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Source: Journal of Wildlife Diseases, 40(1) : 53-59

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-40.1.53

Journal of Wildlife Diseases, 40(1), 2004, pp. 53–59

RETROSPECTIVE DIFFERENTIATION OF CANINE DISTEMPER VIRUS AND PHOCINE DISTEMPER VIRUS IN PHOCIDS

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ABSTRACT: Formalin-fixed paraffin-embedded tissues from one Caspian seal (*Phoca caspica*), one harp seal (*Phoca groenlandica*), one hooded seal (*Cystophora cristata*), and one harbor seal (*Phoca vitulina vitulina*) were used to compare the utility of immunohistochemistry (IHC) versus that of a novel seminested reverse transcriptase polymerase chain reaction (RT-PCR) to detect and differentiate canine distemper virus (CDV) and phocine distemper virus (PDV). Four antibodies made against PDV were able to detect both viruses. Two antibodies made against cetacean morbillivirus (CMV) did not label antigens from either CDV or PDV. A third anti-CMV antibody inconsistently stained CDV antigens but did not label PDV antigens. The seminested RT-PCR was able to detect RNA of the phosphoprotein gene in all positive cases. Nucleotide sequence analyses of seminested RT-PCR products were used to differentiate CDV RNA from PDV RNA. From these data, it was determined that IHC using antibodies generated against PDV provided a rapid means of detection for both CDV and PDV antigens; however, differentiation between CDV and PDV was achieved only with the RT-PCR assay.

Key words: Canine distemper, CDV, immunohistochemistry, phocine distemper, PDV, RT-PCR, seal.

INTRODUCTION

Canine distemper virus (CDV) and phocine distemper virus (PDV) are two closely related members of the genus *Morbillivirus,* family *Paramyxoviridae*, which are negative-sense, single-stranded RNA viruses. Canine distemper virus is a highly contagious pathogen that has a worldwide distribution (Bilexenkrone-Møller, 1993; Gemma et al., 1996) and a wide host range that includes terrestrial carnivores (Deem et al., 2000) and phocids (Osterhaus et al., 1995). Epizootics in Baikal seals (*Phoca sibirica*) in 1987 (Grachev et al., 1989; Osterhaus et al., 1989) and Caspian seals (*Phoca caspica*) in 2000 (Kennedy et al., 2000) were attributed to CDV. Phocine distemper virus was first recognized in 1988 after being isolated from an epizootic in which over 17,000 harbor seals (*Phoca vitulina*) and grey seals (*Halichoerus grypus*) died in the North Sea (Osterhaus and Vedder, 1988). Since that time, evidence of distemper has been found in harbor seals in the eastern Atlantic (Visser et al., 1993; Jauniaux et al., 2001) and in harp (*Phoca groenlandica*), harbor, and hooded (*Cystophora cristata*) seals in the western Atlantic (Daoust et al., 1993; Duignan et al., 1993; Lipscomb et al., 2001). In these cases, infections of CDV and PDV resulted in polysystemic lesions (e.g., bronchopneumonia, lymphoid depletion, encephalitis, and dermatitis) that are indistinguishable on the basis of clinical and pathologic findings alone.

Because of their similar clinical presentations, antigenic similarities, and overlapping host ranges (Bilexenkrone-Møller, 1993), CDV and PDV can be difficult to distinguish by routine methods. Accurate diagnostic assays for these two viruses are of paramount importance considering their global distribution, broad host rang-

es, variety of presentations (Bilexenkrone-Møller, 1993), and history of mass mortality events (Osterhaus and Vedder, 1988; Roelke-Parker et al., 1996). Determining the specific virus responsible for a distemper outbreak is critical to understanding the epizootiology of outbreaks and implementing effective wildlife management strategies. Further complicating the diagnosis of distemper in marine mammals is the inherent difficulty of monitoring and sampling live marine mammals in their wild habitats. Stranding circumstances often necessitate that diagnoses be based on necropsy of carcasses with postmortem changes that render them unsuitable for virologic analysis. Because of these constraints, use of multiple tests has become the best diagnostic strategy for detection of morbillivirus infection, and development of new diagnostic assays is an important means of optimizing this strategy. The goal of this investigation was to develop assays based on reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC) for detection of CDV and PDV. These assays were then evaluated for their ability to differentiate these viruses in formalin-fixed paraffin-embedded (FFPE) phocine tissues.

