On the pathogenesis and shifting dynamics of canine distemper infection in dogs and wild carnivores

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Table of contents

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Abstract

Canine distemper virus (CDV) is an enveloped, single-stranded negative-sense RNA virus belonging to the genus *Morbillivirus*, family *Paramyxoviridae*. Viral particles are composed of six structural proteins encoded by the six genes that compose the genome of CDV. The hemagglutinin (H) and fusion (F) proteins are the surface glycoproteins responsible for attachment and cell membrane fusion, respectively. The H protein drives host species range and cell tropism. It has a high mutation rate, and it is used to classify CDV isolates into distinct lineages, which are defined by sequence differences of 5% or more. These lineages are commonly restricted to specific regions and are named after the geographical locations where they are described.

The disease resulting from CDV infection, distemper, is one of the most lethal diseases in dogs and a wide range of wild carnivores. Many species, including some endangered species, have had their numbers dramatically reduced due to distemper outbreaks. Distemper is associated with varied symptoms and lesions because of the wide range of systems that CDV can infect. Immunosuppression occurs as a result of CDV replicating and causing damage in the immune tissues. It also causes lesions in the respiratory, digestive, integumentary, and nervous systems. An extensive literature review of the virology, pathogenesis, and epidemiology of CDV is included in this thesis (Chapter 1).

The high impact of distemper on canine health prompted the early development of a vaccine that is included in the typical vaccination series for dogs. It is regarded as highly successful. However, existing vaccines are based on CDV lineages that are no longer found in the wild, and they are significantly different from the currently circulating lineages. The H protein is the main target of the infected host's immune system. As a result, it is subjected to high evolutionary pressure to avoid

immune detection, resulting in the emergence of genetic variations. Moreover, reports of distemper in vaccinated dogs have been increasing during the last decade. All together this information has raised concerns about the current effectiveness of the CDV vaccine. In Chapter 2, we addressed this concern by performing a multi-institutional retrospective case-control study in which we compared cases of vaccine breaks with a control vaccinated population that did not develop distemper. The only clear predictor of vaccine break was the time between vaccination and onset of disease, indicating that animals that do not develop a protective response are quickly infected with the virus.

The impact of the variability of CDV H protein is still not well characterized. To address this gap in knowledge, in Chapter 3 we studied the impact of H variability on the pathogenesis of distemper. We used *in vitro* and *ex vivo* approaches to study how H protein variations can affect CDV attachment and replication. We isolated CDV from tissues of infected animals originating from distinct geographical locations in the USA and sequenced their genomes. Several of the innovative approaches proved to be challenging, but we were able to demonstrate clear differences in tropism among the lineages included in the study, suggesting that H protein can have an important role at least in viral replication efficiency in specific cell types.

Even though distemper is one of the longest-known diseases in dogs, the only available treatment is supportive care. There is a clear need for a specific and effective antiviral treatment for distemper. In chapter 4 we investigated the anti-CDV potential of several compounds with proven effectiveness for other viral diseases. We used multiple circulating lineages of CDV to investigate viral RNA replication and cytopathic effect (CPE) inhibition of the antiviral candidates. One candidate, the nucleoside analog GS-441524, had a strong antiviral effect and reduced both vRNA and CPE, while

some other candidates with a similar mechanism of action were able to reduce vRNA production but not prevent cell death. Some compounds with previously reported anti-CDV effects in vaccine lineages, were not effective in our experiment, proving the importance of using a circulating virus for the development of antiviral compounds.

Chapter 1: Introduction

Viral taxonomy and structure

The species of canine distemper virus (CDV) was officially renamed *Canine morbillivirus* in 2016, by the International Committee on Taxonomy of Viruses (ICTV)[1,2]. The last revision of the ICTV establishes that the complete taxonomy of CDV is: realm *Riboviria*, kingdom Orthornavirae, phylum *Negarnaviricota*, class *Monjiviricetes*, order *Mononegavirales*, family *Paramyxoviridae*, subfamily *Orthoparamyxovirinae*, genus *Morbillivirus*, species C*anine morbillivirus*.[3]

