PRODUCTION OF MONOCLONAL ANTIBODIES AND DEVELOPMENT

OF AN ENZYME LINKED IMMUNOSORBANT ASSAY FOR SEAL

HERPESVIRUS TYPE 1

By

BRENT LEE MOORE

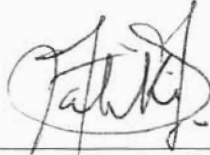Bachelor of Science

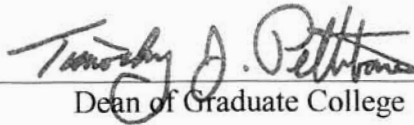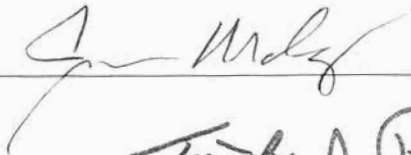Northwestern Oklahoma State University

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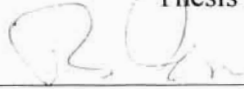2000

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PREFACE

Seal Herpesvirus Type 1 (SeHV-1) causes a debilitating disease in young seals and sea lions in the wild and in rehabilitation centers. Currently, there is only one diagnostic test available for detecting antibodies: the serum/virus neutralization test (SNT). Specific objectives of this project were to 1) produce and characterize monoclonal antibodies to SeHV-1 for use in developing a competitive ELISA to detect SeHV-1 antibody, 2) if objective 1 failed, develop an indirect enzyme linked immunosorbant assay (iELISA) for detection of antibodies to SeHV-1. The results of this study will provide a quicker, more sensitive, and less expensive method for detection of antibodies to SeHV-1 in marine mammal sera.

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| | |
|---|---|
| AMEM | Alpha minimum essential medium |
| cELISA | Competitive enzyme linked immunosorbant assay |
| CrFK cells | Crandell feline kidney cells |
| DMEM | Dulbecco's modification of eagle's medium |
| FBS | Fetal Bovine Serum |
| iELISA | Indirect enzyme linked immunosorbant assay |
| MAb | Monoclonal Antibodies |
| OD | Optical Density |
| PBS | Phosphate buffered saline |
| PBST | Phosphate buffered saline + .05% tween 20 |
| PBST+FBS | PBST + 10% fetal bovine serum |
| SeHV-1 | Seal herpesvirus type 1 |
| SeHV-2 | Seal herpesvirus type 2 |
| SNT | Serum/virus neutralization test |
| TBSE | Tris buffered saline EDTA |
| Vero cells | African green monkey kidney cells |

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

## 1.1. Introduction to Herpesviruses.

Herpesviruses get their name from the Latin word, *herpes*, to creep. The family *Herpesviridae* contains three major subfamilies: *Alphaherpesvirinae, Betaherpesvirinae*, and *Gammaherpesvirinae*. Alpha-herpesviruses typically grow rapidly, lyse infected cells, and establish latent infections primarily in sensory nerve ganglia. Beta-herpesviruses have a comparatively restricted host range, and infect secretory glands, lymphoreticular tissue, kidneys and other epithelial tissues. Their replicative cycle is slow, and cell lysis does not occur until several days after infection. Gamma-herpesviruses have a narrow host range, and typically replicate and become latent in lymphoid cells. Some of these viruses also cause cytocidal infections in epithelial and fibroblastic cells. All Herpesviruses cause lifelong, persistent infections. This usually occurs in the form of latency.

Latent infections are not unique to herpesviruses, but are a defining characteristic of this virus family. Latency is a different pathway from the lytic cycle and does not result in any clinical symptoms. The latent state is characterized by both the lack of efficient expression of all viral genes transcribed during productive infection and the activation of a unique latent-phase

transcriptional program. To return to the lytic cycle, a process called reactivation must be induced, and this replication shift also must be governed by transcriptional regulatory mechanisms (Flint et al., 2000). Excretion of infectious virus may occur continuously or intermittently without disease, or episodes of recurrent clinical disease with concurrent excretion of virus may occur throughout the life of the host (Fenner et al., 1993).

## 1.2. Introduction to Seal Herpesvirus Type I:

### 1.2.1. Seal Herpesviruses.

Seal herpesviruses were first described following an outbreak of a flu-like viral disease in July and August of 1984 (Borst et al, 1986). The outbreak initially began in a seal rehabilitation center off the coast of The Netherlands. This outbreak affected 23 seals in this center, killing 11. The viral agent was described as a new member of the alpha-herpesvirinae subfamily. Antigenically, this new seal herpesvirus is related to both canine herpesvirus (CHV) and feline viral rhinotracheitis virus (FVRV) (Harder et al., 1996). The virus was designated Phocid herpesvirus 1 (PhHV-1) or seal herpesvirus type 1 (SeHV-1) (Osterhaus et al, 1985).

A second herpesvirus, Phocid herpesvirus type 2 (PhHV-2) or Seal herpesvirus type 2 (SeHV-2), was described in a study of California sea lion populations off the California coast (Kennedy-Stoskopf et al, 1986). At the time SeHV-2 was not recognized as a Gamma-herpesvirus, but was rather classified simply as a herpesvirus. SeHV-2 was originally described as a co-infection with a retrovirus in lung tissue and was not believed to be the primary cause of death in

that study (Kennedy-Stoskopf et al, 1986). SeHV-1 and SeHV-2 were separated into distinct viruses based on antigenic and genetic characteristics (Harder et al., 1996). SeHV-2 was shown to be a member of the subfamily *Gammaherpesvirinae* and is much less virulent than SeHV-1. Unlike SeHV-1 there is no evidence of SeHV-2 causing any clinical symptoms in pinnepeds (Zarnke et al, 1997). However, a recent study did indicate a possible correlation between SeHV-2 and a metastatic carcinoma of California sea lions and SeHV-2 (Lipscomb et al., 2000).

A serological study was performed on pinnepeds off the coasts of Alaska and Russia to determine the prevalence of both SeHV-1 and SeHV-2 (Zarnke et al, 1997). Serum antibody prevalence for SeHV-1 ranged from 22% to 77%, whereas SeHV-2 had a lower prevalence ranging from 11% to 50% in various sub-populations. It is evident that both types of seal herpesvirus are present in pinniped populations. Although there have not been any other outbreaks as significant as the initial outbreak in 1984, SeHV-1 is still causing deaths in wild populations and both seal herpesviruses are still being isolated from wild populations (Dierauf and Gulland, 2001). Also, there have been recurring disease outbreaks in a seal rehabilitation center in the Netherlands (Harder et al, 1997).

### 1.2.2. Geographical Distribution.

Seal herpesvirus isolates have been obtained from seals off the European coasts of The Netherlands (Borst et al, 1986) and Germany (Horvat et al, 1989), as well as from seals and sea lions off the coast of California (Kennedy-Stoskopf et al, 1986; Gulland et al, 1997). Both types of SeHV occur in pinnipeds off the

Atlantic and Pacific coasts of the United States (Harder et al., 1996; King et al., 1998). Alaskan, Russian and Antarctic seal populations have also been shown to have antibodies against both SeHV-1 and SeHV-2 (Zarnke et al, 1997; Stenvers et al, 1992). These studies have indicated the presence of SeHV-1 in worldwide seal and sea lion populations.

## 1.3. Seal Herpesvirus Type I Virology:

### 1.3.1. Structural Properties.

Like other alpha-herpesviruses, the seal herpesvirus virion is enveloped and is about 150 in diameter, although size can range from 120nm to 200nm. The capsid is an icosahedron 100nm in diameter, and is composed of 150 hexamers and 12 pentamers. The capsid is surrounded by a tegument which is enclosed by the lipoprotein envelope (Fenner, et al., 1993). Herpesvirus genome consists of a linear dsDNA molecule. The typical alpha-herpesvirus genome size is approximately 150kbp.

### 1.3.2. Cell Cultures and Cytopathic Effect.

SeHV-1 grows readily in Crandell Feline Kidney (CrFK) cells. Cytopathic effects (CPE) are caused by margination and breaking of chromosomes (Flint et al., 2000). Visually, SeHV-1 CPE consists of cell rounding with inclusion bodies and a clumping into grape-like clusters. Syncytia formation was not observed in this study.

### 1.3.3. Clinical Aspects of Infection.

Clinical signs of SeHV-1 disease in seals differ between European and Pacific harbor seals infected with PhHV-1 (Dierauf and Gulland, 2001). SeHV-1

symptoms include elevated body temperature (up to 40°C), inflammation of the oral mucosa, nasal discharge, coughing, vomiting, diarrhea, anorexia and lethargy, and reddening of the conjunctiva are more common in European harbor seals (Visser et al., 1991; Horvat et al., 1989). Additionally, small lesions in the oral mucosa, emphysema, pneumonia, and fatal generalized PhHV-1 usually occurs only in neonates (Borst et al, 1986; Gulland et al., 1997; Harder et al., 1997). The duration of clinical disease ranged from 1 to 6 days (Borst et al, 1986).

