Aleutian mink disease parvovirus (ADV) is a naturally occurring, autonomous parvovirus that is capable of infecting some members of the Mustelidae family. Although this virus originated in mink, its presence in ferrets is cause for concern due to the increasing popularity of these animals as pets. Because of the host specificity and mutability of ADV, infected animals can be asymptomatic or have clinical disease characterized by progressive weight loss, cachexia, malaise, and melena. A common laboratory abnormality is hypergammaglobulinemia. Current methods to diagnose ADV infection in ferrets include counterimmunoelectrophoresis (CIEP), polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA), and DNA in situ hybridization. Virus-specific antibody in serum can be detected by CIEP. PCR can be used to detect target segments of viral nucleic acid in clinical samples. ELISA can be used to detect viral-specific antibodies or viral group-specific antigens. DNA in situ hybridization is also a valuable diagnostic technique that can detect and localize viral DNA in cells, tissues, and organs.

INDEX WORDS: Aleutian mink disease virus (ADV), parvovirus, ferret, enzyme-linked immunosorbent assay (ELISA), counterimmunoelectrophoresis (CIEP), polymerase chain reaction (PCR), DNA in situ hybridization
DEVELOPMENT OF AN ELISA TO DETERMINE ALEUTIAN MINK DISEASE
PARVOVIRUS (ADV) CAPSID PROTEIN ANTIBODY TITERS

by

KATE ELIZABETH PENNICK

Bachelor of Science, The Pennsylvania State University, 1999

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment
of the Requirements for the Degree

MASTER OF SCIENCE

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KATE ELIZABETH PENNICK

Major Professor: Kenneth S. Latimer
Committee: Branson W. Ritchie
Christopher R. Gregory

Electronic Version Approved:
Maureen Grasso
Dean of the Graduate School
The University of Georgia
August 2005
DEDICATION

To Mom, Dad, and Brian. You were there for me whether I touched the wall first or crossed the finish line in agony and tears. Now, as I embark on this part of my academic career, I know that you will always be my number one fans. I love you very much.
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CHAPTER 1

Introduction

Aleutian disease (AD) of mink is caused by infection with Aleutian mink disease parvovirus (ADV), which has more recently been observed as an emerging disease of companion ferrets. The pathogenicity of the virus is not fully understood but a variety of factors control whether or not clinical disease ensues. After viral exposure, ferrets may remain asymptomatic or develop acute illness. The genetic make-up of the host and the strain of ADV are two major factors that control the severity of disease.

The ADV virus spontaneously arose in Aleutian mink, which remain the most susceptible host, followed closely by all other types of mink. In addition, ADV can infect other mustelids, including ferrets. Classic AD in mink is characterized by a heightened humoral response that causes hypergammaglobulinemia. Inability to clear the virus from the body causes immune complex disease. Ultimately, death occurs due to glomerulonephritis and lymphoplasmacytic infiltration of other organs. ADV infection of newborn mink presents in a strikingly different manner, causing acute respiratory distress syndrome. Clinically, adult ferrets with ADV infection have diverse signs of disease. Infected individuals may be sub-clinical or display acute neurological, cardiac, and pulmonary disease. The severity of disease appears to be greater in younger ferrets, but has not been studied in newborn kits.

This thesis represents a compilation of five years of work to develop improved laboratory tests to diagnose ADV infection in ferrets. A case report is included to introduce new information regarding early sub-clinical ADV infection of domestic ferrets. This case report
suggests that ADV infection may be more common in ferrets than previously recognized. Furthermore, there is a good possibility that ADV infection is being overlooked as a cause of death or illness in ferrets because of the lack of testing for this virus.

Prior to the start of this research, counterimmunoelectrophoresis (CIEP) was the only commercially available test to diagnose ADV infection. The CIEP test detects viral-specific antibodies against ADV. Although this test is still available, its clinical value is limited because test results are reported as positive or negative instead of providing an endpoint antibody titer. Because CIEP is performed using a 1:4 dilution of blood or serum, false negative and false positive test results may occur. Over the last five years, a commercial ELISA has been developed to detect anti-ADV NS1 protein antibodies. The NS1 protein is a non-structural protein that is only detected during viral replication. Therefore, it is theorized that a false negative test result could occur if ADV is not replicating. The need for more comprehensive diagnostic tests for ADV in ferrets is evident. Other tests, such as PCR and DNA in situ hybridization, are valuable assays that can identify ADV DNA in various tissues. The new diagnostic tests presented in this thesis should aid in the diagnosis and future research of ADV in ferrets and other mustelids.

The objectives of this research study were as follows:

1. To develop an ELISA for potential commercial use that would detect anti-VP1/VP2 capsid protein antibodies in the serum of ADV-infected ferrets.
2. To validate this ELISA test by comparison to CIEP, including titer accuracy.
3. To develop a DNA in situ hybridization assay for detection of ADV.
4. To evaluate the ability of PCR and DNA in situ hybridization to detect ADV infection in necropsy and biopsy tissues of clinically diseased ferrets.
CHAPTER 2

Literature Review

The companion ferret

The emergence of the ferret (Mustela putorius furo) as a household pet began in the 1970s and has grown to include approximately 10 to 12 million pet ferrets in the United States. Most owners have more than one animal, with an average of 3 to 4 ferrets per household. Popularity of the companion ferret has not wavered despite inaccurate publicity regarding their propensity to contract rabies or attack small children. Like most dogs and cats, ferrets are generally non-aggressive animals that enjoy being handled by people. Ferrets are still banned in California and ownership is restricted in other states.

The companion ferret is similar to the European or steppe polecat. Unlike the North American black-footed ferret, companion ferrets are not native to the United States. The common pet ferret has a long-bodied stature with short legs and a long tail. This animal was brought to the Americas approximately 300 years ago and has remained domesticated to date.

Ferrets were used for fur production during the early 1900s because they had a wide variety of coat colors. Unlike mink, ferrets had a musky smell and the hair often fell from the pelt, limiting its value in the fur industry. Other notable uses for ferrets include rabbit hunting, ratting, and biomedical research. Ferrets continue to serve as a model to study the pathogenesis of viral infections. Research of human diseases such as influenza (type A strains), subacute sclerosing panencephalitis, and vesicular stomatitis has been conducted using ferrets. Viral strains native to other animals such as infectious bovine rhinotracheitis, canine distemper, and canine parainfluenza, have been isolated in ferrets for research purposes.
Ferrets have served as models to demonstrate the association between host defense and viral strain pathogenicity for canine parvovirus (CPV), feline parvovirus (FPV), raccoon parvovirus (RPV), and mink enteritis virus (MEV).\textsuperscript{19-22}

Another mink-derived parvovirus, Aleutian mink disease parvovirus (ADV), has become a problem in companion ferrets more recently. Although ADV virus originated in mink, it can naturally infect ferrets and cause disease.\textsuperscript{23} Because there is no vaccine or cure for ADV infection, the possibility of a severe disease outbreak among the pet ferret population is a concern.

**Aleutian mink disease parvovirus**

In 1946, breeders on an Oregon mink ranch began to see a new blue-gray coat color that arose from a spontaneous genetic mutation.\textsuperscript{24} The unusual pelt color became highly valuable to the fur industry and subsequently lead to increased breeding of these mink. Unfortunately, this genetic mutation was also associated with Chediak-Higashi syndrome, which predisposed the Aleutian mink to infection.\textsuperscript{25-26} Mink farms subsequently began to see increasing illness and mortality, characterized by hypergammaglobulinemia and proteinemia.\textsuperscript{27} The disorder, originally termed Aleutian disease (AD), was caused by persistent immune complex formation due to an antibody response to ADV.\textsuperscript{25, 28-32} The prolonged formation of immune complexes in glomeruli lead to renal failure, debilitation, and death. The first clinical description of AD in mink was published in 1956.\textsuperscript{33}

Researchers were able to pinpoint the replication kinetics and localize the virus \textit{in vivo}, but confirmation that the disease was caused by a parvovirus did not occur until 1980.\textsuperscript{34} Subsequently, a cell culture adapted strain of ADV was developed and designated ADV-G.\textsuperscript{34} This nonpathogenic virus enabled further laboratory research into ADV structure and function.
During the mid-1980s, ADV also was discovered to cause acute, fatal, interstitial pneumonia in <2 week-old kits born to seronegative dams.\textsuperscript{35}

Currently, ADV is known to naturally infect mink and ferrets.\textsuperscript{36-38} Striped skunks, raccoons, otters, weasels, fishers, martens, and foxes also are capable of contracting ADV infection based on seropositivity to the virus and/or ADV-like histopathological lesions.\textsuperscript{36-40} One experiment used PCR to identify ADV viremia in raccoons inoculated with mink ADV strains.\textsuperscript{36} Anti-ADV antibodies have been experimentally induced in cats, dogs, mice, and rabbits without causing histologic lesions or overt clinical disease.\textsuperscript{35}

