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International AIDS Vaccine Initiative Vectors Lab/

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# Recombinant Canine Distemper Virus as a Possible Vector for the Delivery of Simian Immunodeficiency Virus Antigens


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# Abbreviations

| | |
|---|---|
| (-) sense ss RNA Virus | negative sense single strand RNA Virus |
| AAV | adeno-associated virus |
| Ad5 | adenovirus 5 |
| AIDS | acquired immunodeficiency syndrome |
| AP | alkaline phosphatase |
| BCA | bicinchoninic acid |
| bNAb | broadly neutralizing antibody |
| BSA | bovine serum albumin |
| CCR5 | C-C chemokine receptor type 5 |
| CD4 | cluster of differentiation 4, cell surface receptor |
| CDV | canine distemper virus |
| CnBr | cyanogenbromide |
| CPE | cytopathic effect |
| cryoEM | cryo-electron microscopy |
| CTL | cytotoxic T lymphocyte |
| CXCR | C-X-C chemokine receptor type 4 |
| DNA | deoxyribonucleic acid |
| ddH2O | double distilled water |
| DMEM | Dulbecco`s modified Eagle medium |
| dNTP | deoxynucleotide |
| ELISA | enzyme-linked immunosorbant assay |
| Env | envelope protein |
| FBS | fetal bovine serum |
| Gag p55 | glucosaminoglycane, HIV/SIV protein with a molecular weight of 55kDa |

| | |
|---|---|
| GALT | gastrointestinal-associated lymphoid tissue |
| GFP | green fluorescent protein |
| gp140/21 | glycoprotein with a molecular weight of 140/21 kDA |
| gRNA | genomic ribonucleic acid |
| His6 | sixfold histidine |
| HIV | human immunodeficiency virus |
| HRP | horseradish peroxidase |
| IAVI | International AIDS Vaccine Initiative |
| Ig | immunoglobulin |
| IL-2 | interleukin 2 |
| IN | integrase |
| LPS | lipopolysaccharide |
| mAB | monoclonal antibody |
| MEM | modified Eagle's medium |
| MHC | major histocompatibility complex |
| MOI | multiplicity of infection |
| MV | measles virus |
| NHP | nonhuman primate |
| NIH | National Institute of Health |
| OD | optical density |
| p.i. | post inocculation/infection |
| PBMC | peripheral blood mononuclear cell |
| PBS | phosphate buffered saline |
| PEG | polyethylene glycol |
| PFU | plaque forming unit |

| | |
|---|---|
| PMA | phorbol 12 - myristate 13 – acetate |
| PR | protease |
| qRT-PCR | quantitative real time - polymerase chain reaction |
| rAAV | recombinant adeno-associated virus |
| rCDV | recombinant canine distemper virus |
| rCDV-eGFP | recombinant canine distemper virus expressing enhanced green fluorescent protein |
| rMV | recombinant measles virus |
| RNA | ribonucleic acid |
| RPMI | Rosewell Park Memorial Institute medium |
| RT | reverse transcriptase |
| SDS | sodium dodecyl sulfate |
| SHIV | simian/human immunodeficiency virus chimera |
| SIV | simian immunodeficiency virus |
| SPG | sucrose phosphate buffer with glutamine |
| TCID50 | tissue culture infective dose 50 |
| Tris | trisaminomethane |
| VEEV | Venezuelan equine encephalitis virus |
| VN | virus neutralization |
| VSV | vesicular stomatitis virus |

# 1  Introduction

Since its discovery over 30 years ago (1), the Human immunodeficiency virus (HIV) has become one of the most important infectious agents in modern medicine, and the fight against HIV/AIDS (acquired immunodeficiency syndrome, the resulting illness) has proven to be immensely challenging (2, 3). Over 60 million people have so far been infected, with roughly 7500 new infections occurring every day. More than 25 million have died due to infection with HIV (4).

Due to the widespread effects of HIV infection on the human body, the development of a medical cure for HIV/AIDS is highly unlikely. Prevention is key for this disease, and the ultimate hope in combating HIV remains the creation of an effective vaccine. However, developing such a vaccine has proven very challenging due to the effective and peculiar way of action by which HIV affects and alters the host's immune system (5). A variety of different approaches are currently being investigated, one of which is the use of nonpathogenic viral vectors to deliver HIV antigens and enable the body to mount an immune response against them (5). This work aims to evaluate such a newly developed vector system in primary safety and efficacy studies.

Viral vectors for vaccine development are chosen based on pathogenicity to the target species, safety in use and manufacturing, but also efficacy in delivering the inserted viral antigens to the appropriate components of the host's immune system to be processed. As an infectious agent, HIV directly targets the host's immune system, mainly so-called T-cells. The virus enters the body via mucosal surfaces, and these also are the focus of primary contact between immune cells and the virus, as well as initial rapid virus replication. Therefore, creating a localized immune response in those critical mucosal surface areas could be very helpful in preventing HIV from gaining a foothold in the host's body (6). A

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recombinant viral vector containing HIV antigens would ideally accumulate and replicate in the same areas as live HIV, and choose the same cell types for replication, therefore leading to activation of the immune system at the virus's point of entry, and eliciting a local and generalized immune response not only to the vector backbone, but also to the transported insert (7). The members of the paramyxovirus family, particularly members of the morbillivirus genus, share a variety of important characteristics with HIV – they enter the host's body via mucosal membranes, they preferably infect lymphocytes, and their initial replication occurs in epithelial cells and lymphocytes on mucosal surfaces (8). While Measles virus is a human-pathogen member of this group, its' very close relative Canine distemper virus (CDV) is completely non-pathogenic to humans and most non-human primates, but still shares those similarities with HIV. Attenuated vaccine strains of CDV have been established and used for several decades, with an excellent safety record in even the most susceptible target species, ferrets (9). Viruses of those strains have successfully been propagated in primate cell lines such as Vero cell, suggesting that these viruses, while non-pathogenic, are indeed capable of infecting primate and human cells (10). Such vaccine strains would therefore provide an excellent backbone for the design of a viral vector vaccine against HIV.

During the investigation of novel vaccine candidates, vaccine safety and efficacy are paramount. Wild-type CDV is non-pathogenic to humans, and the commercially available vaccine strains of CDV have a longstanding safety record even in susceptible species. Any genetic reversion however bears a small risk of inadvertent increase in pathogenicity. To evaluate this risk, novel vaccine candidates are generally tested for safety in a species that would normally be highly susceptible to infection with wild-type virus. In the case of CDV, the species most susceptible to contracting the disease are mustelids, namely ferrets. Infection with wild-type distemper in ferrets usually results in death of the animal. The

course of viral infection in ferrets has been investigated, and replication pathway and cell tropism of CDV in ferrets are similar to those of HIV, as mentioned above (8). Several vaccine strains of CDV are commercially available and have safely been used in ferrets for several years.

A very close, but not human-pathogenic relative to HIV is its primate sibling and presumed precursor, Simian immunodeficiency virus (SIV) (11). In certain primate species such as rhesus macaques, infection with SIV eventually leads to a clinical picture very similar to AIDS. SIV and HIV infect the same cell populations and demonstrate a very similar course of infection. It has been shown that, just as humans infected with HIV, rhesus macaques infected with SIV do indeed mount an immune response against SIV, albeit due to the unique properties of the virus unsuccessfully. The high degree of similarity to HIV, and the lower risk involved for humans working with the virus make SIV an excellent model for HIV research (11).

The gene sequence of Gag p55 from HIV and SIV encodes for a 55 kDa full-length protein that is the precursor of all virus core proteins. This sequence and the resulting proteins are indispensible for virus replication. Gag p55 is the first sequence to be transcribed during replication, and is very well conserved across the various HIV and SIV strains, highlighting its importance for normal virus function (12). Previous studies have shown gag p55 to be highly immunogenic and, importantly, mediating a strong cell-based immune response, which is essential for controlling viral infections. Thus, gag p55 has developed into an antigen of special interest in SIV and HIV vaccine research (13-15).

During this proof-of-concept study, recombinant CDV viruses with and without SIV gag insert, originating from a laboratory-adapted commercially available Canine distemper virus vaccine strain (9) were evaluated for their potential as vaccine vector candidates both *in*

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*vitro* and *in vivo*. The viruses were first propagated in Vero cells to provide a large enough quantity of virus particles to cover further studies. The viability of the recombinant viruses and their ability to infect Vero cells was assessed. The recombinant viruses were then used in two sequential animal studies to assess safety and immunogenicity in a naturally CDV-susceptible species. Effects of the novel vaccine candidate versus empty vector versus plain solvent were compared. Blood samples and swabs were obtained and clinical observations noted throughout the study. Animals were sacrificed at various time points and terminal blood and tissue samples obtained. Samples were analysed using a variety of virological and immunological assays.

The research presented in this thesis shows that a viable and replication-competent recombinant CDV as well as recombinant CDV expressing SIVgag could be safely administered to CDV naïve ferrets. Presence of the recombinant viruses could be demonstrated in various swab and tissue samples, and it was shown that the viruses were able to replicate within the ferrets. Both cellular and humoral immune responses to the recombinant viruses could be demonstrated.

# 2   Literature review

## 2.1   Human immunodeficiency virus – a virus with an impact

The clinical syndrome now known as Acquired immunodeficiency syndrome (AIDS) was first observed in New York City in 1981 (16, 17), and by 1983 a causative agent, then known as HTLV III was found (1, 18-20). Simultaneously, two more very similar viruses were discovered and named lymphadenopathy-associated virus and AIDS-associated retrovirus. In 1986 it was determined that these three viruses were actually one and the same, and the virus was renamed Human immunodeficiency virus (HIV) (21).

HIV is one of the most devastating viruses ever discovered. 6500 people get newly infected with HIV each day (22) and more people die from HIV/AIDS than from any other infectious disease. It is the fourth leading cause of death worldwide (23). At the end of 2005 almost 40 million people were living with HIV, and during the course of 2006 more than 4 million more were infected (4). Studies suggest that less than 30 % of South African children will be living with both their parents by the end of the decade, while 20 % will be full orphans (24). 60% of children with HIV will not reach the age of five (25).

A cure of HIV infections is impossible at this time and preventive and educational measures have slowed down the spread of infection. However it is believed that only a vaccine can end the pandemic of HIV infections (26). Modelling exercises have shown that even a partially effective vaccine with only moderate uptake would be able to protect 30 – 70 million people over the course of 15 years (27).

## 2.2  Human immunodeficiency virus biology

The two types of HIV characterized to date, HIV-1 and HIV-2, belong to the genus Lentivirus within the order Retroviridae (28). Retroviridae are characterised by their unique replication mechanism. The retrovirus particles enveloped in a lipidic membrane contain the viral genome as positive sense single stranded viral RNA and several enzymes required for virus replication. Once the virus particles have entered the host cell, the virus uses its own reverse transcriptase as well as the host cell's replication machinery to convert the genomic RNA into DNA, which is then integrated into the host cell's genome as double stranded DNA (29). The virus can then become latent for several years before replicating further, resulting in incubation periods of up to a decade before the outbreak of clinical disease (30). The pathogenesis of HIV-1 infection and subsequent AIDS is largely characterized by chronic, generalized immune activation (31-34). Persistent viral infections dampen and exhaust the immune system to prevent tissue damage (35). Exhaustion occurs in both T and B cells, and results in reduced responsiveness of the cells, expression of down-regulatory receptors, and reduced function (36, 37). This ultimately leads to the depletion and collapse of virtually the entire immune system, resulting in the so-called Acquired immunodeficiency syndrome (AIDS) (38).

The HIV-1 genome encodes only 15 proteins (39) and the virus relies on various functions of the host cell to establish infections (A simplified depiction of the virus particle and its genome can be seen in Figure 1) (40). HIV gains entry into the host cell by binding to the cellular receptor CD4, present on white blood cells, dendritic cells and some macrophages, as well as a co- receptor, either CXCR4 or CCR5. The virus particle membrane fuses with the membrane of the host cell, and subsequent dissolution of the viral capsid releases the viral genome and viral proteins reverse transcriptase (RT), integrase (IN), protease (PR), and

accessory proteins Vif, Nef and Vpr into the host cell. A double stranded DNA complement of the viral RNA is then synthesised after the viral proteins bind to host cell actin. The entire assembly then travels to the nucleus and enters it through a pore. Inside the nucleus, viral integrase binds to a host cell integration factor and the viral DNA is integrated into the host DNA. As the host cell apparatus transcribes viral DNA into proviral RNA, the viral factor Tat acts as a promoter of elongation. The viral Rev protein then assists the proviral RNA with nuclear export. The Gag protein is the main structural protein of HIV (41). The Gag protein is produced as one polypeptide but gets proteolytically cleaved into the three main structural proteins of the viral capsid and an accessory protein (42). Newly produced particles of both HIV and SIV bud directly from the host cell surface (42) and are covered in a lipid membrane derived from the host cell. From this surface protrude structures called spikes, which the virus requires to bind and enter new target cells. These spikes consist of two different viral glycoproteins forming a complex; gp41, which crosses the lipid membrane, and gp120, one of which sits on top of and around the gp41 "legs" and covers most of them up.
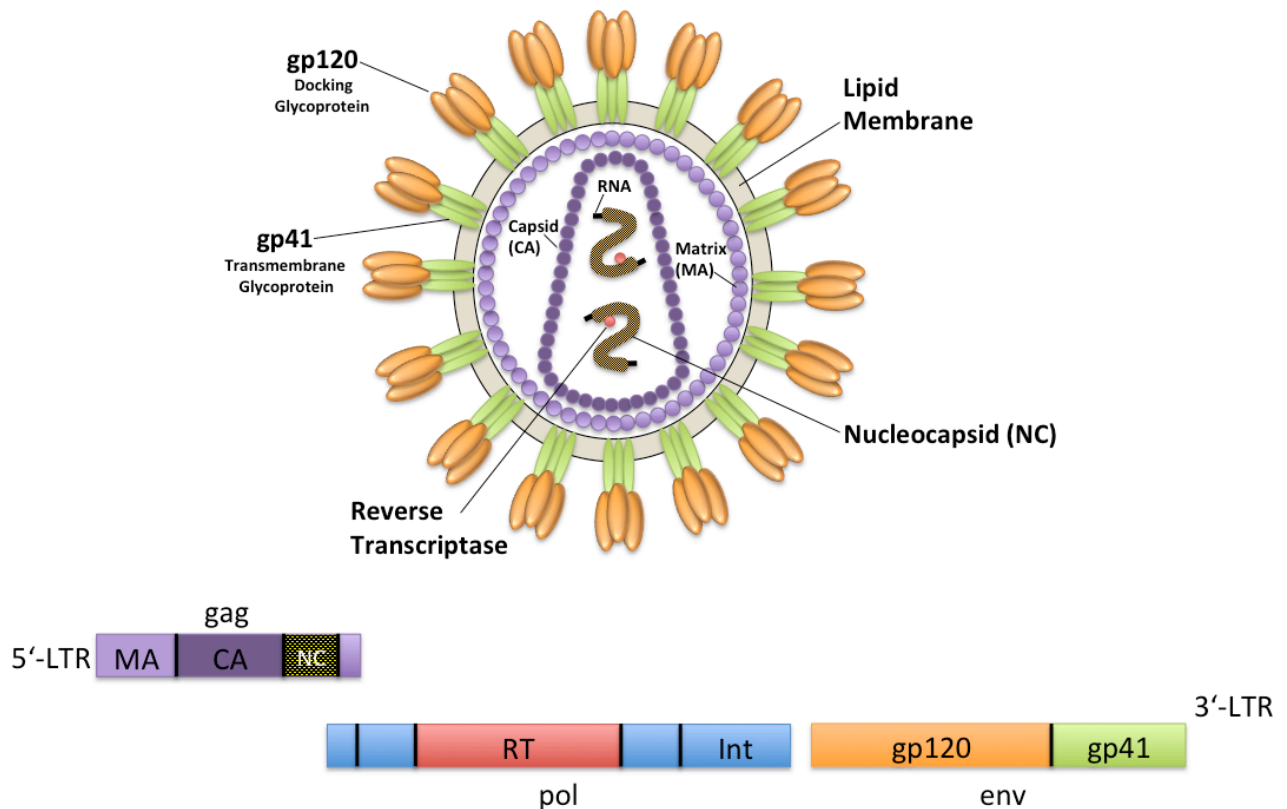
**Figure 1**: **Schematic of a Human immunodeficiency virus virus particle and simplified representation of its genome.** The viral RNA is protected by three layers of glycosaminoglycane protein derivatives: Wrapped in nucleocapsid (NC), which is enclosed by capsid (CA). The capsid is then encased in matrix (MA). All three components are present in many copies. Binding of the virus to the target cell is facilitated by envelope gene products gp41 and gp120. reverse transcriptase (RT) and integrase (Int) are also present in the capsid. Black vertical lines in the genome represent protein boundaries.

Figure based on "Introduction to Modern Virology"; Dimmock, Easton, Leppard; fifth edition, figures 9,8 19.3 and 19.5

While gp41 is fairly well conserved, gp120 has several highly variable loops on its surface. Additionally, gp120 is heavily glycosylated with more than 50 % of its mass contributed by carbohydrates. Both these mechanisms work to effectively disguise the spikes from the host's immune system and make them difficult to access for antibodies (43). Using cryoEM tomography of unstained, unfixed, frozen hydrated HIV and SIV, it was possible to determine that each spike in fact is a hexamer, consisting of three central gp41 molecules that each cross the viral envelope and three gp120 molecules surrounding and covering this gp41 anchor. While gp41 does extend through the gp120 trimer only a very small portion is

exposed. The conserved CD4 binding site of gp120 is in direct vicinity to highly variable loop structures (44). In fact, HIV gp120 shows up to 35 % variation in circulating isolates (45). Within an infected individual, HIV generates a huge variety of quasispecies (46-48) making it difficult for the immune system to lock onto suitable targets to combat infection.

## 2.3   Immunology of human immunodeficiency virus infections

In general, the process of initiating an immune response in the human body is described as follows (as per (49)): Intracellular antigens, which are usually produced by a host cell, are presented on the surface of an antigen-presenting cell via major histocompatibiliy complex (MHC) class 1 molecules. Foreign antigens outside of host cells are taken up by phagocytosis, digested and then presented on the surface of antigen presenting cells via MHC class 2 proteins. Antigen presenting cells then move to the regional lymph nodes. There they differentiate and present the foreign peptides to T cells. The T cells in turn then proliferate and become activated. The resulting effector cells travel to the target tissues and attack the source of the foreign protein. As the invasion is cleared, a portion of the T cells differentiates into memory cells to enable a faster response upon a repeat encounter.

The initial HIV infection only occurs in a small number of cells (46) and only by a small number of virions (50, 51). Most HIV infections in naïve patients are the result of only a single virus (47), and only a very small subset of quasispecies present within an infected individual actually transmit to new hosts (46). HIV1 replicates most effectively in activated CD4+ T-cells when studied *in vitro*. Evidence suggests that HIV even selectively infects HIV-specific CD4+ cells (52). Natural immunity to HIV is not protective. It does not result in virus clearance or protect from superinfection, despite both humoral and cellular responses being generated

(53). Around 0.3 % of HIV infected people however are able to control virus levels, keeping them at low levels without any treatment for several years to decades. These are the so-called elite controllers (54, 55). We will look at the differences in immune responses in this chapter.

HIV can be neutralised by antibodies, which bind to the envelope and block contact with cellular receptors, or by antibodies that bind after attachment of the virus to the host cell and inhibit fusion (56). Sterical and kinetic hindrances however make it difficult for antibodies to access vulnerable sites on virus particles (57). Nearly each individual infected with HIV will at some point produce antibodies against virus proteins but not all of these antibodies are capable of neutralising the virus. The ones that are, all are directed against the envelope spikes. High titers are necessary to achieve neutralisation, and antibodies are generally of narrow specificity (58). Neutralizing antibodies only arise later on in infection, around 6 – 8 months into the process (59-61). Non-neutralizing antibodies may still contribute to the control of virus levels. After binding of the antibody to viral epitopes presented on host cell surfaces, the Fc portions of antibodies can interact with other immune cells and trigger lysis of infected cells and hence reduce virus levels. As the disease progresses this ability is increasingly reduced (58). In some cases patients produce a mixture of various polyclonal, high-affinity, broadly neutralizing antibodies (bNAbs) against several different envelope epitopes (62). Some of these bNAbs have been isolated from patients and are available as monoclonal antibodies for research. One of them was able to block a low dose intra vaginal challenge with SHIV, a recombinant chimera between SIV and HIV exhibiting HIV envelope characteristics (63, 64). These bNAbs react with epitopes on gp120, gp41 and the CD4 binding site on the hexamers (65, 66). Two the bNAbs were thought to be auto reactive, which may have explained why they are so rarely elicited in patients (67-69).

However, other publications suggested this not to be the case (70). Antibody-mediated control of other retroviruses is only possible alongside CD4 and CD8 responses, and is regulated by certain MHC alleles (71, 72). This is of interest in the case of HIV as there are several MHC alleles in humans that enable more effective control of HIV infection (73).

The cellular response to virus exposure is generally broader than the humoral response. The speed and dimension of virus-specific CD4 responses largely determine the result of a retrovirus-infection (74). Strong responses from memory CD4+ T cells result in reduced rather than enhanced virus replication in SIV challenge studies (75). The pathogenesis of HIV2 infection is very similar to that of HIV1, but only rarely causes disease (76). The two are distinguished by a strong CD4 cell response to the virus in the case of HIV2 (76). By the same mechanism SIV in African nonhuman primates demonstrates the same pathogenesis as in Rhesus macaques, yet does not cause disease (77). *In vivo* studies have shown that only a minority of all HIV specific CD4+ cells are actually infected even during times of high virus levels (52). Proper function of CD4+ cells is important for the control of a HIV infection, since this cell population undertakes not only self-control but also control of the majority of other immune cells. A main point here is the control of CD8+ cells via IL-2. The lack of proliferation in CD8+ cells in chronic infection is largely due to the lack of appropriate regulation by CD4 (78). The degree of CD8+ T cell activation seems to predict progression of HIV infection even more accurately than virus levels (79, 80). *In vitro* experiments have shown that CD8+ T cells from infected patients can inhibit replication of HIV-1 (81). During the primary HIV infection, control of the initial surge of virus replication coincides with the appearance of HIV-specific CD8+ T cells (82). In individuals with low virus loads and chronic non-progressive infections, so-called non-progressors, there is evidence of a strong HIV-specific CD8+ response (83, 84). In SIV-infected nonhuman primates, control of virus loads is largely dependent on CD8+ T-

cells. In acute infections with SIV, loss of CD8+ cells results in uncontrolled viremia, lack of specific antibody production and rapid progression. The transient loss of CD8+ cells during chronic infections causes a transient increase in viremia (34). Broad SIV-specific CD8+ responses in monkeys can reduce virus replication after challenge (85, 86). There is a certain level of cytotoxic T-cells detectable after exposure with a subtype of HIV that are cross-clade reactive. This also occurs after exposure during vaccine trials (87, 88). This broad reactivity probably results from the fact that 1) cytotoxic T lymphocyte (CTL) epitopes tend to be well conserved, and 2) T-cell receptors are not as strictly sequence specific as their B-cell counterparts and tolerate a degree of sequence variation within recognized epitopes. Nevertheless, escape mutations in HIV CTL epitopes are not uncommon, suggesting that there is significant pressure exerted by the cellular immune system (89). During chronic HIV infection, it has been show that the presence of gag-specific cellular responses corresponds to lower HIV viral loads (90). Almost all patients demonstrate immune responses against HIV gag, even in the face of CD4+ cell depletion. Gag encodes three major structural proteins of HIV particles, which are proteolytically derived from it, namely capsid, matrix and nucleocapsid (91). All of them are focal points of an anti-HIV immune response and elicit strong reactions (91). Unprocessed gag protein has been found to elicit both CD4 and CD8 responses (91). Gag specific cellular responses are associated with a stronger control of virus levels (78). Especially cytotoxic T cells directed against gag are able to suppress the replication of HIV during early natural infections and thereby reduce viral loads (82, 92, 93). They are potentially even able to prevent the establishment of a persistent infection (94, 95). Gag proteins are highly conserved across clades (12, 96) and are able to self-assemble without the presence of viral RNA or other viral proteins (91). All these properties have made HIV gag an ideal possible immunogen, and most current vaccine candidates include gag (13-

15, 97). There is evidence that vaccine protocols including gag are also capable of inducing mucosal immune responses, even after mucosal delivery (98).


## 2.4   Human immunodeficiency virus at mucosal surfaces – what chances are there?

Pathogens are recognized at mucosal surfaces by antigen presenting cells, which then initiate an immune response. Secretory Immunoglobulin A and G (IgA and IgG) antibodies from B cells can capture pathogens before they enter the body (99), while mucosal CD8+ T cells attack virus-infected cells and can thereby control virus replication and often infection (100). After antigen-specific B and T cells become activated they briefly leave the mucosal surface to enter the lymph- and bloodstream. The majority of them return and become effector cells (101, 102). Immune cell aggregates at different mucosal sites communicate with each other in what is known as the common mucosal immune system. This enables the induction of immune responses at sites distant from the one of first encounter with a pathogen or vaccine antigen. This distant response is stronger than the response created at mucosal sites after systemic vaccination (103-105).

Much research has been conducted to investigate the time course of HIV infections at mucosal surfaces. In the first hours to days after the initial infection with HIV, the virus particles cross the mucosal epithelium and infect lamina propria CD4+ T cells, the most common cell population in this layer. The virus preferentially infects activated or recently activated CD4+ cells but is also able to infect macrophages and dendritic cells. After establishing an infected population at the point of entry, some infected cells are spread via circulation to the regional lymph nodes and subsequently other lymphoid tissues (106). It

takes the virus a few days to reach the degree of replication at which it is self-sustaining, that is, each infected cell results in at least one cell being infected by virus progeny (46). By the end of the first week (107) the virus has reached the largest lymphoid organ in the body, the gastrointestinal-associated lymphoid tissue (GALT). Conditions are now ideal for a massive expansion resulting in the depletion of the vast majority of GALT CD4+ cells, particularly in the lamina propria (108). Peak viral concentrations are reached by day 10-14 (106). This replication burst systemically activates the immune system. The activation however is only partially successful in controlling the virus, but crucially causes the influx of a new supply of uninfected cells, which enables the infection to remain established (109). At this point, latent infections in resting T cells have been established (110, 111) and the virus is escaping elimination pressure by mutating (112, 113). Elimination of HIV specific as well as memory CD4+ cells is ongoing (52, 114). Cellular immune responses involving cytotoxic T cells are only detectable from about two weeks post infection (106), and while they are robust enough to control the infection at the site of entry (106), the overall effect of cellular responses is only limited and unable to eradicate or even contain the infection (109). It is again the components of GALT that suffer the most (115). The ability of the virus to cause a latent, non-replicating infection in resting T cells means this reservoir cannot be attacked unless these cells become activated (110). By about the fourth week post infection, virus levels begin to decline (107). Studies have shown that this is a time where strong immune responses are not yet established, especially in the GALT (115). The conclusion is that this decline of viral load is largely caused by supply shortage of uninfected cells at this time (116, 117). Significant antibody levels are only detectable after viremia has been controlled (118). HIV and SIV infection both result in chronic activation of the immune system, which affects both B cells and T cells (119-121). The degree of immune activation carries more prognostic

value with regards to disease progression than plasma virus levels (79). Immune activation caused by the virus does increase the activation of CD4+ cells, which can then become new virus hosts; but it also activates the immune responses countering the infection. As a result, virus levels are maintained at a balance between new cells being infected and the immune system fighting the infection (106). The events at mucosal surfaces over the acute phase of the infection are directly linked to the degree of immune activation and therefore disease progression. So called elite controllers (infected but maintaining low viral loads and high CD4+ cell counts), still show higher levels of T cell activation than non-infected individuals but present a significantly lower degree of T cell activation than those with a progressing disease (progressors) (122).

The biology of HIV infection at mucosal membranes presents several critical points which present suitable targets for vaccine- and drug development. The efficacy of HIV infection is naturally very low. In SIV studies using intravaginal inoculations, researchers administered around one billion virus particles. When they examined cervical and vaginal tissues for presence of virus between 2 to 24h post inocculation only a fraction of viruses had actually entered the tissues (107, 109). These few dispersed cells then need to establish a productive focus of infection in an at this point rather small number of permissive cells (109). Once the infection is established and the virus is systemically distributed, the productiveness of the virus depends on the availability of target cells, and the limitation of immune responses against the virus (106). Each of these impasses is a potential target for the prevention or limitation of HIV infections. During HIV infection, the virus enters from the lumen, penetrating the protective mucous layer, and infecting dendritic and CD4+ T-cells in the epithelium and the lamina propria. The infected cells start to migrate and can be detected in regional lymph nodes within 24hours (123). However, there is very little evidence of the virus

replicating up to about five days post infection. Then an explosive rise in virus numbers occurs, accompanied by an equally dramatic increase in innate immune responses (107). This leaves a window of several days for vaccine-induced immune responses to prevent the infection from taking hold or to limit its extent. Vaccine induced antibodies of classes IgG and mainly IgA can capture free virus in the lumen of the organ of entry or neutralise virus inside epithelial cells and the lamina propria (124). Studies have shown that the presence of HIV/SIV-specific IgA at (genital) mucosal surfaces can prevent or reduce transmission (125-127). Their production is therefore a main goal of HIV vaccine development.

Infected mucosal cells can also be killed, either by direct or by antibody-mediated cytotoxicity. Early studies into the mucosal events of HIV infections have shown an inflammatory influx of lymphocytes, but also an unexpected loss of CD4+ cells (128, 129), which in the nonhuman primate SIV model was evident within days post infection (130). Phenotypic analysis of intestinal lymphocytes confirmed the specific loss of intestinal CD4+ cells featuring the co-receptor CCR5 (131, 132). Furthermore, flow cytometry experiments showed that the frequency of infection in intestinal CD4+ cells was ten times higher than in CD4+ cells in blood (114, 133). During the acute phase of the infection, the vast majority of all intestinal CD4+ cells is depleted, and the process continues into chronic infection, with the degree of depletion correlating with the onset of clinical AIDS (114, 134). Reconstitution of T-cell levels in the intestinal tract is minimal in patients with HIV. Sootey mangabee monkeys, which are natural non-progressors however show the ability to replenish the intestinal T cell reservoir (135, 136). In long-term non-progressors, CD4+ counts in the intestinal tract seems to be kept up, while virus replication appears controlled (137). So the preservation of CD4+ cells by at least partially preventing infection through a vaccine is an important target.

Studies in SHIV-immunized macaques have shown that the main factor for the protection from a SIV challenge are SIV gag specific CD8+ cells (138, 139). Depletion of CD8+ cells results in a complete loss of protective abilities in SHIV-immunized animals. However, SIV gag specific CD8+ cells only proliferate within local mucosal tissues upon challenge and the overall immune activation is minimal (139). A higher degree of activation of the immune system would result in more target cells available for the virus and a higher rate of retrovirus replication. Hence, the degree of local availability of specific CD8+ cells as well as the degree of activation of the immune system affect virus levels (140). In SHIV immunized macaques, T cell responses to a mucosal SIV challenge have been shown to be well controlled, whereas unvaccinated animals respond with extensive activation (139). So the creation of gag specific CD8+ cells by a vaccine is a valuable target to provide efficacy of the vaccine.

Both, protective humoral and cell-mediated immunity will be required at mucosal entry sites and lymphoid tissues in order to stop HIV or SIV from spreading systemically. A vaccine able to elicit such responses could prevent or minimize virus entry and stop an infection at the mucosal surfaces (124). Evidence suggests that the different immunological sites, both systemic and mucosal, communicate with each other to a good degree. To that effect, intranasal application of vectored HIV proteins in mice resulted in a higher degree of mucosal antibody formation than systemic administration (124). Should HIV manage to overcome these responses, a vaccine-induced systemic immune response would still have an opportunity to combat infection while the virus is in its initial non-replicating phase (140).

## 2.5  Vaccine development – an overview

The first vaccine that successfully protected against a viral infection was developed by Edward Jenner in 1796. He had observed that milkmaids seemed protected against smallpox and concluded without any of the knowledge available today that exposure to cowpox must somehow result in resistance to smallpox. He then inoculated a young boy with fluid from a cowpox lesion and showed that the boy was protected against smallpox (141).

A successful vaccine must fulfil three main requirements: It must be safe to use in the target species. It must elicit a strong and long-lasting immune response in the appropriate or even multiple parts of the immune system. And it must be possible to manufacture the vaccine reliably and affordably at industrial scale. Legal regulations must be fulfilled as well (142).

Generally speaking, there are three main possible classes of vaccines: Inactivated virus vaccines, attenuated live virus vaccines and subunit vaccines. Recently added were DNA vaccines and virus like particles (142).

In the case of inactivated virus vaccines, the virus has been treated, for example by use of chemicals or irradiation, so that it is no longer able to replicate but retains its natural shape (142) (for example hepatitis A). Relatively inexpensive to manufacture but also poorly immunogenic and only resulting in short-term immunity, (143) inactivated virus vaccines also do not induce cellular immunity. Often only very few neutralising antibodies are produced for epitopes which are often only revealed during the course of a viral life cycle (89).

Subunit vaccines are carrier particles such as liposomes, containing one or several soluble antigens, as well as adjuvants (142). They are safe due to the fact that they are not complete viruses, but they also can be expensive to manufacture. Immunity is raised for a medium amount of time (144) and the focus generally lies on inducing humoral immunity, but they can be poorly immunogenic (89).

Attenuated live vaccines are viruses that have been made non-pathogenic to the target organism, for example by repeated passage in tissue culture (142) so that while they remain capable of replicating, mutations are accumulated that reduce pathogenicity. Such vaccines are processed via MHC class I molecules and induce both humoral and cellular immunity, eliciting both CD4 and CD8 responses. They generally induce strong and long-lasting immunity. They are inexpensive and practical to produce on a large scale, and generally have a well-documented safety record (142). Examples are the frequently used MMR and oral polio vaccines. Reversions of live attenuated vaccines to a virulent form have occasionally occurred (e.g. (145)) so safety concerns need to be met.

DNA vaccines are bacterial DNA-plasmids containing immunogenic antigens of the virus in question. They enter the cell and the nucleus, but do not integrate themselves into the host genome. The enzymatic machinery present in the nucleus then expresses the viral proteins or peptides encoded by the plasmids. These are then presented on the surface of the cell in connection with MHC 1 molecules, and will therefore be recognized by the cellular immune system (146). While these systems are quite successful in animal models, they appear to be quite poorly immunogenic in humans and generally require use in a prime-boost regime (89).

Virus-like particles use *in vitro* produced structural virus proteins that combine into particles that appear like virus particles, but cannot replicate or infect (for example papillomavirus). Viral proteins are produced in *in vitro* culture (for example Hepatitis B). These vaccines are processed via the MHC class II pathway and therefor do not elicit vaccine-specific CD8+ cells.

These classical strategies for vaccine development unfortunately do not manage to create protective vaccines against viruses that have a high mutation rate and are thus capable of quickly developing variants that escape the vaccines (55).

Over the last decade, the use of viruses as vectors for antigens from other viruses has been investigated (89, 143, 144). The use of live vectors had initially caused safety concerns, so attempts were made to develop viral vectors that are unable to replicate (147). It is not sure if these vectors will elicit strong and broad enough responses (142). On the other hand, the use of existing live-attenuated vaccine strain viruses as vectors promises to elicit profound responses, while showing an excellent safety record (148). Genetic engineering of existing live vaccine strains however raises safety concerns due to the potential of such measures to change vector virulence or cell tropism, which needs to be kept in mind. Additionally, since these vectors are commonly used as vaccines in their own right, there may be a high level of pre-existing immunity to these vectors, which has the potential to interfere with immune responses to the newly introduced immunogen (142). Research has however shown that for example pre-existing immunity to measles virus does not appear to reduce the response if used as a vector (149). Vectors based on viruses infecting animals rather than humans may be potential candidates for vaccine development as well. They are however usually not very well researched with respect to their characteristics and safety in the human body (142).

Most effective vaccines currently available induce both humoral as well as cellular immunity. Newly developed vaccines may not be fully protective but should be able to greatly reduce virus levels and transmission. It is likely that that combination is necessary to eliminate or at least control an infection (150). The delivery of vaccine viruses via aerosol has been under much investigation over the last few years. The benefits of this would be the induction of immune responses on mucosal surfaces, as well as the possibility to circumvent potential pre-existing immunity (142). Various studies have shown that in animal models as well as humans, intranasal vaccination is able to induce immune responses at distant sites such as the genitourinary tract (151-154). A vaccine generating robust mucosal responses should

hence be able to successfully protect from disease (155). The use of an aerosolized measles-as well as a measles-rubella-vaccine has been shown to be safe and efficient in humans possessing pre-existing antibodies (156-158). Live attenuated vectors seem most promising. If the current developments are successful, they may well be precursors to the development of multivalent vector-based vaccines (142).

## 2.6 Human immunodeficiency virus vaccine design – an overview

An effective and safe vaccine against HIV would solve the problem of the HIV pandemic most effectively (159). An ideal vaccine would confer sterile immunity. This, however, is not even achieved by the majority of vaccines currently in clinical use. Instead, they prevent clinical disease by controlling virus replication and pushing it to subclinical levels (160). Reduced viral set points during HIV infection (i.e. the median plasma levels during chronic infection) are associated with a marked reduction in disease progression as well as transmission (54).

HIV severely affects the host's immune system by killing a large proportion of T-cells in the intestinal tract. The systemic immune activation that results from pathogen entry across damaged mucosal membranes may foster HIV plasma levels (46, 161). HIV prefers CD4+ T cells, and within the first week after infection causes massive depletion of T-cells in the gut (52, 114, 130). Chronic infections result in "exhausted" T and B cells. Modulation of this exhaustion process in the critical early stages of a HIV infection could be a possible target area of HIV vaccine development (161).

Research suggests a major role for CD8+ T-cells in the defence against HIV (34). The appearance of virus-specific cytotoxic cells early on in the infection has resulted in an extensive ability to limit virus replication, and severely reduced the loss of CD4+ cells in SIV

studies (162). Strong cellular immunity also features in long-term non-progressing patients (83). Antigen-specific cytotoxic cells will recognize MHC-bound antigens, but will not respond to free virus particles. They will therefore limit the spread of HIV by killing infected cells and reducing viral load, but will not protect from infection (89, 162).

HIV accrues mutations at an especially rapid rate, which enables it to escape humoral immune responses (163). The ideal for an HIV vaccine would be to raise neutralizing antibody titres high enough to block the infection originating from a huge diversity of sub- and quasispecies before it takes hold. If this proves impossible, immune responses need to be activated quickly enough to prevent the massive T cell depletion and persistent establishment occurring in early HIV infection (150).

Compared with other illnesses, HIV requires very high concentrations of broadly neutralising antibodies to achieve a 50 % neutralisation rate (for example, about 500 – 5,000 x the concentration than would be required for influenza) (49). Several trials attempting to induce protective humoral immunity have failed (57). The immense variability of HIV poses a problem, and finding antibodies that neutralise HIV across several clades has also proven difficult (164). Several broadly neutralizing antibodies have been isolated from human samples and thoroughly investigated. They bind to gp41 or gp120 of the viral envelope, including some that bind directly to the CD4 binding site of gp120 (165). Combinations of these bNAbs result in cross-clade neutralising abilities and protective immunity in new-born macaques (61). Several studies have shown that the transfer of large amounts of neutralising antibodies results in protection from infection in primates (166). Several possible strategies for the design of neutralising antibodies are being investigated (57). However, primary isolates of HIV (i.e being passaged only once on human T-cells) are very hard to neutralize, even using serum from infected patients. No vaccine tested in the twenty years up to 2005

was able to elicit antibodies which could neutralize viruses of the majority of HIV isolates (167).

The route of immunization also appears to influence the immune responses elicited. Since mucosal transmission appears to be the most important route of infection with HIV (168, 169), mucosal immune responses are a main point of investigation. Evidence suggests that antibody presence at mucosal surfaces may be contributing to protection against a HIV infection (126, 170). The presence of systemic as well as mucosal virus-specific CTLs is a regular feature of both HIV and SIV infection (171, 172) and can contribute to reducing the effects of contact with HIV (173). Therefore, the induction of mucosal immune responses by a vaccine may be a key feature for successful immunisation against HIV, and there is evidence to suggest that the mucosal delivery of immunogens induces the strongest humoral mucosal response (174). Delivery of the vaccine does not necessarily have to occur at the site of infection. As mentioned above, evidence has shown that intranasal delivery of immunogens induces mucosal immune responses not only in the intranasal cavity and lung but also in the female genital tract (175-178). Vaccine candidates using this approach have demonstrated success (179, 180).

An HIV vaccine needs to induce both B and T cell responses that are activated at mucosal surfaces during the critical first few hours of a new infection, and can control virus levels in chronic infection (46). HIV develops latent reservoirs very early on in infection, so vaccine-induced immune responses such as virus-specific mucosal immune cells may need to be consistently activated by a successful vaccine to get a head start on the rapid progress of HIV in mucosal membranes (46, 161).

The choice of immungens inserted into the delivery system also plays an important role. Studies suggest that vaccine candidates delivering multiple HIV antigens and containing gp41

and gp120 target both cellular and humoral immunity and result in very good control of the viral infection (181, 182). Vaccine trials targeting Gag and its individual components however were associated with lower plasma levels than those targeting gp41 / gp120 (90). Mutations of HIV may have a negative effect on virus fitness (183), particularly when they occur in Gag proteins since they need to self-assemble into the viral capsid (184, 185). Patients with cytotoxic T cells specific for Gag epitopes present with lower virus loads than patients with gp120-directed CD8+ cells (90). Elite controllers, who maintain very low plasma virus levels without medication seem to be able to do so by pushing the virus to generate mutations that are detrimental to viral fitness (184, 186). Research is being undertaken into the genetic background of patients able to control the early infection, in the hope to find new methods of virus limitation that might be used for vaccine development (187).

The HIV (or SIV) Gag protein is a frequently targeted immunogen in HIV vaccine development. As mentioned above, Gag commonly elicits an immune response in infected individuals and strong responses against gag are associated with lower virus levels (see above and (78, 188). Additionally, gag is capable of self-assembly and exit from the host cell without the need for viral genome or other proteins (189, 190).

SIV mac 239 and 251 (mac for molecular clones) are commonly used as challenge viruses in in Non-human primates vaccine trials since they are most similar to HIV. Like HIV their preferred co-receptor is CCR5, they are resistant to neutralization by antibodies, and T cell depletion and disease course are very similar (191).

## 2.7 Vectors for human immunodeficiency virus vaccines – candidates and preliminary results

No vaccine candidate undergoing clinical studies since the discovery of HIV in 1985 was able to induce neutralizing antibodies effective against the majority of HIV isolates (167). Only two candidates have ever completed clinical trials at the time of this study ((160) and below). The first phase 3 trial started in 1998, using recombinant gp120 from one lab strain and one primary isolate, both produced in mammalian cells and fully glycosylated, adjuvanted with alum (167). Two studies evaluated the trial after successful completion, and both concluded that the vaccine did not elicit protection against a HIV infection (192, 193).

The development of an HIV vaccine was first attempted using classical approaches. Several studies have shown that transfer of large amounts of neutralising antibodies results in protection from infection in primates (166). However, routine commercial production and application of such large amounts of antibodies is unfortunately not practically feasible. Monkeys receiving a live-attenuated SIV vaccine showed extensive ability to control virus replication when being challenged with SIV, as did monkeys already infected with one strain when superinfected with another (194). However, SIV infections could not be entirely blocked in either of these circumstances (194). Furthermore, SIV studies have demonstrated the ability of attenuated SIV to revert back to a pathogenic state. In humans live attenuated HIV appears to be pathogenic and causes late-onset disease (195, 196). This makes the use of attenuated virus for vaccination unsafe in the case of HIV (197). Live attenuated vaccines that show sufficient immunogenicity have also shown enough replication-capabilities to revert to a virulent type (198).

Based on research demonstrating the importance of cellular immunity in controlling viral load (199-201), the second main area of HIV vaccine research alongside the efforts in developing a vaccine inducing neutralising antibodies against HIV is the development of vaccine candidates aimed at eliciting cellular immunity (166). While cellular immunity does not confer complete virus clearance, it is able to significantly limit virus levels and transmission (202, 203).

Subunit vaccine candidates based on epitopes known to stimulate T- and B-cell responses offer one possible solution (204). Combinations of several epitopes in one polypeptide have often been more successful than the expression of single peptides (205-207), which have been shown to antagonize each other's efficacy if administered simultaneously (208). An alternative to this approach is the construction of DNA plasmids incorporating genetic information encoding viral proteins, which are then expressed within the host cell (209, 210). Antigens that are produced and processed within a host cell - as is the case with epitopes included in DNA vaccines - are generally presented on the surface of that cell via MHC I molecules. These molecules get recognised mainly by cells of the cellular immune system and so cause a much stronger cellular than humoral immune response. Antigens that originate from the outside of the host cell, such as free virus particles or extracellular bacteria on the other hand first need to be taken up and processed by antigen presenting cells, who then present these epitopes in connection with MHC II molecules. This leads to activation of T helper cells and to a more pronounced humoral immune response (211). This is the system generally exploited by subunit or peptide vaccines.

One important pathway to the development of cell-based vaccines is the use of viral vectors to express antigenic peptides (212). A large amount of work in this area has been done using adenovirus vectors. Replication defective adenovirus 5 (Ad5) including the genetic

information for HIV-gag elicited high levels of antigen specific cytotoxic T-cells and induced interferon gamma -, interleukin 2- and tumour necrosis factor alpha - production in over 50 % of cases (15, 143, 212-214). Adenovirus vectors are the most immunogenic viral vectors in HIV vaccine development so far (89). However, there is a relatively high level of pre-existing immunity to Ad5 in most populations (142). A recombinant Ad5 vector combination encoding HIV gag, nef and pol in separate vectors was the first cell-targeted vaccine to go into clinical trials, concluding in 2007. The study was meant to evaluate the level of reduction in infection rate as well as virus load for vaccinees that became infected. Analysis of the study results showed that the vaccine program did not protect from infection. Additionally, participants with a high level of pre-existing antibodies to the Adenovirus vector were statistically even more likely to become infected than participants without pre-existing anti vector immunity (215, 216). This follows phase 1 trials evidencing this vaccine program to be safe and immunogenic (217, 218). A possible reason for this might be that the application of this vaccine resulted in activation of memory T-cells against the vector, which would make the recipient of the vaccine more conductive to HIV replication (166). The development of chimeric vector backbones or the use of adenovirus vectors as the second part of a prime-boost regime (often with a DNA prime) are concepts suggested to circumvent this (89). DNA prime - Ad5 boost regimes have elicited significant cellular immune responses in NHPs (214, 219) but have not reduced virus loads or improved survival times after the primates were challenged with SIV (162, 217).

Several other viruses are under development to become vectors for possible HIV vaccine candidates, such as attenuated poxviruses, alphaviruses, measles virus, but also replication-competent viruses (220). The possible influence of the vector as well as vector-directed pre-

existing immune responses as a result of vaccination and / or challenge needs to be kept in mind (221).

Adeno-associated virus (AAV) does not cause any known illness in man, but 70 – 80 % of people possess pre-existing immunity (222). Recombinant AAV (rAAV) has the potential to silence tumour suppressor genes by randomly inserting itself into host DNA (223), a mechanism similar to that of HIV replication and a safety concern. Purified rAAV particles containing SIV antigens have been shown to elicit long term protective antibody and cellular responses after intramuscular injection (224).

Recombinant canarypox vectors have been common candidates for the development of vaccines since they are inherently safe for humans and pre-existing immunity does not pose a problem. In 2009 a phase 3 clinical trial using a canarypox vector encoding HIV antigens (called ALVAC by the developers) as a prime combined with recombinant gp120 boosts was concluded. The vaccine protocol proved safe for the human participants, and in fact showed a 31 % reduction of infection rates. It did, however, have no influence on virus levels or CD4+ cell counts in participants that did become infected. Therefore it had no ameliorating or transmission-reducing effect if infection could not be prevented (225). Further research has shown that the efficacy of the vaccine dropped and titers fell 24 weeks after inoculation (226).

Replication defective virus-like particles mimicking Venezuelan equine encephalitis virus (VEEV) induced protective immunity against SIV (227, 228). Since this is an animal virus, there is no pre-existing anti-vector immunity to be considered (142).

## 2.8   Negative sense single-strand RNA viruses – organisation and biology

Most negative sense single strand RNA ((-) sense ss) RNA viruses display common general features and follow the same replication process. This includes canine distemper virus (CDV) as well as vesicular stomatitis virus (VSV) which has been extensively researched (29). Clarke et al describe the genomic organisation and replication of (-) sense ss RNA viruses as follows, using VSV as an example (229):

The (-) sense ss RNA genome encodes five distinct proteins (Figure 2): N is expressed in large amounts and connects with the RNA to form the nucleocapsid. The P and L proteins together form the viral RNA polymerase, which both replicates the genomic RNA and transcribes genetic information into mRNA. The M protein, which is the most abundant protein within the viral particle, helps with virus budding and regulates host functions during replication. The G protein forms the trimeric spikes on the surface of the particle.
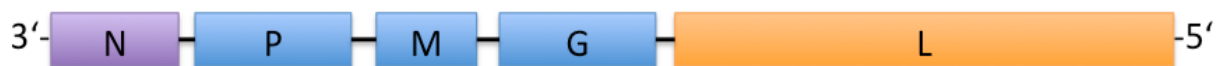


**Figure 2: Schematic of a negative sense single strand RNA virus  genome.** Genes for the five viral proteins (N, P, M, G and L) as well as 5 and 3' ends of the RNA are labelled.

After viral entry and caused by the slightly acidic pH inside the host cell, the viral nucleocapsid disintegrates and the viral genome and polymerase are released from the particle. The polymerase attaches to the 3' end of the genome. The first step of the replication cycle is the production of the five viral protein mRNAs. The polymerase travels from 3' to 5', transcribing between the start and stop codons for each protein. Occasionally the polymerase detaches from the genome before it arrives at the 5`end, and has to start over at the 3`end. This means that of the genes at the 3`end, more mRNA copies are made

and therefore more protein produced than of the ones nearer the 5`end. This corresponds to how much of each protein is actually required to form new virus particles – large amounts of N are required to form the nucleocapsid, and since the gene encoding N sits at the 3′ end of the genome, a multitude of transcriptions are made. The polymerase is only required in small amounts, and finds itself more towards the 5`end of the viral RNA.

Once sufficient mRNA is transcribed, the polymerase switches to genome replication by an unknown mechanism and copies the viral RNA straight from 3`to 5` into a (+) sense strand, which is then used as a template to produce progeny (-) sense RNA. Additional mRNA transcription is being pursued from replicated RNA too.

