EVALUATING THE CLINICAL SIGNIFICANCE OF ALPHAHERPESVIRUSES IN BOTTLENOSE DOLPHINS (*TURSIOPS TRUNCATUS*)

By

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In "The Virologist's Dilemma" (1957), Dr. Robert J. Huebner discussed the conditions necessary for establishing a virus as cause of a specific human disease. While he mentions many important factors it is his ninth topic "Financial support: A consideration so absolutely necessary that it deserves to be called a postulate" that leads me to this: I am very, very grateful for the significant funding, samples, and hard work from the US Navy Marine Mammal Program towards this project. I would specifically like to thank Dr. Stephanie Venn-Watson for the countless hours spent analyzing and *reanalyzing* my data.

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During the routine, conventional PCR-based, viral surveillance of a managed collection of bottlenose dolphins, two distinct alphaherpesviruses, tentatively named *Delphinid herpesvirus 2* (DeHV-2) and *Delphinid herpesvirus 8* (DeHV-8), were detected in the buffy coat of one animal. The 22-year-old, female bottlenose dolphin had a history of elevated transaminases and dehydrogenases, which were normalized following phlebotomy treatment, and a persistent lymphocytosis. Alphaherpesviruses are generally considered to persist in the sensory nerve ganglion or in circulating lymphocytes, thus changes in viral load in circulating blood cells was presumed to be indicative of infection with active virus replication. The presence of the virus was thus evaluated with a quantitative assay so the active virus replication event could be identified by the higher level of virus particles.

Novel, specific TaqMan quantitative PCR assays were developed to measure the viral load of the alphaherpesviruses in the case dolphin. The complete blood counts (CBC) and serum chemistry parameters for the herpesvirus positive samples in the case dolphin were compared to those of the negative samples, in an attempt to correlate viral presence with changes in clinical blood parameters. These assays were also used in a cross-sectional survey to establish a baseline prevalence of the two alphaherpesviruses in a population of clinically healthy bottlenose

dolphins. DeHV-2 has a higher presence in the population (3 animals) than DeHV-8 (which was only detectable in the case animal). The simple presence of DeHV-2 could not be associated with any specific blood parameter changes in the case animal. One positive case animal sample (Tt0719), however, had remarkable trends in four of the blood parameters analyzed: decreases in total white blood cell counts and serum chloride as well as increases in erythrocyte sedimentation rate and hematocrit. Similar trends were not identified surrounding any of the other four DeHV-2 qPCR positive samples. There were an insufficient number of DeHV-8 positives for comparison of blood parameters associated with positive and negative samples.

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CHAPTER 1
INTRODUCTION

Herpesvirales

Most vertebrate species investigated have yielded at least one, and usually several, herpesviruses.[37] More than 200 herpesvirus species have been identified to date.[37] When investigating the lineage of herpesviruses, many major subdivisions mirror the phylogenetic branching order of their hosts.[37] Thus, herpesviruses have co-evolved with their hosts and tend to be host specific.[37] Since there are almost 5,500 different species of mammals[43] alone, it can be expected that the number of herpesviruses that exist in nature well exceeds the 200 species discovered thus far. All herpesviruses fall within the newly established taxonomic order *Herpesvirales*.[9] *Herpesvirales* consists of three families: *Herpesviridae* (which includes the herpesviruses of mammals, reptiles, and birds), *Alloherpesviridae* (which includes the herpesviruses of fish and amphibians), and *Malacoherpesviridae* (bivalve herpesviruses).[9,37] Additionally, the family of *Herpesviridae* contains three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae*.[9,37]

Herpesviruses were traditionally assigned a phylogenetic classification based on their morphology. Current criteria also include DNA sequence similarity, genome arrangement, and relatedness of viral proteins.[37] Herpesvirus characteristics such as their envelope and icosahedral capsid are clearly visible by transmission electron microscopy.[37] Herpesviruses are among the larger viruses with the diameter of mature virions ranging from 120 to 260 nm.[37] Other physical properties include having a single, linear, double-stranded DNA genome (ranging from 124 to 290 kb in length), an envelope with viral glycoprotein spikes, and the tegument, a proteinaceous matrix which surrounds the icosahedral capsid and interfaces with the lipid envelope.[9,37] Four biological characteristics are shared by members of *Herpesviridae.* The first characteristic is self-

generation of a large array of enzymes involving nucleic acid metabolism, DNA synthesis, and processing of proteins.[37] Secondly, the synthesis of viral DNA and the capsid assembly occurs in the nucleus but the final processing of the virion occurs in the cytoplasm.[37] Thirdly, production of the infectious progeny virus is associated with destruction of the infected cell.[37] Lastly, and most notably, all of the herpesviruses examined to date are able to enter into a latent state in their natural host.[37]

Herpesvirus Latency

The ability to remain latent in their natural host is a unique and fundamental characteristic of herpesviruses and has been greatly studied. While the exact mechanism for reactivation is still not fully understood[37], much is known about the latent and active virus states. Latent virus genomes transform to closed, circular molecules where only a few of their genes are expressed.[37] While in latency, they retain their ability to replicate and they can cause disease upon reactivation.[37] Infected cells can all be in a latent state however herpesvirus can also be latent in some cells and simultaneously active in others.[37] When herpesvirus is lytically active in only a subset of infected cells, there are usually no symptoms of the infection in the host.[37] Yet, active proliferation causing disease in the host can still occur while some infected cells remain latent.[37] The ability of herpesviruses to enter latent states and then reactivate allows for chronic infection. Chronic herpesvirus infections occur when infectious progeny are present within the animal system[37] thus the virus is actively proliferating in some infected cells.

The Subfamilies of *Herpesviridae*

Alphaherpesvirinae

There are four genera within *Alphaherpesvirinae*. Viruses within the genera *Simplexvirus* and *Varicellovirus* infect mammals, whereas those of the genera *Mardivirus* and *Iltovirus* infect avian hosts.[9,37] While reptile herpesviruses are also present in the subfamily *Alphaherpesvirinae*,

they have not been assigned a genus.[9,37] Alphaherpesviruses (αHVs) share several biological characteristics including a relatively short reproduction cycle (about 18-20 hrs[41] but can be shorter), a variable host range, rapid spread in cell culture, efficient destruction of infected cells, and the capacity to establish latency in the sensory ganglion of the cranial and spinal nerves.[37]

Betaherpesvirinae

There are also four genera in *Betaherpesvirinae: Cytomegalovirus, Muromegalovirus, Roseolovirus,* and the newly recognized genus *Proboscivirus.*[9] Additional betaherpesviruses (βHVs) exist that are unassigned to a genus within this subfamily, but they are all mammalian herpesviruses.[9,37] βHVs have not been identified for reptiles or birds to date. Biological characteristics shared by βHVs include a long reproductive cycle (about 48-72 hrs)[35], often a restricted host range, slow growth in culture, infected cells frequently become enlarged, and the virus can establish latency in secretory glands, lymphoreticular cells, kidneys, and other tissues.[37]

Gammaherpesvirinae

Two new genera have been added to *Gammaherpesvirinae* to make four genera in this subfamily as well: *Lymphocryptovirus*, *Rhadinovirus*, *Macavirus*, and *Percavirus*.[9] None of the reptilian or avian gammaherpesviruses (γHVs) have been assigned to a genus.[9,37] Furthermore, about 20 unassigned mammalian γHVs have been recognized.[9,37] Biological characteristics for γHV species are loosely described as having a limited host range within the order of their primary host and latency is frequently seen in lymphoid tissue.[37]

Herpesviruses of Humans

Humans are an excellent model of herpesvirus infection because human herpesviruses belong to a variety of genera and are associated with a multitude of clinical presentations. Herpesviruses from each subfamily within *Herpesviridae* are known to infect humans.[9,37] The severity of symptoms from infection can range from subclinical infection to death.[26,47,58] There

are eight known herpesviruses identified with humans as their primary hosts, *Human herpesvirus 1 – 8* (HHV-1 – 8).[9,37] The first three are αHVs: herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), and Varicella-zoster virus (VZV). The fourth is a γHV, Epstein-Barr virus (EBV). The next three are βHVs: cytomegalovirus (CMV), HHV-6, and HHV-7. Lastly, the eighth is a γHV, Kaposi's sarcoma-associated herpesvirus (KSHV).[9,37]

Herpes simplex virus type 1 and 2 are common infections worldwide.[58] A large-scale serosurvey of adults residing in the United States, indicated a seroprevalence of 57.7% and 17% for HSV-1 and HSV-2, respectively.[58] HSV-1 is usually transmitted via direct contact during childhood.[58] In contrast, HSV-2 is almost always sexually transmitted and is the typical etiologic agent for genital herpes.[58] Most HSV-1 and 2 infections are subclinical but they can cause ulcerative orogenital lesions as well as encephalitis and death.[58] The severity of clinical symptoms following primary infection is directly indicative of the frequency and severity of reactivation symptoms.[41]

The seroprevalence for VZV infection of adults living in the United States is extremely high at 99.6%.[26] Varicella primary infection typically results in the highly contagious childhood disease chickenpox.[8] The classic symptom is developing a rash with fluid-filled vesicles surrounded by irregular erythema margins on the head or trunk before spreading to cover the entire body.[8] The virus establishes latency in the dorsal root ganglion of the spinal nerves.[8] Reactivation of VZV causes a disease syndrome called shingles, or herpes zoster, most commonly affecting the elderly.[8] With reactivation, the vesicular cutaneous lesions are typically well-delineated and follow one or more sensory nerve ganglia,[8] instead of the full body rash seen in chickenpox. Varicella zoster virus is known to cause fatalities, and thus a vaccine was created which has been used routinely during childhood immunizations as of 1996.[8,26]

Epstein-Barr virus, of the genus *Lymphocryptovirus*[9,37], is a very successful virus infecting over 90% of humans worldwide.[7] Both Burkitt's lymphoma and infectious mononucleosis are associated with EBV.[7,23] The virus maintains persistent infection in the B cells of the oropharynx.[7] Epstein-Barr virus is most pathogenic in immunocompromised individuals and is typically not treated when diagnosed in those with uncomplicated disease.[7]

Cytomegalovirus seroprevalence for adults in the United States is 58.9% and it is transmitted by close contact.[47] CMV is unique among herpesviruses in that it can also be vertically transmitted.[35] It is the leading cause of congenital illness, affecting 0.2-2.2% of all newborns.[47] Symptoms from CMV infection in newborns include, vision and/or hearing loss, mental retardation, neurologic abnormalities, and death.[47,48] Cytomegalovirus is usually a life-long latent infection for adults and is only pathogenic in immunocompromised patients.[48] However, CMV can also cause heterophile-negative mononucleosis-like infection in adults (attributes to 20 – 50% of the cases).[23,49]

Human herpesvirus 6 and *7* are both ubiquitous viruses.[29,59] The seroprevalence of HHV-6 ranges between 80% and 100% in adults under 40 years old.[30] Furthermore, seroconversion typically occurs by age 2.[29,59] Type 7 seroprevalence is estimated to be higher than 85% in the adult population with most seroconverting as young children as well.[29,59] Both HHV-6 and 7 are in the same genus, *Roseolovirus*, and the pathogenesis of roseolovirus infections is not well characterized.[59] *Human herpesvirus 6* is known to be associated with exanthem subitum, a disease causing fever and rash in infants.[59] Additionally, HHV-6 has been implicated as the cause of multiple sclerosis (MS),[59] and it has been associated with some cases of heterophile-negative mononucleosis-like illness.[23,49]

Kaposi's sarcoma-associated herpesvirus, within the genus *Rhadinovirus,*[9,37] is the rarest of all known human herpesviruses with an adult seroprevalence in the United States of 2.5-7.1%.[14] As the name suggests, KSHV is the etiologic agent of Kaposi's sarcoma, a histologically complex tumor containing multiple cell types within each lesion.[20] Although rare, with the 1980's pandemic of human immunodeficiency virus (HIV), KSHV became the most common neoplasm complicating acquired immunodeficiency syndrome (AIDS).[20]

Seroprevalence data indisputably shows the universal presence of herpesviruses. Most are common and widespread with infections causing minimal, usually mild disease like chickenpox. However, they are often also associated with more severe pathologies such as herpes simplex virus induced encephalitis. The seroprevalence of multiple herpesviruses in humans is not unusual. They are evolutionarily old viruses that have co-evolved with their host.[37] Thus, it is not uncommon to find multiple species within *alpha-*, *beta-*, and *gammaherpesvirinae* present in a host. For example, five herpesviruses (four αHVs and 1 γHV) have been identified in horses (and four more for the family *Equidae*).[9,37] Additionally, four herpesviruses (three αHVs and one γHV) have been identified in cows (and six more for the family *Bovidae*).[9,37] This prevalence is comparable to the eight human herpesviruses and four additional viruses (one βHV and three γHVs) within the family *Hominidae*.[37]

Cetacean Herpesviruses

The presence of herpesviruses in cetaceans (the group of animals consisting of all porpoises, dolphins, and whales) has been recognized since the late 1980s. In 1988, herpesvirus-like viral particles were identified by electron microscopy (EM) in skin biopsies collected during the necropsy of a wild beluga whale (*Delphinapterus leucas*).[32] The juvenile, female beluga whale stranded in the St. Lawrence Estuary, Canada.[32] Biopsies were collected because the beluga had generalized dermatitis that consisted of circular, pale lesions.[32] Pathologic findings

also included several abnormalities that could not be associated with herpesvirus infection including a perforated gastric ulcer with acute exudative peritonitis, verminous broncho-pnemonia, and chronic hepatitis.[32] Herpesvirus-like viral particles were identified by transmission electron microscopy (TEM) within skin biopsies of a second beluga whale with focal dermatitis in 1989.[1] In this instance, the dermatitis developed in captivity a few months after the young adult, female beluga was collected in the Churchill River, Canada and transported to an oceanarium.[1]

In 1992, a herpesvirus was associated with an encephalitis case in a harbor porpoise (*Phocoena phocoena*).[25] A juvenile, female harbor porpoise was found stranded on the coast of Sweden in 1988.[25] Necropsy findings describe white, flat and slightly raised foci on the skin but herpesvirus could not be identified by TEM or immunoperoxidase staining.[25] However, a large amount of herpesvirus-like viral particles were identified, by TEM, in the cerebral cortex tissues.[25] Immunoperoxidase staining of these tissues showed cross-reactivity with HSV-1 and BHV-1 (*Bovine herpesvirus 1*, an alphaherpesvirus) suggesting the herpesviral antigen was an alphaherpesvirus.[25]

In 1994, herpesvirus-like viral particles were again identified by TEM in the skin biopsies of two Peruvian dusky dolphins (*Lagenorhynchus obscures*) with raised black foci on their rostrum.[50] These lesions were seen on two more animals at the same time but viral particles were not detectable by TEM.[50]

In 1999, the first serologic evidence of exposure of cetaceans to herpesviruses was generated. Between 1995 and 1997, beluga whales in the St. Lawrence Estuary were tested by serum neutralization (n=13) and enzyme-labeled immunosorbent assay (ELISA; n=12) using BHV-1 antigen.[34] The researchers concluded that an alphaherpesvirus was likely endemic to this

population since 46% of the animals were positive with the serum neutralization test and 58% demonstrated reactivity by ELISA.[34]

The first cetacean herpesviral genomic sequences, derived from bottlenose dolphins (*Tursiops truncatus*), were not published until 2001.[2] Two distinct alphaherpesviruses were reported in two separate, single bottlenose dolphin strandings along the Atlantic coast.[2] The herpesviruses were associated with acute necrotizing lesions on multiple organs by histology, electron microscopy (EM), and polymerase chain reaction (PCR).[2] The first stranding occurred in 1995 where a juvenile, female bottlenose dolphin was found dead on Hilton Head Island, South Carolina.[2] Herpesvirus particles were identified in the thymus by EM and unfixed lung tissue was used in two consensus PCRs to obtain partial sequence of the DNA-dependent DNA polymerase (Dpol) and terminase genes (GenBank accession numbers AF196646 and AF196647, respectively).[2] In 1999, a second juvenile, female bottlenose dolphin was found alive, stranded on a beach in Delaware, but died shortly thereafter.[2] Herpesvirus particles were identified by EM in tissues from the heart, snout, and tongue.[2] No other tissues were examined. Efforts were also made to obtain Dpol and terminase sequence from the heart tissue, following the same protocols as the first case, but only sequencing of the partial Dpol gene was successful (GenBank accession number AF245443).[2] For the purposes of clarity, these viruses are tentatively named *Delphinid herpesvirus 1* and *2* (DeHV-1 and DeHV-2), respectively. Although not yet recognized by the International Committee on Taxonomy of Viruses (ICTV), they are considered separate species because DeHV-1 (AF196646) has only a 61% amino acid identity to DeHV-2 (AF245443) in the highly conserved Dpol region. The proposed names are derived from the host family and order of virus discovery, in accordance with current ICTV naming conventions for herpesviruses.[9,10]

In 2006, a third novel alphaherpesvirus was reported in a stranded bottlenose dolphin.[31] This juvenile, male dolphin was found alive in 2004 along the west coast of Florida and was transported to a rehabilitation facility.[31] The dolphin had multiple attack wounds and hundreds of 1-3 mm raised, black papules distributed primarily over the dorsal and lateral portions of its body.[31] These papules slowly progressed to superficial, gray erosions and decreased over time.[31] Meanwhile, herpesvirus-like particles were identified using TEM on lesion biopsies.[31] Molecular characterization of the viral particles yielded a partial Dpol herpesviral sequence via consensus PCR (GenBank accession number AF757301).[31] This latest alphaherpesvirus, tentatively named DeHV-3 in this study, has an amino acid identity of 70% to DeHV-1 (AF196646) and 60% to DeHV-2 (AF245443).