**Characterization of ADV**

Aleutian mink disease parvovirus is a member of the genus *Parvovirus*, which is comprised of the autonomously replicating viruses of the subfamily *Parvovirinae*.\textsuperscript{40} This genus includes a variety of species-specific parvoviruses, including canine parvovirus, feline panleukopenia virus, minute virus of mice, porcine parvovirus, raccoon parvovirus, and goose parvovirus.\textsuperscript{40} The ADV virion is 25 nm in diameter and nonenveloped, with an isometric capsid having icosahedral symmetry.\textsuperscript{34, 40} The genome is comprised of \(>90\%\) single-stranded (ss), minus sense (-) DNA.\textsuperscript{34, 41-42} The genome consists of a sequence of 4,748 nucleotides including major left and right open reading frames, smaller mid-genomic open reading frames, and palindromic sequences at both the 5’ and 3’ ends.\textsuperscript{43-44}

Viral proteins are crucial to the survival of a virus in the host; therefore, the identification and function of the proteins that are produced by the ADV are important in the pathogenesis of the disease. Five mRNAs are generated by ADV during replication that code for an array of viral proteins.\textsuperscript{45} Promoter P4 synthesizes R1, R2, R2’, and RX that code for the NS1, NS2, and NS3 non-structural (NS) proteins.\textsuperscript{46-47} Promoter P36 synthesizes R3, which codes for both viral
structural or capsid proteins designated VP1 and VP2. Proteins VP1 and VP2 are coded for within the large right open reading frame, which contains all the required coding sequences necessary for nuclear transport and virion assembly. All of the NS proteins are initiated by differential splicing of the left and middle open reading frame mRNA. The NS1 protein, synthesized from R1 in the large left open reading frame, is necessary for viral genomic replication and excision of viral ssDNA from the replicative double-stranded (ds) DNA form. This protein also controls the capsid protein mRNA at the transcriptional level. Non-structural protein 2, coded for by the R2 and R2’ mRNAs, is also necessary for efficient viral replication. While the function of NS2 is not fully known, the required coding sequence for capsid protein formation is found in the R2 mRNA from which it is made. An additional RX’ mRNA, specific to ADV, codes for a third protein. The actual type and function of the protein has not been determined, but it has been designated as a NS protein because it is spliced from the left open reading frame mRNA. Interestingly, neither the RX’ mRNA nor this third protein is found in other autonomous parvoviruses.

In other parvoviruses, such as minute virus of mice (MVM), the NS2 protein is required for proper capsid assembly and nuclear transport. Three forms of the MVM NS2 protein also can be synthesized by alternative splicing of mRNA. The NS2 protein is found in both the nucleus and cytoplasm in MVM infection, but experiments have determined that the protein is located predominantly in the cytoplasm. A similar experiment may be of use in determining if the location is the same for the ADV non-structural proteins.

Several uncharacterized ADV isolates have been identified, but the most common strains include ADV-Utah 1, ADV-G, and ADV-F. ADV-Utah 1 was the first isolate of ADV, and is highly pathogenic in all types of mink. The pathogenicity of a particular strain of ADV
depends on genetic makeup and host specificity and is regulated by the hypervariable region of the sequence encoding the virus capsid. This explains the generally increased severity of ADV disease in mink versus ferrets. At least one ferret-specific strain of ADV (ADV-F) has been documented and is pathogenic in ferrets but not in mink. The ADV-G strain was developed in the 1980s to facilitate ADV research in cell culture. This virus is apathogenic in vivo, but has provided much of the cellular and molecular information that is known about ADV.

**ADV infection**

Similar to other autonomous parvoviruses, ADV replicates during the S-phase of mitosis of the host cell and regenerates genomic DNA by forming dsDNA intermediates. Approximately $10^5$ copies of both forms of DNA can be found in a given cell at one time. Cellular replication and infectivity of ADV have been studied both in vivo and in vitro. While most research studies of viral replication have been conducted in mink, studies in ferrets have demonstrated that the mode of viral replication is similar. Classic mink AD targets macrophages and dendritic cells for viral replication and sequestration, with peak ADV infection revealing cytoplasmic viral antigen in phagocytic cells of the liver and lymphoid organs. Aleutian mink disease viral DNA also has been found in renal tubular epithelial cells. Viral antigen can be detected throughout ADV infection, but declines with time and may become undetectable.

In newborn mink kits, ADV replicates rapidly in type II alveolar cells. Why these cells are targeted as opposed to macrophages and dendritic cells in adult mink is unknown. Interestingly, clinical disease in newborn mink resembles that of feline panleukopenia, canine parvoviral enteritis, and mink enteritis. Ferret kits have not been studied in detail, but ADV infection appears to be more severe in younger ferrets. Studies of ADV infection in ferret kits may help elucidate the pathogenesis of viral infection.
Persistence of ADV in mink is characterized by elevated numbers of CD8$^+$ lymphocytes and plasma cells.$^{66}$ Long term infection with ADV causes severe immune complex disease, including vasculitis, due to the inability of the virus to be eliminated \textit{in vivo}. A strong antibody response and associated hypergammaglobulinemia result from the antiviral immune response. Antibodies are directed against both structural (capsid) and nonstructural proteins but are unable to clear the virus from the body.$^{67-68}$ The response is also associated with the formation of autoantibodies.$^{69}$ At the cellular level, ADV has been shown to infect macrophages via the Fc (complement) receptor, an action known as antibody-dependent enhancement of infection.$^{70}$ Antibody-antigen complexes containing infectious virus have been demonstrated, which facilitates further infection of macrophages.$^{70}$ While a natural or passive antibody response to ADV can prevent fulminant infection in mink kits or in Crandell Feline Kidney (CRFK) cells \textit{in vitro}, this same response greatly contributes to the disease in adult mink.$^{67}$

More recent studies have focused on understanding the pathogenicity of ADV infection, mechanisms of viral persistence, and development of a vaccine that will confer immunity without stimulating an adverse antibody response to viral proteins. The hypervariable region of the ADV genome that encodes the capsid protein determines the degree of viral pathogenicity. The ADV-G strain has been used to locate two residues of the capsid protein that influence replication of the virus. One experiment created an ADV-G virus where VP2 amino acid residue 534 (histidine, H) was changed to aspartic acid (D), mimicking ADV-Utah. Changing this amino acid residue enabled ADV-G to be infectious both \textit{in vitro} and \textit{in vivo}.$^{71}$ Mink were inoculated with this engineered strain of ADV and developed transient viremia with a strong antibody response at 30 days post inoculation.$^{30}$ Different histopathological changes were observed, including diffuse hepatocellular microvesicular steatosis and abnormal accumulation
of intracellular fat, but the mink did not develop classical AD. Although ADV-G and ADV-Utah differ at only 5 amino acid residues in this region of the capsid protein, single residue changes can greatly alter the pathogenicity of the ADV virus.\textsuperscript{71} Sequences that code for the non-structural ADV proteins also have been evaluated with regard to viral pathogenicity. One experiment demonstrated that substitution of the NS1 and NS2 coding sequences of ADV-G with corresponding sequences from ADV-Utah did not alter the restructured virus from being infective \textit{in vitro} only.\textsuperscript{72-73} Additional studies have suggested genetic immunodominance within the VP2 protein. Thus, the pathogenicity of ADV could be altered by the type and site of mutation within this region of the genome.\textsuperscript{74-75}

Aleutian mink disease parvovirus is the first example of a DNA virus that both induces apoptosis and requires caspase activity for viral replication.\textsuperscript{76} Caspases are cytosolic cysteine proteases. When these enzymes are activated, they cleave substrates for apoptosis and nuclear fragmentation.\textsuperscript{77-78} An \textit{in vitro} experiment has demonstrated that caspases cleave the ADV NS1 protein at two sites during replication in CRFK cells, allowing the proteins to translocate from the cytoplasm to the nucleus for viral replication.\textsuperscript{76} The caspase recognition site is known to be conserved in at least the ADV-G, ADV-Utah, and ADV-SL strains.\textsuperscript{76} Because caspase activity and NS1 protein cleavage are required regardless of \textit{in vitro} or \textit{in vivo} infection, these events are considered to be independent of viral pathogenicity.\textsuperscript{76} In the same study, cleavage of the structural proteins (VP1 and VP2) was unsuccessful. The NS1 protein of other parvoviruses, such as MVM and parvovirus B19 of humans, also promotes caspase dependent apoptosis.\textsuperscript{40, 76} Apoptosis and control of viral replication may partially elucidate the infectivity of ADV and determine whether the immune response plays a role in the severity of viral infection.
The complete immune response to ADV has not been studied in detail. Both mink and ferrets develop an antibody response to the structural and non-structural viral proteins, although antibody against either the two structural or non-structural proteins alone does occur.\textsuperscript{79} This occurrence is likely due to the host-specific response to infection with different ADV strains, or perhaps varying levels of replication depending on the stage of the infection. Since the pathogenicity of the virus is dependent on variations within the hypervariable region of the capsid protein, each individual strain has the potential to affect a host differently. In combination with host-specific responses to the virus, the exact immune response to ADV will vary from animal to animal. This is evident as mink and ferrets respond slightly differently to infection with ADV. Further understanding of this phenomenon has yet to be studied.

