

## A canine distemper outbreak in Alaska: diagnosis and strain characterization using sequence analysis

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**Abstract.** Vaccination with modified-live vaccines has been very effective in reducing the incidence of canine distemper, a disease that can be devastating in unvaccinated populations. A diagnostic submission to the Animal Health Diagnostic Laboratory at Michigan State University, East Lansing, Michigan, involved a case in which several hundred dogs in an Alaskan town died in a suspected canine distemper outbreak. Cytoplasmic and intranuclear eosinophilic inclusion bodies, consistent with canine distemper virus (CDV) infection, were found in urinary bladder, spleen, lung, and salivary gland. Direct fluorescent antibody test gave results that could be considered positive for canine distemper. Because of the condition of the tissues received, the histopathology and fluorescent antibody–staining results were suggestive but not conclusive of CDV. In this study, immunohistochemistry, reverse transcriptase–polymerase chain reaction (RT-PCR), and DNA sequencing were used to confirm the presence of canine distemper virus in these tissues and to perform molecular characterization of the virus. Immunohistochemistry showed the presence of the virus in spleen, lung, and salivary gland. Viral RNA was detected by RT-PCR in brain, spleen, liver, lung, and kidney, both with nucleoprotein and phosphoprotein (P)-gene–specific primers. Sequence alignment and phylogenetic analysis of a 540-bp P-gene fragment of the Alaskan strain with corresponding sequences of 2 vaccine and 7 wild-type CDV strains showed that the virus responsible for the outbreak was closely related to a virulent strain of distemper virus from Siberia.

Canine distemper virus (CDV) is an enveloped negative-strand RNA virus classified under the genus *Morbillivirus* within the family of *Paramyxoviridae*. Canine distemper has a very broad host range, which includes *Canidae*, *Mustelidea*, *Procyonidae*, *Ursidae*, *Viverridae*, and *Felidae*. The clinical picture of CDV infection in dogs is considered to be dependent on virus strain characteristics, age and immune status of the affected animal, and environmental factors. When population immunity is high, many CDV infections are either clinically unapparent or result in a clinical picture that is relatively mild and may go unrecognized as distemper. When infection occurs in highly susceptible young animals, however, severe multisystemic disease can result. The time of onset of the immune response and, likely, also the virulence of the virus are critical factors in the extent of viral invasion of epithelial tissues and of the central nervous system. The degree and extent of the distemper-induced encephalomyelitis varies somewhat between the various strains of virulent virus.<sup>2,3,5,12,13,15</sup>

A large number of dogs that were participating in a sled race in Kotzebue, Alaska, became ill with symptoms that were clinically consistent with canine distemper. Catarrh, mucopurulent nasal discharge, and

neurological signs were noted in the affected animals. During the first week of this outbreak, approximately 200 Alaskan Huskies and other mixed-breed dogs died and approximately an additional 100 or so died in the next 2 weeks. The infection spread from a small community of sled dogs, and within 2–3 weeks affected the local pet population and those of small communities up to 12 miles/20 kilometers away. The vaccination status of the dogs that died was not known. A local veterinarian, who was called to evaluate the situation, suspected that most of the animals were not vaccinated. One team of dogs within the affected area did not develop clinical signs and had a current vaccination history. An initial diagnosis of distemper was made at Washington State University, Pullman, Washington. Tissue samples from 1 of the dogs that had died during the outbreak, a Labrador cross, were sent to the Animal Health Diagnostic Laboratory (AHDL) at Michigan State University, East Lansing Michigan, for confirmatory diagnosis. Because of the condition of the tissues received, the histopathology and fluorescent antibody (FA)–staining results were suggestive but not conclusive of CDV. In this study, we used reverse transcriptase–polymerase chain reaction (RT-PCR) to confirm that the etiologic agent responsible for the outbreak was CDV. Sequencing, followed by sequence alignments, and construction of phylogenetic trees were used to characterize the strain involved at the molecular level.

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## Materials and methods

**Samples.** Tissue samples that were used in this study were obtained from 1 of the affected dogs, a Labrador retriever cross. All samples that were sent frozen to the AHDL were thawed on arrival. The tissues submitted included liver, spleen, heart, brain, salivary gland, bladder, small intestine, lung, and kidney. Portions of these tissues were fixed in 10% neutral-buffered formalin and the remaining portions were re-frozen at  $-70\text{ C}$ .

