DISCOVERY, PHYLOGENETIC ANALYSIS, DIAGNOSTIC TEST DEVELOPMENT, AND SURVEILLANCE OF THE ASTROVIRUSES OF MARINE MAMMALS

By

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To my wife, Karen, and my children, Xavier and Elseya

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LIST OF ABBREVIATIONS

AMV	avian myeloblastosis virus
AIDS	acquired immune deficiency syndrome
BD	Bottlenose Dolphin
Bp	base pairs
BDAstV1	Bottlenose Dolphin Astrovirus 1
BDAstV2	Bottlenose Dolphin Astrovirus 2
BDAstV3	Bottlenose Dolphin Astrovirus 3
BDAstV5	Bottlenose Dolphin Astrovirus 5
BDAstV6	Bottlenose Dolphin Astrovirus 6
BDAstV7	Bottlenose Dolphin Astrovirus 7
BDAstV8	Bottlenose Dolphin Astrovirus 8
BDAstV9	Bottlenose Dolphin Astrovirus 9
BSA	Bovine Serum Albumin
DMSO	dimethyl sulfoxide
cDNA	complementary DNA
CSLAstV1	California sea lion astrovirus 1
CSLAstV2	California sea lion astrovirus 2
CSLAstV3	California sea lion astrovirus 3
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphates
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
FMOC	9-fluorenylmethoxycarbonyl
HAstV1	Human astrovirus 1

HAstV2	Human astrovirus 2
HAstV3	Human astrovirus 3
HAstV5	Human astrovirus 5
HPLC	High performance liquid chromatography
IACUC	Institutional Animal Care and Use Committee
ICTV	International Committee of Taxonomy of Viruses
IgG	Immunoglobulin G
mAb	monoclonal antibody
$MgCl_2$	Magnesium chloride
ML	Maximum likelihood
MLB1	Human Astrovirus MLB1
MMLV	Moloney Murine Leukemia Virus
MWAstV1	Minke Whale Astrovirus 1
MWAstV2	Minke Whale Astrovirus 2
NaCl	Sodium chloride
NEM	negative-staining electron microscopy
OD_{405}	optical density at 405nm
OoAstV1	Orca Astrovirus 1
ORF	open reading frame
PBS	Phosphate Buffered Saline
PCR	polymerase chain reaction
PNPP	P-Nitrophenyl Phosphate
qPCR	quantitative polymerase chain reaction (a.k.a. real-time PCR)
RACE	Rapid Amplification of cDNA Ends
RdRp	RNA-dependent RNA polymerase

RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SARS	Severe acute respiratory syndrome
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electophoresis
SSLAstV1	Steller sea lion astrovirus 1

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DISCOVERY, PHYLOGENETIC ANALYSIS, DIAGNOSTIC TEST DEVELOPMENT, AND SURVEILLANCE OF THE ASTROVIRUSES OF MARINE MAMMALS

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Astroviridae are a family of small nonenveloped positive stranded ribonucleic acid (RNA) viruses associated with enteritis. Knowledge of astrovirus diversity is very limited, with only six astrovirus species from mammalian hosts officially recognized, and additional human, cheetah, rat, dog, and bat astroviruses recently described. We used consensus polymerase chain reaction (PCR) techniques for initial identification of fifteen astroviruses from marine mammals; three from California sea lions (*Zalophus californianus*), one from a Steller sea lion (*Eumetopias jubatus*), nine from bottlenose dolphins (*Tursiops truncatus*), and two from minke whales (*Balaenoptera acutorostrata*). Bayesian and maximum likelihood phylogenetic analysis found that these viruses showed significant diversity at a level consistent with novel species. Some astroviruses from marine mammals clustered within the genus *Mamastrovirus*, whereas others were in a clade outside of known genera. Mamastroviruses identified did not form a monophyletic group. Recombination analysis found that a relatively recent recombination event may have occurred between a human and a California sea lion astrovirus, suggesting that both lineages may have been capable of infecting the same

host at one point. A bottlenose dolphin astrovirus sequence was also consistent with the result of a recombination event.

An enzyme linked immunosorbent assay (ELISA) and a quantitative PCR (qPCR) assay were designed for a bottlenose dolphin *Mamastrovirus* and used to survey seroprevalence, virus prevalence, and virus load. The results showed that animals seroconvert at a young age, and virus prevalence was even higher than what has been seen with astroviruses of terrestrial animals. Virus load correlated with abnormal behavior reported by trainers.

A qPCR assay was designed for an astrovirus from a bottlenose dolphin that did not cluster with known genera. This assay was used for detection of virus prevalence and load. The prevalence was similar to that found in terrestrial mammal astroviruses, but loads were very low. It is unclear whether this virus is actually infecting dolphins.

There is significant diversity amongst marine mammal mamastroviruses. They have a very high prevalence. These findings, together with their similarity to terrestrial astroviruses and recombination frequency, suggest that the marine environment plays an important role in mamastroviral ecology.

CHAPTER 1
INTRODUCTION

Marine Mammals and Virology

The marine environment has amazing diversity. Cellular life first evolved in the oceans, and it is probable that viruses evolved shortly thereafter. The abundance of viruses in marine environments is approximately 15-fold that of bacteria and archaea (Suttle, 2007).

Marine mammals are charismatic animals with a strong appeal to humans. Humans are having a major impact on marine environments, with negative impacts on marine mammal populations (Bejder et al., 2006). Relatively little is understood about the viruses of marine mammals. Diseases such as dolphin morbillivirus have demonstrated the potential to cause significant morbidity and mortality in marine mammal populations (Schulman et al., 1997). The known diversity of marine mammal viruses has just begun to expand rapidly (Nollens et al., 2010, Colegrove et al., 2010, Wellehan et al., 2008). A greater understanding of dolphin virology may positively impact health management of both wild and captive marine mammals.

Additionally, there may be unknown potential viral zoonoses present in the marine environment, presenting risks to human populations. Emerging disease is frequently associated with host switches. One recent meta-analysis of human diseases found that 816 of 1407 (58%) are zoonotic, and of human diseases, zoonotic diseases are significantly more likely to be emerging (Woolhouse and Gowtage-Sequeria, 2005). Most recent emerging diseases have been associated with host switches, including severe acute respiratory syndrome (SARS) coronavirus, H5N1 avian influenza, Hendra virus, Nipah virus, and acquired immunodeficiency syndrome (AIDS). The

aforementioned study also found that viral diseases were much more likely to be emerging, especially ribonucleic acid (RNA) viruses (Woolhouse and Gowtage-Sequeria, 2005). Most emerging diseases have zoonotic origins, and viruses in the marine environment are poorly understood. Understanding of the marine ecosystem is necessary to comprehend viral ecology, and has great significance for human health. Marine mammals are useful sentinels for marine viruses, allowing earlier identification of zoonotic agents and development of diagnostic and epidemiologic strategies. San Miguel Sea Lion Virus has been shown to infect humans and is associated with disease (Smith et al., 1998).

Medicine has traditionally waited for viruses to cause epidemics or epizootics before significant surveillance occurs. With our increased understanding of virus ecology and evolution, it becomes more feasible to identify probable candidates for future novel disease outbreaks, and increase surveillance. An understanding of diverse viruses in wildlife may enable more appropriate epidemiologic responses to new virus infections.

Astroviruses

History

Astroviruses were discovered relatively recently, having first been reported in 1975 from human diarrhea cases (Madeley and Cosgrove, 1975). Once recognized, they were soon associated with childhood enteritis; an early study of a human astrovirus in Oxford, England found that 7% of 6 to 12 month old children had a positive titer, whereas 75% of 5 to 10 year old children were positive (Kurtz and Lee, 1978). Studies of experimental infections in volunteers established them as an etiology of gastroenteritis, although the very high prevalence of antibody titers in adults, indicating

the presence of an acquired immune response, made reliable experimental infection difficult (Kurtz et al., 1979). The first sequence was obtained from human Astroviruses in 1992 (Major et al., 1992, Willcocks and Carter, 1992).

Taxonomy

The family *Astroviridae* is divided into two genera, *Avastrovirus*, found in avian hosts, and *Mamastrovirus*, found in mammal hosts (Monroe et al., 2005). Species are defined by the International Committee on the Taxonomy of Viruses (ICTV) on the basis of the host species (Monroe et al., 2005). In the genus *Avastrovirus*, recognized species include *Chicken Astrovirus*, *Duck Astrovirus*, and *Turkey Astrovirus*. Recognized species in the genus *Mamastrovirus* include *Bovine astrovirus*, *Feline astrovirus*, *Human astrovirus*, *Mink Astrovirus*, *Ovine astrovirus*, and *Porcine astrovirus* (Monroe et al., 2005). There has been significant recent discovery of additional mamastroviruses, including viruses from cheetahs (Atkins et al., 2009), Asian bat species (Chu et al., 2008, Zhu et al., 2009), humans (Finkbeiner et al., 2008, Finkbeiner et al., 2009a, Finkbeiner et al., 2009b, Kapoor et al., 2009), and rats (Chu et al., 2010). Recently, a divergent astrovirus open reading frame (ORF) 1a sequence has been identified from bat guano found under a mixed-species roost in North America (Li et al., 2010). Phylogenetic analysis found that it was weakly supported as basal to other mamastroviruses in a neighbor-joining tree.

Structure and Genomic Organization

Astroviruses are small nonenveloped viruses with a positive stranded RNA genome and a distinct star-like surface morphology seen on electron microscopy. Virus particles have been reported on electron microscopy as 28-34 nanometers in diameter with a round unbroken edge, a six-pointed star with a white center, and triangular

surface hollows (Madeley, 1979). However, astroviruses resemble other small round viruses somewhat morphologically, and a significant rate of misidentification using negative-staining electron microscopy of feces has been reported (Oliver and Phillips, 1988).

Astrovirus genomes are range between 6.8-7.9 kb and contain three open reading frames. ORF1a encodes nsp1a, a 101 kiloDalton (kDa) nonstructural polyprotein, which is cleaved by a viral serine protease and host proteases (Kiang and Matsui, 2002, Geigenmüller et al., 2002). After processing, the amino terminal fragment has a mass of approximately 20kDa (Méndez et al., 2003), and may be a helicase since it shares limited motifs with the pestiviral helicase (Al-Mutairy et al., 2005). The next fragment, containing the serine protease, is a 27kDa protein (Geigenmüller et al., 2002, Méndez et al., 2003). This segment is the best structurally characterized astroviral protein, and is currently the only region where a detailed crystallographic structure is available (Speroni et al., 2009). Following the serine protease fragment, the carboxy-terminal fragment of nsp1a contains a hypervariable region that colocalizes with viral RNA and endoplasmic reticulum. Changes in this hypervariable region have significant effects on viral replication (Guix et al., 2005). It has been proposed, based on sequence analysis, that this region may represent a genome linked viral protein, homologous to VPg in other plus-sense single-stranded RNA viruses (Al-Mutairy et al., 2005).

ORFs 1a and 1b are linked by a translational frameshift. They encode polyprotein nsp1ab, with a mass of 145kDa, which is cleaved to a 57kDa protein encoding an RNA-dependent RNA polymerase (Méndez et al., 2003).

ORF2 encodes the capsid precursor protein. In human astroviruses, the 90kDa membrane bound capsid precursor is formed into viral particles that are subsequently released from the cell membrane by caspase cleavage to form a 70kDa protein (Méndez et al., 2004). Trypsin cleavage further cuts the capsid protein into an early 38.5-41 kDa section forming the main capsid, and a later 25-29 kDa section forming spikes, giving the virus its "star" shape (Méndez et al., 2002). This trypsin cleavage markedly increases infectivity. Neutralizing epitopes have been found on the 25-29 kDa protein (Bass and Upadhyayula, 1997).

Recently, an ORF (ORFX) overlapping the 5' end of ORF2 in a different frame has been identified (Firth and Atkins, 2010). ORFX is conserved amongst *Mamastrovirus* but not *Avastrovirus*. It is unknown whether ORFX is expressed, and if so, what function is serves.

Epidemiology

Most epidemiological data have examined *Human Astrovirus*. As a prevalent enteric disease in children, exposure to *Human Astrovirus* at a young age is typical. In Oxford, England, 75% of 5 to 10 year olds were positive (Kurtz and Lee, 1978). A study in London found that over 50% of children between 5 and 12 months of age had antibody responses to Human astrovirus 1 (HAstV1), and seroprevalence was 90% by the age of 5 (Kriston et al., 1996). In the Netherlands, seroprevalence of HAstV1 was 100% by 5 years of age, and although overall seroprevalence was lower for human astroviruses 2-4, the age of conversion was similar (Koopmans et al., 1998). However, Koopmans et al. (1998) found a later age of onset for HAstV5. A study in Virginia found that the seroprevalence of HAstV1 was 94% at 6 to 9 years of age (Mitchell et al.,

1999). A study in Japan found that by age 3, seropositivity to HAstV1 and HAstV3 approached 100% (Kobayashi et al., 1999).

Human astrovirus quantitative polymerase chain reaction (qPCR) assays have found the prevalence in diarrheic human feces to range from 6% - 9% (Logan et al., 2007, Dai et al., 2010, van Maarseveen et al., in press). One study of fecal electron microscopy of cats found that astroviruses were the most common virus particles seen in cats with diarrhea (Marshall et al., 1987).

Human astrovirus data clearly indicates a cosmopolitan distribution. There is also evidence that *Feline astrovirus* may be cosmopolitan; astroviruses have been documented in cats from Australia (Marshall et al., 1987), England (Harbour et al., 1987), Germany (Herbst and Krauss, 1988), New Zealand (Rice et al., 1993), and the United States (Hoshino et al., 1981).

A human astrovirus qPCR assay was used for a study of clinical correlation of virus load to clinical features. A tendency for longer duration of diarrhea with higher copy numbers was seen. Lower copy numbers were associated with rotavirus coinfection (Zhang et al., 2006).

Pathology

Astroviruses are strongly associated with enteric disease. Unlike other characterized viral causes of enteritis, but much like *Vibrio cholerae*, astroviruses cause a secretory diarrhea without much of a histologic footprint on enterocytes on light microscopy (Koci et al., 2003, Moser et al., 2007, Nighot et al., 2010). Turkey astrovirus 2 (TAstV2) does not increase apoptosis in the intestine (Koci et al., 2003). Human astrovirus 1 capsid protein interacts with apical enterocyte membranes, increasing permeability independent of viral replication. Increased permeability correlates with

disruption of the tight-junction protein occludin and a reduction in actin stress fibers in infected cells, suggesting that tight junctions between enterocytes may become leaky (Moser et al., 2007). With TAstV2 infections, actin rearrangement was also seen, resulting in ultrastructural changes that were not visible on light microscopy (Nighot et al., 2010). Redistribution of the sodium/hydrogen exchanger 3 from the membrane to the cytoplasm was seen, which was likely the cause of the decreased sodium absorption that was found (Nighot et al., 2010).

Following infection of turkey poults, TAstV2 can be recovered from multiple tissues, and animals are viremic (Koci et al., 2003). While diarrhea is the most common clinical sign seen with astroviral infection, a *Mamastrovirus* has been found in a human encephalitis case (Quan et al., 2010), and *Avastrovirus* disease may also be renal or hepatic (Imada et al., 2000, Fu et al., 2009). There is a paucity of information on the pathology and tissue tropisms of astroviruses in sites other than enterocytes.

Future Directions

Astroviruses are known to cause disease in mammals. As small RNA viruses, they are capable of rapid evolution, which is advantageous when invading new habitats such as novel host species. One recent study scored the viruses infecting mammals for biological properties that were considered advantageous to host switching, and found that Astroviridae scored very highly (Pulliam, 2008). Astroviruses are very stable in aquatic environments (Espinosa *et al*., 2008). The marine environment is central in the ecology of caliciviruses, a better studied group of small nonenveloped positive stranded RNA viral human pathogens (Smith *et al*., 1998). The astroviruses of wildlife merit further surveillance, and marine mammals represent an important potential reservoir.

CHAPTER 2
INITIAL CHARACTERIZATION OF PHYLOGENETICALLY DIVERSE ASTROVIRUSES OF MARINE MAMMALS

Introduction

Astroviruses are small nonenveloped viruses with a positive stranded RNA genome and a distinct star-like surface morphology. They were relatively recently discovered and first reported in 1975 (Madeley & Cosgrove, 1975). Based mainly on the host of the virus and the genome structure, the family Astroviridae is divided into two genera. Members of the genus *Avastrovirus* are found in avian hosts, whereas the genus *Mamastrovirus* is found in mammal hosts (Monroe *et al*., 2005). Known astrovirus diversity is very limited, with only three astrovirus species recognized from avian hosts by the International Committee on the Taxonomy of Viruses and six recognized astrovirus species from mammalian hosts (Bovine astrovirus, Feline astrovirus, Human astrovirus (serotypes 1-8), Mink Astrovirus, Ovine astrovirus, and Porcine astrovirus) (Monroe *et al*., 2005). Recently, a divergent human astrovirus from a child with diarrhea (Finkbeiner *et al*., 2008), and a number of astroviruses from vespertillonid and rhinolophid bats have been described (Chu *et al*., 2008). The mamastroviruses have a small, positive sense, single-stranded RNA genome of less than 7,000 base pairs that codes for three open reading frames (ORF) named ORF1a, ORF1b and ORF2. A frame shift between ORF1a and ORF1b allows ORF1 to encode both a protease and an RNA-dependent RNA polymerase (RdRp). ORF2 encodes the viral capsid protein. Cloning of ORF2 in expression vectors has allowed for the in-vitro assembly of virus-like particles (Caballero *et al*., 2004).

While avastroviruses can cause intestinal or renal disease, mamastroviruses predominantly establish infections in the gastrointestinal tract of their hosts. Human

astroviruses are a frequent cause of enteric disease in neonatal, elderly and immunocompromised humans (Dennehy *et al.,* 2001, Gallimore *et al.,* 2005) and one study of fecal electron microscopy of cats found that astroviruses were the most common virus particles observed in cats with diarrhea (Marshall *et al*., 1987). The capsid protein plays a unique role in the pathogenesis of the diarrhea. Astrovirus capsid protein interacts with apical enterocyte membranes, increasing permeability independent of viral replication. Much like *Vibrio cholerae,* astroviruses cause a secretory diarrhea without much of a histologic footprint (Moser *et al*., 2007). Histopathology is therefore an insensitive test for diagnosis of astroviral diarrhea, potentially leading to underdiagnosis of astrovirus infections.

Only very recently has the presence of astroviruses in wildlife hosts been reported. Previous reports in nondomestic hosts include identification of astroviruses in cheetahs and multiple species of bats (Atkins *et al*., 2009)(Chu *et al*., 2008). Here, we report on the first detection of five genetically distinct astroviruses from three marine mammal host species.

Materials and Methods

Animals and Samples

Fecal samples were collected as part of routine health surveillance from two clinically healthy California sea lions (*Zalophus californianus*) and one bottlenose dolphin (*Tursiops truncatus*) housed in open ocean enclosures at the U.S. Navy Marine Mammal Program in San Diego, California (CA). In addition, fecal samples were collected from one stranded, free-ranging California sea lion pup with diarrhea and one stranded, free-ranging Steller sea lion (*Eumetopias jubatus*) pup without outward signs of diarrhea, both housed at The Marine Mammal Center in Sausalito, CA. All samples

were collected between December 2006 and October 2008. Fecal samples were stored in sterile vials and frozen at -80° Celsius (C) until laboratory analysis.

Negative Staining Electron Microscopy and Sample Processing

Upon arrival to the laboratory, each fecal sample was divided into three fractions. The first fraction was sent to the Florida State Diagnostic Lab for negative-staining electron microscopy (NEM). The second fraction was stored at -80˚C for future analysis. The third fraction was suspended at a 1:10 ratio in 0.89% sodium chloride (NaCl) and centrifuged at 4000 x G for 20 minutes at 4°C. The clarified supernatant was collected using a sterile syringe and consecutively passed through 0.8 micrometers (µm), 0.45 µm and 0.22 µm syringe filters to eliminate cellular and bacterial particles. The final filtrate was transferred to a Microsep® concentrator column (Pall Life Sciences) and centrifuged at 1500 x G for 25 to 45 minutes at 4°C. A 140 microliter (µl) aliquot of the concentrated filtrate was used for RNA extraction using a Viral RNA Mini Kit (Qiagen) following the manufacturer's instructions.

Degenerate Polymerase Chain Reaction (PCR)

Degenerate primers designed based on conserved astroviral sequences (Atkins *et al*., 2009) were used in a nested or a semi-nested format to amplify conserved regions of the ORF1b (RdRp) and ORF2 (capsid). For amplification of the partial RdRp gene, primers Astr4380F (5'-GAYTGGRCNCGNTWYGATGGNACIAT-3') and Astr4811R (5'-GGYTTNACCCACATNCCAAA-3') (round #1), and primers Astr4574F (5'-GGNAAYCCMTCWGGICA-3') and Astr4722R (5'-ARNCKRTCATCNCCATA-3') (round #2) were used on all five isolates. For individual viruses, additional degenerate primer combinations were used to obtain more astrovirus RdRp gene sequence (see table 2-

1). For amplification of the partial capsid gene primers Astr4811F (forward, 5'-TTTGGNATGTGGGTNAARCC-3') and Astr5819R (5'-TCATTNGTGTYNGTNANCCACCA-3') (round #1), and primers Astr5159F (5'-TGGAGGGGMGGACCAAAG-3') and Astr5819R (round #2) were used on all five isolates.

For the first round of the PCR assays, fecal RNA was reverse transcribed using a OneStep RT-PCR Kit (Qiagen) at 50˚C for 30 minutes and then denatured at 94˚C for 5 minutes, followed by 36 cycles of denaturation at 94˚C for 30 seconds; annealing at 45˚C for 30 seconds, and extension at 72˚C for 60 seconds, with a final elongation step at 72˚C for 10 minutes. Three µl of product from the first round was used as template in a 20µl nested or semi-nested second round. The second round amplifications conditions using Platinum Taq DNA Polymerase (Invitrogen) were as follows: 5 minutes denaturation at 94˚C, followed by 36 cycles of denaturation at 94˚C (30 seconds), annealing at 45˚C (30 seconds), extension at 72˚C (60 seconds), with a final elongation step at 72˚C for 10 minutes. PCR products from both rounds were run in 1% agarose gels and the DNA bands were visualized under UV light after ethidium bromide staining. Bands of interest were cut from the gel and their DNA extracted using the Qiaquick gel extraction kit (Qiagen). Direct sequencing was performed using the Big-Dye Terminator Kit (Perkin-Elmer) and ABI automated sequencers. All amplicons were sequenced at least twice in both directions, and primer sequences were edited out prior to constructing contiguous sequences.

Sequence Extension

For each isolate, the gap between the upstream ORF1b and downstream ORF2 sequence segment was amplified using specific forward and reverse primers (see Table

2-1) that were designed based on the sequences obtained via degenerate PCR. Again, all amplicons were sequenced at least twice in both directions. Attempts were made to sequence the remaining 5' and 3' sections of the viral genome using a GeneRacer kit (Invitrogen). Specific rapid amplification of complimentary deoxyribonucleic acid ends (RACE) primers were designed, and viral genomic RNA was amplified using the manufacturer's instructions. Briefly, for 3' RACE, RNA was reverse transcribed using AMV reverse transcriptase and amplified with a forward gene specific primer and the GeneRacer 3' Primer. For 5' RACE, RNA was treated with calf intestinal phosphatase, treated with tobacco acid pyrophosphatase to remove the 5' cap structure, and ligated to the GeneRacer RNA oligo. The dephosphorylated, uncapped and ligated RNA was then reverse transcribed and subsequently amplified using a specific reverse gene specific primer (see table 2-1) and the GeneRacer 3' primer. PCR products were run in a 0.7% agarose gel, and bands of interest were sequenced as previously described.

Phylogenetic Analysis

Sequences were compared to those in GenBank (National Center for Biotechnology Information, Bethesda, Maryland), European Molecular Biology Laboratory (EMBL) (Cambridge, United Kingdom), and Data Bank of Japan (Mishima, Shizuoka, Japan) databases using TBLASTX (Altschul *et al*., 1997). The predicted homologous 237-254 amino acid sequences of astroviral RdRp and 186-207 amino acid sequences of astroviral capsid protein were aligned using the following three methods: ClustalW2 (Larkin *et al*., 2007), T-Coffee (Notredame *et al*., 2007), and MUSCLE (Edgar, 2004).

Bayesian analyses of each alignment were performed using MrBayes 3.1 (Ronquist & Huelsenbeck, 2003) with gamma distributed rate variation and a proportion

of invariant sites. Mixed amino acid substitution models were used. The first 10% of 1,000,000 iterations were discarded as a burn in.

Maximum likelihood (ML) analyses of each alignment were performed using PHYLIP (Phylogeny Inference Package, Version 3.66) (Felsenstein, 1989), running each alignment using the program ProML with amino acid substitution models JTT (Jones *et al*., 1992), PMB (Veerassamy *et al*., 2003), and PAM (Kosiol & Goldman, 2005) further set with global rearrangements, five replications of random input order, gamma plus invariant rate distributions, and unrooted. The values for the gamma distribution were taken from the Bayesian analysis. Avian nephritis virus 1 (GenBank accession number AB033998) was designated as the outgroup. The alignment producing the most likely tree was then used to create data subsets for bootstrap analysis to test the strength of the tree topology (200 re-samplings) (Felsenstein, 1985), which was analyzed using the amino acid substitution model producing the most likely tree in that alignment.

Recombination Analysis

A nucleotide alignment was created using MUSCLE on the sequence between primers Astr4380F and Astr5819R of 13 mamastroviruses: the five marine mammal astroviruses from this study, *Ovine astrovirus* (GenBank accession # NC002469), Bat astrovirus AFCD337 (EU847155), *Mink astrovirus* (AY179509), MLB1 astrovirus (FJ222451), Human astrovirus 1 (AY720892), Human astrovirus 3 (AF141381), Human astrovirus 4 (DQ070852), and Human astrovirus 5 (DQ028633). Potential recombination patterns were screened using RDP (Martin & Rybicki, 2000), Geneconv (Padidam *et al*., 1999), MaxChi (Maynard, 1992), Chimaera (Posada & Crandall, 2001), and 3Seq (Boni *et al*., 2007) in the RDP3 suite (Martin *et al*., 2005b) using the step

down correction for multiple comparisons and a P value cutoff of 0.05. Regions of potential recombinant interest were also checked with LARD (Holmes *et al*., 1999), Recscan (Martin *et al*., 2005a), and SiScan (Gibbs *et al*., 2000).

Results

Negative Staining Electron Microscopy

Viral particles were detected in all five fecal samples. The 5 isolates are henceforth referred to as California sea lion astroviruses 1-3 (CSLAstV1, CSLAstV2, CSLAstV3), Steller sea lion astrovirus 1 (SSLAstV1) and bottlenose dolphin astrovirus 1 (BDAstV1). Individual capsids were 30-35 nm in diameter. In all samples, the nonenveloped icosahedral virus particles had distinct star-like surface projections and were consistent in size and morphology with members of the astrovirus family (Figure 2-1).

Degenerate PCR

The primer combination Astr4380F/Astr4811R yielded a band of 431 base pairs (bp) on isolates CSLAstV1, CSLAstV3, SSLAstV1 and BDAstV1. The primers Astr4574F/Astr4722R yielded a band of 148bp on isolates CSLAstV1, CSLAstV2, CSLAstV3 and SSLAstV1. The primer combination 5159F/5819R yielded a band of 660bp on all five isolates.

Sequence extension via specific PCR yielded final contiguous molecules of 1,340 bp (CSLAstV3) and 1,348 bp (SSLAstV1). Additional 3' RACE yielded final contiguous molecules of 3,174 bp (CSLAstV1), 3,505 bp (CSLAstV2), and 3,985 bp (BDAstV1). The contiguous molecules corresponded to the partial capsid gene (ORF1b) and the full-length RdRp gene of reference astroviruses (Figure 2-2). The contiguous sequences were submitted to GenBank under accession numbers FJ890351 (CSLAstV1),

FJ890352 (CSLAstV2), FJ890353 (CSLAstV3), FJ890354 (SSLAstV1) and FJ890355 (BDAstV1).

Comparison with other sequences in GenBank revealed that all five contiguous molecules represented novel astroviruses. TBLASTX results for bottlenose dolphin astrovirus 1 showed the highest identity score with Human astrovirus 3 (GenBank accession # AF141381) at the RdRp and Human Astrovirus 7 (GenBank accession # Y08632) for the capsid precursor. TBLASTX results for California sea lion astrovirus 1 showed the highest score with Ovine astrovirus (GenBank accession # AF141381) at the RdRp and Mink astrovirus (GenBank accession # AY179509) for the capsid precursor. TBLASTX results for California sea lion astrovirus 2 showed the highest score with Human astrovirus 2 (GenBank accession # L13745) at the RdRp and Porcine astrovirus (GenBank accession # AB037272) for the capsid precursor. TBLASTX results for California sea lion astrovirus 3 showed the highest score with Human astrovirus 3 (GenBank accession # AF141381) at both the RdRp and the capsid precursor. TBLASTX results for Steller sea lion astrovirus 1 showed the highest score with Mink astrovirus (GenBank accession # AY179509) at both the RdRp and the capsid precursor.

Phylogenetic Analysis

Bayesian phylogenetic analysis showed the greatest harmonic mean of estimated marginal likelihoods using the MUSCLE alignment for the RdRp (Appendix A) and the T-Coffee alignment for the capsid gene (Appendix B). For the RdRp, the WAG model of amino acid substitution was found to be most probable with a posterior probability of 1.000 (Whelan & Goldman, 2001). For the capsid precursor protein, the WAG model was also most probable with a posterior probability of 0.993, and a posterior probability

of 0.007 for the JTT model. Bayesian trees using the MUSCLE alignment for the RdRp (Figure 2-3) and the T-Coffee alignment for the capsid gene are shown (Figure 2-4).

ML analysis found the most likely tree from the MUSCLE alignment and the PMB model of amino acid substitution for the RdRp, and the T-Coffee alignment and the JTT model of amino acid substitution for the capsid precursor. These parameters were used for bootstrap analysis. Bootstrap values from ML analysis are shown on the trees (Figures 2-3 and 2-4).

Recombination Analysis

The MUSCLE nucleotide alignment was 1479 nucleotides in length. Recombination analysis identified a probable recombination event in CSLAstV3, from parents Human Astrovirus 4 and CSLAstV2, with a P value after corrections for multiple comparisons of 4.911×10^{-6}. It was supported by RDP ($P = 2.197 \times 10^{-4}$), Recscan ($P = 4.359 \times 10^{-3}$), MaxChi ($P = 7.943 \times 10^{-4}$), Chimaera ($P = 4.911 \times 10^{-6}$), SiScan ($P = 3.089 \times 10^{-5}$), LARD ($P = 2.339 \times 10^{-6}$), and 3Seq ($P = 2.634 \times 10^{-3}$). Geneconv did not support this event. This event started at nucleotide 832 of the alignment with a P value for the beginning breakpoint of 8.637×10^{-3} (Fig. 2-7). This initial breakpoint falls early in the coding region for ORF2, at the 15th amino acid. Prior to the breakpoint, CSLAstV3 showed greater homology with Human Astrovirus 4, and greater homology with CSLAstV2 after the breakpoint. The endpoint was not clear- although predicted for nucleotide 1062 of the alignment, the P value was only 0.600. A bootscanning diagram for this event is shown in Figure 2-5.

Differences in branching patterns between the capsid and polymerase in the *Mink Astrovirus*/CSLAstV1/ SSLAstV1 clade were not reconciled by identification of a recombination event in the initial analysis, so other sequences in the alignment were

masked for additional analysis. Weak support was found for a recombination event between nucleotides 727 and 847, without correction for multiple comparisons. This region covers the end of ORF1b and the start of ORF2. This event was supported by RDP (P = 0.02957), Recscan (P = 0.04462), SiScan (P = 7.198 $x10^{-4}$), LARD (P = 2.405 $x10^{-4}$), and 3Seq (P= 0.01088) but not Geneconv (P = 0.074), MaxChi (P = 0.1178), or Chimaera (P= 0.2422).

Discussion

This report documents the first identification of astroviruses in marine mammals. Surprising diversity was identified in these hosts. With the exception of MLB1 (Finkbeiner *et al*., 2008), the astroviruses of humans, the host species that has been investigated most heavily, constitute a single species with eight serotypes. In contrast, the evolutionary distance between the first three astroviruses found in California sea lions is comparable to that seen between recognized species within the Astroviridae. BDAstV1 and SSLAstV1 also appear to be distinct from other astroviruses at a distance consistent with species differentiation.

The genetic distances between these novel viruses was generally slightly greater in the capsid region than in the polymerase region (Fig. 3 and 4), even though we examined the capsid region which is expected to be most conserved. This is consistent with other studies. Capsids are typically under strong positive selective pressure from the host immune system, and one analysis found that most positively selected sites in astroviruses are present in the capsid (Van Hemert *et al*., 2007).

The phylogenetic topology determined in this study is largely in agreement with previous analyses of astrovirus phylogeny (Jonasee *et al*., 2001)(Lukashov & Goudsmit, 2002), although we do not find support for the findings of Chu et al. (2008) (Chu et al.,

2008) that bat astroviruses AFCD11 and AFCD57 are not monophyletic with the other chiropteran mamastroviruses.

Of the six mamastrovirus species recognized by the ICTV, five are from hosts from one mammalian superorder, Laurasiatheria. The other superorder of placental mammals, Euarchontoglires, has only one host (humans) from which an astrovirus is recognized, human. The greater diversity of astroviruses within laurasiatherian hosts may imply a longer host-virus relationship. Bats, from whom additional astroviruses have recently been described, are also members of Laurasiatheria. The astroviruses of bats mostly appear to form a distinct monophyletic group, unlike those of marine mammals, which are distributed across the tree of the known mamastroviruses. Evidence of bat astroviruses outside of the clade of the viruses found by Chu et al. was recently published (Zhu *et al*., 2009).