Experimental ADV infection in mink causes IgM titers to increase from 6 days post infection (PI) through 15 to 18 days PI.\textsuperscript{27} Specific antiviral IgG cannot be detected until ~12 days PI after which the antibody titer steadily increases by 30 days PI.\textsuperscript{27} Concentrations of IgG remain high even after IgM concentration returns to the reference interval.\textsuperscript{27} Specific immunoglobulin responses in ferrets have not been studied to date, although ferrets inoculated with mink ADV strains can be detected from 136 to 180 days PI.\textsuperscript{80-81} Very little is understood about how ADV infection affects cell signaling of the immune response. Knowledge of the activation of effector molecules, such as cytokines, would aid in the understanding of ADV infection in mink and ferrets.

Complete immunity against ADV has yet to be demonstrated, although DNA vaccination with the NS1 gene is partially effective in protecting mink from the development of AD.\textsuperscript{82} In this study, one-year-old female mink were vaccinated with a DNA plasmid construct that codes for the NS1 protein. Following viral challenge one month post vaccination with either DNA
plasmid alone or DNA plus the NS1 protein plasmid, reduced plasma gammaglobulin concentrations and milder disease were observed. One difference among these two groups found that the DNA plus protein vaccinated group had an elevation of CD8\(^+\) lymphocytes at the time of challenge, indicating an anamnestic response.\(^82\) In addition, a majority of monocytes and CD8\(^+\) lymphocytes produced IFN-\(\gamma\). Both groups that received the DNA vaccine also developed antibody titers that were comparable to the unvaccinated control group. Overall, antibodies against both structural and non-structural proteins increased over time in all groups. However, antibody responses for each group differed during various times.\(^82\) These inconsistent responses should be evaluated in more detail to monitor immune responsiveness. While immunological memory is important in the immune response, the researchers of this study did not address the potential downfall of IFN-\(\gamma\) activating macrophages. If macrophages are infected with ADV, their activation would probably hinder the immune response to the virus. Since ADV targets macrophages for viral replication, stimulation of macrophages would likely diminish the protective effects of vaccination with the NS1 protein.

**Transmission of ADV**

Natural horizontal transmission of ADV among mink is considered to occur orally or by aerosolization.\(^61-63\) Furthermore, research has demonstrated that AD can be caused by inoculating naïve mink with whole blood, serum, urine, feces, saliva, and bone marrow from infected mink.\(^24, 62, 64\) Vertical transmission of ADV is more likely to occur with infected dams, although the risk of kits becoming infected is lower if the dam has nonprogressive, subclinical AD.\(^65\) Dams infected before embryo implantation are more likely to experience fetal resorption or have stillborn kits.\(^65\)
In ferrets, natural horizontal transmission of ADV is probably the same as in mink. Vertical transmission of ADV is likely, and although no conclusive research exists, the current trend suggests that it does occur. With the advancement of diagnostics, transmission of ADV among ferrets should be somewhat easy to pinpoint. Control of disease in animal shelters and multiple ferret households would be improved if there was more information regarding viral spread between individual ferrets.

**Clinical signs, gross lesions, and histologic lesions of ADV infection in ferrets**

Published reports of ADV infection in ferrets have been limited over the past 25 years. There are several possible explanations. Either AD has not been prevalent in ferrets until recently, ADV infection has not been considered in the differential diagnosis of a sick ferret, or viral infections largely have been subclinical. In any event, ADV infection is being recognized with increasing frequency in the pet ferret population. Clinical signs of ADV infection in ferrets are listed in Table 2.1. A variety of lesions have been observed grossly during necropsy of ADV-infected ferrets (Table 2.2). However, most of these lesions are nonspecific or are not pathognomonic for ADV infection. An array of histologic lesions have been observed in the tissues and organs of ADV-infected ferrets. Lymphoplasmacytic infiltrates are characteristic of ADV infection; however, other microscopic lesions also have been observed (Table 2.3).

**Laboratory diagnosis of ADV infection in ferrets**

Counterimmunoelectrophoresis remains the most common test to diagnose ADV infection in mink and ferrets. The current commercial CIEP was originally designed for screening mink herds for ADV, but this test is also used to screen for ADV infection in ferrets (Table 2.4). Positive test results are based on the visual observation of a grayish-white
immunoprecipitate in the agarose gel. Endpoint titers, which are critical to evaluate the progression of ADV during chronic infection, are not offered in the commercial CIEP assay.

An ELISA to detect antibodies to the ADV NS1 protein is available, although the potential for a false negative test result exists because only one protein is targeted. Caution should be exercised when using saliva to detect antibody responses to the NS1 protein, as concentrations of IgM and IgG in saliva may not correlate with the severity of clinical disease (Table 2.4).

**Presentation and Treatment**

Currently, there is no effective vaccine or treatment to prevent or eliminate ADV infection in mink and ferrets. In mink that are commercially raised for pelt production, outbreaks of AD have been managed by test and eradication programs.\(^{24,87}\) This approach usually is undesirable for privately owned pet ferrets. Until an effective vaccine or viral-specific treatment regimen is available, ADV infection in ferrets should be managed by routine laboratory testing, proper hygiene and quarantine procedures, and supportive care of clinically ill individuals. Supportive care usually is directed toward correcting electrolyte and fluid imbalances from enteritis and preventing secondary bacterial infections.\(^{24}\) Corticosteroids have been prescribed to decrease the deleterious effects of the immune response associated with ADV.\(^{20}\) However, suppression of the humoral immune response in ADV infection predisposes the patient to other secondary or opportunistic infections.\(^{88}\)

Several experimental treatments have been tried that focus on immune system suppression in mink, but much of the data is anecdotal or based on the responses of individual animals. Levamisole, cyclophosphamide, and interferon inducer (polyinosinic-polycytidilic acid, poly IC) have been used to decrease hypergammaglobulinemia and immune complex
The results of these treatment regimens have been variable and of limited benefit. Most negative side effects were associated with cyclophosphamide administration and included depression, anorexia, cyanosis, and leukopenia. None of these treatments has been tried in ferrets, so their ultimate effect, if any, is unknown. Until an effective vaccine and antiviral treatment are discovered, management of ADV infection will follow more traditional regimens of hygiene and preventive medicine.
Table 2.1. Clinical Signs of Aleutian disease viral infection in domestic ferrets.

- Clinical signs of illness not present (subclinical infection)
- Chronic wasting
- Neurological deficits
  - Posterior paresis or paralysis
  - Urinary incontinence
- Fecal incontinence
- Severe coughing
- Hypergammaglobulinemia
- Anemia
Table 2.2. *Gross post mortem lesions in domestic ferrets with ADV infection.*

<table>
<thead>
<tr>
<th>Gross lesions not present</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>• Subclinical infections</td>
<td></td>
</tr>
</tbody>
</table>

**Respiratory system**
- Serosanguineous pleural effusion
- Pulmonary edema
- Pulmonary ecchymoses
- Lung lobe consolidation and collapse (especially right middle lung lobe)
- Diffuse congestion

**Urinary system**
- Kidneys
  - Pale and enlarged with scattered white nodules
  - Small and shrunken with chronic wasting disease
- Bladder
  - Thickened and congested bladder wall (cystitis)

**Digestive system**
- Liver
  - Scattered white nodules
  - Pale, 1 to 3 mm diameter lesions on surface
- Small intestine
  - Scattered ecchymoses on mucosal surface
  - Melena

**Lymphohematopoietic system**
- Spleen
  - Mild to moderate splenomegaly
- Lymph nodes
  - Enlarged, especially mesenteric lymph nodes
Table 2.3. Histopathological lesions associated with ADV in ferrets.