**Histopathology and fluorescent antibody stain.** Formalin-fixed tissues were paraffin embedded, sectioned at a thickness of  $6\text{ }\mu\text{m}$  and routinely processed with hematoxylin–eosin (HE) stains for histologic examination. A direct FA test<sup>a</sup> was used for canine distemper antigen detection. Frozen tissue sections were cut on a cryostat, placed on glass slides, and fixed in an acetone–methanol mixture (75:25) for 20 min at room temperature. After fixation, and before staining, the slides were dried for 10 min in a dry  $37\text{ C}$  incubator. Slides were then stained with  $50\text{--}75\text{ }\mu\text{l}$  of anti-CDV conjugate<sup>a</sup> for 30 min at  $37\text{ C}$  in a humid chamber. Slides were subsequently washed in FA wash buffer<sup>a</sup> pH 9.0, washed for 10 min in FA buffer, counterstained with Evans Blue, and viewed under a microscope equipped with a UV light source.<sup>b</sup>

**Immunohistochemistry.** Portions of the samples submitted were fixed in 10% neutral-buffered formalin, trimmed, and embedded in paraffin wax. Blocks were mounted on treated slides.<sup>c</sup> Prepared slides were incubated with a 1:10 dilution of a anti-CDV monoclonal antibody.<sup>d</sup> Subsequent steps were performed with reagents included in a commercial kit.<sup>e</sup> After the final rinse, slides were counterstained with Mayer hematoxylin.

**RT-PCR and sequence analysis.** Total RNA was extracted from tissue samples using a commercial kit.<sup>f</sup> Tissues examined included liver, spleen, lung, kidney, and brain. Reverse transcriptase–polymerase chain reaction was performed using a one-Step RT-PCR System.<sup>g</sup> Primers were selected from highly conserved regions of the phosphoprotein (P) and nucleoprotein (N)-genes of CDV using the Primer Express software.<sup>h</sup> P forward primer, 5' AAGAGGTTAAGGGAATCG 3', and P reverse primer, 5' GAGAAAAGCTCATCATCG, amplify a 585-bp fragment of the P-gene with the following cycling conditions: reverse transcription at  $45\text{ C}$  for 30 min and predenaturation at  $94\text{ C}$  for 2 min; followed by 45 cycles at  $94\text{ C}$  for 15 sec,  $55\text{ C}$  for 30 sec, and  $72\text{ C}$  for 1 min, with a final extension at  $72\text{ C}$  for 7 min after the last cycle. N forward primer, 5' CCTAACTATCAAGTTTGG 3', and N reverse primer, 5' CTGAGCATCTTAGTAAGC 3', amplify a 701-bp fragment of the N-gene following

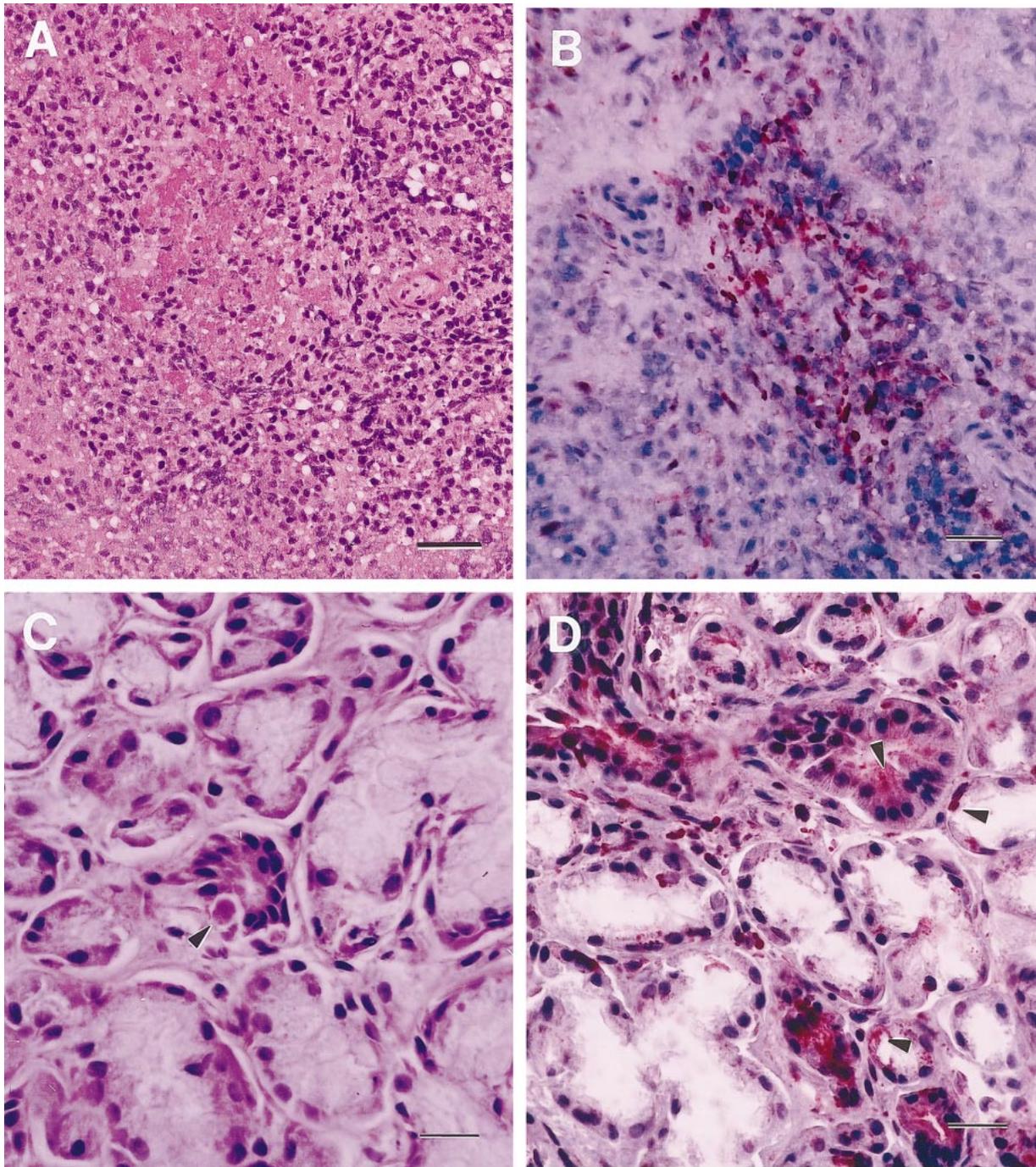
the cycling conditions for P-gene amplification except for a lower annealing temperature at  $50\text{ C}$  for 30 sec. Polymerase chain reaction products were analyzed by agarose gel electrophoresis and UV transillumination of ethidium bromide–stained gels. Polymerase chain reaction products of the expected size were extracted and purified from agarose gels using a commercial kit<sup>f</sup> and submitted with the appropriate sequencing primer to the Genomic Technology Support Facility at Michigan State University for automated DNA sequencing.