When a large enough amount of virus material has been produced, the progeny particles assemble by interaction between genomic RNA, N, M and G proteins. N protein envelops the RNA. The nucleocapsid then, regulated by the M protein, attaches to parts of the cell membrane previously enriched with G protein and the membrane envelopes the new particle. The particle then buds from the cell.

VSV has been researched extensively as a vaccine candidate due to reasons equally applicable to most other (-) sense ss RNA viruses, and particularly the family paramyxoviridae. It lends itself to the design of recombinant viruses for the construction of vector based vaccines due to the simple and discreet organisation of its genome into distinct transcription units, and due to the fact that mRNA is produced following a transcription gradient (230, 231). This means that foreign genes can be inserted relatively easily in between the coding sequences of the viral RNA, and that placement of the foreign insert can control the amount of foreign protein being produced (232, 233). The genome of rVSV is very stable even after the insertion of foreign RNA (230) and during the entire replication process no insertion into the host genome is required (234). It expresses foreign genes without much

variability (229). Additionally, the virus can be propagated to large quantities in a variety of cultured cells (229). There is a negligible level of pre-existing immunity to VSV in the human population, and recombinant viruses are considered to be innocuous to humans upon parenteral application since despite the ability to occasionally infect, they only very rarely causes mild symptoms in humans (233, 235). The insertion of foreign genes into non-segmented (-) RNA viruses also additionally attenuates such viruses (236), so the risk of disease is further reduced.

Since about the mid-nineties, a protocol has been available to produce recombinant VSV by use of cultured cells transfected with plasmids encoding for the N, P and L proteins required for replication, as well as the full length VSV genome (237-239). The N protein then attaches to the full-length genome, and the P and L proteins assemble to form the viral polymerase and initiate virus transcription and replication. Newly formed particles then bud from the cultured cells into the supernatant and can be collected from there. This entire procedure goes by the name of "virus rescue" and the principle can, with slight modifications, be applied to a variety of RNA viruses, including paramyxoviruses (240, 241).

## 2.9 Negative sense single-stranded RNA viruses as vectors for human/simian immunodeficiency virus vaccine development

Recombinant VSV has previously been explored as a vector for HIV/SIV antigens. rVSV expressing HIV Gag and gp41 / gp120 (Env) dramatically slowed progression of AIDS in monkeys after a challenge with SHIV (233, 242). It produced significant humoral and cellular responses, even more so after intranasal application when compared to subcutaneous inoculation (243). VSV is considered a neurotropic virus, which raises safety concerns

regarding mucosal application in humans (142). To circumvent this, rVSV containing a truncated (244) or deleted G gene (242) were designed which, encoding HIV-env, have produced similar humoral and cellular responses as replication competent rVSV (242).

Measles virus (MV) is a paramyxovirus, belonging to the genus morbillivirus. It is closely related to canine distemper virus. It causes skin rashes, fever and potentially neurological symptoms in humans. MV is highly infectious. It primarily enters through the upper respiratory system, and then continues to infect and replicate in the lymphatic organs (245, 246). Live-attenuated MV has an outstanding long-term safety record (247) and leads to long lasting immune responses (248). Recombinant MV (rMV) is generated by rescuing it in cell culture similarly to VSV as described above. This means that plasmids containing the full-length genome of MV are co-transfected with plasmids expressing MV proteins N, P and L which are required for particle production (249). Foreign genes can be included by genetically modifying the full-length genome (250, 251).

Recombinant vaccine strains of MV have successfully expressed HIV/SIV antigens Gag, Pol and Env for example (149, 252). rMV expressing HIV gag has produced both humoral and cellular immune responses in laboratory mice (253). rMv expressing either native or truncated HIV Env gave rise to high titres of antibodies against HIV after a single injection in mice. The antibodies were able to neutralise homologous SHIV as well as heterologous HIV isolates. Significant cellular responses were also detected (253).

Due to near ubiquitous routine measles vaccination programmes, there is a high level of pre-existing immunity in humans to MV vectors (254). The influence of pre-existing antibodies to MV for the use of rMV as a vector has been repeatedly investigated. The use of rMV expressing HIV Env in macaques previously exposed to MV resulted in significant antibody titres against HIV despite pre-existing immunity to MV. The cellular response to HIV antigens

was not reduced either (149). A single injection of rMV containing HIV-Env produced HIV-neutralizing antibodies in animals (149). This prompt response is likely due to the fact that rMV is able to infect dendritic cells and macrophages (255). Functional cellular responses were also elicited in mice when rMV with appropriate polyepitopes was used (256). Measles virus itself has been successfully used as an intranasal vaccine. This opens up the possibility to create rMV vectors that elicit immune responses on mucosal surfaces while at the same time evading any pre-existing systemic immunity (142). One study using mice demonstrated that pre-existing immunity to MV did not affect cellular responses generated after intranasal inoculation with rMV expressing SIV Gag; and that in fact the immune response to the foreign gene was increased under pre-existing anti-vector immunity compared to MV naïve animals (254). Hence a level of immunity directed against a MV vector backbone encoding foreign antigens does not appear to negate its efficacy as a vaccine candidate.

## 2.10 Canine distemper virus as a vector candidate for lymphotropic viruses.

Canine distemper virus (CDV) belongs to the virus family paramyxoviridae, and the genus morbillivirus. Other morbilliviruses include measles virus, peste des petits ruminants virus, rinderpest virus, dolphin morbillivirus, cetacean distemper virus, and phocine distemper virus (245).

Morbilliviruses primarily infect the oral and upper respiratory mucosa. They then spread to lymphatic tissues and induce transient immunosuppression. Potential complications result in central nervous effects but are rare (245, 246).

Ferrets are a natural host for CDV infections, during which they develop a skin rash, fever, respiratory and gastrointestinal signs and immunosuppression. Wild type infection in this

species often also leads to neurological signs and carries a high mortality (257). There has been detailed research into the pathogenesis of CDV infections as well as the response of the ferret immune system. Infected animals develop the above symptoms within days of infection. Accompanying this are leukopenia and a reduced response of the immune system, both in the acute and in the delayed pathway. These signs of generalized immunosuppression appear within a week post infection (258) and persist all through the acute phase of disease (259). Studies using recombinant CDV incorporating green fluorescent protein (GFP) have provided a detailed insight into the cellular process of CDV pathogenesis. Initially, the infection is limited to lymphatic tissues, mainly infecting T- and B- cells. Within the first week of infection, over 70 % of these populations have been shown to be infected (8). Systemic spread of the infection only occurs several days into the process, coinciding with the onset of clinical signs (260). CDV is highly immunogenic and virus clearance occurs through cellular immunity combined with both mucosal and systemic antibody responses, providing subsequent long-lasting immunity (246, 261, 262).

Due to the high susceptibility of this species, novel CDV vaccine candidates have frequently been developed in the ferret model. Initial CDV vaccines followed the traditional approach of using inactivated versus attenuated-live virus. Both of these induced protective immunity, but antibody titres were lower when using the inactivated virus, while attenuated virus induced a degree of immunosuppression and was only effective when given intramuscularly (263). This is mainly an issue for highly susceptible wildlife species of the mustelid family where this resulted in clinical disease (257). Meanwhile, a novel live attenuated vaccine using the Onderstepoort strain has been developed that has been shown to be both safe and efficacious in the face of a virulent CDV challenge when administered to ferrets via

subcutaneous injection (9). This vaccine strain has by now established an impressive safety record even when used in mustelids (264-266).

Several arguments stand in favour of CDV as a vector for HIV/SIV antigens: CDV is non-pathogenic for humans (267), but is able to infect primate cell lines (10). A long established attenuated live vaccine strain is readily available and can be used for the engineering of a recombinant vector (266). A rescue system for the generation of recombinant CDV including foreign genes has been developed and can be employed (268). Despite close relations and some cross-reactivities between the two viruses, pre-existing neutralising immunity to MV does not prevent effective use of CDV as a vector (269-274). Most importantly, the cell tropism, time course and pathway of a CDV infection in the lymphatic system are strikingly similar to infection with HIV/SIV (8, 275, 276), making CDV a highly interesting vector for the delivery of HIV/SIV antigens to locations critical for the pathogenesis of infection.

## 2.11 About this research project

As described above, CDV shares a variety of characteristics with HIV. Most importantly, CDV uses mucosal surfaces as entry sites, is strongly lymphotropic and replicates to a large degree at sites also preferred by HIV. This leads to the hypothesis that due to those similarities, CDV will induce immune responses in cells and at sites important for HIV replication and thus would make a good vaccine vector candidate for the delivery of HIV antigens. CDV is also non-pathogenic to humans and therefor would be a very safe vector to use.

To test this hypothesis, a recombinant CDV was rescued based on an established vaccine strain with an excellent safety and efficacy record. The rescued rCDV was then modified to

include the genomic information of SIVgag. SIVgag was chosen instead of HIVgag due to legal and health and safety considerations. The use of HIV or parts or genetic information of HIV is limited to the very final stages of vaccine development or other very specific research projects.

The research project presented in this thesis aimed to evaluate the rescued rCDV-SIVgag vaccine candidate with view to its general biosafety, and to examine the basic biodistribution and immunogenicity of the candidate.

Rescued rCDV and rCDV-SIVgag were evaluated in ferrets, since they are the species most susceptible to clinical distemper infection and would provide us with the most valuable information with regards to the safety of our recombinant viruses. Additionally, the paternal vaccine strain we employed to generate the recombinant viruses has a longstanding record of use as a vaccine against clinical distemper in ferrets and was shown to be highly immunogenic in this species.

Research was devided into two separate studies. During the first study, a small number of animals was used to evaluate the effects of rCDV –SIVgag on the health and body condition of the ferrets over the course of seven days post intranasal inoculation. Additionally, swab, tissue and blood samples were obtained at regular intervals to assess distribution of the recombinant virus, the possibility of shedding, as well as the presence of cellular and humoral immunity.

In a second study, a larger number of animals was used to gain a deeper insight into biodistribution and immunogenicity of the recombinant virus over the course of 28 days post inoculation. In addition to a group of animals intranasally vaccinated with rCDV-SIVgag and a control group, we included a group of animals that was inoculated with the viral vector

rCDV. All animals also received a booster after 21 days. Health and body condition of the animals were closely monitored. Swab and blood samples were again taken at various time points, and animals were euthanized and tissue samples obtained at weekly intervals. Samples obtained during the study were used in various virological and immunological assays to further explore the process and time course of biodistribution and shedding of both rCDV and rCDV-SIVgag, as well as the development of cellular and humoral immune responses to the recombinant viruses.

# 3 Materials and methods

## 3.1 <u>Origin and production of recombinant viruses rCDV and rCDV-SIVgag</u>

rCDV and rCDV-SIVgag were prepared within the vectors lab at the International AIDS Vaccine Initiative (IAVI) using a commercially available CDV Onderstepoort vaccine strain (Galaxy D, Schering-Plough Animal Health) and provided to me by Chris Parks and Xinsheng Zhang (both IAVI). Briefly, the virus had been adapted to African Green Monkey or "Vero"cells (ATCC) cells by serial passaging. Recombinant viruses were rescued using a helper virus free method described previously (240). For rCDV-SIVgag, the gene encoding SIV gag was inserted upstream of CDV N for maximal expression. Presence of viable infectious recombinant virus particles was confirmed by formation of plaques upon coculture with Vero cells. The presence of SIVgag protein was confirmed using Western Blot of cell lysate using SIVgag positive nonhuman primate serum as probe (see below for method).



**Figure 3: Schematic of recombinant canine distemper virus genome with the gene encoding the simian immune deficiency virus glycosaminoglycan protein (SIVgag) inserted upstream of the N protein.** Genes for the five viral proteins (N, P, M, G and L) as well as 5 and 3' ends of the RNA are labelled.

### 3.1.1 *Propagation of recombinant viruses*

Recombinant viruses were propagated to sufficient yield in Vero cells. Cells were seeded in tissue culture flasks (Corning), at a seeding density of 0.9 - 1.5 x $10^6$ cells per $25cm^2$ flask surface in 5mL / 25 $cm^2$ MegaVir medium supplemented with 2 mM L-Glutamine (both Hyclone). Number of flasks seeded depended on desired virus yield plus one flask to perform cell counts. Cells were incubated at 37 °C, 5 % $CO_2$, and 85 % relative humidity in a ThermoScientific incubator for 24 – 48 h. At that point in time, cells should be in logarithmic growth phase, nearing 70 – 75 % confluency. One of the flasks was then harvested by

removing medium, adding 2 mL of HyQtase (Hyclone), incubating for five minutes, and gently scraping off the loosened cells with a cell scraper (VWR). Cells in this flask were counted in an automated cell counter (Beckman Coulter) to determine the cell numbers to be infected. The remaining cells were infected at a set multiplicity of infection (MOI), which determines the infectious particle : cell ratio. The MOI used for virus propagation was generally 0.02, with an MOI of less than 0.01 not leading to successful infection and selection of a too high MOI resulting in the virus not multiplying sufficiently. To perform infection of Vero cells with virus, medium was aspirated off the remaining flasks, and 2 mL of fresh medium were applied, followed by the amount of virus calculated using the formula (cell count x MOI) / titer of virus used for infection. Once medium and virus were applied the flasks were carefully rocked, placed in an incubator for one hour, being rocked gently every 15 – 20 min. After 1 h, the total volume of medium was brought up to 5 mL / 25 $cm^2$. The flasks were then incubated for the next 24 – 48 h until visual inspection showed the appearance of cytopathic effect (CPE). The cells were then carefully monitored until the maximum CPE was reached. Virus-containing cells and medium were harvested by gently scraping the cells off the culture flask and transferring cells and medium into 15 mL or 50 mL conical tubes (Falcon, VWR). The harvest was subjected to two cycles of snap-freezing in a dry ice / alcohol bath and thawing at 37 °C to break up cell membranes. The virus-stocks were clarified by centrifuging in a Sorvall centrifuge at 800 x g for 10 min at 4 °C to remove cellular debris. Virus was then titrated using Plaque forming units (PFU) or Tissue culture infective dose 50 ($TCID_{50}$) assays, and aliquots were frozen and stored at – 80 °C.

### 3.1.2 Preparation of virus stock

To maintain consistency during our *in vivo* studies, all inoculums of rCDV and rCDV-SIVgag, respectively, were prepared from one batch of tissue-culture-adapted titrated virus. To

achieve this, virus production was gradually upscaled by repeated passaging of virus into larger flask sizes until a sufficient amount of virus could be harvested to cover the requirements of these studies. Virus-infected Vero cell harvests underwent two freeze-thaw cycles in a dry ice / alcohol bath to lyse cell membranes. The lysates were clarified at 800 x g for 10 min at 4 °C. To concentrate the virus, the clarified infection lysates were incubated with polyethylene glycol (PEG, Sigma) overnight at 4 °C. PEG is commonly used in a variety of biochemical settings to concentrate and subsequently precipitate a variety of proteins, including various viruses, from a variety of solvents (277). Virus was precipitated after centrifugation and resuspended in phosphate-buffered saline (PBS, Invitrogen) with 3 % sucrose (Sigma). The concentrated viral solutions were then dialyzed against 3 % sucrose in PBS to remove PEG. Virus was titrated by PFU and $TCID_{50}$ assays. Aliquots of virus stock were stored at – 80 °C.

### 3.1.3  Infectivity assays

To determine the number of virus particles in a set batch of virus, two different assays were employed. Both assays are based on the ability of CDV to cause cytopathic effects in Vero cell cultures. PFU assays make use of the fact that where a virus has infected an individual cell, the infection will likely spread to the cells directly adjacent to it. Cytopathic effects occur in infected cells after an incubation period, forming distinct areas of damaged cells, called plaques. Each plaque is presumed to have arisen from one individual infected cell. The number of plaques in one well of tissue culture plates can therefore be taken as an indication of the amount of virus present in the sample. Serial dilutions of sample virus and setting up assays in duplicates allow conditions in which the number of plaques per well is neither uncountably high nor low to the point of insignificance.

Since differentiation of individual plaques can sometimes be difficult, small plaques may be overlooked when counting while gaps in the monolayer not necessarily due to CPE may accidentally be counted. Therefore, the results of a PFU assay can vary between assessors. A modified version based on the same principles is the TCID$_{50}$ assay. In this assay, serial dilutions of sample virus are set up in multiples, and a 50% endpoint is calculated using the dilutions at which 50% or more and just under 50% of wells show virus-induced cytopathic effects. Endpoint calculations are done following the Reed-Muench formula.

### 3.1.4  Titration of virus using plaque forming units assays

Vero cells were seeded in 6-well plates (Corning), two wells for each virus dilution to be assessed. Seeding was done at 0.6 x 10$^6$ cells / well, so that cells were in logarithmic growth phase and about 70 – 75 % confluent by the next day. Cells were incubated overnight. The following day, old medium was aspirated off the cells and replaced with 0.5 mL fresh medium. 10-fold serial dilutions in medium starting at 1 : 10 were made of the virus batch to be tested, and 100 uL of each dilution added to the corresponding wells in duplicate. Cells were incubated for another hour. The medium containing virus was then taken off. A 1 : 1 mixture of 2 % methycellulose (Sigma) in double-distilled water (ddH$_2$0; MilliQ) with 2 x minimum essential medium (MEM; Invitrogen) supplemented with 10 % fetal bovine serum (FBS; Invitrogen) was prepared and 2 mL of this mixture added to each well to keep CPEs localized. The assays were incubated for a further 2 d, after which the methylcellulose-mixture was aspirated off and the wells washed twice with warm PBS. Cells were then fixed by adding 0.5 mL of 10 % formalin or 16 % paraformaldehyde (Sigma) and incubated for 30 min. The wells were again washed twice with PBS and cells were then stained with Giemsa stain (Sigma) for 2 h. Wells were then rinsed with water and air dried, before plaques were

counted, selecting wells that present 30 – 50 plaques per well to ensure countability. Plaque

counts were averaged over duplicates, and titers expressed as PFU / mL of virus stock.

### 3.1.5  Titration of virus using tissue culture infective dose 50 assays

Vero cells were seeded in 96-well plates at a density of 1.25 x $10^4$ cells / well in 100 uL

medium, and incubated overnight as before. 10-fold serial dilutions of the virus to be tested

were made using Vero cell medium, and 100 uL of virus dilution were added to the existing

100 uL medium in 8 wells per dilution. The cells were then incubated for 2 – 3 d. The

medium was taken off and cells washes with PBS, before being fixed with 10 % formalin or

16 % paraformaldehyde for 30 min. Cells were washed and 100 uL Giemsa stain was added

to each well and left for 2 h. After cells were washed with water and airdried, for each

dilution the number of wells containing CPE was determined. The titer was then calculated

as a 50% endpoint using the Reed-Muench formula.

## 3.2  Animals and housing

32 outbred ferrets (*Mustela putorius furo*) of both sexes (15 neutered males, 17 spayed

females) were purchased from Triple F Farms at 10 weeks of age, and housed in groups of

two to three animals in ventilated cages (Allentown Inc.) until 7 months of age. When

purchased, animals were not vaccinated against any diseases.

Identification of animals was ensured by ear tags as well as subcutaneous implantation of a

microchip that transmits an identification number, and also records and transmits the

animals' subcutaneous temperature (BMDS).

Before beginning of the study, blood samples were taken from all the animals and serum was used in a Virus neutralization assay to confirm absence of neutralizing antibodies against CDV.

Animals were then randomly assigned into study groups, taking care to maintain balanced sex and weight distributions. Animals presenting with extremely high or low weights were assigned to control groups to avoid possible influence of body condition on results of inoculation.

All animal experiments were proposed to the Institutional Animal Care and Use Committee of the State University of New York Downstate Medical Center and reviewed following the Office of Laboratory Animal Welfare's Animal Welfare Assurance regulations. The proposed experiments were accepted under animal use protocol number 04-413-09.

## 3.3   Design of *in vivo* studies

### *3.3.1   Description of study 1 to determine safety of recombinant vectors:*

**Study purpose and design:**

The small-scale pilot study (Table 1) described here served to confirm that rCDV-SIVgag was

safe to be used in a highly CDV-susceptible species, and to evaluate replication competence

and biodistribution of the construct.

| Group | Inoculate | D 0 | D 1 | D 3 | D 4 | D 7 |
|-------|-----------|-----|-----|-----|-----|-----|
| 1 | $2 \times 10^5$ PFU / 100 uL rCDV-gag intranasally | 2 animals rCDV-SIVgag, 1 animal PBS 0.5ml BS | 0.5ml BS | Terminal BS & Tissue Harvest | N/A | N/A |
| 2 | $2 \times 10^5$ PFU / 100 uL rCDV-gag intranasally | 2 animals rCDV-SIVgag, 1 animal PBS 0.5ml BS | 0.5ml BS | 0.5 mL BS | 0.5 mL BS | Terminal BS & Tissue Harvest |

**Table 1: Experimental group setup and timeline in study 1**. Each group consisted of three animals, two experimental and one control. D = day, BS = blood sample. Animals were inoculated intranasally with a set dose of $2 \times 10^5$ plaque forming units (PFU) of recombinant canine distemper virus with glycosaminoglycane insert (rCDV-gag) each on day 0. Tissues harvested on the day of euthanasia included nasal turbinates, mediastinal lymph nodes and Peyer's patches. Animals were clinically observed and had temperatures checked daily. Oral swabs were taken 2 h and 1 d post inoculation, nasal swabs 2 h, 1 d and 3 d post inoculation, and rectal swabs 2 h, 1, 3 and 4 d post inoculation.

### 3.3.2 Description of study 2 to investigate vector biology and immunogenicity

**Purpose and modifications after the first study:**

Following up on the information gathered in study 1, this study was composed to gain a deeper insight into vector replication and shedding, as well as cellular and humoral immune responses post inoculation. To be able to better observe the time course of immune responses, the time frame of the study was extended to 28 d. Additionally, one group of animals was inoculated with vector backbone without gag insert (rCDV).

Based on the results of quantitative real time- polymerase chain reaction (qRT-PCR) and virus culture experiments in study 1, the dose inoculated per animal was increased to $1 \times 10^6$ PFU/100ul in 3 % sucrose intranasally. Mock animals were inoculated with 3 % sucrose alone.

We reduced the frequency of swab and blood samples. Only nasal and rectal swabs were taken, and instead of plasma, which repeatedly formed a gel during *in vitro* tests, we only obtained serum samples in the second part of the study (Figure 4).

| Euthanasia time point | | D 7 | D 14 | D 21 | D 28 |
|---|---|---|---|---|---|
| Inoculum (100 µL intranasally) | Naive (3 % Sucrose) negative control | 2 ferrets | 2 | 2 | 2 |
| | 1 x 10$^6$ PFU rCDV experimental | 3 ferrets | 3 | 3 | 3 |
| | 1 x 10$^6$ PFU rCDV-SIVgag experimental | 3 ferrets | 3 | 3 | 3 |
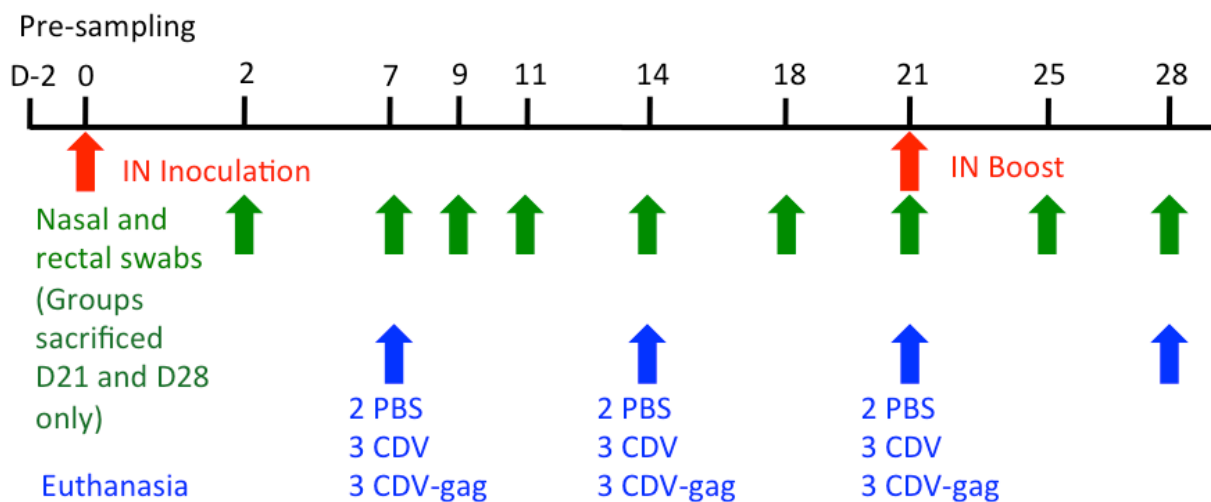


**Figure 4: Experimental group setup and timeline in study 2.** The three groups included 8 negative control animals inoculated with phosphate buffered saline (PBS) as well as 12 animals each for the experimental inocculations using 1 x 10$^6$ plaque forming units of recombinant distemper virus with (rCDV-SIVgag) or without (rCDV) glycosaminoglykane insert, respectively. All inocculations were done intranasally (IN). Red arrows indicate time points of inocculations. Nasal and rectal swabs were taken at regular intervals (green). Animals were clinically observed and had temperatures checked daily. Blue arrows signify time points at which animals were sacrificed. At each time point two control animals and three animals from each experimental group were euthanized. Tissues harvested on the day of euthanasia included nasal turbinates, mediastinal lymph nodes and Peyer's patches.

Animals were assigned into three groups, and received different inoculants via intranasal instillation. Group 1 was a mock-treated negative control group consisting of 8 animals. These animals were only administered PBS with 3 % sucrose. Group 2 was inoculated with 1 x 10$^6$ PFU of the empty recombinant CDV backbone in PBS / 3 % Sucrose, and group 3

received $1 \times 10^6$ PFU of rCDV with SIV-gag as an insert in PBS / 3 % Sucrose. Both groups consisted of 12 animals each.

On day 21 post inoculation (p.i.), all animals present received an intranasal boost with their respective agent.

2 h after inoculation, as well as after 2, 4, 7, 9, 11, 14, 18, 21, 25 and 28 d, we recorded the temperature of all animals present by use of the subcutaneous microchip, and obtained nasal and rectal swabs. On days 7, 14, 21 and 28 all animals present were weighed. After 7, 14, 21 and 28 days, 25 % of the animals in each group were sacrificed and terminal blood collections and necropsies performed. Nasal turbinates, spleens, the large mesenteric lymph node as well as sections of the intestine that appeared rich in Peyer's patches were harvested.

Animals were continuously observed for presence of any clinical symptoms of CDV such as diarrhea, nasal or ocular discharge, respiratory problems, loss of appetite, rough hair coat or inactivity. Any signs noted for any individual animal were recorded.

## 3.4 Experimental procedures

### 3.4.1 Anesthesia of ferrets for study procedures:

Animals were anesthetized for pre-study blood draws as well as intranasal inoculations by intramuscular injection of a mixture of 25 mg $kg^{-1}$ dexmedetomidine hydrochloride (Dexdomitor®, Pfizer Animal Health) and 5 mg $kg^{-1}$ ketamine hydrochloride (Ketaset®,Fort Dodge, now Pfizer), or alternatively 25 mg $kg^{-1}$ ketamine hydrochloride and 2 mg $kg^{-1}$ xylazine hydrochloride (Anased®, Lloyd Laboratories). Dexdomitor, if used, was reversed by

intramuscular administration of 250 mg kg$^{-1}$ atipamezole hydrochloride (Antisedan®, Pfizer Animal Health).

### 3.4.2  Application of recombinant virus by intranasal inocculation:

Upon beginning of the study, a total of 100 uL of inoculums was instilled into the animals' nostrils. Inoculum was drawn up into a 0.5 mL insulin syringe with a 27 G x 1 / 2 " needle (Terumo Medical Corporation). The ferret was anesthetized, held in an upright position, and the syringe and needle were held slightly above the animal's nostrils. A small drop of inoculum was slowly applied to the opening of one nostril. We then waited until the animal had aspirated that droplet, before applying the following one to the other nostril. This alternating procedure was repeated until all of the inoculum had been administered.

### 3.4.3  Procedure to obtain blood samples

Animals were anesthetized as described above, and 0.5 mL of blood was sampled by inserting a 1 mL or 3 mL syringe and a 25 G x 1 " needle (Becton Dickinson and Company) or 23 G x ¾" butterfly blood collection set (Terumo Medical Corporation) into either the cephalic or jugular vein. Blood was then transferred into serum separator tubes (BD Microtainer®, Becton Dickinson and Company), kept upright at 4 °C overnight, and spun in a Sorvall centrifuge at 1200 x g for 15 min the following day. Serum was then taken off and stored in aliquots at - 30 °C until further use.

### 3.4.4  Procedure to obtain swabs

We prepared for swabbing of animals by pre-filling labeled 2.0 mL tubes (Axygen Scientific Inc.) with 1 mL of PBS / sucrose phosphate buffer with glutamine (SPG, 0.2 M sucrose, 7.0

mM $K_2HPO_4$, 3.8 mM $KH_2PO_4$, 5.0 mM glutamic acid) (PBS by Invitrogen, all chemicals by Sigma). Sterile pediatric polyester tipped swabs (Puritan Medical Products Company LLC) were then pre-moistened before swabbing by dipping them into the corresponding tube. Animals were restrained manually, and the outside of their nose and rims of the nostrils, as well as rectum, were swabbed with separate swabs. Swabs were then placed into the corresponding tubes and kept on ice until processing. Samples were processed within 2 to 4 h of acquisition.

### 3.4.5  Euthanasia

Animals were euthanized at their corresponding time points by intraperitoneal injection of at least 100 mg $kg^{-1}$ sodium pentobarbital  (Euthasol®, Virbac Animal Health Inc.) using a 18 G x 1 ½" needle and 3 mL syringe (Becton Dickinson and Company). Death was confirmed by absence of reflexes and cessation of heartbeat and respiration.

### 3.4.6  Collection of post mortem samples

After euthanasia, an intracardial terminal blood draw was performed using Vacutainer® Serum Separator and Whole Blood Tubes and 21 G x 1 ½ " needles and needle holders (all Becton Dickinson and Company). Necropsies were performed and nasal turbinates, spleens, the large mesenteric lymphnodes as well as sections of the intestine that appeared rich in Peyer's patches harvested. The spleen was placed in a 50 mL conical tube (BD Biosciences) filled with Rosewell Park Memorial Insitute medium (RPMI; Hyclone), supplemented with 10 % v / v FBS (Invitrogen), 1 % v / v penicillin/streptomycin (10,000 I.U. $mL^{-1}$ penicillin, 10,000 ug $mL^{-1}$ streptomycin) and 1% v / v L-glutamine solution (200 mM, both Cellgro® by

Mediatech Inc.), on ice, while all other tissues were placed in scintillation vials (VWR), frozen in a dry ice-alcohol bath and kept on dry ice until processing.

### 3.4.7   Processing of tissue samples

Post-mortem tissue samples had to be processed in order to make them useable for further assays. The nasal turbinates, mesenteric lymph nodes, and large intestinal sections were snap-frozen, pulverized into powder and homogenized (Spex Sample Prep Freezer Mill 6870) to make 25 % weight / volume suspensions in SPBG buffer. Homogenised samples were stored at – 80 °C.

### 3.4.8   Titration of serum or plasma samples using virus neutralization assays

Virus neutralization (VN) assays were performed to determine the level of neutralizing antibodies elicited during our *in vivo* studies. The virus neutralizing antibody titer of a serum or plasma is determined as the reciprocal of the highest serum dilution in which virus infectivity is inhibited by 100 %. CDV specific neutralizing antibody titers were determined in Vero cells using 96-well microtiter plates (Corning). Ferret sera were prepared as serial 4-fold dilutions from 1 : 4 to 1 : 16,384 in quadruplicates and incubated with 100 PFU of rCDV virus for 1 h before the serum / virus mixture was loaded onto Vero cells. The plates were evaluated for cytopathic effect after 5 d of incubation. VN titers were expressed as the reciprocal of the highest serum dilution at which CPE was completely inhibited in all 4 wells. A known positive ferret serum was used a positive control and the virus without serum used as negative control.

### 3.4.9 Virus detection in swabs and tissue samples

Virus detection assays using cell culture were performed to evaluate if the samples taken during the *in vivo* studies contained infectious virus particles, and, if suitable, determine PFUs. Vero cells were seeded one day before swabbing in 96-well plates and in 6-well plates for virus detection in the swabs and tissue homogenates, respectively, and reached approximately 80 % confluency at the time of infection. Swab solutions were made into 10-fold serial dilutions in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with antibiotics (Cellgro), glutamine (Hyclone) and 1 % fungizone (Cellgro). 100 uL of undiluted and diluted swab solutions were loaded in quadruplicates at each dilution onto 96-well plates. Tissue homogenates were prepared at a 1 : 10 dilution in DMEM containing 1 % fungizone. Both, neat and diluted tissue homogenates, were inoculated and incubated for 1 h before the medium was changed to DMEM containing 0.75 % methylcellulose (Sigma) and 10 % FBS (Invitrogen). Plates were incubated for 4 d for rCDV and 7 d for rCDV-SIVgag vector exposed tissues before they were fixed with cold methanol. The cells were then stained with ab8352 monoclonal antibody (Abcam) and SIVgag positive monkey serum (kind donation from Arielle Ginsberg, Immunobiology lab, IAVI) for rCDV and rCDV-SIVgag, respectively. After incubation with secondary antibody-horseradish peroxidase (HRP) conjugates (Novus) followed by detection solution (Pierce), plaques were counted using an inverted light microscope.


### 3.4.10 Detection of viral genomic material using quantitative real time-polymerase chain reaction

Quantitative real time – polymerase chain reaction (qRT-PCR) was performed on swab and tissue samples to determine numbers of RNA copies of vaccine virus in these samples.

Standard curves were prepared using logarithmic dilutions of a known amount of synthetic oligonucleotides (Applied Biosystems) for the genes targeted.

100 uL of the resuspended swab was removed for RNA extraction using Qiagen's RNeasy kit. For tissues, each frozen tissue sample was pulverized into frozen powder using a Spex Sample Prep Freezer Mill 6870. Powder from each tissue was weighed and resuspended in PBS / SPG (Invitrogen, Gibco) to make a 20 % suspension, of which 150 uL were used to extract RNA. Extracted RNA was eluted in 100 uL RNase free water. All samples were set up in duplicates. RNA was extracted from samples using a Qiagen RNeasy kit, and following the protocol provided by Qiagen. RNA eluate was either processed right away, or stored at – 80 °C in 1.5 mL Eppendorf tubes.

In the 1st step,10 uL of RNA was used for cDNA synthesis along with the CDV-N forward primer

(5'-CGATGACCGGAAATCGATGGAAGCAATCGCCAAGATGAGGATGCTTACTAAGATGCTCAG TCAACCTGGGAC-3')

 to detect genome copies. In the second step of the assay, qPCR was performed using CDV-N specific primers.

Preparation for reverse transcription was undertaken in the clean room by mixing 2 uL 10 x reverese transcriptase (RT) buffer (Qiagen), 2 uL 5m M deoxynucleotides (dNTPs; Applied Biosystems), 0.4 uL 20 uM forward primer (Applied Biosystems), 1 uL 10 uM RNAseOut (Invitrogen), 1 uL RT enzyme (Qiagen) and 3.6 uL $H_2O$ (Qiagen) (total volume 10 uL) per test, and adding 10 uL per test sample to wells of an optical 96 well plate (Applied Biosystems). The plate was then sealed and moved to an extraction table, where 10 uL of sample RNA were added. Negative controls were run using water and lysis buffer from the RNA extraction kit. The plate was then sealed again and an Optical Cover Compression Pad

(Applied Biosystems) placed on top. The assembly was then centrifuged (ThermoScientific centrifuge) at 200 x g for 1 min. Reverse transcription was performed in a Stratagene Mx3005P Real-Time PCR machine, set to run at 50 °C for 45 min and 95 °C for 2 min.

Subsequently, the produced DNA was amplified using PCR. The test samples were placed on ice, and a mixture of 25 uL 2 x qPCR Master Mix (Qiagen), 1 uL 20 uM forward primer (Applied Biosystems), 1 uL 20 uM reverse primer (Applied Biosystems), 1 uL 1 uM 5′ fluorophore 6-carboxyfluorescein labeled primer (Applied Biosystems) and 2 ul $H_2O$ (Qiagen) (total volume 30 uL per sample) was added to the test wells. The thermocycler was set to run once at 95 °C for 15 min, and then 44 cycles of 94 °C for 15 s followed by 60 °C for 1 min (total run time 1hr 45min). PCR products were analysed and quantified on a Stratagene Mx3005P detector using the Primer Express Software, V 3.0.

Due to the required specialist knowledge to correctly perform this assay, John Coleman, RT-PCR specialist in the IAVI vectors lab, performed the qRT-PCR experiments on all samples.


### 3.4.11 Confirmation of presence of antibodies in serum samples using western blot

Western blot analysis was used to evaluate serum samples for presence of antibodies against SIVgag. The assay was performed as per standard procedures. Briefly, purified recombinant SIVgag p55 (Protein Sciences Corp) was prepared in SDS sample buffer (with reducing agent) (Invitrogen) at a concentration of 35 ug protein per mL of total sample volume, heated for 10 min at 70 °C, and then loaded onto a 4 – 12 % Bis-Tris electrophoresis gel (Invitrogen). The gel was run in 1 x MOPS SDS running buffer (Invitrogen) with antioxidant (Invitrogen) for 46 min at 200 V. It was then blotted onto a nitrocellulose blotting membrane (BioRad) on a dry-blotting apparatus (Invitrogen), using program P3 (20 V) and 7 min transfer time. The membrane was then placed in blocking buffer (Thermo

Scientific) on a rocking platform for 1 h at room temperature. To evaluate serum samples of different animals on the same membrane, we used a screening apparatus that facilitates screening of up to 17 samples on one membrane (Bio-Rad). 500 ul of serum sample per trough was added in a 1 : 500 dilution in blocking buffer. A nonhuman primate serum positive for SIVgag protein, as well as serum obtained from a naïve ferret housed with the study animals prior to the study were included as controls. The assay was incubated for 1 h on a rocking platform at room temperature, and then washed three times by adding PBS (Invitrogen) with 0.05 % Tween 20 (Bio-Rad) to all lanes and rocking for 15 min. Subsequently lanes were incubated with 500 uL per lane Horseradish-Peroxidase-labeled anti-species secondary antibodies (anti-monkey IgG by SantaCruz Biotechnology, Inc., Santa Cruz; and anti-ferret IgG by Novus Biologicals or by Bethyl Laboratories) diluted 1 : 5000 in blocking buffer for 1 h at room temperature on a rocking platform. The blot was then again washed three times and removed from the apparatus. 6 mL of enhanced chemoluminescence (ECL)-Substrate (GE Healthcare Bio-Sciences Corp.) was applied to the membrane and the blot incubated for 5 min before being evaluated using a bioimager (Bio-Rad).

### 3.4.12 Production of recombinant N protein

CDV N protein as an antigen was produced for antibody assays, since the N protein is the most abundantly expressed protein in CDV infected cells and also highly immunogenic (278). Recombinant partial or full length Paramyxovirus N protein for use in immunological assays has been expressed in procaryotic BL21 Escherichia coli (E. coli) several times before) (279, 280). We followed the principles laid out on these publications, making use of a plasmid that has previously worked very well for us in expressing His tagged proteins of similar size. The

full sequence of the CDV Onderstepoort N protein including an N-terminal sixfold Histidine (His$_6$)-SUMO-tag to enable protein purification on Nickel columns was cloned into a pET-28 based plasmid including a multiple cloning site (kind donation from the Shapiro Lab at Columbia University, based on the Champion pET SUMO vector by Invitrogen), transformed into XL10 gold *Escherichia coli* (*E. coli*) (Agilent), and the DNA of several clones isolated using the MiniPrep system (Qiagen). The isolated plasmids were analyzed for inserts by PCR, the results of which were analyzed using agarose gel electrophoresis. Positive clones were sent for sequencing (GeneWiz). 1 clone with a confirmed correct sequence was then used to transform Rosetta 2 (DE3) BL21 *E. coli* (Merckmillipore), which served as the expression strain. The bacteria were then grown in lysogeny broth (LB) medium (Sigma) with kanamycin (50 ug L$^{-1}$, Sigma) and chloramphenicol (34 ug L$^{-1}$, Sigma). Cultures were grown at 37 °C in a shaker (New Brunswick) until their optical density at 600 nm reached 0.6-0.8. Expression of the N protein was then induced by addition of 1 mM L$^{-1}$ Isopropyl β-D-1-thiogalactopyranoside (IPTG, SigmaAldrich). Since all attempts to obtain soluble protein failed, finally growth was continued for a further 24 h at 37 °C to drive expression in inclusion bodies. Cells were harvested in a Sorvall centrifuge for 15 min at 4000 x g. The bacterial pellet was then suspended in lysis buffer (20 mM Tris HCl pH 8, 150 mM NaCl, 20 mM imidazole, 8 M Urea, all Sigma) and sonicated for 4 min on ice to lyse cell walls. The lysate was spun and filtered to remove debris, and then run over a Ni-IMAC column (GE Healthcare). The column was washed using lysis buffer, and bound protein was eluted off the column using lysis buffer containing 500 mM Imidazole. A sample of the eluate was loaded onto an SDS page gel (Invitrogen) and stained using Coomassie (BioRad) to check for expression of the correct protein. A band at 67kD confirmed expression of a protein of appropriate size, containing of N protein at 55kD and the purification tag at 12kD.

Confirmation of presence of N protein was achieved by western blot, using a commercial anti-HIS antibody (Qiagen) as well as a commercial anti-N protein antibody (SantaCruz) and a horseradish peroxidase labeled anti-mouse IgG as a secondary (for protocol, see section on Western Blots, substituting primary and secondary antibodies accordingly). The produced protein solution was then dialysed against ddH$_2$O (MilliQ) to remove urea, since high urea concentrations would interfere with antigen coating of ELISA plates. As expected the protein precipitated during dialysis. The dialysis sample was spun down to collect the N-protein, and the pellet dissolved in Carbonate-Bicarbonate buffer containing 3 % SDS (Sigma). The final protein concentration was determined photometrically at 280 nm. This solution was subsequently used as a coating antigen for ELISA assays, as well as an antigen for western and dot blots.

### 3.4.13 Production of recombinant H protein

CDV H protein is the protein against which most neutralizing antibodies are elicited during natural infection. We hence decided to produce H protein as a backup antigen for antibody assays. CDV H protein is a class 2 transmembrane protein. It's first 58 aminoacids remain inside the viral particle. We therefor decided to only express aminoacids 59 to the stop sequence. Again, to maintain the most natural confirmation, expression in a eukaryotic system was attempted, but likely due to its viral origin, the protein proved to be severely toxic to eukaryotic cells. Hence we proceeded to expression in prokaryotic BL21 DE3 cells. The cloning and expression protocols and controls were identical to the ones described for production of N protein above (3.4.12).

### 3.4.14 Origin and description of simian immunodeficiency virus glycosaminoglycan protein

Purified recombinant SIVgag p55 (Protein Sciences Corp) was used as antigen for all assays designed to determine gag-specific antibody levels. Gag-positive serum samples from Non-human primates (NHPs) was provided by Arielle Ginsberg (IAVI) and were used as positive controls. The SIVgag protein was also detected with a SIVmac251 monkey serum obtained from the NIH AIDS Research & Reference Reagent Program and a HRP-conjugated anti-monkey IgG secondary antibody (Santa Cruz).

### 3.4.15 Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples to be used in intracellular staining and flow cytometry assays. Lymphocyte separation tubes (ACCU-Spin, Sigma-Aldrich) were pre-filled with 15 mL Ficoll-Paque PLUS® (GE Healthcare Bio-Sciences Corp.) and spun in a Sorvall centrifuge for 5 min at 485 x g to bring the gradient medium to the bottom of the tube. Whole blood samples obtained during terminal bleeds were then mixed with PBS in the separation tubes, and the tubes were spun for 30 min at 700 x g, with the acceleration speed set to 7 out of 9, and the deceleration speed set to 1 out of 9, to gradient-separate the PBMCs from other blood components. The interface was then aspirated and placed into 50 mL conical tubes (Becton Dickinson and Company). Those were filled up with PBS, and spun for 10 min at 485 x g with acceleration and deceleration speed set to 9 to wash the PBMCs. Cells were then resuspended in 2 mL ACK lysis buffer (Invitrogen) and incubated for 2 min at room temperature to lyse remaining red blood cells. Washing with PBS was repeated. Cells were then resuspended in RPMI 1640 medium (Invitrogen) and counted using an automated cell counter (Beckman Coulter). While cell

numbers and viability were obtained, cells were spun again at 485 x g for 5 min. They were then resuspended in commercial cell freezing medium (Invitrogen) at a density of $15 \times 10^6$ cells per mL and aliquoted into cryo vials (Nunc A/S) at 1 mL volume per aliquot. Cells were frozen in a controlled rate automated freezer (ThermoScientific) and stored in liquid nitrogen until further use.

### 3.4.16 Evaluating cellular immunological responses by use of stimulation assays, intracellular staining and flow cytometry

To determine the distribution of different subpopulations of PBMCs as well as the level of response to either polyclonal stimulation or SIV gag, previously isolated PBMCs were used for stimulation, intracellular staining and flow cytometry assays. PBMCs were extracted from terminal whole blood samples using the procedure above. Cells were then resuspended in RPMI and counted using an automated cell counter. $0.5 \times 10^6$ PBMCs were stimulated with SIVgag peptide pool (NIH AIDS reagent program) or with phorbol 12-myristate 13-acetate (PMA) / ionocmycin (Biolegend) stimulation as a positive control. Extra sets of cells were left as unstimulated negative controls. Due to the lack of ferret-specific and limited sources of cross-reactive antibodies, PBMCs were only stained for ferret CD8 with a cross reacting antibody (human anti CD8, clone OKT-8 by ebiosciences) and intracellular IFNγ using anti-bovine IFNγ antibody (Abd serotec clone cc302). Ten thousand PBMCs were analyzed of the $0.5 \times 10^6$ stimulated and stained for each sample by flow cytometry. IFNγ+ cells were gated in the CD8+ PBMC population. The gate was set according to cells stained for CD8 alone. Data were analyzed using the Cellquest software (Becton Dickinson FACSCalibur, CA). The frequency of IFNγ+ cells was expressed as the percentage of these cells after background subtraction within the total CD8+ cells counted.

### *3.4.17 Attempt to quantify antibody levels in ferret blood by enzyme-linked immunosorbant assays*

Enzyme-linked immunosorbant assays (ELISAs) offer an option to semi quantitatively assess the amount of antibodies against a specific antigen present in serum samples. Typically, the antigen in question is coated onto the bottom of the wells of 96-well-plates (for example Corning or Nunc) by interaction between the plate material and antigenic peptides or proteins. Antigens can also be "trapped" by coating the plate with (ideally monoclonal) antibodies against this antigen, then adding antigen solution. A blocking step with a protein rich suspension such as bovine serum albumin (BSA, BioRad) or dry milk powder in PBS (Invitrogen) is performed to obstruct unoccupied areas of the plate. ELISAs can then be conducted in two ways. Either, test serum is directly applied to the plate in serial dilutions, and if antibodies against the antigen are present, they will bind to the antigen. The bound antibodies can then be detected by using species-specific anti-Ig antibodies conjugated with a functional group that, if supplied with the correct substrate, will emit a fluorescent signal which can be read in a plate reader (biotek). The higher the concentration of the specific antibody in the serum binding to the antigen, the more secondary antibody will bind and the stronger the fluorescence will be. Alternatively, the assay can be performed in a competitive manner, where antibodies in the test serum block access to antigen for a specific monoclonal antibody. In this case, the higher the antibody level in the test serum, the less monoclonal antibody will be bound and detected, and the lower the fluorescence signal will be.

ELISA test kits for CDV to be used with dog sera are widely available. However, none of them were suitable to detect ferret antibody levels during our studies. We therefore endeavored

to develop our own in house ELISA in order to be able to semi quantitatively determine antibody levels against CDV. Since excessive amounts of work were put into developing this assay, and a variety of issues were encountered, a detailed description of the development attempts can be found in the results section of this thesis.

# 4 Results

## 4.1 Virus propagation

rCDV and rCVDgag were propagated by infection of Vero cells. Infected cells in fully confluent flasks were scraped into the medium and the suspension repeatedly freeze-thawed to release viral particles. The suspension was then clarified by centrifugation to purify virus particles, and supernatant added to naïve 70 % confluent Vero cells. Each passage was individually named. Both rCDV and rCDV-SIVgag grew well in this fashion. To allow for most effective virus propagation, cells were infected while not fully confluent, and a rather low Multiplicity of Infection (MOI) (around 0.02) was used to avoid the formation of interfering defective particles. To further increase virus yield, flask sizes were increased from one passage to the other and/or multiple flasks of Vero cells infected if desired. This allowed for constant maintenance of the original strains selected for this study, and enabled us to grow enough virus per passage to complete the entire study with virus harvested from the same passage, therefore minimizing variation between inoculations.

## 4.2 Viability assays and virus quantification

Along with each virus passage, the harvested virus was assessed for viability and quantified using Plaque Forming Assays. A sample of the harvested virus stock was used in 10-fold serial dilutions to infect Vero-cells in 6-well plates. The infected cells were overlaid with methylcellulose-MEM-solution to aid plaque formation. After a set incubation time cells were washed, fixed and stained with Giemsa stain and plaques were counted. Virus titer for the corresponding batch was calculated and expressed in plaque-forming units per milliliter

(PFU / mL). The constant use of this assay allowed us to accurately observe the amount of virus produced and to ensure possible problems with virus productions were detected early.

During the initial PFU assays, we evaluated the differences between using purified virus, and omitting the centrifugation step and adding the virus non-purified. In direct comparison, initially the same virus harvest titrated at a higher PFU / mL when purified than non-purified. However, this did not hold true in a repetition of the assay. Additionally, assays using purified virus showed a much higher variance between duplicates of dilutions, and it was much harder to distinguish plaques from unaffected areas of the monolayer as compared to non-purified virus assays.

Due to the risk of adverse reactions to cell debris and other particles, we decided that non-purified virus could not be used in the planned animal studies. Therefore, ultimately only purified virus was titrated and stored. Virus harvests used for this study titrated at $5 \times 10^7$ PFU / mL.

## 4.3   <u>Determining distemper exposure status of study animals</u>

The ferrets enrolled in the two studies presented here came into our facilities as certified unvaccinated animals. Prior to beginning the studies, we collected blood samples from each ferret, allowed for clotting at 4 °C overnight, separated off the serum and performed virus neutralization tests for each animal in duplicates. All tests were negative, indicating the absence of neutralizing antibodies against CDV in these animals.