<table>
<thead>
<tr>
<th>Nervous system</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Small areas of mononuclear cell infiltration throughout central nervous system</td>
</tr>
<tr>
<td>Meninges</td>
</tr>
<tr>
<td>• Lymphoplasmacytic infiltrates with occasional histiocytes</td>
</tr>
<tr>
<td>Brain</td>
</tr>
<tr>
<td>• Plasmacytic infiltrates with occasional histiocytes - choroid plexus</td>
</tr>
<tr>
<td>• Lymphoplasmacytic perivascular cuffing of blood vessels - grey and white matter</td>
</tr>
<tr>
<td>• Gliosis</td>
</tr>
<tr>
<td>• Astrocytosis</td>
</tr>
<tr>
<td>• Pukinje cell degeneration</td>
</tr>
<tr>
<td>• Focal malacia</td>
</tr>
<tr>
<td>Spinal cord</td>
</tr>
<tr>
<td>• Lymphoplasmacytic perivascular cuffing - grey and white matter</td>
</tr>
<tr>
<td>• Neuronal degeneration</td>
</tr>
<tr>
<td>• Mononuclear cell infiltration</td>
</tr>
<tr>
<td>• Multifocal malacia</td>
</tr>
<tr>
<td>• Severe meningitis</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lymphohematopoietic system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph nodes</td>
</tr>
<tr>
<td>• Diffuse plasmacytosis</td>
</tr>
<tr>
<td>• Lymphoid hyperplasia, especially involving the mesenteric lymph nodes</td>
</tr>
<tr>
<td>Spleen</td>
</tr>
<tr>
<td>• Extramedullar hematopoiesis, including myeloid hyperplasia and megakaryocytopoiesis</td>
</tr>
<tr>
<td>• Enlarged periarteriolar lymphatic sheaths that occasionally coalesce (hyperplasia of white pulp)</td>
</tr>
<tr>
<td>• Homogeneous eosinophilic material in some germinal centers (negative for amyloid)</td>
</tr>
<tr>
<td>• Hemorrhage</td>
</tr>
<tr>
<td>• Diffuse congestion (accentuated red pulp)</td>
</tr>
<tr>
<td>Bone marrow</td>
</tr>
<tr>
<td>• Increased hematopoiesis</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cardiovascular system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
</tr>
<tr>
<td>• Multifocal lymphoplasmacytic infiltrates with occasional histiocytes</td>
</tr>
<tr>
<td>• Myocardial degeneration</td>
</tr>
<tr>
<td>• Myocardial arteritis</td>
</tr>
<tr>
<td>• Pericardial hyperplasia</td>
</tr>
<tr>
<td>Blood vessels</td>
</tr>
<tr>
<td>• Hyalinization of arteriolar walls</td>
</tr>
<tr>
<td>• Thrombosis</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Respiratory system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
</tr>
<tr>
<td>• Multifocal interstitial lymphoplasmacytic infiltrates with occasional histiocytes, alveolar edema, fibrin deposition, and neutrophil infiltration</td>
</tr>
<tr>
<td>• Diffuse congestion</td>
</tr>
<tr>
<td>• Hemorrhage</td>
</tr>
<tr>
<td>• Atelectasis</td>
</tr>
</tbody>
</table>
Table 2.3 (cont.). Histopathological lesions associated with ADV in ferrets.

<table>
<thead>
<tr>
<th>System</th>
<th>Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Digestive system</strong></td>
<td></td>
</tr>
<tr>
<td>Salivary glands</td>
<td>• Lymphoplasmacytic infiltration</td>
</tr>
<tr>
<td>Stomach</td>
<td>• Lymphocytic infiltration</td>
</tr>
<tr>
<td>Small intestine</td>
<td>• Plasmacytic infiltration with occasional histiocytes</td>
</tr>
<tr>
<td>Liver</td>
<td>• Periportal mononuclear cell infiltration</td>
</tr>
<tr>
<td></td>
<td>• Bile duct dilation and proliferation</td>
</tr>
<tr>
<td></td>
<td>• Hepatic vacuolation</td>
</tr>
<tr>
<td></td>
<td>• Hyalin deposits in hepatocytes</td>
</tr>
<tr>
<td>Pancreas</td>
<td>• Lymphoplasmacytic infiltration</td>
</tr>
<tr>
<td><strong>Urinary system</strong></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>• Glomerular fibrosis and sclerosis</td>
</tr>
<tr>
<td></td>
<td>• Renal cortex plasmacytosis and renal tubule degeneration</td>
</tr>
<tr>
<td></td>
<td>• Foci of lymphocytic infiltration in cortex and medulla</td>
</tr>
<tr>
<td></td>
<td>• Hemosiderosis</td>
</tr>
<tr>
<td></td>
<td>• Widespread multifocal mononuclear cell infiltration</td>
</tr>
<tr>
<td>Bladder</td>
<td>• Bladder wall edema</td>
</tr>
<tr>
<td><strong>Special senses</strong></td>
<td></td>
</tr>
<tr>
<td>Eye</td>
<td>• Severe anterior uveitis</td>
</tr>
<tr>
<td><strong>Endocrine system</strong></td>
<td></td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>• Focal mononuclear cell infiltration</td>
</tr>
<tr>
<td>Thyroid gland</td>
<td>• Lymphoplasmacytic infiltration</td>
</tr>
</tbody>
</table>
Table 2.4. Commercial tests to detect anti-ADV antibody.

<table>
<thead>
<tr>
<th>Test</th>
<th>Company</th>
<th>Description</th>
<th>Results</th>
<th>Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Counterimmunoelectrophoresis</td>
<td>United Vaccines, Inc.</td>
<td>Tests for antibody against ADV virus in ~ 10 µl whole blood or serum (capillary tube preferred)</td>
<td>Reported as positive, negative, or no sample (if tube breaks)</td>
<td>$10.50 first sample</td>
</tr>
<tr>
<td></td>
<td>Madison, WI 800-283-6465</td>
<td></td>
<td>48 hour turnaround</td>
<td>$9.00 each additional sample</td>
</tr>
<tr>
<td>ELISA</td>
<td>Avecon Diagnostics, Inc.</td>
<td>Tests for antibody against ADV non-structural NS1 protein</td>
<td>Reported as positive, weak positive, or negative</td>
<td>$12.00 per individual test (saliva or blood)</td>
</tr>
<tr>
<td></td>
<td>Bath, PA 800-249-5875</td>
<td></td>
<td>Requires serum at 1:10 dilution or undiluted saliva</td>
<td>$10.00 per test for batches of 10 or more specimens</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>48 hour turnaround</td>
<td></td>
</tr>
</tbody>
</table>
References


CHAPTER 3

PERSISTENT VIRAL SHEDDING DURING SUB-CLINICAL
ALEUTIAN MINK DISEASE PARVOVIRAL INFECTION IN A FERRET

Abstract

A two-year-old domestic ferret appeared clinically healthy, but was repeatedly seropositive for Aleutian mink disease parvovirus (ADV) over a 2-year observation period. Reciprocal antibody titers, determined by counterimmunoelectrophoresis, ranged from 1024 to 4096. Viral DNA also was identified in serum, urine, feces, and blood cell fractions by polymerase chain reaction analysis. Ultimately, DNA in situ hybridization revealed ADV DNA in histologic sections of various tissues and organs.

Key words.

Aleutian disease virus, Counterimmunoelectrophoresis, PCR, In situ hybridization
Aleutian mink disease parvovirus is a naturally occurring parvovirus that infects mustelids such as mink and ferrets. The disease was first described in Aleutian mink, which have an increased susceptibility to disease resulting from a Chediak-Higashi-like defect in lysosomal membrane function and reduced cell-mediated immune responses necessary for combating viral infections. Normal or wild type mink are asymptomatic or have mild to moderate clinical signs of disease, chronically elevated antibody titers, and have no detectable viremia or transient viremia depending upon the strain of ADV. Experimental infections of ferrets with mink-derived strains of ADV have resulted in clinical scenarios similar to that of mink with dark coat colors. Natural ADV outbreaks in ferret shelters and multiple ferret households have been increasingly reported. Common clinical signs associated with ADV infection in ferrets include chronic progressive weight loss, cachexia, malaise, and melena. Ataxia leading to posterior paralysis, tremors, and convulsions has also been observed. Little is known about the pathogenesis of natural ADV infection in ferrets or the route by which viral shedding occurs.

Serum from a clinically normal, two-year-old, male, sable point ferret was sent to The University of Georgia College of Veterinary Medicine to test for Aleutian mink disease parvovirus (ADV). Serum samples from the ferret were submitted intermittently for anti-ADV antibody testing over a period of 1.5 years. Counterimmunoelectrophoresis (CIEP) was performed to determine an endpoint antibody titer within the range of 1:4 through 1:16,384. Nine serum samples were consistently positive for anti-ADV antibodies with a reciprocal titer =1024 over a period of 1.5 years (Figure 3.1). Serum was also tested for the presence of ADV DNA using a polymerase chain reaction (PCR) assay. Eight of nine serum samples assayed yielded the appropriately sized band (Figure 3.2).
Additional testing of anticoagulated whole blood was done to determine which fractions were associated with viral DNA. A 1:1 mixture of PBS-diluted, heparinized blood was separated into three layers using gradient centrifugation. A commercial kit was used to extract and purify DNA from the erythrocytes, buffy coat, and plasma fractions. These DNA samples were evaluated by PCR analysis to detect ADV. The acellular plasma pool was negative, but the erythrocyte and buffy coat fractions were positive for ADV DNA. The negative PCR result for the plasma was attributed to possible interference of PCR amplification by residual reagents or anticoagulant.