Sequence analyses, including multiple alignments of nucleotide and predicted amino acid sequences, were performed using the Lasergene biocomputing software.<sup>i</sup> Phylogenetic trees were constructed with the Treecon software package<sup>14</sup> using the Neighbor-joining method and 1,000 bootstrap analyses. Sequence alignment data generated using the Lasergene software were converted into Treecon format using the ForCon<sup>11</sup> software.

## Results

**Histopathology.** Tissues that were examined included brain, heart, liver, kidney, spleen, salivary gland, small intestine, lung, and urinary bladder. All tissues exhibited moderate freeze–thaw artifacts. The transitional epithelial lining of the urinary bladder was artifactually separated over most of the section; however, both cytoplasmic and intranuclear eosinophilic viral inclusions of  $3\text{--}5\text{ }\mu\text{m}$  diameter were present infrequently as single inclusions scattered throughout the epithelial cells. Splenic sections contained mild lymphoid follicular depletion and scattered foci of necrosis and also contained scattered eosinophilic inclusions within mononuclear cells similar to those found in the urinary bladder (Fig. 1A). Epithelial cells lining the ducts of the salivary gland contained occasional cytoplasmic eosinophilic inclusions (Fig. 1C). The lungs had rare intranuclear eosinophilic inclusions within bronchial epithelial cells. These inclusions were suggestive of CDV inclusions based on morphology, cellular location, and tissue distribution. The brain was morphologically normal, with a slight increase in glial cells, but there was no evidence of either mononuclear perivascular cuffing or demyelination of the white matter, which commonly accompany canine distemper infections. Sections of liver, kidney, and small intestine also appeared morphologically normal.

**Fluorescent antibody staining.** Urinary bladder, brain, lung, and kidney were weakly positive for canine distemper. The stain was multifocally distributed in the affected tissues, and the intensity of the fluorescence was adequate for detection; unfortunately, because of freeze–thaw artifacts, the specific cell type staining and cellular location of the staining were equivocal.