As a paramyxovirus, CDV virions are pleomorphic, but are mostly spherical and range from 150 to 350 nm in diameter.[3,4] Virions have an outer envelope covered by two large, 8- to 14-nm-long glycoprotein spikes. These envelope proteins consist of one receptor binding protein, haemagglutinin (H), and one fusion protein (F). The nucleocapsid inside the envelope has a herringbone shape and is helically symmetrical. It is approximately 1 µm long and 18 nm in diameter. The nucleocapsid is composed of three different proteins: the RNA-binding nucleoprotein (N), a phosphoprotein (P) and a large polymerase protein (L), which contains an RNA-dependent RNA polymerase (RdRp) domain.[4,5] These three proteins interact with the viral genome to form a ribonucleoprotein complex (RNP). A sixth membrane-bound unglycosylated matrix protein (M), interacts with the lipid envelope, the cytoplasmatic tail of the H and F proteins, and the RNP.

Most isolated CDV RNA genomes that have been uploaded to the National Center for Biotechnology Information (NCBI) are between 15,600 and 15,690 in length, approximately the same length as porcine and dolphin morbilliviruses, but around 200 nucleotides shorter than that of other related morbilliviruses, such as measles virus, rinderpest virus and small ruminant morbillivirus.[6,7] As in other paramyxoviruses, N monomers are predicted to be associated with precisely 6 nucleotides[8], therefore the virus can only replicate efficiently if the genome is a multiple of six, and that is the case for most of

the described genomes of CDV ("rule of six").[9,10] The genome is organized into six contiguous non-overlapping transcriptional units which are, starting from the 3' end, N-P-M-F-H-L. The 3' end has a 52-nucleotide long leader region and an untranslated region (UTR) with promoter functions that lie before the N open reading frame (ORF). The opposite site, at the 5' end, has a 37 trailer region and a UTR at the end of the L ORF, and their complementary sequences serve a similar function in the antigenome.[11]

The H protein is composed of 607 amino acid residues.[12] It is a type II membrane protein and consists of an N-terminal cytoplasmic tail (residues 1-37), a short transmembrane region (residues 37-58), a stalk region (residues 58-149), and a C-terminal head domain (residues 149-604, ~60kDa).[13] The closely related measles virus has six β-sheets that are arranged in a six-bladed propeller fold to form the H protein's head domain.[14] The H protein forms homodimers by disulfide bonds in the stalk region, and they further associate into tetramers.[15,16] Cysteine residues in positions 139 and 154 of the measles virus H protein are involved in the homodimer formation in a reciprocal and sufficient manner.[16] The H protein is the primary target of neutralizing antibodies,[17] and in response to this selection pressure, has the highest degree of genetic variability. The H gene of CDV, has the highest variability among all morbilliviruses. One publication claims that the number of nucleotide substitutions per site, per year is of 11.65×10^{-4} (range: $5.4–18.1 \times 10^{-4}$), even higher than that of mumps and measles.[18] Other authors have compared all the CDV genes and, despite calculating a smaller rate of 3.3×10^{-4} (range: $2.94–3.65 \times 10^{-4}$), it is still higher than the rest of the genes.[19]

The F protein is a type I membrane protein with a cytoplasmatic tail at the C-terminus and a very hydrophobic external N-terminus, also known as the fusion peptide.[4,20,21] The F protein has a short stalk region supporting a large globular head domain. This globular head has been subdivided into three subdomains (DI-III). The DI and DII subdomains form the base of the head and remain similar in the post-fusion state, while DIII changes drastically.[21] The inactive precursor form of the F protein (F0) is cleaved by cellular proteases (furins) in the Golgi apparatus to create the mature form, which is composed of

two subunits (F1 and F2) linked by disulfide bonds.[22] If the host cell lacks the specific protease, it will produce viral particles with uncleaved F0, making them non-infectious. The F protein forms homotrimers, which are probably formed in the endoplasmic reticulum (ER), where F/H hetero-oligomers are thought to occur as well.[21] The fusion peptide contained in F1 is hidden between two subunits of the trimer to control its fusion capabilities which can occur at neutral pH, in contrast to other viruses which require the low pH of an endosome.[4,20] When activated by signals derived from the H protein, conformational changes in the F protein lead to refolding, and it is believed that the fusion peptide (anchored to the host cell membrane) and the transmembrane domain (embedded in the viral envelope) can then physically interact, disturbing the lipid monolayers and facilitating spontaneous membrane merging.[21]