The first cetacean gammaherpesviruses were also reported in 2006. Gammaherpesviruses were identified with consensus Dpol primers in mucosal lesions of four separate cetacean species: a Risso's dolphin (*Grampus griseus*), a dwarf sperm whale (*Kogia sima*), a Blainville's beaked whale (*Mesoplodon densirostris*), and five Atlantic bottlenose dolphins.[45,46] The majority of samples were from penile and vaginal lesions. However, an identical γHV Dpol sequence was identified from both an oral lesion and a penile lesion of one bottlenose dolphin.[45,46] This dolphin was an adult male that stranded off Islamorada Key, Florida and later died of sepsis.[45] Two more bottlenose dolphins also had the identical gammaherpesvirus Dpol sequence identified. This first bottlenose dolphin gammaherpesvirus will be referred to as DeHV-4 (GenBank accession number AY952777, additional identical sequence accession numbers AY952778, AY952779, AY949831). The other two bottlenose dolphins had a distinct gammaherpesviral sequence, 7-8 amino acids different from DeHV-4 (85-87% amino acid identity). This second gammaherpesvirus will be referred to as DeHV-5 (GenBank accession number AY952776,

additional sequence, with 1 amino acid difference, accession number DQ288667). Since Risso's dolphins are of the family Delphinidae, the gammaherpesviral sequence generated from that dolphin is tentatively named *Delphinid herpesvirus 6* (DeHV-6; GenBank accession number DQ288666). Likewise, the gammaherpesvirus from the dwarf sperm whale is tentatively named *Kogiid herpesvirus 1* (KoHV-1; AY949830) and the gammaherpesvirus from the Blainville's beaked whale is tentatively named *Ziphid herpesvirus 1* (ZiHV-1; AY949828).

Moreover, in 2006, DeHV-1, 2, and 3 Dpol sequences were also obtained from two captive bottlenose dolphins with skin lesions. The viral genomic sequence amplified from a skin lesion of the first dolphin (AY949831) was only 2 amino acids different from DeHV-1. A blood sample from the second dolphin generated herpesviral sequence (DQ295064) identical to DeHV-2, whereas a spleen sample generated sequence (DQ295063) only one amino acid different from DeHV-3. Additionally, a third herpesvirus sequence (AY608707) was amplified from a skin lesion of the dolphin which was 4 amino acids (93% amino acid identity) different from DeHV-1. HSV-1 and 2 only have 5 amino acid differences in the homologous Dpol region so it is likely that sequence AY608707 represents a separate species. Thus, this alphaherpesvirus, AY608707, is tentatively named DeHV-7.

Criteria for Disease Causation

While the presence of herpesvirus in abnormal tissues is an essential argument for association it is not sufficient for establishing said herpesvirus as the cause of a specific lesion or disease. Surpassing association and fulfilling the requirements to establish causation is the spirit of the three Henle-Koch postulates.[40] The first postulate states that the parasite causing the disease in question should occur in every case where the disease is present under circumstances which can account for the pathological and clinical course of the disease.[40] Second, the agent cannot occur in any other disease as a fortuitous or non-pathogenic parasite.[40] Third, after being

isolated and grown in culture, experimental exposure can induce identical disease in a normal host.[40] While the scientific community embraced the postulates they were often found to be too stringent, especially since many pathogens were difficult to culture.[15,40]

With the discovery of viruses, it became even more difficult to fulfill the Henle-Koch postulates when establishing etiologic viral agents for diseases.[15,40] Not only are some viruses difficult to culture, but some viruses that could be cultured were found in both healthy and diseased individuals.[15] Alfred Evans and others highlighted the limitations of the Henle-Koch criteria for viral agents.[15] First, the same pathologic or clinical states can be produced by different etiological agents. Second, the causative agents may vary by geographic areas, age groups, or different patterns of host susceptibility. Third, some diseases require the presence or action of two or more agents. Fourth, a single agent may produce different clinical or pathological responses in different settings. Fifth, any cause or set of causes usually produces a biological gradient of response from no detectable symptoms to mild clinical signs to classic, recognized disease. Finally, the severity of host response following exposure will vary with host characteristics such as genetics, age, immunologic status, socio-economic status, and exposure to other cofactors (infection, drugs, etc).[15] Thus, it is necessary to consider multiple elements when proposing the etiology of a disease. Evans also stressed that even though it may not be possible to fulfill the Henle-Koch postulates in establishing causation, there should be a justification which makes biological sense, using sound reasoning, for the proposed cause.[15]

These sentiments were echoed by David Fredricks and David Relman in their assertion that Koch's postulates inspire scientific rigor to the process of establishing causality, but they are too stringent for today's times of sequence-based identification of viral and microbial agents.[19] Fredricks and Relman elaborated on the limitations of Koch's postulates for disease causation in

light of modern molecular techniques. For instance, highly sensitive PCR assays, theoretically capable of detecting as few as one target molecule, detect the presence of clinically irrelevant quantities of microbes. In recognition of the vastly growing database of microbial pathogens from sequence-based investigation, seven guidelines for establishing causation were proposed. First, nucleic acid sequence for the suspected cause should be found in most cases of the disease and preferentially in tissues with evidenced pathology. Second, fewer, or no, copy numbers of pathogen-associated nucleic acid sequences should occur in host or tissues without disease. Third, with treatment, the copy number should decrease or become undetectable and as such increase during relapse. Fourth, causation is compelling when sequence detection predates the disease or copy number correlates with the severity of the disease. Fifth, the nature of the microorganism should be consistent with known characteristics or the characteristics of those phylogentically-related. Sixth, sequence from tissues should be investigated at the cellular level, including efforts to demonstrate specific in situ hybridization of the sequence to the tissue pathology. Seventh, sequence-based forms of evidence for causation should be reproducible. These guidelines were put forth to establish a compelling argument regarding disease etiology but are not intended for strict adherence.[19]

Correlating Clinical Signs with Bottlenose Dolphin Herpesviruses

During the routine, conventional PCR-based, viral surveillance of a managed collection of bottlenose dolphins, two alphaherpesviruses were detected in the buffy coat of one animal. The 22-year-old, female bottlenose dolphin had a history of elevated transaminases and dehydrogenases, which were normalized following phlebotomy treatment, and a persistent lymphocytosis. Furthermore, the dolphin did not have any skin lesions, unlike the majority of the previous reports of herpesvirus in cetaceans. Thus, the clinical significance of this finding was unclear. Alphaherpesviruses are generally considered to persist in the sensory nerve

ganglion[3,11,21,41] thus the presence in circulating blood cells was presumed to be indicative of infection with active virus replication. However, it has also been suggested that in some hosts, such as the horse, alphaherpesviruses can persist in the white cell fraction.[6,44,55] Regardless of the site of persistence and/or replication, changes in the virus load in the circulating white blood cells, as measured using a quantitative PCR assay, reflect the level of replication of the herpesvirus. This virus load, or virus copy number per sample, cannot be assessed using conventional PCR.

Real-time quantitative PCR (qPCR) assays have been applied in diagnosing or monitoring herpesviral infections including the human herpesviruses[22,27,36,48,54] and *Equid herpesvirus 1* and *4*[12] (EHV-1 and 4). Quantitative PCR has a faster turn-around time and a higher throughput than traditional PCR.[27,48] The TaqMan qPCR method measures the quantity of PCR products with a specific fluorogenic probe, specific primers, and real-time laser scanning in a 96-well plate.[27,36] When these quantities are compared to known standards, the viral load in each sample can be determined. Therefore, TaqMan qPCR assays were developed to measure the viral load of the alphaherpesviruses in the case dolphin. The complete blood counts (CBC) and serum chemistry parameters for the herpesvirus positive samples in the case dolphin were compared to those of the negative samples, in an attempt to correlate viral presence with changes in clinical blood parameters. These assays were also used in a cross-sectional survey to establish a baseline prevalence of the two alphaherpesviruses in a population of clinically healthy bottlenose dolphins.

The Fredricks-Relman criteria for establishing causality of disease require the association of viral load with disease.[19] During the presence of clinical signs, a greater viral load, from lytically active herpesvirus, in the bloodstream is expected. For example, in the case study, time

points with high viral loads could correlate with inflammation. Time points with low or undetectable viral loads would reciprocally correlate with the absence of inflammation and normal blood parameters. Following the causality of disease criteria, the case for DeHV-2 as the cause of inflammation would be strengthened if other dolphins in the population also had inflammation concurrent with increased DeHV-2 viral load. Thus, a qPCR assay was developed as a tool for investigating possible alphaherpesviral diseases because it can quantify the viral load in an animal.

CHAPTER 2
QUALITATIVE AND QUANTITATIVE PCR ASSAYS FOR THE DETECTION OF HERPESVIRUSES

Introduction

Real-time quantitative PCR (qPCR) assays have been applied in diagnosing and monitoring herpesviral infections including the human herpesviruses[22,27,36,48,54] and *Equid herpesvirus 1* and *4*[12] (EHV-1 and 4). Quantitative PCR has a faster turn-around time and a higher throughput than traditional PCR.[27,48] The TaqMan qPCR method measures the quantity of PCR products with a specific fluorogenic probe, specific primers, and real-time laser scanning in a 96-well plate.[27,36] When these quantities are compared to known standards, the viral load in each sample can be determined. Alternative real-time methods also exist that do not use a specific probe. Real-time detection during amplification can be accomplished with SYBR Green, a fluorescent dye that will bind to any double-stranded DNA.[36] However, there is increased nonspecific binding due to the broad nature of the dye.[36] Two specific TaqMan qPCR assays were developed for the detection of *Delphinid herpesvirus 2* and *8* (DeHV-2 and -8). Both of these assays target the DNA-dependent DNA polymerase (Dpol) region of the virus.

Materials and Methods

Sample Collection

The case dolphin was part of a managed collection and was housed in a coastal, open ocean water enclosure. Sixteen serial blood samples were collected between July 12, 2004 and December 17, 2007 from the caudal peduncle vein of the case dolphin using a 20 or 21 gauge 1.5 inch Vacutainer® needle[a] or from a fluke vein using a 21 gauge 1 inch butterfly needle. An EDTA (K_3) Vacutainer® tube containing whole blood was used for collection of total white blood cell fraction (buffy coat). The blood tube was centrifuged at 3,000 rpm at 21°C for 10

min. Approximately 100 µl containing the buffy coat fraction, visible at the interface of the red and white blood cell fraction, was collected via gentle aspiration.

An additional three samples were collected including a mucosal swab from a harbor seal (*Phoca vitulina*), blowhole exudate from an orca (*Orcinus orca*), and an eye swab from a California sea lion (*Zalophus californianus*), and a consensus PCR for herpesvirus was performed.

Consensus PCR

DNA was extracted from the buffy coats via a commercial kit according to manufacturer's instructions (DNeasy Blood and Tissue Kit[b]). PCR was performed on all sixteen case animal samples with nested consensus primers for the DNA-dependent-DNA polymerase (Dpol) of herpesviruses.[51] *Phocid herpesvirus 2* isolate, kindly provided by Dr. Tracey Goldstein, was used as a positive control in the consensus PCR and was also sequenced. Reactions were amplified in a Px2 thermal cycler[c] using Platinum® *Taq* DNA Polymerase[d] in 30µL reactions following manufacturer's instructions. Amplification conditions were as follows: initial denaturation at 94°C for 5 min; 45 cycles of amplification with each cycle consisting of denaturation at 94°C for 30 s, annealing at 46°C for 1 min, and elongation at 72°C for 1 min; a final elongation step was performed at 72°C for 7 min followed by a 4°C hold. Secondary PCR products were resolved on a 1.5% agarose gel made up in 1X TBE and containing 1µg of ethidium bromide per mL. Bands of expected size (170-315bp) were excised and purified using QIAquick Gel Extraction Kit.[b] Direct sequencing was performed with the Big-Dye Terminase Kit[e] using the second round primers, TGV and IYG.[51] This was followed by adding 2µL of 2.2% SDS to each forward and reverse 20 µL sequencing reaction. Reactions were returned to the thermal cycler and heated to 98°C for 5 min, 25°C for 10 min, and held at 4°C. Unincorporated dye terminators were removed with the DyeEx 2.0 Spin Kit[b] and dried via vacuum centrifuge[f] at

1,400 rpm for 25-30 min. Sequencing reactions were submitted to the University of Florida Interdisciplinary Center for Biotechnology Research and analyzed on ABI 3130 DNA sequencers.[g] Primer sequences were edited out prior to further analysis. Sequences were confirmed via translated nucleotide-translated nucleotide and protein BLAST search in the National Center for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Bayesian and Maximum Likelihood Phylogenetic Analysis

A multiple sequence alignment computer program (MUSCLE[13]) was used to align a 58-64 amino acid predicted homologous region of 19 alphaherpesvirus DNA-dependent DNA polymerase sequences. Similarly, MUSCLE was used to align a 55-60 amino acid predicted homologous region of 20 gammaherpesvirus DNA-dependent DNA polymerase sequences. Two separate analyses were run because the predicted amino acid region was too small for the two groups to be analyzed together. References and GenBank accession numbers for the Delphinid herpesviruses are shown in Table 2-1. The MUSCLE alignments were analyzed with MrBayes 3.1[42] with gamma-distributed rate variation plus a proportion of invariant sites, and mixed amino acid substitution models. Four chains were run and statistical convergence was assessed by looking at the standard deviation of split frequencies as well as potential scale reduction factors of parameters. The first 10% of 1,000,000 iterations were discarded as a burn-in. *Iguanid herpesvirus 2* (GenBank accession number AY236869) was designated as the outgroup for the alphaherpesvirus alignment due to its early divergence from other herpesviruses.[33,56] Likewise, *Elephantid herpesvirus 1* (GenBank accession number AF322977) was designated as the outgroup for the gammaherpesvirus alignment.

Maximum likelihood (ML) analyses of each amino acid alignment was performed using computer based phylogenic modeling software, PHYLIP (Phylogeny Inference Package, Version

3.66),[17] and by running each alignment in ProML (protein maximum likelihood) with amino acid substitution models Jones-Taylor-Thornton[24] and Probability Matrix from Blocks (PMB).[52] Both models were set for global rearrangements, five replications of random input order, less rough, gamma plus invariant rate distributions, and unrooted. The value for the alpha of the gamma distribution was taken from the Bayesian analysis, and the proportion of invariant sites was directly taken from the data set. The alignments were also used to create data subsets for bootstrap analysis to test the strength of the tree topology (100 re-samplings),[16] which were analyzed using the amino acid substitution model producing the most likely tree.