## 4.4 Results of study 1 – vector safety

### 4.4.1 Clinical observations

Weights (Table 2) and temperatures (Table 3) of each animal were recorded on the day of inoculation and at each harvest time point. The presence of any clinical signs suggestive of CDV, such as fever, inappetence, nasal or ocular discharge, diarrhea, or rashes was recorded. Since over the course of these studies we planned to use subcutaneous microchips capable of recording body temperature, during this pilot study, a side by side comparison of rectal and chip temperature read-outs was performed to determine if subcutaneous temperatures consistently reflect actual core temperatures, and what the degree of variation would be.

#### 4.4.1.1 Development of body weights

The animals used during this study were still very young and had not yet reached their adult weight. Over the seven-day course of this study, all animals except one continued to gain weight. The one animal that did not had briefly lost 20 g of body weight but had regained its starting weight by the end of the study. So in summary, vaccination with rCDV-gag did not affect body weight.

| Subject # | Weight (kg) | | |
|---|---|---|---|
| | D0 | D3 | D7 |
| 1 (f) | 0.76 | 0.8 | n/a |
| 2 (m) | 1.28 | 1.32 | n/a |
| 3 (m) | 1.30 | 1.34 | n/a |
| 4 (m) | 1.34 | 1.38 | 1.40 |
| 5 (f) | 1.00 | 0.98 | 1.00 |
| 6 (f) | 0.86 | 0.96 | 0.98 |

**Table 2**: **Weight developments of individual animals during study 1.** D = Day, m = male, f = female. Animals #3 and 6 were mock-treated negative controls. Clinical distemper infection would usually cause weight loss. No decrease in body weight can be seen in any of the animals over the course of the study.

### 4.4.1.2 Development of body temperatures

Body temperatures in healthy ferrets vary significantly over the course of the day and with different activity levels. However, generally, temperatures > 40.5 °C are considered above normal. During the course of this study, none of the animals ever presented with temperatures above that level, and temperature curves remained fairly steady throughout, indicating that inoculation with CDV did not cause a systemic reaction leading to the development of fever. When comparing microchip and rectal readings, care had to be taken to ensure that readings were taken at the same time. Variations of more than 1 °C occurred on three occasions, where animals had been woken from a sleep and then became active and warmed up quickly, making the reading noted first significantly lower than the reading taken second. In all other cases though, chip readings remained within 0.5 °C of rectal readings. The average difference between subcutaneous and rectal readings was 0.57°C, with the subcutaneous temperatures regularly being the higher reading. We therefore considered the chip to be an easy, convenient and reliable way to attain an accurate picture of the animals' body temperatures.

| Subject # | Body temperature (°C) | | | | | | | | | |
|-----------|------|------|------|------|------|------|------|------|------|------|
| | D0 | | D1 | | D3 | | D4 | | D7 | |
| | S | R | S | R | S | R | S | R | S | R |
| 1 | 35.5 | 36.7 | 38.5 | 38.2 | 39.4 | euth | n/a | n/a | n/a | n/a |
| 2 | 36.4 | 37.0 | 37.8 | 37.9 | 38.4 | euth | n/a | n/a | n/a | n/a |
| 3 | 38.7 | 39.0 | 38.2 | 38.4 | 38.5 | euth | n/a | n/a | n/a | n/a |
| 4 | 36.0 | 36.6 | 37.0 | 36.7 | 38.5 | 37.8 | 37.5 | 37.8 | 39.7 | 38.8 |
| 5 | 38.1 | 37.8 | 38.4 | 37.8 | 36.7 | 36.4 | 37.5 | 37.1 | 39.8 | 37.9 |
| 6 | 38.6 | 38.1 | 38.1 | 37.2 | 36.0 | 36.0 | 37.5 | 37.2 | 39.5 | 37.3 |

**Table 3: Comparison of simultaneous rectal and subcutaneous chip measurements of body temperature in ferrets over the course of study 1.** D = day, S = subcutaneous, R = rectal. Animals #3 and 6 were mock-treated negative controls. Normal temperatures in ferrets vary between 36 and 40.5°C. Clinical distemper infection would usually cause significantly elevated body temperatures of 40.5-42°C. None of the animals showed temperatures within elevated range at any point during the study. This table also compares results of rectal measurement of body temperature with measurements using microchips implanted subcutaneously. Rectal and subcutaneous measurements differ by a minimum of 0.0°C and a maximum of 2.2°C with an average of 0.57°C. Subcutaneous measurements were usually higher than rectal measurements.

*4.4.1.3   Recording and evaluation of clinical symptoms suggestive of distemper infection*

No animals showed any clinical signs, including diarrhea, discharges, rashes or abnormal behavior, or significant temperature increases during the study, suggesting that the recombinant virus was sufficiently attenuated to not cause clinical symptoms in this highly susceptible species.

### 4.4.2 Swab and tissue samples

#### 4.4.2.1 Detection of recombinant virus in swabs by plaque assay in Vero cells

Briefly, 100 uL of swab sample medium was added onto a monolayer of Vero cells grown in 96 well plates in quadruplicates and incubated for 1 h. Samples were then aspirated and replaced with fresh media. Wells were monitored for plaque formation (Table 4). No plaque formation was observed in any of the samples, indicating that there was not enough virus present in the swab samples to establish a productive infection.

| Group | | Subject # | | Time post infection | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | 2 h | D1 | D3 | D4 | D7 |
| 1 | Vaccinated | 1 | Nasal | 0 | 0 | 0 | n/a | n/a |
| | | | Oral | 0 | 0 | 0 | n/a | n/a |
| | | | Rectal | 0 | 0 | 0 | n/a | n/a |
| | | 2 | Nasal | 0 | 0 | 0 | n/a | n/a |
| | | | Oral | 0 | 0 | 0 | n/a | n/a |
| | | | Rectal | 0 | 0 | 0 | n/a | n/a |
| | Mock | 3 | Nasal | 0 | 0 | 0 | n/a | n/a |
| | | | Oral | 0 | 0 | 0 | n/a | n/a |
| | | | Rectal | 0 | 0 | 0 | n/a | n/a |
| 2 | Vaccinated | 4 | Nasal | 0 | 0 | 0 | 0 | 0 |
| | | | Oral | 0 | 0 | 0 | 0 | 0 |
| | | | Rectal | 0 | 0 | 0 | 0 | 0 |
| | | 5 | Nasal | 0 | 0 | 0 | 0 | 0 |
| | | | Oral | 0 | 0 | 0 | 0 | 0 |
| | | | Rectal | 0 | 0 | 0 | 0 | 0 |
| | Mock | 6 | Nasal | 0 | 0 | 0 | 0 | 0 |
| | | | Oral | 0 | 0 | 0 | 0 | 0 |
| | | | Rectal | 0 | 0 | 0 | 0 | 0 |

**Table 4: Individual results of plaque assays performed on swab samples throughout study 1, given as number of plaques counted**. Nasal, oral and rectal swabs were taken from each study animal 2h, 1, 3, 4 and 7 days post inoculation. Animals in group 1 were euthanized at D3 post inoculation, animals in group 2 at D7 post inoculation. Animals #3 and 6 were mock-treated negative controls. Swabs were then used to conduct plaque assays. No swab samples resulted in plaque formation, indicating that not enough infectious virus was present in any of the samples to result in a productive infection.

*4.4.2.2   Detection of recombinant virus in nasal turbinate and ileum tissues by plaque assay*

Briefly, powdered tissue samples were aliquoted into microcentrifuge tubes and weighed. Aliquots for nasal turbinates weighed between 30 and 70 mg, and for ileal tissue 30 to 150 mg. Approximately 130 uL medium was added to each sample and vortexed. 100 uL of this suspension, as well as a 1:10 dilution of the suspension was added to a monolayer of Vero cells grown in 6-well plates and incubated at 37 °C for 1 h. Samples were then aspirated and replaced with media and plates incubated for 4 d. Wells were monitored for plaque formation (Table 5). No plaques were formed in any of the samples harvested on either D3 or D7, indicating an insufficient amount of virus to create a productive infection.

| Subject # | Nasal Turbinate | | Ileum | |
|---|---|---|---|---|
| | neat | 1:10 | neat | 1:10 |
| 1 | 0 | 0 | 0 | 0 |
| 2 | 0 | 0 | 0 | 0 |
| 3 | 0 | 0 | 0 | 0 |
| 4 | 0 | 0 | 0 | 0 |
| 5 | 0 | 0 | 0 | 0 |
| 6 | 0 | 0 | 0 | 0 |

**Table 5: Results of plaque assays performed on tissue samples during study 1 to detect the presence of infectious particles at D7 post infection. Results given as number of plaques counted.** 30 – 70 mg tissue powder resuspended in 130 uL of medium were used for the nasal turbinate samples. 30 – 150 mg tissue powder resuspended in the same volume were used for the ileum samples. Animals #3 and 6 were mock-treated negative controls. No plaques could be detected from any of the tissue samples, indicating the tissue samples did not contain enough infectious virus to produce a productive infection.

### 4.4.2.3 Detection of viral genetic material in swab and tissue samples obtained during study 1 using quantitative real time – polymerase chain reaction

Briefly, 100 uL of the resuspended swab medium or 200 uL of a 10 % suspension of homogenized tissue in PBS were used for RNA extraction using Qiagen's RNeasy kit. Extracted RNA was eluted in 100 uL RNase free water.

In the first step of qRT-PCR, 10 uL of RNA was used for cDNA synthesis by reverse transcription using the CDV-N forward primer to detect genome copies. In the second step of the assay, qRT-PCR was performed using CDV-N specific primers.

Using swab medium, low levels of CDV genome were detected mainly in two animals (#1 and 4) with the exception of rectal swabs where genomic copies were detectable in one additional animal on D1 post inoculation (Figure 5). These results indicate that a low degree of virus shedding does occur for at least 4 d post inoculation. However, no viral genome could be detected in oral and nasal swabs of animals # 2 and 5, which shared the cage with # 1 and 4, respectively. Animal # 6 is a mock treated animal that was kept in a different cage yet viral copies were detected in one rectal swap. Since no viral genome was detected in swabs of this animal at any other time point, nor was any viral DNA detected in tissue samples from this animal, there is a significant possibility that this positive swab result stems from cross contamination.

qRT-PCR analysis of nasal turbinate samples revealed presence of viral genomic material in one of two experimental animals sacrificed at either time point, suggesting persistent presence of virus in the nasal cavity over the duration of the study. The qRT-PCR assay detected significant levels of CDV genomes in gut mucosal tissue of inoculated ferrets of both experimental groups but not the mock group at D7 (Figure 6). Since no copies were detected in the same tissues at D3, it can be inferred that this represents replication of the inoculum.
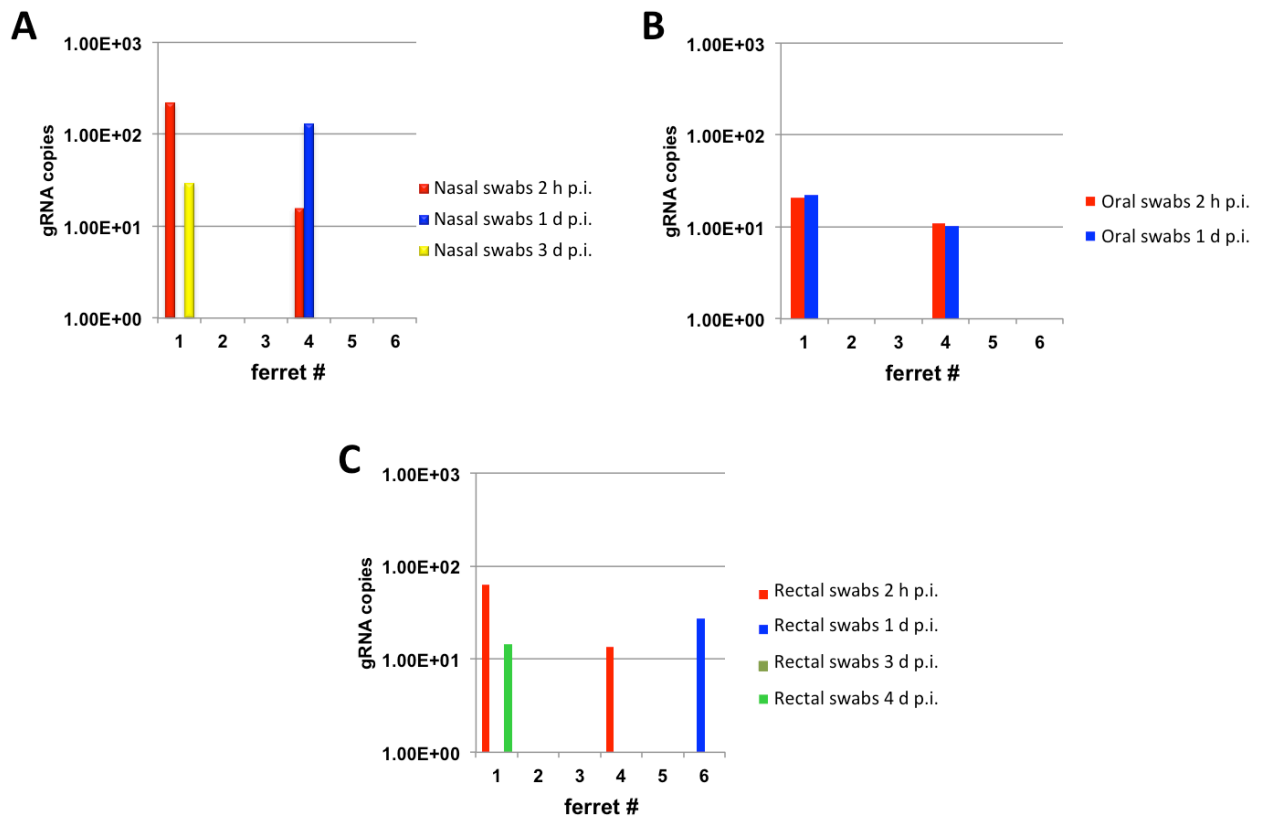
**Figure 5: Graph of numbers of genomic copies of distemper N protein RNA in swab samples by quantitative real time – polymerase chain reaction.** Number of genomic copies of distemper N protein detected in **A** nasal swabs, **B** oral swabs and **C** rectal swabs at 2 h, 1 d, 3 d and 4 d post inoculation. Annotations on the x-axis corresponds to animal IDs. Animal numbers 3 and 6 were mock-treated negative controls. Values below the detection limit of 10 copies of genomic RNA were not plotted. Genomic copies were detectable at different time points in swabs from 50% of the experimental animals. Highest numbers were detected in nasal swabs within 24h of inoculation. Detection of genomic RNA copies in other locations sampled indicates that the recombinant virus does spread throughout the body. On one occasion, genomic copies of N protein RNA were detected in a mock infected animal (rectal swab on d1). Since no positive results were obtained from this animal at any other time point or from any other location, this is likely the result of accidental cross-contamination.
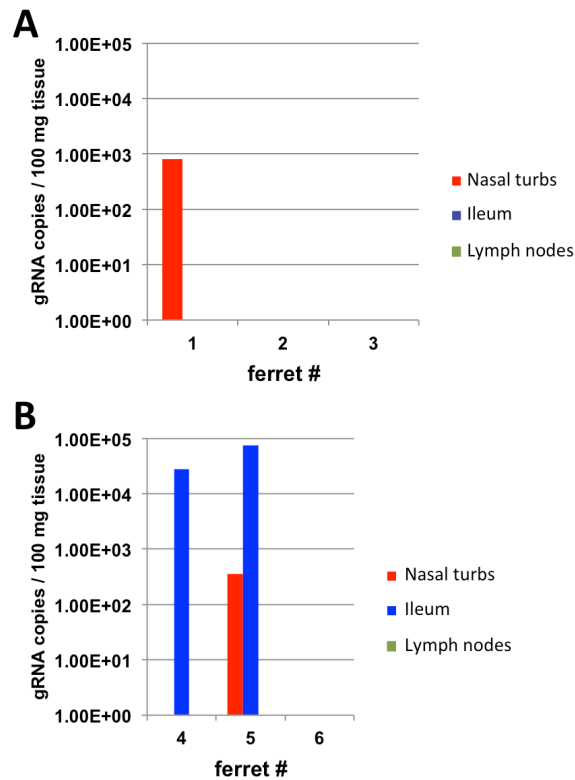
**Figure 6: Genomic copies of distemper N protein RNA in tissue samples analysed by Quantitative Real-Time Polymerase Chain Reaction.** Number of genomic copies of distemper N protein RNA in tissues harvested **A** on D3 and **B** on D7. Annotations on x-axis correspond to animal IDs. Animal numbers 3 and 6 were mock-treated negative controls. Values below the detection limit of 10 copies were not plotted. Copies of viral RNA were detectable in nasal turbinates of one of two experimental animals sacrificed at d3 and d7, indicating persistent presence of viral material in the location of inoculation. Detection of signifikant amounts of viral RNA within the ileal samples of both experimental animals sacrificed at d7 but not d3 indicates replication distant from the inoculation site.

### 4.4.3 Analysis of blood samples

#### 4.4.3.1 Intracellular cytokine staining of PBMCs to detect cellular responses to simian immunodeficiency virus glycosaminoglycan

Briefly, whole blood samples from animals euthanized at D7 were prepared to isolate PBMCs. These were incubated with overlapping peptides covering the gag open reading frame (ORF), PMA/ionomycin, or no stimulant for 9 h at 37 °C. Samples were stained with monoclonal antibodies against CD8 and IFN-γ. Analysis was performed on an LSRII Flow Cytometer using FACSDiva Software. There was no noticeable IFN-γ production after mock

stimulation, and significant IFN-γ production after polyclonal stimulation with PMA. There was an increase of IFN-γ production after stimulation with gag ORF, but there was no significant difference between vaccinated and sham treated animals at this time point. The negative and positive controls worked well in this assay, but no significant gag specific response was detectable at the D7 time point (Figure 7).
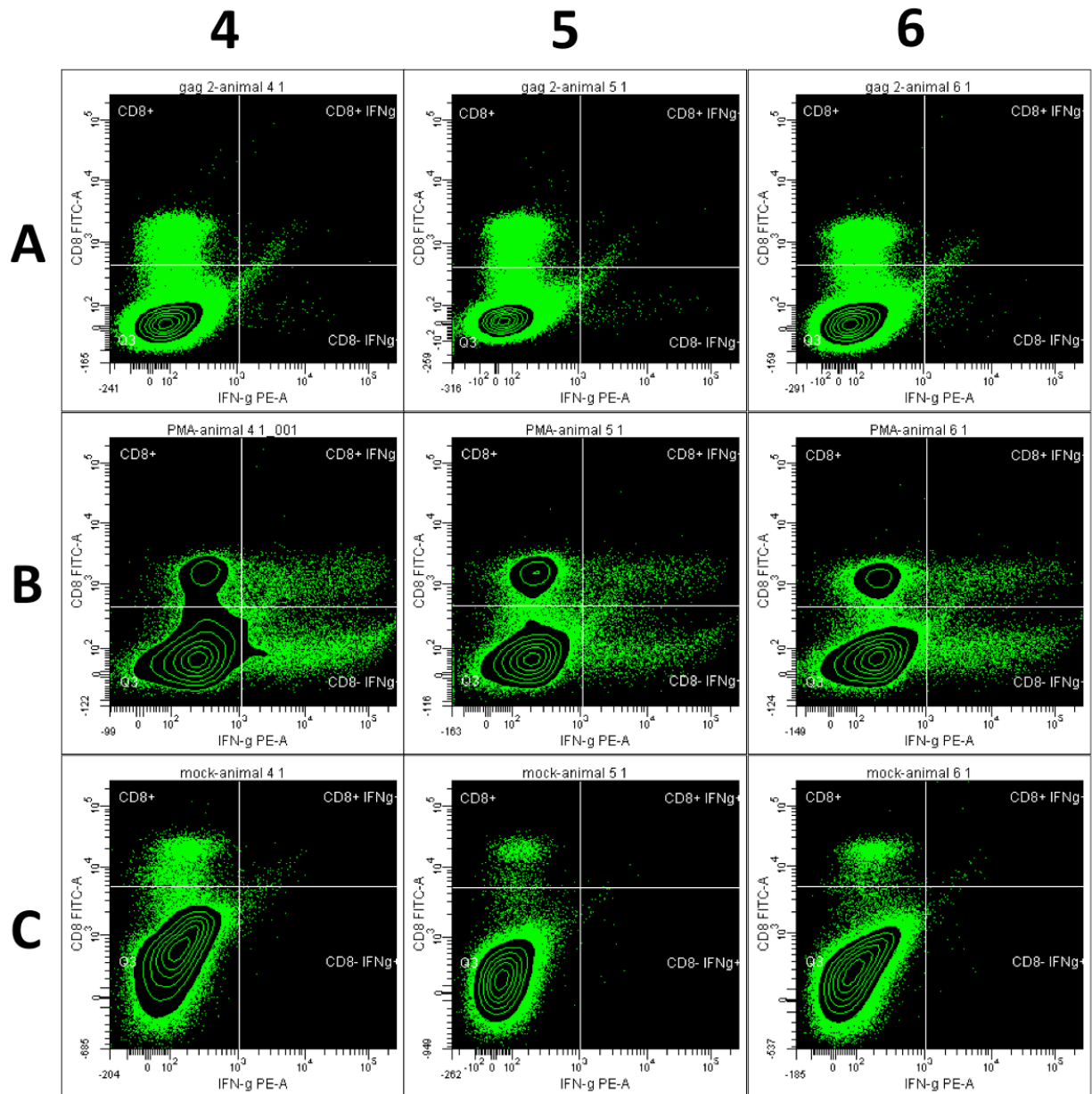
**Figure 7**: **Scatter plots of flow-cytometric analysis of peripheral blood mononuclear cells harvested on D7 of study 1, stained for CD8 and IFN-γ.** Numbers across the top represent animal ID numbers. **A** Glykosaminoglykane stimulated samples of all animals sacrificed at D7. **B** Polyclonally stimulated samples, positive control. **C** Mock-stimulated samples, negative control. Note the increased number of cells in bottom and top right quadrants of PMA stimulated animals, and the slight increase in cell numbers in the top right quadrant in glycosaminoglykane stimulated samples, indicating an increase in cells double-positive for both CD8 as well as IFNg. This suggests the presence of antigen specific CD8 cells at d7 post inoculation.

## 4.5 Results of study 2 – vector biology and immunogenicity

### 4.5.1 Clinical observations

#### 4.5.1.1 Development of body weights

Individual animals mostly maintained their body weight throughout the duration of the study (Figure 8 and Figure 9). There was no obvious tendency for development of body weight within any of the treatment groups.

To minimize possible effects of different body weights on the outcome of the study, animals with highest and lowest weights within the study were assigned into the mock group.

One female animal within the experimental group inoculated with rCDV was found to be consistently loosing weight. This animal was also noticed for presenting with ocular discharge from D7 p.i. onwards as well as occasional soft stool. It was, however, eating, drinking, active and alert over the entire course of the study. It did not present with any temperatures higher than 39.3 °C. Her cage mates did not show any weight loss, increased temperature, decreased appetite or activity level, or any other symptoms possibly related to CDV infection at any time. This animal had initially arrived with a weight closer to her endpoint weight, but had gained 200g until the beginning of the study with no signs of illness. We therefore decided to keep this animal in the study.

Comparing the development of the average of body weights of all animals in one treatment group with the average of weights in the other groups reveals no significant differences between the two experimental groups (rCDV vs rCDV-SIVgag). There were also no tendencies towards weight gain nor weight loss detectable for any of the groups. Body weights remained relatively constant over the course of the study in all treatment groups.

The relatively high level of average body weights for the mock group up to D14 p.i. and then at a lower level up to D21 p.i. is likely due to the influence of two particularly heavy animals assigned to this group, which were sacrificed on D14 and D21.

The increase in average body weights of the rCDV group on D28 is an effect of chance. The animals of this group remaining until D28 happened to be the heaviest animals of this group, and the lack of balance by lighter animals results in the sudden increase.

These very constant data on development of body weight confirm our observations that all animals in the study had normal appetite, were able to get to and take up their food and were eating and drinking over the the entire course of the study.
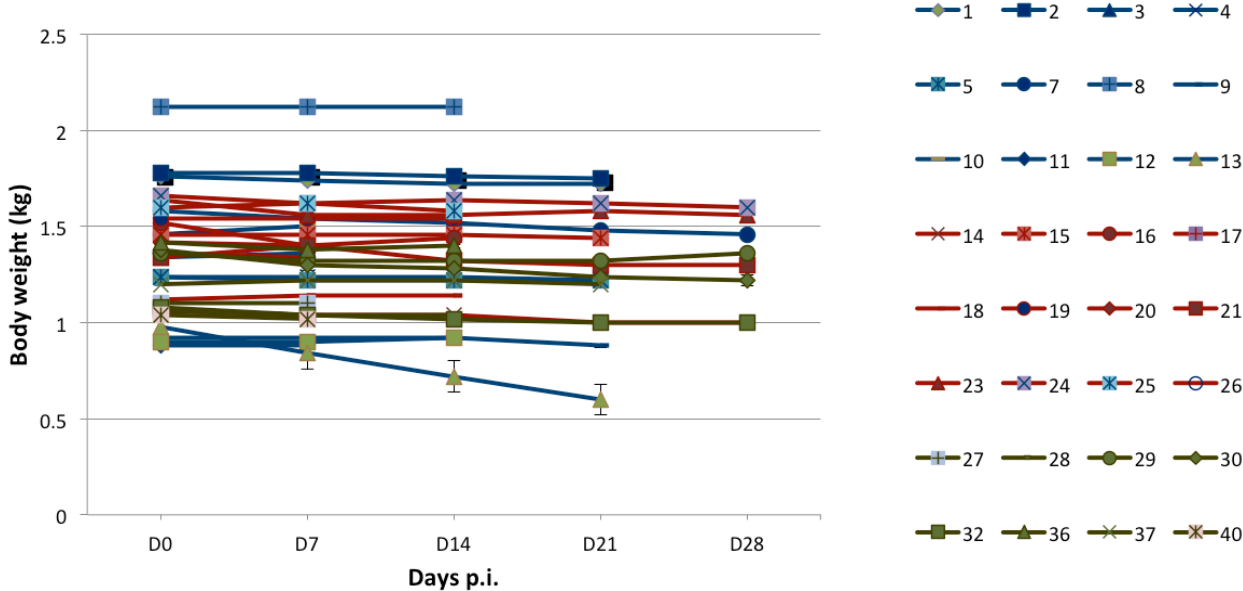


**Figure 8: Body weight of all 32 individual animals over the course of this study.** Blue lines represent animals inoculated with empty vector, red lines represent animals inoculated with recombinant distemper including glykosaminoglykane insert; green lines represent the control group. Individual animals are designated by different labels along the lines. Definite weight loss can only be noted in one animal within the empty vector group. This animal presented no other signs of possible illness.
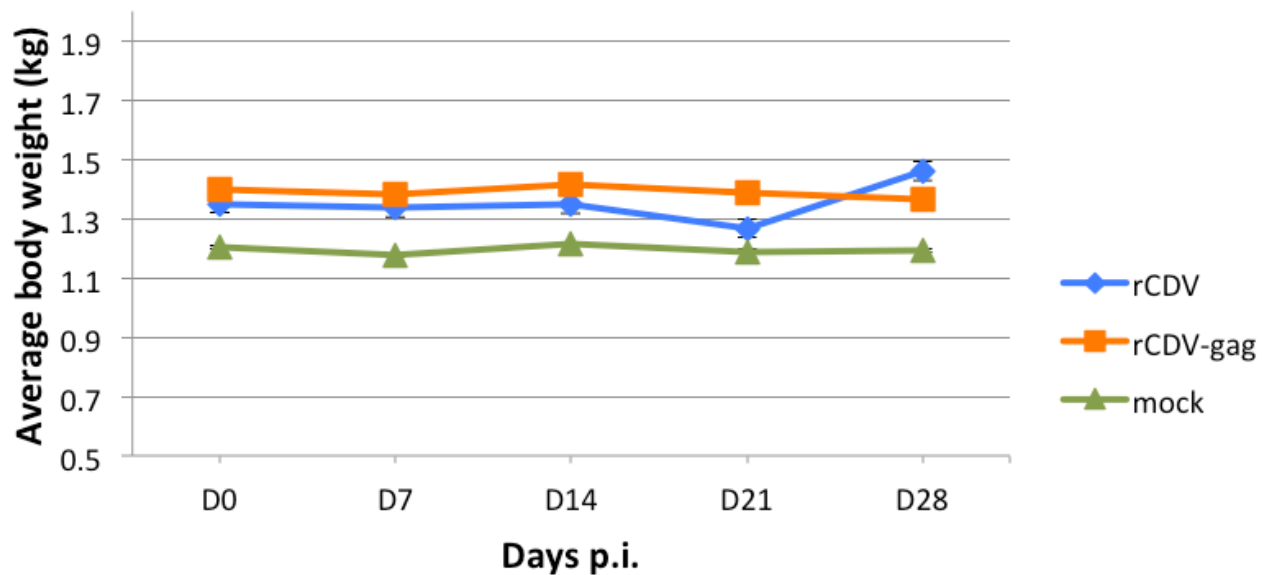
**Figure 9: Average body weights within treatment groups.** Weights of the individual animals within each treatment group were averaged for each time point. Blue line signifies empty vector group, red line recombinant distemper with glykosaminoglykane insert, green line control group. On average no significant changes in body weight can be noted in any of the groups.

### 4.5.1.2 Development of body temperatures

The normal body temperature in ferrets is generally assumed to be between 38 and 40 °C, but varies greatly depending on both the environment and the activity of the animals. In sleeping animals it regularly drops below 38 °C, and in active and excited animals it quickly rises up to 40.5 °C. Activation of the immune system is also known to result in a temporary increase of body temperature of up to 1 °C in mammals. During our study, no animal ever showed temperatures > 40.5 °C (Figure 10). On D0 all animals were anesthetized for inoculation, which limits their ability to maintain normal body temperature and can explain the relatively low values for that reading.

For the graph below, body temperatures of all animals within one treatment group were averaged for each time point and the treatment groups plotted against each other to average effects of activity- or sleep-induced temperature rises or falls. This graph illustrates that the overall body temperatures were within the normal range of 38 – 40 °C at all times

and for all groups. For the first 10 d post inoculation, animals in experimental groups showed

slightly higher temperatures than animals in the mock group, which might indicate activation

of the immune system.
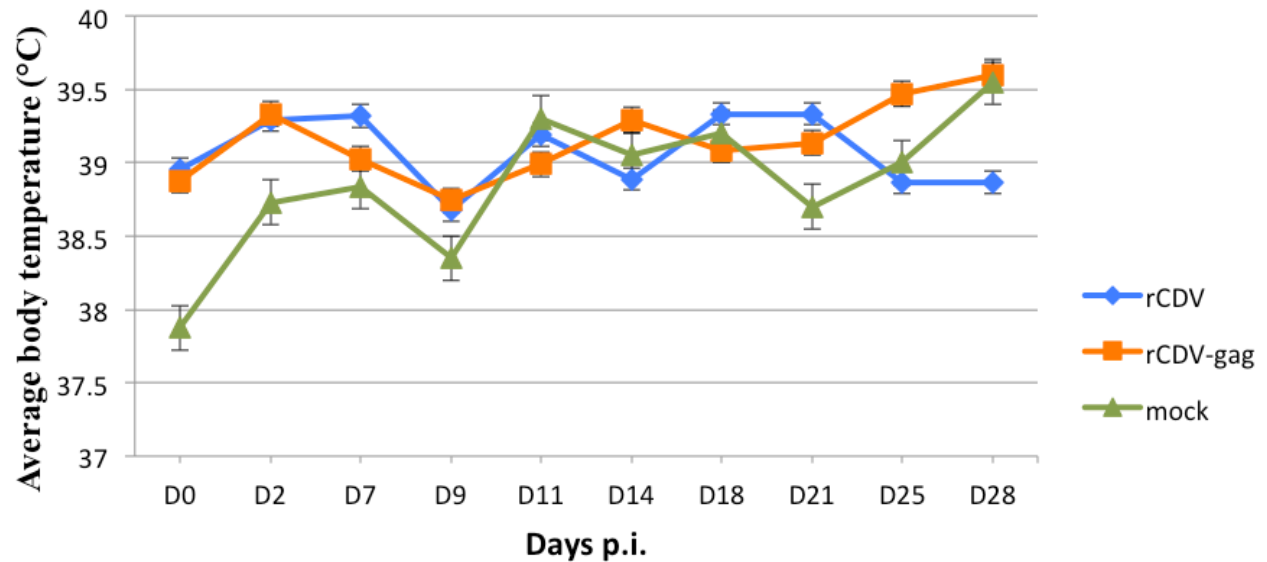


**Figure 10: Average body temperature in treatment groups.** Temperatures of animals within each treatment group were averaged for each time point. Blue line signifies empty vector group, red line recombinant distemper with glykosaminoglykane insert, green line control group. Clinical distemper infection usually causes temperatures of above 40.5°C especially in the first days post infection. This was not observed for any of the groups. Both experimental groups produced average temperatures above the average of the control group for the first ten days post inoculation which could suggest activation of the immune system.

*4.5.1.3  Recording and evaluation of clinical symptoms suggestive of distemper infection*
Animals were observed for symptoms indicative of a CDV infection, such as discharges from

nose or eyes, changes in fecal consistency, appetite or behavior, and central nervous signs

(Figure 11). The only symptoms ever noticed were changes in consistency of feces, and

secretions from nose or eyes and occasional sneezing. Those symptoms were therefore

classified into two major groups, "intestinal symptoms" and "discharges", and plotted for

each group on each day observations were recorded.

One animal in the mock group repeatedly presented with rather soft stool compared to its cage mate, but was otherwise without observations. All other mock animals did not show any symptoms at any given time.

A member of the rCDV group was repeatedly noticed with discharge, and on two occasions also soft stool. This animal also presented with continuous weight loss over the course of the study, but appeared otherwise healthy. Body temperatures remained normal throughout the study.

Aside from those two individuals which repeatedly presented with specific slight observations, symptoms possibly linked to inoculation with CDV were only noted on D7 p.i., which is a relatively short time after the prime inoculation, and on D25 p.i., which equals 4 d post boost inoculation. They were also only noted in animals from experimental groups at those times, but not from the mock group. Four different animals from the rCDV-SIVgag group were observed showing either intestinal symptoms or discharge, while in addition to the frequently noted animal only one other ferret in the rCDV group presented with possible symptoms. It was observed as having slightly watery eyes on D7 before it was sacrificed. Clinical alterations in such relatively close proximity to inoculations could indicate a reaction towards the respective inoculum in some animals. We observed a higher number of individuals in the rCDV-SIVgag group presenting with possible symptoms than in the rCDV group, which might suggest that animals receiving the vector including insert were more affected by the inoculum, although this difference was marginal.
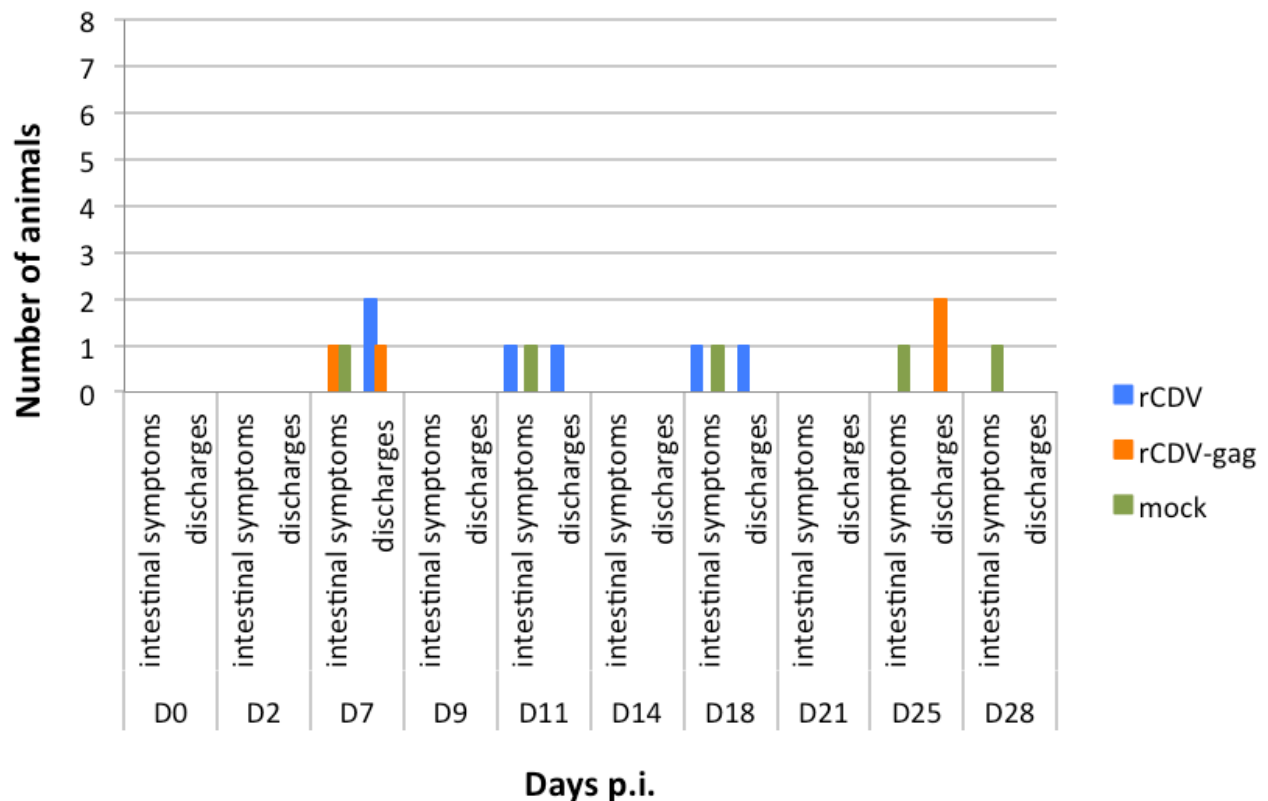
**Figure 11: Number of animals with clinical symptoms per group.** Occurrence of clinical signs of possible CDV infection in experimental groups in number of animals in each group noted with either intestinal symptoms or discharges. No other symptoms such as rash or central nervous signs were noted at any point in the study. Discharges included watery eyes or nose. Blue columns signify animals in the empty vector group, red columns recombinant distemper with glykosaminoglykane insert, green columns control group. All occurances plotted for the control group originated from the same animal and presented as soft stools. It can be noted that no animals showed any severe or continous clinical changes.

### 4.5.2 Analysis of swab samples

#### 4.5.2.1 Detection of infectious particles in swab samples using tissue culture infectious dose 50% assays

While this assay was technically functional and routinely used for virus titrations and detections, and both positive and negative control samples presented the expected results, no CPE could be observed in any of the experimental samples despite some non-specific artifacts in the cell monolayers (data not shown).

### 4.5.2.2 Detection of viral genetic material in swab samples using quantitative real time-polymerase chain reaction

qRT-PCR was performed to look for genomic copies of CDV-N, initially using rectal swabs from all time points (Figure 12). All time points in all samples were analyzed, including those of mock-treated animals. Copy numbers detected in mock control animals were on par with the highest level of detection in rCDV vaccinated animals, suggesting a contamination error. Proper precautions against cross contamination were taken during the conduction of the study by housing ferrets in intra-group pairs in fully sealed and ventilated cages, and ensuring that any and all housekeeping, handling and study procedures were performed in an order that would prevent cross-contamination. Full personal protective equipment was worn at all times, and changed in between study groups. All materials were disinfected or discarded in between study groups. These protocols were maintained at the time of swab collection but seemingly not at the time of aliquoting and/or processing, which was performed on a bench used for CDV work. Likely sources of the CDV contamination include the pipettes used to aliquot samples and forceps used to remove swabs from tubes.
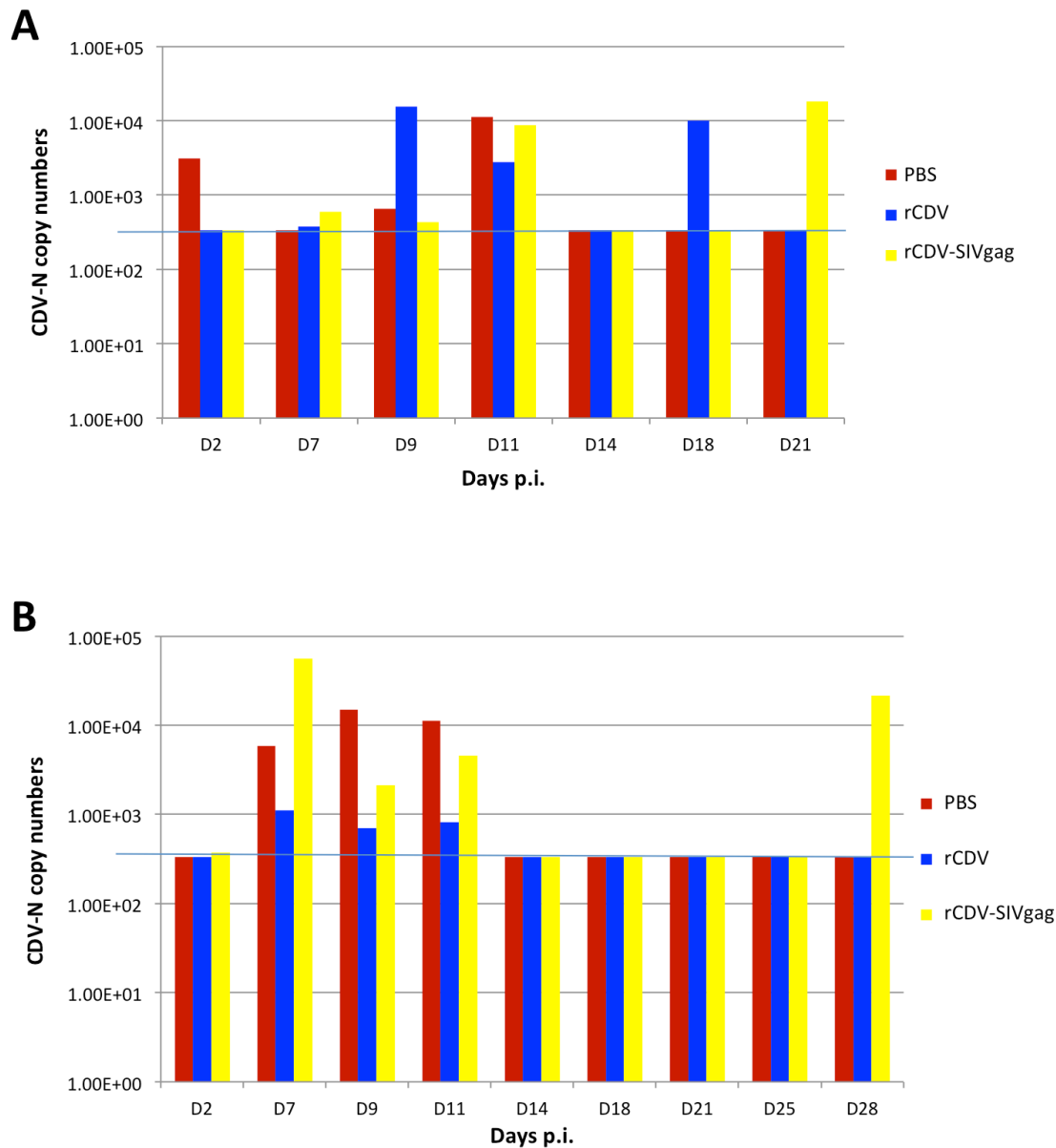
**Figure 12: Numbers of genomic copies of distemper N protein RNA in rectal swabs taken during this study. A** Samples for animals sacrificed on D21. **B** Samples for animals sacrificed on D28. Copy numbers of samples taken from all three groups were averaged for each time point. Blue bars signify empty vector group, yellow bars recombinant distemper with glykosaminoglykane insert, red bars control group. Lightblue cross-bar: Detection limit. Note the consistent presence of high levels of genomic RNA in control animals, indicating likely cross-contamination issues during sample processing. This assay could not be evaluated.

It is impossible to draw any conclusions from this data set because of the apparent contamination. In light of these data suggesting a high likelihood of cross contamination, nasal swab samples were not analyzed for the presence of genomic DNA.

### 4.5.3  Analysis of tissue samples

#### 4.5.3.1  Virus detection and quantification in ferret tissues by plaque assay

This assay was also established previously and routinely used for titration of virus harvests. Positive and negative controls gave the expected results (Figure 13). During the setup of assays analyzing tissue samples from animals euthanized at D7, the viscosity of tissue homogenates presented a problem. Tissue debris made it impossible to collect the correct amounts required for evaluation of neat tissue homogenates. 1 : 10 dilutions were set up instead. For samples gathered on subsequent time points, the homogenates were spun down before removing samples for both the neat sample and the 1:10 dilution assays. For D7 samples, strong responses were observed for Peyer's patches and mesenteric lymph nodes in all samples from animals in the rCDV backbone group. Plaques were also observed in the same tissue samples from the rCDV-SIVgag group, although at a significantly lower level. No plaques were noted in any of the samples from nasal turbinates, or any samples from mock-treated animals.  Zero plaques were observed for D14, D21 and D28 samples. It is unclear why no plaques were observed in tissue samples that contained as many as ten million CDV genome copies per gram tissue. A few different mechanisms are possible. Not every copy of viral genomic information will eventually mature into a fully infectious and functioning viral particle. The recombinant virus may be replication competent but the newly produced particles not as infectious. Cell-associated virus could also have been removed from the assay in the additional centrifugation step prior to plaque assays.
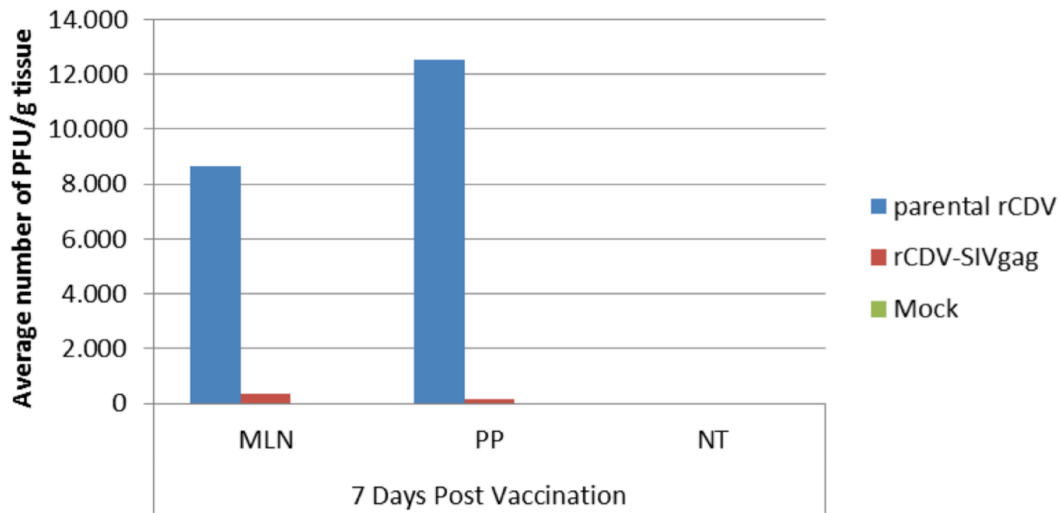
**Figure 13: Virus infectivity titration in ferret tissues.** Results of plaque assays using processed samples of mesenteric lymph nodes (MLN), Peyer's patches (PP), and nasal turbinates (NT). Infectious virus was only detected in tissues collected seven days post vaccination. Blue columns empty vector group, red columns vector including insert group. No plaques were detected for the control group. Average plaque forming units (PFU) were generated from all ferrets in each treatment group. No infectious virus was detected in Nasal turbinates at D7 p.i. in any animal, but at both sites distant from the inoculation site. This suggests the ability of both inoculates to spread and replicate in the ferret body. Samples from animals inoculated with empty vector produced significantly more plaques than those from animals inoculated with vector including insert. This would suggest that, while both inoculates are able to replicate and produce infectious particles distant from the site of inoculation, empty vector seems to replicate more productively.