### 1.3.4. Pathology and Gross Pathology.

Laboratory and necropsy studies on seals dying of SeHV-1 infection typically revealed an enlarged liver (more so in Pacific Harbor seals) (Dierauf and Gulland, 2001), with alterations only observed in the parenchyma and consisting of dystrophic degeneration varying in severity up to massive coagulation necrosis (Borst et al, 1986). Additionally, the presence of typical herpetic intranuclear inclusion bodies in phagocytic cells within the botryomycotic lesions of a captive harp seal has been reported (Daoust et al., 1994).

### 1.4. Diagnostics.

### 1.4.1. Introduction to Diagnosis.

There are several means by which diagnosis of various pathogenic agents can be made. Isolation of the causative agent, in this case a virus, is a direct means of detection that can be performed on tissue specimens that are believed to be infected. Antigen detection is another means of detecting viral proteins directly. These methods include immunofluorescence assays (IFA) and

immunohistochemical (IHC) detection. Another direct detection method involves the viral nucleic acid. Polymerase chain reaction (PCR) or reverse transcription PCR (RT-PCR) are the most common methods of detecting DNA and RNA respectively. Finally, serological diagnosis is used as an indirect means of diagnosis. Serum antibodies directed against the virus can be detected by a number of assays, such as serum/virus neutralization test (SNT) and enzyme linked immunosorbant assay (ELISA).

### 1.4.2. Virus Isolation.

Virus isolation is performed to propagate infectious virus present in clinical specimens such as tissues, secretions and excretions. Specimens suspected of containing virus are processed and inoculated onto a cell culture. If virus is present, it can generally be identified by the appearance of CPE. Characteristics of CPE, such as syncytia formation, clustering of cells, its temporal development of CPE, and the type of cells infected can all help in identifying specific virus. If this is not sufficient, PCR or IFA can be used to make or confirm a viral diagnosis. Additionally, electron microscopy may be utilized to obtain confirmatory diagnosis based on structure, size, and shape of virions.

### 1.4.3. Serum/Virus Neutralization.

The serum neutralization test (SNT) is the current gold standard for serological diagnosis of SeHV-1 and SeHV-2. The SNT is run by using a serum sample that is believed to contain antibodies against a particular virus. This

sample is placed in a 96-well plate along with a known quantity of the virus that is being tested for. If the serum sample contains antiviral antibodies, these antibodies will neutralize the infectious virus. Thus, there will be an absence of CPE in a positive sample. If the serum sample is negative for the given viral antibody, then CPE would be present because there are no antibodies to neutralize the virus.

SNT is a very reliable test in virology. It is based on a specific reaction between serum antibodies and the virus. This is important in that few false positives will occur. Due to its specificity, SNT is the gold standard for diagnosis of many viruses. The time required to run the SNT assay can range from a day for very fast growing viruses to >7 days for slower growing viruses.

### 1.4.4. Polymerase Chain Reaction.

The purpose of PCR is to detect the presence of, and distinguish between, SeHV-1 and SeHV-2 DNA. PCR amplifies a specific nucleic acid sequence using a defined primer set or sets. The amplified product is resolved by gel electrophoresis and visualized by ethidium bromide staining. PCR has been used to detect the presence of SeHV-1 by amplification of a 1.6 kbp product (Harder et al., 1996; King et al., 1998) while no product is amplified from SeHV-2 using the SeHV-1 primers.

### 1.5. Recent Advances in Seal Herpesvirus Research.

One group of researchers in Europe has described the production of monoclonal antibodies (MAbs) against SeHV-1 and SeHV-2 (Lebich et al., 1994). These MAbs have proven useful in classifying new SeHV isolates into types 1

and 2 (Harder et al., 1996; Lebich et al., 1994). Furthermore, at least one anti-SeHV-2 MAb is able to discriminate between American and European SeHV-2 isolates by the failure to bind the American isolate (Harder et al., 1996). Therefore, there is a need to produce MAbs against American isolates of SeHV-1 for use in antigenic typing and diagnosis of SeHV infections in the United States.

In 1998, Harder et al. described the major immunogenic proteins of seal herpesviruses and their relationships to proteins of canine and feline herpesviruses in development of a vaccine. Additionally, a research group from The Netherlands have described a candidate phocid herpesvirus vaccine that protects against feline herpesvirus infections as well (Martina et al., 2002).

## 1.6. Conclusions.

SeHV-1 is a significant cause of disease in seal populations worldwide. Means of diagnosing seal herpesviruses are limited. Research has been performed to develop monoclonal antibodies with some success. It is important to take the monoclonal antibody research to the next step in producing MAbs to North American isolates for antigenic typing and diagnosis of SeHV-1 It is also important to develop a quicker and less expensive diagnostic tool that is amenable to testing large numbers of samples. Although the SNT is the current gold standard, it is more time consuming and labor intensive to perform than an ELISA. Similarly, though it is highly specific, SNT is not as sensitive as an ELISA would be. This is important when dealing with low titer sera, or sera that are toxic to cell cultures. Additionally, since SeHV isolates from geographically

distinct seal populations may vary in the antigenic properties, it is important to have a single test that can detect antibodies to all SeHV-1 isolates.

CHAPTER II

PRODUCTION AND CHARACTERIZATION OF MONOCLONAL

ANTIBODIES TO SEAL HERPESVIRUS TYPE 1

## 2.1. Introduction.

Serum contains many different types of antibodies that are specific for many different antigens. Seldom are more than one tenth of the circulating antibodies specific for one antigen. This causes a variety of problems for diagnostic purposes using immunochemical techniques (Harlow and Lane, 1988). The first isolation of a homogeneous population of antibodies came from studies of B-cell tumors. Clonal populations of these cells can be propagated as tumors in animals or grown in tissue culture. A problem that developed was that *in vivo*, antibodies are synthesized primarily by plasma cells (Harlow and Lane, 1988). These plasma cells cannot be grown *in vitro* as a source of antibodies. However, Kohler and Milstein in 1975 developed a technique that allows the growth of clonal populations of cells secreting antibodies with a defined specificity. In this technique an antibody-secreting cell isolated from an immunized animal is fused with a myeloma cell, a type of B cell tumor. These hybrid cells or hybridomas can be maintained *in vitro* and can continue to secrete antibodies with a defined specificity (Harlow and Lane, 1988).

Due to their exquisite specificity, monoclonal antibodies have become an invaluable tool in the diagnosis of several viruses. The speed, accuracy, and cost efficiency of tests using monoclonal antibodies have placed them in a primary role in the diagnostic field. Since they react with solid-phase antigen, the tests can be run in a single day due to eliminating the need for virus growth in a cell culture as required by SNT.

The advantage of a MAb-based ELISA is that a single conjugate (labeled anti-mouse IgG) can be used to detect anti-viral antibody in sera from a variety of species. This is important in that, a single protocol can be used to test many different species on a single plate, saving time and money.

## 2.2. Materials and Methods.

### 2.2.1. Virus Isolate.

The SeHV-1 isolate used in this study, designated as A92 10/4, was one of eight SeHV-1 isolates obtained from New York harbor seals in 1992-1993 (JT Saliki, unpublished). SeHV-1 virions were purified from a single plaque via plaque purification. This procedure was performed to ensure genetic homogeneity of virus. PCR was used to confirm the identity of virus forming individual plaques as SeHV-1. Virus propagation and antigen preparation were performed once plaque purified virus had been expanded into working stocks.

### 2.2.1.1. Plaque Purification and Expansion.

A single 6-well tissue culture plate was used for virus plaque purification. Crandell Feline Kidney (CrFK) cells were added at a concentration of $1.7 \times 10^4$ cells/well diluted in Dulbecco's modification of Eagle medium (DMEM)

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(Mediatech Inc, Herndon, VA). Fresh DMEM (5 ml) was then added to each well of the 6-well plate. Cells were allowed to incubate for 3 days at 37°C with 5% $CO_2$ to form a monolayer covering at least 90% of the well. Media was then removed from each well, and 100µl of 10-fold serial dilutions of virus [$10^{-2}$ through $10^{-7}$ in Alpha modification of Eagle minimum essential medium supplemented with Earle's salts, L-glutamine and antibiotics (100 U of penicillin and 100 µl of streptomycin per ml), (AMEM) (Mediatech Inc, Herndon, VA) without FBS were added to each well. The plate was incubated at 37°C for 30 minutes while being gently rocked every 10 minutes. Approximately 5 mls of AMEM containing 2% methylcellulose was added to each well. The plate was then incubated for 4 days at 37°C with 5% $CO_2$. Each day, the plate was observed for cytopathic effect (CPE).

