

Recently the pseudotyped virus-based neutralisation assay (PVNA) has shown a much-improved specificity of antibody detection in ruminants, including cattle (Logan *et al*, 2016). This method allows for the expression of PPRV-specific glycoprotein sequences in a deactivated model virus, suitable for use in containment level 2 laboratories. Further, based on glycoprotein sequence specificity, the PVNA can differentiate between morbilliviruses which previous assays could not. Thus, using this method it is possible to determine with greater confidence if cattle are seropositive for PPRV, even in the presence of cross-reactive morbilliviruses. Such a tool is critical in clearly defining parameters of cattle infection with PPRV. Although it has been reported that cattle can maintain antibodies indefinitely following exposure, the relationship between cattle seroconversion and infection of small ruminants is not well understood (Herzog *et al*, 2019). It is important to investigate this relationship to confirm whether cattle infection is indeed spill over from sheep and goats, to inform wider vaccination efforts in small ruminants.

Several experimental studies have described cattle as sub-clinically infected dead-end hosts of PPRV (Schulz *et al*, 2019; Couacy-Hymann *et al*, 2019; Dou *et al*, 2020). Some of these studies (Couacy-Hymann *et al*, 2019; Schulz *et al*, 2019) assessed cattle for clinical signs of disease once experimentally infected with strains of varying virulence and found no evidence of clinical manifestation, except for one calf which developed acute fever (Couacy-Hymann *et al*, 2019). Experimental

studies provide very useful data on infection and transmission within a controlled environment, however there are limitations. Laboratory experiments cannot replicate the natural environment. Further, in the natural setting animals are exposed to a multitude of other pathogens which could influence the competency of their immune response (Kumar *et al*, 2018; Jones *et al*, 2020). Some studies have documented no clinical disease in cattle in their natural environment. Balamurugan *et al* (2013) analysed sera from buffalo, cattle, sheep, and goats across five endemic states in India. The target locations frequently experienced PPR outbreaks, although non-lethal, sub-clinical, or unapparent infection was suspected to occur in mixed livestock communities (Balamurugan *et al*, 2013). Overall seroprevalences were 47% in sheep, 39% in goats, 11% in cattle, and 16% in buffaloes (Balamurugan *et al*, 2013). This study supports the possibility of sub-clinical bovine infection, although clinical analysis was limited to retrospective participant questionnaires. Özkul *et al* (2002) conducted serological and clinical assessment of cattle, sheep, and goats across Turkey. Findings confirmed by cELISA that cattle seropositivity was low (0.9%), and only occurred in farms with clinically ill sheep and goats. No clinical signs of PPRV were observed in any of the cattle sampled (dyspnoea, oculo-nasal discharge, and ulcerative stomatitis), (Özkul *et al*, 2002). However, these studies are no longer recent and are cross-sectional, therefore only representing one point in time. Moreover, there is little research on the relationship between PPRV seroconversion of cattle and clinical disease in cattle, sheep, or goats. No study to date has monitored mixed livestock herds in this context within a natural, longitudinal framework.

This chapter will address the following gaps in current knowledge of the role which cattle play in livestock communities endemic for PPRV. The aims are:

- To investigate seropositivity in cattle using the pseudotype-based virus neutralisation assay (PVNA) in comparison with the IDVet cELISA.
- To investigate patterns of cattle seroconversion in relation to clinical signs consistent with PPRV in cattle.
- To investigate patterns of cattle seroconversion in relation to clinical signs of PPRV in sheep and goats.

3.3. Chapter methods

Details about the design of the study, including field sites, herd and individual selection, sampling and serological testing protocols and sample size calculations are given in Chapter 2.

3.3.1. Study Population

Households were selected for this study on the basis of either i) reported clinical signs of PPR in the weeks prior to the study, or ii) previously detected PPR antibodies during an unrelated study. All households selected had similar management systems, in that they graze their cattle, sheep and goats together at pasture during the day and keep them separated in the boma at night.

3.3.2. Case definition

A positive titre within the PVNA is defined as the point at which there is 90% neutralisation of pseudotype virus , indicated by luciferase counts per minute. The calculated titre translates as the dilution at which the sample can be diluted with antibodies still detectable. A titre below 15 is considered negative because at such a dilution antibodies in a sample should be detected if they are present. Therefore, the lower cut-off point for the PVNA is 15, a titre above which is considered seropositive. For the ELISA the recommended cut-off point is 50%, below which is considered positive and translates to the level of uninhibited light. If a sample inhibits less than 40% the sample is considered negative, therefore the upper cut-off point is 60%. Values falling between 50-60% inhibition are potentially false-positives or weak positives; thus the cut-off point for the purposes of this study were kept at 50% inhibition.

3.3.3. R packages and output

All data and statistical analyses were done using R version 4.0.2. Packages utilised for cleaning and visualisation were ‘dplyr’ and ‘ggplot’. Statistical analysis was done using the ‘glm’ and ‘anova’ functions. Likelihood ratio tests were done by running the ‘chisq’ command within the ‘anova’ function. All other statistical functions were done within the ‘dplyr’ and ‘ggplot’ packages. All R scripts were marked down using the ‘knitting’ function and can be found in the appendix. All data output was saved in a secure folder on University of Glasgow OneDrive with password protection.

3.3.4. Statistical Tests

For the PVNA and ELISA results in section 3.2 a chi square test was done to compare observed and expected results. This was done using the ‘chisq’ function in

R. For logistic regression models in section 3.9 the odds ratio was calculated using the ‘exp(coef())’ command in R. Confidence intervals were calculated for all factors of the models using ‘(exp(confint())’ command in R. To compare models to determine which best fit the data, a likelihood ratio test (LRT) was done using ‘anova()’ command. The ‘chisq’ command was then used to test how the observed models differed from the expected outcomes.

3.3.5. Seroconversion

A generalised linear model (GLM) was used to measure three specific questions:

1) If there is an association between cattle seropositivity and household or sex.
2) If there is an association between cattle seroconversion and clinical signs of PPRV in cattle over time, based on known clinical signs in small ruminants.
3) If there is any association between cattle seroconversion and clinical signs of PPRV in small ruminants over time.

Outcome variables were binomial (positive, negative) and coded as ‘0’ or ‘1’, respectively. Explanatory variables were species and sex. Household was included as a random effect to account for multiple data points for animals which were repeatedly sampled. Models were fitted with both explanatory variables, followed by the exclusion of either variable. An ANOVA was then used to compare each version of the GLM, the result of which was given as a likelihood ratio.

3.3.6. Questionnaire data

The summarised results below are from the answers given by study participants.

- Have you seen any signs of disease in your cattle in the last 6 months?
- Have you seen any sign of disease in your sheep and goats in the last 6 months?
- Are your cattle, sheep and goats confined and grazed together?

3.4. Results

3.4.1 Confirming PPRV seropositivity in cattle using the PVNA.

A total of 947 serum samples were tested using the PVNA throughout the study. Of these, 100 cattle samples were randomly selected from the first and second time points for testing with the cELISA. A summary of serum samples tested with each assay are summarised in the below table.

Table 3.4.1. Summary of samples tested at each time point with the cELISA and PVNA assays.

Time Point	N	Tested with PVNA	Tested with cELISA
September 2018	347	347	50
April 2019	365	365	50
October 2019	292	292	-

Overall, 13% (n = 100) were positive on the ELISA (figure 3.4.1). Results for these individuals were then matched with their corresponding PVNA results, which are plotted against each other in figure 3.1. Samples below the cELISA threshold (40%) and above the PVNA threshold ($>1.5x10^1$) are cattle which were seropositive on both assays (8.7%). Above this shown in the top right are cattle which tested positive on the PVNA test and negative on the cELISA (7%). In total, 4 samples were positive by cELISA alone, although the competition values were weak (ranging from 40-

49%). Pearson correlation indicates a weak negative relationship between PVNA titres and percent competition on the ELISA. This means that as the titre decreases in the PVNA, the percentage competition increases on the ELISA. Due to random selection of these samples from across time periods, some cattle may be represented by multiple data points in the plot.

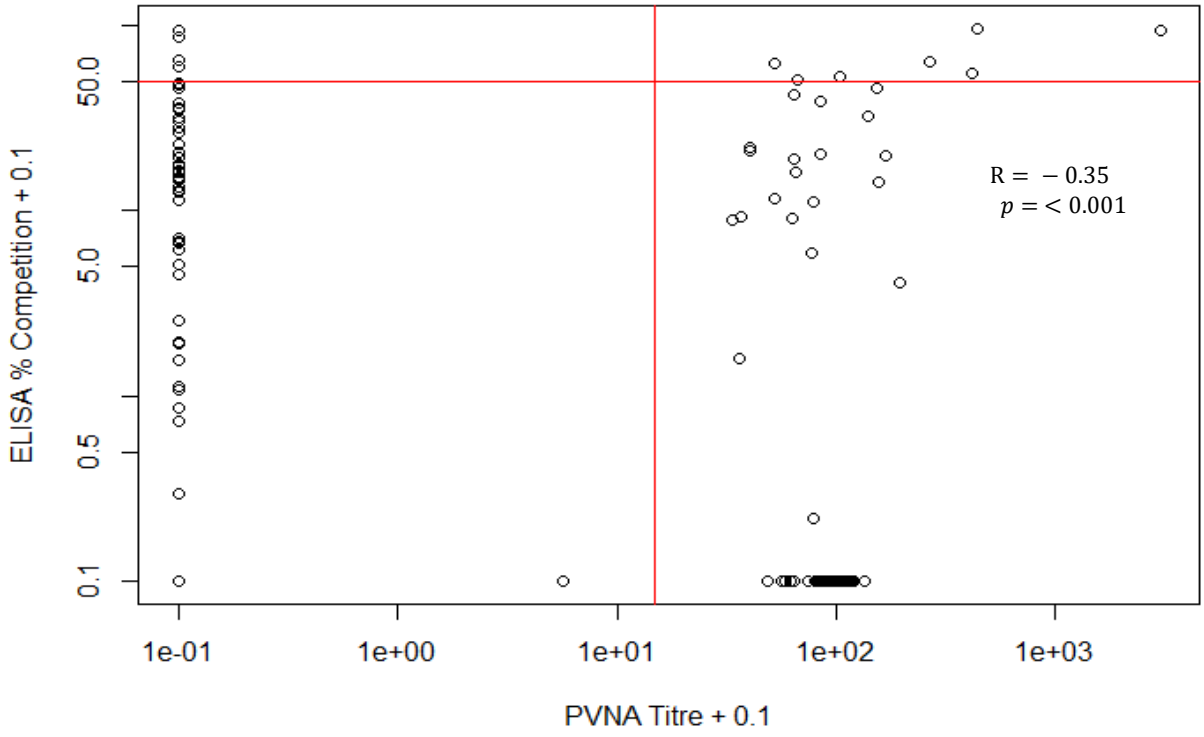


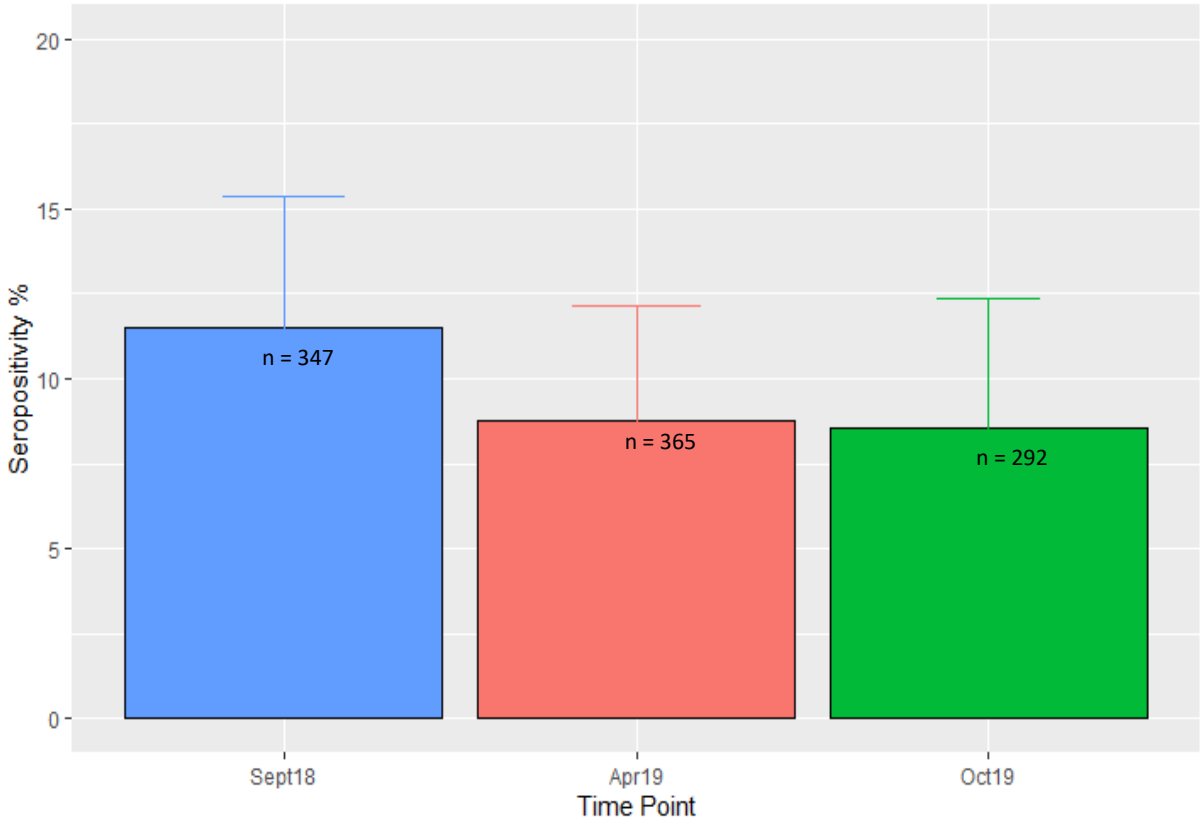
Figure 3.4.1. Plot showing the neutralising antibody titre from the PVNA against the percentage antibody competition using the ELISA. To compare the accuracy of the PVNA used to detect PPRV antibodies, a subset of samples from September 2018 and April 2019 were randomly selected from a sample bag and tested with the PVNA (x axes) and a competition ELISA (y axes), (IDVet, Grabels, France). Cut-off points for seropositivity are shown by the red lines. Correlation calculated with Pearson coefficient.

3.4.2. Assessing the serological evidence of infection of cattle with PPRV

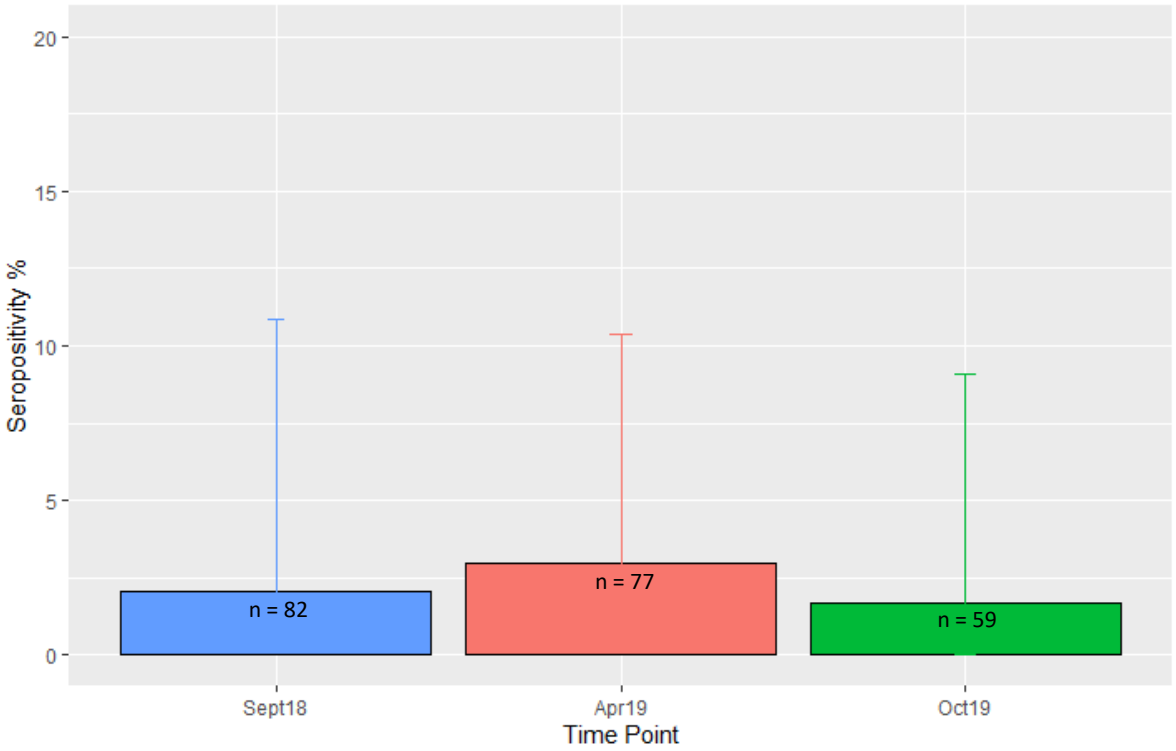
All remaining serum samples were then tested with the PVNA. The number of animals sampled and tested for PPRV neutralising antibodies by the PVNA in each sampling period of the study are summarised in figure 3.4.2(A-D). At all time periods a proportion of cattle were seropositive. At the beginning of the study 12% seroprevalence was detected in cattle, followed by a decline to 8.7% in April 2019, and 8.4% in the third time point (A). Seroprevalence in sheep was 2% at the first time point, which increased to 3% in April 2019 and declined to 1% in the final sampling period (B).

Goats had the lowest seroprevalence overall (C), with only 3.5% seropositive in the final sampling period. Dogs were seropositive at all time points (D).

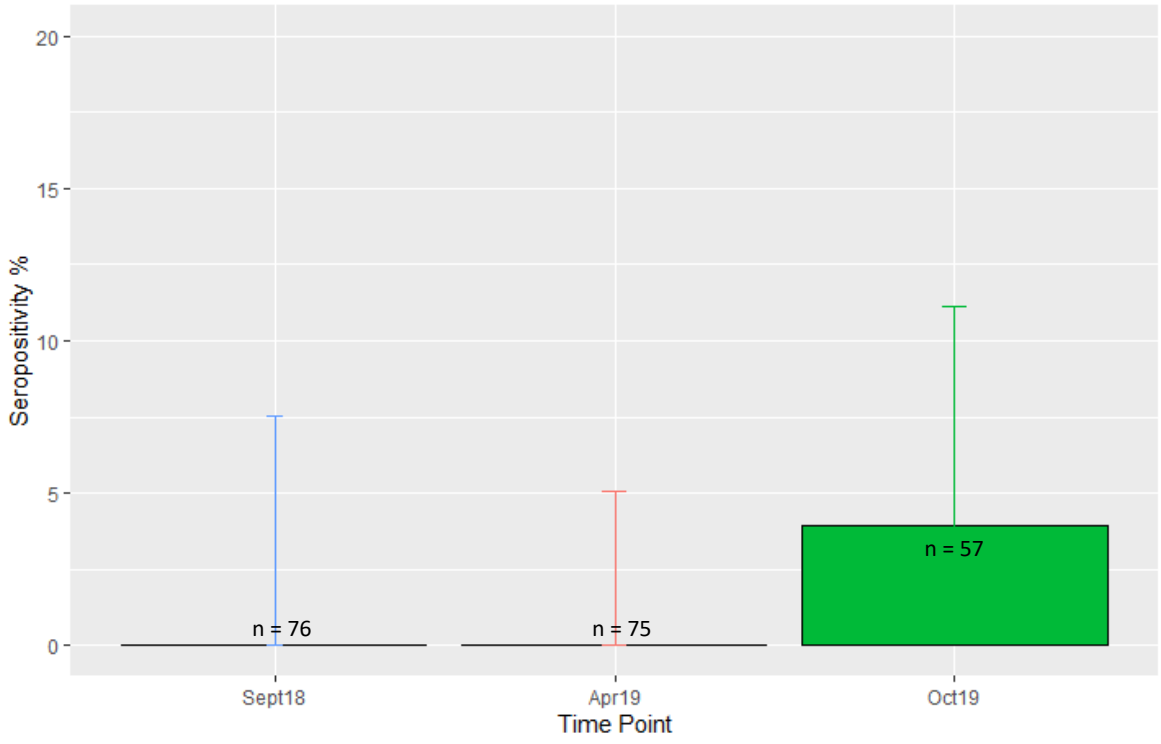
A)



B)


20
15
10
5
0
Seropositivity %
n = 82
n = 77
n = 59
Sept18
Apr19
Oct19
Time Point

C)


20
15
10
5
0
Seropositivity %
n = 76
n = 75
n = 57
Sept18
Apr19
Oct19
Time Point

D)

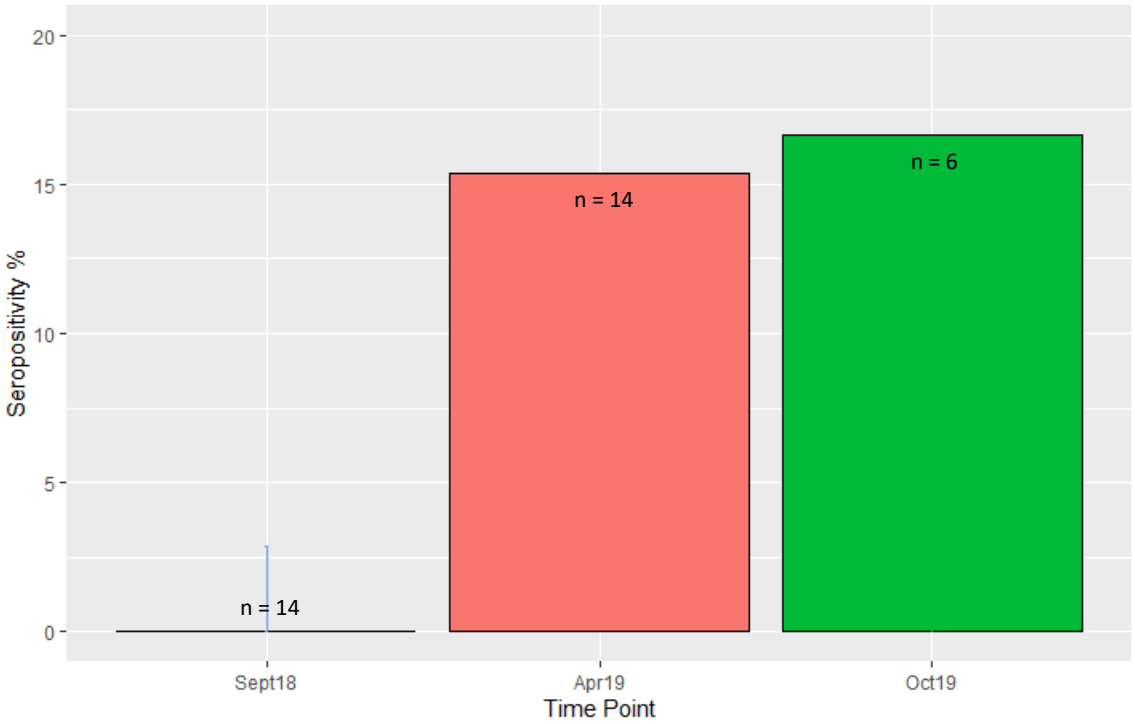


Figure 3.4.2 (A-D). Bar plots illustrating the seroprevalence of animals at each sampling time point. Seropositivity indicates individuals with a neutralising antibody titre against PPRV-specific glycoproteins tested using the PVNA. Cattle are represented in A, sheep in B, goats in C, and dogs in D. April 2019 only includes seropositive samples which were negative in September 2018. October 2019 only includes samples which were negative in the first two time points. Sample sizes are noted above the bars.

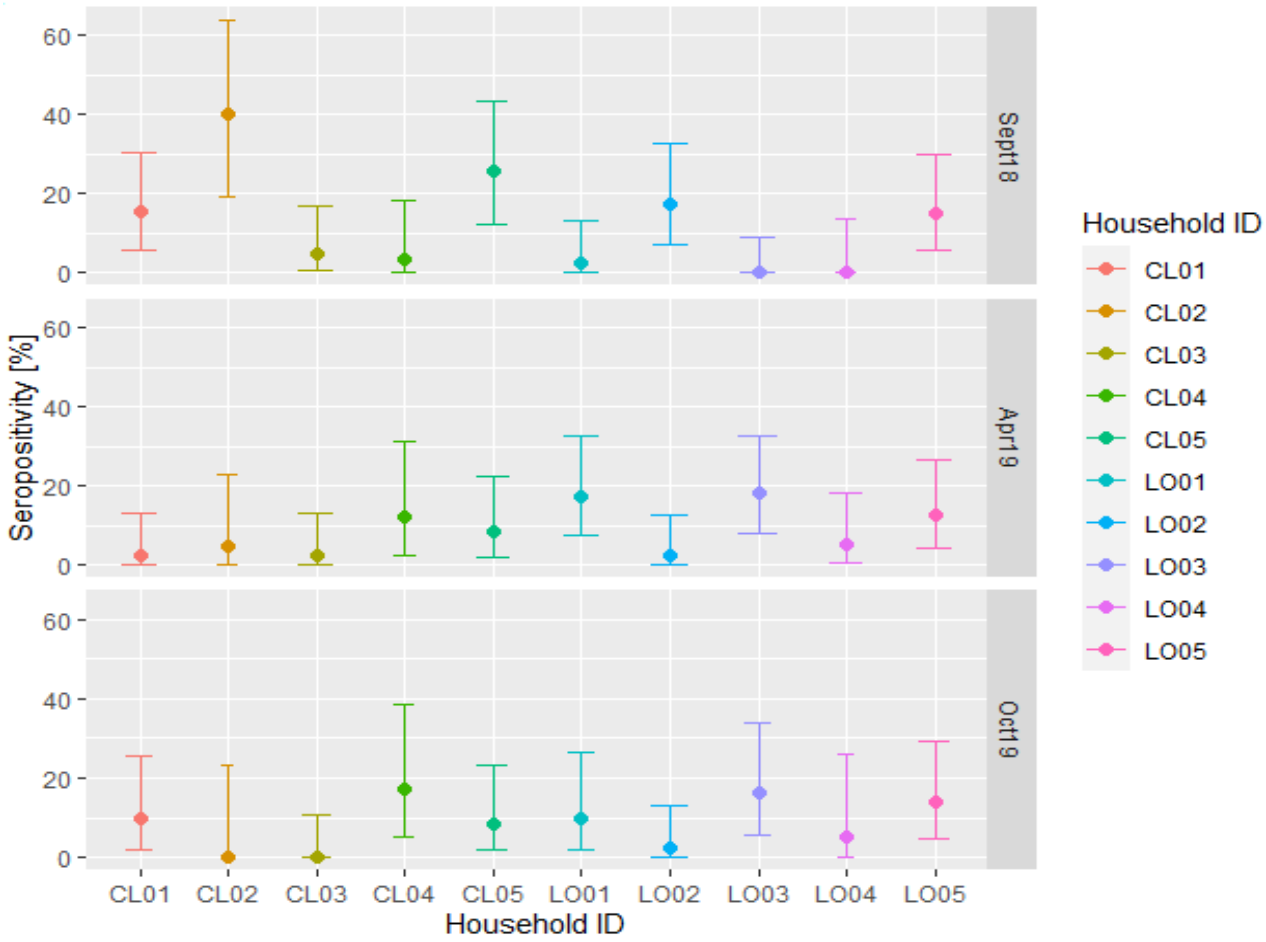
3.4.3. Patterns of Seropositivity

To stratify the seropositivity data shown thus far, cattle seroprevalence was broken down by household (figure 3.4.3 (A-B)). Figure A represents all cattle sampled at all

time points in each household, whereas only cattle repeatedly sampled were plotted in B.

Household differences in seroprevalence are statistically significant (**p=0.0001) as shown in 3.4.4 tables 2 and 3. Household CL02 had a seroprevalence of 35% during the first sampling period in September 2018 (OR = 3.66, p = 0.001). A likelihood ratio test (LRT) showed that other households were not associated with odds of cattle seropositivity. Households combined as a single variable does significantly impact the statistical model in table 2 (LRT = 0.92). The decrease in seroprevalence in other households at subsequent time points is not statistically significant. The overall trend of seroprevalence is varied between households, and between timepoints with no clear pattern. All households have seropositive cattle during at least one sampling period of the study. Seroprevalence varied between male and female cattle with no clear pattern for either sex throughout the study (p = 0.77). A likelihood ratio test (LRT) confirmed that removal of sex as a factor does not impact the GLM (LRT = 0.00).

A


Seropositivity [%]
60
40
20
0
Sept18
Apr19
Oct19
CL01
CL02
CL03
CL04
CL05
LO01
LO02
LO03
LO04
LO05
Household ID
Household ID
CL01
CL02
CL03
CL04
CL05
LO01
LO02
LO03
LO04
LO05

B

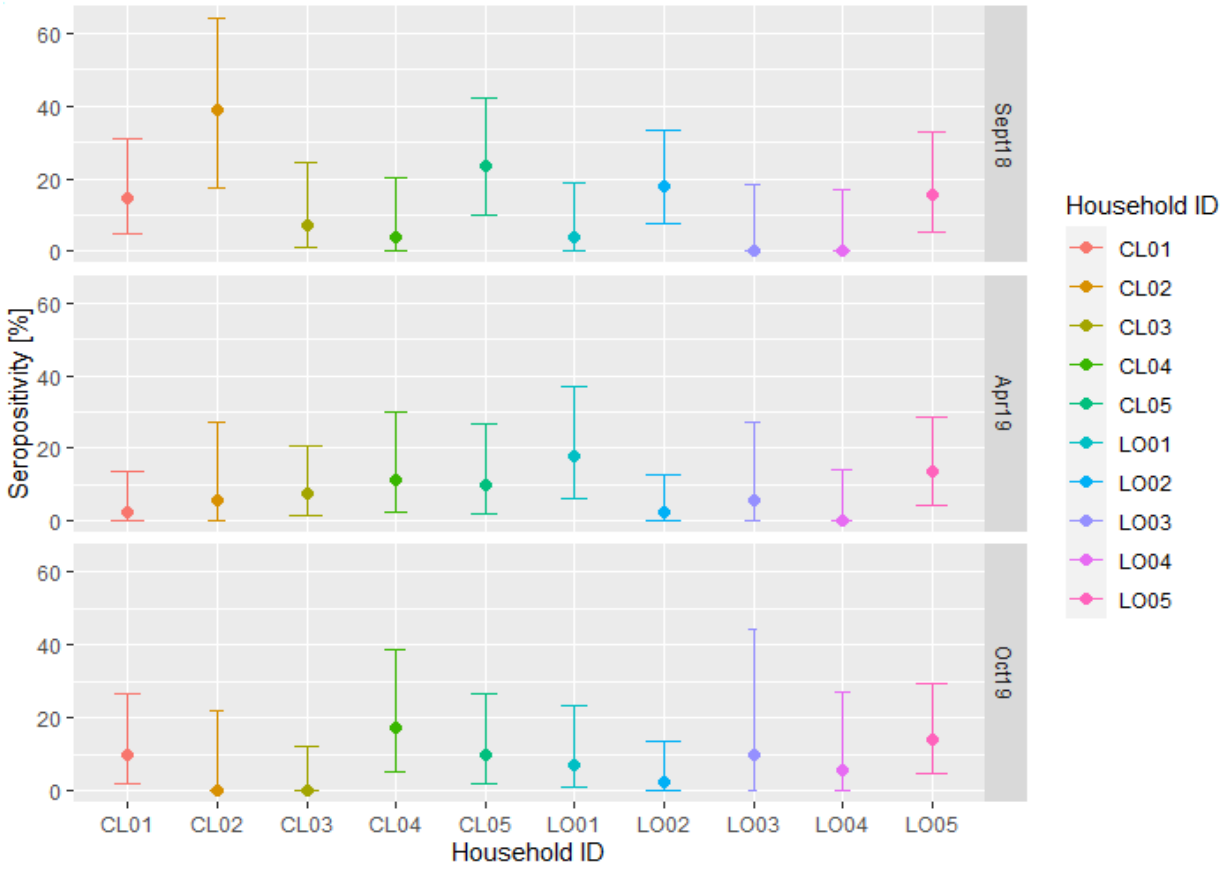


Figure 3.4.3 (A-B). Plot showing cattle seropositivity by participant household during each sampling period. Data points show mean ± SEM. Plot A includes all cattle sampled at each time point. Plot B includes only cattle which were repeatedly sampled at all time points. Sampling periods are in chronological order from top to bottom. Seropositivity is given as a percentage of animals in each household seropositive for PPRV antibodies.