Astroviruses are very stable in aquatic environments (Espinosa *et al*., 2008). Surveys have found a *Human astrovirus* prevalence of up to 61% in some marine shellfish populations, which are good particle concentrators (Elamri *et al*., 2006). The wide diversity seen in astroviruses that were identified from marine mammals implies that the marine environment may play a large role in astroviral ecology. Similarly, the marine environment is central in the ecology of caliciviruses, a better studied group of small nonenveloped positive stranded RNA viruses (Smith *et al*., 1998). An understanding of diverse astroviruses in wildlife may enable more appropriate epidemiologic responses to new astrovirus infections in humans.

Our data suggest that a relatively recent recombination event may have occurred between a human and a marine mammal astrovirus isolate, resulting in CSLAstV3.

Recombination is common in other nonenveloped positive stranded RNA viruses such as picornaviruses and caliciviruses, and as a result, evolution of structural and nonstructural regions of the genome may appear semi-independent (Simmonds, 2006). Previous studies have found evidence for recombination amongst *Human astrovirus* serotypes, as well as turkey astroviruses (Pantin-Jackwood *et al*., 2006)(Simmonds, 2006). The lineage of the region of CSLAstV3 after the area of strong homology with CSLAstV2 is not clear. This may represent a separate recombination event with an as yet unidentified clade of astroviruses. An analysis of the capsid region of a cheetah astrovirus found that it clustered relatively more closely with human astroviruses than was found in the analysis of the polymerase (Atkins *et al*., 2009). If due to a recombination event, this is an apparent opposite event from that seen in CSLAstV3, which has a *Human astrovirus*-like polymerase and a CSLAstV2-like capsid, at least in the 5' end of ORF2. The evidence for recombination in the *Mink Astrovirus*/CSLAstV1/ SSLAstV1 clade is less clear. Data from additional viruses in this clade would be needed to clarify whether a recombination event in these viruses is probable.

Recombination is an important mechanism for rapid evolution of a virus, allowing rapid acquisition of sequence that is less likely to be deleterious than random mutations. The most common mechanism of recombination among nonenveloped positive stranded RNA viruses is switching of the polymerase complex from copying one template to another (Jarvis *et al*., 1992). Nonreplicative RNA recombination has also been shown in picornaviruses, but also requires unencapsulated RNA cotransfected into the same cell (Gmyl *et al*., 1999). Therefore, a recombinant of a human astrovirus and a sea lion astrovirus implies that both viruses infected a sea lion, a human, or a

third host species at the same point. Emerging disease is frequently associated with host switches. One recent meta-analysis of human diseases found that 816 of 1407 (58%) are zoonotic, and of human diseases, zoonotic diseases are significantly more likely to be emerging (Woolhouse & Gowtage-Sequeria, 2005). Most recent emerging human diseases have been associated with host switches, including SARS, Hendra virus, Nipah virus, and AIDS. The aforementioned study also found that viral diseases were much more likely to be emerging, especially RNA viruses (Woolhouse & Gowtage-Sequeria, 2005). The apparent ability of some of these viruses to infect disparate hosts suggests further study of the ecology and host range of astroviruses may be relevant to human health.

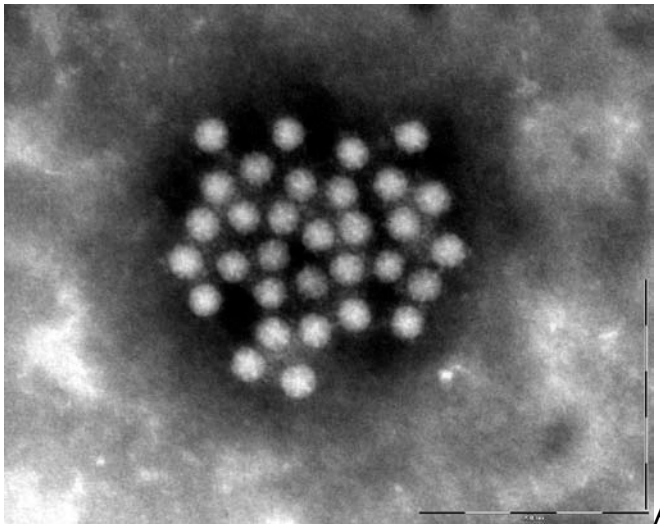
We were unable to clarify the clinical significance of astroviruses for marine mammals from our dataset. Only one sea lion case (CSLAstV3 from a stranded, free-ranging pup) had clinical diarrhea. However, bacterial culture of the diarrheal sample yielded a *Salmonella* sp. culture. Given the clinical signs associated with other mamastroviruses, it would be reasonable to hypothesize that these viruses may cause a secretory diarrhea which is most likely to be clinically important in neonates and weanlings. Further studies are underway to determine the clinical relevance of these viruses to their marine mammal hosts.

In conclusion, we have identified five novel astroviruses from marine mammal hosts. These viruses are diverse and all appear to be consistent with novel species. These viruses are situated across the mamastrovirus tree, and do not form a monophyletic group. There is evidence of recombination between human and marine

mammal astroviruses. Further study of these viruses and their clinical significance in marine mammal populations are indicated.

Table 2-1. Primers used to sequence California sea lion Astroviruses 1-3 (CSLAstV1 to V-3), Steller sea lion astrovirus 1 (SSLAstV1) and bottlenose dolphin astrovirus 1 (BDAstV1)

Primer	5'-3' Sequence	Primer Type	Virus Isolate
Astr4574R	TGNCCWGAKGGRTTICC	Degenerate	CSLAstV1
Astr4542F	TRCCMWSNGGTGARRTCAC	Degenerate	BDAstV1
Astr4811F	TTTGGNATGTGGGTNAARCC	Degenerate	BDAstV1
CSLAstro4805F	AGAGTGGTGGATATGTATAAGG	Specific	CSLAstV1 & BDAstV1
CSLAstro5167R	GCTGCTTGGTTGGCGTGAGCCAT	Specific	CSLAstV1
CSLAstro777F	TGGTGGNTNACNRAYACHAATGA	Specific	CSLAstV1
CSLAstro1121R	CNSCNKYRTYNAVNAYHTGCCA	Specific	CSLAstV1
CSLAstro4588F	CAACCACCAGCAGCCCAGTGGGG	Specific	CSLAstV1
CSLAstro4934R	GCGGAGTAGTTGGTGAACTCCCA	Specific	CSLAstV1
CSLAstro5067F	TCCTTGAAATGACACTGCCGA	Specific	CSLAstV1
CSLAstro6227R	TGWWGRARKTKTCMSCYMAGGCA	Specific	CSLAstV1
CSLAstroGSP-F	GCGTCCTTGAAATGACACTGCCGAAGGA	3'RACE	CSLAstV1
CSL0686GSPseq1	TAAGCCTGACGGCACAACTCACT	3'RACE	CSLAstV1
ZcAstrV-1GSPR2	CACACCGTTCCCAGGCATCACAGA	5'RACE	CSLAstV1
TtAstroGSPF	CCCCTTTGATCGGACCCTCAGCAATCA	3'RACE	BDAstV1
TtAstroGSPR	GCCGGAGGCTTTAACCTCAACGCTAACA	5'RACE	BDAstV1
TtAstVGSPR3	TGGTCTGCTCTGTCACTTCACCCG	5'RACE	BDAstV1
ZcAstrV-2GSPF	CGGCTCAAGCCAAGAGACCTCGCTGG	3'RACE	CSLAstV2
ZcAstrV-2GSPF2	CACCACCACCAACACCACACTTTCA	3'RACE	CSLAstV2
ZcAstrV-2GSPR	GGGAGAACAACTTTACGGTGGACGAG	5'RACE	CSLAstV2
AFEFfor	CTGCAAGCCTTCGAGTTTG	Specific	CSLAstV3
VEVKRev	CCATTGGACTTGACCTCAACA	Specific	CSLAstV3
SSLF1	TGCATCCGTGCAAGACTCTA	Specific	SSLAstV1
SSLR1	TGAAGACTGGGAAAGGGTTG	Specific	SSLAstV1
SSLF2	GTCCACAGTCCGTTTCGTCT	Specific	SSLAstV1
SSLR2	AGTTGCAGACACACGGACAG	Specific	SSLAstV1


A

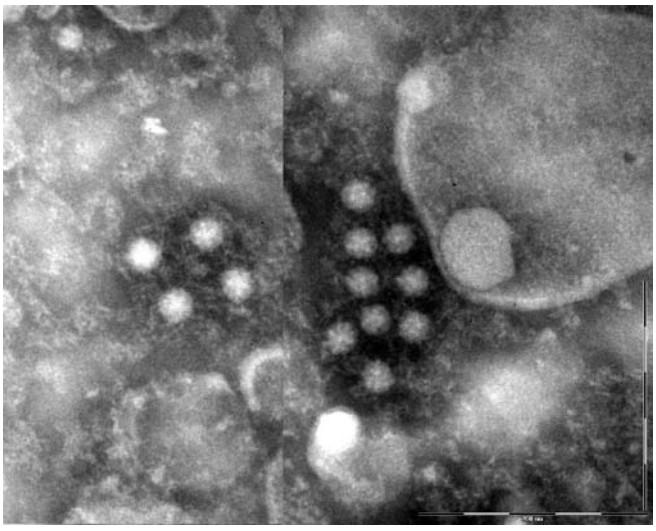

B

Figure 2-1. Negative Staining Electron Microscopy. Negative staining revealed clusters of viral particles were detected in fecal samples from three California sea lions (*Zalophus californianus*), one Steller sea lion (*Eumetopias jubatus*) and one bottlenose dolphin (*Tursiops truncatus*). Individual capsids were 30-35 nm wide. The nonenveloped icosahedral virus particles had distinct star-like surface projections consistent with astroviruses. Only isolates CSLAstV1 (A) and BDAstV1 (B) are shown.

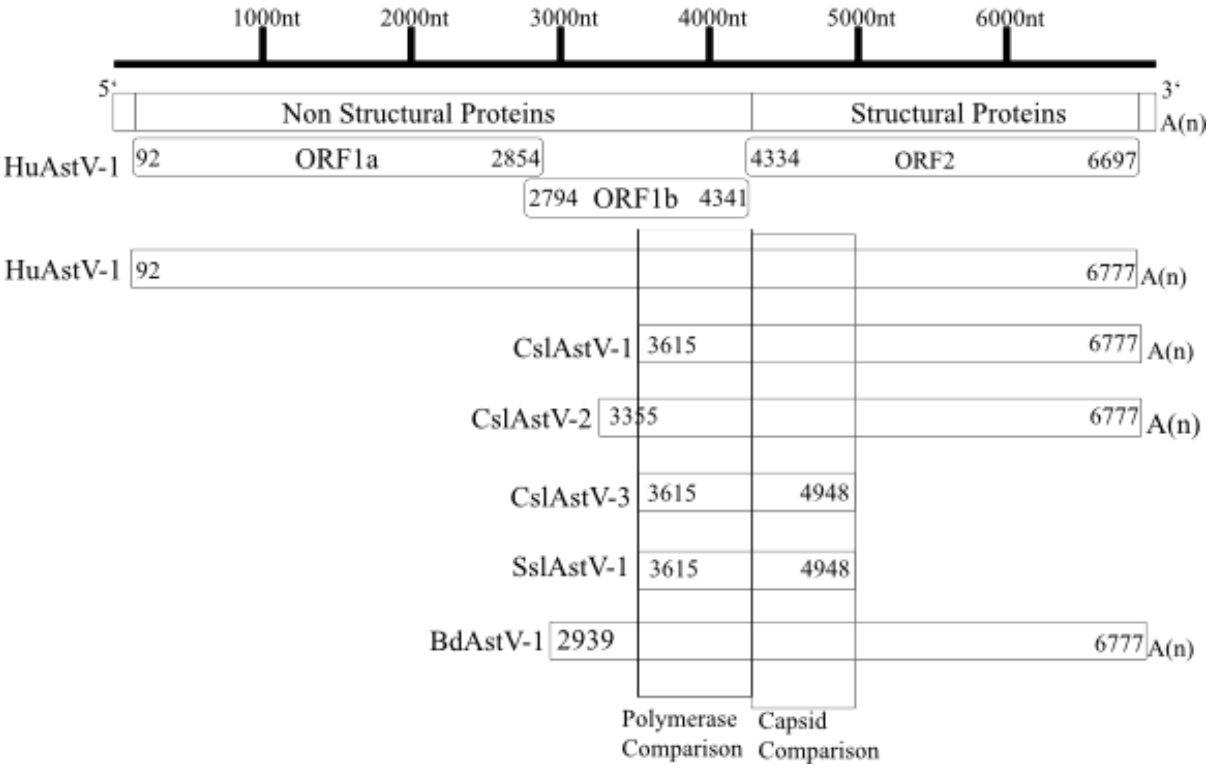


Figure 2-2. HuAstV-1 genome compared to marine mammal astrovirus genome segments. Diagram representing the alignment of the 3,174bp CSLAstV1 genome segment, 3,505bp CSLAstV2 genome segment, 1,340bp CSLAstV3 genome segment, 1,348bp SSLAstV1 genome segment and the 3,985bp BDAstV1 genome segment compared to the full-length genome sequence of Human Astrovirus 1 (6697bp, GenBank accession # AY720892). The relative positions of the ORFs encoding the non-structural and structural proteins are also represented. Partial gene segments of the polymerase and capsid genes used for phylogenetic analysis are indicated.

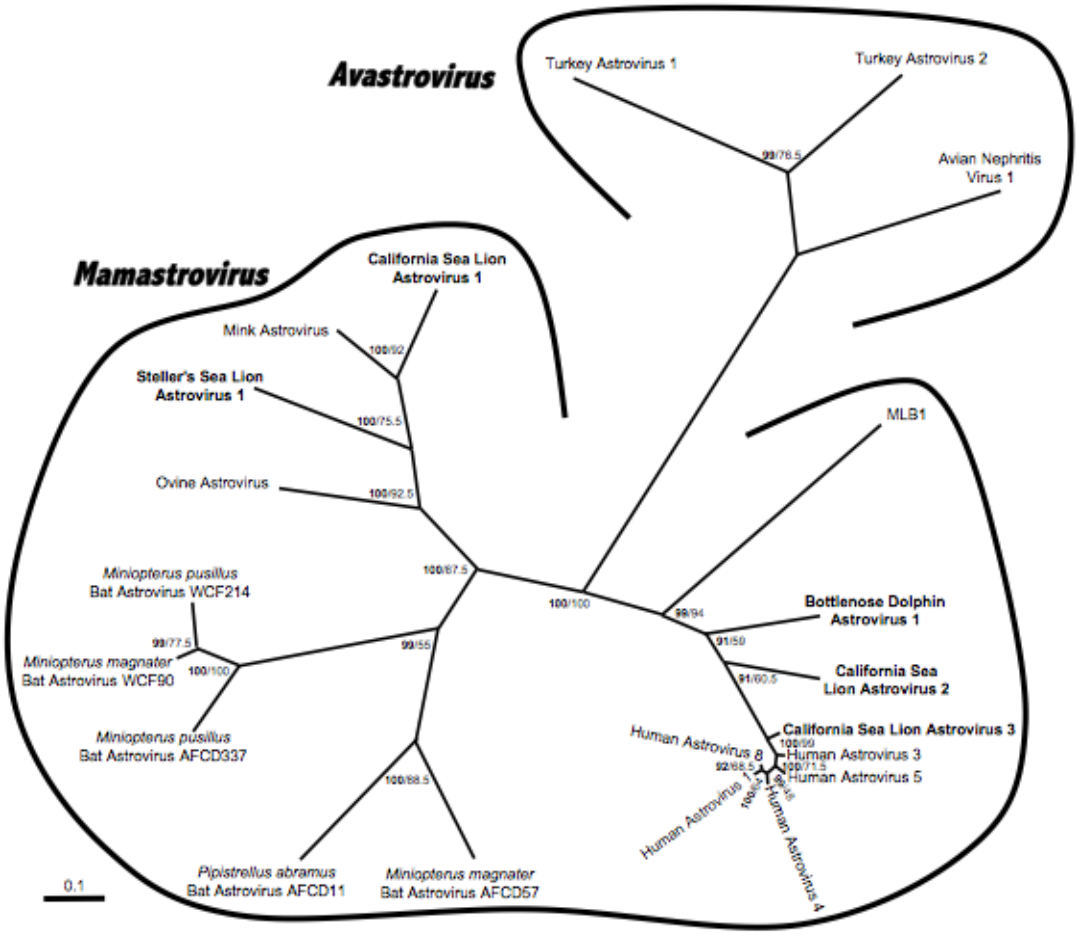


Figure 2-3. Bayesian phylogenetic tree of predicted 237-254 amino acid partial astroviral RNA-dependent-RNA polymerase sequences based on MUSCLE alignment. Bayesian posterior probabilities of branchings as percentages are in bold, and ML bootstrap values for branchings based on 200 re-samplings are given to the right. Avian nephritis virus 1 (GenBank accession number NP_620617) was designated as the outgroup. Virus genera are delineated by brackets. Marine mammal astroviruses are bolded. Sequences retrieved from GenBank include Human astrovirus 1 (GenBank accession # AAW51881), Human astrovirus 3 (AAD28539), Human astrovirus 4 (AAY84778), Human astrovirus 5 (AAY46273), Human astrovirus 8 (AAF85963), Human astrovirus MLB1 (YP002290967), *Miniopterus magnater* bat astrovirus WCF90 (ACF75856), *Miniopterus magnater* bat astrovirus AFCD57 (ACF75852), *Miniopterus pusillus* bat astrovirus AFCD337 (ACF75864), *Miniopterus pusillus* bat astrovirus WCF214 (ACF75862), *Pipistrellus abramus* bat astrovirus AFCD11 (ACF75853), Ovine astrovirus (NP_059945), Mink astrovirus (AAO32082), Turkey astrovirus 1 (CAB95006), Turkey astrovirus 2 (NP_987087), and Avian Nephritis Virus 1 (NP_620617).

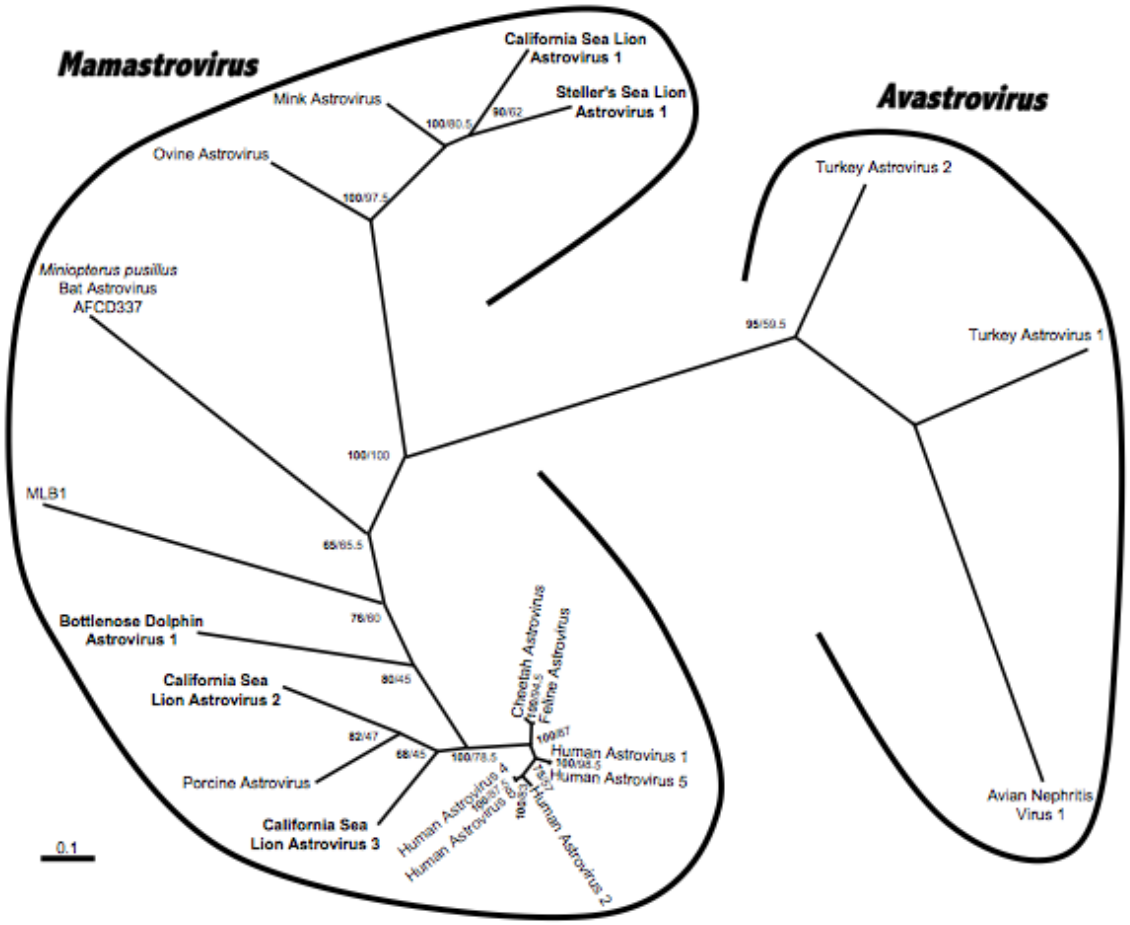


Figure 2-4. Bayesian phylogenetic tree of predicted 186-207 amino acid partial astroviral capsid sequences based on T-Coffee alignment. Bayesian posterior probabilities of branchings as percentages are in bold, and ML bootstrap values for branchings based on 200 re-samplings are given to the right. Avian nephritis virus 1 (GenBank accession number NP_620618) was designated as the outgroup. Virus genera are delineated by brackets. Marine mammal astroviruses are bolded. Sequences retrieved from GenBank include, Human astrovirus 1 (GenBank accession # BAE97460), Human astrovirus 2 (AAA62427), Human astrovirus 4 (BAA93440), Human astrovirus 5 (AAY46274), Human astrovirus 8 (AAF85964), Human astrovirus MLB1 (YP 002290968), Feline astrovirus (AAC13556), Cheetah astrovirus 1 (ACD13861), Porcine astrovirus (CAB95000), *Miniopterus pusillus* bat astrovirus AFCD337 (ACF75865), Ovine astrovirus (NP_059944), Mink astrovirus (NP_795336), Turkey astrovirus 1 (CAB95007), Turkey astrovirus 2 (NP_987088), and Avian Nephritis Virus 1 (NP_620618).

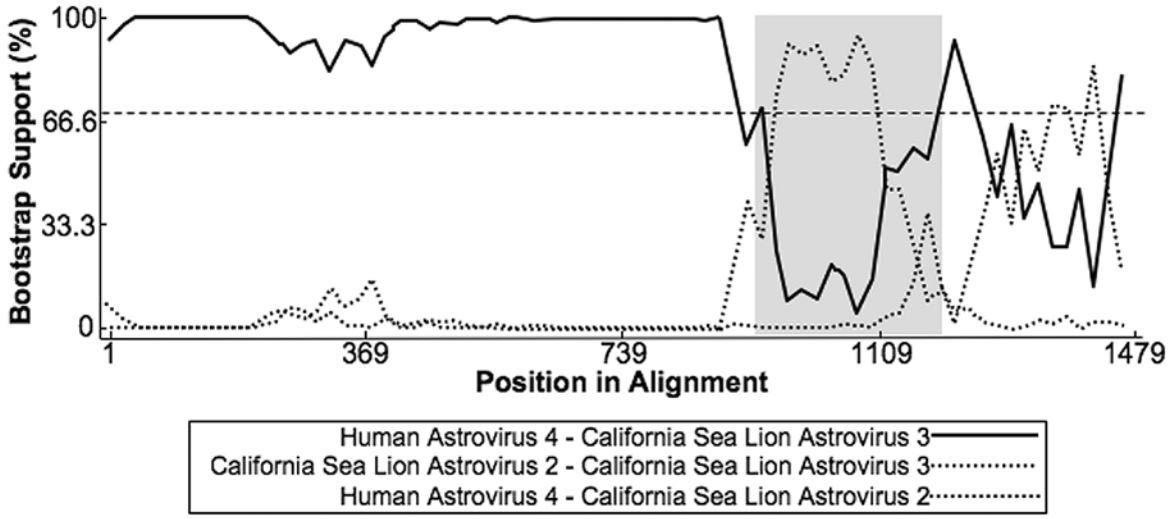


Figure 2-5. Bootscanning analysis of MUSCLE alignment of astroviral sequences produced using the RDP3 suite. The thick line indicates bootstrap support for monophyly of Human astrovirus 4 and CSLAstV3, with a 200bp window size and a step size of 20, with 200 bootstrap replicates. The dotted line indicates support for monophyly of CSLAstV2 and CSLAstV3. The second dotted line indicates bootstrap support for monophyly of Human astrovirus 4 and CSLAstV2. The grey area is the identified possible recombinant region. In addition to the marine mammal viruses found in this study, sequences used in the MUSCLE nucleotide alignment include Human astrovirus 1 (GenBank accession # AY720892), Human astrovirus 3 (AF141381), Human astrovirus 4 (DQ070852), Human astrovirus 5 (DQ028633), Human astrovirus MLB1 (FJ222451), Mink astrovirus (AY179509), Ovine astrovirus (NC_002469), and *Miniopterus pusillus* bat astrovirus AFCD337 (EU847155).

CHAPTER 3
USE OF A PEPTIDE-BASED INDIRECT ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DETECTION OF A HUMORAL IMMUNE RESPONSE TO BOTTLENOSE DOLPHIN ASTROVIRUS 1

Introduction

Astroviruses are small round nonenveloped viruses with a positive stranded RNA genome. They were relatively recently discovered, and were first reported in 1975 (Madeley and Cosgrove, 1975). The family Astroviridae is divided into two genera, *Avastrovirus*, found in avian hosts, and *Mamastrovirus*, found in mammal hosts (Monroe et al., 2005). Human astrovirus is a significant cause of enteric disease in human children (Dennehy et al., 2001). We have recently reported the discovery of diverse astroviruses in marine mammals, including Bottlenose Dolphin Astrovirus 1 (BDAstV1) (Rivera et al., 2010).

As a prevalent enteric disease in children, seroconversion to Human Astrovirus at a young age is typical. An early study of a human astrovirus in Oxford, England found that 7% of 6 to 12 month olds had a positive titer, whereas 75% of 5 to 10 year olds were positive (Kurtz and Lee, 1978). A study in London found that over 50% of children between 5 and 12 months of age had antibody responses to *Human astrovirus 1* (HAstV1), and seroprevalence was 90% by the age of 5 (Kriston et al., 1996). In the Netherlands, age seroprevalence of 7 different human astrovirus serotypes was examined; seroprevalence of HAstV1 was 100% by 5 years of age, and although overall seroprevalence was lower for human astroviruses 2-4, the age of conversion was similar (Koopmans et al., 1998). However, Koopmans et al. found a later age of onset for HAstV5. A study in Virginia found that the seroprevalence of HAstV1 decreased from 67% in infants <3 months of age to 7% by 6 to 8 months of age, consistent with

loss of transplacental antibodies, followed by a marked increase to 94% at 6 to 9 years of age (Mitchell et al., 1999). Mitchell et al. found a similar age distribution but lower seroprevalence for HAstV3. A study in Japan found that by age 3, seropositivity to HAstV1 and HAstV3 approached 100% (Kobayashi et al., 1999).

Attempts at culture of BDAstV1 were unsuccessful, so alternate means of obtaining antigen for serological testing were needed. The capsid (ORF2) contains the characterized neutralizing antibody epitopes in astroviruses (Sánchez-Fauquier et al., 1994, Bass and Upadhyayula, 1997). There are experimental data from human astroviruses suggesting that protease cleavage cuts the capsid protein into an early section forming the main capsid (called VP32 or VP34, VP38.5, or VP41 at different stages of processing by different investigators), and a later section forming the projections (known as VP25, VP26, VP27, VP28 or VP29 at different stages of processing by different investigators). Trypsin cleavage is important in processing of the projections (Méndez et al., 2002). Caspase cleavage of the carboxy-terminal part of the protein is probably involved with release of the virus from the cell (Méndez et al., 2004), and conserved caspase sites suggest this is also likely for other astroviruses. This region is likely cleaved off before exit from the cell, and is not likely to be a target for antibodies. The projections are likely to be external and most exposed to antibodies. Matsui et al. (1993) found that a clone of the region immediately carboxy-terminal to the trypsin cleavage site is recognized by antibodyes from infected individuals.

Synthetic oligopeptides have been used as antigens in successful immunoassays to detect antibodies in the host (Kwang and Torres, 1994, Aizaki et al., 1995, Nilsen et

al., 2003). We hypothesized that as similar approach may work with marine mammal astroviruses. In this study, the utility of a synthetic oligotide ELISA assay for detecting anti-BDAstV1 antibodies was assessed.

Materials and Methods

Animals and Sera

Serum samples were collected from 61 bottlenose dolphins from a managed open-water collection, 31 animals from three different captive closed-water collections, and 64 wild dolphins. Multiple time points were taken from 7 dolphins from the managed open-water collection. Blood samples were collected from the ventral tail vein, the ventral fluke vein, or if taken at post-mortem examination, via cardiocentesis. Samples were typically collected using a 20 or 21 gauge 1.5 inch Vacutainer® needle (Becton Dickinson VACUTAINER Systems, Rutherford, New Jersey 07070) and blood was collected into a Vacutainer® serum separator tube or a Vacutainer® without anticoagulant. The blood samples were centrifuged at 3,000 revolutions per minute (rpm) at 21°C for 10 minutes. Fibrin clots were removed and serum was transferred to cryovials. Archived sera were stored at -80°C.

Astrovirus Peptide Design

Peptides were selected from the predicted capsid protein of BDAstV1. BDAstV1 was aligned with Human Astrovirus 1 using MUSCLE (Edgar, 2004) to determine homologous conserved regions (figure 3-1). Possible antigenic peptides within likely antigenic regions were designed using an antigenic site prediction program (http://immunax.dfci.harvard.edu/Tools/antigenic.html) based on the methods of Kolaskar and Tongaonkar (1990), as well as hydrophilicity analysis using ProtScale (http://expasy.org/tools/protscale.html) based on the methods of Kyte and Doolittle

(1982). The selected peptides were Tt322 (KASEVIVQVVDA), Tt399 (TLAWQQMNKPN), Tt455 (ALAPYWQSLELW), and Tt616 (DMVLLISWV). Two of these peptides (Tt399 and Tt455) were targeted in the area homologous to the antigenic region identified by Matsui et al.(1993).

Peptides were synthesized using 9-fluorenylmethoxycarbonyl (FMOC) chemistry and analyzed through HPLC and mass spectrophotometry at the University of Florida Interdisciplinary Center for Biotechnology Research. Crude peptides were lyophilized. Peptides were reconstituted in water to 1mg/ml and stored at -80C. To improve solubility, Tt455 was reconstituted with 5% dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO), and Tt616 was reconstituted with 3% DMSO.

Monoclonal Antibody

The biotinylated monoclonal antibody (mAb) HL1912, specific for the γ heavy chain of *T. truncatus* IgG, was used as a secondary antibody. The derivation, evaluation, and validation of HL1912 specificity for *T. truncatus* IgG has been described in detail (Nollens et al., 2007).

Optimization of ELISA Parameters

The positive serum sample used was from the index dolphin case 13 days after fecal shedding was detected (28 February 2007). Negative control serum samples were collected from the index animal one week prior to fecal virus shedding (8 February 2007) as well as an immunologically naive neonate bottlenose dolphin that had not yet nursed, and no-serum negative controls were used to optimize the assay signal. All assay parameters were varied (working volume: 50 and 100 µl; peptide concentration: 0.3, 0.5, 1, 5, and 10 µg/ml; serum dilution: 1:50, 1:100, 1:250, and 1:500; HL1912 mAb concentration: 5 µg/ml and 7.5 µg/ml; streptavidin-alkaline phosphatase (AP) dilution:

1:500 and 1:1,000; developing time: 15, 30, 45, and 60 min), and the assay conditions with the highest ratio of optical density at 405 nanometers (OD_{405}) of the sample to OD_{405} of the negative control were chosen.

Peptide Indirect ELISA

Serum samples from 146 dolphins were assayed with the ELISA using peptide antigens. Wells of high protein binding microplate (Nunc Maxisorp, Fisher Scientific, Pittsburgh, PA) were coated with 50 µl of a mixture of peptides Tt322, Tt399, Tt455, and Tt616 in PBS, each at 1 µg/ml, and left to adsorb overnight at 4°C. After this and each subsequent step, all wells were washed three times with phosphate buffered saline (PBS) with 0.05% Tween 20 using an automated ELx405 microplate washer (Biotek Instruments, Winooski, VT). Each subsequent step of the ELISA was incubated with gentle agitation (Nutator; Adams, Fisher Scientific, Pittsburgh, PA) for 1 hr at room temperature. After washing, all wells were blocked with 300 µl of Superblock® blocking buffer (Pierce, Rockford, IL) in PBS and incubated. Dolphin sera were applied in triplicate (1:50 in 1% bovine serum albumin [BSA] in PBS) and incubated. A no-serum negative control and one positive control serum were included on each plate. Biotinylated HL1912 monoclonal antibody (mAb) was added at a concentration of 5 µg/ml (in 1% BSA in PBS) as the reagent for the detection of bound antibodies and incubated. Streptavidin-AP was used as the secondary detection reagent (1:500 in 1% BSA) and incubated. Finally, 1.0 $mg.ml^{-1}$ P-Nitrophenyl Phosphate (PNPP; Sigma, St. Louis, MO) substrate was added and the absorbance at 405 nM (OD_{405}) was recorded after 60 minutes using a Synergy HT microplate reader (Bio-Tek, Winooski, VT). Triplicate OD_{405} readings for each sample were averaged. For analysis, the average

OD_{405} of the 1% BSA negative control was subtracted from the average OD_{405} of all other samples.