### 4.5.3.2 Detection of viral genomic material in tissue samples using quantitative real time – polymerase chain reaction

qRT-PCR on nasal turbinates, Peyer's patches and mesenteric lymph nodes was performed to detect genomic copies of CDV-N in both the rCDV vaccinated as well as the rCDV-SIVgag vaccinated animals (Figure 14 and Figure 15). Genomic RNA levels of CDV-N remained above the detection limit until D21 for nasal turbinates, and throughout the study right up until D28 for Peyer's patches and mesenteric lymph nodes for both rCDV and rCDV-SIVgag samples. Peyer's patches and mesenteric lymph nodes consistently presented higher copy numbers than nasal turbinates, suggesting the intestinal tract as primary site of amplification of the recombinant virus. Genomic RNA levels of CDV-N were about 2 log higher in the rCDV

than in the rCDV-SIVgag group. The peak of virus load was at D7 for all samples, then gradually reducing up until D28. A slight upward trend was noted in D21 samples.



| | Nasal Turbs | Peyer's P | Mes Lym Nod |
|---|---|---|---|
| Day 7 | 1.83E+07 | 3.38E+09 | 9.77E+08 |
| Day 14 | 4.80E+03 | 3.52E+06 | 1.96E+07 |
| Day 21 | 3.80E+04 | 5.78E+06 | 2.49E+07 |
| Day 28 | 8.18E+03 | 2.73E+05 | 1.39E+06 |
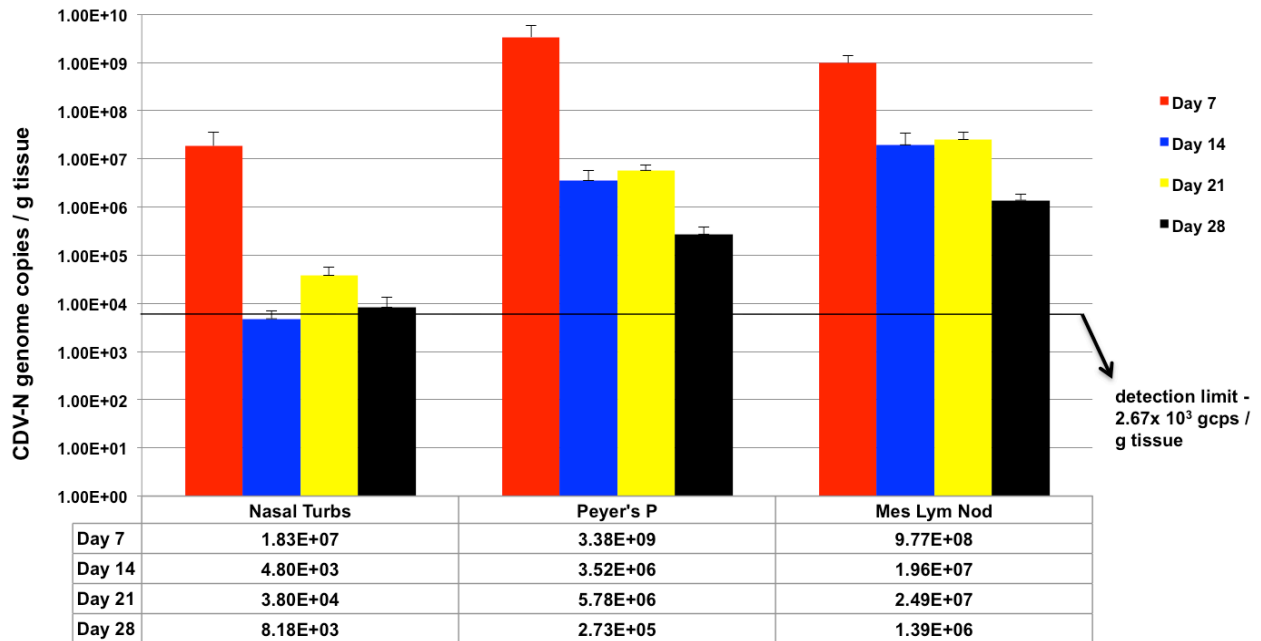
**Figure 14: Numbers of genomic copies of distemper N protein RNA in processed tissue samples from ferrets infected with empty distemper vector.** Samples were taken at weekly intervals (red columns d7, blue columns d14, yellow columns d21, black columns d28). Numbers detected in samples from individual animals were averaged. Genomic copies of N protein RNA were detected in all three tissue types to be analyzed. Numbers were highest for all three tissues at d7, indicating the empty vector spreads and replicates most actively around this time. While numbers in nasal turbinates then dropped, numbers in Peyer`s patches and mesenteric lymph nodes remained several orders of magnitude higher, suggesting a resident replicating virus population at these sites.

| | Nasal Turbs | Peyer's P | Mes Lym Nod |
|---|---|---|---|
| Day 7 | 4.44E+04 | 8.05E+05 | 1.05E+07 |
| Day 14 | 9.33E+03 | 5.47E+05 | 4.59E+06 |
| Day 21 | 2.67E+03 | 9.16E+05 | 3.38E+06 |
| Day 28 | 2.67E+03 | 1.26E+05 | 3.77E+04 |
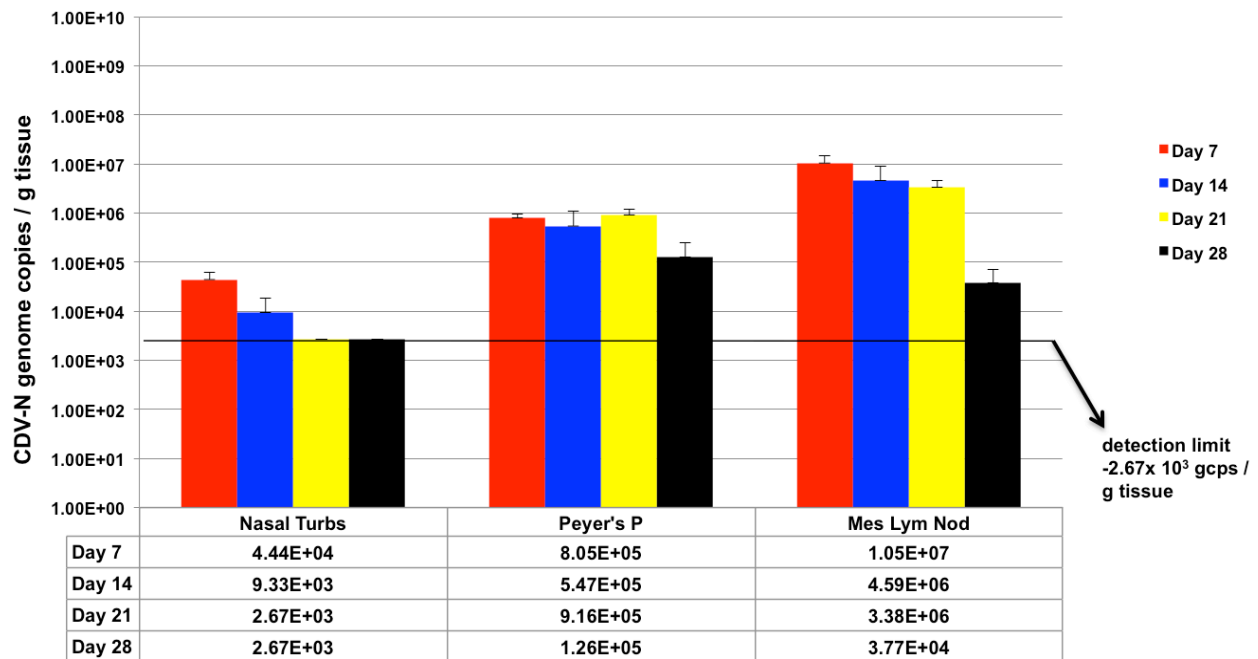
**Figure 15: Numbers of genomic copies of distemper N protein RNA in processed tissue samples from ferrets infected with distemper vector with glykosaminoglykane insert.** Samples were taken at weekly intervals (red columns d7, blue columns d14, yellow columns d21, black columns d28). Numbers detected in samples from individual animals were averaged. Genomic copies of N protein RNA were detected in all three tissue types to be analyzed. Numbers were highest for all three tissues at d7, indicating the vector including insert spreads and replicates most actively around this time. While numbers in nasal turbinates then dropped, numbers in Peyer`s patches and mesenteric lymph nodes remained significantly higher, suggesting a resident replicating virus population at these sites. This follows the pattern determined for the empty distemper vector, indicating that the insertion of glucosaminoglycane does not affect vector viability.

### 4.5.3.3 Detection of genomic copies of simian immunodeficiency virus glycosaminoglycan protein in tissue samples using quantitative real time – polymerase chain reaction

Detection of genomic copies of SIVgag was performed in parallel to detection of CDV-N to demonstrate that the insert was propagated as well (Figure 16). All tissues that were positive for CDV-N were also positive for SIVgag. Graph profiles of copy numbers of SIVgag over the course of time mirrored those of CDV-N. The copy numbers of SIVgag detected in animals vaccinated with rCDV-SIVgag were around 0.5 - 1.0 log lower than the copy numbers of CDV-N in the same animals. This difference could potentially be attributed to the fact that these samples were freeze/thawed several times as multiple assays have been performed and

could reflect degradation of RNA.  The copy numbers of SIVgag detected in Peyer's patches

and mesenteric lymph nodes were above the detection limit until the end of the study (D28),

as was the case for CDV-N.



| | Nasal Turbs | Peyer's P | Mes Lym Nod |
|---|---|---|---|
| Day 7 | 5.88E+03 | 9.62E+04 | 6.62E+05 |
| Day 14 | 2.67E+03 | 1.23E+04 | 6.78E+04 |
| Day 21 | 2.67E+03 | 3.81E+04 | 4.57E+04 |
| Day 28 | 3.31E+03 | 8.88E+03 | 2.56E+04 |
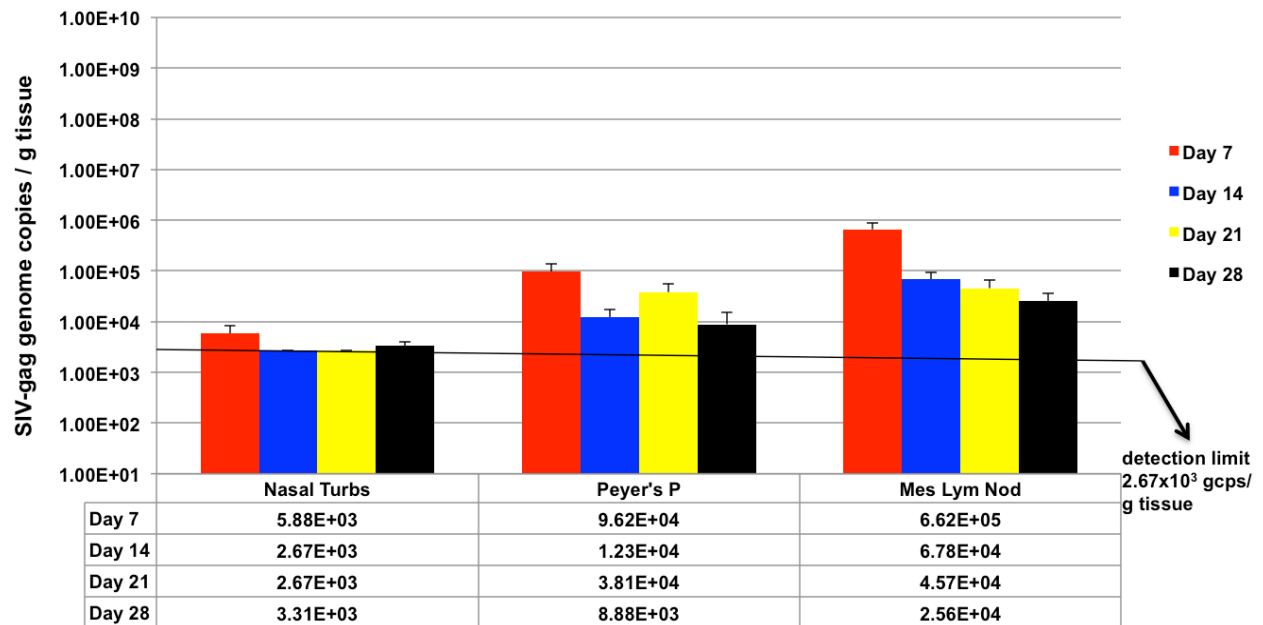
**Figure 16: Numbers of genomic copies of Simian immunodeficiency virus glucosaminoglycane protein RNA in processed tissue samples from ferrets infected with distemper vector with glykosaminoglykane insert.** Samples were taken at weekly intervals (red columns d7, blue columns d14, yellow columns d21, black columns d28). Numbers detected in samples from individual animals were averaged. Genomic copies of glucosaminoglycane RNA were detected in all three tissue types to be analyzed. Numbers were highest for all three tissues at d7, indicating insert is multiplied most actively around this time. While numbers in nasal turbinates then dropped, numbers in Peyer`s patches and mesenteric lymph nodes remained significantly higher, suggesting multiplication of the insert genome at these sites. This follows the pattern determined for the vector backbone, indicating that the insert is conserved during virus spread and replication.

### 4.5.4   Lymphocyte and splenocyte assays

#### 4.5.4.1   Determining cellular responses to simian immunodeficiency virus glycosaminoglycan protein in ferret peripheral blood mononuclear cells using intracellular interferone gamma staining

Proliferation of PBMCs in response to specific peptides or peptide pools is often used as a

measure to determine the extent of cellular immunity against those peptides. PBMCs

isolated from all groups during terminal blood draws were treated with the polyclonal stimulant PMA/Ionomycin, SIVgag peptide pool, or mock treated, and then incubated for 9 h. Cells were subsequently stained with antibodies against IFNg, Ki67 (marker of cell proliferation), and CD8 (Figure 17, Figure 18, Figure 19 and Figure 20). There was a small but significant increase in the percentage of cells from rCDV-SIVgag immunized animals that secreted IFNg after stimulation with the gag peptide pool compared to the same cells without stimulation, and compared to cells from mock animals stimulated with SIVgag. The increase in cell numbers was in the range of 0.2 - 0.4 %. There was an unpredicted increase in Ki67 intensity observed in cells from D28 samples of the parental rCDV group stimulated with SIVgag. This response could possibly have been due to a generalized immune activation at this time point, resulting from the booster vaccinations on D21. This data set gives an indication of a gag specific cellular immune response.
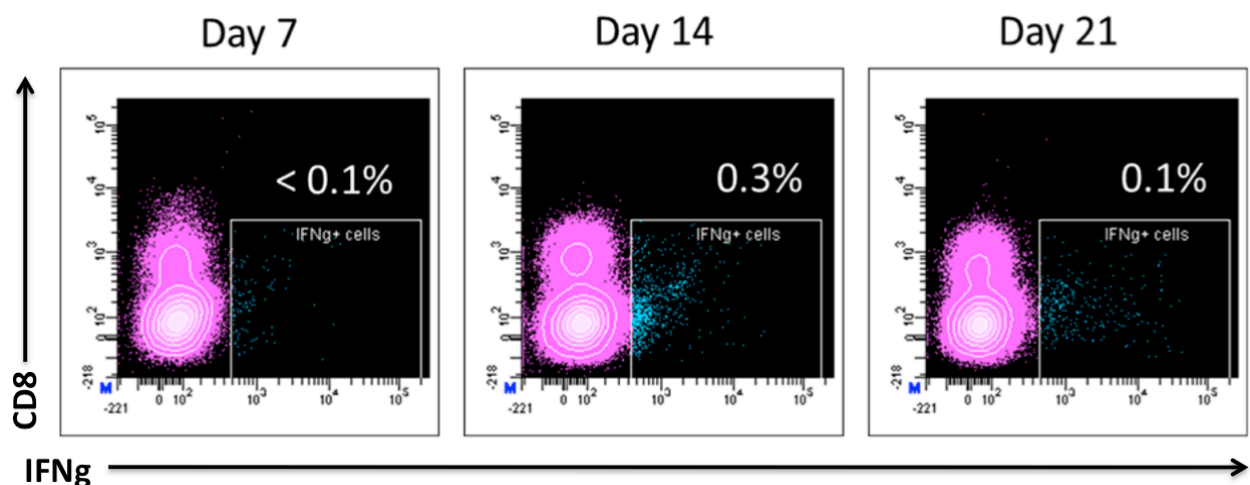


**Figure 17: Flow cytometry of peripheral blood mononuclear cell samples from animals inoculated with vector including glucosaminoglycane insert. Shown are scatter plots of specific cellular immune responses after stimulation with a simian immunodeficiency virus glucosaminoglycane peptide pool.** Of interest was the frequency of interferone gamma positive cells within the CD8 positive population at three different time points (D7, D14, D21). Note the increase of interferone positive cells on d14, with counts on d21 still remaining above those for d7. This suggests that antigen specific CD8 positive cells against simian immunodeficiency virus glucosaminoglycane peptides are being produced and responding to antigen stimulation by d14 post inoculation.
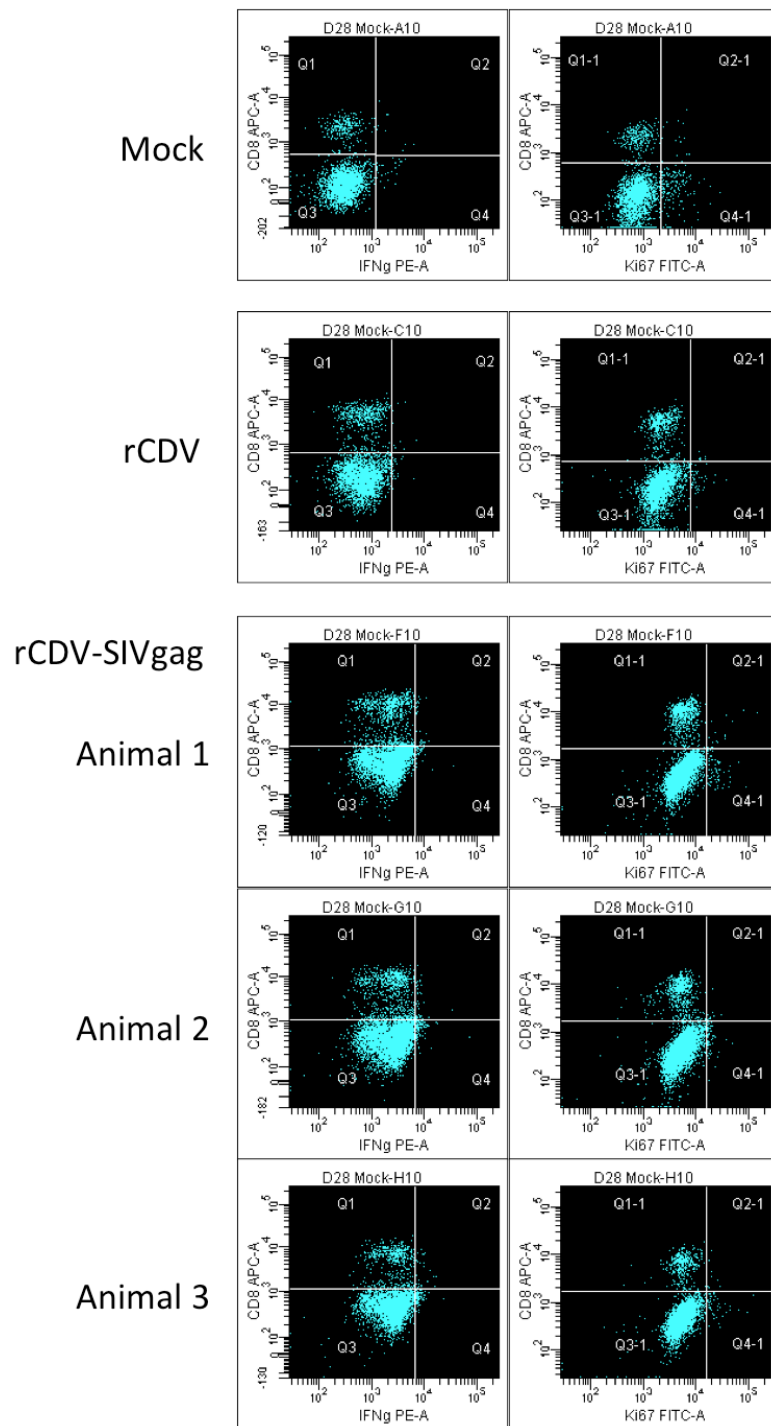
**Figure 18: Flow cytometry results of peripheral blood mononuclear cells from animals sacrificed at D28, mock stimulated only**. Cells were stained for CD8 and either interferone gamma, evidencing antigen specific activation (left column) or KI67, indicating unspecific cell proliferation only (right column). Order of plots from top to bottom: control group (pooled), empty vector (pooled), vector including insert (individual animals). Note that mock stimulation does not result in significant numbers of cells in the right half of the plots, indicating that neither specific nor unspecific stimulation have occurred.
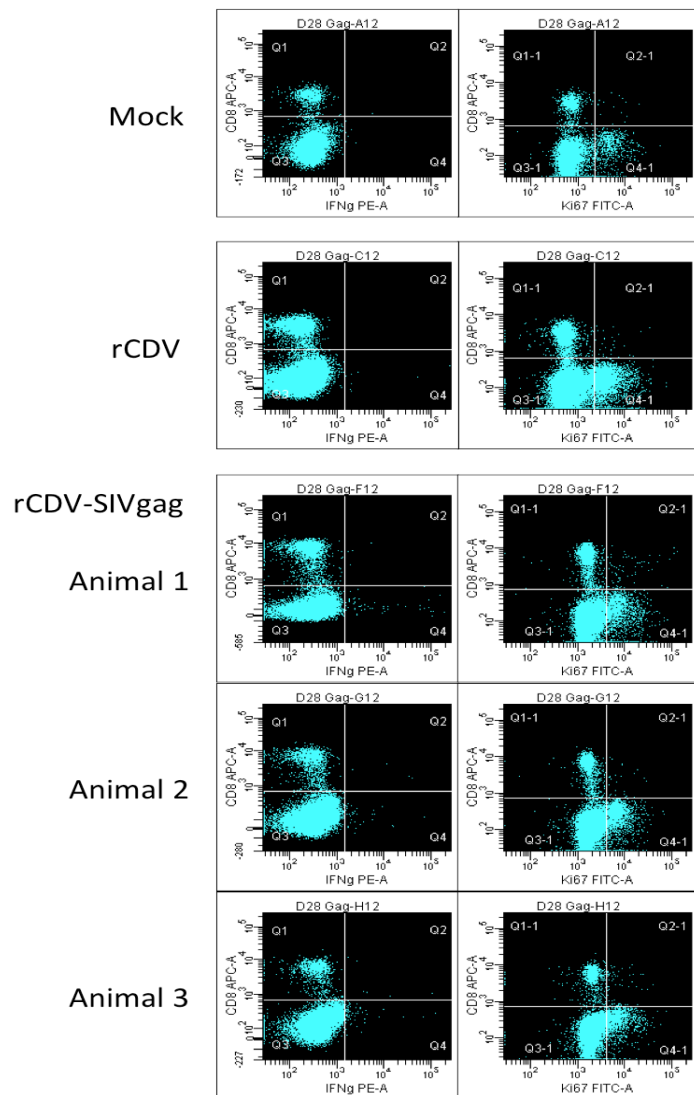
**Figure 19: Flow cytometry results of Peripheral blood mononuclear cells from animals sacrificed at D28, stimulated with simian immunodeficiency virus glucosaminoglycane peptides**. Cells were stained for CD8 and either interferone gamma, evidencing antigen specific activation (left column) or KI67, indicating unspecific cell proliferation only (right column). Order of plots from top to bottom: control group (pooled), empty vector (pooled), vector including insert (individual animals). Note that all samples stained for Ki67 (right column) show an increase of cell frequency in the right half of the plots, indicating the cells were able to proliferate when faced with foreign peptides. Note that this increase is significant in all samples from the vector including insert group, but also in the sample from the empty vector group. This may be caused by generalized activation of the immune system due to the booster inoculations the animals had received on d21. Also note in the left column the small increase in frequency of cells in the right half of plots for all three animals in the vector including insert group. This demonstrates that upon stimulation with glucosaminoglycane, these cells become increasingly positive for interferone gamma. The increase in frequency was between 0.2 and 0.4% in these samples. This suggests that at d28, cells from these animals are able to mount an antigen specific response to glucosaminoglycane peptide exposure.
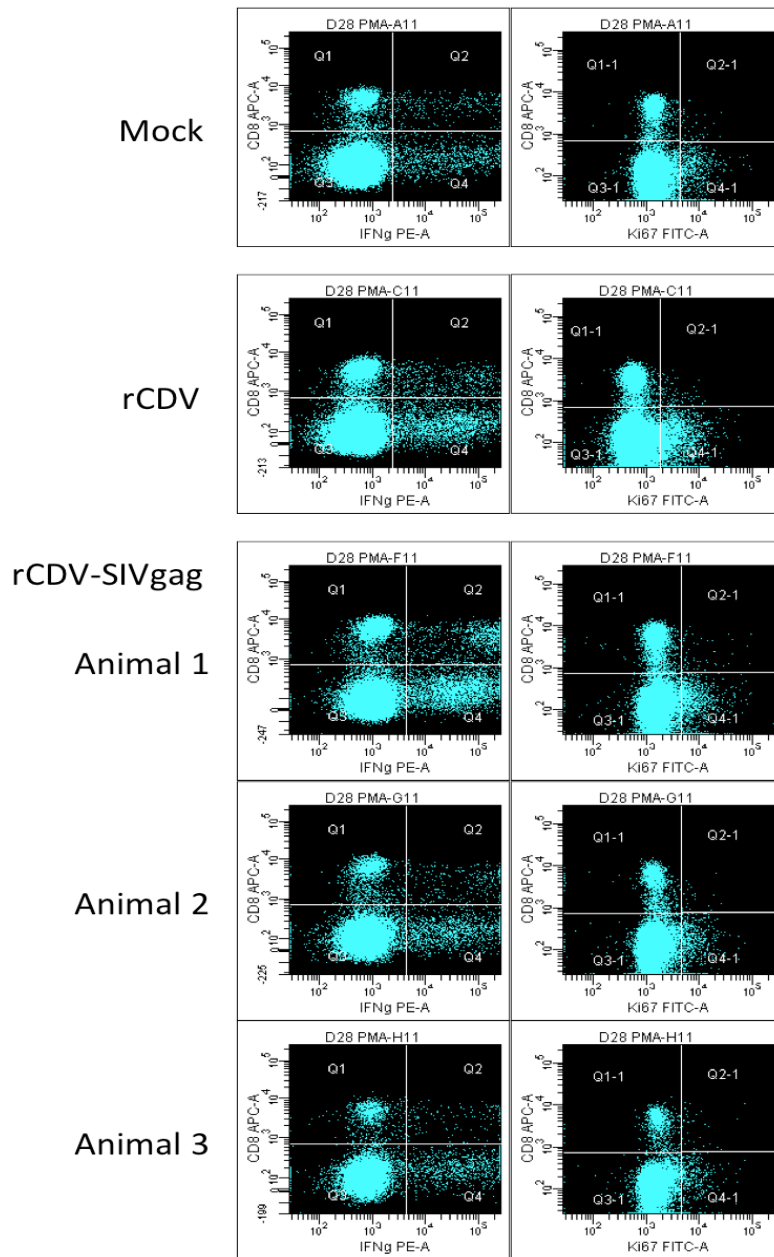
**Figure 20: Flow cytometry results of peripheral blood mononuclear cells from animals sacrificed at D28, stimulated with a polyclonal stimulant (PMA)**. Cells were stained for CD8 and either interferone gamma, evidencing antigen specific activation (left column) or KI67, indicating unspecific cell proliferation only (right column). Order of plots from top to bottom: control group (pooled), empty vector (pooled), vector including insert (individual animals). Note that in all samples, the frequency of cells in the right half of the plots increases dramatically. This indicates that the cells used in this assay were viable and able to respond to stimulation.

### 4.5.5 Analysis of serum samples

#### 4.5.5.1 Detection of neutralizing antibodies in serum samples collected at euthanasia using virus neutralization tests

To evaluate serum samples for the presence of neutralizing antibodies, a virus neutralization (VN) test was employed. To facilitate assay readout, a recombinant CDV expressing enhanced green fluorescent protein (rCDV-eGFP) virus previously established and generously provided by Jason Zhang (IAVI) was used. All samples were set up in quadruplicates of serial 2-fold dilutions. rCDV-eGFP virus grew slower than parental rCDV, so assays were incubated for 5 d before being analyzed. The lowest dilution at which none of the quadruplicate wells showed any more fluorescence upon microscopic examination was taken as the neutralizing antibody titer (Table 6 and Figure 21). The first neutralizing antibodies were detected in samples acquired on D14. On average, VN titers against CDV were 4-fold lower in serum of rCDV-SIVgag vaccinated ferrets than in that of parental rCDV ferrets at each time point. Both viruses however were shown to produce neutralizing antibodies against CDV.

| Time-point | Maximum serum dilution for VN | | |
|:---:|:---:|:---:|:---:|
| | Mock | rCDV | rCDV-SIVgag |
| | 0 | 0 | 0 |
| D7 | 0 | 0 | 0 |
| | 0 | 0 | 0 |
| | 0 | 64 | 16 |
| D14 | 0 | 64 | 16 |
| | 0 | 64 | 16 |
| | 0 | 256 | 64 |
| D21 | 0 | 256 | 64 |
| | 0 | 256 | 64 |
| | 0 | 256 | 256 |
| D28 | 0 | 256 | 64 |
| | 0 | 1024 | 64 |

**Table 6: Detection and titration of neutralizing antibodies against distemper in ferret serum samples**. Serum samples taken at the four euthanasia time points from the animals within each group were analyzed for their ability to neutralize distemper virus. Samples were set up in twofold dilutions for titration. The reciprocal highest dilution at which virus neutralization was achieved is given. No neutralization was achieved in the control group (second column). Neutralizing antibodies were present in all animals in both the empty vector group (third column) and the vector plus insert group (fourth column) from d14 onwards. Titers increased further for both groups by d21, and then held steady or increased by the end of the study. Titers in the vector including insert group ranged two dilution levels below those for the empty vector group, but mirrored their development. This suggests that both empty vector and vector including insert lead to the formation of neutralizing antibodies against the vector backbone within 14d post inoculation.
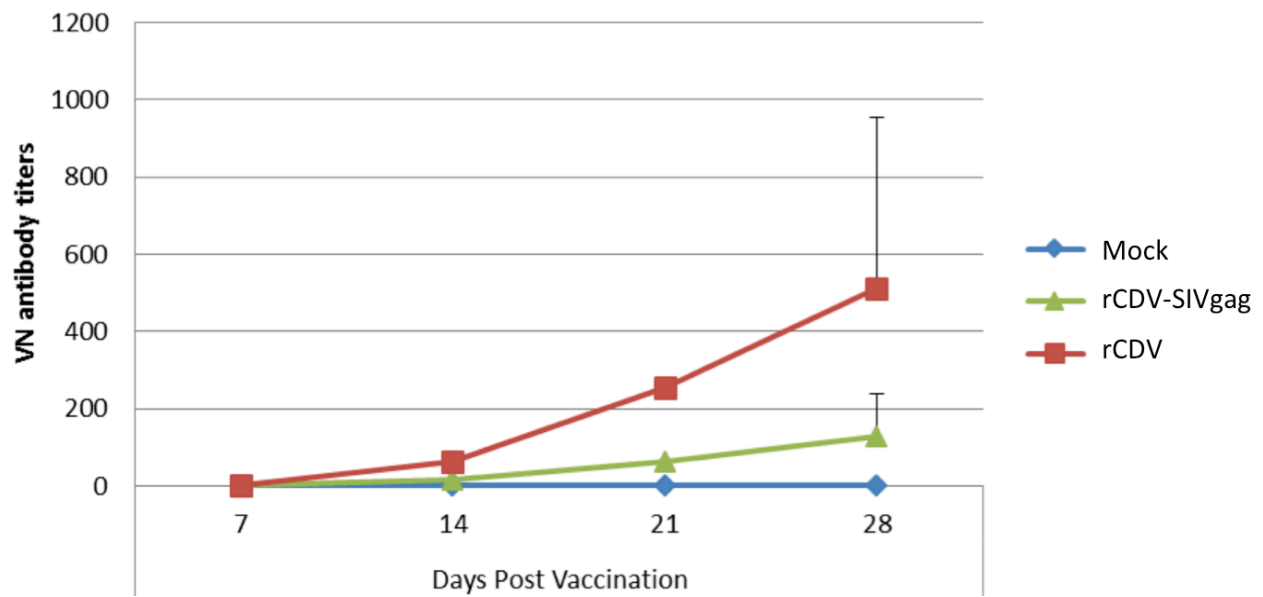
**Figure 21: Average distemper virus neutralizing antibody responses at 7, 14, 21, and 28 days post vaccination.** Titers determined in Table 6 above are averaged within groups, and averages plotted. Blue line symbolizes control group, red line empty vector group and green line vector plus insert group. Again it can be noted that both in empty vector and the vector plus insert groups, neutralizing antibodies against the distemper backbone are present from d14 onwards and continue to increase until the end of the study.

### 4.5.5.2 Detection of viral genomic material in serum samples using quantitative real time – polymerase chain reaction

Serum samples from all animals collected during terminal blood draws were evaluated for the presence of viral genome using qRT-PCR for CDV-N (Figure 22). 2 of 3 animals vaccinated with rCDV and one animal vaccinated with rCDV-gag produced detectable viral copy numbers on D21 of the study. One animal in each group had detectable genomic DNA on D28. Copy numbers detected were 2 to 16x the detection limit for the assay. No genomic copy levels above the detection limit were found at any of the earlier time points. These data suggest that at least in part of the animals, vaccination with both rCDV as well as rCDV-gag results in mild to moderate viremia at those two timepoints after primary vaccination.
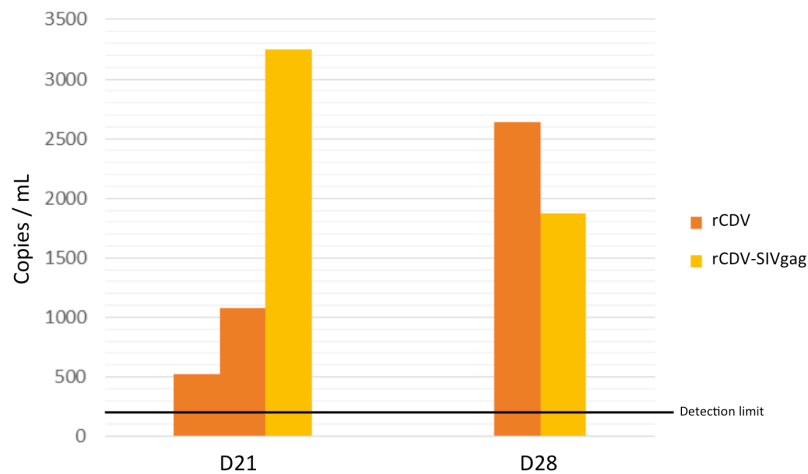
**Figure 22: Numbers of genomic copies of distemper N protein RNA in serum samples taken during euthanasia.** Samples were taken at all four euthanasia time points, but on d7 and d14 none of the samples contained RNA copy numbers above the detection limit. They are therefore not plotted. On D21 the sera of two empty vector (orange columns) and one vector including insert (yellow columns) inoculated animals were positive for viral genomic RNA. On D28 only one animal from each group tested positive. This suggests that during these two timepoints, a mild to moderate viremia is present in at least part of the animals.

### 4.5.5.3 Detection of antibodies against simian immunodeficiency virus glycosaminoglycan protein in serum samples using western blot

We were interested to find out if the animals had been able to develop antibodies recognizing the SIVgag protein (also referred to as p55), whose gene was inserted into the rCDV genome. In the end, development of such antibodies would be a hallmark of the success of this study. One would expect the mock and rCDV inoculated animals to have no antibodies recognizing the gag component p55. The rCDV-SIVgag inoculated animals on the other hand should, if they had mounted an immune response to SIVgag, possess antibodies recognizing p55. Since analysis by ELISA proved intractable (see chapter on ELISA development), we opted for a Western Blot setting. Recombinant p55 was purchased and used as described in the materials and methods section. To understand at which point in time the animals had sufficient antibodies in their sera, samples from all animals sacrificed on D7, 14, 21 and 28 were tested for their ability to detect the 55 kDa SIVgag protein (Figure 23).
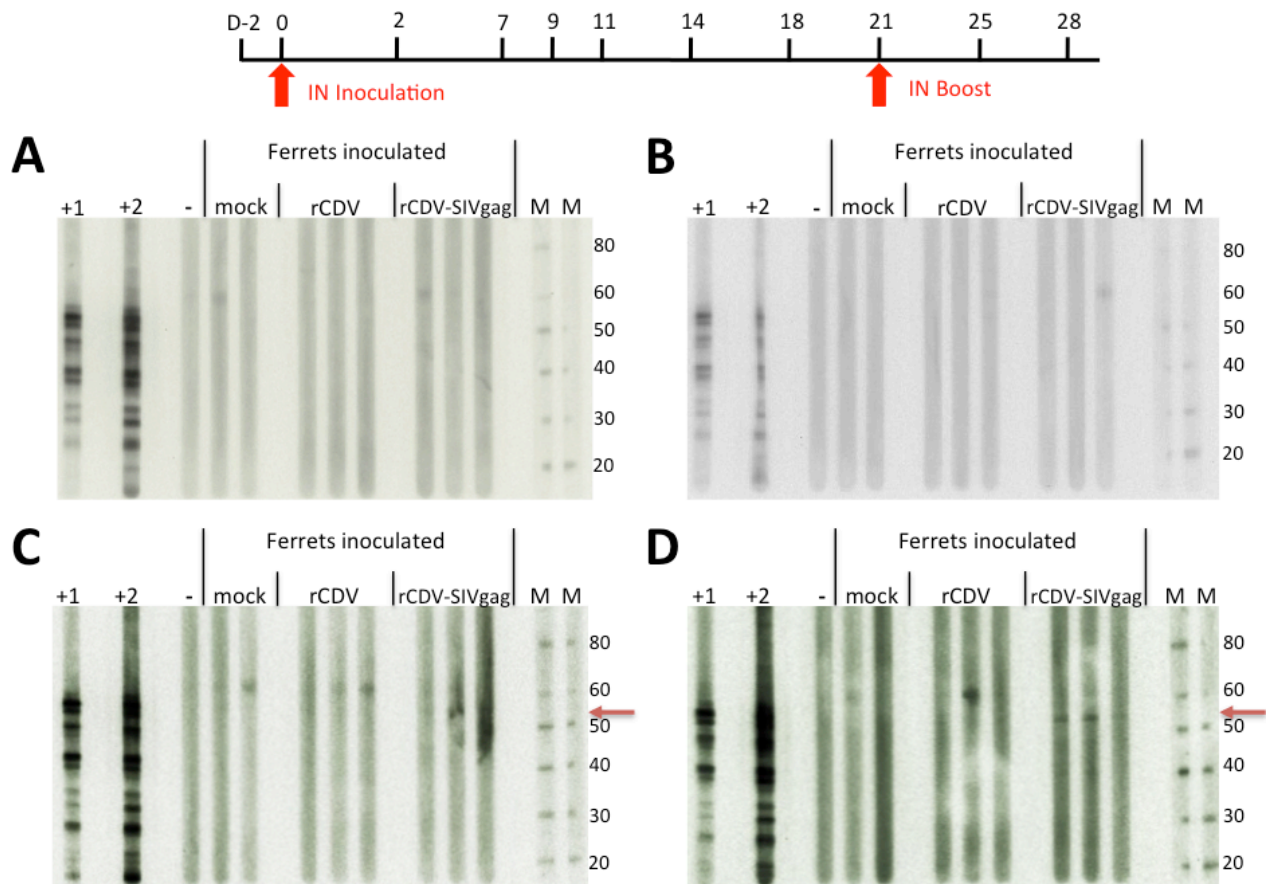
**Figure 23: Western blot of serum samples to assess presence of antibodies able to bind to simian immunodeficiency virus glucosaminoglycane.** A review of the study timeline with inoculation time points at d0 and d21 can be found across the top. Sera were obtained on **A**: D7, **B**: D14, **C**: D21 and **D**: D28 p.i. and tested for their ability to detect p55. Two gag-positive nonhuman primate sera stemming from an SIV infected animal were used as positive controls, giving rise to very strong responses (lanes +1 and +2). Serum of a 7 month-old naïve ferret (lane -) was used as a negative control. Subsequent lanes represent serum samples from two control animals (labelled mock), three animals inoculated with empty vector (labelled rCDV) and three animals inoculated with vector including insert (labelled rCDV-SIVgag). M: MagicMark protein standard, molecular weights given in kDa. Red arrows indicate running height of Simian immunodeficiency virus glucosaminoglycane. Note the darkened lines at the height of the red arrow in all three lanes labelled rCDv-SIVgag in blot D. There is also a slight darkening at the same position for the same lanes in blot C. This indicates that antibodies able to bind simian immunodeficiency virus glucosaminoglycane are present in serum samples from animals inoculated with vector including insert at d28, and likely as early as d21.

As expected the positive non-human primate sera reacted strongly with p55. Laddering in those samples could indicate protein degradation of the commercial sample or previous exposure to other peptides as part of their vaccination protocol. The naïve ferret (negative

control) as well as the mock treated and rCDV inoculated animals showed no response to the antigen at any time point. The mock treated and rCDV inoculated animals served as a control for specificity of p55 detection and possible cross-contamination with rCDV-SIVgag during the study. Bands detected at 60 kDa are likely serum albumin. Analysis of the rCDV-SIVgag inoculated animal sera showed that there are no antibodies against p55 on D7 and D14. Results for D21 varied and they were sometimes positive, sometimes not. This may indicate that the antibody titers are right around the detection limit. On D28 however these animals were all clearly positive for p55 antibodies. It is not clear whether the additional time or booster contributed to this result, but it is likely a combination of both.

To confirm the data, the western blots were repeated using the rCDV-SIVgag inoculated animal sera (Figure 24).
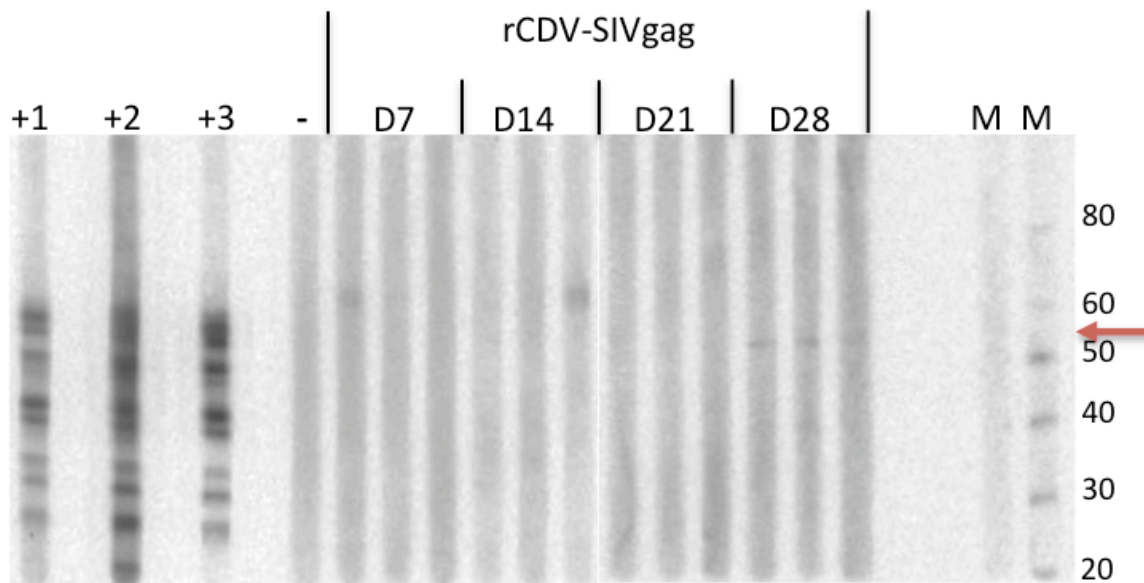
**Figure 24: Western blot of serum samples to assess presence of antibodies able to bind to simian immunodeficiency virus glucosaminoglycane.** The sera from all twelve animals inoculated with vector including insert were tested again for their ability to recognize **simian immunodeficiency virus glucosaminoglycane**. Two glucosaminoglycane positive nonhuman primate sera from simian immunodeficiency virus infected animals and a rabbit polyclonal antibody recognizing the glucosaminoglycane were used as positive controls (lanes +1, +2 and +3, respectively). Serum of a 7 month-old naïve ferret (lane -) was used as a negative control. Three samples each obtained at d7, d14, d21 and d28 are then plotted in sequence and labelled accordingly. M: MagicMark protein standard, molecular weights given in kDa. Red arrow indicates running height of glucosaminoglycane. Note again the darkened lines at level of the red arrow for the d28 samples, as well as the faint darkening at the same level for samples obtained at d21. This indicates that antibodies able to bind simian immunodeficiency virus glucosaminoglycane are present in serum samples from animals inoculated with vector including insert at d28, and likely as early as d21.

In this repeat assay it could again be shown that a robust (with respect to this assay) antibody titer is present after D21 (in the D28 samples). It could therefore be demonstrated that by D28, antibodies reactive to SIVgag p55 are present in ferret serum when immunized intranasally with rCDV-SIVgag.

## 4.6 Development of enzyme-linked immunosorbent assays for the detection and quantification of antibodies in ferret blood samples

To be able to quantify the humoral immune responses developed over the course of the studies conducted, we attempted to establish ELISAs against a set of relevant polypeptides.

As described previously (281), the proteins most interesting with regards to development of antibodies against CDV are the nucleoprotein (N protein, N) and hemaglutinin (H protein, H). The nucleoprotein is the most abundantly expressed of all CDV proteins, and the majority of antibodies produced during clinical CDV infection are directed against N. These antibodies, while very useful for the purpose of diagnosing exposure to CDV, have very little value with regards to combating the infection. Antibodies directed against the H protein carry the strongest capability to neutralize virus particles. The presence and level of these antibodies therefor is indicative of the mounting of an effective immune response.

The studies presented in this thesis employ a CDV backbone including the genetic sequence of the SIVgag protein. This protein is the most immunogenic of all SIV proteins, and is hence commonly used for the diagnosis and qualification of immune responses to an SIV infection. During our studies, one aim of our research was the detection and quantification of humoral responses directed against both the CDV proteins as well as the SIVgag insert.

Detection of CDV antibodies is a very well established method for use with canine samples (278) and in-house test kits are commercially available. Literature on the use of this assay in ferrets and related species is very limited (278, 282). The assays described previously use either whole virus lysate (282) or lysate of recombinant baculovirus infected Heliothis virescens larvae or Sf9 insect cell culture (278). In order to be able to semi-quantify the

overall level of antibody production, as well as the amount of potentially neutralizing antibodies produced, we decided to aim to assess antibody levels to the CDV N and CDV H proteins separately. Both the use of virus culture lysate as well as insect cell or larvae lysate as antigens for an ELISA will result in a vast array of CDV- and non-CDV proteins present for antibody-binding, giving a high chance of unspecific binding and possibly false-positive results. To reduce this risk, we attempted to establish ELISA assays using purified recombinant N and H proteins as antigens. Detailed information on the production, purification as well as confirmation of identity of these proteins can be found in the materials and methods section, 3.4.12 and 3.4.13. ELISA assays using gag p55 as an antigen to assess gag specific antibody levels have previously been established in our lab for the main laboratory animal species. Similar protocols have also been published elsewhere (283). We therefor attempted to modify existing protocols to create a stable assay using ferret blood samples.

The data presented in this chapter are a selection of examples of collected data only. Since we tested a wide variety of different conditions, and each condition was repeated several times, in identical settings or with minor modifications, the presentation of the complete data would by far exceed the limits of this thesis. Since our attempts to create functioning ELISA assays using ferret blood samples were not successful, the purpose of this chapter is not to present data collected during such assays, but instead to detail and exemplify any materials and conditions tested during the development phase.

### 4.6.1  Basic assay setup

Since purified denatured protein was used as an antigen for assay development, an indirect ELISA was considered the most useful method for the development of the CDV ELISA (Figure

25). The SIVgag ELISA protocol already in use in our lab was also based on this principle. Briefly, the antigen, dissolved in a buffer solution, is coated directly onto the peptide-binding surface of the wells of ELISA plates. After an incubation period, the plates are washed to remove excess antigen. The sample sera as well as positive and negative controls are then added to the wells in serial dilutions. Incubation and wash steps are repeated. Commercially available, fluorochrome-labelled secondary antibodies, directed against one or more immunoglobulin (Ig) classes of the test species, are then added to all wells at a constant concentration. After incubation, the plates are again washed, and fluorochrome-specific substrate is added, which will cause development of fluorochrome-specific colour, dependent on the amount of primary and therefore secondary antibody bound to the antigen. The colour intensity is detected using a plate reader and converted into optical density (OD) units, which can then be used to plot the results of the assay and determine antibody titers.
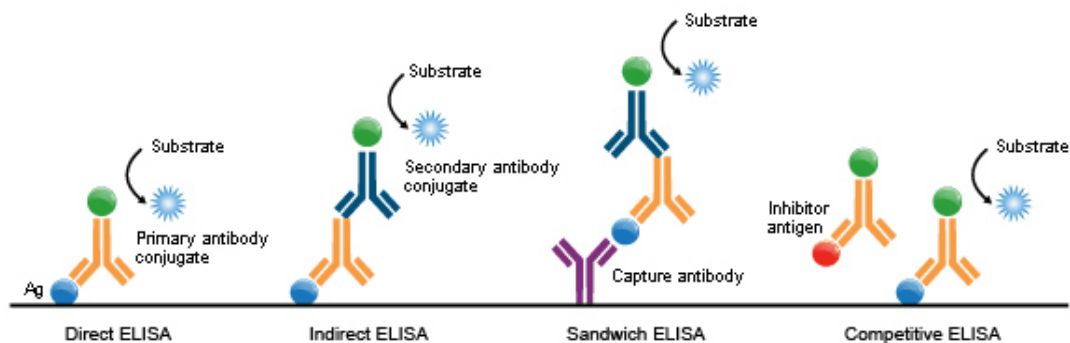


**Figure 25: Schematic representation of the most common types of ELISA.** An indirect ELISA was used in this study.

### 4.6.2 Development of an enzyme linked immunosorbent assay to detect and quantify antibodies against distemper N protein in serum samples

#### 4.6.2.1 Antigen production

We produced purified proteins for use as antigen to minimize the likelihood of unspecific and false-positive binding. We chose *E. coli* BL21-DE3 cells as an expression system since it is a fast, easy and reliable expression system and has previously been used successfully for paramyxovirus N proteins (279, 280) as well as for several other proteins within our lab. Expression in eukaryotic cells in order to stay close to the natural mechanism of N protein production was attempted, however, the protein proved to be severely toxic to eukaryotic cell lines. We initially attempted to express the protein in its native form. This however resulted in consistent precipitation of unpurified product, due to the large size of the protein (58 kDa + 12 kDa His$_6$-tag), and the fact that *in vivo* N protein will self-aggregate to form the nucleocapsid complex with other CDV proteins. To circumvent this issue, we decided to overexpress the protein in cytoplasmic inclusion bodies, from where it can then be purified. In the process of cell lysis and protein purification, the inclusion bodies are dissolved through the use of buffers containing 8 M Urea. High urea content denatures and linearizes the protein, hence maintaining it in solution. Steps to refold the protein into its natural structure were considered, but since these processes generally produce a very low yield, and linearized protein will likely result in making smaller epitopes easily accessible for antibody binding, this step was not taken.