Quantitative PCR

Prior to qPCR analysis, sample concentration and purity (260/280 absorbance ratio) was tested using 1.5µL of DNA extract by spectrophotometry (NanoDrop™ 8000[c]). Specific primers and probes (forward primer, DeHV-2 MGB 50F [5′-GGG AAA TCT GAA GTT AAA CGC TCT A-3′]; reverse primer, DeHV-2 MGB 113R [5′-TGA GAA CTC GCC AGT ATT TGC A-3′]; probe, DeHV-2 76T [6 FAM-CGT TGG CCA TCG GT-MGBNFQ]; forward primer, DeHV-8 MGB 69F [5'-TTA GCA CGC GCG ATT ACC T-3']; reverse primer, DeHV-8 MGB 143R [5'-CGC ACA CGT TCG CAA AGT-3']; probe, DeHV-8 104T [6 FAM-ACC CGT GAA CAG CTG-MGBNFQ]) targeting the Dpol gene were designed for *Delphinid herpesvirus 2* and *8* via commercial software (Primer Express® software v2.0) and synthesized.[g] A 0.9µM working dilution was used for the primers and 0.25µM for the probe in each 20µL reaction using a commercial universal qPCR mix (TaqMan® Fast Universal PCR Master Mix 2X).[g] The maximum amount of DNA template equaled 7uL or 100ng per well. A 7500 Fast Real-Time PCR System[g] was used to amplify the reactions with cycling conditions as follows: initial denaturation at 95°C for 20 s; 50 cycles of 95°C for 3 s followed by 60°C for 30 s. Data

collection was at the annealing/extension phase where the fluorescence crosses the cycle threshold line (C_t), set at 0.3.

Analytical sensitivity of the qPCR assay was tested using a 10-fold serial dilution of cDNA, nested PCR product from the case dolphin. For DeHV-2, the 10-fold serial dilution started at 2 million copies ($1:10^5$ dilutions) and continued to 2 copies ($1:10^{11}$). For DeHV-8, the 10-fold serial dilution started at 2 million copies ($1:10^5$ dilutions) and continued to 20 copies ($1:10^{10}$) followed by two 2-fold dilutions to 5 copies. The virions per μL ratio (viral load) was calculated by multiplying the sample concentration by Avogadro's number and dividing this total by the total of the amplicon length (224bp and 172bp for DeHV-2 and DeHV-8, respectively) multiplied by the average base pair weight ($656.6x10^9$). The resulting mean C_t values for the serial dilutions were used to generate the standard curve.

Results

Consensus PCR

Herpesviral DNA was detected in two samples, labeled Tt0625 and Tt0724, using the consensus PCR assay (Table 2-1). Protein BLAST (BLASTp) analysis of resulting sequence from Tt0625 showed a 100% amino acid identity to DeHV-2 (GenBank accession number AF245443). Sample Tt0724 contained a herpesviral sequence most closely related to DeHV-3 (AY757301) with an 87% amino acid identity. Since the Dpol region is highly conserved, this sequence likely represents a new bottlenose dolphin herpesvirus and is tentatively named DeHV-8. The partial Dpol sequence of DeHV-8 was submitted to GenBank (accession number bankit1249098). The remaining 14 case study samples were negative on consensus PCR (Table 2-2).

Genetically distinct herpesviral sequences were detected in the harbor seal, orca, and California sea lion samples. The translated amino acid sequence from the harbor seal sample was

61% identical to a lymphocryptovirus of a golden-handed tamarin (*Saguinas midas*; GenBank accession number AY166692). The orca sample had an amino acid identity of 87% to DeHV-1 (AF196646). BLASTp analysis of the California sea lion sequence showed a 70% identity to *Mustelid herpesvirus 1* (MusHV-1; AF275657). The partial Dpol sequence of PhHV-2 was also most similar to MusHV-1 with a 72% amino acid identity. These novel DPol herpesviral sequences have been submitted to GenBank under the following accession numbers: *Delphinid HV 8* (DeHV-8, from *Tursiops truncatus*; bankit1249098), DeHV-9 (from *Orcinus orca*; bankit1249103), *Otariid HV 2* (OtHV-2, from *Zalophus californianus*, bankit1248734), *Phocid HV 2* (PhHV-2, bankit1249104), and PhHV-5 (from *Phoca vitulina*; bankit1249107).

Bayesian and Maximum Likelihood Phylogenetic Analysis

The MUSCLE alignments of the 19 alphaherpesviral and 20 gammaherpesviral Dpol sequences are shown in Table 2-3 and 2-4, respectively. Bayesian analysis concluded the Wag model of amino acid substitution was most probable with a posterior probability of 0.930 for the alphaherpesvirus alignment. For the gammaherpesvirus alignment, the Jones model was most probable with a posterior probability of 0.768 followed by the Wag model with a posterior probability of 0.232. ML analysis showed the most likely tree, for both alignments, was from the JTT model of amino acid substitution and these parameters were used in the bootstrap analysis.

The Bayesian tree of the alphaherpesvirus MUSCLE alignment is shown in Figure 2-1 along with the bootstrap values from ML analysis. The delphinid alphaherpesviruses were genetically diverse and formed three distinct phylogenetic clades. DeHV-1 and DeHV-7 clustered as a monophyletic group with DeHV-9, the orca herpesvirus. Additionally, DeHV-3 and DeHV-8 clustered together. DeHV-2 formed a monotypic clade and did not cluster with any of the other alphaherpesvirus used in the analysis. None of the delphinid alphaherpesviruses clearly associated with an established alphaherpesviral genus.

The Bayesian tree of the gammaherpesvirus MUSCLE alignment is shown in Figure 2-2 along with the bootstrap values from ML analysis. All of the cetacean gammaherpesviruses formed a monophyletic group. Within the cetacean gammaherpesvirus clade, ML bootstrap values were weak thus the branching order could not be reliably determined. None of the delphinid gammaherpesviruses clustered with a species in an established gammaherpesvirus genus.

The novel California sea lion herpesvirus (OtHV-2) and three of the phocid herpesviruses (PhHV-2, PhHV-3, and PhHV-4) formed two distinct phylogenetic clades (Figure 2-2). OtHV-1 and PhHV-5 each formed a distinct monotypic clade. None of the pinniped gammaherpesviruses clustered with an established gammaherpesviral genus. The analysis also shows that EHV-7 clusters with the other Equid herpesviruses in the genus *Percavirus*. The ML bootstrap values, however, were weak for this cluster so the confidence interval for branch order within this cluster is low.

Quantitative PCR

The DeHV-2 qPCR assay accurately detected 2 to 2 million virus copies (see Figure 2-3). To reduce false positives, the last serial dilution, 2 copies, was discarded and the detection limit was conservatively set at 10 copies (C_t = 35.90). The standard curve (see Figure 2-4) for the DeHV-2 qPCR assay had a slope of -3.30 and a high correlation coefficient (R^2) of 0.994.

The DeHV-8 qPCR assay accurately detected 5 to 2 million virus copies (see Figure 2-5). Again, the last serial dilution, 5 copies, was discarded and the detection limit was conservatively set at 10 copies (C_t = 36.67). The standard curve (see Figure 2-6) for the DeHV-8 qPCR assay had a slope of -3.1 and a R^2 of 0.990.

Discussion

Two bottlenose dolphin herpesviruses, one of which is a novel alphaherpesviruses, were identified by consensus and specific PCR in the case dolphin. Additionally, three novel herpesviruses (DeHV-8, DeHV-9, OtHV-2) were identified and one novel Dpol herpesviral sequence was generated from the PhHV-2 control isolate by consensus PCR. Current ICTV (International Committee on Taxonomy of Viruses) naming conventions for herpesviruses use host family and order of virus discovery.[9,10] Each of these novel herpesviruses (*Delphinid herpesvirus 8*, *Delphinid herpesvirus 9*, *Otariid herpesvirus 2*, and *Phocid herpesvirus 5*) was tentatively named in accordance with these guidelines. Bayesian and ML phylogenic analysis supports the classification of the isolates as novel alpha- and gammaherpesviruses. It is unclear, based on the limited Dpol sequences, whether the delphinid and pinniped herpesviruses belong to established genera or represent novel genera. More viral genomic sequence would be needed to further clarify their phylogenetic placement.

The DeHV-2 and DeHV-8 qPCR assays should prove useful as primary screening tools for these alphaherpesviruses in blood samples from bottlenose dolphins. The qPCR assays are faster and have a higher throughput than traditional PCR. The DeHV-2 qPCR assay has a greater amplification intensity and a better slope compared to the DeHV-8 qPCR assay. Nevertheless, both qPCR assays are very sensitive with a lower detection limit of 10 virus copies.

Table 2-1. Proposed names, abbreviations, and sources for marine mammal Dpol herpesviral sequences used in this study

Name	Abbreviation	Species	Reference	GenBank accession number
Delphinid HV 1	DeHV- 1	*Tursiops truncatus*	Blanchard et al (2001)	AF196646
Delphinid HV 2	DeHV- 2	*Tursiops truncatus*	Blanchard et al (2001)	AF245443
Delphinid HV 3	DeHV- 3	*Tursiops truncatus*	Manire et al (2006)	AY757301
Delphinid HV 4	DeHV- 4	*Tursiops truncatus*	Smolarek Benson et al (2006)	AY952777
Delphinid HV 5	DeHV- 5	*Tursiops truncatus*	Smolarek Benson et al (2006)	AY952776
Delphinid HV 6	DeHV- 6	*Grampus griseus*	Smolarek Benson et al (2006)	DQ288666
Delphinid HV 7	DeHV- 7	*Tursiops truncatus*	Smolarek Benson et al (2006)	AY608707
Delphinid HV 8	DeHV- 8	*Tursiops truncatus*	This study	bankit1249098
Delphinid HV 9	DeHV- 9	*Orcinus orca*	This study	bankit1249103
Kogiid HV 1	KoHV-1	*Kogia sima*	Smolarek Benson et al (2006)	AY949830
Otariid HV 1	OtHV-1	*Zalophus californianus*	King et al (2002)	AF236050
Otariid HV 2	OtHV-2	*Zalophus californianus*	This study	bankit1248734
Phocid HV 1	PhHV-1	*Phoca vitulina*	King et al (1998)	U92269
Phocid HV 2	PhHV-2	*Phoca vitulina*	This study	bankit1249104
Phocid HV 3	PhHV-3	*Monachus schauinslandi*	Goldstein, Gulland, et al (2006)	DQ093191
Phocid HV 4	PhHV-4	*Mirounga angustirostris*	Goldstein, Lowenstine, et al (2006)	DQ183057
Phocid HV 5	PhHV-5	*Phoca vitulina*	This study	bankit1249107
Ziphid HV 1	ZiHV-1	*Mesoplodon densirostris*	Smolarek Benson et al (2006)	AY949828

Table 2-2. Sampling data and consensus herpesvirus PCR results for case study samples

Sample ID	Collection Date	Consensus PCR
Tt0625	11/8/2006	Positive
Tt0631	12/13/2006	Negative
Tt0634	12/20/2006	Negative
Tt0701	11/22/2006	Negative
Tt0718	10/20/2005	Negative
Tt0719	8/17/2006	Negative
Tt0720	10/25/2006	Negative
Tt0721	1/10/2007	Negative
Tt0724	2/7/2007	Positive
Tt0730	2/21/2007	Negative
Tt0748	2/28/2007	Negative
Tt0777	6/26/2007	Negative
Tt0804	12/17/2007	Negative
Tt0901	7/12/2004	Negative
Tt0902	8/2/2004	Negative
Tt0903	9/22/2006	Negative

Table 2-3. MUSCLE alignment of 19 alphaherpesvirus Dpol amino acid sequences.*

```
IgHV2       AHGYLPCLSIAASITSIGRTMLLKTRDFIHTSWATRENLCSSVSTLPLETVGPD-----YSMKV
GaHV1       MHGMLPCLEVASTVTAIGRDMLLRTKAHIEKEWRSGNQFAEKFLPGSERIQLNQ-----YSVRV
PsHV1       MNGMLPCLEVAATVTAIGRDMLLKTKQYIEENWREYSNIRERFFPAMAHEGVPQ-----YSVAV
GaHV2       SNGLLPCIDVAATVTTIGRNMLLTVRDYIHKQWGTRDALLREFPNLSNFMRPED-----YSVSV
MeHV1       ANGMLPCIDVAASVTTIGRNMLLTVRDYIHDQWGDKSSIMCKFPELENFMQNKE-----YSVDV
HHV3        AQGFLPCLYVAATVTTIGRQMLLSTRDYIHNNWAAFERFITAFPDIESSVLSQK----AYEVKV
DeHV2       AHGLLPCLPVAATVTTIGRDMLLRTRQYLHDRWPTVERLTSDFPEVVSMFIPNT----EYSIRV
PhHV1       SNGLLPCLHIAATVTTIGRSMLLATQSYVESRWATRELLEKDFPGSSSIAIPKK----SYSVNI
BHV2        QRGLLPCLPVAATVTTIGRDMLLATRDYVHSRWVSFDGLVMDFPEAAAIRGEGE-----YSMRI
HHV1        QHGLLPCLHVAATVTTIGREMLLATREYVHARWAAFEQLLADFPEAADMRAPGP-----YSMRI
HHV2        QHGLLPCLHVAATVTTIGRDMLLATRAYVHARWAEFDQLLADFPEAAGMRAPGP-----YSMRI
DeHV9       AQGLLPCLHIAATVTTIGRDMLLQTRDYLHTHWATAERLVEDFDGAAAALLTAP-SAPPYSIHV
DeHV1       AHGLLPCLQIAATVTTIGRDMLLRTRDYLHAHWATAERLVADFDGAAAALLASP-PAPPYSIHV
DeHV7       AQGLLPCLQIAATVTTIGRDMLLQTRDYLHARWATAERLVADFDGAAAALLSSP-PAPPYSIHV
DeHV3       AQGLLPCLPIAATVTTIGRDMLLSTRDYLHSRWATREQLAADFGDAYASPAPISPSASPYSIRV
DeHV8       AQGLLPCLPIAATVTTIGRDMLLSTRDYLHSRWATREQLVADFANVCASPAPGPPSASLYSIRV
EHV3        -NGLLPCLRIAATVTTIGRDMLLGTRDYVHARWATRELLEANFPEARAHRADGP-----YSVRV
EHV1        ANGLLPCLRIAATVTTIGRDMLLKTRDYVHSRWATRELLEDNFPGAIGFRNHKP-----YSVRV
EHV9        ANGLLPCLRIAATVTTIGRDMLLKTRDYVHSRWATRELLEDNFPGATAFRNHKP-----YSVRV
```

* Sequences retrieved from GenBank include: *Bovine HV 2* (BHV-2; AF181249), *Delphinid HV 1* (DeHV-1; AF196646), DeHV-2 (AF245443), DeHV-3 (AY757301), DeHV-7 (AY608707), DeHV-8 (bankit1249098), DeHV-9 (*Orcinus orca*; bankit1249103), *Equid HV 1* (EHV-1; NC_001491), EHV-3 (AF514779), EHV-9 (NC_011644), *Gallid HV 1* (GaHV-1; AF168792), GaHV-2 (NC_002229), *Human HV 1* (HHV-1; X14112), HHV-2 (Z86099), HHV-3 (AB059831), *Iguanid HV 2* (IgHV-2; AY236869), *Meleagrid HV 2* (MeHV-1; NC_002641), *Phocid HV 1* (PhHV-1; U92269), and *Psittacid HV 1* (PsHV-1; NC_005264). IgHV-2 is shown first as the data outgroup.