Initial Validation

Correlation of ELISA results for 46 bottlenose dolphin serum samples using peptides Tt322 and Tt616 as antigen versus ELISA results for the same data set using peptides Tt399 and Tt455 as antigen was examined. The samples were run as described above and the OD_{405} values from the Tt322/Tt616 ELISA and Tt399/Tt455 ELISA were compared by Spearman's nonparametric correlation test using inStat (GraphPad Software, San Diego, CA).

Further Validation

Due to relatively low OD_{405} values and potential concerns about sufficiency of these small peptides as antigens, further validation of the peptide ELISA was pursued. As controls, the amino acid composition was maintained to keep hydrophobicity/mass the same, but the amino acid order was scrambled to remove conformational recognition. The control peptides were: Tt322scr (VVEVVSKAIQAD), Tt399scr (KNNMQALTPQW), Tt455scr (LSLWPYLWAAEQ), and Tt616scr (LVWSILDMV). Peptides were synthesized using FMOC chemistry and analyzed through HPLC and mass spectrophotometry at the University of Florida Interdisciplinary Center for Biotechnology Research. Crude peptides were lyophilized. Peptides were reconstituted in water to 1mg/ml and stored at -80C. For consistency, Tt455scr was reconstituted with 5% DMSO to improve solubility as Tt455 had been, and Tt616scr was reconstituted with 3% DMSO as Tt616 had been.

Correlation of ELISA results for the same 46 bottlenose dolphin serum samples used in the initial validation was examined, using peptides Tt322 and Tt616 as antigen

versus ELISA results for the same data set using peptides Tt399 and Tt455 as antigen was examined. The samples were run as described above. The OD_{405} values from the Tt399/Tt455 ELISA vs. Tt399scr/Tt455scr iELISA, and the Tt322scr/Tt616 scr ELISA vs. Tt399/Tt455 ELISA were compared by Spearman's nonparametric correlation test using inStat.

Results

Initial Validation

OD_{405} values for the 46 samples using the Tt322/Tt616 ELISA and Tt399/Tt455 ELISA are displayed in blue on figure 3-2 and in Appendix C. The r value for the Spearman's nonparametric correlation of the Tt322/Tt616 ELISA and Tt399/Tt455 ELISA was 0.6537, with a 95% confidence interval of 0.4414 to 0.7967. The two-tailed P value was <0.0001, indicating that these values correlate strongly. This correlation is expected if antigenic response to these two pairs of peptides represent immune responses to the same agent.

Optimization of ELISA Parameters

The highest ratio of the OD_{405} of sample to negative control was seen with a working volume of 50 µl; peptide concentration of 1µg/ml; serum dilution of 1:50; HL1912 mAb concentration of 5 µg/ml, a streptavidin-AP dilution of 1:500, and a developing time of 60 min.

Peptide Indirect ELISA

Results of the peptide indirect ELISA on 146 dolphin sera are presented in Appendix D. Time series were run on 7 selected dolphins. Dolphins were selected because they had multiple time points available.

Further Validation

OD_{405} values for the 46 samples using the Tt399scr/Tt455scr ELISA are displayed in Appendix C. The corresponding OD_{405} values between the ELISA assay using peptides Tt399scr/Tt455scr and the ELISA assay using peptides Tt399/Tt455 are shown in red on figure 3-2. The corresponding OD_{405} values between the ELISA assay using peptides Tt322scr/Tt616scr and the ELISA assay using peptides Tt399/Tt455 are shown in yellow on figure 3-2. The r value for the Spearman's nonparametric correlation of the Tt399/Tt455 ELISA and Tt399scr/Tt455scr ELISA was 0.4815, with a 95% confidence interval of 0.2138 to 0.6820. The two-tailed P value was 0.0007, indicating that these values correlate strongly. The r value for the Spearman's nonparametric correlation of the Tt322/Tt616 ELISA and Tt322scr/Tt616scr ELISA was 0.7431, with a 95% confidence interval of 0.5713 to 0.8525. The two-tailed P value was < 0.0001, indicating that these values correlate strongly. The r value for the Spearman's nonparametric correlation of the Tt322/Tt616 ELISA and Tt399scr/Tt455scr ELISA was 0.6289, with a 95% confidence interval of 0.4068 to 0.7808. The two-tailed P value was < 0.0001, indicating that these values correlate strongly. The r value for the Spearman's nonparametric correlation of the Tt399/Tt455 ELISA and Tt322scr/Tt616scr ELISA was 0.5371, with a 95% confidence interval of 0.2842 to 0.7201. The two-tailed P value was 0.0001, indicating that these values correlate strongly.

Discussion

The relatively low OD_{405} values seen in this assay raised doubts about assay validity. The correlation of the results of the scrambled peptide assays with the specific peptide assays is expected if antigenic response is nonspecific. One plausible explanation for this is that we failed to identify suitable antigenic epitopes for the virus.

There is little comparative structural data for astroviruses, and none exists for BDAstV1. The studies on antibody epitopes of astroviruses are very limited, and while two of the peptides designed were within a region homologous to a 93 amino acid segment shown to be an antibody epitope (Matsui et al., 1993), the antigenic portion may have been missed, or the BD dolphin may not have homologous epitopes. Alternatively, the short oligopeptides may not have developed the appropriate conformation that enabled antibody recognition of the longer native BDAstV1 capsid protein, as has been seen with other oligopeptide ELISA assays (Plagemann, 2001). In conclusion, this oligopeptide ELISA assay does not appear to be useful for detecting humoral immune response of bottlenose dolphins to BDAstV1.

```
HumAst1_BA MASKSNKQVT VEVSNNGRNR SKSRARSQSR GRDKSVKITV NSKNR--TRR
TtAstVcaps MANDRSKDVS VEVKASGSQR SKSRSRSRSR GRTPAVKVTV NSKAKRFTRR

QSGRGKHQSS QRVRNIVNKQ LRKQGVTGPK PAICQRATAT LGTVGSNTSG TTEIEACILL
PSGRSFRAKN NSVKQQVRNQ LKKQGLTGPA PAVVQTATAT LGTIGPNTGN DAEREISFYL

NPVLVKDATG STQFGPVQAL GAQYSMWKLK YLNVKLTSMV GASAVNGTVL RVSLNPTSTP
NPALTKENTG SNAFGPVQAL AAQYSMWRCS RAEIRFTPLI GPSAISGTAY RCSLNMAGTP

SSTSWSGLGA RKHLDVTVGK NATFNLKPSD LGGPRDGWWL TNTKDNASDT LGPSMEIHTL
SQTSWSGLGS RKHKDMHIGK SGSFKLTKKE LSGPKETWWL TNTNEEGGQT LGPAVEIHSI

GRTMSSYEN- --EQFTGGLF LVELASEWCF TGYAANPNLV NLVKSTDKQV SVTFEGSAGS
GKTVRVFTSQ TGQTYDGPVF LVELRATWEF ANFSANPGLV ALEKGED-TA RINFSGNIGE

PLIMNVPEGS HFARTVLARS TTPTTLARAG ERTTSDTVWQ VLNTAVSAAE LVTPPPFNWL
PLVMKVTGSS DFHARMMRVM GDDATYTRTG EIKASEVIVQ VVDAGTDIIS -STVPGFGWL

VKGGWWFVKL IAGRTRTGTR SFYVYPSYQD ALSNKPALCT GSTPGGMRTR NPVTTTLQFT
IKAGWFFIKK LAGLSRNGDG EYAVYASYAD AQNNRPCI-- --LPSTVTDV TPKPTTLAWQ

QMNQPSLGHG EAPAAFGRSI PTPGE-EFKV ILTFG----- ---------- APMSPNANNK
QMNKPNLGLE TGSYAMSRSM PVPVEGSYKA ILQLDNYAQM IHQLQADYPR PALAPYWQSL

QNWVNKPLDA PSGHYNVKIA K--DVDHYLT MQGFTSIASV DWYTTDFQPS EAPAPIQGLQ
ELWVGKGNDY GGGQHADRIT QVYKVNRGVF LNQF------ ------YDQV MQPEPTLGYS

VLVNSS--KK ADVYAVKQFV TAQTNNKHQV T------TLF LVKVTTGFQV N-NY--LSYF
IFSNTSHGKI GEVLGFQSYH MPGPTESGAE SLGPVAFNVY LGRITLSSKW SVQYKDTAYF

YRASASGDAT TNLLVRGDTY TAG---ISFT Q--------- ---------- VGWYLLTNTS
PRASDEWGKS AAVMMK-DWY GLGPPKPSYT TDGNGVVKPP RFDPDDMVLL ISWVRFSEGR

IVDGALP-PG WIWNNVELKT NTA------- ---------- ----YHMDKG LIHLIMPLPE
NTD--LPVDK WCNTAMDYTY NVTVSGRKLA RDGQVRVPAG VPYWYYQDVQ TINGSDPVVQ

STQMCYEMLT SIPRSRAAGY GYE----SDN TEYLDAPD-F ADQLREDIET DTHIETTEDE
NRESIFEFET EVPVQRSALL SLKKSAPSRA VKYDEEEEVY YTTLPKQGPP TAPWRMVEDD

DDEADRF--- -----DIIDT SDEEDENETD RVTLLSTLVN QGMTMTRATR IARRAFPTLT
DHDSDSSYWD NDMSDDDFES SEEEELQDVD VDVLANTLEN SGFTRKEA-- ----------

DRIKRGVYMD LLVSGASPNS AWSHACEEAR KAAGEINPCT SGSRGHAE
---------- ---------R AYAQAARDAV LKDGPTAKTV KFSDAPQE
```

Figure 3-1 MUSCLE alignment of Human Astrovirus 1 and BDAstV1 capsid amino acid sequences. Yellow = potential RNA binding domain (inside of capsid, likely not antigenic), Green = Caspase cleavage motif, Red = potential trypsin cleavage site, separating VP34 analogue, Italics = antigenic region cloned by Matsui et al.(1993), Bold = selected peptides

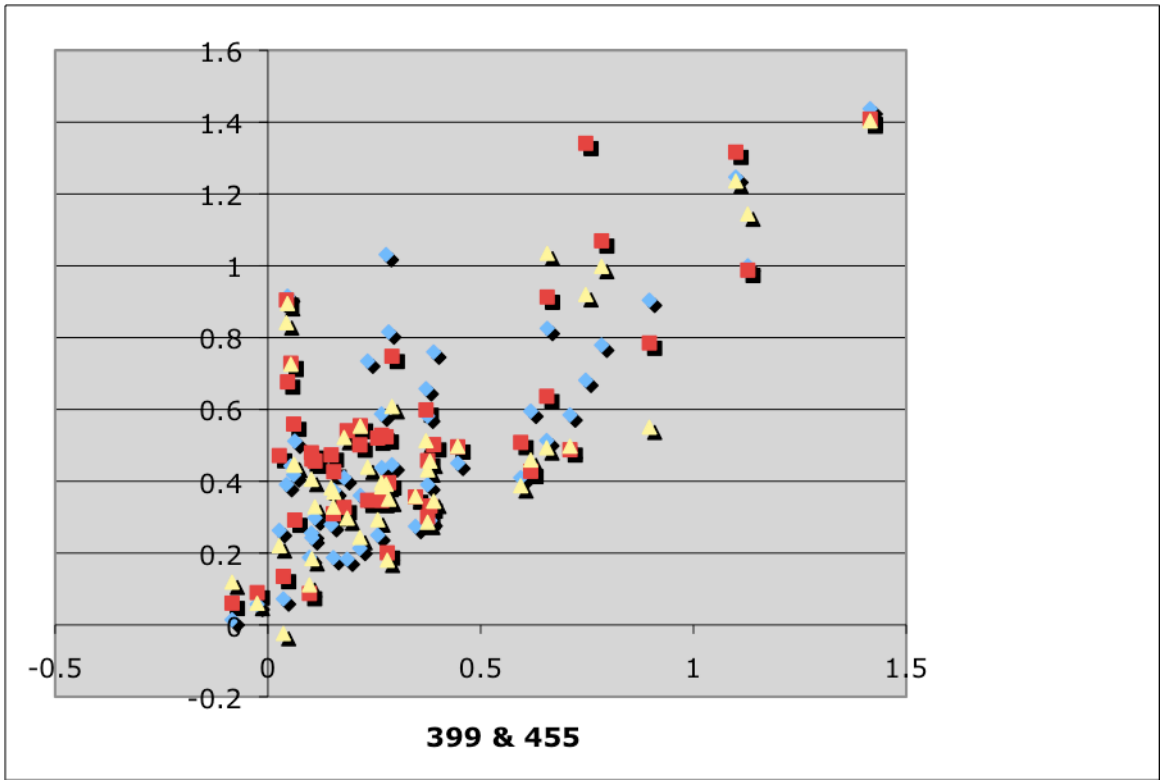


Figure 3-2. Correlation of OD_{405} values between different pairs of peptides. Blue dots represent corresponding OD_{405} values between ELISA assay using peptides Tt322/Tt616 and ELISA assay using peptides Tt399/Tt455. Red dots represent corresponding OD_{405} values between the ELISA assay using peptides Tt399scr/Tt455scr and the ELISA assay using peptides Tt399/Tt455. Yellow dots represent corresponding OD_{405} values between the ELISA assay using peptides Tt322scr/Tt616scr and the ELISA assay using peptides Tt399/Tt455.

CHAPTER 4
USE OF A RECOMBINANT CAPSID PROTEIN- INDIRECT ELISA FOR THE DETECTION OF A HUMORAL IMMUNE RESPONSE TO BOTTLENOSE DOLPHIN ASTROVIRUS 1

Introduction

Astroviruses are small round nonenveloped viruses with a positive stranded RNA genome. They were relatively recently discovered, and were first reported in 1975 (Madeley and Cosgrove, 1975). The family Astroviridae is divided into two genera, *Avastrovirus*, found in avian hosts, and *Mamastrovirus*, found in mammal hosts (Monroe et al., 2005). Human astrovirus is a significant cause of enteric disease in human children (Dennehy et al., 2001). We have recently reported the discovery of diverse astroviruses in marine mammals, including Bottlenose Dolphin Astrovirus 1 (BDAstV1) (Rivera et al., 2010).

As a prevalent enteric disease in children, seroconversion to Human Astrovirus at a young age is typical. An early study of a human astrovirus in Oxford, England found that 7% of 6 to 12 month olds had a positive titer, whereas 75% of 5 to 10 year olds were positive (Kurtz and Lee, 1978). A study in London found that over 50% of children between 5 and 12 months of age had antibody responses to *Human astrovirus 1* (HAstV1), and seroprevalence was 90% by the age of 5 (Kriston et al., 1996). In the Netherlands, age seroprevalence of 7 different human astrovirus serotypes was examined; seroprevalence of HAstV1 was 100% by 5 years of age, and although overall seroprevalence was lower for human astroviruses 2-4, the age of conversion was similar (Koopmans et al., 1998). However, Koopmans et al.found a later age of onset for HAstV5. A study in Virginia found that the seroprevalence of HAstV1 decreased from 67% in infants <3 months of age to 7% by 6 to 8 months of age, consistent with

loss of transplacental antibodies, followed by a marked increase to 94% at 6 to 9 years of age (Mitchell et al., 1999). Mitchell et al. found a similar age distribution but lower seroprevalence for HAstV3. A study in Japan found that by age 3, seropositivity to HAstV1 and HAstV3 approached 100% (Kobayashi et al., 1999).

Attempts at culture of BDAstV1 were unsuccessful, so alternate means of obtaining antigen for serological testing were needed. The capsid (ORF2) contains the characterized neutralizing antibody epitopes in astroviruses. There are experimental data from human astroviruses suggesting that protease cleavage cuts the capsid protein into an early section forming the main capsid (called VP32 or VP34, VP38.5, or VP41 at different stages of processing by different investigators, hereafter called main capsid), and a later section forming the projections (known as VP25, VP26, VP27, VP28 or VP29 at different stages of processing by different investigators, hereafter called arm), giving the “star” shape. Trypsin cleavage is important in processing of the arm (Méndez et al., 2002). Caspase cleavage of the carboxy-terminal part of the protein is probably involved with release of the virus from the cell (Méndez et al., 2004), and conserved caspase sites suggest this is also likely for other astroviruses. This region is likely cleaved off before exit from the cell, and is not likely to be a target for antibodies. The arm projections are likely to be external and most exposed to antibodies. Matsui et al.(1993) found that a clone of the region early after the trypsin cleavage site is recognized by antibodies from infected individuals. In two different studies, the VP26 proteins of HAstV1 and HAstV2 have been found to have neutralizing epitopes (Sánchez-Fauquier et al., 1994, Bass and Upadhyayula, 1997). The BDAstV1 arm region is therefore a good candidate antigen for serodiagnostics.

Materials and Methods

Animals and Sera

Serum samples were taken from 60 bottlenose dolphins from a managed open-water collection in California, 35 animals from three different captive closed-water collections, and 67 wild dolphins from Florida (60 healthy captures and 7 strandings). Multiple time points were taken from 7 dolphins from the managed open-water collection (177 total samples), and multiple time points were examined from an additional 10 calves from the open-water collection (55 total samples). Blood samples were collected from the ventral tail vein, the ventral fluke vein, or if taken at post-mortem examination, via cardiocentesis. Samples were typically collected using a 20 or 21 gauge 1.5 inch Vacutainer® needle (Becton Dickinson VACUTAINER Systems, Rutherford, New Jersey 07070) and blood was collected into a Vacutainer® serum separator tube or a Vacutainer® without anticoagulant. The blood samples were centrifuged at 3,000 rpm at 21°C for 10 minutes. Fibrin clots were removed and serum was transferred to cryovials. Archived sera were stored at -80°C.

BDAstV1 Antigen

The antigen for expression was selected from the predicted capsid protein of BDAstV1. Primers TtAst1spkF1 (ACTGTCCCAGGATTTGGATG) and TtAst1spkR1 (CCACATCCTGCAGTTCCTCT) were designed to amplify the region of BDAstV1 expected to form the outer projections, between the predicted trypsin cleavage site and the predicted caspase cleavage site (Figure 4-1). A 20 µL reaction was used that included 1µL each of forward and reverse primers at 20µM, 2 µL 10x Buffer, 0.8 µl 50mM $MgCl_2$, 0.4 µl 10mM dNTP, 0.5 units polymerase (Platinum *Taq* DNA polymerase, Invitrogen, Carlsbad, CA, United States of America [USA]), 11.7 µL sterile

water, and 3 µl of complementary deoxyribonucleic acid (cDNA) template from 3'RACE amplifications of BDAstV1 as template (Rivera et al., 2010). The mixture was amplified in a thermal cycler (PCR Sprint, Thermo Hybaid, Franklin, MA) with an initial denaturation step at 95°C for 5 minutes, followed by 45 cycles of denaturation at 95°C for 30 sseconds, annealing at 54°C for 30 seconds, and extension at 72°C for 90 seconds, and a final extension at 72°C for 10 min. The PCR product was resolved in 1% agarose. The band was excised and purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA, USA). To confirm product identity, Sanger sequencing was performed directly on an aliquot using the Big-Dye Terminator Kit (Applied Biosystems, Foster City, CA) and analyzed on an ABI 3130 automated DNA sequencer at the University of Florida Interdisciplinary Center for Biotechnology Research Sequencing Facilities to confirm identity. The PCR product was ligated into pDrive plasmid (Qiagen) and cloned into EZ Competent Cells (Qiagen). Individual clones were selected and sequenced as above to confirm identity. The protein was expressed and purified by Genscript Corp., Piscataway, New Jersey, USA. Briefly, the insert had sequence encoding a polyhistidine tag (predicted amino acid sequence MGSSHHHHHHSSGLVPRGSHM) added to the 5' end and was then cloned into an *E. coli* expression vector. The full amino acid sequence is given in figure 4-2. The protein was then expressed and purified. The protein was then run on an SDS-polyacrylamide gel and stained with Coomassie blue to assess purification (Figure 4-3), and further confirmed with western blotting using an anti-polyhistidine-tag antibody (Figure 4-4).

Monoclonal Antibody

The biotinylated monoclonal antibody (mAb) HL1912, specific for the γ heavy chain of *T. truncatus* IgG, was used as a secondary antibody. The derivation,

evaluation, and validation of HL1912 specificity for *T. truncatus* IgG has been described in detail (Nollens et al., 2007).

Optimization of ELISA Parameters

The positive serum sample used was from the index dolphin case (see chapter 2) 13 days after fecal shedding was detected (28 February 2007). Negative control serum samples were collected from the index animal one week prior to fecal virus shedding (8 February 2007) as well as an immunologically naive neonate bottlenose dolphin that had not yet nursed, and no-serum negative controls were used to optimize the assay signal. Assay parameters were varied (protein concentration: 0.1, 0.25, 0.5, 1, 5, and 10 µg/ml; developing time: 10, 15, 20, 30, 45, and 60 min), and the assay conditions with the highest ratio of the OD_{405} of sample to negative control were chosen.

Recombinant Protein ELISA

Serum samples from 146 dolphins were assayed with the recombinant protein ELISA. Wells of high protein binding microplates (Nunc Maxisorp, Fisher Scientific, Pittsburgh, PA) were coated with 50 µl of the cloned protein antigen at 1 µg/ml in PBS and left to adsorb overnight at 4°C. After this and each subsequent step, all wells were washed three times with PBS with 0.05% Tween-20 using an automated ELx405 microplate washer (Biotek Instruments, Winooski, VT). Each subsequent step of the ELISA was incubated with gentle agitation (Nutator; Adams, Fisher Scientific, Pittsburgh, PA) for 1 hr at room temperature. After washing, all wells were blocked with 300 µl of Superblock® blocking buffer (Pierce, Rockford, IL) in phosphate buffered saline (PBS) and incubated. Dolphin sera were applied in triplicate (1:50 in 1% BSA in PBS) and incubated. A no-serum negative control and one positive control serum were included on each plate. Biotinylated HL1912 mAb was added at a concentration of 5

µg/ml (in 1% BSA in PBS) as the reagent for the detection of bound antibodies and incubated. Streptavidin-AP was used as the secondary detection reagent (1:500 in 1% BSA) and incubated. Finally, 1.0 $mg.ml^{-1}$ P-Nitrophenyl Phosphate (PNPP; Sigma, St. Louis, MO) substrate was added and the absorbance at 405 nM (OD_{405}) was recorded after 15 minutes using a Synergy HT microplate reader (Bio-Tek, Winooski, VT). Triplicate OD_{405} readings for each sample were averaged. For analysis, the average OD_{405} of the 1% BSA negative control was subtracted from the average OD_{405} of all other samples.

Comparisons between populations were analyzed using inStat (GraphPad Software, San Diego, CA). For statistical comparison of populations, average values were used for animals with more than one time point. Correlation with age was analyzed using SAS (SAS Institute Inc., Cary, NC).

Results

BDAstV1 Antigen

Of four PCR amplicon clones selected that were sequenced, one was 100% identical with the reference sequence and was selected for expression. The other three had 1 or 2 nucleotide substitutions resulting in 0 to 2 predicted amino acid changes. No substitutions were shared by any of the clones. The predicted molecular mass of the protein was 45176.7 daltons, and the purified expressed protein was consistent with this (Figures 4-3 and 4-4).

Optimization of ELISA Parameters

The highest ratio of the optical density at 405nm (OD_{405}) of sample to negative control was seen with a protein concentration of 1 µg/ml. A developing time of 15 minutes was chosen because of the high ratio of OD_{405} of sample to negative control

and overloading of the limits of reader detection for some samples at longer times. At these parameters, the post-shedding index case OD_{405} was 4.8 times that of the pre-shedding index case OD_{405}, and 114.5 times that of the pre-nursing neonatal dolphin.

Recombinant Protein ELISA

The values of subpopulations (67 wild dolphins, 60 mature animals from the open-water managed collection, 10 calves from the open-water managed collection, and 35 animals from three different captive closed-water collections) were analyzed for normality using the Kolmogorov-Smirnov test (Dallal and Wilkinson, 1986). All populations except calves were found to have non-Gaussian distributions. The mean, median, range, and quartiles of OD_{405} values of the groups of animals are given in table 4-1. The Kruskal-Wallis test with Dunn's post test was used to compare groups. The calves (median value 0.39) were significantly lower than all other groups, with $P<0.001$. No differences were identified (defined by $P>0.05$) between wild animals (median value 1.27) and open-water mature animals (median value 1.05). The values from calves (median value 0.38) were significantly lower than open-water mature animals ($P<0.01$), wild animals ($P<0.001$), and closed system animals (median value 0.94, $P<0.05$). The values from closed system animals were also lower than those from wild dolphins ($P<0.05$). Of wild animals examined, the only two animals with values less than 0.5 were both calves.

Time series of mature open-water collection animals and of calves are given in figures 4-5 and 4-6. Animals 1 and 20 show significant increases in titer at the same time in Winter 2007. There were no significant linear associations with age in mature animals ($R^2=0.10$). Amongst calves, there was a weak linear association with increasing age ($R^2=0.28$, $P < .0001$) (Figure 4-7). Animal 168 also shows a significant

increase in titer in Winter 2007.There were several individual calves with strong associations between age and BDAstV1 antibody levels (Figure 4-8).

Discussion

Our results suggest that, similar to HAstV1 in humans, BDAstV1 exposure in bottlenose dolphins is near-ubiquitous and cosmopolitan. Wild dolphins from the Atlantic oceans and open-water collection animals from the Pacific had significantly higher titers than calves. Titers in closed-water collection animals were lower than wild animals but still significantly higher titers than calves, despite smaller populations in which to maintain virus and the use of ozone and UV sterilization to maintain excellent water quality. Astroviruses are very stable in aquatic environments (Espinosa et al., 2008). Surveys have found a Human astrovirus prevalence of up to 61% in some marine shellfish populations, which are good particle concentrators (Elamri et al., 2006). Wide diversity has been seen in marine mammal astroviruses, implying that the marine environment may play a large role in astroviral ecology (Rivera et al., 2010), and the finding of high prevalence further supports that. The marine environment is central in the ecology of caliciviruses, a better studied group of small nonenveloped positive stranded RNA viruses (Smith et al., 1998).

Clinical diagnosis of astroviral disease is challenging. Astrovirus capsid protein interacts with apical enterocyte membranes, increasing permeability independent of viral replication. Much like *Vibrio cholerae*, astroviruses cause a secretory diarrhea without much of a histologic footprint on enterocytes on light microscopy (Koci et al., 2003, Moser et al., 2007, Nighot et al., 2010). Astrovirus culture is also challenging, with few cell lines capable of supporting Human astrovirus and a high requirement for trypsin (Taylor et al., 1997). Attempts at culture of marine mammal astroviruses to date have

been unsuccessful. Astroviruses resemble other small round viruses somewhat morphologically, and a significant rate of misidentification using negative-staining electron microscopy of feces has been reported (Oliver and Phillips, 1988). Negative-staining electron microscopy of feces for virus detection has also been shown to be comparatively insensitive (van Niewstadt et al., 1988). Histopathology, culture, and negative-staining electron microscopy are therefore insensitive tests for diagnosis of astroviral diarrhea. More sensitive techniques such as PCR are not yet widely applied to marine mammals, leading to probable underdiagnosis of astrovirus infections.

Clinical assessment of diarrhea in dolphins is also challenging. Dolphins normally have poorly formed stool that is excreted in water, making it is difficult to observe and to assess. A secretory diarrhea would not be observed as reliably as it would in a terrestrial animal.

Previous immune exposure may have significant effects on astroviral disease. Human astrovirus is a neonatal disease and has an even lower age of median infection amongst hospitalized children than rotavirus (Dennehy et al., 2001). One study found that 41 of 603 young children with diarrhea (6.8%) had human astrovirus in their feces as determined by antigen ELISA, whereas none of 141 control children did (Dennehy et al., 2001). In a study of naïve turkey poults, 100% of naive turkey poults inoculated with Turkey astrovirus 2 animals exhibited diarrhea by 3 days post-infection. However, experimental infections in volunteer human adults found that only one of eight who ingested Human astrovirus developed diarrhea and vomiting, with a second volunteer developing abdominal discomfort (Kurtz et al., 1979) A second experiment in the same report found that five of nine volunteers ingesting fecal filtrate from the earlier volunteer

developed abdominal discomfort and an increase in antibody titer. Volunteers with no pre-existing anti-Human astrovirus antibodies developed more severe symptoms and were more likely to shed virus (Kurtz et al., 1979). Another study found that none of 17 adult volunteers ingesting a smaller amount of HAstV5 developed clinical signs, and one of 2 volunteers ingesting a larger amount developed vomiting and diarrhea (Midthun et al., 1993). The volunteer developing clinical signs did not have a detectable antibody titer prior to the experiment and had a very high titer following the experiment. Eight of the 19 volunteers showed a fourfold or greater increase in antibody titer. The lower rate of disease in adults combined with the very high seroprevalence in age groups beyond early childhood suggest that acquired immune responses are a significant complicating factor in deciphering the clinical significance of astrovirus infections.

While several of the calves showed strong associations between age and BDAstV1 antibody levels, two of the calves (170 and 171) showed an initial decrease in antibody followed by an increase. This may represent loss of maternal antibodies followed by exposure, as has been seen with HAstV1 in humans by Mitchell et al.(1999).

Examination of the mature animal time series shows that the trends of two of the animals (9 and 31) largely parallel each other, although these animals were housed on different piers. Dolphin 55 shows a moderate titer followed by a persistent elevation for over two years, from December 2005 until April 2008. This elevation coincides temporally with viral shedding by this animal as detected by qPCR (see BDAstV1 qPCR chapter), suggesting persistent infection in this animal. This contrasts with the briefer

elevation seen in animal 1. Persistent infection has been seen with astrovirus infection in humans; persistent gastroenteritis was seen with 8.5% of astrovirus infections in children in Spain (Caballero et al., 2003). A study of children in Bangladesh found that Human astrovirus was more commonly associated with persistent diarrhea than acute diarrhea (Unicomb et al., 1998). Animal 55 may have served as a source of infection for other animals. Animal 1 showed a marked increase in titer between 15 February 2007 and 20 February 2007. Animal 20 showed a marked increase in titer between 25 October 2006 and 23 March 2007. Calf 168 showed a marked increase in titer between 3 January 2007 and 16 March 2007. All three show marked increases in antibody titer in late winter of 2007, indicating a possible common source of infection. Animal 55 was moved into closer contact with animal 20 and calf 168 in late December 2006. Identification of persistently shedding animals may enable management to keep them separate from young calves, where the risk of disease is likely most significant.

In conclusion, this assay is useful for determining humoral immune response of bottlenose dolphins to BDAstV1. Prevalence of exposure appears to be very high and is geographically widespread, similar to Human astrovirus in humans. Both transient and persistent infections with BDAstV1 appear to occur in dolphins. Based on the observed parallels to human astrovirus infection, BDAstV1 is more likely to be clinically significant in calves than other age groups, and this is where management and future investigations should focus.