*Influence of different plates*

Based on the large size of the N protein and the presence of numerous primary amines in its sequence, two plates where chosen for comparison. Corning Costar 3690 High-binding 96well clear polystyrene plates are designed to bind proteins larger than 10 kDa possessing ionic and/or hydrophobic groups. This plate was routinely used for ELISAs during other projects in our lab. Pierce Maleic Anhydride Activated clear 96well plates are coated with maleic anhydride, a compound which covalently binds amine groups in proteins and peptides at pH > 7. In repeat side by side comparisons running exactly the same assay protocol using the same reagents and buffers on both plates, Costar plates consistently produced higher background optical densities (ODs) for negative and stage controls, as well as a less clear distinction between positive and negative samples (Table 7 and Table 8). Nunc Maxisorp (Sigma-Aldrich) plates were also tried but gave results very similar to Costar plates. Maleic Anhydride plates therefor became the preferred choice for N protein ELISAs, although availability and cost occasionally were an issue.

| | 1 | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 | 1:512 | 1:1024 | Blank |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Serum 1 (1) | 0,907 | 0,764 | 0,554 | 0,391 | 0,346 | 0,306 | 0,292 | 0,288 | 0,282 | 0,274 | 0,3 | 0,279 |
| Serum 1 (2) | 1,091 | 0,779 | 0,567 | 0,414 | 0,341 | 0,306 | 0,31 | 0,286 | 0,253 | 0,278 | 0,28 | 0,31 |
| Serum 2 (1) | 0,904 | 0,64 | 0,494 | 0,383 | 0,345 | 0,292 | 0,289 | 0,268 | 0,253 | 0,274 | 0,29 | 0,306 |
| Serum 2 (2) | 0,789 | 0,668 | 0,472 | 0,371 | 0,326 | 0,322 | 0,291 | 0,281 | 0,287 | 0,277 | 0,3 | 0,3 |

**Table 7: Raw data of an enzyme-linked immunosorbant assay on a Maleic Anhydride plate.** N protein is coated at constant dilution across the plate serving as the antigen, duplicates of 2 sera (rows A and B, and C and D, respectively), are diluted 2-fold across the plate, column 12 receives no serum and serves as background control. Secondary antibody is added to all wells at constant dilution. Data are given as optical density readouts. Values of a maximum of 1,1 are generally regarded as rather low readings.

| | 1 | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 | 1:512 | 1:1024 | Blank |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Serum 1 (1) | 1,333 | 1,195 | 1,107 | 1,049 | 1,002 | 1,029 | 1,096 | 1,108 | 1,015 | 0,977 | 1,023 | 1,069 |
| Serum 1 (2) | 1,225 | 1,16 | 1,028 | 1,01 | 0,937 | 0,974 | 0,95 | 0,996 | 1,057 | 1,006 | 1,002 | 1,057 |
| Serum 2 (1) | 1,26 | 1,059 | 0,946 | 0,904 | 0,893 | 0,878 | 0,874 | 0,92 | 0,938 | 0,904 | 1,072 | 0,956 |
| Serum 2 (2) | 1,296 | 1,125 | 0,972 | 0,959 | 0,871 | 0,888 | 0,927 | 0,927 | 0,911 | 0,967 | 0,957 | 0,931 |

**Table 8: Raw data of an enzyme-linked immunosorbant assay on a Costar plate.** The same assay as above gave slightly higher overall readouts but around 3x higher background as evidenced by the values in the blank wells.

### 4.6.2.2 Confirmation of presence of antigen on the plate

During the initial stages of assay development, one priority was to confirm the presence of antigen on the plate after the coating process. This was done by several means to ensure correct function. First, the protein used for coating still contained a $His_6$-tag. So, the use of an anti-$His_4$ and species-appropriate secondary antibody, both at constant concentrations, posed an elegant way of confirming antigen presence. Second, we were able to obtain a large quantity of serum from one CDV vaccinated ferret, which tested positive for antibodies to our antigen preparation using western blot and dot blot techniques. Third, commercial bicinchoninic acid (BCA) assay kits (Pierce) were employed to detect the amount of protein present on the plates. Their functionality however was limited. Examples for the use of these methods can be found in the figures accompanying subsequent sections (for example Table 14 and Table 15). Finally, commercial monoclonal antibodies (mAbs) were available which claimed to detect CDV N protein (clone DV12-2, mouse anti-CDV N, Santa Cruz). Unfortunately, this antibody did not bind to the protein coated on the plate (Table 9). Before this, we had also attempted to use this mAB as a capture antibody, which was also

unsuccessful (4.6.5, Figure 70 and Table 17). Upon further research, we found that the same clone is marketed by other companies (for example Abd Serotec) as a general anti-CDV antibody, and as per their datasheets detects a variety of CDV proteins. This insufficient affinity and specificity caused us to abolish this mAB as a control in ELISA assays.

| | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1280 | 1:2560 | 1:5120 | 1:10240 | 1:20480 | blank |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| F pos HRP | 0,209 | 0,261 | 0,341 | 0,322 | 0,207 | 0,158 | 0,114 | 0,076 | 0,066 | 0,049 | 0,046 | 0,043 |
| | 0,234 | 0,311 | 0,351 | 0,29 | 0,224 | 0,176 | 0,126 | 0,088 | 0,069 | 0,061 | 0,053 | 0,053 |
| Mouse Anti N HRP | 0,052 | 0,046 | 0,044 | 0,045 | 0,045 | 0,054 | 0,048 | 0,046 | 0,048 | 0,062 | 0,047 | 0,045 |

**Table 9: Optical density readouts of enzyme-linked immunosorbant assay using monoclonal antibody DV12-2 (bottom) compared with blot-confirmed positive ferret serum (top) as primary antibodies.** Plate coated with N protein solution at a constant concentration, primaries were added in serial dilution as stated above, column 12 received no primary. Readouts of wells containing DV12-2 are near identical to readouts in the blank wells. This suggests that this antibody does not bind to N protein and cannot be used as a positive control.

### 4.6.2.3 Influence of different antigen preparation and coating conditions

Since it was feared that the high urea content in the antigen stock might interfere with coating, several levels of dialyzed protein stock with urea content between 800 mM and 8 M were produced for evaluation. Attempts to entirely remove the urea component resulted in complete precipitation of the protein. The stepwise addition of sodium-dodecyl-sulfate (SDS) to re-solubilize the protein was successful, however the protein appeared to break down when in SDS-containing buffer, and the coating efficiency decreased to below the level of urea-based buffers. The ratio of background to sample ODs was also higher in plates coated with antigen in SDS buffer compared with urea buffer (Table 10 and Table 11).

|  | 1 | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 | 1:512 | 1:1024 | Blank |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0,372 | 0,331 | 0,352 | 0,276 | 0,268 | 0,237 | 0,266 | 0,247 | 0,236 | 0,232 | 0,257 | 0,293 |
| 1:2 | 0,36 | 0,311 | 0,277 | 0,254 | 0,235 | 0,222 | 0,235 | 0,237 | 0,236 | 0,237 | 0,24 | 0,231 |
| 1:4 | 0,361 | 0,296 | 0,267 | 0,249 | 0,229 | 0,226 | 0,204 | 0,205 | 0,232 | 0,222 | 0,226 | 0,232 |
| 1:8 | 0,348 | 0,326 | 0,265 | 0,244 | 0,223 | 0,226 | 0,202 | 0,202 | 0,211 | 0,222 | 0,223 | 0,213 |
| 1:16 | 0,338 | 0,279 | 0,264 | 0,232 | 0,215 | 0,204 | 0,194 | 0,195 | 0,202 | 0,201 | 0,223 | 0,225 |
| 1:32 | 0,349 | 0,282 | 0,254 | 0,238 | 0,222 | 0,203 | 0,195 | 0,204 | 0,204 | 0,207 | 0,235 | 0,238 |
| 1:64 | 0,331 | 0,277 | 0,258 | 0,244 | 0,196 | 0,197 | 0,201 | 0,222 | 0,203 | 0,235 | 0,237 | 0,241 |
| Blank | 0,106 | 0,098 | 0,095 | 0,089 | 0,086 | 0,084 | 0,082 | 0,088 | 0,085 | 0,089 | 0,085 | 0,086 |

**Table 10: Optical density readouts of a checkerboard assay using N protein in sodiumdodecylsulfate buffer**. Antigen was coated in serial dilutions from row A-G. Row H received no antigen. Serum positive for antibodies to N protein (confirmed by western blot) was then added in serial dilutions across column 1-11. Column 12 received no serum. All wells received secondary antibody at a constant concentration. Overall readout values are low, and no definite titration can be seen down the columns.

|  | 1 | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 | 1:512 | 1:1024 | Blank |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0,676 | 0,561 | 0,52 | 0,435 | 0,411 | 0,355 | 0,346 | 0,343 | 0,328 | 0,337 | 0,327 | 0,351 |
| 1:2 | 0,597 | 0,58 | 0,557 | 0,526 | 0,491 | 0,459 | 0,398 | 0,419 | 0,423 | 0,423 | 0,431 | 0,432 |
| 1:4 | 0,526 | 0,514 | 0,502 | 0,455 | 0,435 | 0,421 | 0,382 | 0,377 | 0,366 | 0,343 | 0,347 | 0,333 |
| 1:8 | 0,51 | 0,487 | 0,42 | 0,403 | 0,375 | 0,37 | 0,35 | 0,324 | 0,319 | 0,316 | 0,31 | 0,298 |
| 1:16 | 0,493 | 0,454 | 0,375 | 0,326 | 0,304 | 0,262 | 0,255 | 0,232 | 0,274 | 0,276 | 0,251 | 0,252 |
| 1:32 | 0,472 | 0,363 | 0,325 | 0,284 | 0,271 | 0,23 | 0,217 | 0,225 | 0,227 | 0,224 | 0,23 | 0,235 |
| 1:64 | 0,472 | 0,366 | 0,319 | 0,274 | 0,235 | 0,233 | 0,237 | 0,229 | 0,232 | 0,236 | 0,219 | 0,225 |
| Blank | 0,131 | 0,109 | 0,099 | 0,091 | 0,09 | 0,086 | 0,085 | 0,085 | 0,085 | 0,087 | 0,086 | 0,093 |

**Table 11 Optical density readouts of a checkerboard assay using N protein in urea buffer.** The same assay as above in with the exception of N protein being resuspended in urea buffer. Note overall higher readouts whilst background readouts (row H no antigen, column 12 no serum) remained similar. Titration of antigen down the columns is more easily visible. This antigen suspension is more suitable for use in subsequent assays.

To enhance the overall OD values obtained during ELISA assays, the effect of different buffers for the dilution of antigen solution on coating were evaluated. For Maleic Anhydride plates, alkaline buffers were required, so a Carbonate-Bicarbonate buffer at pH 9.6 was tried first. As an alternative, PBS pH 7.4 was employed. Since the N protein had a tendency to precipitate in these buffers during coating, Costar plates were additionally coated using 500 mM NaCl and 20 mM Tris-aminomethane (Tris). Since Tris competes with the N protein for binding to the Maleic Anhydride plate, its use with those plates is not recommended. The condition was however still included in our evaluation.

In order to obtain more accurate information about the amounts of antigen actually present after coating under these different conditions, a bicinchoninic acid (BCA) assay (Pierce) was performed for each. This assay measures protein concentrations using a colorimetric method. Results of this assay are again given as OD after readout at 562 nm. The results can then be compared to a standard curve prepared from known amounts of protein to obtain information about the exact amount of protein present. For our purpose it was generally sufficient to identify which conditions yielded the highest ODs and therefore highest amount of protein on the plate. A bovine serum albumin (BSA) standard curve for the two different plates was established alongside the assay, using known amounts of protein for coating. Data from BCA assays evaluating the different coating buffers and conditions are given below (Table 12, Table 13, Table 14 and Table 15).

| | 2000µg/ml | 1500µg/ml | 1000µg/ml | 750µg/ml | 500µg/ml | 250µg/ml | 125µg/ml | 25µg/ml | buffer only | detection only | detection only | detection only |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BSA in Tris 1 | 0,153 | 0,137 | 0,127 | 0,121 | 0,116 | 0,111 | 0,112 | 0,111 | 0,102 | 0,103 | 0,098 | 0,105 |
| BSA in Tris 2 | 0,145 | 0,132 | 0,122 | 0,118 | 0,119 | 0,124 | 0,11 | 0,106 | 0,094 | 0,094 | 0,095 | 0,097 |
| BSA in Carb 1 | 0,16 | 0,146 | 0,122 | 0,13 | 0,116 | 0,111 | 0,106 | 0,115 | 0,095 | 0,107 | 0,097 | 0,102 |
| BSA in Carb 2 | 0,185 | 0,136 | 0,121 | 0,137 | 0,13 | 0,118 | 0,115 | 0,123 | 0,108 | 0,098 | 0,115 | 0,096 |

**Table 12: Bovine serum albumin standard assay using known amounts of albumin coated on a Malein-Anhydride plate, comparing two different buffers used during coating.** Note the near-identical values between carbonate-bicarbonate buffer (rows C and D) and Tris-NaCl buffer (rows A and B). None of these buffers helps to bind more or less protein on the plate than the other

| | 2000µg/ml | 1500µg/ml | 1000µg/ml | 750µg/ml | 500µg/ml | 250µg/ml | 125µg/ml | 25µg/ml | buffer only | detection only | detection only | detection only |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BSA in Tris 1 | 0,171 | 0,161 | 0,139 | 0,155 | 0,156 | 0,14 | 0,133 | 0,124 | 0,1 | 0,119 | 0,122 | 0,161 |
| BSA in Tris 2 | 0,165 | 0,151 | 0,164 | 0,14 | 0,142 | 0,14 | 0,137 | 0,127 | 0,107 | 0,13 | 0,108 | 0,135 |
| BSA in Carb 1 | 0,151 | 0,142 | 0,148 | 0,138 | 0,139 | 0,137 | 0,131 | 0,13 | 0,122 | 0,111 | 0,159 | 0,127 |
| BSA in Carb 2 | 0,218 | 0,174 | 0,143 | 0,141 | 0,165 | 0,141 | 0,151 | 0,12 | 0,127 | 0,124 | 0,153 | 0,13 |

**Table 13: Bovine serum albumin standard assay using known amounts of albumin coated on a Costar plate, comparing two different buffers used during coating.** Readouts between buffers were again very similar.

| | 1:5 | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1280 | 1:2560 | 1:5120 | blank |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N Urea Bicarb 1 | 0,775 | 0,565 | 0,422 | 0,324 | 0,223 | 0,168 | 0,136 | 0,119 | 0,106 | 0,099 | 0,095 | 0,1 |
| N Urea Bicarb 2 | 0,791 | 0,514 | 0,406 | 0,297 | 0,217 | 0,161 | 0,223 | 0,122 | 0,103 | 0,102 | 0,096 | 0,115 |
| N Urea Tris NaCl 1 | 0,18 | 0,238 | 0,336 | 0,259 | 0,196 | 0,166 | 0,143 | 0,114 | 0,109 | 0,111 | 0,112 | 0,106 |
| N Urea Tris NaCl 2 | 0,229 | 0,257 | 0,333 | 0,272 | 0,216 | 0,186 | 0,135 | 0,114 | 0,114 | 0,103 | 0,099 | 0,104 |
| N SDS Bicarb 1 | 0,164 | 0,117 | 0,122 | 0,11 | 0,108 | 0,101 | 0,099 | 0,096 | 0,101 | 0,096 | 0,103 | 0,111 |
| N SDS Bicarb 2 | 0,162 | 0,135 | 0,122 | 0,114 | 0,112 | 0,102 | 0,126 | 0,11 | 0,1 | 0,098 | 0,104 | 0,105 |
| N SDS Tris NaCl 1 | 0,141 | 0,128 | 0,136 | 0,121 | 0,116 | 0,109 | 0,106 | 0,107 | 0,102 | 0,101 | 0,101 | 0,088 |
| N SDS Tris NaCl 2 | 0,278 | 0,133 | 0,128 | 0,125 | 0,12 | 0,113 | 0,142 | 0,112 | 0,111 | 0,108 | 0,113 | 0,107 |

**Table 14: Bicinchoninic acid assay of N protein coated in serial dilutions onto a Malein Anhydride plate to determine amount of protein present on the plate after coating.** Protein resuspended in buffer containing sodiumdodecylsulfate or urea, and coated in buffer containing carbonate-bicarbonate or Tris-NaCl. Note in particular the high readout values and visible titration across dilutions for N protein resuspended in urea and coated in carbonate-bicarbonate buffer (rows 1 and 2). This condition proved most successful for coating antigen onto the plate.

| | 1:5 | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1280 | 1:2560 | 1:5120 | blank |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N Urea Bicarb 1 | 0,48 | 0,42 | 0,284 | 0,25 | 0,245 | 0,183 | 0,146 | 0,131 | 0,125 | 0,102 | 0,119 | 0,125 |
| N Urea Bicarb 2 | 0,522 | 0,474 | 0,327 | 0,265 | 0,219 | 0,18 | 0,146 | 0,134 | 0,117 | 0,103 | 0,119 | 0,105 |
| N Urea Tris NaCl 1 | 0,203 | 0,185 | 0,341 | 0,265 | 0,202 | 0,158 | 0,142 | 0,13 | 0,107 | 0,101 | 0,111 | 0,11 |
| N Urea Tris NaCl 2 | 0,177 | 0,192 | 0,342 | 0,283 | 0,216 | 0,171 | 0,142 | 0,124 | 0,123 | 0,107 | 0,132 | 0,122 |
| N SDS Bicarb 1 | 0,18 | 0,139 | 0,127 | 0,125 | 0,118 | 0,151 | 0,116 | 0,112 | 0,123 | 0,103 | 0,134 | 0,102 |
| N SDS Bicarb 2 | 0,135 | 0,143 | 0,143 | 0,132 | 0,118 | 0,112 | 0,12 | 0,116 | 0,111 | 0,105 | 0,105 | 0,13 |
| N SDS Tris NaCl 1 | 0,178 | 0,176 | 0,181 | 0,169 | 0,139 | 0,13 | 0,118 | 0,113 | 0,123 | 0,107 | 0,119 | 0,152 |
| N SDS Tris NaCl 2 | 0,193 | 0,188 | 0,173 | 0,15 | 0,141 | 0,132 | 0,119 | 0,118 | 0,123 | 0,118 | 0,117 | 0,184 |

**Table 15: Bicinchoninic acid assay of N protein coated in serial dilutions onto a Costar plate to determine amount of protein present on the plate after coating.** Protein resuspended in buffer containing sodiumdodecylsulfate or urea, and coated in buffer containing carbonate-bicarbonate or Tris-NaCl. Note in particular the high readout values and visible titration across dilutions for N protein resuspended in urea and coated in carbonate-bicarbonate buffer (rows 1 and 2). Values for this condition were still significantly lower on a Costar than on a Malein Anhydride Plate, indicating that coating was not as efficient.

As noted previously, coating with N protein dissolved in SDS buffer resulted in overall lower

ODs, with background levels playing a larger part, regardless of the plate type used. Using N

protein in urea buffer, overall ODs were higher and background slightly lower using Maleic Anhydride plates. On both Costar and Maleic Anhydride plates, using N protein in urea buffer, and diluting in carbonate-bicarbonate buffer when coating, resulted in highest ODs.

To further optimize coating conditions, we compared results after coating, using buffer pre-warmed to 40 °C versus buffer at room temperature. Plates were left at room temperature for 1 h after coating and then placed in the fridge overnight in either case. The results of this process were inconsistent despite repeating the same conditions with the same samples. There did not appear to be any benefit from warming the coating buffer.

In summary, the most efficient coating conditions were found to be the use Maleic Anhydride Plates and N protein in urea buffer, diluted in carbonate-bicarbonate buffer for coating.

### 4.6.2.4 Influence of using different coating dilutions

Most suitable dilutions of antigen for coating were determined using checkerboard titrations, which employ serial dilutions of both antigen and primary antibody in perpendicular directions. That is, antigen will be serially diluted down the rows of the plate, and serum across the columns of the plate, or vice versa. This helps to determine the optimal combination of dilutions. Repeated checkerboard assays consistently showed an antigen dilution of 1 : 20 to 1 : 40 gave the best results (Figure 26 and Figure 27). Using these dilutions, ODs were highest at lower dilutions of serum, titration of serum samples was steady, and values within multiples of the same samples were consistent. Dilutions both lower and higher than 1:20 - 1:40 were less consistent and produced less valuable titration curves.

A dilution of 1 : 20 was slightly more consistent than 1 : 40, and since supply of antigen was not an issue, this dilution was used.
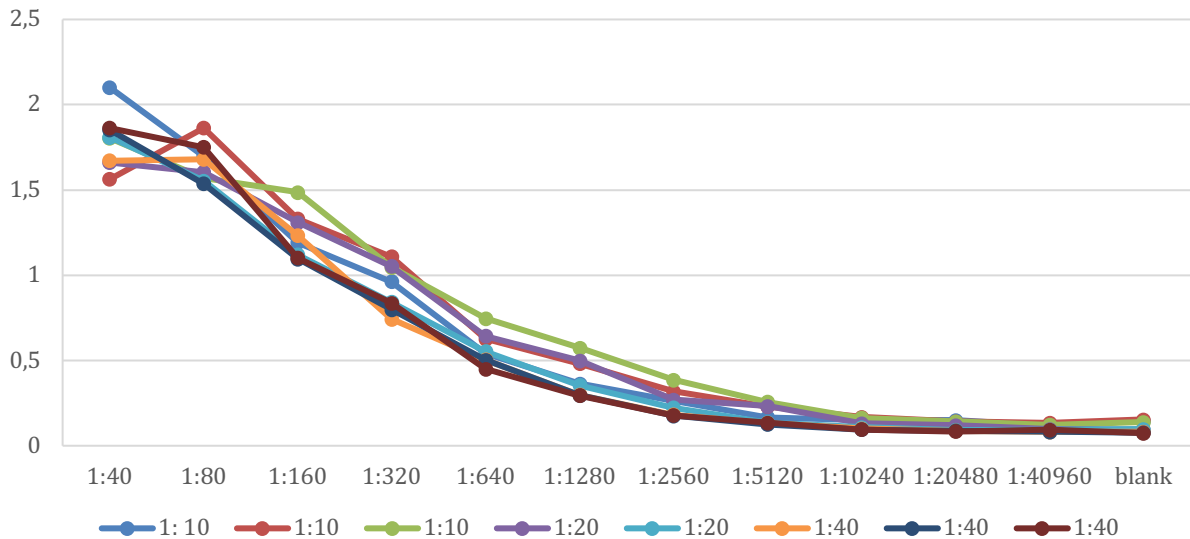
**Figure 26: N protein coating conditions, low dilutions.** Checkerboard titration of 1 : 10, 1 : 20 and 1 : 40 dilutions of N protein for coating, paired with serial 2-fold dilutions of distemper N-positive ferret serum (x-axis). Y-axis denotes readouts generated. Note the higher variation of values for lower serum dilutions for N 1 : 10.
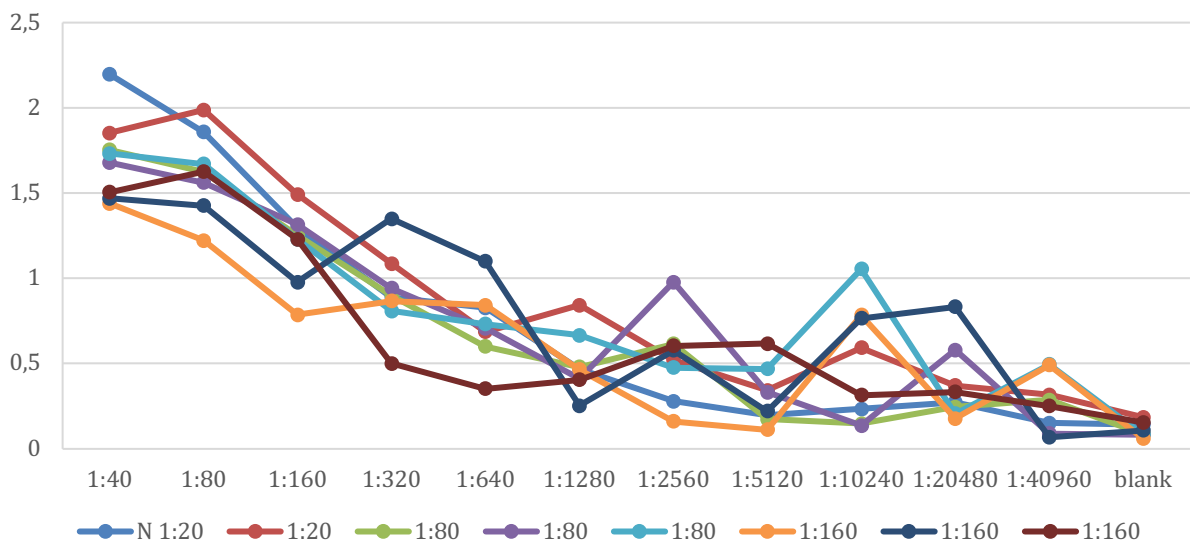


**Figure 27: N protein coating conditions, higher dilutions.** Checkerboard titration of 1 : 20, 1 : 80, and 1 : 1600 dilutions of N protein for coating, paired with serial 2-fold dilutions of distemper N-positive ferret serum (x-axis). Y-axis denotes readouts generated. Note the overall lower readouts and significantly increased variation between duplicates compared to lower dilutions.

### 4.6.2.5 Influence of using different blocking conditions

In order to improve background values and increase specificity of the assay, various methods

of blocking were evaluated using plates coated with a 1 : 20 dilution of N protein and serial

dilutions of a CDV N positive ferret serum. Blocking with rabbit serum maintained overall

ODs compared to blocking with 3 % BSA in PBS which is a standard blocking regime, but increased background values by 200 – 250 %. The use of both goat serum and 10 % dry milk in PBS (w/v) reduced background values to 20 % and 30 % of BSA blocked plates, respectively, but also resulted in much lower overall OD values and a rapid drop of serum OD values, which would lead to much lower titers. A limited number of test runs performed during this study also reduced the differentiation between positive and negative samples, with ODs at starting dilutions of serum samples ranging around 1.5 for positive and 0.6 - 0.7 for negative samples when blocking with BSA (Figure 28, Figure 29 and Figure 30); and changing to 1.0 and 0.7 - 0.8 for blocking with dry milk in PBS, respectively. While blocking with goat serum kept negative sample ODs at about 50 % of positive sample values, goat serum was an important negative control agent during the development of this assay, and was therefore not used for blocking.
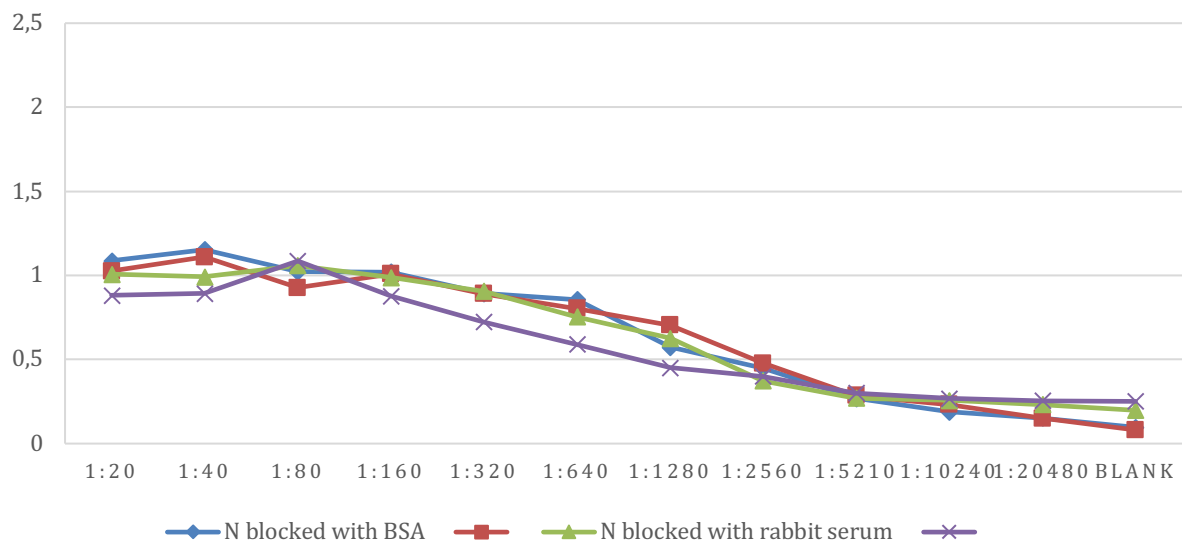


**Figure 28: Comparison of blocking using bovine serum albumin or rabbit serum.** Graph for assay readouts comparing the use of 3% albumin in phosphate buffered saline versus plain rabbit serum for blocking. N protein was coated in constant 1 : 20 dilution, the primary antibody used was distemper N positive ferret serum in serial 2fold dilutions (x-axis). Y-axis denotes readout values. Note increase of "blank" (no primary) readouts by 200 – 250 % with use of rabbit serum.
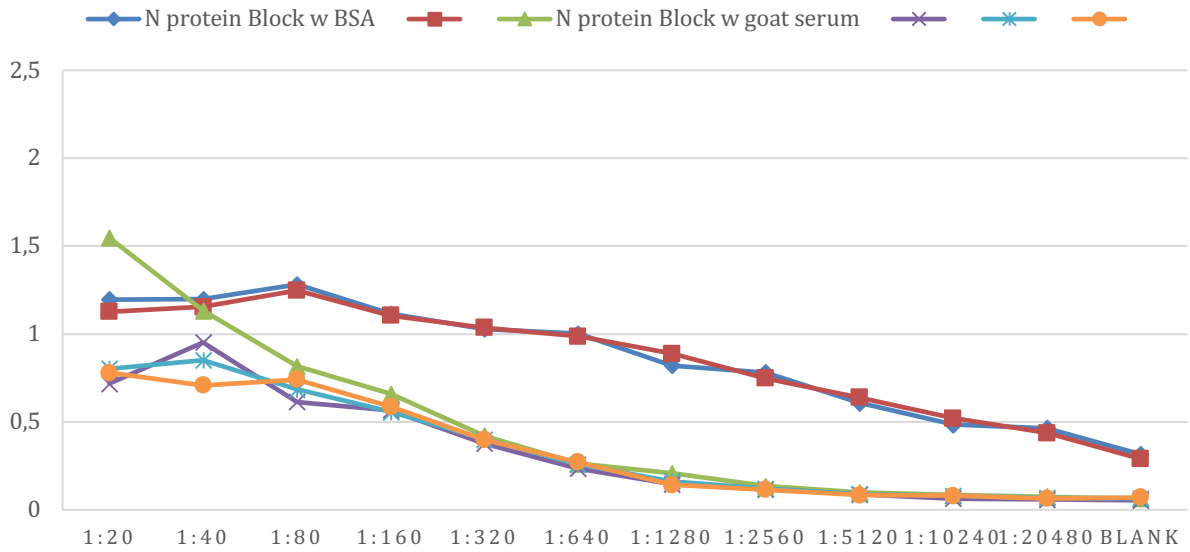
**Figure 29: Comparison of blocking using bovine serum albumin or goat serum.** Graph for assay readouts comparing the use of 3% albumin in phosphate buffered saline versus plain goat serum for blocking. N protein was coated in constant 1 : 20 dilution, the primary antibody used was distemper N positive ferret serum in serial 2fold dilutions (x-axis). Y-axis denotes readout values. Note generally lower readouts and faster decrease of values when blocking with goat serum.
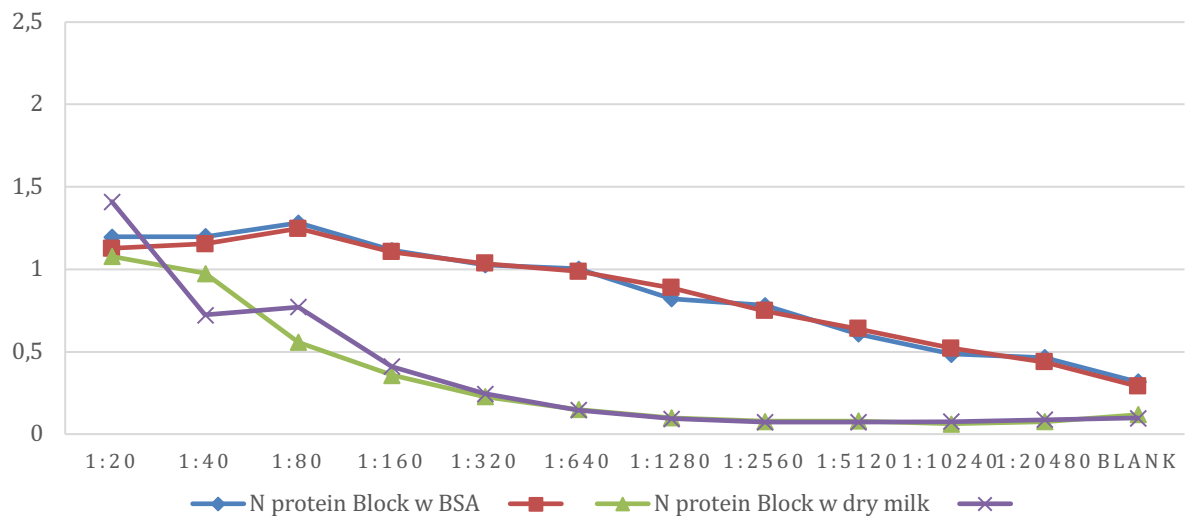


**Figure 30: Comparison of blocking using bovine serum albumin or a 10% dry milk solution.** Graph for assay readouts comparing the use of 3% albumin in phosphate buffered saline versus a 10% solution of dry milk in phosphate buffered saline for blocking. N protein was coated in constant 1 : 20 dilution, the primary antibody used was distemper N positive ferret serum in serial 2fold dilutions (x-axis). Y-axis denotes readout values. Note generally lower readouts with high variability in lower dilutions, and very rapid decrease of values when blocking with dry milk.

*4.6.2.6   Influence of primary antibodies*

Primary antibodies in an indirect ELISA are responsible for binding to and detecting the antigen on the plate. Serum samples to be tested for certain antibodies are employed as primaries; as are specific antibodies used as controls.

*4.6.2.6.1   Effects of using different control primaries:*

A commercially available mouse anti-HIS antibody was used as a control primary to confirm availability of N protein for detection, since the protein carries a HIS tag.

Below is a graph of ODs produced when coating various dilutions of N protein and detecting the protein with anti-HIS antibody (Figure 31).
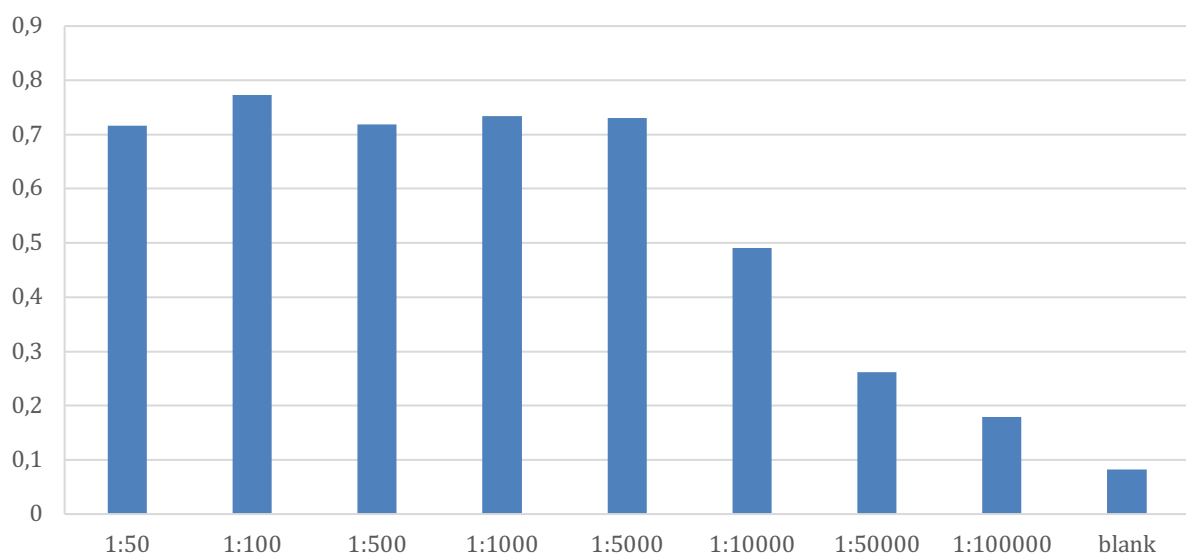


**Figure 31: Use of mouse anti-HIS antibody as a control primary.** Average readouts of various dilutions of N protein (x-axis) were detected with a constant concentration of mouse anti-HIS antibody as a primary. Secondary antibody used was an anti-mouse IgG. "Blank" wells were coated but did not receive primary antibody. This antibody works best at a dilution of 1:100 for the purpose of being a positive control antibody.

A monoclonal antibody stated to be directed against CDV N protein was employed as a control primary to further confirm the presence of N protein on the plate. Problems with the use of this antibody have been outlined above (see 4.6.2.2 "Confirmation of presence of antigen on the plate").

### 4.6.2.7 Determination of suitable dilution of serum samples as primaries

The most appropriate starting dilution for sera was determined using checkerboard titrations (Table 10 and Table 11). 2-fold serial dilutions were performed across the plate in order to get accurate titration results. Due to overall consistently low ODs during assay development, we decided to use only a low starting dilution. The use of serum at starting dilutions of 1 : 10 was discussed, but due to the limited availability and very small volume of samples it was decided to only use this dilution if absolutely necessary. Taking into account the small differences in ODs between 1 : 8 and 1 : 32 dilutions tested, we decided to use a serum starting dilution of 1 : 20 for assays unless otherwise required.

Had assay results suggested that the titer of test samples was higher than what was covered using this setup, either the starting dilution or dilution factor across the plate would have been increased to cover a larger range of titers. Samples were diluted in PBS with 0.02 % Tween (v/v).

#### 4.6.2.7.1 Controls using positive and negative ferret serum

We were able to acquire sera of both CDV vaccinated as well as CDV naïve ferrets for use as control sera for antibody detection (Triple F Farms). To ensure assay consistency, we aimed to use samples of the same serum batches as controls throughout the assays. Positivity or

negativity of the sera were confirmed using dot blot assays, which involve placing a dot of

the protein solution in question onto a nitrocellulose membrane, and then continuing with

blocking, washing, and detection steps as described for western blots (Figure 32). We

employed the antigen solution used for coating, and added samples of the positive and

negative control sera for detection. Bound antibodies were subsequently detected with HRP-

labeled anti-ferret Ig, following the protocol for western blots.
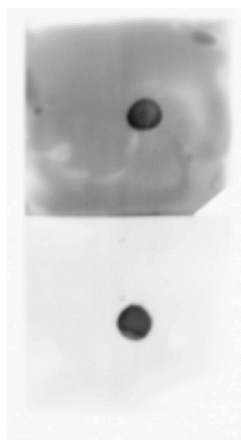


**Figure 32: Dot blot assay to confirm the presence of anti-N antibodies in ferret serum.** Results of
dot blots of serum reported to be positive for distemper N protein antibodies (top) alongside positive
control using anti-HIS antibody as primary (bottom). Both dots gave positive readouts indicating the
presence of antibodies against distemper N protein.

Ferret sera thus confirmed to either contain or be free from antibodies against CDV N

protein were subsequently used to determine the ability of the tested setups to discriminate

between positive and negative samples.

We had obtained a large volume of serum from a terminal bleed of a CDV vaccinated ferret,

which was confirmed by dot blot to contain antibodies to CDV proteins. Aliquots of this

particular serum sample were used as a positive control in most subsequent ELISA assays, to

ensure comparability of results (Figure 33).
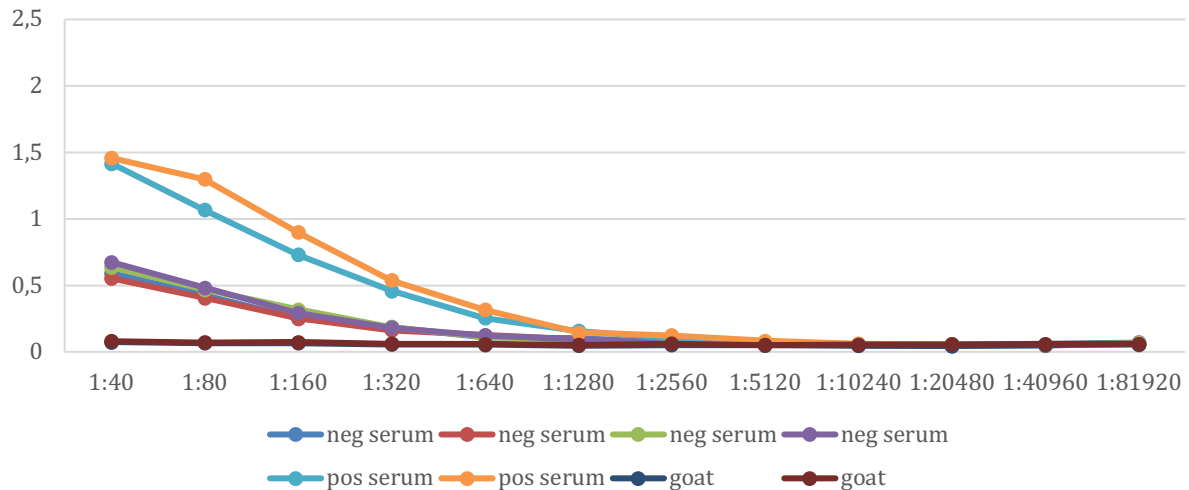
**Figure 33: Comparison of negative versus positive ferret serum during enzyme-linked immunosorbant assay.** Plot of readouts of negative (blue, red, green and purple lines) versus positive (light blue and orange lines) ferret sera on a plate coated with distemper N protein. A polyclonal anti-ferret IgG served as the secondary antibody. Goat serum was used as a negative primary control (dark blue and burgundy lines). The difference between the negative control and importantly the negative serum and the positive serum is obvious until a 1:640 dilution is reached.

### 4.6.2.7.2    Controls using rabbit serum

During the development of the ELISA assay, we frequently encountered low overall OD values with only moderate titration curves visible, and no clear distinction between known-positive and known-negative samples.

To exclude the possibility of components of the ferret serum binding unspecifically, we decided to employ serum of an unrelated species, not susceptible to CDV or related viruses, as a control. Since rabbit serum was readily available in a large enough volume from one animal, and since rabbits are well known for their ability to quickly mount antibodies in large quantities, rabbit serum was an ideal option, in connection with a species-specific antibody (Figure 34).
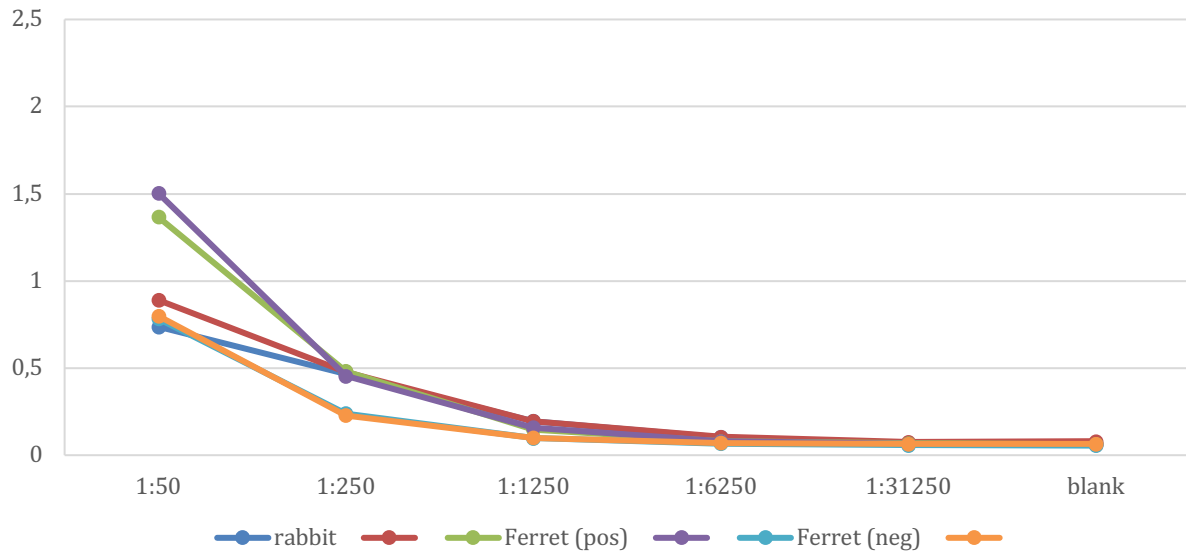
**Figure 34: Use of rabbit serum as a control for unspecific binding in an enzyme-linked immunosorbant assay using distemper N protein.** Rabbit serum (red and blue lines) was tested against a known negative (orange and light blue lines, running on top of each other) and a known positive (green and purple lines) ferret serum to evaluate the use of rabbit serum as a non-related negative control. Unfortunately, these trials demonstrated a considerable reactivity of naïve rabbit serum to the CDV N protein. ODs especially in low dilutions were unacceptably high for a non–related negative control, and titration was evident, indicating that serum components were responsible for creating the signal.

*4.6.2.7.3   Controls using nonhuman primate serum*

Nonhuman primate serum was originally employed as a positive and negative control in the development of gag ELISAs. Once we discovered the results obtained when using rabbit serum and anti-rabbit secondaries, we decided to assess nonhuman primate sera for their response to CDV proteins as well. The interest in evaluating NHP sera in CDV ELISAs was increased further due to a frequently debated antigenic relationship between measles virus – which the majority of NHP colonies are being vaccinated against - and CDV. NHP serum was used in connection with a species-specific secondary antibody (Figure 35).

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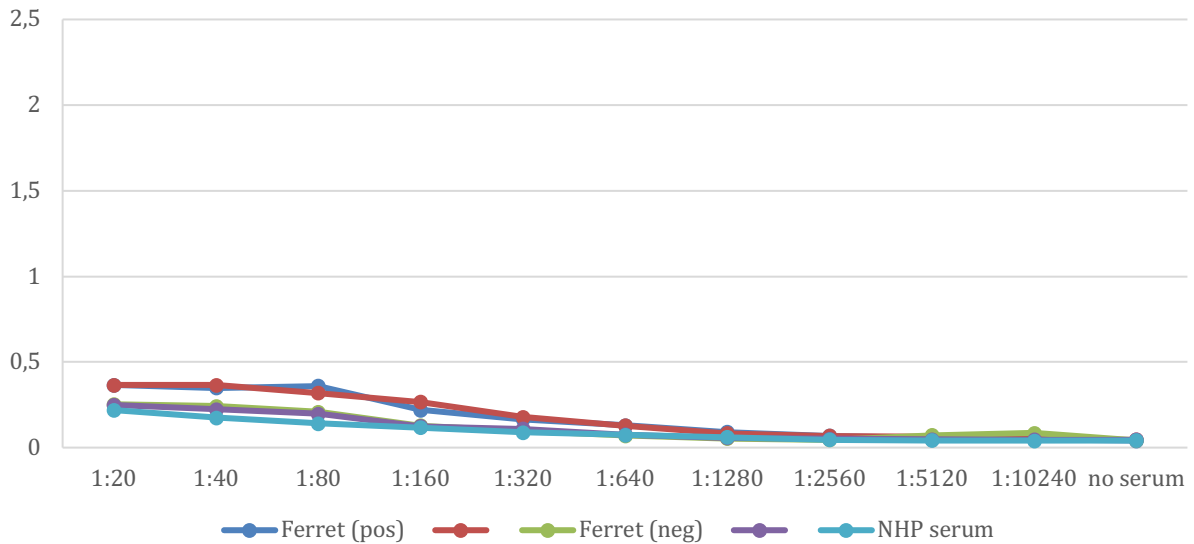
**Figure 35: Use of rabbit serum as a control for unspecific binding in an enzyme-linked immunosorbant assay using distemper N protein.** Nonhuman primate serum (teal line) was tested against a known negative (purple and green lines, running on top of each other) and a known positive (red and blue lines) ferret serum to evaluate the use of rabbit serum as a non-related negative control, nonhuman primate serum gave readouts and titration curves similar to those of distemper negative ferret serum. Again, the presence of a titration curve suggests that serum components were responsible for this reaction.

*4.6.2.7.4   Controls using goat serum*

Goat serum was employed as a negative control since the secondary antibody that was selected for use in ELISA assays is derived from goat serum. So, in addition to a "no serum" blank control, this allowed for a second check of unspecific binding of the secondary.

The use of goat serum as a primary in connection with an anti-ferret secondary served the second purpose of controlling for non-species specific binding and hence false positive detection of our secondary.

Since this combination consistently provided ODs equivalent to zero detection, it was included as a routine negative assay control in the ELISA development. See Figure 33 and Figure 36 for typical examples when using this control.

Upon noticing the consistent difficulty with unspecifically high ODs during the assay development, we evaluated the use of heat-inactivated serum as well as plasma compared to plain serum for use as primary antibody source (Figure 36).
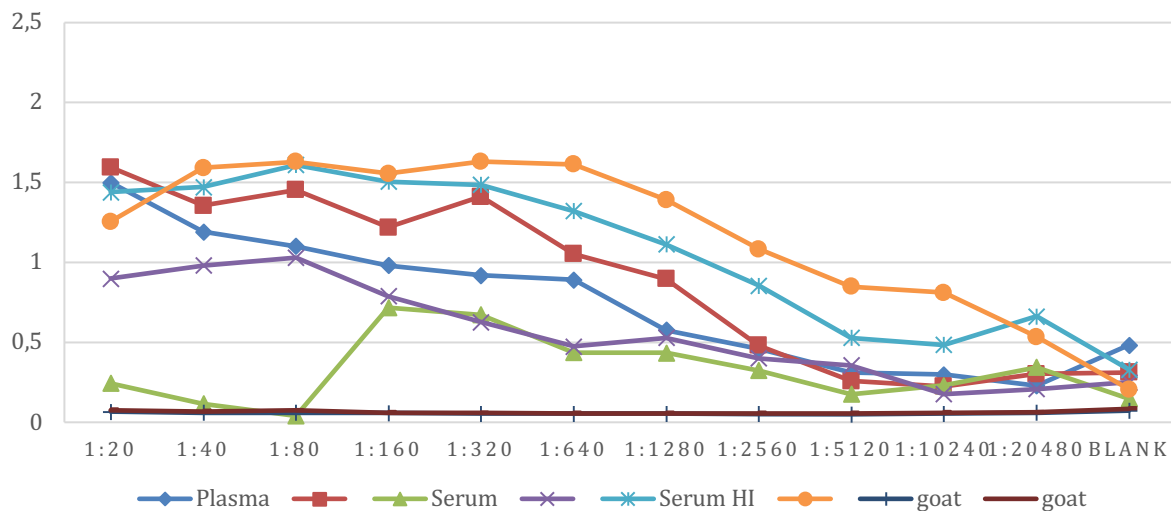


**Figure 36: Comparison of readouts for plasma, serum and heat-inactivated serum (serum HI) from the same animal in an enzyme-linked immunosorbant assay.** This animal was confirmed to be distemper N positive in a previous assay. (Plasma red and blue lines, Serum green and purple lines, Heat-inactivated serum teal and orange lines). Goat serum with anti-ferret secondary served as a negative assay control (dark blue and burgundy lines). This combination gave suitably low readouts. Heat-inactivated Serum resulted in the highest readouts and low variability, but also comparably high background. Plasma demonstrated very high variability.

The comparison of these three different conditions showed that for N protein ELISAs, heat inactivated serum produced the highest ODs. It also however produced the highest background. Plasma demonstrated a very high intra-assay variation.

While the serum samples in this particular assay showed some disturbance in the lower dilutions, serum from this same animal was routinely used as a positive control for N protein ELISAs due to the high ODs and usually nice titrations it produced. See Figure 29 and Figure 30

for examples, where the same serum was used as positive control. The protocol used for this particular assay was the same as was routinely used during later stages of assay development. What caused the irregularities of the serum titration curves is unclear, but symptomatic for the process of developing this assay.

