PHYLOGENETIC CHARACTERIZATION OF CANINE DISTEMPER VIRUSES DETECTED IN NATURALLY INFECTED NORTH AMERICAN DOGS

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The undersigned, appointed by the Dean of the Graduate School, have examined the thesis entitled

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And hereby certify that in their opinion it is worth of acceptance.

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PHYLOGENETIC CHARACTERIZATION OF CANINE DISTEMPER VIRUSES DETECTED IN NATURALLY INFECTED NORTH AMERICAN DOGS

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ABSTRACT

In 2004, dogs with clinical signs suggestive of canine distemper virus (CDV) were subjected to necropsy and/or diagnostic evaluation. From 11 such cases, a diagnosis of CDV infection was established in seven dogs (six puppies and one adult) by the presence of compatible gross and histologic lesions, immunohistochemical labeling for CDV antigen, and detection of CDV RNA by RT-PCR. To further characterize the CDV strains detected, complete gene sequences were determined for the hemagglutinin (H) and fusion (F) protein genes, while partial gene sequencing was performed for the phosphoprotein (P). A total of 4,508 bases were sequenced for the CDV strains detected from each of four cases. Two strains were found to have identical sequences except for 2 bases in the intergenic region of the F and H genes. Phylogenetic analysis strongly suggested an evolutionary relationship between sequences detected in these two strains and those of phocine distemper virus 2 and two other strains of CDV not previously detected in the United States of America. Clear phylogenetic relationships were not established for viruses detected in the two additional cases; however, one strain showed similarity to CDV strains detected in a panda from China. Importantly, the three CDV strains detected were demonstrated to be genetically distant from known vaccine strains and strains previously reported in the continental United States.

I. INTRODUCTION

Canine distemper is one of the best described viral diseases of wild and domestic canidae (dog, fox, wolf, jackal, coyote) and is associated with high morbidity and mortality in immunologically naïve populations (Dungworth, 1993; Murphy et al., 1999). Canine distemper was first described in Spain in 1791 and the virus (canine distemper virus, CDV) was first isolated by Carre in 1905 (Appel & Gillespie, 1972). Canine distemper virus causes primary systemic and central nervous system (CNS) disease (Greene & Appel, 1998).

A. Etiology

CDV is classified in the genus *Morbillivirus* within the Paramyxoviridae family. CDV is closely related to other morbilliviruses such as measles virus (MV) of human and nonhuman primates; rinderpest virus (RPV) of cattle, pigs, goats, sheep, buffalos, elands, giraffes, kudus, and warthogs; pestes des petite ruminant virus (PPRV) of goats, sheep, gazelles and ibexes; phocine distemper virus (PDV) of seals; cetacean distemper virus (CMV) of dolphins and porpoises; and equine morbillivirus (EMV) of horses, humans and pteropus bats (Dungworth,1993; Osterhaus et al., 1995). CDV infects other mammals including species of the families Mustelidae (ferret, mink, skunk, weasel, badger, and marten), Procyonidae (raccoon, coati and kinkajou), Ailuridae (lesser and giant pandas), Ursidae (bear), Viverridae (fossa, mongoose, civet, linsang, binturong, and genet), Hyaenidae (hyena), and Felidae (cheetah, lion, jaguar, margay, tiger, cat and ocelot) (Appel, 1978; Dungworth, 1993).

CDV has a non-segmented, negative sense (3' – 5'), single-stranded RNA genome and has a lipoprotein-enveloped virus particle that is 150 to 300 nm in diameter. The CDV genome is approximately 15,690 nucleotides in length and contains six genes that code for virion proteins (Murphy et al., 1999). The hemagglutinin or "H" glycoprotein is responsible for the viral attachment to the host cell (Murphy et al., 1999). The fusion (F) protein is a glycoprotein that is essential for mediating fusion between the viral particle and host cell membrane. The F protein provides the virus with the necessary mechanism to move from one host cell to another (Murphy et al., 1999). The matrix (M) protein is important for entry of the virus into a susceptible cell and this protein plays an important role in assembly of new viral particles (Murphy et al., 1999). The phospholipid (P) and the large (L) proteins form a functional polymerase complex and are responsible for replication of viral RNA. The P protein also encodes for two non-structural proteins, C and V. The nucleocapsid (NP) protein is responsible for protection of the viral RNA (Murphy et al., 1999).

CDV is susceptible to ultraviolet light, heat and drying, and can be destroyed by temperatures greater than 50º to 60ºC within 30 minutes. Chloroform, ether (<0.5%), formalin solution, phenol (0.75%), and quaternary ammonium disinfectant (0.3%) also will destroy this virus. At freezing temperatures, CDV can survive at -65ºC for at least 7 years (Greene & Appel 1998).

B. Epidemiology

CDV is commonly transmitted by aerosol or droplet exposure originating from respiratory exudates; however, urine and other secretions also contain infectious virus

(Greene & Appel, 1998). Infected dogs can shed the virus for up to 60 to 90 days post-infection (Greene & Appel, 1998). Subclinically or clinically infected dogs can transmit CDV to puppies, which are the most susceptible to CDV infection (Krakowka & Koestner, 1976). Although immunity to CDV is long-lasting, dogs that are not periodically vaccinated may lose protection and can become infected if they are stressed or immunocompromised at the time of virus exposure (Greene & Appel, 1998). It is estimated that 25% to 75% of susceptible animals become subclinically infected when exposed to CDV and clear virus from the body without showing signs of illness (Appel, 1969; Greene & Appel, 1998).

Dogs between 3 and 6 months of age have a greater prevalence to spontaneous CDV infection, which is correlated with loss of maternal antibodies (Greene & Appel, 1998). However, in susceptible populations the disease affects dogs of all ages (Johnson et al., 1995; Patroneck et al., 1995). Although a breed predisposition to this disease has not been proven, Greyhounds, Siberian huskies, Weimareiners, Samoyeds, and Alaskan Malamutes seem to be more commonly affected (Greene & Appel, 1998).

C. Pathogenesis

a. Systemic infection: When dogs are exposed to aerosol or other secretions containing virulent CDV, the virus will localize in bronchial lymph nodes and tonsils. On day two to three post infection, CDV will be present in blood mononuclear cells. Within the first week of infection, the virus replicates in multiple lymphoid tissues such as thymus, spleen, lymph nodes, Kupffer cells of the liver, lamina propria of the intestine and stomach, and mononuclear cells of the bone marrow. Widespread viral replication

will commonly cause clinical signs of pyrexia and severe leukopenia. Damage to lymphoid cells is due to viral replication in T and B lymphocytes. By day eight to nine post-infection, dogs that do not develop an adequate humoral and cellular immune response against CDV will have widespread viral replication in several epithelial and central nervous system (CNS) cells (Appel, 1969). Shedding of the virus is commonly associated with viral replication in epithelial cells (Greene & Appel, 1998). Fourteen days after infection, dogs with adequate CDV antibody titers and cell-mediate immunity will clear the infection. In dogs with poor immune responses, CDV infects multiple tissues such as the skin, exocrine glands, endocrine glands and gastrointestinal, respiratory and genitourinary tract by 9 to 14 days post-infection. At this stage of infection, dogs develop severe clinical signs and often die. Secondary bacterial, protozoal and other viral pathogens will superinfect dogs with CDV (Appel, 1969; Fukushima & Helman, 1984; Greene & Appel, 1998). Dogs with intermediate levels of cell-mediated immune responses and delayed antibody titers often have viral spread to epithelial cells, astrocytes and neurons.

CDV can persist in uveal, neural and integumentary (footpad) tissues. The recovery of affected animals after CDV infection is associated with long-term immunity, which can be interrupted when the animal becomes stressed, immunocompromised or ill from some other disease (Appel, 1969; Green & Appel, 1998). Specific IgG-CDV antibody has been shown to be effective in neutralizing extracellular CDV and inhibiting intercellular spread (Appel, 1969).

b. Central Nervous System Infection: CDV can enter the CNS via the cerebrospinal fluid (CSF) 10 to 14 days post-infection experimentally (Higgins et al.,

1982) or cross the blood-brain barrier (BBB) through infected lymphoid cells, platelets or as free virus (Krakowka et al., 1975). In CSF, free virus or lymphocyte-associated virus spreads to the periventricular and subpial structures. The virus will cause lesions of the subependymal areas, cerebral cortex, optic and other nerve tracts, rostral medullary velum, cerebellar peduncles and spinal cord (Higgins et al., 1982).

Viral antigen is first detected in endothelial cells of the CNS capillaries and venules, as well as in perivascular astrocyte foot processes (Vandevelde et al. 1985; Krakowka et al., 1987a, Mutinelli et al., 1989). Production of antiviral antibody frequently results in immune complex deposition. This facilitates spread of the virus to vascular endothelium, meninges, choroid plexus epithelial cells, and ependymal cells lining the ventricular system (Appel et al., 1982).

Important factors to consider for virulence are dependent on the viral strain, and age and immunocompetence of the dog. These factors determine the course and outcome of CDV infection (Summers et al., 1984) and determine whether infection causes acute or chronic neural lesions. Acute CDV infection can progress to a chronic form in animals that survive (Bernard et al., 1993). Studies have demonstrated that the Snyder Hill strain causes acute poliencephalomyelitis, while the strains A75/17 and R252 cause subacute to chronic demyelinating disease (Summers et al., 1984).

In the acute phase of CDV, the virus can cause severe immunosuppression in young dogs (Krakowka et al., 1980 a & b). The virus infects multiple areas in the gray and white matter. Gray matter infection is characterized by polioencephalomalacia and neuronal degeneration (Lisiak & Vandevelde, 1979; Braund et al., 1979). Neuronal

lesions will induce seizures that can lead to anoxia and selective neuronal necrosis (Lisiak et al., 1979; Braund & Vandevelde, 1979).

Activation of microglial cells infected with CDV is associated with acute myelin destruction (Vandevelde et al., 1983; Zurbriggen & Vandevelde, 1983; Stein et al., 2004). The white matter of the cerebellum, optic nerve and spinal cord is a common site for demyelination (Tipold et al., 1992). The oligodendrocytes or myelin producing cells have been studied to elucidate the pathogenesis of demyelination caused by CDV. Although no light microscopic lesions are observed within the oligodendroglia cells, a recent electron microscopic *in vitro* study of oligodendroglia infected with CDV demonstrated restricted cell degeneration with loss of organelles and lysis of the cell membrane (Vandevelde & Zurbriggen, 2005). *In vitro* biochemical studies of oligodendrocytes have detected a decrease of a specific oligodendroglia myelin-producing enzyme, cerebroside sulfotransferase. Decrease in activity of this enzyme causes down-regulation of myelin synthesis (Zurbriggen et al., 1993 and 1998; Graber et al., 1995; Schobesberger et al., 2002 &1999; Vandevelde & Zurbriggen, 2005).

Disease progression of CDV is observed in animals without an effective immune response (Appel, 1969). Dogs with a delayed immune response tend to develop chronic inflammatory neurological disease (Appel et al., 1982). Severity of inflammation is directly associated with increased tissue damage and progression of neurological signs. At this stage of disease, there is intrathecal production of neutralizing antibodies and antibodies that recognize myelin basic protein (Mitchell et al., 1991; Vandevelde et al., 1986). Antiviral antibodies also can stimulate production of free radicals by macrophages *in vitro* (Griot et al., 1989). Free radicals have been shown to be highly

toxic to oligodendrocytes *in vitro* (Griot et al., 1990). The mechanisms associated with myelin and oligodendrocyte destruction are commonly described as the "innocent bystander" effect (Krakowka et al., 1973; Bollo et al., 1986; Botteron et al., 1992; Axthelm & Krakowka, 1998).

Chronic encephalomyelitis results from a late or insufficient immune response against CDV (Krakowka et al., 1975; Appel et al., 1982). The inflammatory response associated with the demyelinating form of chronic disease causes severe white matter destruction (Vandevelde et al., 1986). In acute cases of encephalitis, the major histocompatibility class antigen II (MHC II) is expressed in the white matter and multifocally throughout the areas of necrosis caused by the virus (Alldinger et al., 1996). In contrast, chronic cases have MHC II expressed in all microglial cells. Additionally, in later stages of chronic encephalomyelitis, auto-antibodies against myelin basic protein are responsible for continued and more wide-spread demyelination and perivascular mononuclear cell infiltration (Alldinger et al., 1996).

Old dog encephalitis (ODE) has been studied in gnotobiotic dogs (Johnson et al., 1987a; Axthelm & Krakowka, 1998). The lesions in ODE are localized in the cerebrum and brainstem (Axthelm & Krakowka, 1998). Unsuccessful attempts to transmit the disease from infected dogs to CDV-susceptible gnotobiotic dogs suggest that the virus is present inside neural cells in a replication-defective form (Axthelm & Krakowka, 1998).

CDV can persist in unaffected sites of the brain of surviving animals (Muller et al., 1995). Persistence of CDV is favored by non-cytolytic spread of the virus and restricted infection of certain cells with reduced viral protein expression, both of which

cause delay immune recognition of this pathogen (Muller et al., 1995; Zubriggen et al., 1995; Vandevelde & Zurbriggen, 1995).

D. Clinical Findings

a. Systemic Signs: The severity of clinical signs observed with CDV is dependent on the virulence of the infecting virus, environmental conditions, as well as the host age and immune status (Greene & Appel, 1998). Fifty to 70% of CDV infections are probably subclinical (Greene & Appel, 1998). Listlessness, anorexia, dehydration, fever and serous occulonasal discharge are some of the clinical signs observed in mild cases of CDV. The nasal discharge frequently progresses to a mucopurulent character, with coughing and dyspnea (Greene & Appel, 1998; Dungworth, 1993). Keratoconjunctivitis sicca due to CDV infection of the tear gland (Dungworth, 1993) is observed in cases of systemic or subclinical infection. Anosmia is a sequelum observed in dogs that have recovered from CDV infection (Myers et al., 1988).