3.4.4. Seroconversion

Seroconversion was ubiquitous throughout the study. In the second and third sampling periods, 7% and 4% of sampled cattle seroconverted, respectively (3.4.4. Table 3). Prior to the beginning of the study in September 2018, study participants reported outbreaks of suspected PPRV in their sheep, goats, and young calves. This is indicated by the red arrow in figure 3.4.4. Seroconversion occurred in 7% of cattle during April 2019, and no clinical signs of PPRV were reported or observed in the weeks prior to this sampling period. In contrast, 4% seroconverted in October 2019 with several households reporting clinical signs of PPRV in small ruminants in the weeks prior. This is indicated by the red arrow in figure 3.4.5. There is therefore no clear pattern of seroconversion to indicate a driver of infection.

3.4.4. Table 1. Summary of cattle seroconversion.

	n	No. Seronegative	No. Seropositive	Total No. Seroconversions
Sep-18	347	307	40	-
Apr-19	365	333	32	23
Oct-19	292	267	25	12

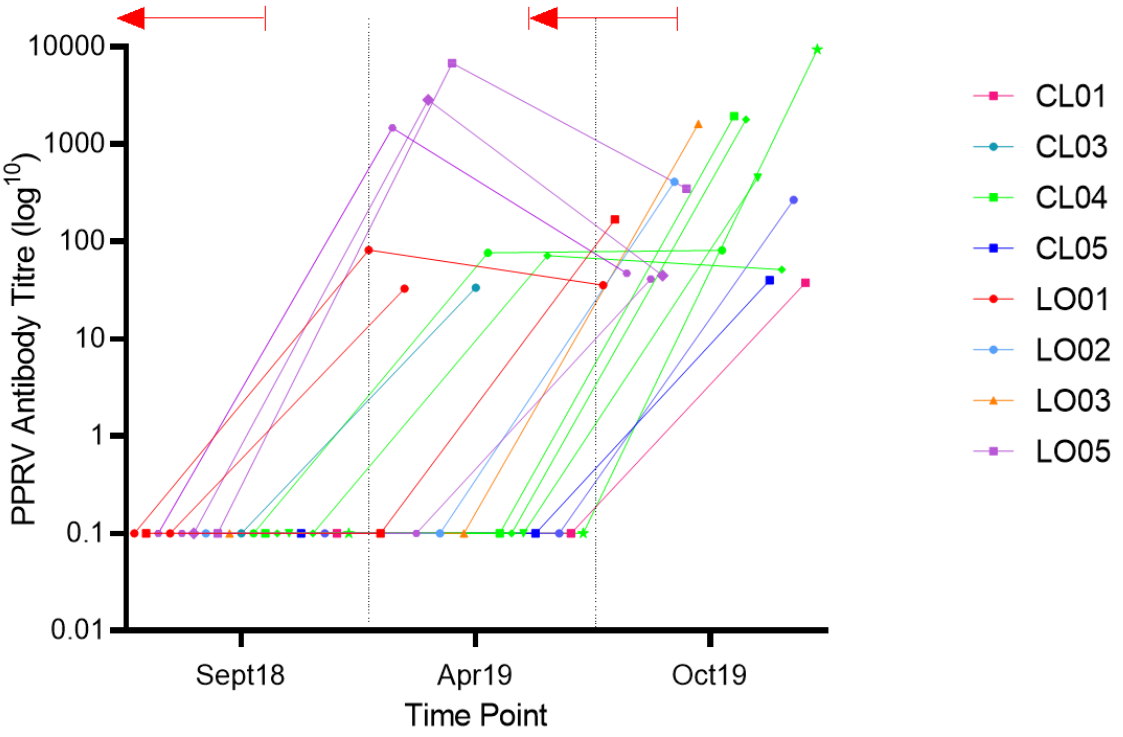


Figure 3.4.4. Plotted seroconversions of animals across sampling periods.

Breakdown of seroconversions of individual animals sampled three times and their antibody titre during respective sampling periods (cattle n=18, goats n=1). Animals are coloured by household. The red arrow above the third sampling period indicate that households reported seeing clinical signs of PPR in their sheep and goats prior to this sampling period (September 2019). The arrow above the first sampling period indicates that households reported seeing clinical signs of PPR in cattle prior to this sampling period (July 2018). Animals in this figure include only those who were seronegative at the beginning of the study.

3.4.4. Table 2. Analysis of cattle seroconversion between September 2018 and April 2019.

Factor Variable	Factor Level	N	Number Seroconverted	Odds Ratio	95%CI	LRT	*p* value
Household	(Ref) CL01	40	1	-	-	0.76	<0.001
	CL02	20	2	3.21	3.53-8.69		0.35
	CL03	40	0	0	2.04-1.83		0.99
	CL04	28	3	5.04	6.37-11.1		0.17
	CL05	35	3	3.25	4.30-7.35		0.31
	LO01	40	6	6.44	1.10-1.33		0.09
	LO02	40	4	4.28	5.90-8.45		0.2
	LO03	40	4	3.65	5.46-7.81		0.2
	LO04	25	1	1	4.18-28.0		0.99
	LO05	40	4	4.04	6.05-8.69		0.22

Sex						0.05	
	Ref						0.99
	Female	225	19	0	0-1.51		0.99
	Male	123	12	1.54	0.7-3.4		0.29

3.4.4. Table 3. Analysis of cattle seroconversion between April 2019 - October 2019.

Factor Variable	Factor Level	N	Number Seroconverted	Odds Ratio	95%CI	LRT	*p* value
Household	CL01	31	1	-	-	0.88	<0.001
	CL02	14	0	1.11	0.27-3.92		0.872
	CL03	32	0	0.54	0.13-1.89		0.354
	CL04	23	3	1.07	0.30-3.55		0.911
	CL05	35	3	1.37	0.46-4.12		0.563
	LO01	30	4	0.86	0.26-2.74		0.809
	LO02	41	2	0.60	0.15-2.09		0.439
	LO03	31	4	0.88	0.27-2.78		0.828
	LO04	19	2	0.68	0.09-3.03		0.65
	LO05	36	1	0	0.38-2.29		0.984
Sex	Ref			-	-	0.08	<0.001
	Female	214	16	1.28	0.37-8.13		0.734
	Male	78	4	0.91	0.24-5.96		0.907

3.4.5. Assessing the clinical evidence of cattle infection with PPRV

The most commonly observed clinical signs in cattle were nasal and ocular discharge. Seroconversion was detected in cattle at each time point but there was no association between cattle seropositivity or seroconversion and either observed or reported clinical signs of PPRV in cattle, sheep, or goats at any of the time points. Clinical signs consistent with PPR were observed and reported in cattle, sheep, and goats during the study but were not associated with seroconversion at any time point.

3.4.5. Table 1. Summary of statistics for seroconversion and observed clinical signs.

Time Point	**Clinical Signs**	**Odds ratio**	***p* value**	**95%CI**
Sept18 Cattle Seropositivity	Cattle	1.29	0.25	0.81-1.92
	Sheep and Goats	0.74	0.45	0.33-1.63
Apr19 Cattle Seroconversion	Cattle	1.32	0.38	0.65-2.42
	Sheep and Goats	1.17	0.80	0.33-4.20
Oct19 Cattle Seroconversion	Cattle	1.28	0.38	0.72-2.26
	Sheep and Goats	0.54	0.27	0.17-1.61

3.4.5. Table 2. Summary of statistics for cattle seroconversion and farmer-reported clinical signs in cattle, sheep, and goats. (NA means no data available).

Time Point	Clinical Signs	Odds ratio	*p* value	95%CI
Sept18 Cattle Seropositivity	Cattle	NA	NA	NA
	Sheep and Goats	NA	NA	NA
Apr19 Cattle Seroconversion	Cattle	NA	NA	NA
	Sheep and Goats	NA	NA	NA
Oct19 Cattle Seroconversion	Cattle	0.3	0.5	-0.9-1.38
	Sheep and Goats	-2.1	0.6	-1.02 – 0.6

3.5. Discussion

Over 1 billion sheep and goats are at risk from PPRV infection, with global spread increasing rapidly and into atypical host species (OIE, 2015; Wang *et al*, 2015; Dou *et al*, 2020). Considering this threat and the aim of eradication by 2030, understanding the patterns of PPRV infection in livestock communities is critical to the success of the vaccination campaign. Domestic cattle live in close proximity to small ruminants in mixed households, with previous serological evidence suggesting that cattle get spill over infection from their sheep and goat counterparts. However, the degree to which cattle infection occurs and the relationship with small ruminant infection are not understood. This understanding has been further limited by the inability of assays to differentiate between related morbilliviruses (Logan *et al*,

2016). To differentiate PPRV-specific antibodies in cattle, sera from study households were tested using the PVNA. The PVNA was positive for all samples which were strongly positive by ELISA. Weakly positive samples on the cELISA were determined negative by PVNA, likely to be false positives due to non-specific binding (de Swart *et al*, 2005; Diallo *et al*, 2001). A similar observation was made by Lembo *et al* (2013) when 38% of positive sera had been missed by the less-sensitive cELISA, when tested with the PVNA. Moreover, the PVNA has been utilised for antibody diagnostics of other pathogens as a substitute for less sensitive and specific assays, such as the ELISA. For example, Suda *et al* (2018) employed a similar mechanism of using a VSV viral core, replacing VSV replication gene with Crimean-Congo Haemorrhagic Fever Virus (CCHF) glycoprotein. When testing patient sera, the CCHF-PVNA had improved sensitivity, and the cross-reactivity with other nairoviruses previously seen with the ELISA was circumvented (Suda *et al*, 2018; Ward *et al*, 1992). Consistent with findings of other research, results here show accurate detection and quantification of PPRV-specific neutralising antibodies in cattle sera. The PVNA therefore offers a robust diagnostic method of serological testing, providing reliable evidence of PPRV infection in cattle.

Previous research using the cELISA has been unable to differentiate between PPRV-specific antibodies and those of related morbilliviruses (Holzer *et al*, 2016). An example of this was shown in a clinical setting by Zhang *et al* (2015), whereby measles-vaccinated human sera cross-neutralised CDV. To test the PVNA's ability to distinguish between morbilliviruses circulating in livestock in this study, all animals were simultaneously tested for CDV antibodies using the PVNA. As shown in figure 3.4.3 there is a clear distinction between antibodies specific to PPRV glycoproteins,

and those specific to CDV. Individuals of all species demonstrated a neutralising response to both viruses throughout this study. Bovines from the Serengeti, where both viruses commonly circulate, were previously found to have CDV and PPRV antibodies using the PVNA (Logan *et al*, 2015). Morbilliviruses are known to reduce the immune competency of infected individuals, thereby increasing their susceptibility to secondary infections (Rojas *et al*, 2016). It is therefore likely that CDV and PPRV co-infected these individuals during this study.

There are, as with any diagnostic method, limitations to the PVNA which require further research. It can be determined with certainty that antibodies detected by the PVNA are PPRV-specific. However, using this method alone it is still unclear whether some individuals are seropositive for both viruses due to co-infection or cross-reactivity between virus epitopes. To segregate these two possibilities, it would serve to either detect viral RNA in blood during peak infection, or to sequence and express purified antibodies from sera in a vector-based assay to determine the source antigen eliciting the humoral response (Ledsgaard *et al*, 2018). Nevertheless, the results in figure 3.4.2 show accurate, specific, PPRV antibody detection using the PVNA, thereby satisfying the first aim of this chapter. This is robust evidence that cattle become infected with and produce a specific immune response to PPRV. This will inform future work for improved seroprevalence-based decisions, and ultimately advancing the understanding and surveillance of PPRV spread in livestock communities and adaptive host-range.

One speculative hypothesis for the unusual findings of PPRV antibodies largely in cattle compared to sheep and goats is that there is a newly emerging morbillivirus

which has high antigenic similarity to PPRV and RPV. It is possible that a novel morbillivirus has evolved from PPRV in the wake of RPV eradication in cattle. This could explain the presence of antibodies to a PPRV pseudotype predominantly in cattle compared to small ruminants, particularly if PPRV continuously circulates in livestock at a low level. If this was truly PPRV circulating at a low level with few small ruminant deaths reported, it would be expected that small ruminants have the higher seroprevalence as they are the natural hosts. Isolation of viral RNA from blood samples for sequencing would have allowed this hypothesis to be examined further, as proposed in the original study design, however limitations on sample collection and laboratory analyses due to outbreaks of FMDV and covid-19 prevented this work from being carried out.

3.5.1. Patterns of association

Prevalence in sheep and goats northern Tanzania has previously been estimated to be between 0-88% (Kivaria *et al*, 2013; Mdetele *et al*, 2021). Higher seroprevalence estimates have been determined for small ruminants from households which had suspected PPR outbreaks, when compared to other households. The results in figure 3.4.2 show a different pattern to those observed in recent work. It is unusual that the results show a low PPRV seroprevalence throughout the study, considering signs of PPRV (naso-ocular lacrimation, diarrhoea, anorexia, sneezing) were reported and observed during the first and third sampling periods, respectively. Further, it is unusual that cattle have a higher PPRV seroprevalence than sheep and goats. The most recent findings across northern Tanzania suggest seroprevalences of 53%, 46%,

and 11% in sheep, goats, and cattle respectively (Herzog *et al*, 2020; Jones *et al*, 2021).

Given that small ruminants are the natural hosts of PPRV, the seroprevalence data would be expected to reflect this. However, the finding that cattle had higher and more widespread seroprevalence than sheep and goats was an unusual finding not consistent with previous work. Research by Herzog *et al* (2019) previously found seroprevalence in Tanzanian cattle, sheep, and goats to be 11%, 26%, and 28% respectively. Other studies have found seroprevalences ranging from 2-18% in cattle (Abraham *et al*, 2005; Abubakar *et al*, 2017). While the range of cattle seroprevalence in this study (8.5-11.5%) resonates with previous findings, it is unusual when compared to sheep and goats. One possible explanation for this is that if an outbreak of PPRV had occurred prior to sampling, small ruminants would have succumbed to clinical disease and likely died. Any surviving sheep and goats would have a neutralising response to the virus. Cattle in the same households would have become infected but survived, thus explaining the higher seroprevalence in cattle compared to small ruminants. However, without available records of small ruminant deaths in participant households, it is difficult to determine if the low seroprevalence was due to deaths. This scenario was supported by three households which reported suspected PPRV infection in their sheep and goats in the weeks prior to the study beginning in September 2018, and six households which reported this just before the final sampling period of October 2019. An outbreak earlier in 2018 could explain the higher seroprevalence at the beginning of the study. Similarly, a brief outbreak prior to the final sampling period would account for the seroconversions by then. Given that new seroconversions occurred in cattle throughout the study, it is likely that infection was occurring regularly at a sub-clinical level in cattle.

The lack of pattern to seropositivity among households suggested that PPRV was ubiquitous in the environment. Recent studies investigating household-level seroconversion found that pastoralist production systems had a higher risk of transmission and seroconversion (Herzog *et al*, 2019). This study did not collect specific information on the management practice of households, although all households were similar in their boma and grazing arrangements, with no difference between households and seroconversion of cattle. One method which could elucidate the directionality of PPRV transmission is a force of infection (FOI) model. Previous work by Herzog *et al* (2020) utilised an age-specific FOI model to demonstrate the age cohorts responsible for transmitting PPRV in mixed-livestock herds. Findings suggested peak infection occurred in cattle aged 2.5-3.5 years (Herzog *et al*, 2020). The low number of seropositive samples collected for this thesis was insufficient to support an FOI model, and age data collected was unreliable. In future, research to explore PPRV and CDV FOI in mixed livestock herds should be done to further understand transmission dynamics. This will allow for better targeting of virus control.

3.5.2. Clinical signs in cattle

Since there is a lack of knowledge on the clinical signs of PPR disease in cattle, recent work has utilised the standardised list of signs in small ruminants provided by the OIE as a guideline (OIE, 2015). Examples of this include experimental infection trials by Couacy-Hymann *et al* (2019) and Abubakar *et al* (2014). To date, studies on clinical signs of PPRV are limited to experimental infection within a clinical setting. At the first and final sampling period of this study, multiple participant

households reported observing clinical signs consistent with PPRV in their animals in the weeks prior to sampling. This included the deaths of many sheep, goats, and some young calves. However, during this period there was an outbreak of FMDV across several households. Active FMDV manifests in cattle as ocular and nasal discharge, salivation, diarrhoea, pyrexia, anorexia, and lesions throughout the oral cavity and feet (Kitching, 2002; Jones *et al*, 2019). Small ruminants can also develop clinical disease. The clinical signs reported and observed during this time overlap with signs of FMDV, therefore it is impossible to differentially diagnose PPRV infection in any livestock species.

If PPRV was the cause of clinical disease during this study, it would be expected that small ruminant morbidity and mortality would reflect this, in addition to increased seroprevalence and higher titres in survivors following infection (Couacy-Hymann *et al*, 2019). This is potentially what happened prior to the start of the first sampling period when some participant households suffered high mortality rates in sheep and goats. This would also account for the unusual seroprevalences observed in cattle. It is possible that some low level of PPRV infection was then maintained in households following this initial outbreak, eliciting new seroconversions in cattle throughout the study. It has been shown that cattle can maintain PPRV infection with antigens present in blood up to 397 days post-infection (Sen *et al*, 2014). However, any link between recurring infections and cattle is speculation and it is difficult to infer anything from these data without further research. Although manifestations of PPRV outbreaks can greatly vary (Jones *et al*, 2019), the lack of association between seroconversion of cattle and clinical signs of PPRV in any species suggests two key points. Firstly, cattle develop sub-clinical infection and do not display clinical signs.

Secondly, the signs observed during the study are likely to have been caused by another pathogen such as FMDV. This is important because this is the first longitudinal study to be conducted in a field setting whereby cattle are monitored in this way, bridging the gap between experimental and field studies.

Further work is needed to monitor cattle during periods of suspected PPRV infection in households. Ideally blood and swab samples could be tested to determine whether infection is current and transmissible, and to assess if FMDV is also active in the sample population. This will further elucidate whether clinical signs are linked to active PPRV infection. This is a further important step in determining the limitations of cattle infection and their role in maintaining PPRV at low levels in mixed livestock systems.

3.5.3. Clinical disease in small ruminants

Signs of PPR disease were observed in small ruminants during sampling, and livestock owners reported such signs between sampling periods. There was no consistent pattern of clinical signs observed in small ruminants between households, although goats did tend to display fewer signs of disease than sheep. Assuming that PPRV was the cause of disease, this would be contrary to previous findings which suggest that goats are more clinically susceptible to PPRV than sheep, and often have higher seroprevalence (ul-Rahman *et al*, 2016; Fakri *et al*, 2017). The proportion of sheep and goats with clinical disease in this study was much smaller than expected during circulation of PPRV, based on some previous research. For example, a recent study by Jones *et al* (2019) found flocks in northern Tanzania with morbidity of up to 67% and mortality of up to 25%. Outbreaks in other geographical locations have up to 100% morbidity and 80% mortality (Misinzo *et al*, 2015; Kgotlele *et al*, 2014).

The range of clinical outcomes of PPRV requires further investigation, as this will be critical for disease surveillance.

3.6. Chapter Conclusion

In summary, this chapter confirms that cattle are susceptible to PPRV infection, detectable using the PVNA which can distinguish seroreactivity to other morbilliviruses. Cattle had a higher seroprevalence than small ruminants of the same households, which was unexpected given that small ruminants are considered primary hosts for PPRV. Although seroprevalence varied over time and between households, infection was ubiquitous and maintained at a low level throughout the study period. No clinical signs of disease were reported in association with seropositivity or seroconversion in infected cattle, suggesting that PPRV infection was sub-clinical or resulted in very mild clinical outcomes. Seroconversion was further not linked with clinical signs of disease in small ruminants, suggesting that PPRV may be circulating below farmer-reported levels of detection and which may pose challenges for clinical surveillance of PPRV in these multi-host communities.

Chapter 4 Assessing the Evidence of CDV Infection in Cattle, Sheep, and Goats

4.1. Summary

Morbilliviruses are known to infect a wide range of host species and evidence is growing that the host range for several morbilliviruses is expanding. Following the finding of widespread infection of Peste des Petits Ruminants virus (PPRV) in atypical hosts in northern Tanzania, this study investigated livestock as hosts for canine distemper virus (CDV). Samples were collected from cattle, sheep, goats, and dogs at each of three time points (n=517, 529, 414, respectively) from 10 households in mixed livestock farming communities where CDV is known to occur. Samples were tested using a pseudotyped virus-based neutralisation assay (PVNA) to measure CDV antibodies, accounting for possible cross-reactivity with other morbilliviruses. Findings confirmed CDV seropositivity in cattle, sheep, and goats with CDV present in all households. Although there was an association between cattle seropositivity and clinical signs of disease in the first sampling period, there was no association between seroconversion and clinical signs of disease in cattle, sheep, or goats. Seroconversion was detected throughout the study irrespective of reported clinical signs of disease. These findings suggest that CDV circulates widely in livestock but does not appear to be an important cause of disease.

4.2. Introduction

Canine distemper virus (CDV) has global distribution and infects the widest host range of the family. The virus was first isolated in 1905 and despite more than a century of scientific study it continues to threaten vulnerable and endangered populations (Carre, 1905; Bresalier & Worboys, 2014). It is primarily a pathogen of

carnivore species, although infections and outbreaks occur across many subphyla and atypical hosts including: *Felidae; Mustelidae; Ursidae; Procyconidae*; *Rodentia*; Primates; *Proboscidea*; *Artiodactyla*; and *Ungulata* (Takeda *et al*, 2020; Martinez-Gutierrez & Ruiz-Saenz, 2016). Outbreaks of CDV have been increasingly reported in non-dog hosts across at least 43 countries thus far (Martinez-Gutierrez & Ruiz-Saenz, 2016), and has been attributed to the propensity of CDV for host-switching. Non-carnivore hosts include species such as Sika deer (*Cervus nippon*) and wild boar (*Sus scrofa*), and there is increasing evidence that CDV is no longer limited to carnivores (Kameo *et al*, 2012; Zhang *et al*, 2017; Logan *et al*, 2016).

CDV has also emerged as a pathogen of conservation concern. For example, the Welgevonden Game Reserve in South Africa lost 93% of its protected lion population due to a CDV outbreak in 2015 (Davidson-Phillips *et al*, 2019). In Tanzania, the Serengeti National Park (SNP) is home to multiple endangered species which have suffered high mortality rates due to sporadic CDV outbreaks. Populations of African wild dogs (*Lycaon pictus*) spotted hyaenas (*Crocuta Crocuta*), and African lions (*Panthera leo*) have been devastated by outbreaks of CDV with mortality reaching 100% in some instances (Weckworth *et al*, 2020; Alexander & Appel, 1994; Haydon *et al*, 2002; Marino *et al*, 2017). The introduction of CDV into protected ecosystems has hindered conservation efforts for many endangered species. It is crucial to identify emerging host species, particularly those which live close to protected areas, for disease control (Young *et al*, 2011). For example, a recent fatal outbreak occurred for the first time in the protected Linnaeus's 2-toed sloth (*Choloepus didactylus*), originating from an unknown external wildlife source in the USA (Watson *et al*, 2020).

Free-roaming and feral dogs have historically been associated with the spread of various pathogens into wildlife and livestock populations (Lushasi *et al*, 2021; Miran *et al*, 2017; Czupryna *et al*, 2020; Young *et al*, 2011; Maboni *et al*, 2019). For example, the fatal 1994 CDV outbreak in the SNP killed approximately 30% of the lion population (Laurensen *et al*, 1998; Munson *et al*, 2008; Viana *et al*, 2015). Virus isolates taken from Serengeti lions (Panthera leo), bat-eared foxes (*Otocyon megalotis*), and spotted hyenas (*Crocuta crocuta*), were sequenced and showed a distinctive similarity to isolates taken from domestic dogs within the region (Carpenter *et al*, 1998; Viana *et al*, 2015). This was supported by the high seroprevalence and disease burden in domestic dogs around the SNP at the time (Roelke-Parker *et al*, 1996). The disease risk to endangered wildlife species from domestic dogs is high, and particularly high pathogenicity has been reported from CDV isolated in the Serengeti region (Weckworth *et al*, 2020; Alexander & Appel, 1994; Roelke-Parker *et al*, 1996). In northern Tanzania, like many other sub-Saharan countries, household owners with livestock are highly likely to also keep domestic dogs (Webster & Ebesole 2019; Knobel *et al*, 2008; Jackman & Rowan, 2007).

A key issue of interest following the eradication of rinderpest has been the creation of a potential ‘niche’, which may have left bovine host species vulnerable to infection with antigenically similar viruses (de Swart *et al*, 2012; Polding & Simpson, 1957). The antigenic similarity between rinderpest and CDV was conceptualised when domestic dogs living with rinderpest-infected cattle showed immunity to CDV (Polding & Simpson, 1957; White *et al*, 1961). Tissue from cattle has previously been found to contain CDV proteins, suggesting that specific SLAM receptors may not be essential for host infection. (Tatsuo & Yanagi, 2002; Alves *et al*, 2015). Thus, CDV infection may be more widespread than previously thought.

Moreover, a study on sporadic meningoencephalomyelitis in Swiss cattle found antibodies which reacted to CDV (Theil *et al*, 1998). Previously, Logan *et al*(2016) detected CDV-specific neutralising antibodies in cattle, sheep, goats, and buffalo sera from Tanzania using the pseudotype-based virus neutralisation assay (PVNA). This showed preliminary evidence that livestock are able to produce CDV-specific antibodies in a natural setting within an endemic area. However, this study was based on archived sera collected from cross-sectional studies on other pathogen research. Data on CDV in livestock is very limited and few studies exist which demonstrate the potential for livestock to host the virus. For example, Bussel & Karzon (1965) successfully infected bovine kidney cells with the Onderstepoort strain of CDV, which supported the possibility that bovine cells could host and maintain the virus. Similarly, Metzler *et al*(1980) infected bovine cells with the R-252-CDV strain of the virus and found persistent infection of cells occurred, suggesting that cattle could be a potential host. Tatsuo *et al*(2001) found that the Onderstepoort strain of CDV was able to infect bovine and canine cells. More recently, Yadav *et al*(2019) found sequence similarity in dog, sheep, goat, and bovine SLAM receptors, and high sequence homology between dog and ruminant nectin-4 receptors. This is evidence that CDV has the potential to infect cattle, sheep, and goats. However, these studies are mostly experimental and their relevance to *in vivo* infection is unclear.

It is not currently known if there is any disease burden associated with infection of livestock. Limited experimental data exist on this topic. For example, Gaskin *et al*(1974) experimentally infected domestic pigs with virulent CDV. The study concluded that all infected animals were susceptible to the virus, which replicated in lymphatic tissue and lungs. Signs of respiratory distress were recorded in infected hosts (Gaskin *et al*, 1974). Although there is preliminary evidence that livestock

species may be susceptible to CDV infection, no studies have been done to assess the parameters of infection of cattle, sheep, or goats. This is an important gap in the literature given the geographical cross-over between domestic dogs, livestock, and susceptible wildlife populations. This chapter builds on these earlier studies to carry out a longitudinal study in mixed dog and livestock-keeping households in northern Tanzania to investigate CDV infection dynamics in cattle, sheep, and goats and to generate preliminary data on clinical outcomes of infection. The study has the following aims:

4.2.1. Key aims:

- To establish whether CDV seropositivity in cattle, sheep, and goats is accurate or due to cross-reactivity.
- To observe if there are patterns of exposure in cattle, sheep, goats. Data will be stratified by household.
- To determine if CDV exposure in cattle, sheep, and goats is associated with clinical signs of disease (consistent with CDV in known host species) using a generalised logistic model (GLM).
- To assess if there is a relationship between the seroconversion in cattle, sheep, and goats, and clinical signs of disease. This will be examined using a GLM.

4.3. Chapter Methods

Details about the design of the study, including field sites, herd and individual selection, sampling and serological testing protocols and sample size calculations are given in Chapter 2.

4.3.1. Study Population

The study population included in this chapter is the same as described in chapter 1. Households were selected for this study on the basis of either i) reported clinical signs of PPR in small ruminants in the eight weeks prior to the study, or ii) previously detected PPRV antibodies during an unrelated study. Cattle which were recruited in September 2018 but were absent during April 2019 due to death or being sold. To mitigate this, other available cattle were recruited in April 2019 including some extra to ensure the sample size would be maintained until the end of the study. All households selected practised similar management systems, in that they grazed their cattle, sheep and goats together at pasture during the day and kept them separated in the boma at night. Dogs were not given identification tags for repeat sampling as they were opportunistically sampled in households at each time point.

4.3.2. Case definition

A positive titre within the PVNA was defined as the point at which there was 90% neutralisation of pseudotype virus, indicated by luciferase counts per minute. The calculated titre translates as the dilution at which the sample can be diluted with antibodies still detectable. A titre below 1:32 is considered negative because at such a dilution, antibodies in a sample should be detected if they are present. Clinical signs consistent with CDV have not previously been assessed in livestock. Therefore, what was recorded and observed are signs consistent with CDV in species known to host the virus and develop clinical disease. This is identical to the list provided in the previous chapter.

4.3.3. R packages and output

All data and statistical analyses were performed using R version 4.0.2. Packages utilised for cleaning and visualisation were ‘dplyr’ and ‘ggplot’. Statistical analysis was carried out using the ‘glm’ and ‘anova’ functions. Likelihood ratio tests were conducted by running the ‘chisq’ command within the ‘anova’ function. All other statistical functions were performed within the ‘dplyr’ and ‘ggplot’ packages. All R scripts were marked down using the ‘knitting’ function and can be found in the appendix. All data output was saved in a secure folder on University of Glasgow OneDrive with password protection.

4.3.4. Statistical Tests

For logistic regression models (section 3.9) the odds ratio was calculated using the ‘exp(coef())’ command in R. This was used to determine if there was any association between i) seropositivity and household, ii) seropositivity and sex, iii) seropositivity and clinical signs of disease, and iv) seroconversion and clinical signs of disease. Confidence intervals were calculated for all factors of the models using ‘(exp(confint())’ command in R. For bivariate models comparing seropositivity with sex and household, a comparison of models was performed to determine which best fit the data. To address this a likelihood ratio test (LRT) was used with the ‘anova()’ command. The ‘chisq’ command was then used to test how the observed models differed from the expected outcomes.

4.3.5. Seroconversion

A generalised logistic model (GLM) was used to measure three specific questions:

4) If there was an association between livestock seropositivity and household.
5) If there was an association between cattle, sheep, and goat seroconversion and observed clinical signs consistent CDV during sampling.
6) If there was any association between cattle, sheep, and goat seroconversion and clinical signs consistent with CDV reported by farmers.

Outcome variables were binomial (positive, negative) and coded as '0' or '1', respectively. Explanatory variables were species and sex. Household was included as a random effect to account for multiple data points for animals which were repeatedly sampled. Models were fitted with both explanatory variables, followed by the exclusion of either variable. An ANOVA was then used to compare each version of the GLM, the result of which was given as a likelihood ratio. There were not enough data on sheep, goats, or dogs to fit a model thus this was only applied to cattle data.

4.3.6. Questionnaire data

The summarised results below are from the answers given by study participants.

- Have you seen any signs of disease in your cattle, sheep, or goats in the last 6 months?
- Have you seen any sign of disease in dogs in the last 6 months?
- Are your cattle, sheep, goats, and dogs confined and grazed together?

This questionnaire is shown in the previous data chapter, and data collected from this was used for both PPRV and CDV.

4.4. Results

4.4.1 Confirming CDV seropositivity in livestock using the PVNA.

Overall, a total of 947 serum samples were tested using the PVNA for this study. A summary of serum samples tested is presented in table 4.4.1. At the beginning of the study, 7% of cattle were seropositive. There was a steady increase in cattle seroprevalence over the course of sampling, reaching 12% in April 2019 and 18% in October 2019.

In contrast, less than 1% of sheep were seropositive at the beginning of the study. This marginally increased to 3% in April 2019, and 5% in October 2019. The lowest seroprevalence was observed in goats which was less than 1% at the beginning of the study, after which time no seropositive goats were detected.

In September 2018, 28% of dogs were seropositive. Dog seroprevalence increased from 31% in April 2019 to 83% in October 2019. At each time point, approximately half of the dogs sampled had previously been vaccinated against CDV with the Onderstepoort vaccine strain. None of these dogs were repeatedly sampled. These data show a parallel increase in the number of seropositive cattle, sheep, and dogs over the course of the study.