After four days of growth, individual plaques were visible. The $10^{-2}$ virus dilution produced CPE in 80% of the monolayer, which was too much for single plaques to be picked. Twenty-four plaques were picked from the $10^{-3}$ (7 plaques), $10^{-4}$ (15 plaques), and $10^{-5}$ (2 plaques) virus dilutions by slowly pipetting 125µl of the plaque and surrounding area with a gentle scraping motion to obtain a small sample of surrounding cells. Each of these plaques were placed directly in individual wells of a 24-well plate with CrFK cells (2 X $10^5$ cells/well in DMEM) in suspension. Plates were incubated at 37°C for 4-5 days to allow CPE in 75% of the monolayer before the virus were harvested.

The five fastest growing viral isolates were then inoculated onto CrFK cells in a T25 flask for initial expansion. Each flask was allowed up to 4 days to

grow to at least 80% CPE before being frozen at -70°C overnight. The flasks were then thawed, the cells scraped, and the contents transferred into separate 15 ml tubes. The tubes were centrifuged at 900 X g for 15 minutes to pellet cell debris and the supernatant fluid aliquoted into snap cap tubes in 1 ml aliquots and frozen at -70°C until further use. The cell pellet was tested by PCR to insure that SeHV-1 was indeed the virus producing CPE. The one purified SeHV-1 isolate showing the fastest and most uniform growth (identified as A92 10/4 ppB4) was used for continued expansion. This virus (150μl) was inoculated into a T75 flask containing a monolayer of CrFK cells and incubated at 37°C for four days. This T75 flask was frozen at -70°C overnight, and virus supernatant harvested as described above. This expansion produced enough virus stock to infect a minimum of 30 T150 flasks, while keeping the virus passage the same throughout the final expansion step. Finally, 33 T150 flasks, containing 2 X $10^6$ CrFK cells in suspension were infected with 300μl each of the SeHV-1 stock. Cultures were incubated for four days to allow virus CPE to involve at least 75% of the cell monolayer, and then frozen at -70°C until further use.

## 2.2.1.2. Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed basically as described by Harder et al (1996) with some modifications to the sequences being amplified. Published PCR primers for the SeHV-1 glycoprotein B gene (Harder et al, 1996) were used to amplify a 1616 bp product for SeHV-1.

The two primers used were:

gB-1: 5'-aca act gta tgg tct gg-3' and

gB-4: 5'-ggt aga aat tca cga tc(c/t) tc-3'

The following PCR components were mixed into a thin-walled PCR tube (Fisherbrand): 5.0μl of 10X buffer, 2.0μl MgCl₂ (25mM stock concentration), 2.0μl each gB-1 and gB-4 primer (15μM each), 1.0μl dNTP (10mM of each), 0.5μl Taq polymerase (2.5 U), and 32.5μl RNAse-free water. Contents were mixed well by vortexing and 5.0μl of DNA solution was added. DNA isolation was performed as developed by Molecular Research Center, Inc (website, http://www.mrcgene.com/dna.htm).

Using a PTC-100 Programmable Thermal Controller from MJ Research Inc, thermocycle conditions were set. These conditions were: one cycle of 94°C for 3 minutes, then 35 cycles of 94°C for 30 seconds, 45°C for 1 minute, and 72°C for 1 minute, followed by one cycle of 72°C for 10 minutes, and finally 4°C to hold the contents indefinitely. After PCR was complete, contents were run along with a fragment size marker on a 2% agarose gel for 15 minutes then observed over UV light for bands.

### 2.2.1.3. Virus Harvesting.

All 33 SeHV-1 infected T150 flasks were thawed to room temperature. Cells were scraped into the medium, transferred to 50ml tubes, and centrifuged at 900 X g for 15 minutes. The supernatant was poured off leaving the pellet with 5mls of supernatant in each tube. Each pellet was sonicated for 2 minutes or until the pellet was completely dissolved using an 80% duty cycle with 70 input setting on a Branson Sonifier 450. The remainder of the supernatant was added back to each 50ml tube for re-centrifugation at 900 X g for 15 minutes. Supernatants

were then transferred into a new T150 flask, and all the cell pellets were then discarded. The pooled supernatant was then placed in polyallomer ultra-centrifuge tubes and centrifuged at 150,000 X g for one hour in an ultra-centrifuge. Supernatant was then poured off and discarded and the virus pellet dried by inverting and gently tapping the tubes on a paper towel. Pellets were then re-suspended in 800 μl of sterile PBS, combined into a single ultra-centrifuge tube, and stored at 4°C overnight.

### 2.2.1.4. Virus Purification.

Two sucrose solutions, 20% and 60%, were used to form a gradient for final purification of SeHV-1 (Lebich et al., 1994). In clear ultracentrifuge tubes, 20 ml of 20% sucrose was pipetted to fill each tube half way. Seventeen mls of 60% sucrose was pipetted slowly using a glass pasteur pipette, below the 20% sucrose. Approximately 1-2 ml of the virus solution was then added slowly on top of the 20% sucrose to avoid mixing. Tubes were centrifuged at 150,000 X g for 1 hour at 4°C.

The virus particle layer remained between the 20% and 60% sucrose layers while cell fragments and organelles remained either on top of the 20% or below the 60%. The virus layer was removed using a pasteur pipette. The tip of the pipette was inserted along the side of the tube and slowly removed the virus band to prevent mixing. Contents were then placed in a new ultracentrifuge tube, and sealed with parafilm to store overnight at 4°C.

## 2.2.1.5. Final Antigen Preparation.

Ultracentrifuge tubes with sucrose gradient purified virus were filled with sterile PBS and centrifuged at 150,000 X g for 1 hour at 4°C. The supernatant was poured off, and tubes were dried by gently tapping on a paper towel. This process was repeated 2 times for each sample to assure removal of all sucrose. The pellet was then re-suspended in 6 mls of sterile PBS and transferred to a 50 ml tube. Contents were then sonicated at 80% duty cycle and 80 input setting for 5 minutes to insure complete dispersion of viral particles. The antigen was stored in 55 µl aliquots at -70°C until needed.

SeHV-2 antigen was prepared using the same method as SeHV-1, except SeHV-2 was grown on African green monkey kidney (Vero) cells. CrFK cell antigen was prepared by growing cells in T150 flasks. The flasks were scraped then transferred to 50 ml tubes and centrifuged at 900 X g for 10 minutes. The supernatant (DMEM) was poured off, the pellets were re-suspended in 5 ml of sterile PBS and sonicated as described previously. Both SeHV-2 antigen and CrFK antigen were stored at -70°C until needed.

## 2.3. Monoclonal Antibody Production and Characterization:

## 2.3.1. Oklahoma State University Hybridoma Center.

All MAb production was performed at the Oklahoma State University Hybridoma Center. Four mice were immunized three times at 14-day intervals. After the final immunization, sera were tested using an indirect enzyme-linked immunosorbant assay (iELISA) against SeHV-1. The mouse showing the highest level of antibody production was selected for monoclonal antibody production.

The initial step in MAb production was the fusion of spleen cells from immunized mice with a myeloma cell line to produce antibody-secreting hybridoma cell lines. The supernatant fluids from these hybridomas were tested for specific anti-SeHV antibody by ELISA technique. Positive hybridomas were expanded and stored in liquid nitrogen.

## 2.3.2. Hybridoma Screening.

Hybridoma screening was performed using an indirect ELISA (iELISA). Immulon-2HB 96 well flat bottom plates (DYNEX, Alexandria, VA) were coated with 100 µl of SeHV-1 antigen and CrFK antigen in alternating columns, each diluted 1:200 in carbonate buffer, 0.05 M, pH 9.6. All plates were stored overnight at 4°C. Plates were washed four times in PBST with 40 second soak times. PBST was not removed following the fourth soak. Plates were taken to the Oklahoma State University Hybridoma Center where the PBST was removed and replaced with 100 µl/well of hybridoma supernatant. Plates were then returned to our laboratory for completion of the ELISA. Each plate was incubated at 37°C for one hour. Plates were washed four times in PBST with 40 second soak times, then dried by inverting each plate and gently tapping on a paper towel. Peroxidase conjugated anti-mouse (100µl) IgG (whole molecule) (Sigma Chemical Co., St. Louis, MO), diluted 1:1000 in PBST was added to each well. Plates where then incubated for one hour at 37°C. Plates were washed and dried, and 95µl of substrate (1mg 3,3',5,5' tetramethylbenzidine (TMB) plus 33µl 3% $H_2O_2$ per 10 ml of substrate buffer) was added to each well and allowed to incubate 4 minutes at room temperature on a plate rotator. The substrate reaction

was stopped by adding 25µl of 2M $H_2SO_4$. Plates were read using a Molecular Devices E-max plate reader at 450 nm wavelength. The positive cutoff was determined to be an optical density (OD) of 0.400 to eliminate low reacting samples.

### 2.3.3. Monoclonal Antibody Characterization.

Characterization of monoclonal antibodies was performed in a two tier format. First, MAbs were characterized as CrFK specific, or SeHV-1 specific. Then, all the SeHV-1 specific binding MAbs were separated into SeHV-1 binding only, or SeHV-1 and SeHV-2 cross-reactive. Monoclonal antibodies with low binding (less than .400 OD value) were still considered in this characterization, but were not further tested for diagnostic purposes.