Unvaccinated dogs or puppies (12 to 16 weeks of age) are most susceptible to the severe, systemic form of disease. Mild fever and serous to mucopurulent conjunctivitis followed by a dry to productive cough are observed in the initial form of systemic CDV (Greene & Appel, 1998). Depression, anorexia, vomiting and diarrhea also are observed in these cases. In rare circumstances, urinary and fecal incontinence (Guilford et al., 1990), priopism, tenesmus and intussusceptions can occur (Greene & Appel, 1998). Severe dehydration and emaciation result from adipsia and fluid loss (Greene & Appel, 1998).

b. Neurological Signs: CNS signs are commonly observed between one and three weeks after affected animals recover from the systemic form of CDV. However, neurologic signs can also occur simultaneously with the systemic illness (Tipold et al., 1992) or, less commonly, months post-infection. The later condition is known as post-infectious encephalitis (Tipold et al., 1992; Greene & Appel, 1998). Neurological signs, whether acute or chronic, are typically progressive. Chronic relapsing neurological deterioration with intermittent recovery and a later, superimposed acute episode of neurological dysfunction can occur (Greene & Appel, 1998). There is no age, sex and breed predilection for the neurological manifestation of CDV and the duration of the signs varies from a few days to more than one month (Tipold et al., 1992).

Neurological signs observed with CDV are variable but often associated with the distribution of infected neural tissue (Tipold et al., 1992). Paresis and ataxia of the limbs are observed when the virus has caused lesions in the spinal cord. Hyperesthesia and cervical rigidity can be found as a result of meningeal inflammation. Head tilt, nystagmus, tendency to fall and cranial nerve signs with conscious proprioceptive deficits are observed when the virus has caused lesions in the cerebellum and vestibular nuclei of the brainstem (central vestibular disease). Ataxia and hypermetria with head bobbing are observed when the virus has infected the cerebellum. Generalized to partial seizures are associated with forebrain lesions; chewing gum type seizures are frequently associated with polioencephalomalacia of the temporal lobes. Uni- or bilateral blindness is seen when the virus has infected any part of the retina, optic nerve, thalamus or occipital area of the cerebrum (Tipold et al., 1992).

In one study conducted by Tipold et al. (1992), 40% of the animals with the neurologic form of CDV infection displayed myoclonus. An electromyographic study demonstrated that the myoclonic rhythm is determined by intrinsic neural mechanisms of the spinal cord where there is local irritation of the lower motor neurons (Breazile et al., 1966). However, another possibility for the production of myoclonus has been suggested. Lesions in the basal nuclei may initiate this sign and establish a "pacemarker" in the spinal cord or brainstem, which then maintains the involuntary muscle activity (de Lahunta, 1983).

c. Ocular Signs: Mild anterior uveitis is commonly observed in dogs with CDV encephalomyelitis (Greene & Appel, 1998). Blindness, dilated unresponsive pupils, optic neuritis, degeneration and necrosis of the retina, retinal detachment, and choroiditis are other lesions caused by CDV infection (Fisher, 1971).

d. Transplacental Infection: Abortions, stillbirths or birth of weak puppies are associated with infection with CDV of pregnant bitches (Greene & Appel, 1998). Puppies infected transplacentally may develop neurologic signs during the first 4 to 6 weeks of age or suffer from permanent immunodeficiencies (Greene & Appel, 1998).

e. Neonatal Infection: Dental impaction, partial eruption, oligodontia, and enamel and dentin hypoplasia are associated with CDV infection in young puppies (Bittegeko et al., 1995). Neonatal (<7 days old) gnotobiotic puppies have developed virus-induced cardiomyopathy after experimental infection with CDV. These animals develop dyspnea, depression, anorexia, collapse and prostration (Higgins et al., 1981).

f. Bone Lesions: Experimental and natural infection of young puppies with CDV can cause metaphyseal osteosclerosis. Large-breed dogs between 3 and 6 months of age

are most frequently affected (Baumgartner et al., 1995). Paget's disease, which is a chronic skeletal disorder characterized by excessive remodeling and deformity of bone, has been associated with slow-virus infections by Paramyxoviruses or CDV in dogs (Mee et al., 1993). Detection of CDV RNA within bone cells of dogs with metaphyseal osteopathy suggests that this virus may be the cause of Paget's disease and provides further evidence that CDV might be responsible for the bony abnormalities seen in this disease (Mee et al., 1993).

g. Rheumatoid Arthritis: High levels of antibodies to CDV in serum and synovial fluid have been found in dogs with rheumatoid arthritis (Bell et al., 1991).

h. Cutaneous Lesions: Pustular dermatitis and nasodigital hyperkeratosis are observed in puppies and adult dogs infected with CDV, respectively (Yager et al., 1993)

E. Gross Findings

a. Systemic Disease: Gross lesions are often minimal and usually observed when death occurs from systemic disease. Emaciation, dehydration and muscle wasting are observed externally in dogs that die from severe CDV infection. Oculonasal discharge is frequently observed when the affected animals have an upper respiratory infection. The respiratory tract contains serous to catarrhal or purulent exudate in the nasopharynx, trachea and bronchi. The mucosal vessels of the larynx and trachea are congested. Pulmonary edema and serous pleural effusions are also evident. Interstitial pneumonia, which is characterized by diffuse mottled reddish-tan coloration with firm texture and a smooth liver-like appearance is frequently observed throughout the lung lobes. Viral and bacterial co-infections cause lung lesions consistent with bronchopneumonia and

characterized by consolidation of the cranial and caudal aspects of the lung lobes (Dungworth, 1993).

Gross lesions observed in the digestive tract of dogs infected with CDV are usually non-specific and include gas, clear fluid or blood distending the gastric and enteric lumins (Dungworth, 1993). Megaesophagus has been described in dogs with neurological dysfunction caused by CDV (Barker et al., 1993). Liver lesions consist of an accentuated lobular pattern due to mild fatty change and/or centrilobular congestion (Dungworth, 1993). Oral examination reveals enamel abnormalities. This is often evident with radiographic examination demonstrating enamel, dentin and root hypoplasia, dental infection, partial dental eruption, and oligodontia (Bittegeko et al., 1995). Gross lesions of dogs infected with CDV also occur in other tissues including lymphoid, CNS, ocular, cardiac, bone and skin.

b. Lymphoid Tissue Lesions: It is difficult to identify alterations in lymphoid organs except for the thymus, which is often significantly reduced in size and may even be difficult to find on necropsy examination. In acute systemic disease, the size of the lymph nodes varies from small (lymphoid depletion, necrosis and atrophy) to large (edema). No gross alterations of the lymph organs are often observed in the later stage of the disease due to lymphoid regeneration and hyperplasia (Dungworth, 1993).

c. Central Nervous Lesions: Gross lesions in the CNS are minimal except for occasional meningeal congestion, ventricular dilation and increased cerebrospinal fluid pressure resulting from brain edema (Appel, 1969).

d. Ocular Lesions: Ocular examination of dogs infected with CDV reveals conjunctival congestion, ocular discharge and retinal degeneration (Fischer, 1971).

e. Cardiac Lesions: The heart of neonates infected with CDV will have multifocal whitish areas of necrosis and mineralization (Higgins et al., 1981).

f. Bone Lesions: Examination of long bones in young dogs with systemic disease will reveal single to multiple areas of proliferation near the growth plate (arrest lines parallel to the physis). These alterations are characteristic of metaphyseal osteosclerosis (Baumgartner et al., 1995).

g. Cutaneous Lesions: Digital hyperkeratosis or "hard pad disease" is a gross finding commonly seen in dogs infected with CDV (Yager et al., 1993). There is also hyperkeratosis of the nose. Juvenile to adult dogs are more susceptible to develop these alterations. Frequently, the cutaneous form of CDV is accompanied by the chronic neurologic presentation of this virus (Greene & Appel, 1998). Pustular dermatitis in puppies infected with CDV is commonly observed. However, this lesion is rarely associated with neurologic complications (Yager et al., 1993; Greene & Appel, 1998).

F. Light Microscopic Findings

a. Systemic Disease: Sections of lung show diffuse interstitial pneumonia, characterized by thickening of the alveolar septa due to infiltration with mononuclear cells, neutrophils, fibrin and vascular congestion. Hyperplasia of the alveolar type II epithelial cell and formation of syncytial cells can also be observed (Dungworth, 1993; Greene & Appel, 1998). The alveolar spaces contain sloughed epithelial cells and alveolar macrophages. Multiple, eosinophilic, oval, 1 to 5 μm viral inclusions are observed in the cytoplasm and/or nuclei of type II pneumocytes, bronchiolar epithelial cells, endothelial cells and macrophages. Mild bronchopneumonia, characterized by

variable accumulations of neutrophils, fibrin and bacteria in alveolar spaces and bronchial lumens, to severe necrotizing bronchopneumonia are commonly observed in the lungs of dogs co-infected with *Bordetella bronchiseptica, Escherichia coli* and *Klebsiella pneumoniae* (Dungworth, 1993).

In the digestive tract, viral inclusions are evident in epithelial cells (chiefs, parietal and superficial epithelium) of the gastric mucosa and rarely in the epithelium of the intestine (Dungworth; 1993). Viral inclusions are also present in the epithelium of the biliary and pancreatic ducts. Dogs that recover from CDV have necrosis and cystic degeneration of ameloblastic epithelium of the developing teeth causing enamel hypoplasia. This lesion is grossly characterized by small focal depressions to large areas lacking enamel (Barker et al., 1993).

CDV infection causes severe damage to the lymphoid tissue leading to severe immunodeficiency which may eventually cause death of the dog (Dungworth, 1993). In experimental studies of CDV in dogs, there is severe lymphoid depletion and necrosis in the cortical zone of the lymph nodes and thymus, white pulp of the spleen, and bone marrow (Dungworth, 1993) by 6 to 9 days post-infection. Hyperplasia of reticuloendothelial cells (mononuclear cells) will occur within 14 days post-infection. Frequently, formation of syncytial cells and eosinophilic intranuclear and intracytoplasmic viral inclusions are evident in lymphoid and mononuclear cells (Dungworth, 1993). Suppurative splenitis and lymphadenitis are observed in a severe systemic disease (Dungworth, 1993). Intracytoplasmic inclusions also are observed in the transitional epithelium of the urinary tract and/or renal epithelial cells of the kidneys.

Hydropic degeneration and cellular swelling are present in affected urinary tract cells (Dungworth, 1993).

b. Acute CNS Lesions: Acute neural disease is a common disease manifestation in neonates and young dogs. Neuronal degeneration and necrosis, as well as myelin degeneration (primary demyelination) occur without significant perivascular inflammation (Braund & Vandevelde, 1979; Lisiak & Vandevelde, 1979; Krakowka et al., 1980 & 1985). Patchy areas of necrosis usually are surrounded by hypertrophic astrocytes and macrophages (Dungworth, 1993). Areas of demyelination are severe in the lateral cerebellar peduncles, the dorsolateral medulla adjacent to the fourth ventricle, and the deep cerebellar white matter (Dungworth 1993; Greene & Appel, 1998). These areas are characterized by spongy vacuolation of the white matter with reactive gliosis. Intracytoplasmic or intranuclear inclusions are found predominantly in astrocytes, microglial cells, ganglion cells, and neurons. The midbrain, basal ganglia, and temporal lobes of the cerebral cortex also are affected (Appel, 1969). The optic tracts, crus cerebri, cranial nerves, and infundibulum are extracerebral areas affected in the acute phase of CDV (Appel, 1969). In some dogs, acute non-inflammatory polioencephalomyelitis can predominantly affect the cerebrum and thalamus (Thomas et al., 1993). Hippocampal necrosis was reported in a five week old puppy with CDV that was presented with grand mal seizures (Braund et al., 1981). Direct viral infection of the vasculature, ischemic changes secondary to seizures, and altered cellular metabolism all lead to acute neuronal necrosis (Palmer, 1972; Braund &Vandevelde, 1979). Experimentally it has been demonstrated that the cellular stress response following CDV infection leads to

translocation of CDV N protein from its normal cellular location to the nucleus where it forms the intranuclear inclusions that are observed (Oglesbee and Krakowka, 1993).

c. Chronic CNS Lesions: Older or more immunocompetent dogs have a tendency to develop the chronic CDV lesions that are consistent with a leukoencephalomyelitis in the caudal brainstem and spinal cord. The chronic neural lesions are more extensive and severe than acute cases of encephalitis, affecting both the white and gray matter (Axthelm & Krakowka, 1998). Widespread perivascular lymphoplasmacytic and histiocytic infiltration within the areas of demyelination, and neuronal degeneration and necrosis are commonly found (Higgins et al., 1982; Axthelm & Krakowka, 1998; Krakowka et al., 1985; Bollo et al., 1986). These changes are accompanied by astrocytic hyperplasia and hypertrophy, microglial cell proliferation, and chronic demyelination. Lesion distribution is more angiocentric and multifocal (Axthelm & Krakowka, 1998). Mononuclear cell infiltration and astrogliosis occur in the gray matter along with neuronal loss, degeneration and necrosis (Axthelm & Krakowka, 1998). Occasional multinucleated giant cells containing intranuclear and intracytoplasmic inclusions are observed (Axthelm & Krakowka, 1998). In the white matter, myelin and axons are separated from one another by mononuclear cells, and accumulation of myelin debris and macrophages (Axthelm & Krakowka, 1998). In more chronic cases there is sclerosing panencephalitis characterized by infiltration and replacement of neural parenchyma by a dense astrocytic network (Axthelm & Krakowka, 1998).

d. Ocular Lesions: Light microscopic lesions observed in the ocular tissues are characterized by retinal and choroidal degeneration with accumulations of melanin pigment. Ganglion cells also may contain viral inclusions (Fischer, 1971).

e. Cardiac Lesions: In neonatal dogs with CDV, the heart contains multifocal to coalescing areas of myocardial necrosis intermingled with areas of mineral accumulation (Higgins et al., 1981).

f. Bone Lesions: The light microscopic lesions observed in the large bones of young animals with CDV are characterized by persistence of the primary spongiosa, loss of the bone marrow cells, and necrosis of osteoclasts. The bone marrow cell lesions vary from mild to severe (Baumgartner et al., 1995).

g. Cutaneous Lesions: Foot pad lesions consist of epidermal basal cell proliferation, and orthokeratotic and parakeratotic hyperkeratosis, along with eosinophilic cytoplasmic inclusion bodies and vacuolar degeneration (Yager et al., 1993; Grone et al., 2003 & 2004). Absence of keratinocyte degeneration has been observed and associated with a noncytocidal persistent infection of footpad keratinocytes by CDV (Grone et al., 2004; Engelhardt et al., 2005).