Scatterplots showing antibody titres against CDV and PPRV are summarised by species in figure 4.4.2 A-D, including all samples collected at each time point. All sampling periods showed at least some cattle were seropositive to CDV and were PPRV-negative. There was no particular household pattern to the titres at any time point. In the first sampling period, titres which were high for CDV (>1000) and low

for PPRV (<100) are considered to have been initiated by CDV and cross-neutralised PPRV. Co-seropositivity in cattle only occurred during the first sampling period, after which time they were seropositive for one virus or the other. These data clearly show evidence of specific seroreactivity to CDV alone.

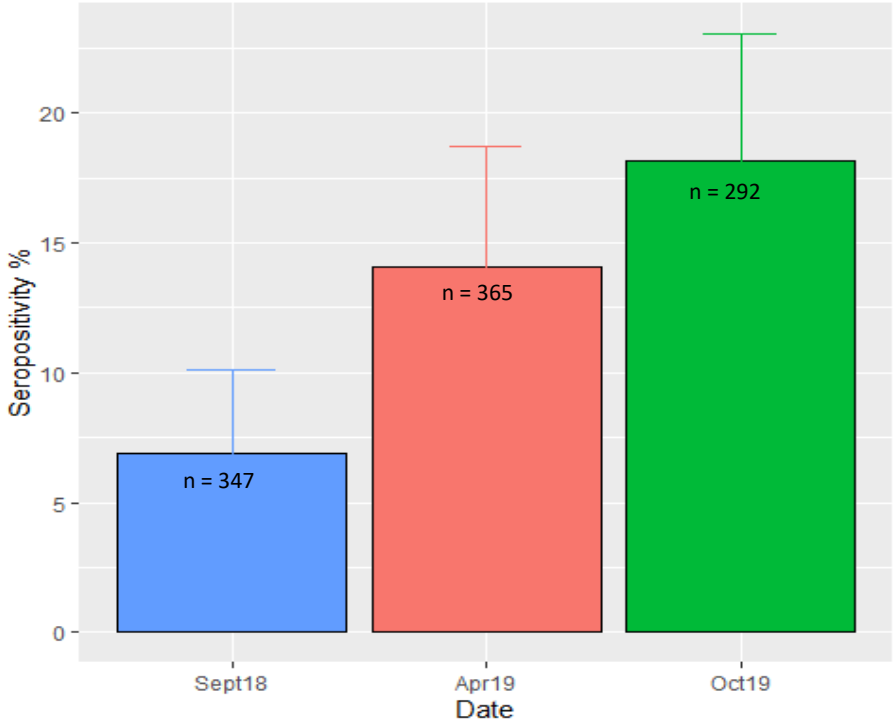
No sheep had CDV-positive titres in the first sampling period. One sample had a high titre for PPRV and was slightly positive for CDV, thus this was likely cross-neutralisation. The second and third sampling periods showed sheep with positive titres explicitly to CDV. There was one goat which had a low positive titre to CDV in the first sampling period. In April 2019 all titres were negative. In the final sampling period, one goat had a high positive titre for PPRV. There was no household pattern to sheep or goat titres.

Dogs showed seropositivity for CDV and PPRV, with the expectation that PPRV titres are indicative of cross-reactivity. The fact that the titres are very high for CDV and low for PPRV suggest this is the case. These plots clearly demonstrate a CDV-specific antibody response at a population level in all species sampled.

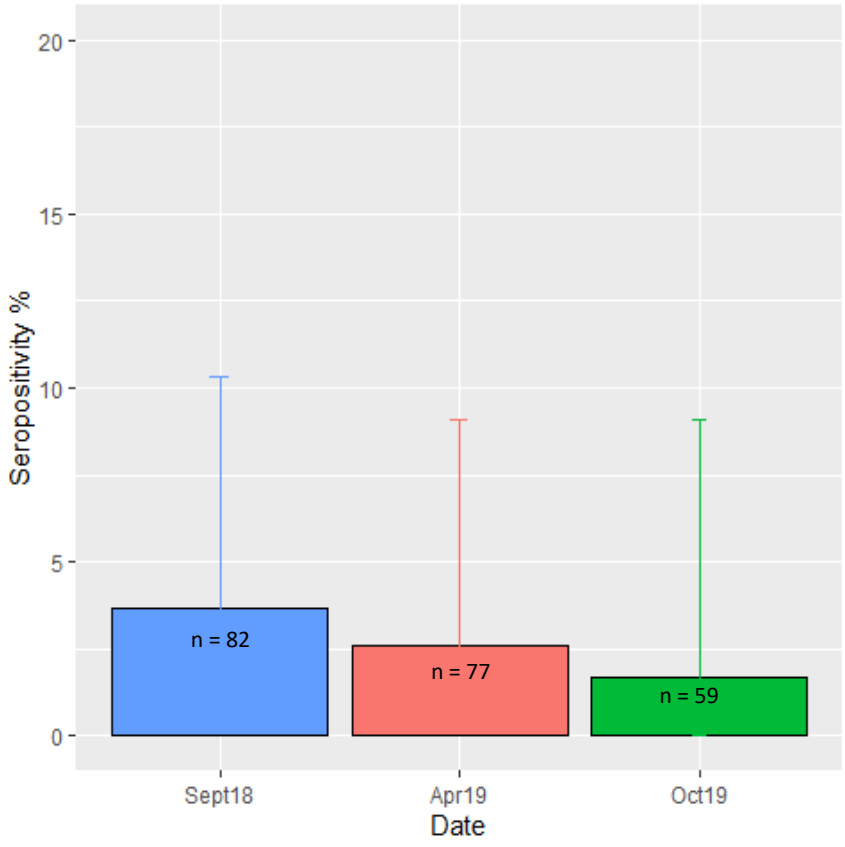
Table 4.4.1. Summary of samples tested with the PVNA at each time point.

Time Point	N	Cattle	Sheep	Goats	Dogs
September 2018	517	347	82	74	14
April 2019	529	365	77	74	13
October 2019	414	292	59	57	6

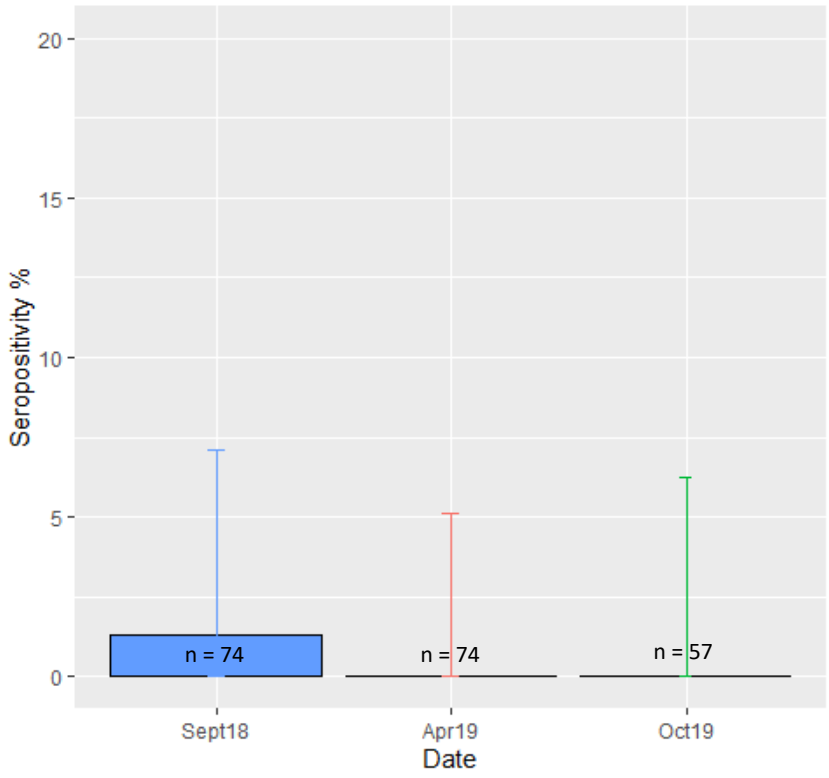
A) Cattle



B) Sheep



C) Goats



D) Dogs

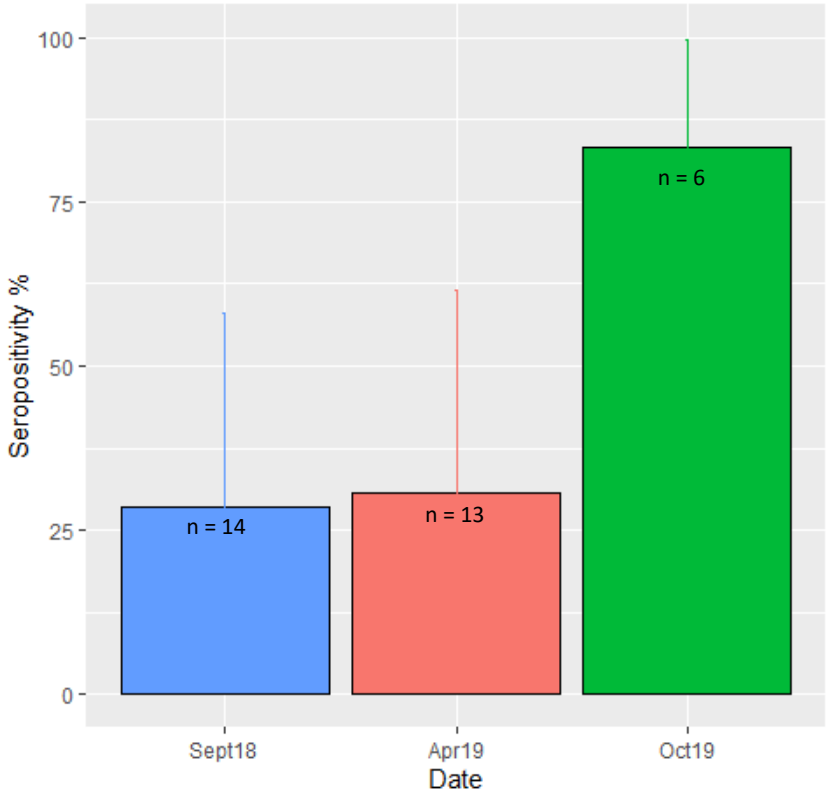
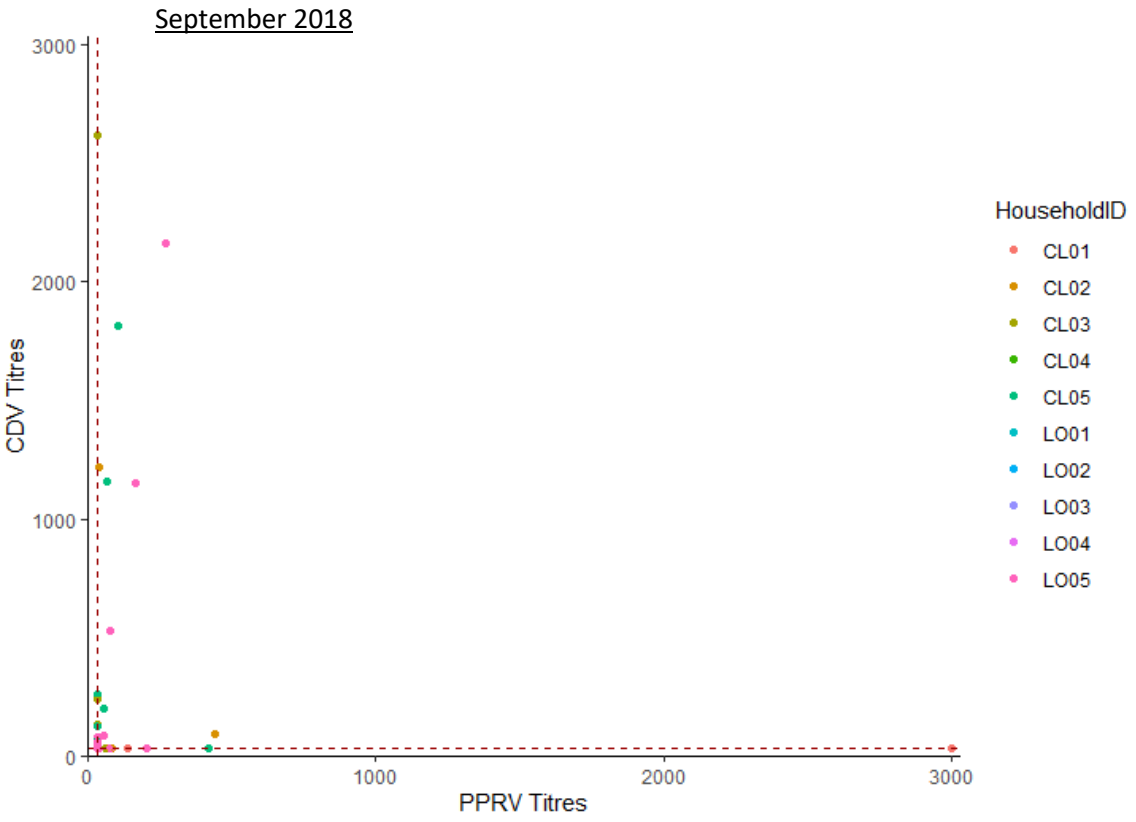
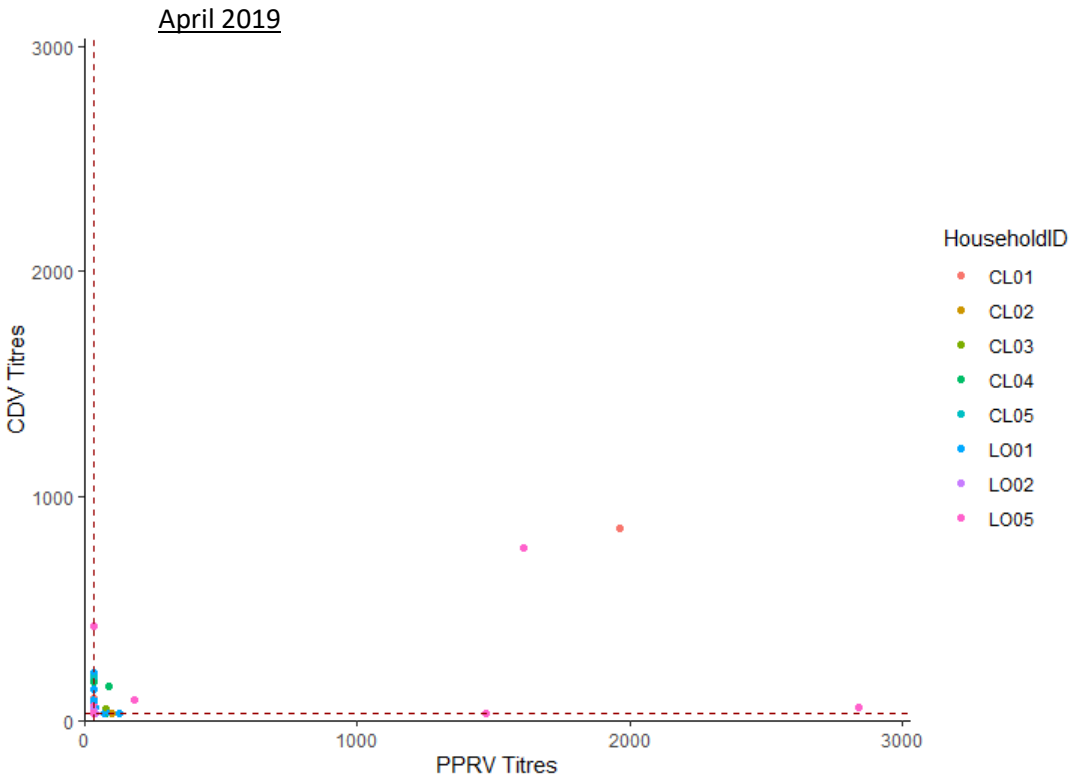


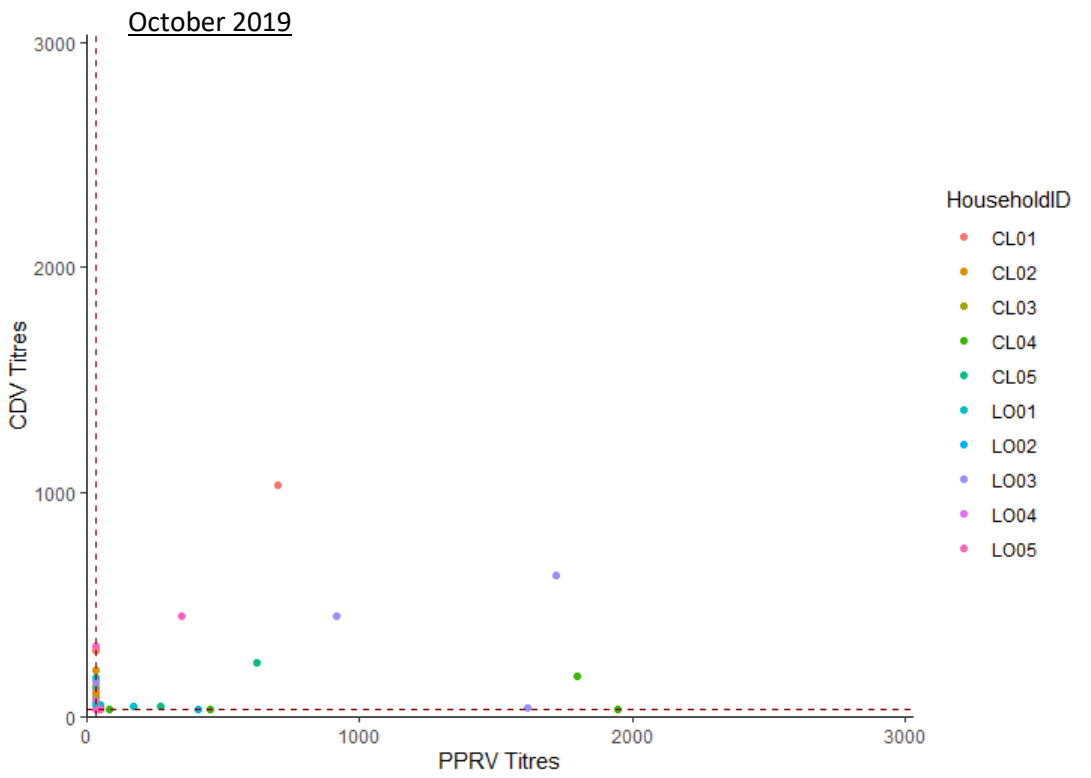
Figure 4.4.1 (A-D). CDV seroprevalence at each time point. For September 2018, all animals sampled are included in the figures. For April 2019, only animals which were seronegative in September 2018 were included. For October 2019, only animals previously seronegative in September and April were included. This therefore shows the true seroprevalence of each time point. Seropositivity indicates individuals with a neutralising antibody titre against CDV-specific glycoproteins tested using the PVNA. Cattle are represented in A, sheep in B, goats in C, and dogs in D. Sample sizes are noted above the bars.

4.4.2. Antibody titres against CDV and PPRV over time

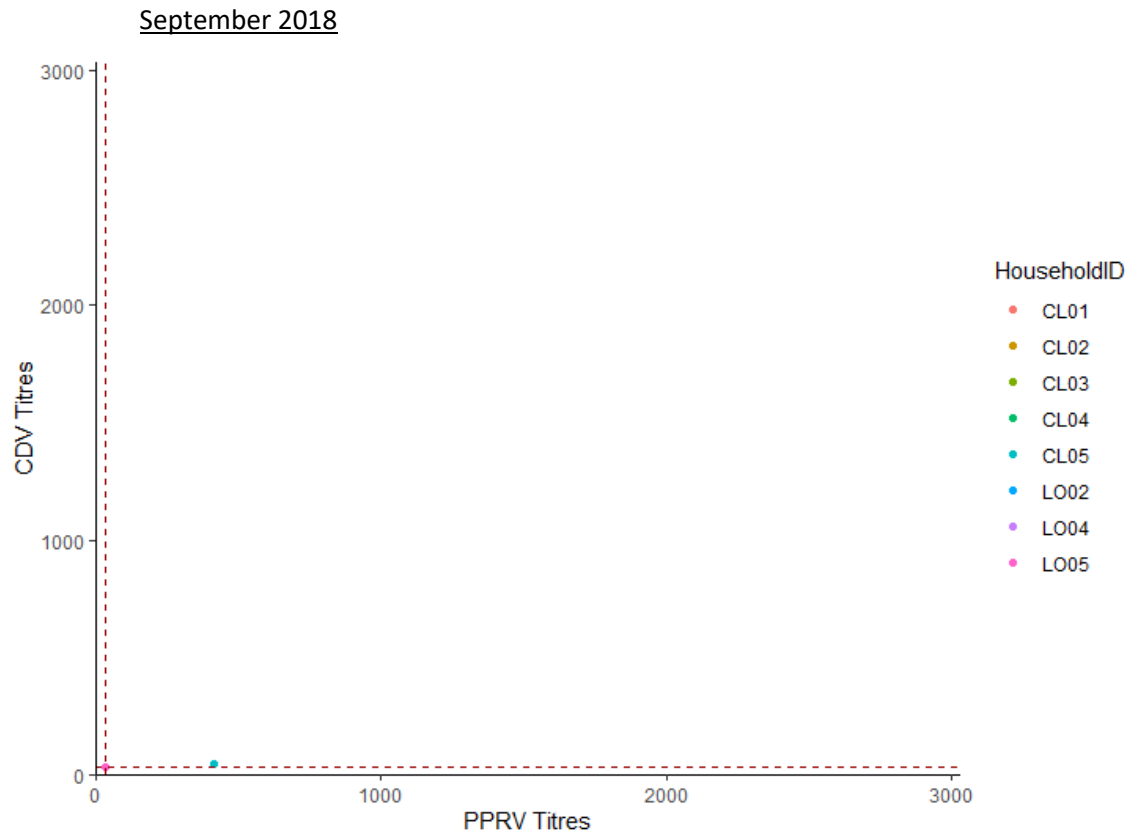
A) Cattle





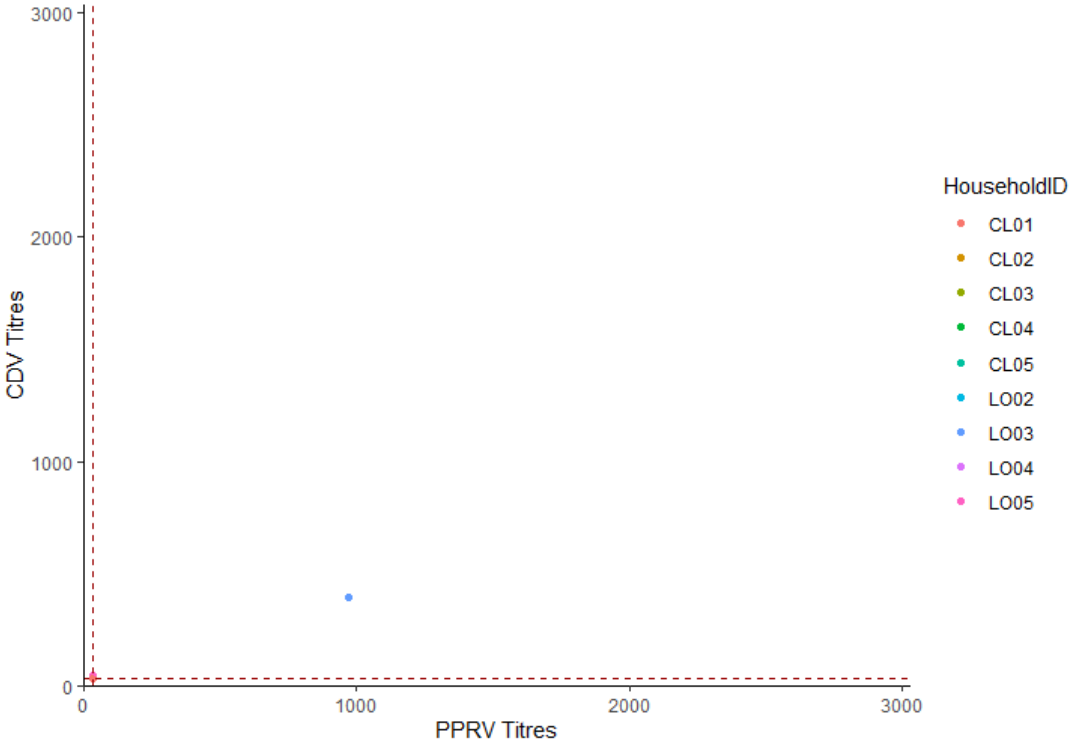

October 2019
CDV Titres
PPRV Titres
0
1000
2000
3000
HouseholdID
CL01
CL02
CL03
CL04
CL05
LO01
LO02
LO03
LO04
LO05

B) Sheep


September 2018
CDV Titres
PPRV Titres
0
1000
2000
3000
HouseholdID
CL01
CL02
CL03
CL04
CL05
LO02
LO04
LO05