The M protein plays a crucial role in viral replication and particle formation. There is currently no complete structural information about morbillivirus M proteins, but crystallographic data of M proteins from other paramyxoviruses indicates high structural conservation.[23–26] The M protein is small (~40kDa), basic, and moderately hydrophobic, and it seems to be organized in subunits consisting of dimers of the M polypeptide.[27,28] Multimerization of these dimers at the inner surface of the host cell plasma membrane is thought to initiate the budding process and provide the membrane curvature.[25,29,30] It is generally thought that the M protein coats the inner surface of the viral envelope, although it appears to be able to assume alternative distribution, such as helices coating the RNP complex.[31] The M protein regulates the assembly and budding of viral particles. It also regulates viral RNA synthesis, and it is thought to be involved in many other processes. The expression of the M protein alone is enough for the formation of virus-like particles (VLP), emphasizing its importance for the completion of the viral cycle.[26,28,32,33] The mechanism by which it helps assemble viral particles is not completely elucidated, but it is believed that it interacts with the cytoplasmatic tails of the H and F proteins, as well as the C-terminal end of the N protein.[34–37] Moreover, the M protein involves the actin cytoskeleton in the

process of RNP transportation and budding, and this is seen not only in CDV, but in many other viruses as well.[37–39] In measles virus, matrix-less recombinants have enhanced fusion activity, leading some people to believe that the M protein itself downregulates cell-to-cell fusion and that M-defective mutants may have enhanced propagation in brains and consequent neuropathogenesis.[40,41]

The nucleoproteins of paramyxoviruses are made of a well conserved folded domain (N_{core}) followed by a long, variable, and intrinsically disordered tail (N_{tail}). The N_{core} forms the main body of the nucleocapsid, and it is involved in its oligomerization. It can be further subdivided in N-terminal and C-terminal domains, which form a cavity of positive charge that allows RNA interaction.[42] The N protein secondary and tertiary structures are highly conserved among paramyxoviruses and even other families of the *Mononegavirales* order[42]. They tend to form rod-like helical structures with herringbone appearance[43,44] that are stable even without the presence of the encapsidated RNA.[45] However, this structure is flexible and the protein's pitch and the number of subunits per turn can vary even within the same protein.[46,47] The expression of N protein alone in mammalian cells promotes the formation of nucleocapsid-like complexes (NCLC) that encapsulate cellular RNA in a variety of structural shapes.[42,48] For measles virus and CMV, the structure of the N protein interacting with RNA[47,49,50] and also with P protein has been solved.[51] The viral genome is embedded in a homopolymer of viral nucleoprotein, forming a structure known as the nucleocapsid. This prevents the formation of double-stranded RNA and the misfolding of single stranded RNA into immunologic active secondary structures that can be targeted by nucleases and siRNA.[42] The nucleocapsid N_{tail} aids in recruitment of RdRp into the viral genome.[42]

The L protein is a large protein with five globular domains linked by flexible segments. It interacts with N and P proteins as part of the nucleocapsid. It transcribes and replicates the viral genome, and carries out all the enzymatic activities needed for this purpose, including RNA synthesis, mRNA cap addition, guanine-N-7-methylation of the cap, and d ribose 2'O-methylation of the first nucleotide.[42,52,53]

The phosphoprotein (P), the last component of the nucleocapsid, is poorly conserved across the *Paramyxoviridae* family, but it is organized in three modules linked by long disordered regions. The N-terminal domain interacts with monomeric N protein and forms complexes that prevent self-assembly of nucleoproteins on RNAs that are not of viral origin.[54–56] The function of the oligomerization domain, which is consecutive to the N-terminal domain, is not well understood. However, it contains phosphorylation sites that might downregulate or upregulate RNA synthesis,[57–63] and it has other functions related to RNA replication and N-P interaction.[42] This domain forms tetramers in most paramyxoviruses, including measles virus.[64] The C-terminal domain recruits the polymerase to the nucleocapsid[65–67]. The P protein is considered a cofactor of L protein and has several functions, including stabilization of L protein[68–70], recruitment to the template, and stabilization of N proteins for encapsidation of nascent RNA.[55,56,71,72]