G. Electron Microscopic Findings

Ultrastructural studies of canine cerebellar cells infected with CDV have demonstrated that at 7 days post infection the glial cells and macrophages contain intracytoplasmic inclusions. These are characterized by small to large aggregates of viral nucleocapsids. After 2 weeks post infection, the majority of cells in the body contain intracytoplasmic inclusions. Three weeks post infection, the nuclei of affected cells contain viral capsids that are arranged in crystalline arrays consistent with immature membranous viral envelop. These membranes are formed due to persistent infection of CDV (Koestner & Long, 1970). Another electron microscopic study described the

morphologic characteristics of the strains R252, Snyder Hill and Onderteport. These morphologic characteristics consist of focal cytoplasmic aggregates of electron-dense bodies (Confer et al., 1975). Most electron microscopic studies agree that oligodendroglial infection is very rare in CVD (Higgins et al., 1982; Summers & Appel, 1987; Vandevelde & Zubriggen, 2005). In order to understand demyelination caused by CDV, multiple studies have been conducted in cells producing myelin (oligodendroglial cells). Ultramicroscopic studies on oligodendroglial cells infected *in vivo* with CDV revealed mild degenerative changes. However, there is no definitive evidence that these cells undergo necrosis or apoptosis (Schobesberger et al., 2002).

H. Diagnosis

a. Clinical Laboratory Findings: CDV infection will cause severe absolute lymphopenia due to lymphoid depletion, necrosis and apoptosis (Greene & Appel, 1998; Schobesberger et al., 2005). CD3 (T cell) and CD21 (B cell) lymphocytes are found to be affected 3 days post-infection with this virus, while apoptosis of lymphocytes and monocytes are observed 6 days post-infection (Schobesberger et al., 2002). Lymphopenia is usually persistent in young animals with systemic and neurologic CDV infection (Greene & Appel, 1998). Thrombocytopenia has also been reported in cases of CDV (Axthelm & Krakowka, 1987). Virus-antibody immune complexes deposited on platelet membranes are responsible for lysis and decrease of megakaryocyte populations in bone marrow (Axthelm & Krakowka, 1987). Regenerative anemia has also been observed in experimentally infected neonatal dogs (Higgins et al., 1981). CDV inclusions can be detected in peripheral blood within lymphocytes, monocytes, neutrophils and

erythrocytes that are stained with Wright-Leishman stain (Greene & Appel, 1998). Serum biochemical changes in acute systemic infection with CDV are often nonspecific (Greene & Appel, 1998). Albumin, alpha and gamma globulin concentrations are typically increased in non-neonates, where as neonates persistently infected with CDV have marked hypoglobulinemia (Greene & Appel, 1998). Dogs with inflammatory and demyelinating CDV lesions will have elevated IgG levels in serum and cerebral spinal fluid (Vandevelde et al., 1985).

b. Radiology: In the lungs, interstitial to alveolar patterns are observed in cases of dogs with CDV viral pneumonia (Greene & Appel, 1998).

c. Cerebrospinal Fluid: Increased immunoglobulin titers (anti-CDV IgG and IgM) in CSF are highly suggestive of CDV infection since these antibodies are locally produced (Greene & Appel, 1998). The IgG titers are often used as an indicator of intrathecal immunoglobulin synthesis (Vandevelde et al., 1985). Increases in protein concentration (>25 mg/dl) and cell count (>10 cells/μl with a predominance of lymphocytes) are frequently observed in inflammatory forms of CDV encephalomyelitis (Greene & Appel, 1998). Increase of albumin is directly associated with the lethality of the viral strain (acute encephalopathy) and terminal breakdown of the blood-brain barrier accompanied by extensive viral antigen in the CSF (Johnson et al., 1987b). In contrast, increases in IgG are associated with inflammatory demyelinating cases of CDV and intrathecal production of antibodies (Bichsel et al., 1984; Sorjonen et al., 1989; Vandevelde et al., 1986; Rima et al., 1991).

d. Diagnostic Tests: Multiple tests are designed to detect the presence of virus or antibodies against CDV. The immunological tests include direct or indirect

immunofluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA) and immunohistochemical stains (IHC). Reverse transcription polymerase chain reaction (RT-PCR) is a molecular test to detect the presence of viral RNA in blood, feces, urine, and fresh and formalin fixed tissues. Serum neutralization, IFAT and ELISA detect the antibody response. Virus isolation is also commonly used in multiple laboratories.

1. Immunofluorescent Techniques to Detect Viral Antigen: Smears of conjunctiva, tonsils, and respiratory epithelium are commonly used to detect CDV using direct or indirect fluorescein-conjugate CDV antibody (Fairchild et al., 1971). Although this test is useful to detect clinical cases of CDV, false-negative results can be obtained when the animal is in the recovery stage of the disease.

2. Serum Neutralization Test (SNT): The serum neutralization test for CDV (initially performed using embryonic hen eggs) have been replaced by a more sensitive microneutralization test for CDV (Appel & Robson, 1973; Greene & Appel, 1998). The former tests were expensive and time-consuming assays (Appel & Robson, 1973).

3. ELISA: An ELISA test has been used to detect serum IgG and IgM antibodies to CDV (Greene & Appel, 1998). High IgM antibody titers are specific for detecting recent CDV infection, although recent vaccination with CDV can also give positive results (Greene & Appel, 1998). A more specific IgM-ELISA test has been designed to differentiate between acute and chronic cases of CDV (Blixenkrone-Moller et al., 1991). IgG titers are ambiguous and indicate either past or present infection or vaccination for CDV. However, IgG titers in CSF are a more reliable indicator of chronic CDV infection (Bichsel et al., 1984; Vandevelde et al., 1986; Sorjonen et al., 1989).

4. Immunohistochemical Staining: Postmortem diagnosis of CDV infection is relatively simple and reliable using anti-CDV antibody in enzyme-based immunohistochemical assays (Ducatelle et al., 1980). In one study, skin biopsies of the dorsal neck region were used to perform antemortem immunohistochemical testing for acute and subacute infection with distemper (Haines et al., 1999).

5. Reverse transcription polymerase chain reaction (RT-PCR): RT-PCR tests amplifying CDV genomic RNA have been used on canine peripheral blood mononuclear cells (Shin et al., 1995), conjunctival exudate, unfixed or formalin-fixed, paraffin-embedded tissues to detect CDV (Stanton et al., 2002). RT-PCR provides a fast and sensitive method for diagnosis of CDV infection in dogs (Shin et al., 1995 Stettler & Zubriggen, 1995; Rodriquez, 1997; Stanton et al., 2002).

6. Virus Isolation: Although viral shedding occurs in many secretions and excretions, viral isolation in cell culture is difficult (Appel, 1969). However, buffy coat cells or tissue from infected animals can be cultivated with mitogen-stimulated canine blood lymphocytes; cultures are examined 72 to 144 hours latter by immunofluorescence (Appel et al., 1992).

I. Treatment

Lack of effective antiviral treatment for infection leads to the need for supportive and symptomatic approaches to treatment (Greene & Appel, 1998). Antibiotics and a clean environment are necessary for dogs with upper respiratory disease caused by secondary infection such as *Bordetella bronchiseptica* (Greene & Appel, 1998). Fluid therapy, anti-emetic and anti-diarrheal medications are necessary in cases of

gastrointestinal disease. Antiepileptic drug therapy and glucocorticoids are useful to alleviate the clinical signs of neurologic disease (Greene & Appel, 1998).

J. Prevention

Vaccination is the best method for preventing CDV infections due to the long-lasting immunity that is obtained. This protection may be adequate unless the animal is exposed to a highly virulent or large quantity of virus, or the animal becomes stressed or immunocompromised. Vaccinations are effective against CDV when administrated as recently as two days before viral exposure (Chappuis, 1995). Antibodies are transferred from the mother to the fetus through the uterus (3%) and colostrum (97%). Maternal antibodies to CDV have a half-life of 8.4 days and become undetectable by 12 to 14 weeks of age. CDV vaccination should occur every 3 to 4 weeks between 6 and 16 weeks of age in puppies that have received colostrum (Chappuis, 1995). Maternal antibodies can interfere with the first vaccination in dogs younger than 8 weeks of age (Chappuis, 1995).

Active immunity against CDV in dogs can be achieved by using a live attenuated vaccine. Inactivated CDV vaccines are used in wild or exotic animals (Chappuis, 1995). In mink and ferret, the modified live attenuated "ferret avirulent" stains (Ondersteport strain) are fully effective (Chappuis, 1995).

a. Vaccine Associated CDV Infection: Reversion to virulence in dogs and ferrets has been experimentally demonstrated *in vivo* with vaccine virus that was attenuated *in vitro* by serial passage in pulmonary macrophages (Appel, 1978). Post-vaccinal encephalitis has been reported in dogs, ferrets and wild animals (Hartley, 1974; Bush et al., 1976; Itakura et al., 1979; Cornwell et al., 1988; Gill et al., 1988; McCandlish et al.,

1992; Williams et al., 1992). Concurrent parvoviral infection, anesthesia, and glucocorticoid therapy can alter the response of distemper vaccination in dogs (Greene & Appel, 1998). Clinical signs in dogs vaccinated with modified-live CDV vaccines are observed between 7 and 20 days post-vaccination. These clinical signs are similar to those observed in natural infection with CDV (Cornwell et al., 1988). However, histologic lesions are more severe in the pontomedullary gray matter (Hartley, 1974; Williams et al., 1992). Neuronal necrosis with secondary Wallerian degeneration and viral inclusions are the hallmarks for vaccine-induced encephalitis (Hartley, 1974; Williams et al., 1992). Virus isolation in cell culture is a reliable method for differentiating vaccine from wild-type strains of CDV (Williams et al., 1992). Vaccine strains preferentially infect macrophages and epithelial cells. Wild-type CDV is difficult to isolate except when using mitogen-stimulated lymphoid cells and alveolar macrophages (Evans et al., 1991; Appel et al., 1992).

K. Public Health Considerations

Strict vaccination programs are necessary to control CDV since this infectious agent causes a highly contagious disease in dogs and other species (Greene & Appel, 1998). Chronic progressive distemper encephalitis in dogs has been of wide interest to many researchers due to histologic similarities with multiple sclerosis and subacute sclerosing panencephalitis seen with latent measles virus infection. The study of CDV is useful for understanding the pathogenesis of other demyelinating disorders in humans (Greene & Appel, 1998).

L. Phylogenetics

Phylogenetics is a field of the taxonomy that deals with identifying and understanding the evolution of life (Weston, 1994). The most convenient way to visualize evolutionary relationships among a group of organism is a phylogenetic tree (Weston, 1994; Page, 1996). A phylogenetic tree is composed of nodes, which represent each one of the taxonomic units (species, strains) and branches, which are the relationships between the taxonomic units in terms of descent and ancestry (Weston, 1994). The branching pattern of the tree is called topology (Weston, 1994). The length of the branches is proportional to the degree of genetic changes. There are trees with scaled and unscaled branches. In addition, the phylogenetic trees can be rooted or unrooted. For rooted trees, the root is the common ancestor. For each species or strain, there is a unique path that leads from the root to that species. The direction of each path corresponds to evolutionary time (Weston, 1994). An unrooted tree specifies the relationships among species, but does not define the evolutionary path (Weston, 1994). A clade is defined as a group of monophyletic genomic sequences that are included in the phylogenetic analysis and have descended from a particular common ancestral sequence (Weston, 1994).

The steps in phylogenetic analysis include: alignment, selecting the methods of analysis, building the tree and examination of the tree. First, the alignment is used to visualize where changes have occurred; second, selecting the method of analysis permits the user to examine phylogenetic relationships between sequences; third, the tree is built with the generated data resulting from the selected method of phylogenetic analysis. The final step consists of evaluation of the tree from which conclusions can be inferred (Page, 1996).

Phenetic and cladistic methods are the two major groups of analyses to examine phylogenetic relationships between sequences (Weston, 1994). In phenetic methods, the trees are calculated by similarities of sequences and based on distance methods. The resulting tree is called dendrogram. Distance methods compress all of the individual differences between pairs of sequences into a single number. Starting from the alignment, pairwise distances are calculated between DNA sequences as the sum of all base pair differences between two sequences (the most similar sequences are assumed to be closely related). This creates a distance matrix, which has the following consideration:

1. All base [Adenine (A), Thymine (T), Cytosine (C) and Guanine (G)] changes can be considered equally or a matrix of the possible replacements can be used.
2. Insertions (addition of one or more nucleotides) and deletions (removal of one or more nucleotides) are given a larger weight than replacements (transitions, which are substitutions from A to G; G to A; C to T or T to C, or transversions, which are substitutions from G to C; C to G; T to A; or A to T). Insertions or deletions of multiple bases at one position are given less weight than multiple independent insertions or deletions.
3. It is possible to correct for multiple substitutions at a single site.

From the obtained distance matrix data, a phylogenetic tree is calculated with clustering algorithms. These cluster methods construct a tree by linking the least distance pairs of taxa (data), followed by successively more distant taxa. The distance matrix method uses the UPGMA clustering (unweighted pair group methods using arithmetic averages) and Neighbor joining. Neighbor joining tries to correct the UPGMA method assuming that the rate of evolution is the same in all taxa (Weston, 1994). When amino acid sequences

are analyzed, the distances between them are calculated using the percentage of accepted mutation or PAM250. PAM250 is also the distance matrix.

In cladistic methods, trees are calculated by considering the various possible pathways of evolution and are based on parsimony or likelihood methods. The resulting tree is called cladogram. Cladistic methods use each alignment position as evolutionary information to build a tree. In parsimony, for each position in the alignment, all possible trees are evaluated and are given a score based on the number of evolutionary changes needed to produce the observed sequence changes. For this reason, the most parsimonious tree is the one with the fewest evolutionary changes for all sequences to derive from a common ancestor. This is a more time-consuming method than the distance methods. In maximum likelihood, for each position in the alignment all possible trees are evaluated and calculated using an explicit model of evolution.

The resulting phylogenetic data is subjected to bootstrap analysis, which is used to produce a majority rule consensus. This statistical method is based on repeated random sampling with replacement from an original sample to provide a collation of new pseudoreplicate samples, from which sampling variance can be calculated (Felsenstein, 1985; Altschul et al., 1997). Phylogenetic analyses have been widely used to detect genetic differences and similarities among CDV strains affecting dogs or other species around the world (Barrett et al., 1991 & 1993; Bolt et al., 1997; Lednicky et al., 2004).