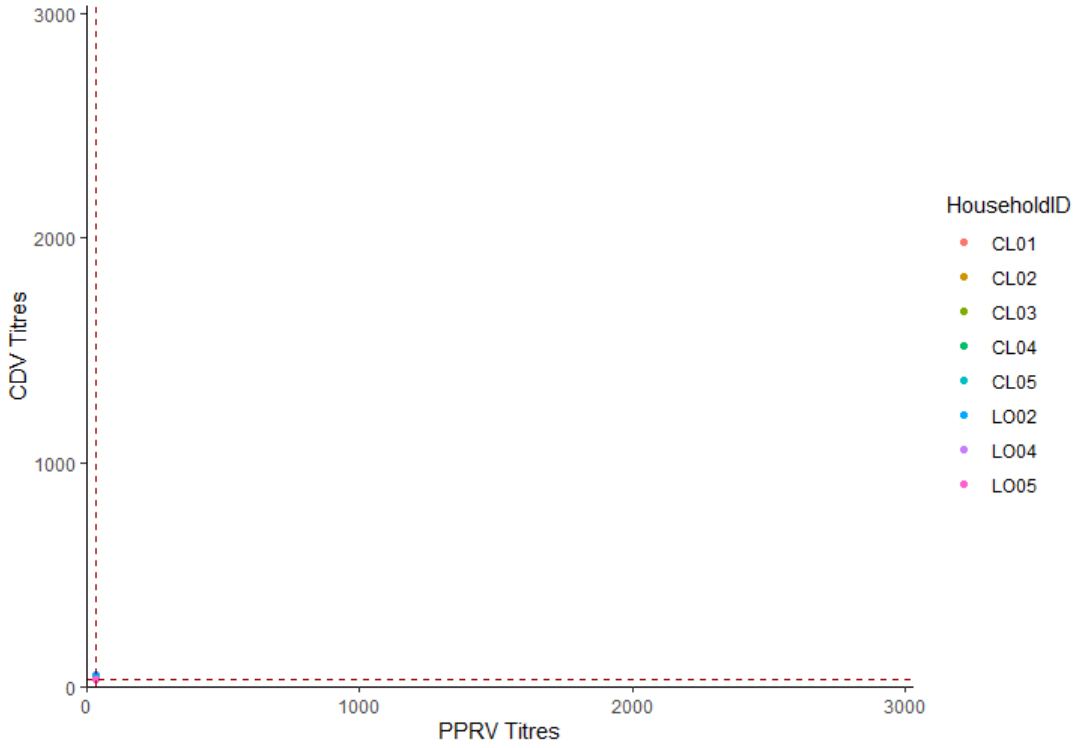
April 2019

October 2019

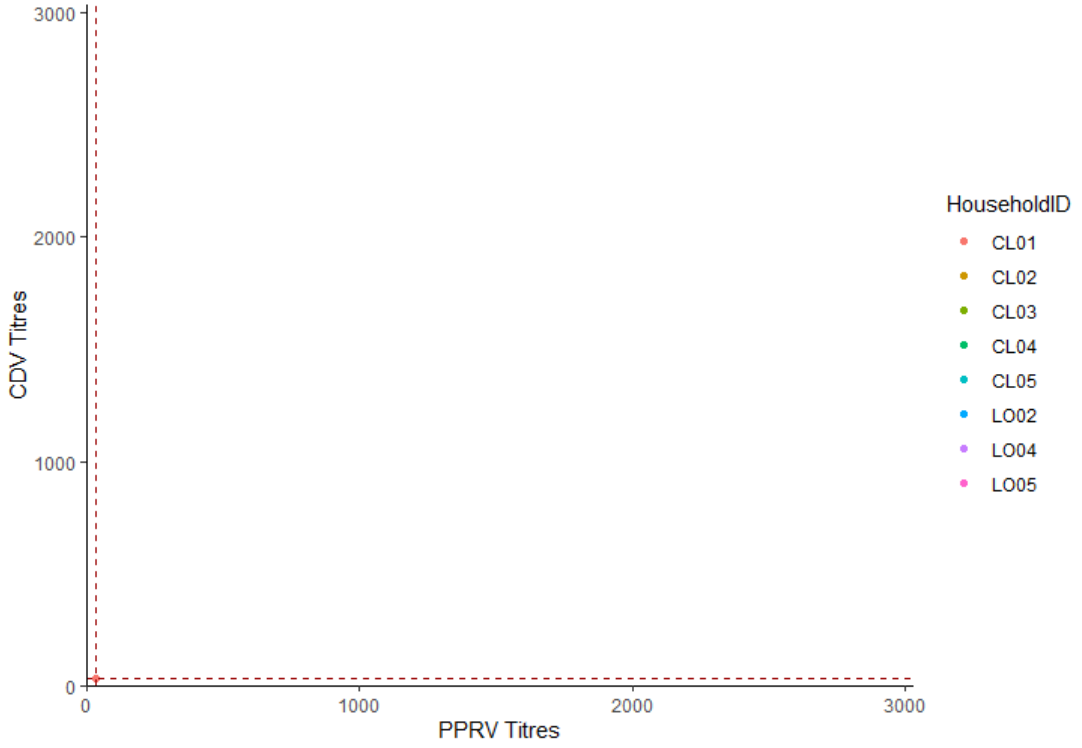


C) Goats

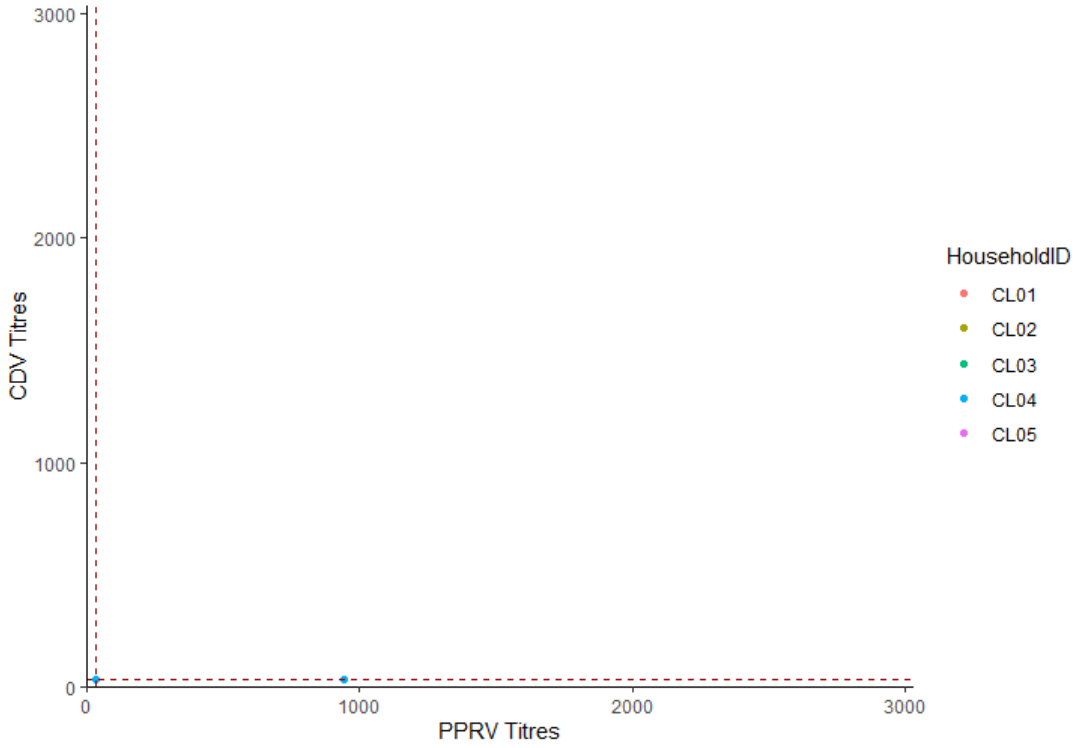
September 2018



April 2019

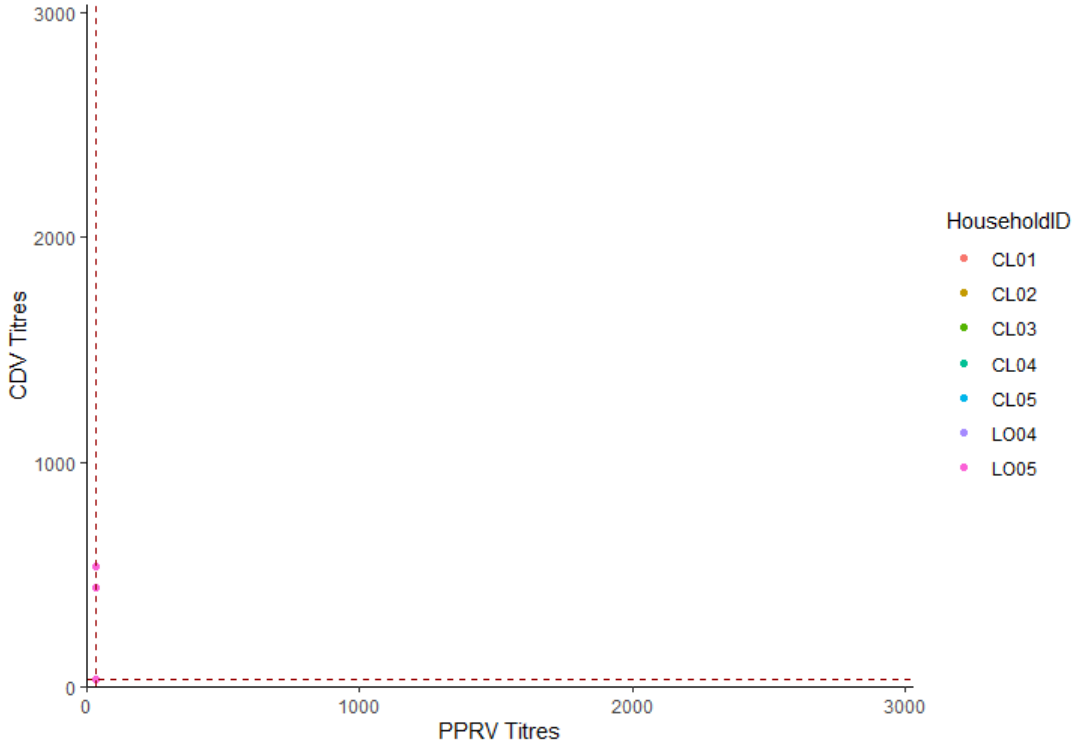


October 2019

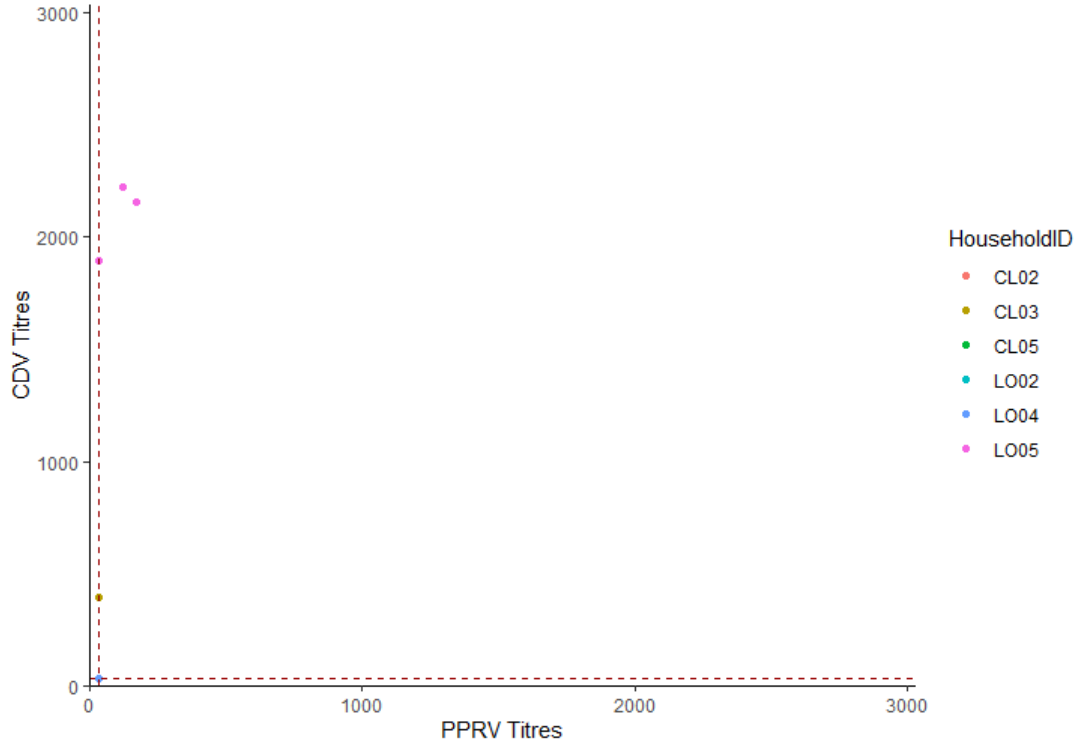


D) Dogs

September 2018



April 2019



October 2019

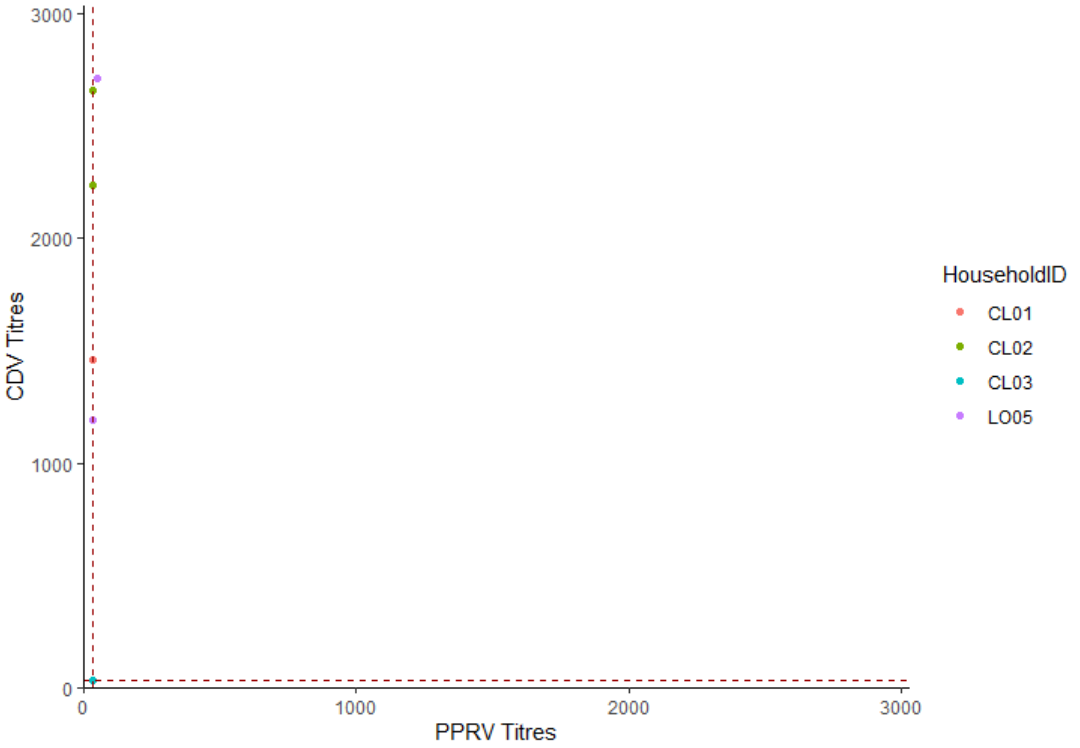


Figure 4.4.2 (A-D). Calculated antibody titres of cattle (A), sheep (B), goats (C), and dogs (D) against CDV and PPRV from each sampling period using the PVNA. Each figure includes all samples collected for that species at that time point. Data points reflect individual animals and are coloured by household. Red dotted lines indicate the titre cut-off point of 32, above which a titre is considered positive. Any data points above the horizontal line are seropositive for CDV. Data points to the right of the vertical line are seropositive for PPRV. Plots demonstrate CDV-specific seropositivity in animals sampled.

4.4.3. Patterns of Seropositivity

To observe if there was a pattern in the seroprevalence data, results were stratified by household and sex for cattle, sheep, goats, and dogs. Overall seroprevalence in cattle was ubiquitous among households and increased over time (fig.4.4.1 A). Six households had low seroprevalences in September 2018, followed by an increase in prevalence in either April (CL01, CL04, LO01, LO02) or October 2019 (LO03 and LO04). All households had seropositive cattle at some stage of the study with no particular pattern to exposure. The highest prevalence in cattle of any household throughout the study was LO03 (42%) during the third sampling period. Household seroprevalence was limited to repeat cattle samples only, although there was no observable or statistical difference in the data.

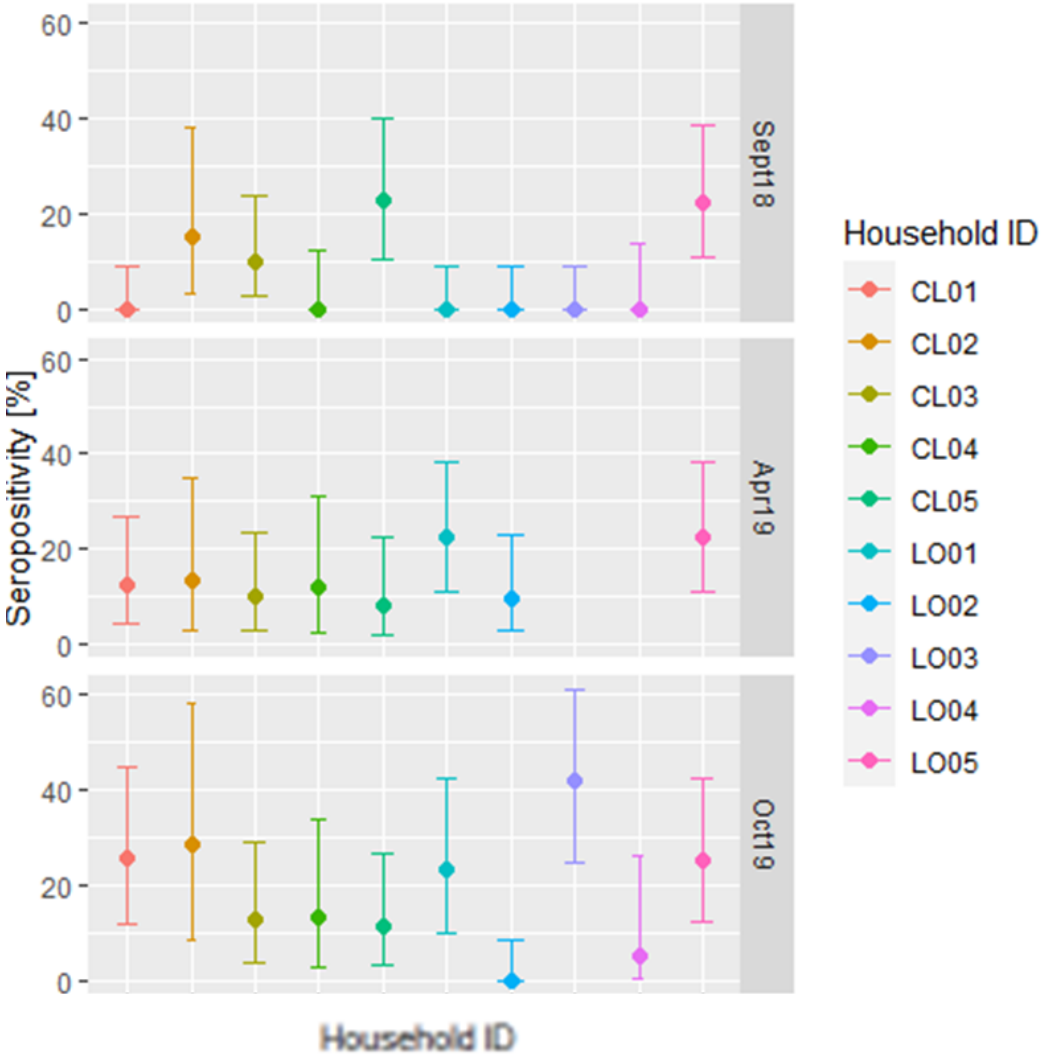
The low number of seropositive sheep was distributed across several households throughout the study, although no particular pattern was observed (fig.4.4.1.B). The

sample size had greatly decreased by October 2019, with n = 1 in LO03, thus seroprevalence was 100% in this household. Two households had seropositive goats overall, one goat during the first sampling period (CL05), and one goat during the third sampling period (CL03). Due to the low seroprevalence of goats in this study, no pattern was observed at the household level.

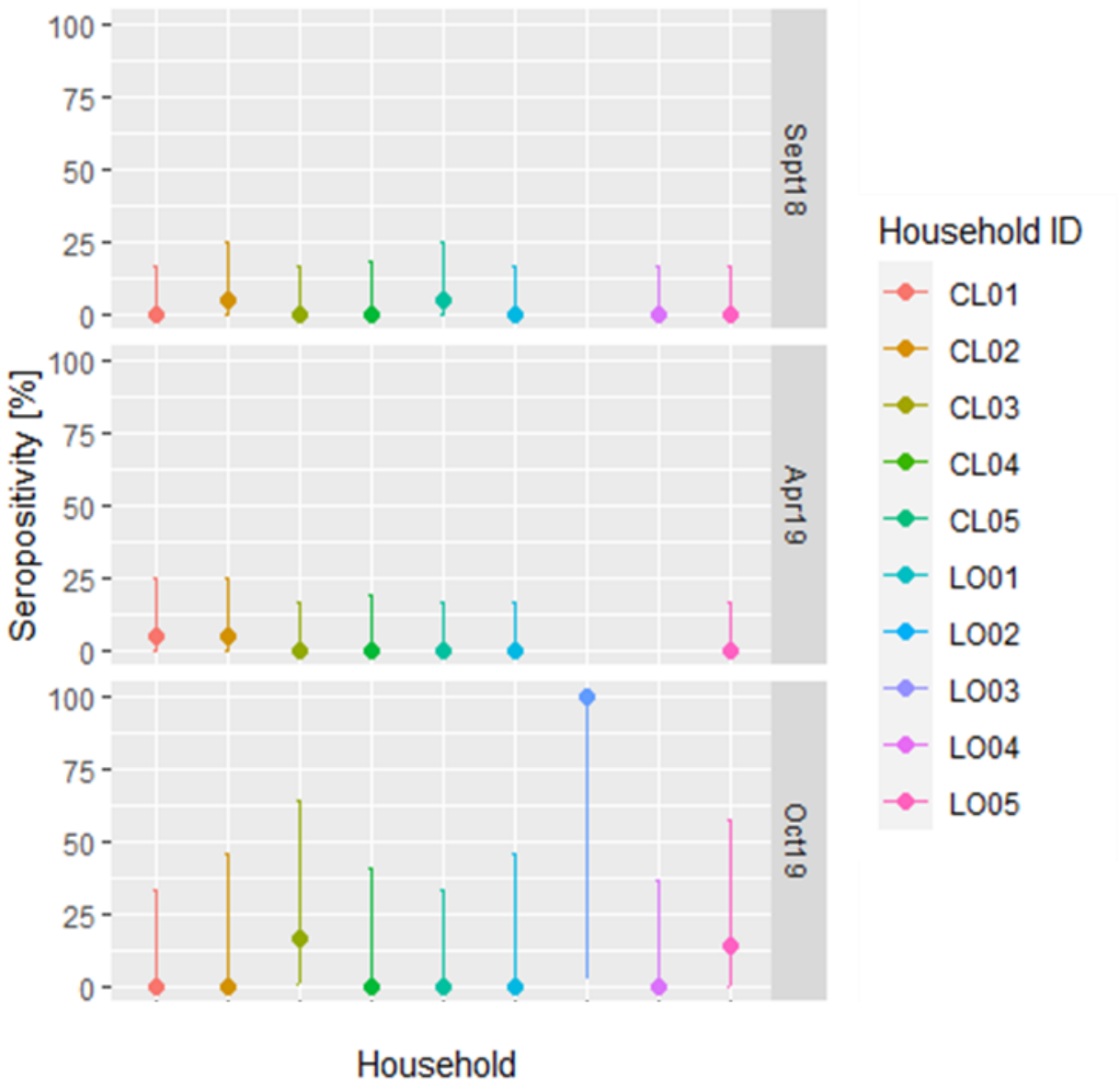
Sample numbers were low for dogs as they opportunistically sampled upon availability. However, all households were expected to have some seropositive dogs, either due to vaccination or natural infection as they are typical hosts of CDV. According to the household questionnaires, approximately 50% of dogs sampled had been vaccinated. Throughout the study, 80% of households had seropositive dogs with the exception of LO02 and LO04 which did not have available dogs during sampling. Overall, there was no particular pattern to household-level dog seroprevalence.

All species demonstrated seropositivity to varying degrees, with cattle showing the most widespread seroprevalence throughout households. There was a general increase in species seroprevalence during the third sampling period, although there appears to be no notable pattern between species and household. There was no statistical association between seropositivity and sex in any of the species.

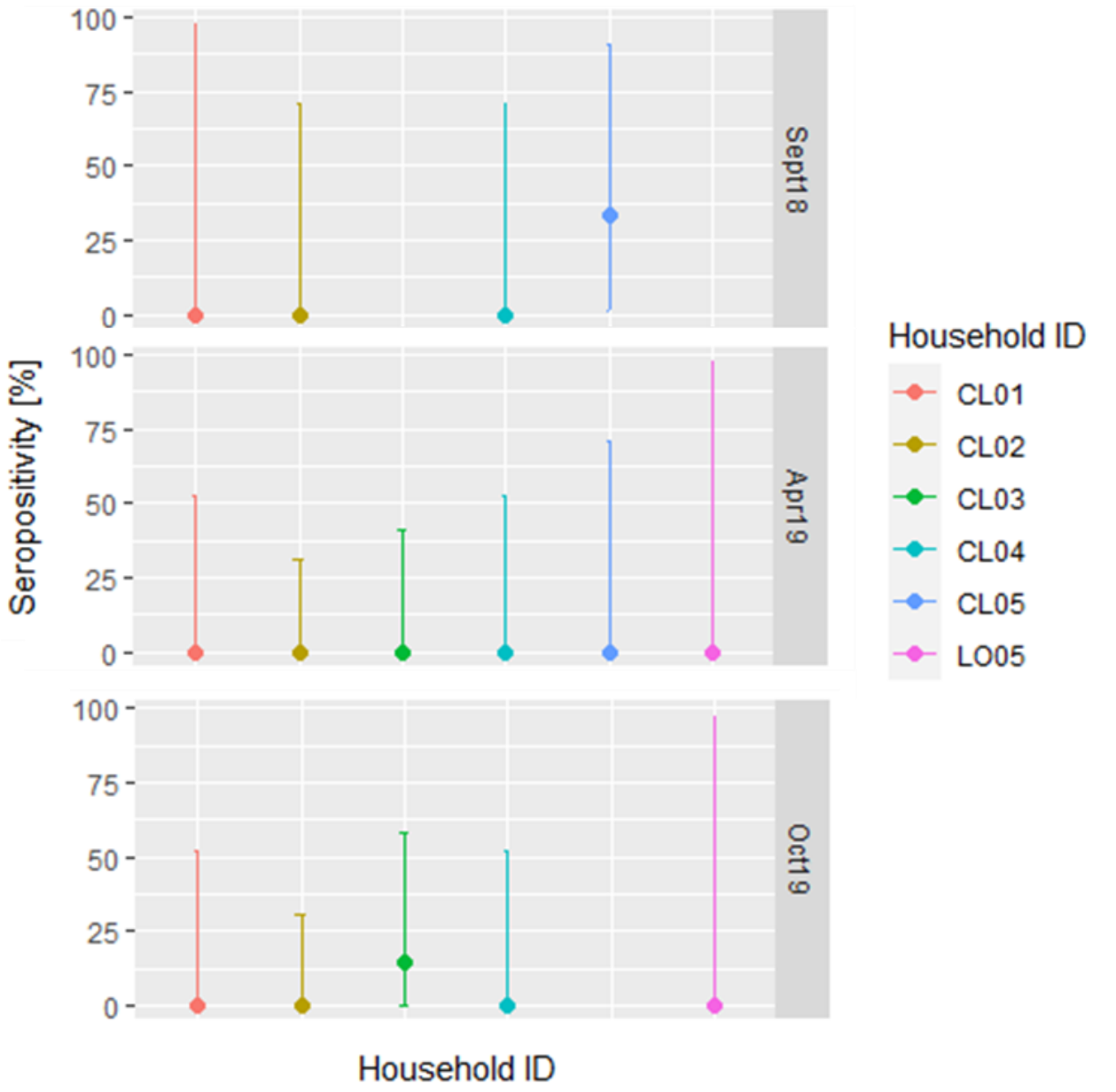
A) Cattle


Seropositivity [%]
60
40
20
0
Sept18
Apr19
Oct19
Household ID
Household ID
CL01
CL02
CL03
CL04
CL05
LO01
LO02
LO03
LO04
LO05

B) Sheep


Seropositivity [%]
100
75
50
25
0
Sept18
Apr19
Oct19
Household
Household ID
CL01
CL02
CL03
CL04
CL05
LO01
LO02
LO03
LO04
LO05

C) Goats


Seropositivity [%]
100
75
50
25
0
Sept18
Apr19
Oct19
Household ID
Household ID
CL01
CL02
CL03
CL04
CL05
LO05

D) Dogs

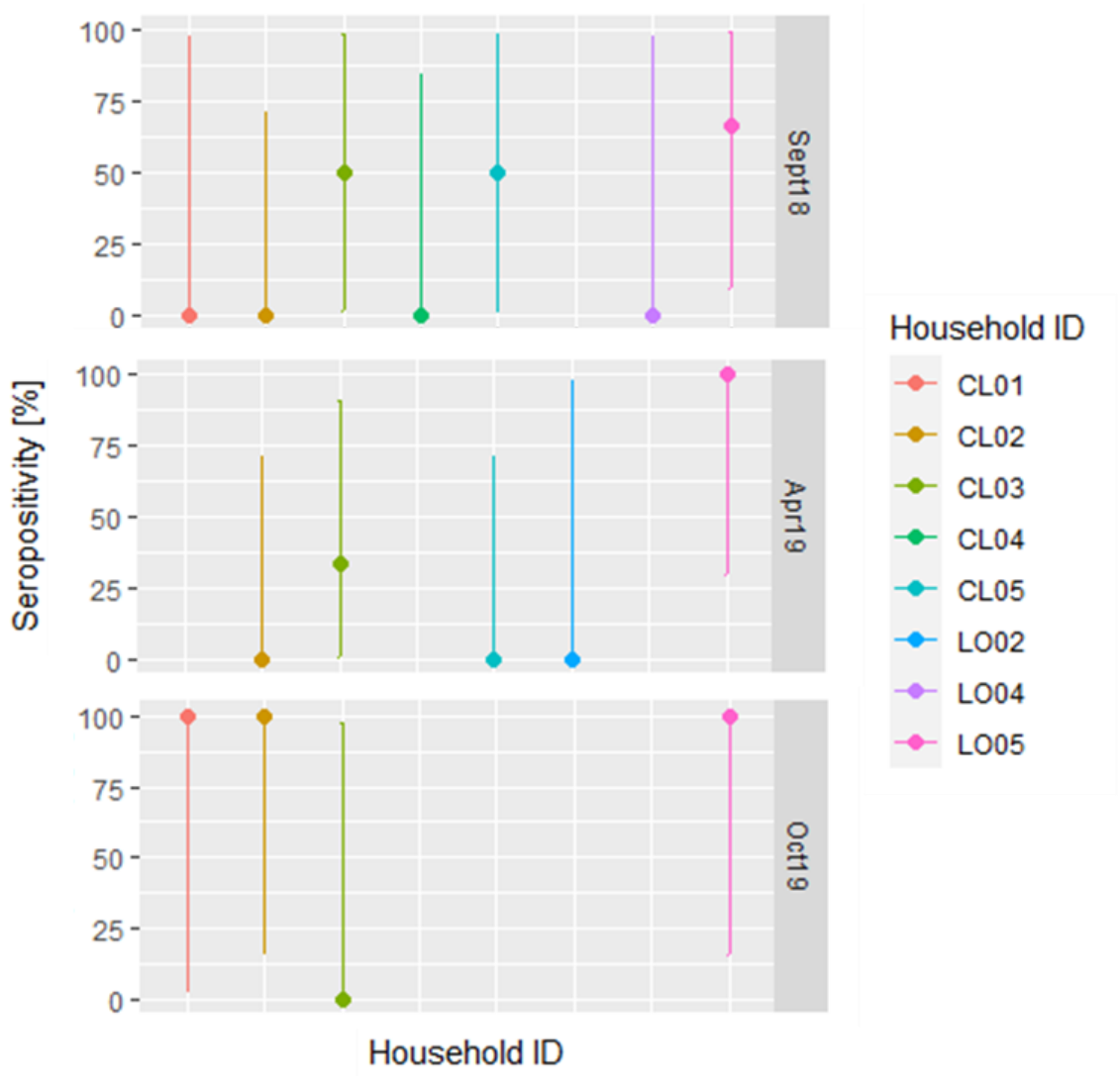


Fig. 4.4.3. (A-D). Plot showing cattle seropositivity by participant household over time. Data points show mean ± SEM. Plot A included all cattle sampled at each time point. All dog samples obtained opportunistically were included in plot D. Sampling periods were in chronological order from top to bottom. Seropositivity was given as a percentage of animals in each household seropositive for CDV antibodies.

4.4.4. Seroconversion

Cattle seroconversion was observed throughout the study, described in the table below. In April 2019, 12% of sampled cattle seroconverted. This number decreased to 5% in the third sampling period. The plot shown in 4.4.4 A describes the pattern of seroconversion of cattle over the course of the study, stratified by household. Three households (LO02, LO03, LO04) did not have any cattle which seroconverted during the study. All other households demonstrate seroconversion in cattle which was ubiquitous. The bivariate model described in 4.4.4 table 1 showed a likelihood ratio of 18.6 (p = 0.028). This indicates that household was a significant variable associated with seroconversion between September 2018 and April 2019. However, no particular household was individually associated with seroconversion. Between April 2019 and October 2019, household was no longer associated with seroconversion (LRT = 0.10, p = 0.94). Sex was not associated with seroconversion at any point during the study.

Although seroprevalence was low, 3% and 6% of sheep seroconverted in the second and third sampling points, respectively. This number was not high enough to include in the GLM analysis. The sheep which seroconverted were from household CL03. Given the small number of sheep it was statistically impossible to determine if there was any relationship between household and seroconversion. No goats seroconverted during the study; thus, no plot was shown. Overall, seroconversion occurred in cattle and sheep across households throughout the study.

4.4.4. Table A. Cattle seroconversion and total number of seropositives over time.

A)	n	No. Seronegative	No. Seropositive	Total No. Seroconversions
Sept-18	347	323	24	-
Apr-19	284	244	40	30
Oct-19	292	239	53	12

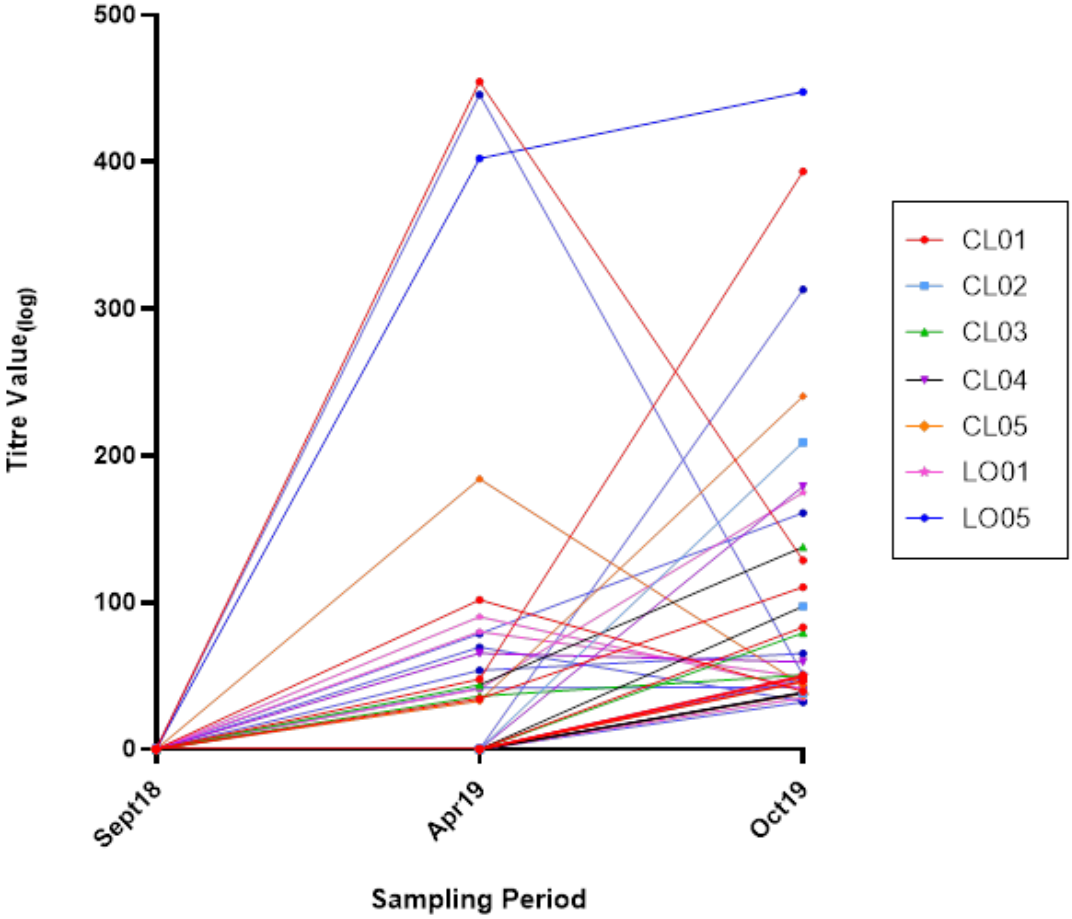
4.4.4. Table B. Sheep seroconversion and total number of seropositives over time.

B)	n	No. Seronegative	No. Seropositive	Total No. Seroconversions
Sept-18	82	81	1	-
Apr-19	67	65	2	2
Oct-19	59	56	3	2

4.4.4. Table C. Goat seroconversion and total number of seropositives over time.

C)	n	No. Seronegative	No. Seropositive	Total No. Seroconversions
Sept-18	76	75	1	-
Apr-19	70	70	0	0
Oct-19	57	57	0	0

A) Cattle


500
400
300
200
100
0
Titre Value(log)
Sept18
Apr19
Oct19
Sampling Period
CL01
CL02
CL03
CL04
CL05
LO01
LO05

B) Sheep

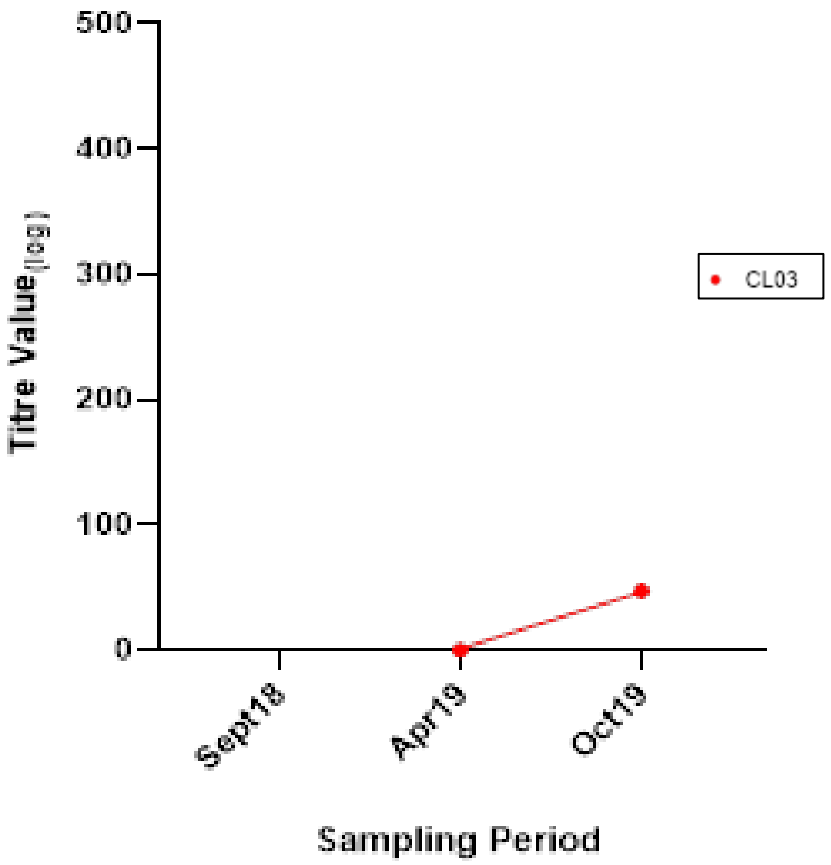


Figure 4.4.4. (A-B). Pattern of seroconversion over the course of the study, reflected by the log titre of CDV-neutralising antibodies. Households of animals is given, and colour coded.