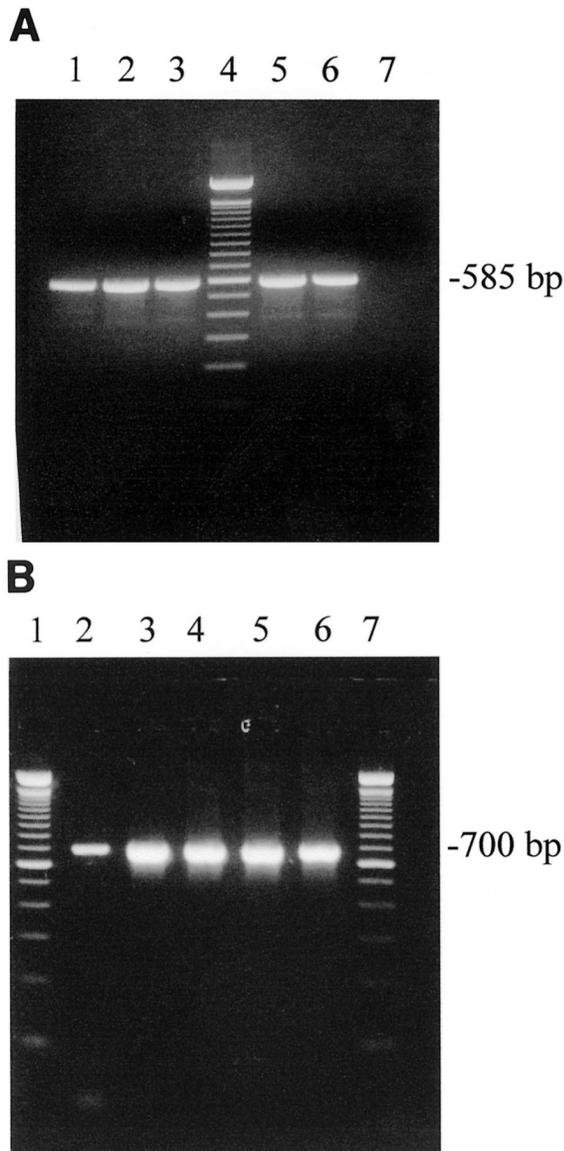


**Figure 1.** Tissues from a dog infected with canine distemper. **A**, spleen. The white pulp of the spleen has lymphoid depletion and fibrin accumulation. HE, Bar = 100  $\mu\text{m}$ . **B**, spleen. Red stain indicates canine distemper antigen within mononuclear cells; both cytoplasmic and intranuclear staining is present. Immunohistochemical stain, Bar = 50  $\mu\text{m}$ . **C**, salivary gland. Multiple presumptive distemper inclusions are present within epithelial cells lining acini and ducts (arrowhead). HE, Bar = 50  $\mu\text{m}$ . **D**, salivary gland. Strong cytoplasmic red staining of epithelial cells (arrowheads), indicating presence of canine distemper antigen. HE, Bar = 50  $\mu\text{m}$ .

*Immunohistochemistry.* Positive results were obtained from the spleen, salivary gland, and lung (Fig. 1B, 1D). The stain was diffusely distributed in the examined tissues. Several areas of the tissues had focal areas of intense staining, possibly suggesting elevated

levels of viral antigen. The results obtained from this technique appeared to provide a more definitive diagnosis than either routine histology or fluorescent antibody staining.

*RT-PCR and sequence analysis.* All tissues exam-



**Figure 2.** RT-PCR-based amplification of CDV sequences from different tissues. **A**, P-gene-specific primers. Lane 1, brain; lane 2, spleen; lane 3, liver; lane 4, molecular weight (MW) marker; lane 5, lung; lane 6, kidney; lane 7, negative control, using distilled H<sub>2</sub>O as the template. **B**, N-gene-specific primers. Lane 1, MW marker; lane 2, brain; lane 3, spleen; lane 4, liver; lane 5, lung; lane 6, kidney; lane 7, MW marker.