II. STATEMENT OF HYPOTHESIS

The hypothesis for this study was that variant or novel wild-type strains of CDV were responsible for recent clinical cases of CDV. To test this hypothesis we phylogenetically characterized four naturally occurring cases of CDV in dogs submitted to the Veterinary Medical Diagnostic Laboratory of the University of Missouri-Columbia between the months of June and October of 2004. The complete gene sequence of the fusion (F) and hemagglutinin (H) genes, and part of the phosphoprotein (P) gene were determined. Nucleotide and amino acid sequences were used to phylogenetically determine relationships with viruses previously detected in the United States, Asia or Europe.

III. MATERIAL AND METHODS

A. Case Material

Between June and October of 2004, 15 dogs (11 cases) were submitted to the University of Missouri's Veterinary Medical Diagnostic Laboratory (VMDL) for routine necropsy examination following death or euthanasia. The term "case" is considered to contain up to three dogs. Thirteen dogs came from unrelated breeding premises and two dogs came from individual family households within the state of Missouri. The dogs had no history of recent travel and were presented to the referring veterinarians with a clinical history suspicious of CDV infection **(Table 1)**.

B. Inclusion Criteria

Based on the inclusion criteria of histologic lesions suggestive of CDV, positive immunohistochemical staining, and RT-PCR testing, four cases (seven dogs) from the initial group of 11 cases (15 dogs) were selected for further analysis. **Table 1** shows selected cases with bold letters.

C. Gross and Light Microscopic Examination

For each dog, a necropsy examination was performed and multiple tissues were fixed for 48 to 72 hours in 10% neutral buffered formalin (Fisher, Saint Louis, MO). Fixed tissues were trimmed (3 mm in thickness) and dehydrated using a series of graded alcohols (Fisher, Saint Louis, MO). These samples were dehydrated with xylene (Fisher, Saint Louis, MO) and infiltrated with paraffin (Surgipath Co., IL) followed by

Table 1: Suspected canine distemper virus cases between June and October 2004

Case number and age	Vaccination	Clinical signs and Gross necropsy findings	Histology	RT-PCR	IHC	Final Diagnosis
11277 12 wks old	Yes, unknown vaccine	Pneumonia and enteritis	Suppurative bronchopneumonia	Pos.[a]	ND[b]	Pneumonia caused by *Bordetella bronchiseptica*
14306 8 wks old	No information	Diarrhea, enteropathy and pulmonary congestion	Enteritis with lymphoid depletion and necrosis	Pos.	ND	Parvoviral enteritis
15178 10 wks old	No information	Emaciation, and dehydration	Enteritis, dermatitis, and lymphoid depletion	Pos.	ND	Enteritis caused by *Escherichia coli* and enteric parasites
15849 10 wks old	No information	None	Mononuclear Inflammation and perivascular cuffing in the spinal cord	Pos.	ND	Possible Dysautonomia
18133 8 wks old	**Yes, Vanguard5 vaccine at 6 and 8 wks**	**Thymic atrophy, lymphadenopathy (mesenteric)**	**Demyelinating encephalitis with viral inclusions, splenic lymphoid depletion and necrosis and enteritis**	**Pos.**	**Pos.**	**CDV confirmed with *Escherichia coli* and *Klebsiella pneumoniae* enteritis**
17569 8 wks old	Yes, unknown vaccine at 6 wks of age	Not performed	Not performed	Pos.	ND	CDV suspected
19213 6-8 wks old	Yes, unknown vaccine at 6 and 8 wks	Diarrhea	Enteropathy, lymphoid necrosis, hepatic lipidosis.	Pos.	ND	Coccidiosis and *Escherichia coli* infection
19876 4.5 years old	**Yes, unknown vaccine**	**Pulmonary congestion**	**Demyelinating encephalitis, lymphoid depletion, bronchitis, with viral inclusions**	**Pos.**	**Pos.**	**CDV confirmed**
20684 10 wks old	No information	Bronchopneumonia and nasal discharge	Suppurative bronchopneumonia and lymphoid hyperplasia	Pos.	ND	Pneumonia caused by *Bordetella bronchiseptica*
21261 6-8 wks old	**Yes, Galaxy vaccine at 6 and 8 wks**	**Pneumonia, thymic atrophy, footpad hyperkeratosis**	**Pneumonia, enteritis, thymic atrophy, footpad hyperkeratosis with inclusions**	**Pos.**	**Pos.**	**CDV confirmed with *Escherichia coli* septicemia**
25259 8 wks old	**Yes, Vanguard5 vaccine at 6 and 8 wks**	**No lesions**	**Demyelinating encephalitis, bronchopneumonia with viral inclusion**	**Pos.**	**Pos.**	**CDV confirmed**

[a]Pos., Positive test
[b]ND, Test not performed

embedding. Embedded tissues were cut 4 μm in thickness, placed on clean slides, and deparaffinized in a microwave before staining. Deparaffinized slides were rehydrated through graded alcohols to water and stained with hematoxylin (Anatech Co., MI) and eosin (Surgipath Co., IL) stains. Stained sections were cover slipped for light microscopic examination.

D. Immunohistochemical Staining

For immunohistochemical staining, sections of paraffin embedded tissue were mounted on treated slides and steamed in 0.1M citrate buffer pH 6.0 (Dako, Carpenteria CA). Steamed slides were incubated at room temperature for 30 min with a 1:800 dilution of anti-CDV monoclonal antibody (DV2-12, Custom Monoclonal, Inc., West Sacramento, CA) targeting the nucleocapsid protein. Envision Plus was used as the detection system with DAB Plus (DAKO-Cytomation, Carpenteria, CA) as the chromagen. Slides were counterstained with Mayer hematoxylin (Newcomer Supply, Middleton, WI).

E. Nucleic Acid Extraction and RT-PCR Amplification

Non-fixed tissues collected at necropsy from the 11 cases were homogenized in phosphate buffered solution (PBS), pH 7.4 for the extraction of RNA using the NucleoSpin RNA II kit (BD Biosciences, Inc. Palo Alto, CA). In a labeled clean 1.7ml tube, 100 μl of the sample and 350 μl of Buffer RA 1 were combined and vortexed. An additional 350 μl of 70% alcohol was added and vortexed. The sample was poured into the column with the vacuum applied, followed sequentially by 350 μl of Buffer MDB and

850 µl of Buffer RA 3. The column was centrifuged for 3 minutes at 13,000 rpm and placed into a collection tube. Finally, 50 µl of nuclease-free water was added to the column, and centrifuged for 1 minute at 13,000 rpm. The column was removed and discarded. The microcentrifuge tubes with the RNA extraction were stored at -20°C.

RNA amplification by RT-PCR was performed with oligonucleotides primers previously reported for the diagnosis of CDV infection of dogs (Frisk et al., 1999). The final product of this amplification was 287 base pairs of the phospholipid protein gene of CDV.

For nucleotide sequence analysis, RT-PCR amplification of RNA purified directly from tissues collected at necropsy from the four cases selected was performed with the oligonucleotide primers shown in **Table 2.** These primers targeted part of the phosphoprotein (P) gene as well as complete fusion (F) and hemagglutinin (H) genes. All RT-PCR amplifications were performed using each primer at a final concentration of 0.6 µM in a 20-µl reaction mixture with 0.8 µl of Qiagen one-step RT-PCR enzyme mix in the manufacturer's buffer containing deoxynucleoside triphosphates (0.4 µM each, final concentration). Thermocycling conditions for amplification were 50ºC (40 min) and 95ºC (12 min), followed by 10 cycles of denaturation (95ºC, 30 sec), annealing (68ºC, 20 sec), and extension (72ºC, 90 sec), with the annealing temperature in these cycles reduced by 1ºC each cycle. An additional 35 cycles of denaturation (95ºC, 30 sec), annealing (54ºC, 20 sec), and extension (72ºC, 90 sec) were performed, followed by a final extension (72ºC, 7 min). Amplification products were separated in a 1.5% agarose, 1 X tris-acetate-EDTA gel and visualized by ethidium bromide staining and ultraviolet transillumination (Sambrook et al., 1989). For each step in RT-PCR amplification and

Table 2: Oligonucleotide primers used for RT-PCR amplification and subsequent nucleotide sequencing

Primer Number	Orientation	Sequence (5´ to 3´)	Nucleotide position[a]	Target region
1	sense	ACCAGGACCTGGAATACG	2106-2123	P gene
2	antisense	GAGAAAAGCTCATCATCG	2721-2738	
3	sense	ACAGGTCAACCAGGTCCA	4873-4890	F gene
4	sense	GCATCGGAATAGCCAGTC	5200-5217	
5	antisense	CAGTTTTATGACCAAGTA	5427-5444	
6	sense	TCAACAACGAACTCGTCC	5815-5832	
7	antisense	GGGCCAAATATTGACAAC	5909-5926	
8	sense	CATCTGTAGCCAGAACTCC	6293-6311	
9	sense	TATTGCCTCCGATACCTG	6410-6427	
10	antisense	GCAGGTATCGGAGGCAAT	6411-6428	
11	sense	GTCTCCTCAGTGTTCCTA	6757-6774	
12	antisense	AATGTCCGTTGGTAGCGTC	6826-6844	
13	antisense	AATGCCGGATCGACCTTA	6857-6874	
14	sense	GTCCTTCTCATCCTACTGG	7199-7217	H gene
15	sense	ACTTCCGCGATCTCCACT	7372-7389	
16	antisense	AGTGGAGATCGCGGAAGT	7372-7389	
17	antisense	ACACTCCGTCTGAGATAGC	7742-7760	
18	sense	TCTCAGACGGCGTGTATG	7746-7763	
19	sense	TCGACACTCGAGAGATTC	7806-7823	
20	antisense	GCATGTCATTCAGCCACC	7851-7868	
21	sense	CATCTTATGGGCGGTTGA	8289-8306	
22	antisense	GTGAACTGGTCTCCTCTA	8378-8395	
23	antisense	TGCCTAAGGCCAATTGAG	8921-8938	
24	antisense	CTGTAAGGGATTTCTCAC	8950-8967	

[a]Nucleotide position based on the genome of strain A75/17 (GenBank accession number AF164967)

analysis, strict protocols were followed to prevent cross-contamination of samples (e.g., use of dedicated pipettes for each step, use of aerosol-barrier pipette tips for all pipetting steps, changing gloves between steps, and performing all postamplification procedures in a room that was physically separate from that used for RT-PCR assembly). Additionally, RNA free water was used to prevent RNase contamination of the samples. Negative control reactions (no template added) were demonstrated to be negative before subsequent analysis of amplification of products.

F. Nucleotide Sequencing

The amplification products were excised from the agarose gel, mixed with 200 µl of membrane binding solution (about 1 µl per mg of gel) Wizard SV kit (Promega, Inc., Madison, WI), and incubated at 50-65ºC until the gel slice was completely dissolved (approximately 10 min). These specimens were vortexed periodically and left 2 minutes at room temperature. The dissolved gel was added into a SV minicolumn (which was inserted into a collection tube), incubated for 1 minute at room temperature, and centrifuged at 16,000 rpm for 1 minute. The flow-through was discarded and a new collection tube was reinserted. Next, 700 µl of a membrane wash solution was added to and the column was then centrifuged at 16,000 rpm for 1 minute. The resulting flow-through was discarded and a clean collection tube was used. Another 500 µl of the same membrane wash solution was added and centrifugation was repeated at 16,000 rpm for 5 minutes. The column was transferred to a clean 1.5 ml microcentrifuge tube and 30 µl of nuclease-free water was added. This tube was labeled, incubated at room temperature for

1 minute, and centrifuged at 16,000 rpm for 1 minute. The samples were centrifuged under vacuum for 15 minutes. For sequencing, a total reaction volume of 12 µl with 5 µmol of primer, nuclease free-water, and 20 to 40 ng of purified DNA products were assembled and submitted to the DNA core facility at University of Missouri-Columbia for automated sequencing.

For each of the four selected cases for this study, overlapping sequences, excluding the oligonucletide primer sequences for the respective fragments, were assembled into contiguous sequences of 540 base pairs of the partial P gene and 3968 base pairs for the complete F and H genes (plus the intergenic region). For all sequences obtained, a minimum of four overlapping sequencing reactions were performed and subsequently analyzed. Nucleotide sequence assembly and alignments were performed with DNAStar software (DNAStar, Inc., Madison, WI), and BLAST analysis (Altschul et al., 1997) was used to search the public domain database. The CDV nucleotide sequences, which were obtained in this study were deposited in the National Center of Biotechnology information database and assigned GenBank accession numbers AY964107 (case 18133, partial P gene), AY964108 (case 18133, F and H genes), AY964109 (case 19876, partial P gene), AY964110 (case 19876, F and H genes), AY964111 (case 21261, partial P gene), AY964112 (case 21261, F and H genes), AY964113 (case 25259, partial P gene), and AY964114 (case 25259, F and H genes).

G. Alignments and Phylogenetic Analysis

Nucleotide and deduced amino acid sequences were aligned with CLUSTAL X (version 1.8) software. Sequences for comparison were obtained from GenBank and

corresponded to nucleotide sequences of CDV P, F, and H gene and deduced protein sequences. Phylogenetic analyses of nucleotide and amino acid alignments were performed using distance matrix methods (DNADIST or PRODIST followed by NEIGHBOR) and maximum parsimony methods (DNAPARS or PROTPARS) within the PHYLIP software package (Felsenstein, 1985 & 1993). Data sets were subjected to bootstrap analysis, based on 100 re-samplings of the original data set, using the SEQBOOT program to produce a majority-rule consensus tree. Completed tree files were visualized using TreeView 1.5 (Page, 1996).

IV. RESULTS

A. Case Material

Between June and October of 2004, 15 dogs with clinical signs suggestive of CDV infection were submitted to the Veterinary Medical Diagnostic Laboratory at the University of Missouri-Columbia for necropsy and virologic testing **(Table 1)**. Inclusion criteria for phylogenetic study included histologic lesions suggestive of CDV infection, and positive immunohistochemical staining and RT-PCR testing. RT-PCR tests were requested for each one of those cases and the results were positive. A diagnosis for CDV infection was given in only four cases (seven dogs), based on inclusion criteria. These four cases (seven dogs) were selected for further genetic analysis.