4.4.4. Table 1. Bivariate Model to assess for any relationship between cattle seroconversion and household between September 2018 to April 2019.

Factor Variable	Factor Level	N	Number Seroconverted	Odds Ratio	95%CI	LRT	*p* value
Household	(Ref) CL01	40	6	-	-	18.6	0.028*
	CL02	20	1	-1.26	-4.23 – 0.60		0.26
	CL03	40	3	-0.72	-2.34 – 0.69		0.33
	CL04	28	1	-1.44	-4.41 – 0.40		0.19
	CL05	35	4	-0.34	-1.78 – 0.99		0.68
	LO01	40	4	-0.64	-2.07 – 0.68		0.35
	LO02	40	7	0.18	-1.01 – 1.40		0.76
	LO03	40	0	-16.8	-2.93 - 32.62		0.98
	LO04	25	5		-1.48 – 1.13		0.81

	LO05	40	0	-16.83	-4.67 – 63.63		0.99
Sex	Ref					6.00	0.11
	Female	225		21.92	-2.16 – 10.10		0.99
	Male	123		0.54	-0.31 – 1.38		0.20

4.4.4. Table 2. Bivariate Model to assess for any relationship between cattle seroconversion and household between April 2019 and October 2019

Factor Variable	Factor Level	N	Number Seroconverted	Odds Ratio	95%CI	LRT	*p* value
Household	(Ref) CL01	40	3	-	-	0.10	0.94
	CL02	20	0	-16.33	NA -79.95		0.99
	CL03	40	0	-16.32	NA – 46.38		0.98
	CL04	28	4	0.67	-0.93 – 2.39		0.40
	CL05	35	3	-0.13	-1.88 – 1.62		0.87
	LO01	40	3	0.03	-1.72 – 1.79		0.96
	LO02	40	1	-1.45	-4.48 – 0.65		0.21
	LO03	40	5	0.58	-0.91 – 2.24		0.45
	LO04	25	1	-0.65	-3.70 – 1.48		0.58

	LO05	40	5	0.40	-1.08 – 2.06		0.59
Sex	Ref					16.09	0.06
	Female	225		0.29	-1.03 – 2.17		
	Male	123		0.43	-1.06 – 2.37		

4.4.5. Assessing the clinical evidence of cattle infection with CDV.

Clinical data collected on livestock were based on signs of disease in typical hosts of CDV, either observed during sampling or reported by the farmer during the questionnaire. It was important to determine if there was any relationship between clinical signs of disease and seropositivity or seroconversion. For this analysis a bivariate model was conducted for each species. During the first sampling period there was a positive association between observed clinical signs of disease in cattle and seropositivity (p = 0.004, 0.11-0.62). This means that cattle displaying at least two signs of disease, consistent with CDV in known hosts, were 0.37 times more likely to be seropositive for CDV than cattle which did not show any signs.

However, the model showed that clinical signs observed in cattle during the second and third sampling periods had no relationship with seroconversion (p = 0.64, 0.32-0.46; p = 0.23, 0.16-0.64, respectively). This means that seroconversion was occurring in cattle throughout the study irrespective of clinical disease. Signs of disease in cattle reported by farmers over the 6 months prior to the beginning of the study showed no association with seroconversion. In the second sampling period, no signs of disease were reported in cattle by any of the farmers. In October 2019, some farmers reported signs of disease in cattle in the previous 6 months, however this had no association with seroconversion (p = 0.22, -0.08- -0.01).

Sheep and goats which were seropositive in September 2018 were not associated with observed clinical signs of disease during sampling (p = 0.99, -2.80- -1.44; p = 0.99, -0.26- -1.44, respectively). Further, there was no association between seropositivity, and signs of disease reported by farmers in the 6 months prior to the study commencing in September 2018 (p = 0.99, -1.64- -0.62; p = 0.99, -2.59- -1.25,

respectively). Due to the low number of seroconversions in sheep and absence of seroconversion in goats, statistical analyses using this model could not be performed for observed or reported clinical signs. Overall, there was no relationship between serological outcome and clinical signs of disease in sheep or goats.

4.4.5. Table 1. Bivariate Model to assess for any relationship between seropositivity (first sampling period) or seroconversion (second and third sampling period) of animals and observed clinical signs of disease.

Time Point	**Clinical Signs**	**Odds ratio**	***p* value**	**95%CI**
Sept18 clinical signs	Cattle	0.37	0.004**	0.11-0.62
	Sheep	-14.48	0.99	-2.8 - -1.44
	Goats	-15.7	0.99	-0.26 – 0.64
Apr19 seroconversion clinical signs	Cattle	0.09	0.64	0.32-0.46
	Sheep	NA	NA	NA
	Goats	NA	NA	NA
Oct19 seroconversion clinical signs	Cattle	0.24	0.23	0.16-0.64
	Sheep	NA	NA	NA
	Goats	NA	NA	NA

4.4.5. Table 2. Bivariate Model to assess for any relationship between seropositivity (first sampling period) or seroconversion (second and third sampling period) of animals and farmer-reported clinical signs of disease.

Time Point	Clinical Signs	Odds ratio	*p* value	95%CI
Sept18 seropositivity clinical signs	Cattle	0.56	0.21	-0.39 – 1.41
	Sheep	-14.45	0.99	-1.64- -0.62
	Goats	-14.69	0.99	-2.59- -1.25
Apr19 seroconversion clinical signs	Cattle	NA	NA	NA
	Sheep	NA	NA	NA
	Goats	NA	NA	NA
Oct19 seroconversion clinical signs	Cattle	-0.02	0.22	-0.08 - -0.01
	Sheep	NA	NA	NA
	Goats	NA	NA	NA

4.6. Discussion

There is a dearth of research into the potential for cattle, sheep, and goats to host CDV. It is important to understand whether livestock are hosts for CDV and become infected in their natural environment. Further, it is critical to establish if they exhibit any signs of disease identified in typical host species, such as: nasal and ocular discharge; anorexia; coughing; sneezing, and diarrhoea. Disease associated with CDV infection could indicate potential virus shedding. This study aimed to confirm

if cattle, sheep, and goats are positive for CDV-specific neutralising antibodies and assess the patterns of seropositivity between species and households. Further, patterns of seroconversion were investigated to identify when livestock became infected during the study, and if this differed between households. Finally, this study utilised clinical data to assess if there is any relationship between infection and clinical signs of disease.

4.6.1. Confirmation of CDV seropositivity.

A major finding of this research was that cattle, sheep, and goats were positive for CDV-neutralising antibodies during at least one stage of this study. This mirrors findings from Logan *et al*(2016), although they concluded that it was potentially cross-neutralisation occurring from PPRV exposure in cattle. This study found distinct populations of antibodies in all species which were CDV-positive and PPRV-negative. Based on the presented data, this chapter concludes that CDV infection elicited specific antibody responses detectable with the PVNA. The majority of cattle seropositives occurred during the first sampling period, suggesting that CDV was circulating in the weeks prior to the beginning of the study. The data also showed that some animals had neutralising antibodies to both CDV and PPRV. It is impossible to tell whether this was due to co-infection or cross-reactivity at this stage and future work should investigate this further. However, there are some possible indicators of both scenarios from the data presented. For instance, if a sample neutralises both CDV and PPRV but the titre for CDV is much higher than that for PPRV, it could be assumed that the response was elicited by CDV and that PPRV has been cross-neutralised. In the alternative scenario of co-infection, CDV and

PPRV would likely show high titres against both viruses, relative to negative and cross-reactive samples. This is because morbillivirus infection results in long-term immune protection thus the titre should remain high for both viruses (Logan *et al*, 2016; Theil *et al*, 1998). However, without proof this is purely speculation. The antibodies neutralising both CDV and PPRV in dogs is consistent with data from Logan *et al*(2016), which showed ~50% dogs which were sampled in the UK elicited a neutralising response to PPRV (Logan *et al*, 2016). Since PPRV in absent in the UK, the only explanation for this is that antibodies raised by CDV sero-reacted with a conserved region of the morbilliviral nucleocapsid. To investigate the issue of cross-neutralisation and co-infection further, sequencing of RNA samples could reveal which viruses were actually present in seropositive animals, although the likelihood of finding virus is low without active infection occurring during sampling. Moreover, an immunogen peptide-binding assay could help to further determine which specific antigens the antibodies react to (Li *et al*, 2018; Muñoz-Alía & Russell, 2019).

4.6.2. Patterns of seropositivity.

The peak of seroprevalence for all species during the final sampling period suggested that the virus was circulating between the second and third sampling periods. However, based on farmer questionnaire data there were no signs of disease consistent with CDV in any of the species during this time. This indicated that circulating infection did not have any clinical manifestation, reflected by the lack of any statistical relationship shown in the models. The fact that the virus is circulating at a sub-clinical level in livestock could indicate two possibilities. Firstly, this could

reflect the early stages of adaptation to a new host species whereby the virus has not yet adapted to overcome the immune response of livestock, therefore no noticeable clinical disease developed (Longdon *et al*, 2014). Secondly, it is possible that livestock play a role as a reservoir for CDV, maintaining the infection at a sub-clinical level which could be highly pathogenic to other species (Wasik *et al*, 2019). In a wider context, predatory wildlife species, such as hyaenas, consume approximately 4.5% of the Serengeti livestock population each year (Holmern *et al*, 2016). If those livestock are infected, depredating carnivores could succumb to severe disease after consumption of infected tissue. Given that carnivore predation is a major cause of livestock deaths in northern Tanzania (Mbise & Røskaft, 2021), this would potentially be a threat to conservation.

The fact that cattle were the most seroprevalent livestock species in this study could have been due to the larger sample size which increased the likelihood of finding seroconverting individuals. The sample sizes of sheep and goats were smaller, and fewer individuals remained in the household throughout the study as a result of small ruminant trade. Most cattle were retained in each household throughout the study, thus repeat sampling was possible for more cattle than small ruminants. This would partially explain the lack of seroconversion in small ruminants compared to cattle. For example, a study with a large sample size of small ruminants may find higher seroprevalences than shown in this study. It is also possible, however, that cattle were more susceptible to CDV than sheep and goats, although there is no current data available to support this. In the previous chapter cattle demonstrated a greater proportion of seropositivity to PPRV than in sheep and goats. Although an unusual finding, cattle have been suggested to be immunologically robust, dead-end hosts for

PPRV (Yadav *et al*, 2021; Schulz *et al*, 2019). It is possible that this is also the case for CDV, although further research is needed to clarify this.

As no particular pattern was observed for seropositivity in each species, the data indicate that CDV was ubiquitous in the environment. There are two possible explanations for this. Firstly, previous data show prolonged low-level exposure of domestic dogs to CDV in northern Tanzania as it continuously circulates amongst canid and non-canid hosts (Weckworth *et al*, 2020). Secondly, dogs which were infected would be expected to shed the virus, but low-level vaccine shedding could have occurred in some dogs which had received the vaccine, also exposing livestock in close proximity (Wilkes *et al*, 2014). Unfortunately, swabs taken to detect shedding of CDV RNA could not be analysed due to the Covid-19 pandemic, and information on dog vaccination is unreliable. Moreover, although there is little available data on CDV in domestic cats within this area, cats freely roam and are susceptible in a similar way to domestic dogs. Domestic cats, however, are not vaccinated against CDV and may be an additional source of infection. In combination, these scenarios could explain a source of constant exposure seen in livestock.

4.6.3. Clinical signs of disease.

A key finding of this study was the positive association between seropositive cattle at the beginning of the study and clinical signs of disease. However, when clinical data were compared with seroconversion events occurring in the second and third sampling periods, there was no relationship between these variables. This finding can

likely be explained by co-infection. When looking at seropositive cattle in September 2018, it is impossible to determine exactly when they became infected with CDV, and which other infectious agents were circulating before the study began. The clinical signs observed in cattle (nasal discharge, ocular discharge, diarrhoea, and anorexia) are attributable to a range of potential infections such as bovine viral diarrhoea, contagious bovine pleuropneumonia, helminth infection, and foot and mouth disease virus (di Tiodoro *et al*, 2020; Ahmed *et al*, 2019; Baker, 1995). The fact that there was no relationship between seroconversion and clinical signs of disease in cattle suggested that the correlation observed in the first sampling period was confounded by other infections. Based on these data, cattle are only sub-clinically infected and may be dead-end hosts for the virus, as suggested for PPRV (Schulz *et al*, 2019). However, the lack of clinical disease does not necessarily mean that cattle do not shed the virus. This is an important factor worthy of further research, particularly given the geographical overlap of livestock with protected wildlife species in Tanzania. It is difficult to determine the extent of CDV infection in sheep and goats due to the lack of data. Based on these findings, there were not enough data to definitively confirm whether sheep and goats develop clinical disease from CDV infection.

4.7. Chapter conclusion

This chapter showed that cattle, sheep, and goats can become infected with CDV and mount an explicit immune response to this virus. Both cross-neutralisation and co-infection may explain co-neutralising antibodies to CDV and PPRV, although this

was indeterminable from these data. Infection was ubiquitous in the environment, indicated by the lack of patterns to the infections, either between households or sampling period. Further, there was no indication from the data that livestock develop clinical disease from CDV infection. It is likely that CDV has emerged into livestock but that they are dead-end hosts and are unlikely to shed the virus, although this should be confirmed. This is preliminary work investigating CDV infection in atypical livestock hosts, and further research is required to clarify co-exposure and cross-reactivity between morbilliviruses. This chapter provides a useful insight into the emergence of CDV into new hosts in a location with critically endangered wildlife subject to the threat of CDV outbreaks. It could therefore be useful to consider livestock as a sentinel for CDV surveillance. Moreover, future work should consider the possibility that livestock may become more significant in the circulation of CDV in the future.

Chapter 5: Genetic Variation and CDV Outbreaks in African Wild Dogs (*Lycaon pictus*) of the Serengeti

5.1 Summary

African wild dogs (AWD) are one of the most critically endangered carnivore species of sub-Saharan Africa. The Serengeti National Park is home to approximately 120 AWD, although recurring outbreaks of canine distemper virus (CDV) have caused devastation to pack numbers, risking their genetic fitness. Previous work by Marsden *et al* (2012) assessed the structure and diversity of archived samples of from the Serengeti population using microsatellite and MHC markers. To date, little work has been done to investigate the population diversity and structure over time in the Serengeti. Further, it is unknown if the DRB immune marker has been subject to selection prior to outbreaks in this area, or if this is in any way related to disease susceptibility. This chapter will build on previous work by Marsden *et al* (2012) by incorporating early data with more recent samples including those from a CDV outbreak in 2017. The chapter will achieve this by addressing the following aims: i) confirm how the 2017 outbreak of CDV was the cause of high mortality in the Serengeti Nyasaori pack, ii) if DRB diversity has changed over time, iii) if neutral marker diversity has changed over time, leading to genetic structuring, iv) comparing neutral and immune markers to assess if there are changes due to factors beyond demographic change, and v) if there is any evidence of selection at the DRB in association with CDV outbreaks.

A pseudotype-based virus neutralisation assay was used to detect CDV-specific neutralising antibodies in sera of wild dogs which survived the 2017 outbreak. RT-

qPCR was used to detect the CDV H gene in tissue samples from deceased wild dogs, as the H gene is highly specific to the virus. Results showed an overwhelming proportion of PCR positives and lack of protective immune response in deceased and surviving animals, respectively. DRB allele frequency data showed changes in diversity. Analysis of heterozygosity for neutral and immune markers showed variation in both over time. Variation at the DRB coincided with the timing of CDV outbreaks. The test for bottlenecks using the TPM model showed no evidence of excess heterozygosity or bottleneck. Bayesian analysis of microsatellites found some genetic structuring over time (K = 2). Positive Tajima's D (TD) statistic indicated balancing selection during at least two time points at the DRB, in keeping with CDV outbreaks. It is still unknown if DRB alleles are linked to disease susceptibility, though this should be the focus of further investigation. This chapter provides useful data on the Serengeti wild dog population and will inform future work.

5.2 Chapter introduction

5.2.1 African wild dog population decline

African Wild Dogs (AWD) are a critically endangered carnivore species of sub-Saharan Africa (Creel *et al*, 2004). Populations have declined in number over recent decades (Marsden *et al*, 2013; Tensen *et al*, 2019). For example, Wolf & Ripple (2017) constructed a contraction map of viable wild dog populations and found that their habitat had decreased by 93% as of 2016, compared to their historic ranges. This has been attributed to habitat loss, increased competition for prey with other threatened carnivore species, and infectious diseases such as canine distemper virus

(CDV) and rabies virus (Wolf & Ripple, 2017; Tensen *et al*, 2019). According to the 2012 International Union for Conservation in Nature (IUCN) Red Data list updated in 2020, there are approximately 1,409 mature individuals remaining in the wild (Marsden *et al*, 2012; Woodroffe 2021). The reduced geographical range of populations and consequent increase of physical competition for prey with other carnivore species, in addition to the highly social nature of wild dogs, means that pathogen transmission and exposure to infections may also increase (Cleaveland *et al*, 2006; Woodroffe *et al*, 2004). Outbreaks of disease have had a devastating impact on the survival of this species. In 1991 a CDV epidemic occurred in the Maasai Mara National Reserve of Kenya, which caused mortality of 50% in domestic dogs, concurrent with the disappearance of African wild dog packs (Alexander & Appel, 1994; MacDonald, 1992; Goller *et al*, 2010). In 1994, an outbreak of CDV in the Serengeti National Park caused mass deaths of lions (*Panthera leo*) and other wildlife species, including African wild dogs (Goller *et al*, 2010). Further, the deaths of entire wild dog packs, partially due to CDV, have been reported in Botswana's Chobe National Park and Moremi Game Reserve (1994-1995), and in Kruger National Park (1981) (Woodroffe & Ginsberg, 1999).

The Serengeti National Park (SNP) has suffered from disease outbreaks in its wild dog population since the Park's establishment in 1951 (Creel & Creel, 2002). Between 1970 and 1991, the wild dog population completely disappeared, thought at the time to be extinct following outbreaks of CDV and rabies (Creel & Creel, 2002; Schaller, 1972; Gascoyne *et al*, 1993; Kat *et al*, 1995). In 1994 an outbreak of CDV caused massive loss of carnivores of the SNP, including lions, hyaenas, and wild dogs. The virus was sequenced from a deceased lion by Roelke-Parker *et al* (1996), which matched isolates from subsequent CDV outbreaks in Serengeti wild dogs

between 2007 and 2018 (Goller *et al*, 2010; Viana *et al*, 2015; Weckworth *et al*, 2020).

In an effort to rebuild the wild dog population in Tanzania, a translocation programme was created by The Tanzanian Wildlife Research Institute (TAWIRI), with the first pack translocations to the Serengeti occurring in 2015 (Wild Dog Report 2018, TAWIRI). Nyasaori pack of wild dogs in the Serengeti was considered one of the most successful stories of wild dog translocation, TAWIRI reported 25 adults with several litters of pups in 2017 (Wild Dog Report 2018, TAWIRI). In December 2017 at least 19 members of the pack died from a suspected CDV outbreak. Outbreaks over recent decades have had a devastating impact on Serengeti wild dogs, and much research has been done to help conserve the species in this area. To date, many studies have investigated factors affecting the survival of wild dogs, such as: increased competition for food; conflict with humans; vaccine studies; and threats from disease. For example, a questionnaire survey by Mbise *et al* (2020) assessed human willingness to coexist with wild carnivores of the SNP, the lack of which poses a risk to wild dogs from hunting. Several studies have looked at safe vaccination of wild dogs, such as Woodroffe (2021), Calatayud *et al* (2019), and Burrows *et al* (1994). Cleaveland *et al* (2007) carried out an epidemiological study to understand how outbreaks impact conservation efforts in the SNP. Although high mortality rates have occurred during CDV outbreaks, disease outcomes can vary greatly between individuals, the reasons for which are not well understood (Woodroffe, 2021; Weckworth *et al*, 2020).

The genetic structure of a population which has suffered outbreaks and mass death is likely to change. Changes in structure can occur due to a lack of gene flow, genetic bottleneck, and inbreeding when numbers decline (Marsden *et al*, 2010). However, few studies have investigated the genetic structure of the surviving Serengeti population. A study by Marsden *et al* (2012) investigated the structure of wild dog populations in different locations, using data from two time periods. Further, the study assessed evidence of selection on MHC class II gene alleles. The Serengeti data from the analyses included samples taken before 1991 and 2001-2009. The results provided insight into the effect of decreasing population size on gene flow. The study by Marsden *et al* (2012) has provided a critical foundation for the current longitudinal, retrospective investigation into the structure and diversity of the Serengeti wild dog population. In addition to the data provided by Marsden *et al*, more recent data from 2011-2016 and 2017 have been analysed in this study. Within the longitudinal framework, this study utilised microsatellite data to look at differentiation between time periods to determine how population structure has changed over time. In addition, this study assessed whether there is any evidence of selection over time by utilising MHC-DRB1 allele data from four time periods up until late 2017. The latest time period during 2017 includes mortality data collected during a CDV outbreak which killed many wild dogs in the Serengeti. The purpose of the mortality data is to assess if there is any pattern with specific alleles and disease outcome. This is important in further understanding if there is a potential link between allele inheritance and disease susceptibility.

5.2.2 Neutral Markers

It is important to understand the genetic variation of populations and the differentiation between them to ultimately inform decisions on conservation measures (Marsden *et al*, 2012). Populations undergo neutral evolution over time, partly shaped by changing demographic factors (Bos *et al*, 2008; Ramirez *et al*, 2006). To measure such changes, most studies have focused on mitochondrial DNA and microsatellite markers, due to their highly polymorphic and neutral nature (Bos *et al*, 2008; Charlesworth *et al*, 2003). Previous work has utilised such markers to explore population structure and variation in African wild dogs (Tensen *et al*, 2019; Marsden *et al*, 2013). For example, Tensen *et al* (2019) analysed microsatellite data from a managed metapopulation of South African wild dogs. Due to geographic isolation, the wild dogs were manually translocated between packs to mimic natural dispersal. The study found that 95% of genetic diversity would have been lost without such management. This work highlights the importance and usefulness of neutral loci in wild dogs populations. Similar work has been done on other endangered canids such as the Iberian wolf *Canis lupus signatus* (Ramirez *et al*, 2006), and the African wolf, *C. lupaster* (Mallil *et al*, 2020). Neutral loci therefore provide key information on population stability and gene flow, consequently informing conservations decisions. However, it is difficult to infer the genetic fitness of a population based on neutral genetics alone (Stouthammer & Nunney, 2014; Woodroffe & Ginsberg, 1999; Bos *et al*, 2008). This is because neutral markers are not subject to selection pressure and therefore they do not represent adaptive change. Further, variation at neutral loci and variation at adaptive loci are not always directly linked, thus one does not necessarily predict the other. Therefore, both are needed to

fully understand genetic change and departure from genetic variation within a population (Holderegger *et al*, 2005).

5.2.3 Immune Markers

Studies have often utilised immune cell receptor genes as adaptive markers to study genetic variation and natural selection (Sommer, 2005; Schaffner & Sabeti, 2008; Vychodilova *et al*, 2018; Kloch *et al*, 2021). Immune markers are more suitable for studying factors affecting the population in a rapid, transient way, for example in *in situ* contexts such as disease outbreaks (Elena *et al*, 1996; Bos *et al*, 2008). They are good indicators of genetic fitness of a population, because of their association with disease susceptibility and an individual's ability to respond to infection (Loots *et al*, 2018; Osborne *et al*, 2015). To date, many genetic studies have investigated the Major Histocompatibility Complex (MHC) to elucidate the loci associated with survival or death of animals (DeCandia *et al*, 2018; Sommer, 2005; Jeffery & Bangham, 2000; Thursz *et al*, 1999). The wealth of research on MHC associations with susceptibility and resistance to disease make it the most widely used proxy for studying adaptive variation (Marsden *et al*, 2012; Piertney & Oliver, 2006).

The MHC is a transmembrane complex present on all nucleated cells of the body that functions in antigen presentation (Benacerraf, 1981). There are two main distinguished classes. MHC class I molecules are involved with intracellular peptides broken down by the cell's proteosome (Osborne *et al*, 2015). Viral particles which are proteolytically processed in this way have their antigens secreted to the cell surface, where MHC I presents the peptides on the cell surface to kickstart an immune response (Osborne *et al*, 2015). MHC class II molecules only occur on the

surface of antigen-presenting cells (such as dendritic cells and macrophages) and are involved with antigen uptake from bacteria, parasites, and other extracellular pathogens (Saddegh-Nasseri & Germain, 1991).

In canids, the MHC is referred to as the Dog Leukocyte Antigen (DLA). The DLA is made up of subunits DRB1, DQA1, and DQB1, the genes for which have been associated with the efficiency of the immune response and disease outcome (Bexfield *et al*, 2012; Kennedy *et al*, 2002). Such genes can be highly polymorphic, though this varies between species (Kennedy *et al*, 2011). Their polymorphic nature in some canids means that they have been used to research diversity within populations of such species (Marsden *et al*, 2012; Marsden *et al*, 2009; Wagner *et al*, 1996). For example, Kennedy *et al* (2011) assessed DLA haplotype diversity in a population of Ethiopian wolves (*Canis simensis*) which were vaccinated during a rabies outbreak. The study showed that dogs with all DLA haplotypes responded well to vaccination, except for those with one particular haplotype. However, inferences about the less responsive haplotype in terms of selection could not be made due to a small sample size (Kennedy *et al*, 2011). Marshall *et al* (2016) assessed the DRB1 diversity in the Newfoundland red fox (*Vulpes vulpes deletrix*), which was previously linked to susceptibility to infection with the endemic heartworm (*Angiostrongyles vasorum*), (Marshall *et al*, 2016; Castillo *et al*, 2010). The large number of alleles and high nucleotide divergence at the DRB1 locus showed that DRB1 was likely to have undergone balancing selection in the past. These studies demonstrate the usefulness of the MHC in measuring natural selection, but they were cross-sectional studies, thus limited to diversity at only one point in time. This is an important gap in previous research, as diversity varies greatly both temporally and spatially (Marsden *et al*, 2010). To address this, Marsden *et al* (2012) assessed diversity at the MHC in

African wild dogs from four East African locations at two time points. Analysis of MHC DRB-1 using a Fixed Effects Likelihood test showed evidence of purifying selection at two peptide-binding residues, important in peptide binding processes of the MHC complex (Consuegra *et al*, 2005; Marsden *et al*, 2012). In addition to investigating temporal adaptive diversity, studies are increasingly doing this within a neutral framework (Ujvari & Belov, 2011; Ejsmond & Radwan, 2011; Savage *et al*, 2018). For example, the study by Marsden *et al* (2012) showed a correlation between neutral and adaptive selection, strongly influenced by demographic change (Marsden *et al*, 2012).

This informs of both population differentiation in relation to changing demographic factors and changing diversity at the MHC under forces of selection, such as disease outbreaks. Sudden outbreaks of disease can risk diversity loss in a population if mortality rates are high (O'Brien & Evermann, 1988; Schmeller *et al*, 2020). Recent CDV outbreaks within the Serengeti have greatly reduced population numbers, thus there is an increased risk of diversity loss, population structuring, inbreeding, and reduced fitness. Therefore, there is a need to conduct a new, more in-depth investigation into the temporal changes in neutral and adaptive variation in this population. This chapter will address these gaps in research and contribute to the knowledge on which conservation decisions may be based.

Therefore, this aims of this chapter are as follows:

i. To confirm that the high mortality of wild dogs from the Nyasaori pack was due to a CDV outbreak in the Serengeti.

ii. To test if the diversity or variation of immune markers has changed over time. This will be achieved by measuring changes in DRB allele and nucleotide diversity, and changes in heterozygosity. This will also be compared to the CDV outbreak data.

iii. To test if population structure has changed over time due to demographic change or selection. This will be achieved by measuring changes in neutral marker haplotype diversity, heterozygosity, and population differentiation over time.

iv. Compare variation in microsatellite and DRB markers to determine whether there were differences beyond demographic changes that might be due to selection at the MHC. This will be achieved by comparing heterozygosity and the inbreeding coefficient (F*is*) for DRB and microsatellites to determine how much their variation deviates from neutrality at each time point. Fisher's exact test will determine any significant deviation from the HWE between microsatellites and DRB.

v. Assess if there was any evidence of selection at DRB alleles in relation to CDV outbreaks, including the laboratory-confirmed 2017/2018 Serengeti outbreak in the Nyasaori pack. This will be done by assessing variation in DRB heterozygosity and changes in allele frequency and by using statistical tests for selection.

5.3 Chapter methods

5.3.1 Samples

Samples were obtained from the Serengeti-Mara ecosystem from four time periods: samples from 1) before the 1991 CDV outbreak and 2) from animals returning to the Serengeti between 2001 and 2009 were those described previously in Marsden *et al.* (2011; 2012); samples from 3) from the Tanzanian Wildlife Research Institute (TAWIRI) monitoring and translocation programme between 2011 and 2017; and 4) from the 2017 CDV outbreak were provided by Ernest Mjingo Eblate and Emmanuel Mpolya Masenga (TAWIRI).

5.3.2. CDV RNA extraction

To confirm the presence of CDV in the tissue of deceased wild dogs from the 2017 outbreak, RNA extraction was done on tissues most relevant to the immune response. This is primarily tissue targeted by the virus (lung tissue), and the tissue producing primary immune cells during infection (spleen and lymph nodes), (Heinen *et al*, 1986; Delemarre *et al*, 1990). Small sample scrapings (1g) were taken from spleen, lymph node, or lung tissue using an 11-blade scalpel (Swann-Morton) and kept at -80°c in 10mL Bijou tubes (Greiner Bio-One). Total RNA extraction was carried out using the RNeasy extraction kit (Qiagen) and RNA yield was quantified using a Nanodrop 1000 (Thermofisher Scientific, UK).

5.3.3. CDV cDNA sequencing

To confirm the strain of CDV causing wild dog mortality in the 2017 outbreak, extracted RNA was converted into cDNA and sequenced. Extracted RNA was used

to synthesise the first strand of cDNA (ProtoScript First Strand cDNA synthesis kit, New England Biolabs) before being subjected the following PCR conditions:

- Initial denaturing at 95°c for 15 minutes.
- Denaturing at 94°c for 30 seconds.
- Annealing at 63°c for 1 minute.
- Extension at 72°c for 1 minute.

This cycle was repeated 30 times using primers targeting the CDV H gene (5'- CAC CAA GTC ATA GAT GTC TTG ACA C-3' and 5'- ACT ACT AGA ATA CCA TCT TGT GAA C-3'). PCR products were then visualised by agarose gel electrophoresis; a positive reaction was confirmed by the presence of a ~700bp product. The products of each positive reaction were then excised and extracted from the agarose by using a QIAquick gel extraction kit (Qiagen). The purified products were then sent for Sanger sequencing externally on an ABI3730 sequencer (Eurofins Genomics) using forward primer DRBIn1 (CCGTCCCCACAGGACATTTC) and reverse primer DRBIn2M13r (CAGGAAACAGCTATGACC TGTGTCACACAC CTCAGCACCA).

5.3.4. Detection of CDV antibodies

Following the deaths of the Nyasaori pack in Serengeti, 12 samples from the Ngorongoro pack were tested for protective antibodies. This was done using the pseudotype-based virus neutralisation assay as described by Logan *et al* (2016). Serum samples were serially diluted from 1:8 to 1:32768 and incubated with pseudotyped VSV expressing CDV glycoproteins for 1 hour. This allowed time for any neutralizing antibodies to neutralize the pseudotyped virus. HEK239 cells

expressing a dog SLAM receptor were then added and incubated for 48 hours. Steadylite Plus solution was then added to activate firefly luciferase expressed within the pseudotyped virus. A viral titre was calculated by the point at which 90% luciferase activity is still inhibited.

5.3.5. AWD DNA extractions

For the purpose of genotyping individual wild dogs, DNA was taken from archived blood, serum, or tissue samples. DNA extractions and PCR reactions for samples collected after 2007 were carried out by Elizabeth Kilbride; those collected prior to that were used in Marsden *et al*. (2011; 2012). For animals collected between 2011 and 2017, DNA was extracted from blood stored in EDTA from live animals (n=31) or tissue samples from animals that had died (n=13), using Qiagen Tissue and Blood Extraction Kits (Qiagen Inc., Paisley). For the 2017 CDV outbreak, DNA was extracted from spleens from animals that had died (n=15) and from serum samples from animals that had survived the outbreak (n=42). For all sets, a negative extraction control was included (water only) to test for contamination on each day that samples were extracted. All procedures carried out by UPA.