Table 2-4. MUSCLE alignment of 20 gammaherpesvirus Dpol amino acid sequences.*

```
ElHV1       SKGMFPCLAIAESVTAQGRQLLAVTKQYICDRFNDWTFLTQIA-PE--LVDCPVDSNRFKIDV
PhHV3       SGGMFPCVKIAETVTLQGRTMLDRSKHFI-EQLSAETLEAVTG-KT--I-QRENDAS-FKV--
PhHV4       SGGMFPCVKIAETVTLQGRTMLDRSKHFI-EQLSAETLEAATG-KT--I-QREDDAC-FK---
OvHV2       ASGMLPCLMIAETVTLQGRTMLEKTKQFV-ENLDVQSLQQICPTQTLKIHAQHPTPR-FTV--
HHV4        ANGLFPCLSIAETVTLQGRTMLERAKAFV-EALSPANLQALAP-SPDAWAPLNPEGQ-LRV--
PhHV5       SSGLLPCIKIAETITLQGRTMLEKSKLFI-ENLSFMEVQNLVP-SY--E-LQTGNGN-FRI--
OtHV1       ATGIMPCLKIAETVTLQGRTMLEQSRQFI-EAIGVDDVSTLMK-QR--V-VAAPTAR-LHV--
TrHV1       SSGLLPCIKIAETVTFQGRAMLEKSKRFV-EAITPERLRELVP-EP--F-SHEPGAR-FQ---
KoHV1       ASGLLPCLKIAETVTSQGRCMLERSKKFI-EAINHSKLEELIG-RA--VPDTCENAD-FRV--
ZiHV1       ASGLMPCLKIAETVTLQGRCMLERSKKFI-EAIDYRRLEELIG-QT--VTDADENSE-FKV--
DeHV5       ASGLLPCLKIAETVTLQGRRMLERSKKFI-EAIDRRKLEELVG-HV--VAGADGDAE-FKV--
DeHV6       ASGLMPCLKIAETVTLQGRCMLERSKMFI-EAINHRRLEELIG-HA--VADADRDAE-FKV--
DeHV4       ASGLLPCLKIAETVTLQGRRMLERSKRFI-EAINHRRLEELIG-HA--VAGADGNAE-FRV--
BHV4        ASGILPCIPIAETVTLQGRTMLEKSKAFV-EMITPERLSDIVS-YP--V-PCDPDAS-FRV--
HHV8        ASGILPCLNIAETVTLQGRKMLERSQAFV-EAISPERLAGLLR-RP--I-DVSPDAR-FKV--
EHV2        ASGILPCLKIAETVTFQGRRMLENSKRYI-EGVTPEGLADILG-RR--V-ECAPDAS-FKV--
EHV5        ASGILPCLKIAETVTFQGRRMLERSKRYI-EAVTPEGLAAILQ-RP--VAACDPEAS-FKV--
EHV7        ASGILPCLKIAETVTFQGRRMLERSKRYI-EAVTPEGLAAILH-RP--V-ACAPDAS-FKV--
PhHV2       ASGILPCLKIAETVTFEGRRMLDRSKKFI-EDISPVDLERLLC-RP--I-TCAPDAN-FRV--
OtHV2       SSGILPCLKIAETVTFEGRRMLERSKKFI-EDISPLDLERLLS-RP--V-VCSEDAN-FRV—
```

* Sequences retrieved from GenBank include: *Bovine HV 4* (BHV-4; AF318573), *Delphinid HV 4* (DeHV-4; AY952777), DeHV-5 (AY952776), DeHV-6 (*Grampus griseus*; DQ288666), *Elephantid HV 1* (ElHV-1; AF322977), *Equid HV 2* (EHV-2; U20824), EHV-5 (AF141886), EHV-7 (EU165547), *Human HV 4* (HHV-4; DQ279927), HHV-8 (U93872), *Kogiid HV 1* (KoHV-1; AY949830), *Otariid HV 1* (OtHV-1; AF236050), OtHV-2 (bankit1248734), *Ovine HV 2* (OvHV-2; DQ198083), *Phocid HV 2* (PhHV-2; bankit1249104), PhHV-3 (DQ093191), PhHV-4 (DQ183057); PhHV-5 (bankit1249107), *Trichechid HV 1* (TrHV-1; DQ238847), and *Ziphid HV 1* (ZiHV-1; AY949828). ElHV-1 (GenBank accession number AF322977) is shown first as the data outgroup.

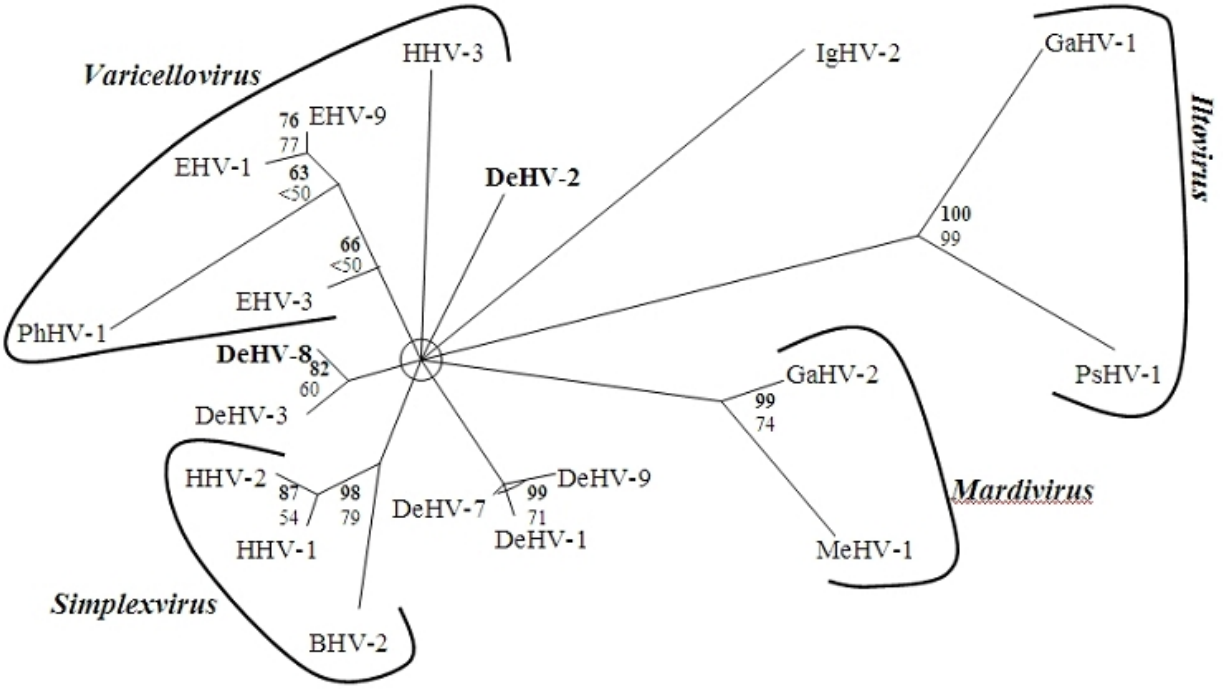

Figure 2-1. Bayesian phylogenetic tree of the 19 predicted partial 58–64 amino acid alphaherpesviral DNA-dependent DNA polymerase sequences based on MUSCLE alignment. Bayesian posterior probabilities of branchings as percentages are in bold and ML bootstrap values for branching, based on 100 re-samplings, are given below them. Iguanid HV 2 (GenBank accession number AY236869) was used as the outgroup. Herpesvirus genera are delineated brackets. Areas of multifurcation are marked by arcs. Bottlenose dolphin herpesviruses used in quantitative PCR assays are bolded. Sequences retrieved from GenBank include: Bovine HV 2 (BHV-2; AF181249), Delphinid HV 1 (DeHV-1; AF196646), DeHV-2 (AF245443), DeHV-3 (AY757301), DeHV-7 (AY608707), DeHV-8 (bankit1249098), DeHV-9 (Orcinus orca; bankit1249103), Equid HV 1 (EHV-1; NC_001491), EHV-3 (AF514779), EHV-9 (NC_011644), Gallid HV 1 (GaHV-1; AF168792), GaHV-2 (NC_002229), Human HV 1 (HHV-1; X14112), HHV-2 (Z86099), HHV-3 (AB059831), Iguanid HV 2 (IgHV-2; AY236869), Meleagrid HV 2 (MeHV-1; NC_002641), Phocid HV 1 (PhHV-1; U92269), and Psittacid HV 1 (PsHV-1; NC_005264).

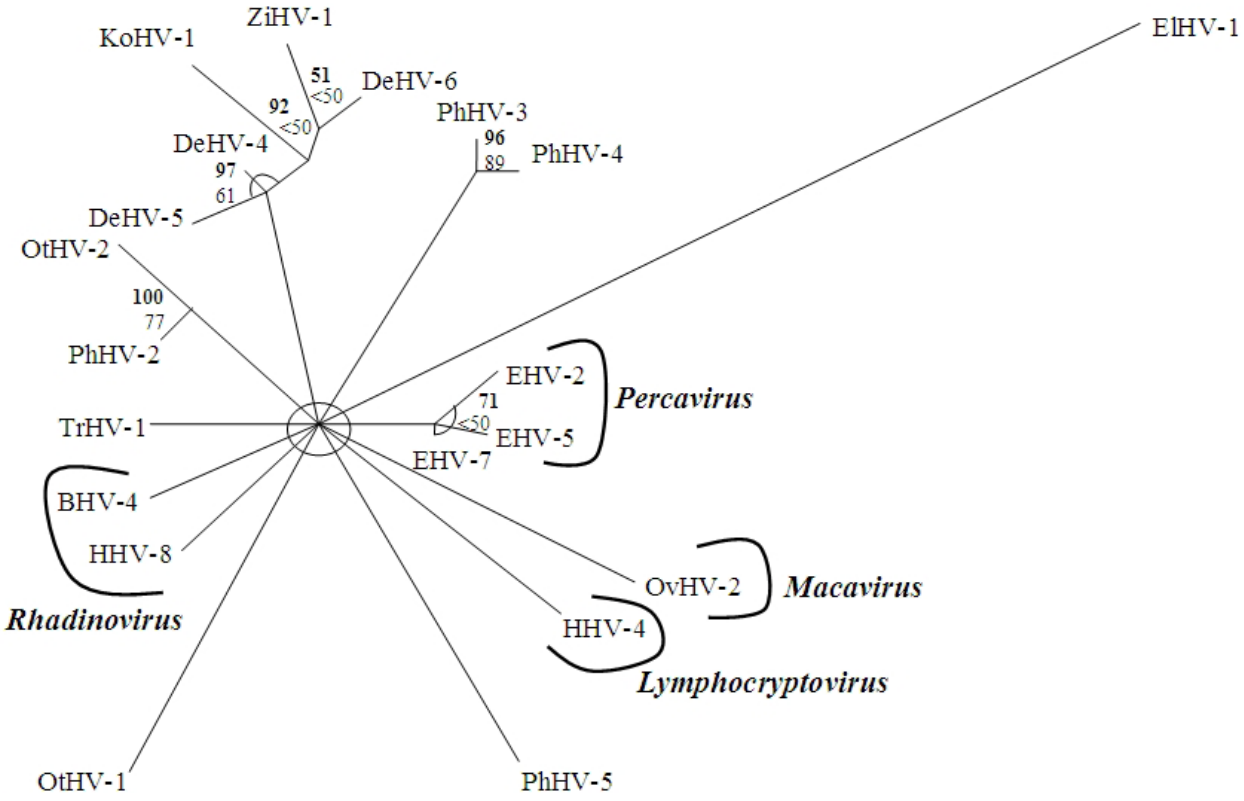

Figure 2-2. Bayesian phylogenetic tree of the 20 predicted partial 55–60 amino acid gammaherpesviral DNA-dependent DNA polymerase sequences based on MUSCLE alignment. Bayesian posterior probabilities of branchings as percentages are in bold and ML bootstrap values for branching, based on 100 re-samplings, are given below them. Elephantid HV 1 (GenBank accession number AF322977) was used as the outgroup. Herpesvirus genera are delineated brackets. Areas of multifurcation are marked by arcs. Sequences retrieved from GenBank include: Bovine HV 4 (BHV-4; AF318573), Delphinid HV 4 (DeHV-4; AY952777), DeHV-5 (AY952776), DeHV-6 (Grampus griseus; DQ288666), Elephantid HV 1 (ElHV-1; AF322977), Equid HV 2 (EHV-2; U20824), EHV-5 (AF141886), EHV-7 (EU165547), Human HV 4 (HHV-4; DQ279927), HHV-8 (U93872), Kogiid HV 1 (KoHV-1; AY949830), Otariid HV 1 (OtHV-1; AF236050), OtHV-2 (bankit1248734), Ovine HV 2 (OvHV-2; DQ198083), Phocid HV 2 (PhHV-2; bankit1249104), PhHV-3 (DQ093191), PhHV-4 (DQ183057); PhHV-5 (bankit1249107), Trichechid HV 1 (TrHV-1; DQ238847), and Ziphid HV 1 (ZiHV-1; AY949828).

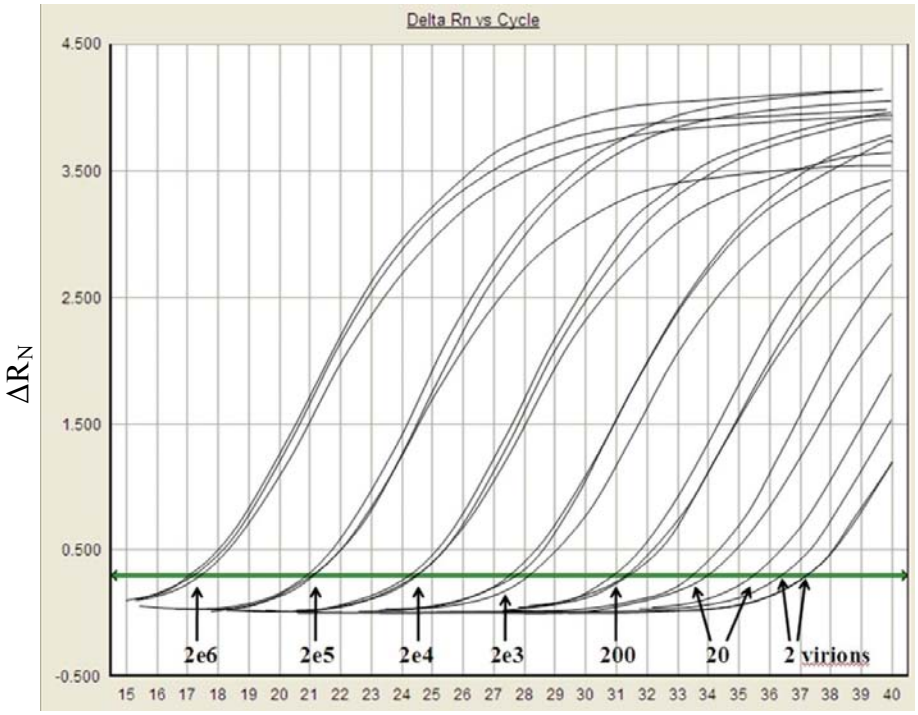

Figure 2-3. DeHV-2 10-fold serial dilutions in triplicate. Cycle threshold line set at 0.3.

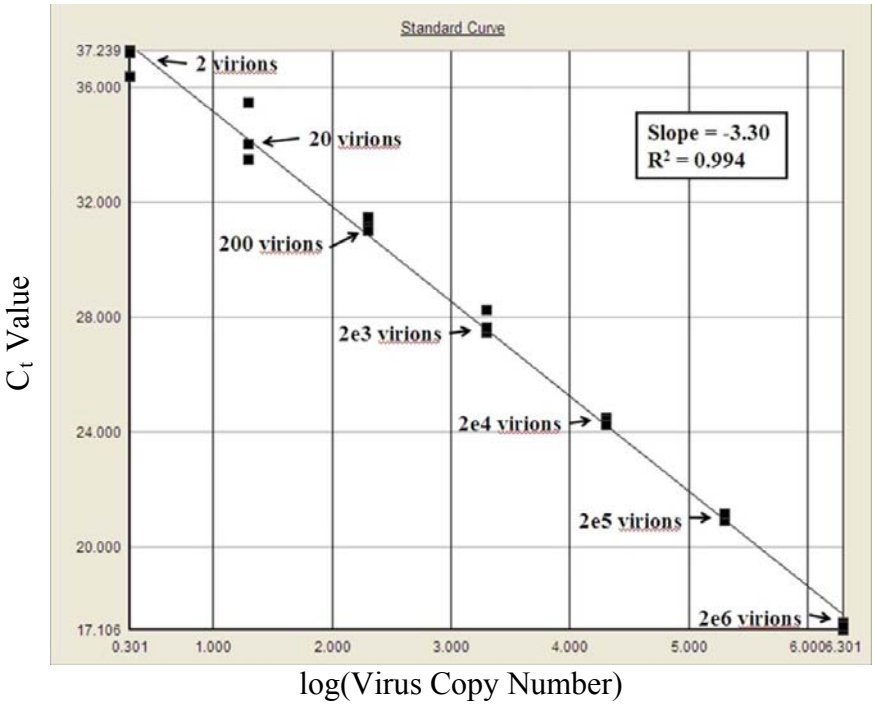

Figure 2-4. DeHV-2 standard curve in triplicate. Cycle threshold line set at 0.3.