Four urine samples and two fecal samples were submitted during the course of the study to determine the likelihood of viral shedding through the urinary and gastrointestinal tracts, respectively. Deoxyribonucleic acid was purified from these specimens using commercially available kits. Following PCR amplification of extracted DNA, two of four urine samples and both fecal samples were positive for ADV. The 600 base PCR sequences from urine and serum samples collected 9 months apart were identical when aligned using commercially available software. When compared to the published sequence for ADV isolated from a ferret, eight single-base differences were identified, with an adenosine to thymine substitution at position 171 (nt 3212) causing a substitution mutation from serine to threonine at amino acid position #325.

The ferret remained clinically normal for 1.5 years following diagnosis of ADV before deteriorating health was first observed. Ultimately, the ferret was euthanized and a necropsy was performed. Nonspecific observations on gross examination included mild atelectasis of one lung due to thoracic effusion. The pericardium was slightly thickened. Soft, pale, round masses were present in the intestinal tract, but specific locations were not noted. Sections of spleen, liver, lung, and kidney were frozen for subsequent DNA analysis. Organ samples were also collected.
and preserved in 10% neutral buffered formalin for routine histopathological evaluation and DNA in situ hybridization (ISH) to detect ADV DNA.

Deoxyribonucleic acid was extracted and purified\(^b\) from frozen tissues and tested by a PCR-based assay as described for serum. Aleutian mink disease viral amplicons of 695-bp were obtained from spleen, liver, lung, and kidney. Additionally, CIEP detected a reciprocal titer of 4096 in the pleural fluid. Aleutian disease viral DNA was not detected by PCR.

Formalin fixed tissues were submitted to The University of Georgia for histopathologic examination. Mild lymphoplasmacytic pericarditis, cholangiohepatitis, and interstitial nephritis were suggestive of infection with ADV (Figure 3.3).\(^{11-14}\) Splenic extramedullary hematopoiesis, pulmonary edema, and fibrinopurulent alveolitis were also present. A homogeneous population of lymphoblasts was present in the spleen, epicardium, and subcapsular area of the kidney. Lymphoma was suspected to be the cause of the recent clinical signs that led to euthanasia, but was not attributed to the ADV infection.

To determine the cellular location of viral DNA in the ferret, ISH was performed.\(^{15}\) Tissue sections were hybridized with a digoxigenin-labeled,\(^5\) PCR-generated probe that was amplified from purified ADV-G plasmid DNA. Sites of probe hybridization were visualized by high affinity immunohistochemistry using antidigoxigenin antibody\(^6\) conjugated to alkaline phosphatase and a chromogen solution\(^6\) of nitroblue tetrazolium dye. The presence of DNA:DNA hybrids was identified by the deposition of blue-black, insoluble pigment (formazan). Aleutian mink disease parvovirus DNA was detected in sections of spleen, liver, lung, kidney, and pericardial fat (Figure 3.4). Reactivity was localized to the nucleus and cytoplasm of follicular dendritic cells and macrophages.\(^{16}\)
The ferret of this report had chronically increased levels of anti-ADV antibody compatible with viral persistence and chronic antigenic stimulation. However, antibody test results do not indicate whether the stimulus for chronic antibody production is due to ongoing viral replication or sequestration of virus. Typically, the increased levels of anti-ADV antibody in mink are non-neutralizing and may be ineffective in clearing the virus.\textsuperscript{16}

The continued detection of ADV DNA in the blood, urine, and feces provides evidence for persistent viral replication in this ferret. Although environmental contamination might be responsible for PCR-positive test results on the fecal specimens, active viral infection would be a more likely cause of viremia and viral shedding in the urine. The viral DNA detected in cellular fractions of whole blood probably was cell surface-associated as antigen-antibody complexes. Aleutian mink disease parvovirus in the systemic circulation is often present in antigen-antibody complexes and may bind to cellular components of blood.

Gross necropsy findings in ADV infection usually are unremarkable and may include hepatomegaly, splenomegaly, and mesenteric lymphadenopathy.\textsuperscript{11-12} Routine histopathology may reveal lymphoplasmacytic inflammation; however, similar infiltrates may be observed in the absence of ADV infection. In contrast, DNA ISH can confirm the presence of ADV and localize sites of viral infection.

The amplified viral DNA from serum and urine was sequenced to confirm that the amplicons originated from ADV, to identify the strain of ADV present, and to determine if mutations had occurred in the genetic code for a portion of the major capsid protein over the course of infection. The 365 base segment from nt3043 to nt3407, including the hypervariable region of the ADV capsid encoding ORF, was similar to the ADV-F sequence, but had eight point mutations that resulted in one amino acid substitution from serine to threonine.\textsuperscript{10}
single mutation resembles the wild type mink Utah1, ADV-G, and ADV-K strains rather than the ADV-F sequence, suggesting a possible new locus to identify pathogenicity among sequenced DNA isolated from ADV positive ferrets. Since the mutations in the hypervariable region of the DNA sequence encoding the capsid protein determine the pathogenicity of a given ADV strain, the persistence of ADV-F in the ferret community is of concern.

Since the ability of the virus to cause disease is highly dependent on the host immune response and genotype, methods to detect ADV by antibody titer and presence of viral DNA are important. Concurrent CIEP and PCR analysis are the best approach for antemortem diagnosis of asymptomatic ADV infection in ferrets.
Acknowledgements

This work was supported in part by donations of ferret enthusiasts to the Emerging Diseases Research Group and the UGARF Animal Health Fund at the University of Georgia. The authors thank Mrs. Danee DeVore and Dr. Thomas A. Kawasaki, Old Bridge Veterinary Hospital, Woodbridge, VA, for the medical history and collection of clinical samples. Dr. Jeff Mauldin, Rome, GA is acknowledged for his technical assistance.
Sources and Manufacturers

a. Histopaque – 1077, Sigma Diagnostics, St. Louis, MO

b. QIAamp DNeasy Tissue Kit, Qiagen, Valencia, CA

c. QIAamp viral RNA mini kit, Qiagen, Valencia, CA (isolates both DNA and RNA by default)

d. QIAamp stool DNA mini kit, Qiagen, Valencia, CA

e. Gene Runner, version 3.05, http://www.generunner.com

f. PCR DIG Labeling Mix, Roche Applied Science, Indianapolis, IN

g. Digoxigenin Detection Kit, Roche Applied Science, Indianapolis, IN
Figure 3.1. Counterimmunoelectrophoresis gel immunoprecipitation of four-fold serial dilutions of ferret serum. Positive (POS) and negative (NEG) control wells are shown for comparison.
Figure 3.2. Gel electrophoresis of PCR products obtained from purified serum and urine DNA. Molecular weight markers (MW) are indicated. Water (N) used in the reaction was tested for purity. The ferret serum and urine samples were compared to known mink negative (NEG) and positive (POS) serum controls. Known ferret serum and urine DNA negative controls (B), respectively, also were included. Lane E is empty.
Figure 3.3. Aleutian disease virus-associated lymphoplasmacytic infiltrates in the renal interstitium. Ferret kidney, hematoxylin and eosin stain.
Figure 3.4. Aleutian disease viral nucleic acid (blue-black formazan pigment deposits) is present within the renal interstitial infiltrates. Ferret kidney, DNA in situ hybridization with formazan chromagen and fast green counterstain.
References


CHAPTER 4

DEVELOPMENT OF AN ELISA TO DETERMINE ALEUTIAN MINK DISEASE PARVOVIRUS (ADV) CAPSID PROTEIN ANTIBODY TITERS

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Abstract

An ELISA was developed for detecting serum antibody against the major capsid proteins of Aleutian disease virus (ADV). Because the viral structural proteins are more commonly expressed, this ELISA was found to be more specific than similar commercial assays. This new ELISA technique may replace the current counterimmunoelectrophoresis (CIEP) test, and could be used in combination with other available methods, such as PCR and DNA in situ hybridization, to evaluate viremia and anti-ADV antibody titers concurrently. Finally, this new ELISA determines a wider range of antibody titers, decreases laboratory test turn around time, and is technically efficient.
**Introduction**

Aleutian mink disease virus (ADV) is an autonomous parvovirus that is known to naturally infect all types of mink and ferrets.\(^1\)-\(^4\) While there is a considerable amount of data regarding ADV infection in mink, little is known about this disease in ferrets. The virus spontaneously arose in the mid-1940s in conjunction with the excessive breeding of mink that were recessive for a gene that caused a blue-grey pelt color.\(^5\) These Aleutian mink subsequently began to exhibit elevated gammaglobulin levels and renal disease characterized by glomerulonephritis.\(^6\) The illness was officially termed Aleutian disease (AD) and was determined to be caused by a persistent viral infection.\(^7\)