### 2.3.3.1. Comparing MAb Binding to CrFK cells vs. SeHV-1 Antigen.

Once the hybridomas were developed, it was important to test MAb supernatant for final characterization. This ELISA was performed as in section **2.3.2**.

### 2.3.3.2. Cross-Reactivity Between SeHV-1 and SeHV-2.

Immulon-2HB 96-well flat bottom plates (DYNEX, Alexandria, VA) were setup in a similar manner as described in **2.3.2**, except that SeHV-1 Ag and SeHV-2 Ag, each diluted 1:200 in carbonate buffer, were coated in alternating columns, then stored at 4°C overnight. MAbs were then placed into three categories: SeHV-1 positive/SeHV-2 negative, SeHV-1 negative/SeHV-2 positive, or cross-reactive.

### 2.3.3.3. Degree of Antigen Binding.

Each candidate MAb was re-tested to determine the optimal dilution for use in a cELISA. All MAbs were tested as described in **2.3.2.**, except that wells were coated only with SeHV-1 Ag. MAbs with low OD value (<0.400) were not considered as potential cELISA candidate.

### 2.3.3.4. Test for a Competitive Monoclonal Antibody.

Immulon-2HB 96-well flat bottom plates (DYNEX, Alexandria, VA) were coated with SeHV-1 antigen diluted 1:200 in carbonate buffer, and incubated overnight at 4°C. Each plate was then washed 4 times in PBST and dried. SeHV-1 positive seal serum samples having titers of 32, 64, and 96 and a negative serum sample (≤4) were diluted in triplicate 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256 then added (50μl) to individual antigen-coated wells. These serum samples were chosen because of their neutralizing titers via serum neutralization test (SNT). PBST was added to the final row as a negative control. Plates were incubated for 30 minutes at 37°C, and 50μl of each candidate MAb diluted 1:10, 1:50, and 1:250 was then added to each respective well without washing the plates. Plates were incubated for an additional 30 minutes, then washed and dried as described before. Anti-mouse IgG conjugate (100 μl) was added to each well and incubated for 1 hour at 37°C. Each plate was washed and dried as previously described, substrate was added (95μl) to each plate, incubated for 5 minutes, reactions were stopped by adding 25μl of 2M $H_2SO_4$, and the plates were immediately read. Competition was determined by a decrease in OD in the sample wells from the MAb only well.

## 2.4. Results:

Plaque purification produced 6 isolates that grew as fast (5-6 days) or faster (4 days) and higher conformation than the non purified virus stock. All 6 plaque purified ( designated as pp.) samples were determined to be SeHV-1 via PCR. Isolate A92 10/4 ppB4 was determined to be the isolate to be expanded due to having the highest consistency in speed and conformation of growth. The A92 10/4 ppB4 was also tested by PCR and found to be SeHV-1 positive after final virus expansion. SeHV-1 antigen was found to be concentrated at 6.65 mg/ml, with a working coated concentration of .03325 mg/ml. SeHV-2 was found to be concentrated at 2.33 mg/ml, and CrFK was found to have a concentration of 6.27 mg/ml.

### 2.4.1. Production of monoclonal antibodies.

Nearly 100% of the initial hybridoma samples (109 out of 114) from the Oklahoma State Hybridoma Center reacted positively by iELISA during the screening phase. After fusion, 102 out of 109 (93.6%) hybridomas that grew out retained SeHV-1 positive reactivity in the iELISA (ranging from an OD of 0.294 to 1.918). The 102 positive MAbs (>0.400) were selected for continuation of characterization.

### 2.4.2. Characterization of monoclonal antibodies.

Ninety-five out of the 102 hybridomas were classified as seal herpesvirus-1 positive, CrFK negative. Seven of the 102 hybridomas were classified as SeHV-1 negative, CrFK positive. These seven hybridomas were eliminated from
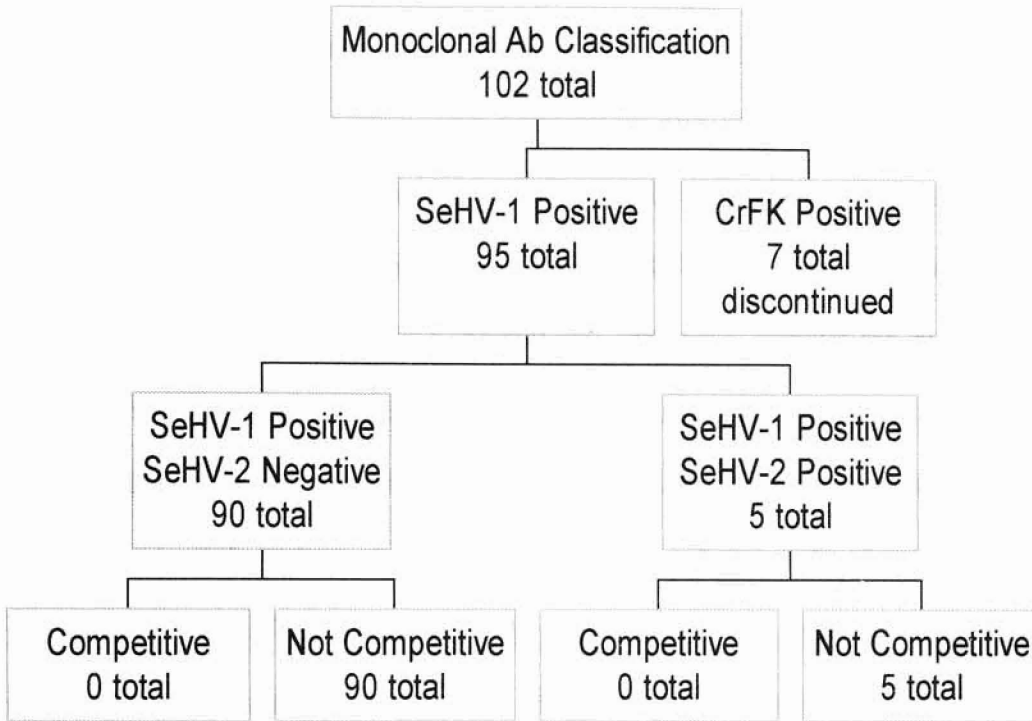
**Figure 2.1 Summary of Monoclonal Antibody Characterization**
This figure summarizes the monoclonal antibody characterization. Note that all MAbs
that were CrFK specific were excluded from further study.
It was also fortunate that some were cross-reactive with SeHV-1 and SeHV-2.
As illustrated, there were no competitive MAbs.

further study. Of the remaining 95 hybridomas, five were classified as SeHV-1 positive, SeHV-2 positive. Ninety hybridomas were classified as SeHV-1 positive, SeHV-2 negative. Final classification of competitive MAb 100% of the not competitive (minimal change in OD value from MAb control and sample wells). Results are summarized in figure 2.1.

## 2.5. Discussion.

In general, particulate antigens make excellent immunogens, because they are readily phagocytized (Harlow and Lane, 1988) as performed in our study. Our study successfully produced monoclonal antibodies against the New York SeHV-1 isolate, A92 10/4 ppB4. High degree of success may be due to quality of antigen. Indeed, a high percentage (93%) of the MAbs in this study were specific for SeHV-1 with only a few binding only to cellular antigen. This high percentage of SeHV-1 specific MAbs may be due in part to the plaque purification (producing virions from a single progeny which lowers the protein variability) and gradient purification (eliminating cell proteins) of virus used for immunization. This would have eliminated most of the CrFK antigens which would normally be present in infected cell or unpurified virus preparation. Of the 95 SeHV-1 positive MAbs, 5 were found to be cross-reactive with SeHV-2. This is unusual in that SeHV-1 is an alpha-herpesvirus, whereas SeHV-2 is a gamma-herpesvirus. This could be explained in that these cross-reactive MAbs are binding to some highly conserved proteins which are common in all subfamilies of herpesviruses (like capsid proteins or enzymes). These MAbs need to be tested using a Western blotting or immunoprecipitation to determine what proteins are

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being recognized, especially with the 5 cross-reactive MAbs. The Western blotting will only be useful if these MAbs recognize sequential amino acid sequences due to the denaturing of protein bands. This testing is important in determining if these MAbs will cross-react with known shared antigenic proteins of feline and canine herpesvirus using an ELISA described by Harder et al. (1998). Additionally, it will be important to test each MAb to determine if any are neutralizing.