Table 4-1. Summary of OD_{405} - control values for groups of dolphins

	Mature open-water collection	Calves open-water collection	Wild	Closed-water collection
Mean	1.19	0.48	1.43	1.03
Median	1.05	0.38	1.27	0.94
Range	0.26-2.82	0.15-1.05	0.07-2.94	0.41-2.26
1st quartile	0.7425	0.29	0.995	0.685
3rd quartile	1.4975	0.6475	1.735	1.18

```
HumAst1_BA MASKSNKQVT VEVSNNGRNR SKSRARSQSR GRDKSVKITV NSKNR--TRR
BDAstV1cap MANDRSKDVS VEVKASGSQR SKSRSRSRSR GRTPAVKVTV NSKAKRFTRR

QSGRGKHQSS QRVRNIVNKQ LRKQGVTGPK PAICQRATAT LGTVGSNTSG TTEIEACILL
PSGRSFRAKN NSVKQQVRNQ LKKQGLTGPA PAVVQTATAT LGTIGPNTGN DAEREISFYL

NPVLVKDATG STQFGPVQAL GAQYSMWKLK YLNVKLTSMV GASAVNGTVL RVSLNPTSTP
NPALTKENTG SNAFGPVQAL AAQYSMWRCS RAEIRFTPLI GPSAISGTAY RCSLNMAGTP

SSTSWSGLGA RKHLDVTVGK NATFNLKPSD LGGPRDGWWL TNTKDNASDT LGPSMEIHTL
SQTSWSGLGS RKHKDMHIGK SGSFKLTKKE LSGPKETWWL TNTNEEGGQT LGPAVEIHSI

GRTMSSYEN- --EQFTGGLF LVELASEWCF TGYAANPNLV NLVKSTDKQV SVTFEGSAGS
GKTVRVFTSQ TGQTYDGPVF LVELRATWEF ANFSANPGLV ALEKGED-TA RINFSGNIGE

PLIMNVPEGS HFARTVLARS TTPTTLARAG ERTTSDTVWQ VLNTAVSAAE LVTPPPFNWL
PLVMKVTGSS DFHARMMRVM GDDATYTRTG EIKASEVIVQ VVDAGTDIIS -STVPGFGWL

VKGGWWFVKL IAGRTRTGTR SFYVYPSYQD ALSNKPALCT GSTPGGMRTR NPVTTTLQFT
IKAGWFFIKK LAGLSRNGDG EYAVYASYAD AQNNRPCI-- --LPSTVTDV TPKPTTLAWQ

QMNQPSLGHG EAPAAFGRSI PTPGE-EFKV ILTFG----- ---------- APMSPNANNK
QMNKPNLGLE TGSYAMSRSM PVPVEGSYKA ILQLDNYAQM IHQLQADYPR PALAPYWQSL

QNWVNKPLDA PSGHYNVKIA K--DVDHYLT MQGFTSIASV DWYTTDFQPS EAPAPIQGLQ
ELWVGKGNDY GGGQHADRIT QVYKVNRGVF LNQF------ ------YDQV MQPEPTLGYS

VLVNSS--KK ADVYAVKQFV TAQTNNKHQV T------TLF LVKVTTGFQV N-NY--LSYF
IFSNTSHGKI GEVLGFQSYH MPGPTESGAE SLGPVAFNVY LGRITLSSKW SVQYKDTAYF

YRASASGDAT TNLLVRGDTY TAG---ISFT Q--------- ---------- VGWYLLTNTS
PRASDEWGKS AAVMMK-DWY GLGPPKPSYT TDGNGVVKPP RFDPDDMVLL ISWVRFSEGR

IVDGALP-PG WIWNNVELKT NTA------- ---------- ----YHMDKG LIHLIMPLPE
NTD--LPVDK WCNTAMDYTY NVTVSGRKLA RDGQVRVPAG VPYWYYQDVQ TINGSDPVVQ

STQMCYEMLT SIPRSRAAGY GYE----SDN TEYLDAPD-F ADQLREDIET DTHIETTEDE
NRESIFEFET EVPVQRSALL SLKKSAPSRA VKYDEEEEVY YTTLPKQGPP TAPWRMVEDD

DDEADRF--- -----DIIDT SDEEDENETD RVTLLSTLVN QGMTMTRATR IARRAFPTLT
DHDSDSSYWD NDMSDDDFES SEEEELQDVD VDVLANTLEN SGFTRKEA-- ----------

DRIKRGVYMD LLVSGASPNS AWSHACEEAR KAAGEINPCT SGSRGHAE
---------- ---------R AYAQAARDAV LKDGPTAKTV KFSDAPQE
```

Figure 4-1 MUSCLE alignment of Human Astrovirus 1 and BDAstV1 capsid amino acid sequences. Yellow = potential RNA binding domain (inside of capsid, likely not antigenic); Green = Caspase cleavage motif; Red = potential trypsin cleavage site; separating VP34 analogue; Italics = antigenic region cloned by Matsui et al.(1993); Bold = cloned protein

1 **MGSSHHHHHH SSGLVPRGSH M**RNGDGEYAV YASYADAQNN RPCILPSTVT DVTPKPTTLA

61 WQQMNKPNLG LETGSYAMSR SMPVPVEGSY KAILQLDNYA QMIHQLQADY PRPALAPYWQ

121 SLELWVGKGN DYGGGQHADR ITQVYKVNRG VFLNQFYDQV MQPEPTLGYS IFSNTSHGKI

181 GEVLGFQSYH MPGPTESGAE SLGPVAFNVY LGRITLSSKW SVQYKDTAYF PRASDEWGKS

241 AAVMMKDWYG LGPPKPSYTT DGNGVVKPPR FDPDDMVLLI SWVRFSEGRN TDLPVDKWCN

301 TAMDYTYNVT VSGRKLARDG QVRVPAGVPY WYYQDVQTIN GSDPVVQNRE SIFEFETEVP

361 VQRSALLSLK KSAPSRAVKY DEEEEVYYTT LPKQGPPTAP WRM

Figure 4-2. Amino acid sequence of cloned BDAstV1 antigen. Added histidine tag is in bold.

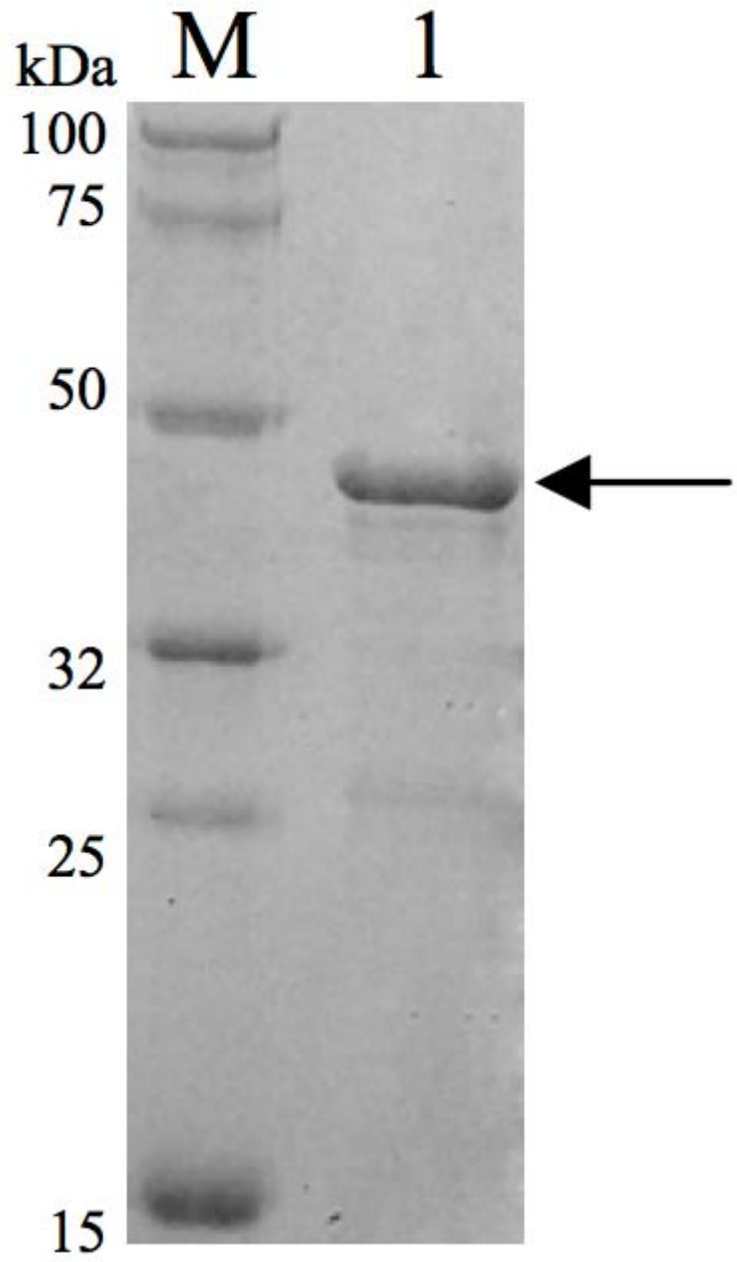


Figure 4-3. Coomassie blue stained SDS-PAGE gel of the expressed BDAstV1 fragment. Markers are given in kilodaltons.

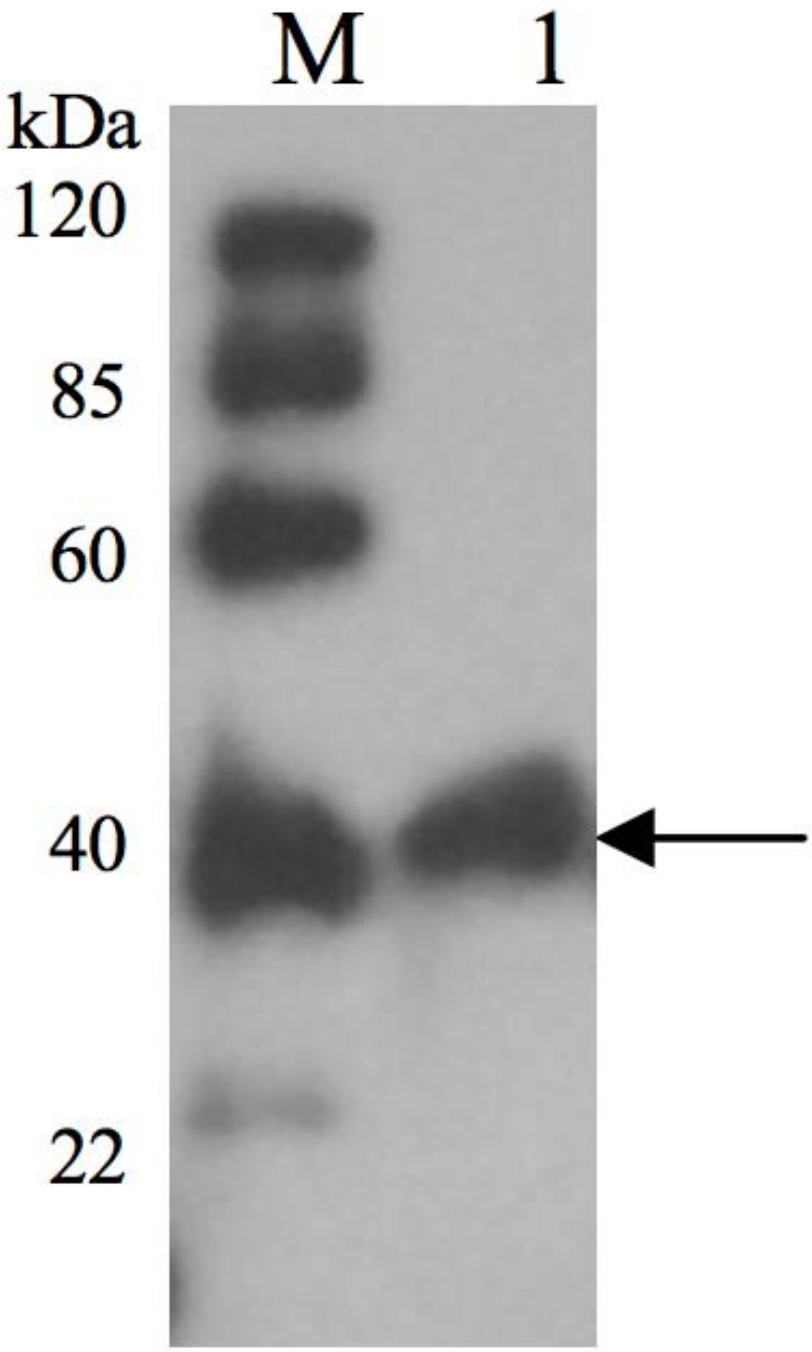


Figure 4-4. Western blot using an anti-polyhistidine antibody. Markers are given in kilodaltons.

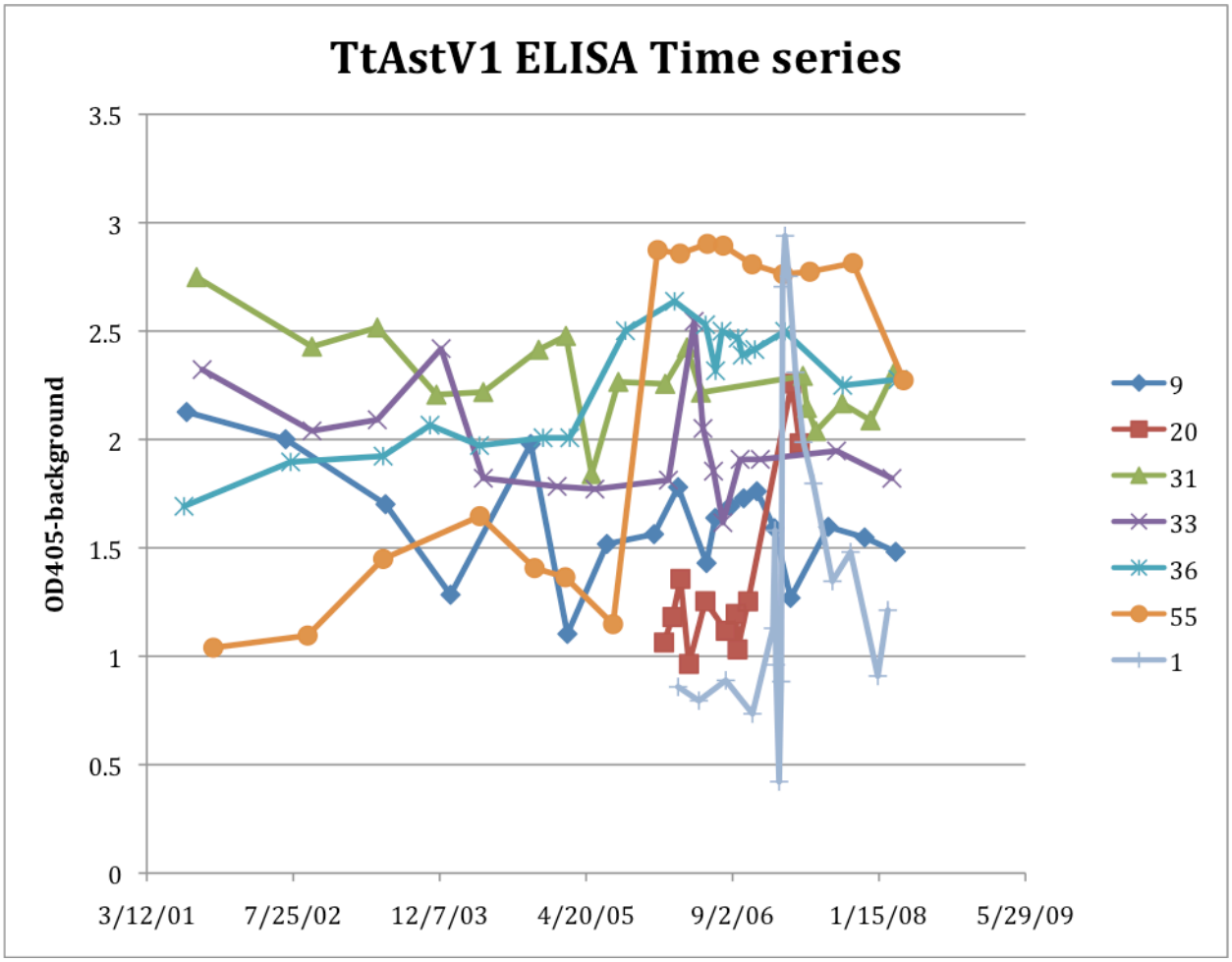


Figure 4-5. ELISA values (OD_{405} – control) of open-water collection mature dolphins over time. Animal ID numbers are given on the right

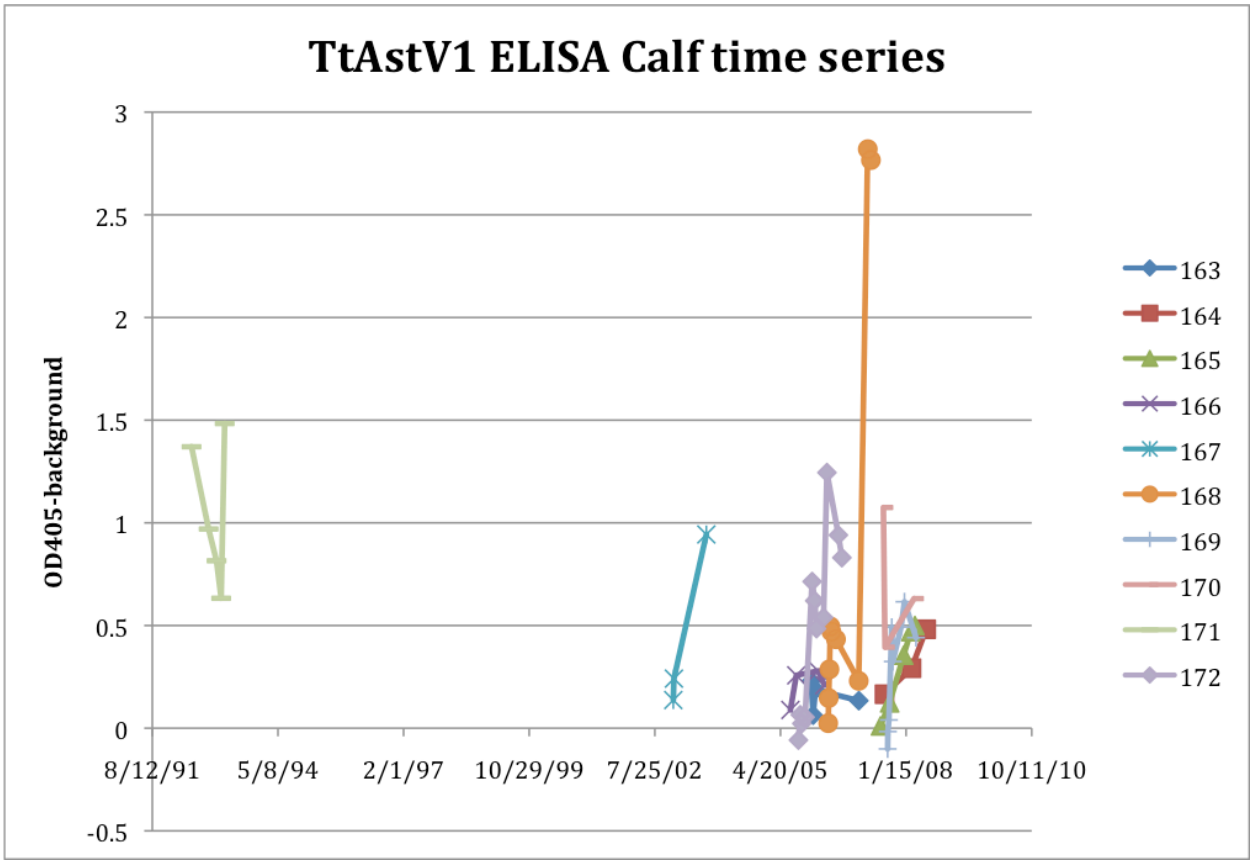


Figure 4-6. ELISA values (OD_{405} - control) of open-water collection calves over time. Animal ID numbers are given on the right

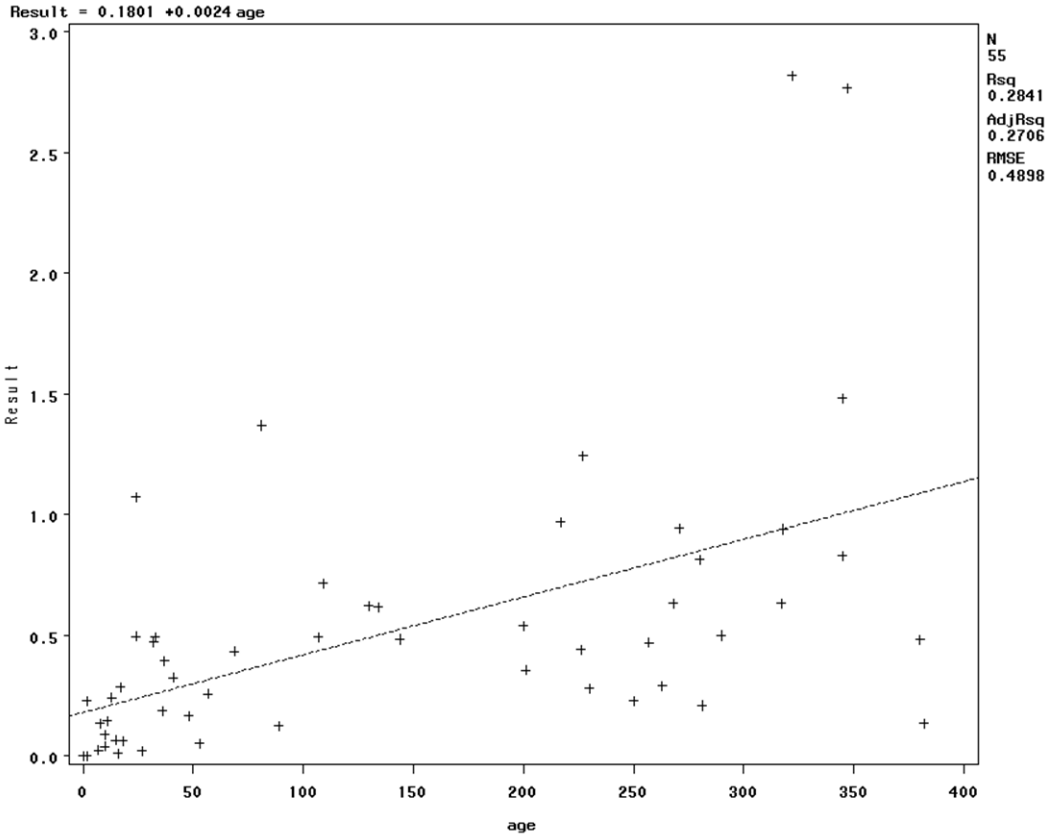


Figure 4-7. ELISA values (OD_{405} - control) vs. days in age among common bottlenose dolphin (*Tursiops truncatus*) calves, including all ten calves.

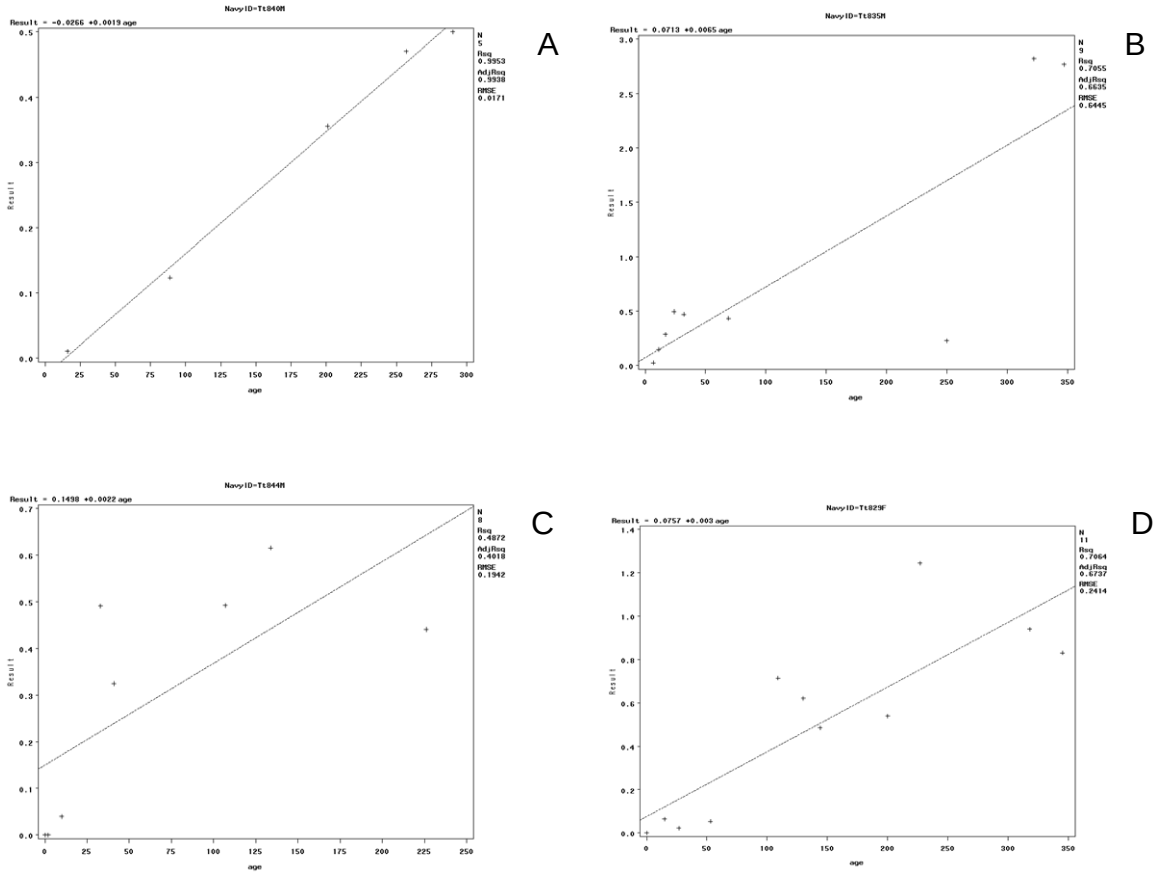


Figure 4-8. ELISA values (OD_{405} - control) vs. days in age among selected individual bottlenose dolphin (*Tursiops truncatus*) calves with significantly increasing titers by days in age. A) calf 165, B) calf 168, C) calf 169, D) calf 172

CHAPTER 5
USE OF A QUANTITATIVE PCR ASSAY FOR THE DETECTION OF BOTTLENOSE DOLPHIN ASTROVIRUS 1

Introduction

Astroviruses are small round nonenveloped viruses with a positive stranded RNA genome. They were relatively recently discovered, and were first reported in 1975 (Madeley and Cosgrove, 1975). The family Astroviridae is divided into two genera, *Avastrovirus*, found in avian hosts, and *Mamastrovirus*, found in mammal hosts (Monroe et al., 2005). Human astrovirus is a significant cause of enteric disease in human children (Dennehy et al., 2001). We have recently reported the discovery of diverse astroviruses in marine mammals, including Bottlenose Dolphin Astrovirus 1 (BDAstV1) (Rivera et al., 2010).

Seroconversion to astrovirus at a young age is typical, and a number of studies have shown that most humans have seroconverted by 5 years of age (Kriston et al., 1996, Kobayashi et al., 1999). Our serological survey of BDAstV1 found that most bottlenose dolphins seroconvert at a young age as well. Given the complications this introduces to serodiagnosis of BDAstV1 infection in populations other than young calves, methods for direct detection of BDAstV1 rather than antibody response have merit.

Quantitative PCR (qPCR, a.k.a. real-time PCR) has been used previously for detection of human astroviruses (Grimm et al., 2004, Royuela et al., 2006, Zhang et al., 2006, Logan et al., 2007, Dai et al., 2010, van Maarseveen et al., in press) and Turkey astrovirus 2 (Spackman et al., 2005) in fecal samples. Quantitative PCR human astrovirus assays have been shown to be significantly more sensitive than culture by more than two orders of magnitude (Royuela et al., 2006). The human qPCR assays

have found prevalences in diarrheic human feces from 6% - 9% (Logan et al., 2007, Dai et al., 2010, van Maarseveen et al., in press). A human astrovirus qPCR assay was used for a study of clinical correlation of virus load to clinical features; a tendency for longer duration of diarrhea with higher copy numbers was seen, and lower copy numbers were associated with rotavirus coinfection (Zhang et al., 2006).

Materials and Methods

Samples

A total of 62 fecal samples were collected from 38 bottlenose dolphins (*Tursiops truncatus*) from a managed open-water collection in California. This was the same managed open-water collection used in the ELISA survey in chapter 3, although samples were not available from all animals surveyed by ELISA. A total of 22 lower gastrointestinal samples were taken from 13 stranded wild cetaceans in New England, including 2 bottlenose dolphins (feces and mesenteric lymph node of each), 2 minke whales (*Balaenoptera acutorostrata*) (colon, duodenum, and feces of one and feces of another), 2 pygmy sperm whales (*Kogia breviceps*) (colon, small intestine, and feces of one and feces of another), 3 short-beaked common dolphins (*Delphinus delphis*) (feces and mesenteric lymph node of one and mesenteric lymph node of the other two), 2 harbor porpoises (*Phocoena phocoena*) (mesenteric lymph nodes), and 2 Atlantic white-sided dolphins (*Lagenorhynchus acutus*) (colon, small intestine, and mesenteric lymph node of one and mesenteric lymph node of another). Samples were stored after collection at -80°C.

RNA Extraction

Samples were maintained on ice at all points during RNA extraction. 0.10g of feces were measured and 900 µl 0.9% NaCl was added to suspend the sample by

vortexing. Samples were spun at 3000 x G for 30 minutes at 4˚C. Supernatant was collected and filtered serially through 800nm, 450nm, and 200nm filters (Millipore, Billerca, MA). Filtrate was concentrated with Microsep concentrator columns (Pall Life Sciences) and centrifuged at 1500 x G for 30 minutes at 4˚C. The concentrated filtrate was used for RNA extraction using a High Pure Viral RNA Kit (Roche, Indianapolis, IN) following the manufacturer's instructions. Tissue samples were cut to 0.10g portions and extracted using RNeasy Mini Kit (Qiagen) following the manufacturer's instructions.

Quantitative PCR

cDNA was synthesized using a MMLV reverse transcriptase kit (Advantage RT-for-PCR, Clontech, Mountain View, CA) using primer TtAstR (CCTGCCATATTCAGGGAACAA) for initial strand synthesis. A BDAstV1 PCR amplicon of the capsid from the index BDAstV1 case, amplified using consensus primers Astr5159F and Astr5819R (Atkins et al., 2009) was used as a positive control for the standard curve, and RNase-free water was used as a negative control. The positive control was quantified by both comparison to a mass ladder standard (Low mass ladder DNA standard, Invitrogen, Carlsbad, CA) on gel electrophoresis as well as spectrophotometry (NanoDrop 8000, Thermo Scientific, Wilmington, DE). The standard curve, run on each plate, used 10-fold serial dilutions, ranging from 10^6 to 10 copies.

Quantitative PCR was performed using forward primer TtAstF (TTGATCGGACCCTCAGCAAT), reverse primer TtAstR, and probe TtAstV-probe (6FAM-AGTGGGACAGCGTATC-MGBNFQ) targeting the Bottlenose dolphin astrovirus 1 (BDAstV1) capsid gene. All samples were run in triplicate and a mean C_t value was calculated. Each 20 µL reaction was 0.9 µM for each primer and 0.25 µM for the probe, and contained 7 µl of extracted DNA and 10 µl of a commercial universal qPCR mix

(TaqMan® Fast Universal PCR Master Mix 2X, Applied Biosystems). A 7500 Fast Real-Time PCR System (Applied Biosystems) was used to amplify the reactions with cycling conditions as follows: initial denaturation at 95 °C for 20 seconds; 50 cycles of 95 °C for 3 seconds followed by 60 °C for 30 seconds. Comparison of years was analyzed using inStat (GraphPad Software, San Diego, CA). Correlation with clinical values, sex, and age was analyzed using SAS (SAS Institute Inc., Cary, NC).

Confirmatory Heminested PCR

Samples with an appropriate curve were confirmed using a BDAStV1-specific heminested PCR and sequencing of PCR products. This confirmatory PCR used forward primer TtAst1SpecF1 (ACCAAATACTGGCAATGATGC) and TtAstR as a first round, and TtAst1SpecF2 (GTTTGGTCCTGTGCAAGCATT) and TtAstR as a second round. For the first round, 3.2 μl of fecal RNA was reverse transcribed using a OneStep RT-PCR Kit (Qiagen, Valencia, CA) at 50˚C for 30 minutes and then denatured at 95˚C for 10 minutes, followed by 45 cycles of denaturation at 95˚C for 30 seconds; annealing at 55˚C for 30 seconds, and extension at 72˚C for 30 seconds, with a final elongation step at 72˚C for 7 min. Two μl of product from the first round was used as template in the 20μl second round. The second round amplification used Platinum Taq DNA Polymerase (Invitrogen) and conditions were as follows: 5 minutes denaturation at 95˚C, followed by 45 cycles of denaturation at 95˚C for 30 seconds, annealing at 55˚C for 30 seconds, and extension at 72˚C for 30 seconds, with a final elongation step at 72˚C for 7 min. PCR products were resolved in 1% agarose gels, excised, and purified using the QIAquick gel extraction kit (Qiagen). Sanger sequencing was performed directly using the Big-Dye Terminator Kit (Applied Biosystems, Foster City, California)

and analyzed on ABI 3130 automated DNA sequencers. Results from the qPCR that were not confirmed by the heminested PCR were considered negative and given as 0.

Results

Quantitative PCR

The BDAstV1 qPCR assay accurately detected 10 to 10^6 cDNA copies. The standard curve for the BDAstV1 qPCR assay had a slope of -3.33 and a correlation coefficient (R^2) of 0.997 (Figure 5-1). Results of the qPCR for the managed open-water collection are given in table 5-1. From the managed open-water collection, 31 of 62 fecal samples (50%) were positive, representing 25 of 38 animals (66%). The mean and median BDAstV1 detected copy numbers were 114 and 1.5 (range 0-5180). There were no significant differences in mean BDAstV1 levels when comparing sex ($P = 0.4$) or linear associations with age ($R^2=0.04$).

The BDAstV1 detected copy numbers found in managed open-water collection samples from 2007 (n=23) and those from 2008 (n=39) were analyzed for normal distribution using the Kolmogorov-Smirnov test (Dallal and Wilkinson, 1986). Both populations were found to have non-Gaussian distributions. For 2007, the mean detected copy number was 303, the median was 54, and the range was 0-5180. For 2008, the mean detected copy number was 3, the median was 0, and the range was 0-44. The Mann-Whitney test was used to compare medians of the different years for significance. The 2007 values were significantly higher than those from 2008, with a two-tailed $P<0.0001$. A plot of detected viral copies detected vs. date is shown (Figure 5-2).

Forty-two fecal samples from 32 animals had paired clinical observations and hematological data. The fecal sample with 5,180 copies detected was a routine sample

collected from a young male dolphin with no abnormal clinical signs identified. Because this count was so high compared to other reported levels (next highest level was 445), this value was not included in comparative analyses involving mean fecal viral levels and clinical parameters.

Animals with abnormal behavior were more likely to have higher mean BDAstV1 copies detected compared to animals with reported normal behavior (Table 5-2). There were no significant differences in mean BDAstV1 copies detected when comparing animals with normal or abnormal appetite or reason for blood sampling (routine or clinical). There were no significant differences in mean serum sodium, potassium, or chloride levels when comparing animals with or without BDAstV1 in their feces (Table 5-3).