MATERIALS AND METHODS

Tissue selection and history

Formalin-fixed paraffin-embedded tissues from a Caspian seal collected from Azerbaijan in 1997 (Forsyth et al., 1998), a hooded seal collected from the New Jersey (USA) coast in 1998 (Lipscomb et al., 2001), a harp seal collected from the Gulf of St. Lawrence (Canada) in 1991 (Daoust et al., 1993), and a harbor seal collected in 1988 from the coast of Northern Ireland (Kennedy et al., 1989) were obtained for evaluation. All samples had been previously determined to be infected with either CDV or PDV by different methods. For the Caspian seal, RT-PCR and nucleotide sequencing of frozen tissue specimens were positive for CDV; IHC on FFPE samples was negative (Forsyth et al., 1998). Southern blotting, RT-PCR, and IHC were used on FFPE tissue of the hooded seal to demonstrate the presence of PDV (Lipscomb et al., 2001). Immunohistochemistry was performed on FFPE tissues to detect PDV antigen in the harbor and harp seals (Kennedy et al., 1989; Daoust et al., 1993). The harbor seal was collected during the 1988 outbreak, in which the virus was isolated and more fully investigated from other seals from this epizootic (Osterhaus and Vedder, 1988). Autolysis of the samples varied from mild to severe, and formalin fixation time varied from days to months. Tissues included lung (harbor, harp, and hooded seals), kidney (harbor and harp seals), brain (harp seal), pancreas (Caspian seal), spleen (Caspian seal), stomach (harp seal), liver (harp seal), skin (harp seal), and esophagus (harp seal). Positive controls were tissues from two dogs infected with canine distemper virus (Stanton et al., 2002). Tissue from one aquarium-kept harbor seal that died of a noninfectious condition served as a negative control.

Immunohistochemistry

Three-micrometer sections of FFPE blocks were cut onto positively charged slides (Probe-On Plus, Fisher Scientific, Springfield, New Jersey). Viral antigens were retrieved by exposing slides immersed in citrate buffer (Antigen Unmasking Solution, Vector Laboratories Inc., Burlingame, California, USA) to microwaves. Nonspecific binding sites were blocked with a commercially available blocking solution (Power Block, BioGenex, San Ramon, California) or 2% goat serum. Slides were then incubated with one of eight primary antibodies. A commercially available mouse monoclonal anti-CDV nucleoprotein antibody (VMRD Inc., Pullman, Washington, USA) was used at a dilution of 1:3,000 as the positive control antibody. Experimental monoclonal antibodies (Mabs) included four antibodies made against PDV (2-1F7, 3-5A12, 2-4D9, and 3-2H1) and three Mabs made against cetacean morbillivirus (CMV: 1-3C1, 1-5B5, and 1-8H1). Protocols were optimized for each primary antibody resulting in dilutions of 1:250, 1:250, 1:50, 1:250, 1:50, 1:250, and 1:50, respectively. A secondary biotinylated goat anti-mouse immunoglobulin-G (IgG) antibody (DAKO Corp., Carpinteria, California) was applied to samples at a dilution of 1:250, followed by signal amplification with avidin-biotin–conjugated peroxidase (Elite Peroxidase, Vector Laboratories Inc.). Finally, the antigen-antibody complex was visualized by its reaction with 3,3′-diaminobenzidine (DAB). Slides were counterstained lightly with hematoxylin and coverslipped. For simplicity, the experimental Mabs mentioned heretofore will be referred to as PDV1, PDV2, PDV3, PDV4, CMV1, CMV2, and CMV3, respectively.

Reverse transcriptase polymerase chain reaction

Extraction of RNA and RT-PCR were performed as previously described (Stanton et al., 2002). Briefly, total RNA was isolated from FFPE blocks containing one or more tissues from a single animal by deparaffinization, phe-

nol/chloroform extraction, and ethanol precipitation. Reverse transcriptase polymerase chain reaction for CDV nucleic acid was performed as a seminested RT-PCR technique. Primers were selected on the basis of three criteria: 1) amplicon size of approximately 150 base pairs (bp), 2) conserved sequences between CDV and PDV at sites of primer hybridization, and 3) variable sequences between primer locations. The following primers were used: CDV1, 5′-AACTGC AGAGTCTTCCCATC-3′ antisense primer (bases 285–304); CDV2, 5′-GGCGAAGATTAT TCCGAAGG-3′ sense primer (bases 135–154); and CDV3, 5′-AATGCTTCATCTAACTGGGG-3′ internal primer (bases 156–175). The targeted amplification sequence was a 149-bp fragment spanning bases 156–304 of the phosphoprotein gene (Onderstepoort strain, M32418).