This study failed to identify any competitive monoclonal antibodies. The reasons for this are unknown at this time. One possible explanation is that the MAbs do not have enough affinity towards the viral proteins. This may be solved by using polyclonal antibodies which use multiple epitopes. A second and more likely possibility is that the mouse recognizes SeHV-1 antigen by different amino acid sequences than seals do. This would allow for both seal sera and mouse MAbs to bind without blocking the other out, thus disallowing competitive interaction and preventing detection of serum antibodies by this assay system. Another possibility involves steric hindrance or blockage of epitope to which the sera binds. In this, MAb IgG binds to a dominant epitope surrounding the epitope(s) to which the serum Ig binds, thus preventing competition. Another possible, though unlikely suggestion is that the monoclonal antibodies were specific to epitopes that are specific to the New York harbor seal isolates and are not present in other regional isolates of SeHV-1. OD values were similar when comparing New York harbor seals with other seal and sea lion species from other geographical locations. It is uncertain if making more monoclonal antibodies than

were produced in this study would have yielded different results. One study was able to produce monoclonal antibodies against European as well as American isolates, including some MAbs that could distinguish between the European and the American isolates (Lebich et al., 1994). Including multiple SeHV-1 isolates may be a solution to the problem in our study.

Monoclonal antibodies produced in this study may still be useful in detecting protein bands via western blot. Western blot testing was beyond the scope of this study, however, it does need to be utilized in future studies of this SeHV-1 isolate.

CHAPTER III


DEVELOPMENT AND EVALUATION OF A PROTEIN A-BASED
INDIRECT ENZYME LINKED IMMUNOSORBANT ASSAY FOR
DETECTION OF SeHV-1 ANTIBODIES

## 3.1. Introduction.

Indirect ELISAs (iELISA) are useful in detecting a wide variety of
pathogens. The basic premise behind the iELISA is to have a plate with antigen,
add the test serum, and have some method to detect the presence of serum
antibodies that bind to the antigen. Detection of serum antibodies requires some
kind of a conjugate, one part of which recognizes the serum antibodies and the
other part providing the means of detection. The latter part usually takes the form
of an enzyme such as a peroxidase or phosphatase. The conjugated enzyme
catalyzes a color reaction that can be quantified based on an optical density (OD).
The relative OD value obtained on control vs. viral antigen are then used to
determine the positive or negative status of serum to a viral specific antigen. The
advantage of the iELISA over most other tests is that it can be used on virtually
any pathogen.

Although horseradish peroxidase-labeled anti-seal conjugate is
commercially available, it is cost prohibitive for routine diagnostic purposes
because it has to be produced on order. To circumvent this obstacle, peroxidase-
labeled Protein A and Protein G were tested as potential conjugates for detection

of anti-SeHV-1 antibodies. Each of these proteins bind specifically to the Fc region of IgG antibodies, but the species specificity of Proteins A and G are broader than that of anti-IgG antibodies (Harlow and Lane, 1988). This allows detection of serum antibodies of a number of different species that can be tested using a single conjugate. In developing an ELISA it is vital to maintain a high level of sensitivity (few false negatives) while allowing for a high degree of specificity (few false positives).

## 3.2. Materials and Methods.

### 3.2.1. Viruses

Eight SeHV-1 isolates were obtained from stranded harbor seals off the New York coast (J. T. Saliki, unpublished). Virus stocks from isolate A92 10/4 ppB4 were grown using CrFK cells. One SeHV-1 isolate was designated for propagation (A92 10/4) then cultured in a 2% methyl-cellulose Alpha MEM media with 2% FBS for plaque purification. Twenty four plaques were then obtained and placed in a 24-well flat bottom tissue culture plate with CrFK cells at a concentration of 250,000 cells/well in 2 mls Dulbecco's Modification of Eagle Medium (DMEM) containing 10% FBS. Virus was then allowed 4 days to replicate to greater than 70% CPE. Six isolated plaques were transferred from the 24 well plate to a T25 flask with 5mls fresh medium and CrFK cells. These plaques were chosen on the basis of degree of CPE and speed of growth. Each T75 Flask was allowed 4 days to attain 80% or greater CPE, then frozen at -70°C overnight. Each flask was thawed at room temperature, cells were scraped and placed in individual 15ml tubes and centrifuged at 1,000 X g for 15 minutes.

Supernatants were poured off into tubes, leaving 5 ml of supernatant on the cell pellet. The pellet was sonicated for 2 minutes to release virions from cells, after which the supernatant was placed back into the 15 ml tubes for re-centrifugation. the final supernatant, containing viable SeHV-1 virions, was stored in 4 ml aliquots at -70°C.

### 3.2.2. Standard iELISA Procedure.

A standard iELISA procedure was used in various optimization experiments. In an Immulon-2HB 96 well flat bottom plate (DYNEX, Alexandria, VA), 100 μl of purified SeHV-1 antigen diluted 1:200 in carbonate buffer (0.05 M, pH 9.6) was added to each well and incubated at 4°C overnight. The plate was washed 4 times in PBS + .05% tween 20 (PBST) with 40 second soak times and dried by inverting the plate and gently tapping on a paper towel. Sample sera (100 μl), diluted in PBST, was added to each well, then incubated for 1 hr at 37°C. The plate was washed and dried as previously described, 100 μl of conjugate added to each well, and incubated as before. The plate was washed and dried as described before, 95 μl of substrate was added (containing 1 mg of 3,3',5,5' tetramethylbenzidine (TMB) and 33μl per 10mls of substrate buffer), then the plate was incubated at room temperature for 5 minutes. The substrate reaction was stopped by the addition of 25μl of 2M $H_2SO_4$, then immediately read for OD value using a Molecular Devices E-Max plate reader at a 450nm wavelength.

### 3.2.3. Protein A and G binding.

Protein A, isolated from the cell wall of *Staphylococcus aureus*, and Protein G, isolated from the cell wall of hemolytic *Streptococcus* strains C and G, were tested for conjugate purposes for an indirect ELISA. Proteins A and G bind specifically to the second and third constant regions Fc region of IgG antibodies (Harlow and Lane, 1988), which makes them ideal candidates for developing an ELISA for marine mammals and other exotic species that peroxidase-labeled anti-IgG conjugate is not commercially available.

### 3.2.4. Species Binding.

Serum samples from various marine mammals were tested to determine how well Protein A and Protein G bind to IgG of each species. Serum samples tested included horse, goat, cow, seal, sea lion, sea otter, dolphin, and polar bear. Goat serum served as a positive control for Protein G, and a negative control for Protein A (Harlow and Lane, 1988). Horse and cow samples bind with both Protein A (++) and G (++++) (Harlow and Lane, 1988). Each serum sample was diluted in 0.05 M carbonate buffer, pH 9.6, starting at 1:4 then a 5 fold dilution series after that to reach a final 1:500 dilution. Each sample was tested in duplicate against Protein A and Protein G. The ELISA described in **3.2.2.** was used to complete this test.

### 3.2.5. Dilution Buffer.

Tris buffered saline + EDTA (TBSE), PBST, Milk Blocker solution (PBST + 5% non-fat dried milk), and PBST + 10% FBS were each tested to determine the optimal dilution buffer for both serum and conjugate dilutions.

Two plates were used for each buffer. Each solution was used to dilute both the serum and conjugate, and each solution was also used to dilute either the conjugate or the serum while using PBST to dilute the other. The procedure described in **3.2.2.** was used in this test.

### 3.2.6. Protein A Dilutions.

To determine the optimal conjugate dilution, four dilutions of protein A in PBST were tested: 1:500, 1:1,000, 1:2,000, 1:4,000. Each dilution was tested under the procedure described in section **3.2.2.**

### 3.2.7. Determination of Optimal Serum Dilution.

To determine the optimal serum dilution to be used for testing of seal sera, serial dilutions of four known positive and four known negative samples were tested. Sera were diluted in PBST in a two-fold dilution series beginning with a 1:4 dilution and ending with a 1:8,192 dilution. The iELISA was performed as described in section **3.2.2**.

### 3.2.8. Substrate Incubation.

The ELISA procedure described in section **3.2.2.** was used with only a few modifications. Sample sera were diluted 1:32, and peroxidase-labeled Protein A was diluted 1:1000, all diluted in PBST. Upon addition of substrate, one column of positive controls and one column of negative controls were stopped by adding 25 µl of 2 M $H_2SO_4$ during each elapsed minute until the 6$^{th}$ minute. The plate was immediately read for OD value after each minute.

## 3.3. Evaluating the iELISA:

Harder et al. (1997) suggest that testing paired serum samples for specific antibodies is probably the method of choice when screening a population (Dierauf and Gulland, 2001). SNT is the current gold standard, so it was important to use this test to evaluate our iELISA.