Results of the qPCR for the wild cetaceans are given in table 5-4. In total, BDAstV1 was found in 7 of 13 (54%) of stranded animals examined. One of two stranded bottlenose dolphins was positive in both the feces and mesenteric lymph node. Both minke whales were positive, as were both pygmy sperm whales. One of 3 short-beaked common dolphins was positive. Neither of the harbor porpoises or the Atlantic white-sided dolphins were positive on qPCR. The mean and median BDAstV1 numbers of detected copies detected in the 7 wild cetacean fecal samples were 9 and 6 (range 0-26).

Confirmatory Heminested PCR

The heminested PCR resulted in a product of 135 bp, as expected. All qPCR positives with more than 10 copies present were confirmed as positive by heminested PCR. Of the 19 positive samples found by qPCR to have less than 10 copies detected, 4 were not confirmed by heminested PCR and were considered negative. No

differences from the reference sequence were seen in any of the 15 positive sequences. Samples with less than 10 detected copies that were confirmed as positive averaged 4.8 detected copies (range 2-8), whereas those that were rejected averaged 1.9 detected copies (range 1-3).

Discussion

The qPCR results further confirm the high prevalence of BDAstV1 in bottlenose dolphins, as serological data had indicated. Fifty percent of fecal samples from the managed collection, many of which were from clinically normal animals, were found to be positive, and 86% of fecal samples from stranded cetaceans were positive. This is higher than values seen with qPCR surveys of human diarrheic samples, where 6%-9% prevalence was found (Logan et al., 2007, Dai et al., 2010, van Maarseveen et al., in press). Differences in assay sensitivity are one possible explanation for this discrepancy. An additional possibility is that the prevalence of astroviruses may be higher in the marine environment. Astroviruses are very stable in aquatic environments (Espinosa et al., 2008). Surveys have found a Human astrovirus prevalence of up to 61% in some marine shellfish populations, which are good particle concentrators (Elamri et al., 2006). Wide diversity has been seen in marine mammal astroviruses, implying that the marine environment may play a large role in astroviral ecology (Rivera et al., 2010), and the finding of high prevalence in dolphins by qPCR further supports that. The marine environment is central in the ecology of caliciviruses, a better studied group of small nonenveloped positive stranded RNA viruses (Smith et al., 1998).

We found BDAstV1 in three odontocete species (bottlenose dolphin, pygmy sperm whale, short-beaked common dolphin) and one mysticete species (minke whale), implying low host fidelity. As small RNA viruses, astroviruses are evolutionarily

fascinating. Due to lack of proofreading by their polymerases, RNA viruses have the fastest mutating genomes found in nature, and many RNA viruses accumulate one mutation per copy, which is thought to be the limit before accumulation of deleterious mutations would lead to extinction (Moya et al., 2004). This provides the ability to adapt rapidly to niches such as novel hosts. Most recent emerging diseases have been associated with host switches, and in humans, RNA viral diseases are much more likely to be emergent (Woolhouse and Gowtage-Sequeria, 2005). We have found evidence of marine mammals playing a role in human astroviral ecology (Rivera et al., 2010). Further understanding of host range and limiting factors of astroviruses is needed.

Virus was found in both Atlantic and Pacific cetacean populations, in agreement with the serological data. With a high prevalence in social species that have large geographic ranges, stability in a marine environment, and known presence in two oceans, it is probable that BDAstV1 is distributed throughout the world's oceans. Virus prevalence differed significantly between the two years surveyed in the managed open-water collection, indicating that there are dynamics to BDAstV1 epidemiology. The qPCR was more useful than serological methods for distinguishing this. Further work is needed to understand the epidemiology of BDAstV1 and the factors impacting it.

It should be noted that our qPCR values are for copies detected, which may be expected to differ from actual virus numbers present. We were unable to cultivate virus (data not shown), and hence were unable to spike control samples directly with virus. We used dilutions of known copy numbers of BDAstV1 PCR amplicon as a standard curve. This control is a DNA template and does not reflect loss during extraction or reverse transcription. The presence of PCR inhibitors or nucleases may result in falsely

low readings. These are common in feces, so this is of special concern for fecal samples. Control 18S rRNA amplification was variable in fecal samples, ansd so equivalency is based solely on use of an equivalent amount of feces used for initial extraction. Further, extraction methods differed between tissue samples and fecal samples, so caution should be used to avoid over-interpretation when comparing different sample types and quantitative data should not be used for this purpose.

Koch's postulates have not been fulfilled for BDAstV1, and the barriers to doing so make this unlikely to happen in the near future, including inability to culture this virus, societal concerns about doing experimental infections in dolphins, and the large expense that would be required to acquire and house specific pathogen-free calves for this. The evidence is fairly strong that this virus infects bottlenose dolphins and is not merely passing through with ingesta; dolphins seroconvert to BDAstV1, some animals shed significant amounts in feces, and BDAstV1 was found in a lymph node of one animal.

Clinical diagnosis of astroviral infection is challenging. Astrovirus culture is challenging, with few cell lines capable of supporting Human astrovirus and a high requirement for trypsin (Taylor et al., 1997). Attempts at culture of marine mammal astroviruses to date have been unsuccessful. Astroviruses resemble other small round viruses somewhat morphologically, and a significant rate of misidentification using negative-staining electron microscopy of feces has been reported (Oliver and Phillips, 1988). Negative-staining electron microscopy of feces for virus detection has also been shown to be comparatively insensitive (Logan et al., 2007, van Nieuwstadt et al., 1988). Culture, and negative-staining electron microscopy are therefore insensitive tests for

diagnosis of astroviral infection. Nucleic acid amplification diagnostic techniques appear to be the best current option, and development of this assay provides this option for BDAstV1.

Diagnosis of astroviral disease is also challenging. Much like *Vibrio cholerae*, astroviruses cause a secretory diarrhea without much of a histologic footprint on enterocytes on light microscopy (Koci et al., 2003). Nighot et al.(2010) found that Turkey astrovirus 2 causes sodium malabsorption. Our data did not show a correlation between BDAstV1 copies detected and electrolyte values. One possible explanation is that the presence of an acquired immune response attenuated disease manifestation. Volunteers experimentally infected with human astrovirus who had no pre-existing anti-human astrovirus antibodies developed more severe symptoms and were more likely to shed virus (Kurtz et al., 1979). The only parameter we found to correlate with BDAstV1 copies detected were trainer reports of abnormal behavior. This situation may differ in previously unexposed calves.

We did not identify a correlation with age and BDAstV1 shedding. Although human astroviral disease prevalence is much higher in young children, there is conflicting data on whether infection prevalence differs by age. Similar virus prevalence was found in adults and children in China (Dai et al., 2010), but higher virus prevalence was found in children than in adults in Europe (van Maarseveen et al., in press).

Dolphin 55 was found to shed virus at two time points. Serological data from dolphin 55 showed a moderate titer followed by a persistent elevation for two years. This elevation coincides temporally with viral shedding by this animal, suggesting persistent infection in this animal. Persistent infection has been seen with astrovirus

infection in humans; persistent gastroenteritis was seen with 8.5% of astrovirus infections in children in Spain (Caballero et al., 2003). A study of children in Bangladesh found that Human astrovirus was more commonly associated with persistent diarrhea than acute diarrhea (Unicomb et al., 1998).

In conclusion, this qPCR assay is useful for detecting and quantitating BDAstV1 in clinical samples from bottlenose dolphins. Prevalence of infection appears to be very high and is geographically widespread, similar to Human astrovirus in humans. Viral load correlates with abnormal behavior. Based on the observed parallels to human astrovirus infection, BDAstV1 is more likely to be clinically significant in calves than other age groups, and this is where management and future investigations should focus.

Table 5-1. BDAstV1 copies detected by qPCR from the managed open-water collection

Animal ID	Date	BDAstV1 copies detected	Animal ID	Date	BDAstV1 copies detected
1	6/18/08	0	40	1/25/07	90
1	10/28/08	5	42	5/29/08	0
1	11/3/08	4	42	7/17/08	0
2	12/20/07	31	46	8/15/08	0
3	3/27/08	0	47	2/14/07	80
5	9/5/07	0	47	2/18/08	0
6	3/2/07	29	52	5/28/08	0
6	9/21/07	0	52	6/25/08	12
7	6/25/08	0	52	8/27/08	0
8	2/14/07	445	55	2/7/07	70
8	9/11/08	5	55	6/21/07	60
9	1/24/07	3	56	2/14/08	0
9	3/20/08	0	56	3/20/08	0
12	1/12/07	16	59	9/17/07	56
14	2/21/07	310	60	5/29/08	0
14	7/29/08	0	60	9/24/08	5
15	5/21/08	0	60	10/28/08	0
15	6/26/08	0	163	5/22/08	0
17	2/8/07	74	163	7/30/08	0
22	2/21/07	5180	168	7/8/08	0
22	12/12/07	11	168	9/24/08	5
23	9/5/07	15	173	1/24/07	54
24	2/1/07	58	174	7/7/07	4
26	8/2/07	46	175	5/28/08	0
27	5/21/08	0	176	1/31/07	8
27	8/27/08	0	176	6/19/08	44
30	8/10/07	336	177	3/12/08	0
30	12/3/08	0	177	3/20/08	0
31	4/16/08	0	178	12/4/08	12
35	1/23/08	0			
39	7/8/08	0			
39	9/24/08	5			
39	10/31/08	22			

Table 5-2. Comparisons of mean BDAstV1 fecal load by animal appetite, behavior, reason for sampling among a population of common bottlenose dolphins (*Tursiops truncatus*)

Health Variable	Sample number	Mean fecal BDAstV1 copies detected	*P* value
Reason for sample			0.53
Initial or follow up	21	29	
Routine	21	58	
Appetite			0.14
Abnormal	4	86	
Normal	38	38	
Behavior			0.05
Abnormal	9	81	
Normal	33	32	

Table 5-3. Comparisons of mean chloride, potassium, and sodium by presence or absence of BDAstV1 in feces among a population of common bottlenose dolphins (*Tursiops truncatus*)

Serum Variable	Mean serum value		*P* value
	BDAstV1-negative feces n=26	BDAstV1-positive feces n=16	
Chloride (mEq/L)	119	120	0.55
Potassium (mEq/L)	3.8	3.7	0.58
Sodium (mEq/L)	153	154	0.41

Table 5-4. BDAstV1 copies detected by qPCR from wild cetaceans.

Animal ID	Species	Sample	Copies detected
179	Minke Whale	Colon	0
179		Feces	13
179		Duodenum	0
180	Minke Whale	Feces	6
181	Pygmy sperm whale	Feces	26
182	Pygmy sperm whale	Feces	6
182		Colon	0
182		Small Intestine	0
183	Short-beaked common dolphin	Mesenteric LN	0
184	Short-beaked common dolphin	Feces	6
184		Mesenteric LN	0
185	Short-beaked common dolphin	Mesenteric LN	0
186	Harbor Porpoise	Mesenteric LN	0
187	Harbor Porpoise	Mesenteric LN	0
188	Atlantic white-sided dolphin	Small Intestine	0
188		Large Intestine	0
188		Mesenteric LN	0
189	Atlantic white-sided dolphin	Mesenteric LN	0
190	Bottlenose dolphin	Feces	0
190		Mesenteric LN	0
191	Bottlenose dolphin	Feces	3
191		Mesenteric LN	2

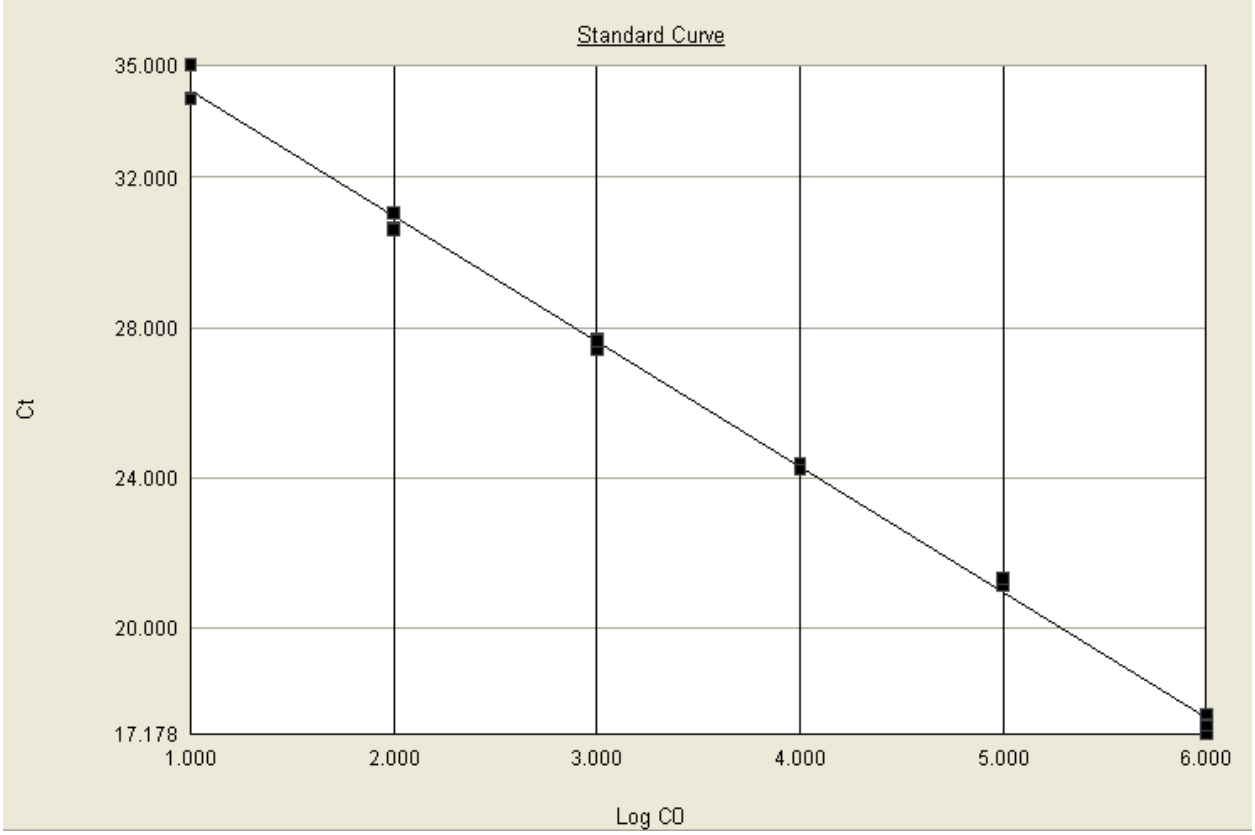


Figure 5-1. The standard curve for the BDAstV1 qPCR. Ct values are plotted on the vertical axis against $\log_{10}$ of the cDNA copy number of the standard curve on the horizontal axis.

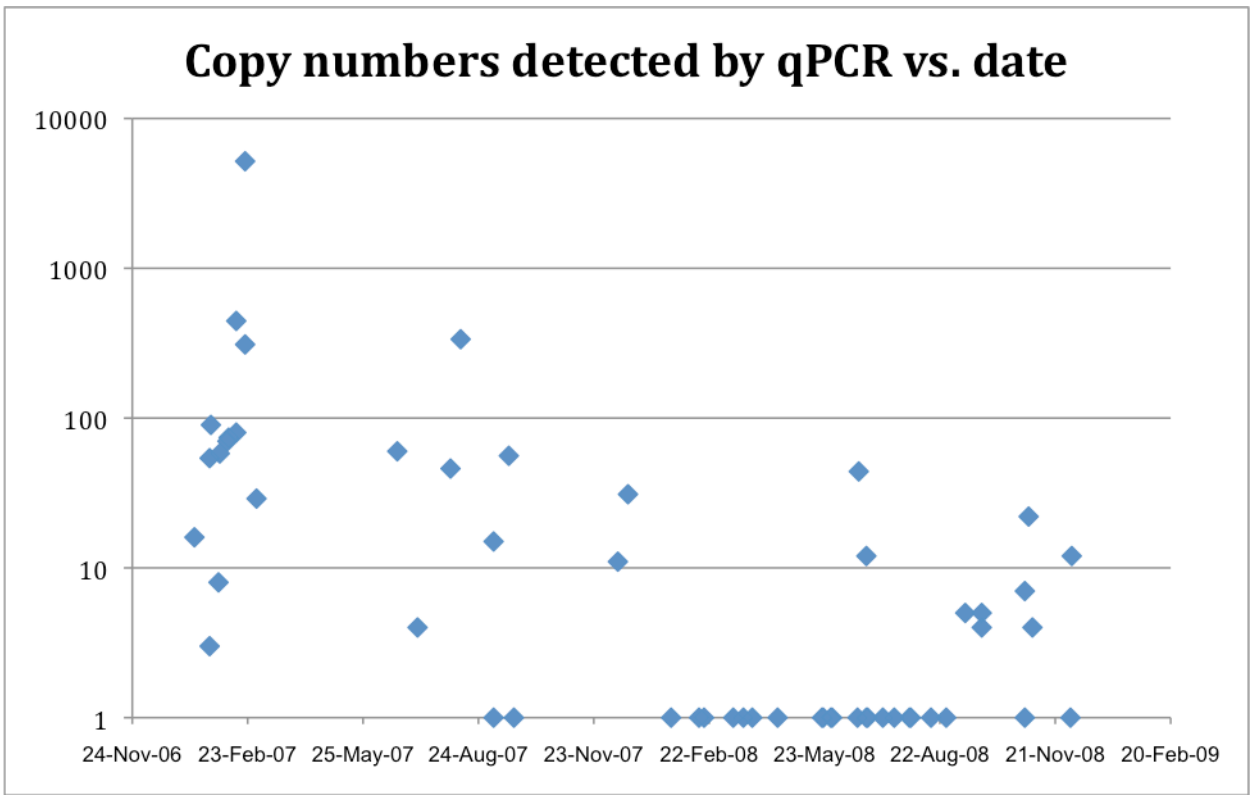


Figure 5-2. BDAstV1 copies detected in from the managed open-water collection plotted on a logarithmic scale on the vertical axis against date on the horizontal axis.

CHAPTER 6
FURTHER INVESTIGATION OF ASTROVIRUS DIVERSITY IN CETACEAN FECAL SAMPLES

Introduction

Astroviruses are small round nonenveloped viruses with a positive stranded RNA genome. They were relatively recently discovered, and were first reported in 1975 (Madeley and Cosgrove, 1975). Human astrovirus is a significant cause of enteric disease in human children (Dennehy et al., 2001).

The family Astroviridae is divided into two genera, *Avastrovirus*, found in avian hosts, and *Mamastrovirus*, found in mammal hosts (Monroe et al., 2005). Recognized species in the genus *Mamastrovirus* include Bovine astrovirus, Feline astrovirus, Human astrovirus, Mink Astrovirus, Ovine astrovirus, and Porcine astrovirus (Monroe et al., 2005). There has been significant recent discovery of additional mamastroviruses, including viruses from cheetahs (Atkins et al., 2009), Asian bat species (Chu et al., 2008, Zhu et al., 2009), humans (Finkbeiner et al., 2008, Finkbeiner et al., 2009a, Finkbeiner et al., 2009b, Kapoor et al., 2009), and rats (Chu et al., 2010). We have recently reported the discovery of diverse astroviruses in marine mammals, including Bottlenose Dolphin Astrovirus 1 (BDAstV1), the first cetacean astrovirus (Rivera et al., 2010).

The cetacea are composed of two clades, the odontocetes (toothed whales) and the mysticetes (baleen whales) (McGowen et al., 2009). There are limited data on cetacean viruses. Most attention has focused on viruses of odontocetes, which are smaller and have been kept in captivity.

Materials and Methods

Samples

A total of 72 fecal samples were collected from 41 bottlenose dolphins (*Tursiops truncatus*) from a managed open-water collection in California. Three fecal samples were taken from 3 stranded wild cetaceans in New England, including 2 minke whales (*Balaenoptera acutorostrata*) and a pygmy sperm whale (*Kogia breviceps*). Two fecal samples were taken from two orca (*Orcinus orca*) from a captive closed-water collection. Samples were stored after collection at -80°C.

RNA Extraction

Samples were maintained on ice at all points during RNA extraction. 0.10g of feces were measured and 900 µl 0.9% NaCl was added to suspend the sample by vortexing. Samples were spun at 3000 x G for 30 minutes at 4˚C. Supernatant was collected and filtered serially through 800nm, 450nm, and 200nm filters (Millipore, Billerca, MA). Filtrate was concentrated with Microsep concentrator columns (Pall Life Sciences) and centrifuged at 1500 x G for 30 minutes at 4˚C. The concentrated filtrate was used for RNA extraction using a High Pure Viral RNA Kit (Roche, Indianapolis, IN) following the manufacturer's instructions.

Consensus PCR

Samples were tested for the presence of astroviruses using BDAStV1-specific PCRs and sequencing of PCR products. The first nested PCR targeted the RdRp and used forward primer Astr4380F and reverse primer Astr4811R in the first round, and Astr4574F and reverse primer Astr4722R in the second round (Atkins et al., 2009). The second heminested PCR targeted the capsid and used forward primer Astr4811F and reverse primer Astr5819R in the first round, and Astr5159F and reverse primer

Astr5819R in the second round (Atkins et al., 2009). PCR products were resolved in 1% agarose gels, excised, and purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA). Sanger sequencing was performed directly using the Big-Dye Terminator Kit (Applied Biosystems, Foster City, California) and analyzed on ABI 3130 automated DNA sequencers. PCR products that resulted in mixed sequences using consensus primers were either resequenced using specific primers or cloned using a TA plasmid ligation kit into an *E. coli* vector (Invitrogen, Carlsbad, CA), extracted using a QIAprep Spin Miniprep Kit (Qiagen) and sequenced as above.

Sequence Extension

For each novel virus, attempts were made to obtain the remaining 3' sections of the viral genome by 3' rapid amplification of cDNA ends (3'RACE) using a kit (GeneRacer, Invitrogen) according to manufacturer's instructions. Briefly, RNA was reverse transcribed using AMV reverse transcriptase and amplified with a forward gene specific primer (Table 6-1) and the Gene Racer 3' Primer. PCR products were run in a 0.7% agarose gel, and bands of interest were sequenced as previously described.

Phylogenetic Analysis

Sequences were compared to those in GenBank (National Center for Biotechnology Information, Bethesda, Maryland), EMBL (Cambridge, United Kingdom), and Data Bank of Japan (Mishima, Shizuoka, Japan) databases using BLASTX (Altschul et al., 1997). The predicted homologous 100-133 amino acid sequences of astroviral RdRp and 388-429 amino acid sequences of astroviral capsid protein were aligned using the following three methods: ClustalW2 (Larkin et al., 2007), T-Coffee (Notredame et al., 2007), and MUSCLE (Edgar, 2004).

Bayesian analyses of each alignment were performed using MrBayes 3.1 (Ronquist & Huelsenbeck, 2003) with gamma distributed rate variation and 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.

Maximum likelihood (ML) analyses of each alignment were performed using PHYLIP (Phylogeny Inference Package, Version 3.66) (Felsenstein, 1989), running each alignment using the program ProML with amino acid substitution models JTT (Jones et al., 1992), PMB (Veerassamy et al., 2003), and PAM (Kosiol & Goldman, 2005) further set with global rearrangements, five replications of random input order, gamma plus invariant rate distributions, and unrooted. The values for the gamma distribution were taken from the Bayesian analysis. Avian nephritis virus 1 (GenBank accession number AB033998) was designated as the outgroup. The alignment producing the most likely tree was then used to create data subsets for bootstrap analysis to test the strength of the tree topology (200 re-samplings) (Felsenstein, 1985), which was analyzed using the amino acid substitution model producing the most likely tree in that alignment.

Results

Consensus PCR

Results of the consensus PCR assays on bottlenose dolphins are presented in table 6-2. On the RdRp assay, 18 of 72 samples (25%) from the bottlenose dolphins resulted in astroviral sequences, representing 13 of 41 animals (32%). On the capsid assay, 6 of 72 samples (8%) from the bottlenose dolphins resulted in astroviral

sequences, representing 6 of 41 animals (15%). Results of the consensus PCR assays on other cetaceans are presented in table 6-3. On the RdRp assay, both minke whale samples and one orca sample resulted in astroviral sequences. The capsid assay did not result in any astroviral product. The bottlenose dolphins represented 9 distinct virus types, the minke whales represented two distinct virus types, and one orca represented another distinct virus type. Four of 72 samples (6%) had multiple astrovirus types present. A bottlenose dolphin sample from animal 27 showed 99% homology (806/811) with the reference BDAstV1 sequence (GenBank accession # FJ890355) over the first 811 nucleotides, and only 67% homology (258/387) over the final 387 nucleotides. A MUSCLE alignment is given in Figure 6-1. Sequences were submitted to GenBank under accession numbers HQ668121-HQ668143.

Sequence Extension

Sequence extension only resulted in product extension on BDAstV3 from case 23 and MWAstV2 from case 40. Additional 3'RACE yielded final contiguous molecules of 2,942 bp (BDAstV3) and 3,080 bp (MWAstV2). The contiguous molecules corresponded to the partial RdRp gene (ORF1b) and the full-length capsid gene (ORF2) and 3'-UTR of reference astroviruses. The contiguous sequences were submitted to GenBank under accession numbers HQ668129 and HQ668143.

Phylogenetic Analysis

BLASTX results for the RdRp of bottlenose dolphin astroviruses 2 and 5 (BDAstV2, BDAstV5) showed the highest identity score with Turkey astrovirus 2 (GenBank accession # Q9ILI5). BLASTX results for bottlenose dolphin astrovirus 3 (BDAstV3) showed the highest identity score with Human astrovirus 3 (ADC53753) for the RdRp and with California sea lion astrovirus 2 (ACR54274) for the capsid. BLASTX

results for the RdRp of bottlenose dolphin astrovirus 4 (BDAstV4) showed the highest identity score with a *Miniopterus magnater* bat astrovirus (ACF75849). BLASTX results for the RdRp of bottlenose dolphin astrovirus 6 (BDAstV6) showed the highest identity score with Turkey astrovirus 2 (ABX46574). BLASTX results for the RdRp of bottlenose dolphin astrovirus 7 (BDAstV7) showed the highest identity score with Duck hepatitis virus 3 (ACF19905). BLASTX results for the RdRp of bottlenose dolphin astrovirus 8 (BDAstV8) showed the highest identity score with Duck astrovirus C-NGB (YP_002728002). BLASTX results for the RdRp of bottlenose dolphin astrovirus 9 (BDAstV9) showed the highest identity score with California sea lion astrovirus 1 (ACR54271). BLASTX results for the RdRp of minke whale astrovirus 1 (MWAstV1) showed the highest identity score with Duck hepatitis virus 2 (ACF19904). BLASTX results for the RdRp of minke whale astrovirus 2 (MWAstV2) showed the highest identity score with Human astrovirus 1 (ACN78557). BLASTX results for the RdRp of orca astrovirus 1 (OoAstV1) showed the highest identity score with a *Miniopterus magnater* bat astrovirus (ACF75839).

Bayesian phylogenetic analysis showed the greatest harmonic mean of estimated marginal likelihoods using the MUSCLE alignment for the RdRp (see Supplemental Material: RdRp alignment) and the T-Coffee alignment for the capsid gene (see Supplemental Material: Capsid alignment). For the RdRp, the BLOSUM model of amino acid substitution was found to be most probable with a posterior probability of 0.995 (Henikoff & Henikoff, 1992), followed by the WAG model of amino acid substitution with a posterior probability of 0.005 (Whelan & Goldman, 2001). For the capsid precursor protein, the WAG model was most probable with a posterior probability of 1.000.

Bayesian trees using the MUSCLE alignment for the RdRp (Figure 6-2) and the T-Coffee alignment for the capsid gene are shown (Figure 6-3). While BDAstV3 and MWAstV2 cluster within the genus *Mamastrovirus*, the other viruses identified do not cluster within known genera.

ML analysis found the most likely tree from the MUSCLE alignment and the PMB model of amino acid substitution for the RdRp, and the T-Coffee alignment and the JTT model of amino acid substitution for the capsid precursor. These parameters were used for bootstrap analysis. Bootstrap values from ML analysis are shown on the trees (Figures 6-2 and 6-3).

Discussion

This survey of astroviruses in cetacean fecal samples has identified significant diversity. The majority of viruses identified do not nest within the recognized astroviral genera, and form a well-supported monophyletic group. Significant diversity is present in this group, and the distance between BDAstV4 and BDAstV7 is greater than that between most *Mamastrovirus*/*Avastrovirus* pairs. It is possible that this novel astrovirus clade may represent more than one genus.

Both of the novel mamastroviruses (BDAstV3 and MWAstV2) identified here were successfully sequenced from the conserved RdRp region through to the 3' end. The phylogenetic analyses of both the RdRp and the capsid found that these viruses represent fairly early divergences within *Mamastrovirus*, in the clade containing Human astrovirus and MLB1. Given the relationship to known mamastroviruses, it is probable that these viruses utilize mammalian hosts and likely represent true infection in these cetaceans.

Neither consensus capsid PCR nor 3'RACE yielded any product from the samples that had resulted in non-*Mamastrovirus* astrovirus sequences. Possible explanations for failure of amplification using 3'RACE include a distance from the known sequence to the 3' end that was too long for efficient amplification, and lack of sufficient copy numbers of intact template present. The failure of amplification using consensus primers is likely due to lack of conservation of the template sequences in the divergent viruses. The RdRp and the 3' portion of the capsid are some of the most conserved regions in astroviruses (van Hemert et al., 2007). Recently, an ORF (ORFX) overlapping the 3' end of the capsid in a different frame has been identified (Firth and Atkins, 2010). ORFX is conserved among *Mamastrovirus* but not *Avastrovirus* It is unknown whether ORFX is expressed, and if so what function it has, but if it is relevant for mamastroviral biology then it may provide selective pressure for conservation of this region in mamastrovirsues and not other astroviruses.

A divergent astrovirus sequence has recently been identified from bat guano found under a mixed-species roost (Li et al., 2010). Random amplification and pyrosequencing produced a sequence homologous to a region of ORF1a that was weakly supported as basal to other mamastroviruses in a neighbor-joining tree. Unfortunately, sequence from homologous regions is not available to compare this to the non-*Mamastrovirus* astroviruses from cetacean feces.

The BDAstV1 sequence from animal 27 showed very strong homology with the reference BDAstV1 sequence over the first 811 nucleotides sequenced, and only 67% homology over the final 387 nucleotides. This is strongly suggestive of recombination. The juncture between these regions is after the RdRp, and near the 5' end of the

capsid/ORFX. Recombination appears to be common in astroviruses (Belliot et al., 1997, Walter et al., 2001, Pantin-Jackwood et al., 2006, Strain et al., 2008, Rivera et al., 2010, Ulloa and Gutiérrez, 2010). Recombination appears to be especially common near the end of RdRp/start of the capsid (Walter et al., 2001, Pantin-Jackwood et al., 2006, Strain et al., 2008, Rivera et al., 2010), as was seen here. The less homologous final 387 nucleotides are still closer to the reference BDAstV1 than to other known astroviruses on a BLASTX search, and the probable parent sequence remains to be discovered.

Comparison of the relationships of viruses in the RdRp and capsid regions reveals a number of discrepancies. The diversity and high rate of recombination of astroviruses is diagnostically and epidemiologically challenging. Further understanding of the diversity, host range, and biology of the astroviruses is needed to improve diagnostic testing and assess risks.

It should be noted that CSLAstV2was found in dolphin 36. This is clear evidence of host switching over an even larger phylogenetic distance than was identified with BDAstV1 between cetacean species. Emerging disease is frequently associated with host switches. One recent meta-analysis of human diseases found that 816 of 1407 (58%) are zoonotic, and of human diseases, zoonotic diseases are significantly more likely to be emerging (Woolhouse & Gowtage-Sequeria, 2005). Most recent emerging human diseases have been associated with host switches, including SARS, Hendra virus, Nipah virus, and AIDS. The aforementioned study also found that viral diseases were much more likely to be emerging, especially RNA viruses (Woolhouse & Gowtage-

Sequeria, 2005). The ability of astroviruses to infect disparate hosts suggests further study of their ecology and host range may be relevant to human health.

MWAstV1 and MWAstV2 were found in samples from mysticetes. Very little is known of mysticete viruses. There is some serologic evidence of viral infection, but concerns about cross-reactivity with unknown agents in poorly-studied host species limit conclusions that can be drawn from this. Adenoviruses have been isolated from feces of a sei whale (*Balaenoptera borealis*) and a bowhead whale (*Balaena mysticetus*) that were not further identified, and serologic testing of the bowhead whale found no neutralizing antibodies, suggesting this may not have been infecting the whale (Smith and Skilling, 1979, Smith et al., 1987). An enterovirus was also isolated from feces of a gray whale (*Eschrichtius robustus*) (Smith and Skilling, 1979). Paramyxovirus-like particles have been seen on electron microscopy in two fin whales (*Balaenoptera physalus*) with lesions histologically consistent with morbilliviral disease (Jauniaux et al., 2000). The only sequence data available prior to this study was from a poxvirus in a skin lesion on a bowhead whale (Bracht et al., 2006).

Further study is needed to understand these viruses, especially those outside of known genera. A number of studies have recently begun to look at viral diversity in feces; the majority of eukaryotic viruses have often been viruses of food items (Zhang et al., 2006, Li et al., 2010). Study of fish or other prey species of cetaceans to look for the presence of these viruses, study of virus loads, and searching for acquired immune responses in cetaceans are indicated to delineate any potential role these viruses play in cetacean health. While most mamastrovirus infections are enteric, a mamastrovirus has been found in a human encephalitis case (Quan et al., 2010), and avastrovirus

disease may also be renal or hepatic (Imada et al., 2000, Fu et al., 2009). The search for these viruses should not be limited to the gastrointestinal tract.

In conclusion, we have discovered several novel astroviruses present in cetacean feces. Phylogenetic analysis revealed that several of these did not cluster within known genera. Further study is needed to understand the ecology and clinical significance of these viruses.