Amplicons were separated by agarose gel electrophoresis and visualized by ethidium bromide staining and ultraviolet transillumination. Bands of the appropriate molecular mass were excised from gels, and DNA was extracted (Qiagen Gel Extraction Kit, Valencia, California) and subjected to direct sequencing by oligonucleotide-directed dideoxynucleotide chain termination cycle sequencing reactions. Beta-actin mRNA served as a control for the extraction of amplifiable RNA from tissue blocks, as previously described (Stanton et al., 2002).

Molecular phylogenetic analysis

Six PDV and 32 CDV sequences that included the region amplified by the seminested RT-PCR were collected from GenBank (www.ncbi.nlm.nih.gov/Genbank/) and used with the four sequences obtained from this study for phylogenetic analysis. Sequences were aligned and formatted by ClustalX (v1.81; Thompson et al., 1997). With PAUP* (v4.0b10; Swofford 2000), a maximum parsimony consensus tree was computed using the stepwise-addition option and a heuristic search method with 1,000 bootstrap replicates. Groups with a frequency greater than 50% were retained, and rinderpest virus and measles virus sequences were designated as outgroups.

RESULTS

Immunohistochemical and RT-PCR results are presented in Table 1. All four anti-PDV antibodies labeled antigen in all cases but did not differentiate between CDV and PDV. PDV3 was the antibody that stained both viruses most intensely. CMV2 inconsistently bound to CDV in the positive control canine cases and in the CDV-infected Caspian seal tissues, but it did not react with PDV-infected tissues. All other anti-CMV antibodies did not label either PDV or CDV antigens.

Labeling of distemper antigen in pancreas and spleen (the only tissues available) in the Caspian seal was weaker than the virus labeling observed in other tissues of other species; however, autolysis was most severe in the Caspian seal tissues.

Only pulmonary and renal tissues were available in multiple cases. In these tissues the degree of nonspecific staining was not affected by the seal species.

The seminested RT-PCR yielded an amplicon of the appropriate molecular weight in all cases previously identified by other laboratories as positive for either CDV or PDV. Nucleotide sequence analyses of the amplicons allowed for definitive differentiation between CDV- and PDV-infected cases (Figs. 1, 2), and the genetic sequenc-

TABLE 1. Immunohistochemistry (IHC) and RT-PCR results from canine distemper virus or phocine distemper virus infected phocids.

Specimen	Anti-PDV IHC				Anti-CMV IHC			Control IHC[a]	RT-PCR
	PDV1	PDV2	PDV3	PDV4	CMV1	CMV2	CMV3		
Caspian seal	+	±[b]	+	+	−	±	−	+	+(CDV)[c]
Hooded seal	+	+	+	+	−	−	−	+	+(PDV)
Harp seal	+	+	+	+	−	−	−	+	+(PDV)
Harbor seal	+	+	+	+	−	−	−	+	+(PDV)
Pos. control 1	+	+	+	+	−	±	−	+	+(CDV)
Pos. control 2	+	+	+	+	−	±	−	+	+(CDV)
Neg. control 1	−	−	−	−	−	−	−	−	−

[a] Anti-CDV nucleoprotein antibody (VMRD Inc., Pullman, Washington, USA).
[b] Inconsistent staining.
[c] Virus species determined by nucleotide sequence data.