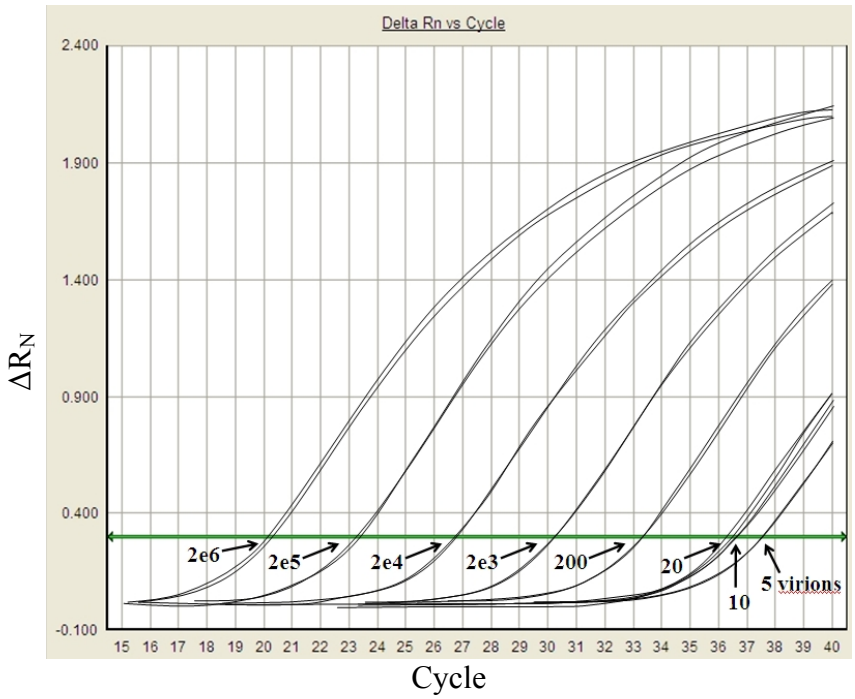

Figure 2-5. DeHV-8 10-fold serial dilutions in duplicate. Cycle threshold line set at 0.3.

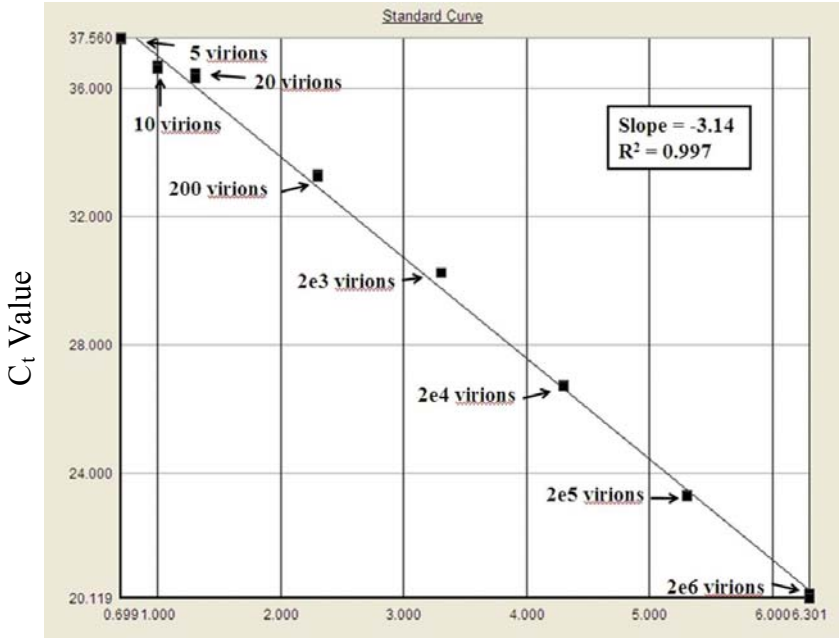

Figure 2-6. DeHV-8 standard curve in duplicate. Cycle threshold line set at 0.3.

CHAPTER 3
USING QUANTITATIVE PCR TO INVESTIGATE CORRELATIONS BETWEEN TWO BOTTLENOSE DOLPHIN HERPESVIRUSES AND HEALTH PARAMETERS

Introduction

Herpesvirus infections are being reported with increased frequency in cetaceans. Currently, four alphaherpesviruses and two gammaherpesvirus have been reported in bottlenose dolphins. Alphaherpesvirus particles have been identified by electron microscopy (EM) and polymerase chain reaction (PCR) in skin lesions of bottlenose dolphins.[2,46] An alphaherpesvirus was also identified, by EM and immunoperoxidase staining, in cerebral cortex tissues of a harbor porpoise with encephalitis.[25]

Herpesviruses must be lytically active to cause disease.[37] While herpesviruses can be actively proliferating in some cells and latent in others, clinical signs are usually only apparent when the virus is lytically active in the majority of cells.[37] The amount of viral DNA circulating in the white blood cells correlates with the level of virus replication at the site of infection. Thus, an alphaherpesvirus is more active and clinical signs can be expected when a greater number of viral particles are in the bloodstream.

During the routine, conventional PCR-based, viral surveillance of a managed collection of bottlenose dolphins, two alphaherpesviruses (tentatively named DeHV-2 and DeHV-8) were detected in the buffy coat of one animal. The 22-year-old, female bottlenose dolphin had a history of elevated transaminases and dehydrogenases, which were normalized following phlebotomy treatment, and a persistent lymphocytosis. Furthermore, the dolphin did not have any skin lesions, unlike the majority of the previously reported cases of herpesvirus infections in cetaceans. Thus, the clinical significance of this finding was unclear. Alphaherpesviruses are generally considered to persist in the sensory nerve ganglion[3,11,21,41] thus the presence in circulating blood cells was presumed to be indicative of infection with active virus replication.

However, it has also been suggested that in some hosts, such as the horse, alphaherpesviruses can persist in the white cell fraction.[6,44,55] Regardless of the site of persistence and/or replication, changes in the virus load in the circulating white blood cells, as measured using a quantitative PCR assay, reflect the level of replication of the herpesvirus. This virus load, or virus copy number per sample, cannot be assessed using conventional PCR.

Real-time quantitative PCR (qPCR) assays have been applied in diagnosing or monitoring herpesviral infections including the human herpesviruses[22,27,36,48,54] and *Equid herpesvirus 1* and *4*[12] (EHV-1 and 4). Quantitative PCR has a faster turn-around time and has a higher throughput than traditional PCR.[27,48] The TaqMan qPCR method measures the quantity of PCR products with a specific fluorogenic probe, specific primers, and real-time laser scanning in a 96-well plate.[27,36] When these quantities are compared to known standards, the viral load in each sample can be determined. Therefore, TaqMan qPCR assays were developed to measure the DeHV-2 and DeHV-8 viral load in the case dolphin. The complete blood counts (CBC) and serum chemistry parameters for the herpesvirus positive samples in the case dolphin were compared to those of the negative samples, in an attempt to correlate viral presence with changes in clinical blood parameters. These assays were also used in a cross-sectional survey to establish a baseline prevalence of the two alphaherpesviruses in a population of clinically healthy bottlenose dolphins.

Materials and Methods

Sample Collection

Sixteen serial blood samples were collected between July 12, 2004 and December 17, 2007 from the case dolphin, a 22-year-old (at the start of the study) female bottlenose dolphin with persistent lymphocytosis. As part of a cross-sectional survey, an additional 55 blood samples, collected from 55 clinically healthy bottlenose dolphins, were collected randomly

between July 2nd and September 25, 2007. Daily food consumption and behavioral abnormalities were noted for each animal. Blood was collected into one Vacutainer® serum separator tube (SST) and two Vacutainer® EDTA (K_3) tube for serum chemistries, complete blood counts (CBC) and buffy coat collection, respectively.

Serum Chemistry and CBC

The SST samples for serum chemistry analysis were centrifuged within two hours of collection. Centrifugation was performed at 3,000 rpm at 21°C for 10 minutes. Fibrin clots were removed and serum was transferred to a 5 ml plastic submission tube. One EDTA Vacutainer® tube containing whole blood was shipped on ice via courier to Quest Diagnostic Laboratories in San Diego, California. Automated CBC analyses were conducted by the reference laboratory with the Coulter® LH 1500 Series.[h] The Fisherbrand Dispette 2®,[c], correlating with the Westergren method, was used to determine sixty minute erythrocyte sedimentation rate (ESR) from 1ml EDTA whole blood.

Quantitative PCR

For qPCR analysis, 16 buffy coat samples from the case dolphin and 55 samples from the clinically healthy animals were extracted, as described in Chapter 2. All samples were run in triplicate and a mean C_t value was calculated. Eukaryotic 18S rRNA (20X)[g] was used as a positive control for each sample in a separate well. Each specific qPCR assay was performed as described in Chapter 2. Using a standard curve and a reference sample on each plate for comparison, a C_t value between 1 and 35.90 was considered positive for DeHV-2 and between 1 and 36.67 for DeHV-8. Alternatively, a C_t value greater than or equal to 35.90, and 36.67 for the DeHV-8 qPCR assay, as well as samples that did not yield a detectable level of fluorescence were considered negative.

Specific PCR

Specific DeHV-8 primers were designed (forward primer, DeHV8-F2b [5'- TTG CTC CCG TGT CTG CCG AT-3']; reverse primer, DeHV8-R2b [5'- AGT AGA GAG ACG CGG AAG GAG G-3']) based on the Dpol herpesviral sequence detected in the case dolphin via consensus PCR. A Px2 thermal cycler was used to amplify 50µL reactions using Platinum® *Taq* DNA Polymerase and following manufacturer's instructions. DeHV-8 specific PCR amplification conditions were as follows: initial denaturation at 94°C for 5 min; 45 cycles of amplification with each cycle consisting of denaturation at 94°C for 30 s, annealing at 65°C for 1 min, and elongation at 72°C for 1 min; a final elongation step was performed at 72°C for 10 min followed by a 4°C hold. The amplification process was repeated in a second 50µL reaction PCR. The final PCR products were resolved on a 1.5% agarose gel made up in 1X TBE and containing 1µg of ethidium bromide per mL. The DeHV-8 assay amplifies a 172bp band. Bands of expected size were excised and prepared for direct sequencing as described in Chapter 2. The specific primers were used in the sequencing reactions.

Statistical Analysis

Sixteen parameters for the CBC, including ESR, and 25 parameters for the serum chemistry, were analyzed for each sample. Data were analyzed by use of computer software.[i] Analyses of variance (ANOVAs) were conducted to compare mean clinicopathologic values of DeHV-2 positive and DeHV-2 negative samples. DeHV-2 case animal positives were also compared to clinicpathologic values of the three most recent blood draws preceding and the three immediately following the DeHV-2 qPCR positive. Significance for all analysis was set at $P \leq 0.05$.

Results

Quantitative PCR

DNA concentration and purity data for all samples is shown in Tables 3-1 – 3. Five of the 16 case animal samples (31.2%) were DeHV-2 qPCR positive (see Table 3-4). For the cross-sectional survey, 2 out of 54 samples (3.7%) were DeHV-2 qPCR positive (see Table 3-5). One of the cross-sectional survey samples was excluded from the analysis because the eukaryotic 18S reaction control was negative. Only one case animal sample (6.3%) was positive using the DeHV-8 qPCR assay (see Table 3-6). Additionally, there were no DeHV-8 qPCR positive cross-sectional survey samples (see Table 3-7).

Specific PCR

The amino acid sequence of sample Tt0721 was 100% identical to the DeHV-8 amino acid sequence (from sample Tt0724 using consensus PCR primers) with the specific DeHV-8 primers.

Statistical analysis

The case animal, a female bottlenose dolphin, had a mean age of 24.2 during the study (range 22.1 – 25.0). There were no significant differences when comparing mean CBC and serum chemistry values between DeHV-2 qPCR positive and DeHV-2 qPCR negative samples. For samples with detectable DeHV-2, the 41 CBC and serum chemistry parameters were plotted with the animal's six most recent blood profiles spanning before and after the positive date. When assessing trend CBC and serum chemistry values on days surrounding one of the five positive samples (Tt0719), there was an associated decrease in overall WBC counts (see Figure 3-1) and an increase in erythrocyte sedimentation rate (ESR; see Figure 3-2) and hematocrit (HCT; see Figure 3-3). Also noteworthy was a decrease in serum chloride (see Figure 3-4). Similar trends were not identified surrounding any of the other four DeHV-2 qPCR positive

samples. The cross-sectional survey samples were composed of 23 females (42.6%) and 31 males. Animals' ages ranged from 0.1 to 50.3 years old with a mean of 23.7 years old. There were no significant differences between gender or age of the DeHV-2 qPCR positive and negative cross-sectional survey dolphins ($P = 0.1$ and 0.4, respectively). Only two animals in the cross-sectional survey were DeHV-2 qPCR positive so statistical analysis was not performed.

Discussion

Unlike DeHV-8, DeHV-2 was detected in the cross-sectional survey of the study population. Despite an identical calculated detection limit (10 virus copies), DeHV-2 was detected in two other dolphins whereas DeHV-8 was only detected in the case dolphin and then only in one sample. The DeHV-2 qPCR assay proved to be more sensitive than traditional consensus PCR (see Chapter 2) with three additional DeHV-2 qPCR positive case animal samples. Additionally, low DeHV-8 viral load is suggested by comparison of the DeHV-8 qPCR assay to the traditional PCR assays. With consensus PCR only one sample, Tt0724, was positive however with qPCR only sample Tt0721 was positive and that positive was confirmed with specific PCR. This suggests that even in our case dolphin active DeHV-8 viral load was minimal and virus replication infrequent. Differing levels of prevalence for distinct alphaherpesviruses in a population is common in other species. Seroprevalence of the three human alphaherpesvirus, human simplex virus 1, 2 and Varicella-Zoster, is 57.7%[58], 17%[58], and 99.6%[26], respectively, in adults in the United States. Thus, it is not unexpected to see varying prevalence between these two viruses.

Prevalence of DeHV-2 and 8 will likely differ between dolphin populations as well. A serological survey in China of *Bovine herpesvirus 1* (BHV-1) showed a nationwide seroprevalence of 35.8%, while the prevalence for individual provinces ranged from 12.1% to 77.8%.[60] This highlights the need for attaining baseline values for distinct populations. These

baseline prevalences are needed for the clinical interpretations of the detection of an alphaherpesvirus.

The simple presence of DeHV-2 could not be associated with any specific blood parameter changes in the case animal. One positive case animal sample (Tt0719), however, had noteworthy changes in four of the blood parameters analyzed (total WBC count, ESR, hematocrit, and serum chloride). However, these changes were only associated with one of the five DeHV-2 positive samples and they can therefore not be correlated with the increased circulating herpesviral load. Even if unrelated to DeHV-2 replication, the observed changes do suggest a clinical event because they were often significant differences and had values outside the normal reference ranges for the population.

The total WBC count for females between 10 and 30 years old in this population ranges from 5, 381 – 11,336 cells/µL.[53] While the case animal did not significantly exceed this range, her WBC count was about 4,000cells/µL higher 24 days prior and 11 days after the positive sample date. This is clearly a significant decrease in WBCs. The decrease in WBCs was coupled with a significant increase in ESR (normal range: 0-19mm/h)[53] to 45mm/h. An increase of that proportion is highly suggestive of inflammation.[28,39] An increase in the hematocrit (47%) was also noted in Sample Tt0719 which also exceeded the normal range of 38 – 46% for animals in this age group within the population.[53] The HCT increase suggests the animal was less hydrated at the time of sample collection than normal.[4] The fourth affected parameter was serum chloride. The normal range for the population is between 115 and 125mEq/L. Sample Tt0719 had a chloride level of only 113mEq/L and the animal's chloride level further dropped to 111mEq/L 11 days later before slowly returning to normal. The hypochloremia could be an indicator of

metabolic alkalosis, as associated with ileus in cattle and horses, or an effect of vomiting/diarrhea.[5]

Even if a true clinical disease process was present at the time of the sample collection, the association of clinical signs with active herpesviral proliferation cannot be confirmed. It remains unclear whether the disease process demanded an immunologic response that created an opportunity for latent DeHV-2 to proliferate, whether DeHV-2 proliferation was responsible for the subsequent abnormalities in the homeostasis of the body, or whether the two events are causally linked at all.