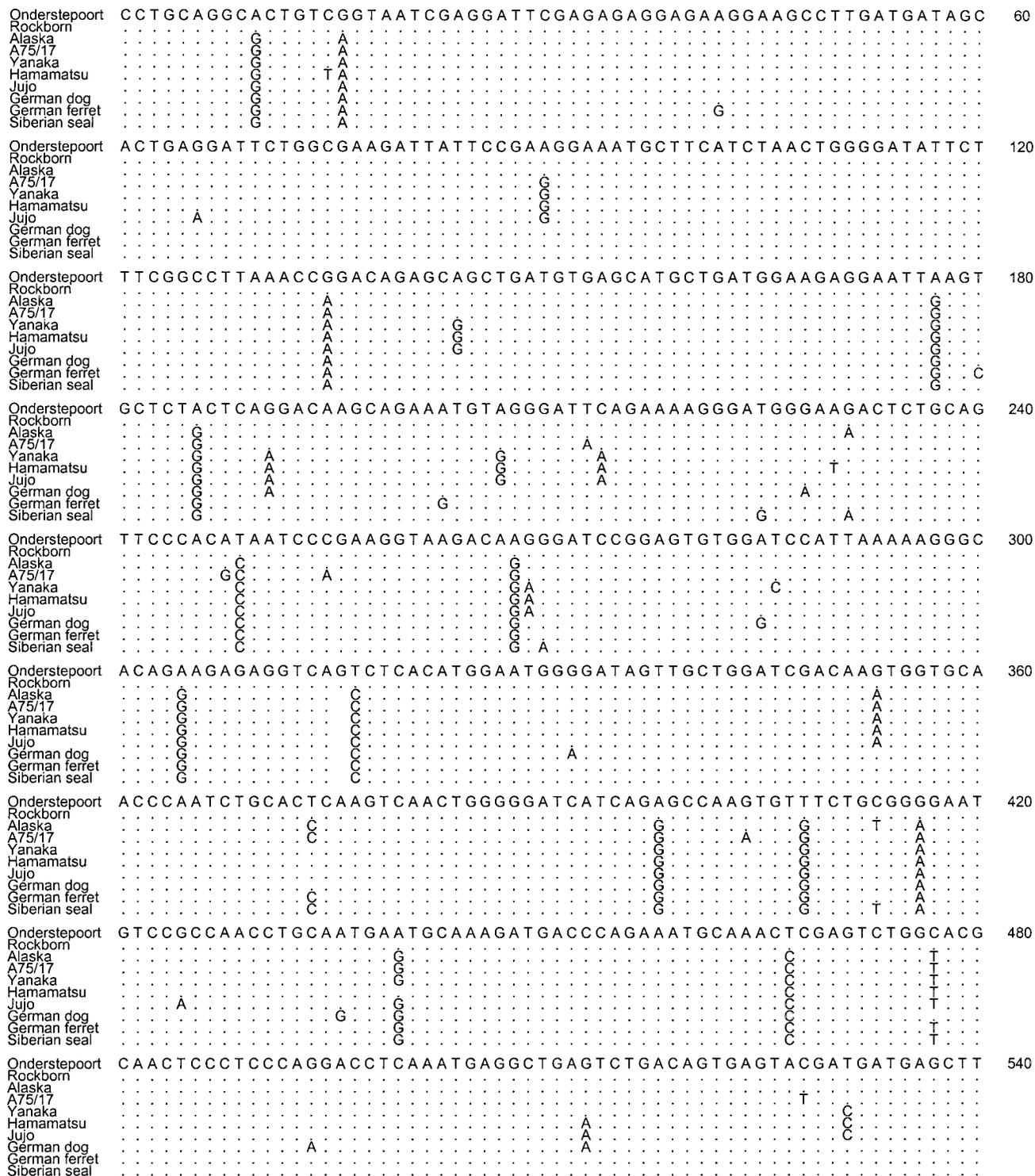
ined were positive for both RT-PCR assays and products of the expected size were obtained (Fig. 2A, 2B). Initial analysis of amplicon sequence data by the similarity search tool, BLAST,<sup>1</sup> confirmed the detection of CDV in the tissues. The Alaska P-gene 540-bp sequence data (CDV mRNA sense) was aligned with the corresponding sequences of the following CDV strains obtained from GenBank: Onderstepoort and Rockborn vaccine strains; virulent strains A75/17, Yanaka, Hamamatsu, Jujo; and CDV isolates from a German dog, a German ferret, and a Siberian seal (Fig. 3). The Si-

berian seal sequence exhibited the highest percent nucleotide identity of 99.4% with the Alaskan sequence with only 3 nucleotide differences between them. In this particular P-gene fragment, the Alaskan and Siberian strains shared 18 common nucleotide sequence changes from the vaccine strains, from a total of 19 and 20 sequence changes observed in the Alaskan and the Siberian sequence, respectively. The corresponding amino acid sequence for each CDV strain was derived and analyzed similarly by sequence alignment (Fig. 4). Again, the Alaskan sequence showed the highest sequence match with that of the Siberian seal, at 98.9%. These 2 strains shared 11 common amino acid changes from the vaccine sequences out of 12 total changes for each. Phylogenetic trees were generated from both the nucleotide (Fig. 5A) and amino acid (Fig. 5B) sequence alignment data, with the corresponding sequence of a phocid distemper virus (PDV) included as the out-group sequence. The results show that the Alaskan strain is genetically most closely related to the Siberian seal isolate.

### Discussion

Classic distemper outbreaks, such as the one presented in this study, have become increasingly rare in the continental United States since the 1950s and 1960s. Improved vaccination awareness has greatly reduced the frequency and magnitude of the disease occurrence. Studies have been conducted in the continental United States investigating the incidence of distemper, and there have been reports, based on survey results from private practitioners, of a slight increase in the frequency of the disease in certain areas of Indiana from 1992 to 1993.<sup>10</sup> Outbreaks of this disease have been reported in other countries around the world such as Great Britain, France, Germany, and Brazil.<sup>3</sup> The most common factor that has been associated with these outbreaks is the lack of appropriate vaccination. Another possibility, which is encountered occasionally, is the residual virulence of a vaccine strain. Regardless of the previously stated possible underlying causes for disease, distemper has proven to be an important worldwide disease involving many different species.<sup>5</sup>

Infection of susceptible dogs with virulent CDV results in a multisystemic disease with high morbidity and significant mortality. In this paper, the laboratory confirmation of an outbreak of distemper in Alaskan sled dogs is described. Histologic examination revealed the presence of viral inclusion bodies suggestive of CDV infection in urinary bladder, spleen, lungs, and salivary glands. Fluorescent antibody testing identified viral antigen in multiple tissues. The results could not be considered definitive, however, because the intensity of the FA stain was rather weak. Fur-