### 4.6.2.8 Fluorochrome based detection of bound primary antibody by secondary antibodies

#### 4.6.2.8.1 Effects of different secondaries tested

During assay development, we tested several secondary antibodies that were described to be anti-ferret secondaries, or directed against related species but crossreactive with ferret Igs. Initially, we had planned to create a repertoire of anti-ferret secondaries labelled with a variety of fluorochromes, so that we could use them in various antibody assays, and in parallel if required. In order to achieve this, we ordered plain anti-ferret IgG, IgM and IgA (KPL) and conjugated them with various fluorochromes using DyLight antibody conjugation kits (Pierce) and following manufacturer's instructions. However, when employed in an ELISA setup, none of these conjugated antibodies worked, giving zero-detection ODs in every assay (Table 16).

| | 1:100 | 1:200 | 1:400 | 1:800 | 1:1600 | 1:3200 | 1:6400 | 1:12800 | 1:25600 | 1:51200 | 1:102400 | Blank |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N 1:10 | 0.027 | 0.022 | 0.026 | 0.027 | 0.023 | 0.022 | 0.023 | 0.024 | 0.026 | 0.026 | 0.025 | 0.020 |
| N 1:20 | 0.029 | 0.024 | 0.026 | 0.032 | 0.023 | 0.024 | 0.023 | 0.024 | 0.022 | 0.023 | 0.027 | 0.024 |
| N 1:40 | 0.024 | 0.023 | 0.024 | 0.027 | 0.026 | 0.032 | 0.03 | 0.022 | 0.024 | 0.028 | 0.025 | 0.023 |
| N 1:80 | 0.027 | 0.022 | 0.025 | 0.031 | 0.028 | 0.022 | 0.025 | 0.027 | 0.043 | 0.024 | 0.025 | 0.024 |

**Table 16: Readouts of enzyme-linked immunosorbant assay using DyeLight conjugated secondary antibodies.** Several different dilutions of N protein (vertical index) and 2-fold serial dilutions of ferret serum (horizontal index) were used and detected with a constant concentration of DyLight 493 – conjugated anti-ferret IgG. This secondary did not function.

We then proceeded to test various commercially available pre-labeled secondary antibodies. Each antibody was tested at the low end and the high end of their recommended dilution ranges. Secondaries were first evaluated for general function using our so far established standard setup (antigen diluted 1 : 20, and the standard positive control serum applied in serial 2-fold dilutions starting at 1 : 40). We then tested the lowest level of detection for each antibody by using the same serum at dilutions starting from 1 : 100. Subsequently, secondary antibodies were evaluated for consistency of detection by running quadruplicates of the same assay using our standard positive control serum. Finally, the secondaries were tested for their ability to differentiate the standard known-positive from a known-negative serum.

Commercially available secondaries assessed included: Alkaline phosphatase (AP) conjugated anti –ferret IgG by Rockland, KPL and Lifespan; horseradish peroxidase (HRP) conjugated anti – ferret IgG by KPL, Lifespan, Acris, Genetex, Novus and Bethyl; as well as HRP conjugated IgM and IgG/IgA/IgM, both by Acris (Figure 37, Figure 38, Figure 39, Figure 40 and Figure 41).

It became evident very quickly that AP conjugated secondaries did not give any meaningful OD readouts, while most HRP conjugated sets gave convincingly high OD's in identical setups. Ultimately, we had determined two equally well performing secondaries, and chose to use the slightly less expensive one of the two as our routine secondary anti-ferret antibody.
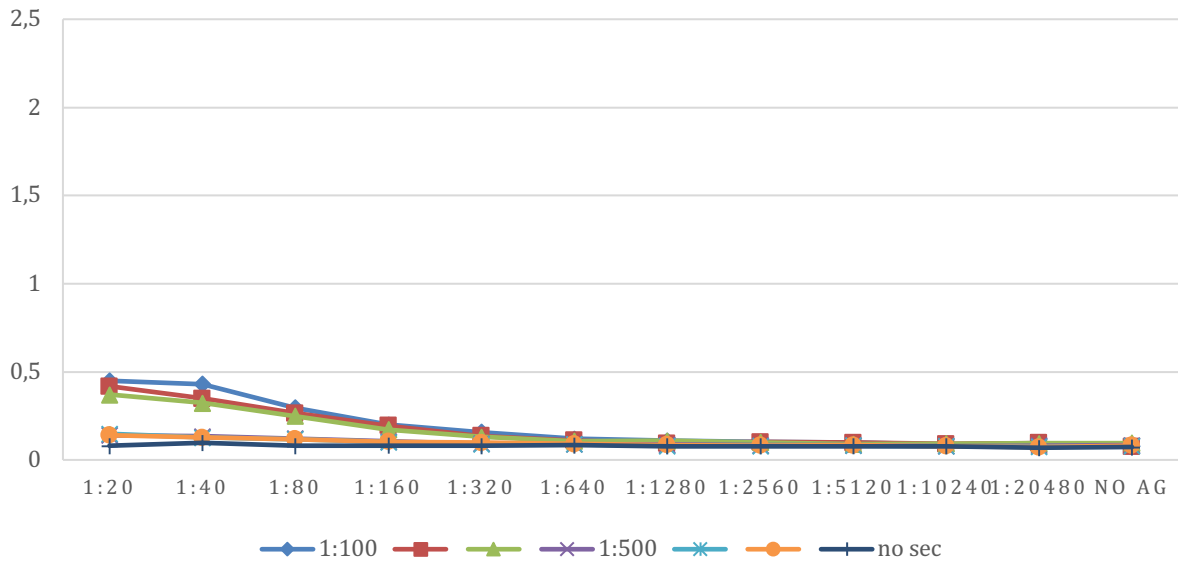
**Figure 37: Readouts of enzyme-linked immunosorbant assay using KPL anti-ferret IgG, alkaline phosphatase conjugated, as a secondary antibody.** Functional evaluation of an AP conjugated anti-ferret IgG (KPL) against N protein and CDV N positive ferret serum, two different dilutions, 1 : 100 (red, blue and green lines) and 1 : 500 (purple, light blue and orange lines), as suggested by the manufacturer. A row not receiving secondary was used as a negative control (dark blue line). This secondary produced rather low overall readouts.
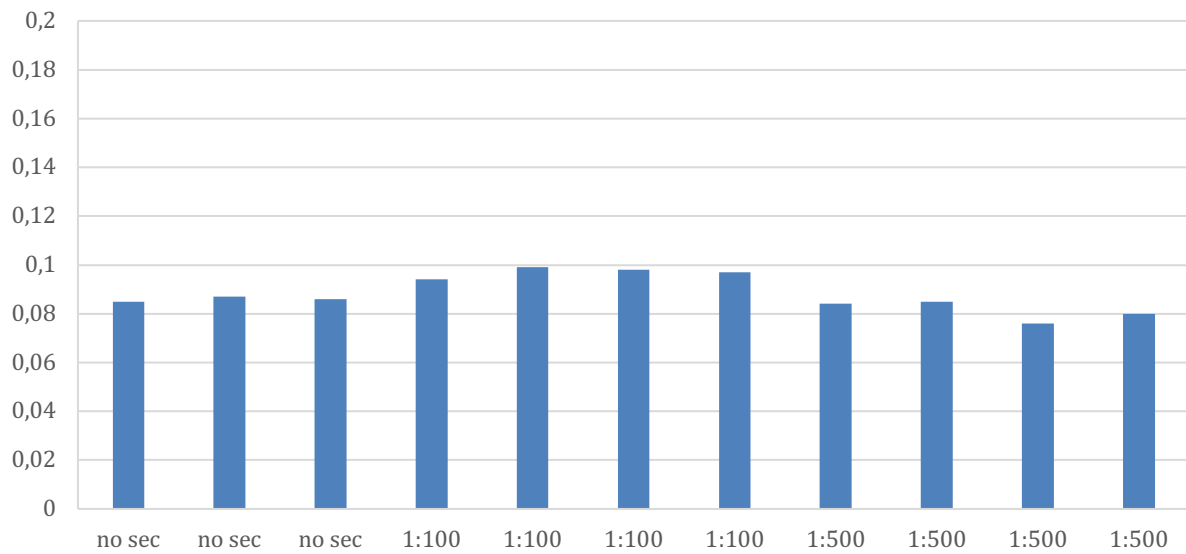


**Figure 38: No serum control, KPL anti-ferret IgG, alkaline phosphatase conjugated.** The same secondary as above (KPL) on wells with antigen only but no serum, to establish background values for no secondary, secondary at 1 : 100 dilution and secondary at 1 : 500 dilution. Background values for this secondary were unremarkable.
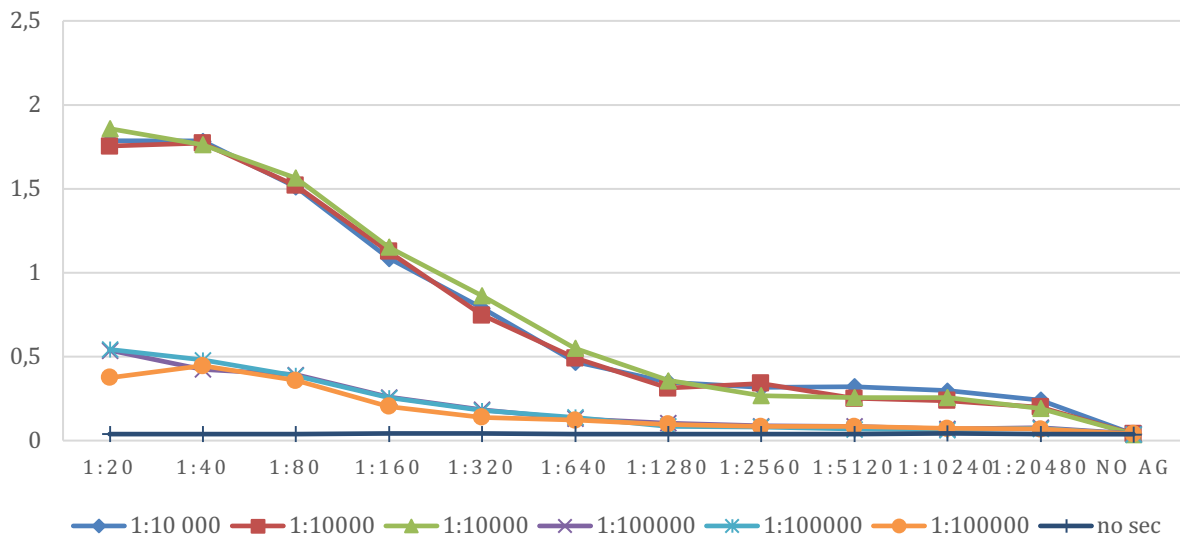
**Figure 39: Functional evaluation of a Bethyl Labs horseradish peroxidase conjugated anti-ferret IgG.** This antibody was tested as a secondary antibody in an enzyme-linked immunosorbant assay using distemper N protein as the antigen and distemper N positive ferret serum as a primary. Two different dilutions, 1 : 10,000 (blue, red and green lines) and 1 : 100,000 (purple, teal and orange lines), were evaluated as suggested by the manufacturer. A row without secondary was used as a negative control (dark blue line). The 1 : 10, 000 dilution resulted in good readout values and an easily visible titration.
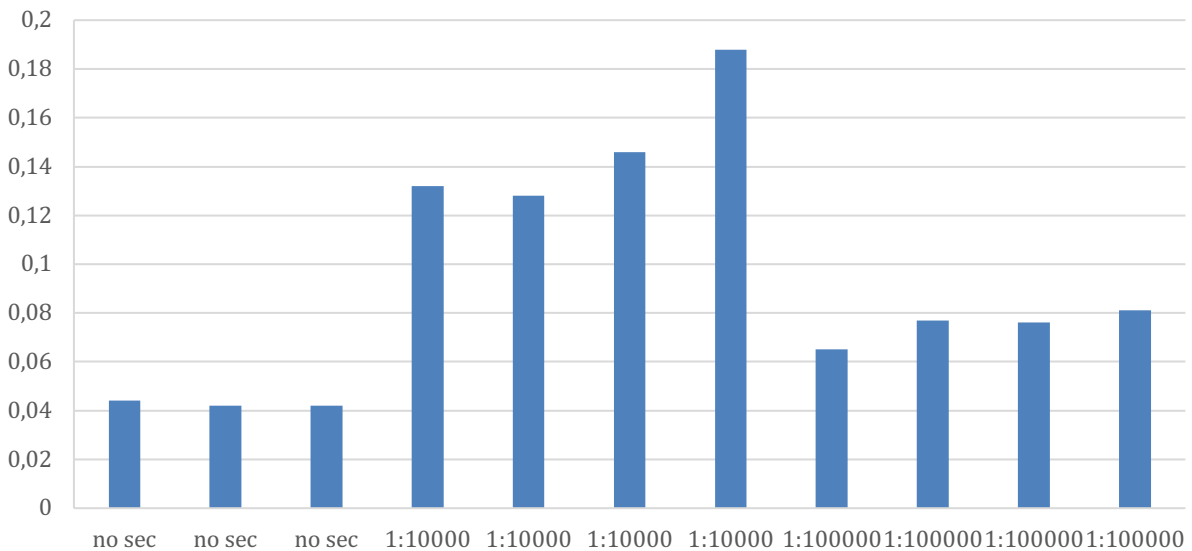


**Figure 40: No serum control assay using Bethyl horseradish peroxidase conjugated anti-ferret IgG.** Same secondary as above (Bethyl) on wells with antigen only but no serum, to establish background values for no secondary, secondary at 1 : 10,000 dilution and secondary at 1 : 100,000 dilution. While the 1 : 10, 000 dilution resulted in background values more than twice as high as the 1 : 100, 000 dilution, the background still only accounted for about 10% of the maximum readout values reached in the assay above.
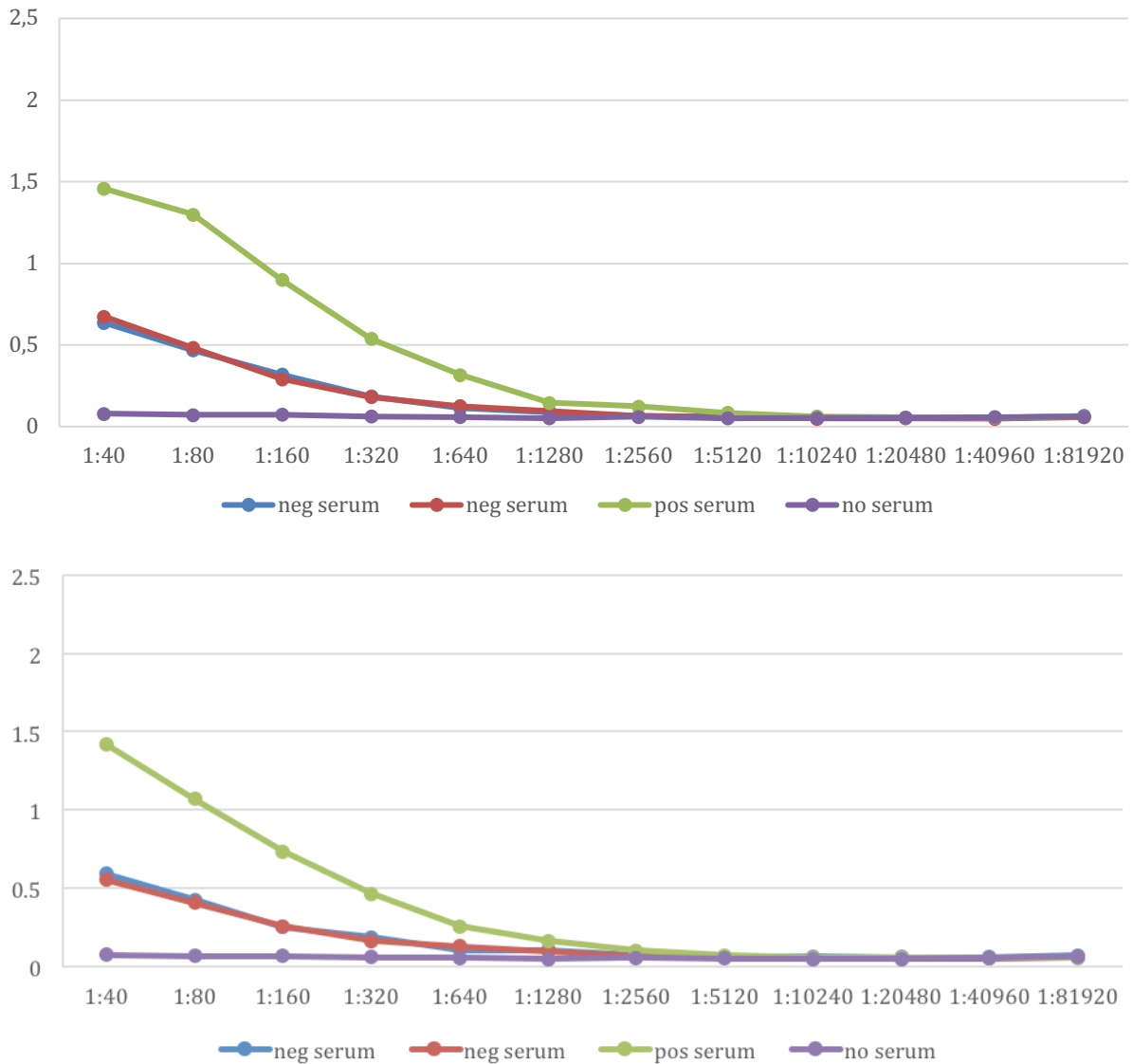
**Figure 41: Direct comparison of horseradish peroxidase-conjugated anti-ferret IgG from Bethyl (top graph) and Novus (bottom graph).** Distemper N protein positive (green line) and negative (red and blue line) samples were used for this enyzme-linked Immunosorbant assay using distemper N protein as antigen. The secondary antibodies were both used at a dilution of 1 : 10,000. A comparison was made with regards do overall readouts, discrimination of positive and negative samples, and "no serum" background readouts. Note the near-identical results for both secondaries.

When evaluating the above figures, it can be noted that, while both the KPL and the Bethyl antibody produce similar titer curves, the ODs achieved are very different (0.5 to 1.8 maximum, respectively). This has a strong effect on the ability of the antibodies to discriminate positive and negative samples.

While the antibody made by KPL numerically produces lower background (0.1 compared to 0.18, respectively), it does account for a higher percentage of total maximum OD (25% compared to 10% respectively). These background values are still very high, but likely not solely due to the secondary antibody, since this problem recurred consistently to various degrees during the entire process of CDV N ELISA development.

Since IgG antibodies are commonly only produced in an established infection, we also assessed secondaries directed against ferret IgM or ferret IgM/A/G, to have the option to confirm the status of infection early on after vaccination. These were only available to us produced by Acris. Based on our experience with anti-IgG secondaries, we chose to evaluate HRP-conjugated sets (Figure 42, Figure 43, Figure 44 and Figure 45).
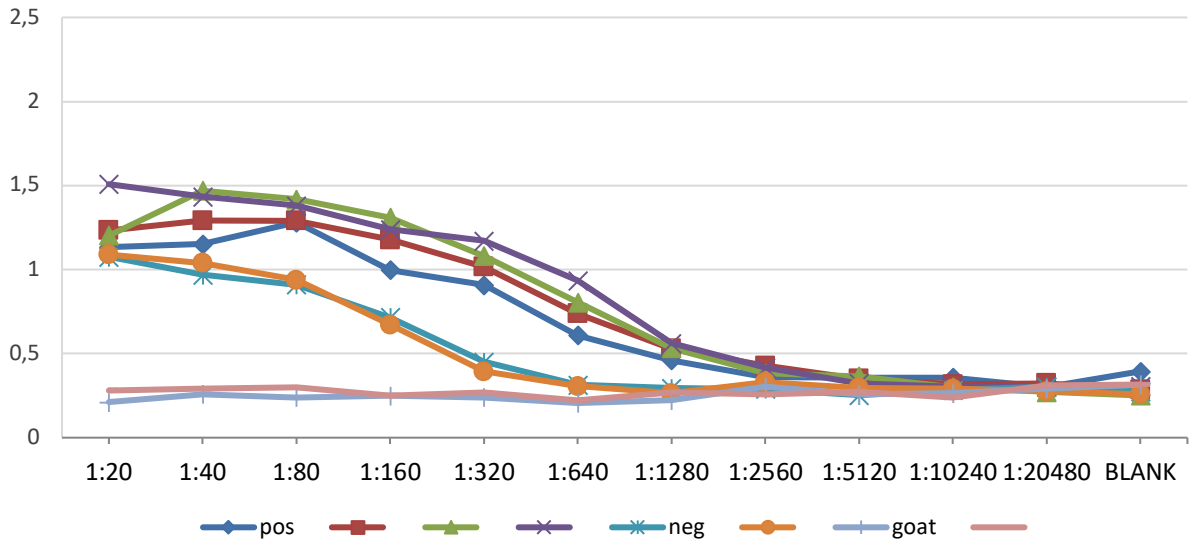
**Figure 42: Test assay using an anti-ferret IgM antibody at a 1 : 10,000 dilution. Enzyme-linked immunosorbant assay** using positive (blue, red, green and purple lines) and negative (teal and orange lines) serum, as well as goat serum as an assay control, with goat anti-ferret IgM (Acris), dilution 1 : 10,000 (light blue and pink lines). Note the lack of distinction between positive and negative samples especially at low primary dilutions. From a dilution of primary of 1 : 80 and upwards, distinction becomes much more evident. Variation between the positive samples (all using the same serum) is considerable.
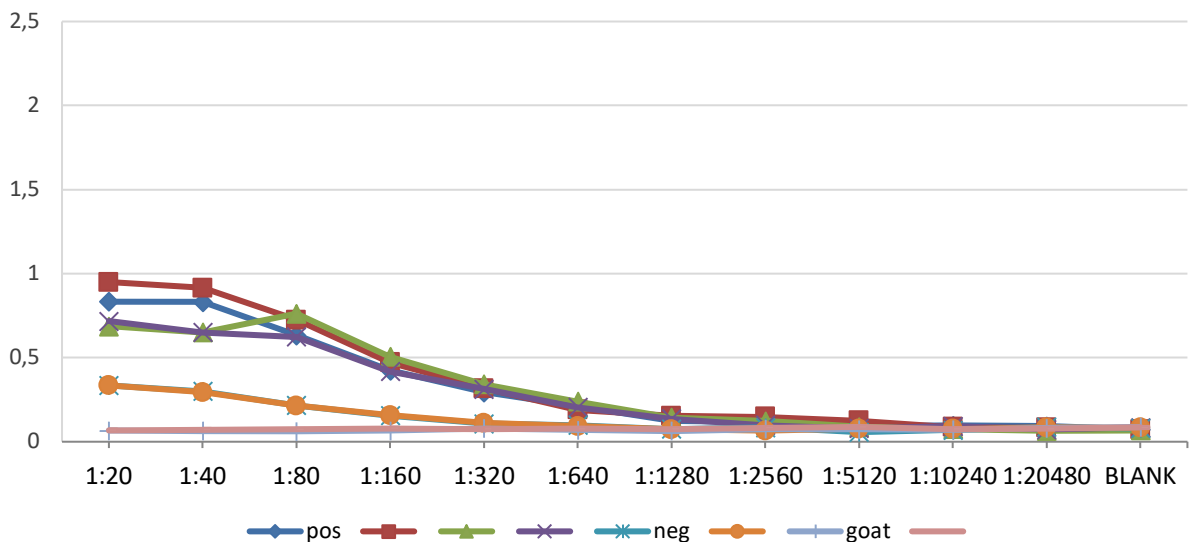


**Figure 43: Test assay using the same anti-ferret IgM antibody at a 1 : 50,000 dilution. Enzyme-linked immunosorbant assay** using positive (blue, red, green and purple lines) and negative (teal and orange lines) serum, as well as goat serum as an assay control, with goat anti-ferret IgM (Acris), dilution 1 : 50,000 (light blue and pink lines). Distinction of positive and negative samples is a lot more visible at this dilution, but overall readout values are much lower.

This secondary does show significant intra-assay variability especially at lower serum dilutions, but is still able to discriminate between positive and negative sera in an ELISA for CDV N antibodies, if used at the higher dilution of 1 : 50,000.
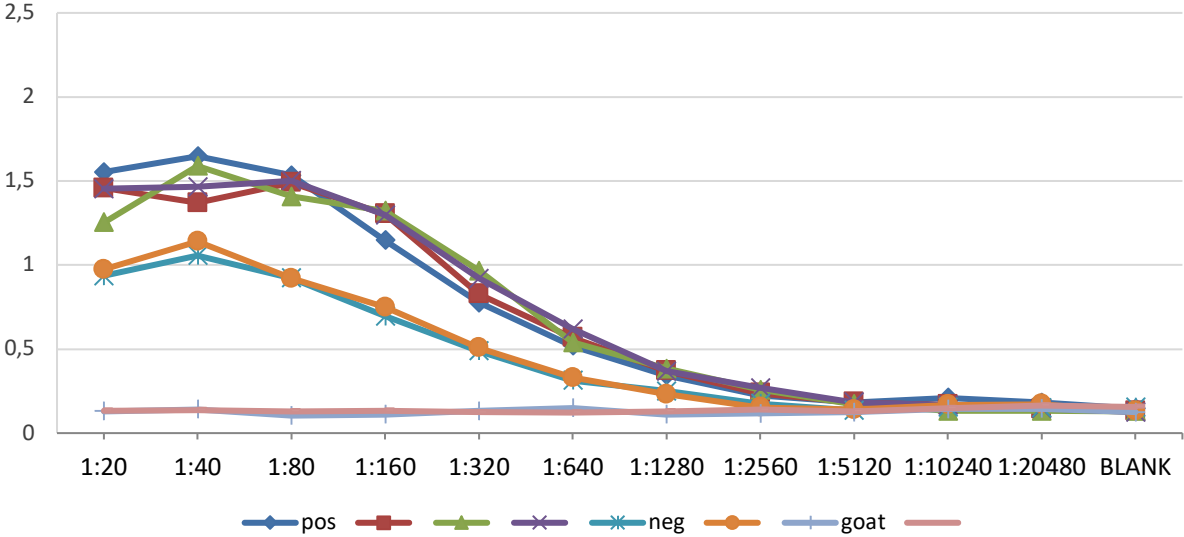


**Figure 44: Test assay using an anti-ferret IgM/G/A antibody at a 1 : 10,000 dilution. Enzyme-linked immunosorbant assay** against distemper N protein using positive (blue, red, green and purple lines) and negative (teal and orange lines) serum, as well as goat serum as an assay control, with goat anti-ferret IgM/G/A (Acris), dilution 1 : 10,000 (light blue and pink lines). This secondary provides acceptable distinction between positive and negative samples for the lower half of primary dilutions, but overall readout values are not particularly high.
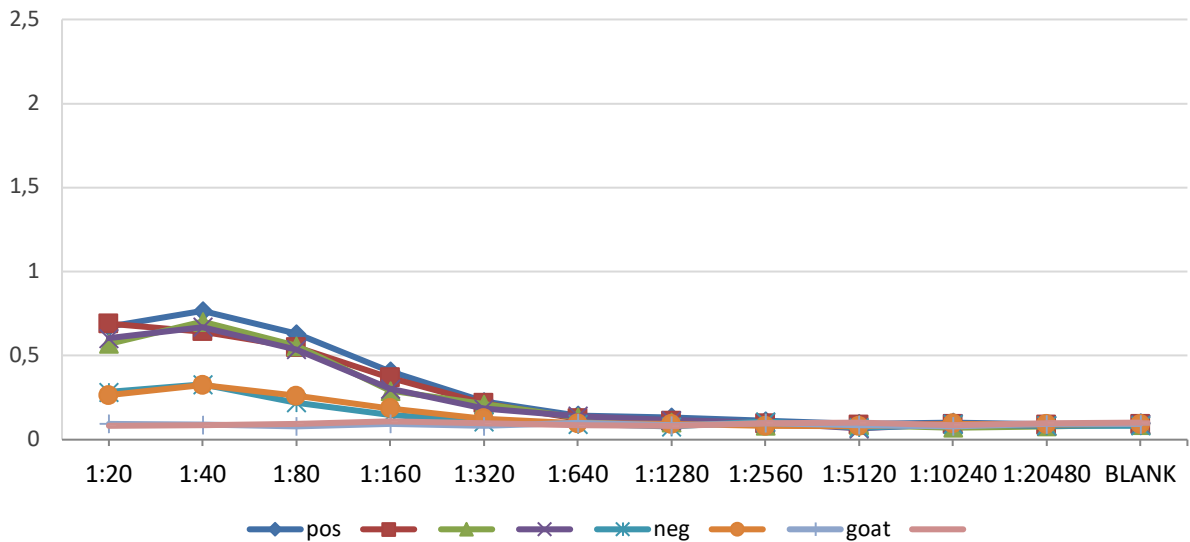
**Figure 45: Test assay using an anti-ferret IgM/G/A antibody at a 1 : 50,000 dilution. Enzyme-linked immunosorbant assay** against distemper N protein using positive (blue, red, green and purple lines) and negative (teal and orange lines) serum, as well as goat serum as an assay control, with goat anti-ferret IgM/G/A (Acris), dilution 1 : 50,000 (light blue and pink lines). Distinction between positive and negative samples is present for low dilutions of primary but fades very quickly, and overall readout values are very low.

This goat anti-ferret IgM/A/G does show an ability to differentiate between positive and negative sera, particularly at lower serum dilutions, but titration curves approach each other very quickly, which would make differentiation of positive and negative sera based on titer impossible.

In summary two of the antibodies tested, HRP-conjugated anti-ferret IgG made by Bethyl and Novus, consistently resulted in near identical results of equally high quality. A direct comparison of the two during the same assay can be found in Figure 41. Note near identical starting ODs, titer curves and background values.

These antibodies were the most suitable anti-IgG secondaries. Since the antibody made by Novus was significantly more affordable, this one was chosen as the selected secondary for

IgG assays. The HRP conjugated anti-ferret IgM by Acris also provided useful results and was occasionally employed to attempt to detect antibodies in the early stages post vaccination.

### 4.6.2.9 Alternative strategies to investigate and reduce unspecific binding

In order to shed light on the reasons behind the significant amount of unspecific binding and variability occurring throughout the assay development, and even with unrelated, CDV naïve serum samples, we performed several control assays using alternative antigen preparations and pretreated serum samples to assess the role plaid by accompanying proteins in the cause of unspecific binding.

#### 4.6.2.9.1 _Effect of using "mock protein" from empty bacterial vectors as coating antigen_

To assess the possibility of serum antibodies reacting to any substances carried into the antigen preparation during the production and purification process, we performed a full transformation, production and purification process using the plasmid pLys we had employed for cloning and expression, but without any insert. The plasmid and transformed BL21 cells were treated exactly as the ones containing the genomic information for the CDV N protein, and incubation, harvest and purification of mock protein also followed the exact protocol employed for N protein production. The eluate was kept in the same buffer and used under the same conditions as the N protein used as an antigen (Figure 46).
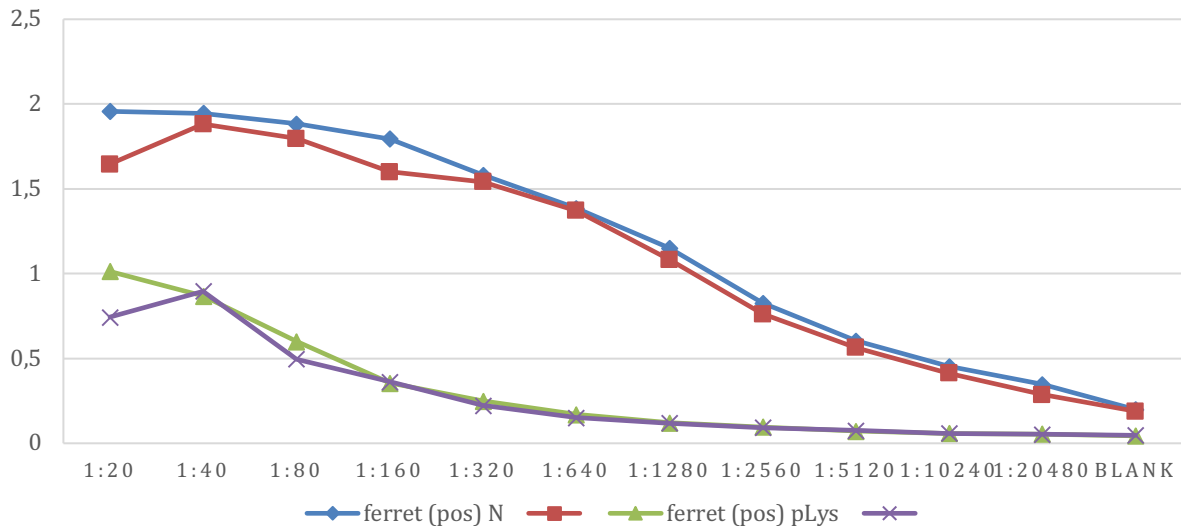
**Figure 46: Coating with N protein versus a mock protein preparation using empty plasmid pLys to assess possible reasons for unspecific binding.** Comparison of assays using identical serum samples (standard positive control) and secondaries, run on plates coated with N protein (red and blue lines) or a mock-preparation using plasmid pLys (green and purple lines). Although the background noise resulting from the preparation from the empty plasmid gives significant readout values for the lowest serum dilutions, it quickly reaches "blank" values and should not influence readout values in actual assays.

The data obtained in this experiment suggest that while unspecific binding to substances in the mock-preparation does contribute significantly to ODs formed at lower serum dilutions, this effect disappears fairly rapidly, and should not influence the results of titer determination or discrimination of positive versus negative sera. It also does not appear to contribute significantly to high background ODs, since blank values are equivalent to those gained when plates filled with plain ddH2O are measured.

### 4.6.2.9.2 *Effect of attempting to reduce unspecific binding by pre-absorbtion of serum samples against bovine serum albumin beads*

Since plates were routinely blocked using BSA, we questioned whether the unspecific and variable binding and relatively high background we observed could be due to serum samples binding to BSA. In order to test this, we coupled BSA to cyanogen bromide (CnBr)-activated

sepharose beads (GE Healthcare) following the manufacturer's instructions, and pre-absorbed serum samples with those beads prior to using them in an ELISA. Any serum components binding to BSA should this way remain bound to the beads, and the post-absorption serum should not show any unspecific binding with the blocking agent during the ELISA (Figure 47).
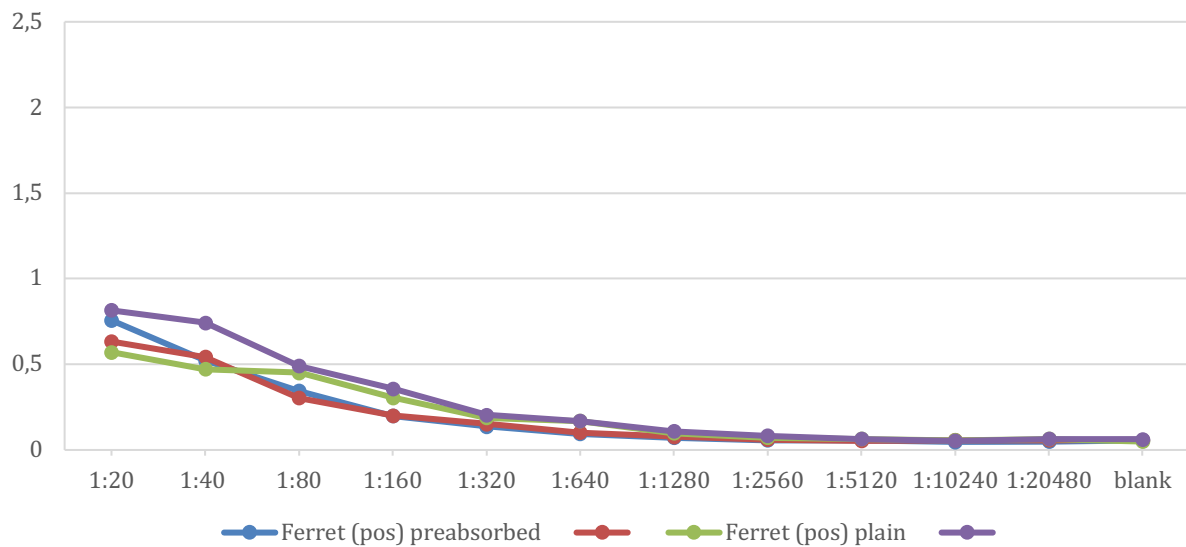


**Figure 47: Enzyme-linked immunosorbant assay using plain serum and serum pre-absorbed with bovine serum albumin beads in an attempt to reduce unspecific binding.** N protein was used as antigen, and the same known N-positive serum was used in serial dilutions in plain (red and blue lines) or pre-absorbed (green and purple lines) form to attempt to remove unspecific signal. There is no visible difference between the two conditions.

Incubating serum samples with BSA-coupled sepharose beads prior to use within the routine ELISA protocol did not make a noticeable difference to the resulting ODs or background values. It can hence be concluded that unspecific binding to the blocking agent is not the cause of the high background and variability during assay development.

*4.6.2.9.3* *Effect of attempting to reduce unspecific binding by pr-eabsorbtion of serum samples*

*against bacterial lipopolysaccharide*

Since the protein employed as antigen during the ELISA development process was derived from bacterial lysate, it was questioned if unspecific binding of serum components to bacterial LPS might be a cause for the high background values and variability of this assay. So a second batch of sepharose beads was coupled to LPS (Sigma-Aldrich), and serum samples were again incubated with the coupled beads prior to use in CDV N protein ELISA (Figure 48).
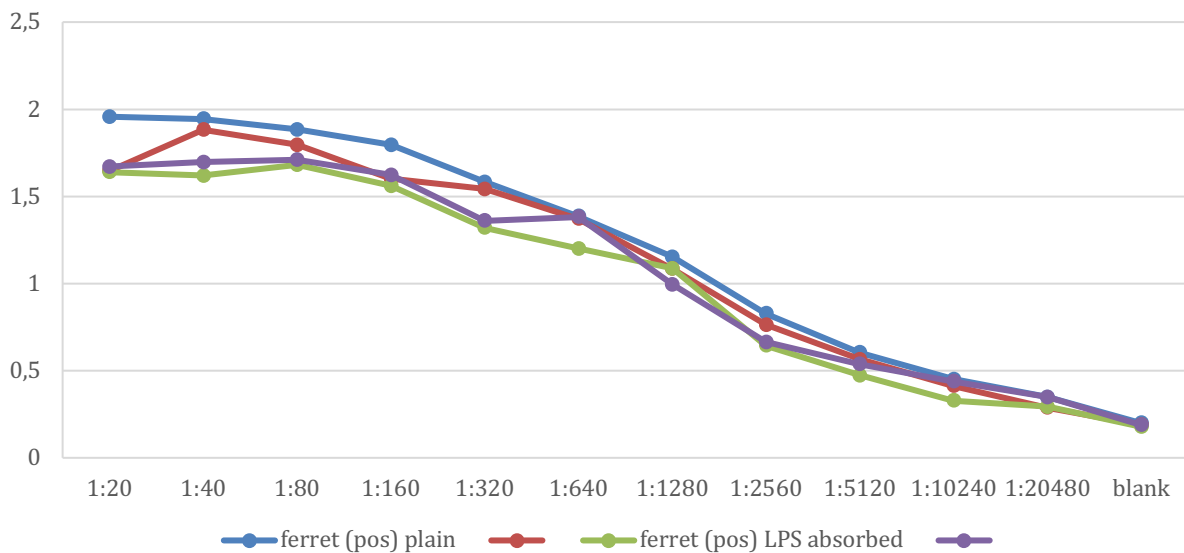


**Figure 48: Enzyme-linked immunosorbant assay using plain serum and serum pre-absorbed with beads coated with bacterial lipopolysaccharide in an attempt to reduce unspecific binding.** N protein was used as antigen, and the same known N-positive serum was used in serial dilutions in plain (red and blue lines) or pre-absorbed (green and purple lines) form to attempt to remove unspecific signal. There is no visible difference between the two conditions.

To further explore the possible influence of LPS on serum ODs, we also used LPS at a concentration of 10 ug / well as coating antigen, replacing N protein in the routine ELISA protocol (Figure 49).
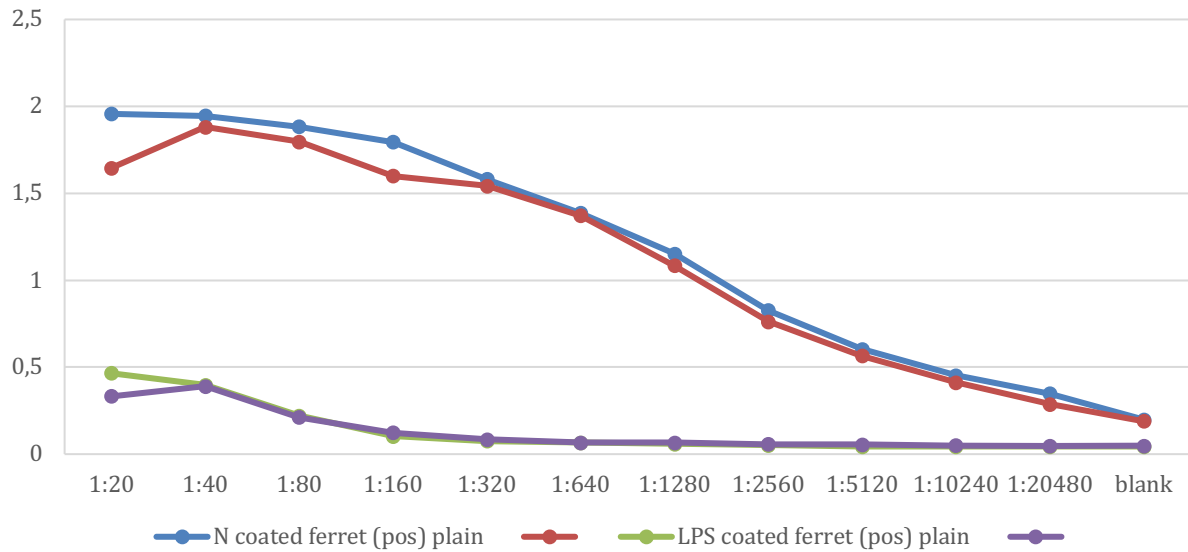
**Figure 49: Assay using N protein positive serum used on a plate coated with either N protein or bacterial lipopolysaccharide to evaluate the source of unspecific signal.** Comparison of readouts achieved when running the standard positive control serum on a plate coated with distemper N protein (red and blue lines) or with bacterial lipopolysaccharide (green and purple lines). There is a very clear distinction between the two conditions, indicating that unspecific binding against bacterial lipopolysaccharide is not an issue.

Pre-treatment of sera with LPS-coupled sepharose beads did not result in any reduction of ODs. Similarly, the use of LPS as a coating antigen did not give rise to significant ODs, and background values were equivalent to those of plain ddH$_2$O. This suggests that unspecific binding to LPS does not play a role in causing the relatively high background ODs and variability encountered in developing this assay.

### 4.6.3 Development of an enzyme-linked immunosorbant assay to detect and quantify antibodies against distemper H protein

While during infection with CDV the largest percentage of antibodies produced is directed against CDV N, which can be used for the detection of an established infection, antibodies directed against the CDV H protein are of interest due to their ability to neutralize viral particles. Antibodies against CDV H are therefore critical for the control of a CDV infection.

We attempted to establish an ELISA assay for the semi-quantitative detection of antibodies against CDV H from ferret blood samples, with the aim to explore responses in both established as well as recent infections, by testing for anti-H IgG and IgM, respectively.

### 4.6.3.1 Antigen production and preparation

CDV H protein production in eukaryotic cells was attempted, but again likely due to its viral origins expression failed due to severe cytotoxicity. CDV H protein was subsequently expressed in *E. coli* BL21 DE3 cells as described previously (284). Presence and purity of the protein were confirmed as for N protein.

### 4.6.3.2 Development of assay protocol and determination of suitable antigen dilutions

Detection of antibodies against CDV H was attempted using an indirect ELISA, as described above (Chapter 4.6.1) and modified from Chan et al., (284). Most effective dilutions of coating antigen were again determined using checkerboard titrations (see Chapter 4.6.2.4 for details). Repeated checkerboard assays showed that a dilution of 1 : 2.5 to 1 : 5 antigen stock : buffer slightly outperformed the other dilutions tested. While all dilutions produced very nice curves, a 1 : 2.5 and 1 : 5 dilution gave very slightly higher maximum ODs and the titration curves dropped slightly slower than the others (Figure 50 and Figure 51). In light of the high similarity of curves produced and in an attempt to conserve antigen, a dilution of 1 : 5 was chosen for further assay development. The possibility of increased background and unspecific binding at lower dilutions was also taken into consideration.
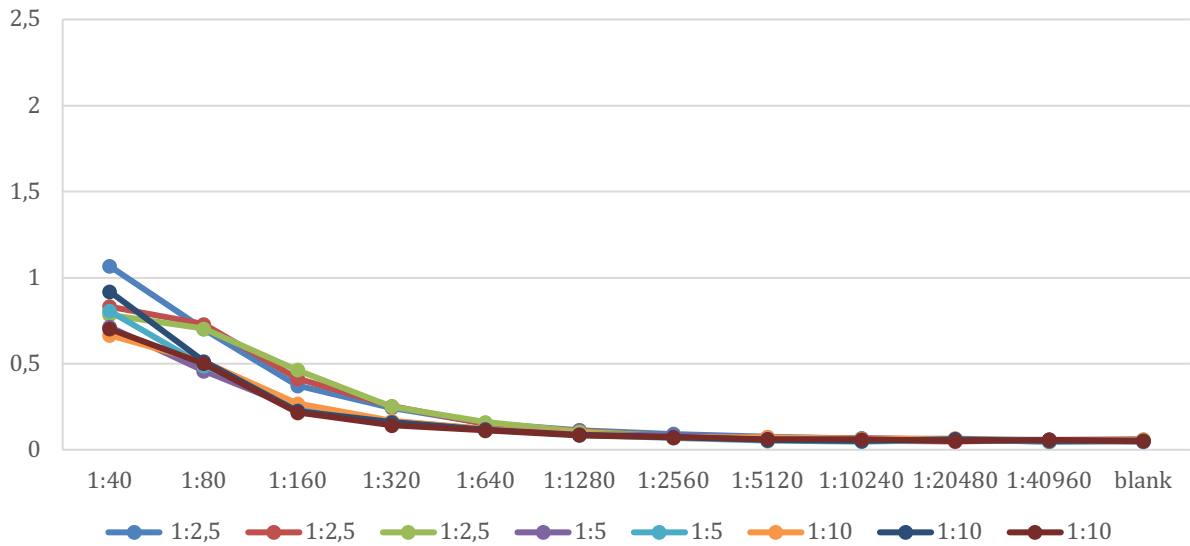
**Figure 50: Determining suitable concentrations of distemper H protein as a coating antigen, low protein dilutions.** Checkerboard titration of distemper H protein diluted 1 : 2.5, 1 : 5 and 1 : 10 against 2-fold serial dilutions of distemper positive ferret serum. Note higher readouts but also higher intra-assay variability of the 1 : 2.5 dilution. Also note overall low readout values. Also compare Fig. 50 for further data on 1 : 5 dilutions.
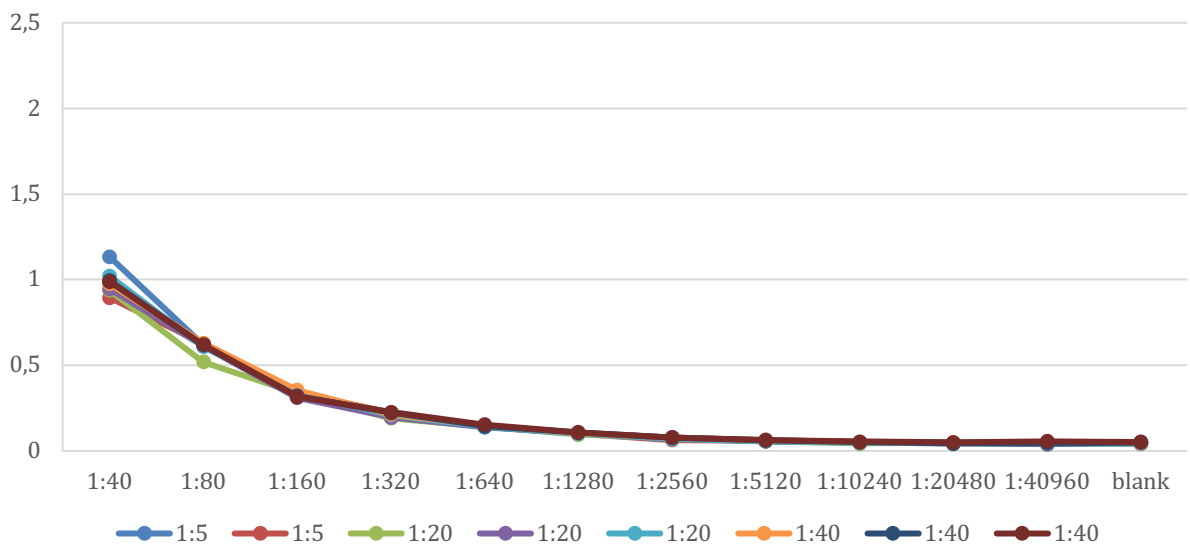


**Figure 51: Determining suitable concentrations of distemper H protein as a coating antigen, high protein dilutions.** Checkerboard titration of distemper H protein diluted 1 : 5, 1 : 20 and 1 : 40 against 2-fold serial dilutions of distemper positive ferret serum. Note slightly higher maximum higher readouts of the 1 : 5 dilution compared to the higher dilutions, but still overall very low values.