The cause of death for the dogs not selected also was evaluated **(Table 1)**. Suppurative bronchopneumonia was diagnosed in dogs of cases 11277 and 20684. The dog of case 14306 was diagnosed with severe parvoviral enteritis. *Escherichia coli* and coccidial enteritis were the cause of death for the dog in case 19213. Dysautonomia was suspected in case 15849, based on the clinical signs described by the submitter and histologic findings. Enteric nematode parasitic disease was the cause of death for dog in case 15178. CDV infection was suspected for the dog of case 17569 since only conjunctival and nasal swabs were submitted for PCR analysis. Although vaccination history was not clear in most of the non selected cases, recent vaccination was suspected as a cause of positive RT-PCR test results. This suspicion was further supported by sequencing partial P protein gene from samples of dogs of cases 17559, 15849, 15178

and 19213 which verified that sequences from these cases were consistent with that of CDV vaccine strains.

B. Clinical Signs

In most of the cases, a completely detailed clinical history was not provided by the submitter's; however, serous oculonasal discharge, salivation, dehydration, decreased jaw tone, tremors, seizures and death were the clinical signs observed in all dogs of case 18133. The dog of case 19876 had central vestibular neurologic dysfunction and myoclonus. Oculonasal discharge, upper respiratory dysfunction, and anorexia were found in the dogs of case 21261. Blindness and disorientation were observed by the referring veterinarian for the dog in case 25259 **(Table 1)**.

C. Gross Findings

Gross findings are summarized in **Table 1**. In case 18133, marked to severe thymic atrophy, meningeal edema and mesenteric lymphadenopathy were observed in the necropsy examination. The right middle and left caudal lung lobes were congested in the dog in case 19876. Severe thymic atrophy and lung consolidation and footpad hyperkaratosis were observed in the two dogs for case 21261. No gross lesions were observed by the referring veterinarian in the dog of case 25259.

D. Light Microscopic Findings

a. Systemic Lesions: The lungs of cases 21261 and 25259 had necrosis, regeneration and hyperplasia of the bronchial and bronchiolar epithelial cells. The

alveolar septa were expanded by fibrin and many neutrophils, lymphocytes, plasma cells and macrophages (interstitial pneumonia). Type II pneumocytes were hyperplastic. There were many 1-5 μm predominantly intracytoplasmic and occasional intranuclear vial inclusions in bronchial **(Figure 1A)**, bronchiolar and alveolar epithelial cells, and macrophages. Additionally, neutrophils, fibrin and cell debris were present in the lumen of many alveolar spaces, bronchi and bronchioles. In case 21261, some medium size blood vessels had fibrinoid vaculitis. Mild interstitial pneumonia and bronchiolitis with intraepithelial viral inclusions were found in multiple sections of the lung of case 19876. Mild multifocal interstitial pneumonia was evident for case 18133.

Lymphoid depletion and necrosis of the white pulp of the spleen was evident in all cases examined **(Figure 1C)**. Thymic atrophy was observed in cases 21261, 25269 and 18133. Viral inclusions were occasionally observed in the cytoplasm and/or nuclei of lymphocytes within the spleen and thymus of case 19876. Lymphoid depletion in the Peyer's patches was present in the small intestine of case 19876.

Many parietal and chief cells of the stomach of cases 21261 and 25259 contained predominantly eosinophilic intracytoplasmic and occasional intranuclear viral inclusions. In cases 21261 and 18133, bacterial enteritis was diagnosed. Transitional epithelial cells of the urinary bladder of cases 21261, 18133 and 25259 contained abundant 1 to 5 μm in, eosinophilic, intracytoplasmic inclusions **(Figure 1E)** and cytoplasmic vacuolation.

b. CNS Lesions: CNS lesions in cases 25259 and 18133 were histologically similar and they were consistent with a subacute to acute mildly inflammatory encephalitis. The white matter of the cerebrum and brainstem showed demyelination and necrosis. These areas were surrounded by reactive microglia cells and astrocytes. The

leptomeningeal surface of the medulla oblangate of case 19876 had a small irregularly-shaped area of demyelination. The gray matter of cases 25259 and 18133 had

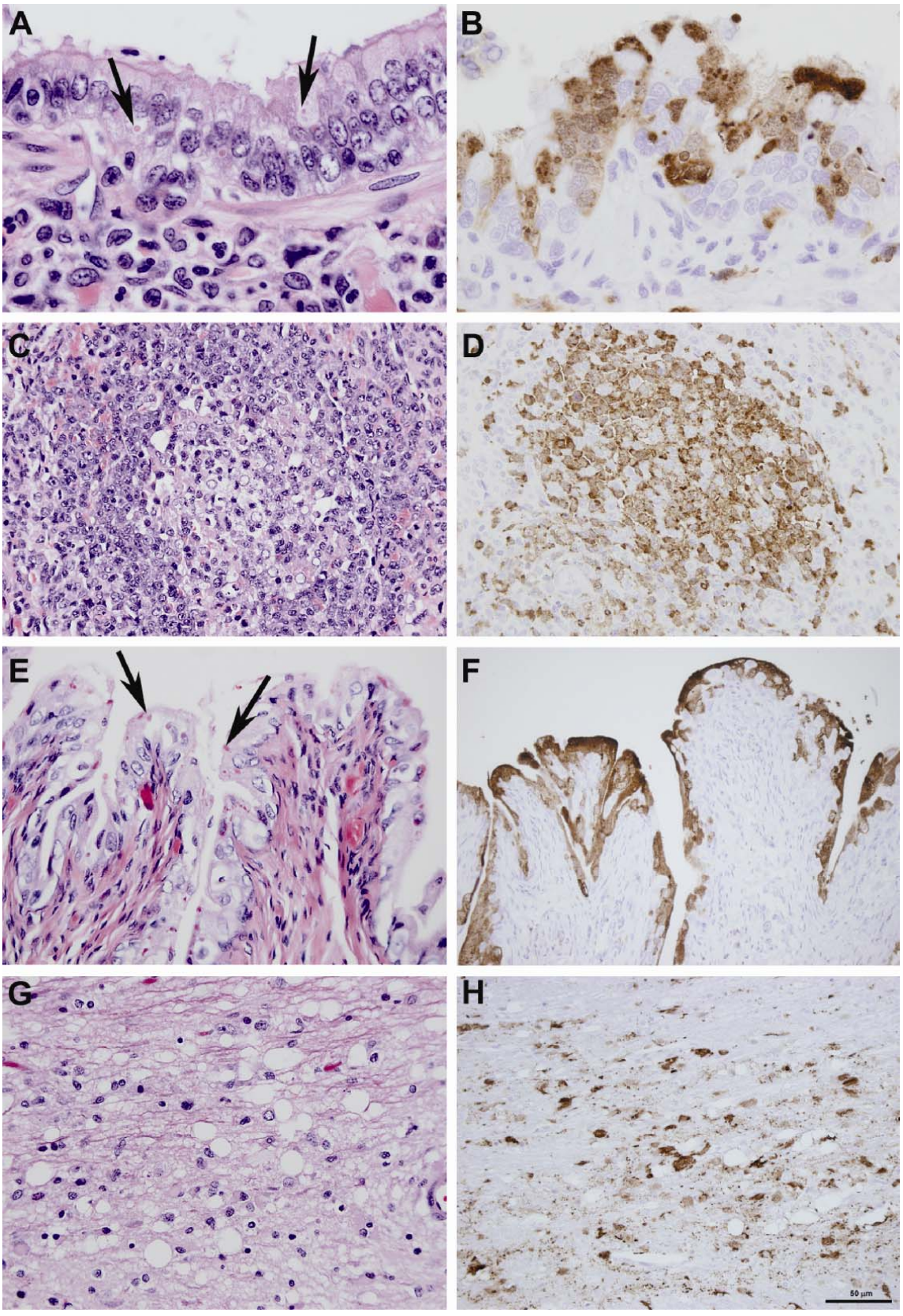
A
B
C
D
E
F
G
H
50 µm

Figure 1: Tissues from four dogs that were naturally infected with canine distemper virus (CDV). **A**. Bronchus (case 25259). The bronchial epithelial cells have multiple 2-5 μm eosinophilic intracytoplasmic viral inclusions (arrows) H&E. **B**. The brown staining in the cytoplasm and nuclei of the epithelial cells of the bronchus from figure A indicates positive staining for canine distemper antigen. **C.** Spleen (case 18133). Within the white pulp, there is lymphoid depletion and necrosis in the germinal center H&E. **D.** Monoclonal IHC for CDV within the mononuclear cells of the white pulp of the spleen shown in figure C. **E.** Urinary bladder (case 21261). Note the 2-5 μm eosinophilic intracytoplasmic and intranuclear viral inclusions in affected transitional epithelial cells (arrows) H&E. **F**. There is diffuse monoclonal IHC staining in the cytoplasm and nucleus of affected transitional epithelial cells from the urinary bladder of panel E. **G.** Brain (case 19876). Note the vacuolation of the neuropil with mild histiocytic and microgial infiltration H&E. **H.** The brown staining indicates canine distemper antigen within the neuropil, and nucleus and cytoplasm of histiocytic and microglia cells of panel G. **Bar = 50 μm.**

multifocal areas containing degenerate to necrotic neurons that were often surrounded by gliosis, reactive astrocytes, and they were also infiltrated by few macrophages and lymphocytes. Multiple 1 to 5 μm, eosinophilic, intranuclear and intracytoplasmic inclusions were also observed in affected neurons, astrocytes and glia cells. Non-staining vacuoles were present scattered throughout the neuropil of case 19876 **(Figure 1G)**. These vacuoles were surrounded by mild accumulations of histiocytes and gliosis. Occasional swollen axons and gemistocytes were also present in the white matter. No CNS lesions were observed in case 21261 **(Table 1)**.

E. Immunohistochemical Staining

Positive monoclonal immunohistochemical (IHC) staining for CDV antigen was observed in multiple tissue sections of lung, spleen, thymus, Peyer's patches of the intestine, stomach, urinary bladder and brain. Intranuclear and intracytoplasmic staining for CDV was present in epithelial cells of a bronchus in case 25259 **(Figure 1B)**. Positive intranuclear and intracytoplasmic staining within the mononuclear cells of the white pulp of the spleen of case 18133 **(Figure 1D)**. Diffuse immunohistochemical staining for CDV was found in transitional epithelial cells of the urinary bladder of case 21262 **(Figure 1F)**. Positive staining for CDV was observed within the neuropil, and cytoplasm and nuclei of microglia cells and histiocytes **(Figure 1H).**

F. RT-PCR and Sequence Analysis

Extracted RNA from necropsy tissue samples of all 11 cases (15 dogs) were positive for CDV, yielding an amplification product of the expected size of (287 base

pairs) using oligonucleotide primers previously described for diagnosis of CDV infection in dogs (Frisk et al., 1999). Samples of the same RNA from dogs representing each of the four cases selected (7 dogs) were subsequently subjected to additional RT-PCR amplification using oligonucleotides primers **(Tables 2)** designed to amplify the F and H genes, plus the intergenic space region, and the central region of the P gene. Overlapping amplification products ranging in size from 562 to 1228 base pairs **(Table 3)** were produced, using multiple combinations. Assembly of the nucleotide sequence data yielded full gene sequences for the F and H genes plus the intergenic region (3968 base pairs) and partial sequence (540 base pairs) for the P gene from each of the four cases shown in bold in **Table 1**.

Alignment of the H gene nucleotide and predicted amino acid sequences demonstrated that detected CDV in cases 18133 and 21261 were 100% identical for the nucleotide sequence. In contrast, predicted H gene amino acid sequences from these cases were 93.5% and 96.7% identical to cases 19876 and 25259, respectively, while case 19876 was 95.3% identical to 25259 **(Table 4)**. Similar levels of nucleotide sequence identity were observed between the detected CDV in these cases **(Table 4)**. Alignment of sequences to CDV sequences from the public databases **(Figure 2)** demonstrated identity levels ranging from 93.2% to 98.6% for the predicted amino acid sequences with similar levels of identity for the nucleotide sequences **(Table 4)**. Phylogenetic analysis of these H gene amino acid sequences plus others from the databases **(Figure 3)** demonstrated that cases 18133 and 21261 were grouped in a distinct clade with PDV-2 and CDV detected in dogs from China and Greenland. A bootstrap value of 100 for this clade suggests robust phylogenetic grouping. Phylogenetic analysis of the predicted H gene amino acid

Table 3: Oligonucleotide primer combination and expected product size

Primer combination	**Product size**
P1+P2[a]	632 bp
P3+P5	571 bp
P4+P10	1228 bp
P6+P13	1059 bp
P3+P7	1053 bp
P8+P12	551 bp
P9+P16	979 bp
P11+P16	632 bp
P11+P20	1111 bp
P14+P17	561 bp
P15+P22	1023 bp
P18+P22	649 bp
P18+P23	1192 bp
P18+P24	1221 bp
P19+P23	1132 bp
P21+P23	649 bp

[a]Primer designations are shown in the Table 2

Table 4: Nucleotide and deduced amino acid sequence identities of the canine distemper virus hemagglutinin (H) protein genes

Virus	% identity[a]						
	18133 [b]	19876	25259	Dog (Chn)	Panda (Chn)	Mink (Dnk)	A75/17
18133[b]		**93.5**	**96.7**	**98.1**	**95.7**	**95.0**	**95.5**
19876	93.5		**95.3**	**93.2**	**95.8**	**95.8**	**95.8**
25259	96.7	95.3		**95.8**	**98.6**	**96.8**	**97.3**
Dog (Chn)	98.1	93.2	95.8		**95.8**	**94.8**	**95.2**
Panda (Chn)	95.7	95.8	98.6	95.8		**97.5**	**98.0**
Mink (Dnk)	95.0	95.8	96.8	94.8	97.5		**97.4**
A75/17	95.5	95.8	97.3	95.2	98.0	97.4	

[a]Values for deduced amino acid sequence identities are in boldface.
[b]Values shown are identical for cases 18133 and 21261.