### 3.3.1. Serum Neutralization of Archived Samples.

Serum samples from seal and sea lion species from parts of Hawaii, California, and pacific islands (505 total) were tested by serum neutralization for presence of SeHV-1 antibodies. In a 96-well flat bottom tissue culture plate, 25 $\mu$l of serum-free DMEM and 25 $\mu$l of undiluted serum were added to the bottom two rows of each plate, each sample in duplicate. Sera were mixed using a multichannel pipetter set at 25 $\mu$l, transferring 25 $\mu$l of diluted serum from row G through row A to obtain a serial 2-fold dilution series of sera. Using DMEM without FBS, virus was diluted to contain approximately 100 $TCID_{50}/25$ $\mu$l, and 25 $\mu$l added to each well except the cell control row (row H). This resulted in a final 2-fold dilution series (A-G) of 1:4 through 1:256. The plates were incubated for 1 hour at 37°C with 5% $CO_2$. During this incubation, CrFK cells were trypsinized and diluted to $10^5$ cells/ml in DMEM. At the end of the 1 hour incubation, 150$\mu$l of the cell suspension (1.5 X $10^4$ cells) were added to all wells. The plates were read after 4 days of incubation at 37°C with 5% $CO_2$. Negative serum samples were identified by the presence of cytopathic effect (CPE) due to their lack of virus neutralizing antibodies. Sera that showed CPE in both wells at a 1:4 dilution were considered negative. Positive samples lacked CPE due to

presence of anti-SeHV-1 neutralizing antibodies. The titer was determined by taking the reciprocal of the dilution of the last pair of wells that contained no

**Table 3.1 Geographical distribution of seal and sea lion serum samples**

| Species | Common Name | Location | ELISA | | SNT | | | |
|---|---|---|---|---|---|---|---|---|
| | | | SeHV-1 Positive | SeHV-1 Negative | SeHV-1 Positive | SeHV-1 Negative | non reportable | total reportable |
| *Phoca* | Harbor Seal | Alaska | 263 | 24 | 253 | 34 | 9 | 287 |
| *Zalophus* | California Sea lion | New York | 0 | 4 | 0 | 1 | 3 | 4 |
| *Zalophus* | South Sea lion | New York | 23 | 41 | 21 | 46 | 34 | 64 |
| *Phoca* | Elephant Seal | New York | 0 | 0 | 0 | 0 | 4 | 0 |
| *Phoca* | Hooded Seal | Alaska | 0 | 1 | 0 | 1 | 0 | 1 |
| *Phoca* | Weddells Seal | New York | 10 | 7 | 9 | 8 | 7 | 17 |
| *Phoca* | Grey Seal | Maryland | 2 | 0 | 2 | 0 | 1 | 2 |
| *Phoca* | Monk Seal | Hawaii | 0 | 15 | 2 | 13 | 0 | 15 |
| Unknown | Pinnepeds | Hawaii | 13 | 22 | 14 | 21 | 19 | 35 |
| **totals** | | | 311 | 114 | 301 | 124 | 80 | 425 |

CPE. Titers of 6 and 8 were considered suspect. Samples that showed titers $\geq 8$ were considered positive. Toxic samples could not be reported because the cells were unable to grow due to serum toxicity.

**3.4. Results.**

Protein A bound to serum antibodies with nearly a ten fold greater efficiency than protein G. Protein A had an optical density (OD) of nearly 2.000, whereas Protein G had approximately 0.200 OD value. Each conjugate did show a statistically significant difference in detecting positive, negative, and blank (PBST only) samples. This is illustrated in figure 3.1. However, when comparing each protein to various species, Protein A showed a much higher

binding ability and consistency to the tested species than Protein G, as illustrated in figures 3.2a and 3.2b. Protein A at a dilution of 1:1000 was determined to be optimal and was used in all subsequent ELISA testing.

When comparing dilution buffers, PBST allowed for the greatest binding of Protein A and serum samples, without significantly increasing the background. TBSE allowed binding, but yielded more background in the blank (PBST only) wells. Milk blocker, when used as a diluting solution prevented any Protein A binding, but did allow serum to bind when using PBST as a diluting buffer for Protein A. PBST + 10% FBS also allowed both Protein A and serum binding without having significant background reactions.

The final optimal conjugate dilution was determined to be a 1:1000 dilution in PBST. This dilution provided a linear curve with the serum dilution. Results are illustrated in figure 3.3. All other dilutions tested could be useful, but provided either an inconsistent curve, or high background reactions.

Optimal substrate incubation period was determined to be 2-3 minutes. Incubation shorter than 2 minutes did not allow enough time for adequate reaction. Incubation longer than 3 minutes created significant background in negative sera. Results are illustrated in figure 3.4.

### 3.4.1. SNT Results.

Of the 505 seal and sea lion serum samples, 298 tested positive (titers >8), 51 tested suspect (titers of 6 and 8), 127 samples tested negative, and 29 samples were toxic to the cell culture. Trends in titers between geographical regions were negligible since there were too few samples from one population to detect trends,

with the exception of Alaskan harbor seals. Titers ranged from 4 through 192, with most falling between 4 and 32 (data not shown).

### 3.4.2. iELISA Results.

Of the 505 seal and sea lion serum samples tested, 386 (76.4%) tested positive and 119 (23.6%) tested negative against SeHV-1 antigen. Most negative (< 0.600 OD) serum samples had minimal (OD's ≤ 0.200). Few negative serum samples produced OD's of 0.201 to 0.399. Four samples tested between 0.400 and 0.599. Samples showing >0.600 OD were considered positive. All positive samples were greater than 0.800 OD, most of which exceeded a 0.900 OD value. Interpreted results are described in table 3.1. Only serum samples that had reportable results by the SNT and ELISA (425 total) are represented in detail. Serum samples that had toxic or suspect results were classified as non-reportable. Specific trends were difficult to define as described in section **3.4.1**. However, the Alaskan harbor seal population showed a slightly higher prevalence of SeHV-1 as determined by ELISA than by SNT (91%). This can be attributed to the possibility of false positives from the ELISA from low titer (<4) reports from the SNT, or false negative labeling by the SNT.

### 3.4.3. Comparison of iELISA and SNT.

Results of comparisons are described in table 3.2 below. When 505 serum samples were titered by SNT, endpoint titers could not be determined for 29 samples that were either toxic or contaminated, 11 of which tested positive on the ELISA. Additionally, there were 51 samples that tested as suspect by SNT (titers
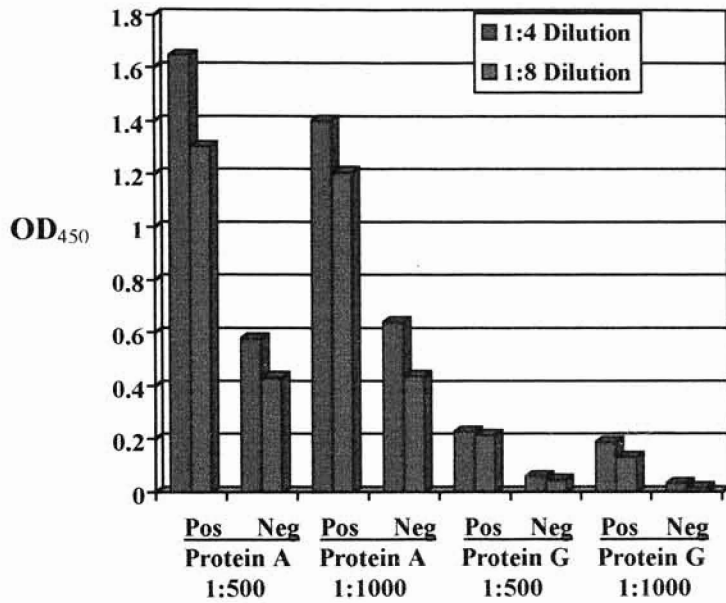
**Serum Classification**