Table 6-1. Additional primers used for 3' RACE of novel astroviruses from cetacean feces

3'RACE Primers	5'-3' Sequence
TtAst2RACE3':	GTCGCGGCAATGTACGGAGAATGGA
TtAst3RACE3':	GACAGAAAGGTGGTAAYCCCTCCGGACA
TtAst4RACE3':	CCGCAACTACCAACCTGCGGAAAAAGA
TtAst5RACE3':	GGTTACGGCCATGTATGCGGAATGGA
TtAst5RACE3'.2:	TCGTCCCATCATATCGTGCCCAATCA
TtAst6RACE3':	RGTCGCAGCAATGTATGGGGAATGGA
TtAst7RACE3':	GCATCGAATGCCCAATGGTGGAAACA
TtAst8RACE3':	CCAGCAGAGCTCAAAGCCACCTACCG
TtAst8RACE3'.2:	RCGTCGGACACTACCCAACACCAGCA
BaAst1RACE3':	TCCTCACGGCCTACGAAAACGCACAC
BaAst2RACE3':	AGACACGCGGGAATCCTTCTGGTCAA
OoAst1RACE3':	CGTTGAAGCCAAAACATGGGCCAACA
Primers for closing sequence gaps of 3'RACE products	
TtAstV3seq1	TGTCAGAGCAGCGGTATCAC
TtAstV3F1720	CACCTTTTGGCTGGCTTATT
TtAstV3R3'1	CTTATCTGCTGCAACCACCA
BaAstV2seq1	AAGTCACACCCAGCTTGGAC
BaAStV2F938	GCCTAAGCCAGCACTCTCAC
BaAstV2capR3'1	ACAATCCAATGGTGGTCTGG

Table 6-2. Results of astroviral consensus PCR and sequencing of bottlenose dolphin fecal samples.

Animal ID	Date	RdRp PCR	Capsid PCR	Animal ID	Date	RdRp PCR	Capsid PCR
1	2/15/07	BDAstV1	BDAstV1	36	6/29/08	CSLAstV2	-
1	6/18/08	-	-	37	4/9/08	-	-
1	10/28/08	BDAstV2	-	39	7/8/08	BDAstV6	-
1	11/3/08	BDAstV8	-	39	9/24/08	-	-
2	12/20/07	-	-	39	10/31/08	-	-
3	3/27/08	-	-	40	1/25/07	-	-
5	9/5/07	-	-	42	5/29/08	-	-
6	3/2/07	-	-	42	7/17/08	-	-
6	9/21/07	-	-	46	8/15/08	-	-
7	6/25/08	BDAstV5, BDAstV6	-	47	2/14/07	-	-
8	2/14/07	-	BDAstV1	47	2/13/08	-	-
8	9/11/08	-	-	47	2/18/08	-	-
9	1/24/07	-	-	52	5/28/08	-	-
9	3/20/08	-	-	52	6/25/08	-	-
12	1/12/07	-	-	52	8/27/08	-	-
12	12/9/08	-	-	55	2/7/07	-	-
14	2/21/07	-	-	55	6/21/07	-	-
14	7/8/08	-	-	56	2/14/08	-	-
14	7/24/08	BDAstV2	-	56	3/20/08	-	-
14	7/29/08	-	-	59	9/17/07	-	-
15	5/21/08	BDAstV4	-	60	5/29/08	-	-
15	6/26/08	-	-	60	9/24/08	BDAstV3	-
17	2/8/07	-	-	60	10/28/08	BDAstV6, BDAstV8	-
21	11/30/07	-	-	163	5/22/08	-	-
22	2/21/07	BDAstV1	BDAstV1	163	7/30/08	-	-
22	12/12/07	-	-	163	7/1/08	-	-
23	9/5/07	BDAstV1, BDAstV1b, BDAstV3	BDAstV1, BDAstV3	168	7/8/08	-	-
24	2/1/07	-	-	168	9/24/08	-	-
24	4/9/08	-	-	173	1/24/07	-	-
26	8/2/07	-	-	174	7/7/07	BDAstV2	-
27	5/21/08	BDAstV1 (100%)	Recomb. (68% ID)	175	5/28/08	-	-
27	8/27/08	BDAstV7	-	176	1/31/07	-	-
30	8/10/07	BDAstV1	BDAstV1	176	6/19/08	-	-
30	12/3/08	BDAstV5	-	177	3/12/08	-	-
31	4/16/08	-	-	177	3/20/08	BDAstV2, BDAstV6, BDAstV9	-
35	1/23/08	-	-	178	12/4/08	-	-

Table 6-3. Results of astroviral consensus PCR and sequencing of other cetacean samples.

Animal ID	Species	RdRp PCR	Capsid PCR
179	Minke Whale	MWAstV1	-
180	Minke Whale	MWAstV2	-
181	Pygmy Sperm Whale	-	-
192	Orca	OoAstV1	-
193	Orca	-	-

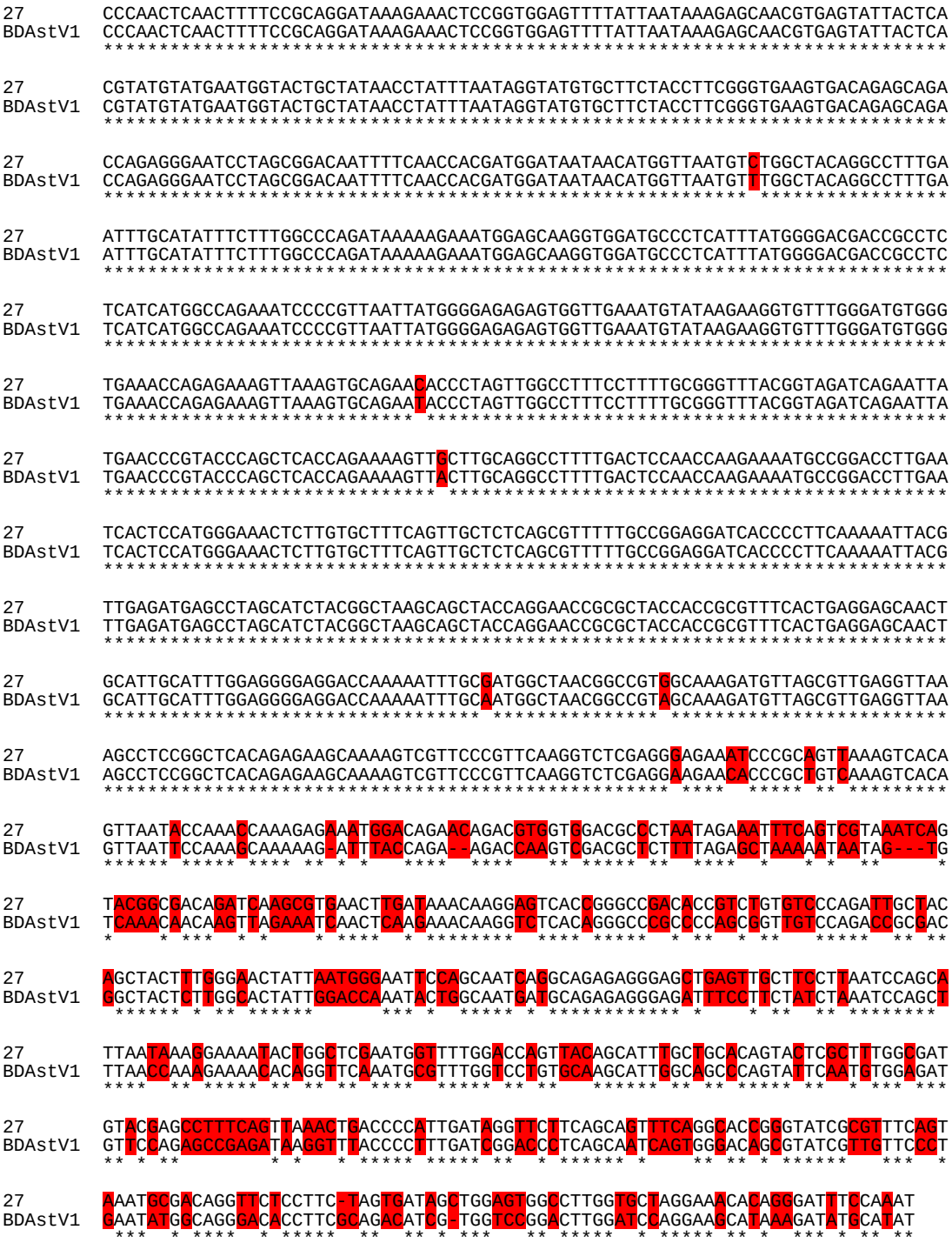


Figure 6-1. MUSCLE alignment of sequence from animal 27 and reference BDAstV1. Differences are highlighted in red.

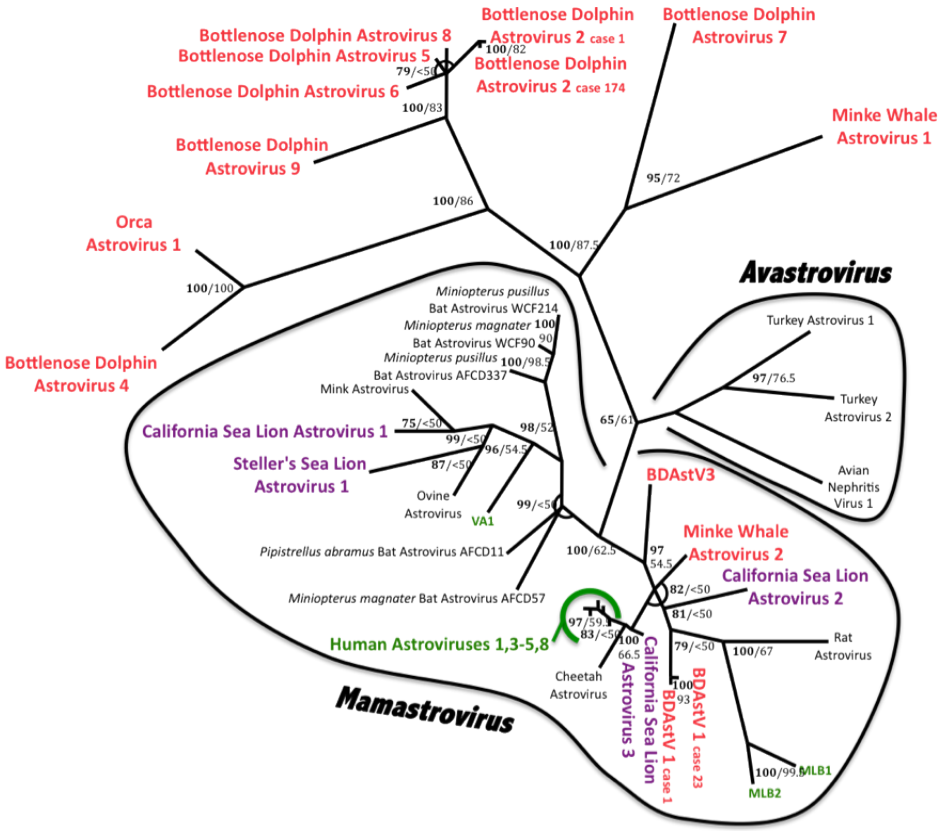


Figure 6-2. Bayesian phylogenetic tree of predicted 100-133 amino acid partial astroviral RNA-dependent-RNA polymerase sequences based on MUSCLE alignment. Bayesian posterior probabilities of branchings as percentages are in bold, and ML bootstrap values for branchings based on 200 re-samplings are given to the right or below. Avian nephritis virus 1 (GenBank accession number NP_620617) was designated as the outgroup. Virus genera are delineated by brackets. Marine mammal astroviruses are bolded and in red (cetaceans) or purple (pinnipeds). Human astroviruses are in green. Areas of multifurcation are marked by arcs. Sequences retrieved from GenBank include Human astrovirus 1 (GenBank accession # AAW51881), Human astrovirus 3 (AAD28539), Human astrovirus 4 (AAY84778), Human astrovirus 5 (AAY46273), Human astrovirus 8 (AAF85963), Human astrovirus MLB1 (YP002290967), Human astrovirus MLB2 (ACX69833), Human astrovirus VA1 (ACR23347), Rat astrovirus (ADJ38390), Cheetah astrovirus, (*Miniopterus magnater* bat astrovirus WCF90 (ACF75856), *Miniopterus magnater* bat astrovirus AFCD57 (ACF75852), *Miniopterus pusillus* bat astrovirus AFCD337 (ACF75864), *Miniopterus pusillus* bat astrovirus WCF214 (ACF75862), *Pipistrellus abramus* bat astrovirus AFCD11 (ACF75853), Ovine astrovirus (NP_059945), Mink astrovirus (AAO32082), Turkey astrovirus 1 (CAB95006), Turkey astrovirus 2 (NP_987087), and Avian Nephritis Virus 1 (NP_620617).

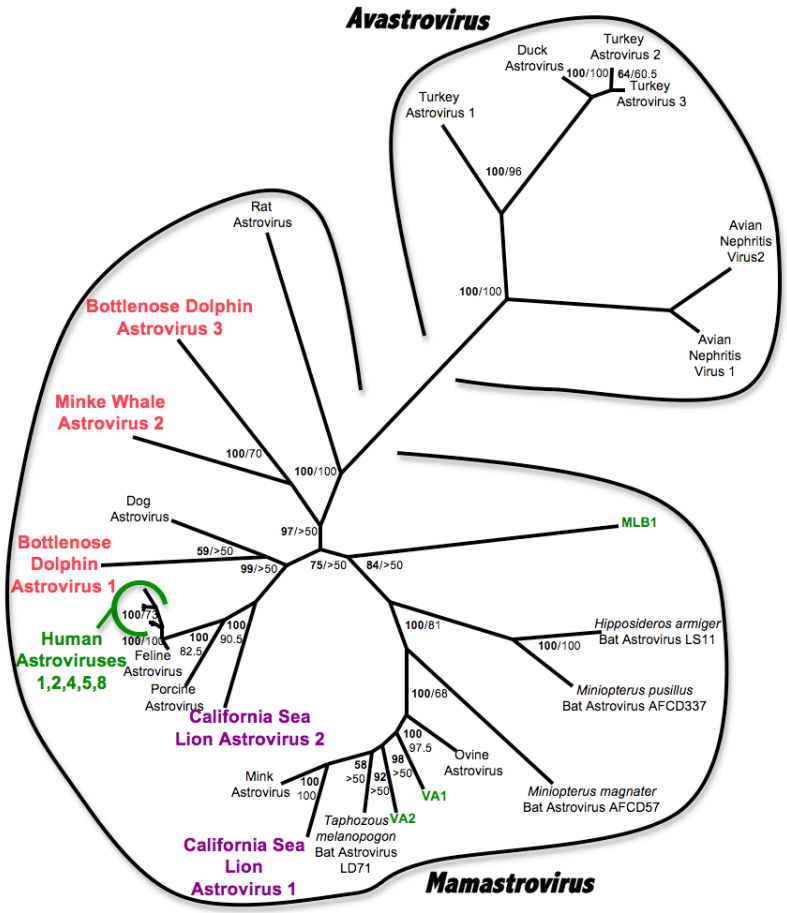


Figure 6-3. Bayesian phylogenetic tree of predicted 388-429 amino acid partial astroviral capsid sequences based on T-Coffee alignment. Bayesian posterior probabilities of branchings as percentages are in bold, and ML bootstrap values for branchings based on 200 re-samplings are given to the right. Avian nephritis virus 1 (GenBank accession number NP_620618) was designated as the outgroup. Virus genera are delineated by brackets. Marine mammal astroviruses are bolded and in red (cetaceans) or purple (pinnipeds). Human astroviruses are in green. Sequences retrieved from GenBank include Human astrovirus 1 (GenBank accession # BAE97460), Human astrovirus 2 (AAA62427), Human astrovirus 4 (BAA93440), Human astrovirus 5 (AAY46274), Human astrovirus 8 (AAF85964), Human astrovirus MLB1 (ACI62175), Human astrovirus VA1 (ACR23349), Human astrovirus VA2 (ACX83591), Rat astrovirus (ADJ38391), Feline astrovirus (AAC13556), Dog astrovirus (CAR82569), Porcine astrovirus (CAB95000), *Hipposideros armiger* Bat Astrovirus LS11 (ACN88714), *Miniopterus pusillus* bat astrovirus AFCD337 (ACF75865), *Miniopterus magnater* Bat Astrovirus AFCD57 (ACU30844), *Taphozous melanopogon* Bat Astrovirus LD71 (ACN88712), Ovine astrovirus (NP_059944), Mink astrovirus (NP_795336), Turkey astrovirus 1 (CAB95007), Turkey astrovirus 2 (NP_987088), Turkey astrovirus 3 (AAV37187), Avian Nephritis Virus 1 (NP_620618), Avian Nephritis Virus 2 (BAB21617), and Duck Astrovirus (ACN82429).

CHAPTER 7
USE OF A QUANTITATIVE PCR ASSAY FOR THE DETECTION OF BOTTLENOSE DOLPHIN ASTROVIRUS 6

Introduction

Astroviruses are small round nonenveloped viruses with a positive stranded RNA genome. They were relatively recently discovered, and were first reported in 1975 (Madeley and Cosgrove, 1975). The family Astroviridae is divided into two genera, *Avastrovirus*, found in avian hosts, and *Mamastrovirus*, found in mammal hosts (Monroe et al., 2005). Human astrovirus is a significant cause of enteric disease in human children (Dennehy et al., 2001). We have recently discovered diverse astroviruses in marine mammals, some of which do not cluster phylogenetically with known astroviral genera, including Bottlenose Dolphin Astrovirus 6 (BDAstV6).

Seroconversion to astrovirus at a young age is typical, and a number of studies have shown that most humans have seroconverted by 5 years of age (Kriston et al., 1996, Kobayashi et al., 1999). Our serological survey of BDAstV1 found that most bottlenose dolphins seroconvert at a young age as well; serological data on BDAstV6 is not available. Given the complications this introduces to diagnosis in populations other than young calves, methods for direct detection of BDAstV6 rather than antibody response have merit.

Quantitative PCR (qPCR, a.k.a. real-time PCR) has been used previously for detection of human astroviruses (Grimm et al., 2004, Royuela et al., 2006, Zhang et al., 2006, Logan et al., 2007, Dai et al., 2010, van Maarseveen et al., in press) and Turkey astrovirus 2 (Spackman et al., 2005) in fecal samples. Quantitative PCR human astrovirus assays have been shown to be significantly more sensitive than culture by more than two orders of magnitude (Royuela et al., 2006). The human qPCR assays

have found prevalences in diarrheic human feces from 6% - 9% (Logan et al., 2007, Dai et al., 2010, van Maarseveen et al., in press).

Materials and Methods

Samples

A total of 62 fecal samples were collected from 38 bottlenose dolphins (*Tursiops truncatus*) from a managed open-water collection in California, and 22 lower gastrointestinal samples were taken from 13 stranded wild cetaceans in New England, including 2 bottlenose dolphins (feces and mesenteric lymph node of each), 2 minke whales (*Balaenoptera acutorostrata*) (colon, duodenum, and feces of one and feces of another), 2 pygmy sperm whales (*Kogia breviceps*) (colon, small intestine, and feces of one and feces of another), 3 short-beaked common dolphins (*Delphinus delphis*) (feces and mesenteric lymph node of one and mesenteric lymph node of the other two), 2 harbor porpoises (*Phocoena phocoena*) (mesenteric lymph nodes), and 2 Atlantic white-sided dolphins (*Lagenorhynchus acutus*) (colon, small intestine, and mesenteric lymph node of one and mesenteric lymph node of another). Samples were stored after collection at -80°C. This was the same sample set used in Chapter 5.

RNA Extraction

Samples were maintained on ice at all points during RNA extraction. 0.10g of feces were measured and 900 µl 0.9% NaCl was added to suspend the sample by vortexing. Samples were spun at 3000 x G for 30 minutes at 4˚C. Supernatant was collected and filtered serially through 800nm, 450nm, and 200nm filters (Millipore, Billerca, MA). Filtrate was concentrated with Microsep concentrator columns (Pall Life Sciences) and centrifuged at 1500 x G for 30 minutes at 4˚C. The concentrated filtrate was used for RNA extraction using a High Pure Viral RNA Kit (Roche, Indianapolis, IN)

following the manufacturer's instructions. Tissue samples were cut to 0.10g portions and extracted using a RNeasy Mini Kit (Qiagen) following the manufacturer's instructions.

Quantitative PCR

cDNA was synthesized using a MMLV reverse transcriptase kit (Advantage RT-for-PCR, Clontech, Mountain View, CA) using consensus astroviral primers Astr4722R and Astr4811R (Atkins et al., 2009) for initial strand synthesis. A BDAstV6 PCR amplicon of the polymerase from the index BDAstV1 case, amplified using consensus primers Astr4380F and Astr4722R (Atkins et al., 2009) was used as a positive control for the standard curve, and RNase-free water was used as a negative control. The positive control was quantified by both comparison to a mass ladder standard (Low mass ladder DNA standard, Invitrogen, Carlsbad, CA) on gel electrophoresis as well as spectrophotometry (NanoDrop 8000, Thermo Scientific, Wilmington, DE). The standard curve, run on each plate, used 10-fold serial dilutions, ranging from 10^6 to 10 copies.

Quantitative PCR was performed using forward primer TtAstV6qPCRF (GTTRGTCGCAGCAATGTATG), reverse primer TtAstV6qPCRR (YCCCTAAGCTCGTCAAGTGT), and probe TtAstV6probe (6FAM-TGGATTTTTRAGAATGTCGGA-MGBNFQ) targeting the BDAstV6 polymerase gene. All samples were run in triplicate and a mean C_t value was calculated. Each 20 µL reaction was 0.9 µM for each primer and 0.25 µM for the probe, and contained 7 µl of extracted DNA and 10 µl of a commercial universal qPCR mix (TaqMan® Fast Universal PCR Master Mix 2X, Applied Biosystems). A 7500 Fast Real-Time PCR System (Applied Biosystems) was used to amplify the reactions with cycling conditions as

follows: initial denaturation at 95 °C for 20 seconds; 50 cycles of 95 °C for 3 seconds followed by 60 °C for 30 seconds.

Confirmatory Heminested PCR

All samples from the managed open-water collection were confirmed using a consensus astrovirus heminested PCR and sequencing of PCR products, protocol using forward primer Astr4380F and reverse primer Astr4811R in the first round, and Astr4380F and reverse primer Astr4722R in the second round (Atkins et al., 2009). PCR products were resolved in 1% agarose gels, excised, and purified using the QIAquick gel extraction kit (Qiagen). Sanger sequencing was performed directly using the Big-Dye Terminator Kit (Applied Biosystems, Foster City, California) and analyzed on ABI 3130 automated DNA sequencers. Results from the qPCR that were not confirmed by the heminested PCR were considered negative and given as 0.

Results

Quantitative PCR

The DeHV-2 qPCR assay accurately detected 10 to 10^6 cDNA copies. The standard curve for the BDAstV6 qPCR assay had a slope of -3.34 and a correlation coefficient (R^2) of 0.996 (Figure 1).

Results of the qPCR for the managed open-water collection are given in table 7-1. From the managed open-water collection, 5 of 62 fecal samples (8%) were positive, representing 5 of 38 animals (13%). The mean and median BDAstV6 detected copy numbers were 0.2 and 0 (range 0-4). None of the wild stranded cetaceans were found to have BDAstV6.

Confirmatory Heminested PCR

The heminested PCR resulted in a product of 400 bp after primers were edited out, as expected. All qPCR negatives were confirmed as negative by heminested PCR. Of the 10 samples found positive by qPCR, 5 were not confirmed by heminested PCR and were considered negative. Surprisingly, samples that were confirmed as positive averaged 2.4 detected copies (range 2-4), whereas those that were rejected averaged 7.0 detected copies (range 4-11).

Discussion

The qPCR results find a much lower prevalence of BDAstV6 than BDAstV1 in bottlenose dolphins. Eight percent of fecal samples from the managed collection were found to be BDAstV6 positive, as compared to 50% for BDAstV1, and none of the fecal samples from stranded cetaceans were positive, as compared to 86% for BDAstV1. The BDAstV6 prevalence found is similar to values seen with qPCR surveys of human diarrheic samples, where 6%-9% prevalence was found (Logan et al., 2007, Dai et al., 2010, van Maarseveen et al., in press). Differences in assay sensitivity are one possible explanation for this discrepancy.

It should be noted that our qPCR values are for copies detected, which may be expected to differ from actual virus numbers present. We were unable to cultivate virus (data not shown), and hence were unable to spike control samples directly with virus. We used dilutions of known copy numbers of BDAstV6 PCR amplicon as a standard curve. This control is a DNA template and does not reflect loss during extraction or reverse transcription. The presence of PCR inhibitors or nucleases may result in falsely low readings. These are common in feces, so this is of special concern for fecal

samples. Equivalency is based solely on use of an equivalent amount of feces used for initial extraction.

Koch's postulates have not been fulfilled for BDAstV6, and the barriers to doing so make this unlikely to happen in the near future, including inability to culture this virus, societal concerns about doing experimental infections in dolphins, and the large expense that would be required to acquire and house specific pathogen-free calves for this. It is possible that BDAstV6 is not actually a virus of dolphins but is actually a fish virus passing through with ingesta; BDAstV6 has only been found in feces so far, and the amounts shed are fairly small. As a very divergent astrovirus, it is difficult to predict what would be expected for host range or tissue tropism. While most mamastrovirus infections are enteric, an astrovirus has been found in a human encephalitis case (Quan et al., 2010), and avastrovirus disease may be renal or hepatic.

Clinical diagnosis of astroviral infection and astroviral disease is challenging. Astrovirus culture is challenging, with few cell lines capable of supporting Human astrovirus and a high requirement for trypsin (Taylor et al., 1997). Attempts at culture of marine mammal astroviruses to date have been unsuccessful. Astroviruses resemble other small round viruses somewhat morphologically, and a significant rate of misidentification using negative-staining electron microscopy of feces has been reported (Oliver and Phillips, 1988). Negative-staining electron microscopy of feces for virus detection has also been shown to be comparatively insensitive (Logan et al., 2007, van Nieuwstadt et al., 1988). Much like *Vibrio cholerae*, astroviruses cause a secretory diarrhea without much of a histologic footprint on enterocytes on light microscopy (Koci et al., 2003), and there are no pathognomonic lesions. Culture, negative-staining

electron microscopy, and histopathology are therefore insensitive tests for diagnosis of astroviral infection. Nucleic acid amplification diagnostic techniques appear to be the best current option, and development of this assay provides this option for BDAstV6.

In conclusion, this qPCR assay is useful for detecting and quantitating BDAstV6 in clinical samples from bottlenose dolphins. Prevalence in dolphin feces appears to be lower than that of BDAstV1 and similar to Human astrovirus in human feces. The host range, tissue tropism, and disease implications of BDAstV6 are still unknown, and this is where future investigations should focus.

Table 7-1. BDAstV6 copies detected by qPCR from the managed open-water collection.

Animal ID	Date	Copies detected
1	18-Jun-08	0
1	28-Oct-08	0
1	3-Nov-08	2
2	20-Dec-07	0
3	27-Mar-08	0
5	5-Sep-07	0
6	2-Mar-07	0
6	21-Sep-07	0
7	25-Jun-08	2
8	14-Feb-07	0
8	11-Sep-08	0
9	24-Jan-07	0
9	20-Mar-08	0
12	12-Jan-07	0
14	21-Feb-07	0
14	29-Jul-08	0
15	21-May-08	0
15	26-Jun-08	0
17	8-Feb-07	0
22	21-Feb-07	0
22	12-Dec-07	0
23	5-Sep-07	0
24	1-Feb-07	0
26	2-Aug-07	0
27	21-May-08	0
27	27-Aug-08	0
30	10-Aug-07	0
30	3-Dec-08	0
31	16-Apr-08	0
35	23-Jan-08	0
39	8-Jul-08	4
39	24-Sep-08	0
39	31-Oct-08	0
40	25-Jan-07	0
42	29-May-08	0
42	17-Jul-08	0
46	15-Aug-08	0
47	14-Feb-07	0
47	18-Feb-08	0
52	28-May-08	0
52	25-Jun-08	0
52	27-Aug-08	0
55	7-Feb-07	0
55	21-Jun-07	0
56	14-Feb-08	0
56	20-Mar-08	0
59	17-Sep-07	0
60	29-May-08	0
60	24-Sep-08	0
60	28-Oct-08	2
163	22-May-08	0
163	30-Jul-08	0
168	8-Jul-08	0
168	24-Sep-08	0
173	24-Jan-07	0
174	7-Jul-07	0
175	28-May-08	0
176	31-Jan-07	0
176	19-Jun-08	0
177	12-Mar-08	0
177	20-Mar-08	2
178	4-Dec-08	0

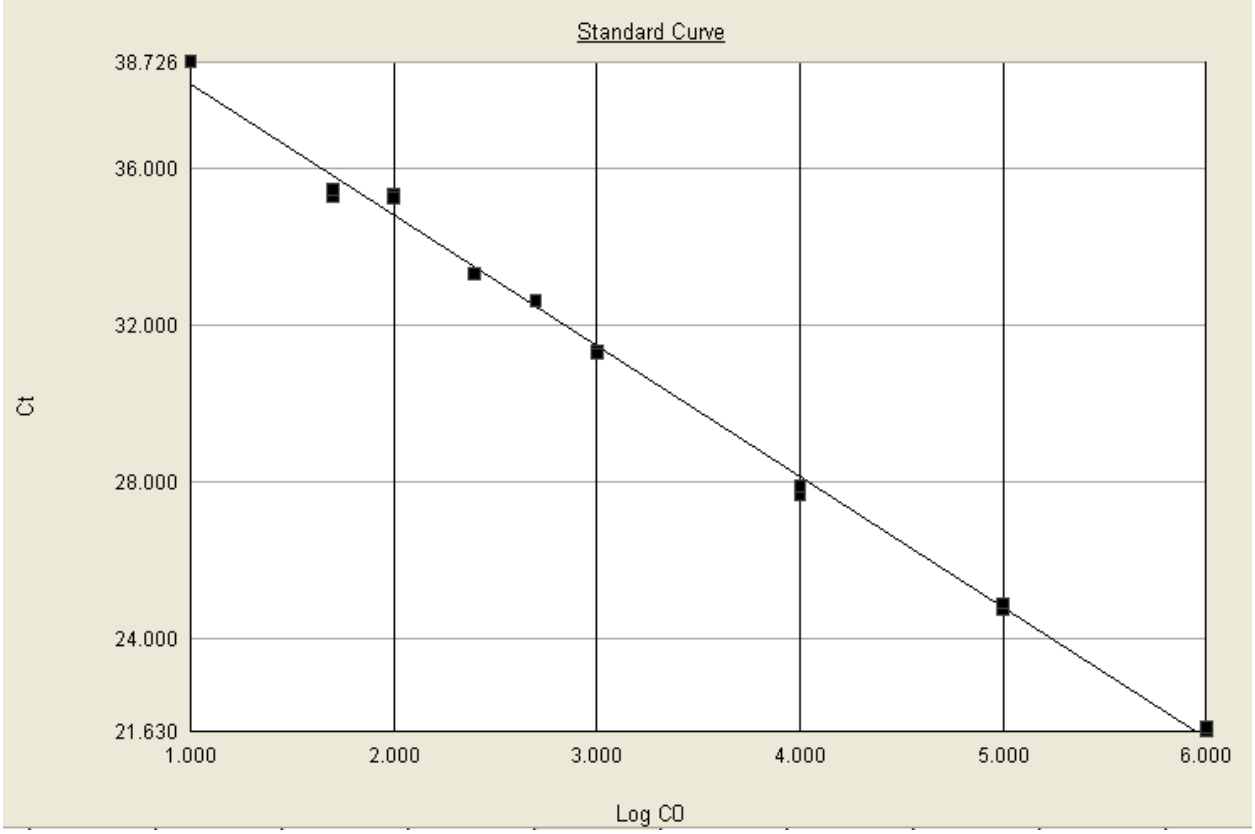


Figure 7-1. The standard curve for the BDAstV6 qPCR. Ct values are plotted on the vertical axis against $\log_{10}$ of the cDNA copy number of the standard curve on the horizontal axis.

CHAPTER 8
CONCLUSIONS

This work demonstrates that astroviruses are prevalent in the marine environment, and specifically in marine mammals. As in humans, the seroprevalence study showed that dolphins develop antibodies to BDAstV1 at a young age. The BDAstV1 virus prevalence by qPCR suggests, however, that active infection and shedding are potentially even more common in dolphins than has been seen with any astrovirus in terrestrial species. Astroviruses are very stable in aquatic environments (Espinosa *et al*., 2008), and this is likely to contribute to a higher pathogen prevalence.

We have identified evidence of historical recombination in CSLAstV3 and in BDAstV1. Recombination appears to be common in astroviruses (Belliot et al., 1997, Walter et al., 2001, Pantin-Jackwood et al., 2006, Strain et al., 2008, Rivera et al., 2010, Ulloa and Gutiérrez, 2010). Throughout biology, hybridization is a factor allowing rapid non-detrimental change, allowing species to invade novel habitats (Nolte et al., 2005, Rieseberg et al., 2007). Recombination of viruses may provide a hybrid advantage for crossing host species.

The host fidelity of astroviruses does not appear to be stringent. BDAstV1 was identified in a variety of cetacean species, both odontocetes and mysticetes. CSLAstV2 was found able to infect both pinnipeds and cetaceans. The recombination between astroviruses of humans and marine mammals as evidenced by analysis of CSLAstV3 indicates that a human and a marine mammal virus can infect the same host.