**Figure 3.** Nucleotide sequence alignment of a 540-bp P-gene fragment (mRNA sense) of the following canine distemper virus strains and isolates (GenBank accession numbers are in parentheses): vaccine strains, Onderstepoort (#NC\_001921) and Rockborn (#AF181446); isolate from an Alaskan dog, Alaska (this study); wild-type virulent strains, A75/17 (#AF164967), Yanaka (#AB028914), Hamamatsu (#AB028915), and Jujo (#AB028916); and isolates from a German dog (#AF259549), a German ferret (#AF259550), and a Siberian seal (#AF259551). Nucleotides identical to the sequence of the Onderstepoort strain are shown in dots.

Onderstepoort	P A G T V G N R G F E R G E G S L D D S T E D S G E D Y S E G N A S S N W G Y S F G L K P D R A A D V S M L M E E E L S	60
Rockborn	.....	
Alaska	..... A . S . . . . .	
A75/17	..... A . S . . . . .	
Yanaka	..... A . S . . . . .	
Hamamatsu	..... A . S . . . . .	
Jujo	..... A . S . . . . .	
German dog	..... A . S . . . . .	
German ferret	..... A . S . . . . .	
Siberian seal	..... A . S . . . . .	
Onderstepoort	A L L R T S R N V G I Q K R D G K T L Q F P H N P E G K T R D P E C G S I K K G T E E R S V S H G M G I V A G S T S G A	120
Rockborn	.....	
Alaska	..... G . . . . . G . . . . . A . . . . . N . . . . .	
A75/17	..... R . . . . . G . . . . . G . . . . . A . . . . . N . . . . .	
Yanaka	..... K . . . . . K . . . . . E . . . . . P . . . . . G . . . . . A . . . . . N . . . . .	
Hamamatsu	..... K . . . . . K . . . . . M . . . . . E . . . . . G . . . . . A . . . . . N . . . . .	
Jujo	..... K . . . . . K . . . . . E . . . . . G . . . . . A . . . . . N . . . . .	
German dog	..... K . . . . . G . . . . . G . . . . . A . . . . . E . . . . .	
German ferret	..... S . . . . . G . . . . . G . . . . . A . . . . .	
Siberian seal	..... E . . . . . G . . . . . G . . . . . A . . . . .	
Onderstepoort	T Q S A L K S T G G S S E P S V S A G N V R Q P A M N A K M T Q K C K L E S G T Q L P P R T S N E A E S D S E Y D D E L	180
Rockborn	.....	
Alaska	..... P . . . . . G . . . . . V E . . . . . S . . . . . P . . . . .	
A75/17	..... P . . . . . G . . . . . N . . . . . E . . . . . S . . . . . P . . . . .	
Yanaka	..... G . . . . . E . . . . . S . . . . . P . . . . .	
Hamamatsu	..... G . . . . . E . . . . . P . . . . .	
Jujo	..... G . . . . . E . . . . . H . . . . . S . . . . . P . . . . .	
German dog	..... G . . . . . E . . . . . V S . . . . . P . . . . . K . . . . .	
German ferret	..... P . . . . . G . . . . . E . . . . . S . . . . . P . . . . .	
Siberian seal	..... P . . . . . G . . . . . V E . . . . . S . . . . . P . . . . .	

**Figure 4.** Alignment of deduced amino acid sequences of canine distemper virus strains and isolates corresponding to the 540-bp P-gene fragment nucleotide sequences in Fig. 3 (GenBank accession numbers are in parentheses): vaccine strains, Onderstepoort (#NP.047202) and Rockborn (#AAD53535); isolate from an Alaskan dog, Alaska (this study); wild-type virulent strains, A75/15 (#AAD49700), Yanaka (#BAA93471), Hamamatsu (#BAA93472), and Jujo (#BAA93473); and isolates from a German dog (#AAG15481), a German ferret (#AAG15484), and a Siberian seal (#AAG15487). Amino acids identical to the sequence of the Onderstepoort strain are shown in dots.