**Figure 3.1 Protein A vs. Protein G Binding Results**
Comparison of Protein A vs. Protein G detection of known positive (Pos) and negative (Neg) samples against SeHV-1. The blank column values were subtracted out of each value. In each Dilution, the positive sample produced an OD value that was at least 2 times greater than the negative sample.
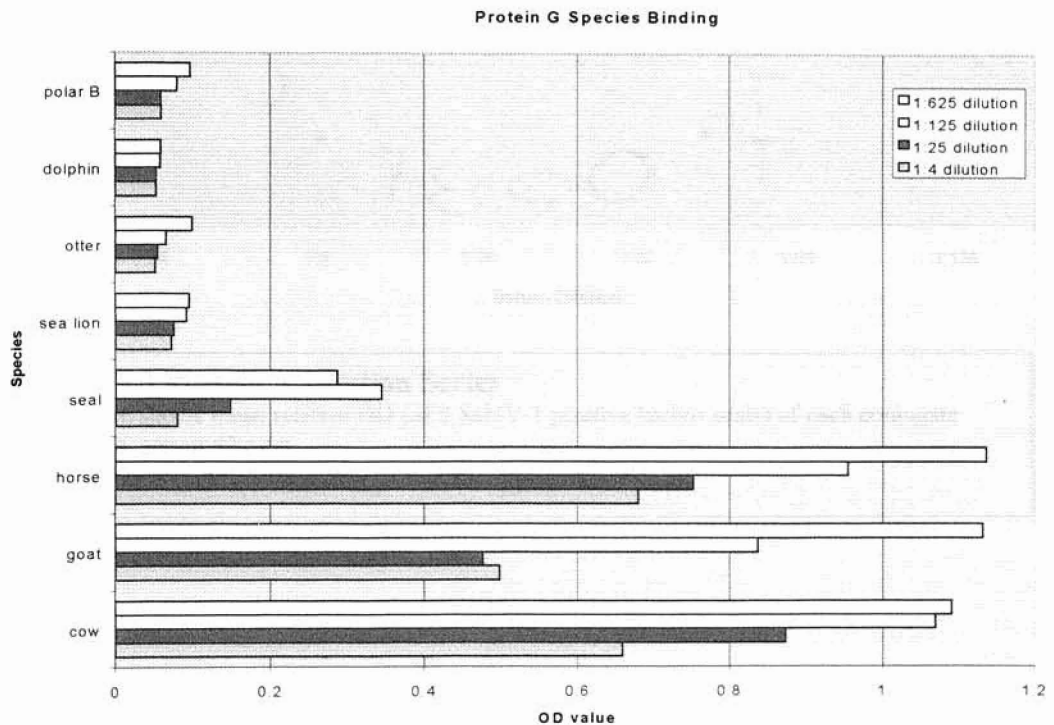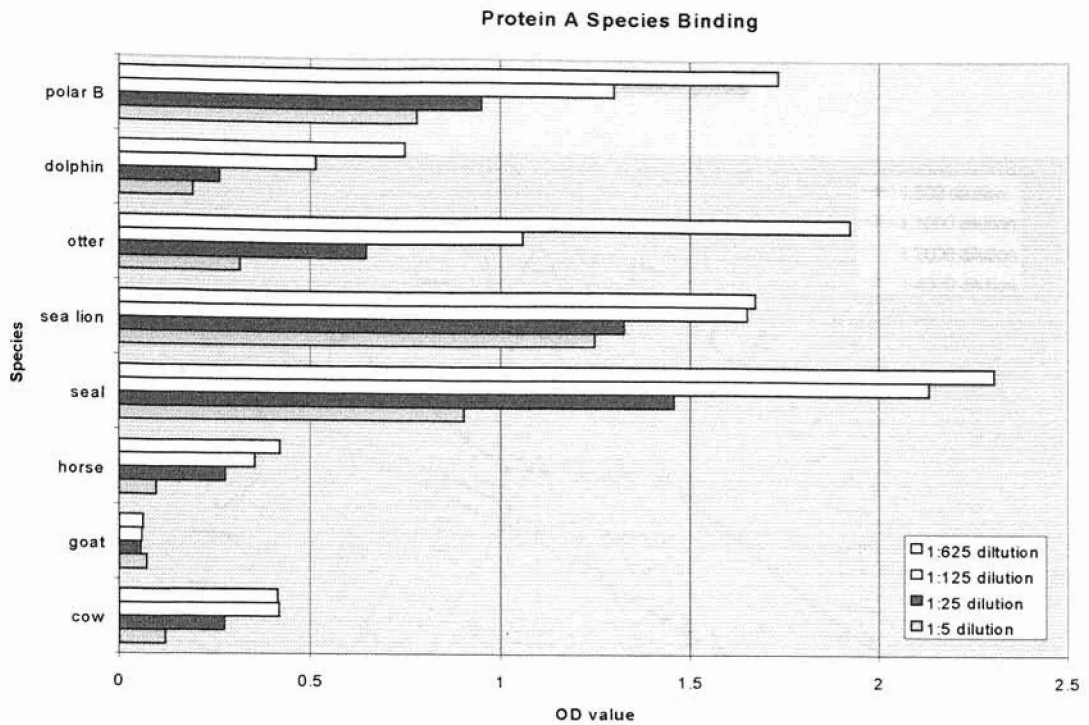
**Figure 3.2a (top) and 3.2b (bottom) Protein A and Protein G Species Binding Results**
These figures describe the comparison of Protein A and Protein G binding reactivity towards various mammal sera. Goat serum was used as a positive control for Protein G, and a negative control for Protein A. Each bar is an average of two different animal sera. Horse and cow samples were positive controls for Protein A as well as known reactivity with Protein G.
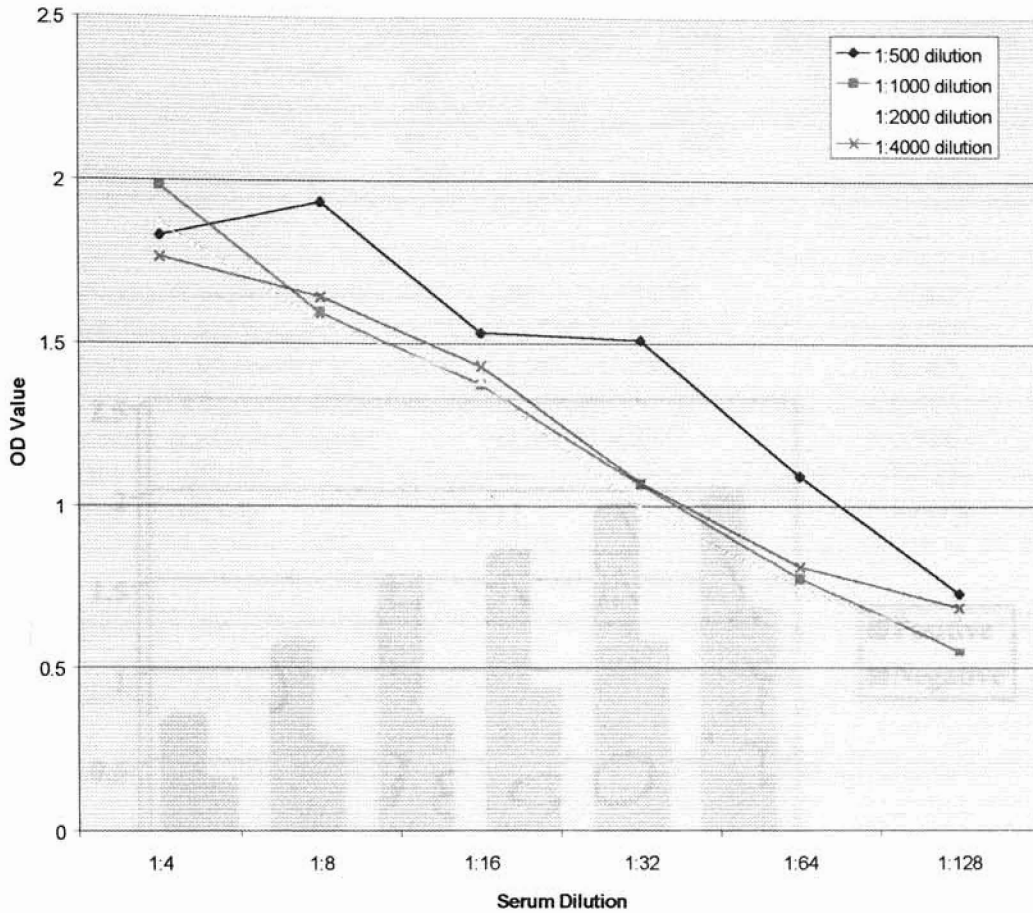
**Figure 3.3 Protein A Dilution Series**
This figure shows mean relative OD (of 2 SeHV-1 positive harbor seals) of each conjugate dilution vs. serum dilution.
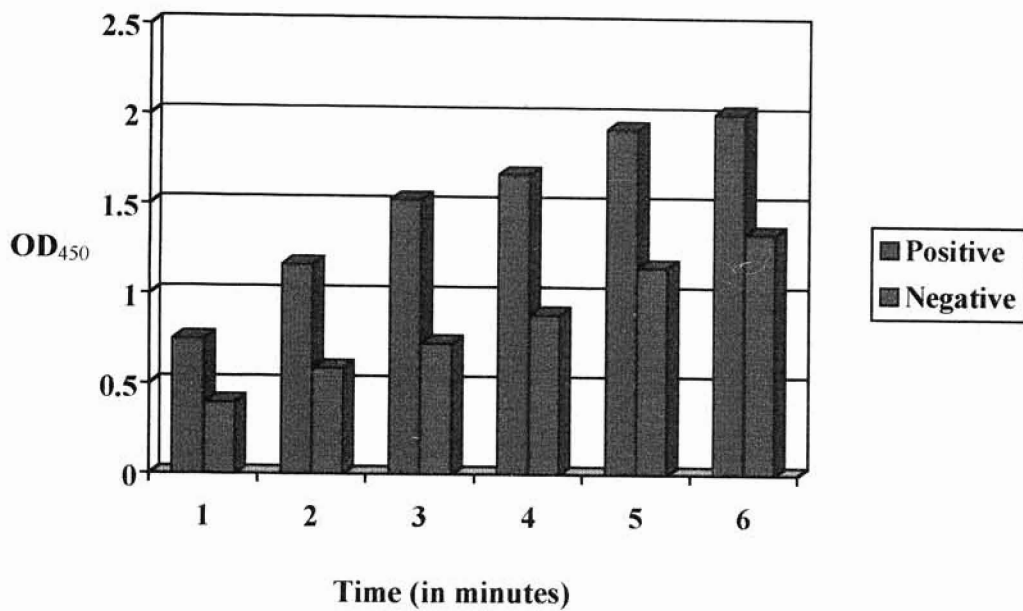
**Figure 3.4 Substrate Incubation Time Results**
This figure shows the relative OD value of each serum sample over time (in minutes) when comparing positive vs. negative samples.