The nomenclature of astroviruses is problematic. Astroviral species are defined by the ICTV on the basis of host species of origin (Monroe et al., 2005). We have identified significant diversity of astroviruses in bottlenose dolphins, California sea lions,

and minke whales. Similarly, others have recently identified significant diversity in astroviruses of humans (Finkbeiner et al., 2008, Finkbeiner et al., 2009a, Finkbeiner et al., 2009b, Kapoor et al., 2009) and individual bat species (Chu et al., 2008, Zhu et al., 2009). Kapoor et al. (2009) have suggested that astroviruses be grouped and named based on both host species and a genetic distance criteria using either the capsid or RdRp loci. The apparently high recombination rate of astroviruses complicates this. Recombination appears to be especially common near the end of RdRp/start of the capsid (Walter et al., 2001, Pantin-Jackwood et al., 2006, Strain et al., 2008, Rivera et al., 2010), as was seen here. Capsid and RdRp genes may have very different ancestries, and naming according one gene or the other may not accurately indicate virus behavior. Use of a multiple gene nomenclature, as has been done with hemagglutinin and neuraminidase genes for influenza viruses, may be a potential solution.

The data generated also shows that significant diversity of the Astroviridae beyond *Avastrovirus* and *Mamastrovirus* exists. More study is indicated to determine the diversity, host range, and clinical significance of these viruses. The very high astrovirus prevalence seen in marine mammals, the great diversity seen in astroviruses from these species, the stability of astroviruses in the marine environment, and the widespread geographic distribution of marine mammal astroviruses all suggest that the marine environment plays a central role in astrovirus biology. We have shown that host fidelity of these viruses is limited. Given that most recent emerging diseases have been associated with host switches, and the marine environment appears to be a significant

reservoir of astroviral diversity, there is a need to further understand marine astroviruses.

APPENDIX A

MUSCLE ALIGNMENT OF PARTIAL ASTROVIRAL RIBONUCLEIC ACID (RNA) DEPENDENT RNA POLYMERASE AMINO ACID SEQUENCES

```
           1                                                  50
AvNeph1    PVQLFQRMRELRKFFLTRRSR-RRYGKLLDWYNAQLTDRITLLPTGEVTH
Turkastr1  PPELFRRIKLMRFFLLDPKYKTPENRDRYNWYVENLIDKVVLLPTGEVCK
Turkastr2  PKSLFWRIRQIRFFFLHDSHKTPKMRRLYNWYVKNLLEKIILLPTGEVCQ
MLB1       PPTLLMHIKKLRFTLMGSMSR--KYENVYKWYCRNLINRFVVLPSGEVTA
BdAstV-1   PTQLFRRIKKLRWSFINKEQR-EYYSRMYEWYCYNLFNRYVLLPSGEVTE
CslAstV-2  PTPLLLHIKKLRWSMINEVQR-KKYQSLHDWYCHNLVHRKVVLPSGEITE
CslAstV-3  PPALFRHIKEIRWNFINKEQR-EKYRHVHEWYVDNLLKRNVLLPSGEVTV
Humastr3   PPALFRHIKEIRWNFINKDQR-EKYRHVHEWYVDNLLNRHVLLPSGEVTV
Humastr5   PPSLFRHIKEIRWNFINKDQR-EKYRHVHEWYVDNLLNRHVLLPSGEVTL
Humastr4   PPALFKHIKEIRWNFINKDQR-EKYRHVHEWYVDNLLNRHVLLPSGEVTL
Humastr1   PPALFKHIKEIRWNFINKDQR-EKYRHVHEWYVDNLLNRHVLLPSGEVTL
Humastr8   PPALFKHIKEIRWNFINKDQR-EKYRHVHEWYVDNLLNRHVLLPSGEVTL
BatAstPa   PPEVFFQIKDIRFGLLSPEYRTVRNRSVYKWYCENLINRDVVLPSGEITH
BatAstMm2  PNQVFHKIKDIRFNFLSKEYRTKENREIYDWYCKNLTNRVVLLPSGEVTK
BatAstMp   PNEVFRHIKWFRFNMLDPVYKTDLNRSVYSWYVDQMLHRYVLLPSGEVTI
BatAstMm   PVEVFRHIKDFRFSMLDPVYKTDLNRSVYDWYVSQLVYRYVLLPSGEITI
BatAstMp2  PVEVFRHIKNFRFMMLDPVYKTDMNKSIYDWYVGQLMYRYVLLPSGEITI
SslAstV-1  PVEVFLAIKQVRFSFLADEYKTLENYDIYSWYCHNLVHRFVCMPSGEITL
Ovineastr  PSQIFKHIKNFRFSMLAKEYQTPELRNMYHWYVDNILRRYVCMPSGEITI
Minkastro  PREIFAKIKSFRFSCLAEEFQTDANRAMYQWYCDSLLDRYVLMPSGEVTR
CslAstV-1  PREVFMHIKKFRFSCLADEYKTPELESMYDWYCNALLERYVLLPSGEVTL

           51                                                 100
AvNeph1    VKKGNPSGQFSTTVDNNLVNEWLTAFEFGYQHLENHGIIPTVRDYRANVD
Turkastr1  IYGGNPSGQFSTTVDNNFVNVWLTVFELAYLFYKEHNRLPTICEIKKHTD
Turkastr2  VKKGNPSGQFSTTVDNNMINVWLTTFEVSYLFFKQRGRLPTEKELQENCS
MLB1       QQRGNPSGQMSTTMDNNMINYWLQAFEYKFLN-------LPEEE-WMHFD
BdAstV-1   QTRGNPSGQFSTTMDNNMVNVWLQAFEFAYFF-------GPDKKKWSKVD
CslAstV-2  QHRGNPSGQFSTTMDNNCVNLWIQAFEFAYMI-------GPDKELWKKYD
CslAstV-3  QTRGNPSGQFSTTMDNNMVNVWLQAFEFAYFN-------GPNKELWKNYD
Humastr3   QTRGNPSGQFSTTMDNNMVNFWLQAFEFAYFN-------GPNKELWKTYD
Humastr5   QTRGNPSGQFSTTMDNNMINFWLQAFEFAYFN-------GPNKDLWKTYD
Humastr4   QTRGNPSGQFSTTMDNNMVNFWLQAFEFAYFN-------GPNKDLWKTYD
Humastr1   QTRGNPSGQFSTTMDNNMVNFWLQAFEFAYFN-------GPDKDLWKTYD
Humastr8   QTRGNPSGQFSTPMDNNMVNFWLQAFEFAYFN-------GPDKDLWKTYD
BatAstPa   QDRGNPSGQVSTTMDNNMINTFLQAFEFIYLN---NLTIETAKELWESYD
BatAstMm2  QLNGNPSGQVSTTMDNNMVNTFCQAFEFMFVN---GLTIDEAKKKWVDYD
BatAstMp   QDRGNPSGQISTTMDNNLVNSFLQAFEFAFIH--PELDLDELTELYKQCD
BatAstMm   QDRGNPSGQISTTMDNNLVNTFLQAFEFAYVN--PELSLDELDVLYAQCD
BatAstMp2  QDRGNPSGQISTTMDNNLVNTFLQAFEFAYMN--PELSSDELDTLYAQCD
SslAstV-1  QERGNPSGQVSTTMDNNMCNVFFQAFEYAWLH--PCKTLDELHEDWERVD
Ovineastr  QHKGNPSGQVSTTMDNNLVNVFLQAFEYAYLH--PEKSMDELRKDWESYD
Minkastro  QTKGNPSGQISTTMDNNLCNVFFQAFEYAYIH--PEKSIEELRESWDRCD
CslAstV-1  QTKGNPSGQISTTMDNNLCNVFFQAFEFAYIN--PDLSMQELCDAWERCD
```

```
          101                                                 150
AvNeph1   FLCYGDDRLLAFNPSFVN-YDPQVTIDM----YKNIFGMWVKPENIKLFD
Turkastr1 WICYGDDRLLAVDKRFINSYDTAAVIAM----YKDVFGMWVKPDNIKVFP
Turkastr2 MICYGDDRLLSIRKGFVE-YEPDTVIDM----YKNIFGMWVKRNNIKIQD
MLB1      TLIYGDDRISTY-RSIPNEYT-KTIVDM----YKNVFGMWVKPEKVKVSE
BdAstV-1  ALIYGDDRLSSW-PEIPVNYG-ERVVEM----YKKVFGMWVKPEKVKVQN
CslAstV-2 TLVYGDDRLSTT-PKIVDNYE-EKVIEM----YKNIFGMWVKPGKVKISE
CslAstV-3 TIVYGDDRLSTT-PVVPDNYE-ERVIEM----YREIFGMWVKPGKVVCKD
Humastr3  TVVYGDDRLSTT-PSVPDNYE-ERVIAM----YRDIFGMWVKPGKVICRE
Humastr5  TVVYGDDRLSTT-PSVPENYE-ERVIDM----YRDIFGMWVKPGKVICRE
Humastr4  TVVYGDDRLSTT-PSVPNNYE-ERVITM----YRDIFGMWVKPGKVICKD
Humastr1  TVVYGDDRLSTT-PSVPDDYE-ERVITM----YRDIFGMWVKPGKVICRN
Humastr8  TVVYGDDRLSTT-PSVPDNYE-ERVITM----YRDIFGMWVKPGKVICRD
BatAstPa  SLVYGDDRVTST-PLVPSNYV-ERVVGM----YADIFGMWVKPDNVKVSN
BatAstMm2 TIVYGDDRITSS-PLVPPDYS-DRVIRM----YKDIFGMWVKPENVKISD
BatAstMp  SAVYGDDRLSSW-PCVPDDYV-HQVVCM----YEHVFGMWVKPEKVKISD
BatAstMm  SLIYGDDRLSSW-PVIPEDYV-HKVSCM----YEHVFGMWVKPEKVKVSD
BatAstMp2 SLIYGDDRLSSW-PCVPEDYE-TRVSGM----YEHVFGMWVKPDKVKVSD
SslAstV-1 SLVYGDDRLSFV-PDVPSDYV-DKVVAM----YETVFGMWVKPTKVVVSD
Ovineastr SLIYGDDRLTTS-PSVPNDYV-TRVVAM----YKDIFGMWVKPEKVKVSH
Minkastro SLIYGDDRLTTF-DHVPPDYV-DRVVHM----YKDVFGMWVKPEKVIVSD
CslAstV-1 SLIYGDDRLTTF-PSIPSDYV-NRVVDMYKDIYKDIFGMWVKPDKVVVQD

          151                                                 200
AvNeph1   SPTGSSFCGFTLVKPH-GQWVGVV-NVNKLLQSLKTPTRRLPDLESLWGK
Turkastr1 SLEGVSFCGMVWTKRK-GQYVGKP-NVDKILSTLSDPVSRLPDIQSLWGK
Turkastr2 TPEGLSFCGLTIVKSSTGAYVGVP-NVNKILSTLENPVRRLPDVESLWGK
MLB1      SLEGLSFCGFTYTPNG-----PVPSEPYKLMASLLKPATKLPDLIALHGK
BdAstV-1  TLVGLSFCGFTVDQ----NYEPVPSSPEKLLAGLLTPTKKMPDLESLHGK
CslAstV-2 TLVGLSFCGFTVDQ----NLEPIPTAPEKLMASLLKPSTKLPDLESLHGK
CslAstV-3 TIVGLSFCGFTVNE----DLEPVPTSPEKLMASLLKPYKVLPDLESLHGK
Humastr3  SIIGLSFCGFTVNS----DLEPVPTSPEKLMASLLKPYKVLPDLESLHGK
Humastr5  SIVGLSFCGFTVNA----DLEPVPTSPEKLMASLLKPYKILPDLESLHGK
Humastr4  SIVGLSFCGFTVNE----NLEPVPTSPEKLMASLLKPYKILPDLESLHGK
Humastr1  SIVGLSFCGFTVNE----NLEPVPTSPEKLMASLLKPYKILPDLESLHGK
Humastr8  SIVGLSFCGFTVNE----NLEPVPTSPEKLMASLLKPYKILPDLESLHGK
BatAstPa  TVNGLSFCGFTNNLIS-NMYLPVPTNVNKLVASLITPVKKLQDIESLAGK
BatAstMm2 TLVGLSFCGFTNIREK-GMYLPVPSNCEKLVAALVRPVKKLPDIEALAGK
BatAstMp  TLVGLTFCGFTIFKDG-DLYLPVPVDAWKFISSTLHPVKALPDFDALVGK
BatAstMm  TLEGLTFCGFTVIRSG-GFYLPIPVDAWKFISSTICPTKQLPDFDALVGK
BatAstMp2 TLEGLTFCGFTVIRSG-GLYLPIPFDAWKFISSTLCPTKQLPDFDALVGK
SslAstV-1 TPVGLTFCGFTVSP----DLLPMPTNTEKLIAALVTPTRKLKDMDALYAK
Ovineastr SPVGLSFCGFVITHQD-GQYLPVPAEEAKLLASLLRPTKKLENMDALYGK
Minkastro TPVGLSFCGFTVGP----DLMPVPTDCDKLVASLVTPTKKLQDIVALYSK
CslAstV-1 TPIGLSFCGFTVNQ----DFMPVPTECDKLIASLVTPTKKLADIYSLYSK
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          201                                                 250
AvNeph1   LVSLKIMCYHSDPEAVS--YLSNQIRRVEEYARAEGIELP-EVGPDFYRK
Turkastr1 LVSLRLLCENESDEVVD--YLDKQIESVSRHAKEAGIALP-KIGPDFYAE
Turkastr2 LVSLRILCENAPSNVKH--FLDEQISNVEEFAARENIQLP-EVGPDFYSR
MLB1      LLCFQLLMAN-DTAHPFYGYIEQCLQYTHRALSDVS--LPRRFTRRQLEY
BdAstV-1  LLCFQLLSAFLPEDHPFKNYVEMSLASTAKQLPGTA--LPPRFTEEQLHC
CslAstV-2 LLCYQLLSTFLDEEHPFKGYVEQCLARTSKQLRDSG--LPARFTEEQLRR
CslAstV-3 LLCYQLLAAFMAEDHPFKVYVEHCLSRTAKQLRASG--LPARLTEEQLHR
Humastr3  LLCYQLLAAFMAEDHPFKVYVEHCLSRTAKQLRESG--LPARLTEEQLHR
Humastr5  LLCYQLLAAFMAEDHPFKVYVEHCLSRTAKQLRDSG--LPARLTEEQLHR
Humastr4  LLCYQLLAAFMAEDHPFKVYIEHCLSRTAKQLRDSG--LPARLTEEQLHR
Humastr1  LLCYQLLAAFMAEDHPFKVYVEHCLSRTAKQLRDSG--LPARLTEEQLHR
Humastr8  LLCYQLLAAFMAEDHPFKVYVEHCLSRTAKQLRDSG--LPARLTEEQLHR
BatAstPa  VLSFKVLMHNLPDDDPGKIFILNCESALRRHMDAVG-QPWVNFTTSMLDF
BatAstMm2 VLSYKVLTHNLPDDDPSKQFVLACELSINKHLRARG-VDPITFTREMLDF
BatAstMp  ILSYQILTHNLPDDDPVKTWFEEAHASLTLHNRVHGGDPLPVMSRDMRDF
BatAstMm  ILSYQILTHNLPDDDPVKKWFEEAHSALVMHNRVSGGDPLPTITRDMRDF
BatAstMp2 ILSYQILTHNLPDDDPVKTWFEEAHSALVVHNRVSGGDPLPTITRDMRDF
SslAstV-1 LQCYGILGHNLPTDDEFKNYIYLALEVLARHIRAAGGEEPVRFTDRMLDA
Ovineastr LLCYRILNHNLPNDNKFRNYILVALEVMARHYSSRGEEPPFYVTESMLDK
Minkastro VLCYRILGHNLSDEHEFKRYVRVALEVLARHIRNLGGEEPVHVTERLLDK
CslAstV-1 VLCYNILGYNLEDEHEFKNYARIALEVLARHIRNMGGEEPVHVTEKMLDV

          251       263
AvNeph1   IW----------
Turkastr1 IW----------
Turkastr2 IW----------
MLB1      IWRGGPNDDYG--
BdAstV-1  IWRGGPKICNG--
CslAstV-2 IWRGGPKTCDG--
CslAstV-3 IWRGGPKKCDG--
Humastr3  IWRGGPKKCDG--
Humastr5  IWRGGPKKCDG--
Humastr4  IWRGGPKKCDG--
Humastr1  IWRGGPKKCDG--
Humastr8  IWRGGPKKCDG--
BatAstPa  LWSGGPN------
BatAstMm2 LWRGGPN------
BatAstMp  LWSGGPKKDGRST
BatAstMm  LWS----------
BatAstMp2 LWR----------
SslAstV-1 LWRGGPK------
Ovineastr LWRGGPKFDYG--
Minkastro LWRGGPK------
CslAstV-1 LWRGGPKRRDG--
```