With only one DeHV-8 positive sample, statistical associations between DeHV-8 viral load and clinical signs could not be completed. Despite the more frequent detection of DeHV-2 in the study population, clinical symptoms could not be associated with DeHV-2 presence. Our data demonstrate that herpesviruses are common in bottlenose dolphins and one should be cautious in attributing clinical signs with herpesvirus detection as they are often present asymptomatically.

Table 3-1. Spectrophotometric analysis of DeHV-2 qPCR case study samples. DNA purity expressed as the absorbance at 260 nm:280 nm.

Sample ID	Collection Date	ng/µl	$\frac{260}{280}$
Tt0625	11/8/2006	13.05	1.67
Tt0631	12/13/2006	32.02	1.83
Tt0634	12/20/2006	93.57	1.97
Tt0701	11/22/2006	22.35	1.84
Tt0718	10/20/2005	0.70	0.80
Tt0719	8/17/2006	8.60	1.70
Tt0720	10/25/2006	11.45	1.63
Tt0721	1/10/2007	6.94	1.64
Tt0724	2/7/2007	102.07	1.99
Tt0730	2/21/2007	11.53	1.89
Tt0748	2/28/2007	9.11	1.62
Tt0777	6/26/2007	187.66	2.06
Tt0804	12/17/2007	119.01	1.98
Tt0901	7/12/2004	12.22	1.47
Tt0902	8/2/2004	11.81	1.50
Tt0903	9/22/2006	7.36	1.55

Table 3-2. Spectrophotometric analysis of DeHV-8 qPCR case study samples. DNA purity expressed as the absorbance at 260 nm:280 nm.

Sample ID	Collection Date	ng/µl	$\frac{260}{280}$
Tt0625	11/8/2006	20.08	1.73
Tt0631	12/13/2006	32.02	1.83
Tt0634	12/20/2006	93.57	1.97
Tt0701	11/22/2006	22.35	1.84
Tt0718	10/20/2005	0.70	0.80
Tt0719	8/17/2006	4.28	1.48
Tt0720	10/25/2006	11.45	1.63
Tt0721	1/10/2007	6.94	1.64
Tt0724	2/7/2007	260.54	1.99
Tt0730	2/21/2007	8.94	2.36
Tt0748	2/28/2007	9.11	1.62
Tt0777	6/26/2007	206.73	2.08
Tt0804	12/17/2007	119.01	1.98
Tt0901	7/12/2004	12.22	1.47
Tt0902	8/2/2004	11.81	1.50
Tt0903	9/22/2006	7.36	1.55

Table 3-3. Spectrophotometric analysis of the cross-sectional survey samples. DNA purity expressed as the absorbance at 260 nm:280 nm.

Sample ID	Collection Date	Media*	ng/µL	$\frac{260}{280}$
Tt0812	8/23/2007	EDTA	19.85	1.63
Tt0813	8/7/2007	EDTA	16.70	1.62
Tt0814	7/24/2007	EDTA	18.45	1.53
Tt0815	7/20/2007	EDTA	19.62	1.61
Tt0816	7/2/2007	EDTA	17.75	1.68
Tt0817	7/27/2007	EDTA	11.25	1.43
Tt0818	7/2/2007	EDTA	45.40	1.92
Tt0819	8/7/2007	EDTA	17.50	1.62
Tt0820	7/25/2007	EDTA	8.72	2.04
Tt0821	8/14/2007	EDTA	21.24	1.76
Tt0822	9/21/2007	EDTA	22.22	1.94
Tt0823	7/12/2007	EDTA	20.03	1.89
Tt0824	9/13/2007	EDTA	14.51	2.04
Tt0825	7/5/2007	EDTA	31.44	1.96
Tt0826	8/29/2007	EDTA	7.61	1.56
Tt0827	7/13/2007	EDTA	40.00	1.86
Tt0828	9/4/2007	EDTA	14.74	1.81
Tt0829	8/8/2007	EDTA	31.78	1.91
Tt0830	9/6/2007	EDTA	21.98	2.04
Tt0831	8/24/2007	EDTA	16.61	2.01
Tt0832	7/5/2007	EDTA	31.21	1.88
Tt0833	7/11/2007	EDTA	26.43	1.93
Tt0834	7/28/2007	EDTA	26.13	1.83
Tt0835	8/23/2007	EDTA	13.82	1.83
Tt0836	7/5/2007	EDTA	12.78	1.67
Tt0837	7/5/2007	EDTA	20.27	1.84
Tt0838	8/9/2007	EDTA	5.33	1.85
Tt0839	9/19/2007	EDTA	20.33	1.97
Tt0840	8/7/2007	EDTA	12.70	1.77
Tt0841	9/4/2007	EDTA	12.57	1.85
Tt0842	7/12/2007	EDTA	9.68	1.86
Tt0843	8/22/2007	EDTA	17.86	1.89
Tt0844	9/24/2007	EDTA	19.27	1.94
Tt0845	9/11/2007	EDTA	47.00	1.85
Tt0846	9/20/2007	EDTA	10.43	1.99
Tt0847	7/2/2007	EDTA	8.89	1.69
Tt0848	7/18/2007	EDTA	10.62	1.72
Tt0849	9/11/2007	EDTA	26.69	1.88
Tt0850	8/3/2007	EDTA	28.91	1.94

Table 3-3. Continued

Sample ID	Collection Date	Media*	ng/µL	$\frac{260}{280}$
Tt0851	8/30/2007	EDTA	29.44	1.85
Tt0852	7/2/2007	EDTA	13.78	1.98
Tt0853	9/13/2007	EDTA	6.40	1.74
Tt0854	7/5/2007	EDTA	25.46	1.79
Tt0855	7/16/2007	EDTA	25.03	2.04
Tt0856	8/10/2007	EDTA	13.20	1.97
Tt0857	8/21/2007	EDTA	40.88	1.91
Tt0858	9/25/2007	EDTA	20.53	1.86
Tt0859	9/12/2007	EDTA	11.89	2.01
Tt0860	7/31/2007	Na Hep	16.69	1.85
Tt0861	8/10/2007	EDTA	10.34	1.67
Tt0862	8/7/2007	EDTA	10.24	1.81
Tt0863	8/22/2007	EDTA	10.29	2.14
Tt0864	7/27/2007	EDTA	13.28	1.95
Tt0865	7/2/2007	EDTA	31.39	1.92
Tt0866	8/21/2007	EDTA	10.20	1.61

*Media refers to the anticoagulant used in the blood collection tubes.

Table 3-4. DeHV-2 qPCR case study data

Sample ID	Collection Date	ng DNA*	C_t Value†	Viral Presence‡
Tt0625	11/8/2006	91.35	34.9	+
Tt0631	12/13/2006	100.90	36.8	ND
Tt0634	12/20/2006	93.57	35.6	ND
Tt0701	11/22/2006	100.60	35.0	ND
Tt0718	10/20/2005	4.90	35.90	+
Tt0719	8/17/2006	60.20	33.3	+
Tt0720	10/25/2006	80.20	34.6	ND
Tt0721	1/10/2007	48.58	36.4	ND
Tt0724	2/7/2007	102.07	35.4	ND
Tt0730	2/21/2007	80.71	32.0	+
Tt0748	2/28/2007	63.77	36.5	ND
Tt0777	6/26/2007	187.66	33.4	ND
Tt0804	12/17/2007	119.01	33.4	ND
Tt0901	7/12/2004	85.54	36.0	ND
Tt0902	8/2/2004	82.67	32.4	+
Tt0903	9/22/2006	51.52	35.8	ND

*DNA equals the total DNA of the sample used in each well of the qPCR.
† C_t value for each of the three sample replicates was averaged to calculate the C_t value.
‡ + indicates positive viral load (the Ct value for the sample was before the 35.90 detection limit); ND = The Ct value was higher than the 35.90 detection limit and thus not detectable.

Table 3-5. DeHV-2 qPCR cross-sectional survey data

Sample ID	Collection Date	ng DNA*	C_t Value†	Viral Presence‡
Tt0812	8/23/2007	99.25	35.5	ND[|]
Tt0813	8/7/2007	100.20	ND	ND
Tt0814	7/24/2007	101.48	38.4	ND
Tt0815	7/20/2007	98.10	ND	ND
Tt0817	7/27/2007	78.75	ND	ND
Tt0818	7/2/2007	102.15	42.3	ND
Tt0819	8/7/2007	96.25	44.6	ND
Tt0820	7/25/2007	61.04	40.2	ND
Tt0821	8/14/2007	100.89	39.0	ND
Tt0822	9/21/2007	99.99	33.8	ND
Tt0823	7/12/2007	100.15	35.8	ND
Tt0824	9/13/2007	101.57	ND	ND
Tt0825	7/5/2007	94.32	33.4	ND
Tt0826	8/29/2007	53.27	ND	ND
Tt0827	7/13/2007	100.00	32.4	ND
Tt0828	9/4/2007	103.18	ND	ND
Tt0829	8/8/2007	103.29	33.2	ND
Tt0830	9/6/2007	98.91	35.90	ND
Tt0831	8/24/2007	99.66	37.0	ND
Tt0832	7/5/2007	101.43	34.8	ND
Tt0833	7/11/2007	99.11	33.0	ND
Tt0834	7/28/2007	97.99	38.8	ND
Tt0835	8/23/2007	96.74	ND	ND
Tt0836	7/5/2007	89.46	37.1	ND
Tt0837	7/5/2007	101.35	35.6	ND
Tt0838	8/9/2007	37.31	42.9	ND
Tt0839	9/19/2007	101.65	37.4	ND
Tt0840	8/7/2007	88.90	42.5	ND
Tt0841	9/4/2007	87.99	38.8	ND
Tt0842	7/12/2007	67.76	36.5	ND
Tt0843	8/22/2007	98.23	39.6	ND
Tt0844	9/24/2007	96.35	ND	ND
Tt0845	9/11/2007	94.00	32.4	ND
Tt0846	9/20/2007	73.01	ND	ND
Tt0847	7/2/2007	62.23	ND	ND
Tt0848	7/18/2007	74.34	ND	ND
Tt0849	9/11/2007	100.09	39.6	ND
Tt0850	8/3/2007	101.19	35.6	+
Tt0851	8/30/2007	103.04	36.0	+
Tt0852	7/2/2007	96.46	ND	ND

Table 3-5. Continued

Sample ID	Collection Date	ng DNA*	C_t Value†	Viral Presence‡
Tt0853	9/13/2007	44.80	ND	ND
Tt0854	7/5/2007	101.84	40.4	ND
Tt0855	7/16/2007	100.12	34.5	ND
Tt0856	8/10/2007	92.40	ND	ND
Tt0857	8/21/2007	102.20	33.0	ND
Tt0858	9/25/2007	102.65	40.2	ND
Tt0859	9/12/2007	83.23	ND	ND
Tt0860	7/31/2007	100.14	ND	ND
Tt0861	8/10/2007	72.38	ND	ND
Tt0862	8/7/2007	71.68	43.0	ND
Tt0863	8/22/2007	72.03	ND	ND
Tt0864	7/27/2007	92.96	36.4	ND
Tt0865	7/2/2007	102.02	ND	ND
Tt0866	8/21/2007	71.40	ND	ND

*ng DNA equals the total sample DNA used in each well of the qPCR.
† C_t for each of the three sample replicates was averaged to calculate the C_t value.
‡ + indicates positive viral load (the Ct value for the sample was before the 35.90 detection limit);
¦ND = C_t was not detected for any sample replicate or the C_t value was greater than the 35.90 (10 copies) detection limit.

Table 3-6. DeHV-8 qPCR case study data

Sample ID	Collection Date	ng DNA*	C_t Value†	Viral Presence‡
Tt0625	11/8/2006	104.00	ND¦	ND
Tt0631	12/13/2006	100.90	43.5	ND
Tt0634	12/20/2006	93.57	ND	ND
Tt0701	11/22/2006	100.60	39.7	ND
Tt0718	10/20/2005	4.90	38.6	ND
Tt0719	8/17/2006	29.96	ND	ND
Tt0720	10/25/2006	80.20	ND	ND
Tt0721	1/10/2007	48.58	36.5	+
Tt0724	2/7/2007	260.54	39.6	ND
Tt0730	2/21/2007	62.58	ND	ND
Tt0748	2/28/2007	63.77	ND	ND
Tt0777	6/26/2007	206.73	ND	ND
Tt0804	12/17/2007	119.01	ND	ND
Tt0901	7/12/2004	85.54	ND	ND
Tt0902	8/2/2004	82.67	ND	ND
Tt0903	9/22/2006	51.52	ND	ND

*ng DNA equals the total sample DNA used in each well of the qPCR.
† C_t for each of the three sample replicates was averaged to calculate the C_t value.
‡ + indicates positive viral load (the Ct value for the sample was before the 36.67 detection limit)
¦ND = C_t was not detected for any sample replicate or the C_t value was greater than the 36.67 (10 copies) detection limit.

Table 3-7. DeHV-8 qPCR cross-sectional survey data

Sample ID	Collection Date	ng DNA*	C_t Value†	Viral Presence‡
Tt0812	8/23/2007	99.25	ND§	ND
Tt0813	8/7/2007	100.20	ND	ND
Tt0814	7/24/2007	101.48	ND	ND
Tt0815	7/20/2007	98.10	ND	ND
Tt0817	7/27/2007	78.75	43.6	ND
Tt0818	7/2/2007	102.15	ND	ND
Tt0819	8/7/2007	96.25	ND	ND
Tt0820	7/25/2007	61.04	ND	ND
Tt0821	8/14/2007	100.89	ND	ND
Tt0822	9/21/2007	99.99	ND	ND
Tt0823	7/12/2007	100.15	ND	ND
Tt0824	9/13/2007	101.57	43.1	ND
Tt0825	7/5/2007	94.32	ND	ND
Tt0826	8/29/2007	53.27	44.6	ND
Tt0827	7/13/2007	100.00	ND	ND
Tt0828	9/4/2007	103.18	41.6	ND
Tt0829	8/8/2007	103.29	39.3	ND
Tt0830	9/6/2007	98.91	ND	ND
Tt0831	8/24/2007	99.66	39.7	ND
Tt0832	7/5/2007	101.43	ND	ND
Tt0833	7/11/2007	99.11	38.4	ND
Tt0834	7/28/2007	97.99	ND	ND
Tt0835	8/23/2007	96.74	48.7	ND
Tt0836	7/5/2007	89.46	ND	ND
Tt0837	7/5/2007	101.35	ND	ND
Tt0838	8/9/2007	37.31	42.5	ND
Tt0839	9/19/2007	101.65	39.4	ND
Tt0840	8/7/2007	88.90	47.3	ND
Tt0841	9/4/2007	87.99	40.3	ND
Tt0842	7/12/2007	67.76	39.7	ND
Tt0843	8/22/2007	98.23	ND	ND
Tt0844	9/24/2007	96.35	ND	ND
Tt0845	9/11/2007	94.00	ND	ND
Tt0846	9/20/2007	73.01	43.1	ND
Tt0847	7/2/2007	62.23	38.6	ND
Tt0848	7/18/2007	74.34	ND	ND
Tt0849	9/11/2007	100.09	ND	ND
Tt0850	8/3/2007	101.19	ND	ND
Tt0851	8/30/2007	103.04	ND	ND
Tt0852	7/2/2007	96.46	ND	ND
Tt0853	9/13/2007	44.80	40.4	ND

Table 3-7. Continued

Sample ID	Collection Date	ng DNA*	C_t Value†	Viral Presence‡
Tt0854	7/5/2007	101.84	ND	ND
Tt0855	7/16/2007	100.12	ND	ND
Tt0856	8/10/2007	92.40	ND	ND
Tt0857	8/21/2007	102.20	ND	ND
Tt0858	9/25/2007	102.65	ND	ND
Tt0859	9/12/2007	83.23	43.8	ND
Tt0860	7/31/2007	100.14	ND	ND
Tt0861	8/10/2007	72.38	ND	ND
Tt0862	8/7/2007	71.68	38.7	ND
Tt0863	8/22/2007	72.03	ND	ND
Tt0864	7/27/2007	92.96	ND	ND
Tt0865	7/2/2007	102.02	ND	ND
Tt0866	8/21/2007	71.40	ND	ND

*ng DNA equals the total sample DNA used in each well of the qPCR.
† C_t for each of the three sample replicates was averaged to calculate the C_t value.
‡ + indicates positive viral load (the Ct value for the sample was before the 36.67 detection limit)
¦ND = C_t was not detected for any sample replicate or the C_t value was greater than the 36.67 (10 copies) detection limit.