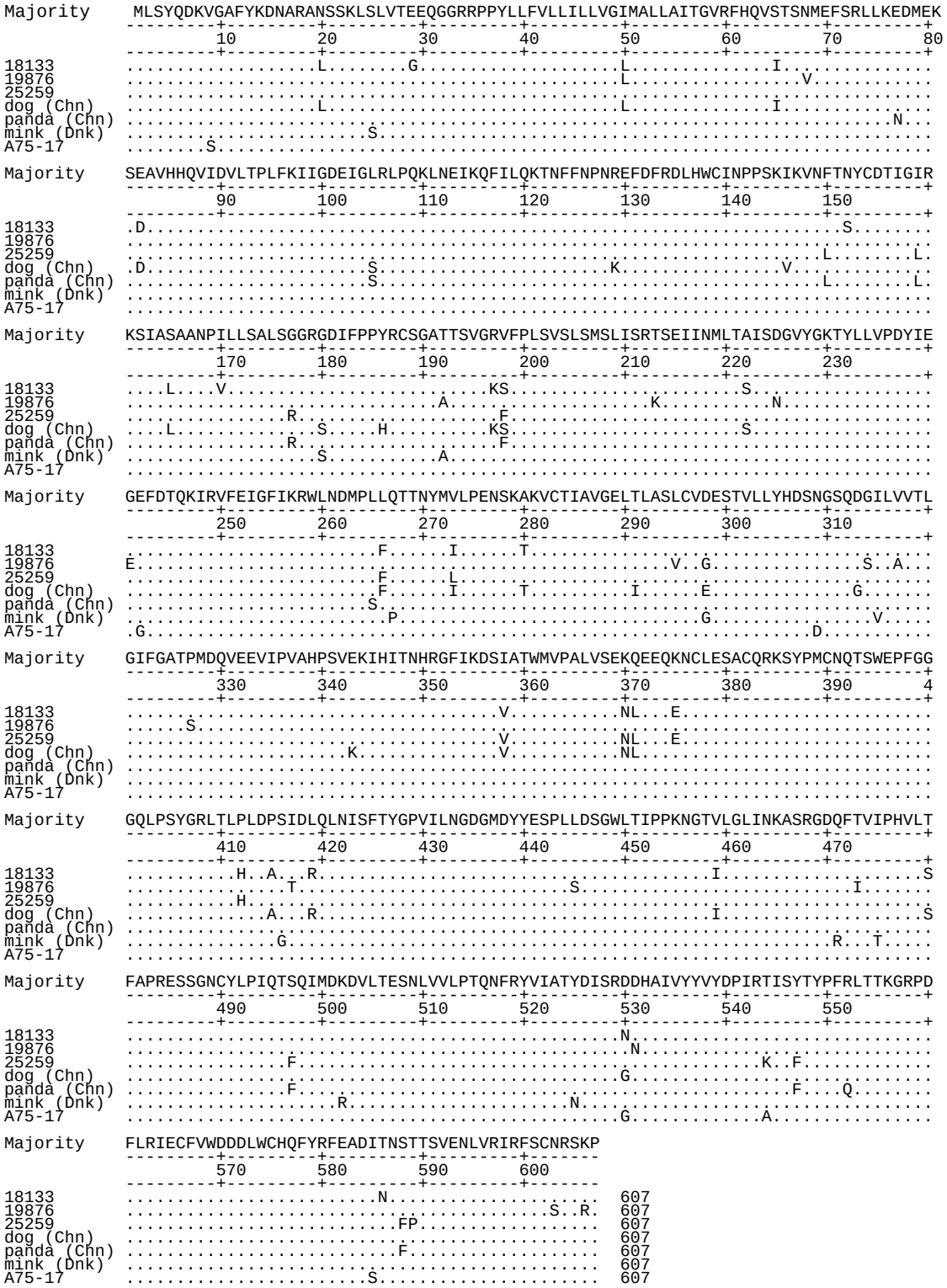

Figure 2: Alignment of deduced amino acid sequences from the hemagglutinin (H) gene of CDV strains. Only amino acids which differ from the majority sequence are shown. Identical residues are shown by dots.

sequences of case 25259 placed the virus detected closest to those from a lesser panda and giant panda, both of which were located in China. Analysis of the predicted H gene amino acid sequence from case 19876 did not clearly group the virus detected from this case with any available CDV sequences **(Figure 3)**.

As observed for the H gene sequences, alignment of F gene sequences from cases 18133 and 21261 demonstrated identical nucleotide sequences through the coding region **(Figure 4)**. Of the 4508 bases sequenced from these two isolates, only two nucleotide changes were noted and both were in the intergenic regions between the F and H gene sequences. Predicted F gene amino acid sequences from cases 18133 and 21261 were 91.2% and 94.9% identical to cases 19876 and 25259, respectively, while cases 19876 was 94.1% identical to 25259 **(Table 5)**. Following nucleotide sequence alignment, similar levels of identity were observed between the CDV isolates detected in these cases **(Table 5)**. Alignment of sequences to those in the public database **(Figure 4)** demonstrated a maximum identity level of 96.7% to phocine distemper virus type 2 (PDV-2), for the predicted amino acid sequences, and 97.3% for the nucleotide sequence **(Table 5)**. Considerably fewer full-length CDV F gene sequences were available for phylogenetic analysis when compared to the number available for the H gene. However, for cases 18133 and 21261 phylogenetic analysis of F gene amino acid sequences supported results for those of the H gene by grouping these sequences with those of PDV- 2, again having a bootstrap value of 100 **(Figure 5)**. In contrast, clear phylogenetic relationships were not established for F gene amino acid sequences from cases 19876 and 25259 with those available for comparison.

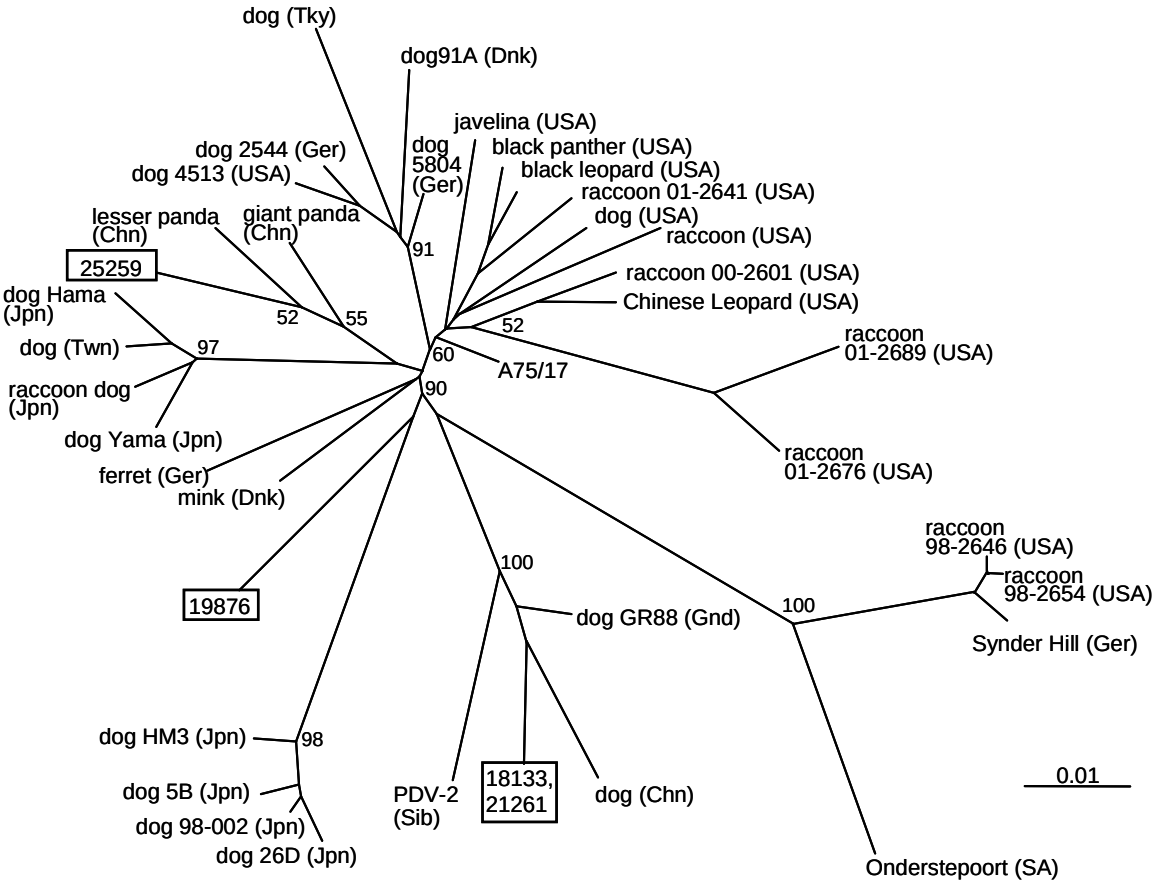

Figure 3: Phylogenetic tree for the complete hemagglutinin (H) protein gene sequences of representative canine distemper viruses plus those detected in cases 18133, 21261, 19876 and 25259 (shown in boxes). The unrooted tree was generated using the distance matrix program NEIGHBOR with the jumble option evoked. Distance values were calculated by the DNADIST program within the PHYLIP software package using the Kimura 2-parameter. Only bootstrap values greater than 70 are shown, and the branch lengths are propionate to genetic distances. The country of origin of Canine distemper viruses is indicated by a three letter abbreviation following the isolate designation; Chn, China; Jpn, Japan; Ger, Germany; SA, South Africa; USA, United States of America; Dnk, Denmark; Trk, Turkey; Twn, Taiwan; Gnd, Greenland; Sib, Siberia. Canine distemper viruses used for comparison with the country of origin and year of isolation (if known), plus the accession numbers are as follows: A75/17 (USA, 1975) AF164967; black leopard (USA, 1991) Z47763; black panther, strain A92-6 (USA, 1992) Z54166; Chinese Leopard, strain A92-27/4 (USA, 1992) Z54156; dog (USA, 1989) Z47762; dog 4513 (Ger) Z77673; dog (Taiwan) AY378091; dog Hamamatsu (Japan, 1992-1994) D85754; dog Yanaka (Japan, 1992-1994) D85755; dog 5804 (Germany, 1990) AY386315; dog 91A (Denmark, 1991) AF478544; dog 91B (Denmark, 1991) AF478546; dog 2544 (Germany, 1995) Z77672; dog (Turkey) AY093674; dog (China) AF172411; dog GR88 (Greenland, 1988) Z47760; dog 26D (Japan, 1999) AB040766; dog 5B (Japan,

Figure 3 (continued): 1999) AY297453; dog 98-002 (Japan, 1998) AB025270; dog HM3 (Japan, 1999) AB040767; dog KDK-1 strain (Japan, 1991) AB025271; dog Ueno (Japan, 1992-1994) D85753; ferret (German, 1989) X84999; giant panda (China) AF178038; javelina (USA, 1989) Z47764; lesser panda (China) AF178039; mink (Denmark, 1986) Z47759; Onderstpoort (South Africa) AF378705; PDV-2 Siberian seal (Siberia, 1988) X8499; raccoon 98-2646 (USA, 1988) AY542312 (also represents raccoon 98-2655 (USA, 1998) AY548109, and raccoon 98-2666 (USA, 1998) AY548110); raccoon 01-2689 (USA, 2001) AY465925 (also represents raccoon 01-2690 (USA, 2001); raccoon 00-2601 (USA, 2000) AY443350; raccoon 01-2641(USA, 2001) AY526496; raccoon 01-2676 (USA, 2001) AY498692; raccoon 98-2654 (USA, 1998) AY466011; raccoon (USA, 1989) Z47765; raccoon dog (Japan, 1996) AB016776; Snyder Hill (Germany) AF259552.

Table 5: Nucleotide and deduced amino acid sequence identities of the canine distemper virus fusion (F) protein genes

	% identity[a]				
virus	18133 [b]	19876	25259	PDV-2	A75/17
18133 [b]		**91.2**	**94.9**	**96.7**	**92.9**
19876	92.7		**94.1**	**91.5**	**93.7**
25259	96.4	94.6		**95.2**	**96.1**
PDV-2	97.3	93.2	96.4		**94.4**
A75/17	94.0	94.3	96.4	95.0	

[a]Values for deduced amino acid sequence identities are in boldface.
[b]Values shown are identical for cases 18133 and 21261.

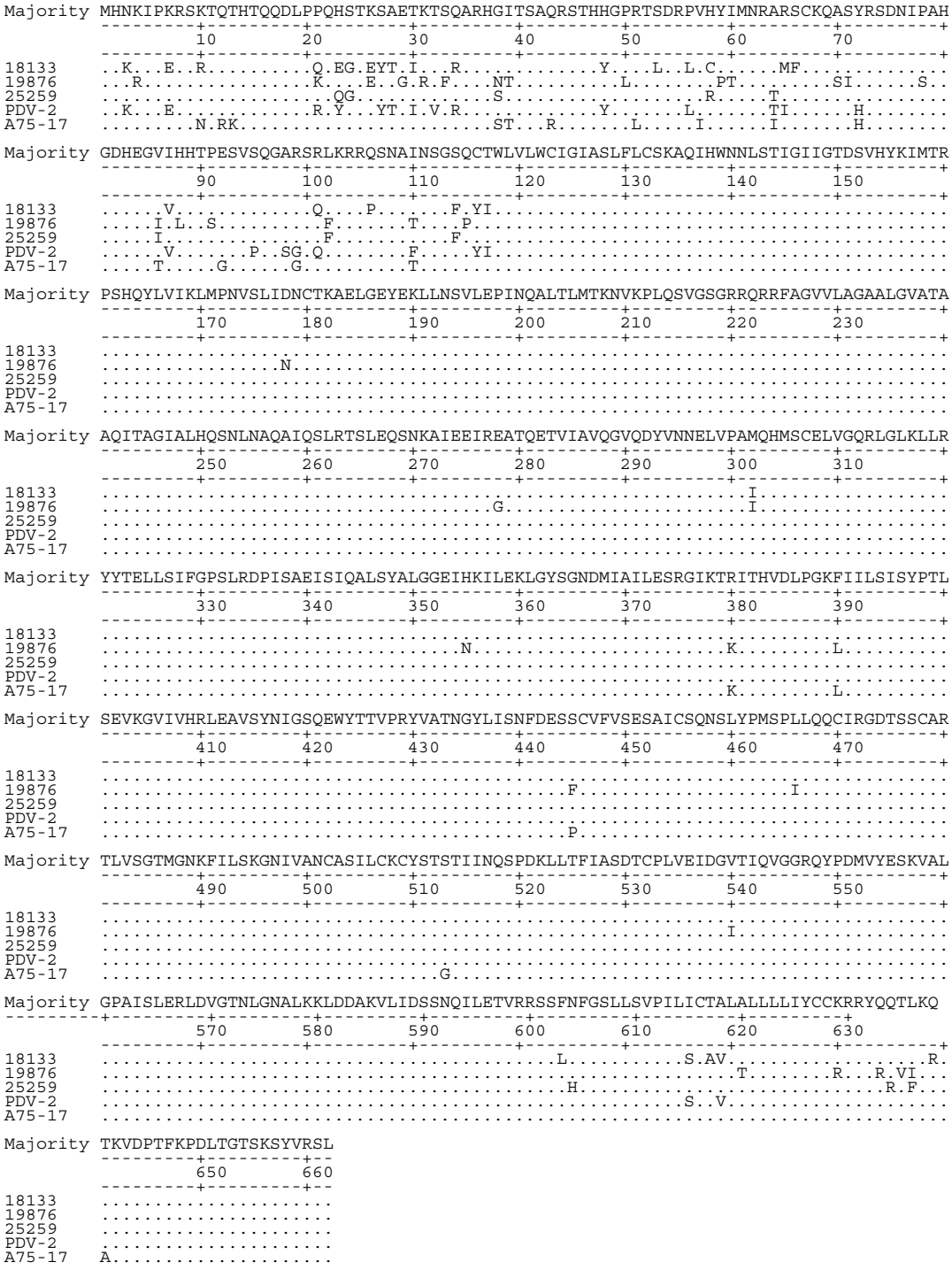

Figure 4: Alignment of deduced amino acid sequences from the fusion (F) gene of CDV strains. Only amino acids which differ from the majority sequence are shown. Identical residues are shown by dots.