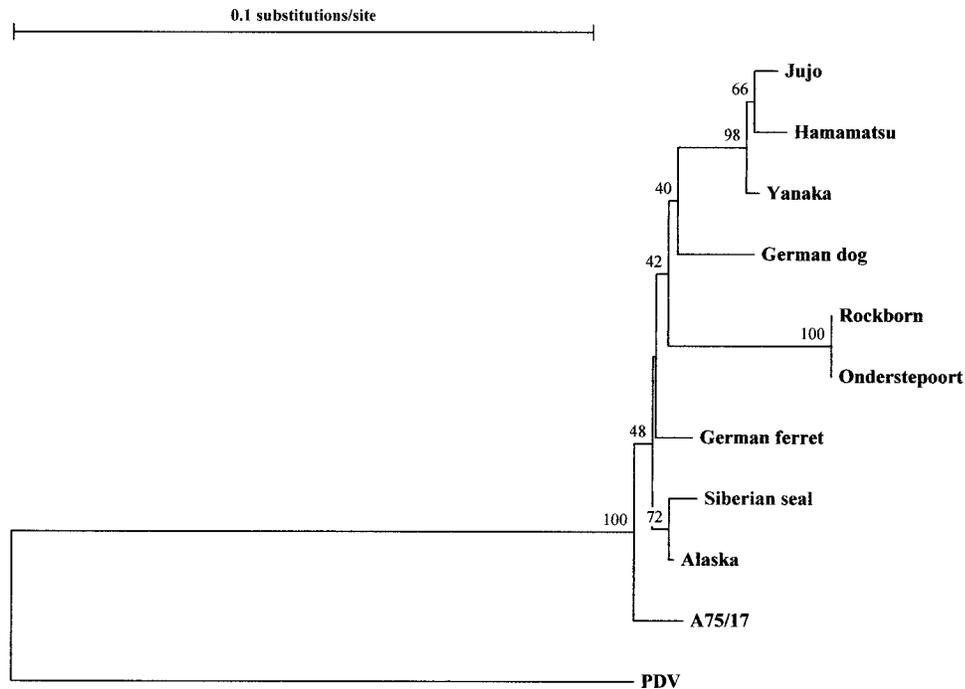
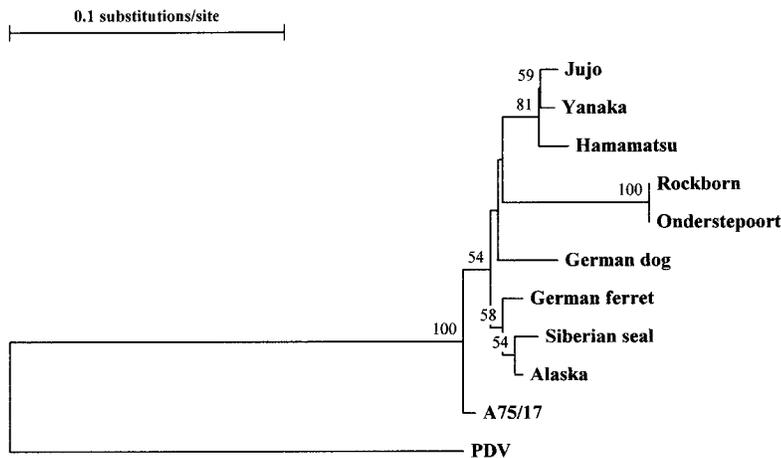
thermore, the specificity of the FA results was difficult to assess in tissues that were suboptimal in quality because of freeze–thaw artifacts.

A combination of immunohistochemistry and RT-PCR was used to obtain a more definitive diagnosis. The immunohistochemical staining was positive and specific on sections of spleen, lung, and salivary gland. Amplification of portions of the P- and N-genes by RT-PCR clearly identified the presence of distemper RNA in the tissues submitted.

Because outbreaks of virulent distemper are relatively rare in the United States, it was decided to further characterize the strain responsible for this outbreak. One possible approach to differentiate between virulent and attenuated strains is to attempt to isolate the virus in cell culture. Virulent strains are rather difficult to grow in cell culture, whereas vaccine strains would grow readily and induce syncytium formation in infected cells. Virulent strains can be grown in primary cultures of alveolar macrophages or mitogen-stimulated monocytes.<sup>6</sup> The isolate can then be characterized further with monoclonal antibodies.<sup>2,4</sup> Because the tissue quality was suboptimal and monoclonal antibodies were not available, the approach considered in this study consisted of direct sequencing

of the PCR amplification product corresponding to a region of the P-gene and comparison with previously published sequences. As expected, the alignment results showed that the Alaska strain differed significantly from the attenuated Onderstepoort and Rockborn vaccine strains with 19 nucleotide changes, 13 of which were shared in common by all 7 other wild-type virulent strains depicted in Fig. 4. Clearly, the Alaska strain was shown instead to have a high degree of homology in this region with published sequences of other virulent CDV strains.