5.3.6. DRB genotyping

The DRB locus was targeted using forward primer DRBIn1 (CCG TCC CCA CAG GAC ATT TC), and reverse primer DRBIn2M13r (CAG GAA ACA GCT ATG ACC TGT GTC ACA CAC CTC AGC ACCA), as described in Marsden *et al*. (2012). PCR reactions were carried out by Elizabeth Kilbride, under the following conditions:

- Denaturing at 95°c for 15 minutes.

- Denaturing for a further 30 seconds at 95°c.
- Annealing at 62°c for 1 minute.
- Extension at 72°c for 1 minute.

This cycle was repeated 14 times with temperatures reduced by 0.5°c each cycle.

Then:

- Denaturing at 95°c for 30 seconds.
- Annealing at 60°c for 1 minute.
- Extension at 72°c for 1 minute.
- Extension for a further 10 minutes at 72°c.

Reactions were carried out using the PTC-200 DNA engine machine (MJ Research Inc).

PCR products were visualised on agarose gels and those of the target size (270bp, oligonucleotide data unavailable) were sent for Sanger sequencing on an ABI 3730 at the University of Dundee Sequencing service. Sequence clean ups and genotyping were performed by Barbara Mable. Chromatograms were assembled and base-calling errors corrected using Sequencher version 5.6 (Gene Codes Inc, Ann Arbor). IUPAC ambiguity codes were used to identify heterozygous positions based on double peaks in the chromatograms and DRB haplotypes were resolved by aligning to the set of sequences described in Marsden *et al*. (2012) and available on NCBI (Accession numbers: JQ085961, JQ085962, JQ085960, JQ085963- JQ085964). Where heterozygotes could not be resolved based on previously identified haplotypes, putative haplotypes were given a new name. Sequences were aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and optimised using Se-Al, version 2.0 (Rambaut 2002, Sequence Alignment Editor) and Macclade 4.5 (Maddison & Maddison 2000, Sinauer, Sunderland, MA).

5.3.7. DRB haplotype network

Comparison of allele frequencies was visualised at a population level for each time point using PopART version 1.7 (Leigh & Bryant, 2015, Clement *et al*, 2002). A median-joining network of DRB allele sequence data was done with the algorithm described by Bandelt, Forster & Rohl (1999). The distance (shortest path between two sequences) between input sequences was reflected by the distance between nodes, with line markers to indicate mutations in successive input sequences, as described by Leigh & Bryant (2015). For comparison of the Serengeti population in a wider context, DRB allele data from the wild dog population in Laikipia, South Africa, were used as a control in the haplotype network.

5.3.8. DRB Diversity

Samples from the Serengeti population were tested for changes in diversity over the four time periods at the DRB (Pi, Theta, Tajima's D, H_o, H_e, and F_{is}). This was done using DnaSP version 6 (Rozas *et al*, 2017).

Microsatellite Methods

5.3.9. AWD microsatellite genotyping

Of the 10 original microsatellite loci, one locus (FH2785) was excluded because it showed inconsistent amplification in the newly collected samples (2011-2017 and outbreak samples). Serum samples were also excluded because they showed poor amplification in initial tests. The final dataset for microsatellites was based on: 1) 20 samples from before 1991 (from Marsden *et al*. 2011); 2) 13 samples from 2001-2009 (from Marsden *et al*. 2011); 3) 37 samples from 2011-2017 (7 samples did not

amplify); and 4) 14 samples from animals that had died during the 2017 outbreak (1 sample did not amplify).

DNA samples from 2011-2016 and those from individuals who had died from the 2017 outbreak were genotyped at the 10 microsatellite loci that had been used for the samples collected during the earlier two timepoints, as described in Marsden *et al* (2011; 2012): PEZ08, PEZ12, PEZ15,1999); FHC2010, FHC2054, FHC2611, FHC2658, FHC2785, FHC3399, FHC3965 (Table 1). The forward primer of each pair was labelled with ABI fluorescent dyes: NED (yellow), 6-FAM (blue) or HEX (green). Samples were amplified alongside negative controls by multiplex PCR using Qiagen Multiplex PCR mix.

The reagent concentrations recommended by the manufacturer were followed. Multiplex PCR was performed as described in Marsden *et al*. (2012) on PTC-200 (MJ Research) thermocyclers with the following touchdown protocol:

- 15 min at 95°C; 12 touchdown cycles of 94°C for 30 s.
- 1 min at 30 s annealing, starting at 60°C and reducing at 0.5°C per cycle; and
- 72°C for 1 min.
- 33 cycles of 89°C for 30 s, 55°C for 1 min, and 72°C for 1 min.
- Extension of 60°C for 30 minutes.

Microsatellite fragments were resolved using an ABI 3730 with a ROX 500 size standard by The Sequencing Service, University of Dundee. Low concentration or poorly amplifying DNA samples were amplified and genotyped in duplicate. Negative extraction controls, along with negative PCR controls (water only) were also tested for amplification, to test for contamination. Microsatellite size binning

and genotype calling were performed by Barbara Mable, using GENEMAPPER 4.7 (Applied Biosystems).

Table 5.3.9. Information on the microsatellite primers and dyes used for this study, taken from Marsden *et al*. (2012).

Primer	Sequence (5'- 3')	Dye and Primer Mix	Repeat Number	Size Range
FH2611F FH2611R	GAAGCCTATGAGCCAGATCA TGTTAGATGATGCCTTCCTTCT	NED Multiplex 1	20	
PEZ12F PEZ12FR	GTAGATTAGATCTCAGGCAG TAGGTCCTGGTAGGGTGTGG	NED Multiplex 1	7	
PEZ08F PEZ08R	TATCGACTTTATCACTGTGG ATGGAGCCTCATGTCTCATC	6-Fam Multiplex 2	10	
FH2785F FH2785R	ATGGCAGGTCAAGAGTATGG GATAGATCCAAGCCAACACC	6-Fam Multiplex 2	12	
FH3965F FH3965R	GTCGCTCAGCAGTTAAGCTC GAATCCTGGCTCTGCTACTTAC	6-FAM Multiplex 1	20	

FH2054F FH2054R	GCCTTATTCATTGCAGTTAGGG ATGCTGAGTTTTGAACTTTCC	Hex Multiplex 1	7	
FH2658F FH2658R	TCTTAGAAATTGCTGGTGGG TAAGAAACTGCCAGTCTGTGG	Hex Multiplex 1	11	
FH3399F FH3399R	TCTCTATGCCTGCAGTTTCC TTCTGATGCCCTCATAAAGC	Hex Multiplex 1	32	
FH2010F FH2010R	AAATGGAACAGTTGAGCATGC CCCCTTACAGCTTCATTTTCC	Ned Multiplex 2	5	
PEZ 15F PEZ15R	CTGGGGCTTAACTCCAAGTTC CAGTACAGAGTCTGCTTATC	Hex Multiplex 2	9	

5.3.10. File conversion and formatting

Files were converted to ‘Genepop’ format using ‘.txt’ formatted data, imported into R with the ‘adegenet’ package. For structure analysis of microsatellites, PGD Spider version 2.1.1.5 (Lischer, 2012) was used to convert files from text to STRU format. To analyse variation of microsatellites, data was converted manually using GenAIEx 6.5 (Peakall *et al*, 2012).

5.3.11. Microsatellite Variation

To test for a bottleneck effect on the population using the microsatellite alleles (as a function of K), an mRatio test was conducted using the 'diveRsity' package in R Studio. An M-ratio test was used to calculate heterozygosity excess and bottlenecks in the microsatellite data. A model was utilised to do this using the 'mRatio' package in R – Two Phase Model (TPM). The null hypotheses of excess heterozygosity under the TPM model assumes genetic-drift equilibrium, whereby there is no excess or deficit of any allele in the population.

The TPM model is as follows: the probability (p) of mutations; mutations are of >1 unit (two-phase), and the probability of such mutations is 1-p.

For the TPM model a Wilcoxon Sign rank test was used to calculate how much the observed heterozygosity deviated from what would be expected in a mutation-drift equilibrium. This is important to determine if a population bottleneck has occurred at any of the time points, which can occur following sudden population decrease. The Wilcoxon Sign rank test used 95% confidence intervals, so values falling below the 0.05 threshold would be considered a significant deviation from the null hypothesis. Values above the threshold are consistent with the null hypothesis.

5.3.12. STRUCTURE analysis

To determine if the population became differentiated over time, a Bayesian clustering analysis was done. STRUCTURE version 2.3.4 was used to compute the best fitting number of populations for the microsatellite data (K). The value 'K' refers to the optimum number of populations supported by the data with minimal deviation from the Hardy-Weinberg equilibrium. The parameter set used included

10,000 burn-in iterations, 10,000 MCMC iterations, and K values from 1 to 7, with 10 runs per K value. The parameters sampled were: LnP(D) to estimate the log probability of K; Evanno's method of inferring L(K), (Evanno *et al*, 2005); the FST fixation index to measure variation among populations; and the log likelihood of the data given allele frequencies (P) and population (Q). The output file was then processed in the Structure Harvester server (Dent & vonHoldt, 2012). The processed output file was exported to R Studio for graphical and statistical analysis using 'evannoMethodStructure'.

5.3.13. Principal Component Analysis

Principle component analysis (PCA) was carried out to visualise the clustering of microsatellites in the Serengeti population across the four time periods. The 'Adegenet' and 'Ade4' packages in R Studio (Rstudio, PBC, Boston, USA) calculated Eigenvalues to show the variance occurring between principal components on the PCA axes. The screeplot and cumulative variance were included to further demonstrate this variation.

5.4. Results

5.4.1. Confirmation of CDV Outbreak in Nyasaori Pack

In total, 93% (14/15) of the samples from the Serengeti Nyasaori pack sampled during the 2017 outbreak tested positive for CDV by PCR,. The exception was a young pup which died during the outbreak but had no detectable virus in sampled tissues. Unfortunately, RNA samples were only available from tissue from deceased animals and not seropositive animals which survived. Three serum samples from the individuals from the Ngorongoro area that had survived the outbreak showed very

high titres against CDV. This demonstrated a general lack of a protective immune response to the virus compared to other packs and was consistent with the high mortality seen in the Serengeti Nyasaori pack.

5.4.1. Table 1. PCR results for wild dogs following CDV outbreak. Pups are defined as <14 months old, sub-adult is between 14-30 months old and adults are >30 months old.

ID No.	Age	Pack	RT-PCR results
Lpi-2729	Adult	Nyasaori	PCR positive
Lpi-2751	Pups	Nyasaori	PCR positive
Lpi-2752	Adult	Nyasaori	PCR positive
Lpi-2753	Adult	Nyasaori	PCR positive
Lpi-2754	Sub-Adult	Nyasaori	PCR positive
Lpi-2755	Adult	Nyasaori	PCR positive
Lpi-2756	Sub-Adult	Nyasaori	PCR positive
Lpi-2757	Sub-Adult	Nyasaori	PCR positive
Lpi-2758	Adult	Nyasaori	PCR positive
Lpi-2759	Sub-Adult	Nyasaori	PCR positive
Lpi-2760	Sub-Adult	Nyasaori	PCR positive
Lpi-2761	Adult	Nyasaori	PCR positive
Lpi-2762	Sub-Adult	Nyasaori	PCR positive
Lpi-2763	Adult	Nyasaori	PCR positive
Lpi-2764	Puppy	Nyasaori	PCR negative

5.4.1. Table 2. PVNA results for wild dogs following CDV outbreak. A titre of <32 is considered negative, while a titre above this is positive. Pups are defined as <14 months old, sub-adult is between 14-30 months old and adults are >30 months old.

ID No.	CDV serostatus	CDV titre	Age	Pack
Lpi-2782	Neg	<32	Adult	Mwiba
Lpi-2783	Neg	<32	Adult	Nyasaori
Lpi-2732	Neg	<32	Sub-Adult	Nyasaori
Lpi-2733	Neg	<32	Adult	Nyasaori
Lpi-2734	Neg	<32	Puppy	Nyasaori
Lpi-2735	Neg	<32	Adult	Nyasaori
Lpi-2736	Pos	63	Sub-Adult	Nyasaori
Lpi-2737	Neg	<32	Adult	Nyasaori
Lpi-2738	Neg	<32	Adult	Nyasaori
Lpi-2748	Neg	<32	Adult	Nyasaori
Lpi-2784	Pos	8721	Adult	Ngorongoro
Lpi-2785	Pos	7365	Adult	Ngorongoro
Lpi-2786	Pos	2242	Adult	Ngorongoro
Lpi-2789	Neg	<32	Adult	Vodacom
Lpi-2787	Neg	<32	Sub-Adult	Vodacom
KT-MAIN	Neg	<32	Adult	Kikwete main
Lpi-2767	Neg	<32	Sub-Adult	Grumeti

5.4.2. Genetic Diversity at DLA-DRB1

Although the overall number of DRB alleles per individual was quite stable over time, there was some fluctuation in the relative frequency of individual alleles (Table 5.4.2). Only 4 DRB alleles were identified in the pre-1991 population (90204, 90601, 90801, 91101) across the 16 individuals sampled, with 90204 and 90601 present at the highest frequency. Interestingly, the two alleles which were at a high frequency in pre-1991 samples were greatly reduced in frequency or disappeared completely in animals that had returned to the Serengeti between 2001 and 2009 (90204 and 90601, respectively) and two alleles previously absent in the pre-1991 samples were found at moderate to high frequencies (90202 at 0.13, and 90203 at 0.40, respectively).

Between 2011-2017 more individuals were sampled but three completely new alleles were identified, albeit at very low frequency (new1 at 0.02, new2 at 0.01, and new3 at 0.01) but all other alleles had been found in at least one of the previous timepoints. Compared to 2001-2009 samples, there was an increase in the frequency of three particular alleles (90202, 90204, 90601). Interestingly, 90601, which was not found in 2001-2009 was found at relatively high frequency.

Although the 2017 serum samples did not amplify well, six new alleles were identified, three of which showed single nucleotide mutations compared to alleles 90601 and 90201.

Although the animals that died during the CDV outbreak in 2017 were from a single pack, they showed over-representation of an allele that had been relatively rare in other sample sets, including the serum samples (90801) and a decrease in frequency of alleles that had remained relatively stable (90204 and 90601), although the latter was also at low frequency in the serum samples. Allele 90202 gradually increased in

frequency over time since 2001, reaching a frequency of 0.21 in the 2017 sera and 0.33 in the 2017 tissue samples.

Alleles of particular note are: 1) 90202, which was not present in the pre-1991 samples but was at high frequency in later timepoints; 2) 90204, which was at high frequency pre-1991 but was substantially reduced in frequency in individuals that returned after the outbreak (2007-2011), rose again in frequency by 2011-2017 and then was at very low frequency both in individuals that survived and died during the 2017 CDV outbreak; 3) 90604, which showed a similar pattern to 90204; and 4) 90801, which was relatively stable across timepoints but was over-represented in the pack that died during the 2017 CDV outbreak.

Table 5.4.2. Summary of DRB allele frequency over time. The number of heterozygotes (N) and frequency (Freq) at different timepoints are shown. The number and frequency of heterozygotes is also given, along with the number of individuals sampled, gene copies and total number of alleles at each time point.

DRB Alleles	Serengeti <1991		Serengeti 2001-2009		Serengeti 2011-2017		Serengeti 2017 Outbreak		Serengeti 2017 Sera	
	N	Freq	N	Freq	N	Freq	N	Freq	N	Freq
90101	0	0	0	0	0	0	0	0	1	0.02
90201	0	0	0	0	0	0	1	0.03	1	0.02
90202	0	0	4	0.13	14	0.17	10	0.33	5	0.09
90203	0	0	12	0.4	11	0.13	0	0	1	0.04
90204	13	0.41	5	0.17	28	0.34	1	0.03	1	0.02
90601	12	0.38	0	0	13	0.16	1	0.03	5	0.09
90602	0	0	0	0	0	0	0	0	0	0
90701	0	0	0	0	2	0.02	0	0	0	0
90801	6	0.19	5	0.17	8	0.1	17	0.57	2	0.08
91101	1	0.03	1	0.03	1	0.01	0	0	0	0
new1	0	0	0	0	2	0.02	0	0	0	0
new2	0	0	0	0	1	0.01	0	0	0	0
new3	0	0	0	0	1	0.01	0	0	0	0
new7	0	0	0	0	0	0	0	0	1	0.02
new8	0	0	0	0	0	0	0	0	2	0.08
new9	0	0	0	0	0	0	0	0	1	0.02
90601b_1 mutation	0	0	0	0	0	0	0	0	1	0.02
90201b_1 mutation	0	0	0	0	0	0	0	0	1	0.02
90201c_1 mutation	0	0	0	0	0	0	0	0	2	0.08
No. Gene copies	32	32	30	30	82	82	30	30	56	56
No. Individuals	16	-	15	-	41	-	15	-	28	-
No. Heterozygotes	12	0.75	13	0.87	34	0.83	11	0.73	4	0.14
No. alleles	4	-	6	-	11	-	5	-	13	-

5.4.3. DRB Haplotype Network

The haplotype network shows the ancestry of haploid genotypes which have occurred in the population at different time points. The network shows the ancestral haplotype to be 90204, as this haplotype occurred across all time points (reflected by the presence of all time points), and several subsequent haplotypes have mutated from it (Bandelt, Forster & Rohl, 1999). Although haplotypes 90201 and 90204 were shared between time periods, the frequency greatly decreased in the 2017 outbreak group. The top of the network shows three distinct haplotypes (DLA_DRB_1601b, DLA_DRB_90401, and DLA_DRB_04801) at low frequencies, with at least ten mutational changes from the ancestral haplotype 90204 However, they occurred in few individuals and differ from each other by 1 to 2 mutational changes. This is consistent with the data shown in table 5.4.2. The upper half of the network shows haplotypes 90601 and 90801 as higher in frequency overall among the populations, though the 2017 outbreak group differs. The 90601-haplotype occurred at a very low frequency in this group and the 90801 haplotype frequency was highest in this group.

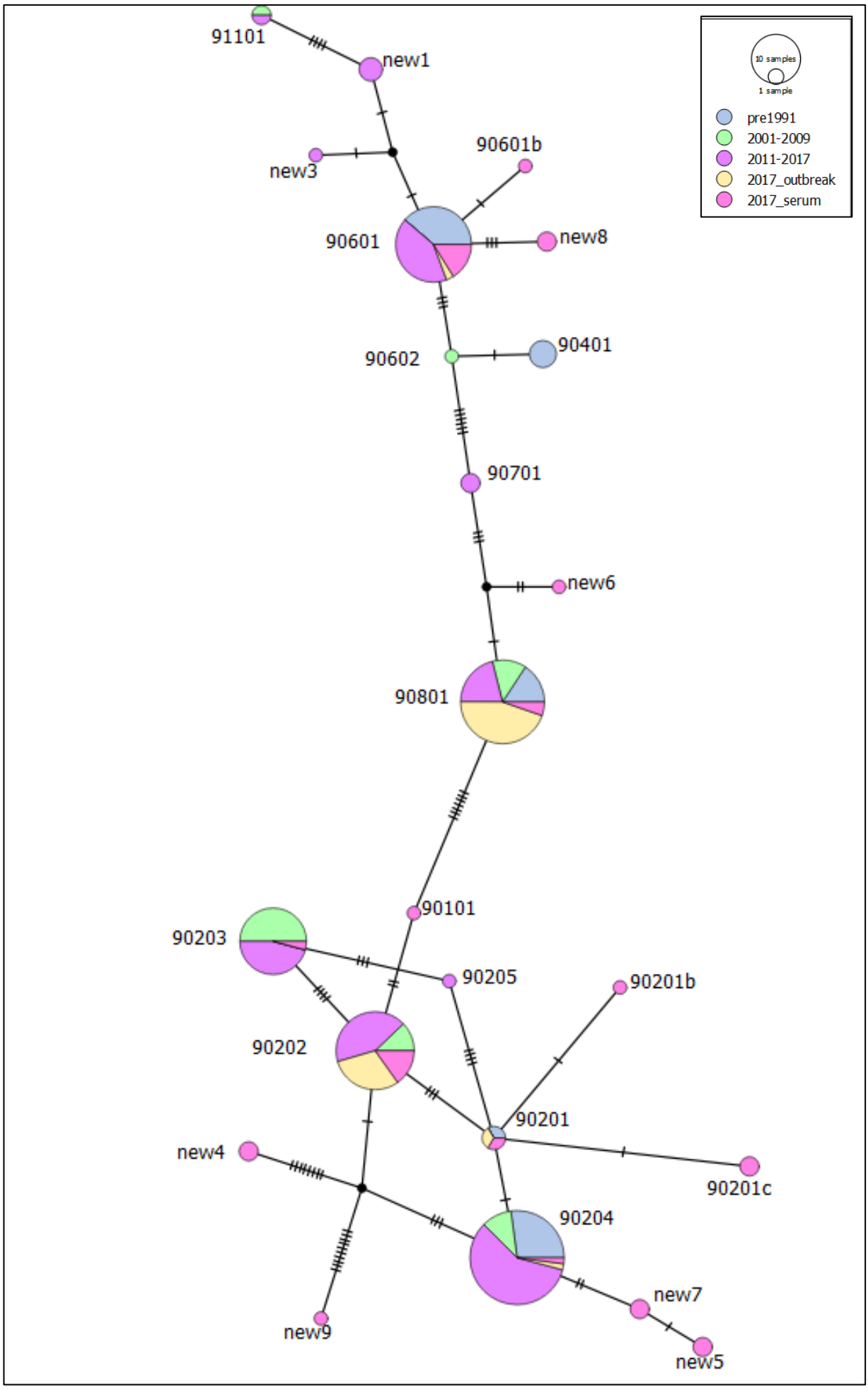

10 samples
1 sample
pre1991
2001-2009
2011-2017
2017_outbreak
2017_serum
91101
new1
new3
90601b
90601
new8
90602
90401
90701
new6
90801
90101
90203
90205
90201b
90202
90201
new4
90201c
90204
new7
new9
new5

Figure 5.4.3. Median-joining haplotype network showing the relative frequency of DRB haplotypes in the Serengeti population of African wild dogs over time. Haplotypes from each time period are coloured, as shown in the key. Individual haplotypes are represented by a circle, coloured to show which populations it occurs in. The size of the circle indicates the relative frequency of the haplotype. Ticks on the branches denote the number of nucleotide mutations separating the haplotypes. This shows how haplotypes cluster in time and space.

5.4.4. DRB Sequence Diversity

DRB diversity was lowest in pre-1991 samples. Nucleotide diversity (π = 0.051) was higher in the pre-1991 samples compared to other sample sets. The value of θ and the number of segregating sites indicate that sequence diversity was quite high in the population at this time. The high Tajima's D value of 3.99 (p = <0.001), consistent with π exceeding θ, means that sequence diversity was higher than expected in neutral circumstances, consistent with either population contraction or balancing selection. The test for selection was not significant for 2001-2009 samples (p = 0.1).

Between samples from 2001-2009 and 2011-2017 there is little difference in π and θ values, meaning there was little change in DRB diversity between these periods. The number of haplotypes between 2011-2017 increased from the previous time period but with no significant difference between them. Tajima's D is significantly greater than 0 (p = < 0.01) during 2011-2017, due to population contraction or balancing selection, similar to what was observed for pre-1991 samples .

The haplotype diversity of 2017 outbreak samples slightly decreased since the previous time period (0.574). The Tajima's test for selection was not significant for

these samples (TD = 1.77, p = >0.05). Though the number of samples in the outbreak tissue group was half that of the 2011-2017 sample set, the number of haplotypes and segregating sites increased. The TD value for the 2017 outbreak sera is smaller than any other time period and indicates a higher level of low frequency polymorphisms compared to other time points. However, the low yield of quality DNA from these serum samples makes this result unreliable.

Only pre-1991 and 2011-2017 samples were statistically significant in the test for selection. Based on these data, the pre-1991 samples were either subject to the strongest selection pressure overall or reflect the largest bottleneck in the population. Overall, though some small changes occurred there was little difference in sequence diversity between time points.

Table 5.4.4. Summary of DRB sequence diversity over time, including number of samples (N), number of segregating sites (S), number of haplotypes (N haps), haplotype diversity (Hd), pairwise differences between sequences (Pi), nucleotide diversity (Theta), and Tajima's D (TD).

Time Period	N	S	N haps	Hd	Pi (π)	Theta (θ)	TD
<1991	20	30	4	0.728	0.0510	0.0229	3.99***
2001-2009	56	30	5	0.743	0.0386	0.0241	1.95
2011-2016	32	22	11	0.805	0.0361	0.0158	3.55***
2017 Outbreak Tissue	15	30	13	0.574	0.0368	0.0238	1.77
2017 Outbreak Sera	32	32	5	0.874	0.0444	0.0335	1.18

5.4.5. DRB Variation over time.

For DRB, there was a significant increase in DRB heterozygosity between 2001-2009 and the 2017 outbreak (Fisher's exact test, p = <0.05). This correlates with the low F*is* value, indicating low inbreeding and high levels of heterozygosity. The DRB heterozygosity significantly decreased between 2001-2009 and 2011-2017 (Fisher's exact test, p= <0.05). Between 2011-17 and the 2017 outbreak group there was a significant decrease in the expected heterozygosity (Fisher's exact test, p = <0.05) and the observed value was greater. The low Fis value for the outbreak group correlates with this, indicating very little inbreeding.

5.4.6. Comparison of DRB diversity with CDV outbreak

14/15 deceased wild dogs were PCR positive for CDV (5.4.1 Table 1). In surviving dogs, 1/9 sera was positive for CDV neutralising antibodies though this was a low titre (5.4.1 Table 2). This suggests a high infection rate and an inability to mount an immune response during the outbreak. When observing the DRB allele frequencies, 90202 and 90801 greatly increased in frequency during 2017 compared to previous time points. In contrast, alleles 90601 and 90204 decreased to very low frequencies in the 2017 outbreak. Using this data, the key observable difference between deceased and surviving animals during the 2017 outbreak were the differing 90601 and 90801 frequencies. Based on the DRB sequence and allele diversity results, outbreak sera had an excess of low frequency polymorphisms and could have undergone balancing selection. The high level of low frequency polymorphisms were not observed in deceased outbreak animals. When looking at variation at the DRB, deceased animals showed an excess of heterozygosity in the Fisher's exact test (p =

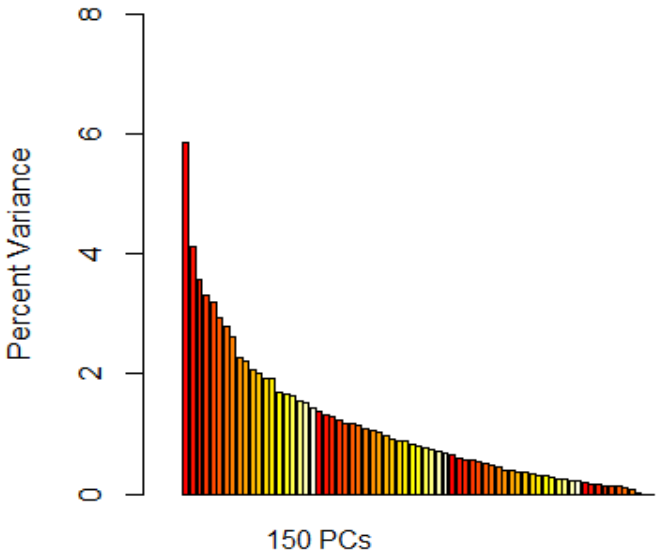
0.03) and a had a low inbreeding coefficient (F*is* = -0.26) compared to previous time points. This could not be shown for sera due to low quality DNA. Overall, there were small key differences between wild dogs which died during the 2017 CDV outbreak, those which survived, and other time points.

5.4.7. Principle Component Analysis of Microsatellites.

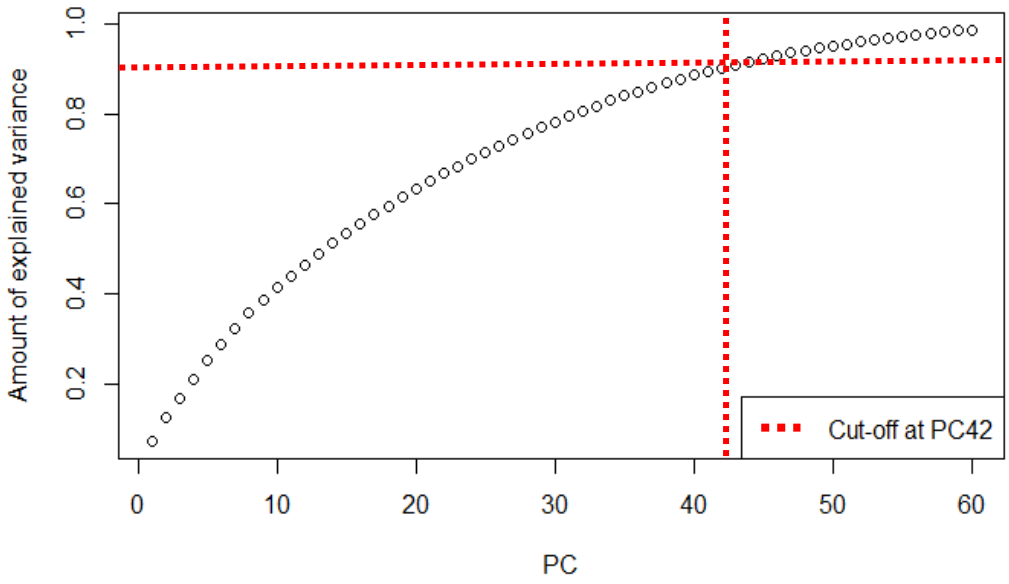
Analysis of microsatellite clustering was done using 10 loci represented in the following results. PCA1 and PCA2 (5.3.6 figures 3-4) accounted for the highest level of variance in the data (5.86% and 4.11%, respectively) but they still explained only a very small amount of the variation. Components 3 and thereafter account for less than 4% of the variation (5.4.7. C-D). The red lines in figure 5.4.7. B indicate that 90% of the variance can be retained by reducing the number of components to 42. This means that out of the 150 principal components, the first 42 can be used to explain 90% of the data variance. The remaining components explain the final 10% of the variance and were the least useful for separating data clusters.

The scatterplot (Figure 5.4.7. C) showed 3 major clusters of individuals: one cluster included the pre-1991 and 2001-2009 samples; one cluster included the 2011-2017 samples; and the final cluster comprised deceased individuals from the 2017 outbreak. The clusters were consistent with chronology of samples, showing overlap consistent with gene flow between pre-1991 until 2011-2017. There was also overlap between the microsatellites from 2011-2017 and the 2017 outbreak. Microsatellites from pre-1991 and the 2017 outbreak were distinct from one another. PCA 3 and 4 (figure 5.4.7. D) showed greater overlap with no clear clustering of any group.