Experimental inoculation of ferrets with ADV started in the 1960s. Although ferrets were capable of being infected with highly pathogenic mink strains of ADV, they did not develop clinical signs or have histopathological lesions that were as severe as in mink.\(^8\) In the 1980s, ADV was officially characterized as a parvovirus and a cell culture adapted strain (ADV-G) was developed that is cytopathic *in vitro*, but non-pathogenic *in vivo*.\(^1\) Around this time, researchers also discovered that the virus causes fulminant interstitial pneumonia in newborn mink kits by infecting alveolar type II cells.\(^9\) In contrast, classic adult AD targets macrophages and dendritic cells in various tissues and organs.\(^10\)-\(^12\) Infection with ADV has not been studied in newborn ferrets; however, some investigations have found that ADV infection is worse in younger ferrets than in adults.\(^13\)

Adult AD in mink has been classified into three types of disease: progressive AD as originally described in Aleutian mink, persistent nonprogressive infection, or nonpersistent,
nonprogressive infection with clearance of the virus. Thus far, ADV infection has been found to cause similar disease in adult ferrets. Clinical signs of AD in ferrets range from sub-clinical infection to hypergammaglobulinemia accompanied by neurological, cardiac, and respiratory disease.

Current diagnostic tests for ADV include counterimmunoelectrophoresis (CIEP) to detect antibody against the viral capsid proteins and ELISA to detect antibody against the non-structural NS1 protein. The commercially available CIEP test is limited in that it only uses a 1:4 dilution of the serum specimen, giving a positive or negative test result, but a specific antibody titer is not reported. The purpose of this study was to develop a new ELISA test to detect ADV antibody more specifically and quantitatively.

**Materials and Methods**

*Counterimmunoelectrophoresis.* Anti-ADV antibodies were detected based on a previously published counterimmunoelectrophoresis (CIEP) assay. The antigen was derived from semi-purified cell culture supernatant from Crandell feline kidney (CRFK) cells infected with ADV-G. The concentrate of this supernatant was evaluated as a positive control when tested against mink serum containing anti-ADV antibodies at a known titer. Serial 4-fold dilutions of serum samples subsequently were tested at various dilutions ranging from 1:4 to 1:16,384 to determine an endpoint antibody titer. The serum sample was diluted in phosphate-buffered balanced salt solution (PBS) containing 1% normal rabbit serum. Ten µL of each dilution was loaded across from 10 µL of commercial ADV antigen or ADV baculovirus-generated antigen (ADV Bac) in a 0.7% high molecular weight agarose gel prepared with high resolution buffer. Samples were electrophoresed for 40 minutes at 30V. The gel subsequently was soaked overnight in 0.9% saline, dried on a glass plate, and stained for 7 minutes with
0.25% Coomassie blue dye in destaining solution (40% methanol, 10% acetic acid). Excess Coomassie blue dye was removed with destaining solution until immunoprecipitates were detectable by visual inspection as distinct blue bands.

*Polymerase Chain Reaction.* Serum samples also were tested for the presence of ADV DNA using a polymerase chain reaction (PCR)-based assay. The oligonucleotide primers were complementary to a cell culture-derived molecular clone of mink ADV, designated ADV-G. Primer pairs were designed to amplify a 692-bp fragment of the highly conserved VP-2 region of the viral genome that encodes the major capsid protein. The level of detection of the PCR-based assay was <1 fg of viral DNA in 2.5 µL of serum. The PCR products were evaluated in a 2.5% agarose gel containing 0.5 µg/mL ethidium bromide (EtBr) prepared with 0.5X TRIS borate-EDTA buffer with an additional 0.5 µg/mL EtBr. The gel was electrophoresed for 1.5 hours at 75V, and PCR products were visualized by transillumination using an ultraviolet lamp.

*DNA in situ Hybridization.* In order to determine the cellular location of viral DNA in ferret tissues, DNA in situ hybridization (ISH) was performed. Sections of paraffin-embedded tissues were mounted on capillary gap slides, deparaffinized, and subsequently rehydrated through graded alcohol solutions to 1X Autobuffer. After tissue digestion at 37°C for 10 minutes with 0.25% pepsin dissolved in 1X Autobuffer, pH 2.0, the sections were pre-hybridized with 100% formamide for 5 minutes at 105°C. Tissues were hybridized in the probe solution for 60 minutes at 37°C. This hybridization solution contained a digoxigenin labeled PCR-generated probe amplified from purified ADV-G plasmid DNA using the same primer pairs as those in the previously described PCR-based assay. Antidigoxigenin antibody conjugated to alkaline phosphatase was then applied (500:1 dilution) to the tissue sections, and they were
incubated for an additional hour at 37°C. The sections were then incubated in chromagen solution\(^h\) (nitroblue tetrazolium dye solution) for 1 hour at 37°C to detect sites of probe hybridization. The slides were rinsed, counterstained in 1% fast green dye for 5 minutes, coverslipped, and examined microscopically. The presence of DNA:DNA hybrids was identified by the deposition of blue-black pigment (formazan).

**ADV-Baculovirus Particle Isolation.** A 2.3-kb cDNA clone encoding ADV structural capsid proteins VP1 and VP2 was inserted into the polyhedron gene of *Autographa californica* nuclear polyhedrosis virus (AcNPV) and expressed by the recombinant virus AcADV-1 in *Spodoptera frugiperda* (Sf9) cells.\(^{22}\) This technique was completed as part of an initial experiment to express ADV capsid proteins in a baculovirus system. Supernatant from a successful infection, as well as the fluorescent labeled mink ADV antibody solution was graciously donated by Dr. Marshall Bloom and Jim Wolfinbarger at Rocky Mountain Laboratories, Hamilton, MT.

Spinner cultures of Sf9 insect cells\(^i\) were seeded in Graces insect medium\(^i\) (containing 10% fetal bovine serum, 0.002 mg/ml gentamycin) at a concentration of 2.0×10\(^6\) cells/ml and infected with 10 ml of ADV baculovirus stock (multiplicity of infection = 1 plaque forming unit/cell). Cells were spun at 97 rpm at 28°C and monitored daily for viability using 0.04% trypan blue dye solution. When cell death was observed to be ~50%, the cell culture was checked for adequate infection with ADV using fluorescent antibody staining. Cytospin slides containing 0.1 ml culture in 0.9 ml media were prepared and fixed for 15 minutes in ethanol. Normal mink serum, diluted 1:15 in PBS, was applied and the slides were incubated at ambient temperature for 30 minutes in a dark, moist environment. Anti-ADV antibody conjugated to fluorescein isothiocyanate (FITC)\(^j\) was added and the slides were incubated for another 30
minutes. The slides were rinsed twice with PBS, once with 70% ethanol, and coverslipped for examination by fluorescence microscopy. The presence of ADV was identified by green fluorescence within and on the surface of the cultured cells.

After confirming viral infection of the cell culture, the spinner flask was chilled on ice for 15 minutes. The cell suspension was centrifuged at 4°C and 16,264 x g for 15 minutes, and the pellet was resuspended in 50 ml cold lysis buffer (50 mM Tris, 150 mM NaCl, 0.2% Triton-X 100) containing fresh protease inhibitor tablets. Four freeze-thaw cycles in liquid nitrogen were followed by 3 sonication cycles at 6 watts for 15 seconds each to further lyse the cells. The lysate was centrifuged for 30 minutes as detailed above. Chloroform was added at a 50:50 v/v ratio to the supernatant, sonicated for 30 seconds at 7 watts, and centrifuged again for 30 minutes. This extraction procedure was repeated twice on the aqueous upper phase of the cell culture supernatant. Polyethylene glycol was added to the final supernatant (1:10 w/v ratio) and stirred slowly overnight in a 4°C cold room. The remaining solution was centrifuged at 8,635 x g for 30 minutes at 4°C, and the pellet was resuspended in 2.5 ml 50 mM Tris-250 mM NaCl with protease inhibitor tablets. The suspension was recentrifuged under the same conditions and purified using a 0.22 µm filter.

Four 10 µl samples were taken during the viral particle isolation and added to 10 µl of 2X Laemmli sample buffer with 6% beta-mercaptoethanol for SDS-PAGE analysis to assess sample purity. Samples were incubated at 99°C for 10 minutes prior to electrophoresis in a 12% polyacrylamide gel at 150V for 1 hour. The gel was stained with Fairbanks A Coomassie blue solution and subsequently destained with Fairbanks D solution according to a previously published protocol. A sample of the final solution also was examined by negative contrast electron microscopy to verify the presence of empty virion capsids. The relative amount of
protein was determined using 260/280 nm spectrophotometric analysis to compare protein versus DNA concentration. The sample was diluted 1:100 in buffer (2.5 ml 50 mM Tris-250 mM) prior to measuring the absorbance.