The origin of the strain of CDV infecting these dogs remains unclear. Phylogenetic analyses showed that this strain is different from other virulent strains but is most closely related to a strain from a Siberian seal. This is consistent with the very high sequence identities observed between these 2 strains: 99.4% at the nucleotide level and 98.9% at the amino acid level. Eighteen of the 19 nucleotide changes found in the Alaskan strain, in comparison with the vaccine strains, were also found in the Siberian seal isolate. The Siberian seal isolate dates back to 1987–1988 when CDV was identified as the causative agent of an epizootic in Lake Baikal seals, resulting in significant mortality.<sup>4,9</sup> A later study showed that the same virus

**A****B**

**Figure 5.** Phylogenetic analysis of CDV strains and isolates. **A**, on the basis of the nucleotide sequence of a 540-bp P-gene fragment. **B**, on the basis of the amino acid sequence of the same 540 bp fragment. A PDV strain (#D10371) was included as the out-group. Bootstrap values for each node are expressed as percentage and given if 40% or higher.

continued to circulate in Lake Baikal seals, years after the epizootic, with only 2–3 nucleotide differences in comparison with the original isolate.<sup>8</sup> The authors of that study looked at a region of the P-gene, which overlaps much of the region analyzed in this study. Similarly, 3 nucleotide differences were observed in

the Alaskan sequence compared with that of the Siberian isolate of 1987–1988. On the basis of these observations and the fact that CDV is highly notorious for interspecies transmission,<sup>7</sup> it is speculative whether these 2 viruses are epizootiologically linked in any way at all. Seals, because of their aquatic habits, abil-

ity to swim long range, and gregarious nature, may well be the link between the distemper outbreaks in Lake Baikal and Kotzebue, Alaska.

The present standard for diagnostic investigation of suspected distemper cases involves postmortem microscopic analysis of tissues by way of specialized stains and immunological techniques. *In vivo* diagnostic tests, such as the RT-PCR, will be very useful in monitoring this disease. Sequence comparisons, which result from the direct sequencing of PCR products, are an important tool for further understanding of the molecular epidemiology of CDV infections.

### Sources and manufacturers

- a. VMRD Inc., Pullman, WA.
- b. Nikon Labophot-2, Nikon Inc., Melville, NY.
- c. Fisher Scientific, Pittsburgh, PA.
- d. Dr. M. Vandeveld, University of Bern, Switzerland.
- e. Broad Spectrum Histostain SP, Zymed Laboratories, South San Francisco, CA.
- f. QIAGEN Inc., Valencia, CA.
- g. Invitrogen life technologies, Carlsbad, CA.
- h. Applied Biosystems, Foster City, CA.
- i. DNASTAR Inc., Madison, WI.

### References

1. Altschul SE, Gish W, Miller W, et al.: 1990, Basic logical alignment search tool. *J Mol Biol* 215:403–410.
2. Appel MJG, Yates RA, Foley AL, et al.: 1994, Canine distemper virus epizootic in lions, tigers and leopards in North America. *J Vet Diagn Invest* 6:277–288.
3. Blixenkron-Moller M, Svansson V, Appel MJG, et al.: 1992, Antigenic relationships between field isolates of morbilliviruses from different carnivores. *Arch Virol* 123:279–294.
4. Grachev MA, Kumarev VP, Mamaev LV, et al.: 1989, Distemper virus in Baikal seals. *Nature* 338:209.
5. Greene CE, Appel MJ: 1998, Canine distemper. *In: Infectious diseases of the dog and cat*, ed. Greene CE, 2nd ed., pp. 9–22. WB Saunders, Philadelphia, PA.
6. Harder TC, Kenter M, Vos HW, et al.: 1996, Canine distemper virus from diseased large felids: biological properties and phylogenetic relationships. *J Gen Virol* 77:397–405.
7. Harder TC, Osterhaus ADME: 1997, Canine distemper virus—a morbillivirus in search of new hosts? *Trends Microbiol* 5:120–124.
8. Mamaev LV, Visser IKG, Belikov SI, et al.: 1996, Canine distemper virus in Lake Baikal seals (*Phoca sibirica*). *Vet Rec* 138: 437–439.
9. Osterhaus ADME, Groen J, de Vries P, et al.: 1988, Canine distemper virus in seals. *Nature* 335:403–404.
10. Patronek GJ, Glickman LT, Johnson R, et al.: 1995, Canine distemper infections in pet dogs: II. A case-control study of risk factors during a suspected outbreak in Indiana. *J Am Anim Hosp Assoc* 31:230–235.
11. Raes J, Van de Peer Y: 1999, ForCon: a software tool for the conversion of sequence alignments. *EMBnet.news* 6(1). Available at: <http://vulcan.rug.ac.be/~jerae/ForCon/index.html>.
12. Stettler M, Beck K, Wagner A, et al.: 1997, Determinants of persistence in canine distemper viruses. *Vet Microbiol* 30:83–93.
13. Stettler M, Zurbriggen A: 1995, Nucleotide and deduced amino acid sequences of the nucleocapsid protein of the virulent A75/17-CDV strain of canine distemper virus. *Vet Microbiol* 44:211–217.
14. Van de Peer Y, De Wachter R: 1994, TREECON for Windows; a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Comput Applic Biosci* 10:569–570.
15. Vandeveld M, Zurbriggen A: 1995, The neurobiology of canine distemper virus infection. *Vet Microbiol* 44:271–280.