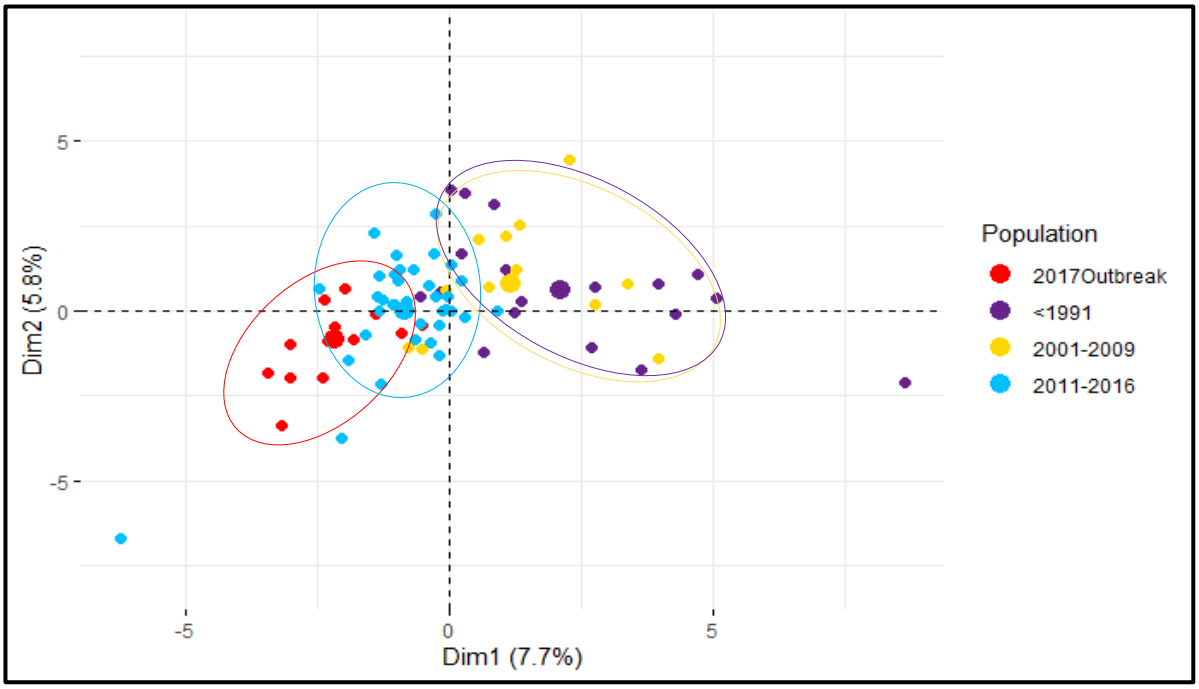
(A)


Percent Variance
0
2
4
6
8
150 PCs

(B)


Amount of explained variance
0.2
0.4
0.6
0.8
1.0
0
10
20
30
40
50
60
PC
Cut-off at PC42

(C)


Population
2017Outbreak
<1991
2001-2009
2011-2016
Dim1 (7.7%)
Dim2 (5.8%)
5
0
-5
-5
0
5

(D)

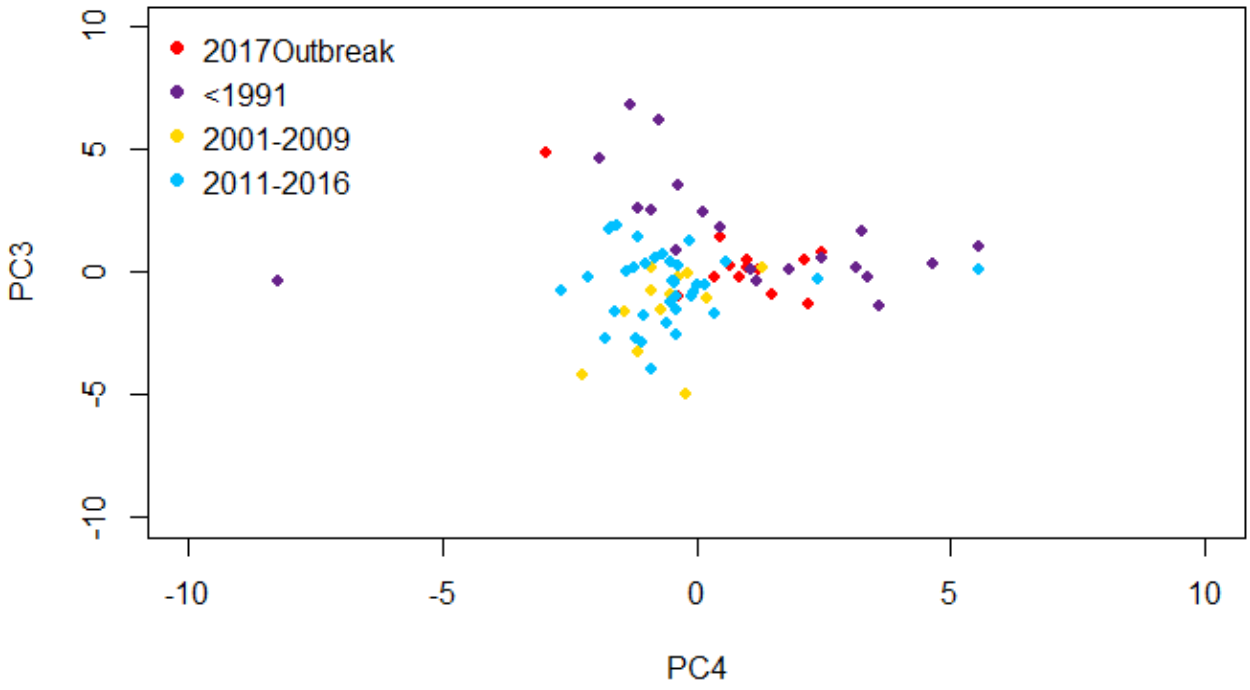

2017Outbreak
<1991
2001-2009
2011-2016
PC3
PC4
10
5
0
-5
-10
-10
-5
0
5
10

Figure 5.4.7. (A-D). **Principle component analysis.** Plot A is a screeplot showing Eigenvalues (y axis) of principle components resolved from microsatellite variation from African wild dog populations. Plot B shows the cumulative variance among principal components. The number of principal components is given on the x-axis, with a maximum of 60. The red lines indicate the cut-off point for 90% variance (y axis), and where this meets the curve (x axis).

Plots C and D visualise the clustering of microsatellites at 9 loci in 84 individuals from the Serengeti population across 4 time periods. Specifically, plot C shows the first (Dim1) and second (Dim2) dimensions of the principle components, whilst plot B shows PCA3 and PCA4. Ellipses show the overlap between time periods whilst covering 95% of the individuals within each group.

5.4.8. STRUCTURE analysis of microsatellites

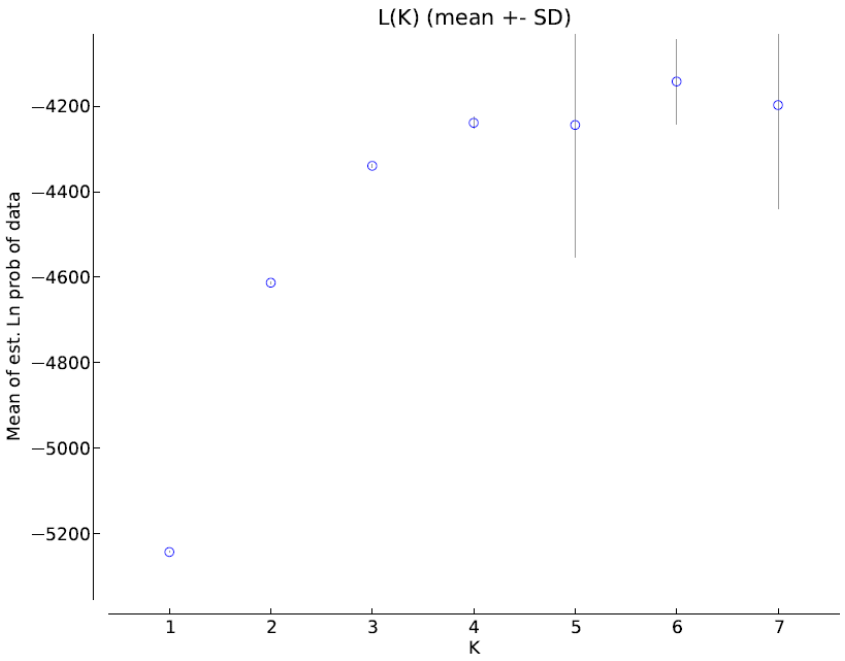
The microsatellite data supported an overall value of k=2 where the deepest level of population differentiation was observed. For k=2 (Table 5.4.8), the pre-1991 and 2001-2009 individuals were genetically distant from those sampled after 2011, with one sample from 2001-2009 showing evidence of admixture (i.e., a mixture between the two genetic clusters indicated by two colours in one column). This individual (from the Parimangati pack) also showed admixture at k=3 (Figure 5.4.8. D). At k = 3 the pre-1991 samples were genetically distinct from later samples, and there was division between 2011-2017 and 2017 outbreak samples but there was extensive admixture. For figures 5.4.8. D and 5.4.8. E where K=3 and 4 respectively, there was greater admixture in all clusters between 2001 and the 2017 outbreak but some distinction between pre-2009 and post-2011 samples. At k=4 (Figure 5.4.8. E) there

was more of a distinction between pre-1991 and 2001-2009 samples but also more sub-structuring within the 2011-2017 samples, and more distinction between that set and the 2017 outbreak samples. As highlighted in the summary table (Table 5.4.8), this means that the most probable number of genetic populations which fit the microsatellite data was 2. There was a lower level of subdivision at K=3, after which successive K values showed connectivity through admixture, except for pre-1991 samples.

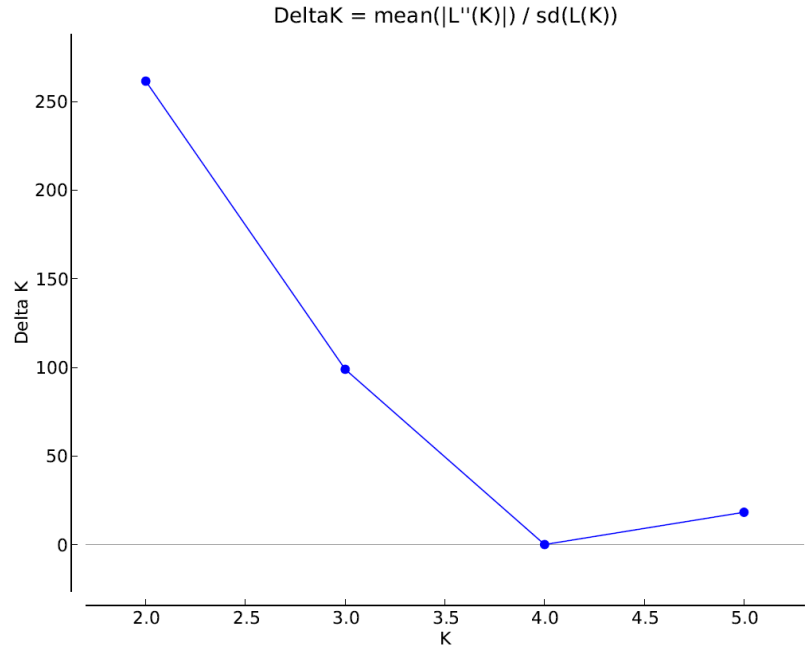
Table 5.4.8. K statistics summary showing K=2 as the least negative ΔK value and maximum likelihood.

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	10	-5243.430000	1.042486	—	—	—
2	10	-4613.040000	1.842824	630.390000	356.730000	193.577908
3	10	-4339.380000	3.036372	273.660000	173.040000	56.989063
4	10	-4238.760000	12.974351	100.620000	105.720000	8.148385
5	10	-4243.860000	310.332396	-5.100000	106.920000	0.344534
6	10	-4142.040000	98.582466	101.820000	156.990000	1.592474
7	10	-4197.210000	242.247442	-55.170000	—	—

A)



B)



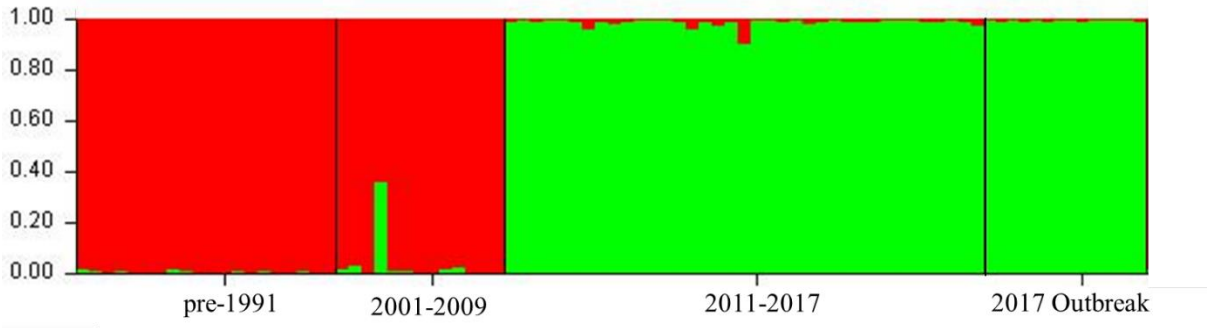
5.4.8. (A-B). Plot of LnPD against K (A), and Delta K (B) for each population.

Plotted are the exponentiated mean values of each inferred number of populations K (x-axis). The K value is a measure of population differentiation over time by showing the most likely number of populations based on the genetic input. The

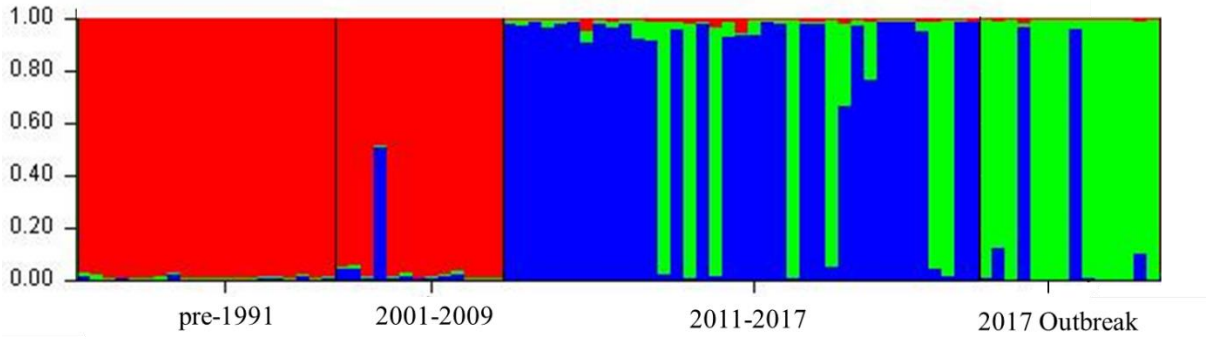
maximum likelihood of K is the least negative value of LnPD. Plot B is of delta K (ΔK) values for each population K, relating to the rate of change in the log probability of data for K1-7. K scaled by the standard deviation of LnP(D).

Figure 5.4.8. (C-E). STRUCTURE plots for microsatellites.

C)



D)



E)

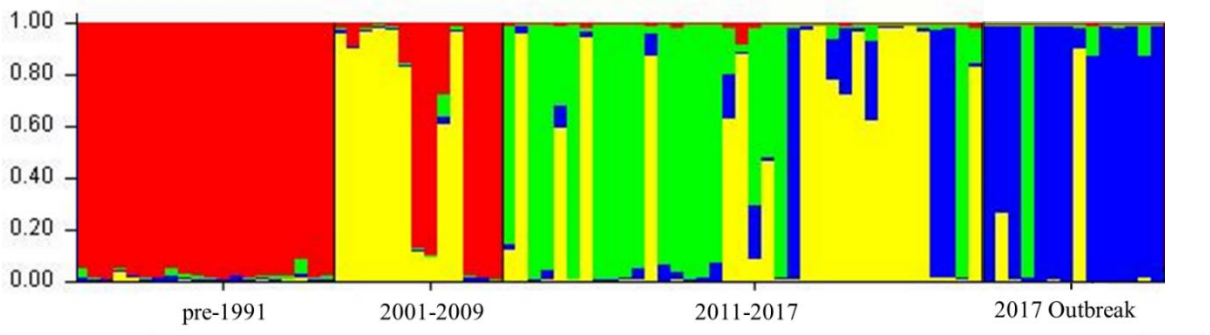


Figure 5.4.8. (C-E). Bayesian clustering from STRUCTURE for the microsatellite data, showing the proportion of inferred ancestry of microsatellites (Q) for populations, where K=2 (C), K=3 (D), and K=4 (E). Each column is a genetically distinct cluster, mapped onto each time point to test how they fit.

5.4.9. Microsatellite variation over time.

Overall, there was no significant deviation of microsatellite heterozygosity from what was expected under the HWE at any time point. The F*is* value for pre-1991 samples (Table 5.4.10) reflected the marginally lower number of heterozygotes than expected, though this was not significant. The F*is* value was weakly positive in the 2001-2009 and 2017 outbreak tissue samples.

Under the TMP model, the Wilcoxon sign rank test indicated that there was no significant evidence of a population bottleneck at any time period as values were above the 95% confidence threshold of 0.05 and therefore consistent with the null hypothesis. (Table 5.4.9). The model show that heterozygosity was not in excess at any time point.

Table 5.4.9. M-ratio test for population bottlenecks using the number of samples per locus (N) and average number of alleles (K) to test the probability of excess heterozygosity (TPM Wilcoxon Sign).

Population	Mean N	Mean K	Mean H_e	TPM Wilcoxon Sign
Pre-1991	39.56	8.56	0.78642	0.28781
2001-2009	24.44	5.89	0.75726	0.45477
2011-2016	67.44	8.78	0.74840	0.28514
2017 Outbreak	26.78	4.78	0.66816	0.30179

5.4.10. Comparison of Neutral and Adaptive Heterozygosity

The observed level of heterozygosity of both microsatellites and MHC in the pre-1991 samples were lower than what is expected under the Hardy-Weinberg equilibrium, though this was not a statistically significant deviation (p = 0.08). After the return of wild dogs to the Serengeti in 2001-2009, variance increased at the MHC in the population, although microsatellites did not deviate from neutrality at this time (p = 0.04).

The level of MHC and microsatellite heterozygosity did not deviate from neutrality between 2011-2017 (p = 0.09). The negative inbreeding coefficient (F*is* = -0.011) is minor and reflects the slight difference in expected heterozygosity versus what was observed.

The samples from animals deceased during the 2017 outbreak showed an excess of DRB heterozygosity than expected in this time period (p = 0.03), consistent with the negative inbreeding coefficient (F*is* = -0.26). Microsatellites did not deviate from neutrality at this time. There was no significant change in microsatellite heterozygosity between any of the time periods.

The microsatellite and DRB heterozygosity differed significantly between 2001-2009 and in the 2017 outbreak tissue (Fisher's exact test, p = 0.04 and p = 0.03, respectively).

Table 5.4.10. Summary of Comparison of Neutral and Adaptive Heterozygosity

	Microsatellites				**DRB**				
Time Period	**N**	**H_o**	**H_e**	**F_{is}**	**N**	**H_o**	**H_e**	**F_{is}**	**Fisher's Exact Test (p)**
<1991	20	0.76	0.79	0.035	20	0.67	0.74	0.09	0.08
2001-2009	13	0.75	0.76	0.005	13	0.92	0.75	-0.24	0.04
2011-2017	37	0.76	0.75	-0.011	37	0.78	0.80	0.03	0.09
2017 Outbreak Tissue	15	0.66	0.66	0.006	15	0.73	0.57	-0.26	0.03

Table 5.4.10. Summary table showing the observed (Ho) and expected (He) heterozygosity of the total sample number (N) for microsatellites and DRB. The inbreeding coefficient is given as a value of F*is*, ranging from -1 (excess

heterozygosity) to +1 (total inbreeding). Fisher's exact test determines the significant level of deviations from the neutrality of Hardy Weinberg Equilibrium between microsatellites and DRB.

5.5. Discussion

5.5.1. Confirmation of CDV outbreak in the Nyasaori Pack

From the data presented it is clear that there was an outbreak of a highly infectious strain of CDV (identified as 1994 Serengeti strain, data unavailable) in the Nyasaori pack in 2017. The lack of detectable neutralising antibody responses, high number of deaths, and PCR positive results compared to other packs collectively suggests this was a highly pathogenic strain of the virus in an immunologically naïve pack of wild dogs. Unfortunately, no samples were obtained from other packs for a CDV sequence comparison to determine if multiple strains circulated with differing pathogenicity. The fact that wild dogs which were able to mount an immune response were from the Ngorongoro pack, and therefore geographically separate from the Nyasaori pack, could indicate different virus pathogenicity if more than one strain was circulating at the time. Due to a lack of information on the CDV strain which infected the Ngorongoro pack, this cannot be confirmed but could explain the contrast in disease outcome between the packs.

5.5.2. Has diversity of immune markers changed over time?

5.5.2.1 DRB Diversity

The fluctuation of allele frequencies over time may reflect different selection pressures and demographic changes in the wild dog population. Wild dogs from the Serengeti were reduced to just 30 individuals by 1976, the exact reason for which remains undetermined but susceptibility to and outbreak of disease has been suggested as a probable explanation (Ginsberg *et al*, 1995; Burrows *et al*, 1994). The decline of an already small population size would result in greatly reduced genetic variation and increased risk of extinction (Ginsberg *et al*, 1995), which could have made the population more susceptible to diseases such as rabies and CDV. Investigative studies carried out around this time were varied in their interpretation. For example, Burrows *et al* (1994) suggested that human intervention with vaccination efforts could have compromised wild dog survival by causing stress-induced susceptibility to disease. Further to this, the last known remaining wild dogs in the SNP were found to have died of acute viral encephalitis caused by rabies (Kat *et al*, 1995; Creel *et al*, 1997). Contrary to this, Creel *et al* (1997) conducted serological analysis of wild dogs and found that exposure to such viruses alone did not mean the population was less able to remain viable and healthy. However, these studies had limited demographic and ecological data for the wild dogs, thus a lack of empirical evidence has made their decline difficult to research (Creel *et al,* 1997; MacDonald *et al*, 1992). When the Serengeti wild dogs disappeared in 1991, the alleles at highest frequency in pre-1991 samples greatly reduced in frequency or disappeared completely in wild dogs which then returned to the Serengeti between 2001-2009. These alleles were replaced with two alleles previously absent in the pre-1991 samples which then became fixed at moderate to high frequencies. This suggests possible expansion of MHC diversity with the integration of new alleles into the population as wild dogs returned to the Serengeti following a CDV outbreak

in north-eastern Tanzania in 2007 (Goller *et al*, 2010). This would also explain the occurrence of three new alleles at very low frequency in the 2011-2017 samples. At the nucleotide level, the low number of sequence polymorphisms and alleles at high frequency in the pre-1991 samples (reflected by the positive Tajima's D statistic) may be related to increased disease susceptibility at this time before wild dogs disappeared from the Serengeti. However, there is some evidence that populations can remain viable and stable with low MHC diversity (Slade *et al*, 1992; Ellegren *et al*, 1993; Pizarro *et al*, 2021). For example, Ploshnitsa *et al* (2011) hypothesised that strong positive selection on the MHC may reduce allele variation but may be beneficial if certain alleles favour survival (Phillips *et al*, 2018; Pizarro *et al*, 2021). For wild dog immunogenetics this is, however, a gap in knowledge which can only be speculated without further research. Investigating the polymorphism sequences could also help to elucidate differences among individuals.

Alleles 90204 and 90604 were previously at similar high frequencies pre-1991 and were reduced in frequency in individuals that returned after the outbreak (2007-2011). These alleles rose again in frequency by 2011-2017, and then were at very low frequencies both in individuals that survived and died during the 2017 CDV outbreak. This fluctuation could be linked to selection on this particular allele, though it was not related to the CDV outbreak. It is also possible that the fluctuations were due to founder effects as the sample sizes are low and could therefore be due to chance alone.

The slight increase in allele diversity in the 2011-2016 compared to the previous time points but slight decrease in nucleotide diversity (particularly based on theta) could also reflect the impact of this assisted migration (i.e., augmentation of genetic diversity but with a signature of expansion after a bottleneck, reflected in the

increase in Tajima's D). However, this also could reflect ongoing balancing selection on the DRB, since Tajima's D remained positive in the 2001-2009 samples as well.

Interestingly allele 90801 had consistently been at a stable frequency throughout the time periods, but it was overrepresented in the wild dogs which died during the 2017 outbreak. This could be due to chance as the deceased animals provided only a small sample set. However, considering surviving animals had this allele at a relatively low frequency in comparison, it is possibly due to diversifying selection favouring this particular allele (Ekblom *et al*, 2007; Manlik *et al*, 2019). Further, the serum samples may not accurately represent allele frequencies given the low quality of DNA and difficulty in amplifying it. It is likely that the new alleles at very low frequency which occurred in the serum samples were artifacts, and the true allele diversity in this group could in fact be quite different than the observed results. It is therefore difficult to infer much from the new alleles found in the 2017 serum samples, however it is possible to draw some conclusion from the known alleles detected. The sequence diversity for the 2017 outbreak sera is reflective of the new low frequency polymorphisms (Miller-Butterworth *et al*, 2021; Marsden *et al*, 2013; Marsden *et al*, 2012; Marsden *et al*, 2009).. The results were consistent with either a selective sweep or recent population bottleneck. However, measures of diversity and heterozygosity may be inaccurate in these samples if allelic dropout has occurred with low DNA yield, which has been addressed by previous studies (Leigh *et al*, 2012; Sommer *et al*, 2013). Overall, there is stable diversity throughout the time periods though frequencies fluctuate, which is expected of an adaptive marker subject to selection pressure.

5.5.2.2. DRB variation over time

Prior to their disappearance in 1991, wild dogs had lower heterozygosity at the DRB than expected, which has been observed in populations with declining numbers and those experiencing bottlenecks (Ploshnitsa *et al*, 2011). Considering the low level of inbreeding at this time (9%), the loss of variability is likely due to other attributing factors such as disease or predator competition (Phillips *et al*, 2018). After the discovery of wild dog dens in the Ngorongoro area of Tanzania in 2001, the population was considered to have returned (Fyumagwa & Wiik, 2001). The significant increase in DRB variation between 2001-2009 in consistent with findings from Marsden *et al.* (2012). Given that sporadic outbreaks of disease occurred in wild dogs between 2007-2018, the maintained high level of variation suggests that animals which survived outbreaks mixed with migrating wild dogs, thus introducing new alleles into the population.

5.5.2.3. Comparison of DRB variation and CDV outbreak

The lack of deviation from the expected heterozygosity between 2011-2017, could suggest a relaxation of selection pressure on the DRB. During this time (2015) there was a translocation programme by TAWIRI which introduced new packs to the wider Serengeti area to boost population size and genetic stability (TAWIRI Wild Dog Report, 2018). This would explain the increased level of outbreeding observed in the 2017 outbreak samples, though the samples were limited to deceased individuals. This is a key limitation of the study, as it would be incredibly useful to compare the variation within surviving individuals to those deceased, and to the serology data.

5.5.3. Microsatellites

5.5.3.1. Has variation of neutral markers changed over time?

Based on the cluster analyses, the pre-1991 samples were distinct from later time points but overlap with 2001-2009 samples. This is consistent with findings from Marsden *et al* (2012) who demonstrated overlap between pre-extinction and recolonisation of Serengeti samples. This supports the hypothesis that some pre-1991 Serengeti wild dogs likely survived, and their descendants were included in later samples (Marsden *et al*, 2012). In terms of microsatellite variation, there was no significant deviation from HWE in either pre-1991 or 2001-2009 samples, suggesting that enough gene flow was occurring in the population to prevent a high inbreeding coefficient (Arauco-Shapiro *et al*, 2020). The STRUCTURE analyses at k=2 suggested that samples from pre-1991 and 2001-2009 form a single genetic cluster, as described in Marsden *et al*. (2013), with increasing differentiation thereafter.

The cluster analyses showed samples from 2011-2017 as being partially separate from previous samples, which can be explained by the translocation of packs into the Serengeti, thus introducing new markers into the population. The presence of some 2001-2009 samples within the same cluster as 2011-2017 suggests that gene flow was occurring between previously resident and translocated individuals. This was consistent with the F_{is} evidence of outbreeding between 2011-2017 and the strong differentiation between pre-1991 and 2011-2017/2017 outbreak samples, indicating that the population was structured (K=2). This structuring also supports findings by Marsden *et al* (2013).

The partial segregation of the 2017 outbreak samples from other clusters corresponds to the chronology of the samples. For example, the cluster has some overlap with 2011-2017 samples but it is completely separate from pre-1991 and 2001-2009, and

may be explained by the variance in PCA1, which accounts for factors explaining the most variance between microsatellite clusters. Though the 2017 outbreak individuals provided insight into the level of population differentiation, without the use of serum samples of surviving wild dogs from this time it is impossible to obtain a complete picture. Overall, microsatellite variation fluctuated over time attributable to demographic change. Despite structuring occurring between the time of the disappearance of Serengeti wild dogs and translocation of new packs, microsatellite variation was maintained with little inbreeding.

5.5.3.2. Comparison of Neutral and Adaptive Variation

The small fluctuations in neutral variation over time suggest some stability in the Serengeti wild dogs population despite threats to their survival. Before they were thought to have disappeared in 1991 the population retained a low level of inbreeding based on F_{is} of microsatellites and DRB, with a small number of DRB alleles present in the population (n = 4). The similar pattern between neutral and adaptive markers at this time suggest that the lower H_o, though not significant, was a result of a sudden population decline. If this result had been a significant departure from neutrality it would be consistent with a genetic bottleneck as much of the population had disappeared by this time. Based on these data and the test for genetic bottleneck, the population retained variation at both neutral and adaptive markers (Ciborowsi *et al*, 2017; Rödin-Mörch *et al*, 2019; Buzan *et al*, 2021).

The period following (2001-2009) had a similar pattern at neutral markers but the significant departure from HWE of adaptive markers in comparison is consistent with balancing selection (Charlesworth, 2006; Cicconardi *et al*, 2017). It is possible that the individuals which returned to the Serengeti were heterozygous for

advantageous DRB alleles, favoured by their efficiency in pathogen antigen peptide presentation (Marsden *et al*, 2009). Which alleles these may be in particular is not currently known, although the shift in frequencies of 90202, 90203, 90204, and 90601 should be investigated to address this gap in knowledge.

The departure from expected neutrality of microsatellite markers between 2011-2017 is a signature of admixture which occurred when wild dogs from other packs were translocated to the Serengeti, contributing additional markers to the population. During this time period DRB variation did not deviate significantly from neutrality and allelic diversity more than doubled compared to previous time periods (n = 11). This reflects the addition of new alleles into the population during the translocation programme which likely included homozygous pack members. It is possible that certain alleles were favoured, for example 90202 and 90602, thus homozygotes with such favoured alleles may have prevailed more successfully than heterozygotes in the population. This could explain the high level of allele diversity but slightly lower heterozygosity than expected under HWE. Due to incomplete pack information from 2011 onwards and unsuccessful amplification of serum samples, the DRB alleles could not be quantitatively interpreted, but did provide an retrospective insight into patterns of selective markers.

The group of deceased wild dogs from the 2017 outbreak mirrored the pattern observed in 2001-2009 samples, as neutral markers did not deviate from neutrality but there was an excess of DRB heterozygosity. It was difficult to make inferences about the DRB variation without the serum data from surviving wild dogs, however some observations could be made. The surge in frequencies of alleles 90202 and 90801 in deceased animals may be related to the excess heterozygosity in this group. It is

possible that animals heterozygous for these two alleles were at a selective disadvantage when outbreak occurred, though this is only speculation without further investigation. Another possibility is that DRB allelic richness was depleted after the recent translocation of packs from other areas, if the translocated areas were somewhat isolated from one another (Tensen *et al*, 2016; Marsden *et al*, 2013). However, there was no available pack information to clarify this and without further research this is purely speculation. Excess heterozygosity has previously been observed following recent bottlenecks, for example in Finnish and Russian Karelian wolf populations (*Canis lupus*), (Niskanen *et al*, 2014). However, based on the TPM model there was no statistical evidence of any bottleneck or excess heterozygosity in the population at any time point, which was consistent with findings from Marsden *et al* (2012). One limitation of this study was that the sample size was relatively small for a TPM model, compared to previous work (Maltagliati, 2002; Sharma *et al*, 2015).

5.5.4. Is there evidence for selection of DRB alleles associated with CDV outbreaks?

Based on Tajima's D statistic the strongest selection pressure on the population was pre-1991 (TD = 3.36***), which is likely due to the disappearance of wild dogs around this time. The negative F*is* value for immune markers in 2001-2009 samples indicates an excess of heterozygotes in the population, consistent with a recent reduction in population size (Oliver *et al*, 2008). Given that there was no significant deviation from neutrality for microsatellites at this time, this is also an indication of selection. Since the level of F*is* was negative, it is possible that DRB heterozygotes were favoured.

The samples from 2011-2017 also showed evidence of selection at the DRB (TD = 3.55***). As allele diversity was high between 2011-2017 and the population was increasing with translocated packs, it makes sense to interpret this as strong balancing selection. Moreover, the fact that DRB heterozygosity did not deviate from neutrality at this time reinforces the idea that selection could have been acting on homozygotes of particular alleles (Carlson *et al*, 2005).

Although not statistically significant, the positive Tajima's D results for other time points could reflect slight population contraction (Zhang *et al*, 2018; Cortazar-Chinnaro *et al*, 2018; Ginsberg *et al*, 1995; Marsden *et al*, 2012). In the 2017 outbreak tissue, microsatellites did not deviate from neutrality, although the F*is* value for DRB suggested a high level of heterozygosity. However, when looking at Tajima's D, there was no evidence of selection occurring. This was likely to be explained by either a decline in population size, or more likely that the sample size was not large enough to find any significance in the test for selection (Marsden *et al*, 2012). Overall the data showed that changes in variation were possibly due to factors beyond demographic change such as balancing or diversifying selection, demonstrated by the differences in F*is* values for microsatellites and DRB markers. Similar patterns have been found in other species, such as water voles and great snipes (Bryja *et al*, 2007; Ekblom *et al*, 2007). The data showed evidence of selection at the DRB, although further investigation is needed to assess if there is any link between selection and particular alleles. The evidence of selective sweeps coincide with known fatal outbreaks of (but not limited to) CDV, but the implications of DRB alleles in disease susceptibility are completely unknown.