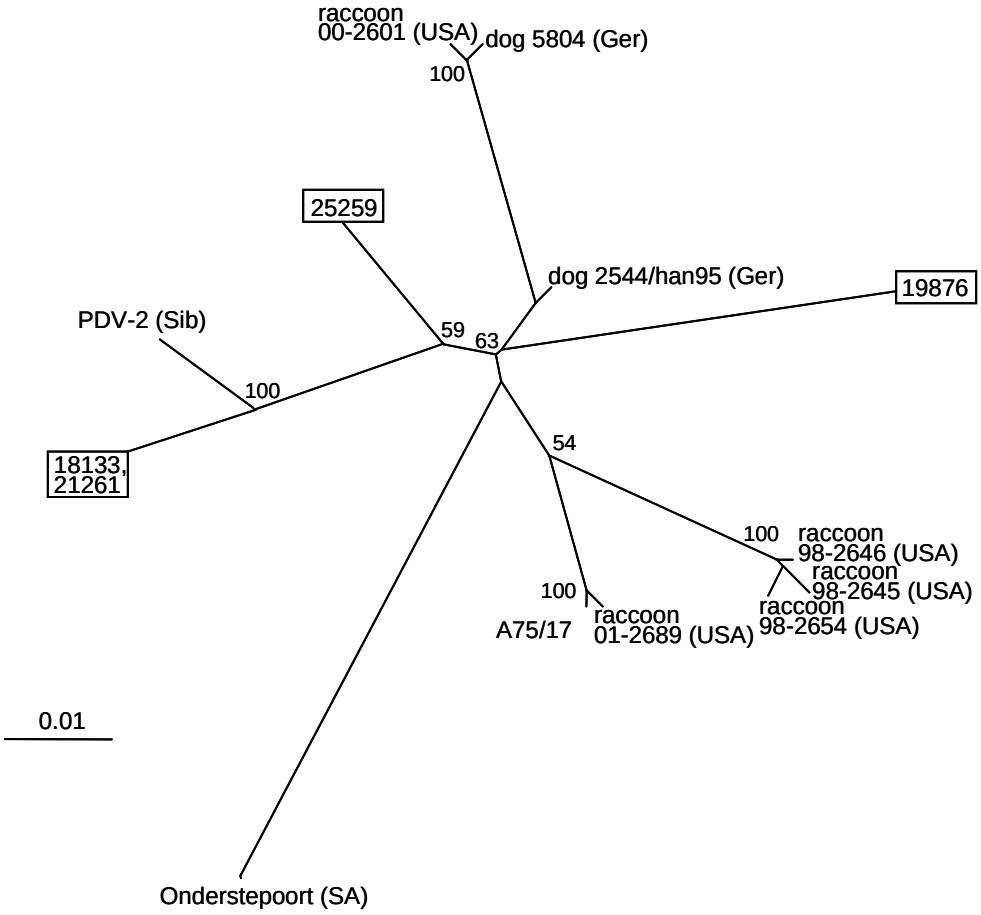

Figure 5: Phylogenetic tree for the complete fusion (F) protein gene sequences of representative canine distemper viruses plus those detected in cases 18133, 21261, 19876 and 25259 (shown in boxes). The unrooted tree was generated using the distance matrix program NEIGHBOR with the jumble option evoked. Distance values were calculated using the PROTDIST program with the Dayhoff percent accepted mutation matrix. Only bootstrap values greater than 50 are shown, and the branch lengths are propionate to genetic distances. The country of origin of Canine distemper viruses is indicated by a three letter abbreviation following the isolate designation: Ger, Germany; SA, South Africa; USA, United States of America; Sib, Siberia. Canine distemper viruses used for comparison and their accession numbers are as follows: A75/17 AF164967; dog 5804 (Germany) AY386315; dog 2544/han95 (Germany) AJ007711; raccoon 00-2601 (USA) AY443350; raccoon 01-2689 (USA) AY289615; raccoon 98-2646 (USA) AY542312; raccoon 98-2654 (USA) AY466011; raccoon 98-2645 (USA) AY445077; Onderstepoort, South Africa AAC26994; PDV-2, Siberia L07075

Alignment of the partial P gene nucleotide and predicted amino acid sequences demonstrated that CDV detected in cases 18133 and 21261 were again 100% identical in nucleotide sequence for a 540 base fragment of this gene **(Figure 6)**. Predicted partial P gene amino acid sequences from these cases were 96.1% and 97.2% identical to cases 19876 and 25259, respectively, while case 19876 was 97.8% identical to case 25259 **(Table 6)**. Following alignment, similar levels of nucleotide sequence identity were observed between the CDV detected in these cases. Alignment of sequences to those in the public database **(Figure 6)** demonstrated identity levels ranging from 96.8% to 99.4% at the predicted amino acid sequences with similar levels of identity of the nucleotide sequences **(Table 6)**. Phylogenetic analysis of these partial P gene amino acid sequences plus other from the databases **(Figure 7)** supported a relationship between cases 18133 and 21261 and PDV-2, although relatively low bootstrap values were found for analysis of the P gene amino acid sequences. In analysis of the partial P gene amino acid sequences, cases 25259 and 19876 were most closely related to cases 18133 and 21261 as well as PDV-2, A75/17 and stains detected in a dog from Alaska and a German ferret.

Within the non-selected cases, phylogenetic analysis of the partial P gene protein was performed in some of these dogs. Sequence analysis results of dogs in cases 17569, 15849 and 15178 were closely related to the A75/17 strain (data not shown). The sequence analysis result of dog in case 19213 was closely related to Ondestepoort strain.

Table 6: Nucleotide and deduced amino acid sequence identities of the canine distemper virus phosphoprotein (P) gene fragments

Virus	% identity[a]						
	18133 [b]	19876	25259	PDV-2	Alaska dog	German ferret	A75/17
18133 [b]		**96.1**	**97.2**	**96.7**	**97.8**	**96.1**	**96.1**
19876	96.8		**97.8**	**97.2**	**98.3**	**96.7**	**96.7**
25259	98.4	97.6		**98.3**	**99.4**	**98.9**	**98.9**
PDV-2	98.6	97.0	98.6		**98.9**	**98.3**	**97.2**
Alaska dog	99.2	97.6	99.2	99.4		**98.3**	**98.3**
German ferret	98.0	96.8	98.8	98.6	98.8		**97.8**
A75/17	97.8	97.2	99.0	98.0	98.6	98.2	

[a]Values for deduced amino acid sequence identities are in boldface.
[b]Values shown are identical for cases 18133 and 21261.

```
Majority   PAGAVSNRGFERGEGSLDDSTEDSGEDYSEGNASSNWGYSFGLKPDRAADVSMLMEEELSALLRTSRNVGIQKRDGKTLQ
              ---------+---------+---------+---------+---------+---------+---------+---------+
                    10        20        30        40        50        60        70        80
           ---------+---------+---------+---------+---------+---------+---------+---------+
18133      ................................................................................
19876      .........L.......................................................N.....H........
25259      ................................................................................
sib seal   ..........................................................................E.....
Alask dog  ................................................................................
ferret Ger ...................................................................S............
A75-17     ................................................................................

Majority   FPHNPEGKTGDPECGSIKKGTGERSASHGMGIVAGSTNGATQSAPKSTGGSSGPSVSVENVRQPAMSAKMTQKCKPESGT
           ---------+---------+---------+---------+---------+---------+---------+---------+
                    90       100       110       120       130       140       150       160
           ---------+---------+---------+---------+---------+---------+---------+---------+
18133      .......................K.........................V...............V..............
19876      ................................................................................
25259      .........................................................A......................
sib seal   .....................................S..........................................
Alask dog  ................................................................................
ferret Ger .....................................S...................A......................
A75-17     ..R...................................................N..A......................

Majority   QLPPRTSNEAESDSEYDDEL
           ---------+---------+
                   170       180
           ---------+---------+
18133      ......P.............
19876      ....................
25259      ....................
sib seal   ....................
Alask dog  ....................
ferret Ger ....................
A75-17     ....................
```

Figure 6: Alignment of deduced amino acid sequences from partial phospholipid (P) gene of canine distemper strains. Only amino acids which differ from the majority sequence are shown. Identical residues are shown by dots.

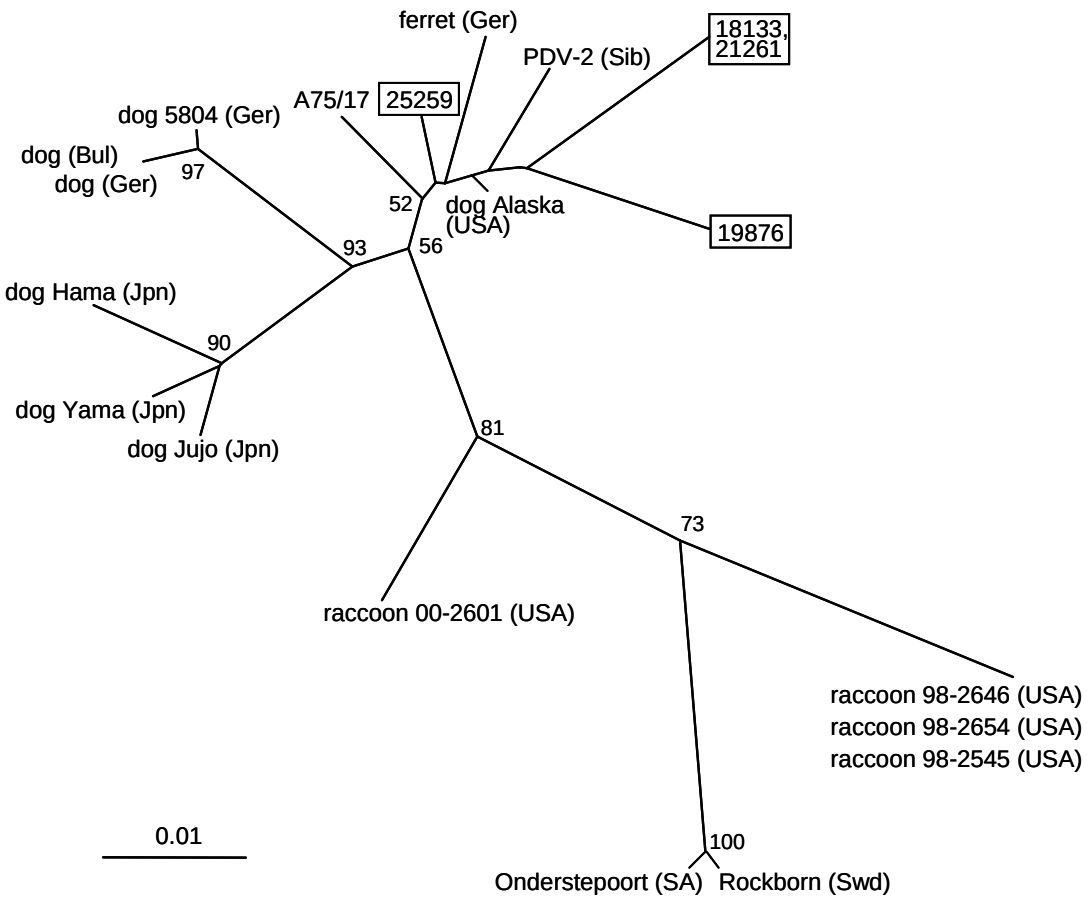

Figure 7: Phylogenetic tree for partial phosphoprotein (P) protein gene sequences of representative canine distemper viruses plus those detected in cases 18133, 21261, 19876 and 25259 (shown in boxes). The unrooted tree was generated using the distance matrix program NEIGHBOR with the jumble option evoked. Distance values were calculated using the PROTDIST program with the Dayhoff percent accepted mutation matrix. Only bootstrap values greater than 50 are shown, and the branch lengths are propionate to genetic distances. The country of origin of Canine distemper viruses is indicated by a three letter abbreviation following the isolate designation; Bul, Bulgaria; Jpn, Japan; Ger, Germany; SA, South Africa; USA, United States of America; Sib, Siberia; Swd, Sweden. Canine distemper viruses used for comparison and their accession numbers are as follows: A75/17 AF164967; dog Alaska (USA); dog (Bulgaria) AF259549; dog Hamamatsu (Japan) AB028915; dog Jujo (Japan) AB028916; dog Yanaka (Japan) AB028914; dog (Germany) AF259549; dog 5804 (Germany) AY386315; ferret (Germany) AF259550; PDV-2 (Siberia) AF259551; raccoon 98-2645 (USA) AY445077; raccoon 98-2646 (USA) AY542312; raccoon 98-2654 (USA) AY466011; raccoon 00-2601 (USA) AY443350; Rockborn (Sweden) AF181446

V. DISCUSSION

Over 5 months (June to October of 2004), 11 cases (15 dogs) were submitted to the Veterinary Medical Diagnostic Laboratory of the University of Missouri-Columbia for necropsy and detailed study of CDV infection. CDV infection was suspected in these dogs based on clinical signs. Although RT-PCR test was positive in all of these cases, only four cases (seven dogs, six puppies and one adult dog) were selected, based on the inclusions criteria including histologic lesions and positive RT-PCR and IHC results.