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Baikal CDV:    ATATTCTTTCGGCCTTAAACCAGACAGAGCAGCTGATGTGAGCATGCTGATGGAA
P. caspica:    ----T----C--------A--------G--------------------G-----A
C. cristata:   ----A----T--------C--------A--------------------A-----G
P. vitulina:   ----A----T--------C--------A--------------------A-----G
P. groenlandi: ----A----T--------C--------A--------------------A-----G
Ulster PDV:    ATATACTTTTGGCCTTAACCCAGACAGAGCAGCTGATGTGAGCATGCTAATGGAG


Baikal CDV:    GAGGAATTGAGTGCTCTGCTCAGGACAAGCAGAAATGTAGGGATTCAGAAAAGG
P. caspica:    --G--A----G---------CAAG---A--AGA----TA---ATT--G-GA-G-
C. cristata:   --A--G----C---------TGGC---G--CAC----CC---GGC--A-AG-G-
P. vitulina:   --A--G----C---------TGGC---G--CAC----CC---GGC--A-AG-G-
P. groenlandi: --A--G----C---------TGGC---G--CAC----CC---GGC--A-AG-A-
Ulster PDV:    GAAGAGTTGACTGCTCTGCTTGGCACAGGCCACAATGCCGGGGGCCAAAAGAGG
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FIGURE 1. Comparison of 109-bp sequences from the phosphoprotein gene of canine distemper virus (CDV) and phocine distemper virus (PDV). Row 1: published sequence from CDV isolated from Lake Baikal seal in 1987 (GenBank AF259551). Row 2: CDV sequence amplified during this study from a Caspian seal collected in 1997 on the coast of Azerbaijan (AY332387). Row 3: PDV sequence amplified during this study from a hooded seal collected in 1998 on the coast of New Jersey (AY332389). Row 4: PDV sequence amplified during this study from a harbor seal collected in 1988 on the coast of Ireland (AY332390). Row 5: PDV sequence amplified during this study from a harp seal collected in 1991 from the Gulf of St. Lawrence (Canada; AY332388). Row 6: published sequence from Ulster 88 strain of PDV isolated from the 1988 PDV epizootic (GenBank D10371). Dashed lines represent conserved bases among the sequences.

es obtained also provided for further analysis of the infecting viruses. Nucleotide sequence analyses of amplicons from PDV-infected cases demonstrated a high degree of nucleotide sequence identity. The only variation among amplicons from the PDV-infected cases tested was a one nucleotide difference found in the amplicon from tissues of the harp seal (AY332388). There was 100% agreement between the nucleotide sequence of the PDV amplicon from the harbor seal collected on the coast of Ireland in 1988 (AY332390) and that from the hooded seal collected on the US coast in 1998 (AY332389).

Phylogenetic relatedness and groupings inferred from maximum parsimony analysis of multiple sequence alignments of CDV and PDV phosphoprotein gene sequences indicate that CDV and PDV sequences can be distinguished by this RT-PCR (Fig. 2). All PDV sequences in this study grouped into a well-supported (bootstrap value of 100) clade separate from all other morbillivirus sequences. Individual variation within the PDV sequences was not sufficient to provide phylogenetic data on strain variations. However, there was sufficient polymorphism within the CDV sequences to allow for differentiation of several, but not all, CDV isolates. Although the sequence from the Caspian seal (AY332387) could not be distinguished from that of the Siberian seal, it could be distinguished, with varying degrees of statistical confidence, from isolates from the Serengeti, Illinois (USA), Japan, and two-vaccine strains.

DISCUSSION

Canine distemper virus and PDV have many attributes that make them difficult viruses for investigators to differentiate. Both viruses induce a broad range of possible clinical signs and virtually indistinguishable postmortem lesions (Bilexenkrone-Møller, 1993). Both are environmentally unstable RNA viruses, making postmortem isolation difficult. Further complicating matters is the genetic and antigenic similarity of these two viruses (Bilexenkrone-Møller, 1993). Finally, lengthy postmortem intervals and extensive autolysis exacerbate these problems when attempting to diagnose and differentiate these viruses in dead, stranded marine mammals. All of the cases used in this study had been independently investigated