5.6. Conclusion to chapter

In summary, this chapter achieved the first aim by showing that an outbreak of a highly pathogenic strain of CDV caused mortality in the Nyasaori pack of African wild dogs in 2017. The second aim of this study was to demonstrate if diversity and variation changed over time at the adaptive MHC marker DLA-DRB-1, and if there was any link between these patterns and the CDV outbreak data. Results show adaptive markers fluctuated over time in their diversity with the introduction of new pack members, and changes in allele frequencies coincided with timing of disease outbreaks. The third aim of this study was to assess if the population had differentiated over time by measuring changes in the variation of neutral markers. Findings suggest that population differentiation did occur between earlier samples (pre-1991, 2001-2009) and those from 2011 onwards, following translocation of animals. This was consistent with findings from Marsden *et al* (2012). However, the data showed that neutral variation was maintained in wild dogs over time since their reintroduction to the Serengeti. Diversity and variation of neutral markers fluctuated over time. The fourth aim of the study was to compare neutral and adaptive markers to determine if changes in variation might be attributed to factors beyond demographic change, such as selection. The data show that fluctuations in microsatellite variation coincided with population expansion, and changes in DRB variation reflected selective pressure in the population at the time. The final aim of this chapter was to test for selection at the DRB. There was evidence of strong balancing selection of the DRB at two time points. Future work should further investigate DRB alleles and whether there is any relationship with disease susceptibility to address this gap in research.

Chapter 6. Discussion

6.1. Overview

The thesis aimed to investigate the infection dynamics of cattle with two emerging morbilliviruses: peste des petits ruminants virus (PPRV) and canine distemper virus (CDV). The thesis also aimed to assess changes in genetic diversity of the Serengeti African wild dog population following decades of decline and CDV outbreaks. PPRV is a notorious plague of sheep and goats, but has emerged into new hosts in recent years with high mortality rates, economic burden, and ecological concern (Pruvot *et al*, 2020). Cross-reactivity between antibodies against distinct morbilliviral species may render prevalence estimates unreliable in areas where multiple morbilliviruses co-circulate (Herzog *et al*, 2020). No study to date has monitored the infection status and clinical signs of disease in cattle in a natural, mixed-livestock setting over time. Therefore, the role of cattle in PPRV epidemiology is unclear.

CDV is a virus which has emerged into nearly all phyla, including non-human primates. It typically causes sporadic outbreaks among domestic dogs but has a high mortality rate in many protected species, including African wild dogs (Bucci *et al*, 2022). In mixed-livestock households with domestic dogs in endemic areas, the virus may be spreading into livestock species without associated clinical disease. There are no studies that have investigated CDV infection in livestock, or the role they could play in the epidemiology of the virus. In an area which is endemic with CDV and overlaps with protected susceptible wildlife species, potential hosts must be investigated and understood (Duque-Valencia *et al*, 2019). Further, the decline of one such species, the African wild dog, is partially attributed to CDV outbreaks, the

impact of which is important to understand for their survival and conservation (Marsden *et al*, 2012).

This thesis reports findings from the pseudotype virus neutralisation assay (PVNA) to detect specific antibodies to two different viruses, PPRV (chapter three) and CDV (chapter four) and investigates the infection patterns and clinical disease associated with each virus. This thesis also reports the findings on genetic structuring, diversity, and evidence of selection in African wild dogs which suddenly declined during a CDV outbreak in 2017 (chapter five).

6.2. PPRV in an atypical host: clarification of the role of cattle

6.2.1. Seroconversion of cattle

Several previous studies have demonstrated PPRV antibodies in cattle (Balamurugan *et al*, 2014; Logan *et al*, 2016; Herzog *et al*, 2020), but uncertainties have remained because of the potential for non-specific binding of antibodies to related morbilliviruses (Logan *et al*, 2016). The PVNA utilised in this research confirmed that cattle in the Serengeti District of Tanzania do have PPRV-specific antibodies, that PPRV is circulating in widely in cattle at low levels, and that cattle are being continuously exposed to the virus (Schulz *et al*, 2019; Couacy-Hymann *et al*, 2019; Herzog *et al*, 2020). This study did not detect any association between seroconversion and clinical signs (either in cattle or small ruminants), suggesting that infection in cattle is either sub-clinical or results in a very mild clinical disease. This is consistent with clinical studies in which cattle were experimentally infected with the virus (Schulz *et al*, 2019; Couacy-Hymann *et al*, 2019). However, several

important limitations in the study preclude a robust conclusion being drawn. For example, only limited data were available.

on small ruminants in this study. This was simply due to the fact that small ruminants are frequently traded in Tanzania and ear-tagged animals were therefore not all sampled as planned. Further work is required to clarify whether signs of PPRV in small ruminants coincides with seroconversion events in cattle, ideally with a larger sample size.

6.2.2. Sub-clinical infection of cattle

The finding that PPRV infection was occurring in cattle throughout the study irrespective of clinical signs of disease adds valuable insight to our understanding of PPRV circulation in northern Tanzania, with implications for surveillance of PPRV in multi-host communities. The likelihood of cattle being another source of PPRV spread through shedding is greatly reduced if they do not develop any disease, as the virus is more likely to shed in bodily fluids associated with disease (Padhi & Ma, 2014) and is consistent with experimental transmission studies in which PPRV-infected cattle have failed to transmit PPRV to in-contact small ruminants (Herzog *et al*, 2020; Schulz *et al*, 2019; Couacy-Hymann *et al*, 2019; Abubakr *et al*, 2017). This thesis is the first to provide longitudinal monitoring of clinical signs of disease together with evidence of infections actively occurring and supports previous findings that infection results in sub-clinical outcomes (Herzog *et al*, 2021; Balamurugan *et al*, 2014).

6.3. An emerging virus in livestock? CDV in cattle, sheep, and goats

6.3.1. Serological evidence

The fact that many cattle had CDV-specific antibodies (including individuals which were PPRV-negative), and that seroconversion to CDV occurred during the study provides strong evidence that they are susceptible hosts for CDV. Whether this is a new phenomenon or has been masked previously by immunity to RPV is yet to be determined, but this finding is important in developing critical knowledge on the emergence of CDV into new host species. The lack of antibody detection in small ruminants likely reflects the low sample size in this study, however it is possible that CDV has a higher affinity for cattle SLAM receptors than for sheep and goat SLAM (Couacy-Hymann *et al*, 2019). Although the functional region of SLAM is conserved among livestock species, caprine and ovine SLAM receptors are more closely related to one another (98.5%) than to bovine (96.4%) (Sarkar *et al*, 2009).

6.3.2. Sub-clinical Infection

This thesis is the first study to monitor clinical signs of CDV disease in livestock, predominantly in cattle. Since there is no literature describing clinical CDV in livestock this study utilised a standard list of signs outlined by WOAH (nasal discharge, ocular discharge, anorexia, diarrhoea, lethargy). While cattle seropositivity was positively associated with clinical signs of CDV in the first sampling period, seroconversion in the second and third sampling periods had no relationship with clinical signs. This is likely to be due to active infection with a different pathogen in the first sampling period confounding results. The signs of disease observed are, unfortunately, similar during infection with a range of

pathogens such as foot and mouth disease virus (FMDV), contagious bovine pleuropneumonia (CBPP), and bovine viral diarrhoea virus (BVDV). This research was unable to confirm whether seroconversion of small ruminants had any relationship with signs of disease due to a lack of data, although future work should consider monitoring clinical signs of CDV in livestock to ensure remaining questions are answered. Particular attention should be paid to the role of cattle in CDV epidemiology. While there was no evidence that CDV caused the clinical signs observed in this research, it is worth investigating the presence of the virus in blood, nasal or ocular swabs, and faeces. The lack of clinical evidence of CDV in this study does not necessarily mean that livestock do not experience an impact upon their health, as CDV is known to cause immunosuppression in hosts, leaving them more susceptible to severe disease outcomes from infection with other pathogens (Von Messling *et al*, 2003; Gonzalez-Astudillo *et al*, 2021). CDV and PPRV may have been present in livestock long before they were detected, likely being masked by severe disease associated with RPV. Further, mass vaccination against RPV may have concealed the immune response to CDV and potentially offered some cross-protection. This could be why PPRV and CDV are only now being detected.

The risk of cattle becoming more efficient hosts of CDV is that spill over infection could have a devastating impact upon protected wildlife species, particularly as cattle frequently come into contact with wildlife in the Serengeti District (Fakri *et al*, 2017; Mdetele *et al*, 2021). This thesis provides the first evidence that cattle, and to a lesser extent sheep and goats, can become naturally infected with CDV in this area. It is therefore that further work explores the possibility that CDV could fully emerge in livestock, posing a threat to virus control.

6.4. Emerging morbilliviruses: Future perspectives

This thesis demonstrates the usefulness of serological surveillance and clinical monitoring of livestock herds for detecting morbillivirus infections in atypical hosts. It also highlights the importance of emerging infectious disease in the wake of pathogen eradication, particularly in the case of PPRV in cattle in an RPV-eradicated world, and CDV in its host range expansion. By extension, this thesis provides relevant contextual insights into future threats to human and animal health. For example, the concerns for morbillivirus emergence, such as CDV, into humans in a measles-free world following its eradication (Baron *et al*, 2017). The context of this thesis is also relevant for current infectious disease emergence in humans, such as monkeypox virus outbreaks in the aftermath of smallpox eradication. Lessons from this study speak to the importance of investigating transmission risk events in mixed-host environments. For example, by establishing the capacity for a circulating virus to interact and bind with novel host receptors (Baron *et al*, 2017). It would also be prudent to assess the level of protection offered by neutralising antibodies to other morbilliviruses. For example, measles antibodies greatly reduce CDV disease burden in primates and domestic dogs, enabling them to develop an immune response and survive the infection (de Vries *et al*, 2014). By understanding the potential for morbilliviruses to adapt into new hosts, establishing transmission event risks, and the surveillance of potential hosts, disease control methods can be better implemented benefitting both human and animal health.

6.5. What genetic change has occurred in African wild dogs of the Serengeti?

The research described in chapter 5 describes a study of genetic change in Serengeti wild dogs since Marsden *et al* (2012), incorporating data from pre-1991 up until 2017 to investigate neutral and adaptive change. As shown in chapter 5, STRUCTURE analysis and delta K values revealed that the population had differentiated into two separate genetic groups, one comprising the older samples (pre-1991, 2001-2009), and the other comprising the 2017 samples. This would be indicative of genetic drift if the groups were totally isolated from one another, however the clusters are connected by admixing samples from 2011-2016. This means that, while differentiation did occur in the population over time, gene flow was maintained. The migration of free-roaming packs and the translocation of animals from other areas into the Serengeti collectively saved the population from further structuring, and possible population collapse (Tensen *et al*, 2019; Chavez *et al*, 2019). The difficulty in obtaining usable data from the 2017 serum samples in this study means that comparisons cannot be made with other sample sets, including the 2017 outbreak tissue. Future work should aim to obtain, if possible, fresh serum samples from current pack members, and pack information from 2011-2016. This would allow for a more complete picture on the genetic status and health of the wild dogs. This thesis provides evidence that the Serengeti African wild dogs have maintained relatively healthy genetic variability despite numerous outbreaks and demographic challenges which greatly reduced their numbers in recent decades. This should inspire hope for their survival and progress in the Serengeti, although further investigation is required to ensure that this population is preserved.

6.6. Adaptive Immunity and disease

The study of immune genes such as the major histocompatibility complex (MHC) has become increasingly popular in pathogen research. Since MHC markers are highly variable, they provide useful information about selection pressure in the event of infection and disease outcomes in a population (Jeffrey & Bangham, 2000; Beineke *et al*, 2008). A loss of diversity at the MHC locus has been shown to correlate with increased disease susceptibility in other species such as the cheetah (O'Brien & Evermann, 1988; Radwan *et al*, 2010). In African wild dogs MHC variation is lower than in other canids (Marsden *et al*, 2009), thus a loss of diversity at this marker could leave this species more susceptible to disease. The work in chapter 5 demonstrates how diversity at the DRB-1 locus fluctuated over time. Combined with the positive Tajima's D statistic, this suggests that the DRB-1 locus was under selective pressure in pre-1991 samples and again between 2011-2016. Alleles which become overrepresented in a population before an outbreak occurs with high mortality should be investigated as a potential link with disease susceptibility. For example, in chapter 5 one particular allele, 90801, was at a stable frequency throughout the time periods until the 2017 CDV outbreak, where it was overrepresented in animals which died. Unfortunately, due to the lack of comprehensive sequence data from serum samples, and information on surviving animals, further comparisons of allele frequencies cannot be made. The link between specific alleles identified and sudden population decline due to outbreaks is a question which remains unanswered.

This study utilised MHC-II markers to investigate how diversity has changed over time, and evidence of selection. Although virus antigen presentation to T-cells occurs via the MHC-I and MHC-II pathways, this research only utilised MHC-II sequence

data with the original purpose of investigating *Toxoplasma gondii* infection, which would mainly require the study of MHC-II. The roles of MHC-I and MHC-II are not mutually exclusive in viral peptide processing, thus they both have relevance when looking at susceptibility to infection (Luckey *et al*, 2019; Sinnathamby *et al*, 2003; Miller *et al*, 2015). It would be useful to study MHC-I markers in wild dogs to further understand their adaptive diversity and inform further work.

7 Appendices

Appendix I: Participant Information (English)

LIVESTOCK HEALTH IN SERENGETI DISTRICT

PARTICIPANT INFORMATION SHEET-Household survey

INTRODUCTION

You are being invited to take part in a research study. But before you decide if you want to take part, it is important for you to understand why the research is being done and what it will involve. Please take the time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Please take time to decide whether or not you wish to take part.

PURPOSE OF THE STUDY: We are studying several diseases of livestock that are present in this area, including trypanosomiasis, foot and mouth disease, and Peste de Petits Ruminants. These diseases cause loss of production and death. Our study aims to understand transmission and control of these diseases, so we can ultimately control them better.

WHY HAVE I BEEN CHOSEN? You have been asked to take part in this study because you are a livestock keeper and a member of this community in the Serengeti District. We think you have valuable information that will help achieve the aims of the study. We will be asking other household leaders to take part.

DO I HAVE TO TAKE PART? You have the right to decide whether you want to take part in the study or not. Participation is voluntary. If you do decide to take part, you will be given this information sheet to keep and be asked to sign a consent form.

You can withdraw at any point if you feel you no longer want to participate in the study.

WHAT WILL HAPPEN TO ME IF I TAKE PART? You will be asked some questions on a questionnaire. You may also be asked to take part in a meeting in the future regarding results. Taking part in this study is voluntary. All information which is collected about you, or responses that you provide, during the course of the research will be kept strictly confidential.

WHAT ARE THE POSSIBLE DISADVANTAGES AND RISKS OF TAKING PART? There is no anticipated risk to taking part in this study other than what is usually encountered in daily life. We will ask to take some of your time. We will ask you some questions, that will take 15 minutes, and collect samples from your livestock that will take 2-3 hours. The information you provide will be kept confidential.

You can indicate your wish to discontinue in any activity at any point during the study even if you have consented to taking part at the beginning.

BENEFITS OF TAKING PART IN THE STUDY: This study will help increase your knowledge of livestock disease prevention and control. You will learn about the health of your livestock and how to protect them from infection. You will also be contributing to an important study that will enable policy decisions to be taken, that will promote the health of your livestock.

This study ultimately aims to improve surveillance, diagnosis, and control of livestock disease in Tanzania, although you are likely to see these benefits only after completion of the study.

WILL MY TAKING PART IN THIS STUDY BE KEPT CONFIDENTIAL? All information which is collected about you, or responses that you provide, during the course of the research will be kept strictly confidential. You will be ultimately identified by an ID number, and any information about you will have your name and address removed so that you cannot be recognized from it. Please note that assurances on confidentiality will be strictly adhered to.

WHAT WILL HAPPEN TO THE RESULTS OF THE RESEARCH STUDY? The results of this study will be available to you and the public after they have been analysed. We will make sure to feedback the results of the current study to the communities and individuals involved. Please note that any results reaching the public domain will contain no personal identifying information. The information and knowledge obtained from the study is expected to contribute to scientific knowledge and inform policy making and surveillance of PPR in this and similar areas affected by this disease.

WHO IS ORGANISING AND FUNDING THE RESEARCH? This study is funded by BBSRC, UK and is being carried out in collaboration with the Nelson Mandela African Institution of Science and Technology, Arusha.

WHO HAS REVIEWED THE STUDY? This study has been reviewed by the Tanzania Commission for Science and Technology and the University of Glasgow ethics committee.

Thank you for taking the time to read this, we look forward to your collaborations on this project.

ANY OTHER INFORMATION: If you have any questions or require any information not included in this sheet, please contact the addresses below:

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Dr Harriet Auty, email address: harriet.auty@sruc.ac.uk, tel:

Appendix II: Participant consent form (English)

CONSENT FORM

Title of Project: Livestock disease research in the Serengeti Ecosystem

Name of Researchers: Ursula Pomeroy-Arthur, Dr Harriet Auty, Dr Tiziana Lembo

	Please initial box
I confirm that I have read and understand the information sheet dated 2019 for the above study and have had the opportunity to ask questions.	☐
I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my rights being affected.	☐
I agree to take part in the above study.	☐

_____	_____	_____
Name of subject	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature
_____	_____	_____
Researcher	Date	Signature

Figure 1. Participant consent form for all households visited at each sampling time point of the study.

Appendix III: Protocol for clinical monitoring

Protocol for Sampling Herds

Longitudinal herds

Some of the longitudinal herds from the FMD/Tryps project have interesting results, so we will visit 5 of these herds and sample them. The longitudinal herds to be sampled are from the following households.

The protocol for these herds is exactly the same as for the clinical herds.

The total number of samples to be taken in the longitudinal herds are:

	Swabs	Tempus tube	Serum tube
Cattle	40	40	40
Sheep	10	10	10
Goats	10	10	10
Total Number per herd	**60**	**60**	**60**
Total Number in 5 clinical herds	**300**	**300**	**300**

Sampling Dogs

It is important to sample domestic dogs which live in close contact with livestock. Each household should have all dogs sampled. The samples for dogs are as follows:

Swabs	**Blood**
Ocular & Nasal (use the same swab)	Tempus tube
Oropharyngeal	Serum tube

Protocol

Animal Selection

Sample ear tagged cattle and add in new animals to keep the total at 40 cattle, as usual for the longitudinal herds. Make sure this includes some baby calves. In addition, select 10 sheep and 10 goats. Ear tag these animals and sample them.

Sampling

Swabs should be taken from the oropharyngeal, ocular, and nasal cavities of each animal (cattle, sheep, and goats). The same swab used to collect ocular secretion can be used immediately to swab the nasal cavity and placed into a tube containing approximately 500uL of RNA later/PBS. A new swab will then be used to sample the back of the animal's mouth, and then placed into the same tube with the ocular/nasal swab. Therefore, there should be one tube of RNA later/PBS with swabs per animal. These tubes should then be placed in a cool container or on ice. The swab protocol is the same for dogs at each household.

Blood samples are to be taken from each animal, one tempus tube for RNA extraction (fill the tube) and one plain tube for serum sample (fill the tube). For these longitudinal herds, please also take a Pax gene tube. These tubes will be placed in the cool container or on ice. Also do this for dogs at each household.

Sample processing

Swabs in RNA later should be stored in the freezer.

Plain blood should be spun down in the centrifuge and serum split into 3 aliquots. Tubes should be labelled on the side of the tube (ear tag number and date) and the lid (ear tag number). Serum should be stored in the freezer and blood clots discarded.

Appendix IV: Clinical data collection form

Household ID: **Village:**

Date:

Clinical Scoring Sheet – Ear-Tagged Cattle

Cattle 1	Clinical Signs	**Samples collected**
Age: Sex: Ear Tag Number: Is this animal a calf kept with sheep and goats? Yes / No	Diarrhoea □ Nasal Discharge □ Ocular Discharge □ Oral Lesions □ Anorexia □ Other?	Ocular/nasal/oropharyngeal swabs □ Whole blood for serum □ Whole blood for Tempus Tube □
Has this animal received any treatments in the last 2 months?		

Cattle 2	Clinical Signs	**Samples collected**
Age: Sex: Ear Tag Number: Is this animal a calf kept with sheep and goats? Yes / No	Diarrhoea □ Nasal Discharge □ Ocular Discharge □ Oral Lesions □ Anorexia □ Other?	Ocular/nasal/oropharyngeal swabs □ Whole blood for serum □ Whole blood for Tempus Tube □
Has this animal received any treatments in the last 6 months?		

Cattle 3	Clinical Signs	**Samples collected**
Age: Sex: Ear Tag Number: Is this animal a calf kept with sheep and goats? Yes / No	Diarrhoea □ Nasal Discharge □ Ocular Discharge □ Oral Lesions □ Anorexia □ Other?	Ocular/nasal/oropharyngeal swabs □ Whole blood for serum □ Whole blood for Tempus Tube □
Has this animal received any treatments in the last 2 months?		

Cattle 4	Clinical Signs	**Samples collected**
Age: Sex: Ear Tag Number: Is this animal a calf kept with sheep and goats? Yes / No	Diarrhoea □ Nasal Discharge □ Ocular Discharge □ Oral Lesions □ Anorexia □ Other?	Ocular/nasal/oropharyngeal swabs □ Whole blood for serum □ Whole blood for Tempus Tube □
Has this animal received any treatments in the last 2 months?		

Figure 1. Format of clinical data collection for cattle.

Appendix V: Raw Microsatellite Data

IDNumber	Population	PopCode	FH2054_0	FH2054_1	FH2611_0	FH2611_1	FH2658_0	FH2658_1	FH3399F_0	FH3399F_1	FH3965_0	FH3965_1	Pez12_0	Pez12_1	FH2010_0	FH2010_1	PEZ08_0	PEZ08_1	PEZ15_0	PEZ15_1
lpi_2729	2017outbreak	1	132	132	195	195	258	258	301	398	377	377	260	260	233	233	221	221	233	233
lpi_2751	2017outbreak	1	132	132	186	186	250	258	NA	NA	377	388	254	262	217	217	221	221	250	250
lpi_2752	2017outbreak	1	127	132	186	186	250	260	301	352	392	393	254	260	217	233	217	217	233	255
lpi_2753	2017outbreak	1	127	127	178	195	250	260	294	398	439	439	256	256	221	230	217	217	233	238
lpi_2754	2017outbreak	1	132	132	186	186	260	260	301	352	377	392	254	262	217	217	217	221	250	255
lpi_2755	2017outbreak	1	132	132	186	186	260	260	301	352	377	392	254	262	217	217	217	221	250	255
lpi_2756	2017outbreak	1	127	132	178	195	258	260	301	301	388	392	254	262	233	233	217	217	233	233
lpi_2757	2017outbreak	1	132	132	NA	NA	250	260	NA	NA	NA	NA	NA	NA	233	233	217	221	233	250
lpi_2758	2017outbreak	1	127	132	195	196	255	258	301	414	377	388	254	260	233	233	209	217	233	273
lpi_2759	2017outbreak	1	132	132	186	195	255	258	301	398	377	388	260	262	217	233	217	221	233	233
lpi_2760	2017outbreak	1	127	132	178	186	258	260	301	398	377	377	254	254	217	233	217	221	250	255
lpi_2761	2017outbreak	1	127	132	178	186	258	260	301	398	388	392	254	254	217	233	217	221	233	233
lpi_2762	2017outbreak	1	127	132	190	195	258	258	301	322	388	392	254	260	221	233	217	221	233	246
lpi_2763	2017outbreak	1	132	132	178	186	250	260	301	352	377	388	254	262	217	233	217	217	233	255
RKW154	pre1991	3	127	140	180	186	258	260	318	414	417	417	258	262	217	233	213	213	238	250
RKW165	pre1991	3	135	140	180	186	258	260	309	318	386	417	254	258	217	233	NA	NA	250	250
RKW166	pre1991	3	135	140	178	178	260	260	341	341	436	436	258	258	217	217	213	213	242	246
RKW5309	pre1991	3	140	140	178	178	256	258	309	342	NA	NA	258	258	217	229	217	229	242	250
RKW5310	pre1991	3	135	140	178	195	260	260	342	414	417	436	254	258	217	217	213	213	246	246
RKW5311	pre1991	3	132	135	178	186	260	260	315	414	417	436	254	258	217	229	213	213	242	250
RKW5312	pre1991	3	127	140	178	186	258	260	318	414	386	417	254	258	217	233	213	217	250	253
RKW5314	pre1991	3	127	140	186	190	258	260	309	414	386	386	258	262	233	233	213	217	250	250
RKW5315	pre1991	3	127	127	178	195	256	256	342	401	390	410	254	258	217	233	217	217	250	253
RKW328	pre1991	3	127	135	186	195	256	258	309	309	413	413	254	254	229	229	217	217	238	238
RKW171	pre1991	3	132	140	195	195	246	256	294	352	413	427	254	254	217	217	217	217	250	253
RKW177	pre1991	3	132	140	186	195	256	258	315	414	417	436	254	258	217	233	213	213	242	250
RKW179	pre1991	3	132	135	186	195	256	258	294	318	430	430	254	254	217	233	217	217	250	253
RKW236	pre1991	3	127	132	183	195	246	265	294	352	390	390	251	254	217	233	217	230	242	250
RKW285	pre1991	3	132	140	178	195	258	260	342	414	417	422	254	258	217	229	213	213	246	250
RKW5308	pre1991	3	135	140	195	195	246	256	322	352	390	390	251	254	217	217	230	230	250	250
RKW294	pre1991	3	135	135	195	195	250	256	309	352	413	427	254	258	217	233	230	230	250	257
RKW444	pre1991	3	127	127	193	196	250	265	318	352	417	436	238	251	221	233	217	217	242	253
RKW446	pre1991	3	132	135	186	195	256	258	294	318	430	430	254	254	217	233	217	240	250	253
RKW447	pre1991	3	132	135	195	195	256	265	294	322	390	430	251	258	217	221	217	230	242	250
S1537	2001_2009	4	127	135	186	195	256	256	352	357	NA	NA	254	258	217	233	217	221	246	250
S1539	2001_2009	4	132	135	186	196	256	260	352	352	NA	NA	251	258	217	233	217	221	250	250
S1541	2001_2009	4	127	132	186	195	256	256	349	352	394	394	258	258	217	233	221	221	246	246
S1543	2001_2009	4	127	135	186	196	256	256	NA	NA	NA	NA	254	258	221	233	209	221	246	250
S1545	2001_2009	4	127	135	186	195	256	256	352	352	394	394	254	258	221	233	209	221	246	250

IDNumber	Population	PopCode	FH2054_0	FH2054_1	FH2611_0	FH2611_1	FH2658_0	FH2658_1	FH3399F_0	FH3399F_1	FH3965_0	FH3965_1	Pez12_0	Pez12_1	FH2010_0	FH2010_1	PEZ08_0	PEZ08_1	PEZ15_0	PEZ15_1
S1547	2001_2009	4	127	132	178	186	256	260	342	352	394	399	258	258	221	233	217	221	246	253
SD1LOS	2001_2009	4	140	140	178	195	256	256	352	414	NA	NA	251	258	217	221	213	213	246	250
SD2LOS	2001_2009	4	132	140	178	195	256	265	318	414	NA	NA	251	258	221	233	213	221	246	253
SFMUT	2001_2009	4	127	132	190	190	260	268	352	408	394	410	251	258	217	217	209	209	246	246
SOL07	2001_2009	4	127	127	186	195	256	256	342	352	394	394	258	262	217	221	221	221	246	250
S1005	2001_2009	4	132	132	171	190	250	265	318	401	390	390	251	251	233	233	217	221	250	253
S1105	2001_2009	4	132	132	171	190	250	265	318	401	390	390	251	251	233	233	217	221	250	253
S1405	2001_2009	4	132	132	171	190	250	265	318	401	390	390	251	251	233	233	NA	NA	250	253
GA1	2011_2016	6	127	127	196	196	260	260	322	398	NA	NA	253	262	217	233	217	230	250	250
GA2	2011_2016	6	132	135	178	186	NA	NA	NA	NA	NA	NA	NA	NA	217	233	217	217	233	250
GA3	2011_2016	6	127	140	178	180	260	260	322	414	439	439	254	256	221	221	221	230	250	250
GA4	2011_2016	6	127	140	190	196	258	258	322	326	377	420	256	260	233	233	221	230	246	273
GA5	2011_2016	6	119	119	190	196	258	258	NA	NA	377	392	NA	NA	217	221	221	230	238	246
GA6	2011_2016	6	127	140	180	195	258	260	322	414	392	420	256	260	221	221	221	230	250	250
GA7	2011_2016	6	140	140	178	186	258	260	352	352	NA	NA	254	260	217	217	221	221	246	250
GA10	2011_2016	6	127	140	190	196	260	265	322	398	392	410	260	262	221	233	217	230	250	250
GA11	2011_2016	6	127	140	195	196	260	260	322	414	420	420	254	262	221	221	217	217	221	250
GA12	2011_2016	6	135	140	178	196	260	265	322	398	392	410	256	260	221	233	217	230	250	255
GA13	2011_2016	6	132	135	178	180	260	260	398	398	392	392	254	262	221	221	221	230	250	255
GA14	2011_2016	6	127	140	186	196	258	260	342	342	392	392	260	262	217	217	217	221	246	250
GA16	2011_2016	6	127	140	180	180	258	260	294	398	388	388	254	260	NA	NA	217	221	233	250
GA17	2011_2016	6	127	132	178	195	258	260	322	398	422	439	254	260	221	221	217	221	250	250
GA18	2011_2016	6	127	127	180	196	250	260	312	414	377	388	261	262	NA	NA	NA	NA	NA	NA
GA19	2011_2016	6	127	140	190	195	258	260	398	414	392	410	260	262	221	233	217	221	238	250
GA20	2011_2016	6	127	140	178	180	260	265	312	411	388	388	254	262	233	233	217	221	255	259
GA21	2011_2016	6	127	140	195	196	258	260	352	352	392	392	254	260	217	221	217	217	250	250
GA22	2011_2016	6	127	140	178	196	258	260	352	352	NA	NA	260	260	217	221	213	217	250	250
GA23	2011_2016	6	119	132	190	196	250	258	322	342	377	392	254	256	217	221	221	230	238	246
GA25	2011_2016	6	127	140	180	195	NA	NA	322	398	NA	NA	256	260	217	233	221	221	250	255
GA31	2011_2016	6	127	140	190	195	250	258	398	414	439	439	254	260	221	233	221	233	238	246
GA32	2011_2016	6	119	128	186	196	258	260	301	322	388	392	254	254	217	233	217	221	233	246
GA33	2011_2016	6	127	135	186	196	258	258	342	357	392	392	256	260	221	233	209	221	246	250
GA34	2011_2016	6	127	135	186	195	NA	NA	352	352	392	392	NA	NA	217	233	221	221	246	246
GA35	2011_2016	6	127	132	186	195	258	258	346	352	392	392	260	260	NA	NA	221	221	246	246
GA36	2011_2016	6	132	135	186	196	258	260	352	352	392	392	254	260	217	233	217	221	250	250
GA37	2011_2016	6	127	135	186	195	258	258	352	352	392	392	256	260	221	233	209	221	246	250
GA38	2011_2016	6	127	132	178	186	258	260	342	352	392	397	260	260	221	233	217	221	246	255
GA39	2011_2016	6	127	135	186	196	258	258	342	342	NA	NA	256	260	221	233	221	221	246	250
GA40	2011_2016	6	127	132	178	178	NA	NA	NA	NA	NA	NA	256	256	233	233	217	221	246	250
GA41	2011_2016	6	NA	NA	178	186	NA	NA	NA	NA	NA	NA	260	260	233	233	217	217	246	246
GA42	2011_2016	6	135	135	178	186	258	260	342	352	392	392	260	260	217	233	217	221	246	250
GA43	2011_2016	6	119	127	186	190	250	260	322	352	388	392	254	260	217	233	209	217	246	255
GA44	2011_2016	6	119	127	186	196	258	260	301	322	388	392	254	254	217	233	217	221	246	246
GA45	2011_2016	6	127	140	190	195	258	260	398	414	410	439	260	260	221	233	217	221	238	246
GA46	2011_2016	6	119	127	NA	NA	250	260	NA	NA	NA	NA	240	240	221	230	221	221	242	250

Table 1. Microsatellite date summarized.

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