**Table 3.2 Agreement between SNT and ELISA for detection of antibody in 425 sample sera** (Martin et al., 1988)

| | | ELISA Positive | ELISA Negative | Total | Apparent Prevalence |
|---|---|---|---|---|---|
| SNT | Positive | 298 | 3 | 301 | 0.708 |
| | Negative | 13 | 111 | 124 | 0.292 |
| | Total | 311 | 114 | 425 | |
| Apparent prevalence | | 0.732 | 0.268 | | |

| | | | |
|---|---|---|---|
| Observed proportional agreement | (298 + 111)/425 | | = 0.962 |
| Chance prop. agreement | (0.732 X 0.708) + (0.268 X .292) | | = 0.597 |
| Observed minus chance agreement | (0.962 – 0.597) | | = 0.365 |
| Max. possible agreement beyond chance level | | (1 – 0.597) | = 0.403 |
| Agreement quotient (kappa) | (0.365/0.403) | | = 0.906 |
| Relative specificity | (111/124) | | = 89.5% |
| Relative sensitivity | (298/301) | | = 99.0% |

that were $\geq 4$ but less than 8), but all 51 suspect serum samples tested positive on the ELISA. Of the remaining 425 samples a total of 298 serum samples were positive by SNT and ELISA (70.1%), 3 (0.7%) were negative by ELISA but positive by SNT, 111 (26.1%) were negative by both ELISA and SNT, and 13 (3.1%) were positive by ELISA but negative by SNT.

## 3.5. Discussion.

Seal Herpesvirus is common world wide with as much as a 77% prevalence in some areas (Zarnke et al., 1997). However, the prevalence of SeHV-1 was greater in our study (90%) than described by Zarnke et al. (1997) (77%). SeHV-1 prevalence in other seal and sea lion populations were difficult to observe due to low numbers of samples. Currently, the SNT is the most commonly used diagnostic test to detect SeHV-1 antibodies. The SNT is labor intensive, and requires 4 days of incubation before results can be obtained. It is

desirable to have a test available that is quicker, less expensive to run, and easily amenable for many samples.

We standardized the ELISA to the SNT because of two criteria: a) the SNT is currently the only diagnostic method of detecting serum antibodies, and b) neutralizing antibodies are presently considered the best predictor of host immune status. The ELISA results were very similar to the SNT results, yielding only 16 conflicting samples (Table 3.1). The cutoff OD line of 0.600 was established by comparing SNT and ELISA results and using 3 times a mean of negatives on an ELISA. Even with that high of a cutoff, there were 13 false positives via ELISA. Western Blot testing will be used in future studies to determine the cause of this phenomenon. For the 3 false negatives, it may be necessary to decrease the dilution to 1:10 or 1:16 to increase sensitivity.

Our indirect ELISA proved to be a rapid, sensitive, relatively inexpensive and specific method of detecting SeHV-1 antibodies. This test also has the ability to test a variety of marine mammal sera using Protein A as a conjugate. In comparison to the SNT, the ELISA offers a high sensitivity (99.0%) and specificity (89.5%) while decreasing the run time from 4 days to less than 5 hours. Additionally, the ELISA is less dependent on the need for serum and tissue culture qualities. Cell cultures have the potential to lose sensitivity to virus infection over a series of passages, and some serum can be toxic to cell cultures. In reference to the serum quality, one primary concern involves sample contamination. Contaminants in the serum sample can degrade antibodies and/or alter the pH to a level that effects antibody binding. Though antibody degradation

cannot be eliminated by this test, altered pH can be nullified by the dilution of 1:32 in PBST.

One problem observed in this ELISA is the low substrate incubation period. This may be solved by diluting serum samples more than the determined 1:32. Additional testing of conjugate dilution may also be necessary. Another possible solution would be to increase the negative cutoff level if more substrate incubation is required.

Agreement between the SNT and ELISA were calculated by estimating the agreement quotient (kappa), following established procedures (Martin et al., 1988). Our relative specificity (89.5%) and specificity (99.0%) are dependant on the SNT values. These values may increase as Western blotting is completed. There is a possibility that these values may increase if false SNT labeling is determined to occur. Our kappa value of 0.906 indicates a high level of absolute agreement between the SNT and ELISA. As indicated earlier, this value may increase if Western blotting determines that the conflicting SNT results are flawed.

All 29 serum samples that resulted in suspect diagnosis by the SNT tested positive by our ELISA. We speculate that the neutralizing titer was minimal, but non neutralizing titers (i.e. antibodies against enzymes, core proteins, etc.) were readily detectable by the ELISA.

In this study we obtained products from one company (Sigma Chemical Co.). Possible explanations for our Protein A vs. Protein G binding and affinity results may be: a) peroxidase label may diminish Protein G binding, or b) possible

problems with ordered batch c) Protein G does not bind to the tested marine mammal sera. Scenarios a and c are unlikely due to their binding described in figures 3.2a and 3.2b, but still remain a slight possibility. Whatever the case, Protein A yielded adequate results.

Tests that were not performed in this study, but will be tested in the future include: a) testing dogs and cats that have a history of canine or feline herpesvirus positive will be tested against seal herpesvirus type 1 antigen to detect cross-reactivity to serum antibodies, b) use the western blot to distinguish between true positive and true negative in comparison to the ELISA and SNT. These tests are important in final diagnosis. If canine and feline samples are cross-reactive, then SNT is required to distinguish whether the samples are SeHV-1 positive and/or feline or canine herpesvirus positive. If it is found that they are not cross-reactive, this developed ELISA could stand alone in the diagnosis of SeHV-1. Though these tests were beyond the scope of this study, Western blot testing, and testing of dog and cat samples will be important in providing final efficacy of this test.

## CHAPTER IV

## SUMMARY AND CONCLUSIONS

Seal herpesvirus type 1 is prevalent in seal and sea lion populations world wide, including populations off the European coasts of The Netherlands (Borst et al, 1986) and Germany (Horvat et al, 1989), Atlantic and Pacific coasts of the United States (Harder et al., 1996; King et al., 1998Kennedy-Stoskopf et al, 1986; Gulland et al, 1997), Alaska and Russia (Zarnke et al, 1997) and Antarctica (Stenvers et al, 1992). All these seal populations also carry antibodies against SeHV-2. Due to the wide range of this disease, it is important to have an accurate means of diagnosing SeHV-1.

Currently, there is only one means of serological diagnosis, serum neutralization. The SNT is the current gold standard, but it is labor intensive, requires 4 days before results can be determined, and is highly dependent on the quality of serum to be tested. Therefore, it is important to have a test that can minimize these shortcomings while maintaining a high level of sensitivity and specificity. The ELISA described in this study maintains a high specificity (89.5%) and a high sensitivity (99%) and only requires less than 5 hours to run. This ELISA is easily amenable to testing a far larger number of samples than an SNT would be. This ELISA is also usable on many "poor" serum samples that cannot be tested by SNT.

It was unfortunate that we were unable to develop a competitive MAb. Further testing can still be performed to determine future values. It will be interesting to see if some MAb's may be neutralizing, which may have value in the SNT for control purposes. IFAs and other immunohistochemical tests may also utilize these MAbs. It will also be interesting to test these by Western blotting to detect feline and canine herpesvirus proteins that cross-react with SeHV-1, which would be consistent with Martina et al. (2002). Western blotting will be limited to MAbs that detect linear amino acid sequences and will not be able to detect conformational amino acid sequence-recognizing MAbs.

Though a cELISA would have allowed for testing of any species without changing any of the procedure, the Protein A based iELISA allows testing for several marine mammal serum samples on the same plate (Fig. 3.2a). Because of this, the described iELISA maintains the advantages that a cELISA would have yielded while being less complicated than the cELISA and possibly being more reliable.

This study yielded a diagnostic method that nearly matched the gold standard results and possibly surpassed the reliability by distinguishing suspect samples into positive or negative status. This new ELISA was also able to detect serum antibodies against SeHV-1 that were toxic and unreadable on the SNT. Further testing via western blot needs to be performed to evaluate the overall performance of both the SNT and ELISA. This will allow added confidence levels to either test. If it is found that the conflicting results favor the ELISA, then the ELISA could be considered superior to the SNT. Also, testing dog and

cat samples with histories of canine and feline herpesvirus infections is important to evaluate additional uses for this test, and may suggest that this ELISA can either stand alone, or require SNT for differential diagnosis of SeHV-1, feline, and canine herpesviruses. This testing will be limited to Protein A binding to cat and dog IgG antibodies. If it is found that this ELISA is cross-reactive towards canine and feline herpesviruses to a high degree (OD >0.600), then it would require SNT testing to properly identify the correct pathogen. If there is minimal cross-reactivity, then this ELISA could stand alone in detection of SeHV-1 antibodies.

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