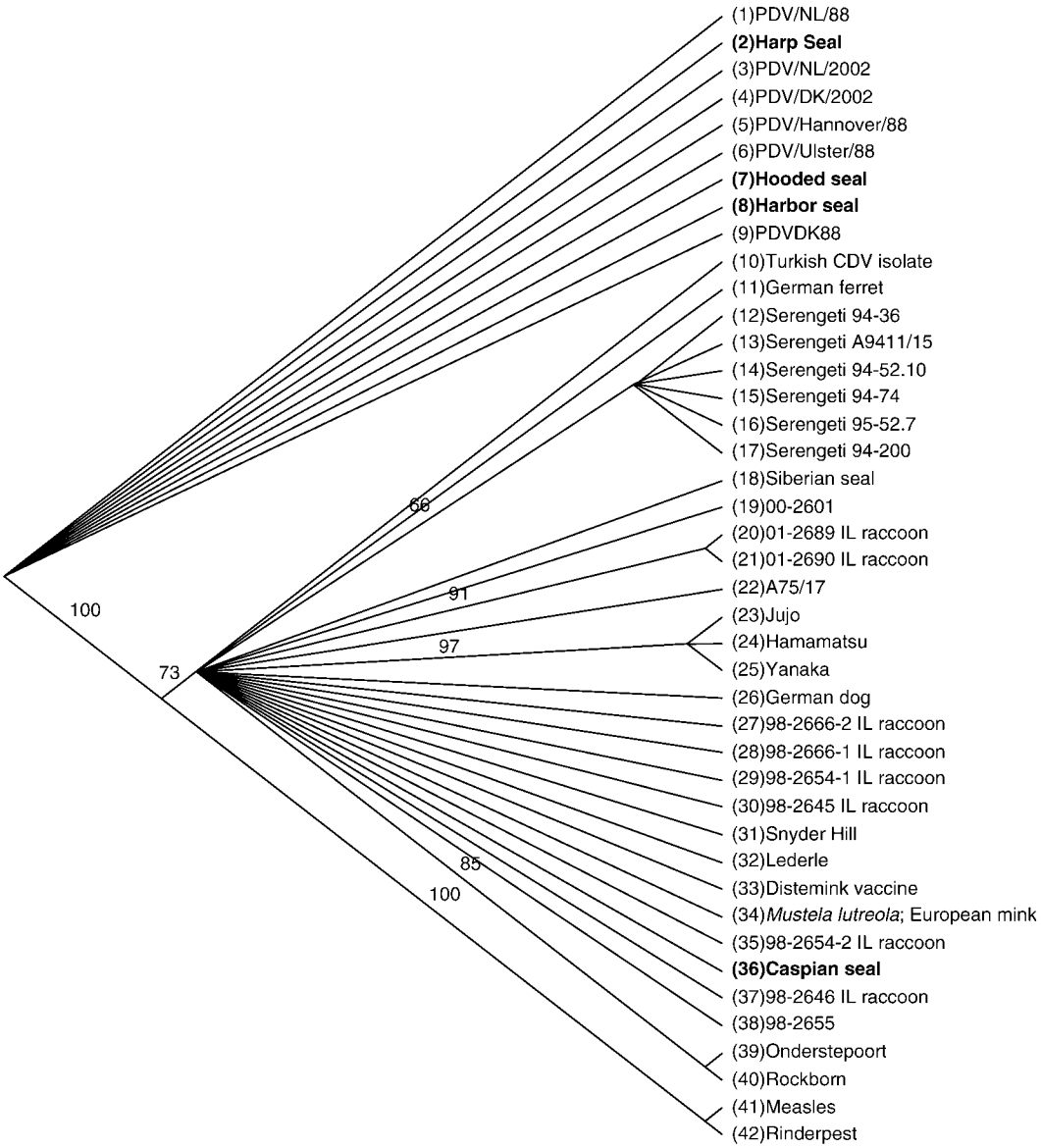

FIGURE 2. Bootstrap consensus maximum parsimony phylogenetic tree inferred from sequences of the morbilliviral phosphoprotein gene and displaying the associations of the one seal canine distemper virus (CDV) and three seal phocine distemper virus (PDV) sequences with those of other CDVs isolated from other host species from other parts of the world. GenBank numbers from top to bottom are (1) AF525289, (2) AY332388, (3) AF525288, (4) AF525287, (5) X65512, (6) D10371, (7) AY332389, (8) AY332390, (9) X75960, (10) AF384686, (11) AF259550, (12) Z46431, (13) U53715, (14) U53712, (15) U53711, (16) U53713, (17) U53714, (18) AF259551, (19) AY263374, (20) AY286488, (21) AY264266, (22) AF164967, (23) AB028916, (24) AB028915, (25) AB028914, (26) AF259549, (27) AY286487, (28) AY286486, (29) AY286484, (30) AY286482, (31) AY286481, (32) AY286480, (33) AY130857, (34) AY130856, (35) AY286485, (36) AY332387, (37) AY286483, (38) AY263373, (39) AF378705, (40) AF181446, (41) M89920, (42) X68311. Rinderpest virus and measles virus sequences were designated as outgroups.

at the time of collection. The method of investigation and the type of tissue used for that work varied from case to case. In all cases, the infecting virus was identified as either CDV or PDV; however, in only two cases were the viruses genetically characterized. During this study, methods were developed to consistently detect and genetically characterize these two viruses in FFPE tissues.