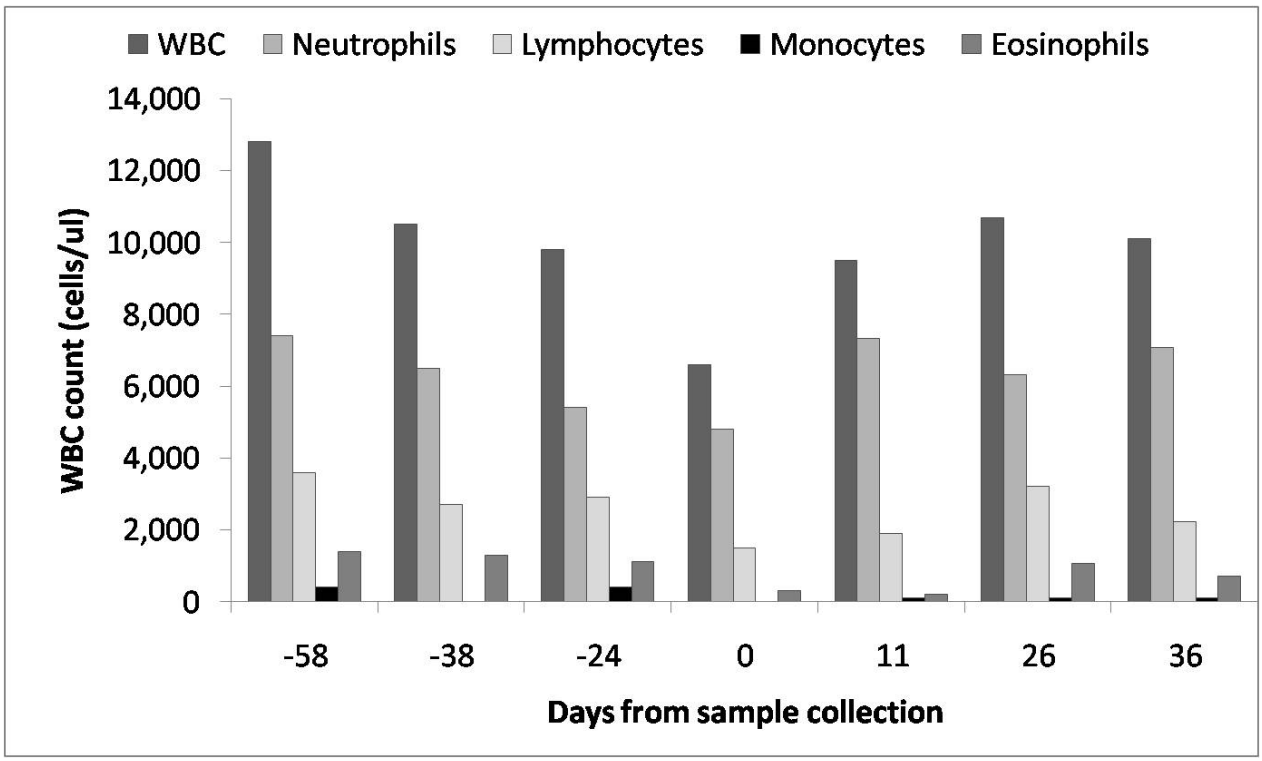

Figure 3-1. WBC counts surrounding sample Tt0719 collection date.

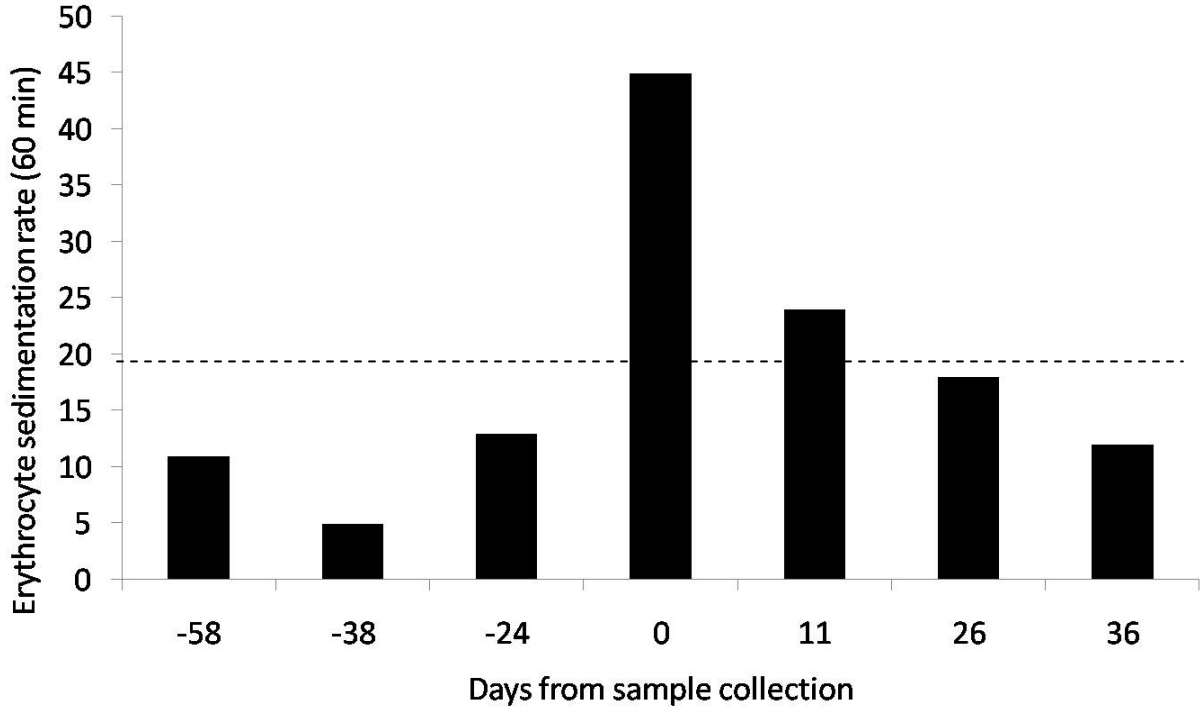

Figure 3-2. ESR values surrounding sample Tt0719 collection date. Dashed line marks high normal.

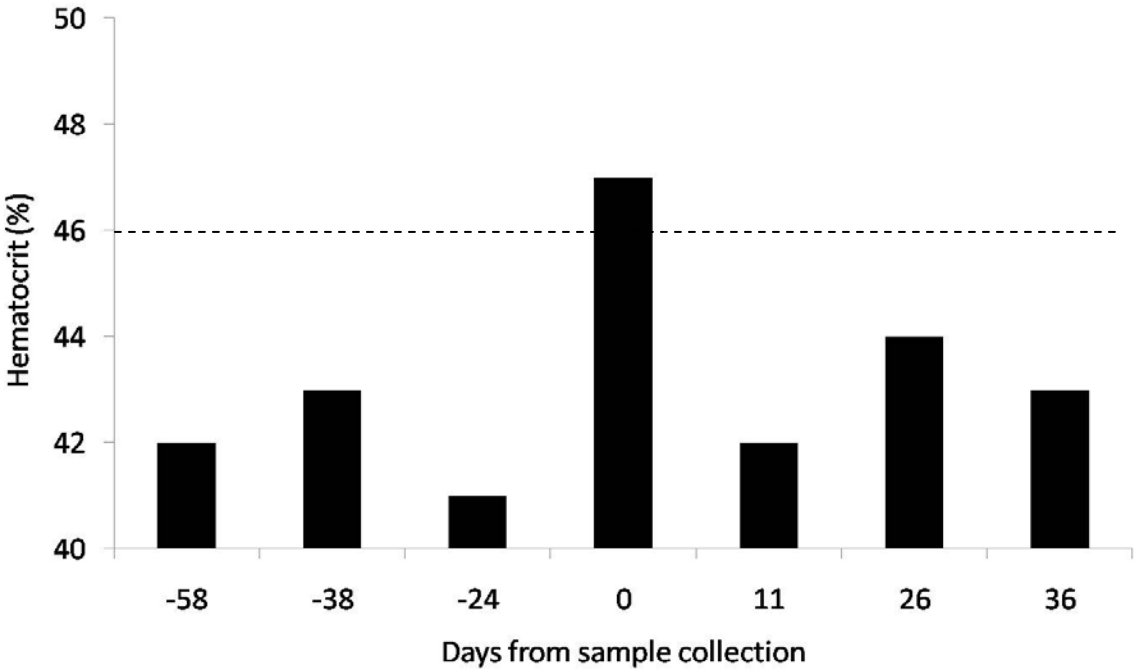

Figure 3-3. Hematocrit values surrounding sample Tt0719 collection date. Dashed line marks high normal.

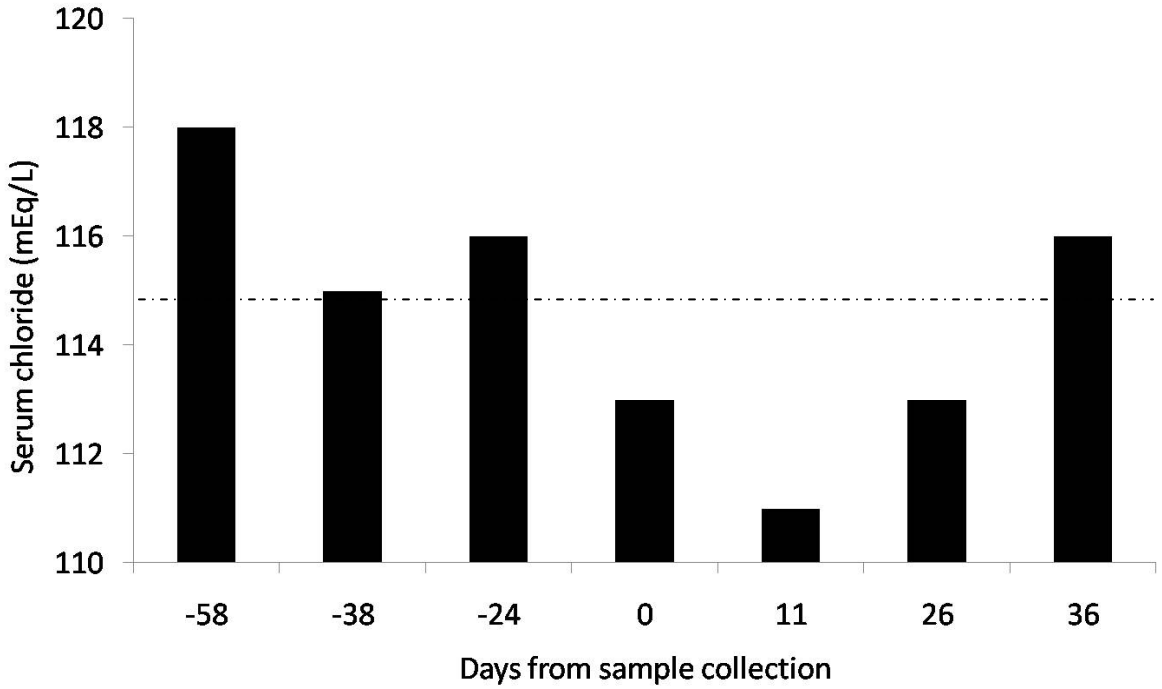

Figure 3-4. Sodium chloride values surrounding sample Tt0719 collection date. Dashed-dot line marks low normal.

CHAPTER 4
DISCUSSION

Two bottlenose dolphin herpesviruses, one of which is a novel alphaherpesviruses, were identified by consensus PCR in the case animal. Additionally, three novel marine mammal herpesviruses were identified (DeHV-8, DeHV-9, OtHV-2) and one novel Dpol herpesviral sequence from the PhHV-2 control isolate was generated by consensus PCR. Bayesian and ML phylogenic analysis supports the classification of the isolates as novel alpha- and gammaherpesviruses. It is unclear, based on the limited Dpol sequences, whether the novel delphinid and pinniped herpesviruses belong to established genera or represent novel genera.. None of the delphinid herpesviruses clearly associate with any established genus either. Further studies assessing phylogenetic relationships amongst marine mammal herpesviruses should aim to attain more sequence data for phylogenetic analysis. More complete genome segments for comparison should yield greater phylogenetic resolution[18] and will allow for the alpha- and gammaherpesviruses to be included in the same Bayesian and ML analysis.

The novel qPCR assays developed for DeHV-2 and DeHV-8 are faster and have a higher throughput than traditional PCR. Both qPCR assays are very sensitive and had a wide detectable range with a lower limit of 10 virus copies. The assays were successful in detecting lytically active alphaherpesviruses as shown by the detection of changes in viral load in the case animal at varying time points during the study.

The simple presence of DeHV-2 could not be associated with any specific blood parameter changes in the case animal. One positive case animal sample (Tt0719), however, had remarkable trends in four of the blood parameters analyzed: decreases in total WBC counts and serum chloride as well as increases in ESR and HCT. These trends are not well supported as relating to the increased herpesviral load since they were only present with one of the five

positives. Yet, the trend changes do suggest a clinical event because they were often significant differences and had values outside the normal reference ranges for the population. One way to demonstrate the probability of inflammation would be to run fibrinogen or acute-phase protein levels on sample Tt0719.[28,39] It would also be helpful to have a more thorough analysis of animal's eating habits to better grasp the theories of dehydration and metabolic alkalosis. The majority of water intake for bottlenose dolphins is the pre-formed and metabolic water in their food.[57] If the animal was not eating normally or regurgitating food the clinical significance of the changes would be greatly increased. Even if a true clinical disease process was present it remains unclear whether the disease process demanded an immunologic response that created an opportunity for latent DeHV-2 to proliferate, whether DeHV-2 proliferation was responsible for the subsequent abnormalities in the homeostasis of the body, or whether the two events are causally linked at all.

While an attempt was made to compare the blood parameters associated with samples that did or did not contain detectable DeHV-2 levels, there were very few positives samples available. There were an insufficient number of positives for statistical analysis of DeHV-2 and 8 within the population and insufficient DeHV-8 positive dates in the case animal. Future work should focus on collecting more positive samples with high and varying viral loads for comparisons. This would allow for a more robust analysis of the positives and negatives as well as for quantitative correlations between virus load and changes in blood parameters. If changes in blood parameters can be correlated with changes in viral load, one of the Fredricks-Relman criteria would be satisfied and a case for establishing causality of disease would be strengthened.

The qPCR assays detected a higher baseline prevalence of DeHV-2 (3 animals) than DeHV-8 (only the case dolphin). Attaining baseline values for distinct populations can assist

with clinical interpretations of virus presence as they are expected to vary between populations. Additionally, with five more bottlenose dolphin herpesviruses identified thus far, population prevalence rates for the other dolphin herpesviruses should also be investigated. Herpesviruses are common in bottlenose dolphins and one should be cautious in attributing clinical signs with herpesvirus detection as they are often present asymptomatically. Assessment of the clinical significance of herpesvirus presence should be completed before use of potentially harmful antivirals for treatment in unstudied species.

APPENDIX A
TROUBLESHOOTING

Inconsistent Amplification of Standards

Problem Identified

The *Tursiops truncatus herpesvirus 2* (DeHV-2) complementary deoxyribonucleic acid (cDNA) serially diluted standards, made from the consensus Polymerase Chain Reaction (PCR) product, was amplifying inconsistently in the quantitative PCR (qPCR) assay. While the amplification efficiency remained acceptable ($\Delta Rn > 3.5$), threshold values shifted. For example, the cycle threshold (C_t)value of 2 million copies which equaled 18 on 1/11/08, was delayed to cycle 20.5 the next day.