**ADV-Enzyme-linked Immunosorbent Assay (ELISA) for Antibody Detection.** All incubations occurred in a humid, 37°C chamber. Rows B through H of a 12 x 8 well microtiter plate were coated with 100 µl of viral particles at a working concentration of 0.1 ng/ml of viral particles in PBS. Row A was coated with PBS as a background control. The plate was incubated overnight (~18 hours) to allow the viral particles to adhere to the plate surface. After washing twice with 200 µl of PT buffer (PBS, 0.5% Tween 20), 200 µl of PTM blocking solution (PBS, 0.5% Tween 20, 5.0% dry milk) was added to all wells and the microtiter plate was incubated for 2 hours. During this period, dilutions of patient serum samples were made in a separate microtiter plate. Serial, 5-fold dilutions were made in 200 µl PTM from a starting dilution of 1:10 (25 µl serum in 225 µl PTM), up to a final dilution of 1:156,250.

After blocking, the wells were washed 4 times with PT solution. Each well in the background row received 100 µl PTM as a control; 100 µl of each previously made dilution was transferred by pipet to the remaining rows. The microtiter plate was incubated for 2 hours to allow antigen-antibody complex formation and washed 6 times with PT solution. Secondary antibody was added by pipeting 100 µl of protein A conjugated to horseradish peroxidase (HRP) and incubated for 30 minutes. Six additional washes were performed to remove any unbound proteins. The wells with Protein A conjugate binding were identified by chromagen development using 3,3’, 5,5’-tetramethylbenzidine (TMB) solution (4 mg/ml in dimethyl sulfoxide diluted in a stock solution of 4.85% 2M sodium acetate in water) plus of 30% hydrogen peroxide (0.0485% final concentration). All wells received 100 µl of the TMB
solution and were incubated at ambient temperature for 15 minutes. Addition of 50 µl of 2M sulfuric acid to each well terminated the reaction and produced a yellow colored solution. Absorbance measurements were determined at 450 nm in a standard 96 well plate reader.

The mink positive control serum, along with several ferret serum samples that were CIEP positive were re-evaluated by ELISA using the commercial Danad antigen and the ADV Bac VP1/VP2 antigen created for this experiment. Donated mink serum from a controlled ADV infection experiment was used as the positive control for all tests. This serum was used for all CIEP assays related to this study. Known ferret serum lacking anti-ADV antibodies was used as the negative control. Although the exact concentration of the commercial antigen used for CIEP was unknown (this antigen is only tested during manufacturing to react with an anti-ADV antibody titer of at least 2,048), a maximum dilution of 1:256 was required to precipitate antigen-antibody complexes in the positive control mink serum using the CIEP method.

Results

The Sf-9 cells were successfully infected with baculovirus that contained the cDNA clone encoding ADV viral capsid proteins VP1 and VP2. Viral infection was identified by monitoring the cultures for cell death. Approximately 4 days post-infection, 50% cellular mortality was observed microscopically following the addition of trypan blue dye. Nonviable cells stained blue and were not refractile, while viable cells resisted trypan blue dye staining and appeared refractile. Prior to harvesting the cells, adequate cellular infection was confirmed on cytospin preparations by fluorescent antibody staining (Figure 4.1). Viral infection and replication were identified by marked cytoplasmic and cell-surface staining involving ~50% of the cell population. Purity of the final supernatant was confirmed by SDS-PAGE (Figure 4.2). Negatively stained electron microscopic preparations revealed that empty, intact virions were
being produced by the baculovirus system (Figure 4.3). Spectrophotometric absorbance at 260 and 280 nm indicated ~37 µg/ml total protein concentration.

The undiluted VP1/VP2 antigen produced in this experiment demonstrated the same CIEP results as those of the commercial Danad antigen concentrate. The CIEP test was not capable of detecting antibodies against either Danad antigen or ADV Bac antigen when specimens were diluted to the concentration used for the ELISA test.

All previously known CIEP positive anti-ADV antibody samples tested were also positive using the ELISA assay developed for this study. The ELISA test had increased sensitivity and required less antigen for the assay. The Danad antigen detected anti-ADV antibodies at a dilution of 1:1000 in PBS and the ADV Bac particles detected anti-ADV antibodies at a dilution of 0.1 ng/ml. A positive test result was recorded if the test serum absorbance reading was above the highest absorbance reading obtained from the negative control serum. The absorbance for each separate dilution was determined by taking the average of the individual absorbances for the triplicate wells of those dilutions. At these antigen concentrations, reciprocal antibody titers were detected up to a dilution of 1:1,250 for all ferret serum samples that had previous positive CIEP reciprocal titers at a 1:1,024 dilution. When re-evaluated using CIEP, the known positive control serum fluctuated between a reciprocal titer of 256 and 1,024 when using the undiluted Danad and undiluted ADV Bac antigens, respectively. The ELISA was more sensitive using less antigen than was required for the CIEP test, with the Danad (1:1000 dilution) and ADV Bac (0.1 ng/ml dilution) antigens detecting reciprocal antibody titers of 1,250 and 6,250, respectively.

Further work with the ELISA procedure revealed that the incubation times could be reduced without losing test sensitivity. Plate incubation times with these antigens (either Danad...
or ADV Bac antigen) were reduced from overnight to 2 hours. Blocking, serum sample, and secondary antibody incubations were reduced to 60 min. The color development step remained unchanged.

In the research conducted for this thesis, ferrets that were test-positive for viral capsid protein antibodies using CIEP and ELISA were tested for the presence of ADV DNA by PCR. During this experiment, several ferrets have tested seropositive for ADV capsid protein antibodies by CIEP and/or ELISA, but were test-negative by PCR. Urine and/or fecal DNA from a few of these select ferrets were PCR test-positive instead (Figures 3.2 and 4.4). One specific ferret that was test-positive by CIEP, ELISA, and PCR also was test-positive for ADV DNA by DNA \textit{in situ} hybridization. Complete studies to monitor results for all of these tests was limited at this time to only this one animal.

\textbf{Discussion}

Viral replication levels in mink infected with highly pathogenic Utah-1 strain may reach \( >10^8 \) infectious doses per gram of lymphoid tissue by \( \sim 10 \) days post infection.\(^{14}\) Mink with chronic AD may generate \( >30 \) mg/ml gammaglobulin concentrations during the course of viral infection.\(^{24}\) Although ADV infection in ferrets is generally less pathogenic than in mink, viral replication most likely occurs during the initial days of infection as well. Classic AD in mink is characterized by a dramatic decrease in viral replication and viral load as the infection progresses, usually without complete clearance of the virus.\(^{24}\)

In mink, an increase in IgM antibody titers is detected as early as 6 days PI.\(^6\) Increased antibody titers persist for at least 3 months post infection (PI).\(^6\) Although IgM concentrations peak around 15 to 18 days PI, the IgG response only becomes apparent at 12-15 days PI.\(^6\) A consistent increase in IgG titers is apparent by 30 days PI.\(^6\) A three-fold increase in IgG
concentration can be seen by 3 months after ADV infection of mink. In persistently infected mink with progressive AD, IgG titers may increase 10 fold. Approximately 80% of the IgG response consists of anti-ADV antibodies. Although neutralizing antibody is hard to demonstrate, attempts to immunize adult mink with ADV antibody accentuated the development of immune-complex disease. IgG immune responses of ferrets have been evaluated following both natural and experimental ADV infection. The antibody titer correlated with the type of viral protein that elicited the antibody response, but there was no difference in antibody titer based upon mode of viral infection. Experiments have demonstrated that mink and ferrets produce an equal distribution of antibody to the four viral proteins VP1, VP2, NS1, and NS2. Ferrets that were infected with ferret ADV strains exhibited lower antibody titers in comparison to the mink infected with mink ADV strains. A statistically significant trend revealed that ferrets with reciprocal antibody titers of 2,560 or above reacted with both structural and non-structural proteins. In contrast, ferrets with reciprocal titers of 640 and below reacted preferentially with non-structural proteins. In ferrets with lower anti-ADV antibody titers, the likelihood of detecting capsid protein antibody is still at least 53%, based on the results generated from these 70 ferrets. Reciprocal ADV antibody titers of at least 640 increase the detection of ADV infection over 62%. Ferrets with anti-ADV antibody titers = 10,240 are over 84% more likely to be producing antibodies to at least the capsid proteins. Further experiments will be necessary to explore the antibody responses to specific viral-associated proteins. This is important because serum antibody titers are used routinely as a diagnostic test for ADV.