APPENDIX B
T-COFFEE ALIGNMENT OF PARTIAL ASTROVIRAL CAPSID AMINO ACID SEQUENCES

```
           1                                                          60
AvNeph1    MAGGATAPAG--------------------AKPKQPKQKQKKPSSQARKKPSQKQKAM-
Turkastr1  ------------MS--APAGKAGPKAQ-----KKCKVVTQ---KTKTVPKKTKQQKPRK
Turkastr2  MAAMADKVVVKKTTT--RRRGRSNSRSR-----SRSRSRSRTKKTVKIIEKKPEK-SILK
cheetahcap MASKPGKEVTVEVNN-TNGRSRSKSQPR-----SRSRGR--GKT-VKITVNSKGGSRG-R
felineastr MASKPGKEVTVEVNN-TNGRSRSKSQPR-----SRSRGR--GKT-VKITVNSKGGSRG-R
Humastr1   MASKPSKQVTVEVN---NGRSRSRSRPR-----SQSRGR--DKS-VKITVNSRNKGR--R
Humastr2   MASKSDKQVTVEVNN--NGRNRSKSRAR-----SQSRGR--GRS-VKITVNSHNKGR--R
Humastr4   MASKSDKQVTVEVNN--NGRSRSKSRAR-----SQSRGR--GRS-VKITVNSNNKGR--R
Humastr5   MASKPSKQVTVEVN---NGRSRSRSRPR-----SQSRGR--DKS-VKITVNSRNKGR--R
Humastr8   MASKSDKQVTVEVNN--NGRSRSKSRAR-----SQSRGR--GRS-VKITVNSHNKGR--R
Porcineast MASKSGKDVTVKVEN-TNGRGRSRSRSR-----SRSRAR--NKN-VKITINSKPGASG-G
Ovineastro --M-AEKPQQKAVAS--AAKQLAKEVVK-LDKITKSNGKQHPQKNVPARKWRPRQA----
Minkastro  MAS-ANQAAKAEAKK--VIEKVAKEVI----KETKNSAQRN---QGPGKRWNSKKGRHM-
BdAstV-1   MANGRSKDVSVEVKA--SGSQRSKSRSR-----SRSRGR--TPA-VKVTVNSKAKRFTRR
CslAstV-1  MAH-ANQAAKSEAKK--EVKKVVKELVKDVAKEAKKDAQRR---SAPNRRWKGQRG----
CslAstV-2  MASASGKNVTVEVKN-TG--SRSKSRGR-----SQSRGR--SKN-VKITVNSKPNRK--Q
CslAstV-3  MASKSDKKVTVEVKSNGNGRSRSKSRSR-----SQGRGR--KSD-VKITVNSKPRGG--G
MLB1       -MANASKGVTVNINN-----AKRKPRFT-----NNQRAR--STR-PNFTPAPKFR-----
BatAstMp   NNNITSAAPTADATP--SG--------VSTTTAPRAPRRRRRSRRVRFVNRPLIENDVF-
SslAstV-1  MAT-AGQAAKAEAKK--EVKKLVKEVKKEVKQERKNNHAG----------QGRRGRGQ-

           61                                                         120
AvNeph1    --KPVKQE-LRKVEKQVR--VLKARTNGPKVNDTMKTTVTVGTLVGQTQSGLNRQLRVSF
Turkastr1  VRLQKVE-------RQVKTL--KAKTRGPKISDTFSTVVTVGRIIGNNDDSLTRQLKVFV
Turkastr2  KIDQAERRDA----KQLRRI--RKKVQGPPVNSRMTTVVTLGQITGNKDNTLERKHKCFL
cheetahcap QNGRGKRQSTQRVRNIVNKQLRKQGVTGPKPAICQRATATLGTVGSNTSGTTEIEACILL
felineastr QNGRGKRQSAQRVRKIVNKQLRKQGVTGPKPAICQRATATLGTVGSNTSGTTEIEACILL
Humastr1   QTGRNKHQSNQRVRNIVNKQLRKQGVTGPKPAICQRATATLGTVGSNTSGTTEIEACILL
Humastr2   QNGRNKYQSNQRVRKIVNKQLRKQGVTGPKPAICQRATATLGTIGTNTTGATEIEACILL
Humastr4   QNGRNKYQSNQRVRKIVNKQLRKQGVTGPKPAICQTATATLGTIGSNTTGATEIEACILL
Humastr5   QNGRNKHQSNQRVRNIVNKQLRKQGVTGPKPAICQRATATLGTVGSNTSGTTEIEACILL
Humastr8   QNGRNKYQSNQRVRKIVNKQLRKQGVTGPKPAICQTATATLGTIGSNTTGATEIEACILL
Porcineast QRRRGKPQSDKRVRSIVKQQLDKSGVTGPKPAIRQRATATLGTIGSNSSGKTELEACILT
Ovineastro ------KPNNRRVTHKIKRELHKQGLEGPASRFRVTVSATIGKVGPNKEQGPELQIATFL
Minkastro  PKNNNNKGMKRTVDNEVKQKLKKEGLEGPRSRFSVRVSATIGKIGPNKEQGPELQIATFL
BdAstV-1   PSRRSFRAKNNSVKQQVRNQLKKQGLTGPAPAVVQTATATLGTIGPNTGNDAEREISFYL
CslAstV-1  ------KQTKQTVHKEANKKLRKEGLEGPRPRFSVRVSATIGKVGPNKEQGPELQIATFL
CslAstV-2  RRTGPRGGSSKRVARLVKQHLDKSGATGPKPAIAQKATATLGVVGANTSGNTELEMCLMT
CslAstV-3  RTGRGARQSNQRVARIVRKQLDKSGVKGPKPAVKQRATATLGTVGSNTSGNTELESCIFT
MLB1       --------KRRFIPNRNRRRRQNTSTTGPKPAVSQTITATLGTVGSNLSDVVETECAVFL
BatAstMp   --SGRRRFPRRFITRAVKREIKREGLEGPKVSVQQKITSTFGMIGPNTTDNAELELNFFL
SslAstV-1  RNNNHGRETKRTVDREVNRKMKREGLEGPKSRFTVRVSATIGRLGPNTTQGPELQLSAFM
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           121                                                       180
AvNeph1    NPLLMKSTEGGST-TPLSIRASMYEMWKPLSVEIFATPLSGFSSVVGSVGFMVITL-NGL
Turkastr1  NPLLMKNQDSGSTSSPLSIRASQYGLWKIAKLHVYFTPLAGSANVIGTVSFASLEQ-ES-
Turkastr2  NPLLMKSQETGQTATPLSVRASQYNLWKLSRLHVRLIPLAGKANILGSVVFLDLEQEAN-
cheetahcap NPVLVKDATGSTQFGPVQALGAQYSMWKLKYLNVKLTSMVGASAVNGTVVRVSLNP-TS-
felineastr NPVLVKDATGSTQFGPVQALGAQYAMWKLKYLNVKLTSMVGASAVNGTVVRVSLNP-TS-
Humastr1   NPVLVKDATGSTQFGPVQALGAQYSMWKLKYLNVKLTSMVGSSAVNGTVVRVSLNP-TS-
Humastr2   NPVLVKDATGSTQFGPVQALGAQYSMWKLKYLNVKLTSMVGASAVNGTVLRISLNP-TS-
Humastr4   NPVLVKDATGSTQFGPVQALGAQYSMWKLKYLNVRLTSMVGASAVNGTVVRISLNP-TS-
Humastr5   NPVLVKDATGSTQFGPVQALGAQYSMWKLKYLNVKLTSMVGSSAVNGTVVRVSLNP-TS-
Humastr8   NPVLVKDATGSTQFGPVQALGAQYSMWKLKYLNVRLTSMVGASAVNGTVVRISLNP-TS-
Porcineast NPILVKDNTGNNTFGPIVALGAQYSLWRIRFLRIKFTPMVGQSAVTGTVVRASLNP-TA-
Ovineastro HPSLVKEPNDGSNFGPLQAAAAQWGLWRISDLEVRFTPLVGSSAVTGSVTRASLNL-TQ-
Minkastro  HPSLMKEPNDGTNFGPLQAAAAQWGLWRLSSLEVKCTPLVGSSAVTGSIYRMSLNL-TQ-
BdAstV-1   NPALTKENTGSNAFGPVQALAAQYSMWRCSRAEIRFTPLIGPSAISGTAYRCSLNM-AG-
CslAstV-1  HPGLMKEPNDGTNFGPLQPPAAQWGMWRIASLSVRFTPLVGPSPVTGSVYRVSLNL-TQ-
CslAstV-2  NPCLVKDNTGNNAFGPVQALGAQYTMWRIKNLTVKLTPLVGSSAIVGTVVRMSLNS-TS-
CslAstV-3  NPCLVKDSTGSAQFGPIQALGAQYSLYKLSYLNVTLTPLVGASAVSGTVVRVSVNP-TA-
MLB1       NPIIAKDSGASATFGPLQSLGAQYALWRLKWLEVRLQPLVGNSAVSGTVARVSLNM-TT-
BatAstMp   HPALAKEANDGTAFGPLQALAAQYSLWKIKYLTLRFTPMVGASAVSGTVVRASLNL-SQ-
SslAstV-1  HPSLMKEPNDGTNFGPLQAAAAQWGLWQLSSMTVRFTPLVGPSAVTGSCYRASLNL-TQ-

           181                                  220
AvNeph1    E-ASADSIDTIKARRHVQMALGRPYRLKLSARELAGPREG
Turkastr1  GVATAESPDTIKAKYHAEVPIGSRFVWKVPPRMLTGPREG
Turkastr2  T-AGPESVDTIKARPHVEVPIGSKTVWKVHPRSALGPRQG
cheetahcap T-PSSTSWSGLGARKHLDVTVGKNAVFKLRPXDLGGPRDG
felineastr T-PSSTSWSGLGARKHLDVTVGRDAVFKLRPSDLGGPRDG
Humastr1   T-PSSTSWSGLGARKHLDVTVGKNAVFKLKPADLGGPRDG
Humastr2   T-PSSTSWSGLGARKHMDVTVGRNAVFKLRPSDLGGPRDG
Humastr4   T-PSSTSWSGLGARKHLDVTVGKNAVFKLKPSDLGGPRDG
Humastr5   T-PSSTSWSGLGARKHLDVTVGKNAVFKLKPADLGGPRDG
Humastr8   T-PSSTSWSGLGARKHLDVTVGKNAVFKLKPSDLGGPRDG
Porcineast T-PSSTGWSGLGARRHIDIVVGKAATFNLKASDLSGPREG
Ovineastro S-PGATSWGGLGARKHLDVPTGVSKVWKLRRGDLTGPRQT
Minkastro  S-PGNASWGGLGARKHKDIPAGKSVSWKLQRGDLAGPRQT
BdAstV-1   T-PSQTSWSGLGSRKHKDMHIGKSGSFKLTKKELSGPKET
CslAstV-1  S-PGNSSWGGLGARRHMDIPVGRQVTWKLTKGELYRPRQT
CslAstV-2  T-PSSTSWSGLGARLHADAVVGRSATFRLKPRDLAGPREG
CslAstV-3  T-PSSTSWSGLGARYHMDVMVGRKAVFKLRANQLNGPREG
MLB1       G-PTLNSWSGLGARIHKDVRVGSNLVWRIKQRLVSGPCET
BatAstMp   S-PGGSNWSGLGTRLHIDMHPGQVATFHLRGDQVGGPRDG
SslAstV-1  S-PGNASWGGLGARKHVDISVGRTVSWKLSRGDLAGPRQT
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APPENDIX C

OPTICAL DENSITY AT 405 NANOMETERS (OD_{405}) VALUES FOR 46 DOLPHIN SERUM SAMPLES USING A BOTTLENOSE DOLPHIN ASTROVIRUS 1 (BDASTV1) PEPTIDE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) AGAINST SELECTED PEPTIDE PAIRS

Animal ID	**Date**	**plate1 399 455**	**plate2 322 616**	**plate3 399scr 455scr**
Blank		0.555	0.362	0.454
1	9/28/06	0.155	0.375	0.427
2	7/26/06	0.217	0.361	0.555
3	8/3/06	0.061	0.418	0.559
4	8/10/06	0.111	0.298	0.456
5	11/2/06	0.149	0.280	0.474
6	8/24/06	0.896	0.904	0.785
7	8/11/06	0.102	0.256	0.480
8	8/3/06	0.044	0.391	0.905
21	8/24/06	0.747	0.682	1.341
24	8/8/06	0.046	0.916	0.677
25	1/26/05	0.027	0.263	0.472
26	7/6/06	0.292	0.446	0.748
27	10/3/06	1.099	1.247	1.317
28	9/26/06	0.154	0.188	0.309
29	9/26/06	0.278	1.031	0.524
30	9/22/06	0.054	0.443	0.729
31	9/27/06	0.179	0.410	0.327
33	2/13/06	-0.084	0.015	0.061
33	12/28/06	-0.025	0.058	0.090
34	8/7/06	0.390	0.760	0.502
35	7/31/06	0.216	0.213	0.501
36	8/16/06	0.655	0.514	0.637
37	7/6/06	0.186	0.183	0.541
38	8/29/06	0.267	0.588	0.528
39	4/15/06	0.098	0.188	0.088
40	8/23/06	0.381	0.291	0.331
136	1/9/07	0.656	0.826	0.913
137	6/25/00	0.446	0.450	0.496
138	8/11/06	0.281	0.194	0.201
139	8/23/00	0.594	0.410	0.508
140	12/18/01	0.234	0.735	0.347
141	11/9/93	1.128	1.000	0.988
142	3/2/92	0.372	0.658	0.599
143	3/4/93	1.415	1.437	1.409
144	9/23/92	0.618	0.595	0.428
145	11/19/93	0.710	0.585	0.488
146	8/9/95	0.784	0.779	1.070
147	1/18/07	0.103	0.243	0.462

149	1/18/07	0.258	0.250	0.520
150	1/24/07	0.284	0.816	0.396
151	1/17/07	0.347	0.275	0.356
152	1/30/06	0.036	0.073	0.135
153	1/18/07	0.063	0.512	0.292
154	12/28/06	0.375	0.391	0.301
155	1/10/07	0.375	0.582	0.458
156	1/17/07	0.268	0.438	0.347

APPENDIX D

OD_{405} VALUES FOR 146 DOLPHIN SERUM SAMPLES USING A BDASTV1 PEPTIDE ELISA AGAINST ALL FOUR PEPTIDES (TT322, TT399, TT455, TT616)

Collection	**Animal ID**	**Date**	**OD_{405}**
Managed			
Open-Water	1	9/28/06	0.593
	2	7/26/06	0.833
	3	8/3/06	0.544
	4	8/10/06	0.580
	5	11/2/06	0.604
	6	8/24/06	1.208
	7	8/11/06	0.618
	8	8/3/06	0.565
	9	9/18/06	1.577
	10	5/25/05	0.666
	11	8/17/06	0.486
	12	9/18/06	0.972
	13	7/7/05	0.674
	14	9/28/06	0.513
	15	7/6/06	0.496
	16	4/12/06	0.421
	17	8/17/06	0.655
	18	8/22/06	0.428
	19	8/24/06	0.628
	20	9/11/06	0.547
	21	8/24/06	1.670
	22	9/14/06	0.819
	23	8/30/06	0.603
	24	8/8/06	0.399
	25	1/26/05	0.404
	26	7/6/06	0.572
	27	10/3/06	1.080
	28	9/26/06	0.515
	29	7/20/06	0.520
	30	9/22/06	0.486
	31	9/27/06	1.059
	32	9/12/06	1.620
	33	2/13/06	0.469
	33	12/28/06	0.858
	34	8/7/06	1.502
	35	7/31/06	1.074
	36	8/16/06	1.631
	37	7/6/06	1.218
	38	8/29/06	1.265
	39	4/5/06	0.449
	40	8/23/06	1.065

	41	9/15/06	0.724
	42	8/11/06	1.324
	43	4/11/06	0.397
	44	9/18/06	0.959
	45	8/8/06	0.518
	46	9/14/06	0.787
	47	8/8/06	0.801
	48	9/13/06	0.818
	49	9/19/06	1.003
	50	8/29/06	0.564
	51	11/2/06	0.718
	52	11/7/06	0.830
	53	7/6/06	0.676
	54	7/26/06	0.730
	55	9/22/06	0.487
	56	9/29/06	2.498
	57	7/26/06	0.860
	58	7/31/06	0.739
	59	1/26/06	0.633
	60	9/18/06	0.249
	61	8/24/06	0.002
Wild	62		1.702
	63		0.780
	64		1.463
	65		0.999
	66		0.872
	67		2.147
	68		1.423
	69		0.770
	70		1.272
	71		1.285
	72		1.341
	73		1.227
	74		1.029
	75		0.813
	76		1.393
	77		1.275
	78		1.318
	79		0.843
	80		0.951
	82		0.969
	83		1.254
	84		1.068
	85		2.110
	86		1.020

	87		0.951
	88		1.627
	89		1.246
	90		0.664
	91		0.793
	92		1.414
	93		1.238
	94		0.418
	95		0.701
	96		0.954
	97		1.035
	98		1.521
	99		0.488
	68		0.959
	100		0.909
	101		0.638
	102		0.698
	103		8.830
	104		1.602
	105		0.698
	106		6.850
	107		0.466
	108		0.675
	109		0.241
	110		0.860
	111		0.838
	112		0.523
	113		0.772
	114		0.781
	115		0.371
	116		0.346
	117		0.320
	118		2.267
	119		1.195
	120		3.147
	121		1.903
	122		0.075
	123		2.242
	124		1.042
	125		2.018
Captive collection 1	126	5-Jun-06	1.089
	127	16-Jul-06	0.788
	128	3-Feb-07	0.937
	129	1-Nov-07	1.087

	130	5-Apr-06	4.870
	131	5-Sep-06	0.587
	132	17-Oct-06	1.822
	133	25-Oct-05	0.442
	134	13-Mar-06	0.351
	135	2-May-05	0.563
Captive collection 2	136	1/9/07	1.834
	137	6/25/00	1.160
	138	8/11/06	0.729
	139	8/23/00	1.096
	140	12/18/01	1.060
	141	11/9/93	2.185
	142	3/2/92	1.374
	143	3/4/93	2.062
	144	9/23/92	1.251
	145	11/19/93	1.185
	146	8/9/95	1.855
Captive collection 1	147	1/18/07	0.363
	148	1/18/07	0.067
	149	1/18/07	0.334
	150	1/24/07	0.335
	151	1/17/07	0.209
	152	1/30/06	0.356
	153	1/18/07	0.222
	154	12/28/06	0.509
	155	1/10/07	0.156
	156	1/17/07	0.263

Time series

Animal	**Date**	**Run1**	**Run2**
4	28-Feb-06	1.034	
4	10-May-06	1.026	
4	10-Aug-06	0.665	
4	9-Nov-06	0.815	
4	18-Jan-07	1.270	
4	25-Jan-07	1.249	
4	28-Jan-07	0.792	
4	8-Feb-07	0.994	
4	15-Feb-07	1.003	
4	20-Feb-07	0.953	
4	28-Feb-07	0.783	

4	13-Mar-07	0.790	
4	5-Apr-07	1.577	
4	2-May-07	1.468	
4	5-Jun-07	1.543	
4	9-Aug-07	1.401	
4	10-Oct-07	0.833	
4	11-Jan-08	0.998	
4	14-Feb-08	0.851	
9	26-Jul-01	2.140	1.957
9	29-Jul-02	2.004	1.785
9	5-Jun-03	1.694	1.304
9	13-Jan-04	1.725	1.346
9	13-Oct-04	1.847	1.737
9	15-Feb-05	1.618	1.565
9	30-Jun-05	1.821	1.974
9	8-Dec-05	2.043	1.696
9	28-Feb-06	1.814	1.753
9	5-Jun-06	1.927	1.735
9	5-Jul-06	1.800	1.669
9	25-Aug-06	1.813	1.592
9	10-Oct-06	1.739	1.867
9	24-Nov-06	1.514	1.889
9	23-Jan-07	1.565	1.734
9	19-Mar-07	1.018	1.211
9	26-Jul-07	1.034	1.124
9	27-Nov-07	1.341	1.599
9	12-Mar-08	1.106	1.313
21	11-Jan-06	0.922	0.937
21	9-Feb-06	0.795	0.611
21	8-Mar-06	1.096	0.729
21	6-Apr-06	1.302	0.495
21	1-Jun-06	8.110	5.463
21	10-Aug-06	2.188	1.299
21	13-Sep-06	1.700	1.077
21	19-Sep-06	1.453	0.719
21	25-Sep-06	2.796	1.731
21	23-Mar-07	1.093	0.598
21	19-Apr-07	1.232	0.671
32	26-Jul-01	1.058	
32	29-Jul-02	0.549	
32	5-Jun-03	1.079	
32	13-Jan-04	0.530	
32	13-Oct-04	0.655	
32	15-Feb-05	1.208	
32	30-Jun-05	1.270	
32	8-Dec-05	0.327	

32	28-Feb-06	0.691
32	5-Jun-06	0.705
32	5-Jul-06	1.085
32	25-Aug-06	0.150
32	10-Oct-06	0.609
32	24-Nov-06	0.952
32	23-Jan-07	0.629
32	19-Mar-07	0.875
32	26-Jul-07	0.913
32	27-Nov-07	0.991
34	17-Sep-01	0.615
34	27-Sep-02	0.709
34	8-May-03	0.494
34	12-Dec-03	0.355
34	4-May-04	0.470
34	11-Jan-05	0.324
34	20-May-05	0.523
34	26-Jan-06	0.414
34	24-Apr-06	0.348
34	24-May-06	0.442
34	29-Jun-06	0.204
34	31-Jul-06	0.130
34	28-Sep-06	0.343
34	6-Dec-06	0.218
34	23-Aug-07	0.266
34	8-Feb-08	0.176
36	17-Jul-01	0.651
36	16-Jul-02	0.534
36	28-May-03	0.567
36	4-Nov-03	0.603
36	21-Apr-04	0.652
36	24-Nov-04	0.723
36	23-Feb-05	0.732
36	1-Sep-05	0.527
36	16-Feb-06	0.573
36	2-Jun-06	0.671
36	6-Jul-06	0.612
36	28-Jul-06	0.465
36	21-Sep-06	0.726
36	5-Oct-06	0.655
36	17-Nov-06	0.661
36	27-Feb-07	0.263
36	13-Sep-07	0.403
36	20-Mar-08	0.403
56	25-Oct-01	1.412
56	12-Sep-02	0.835

56	28-May-03	1.359
56	22-Apr-04	1.330
56	26-Oct-04	1.432
56	8-Feb-05	1.307
56	21-Jul-05	1.461
56	20-Dec-05	1.293
56	7-Mar-06	1.281
56	8-Jun-06	1.680
56	1-Aug-06	1.348
56	8-Nov-06	1.401
56	22-Feb-07	1.273
56	24-May-07	1.159
56	18-Oct-07	1.587
56	8-Apr-08	0.795

LIST OF REFERENCES

Aizaki, H., Watanabe, Y., Sarashina, T., Sakurai, T., Nagamori, S., Suzuki, T., Matsuura, Y., Saito, I., Miyamura, T., 1995. Genotyping of hepatitis C virus by a simple ELISA method. Clin. Diagn. Virol. 3, 105-109.

Al-Mutairy, B., Walter, J.E., Pothen, A., Mitchell, D.K., 2005. Genome Prediction of Putative Genome-Linked Viral Protein (VPg) of Astroviruses. Virus Genes. 31, 21-30.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389-3402.

Atkins, A., Wellehan, J.F.X. Jr., Childress, A.L., Archer, L.L., Fraser, W.A., Citino, S.B., 2009. Characterization of an outbreak of astroviral diarrhea in a group of cheetahs (Acinonyx jubatus). Vet. Microbiol. 136, 160-165.

Bass, D.M., Upadhyayula, U., 1997. Characterization of human serotype 1 astrovirus-neutralizing epitopes. J. Virol. 71, 8666-8671.

Bejder, L., Samuels, A., Whitehead, H., Gales, N., Mann, J., Connor, R., Heithaus, M., Watson-Capps, J., Flaherty, C., Krützen, M., 2006. Decline in relative abundance of bottlenose dolphins exposed to long-term disturbance. Conserv. Biol. 20, 1791-1798.

Belliot, G., Laveran, H., Monroe, S.S., 1997. Detection and genetic differentiation of human astroviruses: phylogenetic grouping varies by coding region. Arch. Virol. 142, 1323-1334.

Boni, M.F., Posada, D., Feldman, M.W., 2007. An exact nonparametric method for inferring mosaic structure in sequence triplets. Genetics. 176, 1035-1047.

Bracht, A.J., Brudek, R.L., Ewing, R.Y., Manire, C.A., Burek, K.A., Rosa, C., Beckmen, K.B., Maruniak, J.E., Romero, C.H., 2006. Genetic identification of novel poxviruses of cetaceans and pinnipeds. Arch, Virol. 151, 423-438.

Caballero, S., Guix, S., Ribes, E., Bosch, A., Pinto, R.M., 2004. Structural Requirements of Astrovirus Virus-Like Particles Assembled in Insect Cells. J. Virol. 78, 13285-13292.

Chu, D.K., Chin, A.W., Smith, G.J., Chan, K.H., Guan, Y., Peiris, J.S., Poon, L.L., 2010. Detection of novel astroviruses in urban brown rats and previously known astroviruses in humans. J. Gen. Virol. 91, 2457-2462.

Chu, D.K., Poon, L.L., Guan, Y., Peiris, J.S., 2008). Novel astroviruses in insectivorous bats. J. Virol. 82, 9107-9114.

Colegrove, K.M., Wellehan, J.F. Jr,, Rivera, R., Moore, P.F., Gulland, F.M., Lowenstine, L.J., Nordhausen, R.W., Nollens, H.H.. 2010. Polyomavirus infection in a free-ranging California sea lion (Zalophus californianus) with intestinal T-cell lymphoma. J Vet Diagn Invest. 22, 628-632.

Dai, Y.C., Xu, Q.H., Wu, X.B., Hu, G.F., Tang, Y.L., Li, J.D., Chen, Q., Nie, J., 2010. Development of real-time and nested RT-PCR to detect astrovirus and one-year survey of astrovirus in Jiangmen City, China. Arch. Virol. 155, 977-982.

Dallal, G.E., Wilkinson, L., 1986. An Analytic Approximation to the Distribution of Lilliefors's Test Statistic for Normality. The American Statistician. 40, 294-296.

Dennehy, P.H., Nelson, S.M., Spangenberger, S., Noel, J.S., Monroe, S.S., Glass, R.I., 2001. A prospective case-control study of the role of astrovirus in acute diarrhea among hospitalized young children. J. Infect. Dis. 184, 10-15.

Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792-1797.

Elamri, D.E., Aouni, M., Parnaudeau, S., Le Guyader, F.S., 2006. Detection of human enteric viruses in shellfish collected in Tunisia. Lett. Appl. Microbiol. 43, 399-404.

Espinosa, A.C., Mazari-Hiriart, M., Espinosa, R., Maruri-Avidal, L., Méndez, E., Arias, C.F., 2008. Infectivity and genome persistence of rotavirus and astrovirus in groundwater and surface water. Water Res. 42, 2618-2628.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution. 39, 783-791.

Felsenstein, J., 1989. PHYLIP-Phylogeny inference package. Cladistics. 5, 164-166.

Finkbeiner, S.R., Holtz, L.R., Jiang, Y., Rajendran, P., Franz, C.J., Zhao, G., Kang, G., Wang, D., 2009a. Human stool contains a previously unrecognized diversity of novel astroviruses. Virol. J. 6, 161.

Finkbeiner, S.R., Kirkwood, C.D., Wang, D., 2008. Complete genome sequence of a highly divergent astrovirus isolated from a child with acute diarrhea. Virol. J. 5, 117.

Finkbeiner, S.R., Li, Y., Ruone, S., Conrardy, C., Gregoricus, N., Toney, D., Virgin, H.W., Anderson, L.J., Vinjé, J., Wang, D., Tong, S., 2009b. Identification of a novel astrovirus (astrovirus VA1) associated with an outbreak of acute gastroenteritis. J. Virol. 83, 10836-10839.

Firth, A.E., Atkins, J.F., 2010. Candidates in Astroviruses, Seadornaviruses, Cytorhabdoviruses and Coronaviruses for +1 frame overlapping genes accessed by leaky scanning. Virol. J. 7, 17.

Fu, Y., Pan, M., Wang, X., Xu, Y., Xie, X., Knowles, N.J., Yang, H., Zhang, D., 2009. Complete sequence of a duck astrovirus associated with fatal hepatitis in ducklings. J. Gen. Virol. 90, 1104-1108.

Gallimore, C. I., Taylor, C., Gennery, A.R., Cant, A.J., Galloway, A., Lewis, D., Gray, J.J., 2005. Use of a heminested reverse transcriptase PCR assay for detection of astrovirus in environmental swabs from an outbreak of gastroenteritis in a pediatric primary immunodeficiency unit. J. Clin. Microbiol. 43, 3890–3894.

Geigenmüller, U., Chew, T., Ginzton, N., Matsui, S.M., 2002. Processing of nonstructural protein 1a of human astrovirus. J Virol. 76, 2003-2008.

Gibbs, M. J., Armstrong, J.S., Gibbs, A.J., 2000. Sister-Scanning: a Monte Carlo procedure for assessing signals in recombinant sequences. Bioinformatics 16, 573-582.

Gmyl, A.P., Belousov, E.V., Maslova, S.V., Khitrina, E.V., Chetverin, A.B., Agol, V.I., 1999. Nonreplicative RNA recombination in poliovirus. J. Virol. 73, 8958-8965.

Grimm, A.C., Cashdollar, J.L., Williams, F.P., Fout, G.S., 2004. Development of an astrovirus RT-PCR detection assay for use with conventional, real-time, and integrated cell culture/RT-PCR. Can. J. Microbiol. 50, 269-278.

Guix, S., Caballero, S., Bosch, A., Pintó, R.M., 2005. Human astrovirus C-terminal nsP1a protein is involved in RNA replication. Virology. 333, 124-131.

Harbour, D.A., Ashley, C.R., Williams, P.D., Gruffydd-Jones ,T.J., 1987. Natural and experimental astrovirus infection of cats. Vet Rec. 120, 555-557.

Henikoff, S., Henikoff, J.G., 1992. Amino acid substitution matrices from protein blocks. Proc. Natl. Acad. Sci. USA 89, 10915-10919.

Herbst, W., Krauss, H., 1988. Electron microscopy in the diagnosis of enteritis in cats. J Small Anim Pract. 29, 5-9.

Holmes, E.C., Worobey, M., Rambaut, A., 1999. Phylogenetic evidence for recombination in dengue virus. Mol. Biol. Evol. 16, 405-409.

Hoshino, Y., Zimmer, J.F., Moise, N.S., Scott, F.W., 1981. Detection of astroviruses in feces of a cat with diarrhea. Arch Virol. 70, 373-376.

Imada, T., Yamaguchi, S., Mase, M., Tsukamoto, K., Kubo, M., Morooka, A., 2000. Avian nephritis virus (ANV) as a new member of the family Astroviridae and construction of infectious ANV cDNA. J. Virol. 74, 8487-8493.

Jarvis, T.C., Kirkegaard, K., 1992. Poliovirus RNA recombination: mechanistic studies in the absence of selection. EMBO J. 11, 3135-3145.

Jauniaux, T., Charlier, G., Desmecht, M., Haelters, J., Jacques, T., Losson, B., Van Gompel, J., Tavernier, J., Coignoul, F., 2000. Pathological findings in two fin whales (*Balaenoptera physalus*) with evidence of morbillivirus infection. J. Comp. Pathol. 123, 198-201.

Jonassen C.M., Jonassen, T., Saif, Y.M., Snodgrass, D.R., Ushijima, H., Shimizu, M., Grinde, B., 2001. Comparison of capsid sequences from human and animal astroviruses. J. Gen. Virol. 82, 1061-1067.

Jones, D.T., Taylor, W.R., Thornton, J.M., 1992. The rapid generation of mutation data matrices from protein sequences. Comput. Appl. Biosci. 8, 275-282.

Kapoor, A., Li, L., Victoria, J., Oderinde, B., Mason, C., Pandey, P., Zaidi, S.Z., Delwart, E., 2009. Multiple novel astrovirus species in human stool. J. Gen. Virol. 90, 2965-2972.

Kiang, D., Matsui, S.M., 2002. Proteolytic processing of a human astrovirus nonstructural protein. J Gen Virol. 83, 25-34.

Kobayashi, S., Kobayashi, M., Araki, K., Shinozaki, T., Yanagawa, Y., 1999. Antibody prevalence against astrovirus according to age groups. Kansenshogaku Zasshi. 73, 578-583.

Koci, M.D., Moser, L.A., Kelley, L.A., Larsen, D., Brown, C.C., Schultz-Cherry, S., 2003. Astrovirus induces diarrhea in the absence of inflammation and cell death. J. Virol. 77, 11798-11808.

Kolaskar, A.S., Tongaonkar, P.C., 1990. A semi-empirical method for prediction of antigenic determinants on protein antigens. FEBS Lett. 1990 Dec 10;276(1-2), 172-4.

Koopmans, M.P., Bijen, M.H., Monroe, S.S., Vinjé, J., 1998. Age-stratified seroprevalence of neutralizing antibodies to astrovirus types 1 to 7 in humans in The Netherlands. Clin. Diagn. Lab. Immunol. 5, 33-37.

Kosiol, C., Goldman, N., 2005. Different versions of the Dayhoff rate matrix. Mol. Biol. Evol. 22, 193-199.

Kriston, S., Willcocks, M.M., Carter, M.J., Cubitt, W.D., 1996. Seroprevalence of astrovirus types 1 and 6 in London, determined using recombinant virus antigen. Epidemiol. Infect. 117, 159-164.

Kurtz, J., Lee, T., 1978. Astrovirus gastroenteritis age distribution of antibody. Med. Microbiol. Immunol. 166, 227-230.

Kurtz, J.B., Lee, T.W., Craig, J.W., Reed, S.E., 1979. Astrovirus infection in volunteers. J. Med. Virol. 3, 221-230.

Kwang, J., Torres, J.V., 1994. Oligopeptide-based enzyme immunoassay for ovine lentivirus antibody detection. J. Clin. Microbiol. 32, 1813-1815.

Kyte, J., Doolittle, R.F., 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157, 105-132.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. ClustalW and ClustalX version 2. Bioinformatics 23, 2947-2948.

Li, L., Victoria, J.G., Wang, C., Jones, M., Fellers, G.M., Kunz, T.H., Delwart, E., 2010. Bat guano virome: predominance of dietary viruses from insects and plants plus novel mammalian viruses. J. Virol. 2010 84, 6955-6965.

Logan, C., O'Leary, J.J., O'Sullivan, N., 2007. Real-time reverse transcription PCR detection of norovirus, sapovirus and astrovirus as causative agents of acute viral gastroenteritis. J. Virol. Methods. 146, 36-44.

Lukashov, V.V., Goudsmit, J., 2002. Evolutionary relationships among Astroviridae. J. Gen. Virol. 83, 1397-1405.

Madeley, C.R., 1979. Comparison of the features of astroviruses and caliciviruses seen in samples of feces by electron microscopy. J. Infect. Dis. 139,519-523.

Madeley, C.R., Cosgrove, B.P., 1975. 28 nm particles in faeces in infantile gastroenteritis. Lancet 2, 451-452.

Major, M.E., Eglin, R.P., Easton, A.J., 1992. 3' terminal nucleotide sequence of human astrovirus type 1 and routine detection of astrovirus nucleic acid and antigens. J Virol Methods. 39, 217-225.

Marshall, J.A., Kennett, M.L., Rodger, S.M., Studdert, M.J., Thompson, W.L., Gust, I.D., 1987. Virus and virus-like particles in the faeces of cats with and without diarrhoea. Aust. Vet. J. 64, 100-105.

Martin, D., Rybicki, E., 2000. RDP: detection of recombination amongst aligned sequences. Bioinformatics. 16, 562-563.

Martin, D.P., Posada, D., Crandall, K.A., Williamson, C., 2005a. A modified bootscan algorithm for automated identification of recombinant sequences and recombination breakpoints. AIDS Res. Hum. Retroviruses. 21, 98-102.

Martin, D.P., Williamson, C., Posada, D., 2005b. RDP2: recombination detection and analysis from sequence alignments. Bioinformatics. 21, 260–262.

Matsui, S.M., Kim, J.P., Greenberg, H.B., Young, L.M., Smith, L.S., Lewis, T.L., Herrmann, J.E., Blacklow, N.R., Dupuis, K., Reyes, G.R., 1993. Cloning and characterization of human astrovirus immunoreactive epitopes. J. Virol. 67, 1712-1715.

Maynard Smith, J., 1992. Analyzing the mosaic structure of genes. J. Mol. Evol. 34, 126-129.

McGowen, M.R., Spaulding, M., Gatesy, J., 2009. Divergence date estimation and a comprehensive molecular tree of extant cetaceans. Mol. Phylogenet. Evol. 53, 891-906.

Méndez, E., Fernández-Luna, T., López, S., Méndez-Toss, M., Arias, C.F., 2002 Proteolytic processing of a serotype 8 human astrovirus ORF2 polyprotein. J. Virol. 76, 7996-8002.

Méndez, E., Salas-Ocampo, M.P., Munguía, M.E., Arias, C.F., 2003. Protein products of the open reading frames encoding nonstructural proteins of human astrovirus serotype 8. J Virol. 77, 11378-11384.

Méndez, E., Salas-Ocampo, E., Arias, C.F., 2004. Caspases mediate processing of the capsid precursor and cell release of human astroviruses. J. Virol. 78, 8601-8608.

Mitchell, D.K., Matson, D.O., Cubitt, W.D., Jackson, L.J., Willcocks, M.M., Pickering, L.K., Carter, M.J., 1999. Prevalence of antibodies to astrovirus types 1 and 3 in children and adolescents in Norfolk, Virginia. Pediatr. Infect. Dis. J. 18, 249-254.

Monroe, S.S., Carter, M.J., Hermann, J., Mitchell, D.K., Sanchez-Fauquier, A., 2005. Astroviridae, pp. 859-864. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, E., Ball, L.A. (ed.), Virus Taxonomy: Classification and Nomenclature of Viruses. Academic Press, San Diego.

Moser, L.A., Carter, M., Schultz-Cherry, S., 2007. Astrovirus increases epithelial barrier permeability independently of viral replication. J. Virol. 81, 11937-11945.

Moya, A., Holmes, E.C., Gonzalez-Candelas, F., 2004. The population genetics and evolutionary epidemiology of RNA viruses. Nat. Rev. Microbiol. 2, 279-288.

Nighot, P.K., Moeser, A., Ali, R.A., Blikslager, A.T., Koci, M.D., 2010. Astrovirus infection induces sodium malabsorption and redistributes sodium hydrogen exchanger expression. Virol. 401, 146-154.

Nilsen, A., Ulvestad, E., Marsden, H., Langeland, N., Myrmel, H., Matre, R., Haarr, L., 2003. Performance characteristics of a glycoprotein G based oligopeptide (peptide 55) and two different methods using the complete glycoprotein as assays for detection of anti-HSV-2 antibodies in human sera. J. Virol. Methods. 107, 21-27.

Nollens, H.H., Green, L.G., Duke, D., Walsh, M.T., Chittick, B., Gearhart, S., Klein, P.A., Jacobson, E.R., 2007. Development and validation of monoclonal and polyclonal antibodies for the detection of immunoglobulin G of bottlenose dolphins (*Tursiops truncatus*). J. Vet. Diagn. Invest. 19(5), 465-70.

Nollens, H.H., Wellehan, J.F., Archer, L., Lowenstine, L.J., Gulland, F.M., 2010. Detection of a respiratory coronavirus from tissues archived during a pneumonia epizootic in free-ranging Pacific harbor seals *Phoca vitulina richardsii*. Dis Aquat Organ. 90, 113-120.

Nolte, A.W., Freyhof, J., Stemshorn, K.C., Tautz, D., 2005. An invasive lineage of sculpins, *Cottus* sp. (Pisces, Teleostei) in the Rhine with new habitat adaptations has originated from hybridization between old phylogeographic groups. Proc Biol Sci. 272, 2379-2387.

Notredame, C., Higgins, D.G., Heringa, J., 2000. T-Coffee: A novel method for fast and accurate multiple sequence alignment. J. Mol. Biol. 302, 205-217.

Oliver, A.R., Phillips, A.D., 1988. An electron microscopical investigation of faecal small round viruses. J. Med. Virol. 24, 211-218.

Padidam, M., Sawyer, S., Fauquet, C.M., 1999. Possible emergence of new geminiviruses by frequent recombination. Virol. 265, 218-225.

Pantin-Jackwood, M.J., Spackman, E., Woolcock, P.R., 2006. Phylogenetic analysis of Turkey astroviruses reveals evidence of recombination. Virus Genes. 32, 187-192.

Plagemann, P.G., 2001. Complexity of the single linear neutralization epitope of the mouse arterivirus lactate dehydrogenase-elevating virus. Virol. 290, 11-20.

Posada, D., Crandall, K.A., 2001. Evaluation of methods for detecting recombination from DNA sequences: Computer simulations. Proc. Natl. Acad. Sci. 98, 13757-13762.

Pulliam, J.R.C. 2008. Viral host jumps: Moving toward a predictive framework. Ecohealth 5, 80-91.

Quan, P.L., Wagner, T.A., Briese, T., Torgerson, T.R., Hornig, M., Tashmukhamedova A., Firth, C., Palacios, G., Baisre-De-Leon, A., Paddock, C.D., Hutchison, S.K., Egholm, M., Zaki, S.R., Goldman, J.E., Ochs H.D., Lipkin, W.I., 2010. Astrovirus encephalitis in boy with X-linked agammaglobulinemia. Emerg. Infect. Dis. 16, 918-925.

Rice, M., Wilks, C.R., Jones, B.R., Beck, K.E., Jones, J.M., 1993. Detection of astrovirus in the faeces of cats with diarrhoea. N Z Vet J. 41, 96-97.

Rieseberg, L.H., Kim, S.C., Randell, R.A., Whitney, K.D., Gross, B.L., Lexer, C., Clay, K., 2007. Hybridization and the colonization of novel habitats by annual sunflowers. Genetica. 129, 149-165.

Rivera, R., Nollens, H.H., Venn-Watson, S., Gulland, F.M.D., Wellehan, J.F.X., 2010. Characterization of phylogenetically diverse astroviruses of marine mammals. J. Gen. Virol. 91, 166-173.

Ronquist, F., Huelsenbeck,J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 19, 1572-1574.

Royuela, E., Negredo, A., Sánchez-Fauquier. A., 2006. Development of a one step real-time RT-PCR method for sensitive detection of human astrovirus. J. Virol. Methods. 133, 14-19.

Sánchez-Fauquier, A., Carrascosa, A.L., Carrascosa, J.L., Otero, A., Glass, R.I., Lopez, J.A., San Martin, C., Melero, J.A., 1994. Characterization of a human astrovirus serotype 2 structural protein (VP26) that contains an epitope involved in virus neutralization. Virol. 201, 312-320.

Schulman, F.Y., Lipscomb, T.P., Moffett, D., Krafft, A.E., Lichy, J.H., Tsai, M.M., Taubenberger, J.K., Kennedy, S., 1997. Histologic, immunohistochemical, and polymerase chain reaction studies of bottlenose dolphins from the 1987-1988 United States Atlantic coast epizootic. Vet. Pathol. 34:288-295.

Simmonds, P., 2006. Recombination and selection in the evolution of picornaviruses and other Mammalian positive-stranded RNA viruses. J. Virol. 80, 11124-11140.

Smith, A.W., Skilling, D.E., 1979. Viruses and virus diseases of marine mammals. J. Am. Vet. Med. Assoc. 175, 918-920.

Smith, A.W., Skilling, D.E., Benirschke, K., Albert, T.F., Barlough, J.E., 1987. Serology and virology of the bowhead whale (*Balaena mysticetus* L.). J. Wildl. Dis. 23, 92-98.

Smith, A.W., Skilling, D.E., Cherry, N., Mead, J.H., Matson, D.O., 1998. Calicivirus emergence from ocean reservoirs: zoonotic and interspecies movements. Emerg. Infect. Dis. 4, 13-20.

Spackman, E., Kapczynski, D., Sellers, H., 2005. Multiplex real-time reverse transcription-polymerase chain reaction for the detection of three viruses associated with poult enteritis complex: turkey astrovirus, turkey coronavirus, and turkey reovirus. Avian Dis. 49, 86-91.

Speroni, S., Rohayem, J., Nenci, S., Bonivento, D., Robel, I., Barthel, J., Luzhkov, V.B., Coutard, B., Canard, B., Mattevi, A., 2009. Structural and biochemical analysis of human pathogenic astrovirus serine protease at 2.0 Å resolution. J Mol Biol. 387, 1137-1152.

Strain, E., Kelley, L.A., Schultz-Cherry, S., Muse, S.V., Koci, M.D., 2008. Genomic analysis of closely related astroviruses. J. Virol. 82, 5099-5103.

Suttle, C.A., 2007. Marine viruses- major players in the global ecosystem. Nat. Rev. Microbiol. 5, 801-12.

Taylor, M.B., Grabow, W.O., Cubitt, W.D., 1997. Propagation of human astrovirus in the PLC/PRF/5 hepatoma cell line. J. Virol. Methods. 67, 13-18.

Ulloa, J.C., Gutiérrez, M.F., 2010. Genomic analysis of two ORF2 segments of new porcine astrovirus isolates and their close relationship with human astroviruses. Can. J. Microbiol. 56, 569-577.

Unicomb, L.E., Banu, N.N., Azim, T., Islam, A., Bardhan, P.K., Faruque, A.S., Hall, A., Moe, C.L., Noel, J.S., Monroe, S.S., Albert, M.J., Glass, R.I., 1998. Astrovirus infection in association with acute, persistent and nosocomial diarrhea in Bangladesh. Pediatr. Infect. Dis. J. 17, 611-614.

van Hemert, F.J., Lukashov, V.V., Berkhout, B., 2007. Different rates of (non)-synonymous mutations in astrovirus genes; correlation with gene function. Virol. J. 4, 25.

van Maarseveen, N.M., Wessels, E., de Brouwer, C.S., Vossen, A.C., Claas, E.C., 2010. Diagnosis of viral gastroenteritis by simultaneous detection of Adenovirus group F, Astrovirus, Rotavirus group A, Norovirus genogroups I and II, and Sapovirus in two internally controlled multiplex real-time PCR assays. J. Clin. Virol. *In press*. Available online 2010 Sep 7.

van Nieuwstadt, A.P., Cornelissen, J.B., Vreeswijk, J., 1988. Solid phase immune electron microscopy for diagnosis of transmissible gastroenteritis in pigs. Res.Vet. Sci. 44, 286-294.

Veerassamy, S., Smith, A., Tillier, E.R., 2003. A transition probability model for amino acid substitutions from blocks. J. Comput. Biol. 10, 997-1010.

Walter, J.E., Briggs, J., Guerrero, M.L., Matson, D.O., Pickering, L.K., Ruiz-Palacios, G., Berke, T., Mitchell, D.K., 2001. Molecular characterization of a novel recombinant strain of human astrovirus associated with gastroenteritis in children. Arch. Virol. 146, 2357-2367.

Walter, J.E., Mitchell, D.K., 2003. Astrovirus infections in children. Curr. Opin. Infect. Dis. 16, 247-253.

Wellehan, J.F., Johnson, A.J., Childress, A.L., Harr, K.E., Isaza, R. 2008. Six novel gammaherpesviruses of Afrotheria provide insight into the early divergence of the Gammaherpesvirinae. Vet Microbiol. 127, 249-257.

Whelan, S., Goldman, N., 2001. A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. Mol. Biol. Evol. 18, 691-699.

Willcocks, M.M., Carter, M.J., 1992. The 3' terminal sequence of a human astrovirus. Arch Virol. 124:279-289.

Woolhouse, M.E., Gowtage-Sequeria, S., 2005. Host range and emerging and reemerging pathogens. Emerg. Infect. Dis. 11, 1842-1847.

Zhang, T., Breitbart, M., Lee, W.H., Run, J.Q., Wei, C.L., Soh, S.W., Hibberd, M.L., Liu, E.T., Rohwer, F., Ruan, Y., 2006. RNA viral community in human feces: prevalence of plant pathogenic viruses. PLoS Biol. 4, e3.

Zhang, Z., Mitchell, D.K., Afflerbach, C., Jakab, F., Walter, J., Zhang, Y.J., Staat, M.A., Azimi, P., Matson, D.O., 2006. Quantitation of human astrovirus by real-time reverse-transcription-polymerase chain reaction to examine correlation with clinical illness. J. Virol. Methods. 134, 190-196.

Zhu, H.C., Chu, D.K., Liu, W., Dong, B.Q., Zhang, S.Y., Zhang, J.X., Li, L.F., Vijaykrishna, D., Smith, G.J., Chen, H.L., Poon, L.L., Peiris, J.S., Guan, Y., 2009. Detection of diverse astroviruses from bats in China. J. Gen. Virol. 90, 883-887.

BIOGRAPHICAL SKETCH

Jim Wellehan grew up in Auburn, Maine. He graduated from Bowdoin College in 1992 with a B.A. in biochemistry. In 2001, he graduated from the University of Minnesota as a Doctor of Veterinary Medicine, and a Master of Science in Veterinary Molecular Biosciences on "*Mycoplasma* infections in passerine birds in Minnesota". This was followed by an internship in Avian/Exotic/Wildlife Medicine and Surgery at Ontario Veterinary College in 2001/2002, and a residency in Zoological Medicine at the University of Florida from 2002-2005. After becoming a diplomate of the American College of Zoological Medicine and serving as a clinical instructor at Disney's Animal Kingdom in 2005/2006, he returned to the University of Florida to begin Doctor of Philosophy studies and served as an attending clinician on the Zoological Medicine Service at the College of Veterinary Medicine. He became a diplomate of the American College of Veterinary Microbiologists in both the Virology and Bacteriology/Mycology subspecialties in 2006. He is currently a Clinical Assistant Professor at the University of Florida College of Veterinary Medicine and serves as an attending clinician for the Zoological Medicine Service, as service chief for the Clinical Microbiology Laboratory at the University of Florida Veterinary Hospitals, and as codirector of the Marine Animal Disease Laboratory. He is very happily married to Dr. Karen Schaedel, and they have two children, Xavier and Elseya.