A complete clinical history was not available for all of the cases included in this study. However, gross examination and histologic findings observed in all four cases were consistent with the acute systemic form of CDV infection. Some clinical signs described by referring veterinarians included, serous nasal discharge, anorexia, dehydration and salivation. These signs are the result of viral tropism to multiple organs (Appel, 1969). In all cases, light microscopic findings in multiple tissues (lungs, lymphoid tissue, stomach, urinary bladder, and skin) demonstrated the presence of CDV inclusions and viral induced lesions. Neurologic signs were found in three cases and also were consistent with the acute form of CDV infection. Descriptions included decreased jaw tone, tremors, seizure, blindness and disorientation. The origin of these clinical signs was explained by the light microscopic findings consistent with multifocal areas of polioencephalomyelitis, neuronal degeneration and necrosis, and demyelination. Neuronal lesions are known causes of seizures that can lead to anoxia and selective neuronal necrosis (Lisiak & Vandevelde, 1979; Braund & Vendevelde 1979). Case 19876 was presented to the University of Missouri-Columbia Veterinary Teaching Hospital with signs of central vestibular dysfunction and myoclonus. Central vestibular dysfunction is

often associated with demyelinating lesions (Vandevelde & Zubriggen, 1995). Light microscopic findings were consistent with the observed clinical signs and were characterized by multifocal demyelination lesions, vacuolar degeneration of the neuropil, and viral inclusions in glial cells within the brainstem and medulla.

Virulence of the viral strain, environmental conditions, and host age and immune status are associated with the severity of clinical signs (Summers et al., 1984; Greene & Appel, 1998). These factors could have played an important role in this study, since the majority of dogs were puppies between 6 and 8 weeks of age that were raised in breeding facilities. A history of vaccination for CDV was described in three of the four selected cases for this study **(Table 1)**. However, serologic analysis to determine the immune status of these dogs was not performed. Lesions suggestive of vaccine-induced encephalitis (Hartley, 1974; Williams et al., 1992) including neuronal necrosis with secondary Wallerian degeneration and intraneuronal viral inclusions of the pontomedullary gray matter were not observed in these dogs.

Because virus isolation was not attempted in this study, presence of CDV infection was confirmed by RT-PCR and immunohistochemical staining of several tissues for each dog from all cases. Immunohistochemical staining results were similar to those described previously (Ducatelle et al., 1980; Haines et al., 1999). Intracytoplasmic and intranuclear immunostaining was positive for CDV in bronchial epithelial cells, type II pneumocytes, alveolar macrophages, lymphoid cells of the spleen, thymus, Peyer's patches, transitional epithelial cells of the urinary bladder, neuropil, neurons, astrocytes and other glial cells.

Nucleotide sequencing of the F and H protein genes and a portion of the P protein gene was performed following RT-PCR amplification of RNA purified directly from tissues collected at necropsy. A total of 4508 base pairs were sequenced for each of four cases. Dogs in cases 18133 and 21261 were identical except for two base differences in the intergenic region of the F and H protein genes. However, no epidemiologic link or common source of viral exposure was established between the two premises. Comparing CDV in dogs of cases 18133 and 21261 to that detected in cases 19876 and 25259 demonstrated that in the conserved P gene, identities range from 96.1% to 97.2% while in the more divergent F and H genes, identity values were lower. Phylogenetic analysis of the P, F, and H gene nucleotide and predicted amino acid sequences demonstrated that three genetically distinct strains had been detected among the four cases examined in this study. None of the strains detected were closely related to either known vaccine strains or lineages previously detected in North America.

Phylogenetic analyses of CDV P, F and H protein gene nucleotide and amino acid sequences have been performed to study the evolutionary relationship between CDV strain isolates and to find genetic variations between wild-type and CDV vaccine strains. In cases originating in Europe, analysis of the H protein gene of wild-type CDV isolates demonstrated that sequencing was a sensitive tool to precisely characterize current CDV field viruses (Haas et al., 1997).

The P gene is highly conserved among members of CDV clades and has been used for phylogenetic analysis by other (Barrett et al., 1993; Carpenter et al., 1998; Van de Bildt et al., 2002; Rzeutka & Mizak, 2003). However, relatively few CDV P protein gene sequences of the length sequenced in this study are available in the public databases

for phylogenetic comparison. Sequence analysis of a fragment of the P protein gene of a Polish distemper virus isolate demonstrated genetic diversity compared to other field isolates (Barrett et al., 1993; Carpenter et al., 1998; Van de Bildt et al., 2002; Rzeutka & Mizak, 2003).

The F protein gene is conserved among morbillivirus species and has also been used to determine phylogenetic relationships among these viruses (Barrett et al., 1991). Phylogenetic studies of the F protein gene have been widely used to determine mutation in the cleavage site for the F protein gene. Mutation in the cleavage site directly affected the virulence of Newcastle disease and Measles virus (Liermann et al., 1998). However, mutations at this cleavage site have not been observed in wild-type or CDV vaccine isolates (Liermann et al., 1998). A region of the F protein gene that encodes a protein cleavage site [subtilisin-like endoprotease motif (R-X-K/R-R)] and the fusion domain were examined by Lednicky et al. (2004) to differentiate CDV from an unrelated morbillivirus of raccoons.

The H protein gene is the most variable among CDV isolates, perhaps due to the role this protein plays in the host immune reaction. This protein is also the major determinant of tropism and cytopathogenicity (von Messling et al., 2003) making this gene useful for phylogenetic analysis (Harder et al., 1996; Bolt et al., 1997; Haas et al., 1997; Mochizuki et al., 1999;Hashimoto et al., 2001). A large panel of monoclonal antibodies against the N, P, F and H viral components of CDV confirmed that H protein has the highest antigenic variation, while the N, F and P proteins have insignificant antigenic changes (Blixenkrone et al., 1992). Because the H protein gene is the most variable, more complete H gene sequences are available in public database for

phylogenetic analyses compared to complete F and partial P protein gene sequences. Phylogenetic analysis of the H protein gene obtained from vaccine strains and field isolates of CDV in Japan demonstrated that wild-type CDV was distinct from vaccine strains, as well as European and North American CDV isolates (Iwatsuki et al., 1997; Mochizuki et al., 1999). Similarly in Denmark, phylogenetic analysis of the H protein gene characterized the variability of recent field isolates of CDV from different host and geographical areas of that country (Bolt et al., 1997).

Analysis of complete H protein gene sequences in this study provided the most robust results of the three genes examined. Analysis of the H gene sequences inferred an evolutionary relationship between cases 18133 and 21261 as well as H protein gene sequences of CDV strains detected in dogs from China and Greenland. The relationship of cases 18133 and 21261 to PDV-2 was further supported by phylogenetic analysis of complete F protein gene amino acid sequences and partial P gene amino acid sequences. Analysis of H protein gene amino acid sequences also suggested that cases 25259 may be related to CDV isolates previously detected in a lesser and giant panda from China, although lower bootstrap values made this evolutionary inference tentative. Unfortunately, sequences for the panda strain isolates were not available to perform F and P protein gene analyses and thus this relationship could not be further supported. Analysis of the P, F and H protein gene sequences did not strongly infer a relationship for CDV detected in case 19876. This suggests the CDV strain detected in this dog may be divergent from previously characterized CDV isolates.

Given the relationships suggested by phylogenetic analysis of the H protein gene sequences, CDV strains infecting dogs in this study may have originated form non-canine

species or may have been transmitted from dogs to another species. Interspecies transmission has been well documented (Haas et al., 1996; Harder & Osterhous, 1997; Deem et al., 2000). CDV isolates from captive large felids in the United States were thought to have originated from feral, non-felid carnivores (Harder et al., 1996). Studies on sequences of partial P and H protein genes of large felids demonstrated that virulent strains of CDV have emerged among these captive felids within the last few years and that CDV has frequently crossed host species among Serengeti carnivorous (Carpenter et al., 1998). A seroepidemiologic survey of CDV infection in Asian Felids revealed that the prevalence of antibodies for CDV varied depending on region and, in some cases, exposure of felid to dogs (Ikeda et al., 2001). The serologic pattern in cats with antibodies to CDV indicated that cats had likely been exposed to field strains rather than typical CDV vaccine strains (Ikeda et al., 2001).

Analysis of the H protein gene of PDV-2, which was isolated from an epizootic of disease in Siberian seals in 1987-88, demonstrated a relationship to CDV isolates from a German dog and ferret (Mamaev et al., 1995). These results suggest that transmissions from one of these hosts may have been responsible for the widespread infection seen in the freshwater seals. In addition, a follow-up study of the P protein gene also demonstrated that the same virus continued to circulate in seals of the Lake Baikal after the initial epizootic (Mamaev et al., 1995 and 1996).

Recent studies have characterized CDV strains in both dogs and non-canine species within North America. The phylogenetic studies of partial P and F protein gene sequences and complete H protein gene sequences of CDV strains isolates from raccoons concluded that these viruses were distinct from, yet strongly related to, known American

lineage CDV isolates such as Snyder Hill, Lederle and CDV A75/17 (Lednicky et al., 2004). A CDV outbreak killing several hundred sled dogs was recently described in Alaska (Maes et al., 2003). Sequence analysis of 540 base pairs of the P protein gene suggested a close relationship of the strain in the sled dogs to a strain of distemper virus isolated from Siberian seal (PDV-2) (Maes et al., 2003). Pairwise alignment of CDV partial P protein gene from the cases of the present study also demonstrated that the P protein gene sequences, as well the H and F sequences of cases 18133 and 21261, were potentially related to PDV-2 and thus may also be related to the virus detected in the Alaskan dogs in 2003. Unfortunately, only partial sequence of the P protein gene was available for the CDV outbreak in Alaskan dogs and thus a more definitive link between these cases could not be further explored.

In dog populations with a high rate of vaccination against canine diseases, clinical cases of CDV typically occur as epidemics. Speculations put forth to explain these cases are varied but often include vaccine failures, reversion of attenuated CDV vaccine strains to virulence (Appel, 1978), or the emergency of new strains which are sufficiently divergent to evade immune protection elicited by the vaccine used. In the cases studied herein, vaccination with modified live vaccine was performed recently in three of the four selected cases (6 dogs). Importantly, the CDV strains detected were clearly distinct from known vaccine strains. The CDV strain Lederle, which was used as a vaccine in the past (Harder & Osterhaus, 1997) and may still be circulating in wildlife, is related to the CDV strain Snyder Hill (Lednicky et al., 2004) and thus distant to the strains detected in the present study. The widely used Galaxy-D CDV vaccine (Schering-Plough, Kenilworth, NJ) originates from the Onderstepoort strain, which is also genetically distant from the

CDV strains detected in each of the four cases in this study. To further explore the possibility of vaccine virus reverting to virulence, partial sequencing (979 bases, data not shown) of the H protein gene was performed from the same production lot of the CDV vaccine (Vanguard 5, Pfizer, Inc., Exton PA) used in dogs from case 18133. The nucleotide sequence detected from this vaccine was 97.7% identical to strain A75/17 (which was the closest match in GenBank over the region sequenced) but only 95.0% identical to the virus detected in cases 18133 and 21261. Together, these observations and the histologic lesions observed in the brain of these dogs suggest that a recent reversion of vaccine virus to virulence was not likely to be the cause of clinical disease seen in the dogs for which vaccine had been recently used. However, the presence of a minor though virulent strain present as a contaminant of the vaccine stock can certainly not be excluded.

In summary, the CDV strains detected in this study were distinct from viruses previously detected in the continental United States and most closely match strains from Asia and Europe. For one case (19876), the CDV detected failed to closely match any other strains following phylogenetic analysis of the F and H protein genes. In three of the four selected cases (18133, 21261 and 25259), a recent history of CDV vaccination was reported yet none of the viruses detected were related to known vaccine strains. The appearance of novel, divergent CDV strains of North America may require renewed efforts by vaccine manufactures to ensure adequate protection following immunization of dogs against CDV.

VI. SUMMARY

Samples from fifteen dogs (11 cases) were submitted to the Veterinary Medical Diagnostic Laboratory of the University of Missouri-Columbia between June and October 2004 for necropsy and/or diagnostic examination. These dogs presented to referring veterinarians with clinical signs suspicious of CDV infection. Gross and histologic examination, immunohistochemical and RT-PCR tests were performed on tissues from 14 dogs (10 cases). In one dog (case 17569), only RT-PCR test was performed since only oculonasal swabs were submitted. Further genetic sequences and phylogenetic analysis of the complete H and F, and partial P protein genes were performed in four selected cases (case numbers 18133, 21261, 19876 and 25259).

The phylogenetic analysis of the H and F protein gene sequences demonstrated that cases 18133 and 21261 were almost 100% identical. However, there was no known epidemiologic link between these cases. In addition, cases 18133 and 21261 were potentially related to phocine distemper virus type 2 (PDV-2). The H protein gene sequence for case 25259 suggested a close relationship to a CDV isolate detected in a lesser and giant panda. Unfortunately, the F protein gene sequences for the lesser and giant panda viral isolates were not available to confirm this relationship. Analysis of H, F and P genes sequences did not infer a relationship for CDV detected in case 19876 with other known isolates. These data suggest that this viral strain is genetically divergent from previously characterized CDV isolates.

In this study, phylogenetic analysis of the H protein gene was more robust than phylogenetic analyses of F and P protein gene. F protein gene sequences confirmed the

results obtained in H protein gene sequences. Compared to F and H protein gene sequences, the P protein gene sequences were more conserved between the different viral strains, as previously reported in other studies. Importantly, the histologic lesions and phylogenetic analyses failed to support a reversion of vaccine virus to virulence. The CDV strains detected in the selected cases in this study were distinct from viruses previously detected in USA and most closely match strains from either Asia or Europe.

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