Based on the results of this experiment, ELISA apparently provides better detection of anti-ADV capsid protein antibodies than CIEP. The CIEP test is limited to detection of anti-ADV capsid protein antibodies up to a maximum reciprocal titer of 16,384, versus 156,250 for
The disadvantages of the CIEP test include a long turnaround time (test period ~36 hours) and a more complex laboratory procedure. Because gel preparation is necessary, a maximum of 8 individual serum samples can be assessed during one test period. Gel preparation and sample loading are tedious and labor intensive. A positive test result occasionally is difficult to confirm due to uneven drying of the gel and/or irregular staining of the 3 mm diameter immunoprecipitation bands. Final reciprocal titers are determined by visual inspection that may be susceptible to observer bias.

When evaluating a ferret for potential ADV infection, the stage of disease is important in determining how to test for the virus. The ELISA developed in this study can detect ADV by recognizing antibodies produced against the capsid structural proteins. These antibodies should be present whether or not ADV is replicating. In comparison to CIEP, the ELISA appears to provide more sensitive detection of anti-ADV capsid protein antibodies. The current experiment intended to use the ADV Bac VP1/VP2 capsid proteins as the antigen for the ELISA, but further testing found that the commercially available Danad antigen worked equally well. Experimental development of the ELISA antigen used a baculovirus system to produce empty virions that expressed the VP1 and VP2 proteins of the capsid. The Danad antigen is composed of complete or partial ADV-G viral particles, which includes the VP1 and VP2 capsid proteins. While these two antigens are not exactly the same, both detect antibodies against the ADV capsid proteins.

As companion ferrets become more popular, reliable diagnostic tests for ADV are essential. Although ADV has been studied for more than 40 years in mink, the epidemiology of AD has received less attention in ferrets. The ELISA procedure presented in this thesis is more cost effective and less technically demanding. Turnaround time has decreased from >24 hours to about 4 hours. Furthermore, the number of test samples that can be processed is limited only by
available labor. The CIEP test ultimately should be replaced by ELISA, in combination with PCR and DNA in situ hybridization testing. This will allow the clinician to monitor the serum antibody response of ferrets that are suspected to have ADV exposure or infection and detect the presence of viral DNA if infection is present. The commercially available CIEP does not evaluate serial dilutions of the patient’s serum. This prevents detailed evaluation of the ferret’s antibody response to ADV, which can vary from initial viral exposure until the development of acute disease. False positive or false negative test results also can occur from detection of non-specific proteins or from the presence of a very high serum ADV antibody titer that overwhelms the antigen-antibody zone of equilibrium required for formation of immunoprecipitates.

The ELISA provides an endpoint reciprocal antibody titer for ferrets that are exposed to or infected with ADV. False positive test results are unlikely, due to the specificity of the ADV viral capsid structure. False negative test results also are unlikely unless the animal is only producing antibody against the non-structural viral proteins. In this case, the commercially available ELISA for NS1 protein detection in serum in combination with PCR testing could be employed to detect viral DNA and replication. An ideal diagnostic approach would be to routinely perform both ELISA tests and PCR analysis every 1-3 months for ferrets in which ADV infection is suspected.

ADV DNA can be detected in formalin fixed, postmortem or biopsy tissues by DNA in situ hybridization. This technique uses a viral-specific probe to localize ADV DNA in tissue sections. This technique has the ability to confirm viral infection and identify infected cells, tissues, and organs.

Data collected from this experiment has demonstrated that ferrets seropositive for ADV by CIEP and/or ELISA may be test-negative by PCR. Furthermore, ferrets that were
seronegative by CIEP have been found to have ADV DNA in tissues after DNA *in situ* hybridization. At the cellular level, DNA *in situ* hybridization has detected ADV viral nucleic acid in the cytoplasm of macrophages in the liver, kidney, pericardium, and lymphatics of an asymptomatic ferret.\(^{27}\) Follicular dendritic cells in the spleen also contained ADV viral nucleic acid. In this particular ferret, the presence of ADV was documented 5 years post infection, indicating long term viral infection. PCR detected viral DNA in serum, urine, feces, and blood.\(^{27}\) DNA *in situ* hybridization also has been used in other studies to differentiate the replicative form of ADV from sequestered ADV DNA in cells of infected mink.\(^{22}\) DNA *in situ* hybridization would be extremely useful in a retrospective study of mustelids with unspecified immune system disease to determine which individuals are infected with ADV. The antibody response, which is the major factor in adult, persistent AD in mink and ferrets, cannot be detected using this method. Furthermore, DNA *in situ* hybridization is capable of providing excellent proof of viral DNA presence, especially if combined with ELISA and PCR data.
Sources and Manufacturers

a. Danish Furbreeders’ Laboratory, Glostrup, Denmark
b. Agarose High – mₖ, Bio-Rad Laboratories, Hercules, CA
c. Gelman Sciences, Ann Arbor, MI
d. Seakem GTG agarose, BioWhittaker Molecular Applications, Rockland, ME
e. ProbeOn™ Plus Microscope Slides, Fisher Scientific, Pittsburgh, PA
f. Biomedia Corp., Foster City, CA
g. PCR DIG Labeling Mix, Roche Applied Science, Indianapolis, IN
h. Digoxigenin Detection Kit, Roche Applied Science, Indianapolis, IN
i. Invitrogen, Carlsbad, CA
j. FITC-MAD antibody solution, courtesy of Dr. Marshall Bloom, Rocky Mountain Laboratories NIH, NIAID, Hamilton, MT
k. Sorvall RC2-B centrifuge, GSA rotor, Kendro Laboratory Products, Asheville, NC
l. Roche Diagnostics, Indianapolis, IN
m. Fisher Sonic Dismembrator 60, Fisher Scientific, Pittsburgh, PA
n. Sorvall RC2-B centrifuge, SS-34 rotor, Kendro Laboratory Products, Asheville, NC
o. Immulon 2HB (U-shaped), Thermo Electron Corp., Waltham, MA
p. Zymed, San Francisco, CA
q. Sigma, St. Louis, MO
r. Beckman Coulter AD 340C Absorbance Detector, Fullerton, CA
s. Personal communication, Dr. Claus M. Willadsen, Danish Furbreeders’ Laboratory
Figure 4.1. Virus-infected cells glow bright green with fluorescence microscopy.

Fluorescein isothiocyanate (FITC) labeled anti-ADV antibody; bar = 25 µm.
Figure 4.2. SDS-PAGE of samples taken during the ADV baculovirus purification procedure. Lane 1 represents the protein size marker. Lanes 2 and 3 depict samples taken after the first centrifugation and sonication. The sample in lane 4 shows the purity of the viral proteins after subsequent chloroform extraction. Lane 5 is the final, filtered supernatant used as the viral protein antigen for the ELISA.
Figure 4.3. Electron micrograph of a negatively-stained sample of the final viral antigen preparation. Notice the numerous baculovirus virions. The Sf9 spinner cell culture supernatant has been filtered and the virions have been concentrated. The engineered baculovirus expresses the VP1 and VP2 capsid proteins. Bar = 100 nm.
Figure 4.4. Gel electrophoresis of DNA PCR products obtained from feces, spleen, liver, lung, and kidney. Molecular weight markers (MW) are indicated. Water used in the reaction was tested for purity (A). Known ferret positive and negative fecal (B, C) and tissue-derived samples (D, E) were used for comparison.
References


CHAPTER 5

Conclusion

An enzyme-linked immunosorbent assay (ELISA) test was developed to detect the VP1 and VP2 capsid proteins of Aleutian mink disease parvovirus (ADV). This assay was accurate, had a faster turnaround time, and was less labor intensive than existing tests. Although a commercial ELISA is currently available that detects the ADV NS1 protein, the test developed in this study provides a broader range of anti-ADV antibody detection without sacrificing specificity. Theoretically, the likelihood of detecting antibodies to the VP1 and VP2 capsid proteins in an exposed or infected ferret will be greater than for the NS1 protein. Furthermore, the ELISA developed herein study could substitute for the currently available counterimmunoelectrophoresis (CIEP) test. Aleutian mink disease parvoviral infection is a potential problem in domestic ferrets; therefore, prevention of ADV dissemination requires rapid disease surveillance and the ability to screen large populations of ferrets for this virus. The commercial ADV tests, along with the newer and improved diagnostic techniques described in this thesis, should improve the detection of ADV infection and help to elucidate the pathogenesis of this disease in ferrets.

The combined use of ELISA tests that determine antibody titers and detect non-structural viral proteins will reveal more about the epidemiology of ADV among ferrets. To date, many aspects of ADV infection in ferrets have not been addressed. Further research should be done to determine how ADV stimulates and controls the immune response as well as the effects of this virus on cellular signaling. The role of cytokines in ADV infection has received little investigation. Complete understanding of these facets of Aleutian disease may enhance the development of an effective vaccine. If a genetically engineered vaccine is produced, it may
require multiple cloned DNA sequences to produce both viral and non-viral proteins as potential antigens.