All four antibodies directed against PDV antigens reacted with both CDV and PDV. Subjectively, PDV3 stained the most intensely, whereas the intensity of the staining was similar among the other three antibodies. The use of these antibodies for immunohistochemical detection of CDV and PDV provided a rapid means of identifying the presence of one of these viruses. Antigen was even detected by these Mabs in the severely autolyzed Caspian seal tissues, which were negative by previous immunohistochemical assays, with Mabs directed toward the hemagglutinin protein of PDV (Kennedy et al., 2000). Immunohistochemical assays such as these also allow for study of the pathogenesis of these viruses via tissue tropism analysis in different marine mammal species. For example, intense staining was found in the epithelium of numerous bronchioles of the harbor and harp seals, but only a few pelvic urothelial cells were positive in these same animals. Also in the harp seal, the brain was positive, indicating simultaneous epithelial and nervous tissue infection.

The seminested RT-PCR used in this assay amplified both CDV and PDV RNA from FFPE tissues. This test was useful to confirm cases that were weakly positive by IHC because of severe autolysis. When combined with nucleotide sequence analysis, this test also provided a definitive means for differentiating CDV and PDV. Unfortunately, the small product size of this novel RT-PCR limited its ability to determine strain variations, and analysis of large fragments of the genomes would be required for more definitive phylogenetic characterization.

Reverse transcriptase polymerase chain reaction assays that make use of FFPE tissues are limited to shorter target sequences and a paucity of amplifiable RNA. Inability to amplify longer genetic sequences is a consequence of formalin fixation, which results in chemical modifications of RNA that prevent full-length enzymatic reactions (Masuda et al., 1999). The practical reality is that target sequence size is limited to approximately 150 bp in order to maintain consistent detection of RNA. In the current study, the 149-bp amplicon from the phosphoprotein gene contains enough polymorphism between CDV and PDV to confidently differentiate these viruses. Unfortunately, the amplicon is neither large enough nor sufficiently polymorphic to fully characterize different intraspecific strains, although it can still provide evidence to guide other attempts at strain determination. For example, it was possible to determine that the Caspian seal was infected with a strain of CDV that is likely distinct from the Rockborn or Onderstepoort vaccine strains or from viruses isolated in the Serengeti or Japan. Also, the results of multiple sequence alignments of the RT-PCR products from PDV-infected tissues from several different species of seals indicated that there is high nucleotide sequence identity within this region of the phosphoprotein gene among PDV isolates that had been obtained a decade apart and on opposite sides of the Atlantic ocean (Kennedy et al., 1989; Lipscomb et al., 2001). Examination of additional PDV isolates is required to determine whether PDV is truly less diverse or whether this result is a function of the limited number of isolates examined.

The IHC assays provided a fast and reliable method of detecting the presence of CDV or PDV antigens, but the Mabs used were unable to distinguish between these two viruses. In contrast, the seminested RT-PCR protocol provided distinct, easily interpreted amplicons, even when IHC staining was difficult to evaluate because of autolysis. In addition, direct sequencing of the products of this seminested RT-PCR provided nucleotide sequence data that allowed for virus differentiation. The ability to differentiate these two morbilli-

viruses from FFPE tissue samples can provide insight into the epizootiology of distemper outbreaks in phocids and assist in deciphering global distribution patterns.

ACKNOWLEDGMENTS

This study was supported by a grant from the Geraldine R. Dodge Foundation. We thank S. Kennedy (Department of Agriculture for Northern Ireland, Belfast, Northern Ireland) and P.-Y. Daoust (University of Prince Edward Island, Canada) for provision of seal tissue specimens. This is Scientific Contribution 2107 from the Storrs Agricultural Experiment Station. Our gratitude is also extended to the Athens Diagnostic Laboratory for the use of the canine tissues and to R. Gast, Woods Hole Oceanographic Institution, for her assistance with molecular phylogenetic analyses and her constructive commentary on the manuscript.

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Received for publication 11 July 2002.