Troubleshooting Approach

Degradation of the qPCR probe and/or the template cDNA was the anticipated cause. Troubleshooting approaches included the following:

- Making smaller aliquots of the probe, to reduce the number of freeze/thaw cycles.
- Cloning of the DeHV-2 DNA-dependent DNA polymerase (Dpol) cDNA into a plasmid to increase stability and constructing standard curve serial dilutions using purified plasmid DNA rather than cDNA. For consistency between the DeHV-2 and DeHV-8 assays, we planned to construct a plasmid based standard curve serial dilution for DeHV-8 as well.

Materials and Methods

Purified cDNA, from secondary consensus PCR product, was cloned into the pDrive Cloning Vector using a PCR Cloning^{plus} Kit.[b] Five DeHV-2 clones were selected for plasmid purification using the PureLink™ Quick Plasmid Miniprep Kit.[d] Twenty-four clones were selected for DeHV-8 plasmid purification. Insertion of the sequence was confirmed by PCR of

the purified plasmids and sequencing the resulting bands. M13 forward (-20) and M13 reverse primers were used with the Platinum® *Taq* DNA Polymerase[c] in 10µL reactions following manufacturer's instructions with cycler conditions as follows: initial denaturation at 94°C for 5 min; then 40 cycles of denaturation at 94°C for 30 s, immediately followed by annealing at 46°C for 45 s, and extension at 72°C for 45 s; final extension was carried out at 72°C for 10 min followed by a 4°C hold. Sequencing of the purified plasmids (using the M13 primers) and creation of the serial dilutions followed the same protocol outlined in Chapter 2. The plasmid serial dilutions were then run in tandem with a freshly amplified cDNA serial dilution.

Results

Making smaller aliquots of the probe did make the amplification more consistent. However, the variability in C_t value for each dilution was still not within acceptable limits. Thus, creating plasmids from the cDNA was pursued.

The purified plasmids yielded a higher DNA concentration and higher purity ratios than the cDNA (see Table A-1). All five DeHV-2 clones were confirmed via sequencing to contain the insert but none of the DeHV-8 clones contained inserts. The DeHV-2 clone with the highest concentration of DNA (Tt0625-1pp shown in Table A-1) was then used to create the serial dilutions. Once used in the qPCR, the plasmid dilutions, which were calculated to have similar virions/µL as the cDNA dilutions, consistently generated C_t values about 2 cycles behind the cDNA counterpart (see Figure A-1).

Discussion

The difference in length between the DeHV-2 and DeHV-8 Dpol segment is 43bp. Since both products are small, 224bp for DeHV-2 and 172bp for DeHV-8, this difference is significant and believed to be the reason for the lack of insertion into the plasmid vector. While the cloning kit used did not specify a minimum PCR product size, other kits are known to have a minimum

of 200bp. Additionally, after qPCR analysis, sample Tt0724 had a viral copy number so low that it could not be detected. The low amount of template may have also contributed to the inability to insert the DeHV-8 sequence into the plasmid.

Restriction Enzymes

Problem Identified

The C_t values of the plasmid standards lagged 2 cycles behind those of the cDNA dilutions, despite similar concentrations and less protein in the plasmid product, it was postulated that the secondary or tertiary structure of the plasmid was inhibiting the binding of the primers and probe to the insert. The effects of secondary and tertiary structure are being recognized with increasing importance. In epigenetic studies of twins, DNA methylation has caused disease, such as Beckwith-Wiedemann syndrome, in one twin while the identical twin is unaffected.[38]

Troubleshooting Approach

A restriction enzyme was utilized to cut the plasmid in an attempt to linearize it. A linear plasmid should resolve hindrance issues from secondary and tertiary structures. The sequence was thus analyzed for a restriction enzyme and site that would only cut once.

Materials and Methods

Recovery of plasmids stored in glycerol. DeHV-2 plasmids were stored, individually, in 5 mLs of glycerol at -80°C. To recover the plasmids, 0.5 mL of frozen stored plasmid was added to 5 mLs LB broth and placed in an incubated shaker overnight. It took eight more nights before one of the five tubes turned cloudy and was spread onto an LB agar plate to grow up for two days in the incubator and then plaque purified (as described above). Since the recovery of the plasmids was poor, white colonies were also picked for storage as well as purification. To store the plasmids, one white colony was put into 5 mLs LB broth and shaken in an incubator for three hours before adding 5 mLs of glycerol, dividing into 2 mL aliquots and storing at -80°C.

Restriction enzyme digestion. Purified plasmids underwent spectrophotometric analysis (as described in Chapter 2) to assess purity and concentration prior to use in the restriction enzyme assay. The first plasmid, Tt0625-2pp, used in the restriction enzyme digestion had a concentration of 2µg (5 U BamH I used) while 4µg was used for the second plasmid, Tt0625-3pp, (10 U BamH I used). BamH I[d] was chosen for the digestion because it only cuts once, just outside of the insert. Digestion was performed, according to manufacturer's instructions, using the buffer supplied with the restriction enzyme in 60µL reactions. A Px2 thermal cycler[b] was used to incubate the reactions at 37°C for 60 min then to heat the reactions to 60°C for 10 min to stop the digestion. The product was resolved on an agarose gel and gel extracted for purification (as described in Chapter 2). Spectrophotmeteric analysis was then performed prior to creating a standard curve for qPCR (as described in Chapter 2). Lastly, the restriction enzyme serial dilutions (RE serial dilutions) were run concurrently with the plasmid and cDNA serial dilutions.

Results

The digested plasmids had much lower concentrations and higher protein presence than prior to digestion as well as compared to the cDNA (see Table A-1). Since the restriction enzyme digestion produced a cleaner band with only 2µg of purified plasmid in the reaction (see Figure A-2), Tt0625-1re was used to create the dilutions. The results from all three serial dilutions, run simultaneously, are shown in Figure A-3. The plasmid dilutions and the cDNA dilutions are almost identical to the previous run except for a misfire of the last cDNA standard. Thus, the plasmid standard at this dilution, $1{:}10^8$, actually has a lower C_t value (more virions/µL) than the $1{:}10^8$ cDNA standard. At every other dilution, the plasmid rises about 2 cycles after its cDNA counterpart. The RE serial dilution amplification was poor even though it had similar virions/µL at each dilution factor as the others. The first C_t value for the RE serial dilution is approximately 20 cycles behind the first C_t value of the two other standards. Furthermore, all the RE standards

came up after the $1:10^8$ dilution, the last of the four 10-fold dilutions shown, of the other two standards.

Discussion

The significant decrease in the recovery and purity of the DNA after the restriction enzyme digestion clearly inhibited the qPCR reaction. A different purification method post-digestion, such as a column-based method that would bypass the agarose gel step, may preserve more of the yield and purity but it seems dubious to expect a significant change in recovery when almost 90% of the DNA was lost and 40% of the purity. Therefore, attempts to make plasmid based standards were abandoned.

Table A-1. Spectrophotometric analysis

Sample ID	Sample type	ng/μL	260/280
Tt0625-1c	cDNA (PCR product)	26.0	1.83
Tt0625-2c	cDNA (PCR product)	20.8	1.73
Tt0625-1pp	purified plasmid	82.8	1.96
Tt0625-2pp	purified plasmid	90.9	1.94
Tt0625-3pp	purified plasmid	103.6	2.03
Tt0625-1 re	BamH I digested plasmid	12.3	1.54
Tt0625-2re	BamH I digested plasmid	7.5	1.28

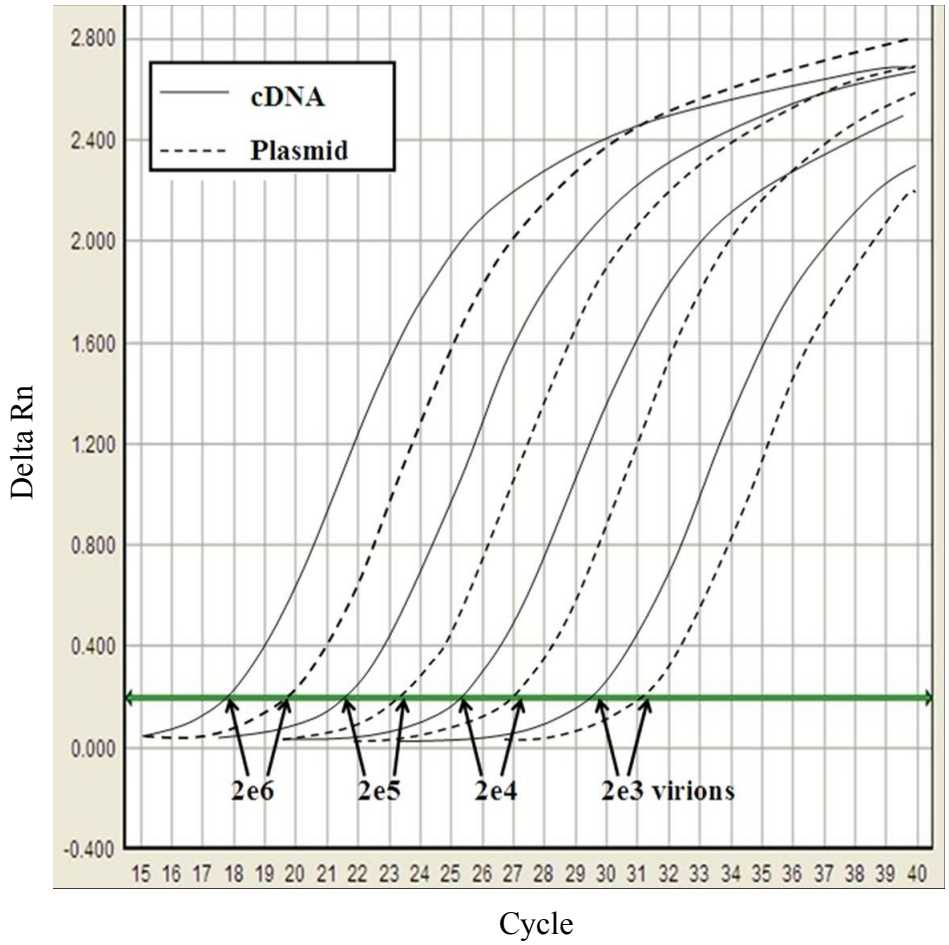

Figure A-1. DeHV-2 qPCR amplification comparison of cDNA and plasmid serial dilutions. Threshold line shown at 0.3.

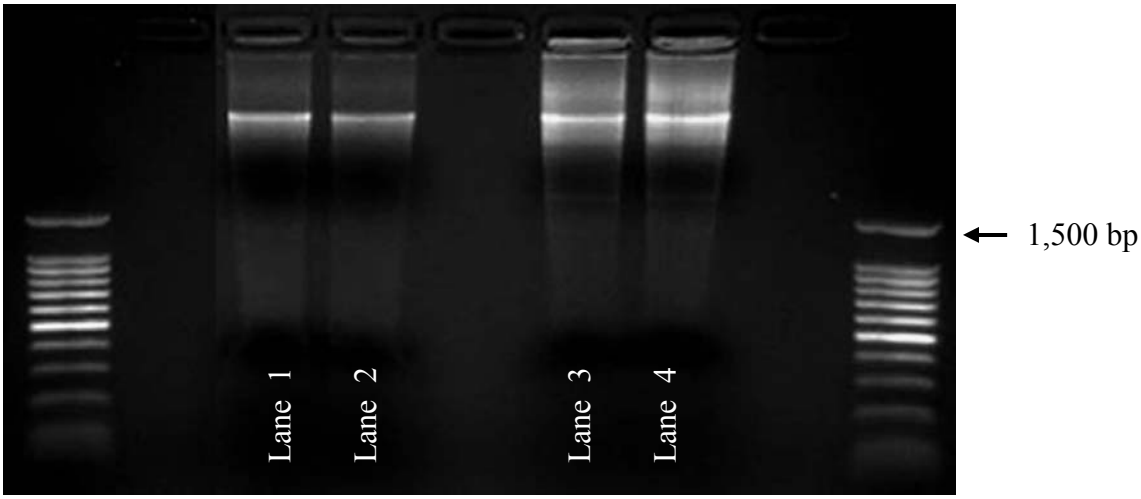

Figure A-2. Gel electrophoresis illustrating the plasmid after restriction enzyme digestion. Lane 1&2 = Tt0625-1re, Lane 3&4 = Tt0625-2re, Outside lanes = Benchtop 100bp DNA ladder.j

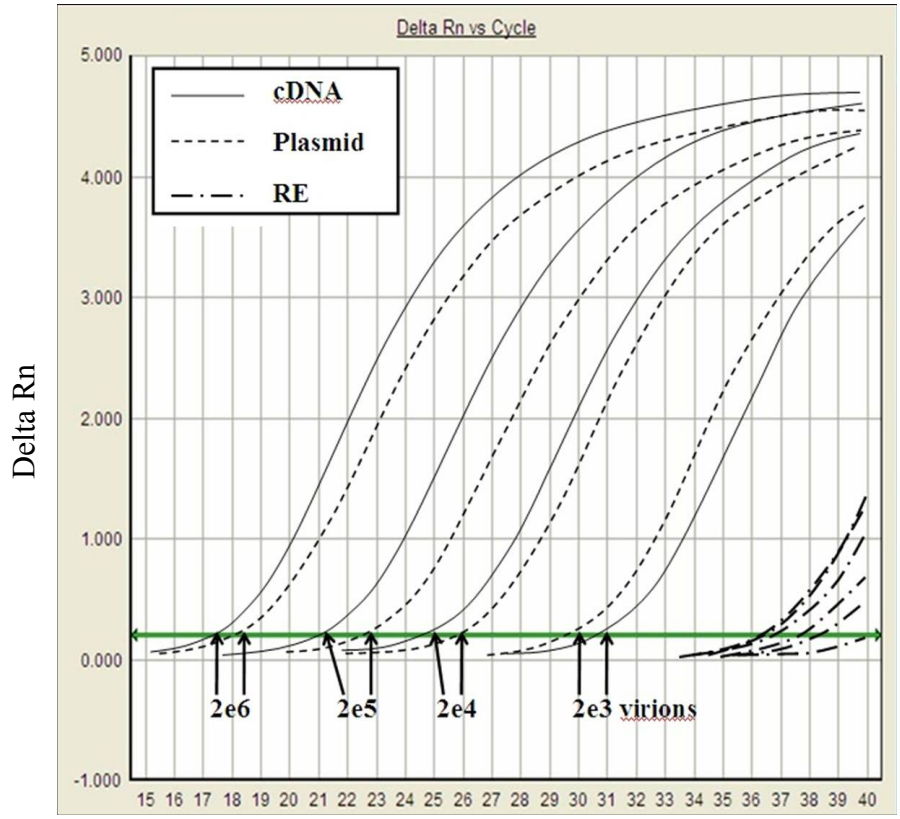

Figure A-3. DeHV-2 qPCR amplification comparison of cDNA, plasmid, and RE serial dilutions (RE serial dilution reflects the purified plasmid digested by the restriction enzyme BamH I). Threshold line shown at 0.3.

SOURCES AND MANUFACTURERS

a. Becton, Dickinson, and Co., Franklin Lakes, NJ.
b. Qiagen Inc., Valencia, CA.
c. Thermo Fisher Scientific, Inc., Waltham, MA.
d. Invitrogen Corp., Carlsbad, CA.
e. PerkinElmer Inc., Branchburg, NJ.
f. Eppendorf AG, Hamburg, Germany.
g. Applied Biosystems Inc., Foster City, CA.
h. Beckman Coulter Inc., Fullerton, CA.
i. PROC GLM Overview, SAS Online Doc, version 8, SAS Institute Inc., Cary, NC.
j. Promega Corp., Madison, WI.

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BIOGRAPHICAL SKETCH

Heather Daniel was born and raised in Tampa, FL. She was a girl scout for thirteen years and earned the gold award as a senior. She received the Hillsborough Community College Honors Institute Presidential scholarship and graduated with an Associate of Arts degree in 2003. Heather graduated from the University of Florida in 2007 with a dual Bachelor of Science, majoring in Wildlife, Ecology & Conservation as well as Animal Sciences. She graduated, for the second time from University of Florida, in 2009 with a Master of Science from the College of Veterinary Medicine. She currently works full-time at the college in Aquatic Animal Health.