*4.6.3.3 Determination of suitable positive and negative controls*

Within the same dot blot described in Chapter 4.6.2.7.1 we also tested serum samples from CDV vaccinated or naïve ferrets for their reactivity to CDV H protein. Samples shown to be clearly positive and clearly negative were subsequently used as positive and negative controls during assay development. For examples of their use, see Figure 52 here and in Chapter 4.6.2.8, "Secondaries". Distinctions of positive and negative sera were clearly visible for lower dilutions of sera, however, titration curves approached each other quickly, making differentiation based on titer difficult.
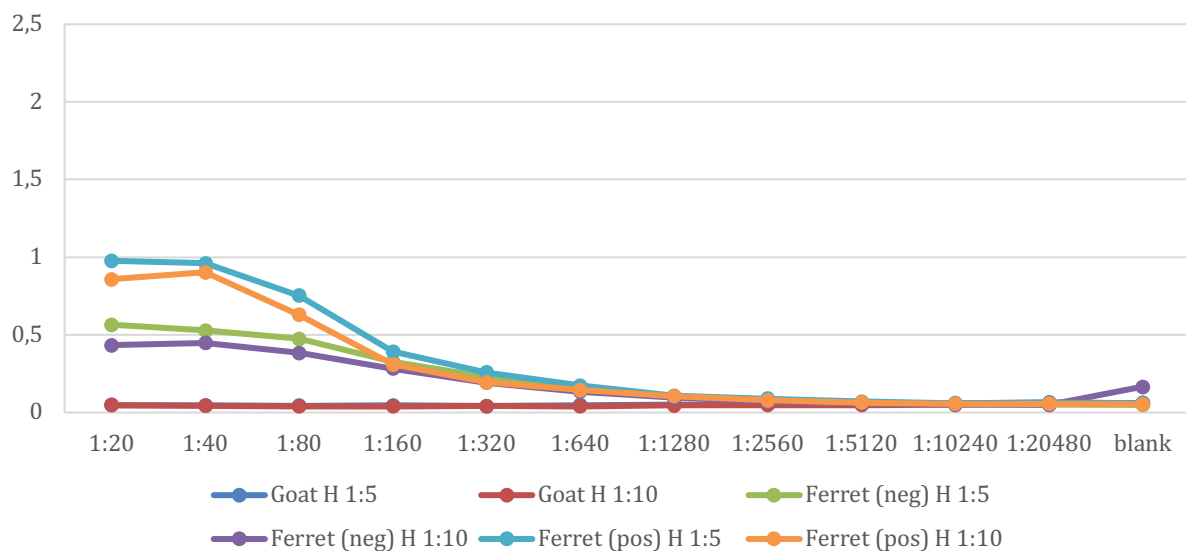


**Figure 52: Evaluation of known positive versus negative ferret sera in an enzyme-linked immunosorbant assay using two different dilutions of distemper H protein.** Antigen dilutions used were 1 : 5 and 1 : 10. Goat serum was used as a negative control (blue and red lines). Positive serum 1 : 5 teal line, 1 : 10 orange line. Negative serum 1 : 5 green line, 1 : 10 purple line. Distinction between positive and negative samples is visible but only in very low serum dilutions, and the overall readouts are very low.

*4.6.3.4 Determination of suitable Inter-species controls to control against unspecific binding*

To control for unspecific binding of ubiquitous serum components, serum samples of unrelated species were used as negative assay controls. Samples from rabbit and goat were

employed for reasons detailed above (see Chapter 4.6.2.7). After the results of testing rabbit serum as a negative control cast doubt on the viability of the assay, nonhuman primate serum was not tested.
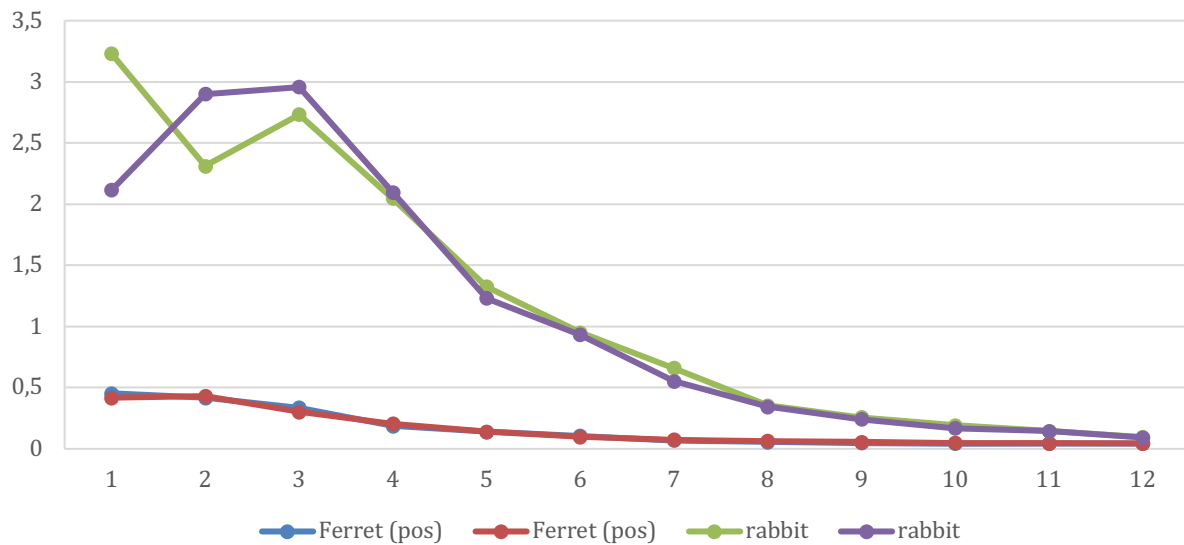
**Figure 53: Evaluating the use of naïve rabbit serum as a negative control in distemper H protein enzyme-linked immunosorbant assays.** Comparison of readouts for a known-positive ferret serum and serum of a distemper-naïve rabbit with species-specific secondaries in an assay using distemper H protein as the antigen. Note the y-axis maximum is 3.5 for this graph. Also note the serum from a distemper negative rabbit gave up to seven times higher readouts than a blot-positive ferret serum. Titration is also evident. Rabbit serum will not be useful as a negative control due to this high unspecific binding.

The use of CDV naïve rabbit serum in CDV H ELISAs consistently resulted in ODs five to eight times higher than the CDV positive ferret control serum (Figure 53). The reasons for this are not known.

4.6.3.4.2   *Effects of using goat serum as a control*

Goat serum was again used with an anti-ferret secondary as described above (Chapter 4.6.2.7.4). This gave very low ODs as was expected for this negative control (Figure 54).
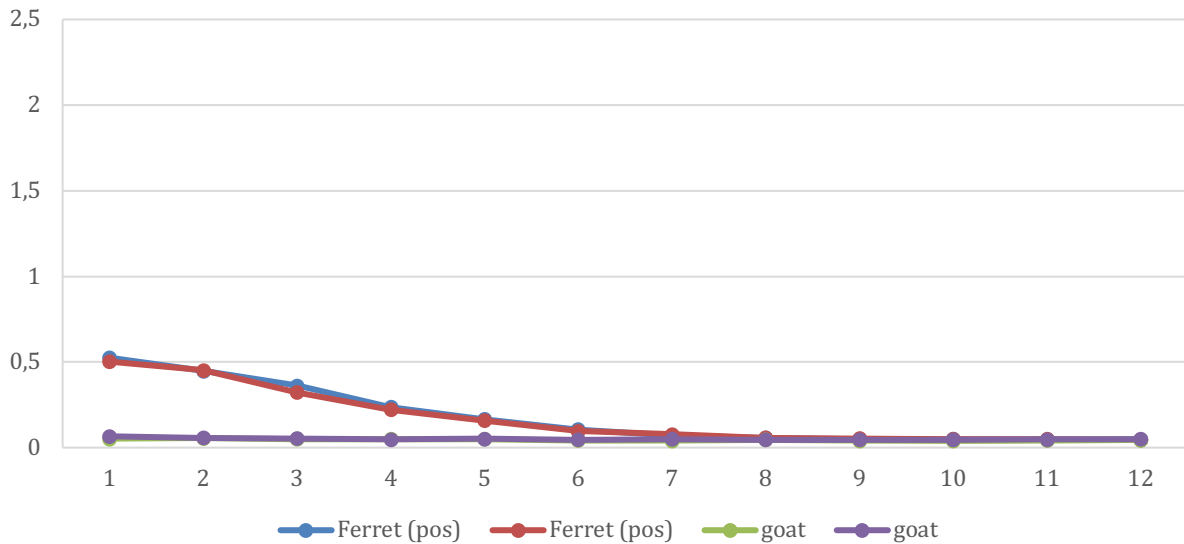
**Figure 54: Evaluating the use of naïve goat serum as a negative control in distemper H protein enzyme-linked immunosorbant assays.** Comparison of readouts for a known-positive ferret serum and serum of a distemper-naïve goat with species-specific secondaries in an assay using distemper H protein as the antigen. Note the consistent near-zero readouts for the goat serum. This serum will be useable as a negative control.

### 4.6.3.5   Use of serum versus heat-inactivated serum versus plasma

As with CDV N protein, we also assessed the CDV H ELISA for differences between the use of serum, heat-inactivated serum or plasma as a source of primary antibodies (Figure 55). Heat-inactivated serum produced slightly higher ODs for a known positive sample. Sera were routinely heat-inactivated for storage so ELISAs against CDV H would just be performed after heat inactivation.
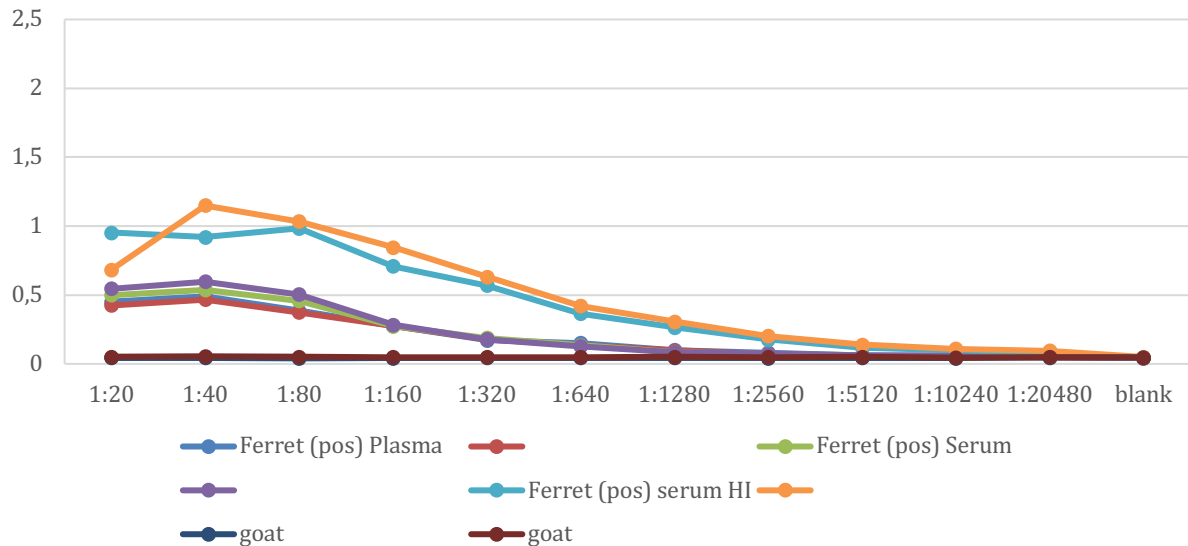
**Figure 55: Comparison of readouts for plasma, serum and heat-inactivated serum (serum HI) from the same animal in an enzyme-linked immunosorbant assay using distemper H protein as an antigen.** The ferret was confirmed to be distemper H positive in a previous blot assay. (Plasma red and blue lines, serum green and purple lines, heat-inactivated serum teal and orange lines.) Goat serum with anti-ferret secondary served as a negative assay control (dark blue and burgundy lines). This combination gave suitably low readouts. Heat-inactivated serum resulted in the highest readouts and low variability in higher serum dilutions. Plasma and serum showed low variability but much lower readouts.

### 4.6.3.6   Comparison of different secondary antibodies

To find the most suitable secondary antibodies, we tested all the anti-IgG secondaries evaluated above (Chapter 4.6.2.8) for their use in H protein ELISAs. The HRP conjugated goat-anti ferret IgGs made by Bethyl and Novus gave best results again, very similar to their behavior in CDV N ELISAs (Figure 57). Of these two brands, Novus anti-ferret IgG was chosen as standard secondary for economic reasons. The overall lower ODs in these assays compared to the CDV N assays are very likely due to the much lower amount of CDV H antibodies being produced during CDV infection.
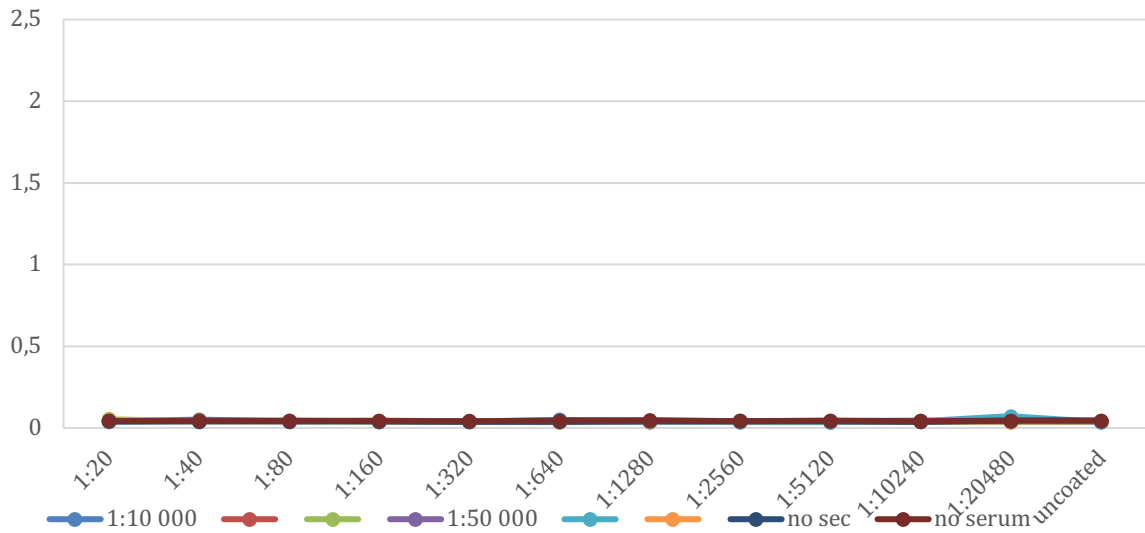
**Figure 56: Enzyme-linked immunosorbant assay against distemper H protein using Lifespan horseradish peroxidase conjugated secondary antibody at two dilutions.** This assay employed a constant concentration of antigen, serial 2-fold dilutions of a known positive serum, and Lifespan goat anti-ferret IgG conjugated to HRP at two different dilutions as recommended by the manufacturer. Note the near-zero readout values for all conditions. This secondary antibody did not function.

The secondary antibody provided by Lifespan did not recognize any ferret antibodies at all, giving ODs equivalent to those of ddH2O and not showing any titration of serum (Figure 56).
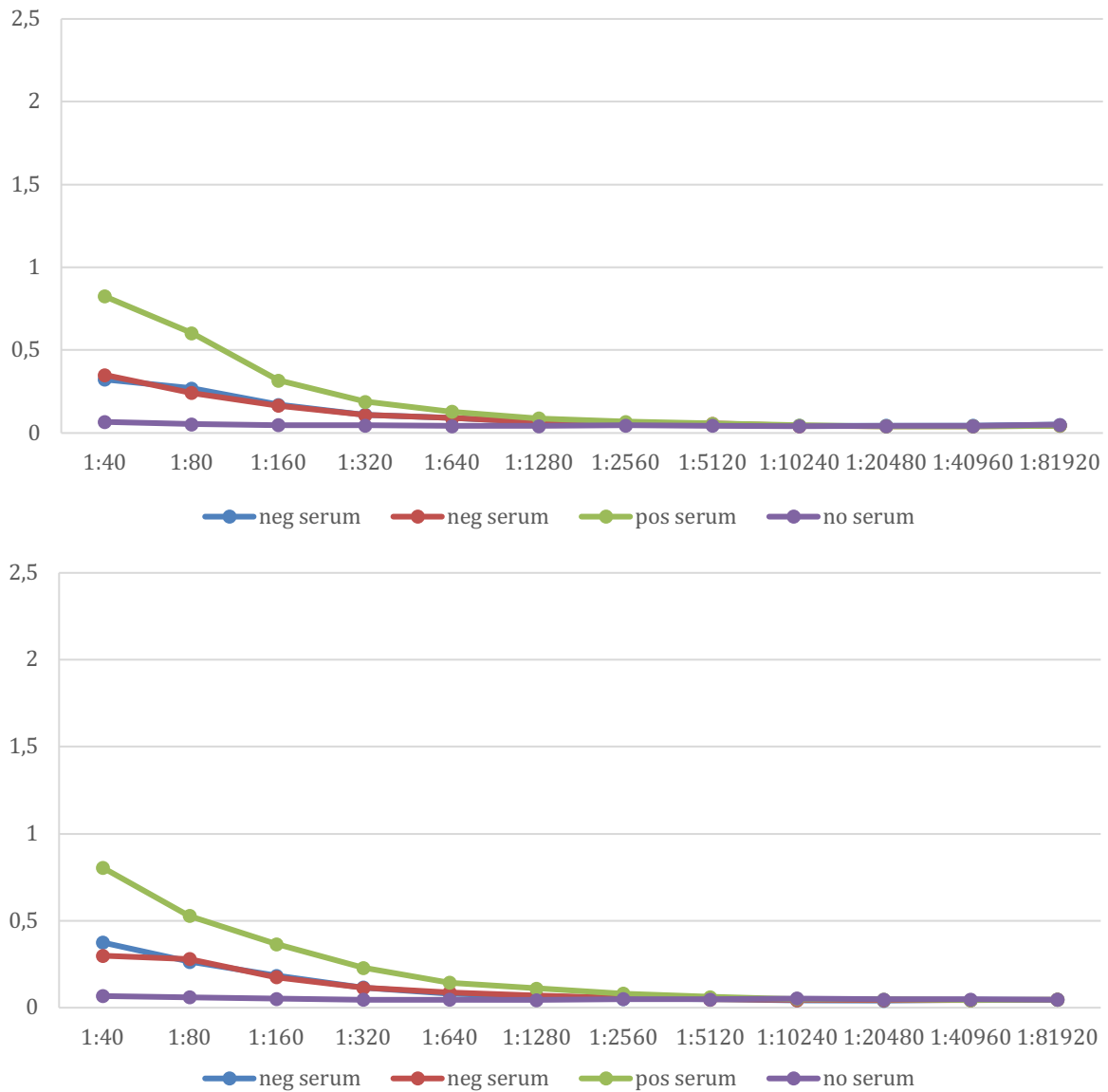
**Figure 57: Direct comparison of horseradish peroxidase-conjugated anti-ferret IgG from Novus (top graph) and Bethyl (bottom graph).** Distemper H protein at constant dilution and confirmed positive (green lines) and negative (blue and red lines) ferret sera in 2-fold dilutions were used for this assay. The secondary antibodies were both used at a dilution of 1 : 10,000 as recommended by the manufacturer. A row with no serum was used as a negative control (purple line). A comparison was made with regards do overall readouts, discrimination of positive and negative samples, and "no serum" background readouts. Note the near-identical results, giving good discrimination of positive and negative samples at lower serum dilutions, and very low background.

We also assessed secondary goat anti-ferret antibodies directed against ferret IgM or ferret IgM/A/G (both made by Acris), to have the option to confirm the status of infection early on after vaccination (Figure 58, Figure 59 and Figure 60).

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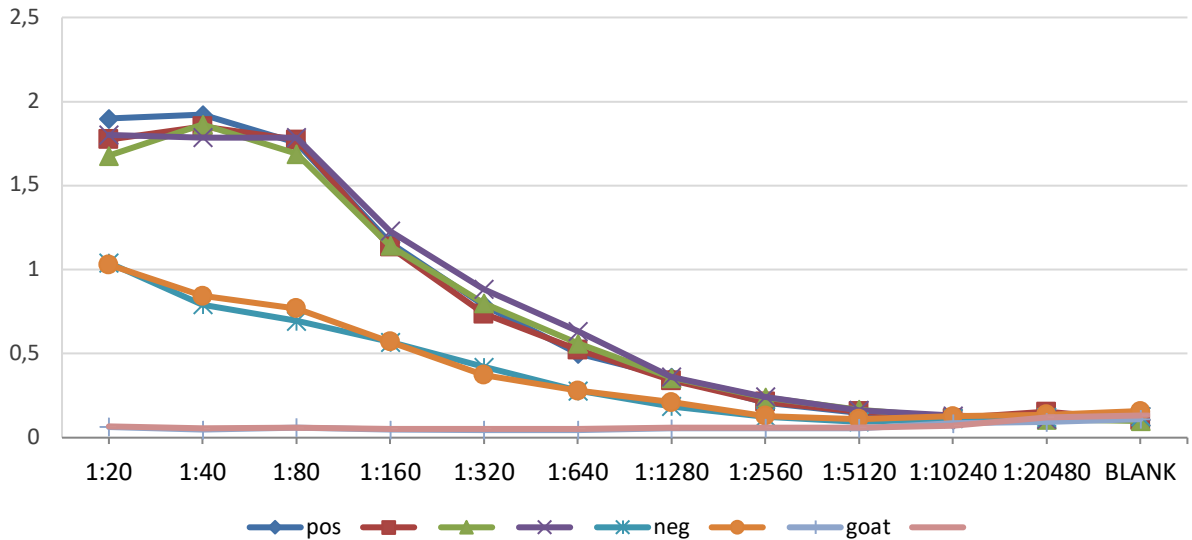
**Figure 58: Test of an anti-ferret IgM secondary antibody at a 1 : 10,000 dilution in an enzyme-linked immunosorbant assay against distemper H protein.** Blot confirmed positive (blue, red, green and purple lines) and negative (teal and orange lines) ferret serum were used as primaries, as well as goat serum as an assay control, with goat anti-ferret IgM (Acris), dilution 1 : 10,000 (light blue and pink lines). This secondary resulted in relatively high overall readouts and a very good distinction between positive and negative samples with comparably low background. It would be very useful for this assay.
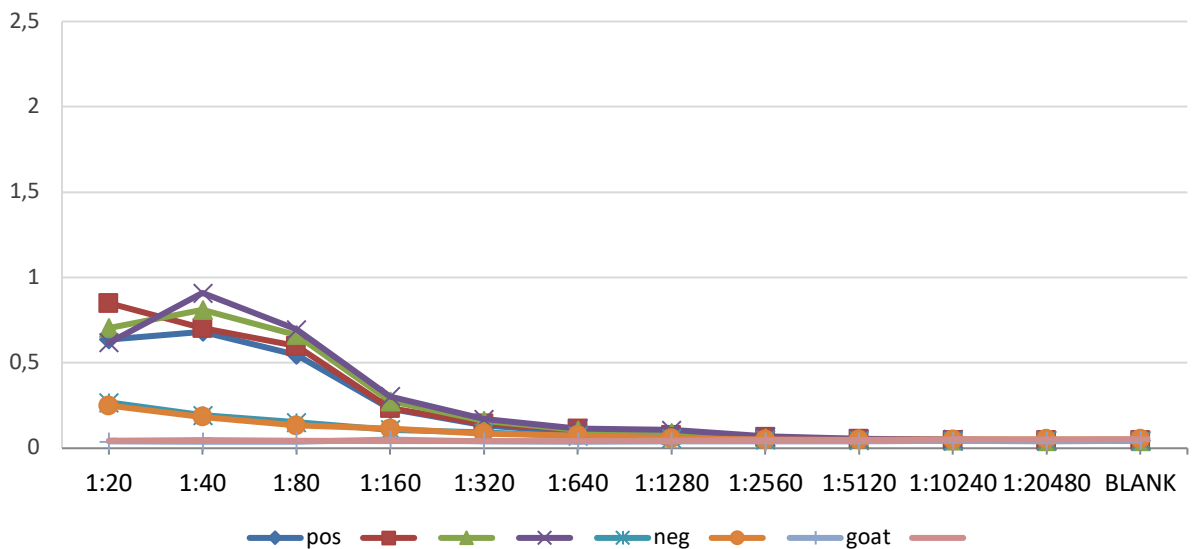


**Figure 59: Test of an anti-ferret IgM secondary antibody at a 1 : 50,000 dilution in an enzyme-linked immunosorbant assay against distemper H protein.** Blot confirmed positive (blue, red, green and purple lines) and negative (teal and orange lines) ferret serum were used as primaries, as well as goat serum as an assay control, with goat anti-ferret IgM (Acris), dilution 1 : 50,000 (light blue and pink lines). This higher dilution of the same antibody as above resulted in acceptable distinction between positive and negative samples, but only for low serum dilutions and with overall low readouts.

The Acris anti ferret IgM secondary antibody in an assay directed against CDV H resulted in good quality ODs and low intra-assay variability while enabling discrimination of positive and negative sera based on titration curve, if maybe not based on titer, if used at the 1 : 10,000 dilution. Used at 1 : 50,000 dilution, the intra-assay variability became high and overall ODs low, resulting in limited use of this condition.
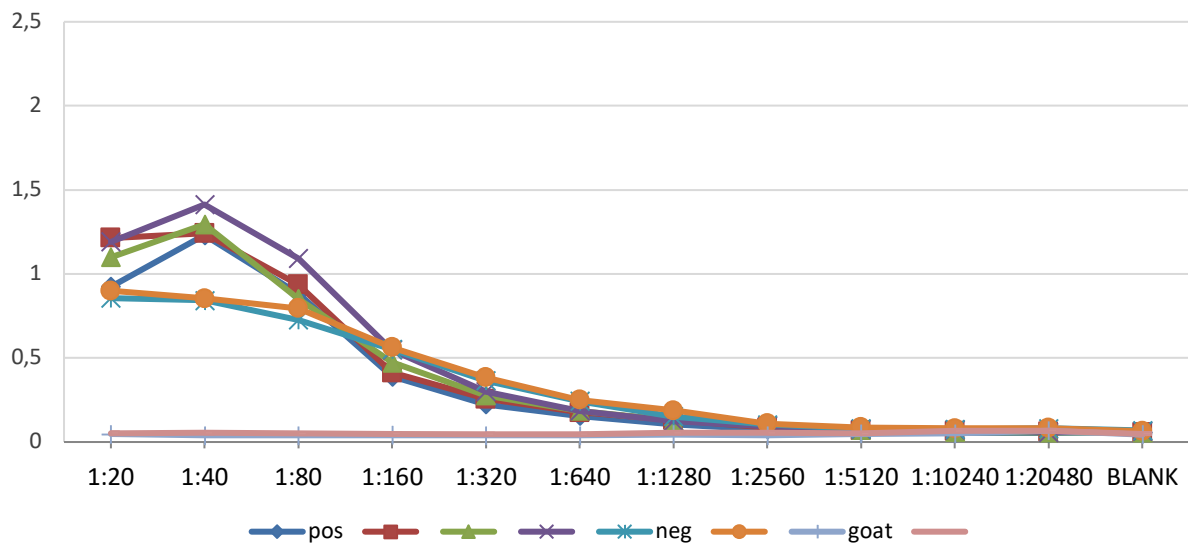


**Figure 60: Test assay using an anti-ferret IgM/G/A antibody at a 1 : 10,000 dilution. Enzyme-linked immunosorbant assay** against distemper H protein using positive (blue, red, green and purple lines) and negative (teal and orange lines) serum, as well as goat serum as an assay control, with goat anti-ferret IgM/G/A (Acris), dilution 1 : 10,000 (light blue and pink lines). This secondary does not distinguish between positive and negative samples.

The anti IgM/A/G antibody made by Acris was not able to discriminate positive or negative sera in an ass using CDV H protein.

### 4.6.3.7  Alternative strategies to pinpoint and reduce unspecific binding

As with CDV N protein (see Chapter 4.6.2.9), we assessed CDV H ELISAs for possible sources of unspecific background, by coating with a mock-preparation of the empty plasmid, which gave identical results to what was already seen in the corresponding section above, with

values for positive serum (at this point not heat inactivated yet) used against CDV H resulting in typical ODs around 0.5, while the values for positive serum used against empty plasmid being a significant (albeit on the graph barely visible) 20% less in the region of 0.4. It also confirmed again the previously seen very high ODs for naïve rabbit serum, even just on a plate coated with a preparation made from an empty plasmid (Figure 61).
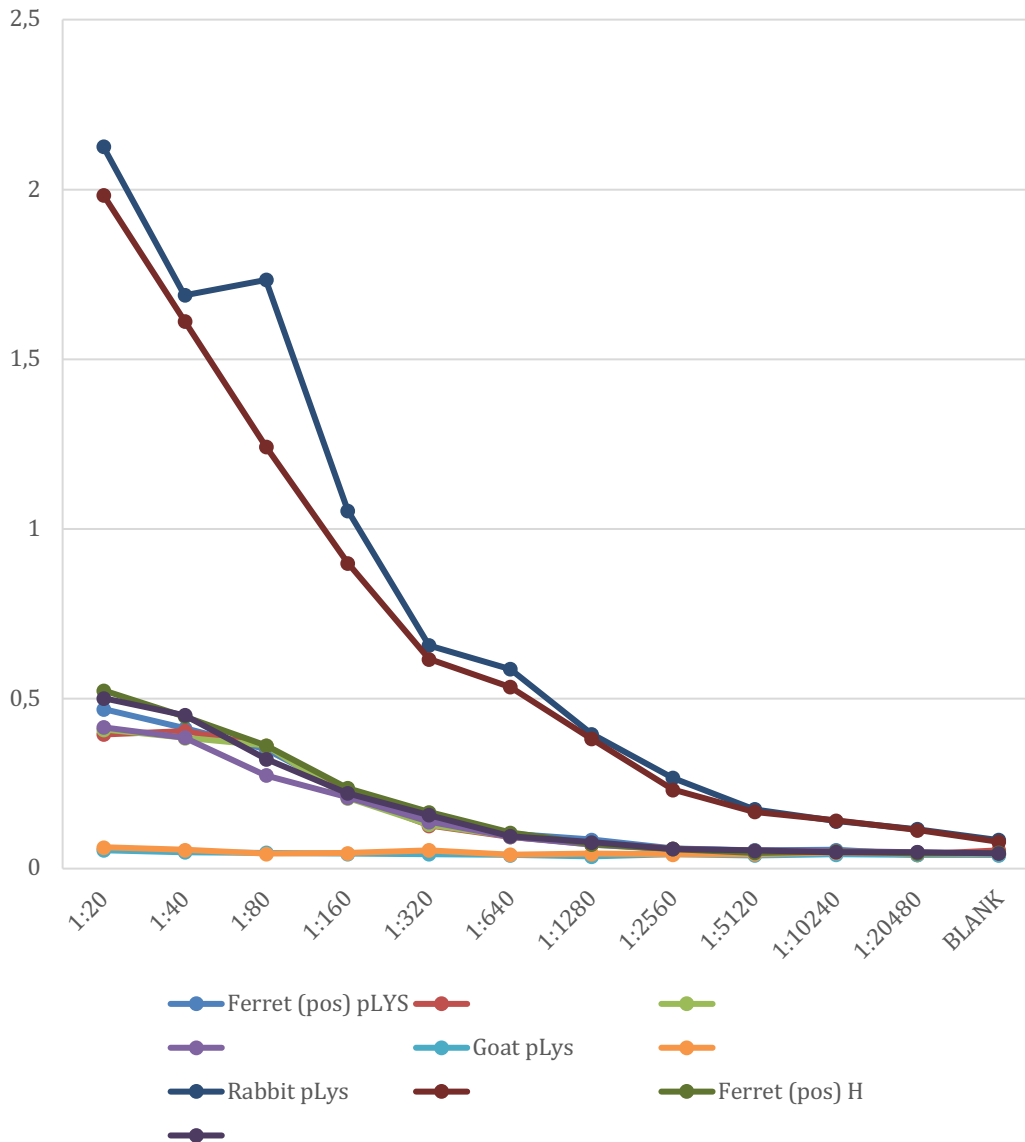
**Figure 61: Enzyme-linked immunosorbant assay using ferret, goat and rabbit serum against a mock protein purification of empty plasmid pLys as an antigen, and using confirmed-positive ferret serum against distemper H protein as an antigen to evaluate origins of unspecific binding.** Of particular interest is the comparison between known-positive ferret serum tested against distemper H protein (dark green and dark purple lines) and the same serum tested against the mock preparation from the empty plasmid (blue, red, green and purple lines), both using and anti-ferret secondary. The goat serum tested against the mock preparation with an anti-ferret secondary (teal and orange lines) and the rabbit serum tested against the mock preparation with an anti-rabbit secondary (dark blue and dark red lines) served as assay controls. Note the rabbit serum gave the highest readouts, indicating a strong unspecific reactivity of rabbit serum against peptides contained in the empty plasmid preparation. Further note the near identical graphs of both conditions using ferret serum. A distinction between samples tested against distemper H protein and those tested against the mock preparation cannot be made. This suggests that the signal obtained when using the distemper H protein as antigen is largely unspecific.

*4.6.3.8   Examples of enzyme-linked immunosorbant assays against distemper H protein*

*using study serum samples*

A test assay was run against the CDV H protein using serum samples gained on D0 and D7 of our first study. Samples from D0 were collected before inoculation, and represent naïve animals. Due to the short time frame of the study, we expected to likely not see IgG being formed yet, so samples were assessed for presence of IgM as well. See Figure 62, Figure 63 and Figure 64 for the results.
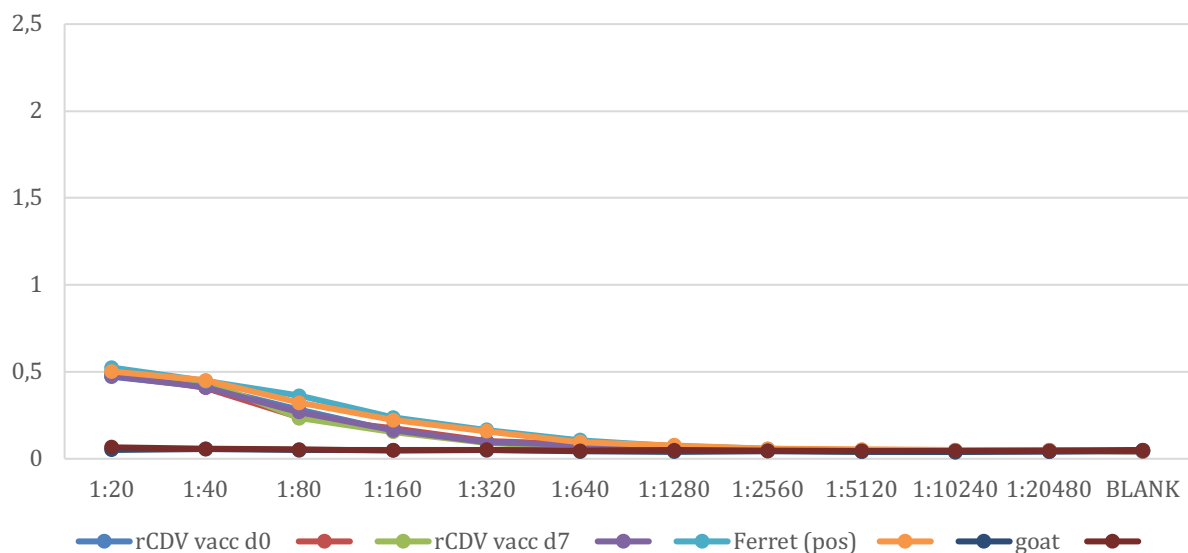


**Figure 62: Enzyme-linked immunosorbant assay against distemper H protein using serum from a recombinant distemper vaccinated ferret, detecting IgG in samples obtained on d0 and d7 post inoculation.** Serum samples from d0 (red and blue lines) and d7 (green and purple lines) were compared with a known-positive ferret serum sample (teal and orange lines), and a goat serum negative control (dark blue and burgundy lines). Note that samples obtained on d0 resulted in near-identical readouts to samples from d7 and positive control samples. The ferrets enrolled in the study were previously confirmed to not possess antibodies against distemper. The d0 samples should have provided much lower readouts.

The results of these trial runs showed surprisingly high ODs for the test samples. ODs for both the D0 as well as the D7 sample were near identical to the OD of the positive control serum. This applied to all animals tested, including the mock-inoculated animals. Since we

had previously assessed our positive control serum via dot blot and found it strongly positive, the assumption was that the ODs for the positive serum were correct. However, the exact same serum had in previous assays (see Figure 52 in Chapter 4.6.3.3 "Positive and negative controls") under the same conditions resulted in ODs of around 1.0, twice as high as in the test assays with study samples. In the assays comparing positive and negative sera, it was the negative sera that presented ODs of around 0.5. Since the entire batch of positive control serum was aliquoted and labelled at the same time after confirming its status, and had still produced ODs of around 0.5 rather than 1.0 in several assays, a mix up of samples could be excluded.

The use of anti-IgM secondary to analyze appearance of early-appearing IgM antibodies revealed a similar picture and confirmed again the variability of ODs reached by the known-positive serum when assayed against CDV H. See Figure 63 and Figure 64 below for a comparison of one mock treated and one rCDV vaccinated animal, tested in the same run, using the exact same positive control.
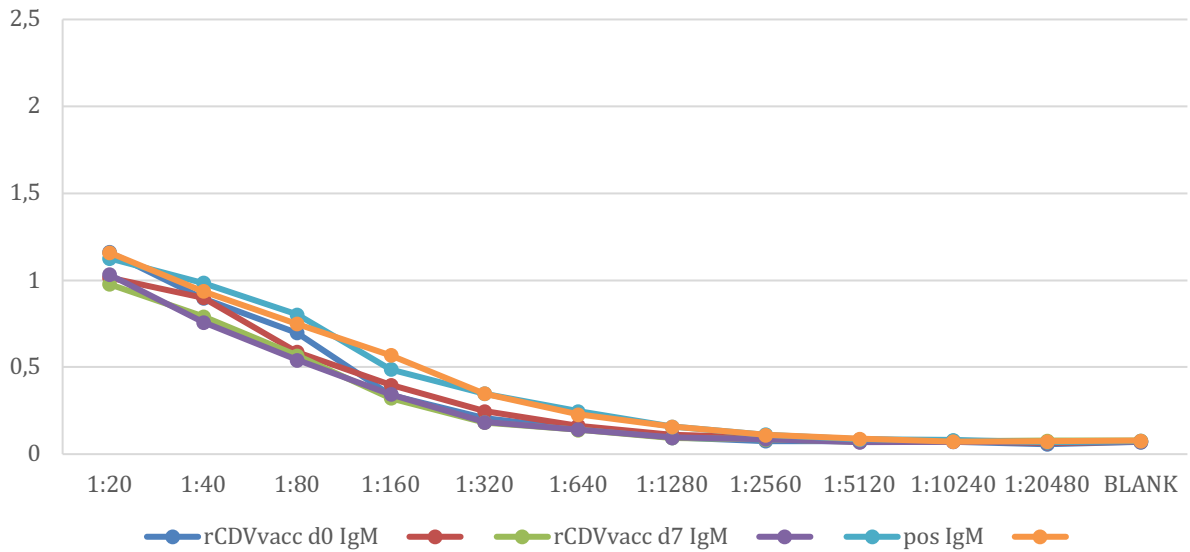
**Figure 63: Enzyme-linked immunosorbant assay against distemper H protein using d0 and d7 serum from a ferret vaccinated with recombinant distemper, detecting IgM.** Serum samples from a ferret vaccinated with recombinant distemper, collected at d0 (red and blue lines) and d7 (green and purple lines) post vaccination, compared with results of a known-positive serum sample (teal and orange lines). Note again the near-identical results for all samples. Since ferrets enrolled in the study were previously confirmed to not possess antibodies against distemper. Samples obtained on d0 should produce much lower readouts.
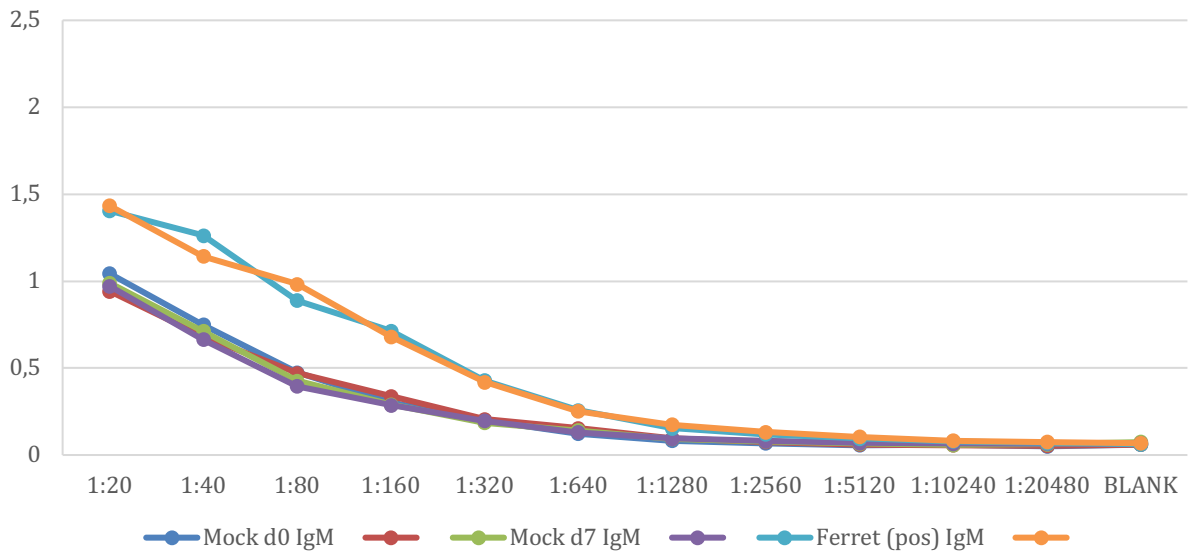


**Figure 64: Enzyme-linked immunosorbant assay against distemper H protein using d0 and d7 serum samples from a mock vaccinated control ferret and an IgM positive control, detecting IgM.** Assay using test sera of a control ferret obtained on d0 (red and blue lines) and d7 (green and purple lines) as well as a known positive serum (teal and orange lines). Note readouts for positive control being higher than in Figs 62 and 63, while remaining readouts are very similar, despite the same control sample being used, and despite the study ferret being a mock-vaccinated control animal.

### 4.6.4 Development of an enzyme-linked immunosorbant assay to detect and quantify antibodies against simian immunodeficiency virus glycosaminoglycan protein

#### 4.6.4.1 Description of assay protocol and antigen dilutions

SIVgag ELISAs were set up according to the standard protocol used in the lab. This protocol was successfully adapted for several laboratory animal species prior to the use in ferrets. It was also, apart from the changed antigen, identical to the protocol for detecting antibodies to HIVgag in sera from pre-clinical studies.

For this protocol, Costar or Nunc Maxisorp plates were coated with SIVgag p55 protein diluted to 2.5 ug / mL in PBS for an hour. After washing, plates were blocked with PBS containing 3 % BSA (w/v) for 1 h. They were then washed and serum samples and controls added in serial dilutions. After incubating the plates were washed and species-specific labeled anti-IgG or IgM secondary antibodies were added. Plates were again incubated and washed, then substrate was prepared and added to all wells, and after appropriate development intervals, plates were read in an automated reader.

#### 4.6.4.2 Origin of antigen and monoclonal antibodies used during the assay

The SIVp55 protein used as an antigen is a full-length recombinant protein derived from a baculovirus expression system and commercially available through Protein Sciences Corp. Occasionally, an unconjugated monoclonal antibody against a proteolytic fragment of gag p55, the smaller protein p27, was used as a positive control or capture antibody. This antibody was the mouse anti-SIV mac251 strain p27 gag mAb, clone SF8-6F5, sourced from Advanced Biotechnologies Inc..

### 4.6.4.3 Determination of suitable secondary antibodies

For ferret samples, we continued to employ the secondaries we had successfully established during CDV ELISA development. Secondaries for use with control sera (Nonhuman primate and rabbit) and monoclonal antibodies were used as recommended by the original assay protocol. Anti-Rhesus macaque IgG by Santa Cruz was used with nonhuman primate serum, and anti-rabbit IgG by Jackson was used with rabbit samples.

### 4.6.4.4 Determination of suitable positive and negative controls

Since no ferret sera confirmed positive for SIVgag were available to use as controls during assay development, we had to resort to positive controls originating from another species. This assay had originally been developed for use with nonhuman primate samples, and was very well established in this context. We were kindly provided with standardized nonhuman primate positive and negative control sera that were used for nonhuman primate assays (kindly supplied by A. Ginsberg, IAVI). In order to be able to validate results gained from ferret samples, we directly compared samples taken from the same animal but at different time points. D0 samples, taken prior to inoculation, served as the negative controls, and were used as a baseline to compare results of later time points of the same animal. A non-study ferret serum was also used as a negative control. Based on experiences during CDV assay development, we decided to include rabbit serum and anti-rabbit secondary as an interspecies negative control, and goat serum with anti-ferret secondary as the standard zero-value control (see Chapter 4.6.2.7 for details).
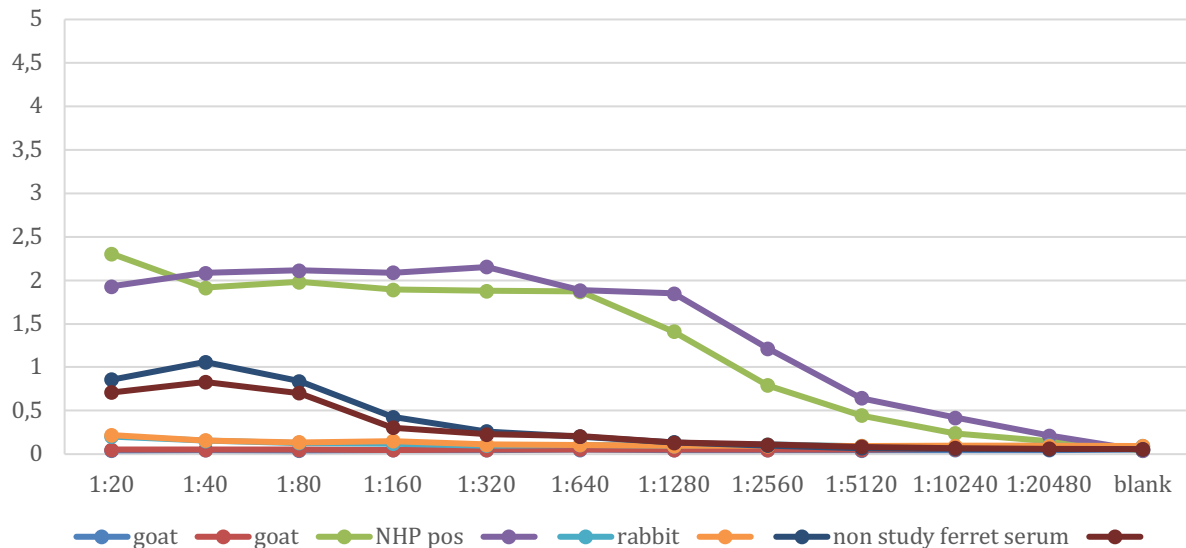
**Figure 65: Comparison of different assay controls in an enzyme-linked immunosorbant assay against simian immunodeficiency virus glucosaminoglycane.** Serum samples employed originated from goat (red and blue lines), a confirmed-positive nonhuman primate (green and purple lines), a naïve rabbit (teal and orange lines) and a naïve ferret (dark blue and burgundy lines). Goat serum as a negative control and a confirmed-positive nonhuman primate serum as a positive control worked very well. Note however the high readouts for the naïve non-study ferret sample. This sample should give much lower values. There appears to be a degree of unspecific binding present.

As can be seen in Figure 65, the positive control as well as both inter-species controls used in this assay worked very well. However, the ferret specific control, which consisted of serum of one unrelated, SIVgag naïve, non-study ferret, showed considerable maximum ODs and titration.

### 4.6.4.5 Effects of using plasma versus serum versus heat-inactivated serum

As for CDV ELISAs, we also attempted to establish which antibody source would best be used as a primary. This assay was established using serum, but since we had experienced two different results of this examination previously, we felt it interesting to check (Figure 66).
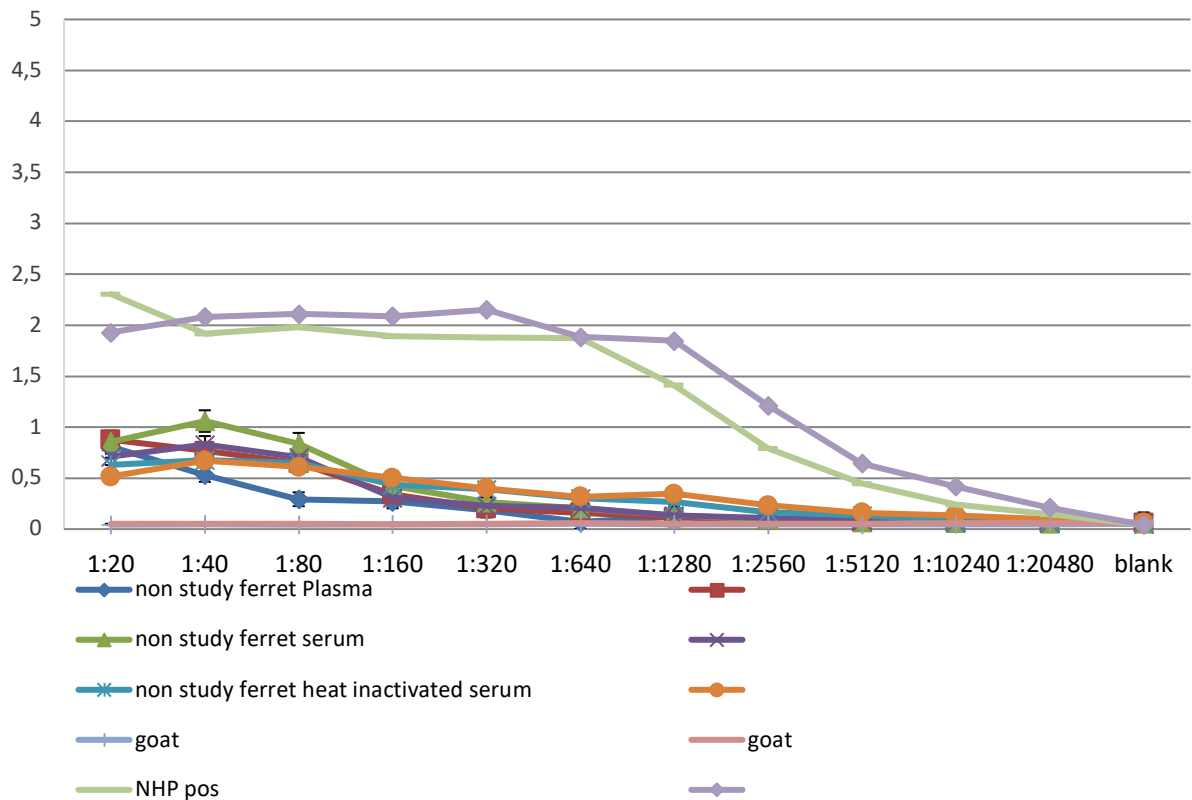
**Figure 66: Enzyme-linked immunosorbant assay against simian immunodeficiency virus glucosaminoglycane, comparing the use of plasma versus serum versus heat-inactivated serum.** Naive ferret plasma (blue and red lines), serum (green and purple lines) and heat inactivated serum (teal and orange lines) were used in twofold dilutions, employing naïve goat serum as a negative control (light blue and pink lines) and glucosaminoglycane antibody positive nonhuman primate serum as a positive control (light green and light purple lines). There was no notable difference between the three assay conditions, but all showed high variability and overall low readouts.

As seen in Figure 66, there were no major differences between the three different substrates, but all substrates showed a high degree of intra-assay variability, which would make it hard to get clear results.

### 4.6.4.6 Exemplary assay data

The data sets in this section serve to highlight the problems of non-reproducibility and reliability we encountered whilst attempting to develop this assay. Figure 67 demonstrates

issues encountered in establishing positive and negative controls. The naïve ferret serum

employed throughout this assay development came from an individual that was not a

member of any of our studies, and resided at a facility that does not have access to SIV, HIV,

or the individual immunogenic proteins thereof. In reference to Figure 68, all the data are

repeats of the same assay, using the same conditions and reagents. Of note are the very

varied ODs obtained for the same samples in the individual assays, the marked differences in

titration curves between assays, and the variable degree of intra-assay variation. The OD of

the naïve ferret negative control also continues to be at a significant level.
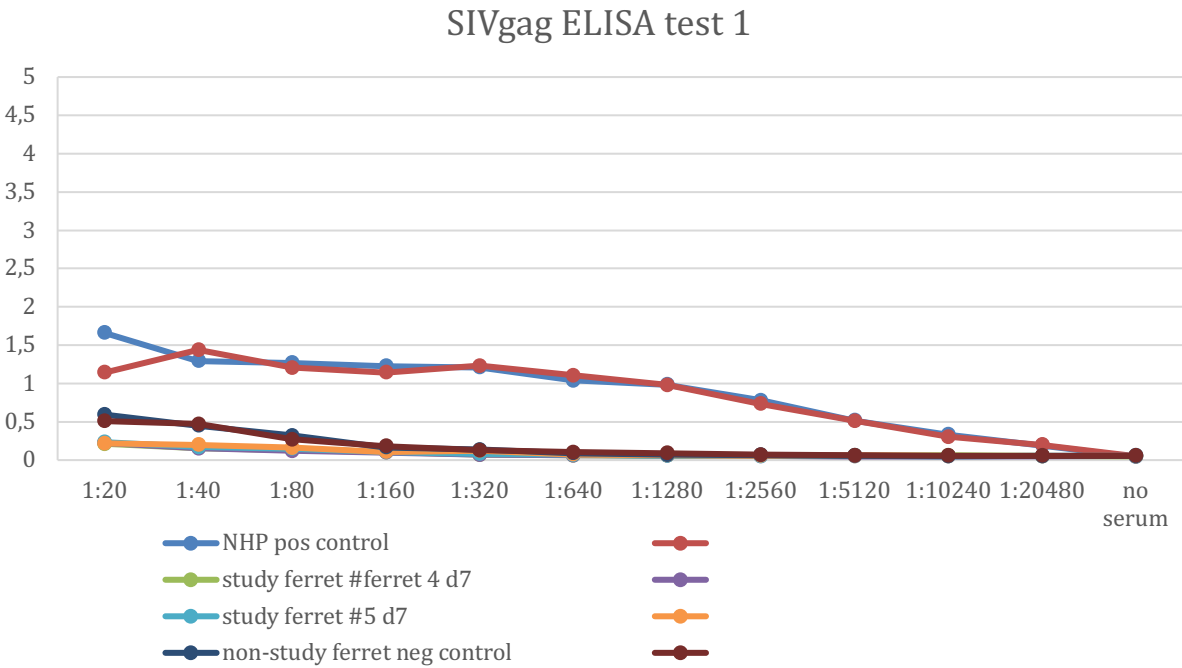


**Figure 67: Experimental enzyme-linked immunosorbant assay against simian imunodeficiency virus glucosaminoglycane.** Test of our assay protocol using serum samples from two different ferrets from this study, taken at D7 (green and purple and teal and orange lines, respectively), one serum of a glucosaminoglycane antibody positive nonhuman primate as a positive control (red and blue lines), and a naïve ferret (dark blue and burgundy lines) as a negative control. Note the high readouts achieved for the naïve ferret which was intended as a negative control.
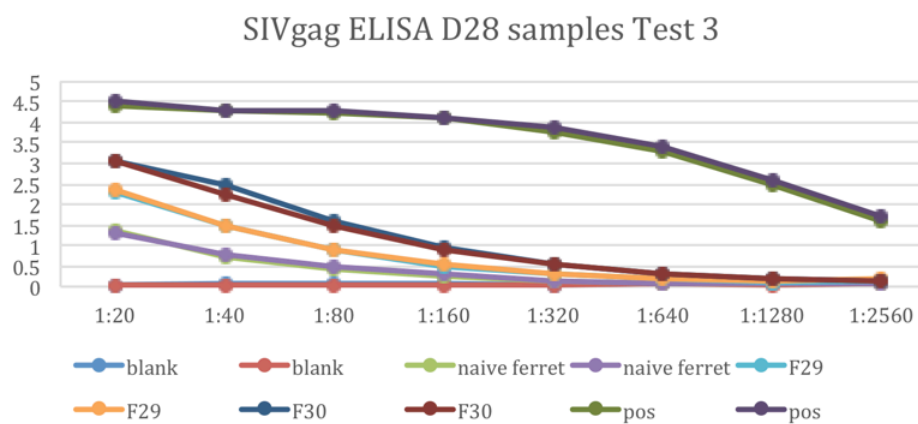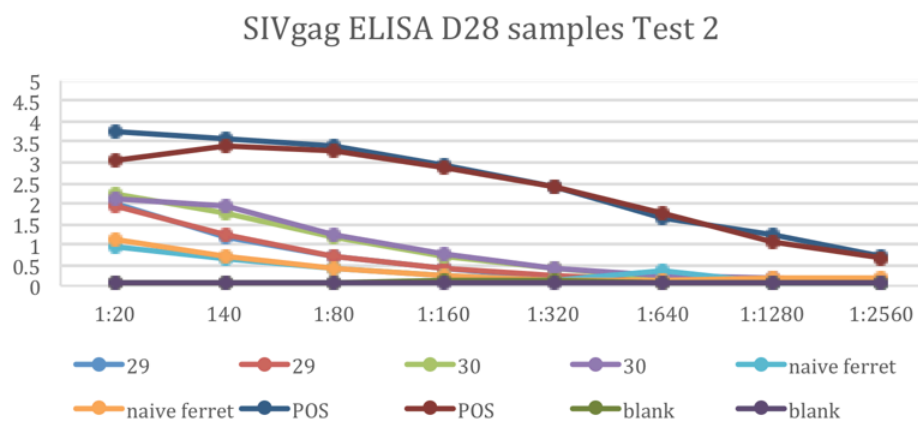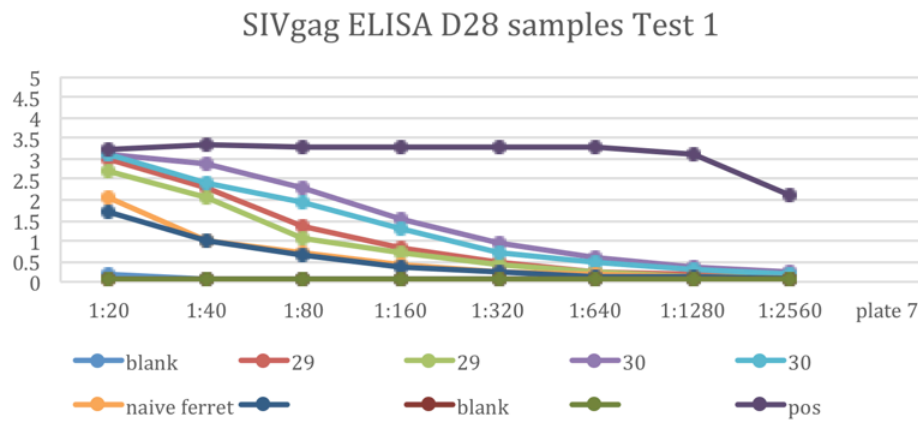
**Figure 68: Comparison of three repetitions of an enzyme-linked immunosorbants assay against simian immunodeficiency virus glucosaminoglycane.** Direct comparison of three identical assays using the same samples taken from ferrets inoculated with vector with glucosaminoglycane insert on d28 post inoculation (labelled 29/F29 and 30/F30 in the graphs). Serum from one naïve ferret (labelled such in the graphs) as well as two rows with no serum (labelled blank in the graphs) were used as negative controls in each of the assays. One confirmed-positive nonhuman primate serum (labelled Pos in the graphs) was used as a positive control in each repetition. Identical materials and methods, equipment and protocols were employed for each repetition. Note the high variability in results for the same samples from repetition to repetition.

## 4.6.5  Crossreactivities observed

During the development of ELISA assays using ferret samples, several unexpected interactions of secondary antibodies with other assay components were discovered.

When performing checkerboard titrations to determine most suitable antigen and serum dilutions while using Rockland AP conjugated anti-ferret IgG as a secondary, it was notable that the assay resulted in unusually constant OD values for each N protein dilution, irrespective of the dilution factor of serum. These ODs were even maintained in the wells that did not contain any serum. The wells that contained no antigen consistently produced very similar near-zero ODs irrespective of the serum dilution. This suggests that Rockland AP conjugated anti-ferret IgG in fact detects CDV N protein or binds non-specifically to the plates (see Figure 69).
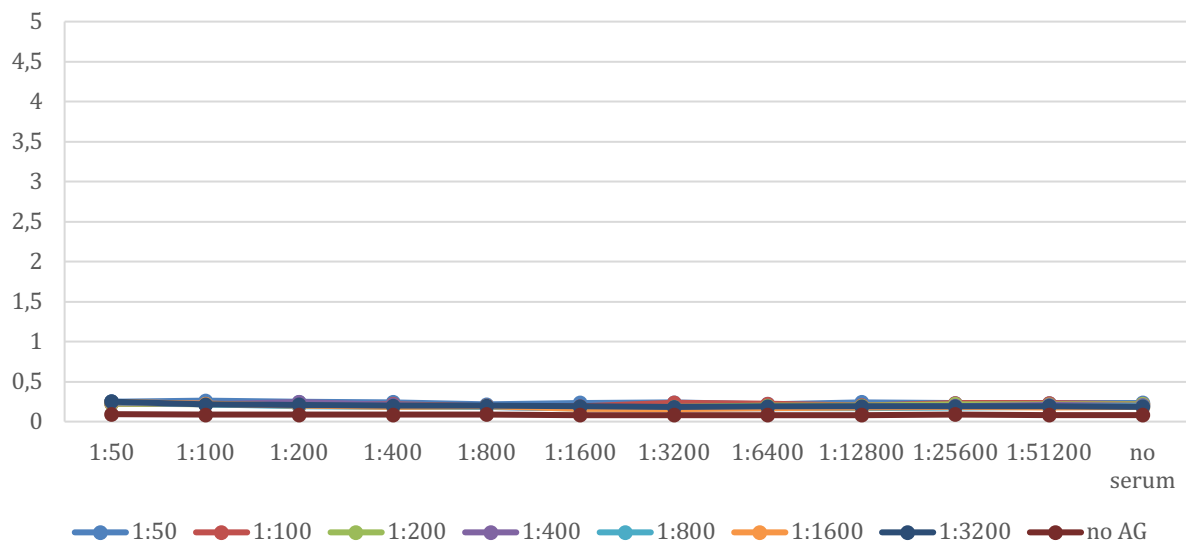


**Figure 69: Exemplary data of Rockland alkaline phosphatase conjugated anti-ferret IgG as a secondary antibody in an enzyme-linked immunosorbant assay against distemper N protein.** Checkerboard titration assay for CDV N using various dilutions of N protein between 1 : 50 to 1 : 3200 (individual data plots) with serial dilutions of a known-positive serum (x-axis) and a constant concentration of the secondary. A row with no antigen (dark red line) was used as a negative control. Note the absence of titration curves, as well as the OD values for "no serum" data points. Also note the identical results irrespective of dilution of antigen. This secondary was not useable as it crossreacted with either antigen or plate.

As an alternative strategy to overcome the issues in assay development, a capture ELISA was attempted to detect antibodies to CDV N protein. For that purpose, ELISA plates were coated with DV12-2 anti-CDV N monoclonal antibody. N protein was then added in various dilutions, followed by serum samples in serial dilutions. The bound antibodies would then be detected using AP conjugated goat anti-ferret IgG (Rockland). Again, the assay produced consistent, very high OD values irrespective of the dilution or even presence of serum, but also regardless of the dilution or even presence of N protein (see Figure 70). This suggests that the secondary antibody did in fact bind and detect the capture monoclonal antibody.
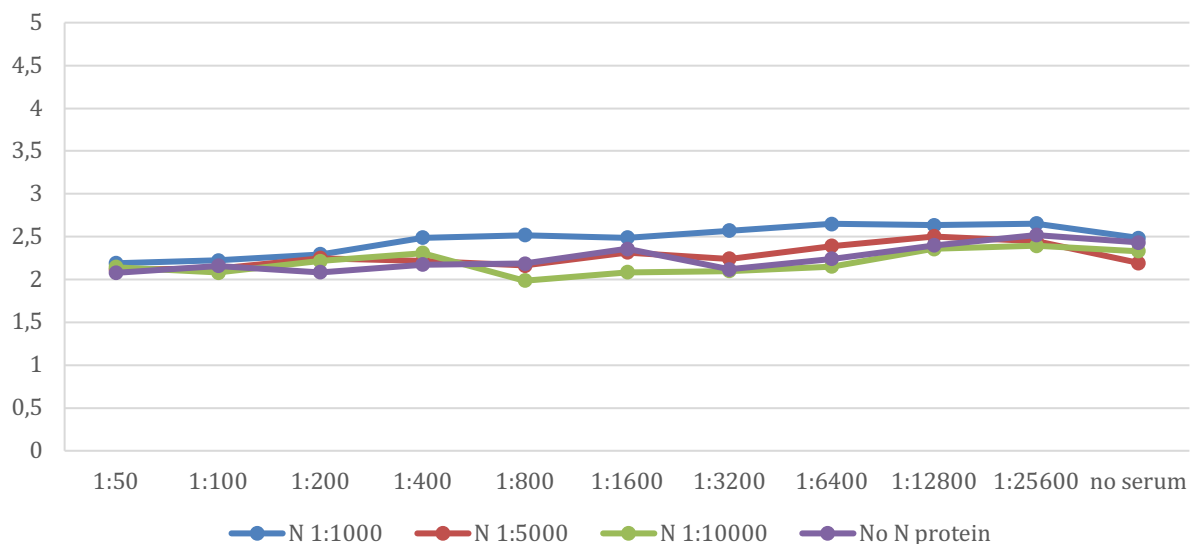


**Figure 70: Attempt to develop a capture enzyme-linked immunosorbant assay employing distemper N protein as the antigen being captured.** Plates were coated with anti-N protein monoclonal antibody DV12-2. Then N protein was added in various dilutions between 1 : 1, 000 and 1 : 10, 000 (individual plots), followed by known-positive ferret serum in serial 2-fold dilutions (x-axis) and secondary antibody (Rockland Alkaline phosphatase-conjugated anti-ferret IgG) at a constant concentration. A row not receiving antigen was included as a negative control (labelled No N Protein). Note the absence of titration across the different serum dilutions, as well as the absence of difference between plots using different amounts of N protein. Also note the high values for the no antigen control. It appears that there are crossreactivities between the capture and secondary antibody.

Since the established protocol for an indirect ELISA for the detection of antibodies to SIV gag could not be transferred successfully, we attempted to develop a five stage capture ELISA for this purpose. The principle of this assay was as follows:

Plate

Anti-ferret IgG (Novus, unconjugated)

Serum samples

Gag p55 peptide pool (gifted from A. Ginsberg, IAVI)

Mouse anti-gag p27 mAB (Advanced Biotechnologies)

HRP-labelled goat anti-mouse IgG (Thermo)

This assay was controlled at every stage. If the plate was coated with capture AB, it produced near-zero values only, if detection secondary AB was missing. It produced ODs at around 50% of the full setup, if anti-gag p27 Antibody was missing. Lack of serum or lack of gag p55 did not affect ODs. This suggested that the secondary detection antibody had partial, and the monoclonal anti-gag p27 even had full affinity to the capture antibody.

| Coated with anti-ferret IgG | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Fx | Fx | Fy | Fy | Fx no gag | Fx no gag | Fx no p27 | Fx no p27 | Fx no sec | Fx no sec | no serum | no serum |
| A | 3,907 | 3,901 | 3,924 | 3,959 | 3,337 | 3,353 | 1,367 | 1,308 | 0,048 | 0,09 | 4,034 | 4,024 |
| B | 3,813 | 3,797 | 3,793 | 3,823 | 3,177 | 3,219 | 1,107 | 1,142 | 0,048 | 0,047 | 3,864 | 3,919 |
| C | 3,773 | 3,737 | 3,764 | 3,789 | 3,072 | 2,991 | 0,961 | 0,997 | 0,047 | 0,047 | 3,741 | 3,98 |
| D | 3,733 | 3,716 | 3,724 | 3,678 | 2,875 | 2,869 | 0,912 | 0,914 | 0,053 | 0,061 | 3,763 | 3,804 |
| uncoated plate | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| | no serum | no serum | Fx no gag | Fx no gag | Fx no p27 | Fx no p27 | Fx no sec | Fx no sec | Fz | Fz | gag and sec only | gag and sec only |
| A | 3,822 | 3,819 | 3,266 | 3,35 | 0,074 | 0,071 | 0,045 | 0,06 | 3,986 | 3,981 | 0,073 | 0,259 |
| B | 3,824 | 3,887 | 3,222 | 3,11 | 0,064 | 0,069 | 0,051 | 0,06 | 3,871 | 4,134 | 0,13 | 0,1 |
| C | 3,88 | 3,834 | 3,081 | 3,074 | 0,067 | 0,067 | 0,053 | 0,055 | 3,91 | 3,853 | 0,048 | 0,049 |
| D | 3,847 | 3,756 | 2,987 | 2,98 | 0,06 | 0,054 | 0,061 | 0,052 | 3,734 | 3,741 | 0,056 | 0,057 |

**Table 17: Attempt to develop a 5-stage capture enzyme-linked immunosorbant assay using simian immunodeficiency virus glucosaminoglycane, raw data.** Principle of this assay: anti-ferret IgG was coated on the plate as capture antibody. Test serum was added (labelled Fx, Fy and Fz in the table), then glucosaminoglycane protein, then mouse anti-p27 monoclonal AB to bind the glucosaminoglycane, finally the bound monoclonal is detected with Horseradish peroxidase-conjugated goat anti-mouse IgG. Control steps employed were as follows: The top plate was coated with anti-ferret IgG, the bottom was plate not coated. Each plate had four controls of two columns each employing no glucosaminoglycane, no monoclonal antibody, no serum and no anti-mouse secondary. Two columns employing only glucosaminoglycane and anti-mouse secondary, and no other stages, were also tested. Each condition was run in quadruplicates. Wells containing no anti-mouse secondary, no capture antibody and no anti-p27, or only glucosaminoglycane and secondary all resulted in near-zero readouts. Omitting the addition of serum or glucosaminoglycane did not reduce readout values. This suggests significant crossreactivities between the anti-mouse secondary, the monoclonal anti-p27 antibody and the capture antibody as well as the plate.

To evaluate this further, we ran the assay without coating the plate with capture antibody. The empty plate was blocked and the assay continued as described. The assay still produced full ODs if all other components were present, but also, if serum or gag p55 were missing. The assay gave a strongly reduced OD, if only gag p55 and detection secondary were present, suggesting a mild interaction between these two components. The assay only gave near zero values if either detection antibody, or gag p27mAB were absent (see Table 17). This would likely suggest that despite the plate being blocked, anti p27 mAB and to a smaller

degree secondary detection antibody still bind to the plate and create very high false

positive ODs.

# 5 Discussion

This thesis describes the first series of *in vivo* studies of a novel candidate for the development of an HIV/SIV vaccine. A Canine distemper virus vector derived from a commercially available CDV Onderstepoort vaccine strain and containing the gene for the highly immunogenic SIVgag protein was delivered to CDV-naïve ferrets, and biosafety, efficacy and immunogenicity of the vaccine candidate in this highly susceptible *in vivo* model were evaluated.

## 5.1  Virus growth and quantification

The recombinant distemper virus with and without gag insert grew well in Vero cell culture. The insertion of foreign genomic information did not negatively affect virus replication to a noticeable degree; titers of rCDV-SIVgag reached $5x10^7$ PFU, which enabled us to use one single virus harvest to inoculate all animals at the desired dose of $2x10^5$ PFU. This reduced the possibility of variations between inoculates. Viruses used for inoculation had previously been sequenced and showed no alteration of protein sequence compared to the paternal Onderstepoort strain, which was important since the paternal strain had been shown to be non-pathogenic when used in ferrets (266). So the recombinant viruses used in this study were not expected to cause clinical distemper when administered to the ferrets.

## 5.2  Screening of ferrets for pre-existing antibodies to canine distemper virus

The ferrets used in these studies were purchased at a very young age and as per our requirements not vaccinated against CDV. They were housed in ventilated cages at our facility until they had reached an age at which maternal antibodies were expected to have faded. Prior to enrolling ferrets in the *in vivo* studies, all animals were blood sampled and

serum samples used in virus neutralisation tests (VNTs) to confirm the absence of antibodies to CDV. VNTs for all animals were negative, which indicated that none of the animals possessed any neutralising antibodies against CDV – maternal or otherwise – that could affect the immunological responses of the ferrets towards inoculation with rCDV constructs.

## 5.3 Study 1 - Biosafety, biodistributrion and basic immunogenicity of the vaccine candidate

The pilot study included six animals, four of which were intranasally inoculated with $2x10^5$ PFU rCDV-SIVgag, and two of which served as controls. Animals were monitored for clinical signs of CDV infection daily over the course of the seven-day study, and swabbed nasally, orally and rectally at 2 h, 1 d, 3 d, 4 d and 7 d post inoculation. Blood samples were taken alongside swab samples. 50 % of animals in each group were sacrificed on day three and day seven of the study, respectively. Post mortems were performed and nasal turbinates, ileum and mesenteric lymph nodes were harvested. This study focused on determining the safety and biodistribution of rCDV with the insert encoding for SIVgag when applied to a highly CDV-susceptible species.

Canine distemper in ferrets is a highly virulent and often lethal disease. It commonly presents with high temperatures, rashes, nasal discharges, diarrhoea, weight loss and in later stages behavioural and neurological changes. During our study, no neurological or behavioural changes were noted, and none of the animals presented with any rashes, continuous weight loss or increased body temperature. Only individual transient cases of mild soft stools and minor nasal discharge were noted, which cleared up quickly without intervention. This demonstrated that our vaccine candidate rCDV-SIVgag did not cause clinical disease in ferrets, a highly susceptible species. This finding is in line with our

expectations, since the parental strain of the recombinant vector is a commercially available Onderstepoort vaccine strain with several years of excellent safety records when used in ferrets, and the SIVgag gene, when expressed alone, does not give rise to a pathogenic product.

Swab samples of nose and rectum and tissue samples of nasal turbinates, spleen, large intestinal lymphnode and Peyer's patches obtained during the study were analysed *in vitro* for the presence of infectious virus particles using plaque assays. No plaques were detected in any of the samples at any point of the study. This suggests that only minimal, if any, amounts of recombinant virus were present in the samples, and secretions of animals inoculated with rCDV-SIVgag did not contain sufficient amounts of recombinant virus to infect other systems such as tissue cultures. The samples were furthermore examined for the presence of genomic copies of rCDV-SIVgag using qRT-PCR. This assay is able to detect genetic material of the recombinant virus even if it stems from replication defective particles, and will have a lower detection limit than assays based on infectivity. Two of the four ferrets inoculated with rCDV-SIVgag, one sacrificed on day 3 and one sacrificed on day 7, showed genomic copies of the inoculate in all swab samples, ranging in number from 10 to over 100 copies. Highest copy numbers were obtained in nasal swabs. One control animal presented with a moderate number of copies in a rectal swab collected on day 1 post inoculation. In light of the fact that this animal did not test positive in any other samples examined, this data is likely a result of post-collection cross-contamination and not a true finding. During qRT-PCR analysis of tissue samples, three out of four ferrets inoculated with rCDV-SIVgag showed presence of genomic copies of the inoculate in at least one tissue. Both experimental animals sacrificed on day 7 showed genomic RNA (gRNA) of the inoculate in the ileum, with copy numbers ranging between $10^4$ and $10^5$. One of the animals sacrificed on

day 7 also showed gRNA in the nasal turbinates, alongside one animal sacrificed at day 3. Copy numbers for these samples ranged from 100 to just under 1,000. Control animals were consistently negative for gRNA in tissue samples.

rCDV-SIVgag was designed to be a replication-competent vector, and in previous *in vitro* assays was shown to actively replicate and induce cytopathic effects in Vero cells. Thus, we expected the virus to replicate in the ferrets after inoculation. Animals were inoculated intranasally. The presence of viral gRNA in nasal turbinates on day 3, especially in connection with positive nasal swabs on that day for that same animal, suggests that replication of recombinant virus is happening early on post vaccination at the inoculation site. A positive rectal swab taken prior to euthanasia in this individual, alongside positive samples of the ileum for both animals sacrificed on day 7 demonstrates that rCDV-SIVgag does spread systemically within seven days after intranasal application and is indeed able to replicate.

Peripheral blood mononuclear cells (PBMCs) were collected from blood samples of all animals sacrificed on day 7, and were subjected to intracellular cytokine staining using monoclonal antibodies to T-cell surface marker CD8 and Interferon gamma. Samples were set up in triplicates, and stimulated with either SIV gag open reading frame peptide pool (Protein Biosciences), PMA/ionomiycin as positive control, or not at all as negative control, respectively, before being stained. Samples were then analysed using Flow Cytometry to determine numbers of CD8-positive T-cells and secretion of IFNg in response to the different stimulants. If the animals had already established cellular immune responses to the SIVgag insert within seven days, the expected result of this assay would be an increase in cells positive for both CD8 and IFNg, as gag-specific T cells are stimulated to expand and IFNg production is induced. In our analysis, there was indeed a slight increase of double-positive cells from animals inoculated with rCDV-SIVgag after stimulation with gag peptides

compared to non-stimulated samples; however, this increase was too small to allow for a definitive analysis.

To summarize, in a seven-day study of ferrets inoculated intranasally with rCDV-SIVgag, the recombinant vector with insert did not induce any adverse reactions or signs of clinical disease. Systemic distribution and replication of the antigen was demonstrated, but cytopathic recombinant virus could not be cultured from any samples throughout the study, indicating that shedding of the recombinant virus was minimal and not sufficient to infect other systems. Cellular immune responses to gag peptides could not definitely be shown, but a low degree of response to stimulation after seven days was present.

## 5.4  Study 2 – Replication abilities of vaccine candidates in host tissues and cellular and humoral immune responses

The second study described in this thesis was designed to further evaluate the replication ability of the construct as well as the immunological responses generated after vaccination. To evaluate effects of the vector backbone versus effects of the full insert, three groups of animals were used. One group was inoculated with rCDV-SIVgag, one with just rCDV, and one was mock-inoculated with PBS. The study was extended to 28 days, with animals being intranasally vaccinated on d 0 and boosted again on d 21. Clinical observations were performed daily. Nasal and rectal swabs were obtained and temperatures controlled every two to three days, and weights were taken weekly. Two animals of each group were sacrificed every seven days. Terminal blood draws were performed prior to euthanasia. Nasal turbinates, mesenteric lymph nodes and Peyer's patches were harvested for further analysis.

Over the course of the study, none of the enrolled animals ever presented with body temperatures outwith the normal range of 38-40 °C. Body weights remained stable during the study, and there was no significant difference in body weight noted between members of different groups. Observation for clinical signs included nasal and ocular discharge, diarrhoea, lethargy, behavioural or neurological abnormalities in addition to fever and weight loss. Of note were mild discharges and soft stools in a small number of experimental animals but not the mock group on day 7 and on day 25, which corresponds to four days post booster inoculation. Four animals that had received rCDV-SIVgag were affected over the course of the study, while only two animals from the rCDV group showed any clinical changes. These changes were not persistent. None of these animals progressed to more severe respiratory symptoms or manifest diarrhoea, and none of the study animals showed any other clinical signs linked to a CDV infection. While this may indicate that animals receiving rCDV-SIVgag were slightly more affected, neither rCDV nor rCDV-SIVgag caused clinical distemper in ferrets over the course of 28 days.

Virus culture and titration was attempted from swab samples as well as tissue samples to evaluate the presence and distribution of infectious recombinant virus particles. No cytopathic effects indicating the presence of infectious particles were observed using any of the swab samples. Tissue samples obtained from Peyer's patches and mesenteric lymph nodes from animals sampled on day 7 produced cytopathic effects for all animals that had received rCDV, and one animal that had received rCDV-SIVgag. From mesenteric lymph nodes 8,500 and under 500 plaques per gram tissue were generated for animals inoculated with rCDV and rCDV-SIVgag, respectively. For Peyer's patches, these numbers were 12,500 (rCDV) and under 200 (rCDV-SIVgag) plaques per gram tissue. Samples obtained from nasal turbinates of the same animals did not result in formation of plaques. No cytopathic effects

were observed for experimental animals at any of the other time points, nor at any point at all in mock inoculated animals. This suggests that live infectious virus particles are present in lymphatic tissues distant from the inoculation site one week post first inoculation, but not after the booster inoculation. Inoculation with rCDV resulted in higher numbers of infectious particles being produced than inoculation with rCDV-SIVgag, indicating that rCDV-SIVgag may be further attenuated than the parental strain, but still able to give rise to infectious particles distant from the inoculation site. Mock-inoculated animals did not show any evidence of infectious particles.

Quantitative real time polymerase chain reaction (qRT-PCR) was employed to detect genomic copies of CDV N protein in swab and tissue samples obtained over the course of the study. Whilst performing the assay using rectal samples, it became evident that copies of genomic DNA could be detected in all samples obtained, including the samples taken from mock-inoculated animals. We determined the likely reason for this to be cross-contamination caused by errors in the post-collection phase while preparing the swabs for subsequent assays. We therefore did not proceed to analyse the nasal swab samples that had been processed in the same way. Tissue samples taken from Peyer's patches, mesenteric lymph nodes and nasal turbinates of terminated ferrets were processed ensuring strict sterility and separation between groups to avoid this issue in subsequent analyses. Genomic copies encoding CDV N protein were detected in significant numbers in all three tissues and across all time points, for both the rCDV and the rCDV-SIVgag group. Copy numbers consistently reached a maximum on day 7 and then proceeded to gradually decline. In animals inoculated with rCDV, genome numbers in all tissues demonstrated a slight peak on day 21, which was only present in Peyer's patches for animals inoculated with rCDV-SIVgag. The animals harvested on day 21 had not received a booster inoculation. Copy

numbers detected were consistently higher in animals inoculated with rCDV than those inoculated with rCDV-SIVgag. The highest number of copies in the rCDV group was detected in Peyer's patches, reaching 3.38 x $10^9$ copies per gram on day 7; equivalent samples of rCDV-SIVgag inoculated animals reached a maximum of 9.16 x $10^5$ copies per gram. Samples obtained from mesenteric lymph nodes resulted in the highest number of copies in the rCDV-SIVgag inoculated ferrets, reaching a maximum of $10^7$ copies per gram. This was still lower than the equivalent samples of the rCDV treated animals reaching 9.77 x $10^8$ copies / g. Samples obtained from nasal turbinates gave the lowest number of genome copies for both groups, resulting in a maximum of 1.83 x $10^7$ (rCDV) and 4.44 x $10^4$ (rCDV-SIVgag) copies / g, respectively. Taken together, these findings confirm again that, while rCDV-SIVgag appears to be more attenuated than rCDV, both constructs are indeed able to replicate not only at the inoculation site, but even more so at remote lymphatic tissues, fulfilling the expectation that the constructs will maintain the lymphotropic characteristics of the parental strain. The gradual decrease of genome copies detected over the course of time also suggests a level of control of infection within the ferrets, likely due to the development of an immune response.

Parallel to the qRT-PCR for CDV N genome, samples from animals inoculated with rCDV-SIVgag were also analysed using qRT-PCR for SIVgag genome. The course of genomic copy numbers over time and between different tissues paralleled that of CDV N in this group. Genomic copy numbers ranged from about 0.5 to 1 orders of magnitude lower than copy numbers for CDV N in the same animals. The detection of genomic copies of SIVgag not only in nasal turbinates but also in remote lymphatic organs demonstrates that during replication and distribution of the vector, the insert is conserved and does not disappear. The lower copy numbers of SIVgag compared with CDV N suggest that likely not every replicated

particle retains the insert, but there are still significant numbers of SIVgag genome present in lymphatic tissues over the course of the study.

Serum samples of all animals, obtained just prior to euthanasia, were also evaluated for the presence of viral genomes using qRT-PCR. Genomic DNA encoding for the CDV N protein could not be detected in any of the control animals at any time point. No copies were detected in the experimental groups on days 7 and 14. On day 21, genomic DNA encoding for CDV N was evident in two animals inoculated with rCDV, with copies numbers of 500 and 1,200 copies / mL respectively, and in one animal inoculated with rCDV-SIVgag, where 3,250 cppies / mL could be detected. On day 28 one animal of the rCDV group and one animal of the rCDV-SIVgag group presented with detectable copies of CDV N, reaching 2,150 copies / mL and 1,890 copies / mL respectively. This indicates that both inoculates cause a mild viremia in some but not all inoculated animals around day 21 to 28.

PBMCs from blood samples obtained upon euthanasia were employed in intracellular cytokine staining assays to evaluate the level of cellular responses to stimulation with a polyclonal positive control stimulant, mock stimulation, or stimulation with SIV gag peptides. PBMCs were isolated from samples using Ficoll gradient separation, and subsequently aliquots were incubated with the described stimulants. Stimulated cells were then stained for T-cell surface marker CD8, cell proliferation marker Ki67, and the cytokine Interferon gamma (IFNg), which is released during cellular immune responses to an antigen. Samples were then analysed using flow cytometry. Stimulation with SIVgag peptides resulted in 0.2 - 0.4 % increased secretion of IFNg in samples from the rCDV-SIVgag group compared to unstimulated samples or samples from the Mock group. The highest increases were noticed on day 14 and day 28 post inoculation. There was an unpredicted increase of the proliferation marker Ki67 in gag-stimulated samples obtained from rCDV-inoculated ferrets

on day 28, which can likely be attributed to a general activation of the immune system following the booster inoculations on day 21. That is consistent with the highest levels of IFNg release on day 28 for gag-stimulated samples from the rCDV-SIVgag group.

Virus neutralisation assays were performed on serum samples of all animals collected during terminal blood sampling to assess the presence and levels of antibodies against CDV. Antibodies could not be detected at any time point in the control group, but were present in both the group inoculated with rCDV and the group inoculated with rCDV-SIVgag from day 14 onwards. Titres for animals receiving rCDV were four times higher than those of animals receiving rCDV-SIVgag in samples taken on day 14 and 21. This correlation became less strict in samples taken on day 28, and titres of the majority of animals sampled on that day were no higher than those of animals sampled on day 21. Since different animals were sampled at each time point, it cannot be stated that antibody titres reach a plateau on day 21, but there does not appear to be a trend for significantly higher titres on day 28. While rCDV does appear to induce a significantly stronger humoral response towards CDV, neutralising antibodies to CDV are definitely present and developing at the same speed in animals inoculated with rCDV-SIVgag.

Western blot assays were used to examine the same serum samples for the presence of antibodies to SIVgag. To this end, a commercially available SIVgag p55 protein of the size of 55 kDa was blotted onto a nitrocellulose membrane, and serum samples from the study along with known positive and negative control sera were used as primary antibodies during the blot. The appearance of a dark band at the appropriate level of 55 kDa upon detection indicated the presence of antibodies to SIVgag in the sample. Controls in this assay worked very well and very reliably. No bands could be detected for mock-treated or rCDV inoculated ferrets at any time, nor for any of the rCDV-SIVgag inoculated ferrets at day 7 or 14. Bands

were however definitely and repeatedly present at the 55 kDa level in samples from rCDV-SIVgag inoculated animals on day 28, and faint bands appeared in some repeats of the assay in samples obtained on day 21. This indicates that binding antibodies against SIV gag are indeed being produced in ferrets after inoculation with rCDV-SIVgag. While detection of antibodies on day 21 is variable, they are consistently present on day 28.

In order to be able to quantify humoral immune responses directed towards the vector backbone as well as the insert, we attempted to develop ELISA assays using CDV N and H proteins as well as SIV gag. Antibodies directed against CDV N are most abundantly produced during CDV infection (285) and are routinely used for titre determination in other species (278, 286, 287), while those directed against CDV H have the strongest neutralising ability (288), which is why humoral responses to both these antigens were of interest to us. A detailed description of the assay development process can be found in Chapter 4.6, "Development of Enzyme-linked immunosorbent assays for the detection of antibodies in ferret blood samples". Initial attempts to establish the ELISA assays with CDV proteins were made using previously described protocols (Chapter 4.6) and purified CDV proteins produced in *E. coli*. We decided to produce and employ purified proteins rather than the previously described whole virus lysate or insect larvae lysate in order to reduce the possibility of unspecific binding. Assays performed using these methods did however not achieve useful results. High background values and unclear discrimination between known positive and negative samples combined with overall low signals were noted repeatedly. Abundant modifications were undertaken at all stages of the assay. Antigen purity, availability and binding capabilities were confirmed. Protein- and purification tag-specific monoclonal antibodies were used as primaries in place of serum to evaluate assay mechanics. Control sera were employed in dot blot assays using the purified proteins as antigen, to confirm

positivity and negativity. A variety of secondary antibodies were tested. The use of serum, heat-inactivated serum as well as plasma was compared. Different blocking protocols were employed. Different assay strategies were tested. Mock assays were run using bovine serum albumin (BSA) or lipopolysaccharides (LPS) as coating antigens to evaluate the possibility of background being caused by unspecific binding to accompanying antigen in the purified protein solution. Test samples were pre-absorbed against BSA or LPS before being used in the assay with the same goal. Serum samples of non-CDV susceptible species were used as another negative control. However, it was noted that these foreign species samples produced very high signals. We are not sure as to the reasons for this result. The issue of high background, false positive results for naïve samples from other species, and insufficient discrimination between positive and negative samples could not be solved despite our best efforts.

For ELISAs using SIV gag as an antigen, we based the assay development on protocols already in existence in our lab, and routinely used with serum samples from various laboratory animal species. This assay produced higher signals than the assays using CDV proteins, but showed a very high degree of variability between repeats of identical assays, despite using not only the same protocol but also the same reagents, materials and samples. This occurred for repetitions performed by the same as well as different researchers. A capture assay was attempted as an alternative assay strategy, but failed due to unexpected cross-reactivities of reagents at various stages of the assay. On various occasions, naïve ferret serum used as negative control produced higher signals than any of the assay sera. The reasons for this were not clear. Due to increasing pressure to obtain information about humoral responses to SIV gag, we decided to change to a different technique at this point,

and successfully employed the Western Blots described above to qualitatively assess the presence of SIV gag antibodies.

To summarise, the series of *in vivo* studies described in this thesis demonstrated that a recombinant CDV vector containing the SIV gag gene can safely be used in a highly CDV susceptible species. The vector does not cause clinical CDV, but is able to distribute and replicate within the host. Replication is reduced for rCDV-SIVgag compared to the empty vector, but rCDV-SIVgag retains the lymphotropic characteristics of the vector. Mild viremia was detected for both vaccine candidates during the second half of the 28 day study, but no live virus could be cultured from any of the swab samples taken, indicating that no significant shedding of infectious particles occurs. Humoral responses against both CDV and SIV gag could be demonstrated, and there was evidence that cellular responses to SIV gag were being established.

## 5.5   Further research

Since the end of the studies described here the vaccine candidate rCDV-SIVgag has been employed in further studies. The qRT-PCR methodology to detect viral particles has been further improved and a paper published on its use (289). From personal communication with Xingsheng Zhang and Joanne DeStefano, both at IAVI, I have learned that rCDV-SIVgag has successfully passed further stages of *in vivo* evaluation in ferrets, including studies using rCDV expressing GAG and rCDV expressing SIV ENV simultaneously. Both vectors were able to replicate equally well when applied singly or in combination, and genomic RNA of the vector was detectable in the gut-associated lymphatic tissue for a significant time period. The recombinant CDV vectors have since advanced to being administered to Rhesus

macacques, which were then to be challenged with SIV. The vectors have proven safe for use in primates, and have been shown to control virus loads and confer a significant degree of protection upon challenge. A degree of immunity could also be detected. At the current stage, rCDV vectors expressing the complete SIV proteome are undergoing evaluation in nonhuman primates in an effort to increase immunogenicity. These vectors will be undergoing challenge shortly, and a significant degree of protection from challenge is expected. This stage of nonhuman primate challenge studies represents one of the final stages of pre-clinical testing of novel vaccine candidates before proceeding into clinical studies. The progressing of a vaccine candidate to that stage makes it a very successful construct. Additionally, rCDV vectors also hold potential as possible therapeutic mechanism in HIV infections. CDV overlaps with SIV/HIV in tissue and cell tropisms. Therefor the vectors may be able to target SIV/HIV infected cells, and if replicating sufficiently, they may be able to utilise viral or immunological mechanisms to destroy these cells, leading to a reduction in virus load.

# 6  Summary

Recombinant canine distemper virus as a possible vector for the delivery of SIV antigens – establishment and evaluation of biosafety and immunogenicity of rCDV and rCDV-SIVgag in the ferret model

HIV/AIDS has been termed one of the most devastating diseases of mankind. Due to the unique pathogenesis of this infection, medical therapy can only slow down the disease progress but is unable to cure it. Preventative measures including improved education, supply and use of condoms and microbicides, and the use of antiretroviral drugs during pregnancy and breastfeeding of infected women have slowed the infection rates globally, but ultimately only a vaccine is believed to be able to control the pandemic. Simian immunodeficiency virus (SIV), a close relative of HIV that leads to comparable disease processes in Rhesus monkeys, has been employed to study pathogenesis, treatment and prevention of HIV infection, since it is non-pathogenic to humans. Development of potential vaccine candidates is posing a significant challenge to the research community. Various pathways have been explored; with viral vectors having shown considerable success over the last few years. Mucosal delivery of vector based vaccines is stipulated to elicit improved immune responses at mucosal virus entry sites. Ideal viral vectors share their main replication characteristics such as cell tropism and location of replication with the pathogenic virus in question, which enables them to deliver the included foreign antigens to the site of natural infection and replication. Members of the genus morbillivirus enter the host through mucosal membranes, infect preferably T cells in the gastrointestinal lymphoid tissue and cause significant immunosuppression in a susceptible host. This research study employs a live attenuated vaccine strain of canine distemper virus (CDV), a morbillivirus non-pathogenic to humans but able to replicate in primate cells, that was rescued using reverse

genetics and subsequently genetically engineered to contain the genetic information encoding the SIV gag protein, which is the most immunogenic of HIV/SIV proteins. The two recombinant viruses were evaluated *in vitro* to ensure infectivity and ability to replicate. The presence of the 55 kDa protein gag was confirmed. The recombinant viruses were then intranasally applied to ferrets, a species highly susceptible to canine distemper infection, to evaluate the safety and efficacy of the recombinant viruses. The ferrets were closely observed for clinical signs of distemper. Blood and tissue samples were taken at various time points post inoculation, and evaluated for the presence of recombinant virus and levels of immune responses elicited. The recombinant canine distemper virus vector containing SIV Gag proved to be clinically safe when administered to a highly susceptible species. The recombinant virus was able to replicate in the host, and both humoral and cellular immune responses to the virus backbone as well as the insert were evident. This suggests that recombinant canine distemper virus containing SIV antigens is a promising candidate for the development of an effective vaccine. Further studies have been conducted since the completion of the work included in this thesis, and recombinant CDV containing SIV gag has been successfully employed in several additional *in vivo* studies in ferrets as well as subsequently nonhuman primates. Data obtained in these studies suggest that the construct induces significant immune responses. At the time of writing, the construct is being employed in nonhuman primate challenge studies using a chimera of SIV and HIV as the challenge virus, to confirm the ability of the construct to create immune responses that are effective in an actual virus encounter. Based on the data gained so far, successful completion of this step is expected.

# 7 Zusammenfassung

Das Hundestaupevirus als möglicher Vektor für SIV-Antigene – Etablierung rekombinanter Vektoren mit und ohne SIVgag-Insert, und Evaluierung dieser Vektoren auf klinische Sicherheit und Immunogenität im Frettchenmodell

(Rebecca Köhnke)

Das Humane Immunodefizienz-Virus (HIV) und das durch die Infektion letztendlich verursachte erworbene Immundefizienzsyndrom (AIDS) werden oft als eine moderne Plage der Menschheit bezeichnet. Aufgrund der besonderen Biologie des HI-Virus kann mit medikamentöser Behandlung nur das Fortschreiten der Erkrankung verlangsamt, nicht aber das Virus eliminiert werden. Vorbeugende Maßnahmen wie verbesserte Aufklärung über die HIV-Infektion, weiter verbreitete Verwendung von Kondomen und Mikrobiziden, oder die Anwendung antiretroviraler Medikamente während Schwangerschaft und Stillzeit infizierter Frauen haben zwar weltweit die Neuinfektionsrate vermindert, aber es wird davon ausgegangen, dass letztendlich nur ein Impfstoff für ein Ende des Ausbreitens der Epidemie sorgen kann.

Um die Pathogenese, Behandlung und Prävention einer HIV-Infektion wissenschaftlich zu untersuchen, wird häufig auf das simian immunodeficiency virus (SIV) als Modellorganismus zurückgegriffen. Dieses Virus ist ein enger Verwandter des HI-Virus, der nicht für Menschen pathogen ist, aber in Rhesusaffen eine vergleichbare Erkrankung auslöst. Die Entwicklung möglicher Impfstoffkandidaten hat sich allerdings auch trotz verfügbarer Modellsysteme als schwierig erwiesen. Zahlreiche verschiedene Ansätze sind in der Entwicklung, wobei die Verwendung viraler Vektoren sich über die letzten Jahre als erfolgversprechend erwiesen

hat. Speziell die Applikation solcher Vektoren über die Schleimhäute hat wiederholt zu der verstärkten Ausbildung mukosaler Immunantworten geführt und ist deshalb von Interesse auch im Bereich der HIV-Impfstoffentwicklung. Ein geeigneter Vektor verfügt über ähnliche Charakteristiken wie das pathogene Virus, gegen das geimpft werden soll, vor allem in Bezug auf Zelltropismus und Ort der Virusreplikation im Körper. Dadurch werden die im Vektor inkorporierten Fremd-Antigene direkt an die während einer natürlichen Infektion relevanten anatomischen Strukturen geführt.

Natürliche Infektionen mit Mitgliedern des Genus Morbillivirus beginnen in der Regel mit dem Eindringen des Virus über die Schleimhäute. Daraufhin infiziert das Virus bevorzug T-Zellen, vor allem im gastrointestinalen lymphatischen System, und sorgt damit für eine Immunsuppression des natürlichen Wirtes. Im Rahmen der hier vorgestellten Untersuchungen wurde ein attenuierter Stamm des zu den Morbilliviren gehörenden Hundestaupevirus als Vektor für SIV-Antigene evaluiert. Das Hundestaupe-Virus ist zwar *in vitro* fähig Primatenzellen zu infizieren, ist aber für den Menschen nicht pathogen. Das Virus wurde als rekombinantes Virus aus Zellkultur isoliert und genetisch modifiziert, sodass die Erbinformation für das SIV gag Protein in den Vektor eingebaut wurde. Gag ist ein wichtiges virales Strukturprotein und das immunogenste alle SIV-Proteine. Im Rahmen dieser Forschungsarbeit wurden die Replikationsfähigkeit und Infektiosität der rekombinanten Vektoren *in vitro* sowie das Vorhandensein der Gag-Sequenz überprüft. Die rekombinanten Vektoren wurden dann auf intranasalem Wege an Frettchen verabreicht, die für natürliche Staupeinfektion sehr empfänglich sind. Während des gesamten Versuchsverlaufs wurde der klinische Zustand der Frettchen genau auf das Vorhandensein von Staupesymptomen überprüft. Wiederholt wurden Blut- und Gewebeproben genommen, die auf Virusgehalt und Hinweise auf Immunreaktionen untersucht wurden. Das rekombinante Staupevirus mit SIV

Gag-Insert hat sich in dieser Studie in einer hochempfindlichen Spezies als klinisch unbedenklich, aber dennoch replikationsfähig erwiesen. Die Blut- und Gewebeproben weisen Anzeichen humoraler und zellulärer Immunreaktionen sowohl auf den Vektor selbst als auch auf das Insert auf. Dies deutet darauf hin, dass rekombinantes Staupevirus mit SIV gag Insert ein erfolgversprechender Kandidat für weitere Entwicklungsschritte ist. Seit Beendigung der hier beschriebenen Arbeiten sind im Gastinstitut mehrere weitere Studien mit diesem Kandidaten durchgeführt worden, um Effektivität und Immunogenität sowohl in Frettchen als auch in Rhesusmakaken weiter zu eruieren. Diese Studien waren ebenfalls erfolgreich, sodass zum Zeitpunkt des Schreibens dieser Dissertation in einer weiteren Studie Rhesusmakaken mit dem rekombinanten Staupevirus mit SIV gag Insert inokuliert wurden, um sie dann mit einer pathogenen HIV/SIV-Chimäre zu infizieren. Diese sogenannten Challenge-Studien belegen letztendlich die Existenz einer belastbaren Immunität im Falle einer Infektion, und sind eine der wichtigsten Hürden in der Entwicklung von Impfstoffen. Basierend auf den bisher gewonnenen Daten wird von einem erfolgreichen Abschluss dieser Studie ausgegangen.

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# 9 Publikationsverzeichnis

Teile dieser Arbeit wurden mit vorhergehender Genehmigung bereits veröffentlicht in:

Xinsheng Zhang, Olivia Wallace, Kevin J. Wright, Martin Backer, John W. Coleman, Rebecca Koehnke, Esther Frenk, Arban Domi, Maria J. Chiuchiolo, Joanne DeStefano, Sandeep Narpala, Rebecca Powell, Gavin Morrow, Cesar Boggiano, Timothy J. Zamb, C. Richter King, Christopher L. Parks

**Membrane-bound SIV envelope trimers are immunogenic in ferrets after intranasal vaccination with a replication-competent canine distemper virus vector**

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Mein Anteil an dieser Studie bestand in der Entwicklung der nötigen Tierversuche, sowie Hilfe bei der Erstellung des Manuskriptes.

# 10 Danksagung

# 11 Selbstständigkeitserklärung

**Selbständigkeitserklärung:**

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen Anspruch genommen habe.

Berlin, den 19.07.2018

Rebecca Köhnke