Canine distemper virus also produces two nonstructural proteins encoded in the P gene: C and V-proteins. Their roles are mainly focused on the modulation of viral replication and the host immune system. The small C protein is a result of a different reading frame from the P gene, while the V protein is a result of mRNA editing: the addition of an extra G nucleotide (site-specific stuttering) results in a different amino acid sequence downstreamx the insertion, but a homolog N-terminal region.[4] Although dispensable for viral growth in cell culture, they are proven to be essential in host infectivity.[73] The V protein is known to prevent STAT 1 and STAT 2 activation and nuclear translocation, effectively blocking the type I IFN signaling pathway.[74,75] In measles, V protein also blocks the innate immune response pathways that lead to the production of type I IFN and other cytokines, including the inhibition of IRF3 and IRF7 transcription[76] (for a more complete explanation, read Pfeffermann *et al*., 2018[77]). The C protein of measles virus blocks also IFN-β transcription[78] and dimerization of STAT[79].

Infection cycle

The infection cycle of CDV starts when the hemagglutinin of the viral particle adheres to viral receptors in a susceptible cell. The viral receptors described so far are signaling lymphocytic activation molecule (SLAM), an adhesion molecule present in the surface of macrophages, dendritic cells T and B lymphocytes; and Nectin-4, present in epithelial cells.[80] Other molecules have been also speculated to act as receptors for CDV.[80] For example, the cellular receptor involved in the infection of neurons and glial cells remains to be identified.[4,81,82]

The signaling lymphocytic activation molecule is a glycosylated transmembrane protein member of the immunoglobulin superfamily, that interacts with other SLAM on an adjacent cell.[83,84] It was first recognized as a receptor for measles virus by Tatsuo *et al*. in 2000,[85] and for other morbilliviruses including CDV the year after.[86] It is an important costimulatory molecule in T- and B-cell activation. Thus, mere binding of measles virus particles or envelope proteins to SLAM on the cell surface might affect the signals induced through SLAM, thereby impairing lymphocyte activation.[87] The ectodomain of SLAM contains a membrane-distal immunoglobulin variable (IgV) domain and a membrane-proximal truncated immunoglobulin constant-2 (IgC2) domain. The cytoplasmic domain contains at least two immunoreceptor tyrosine-based switch motifs.[88] All morbilliviruses bind to the V domain of SLAM.[89] Twenty-one amino acid residues in the IgV domains of the SLAM bind the CDV-H. Homology of this region has been shown to allow infection across multiple host species.[90]

The nectin family of proteins promotes cell-cell adhesion. In particular, Nectin-4, together with cadherins, participates in the formation and maintenance of adherens junctions and tight junctions of epithelial cells and promotes apical-basal polarity.[91,92] It was described as a receptor for measles virus in 2011 by two different groups[93,94] and for CDV a year after.[95] All nectin proteins contain an extracellular domain with three Ig-like loops (V and two C2-type domains), a transmembrane domain, and a

cytoplasmatic domain that binds afadin, an actin filament-binding protein.[96] Nectin-4 forms *cis*-dimers with molecules of the same plasma membrane.[89] Morbilliviruses bind to the V domain.[97]

Once adhesion has occurred, the interaction of the hemagglutinin with a specific receptor triggers conformational changes in the H stalk domain which themselves cause conformational changes in the fusion protein.[98–102] These changes bring into close contact and disturb the cell's plasma membrane and the viral envelope, resulting in the merge of both lipid layers and the release of the RNP into the cytoplasm of the infected cell.[21,103]

The viral RNA-dependent RNA polymerase (RdRp) is composed of the L and P proteins, and it is contained in the viral particle. It is necessary and sufficient to start transcription of the negative sense viral genome. The P protein is responsible for recruiting the RdRp on the nucleocapsid by binding the N_{tail} of the nucleoprotein. The 3' end is released by a series of conformational changes and interactions between P and N proteins, which allows the transcription of the genome.[42] The permanent association of the RNP with RNA might be the reason for a very low recombinant rate because RNA polymerase cannot jump to different templates of RNA.[104] After each gene there is a conserved polyadenylation termination signal that interrupts the synthesis of mRNA.[105,106] The RdRp can then detach from the genome template completely, or it can recognize the starting signal from the downstream gene. As a result, a gradient of (+)mRNA abundance correlating with proximity to the 3' end of the viral genome is created. [42,107,108] Therefore, the most abundant mRNA corresponds to the N gene (nucleoprotein) and the least abundant corresponds the L gene (polymerase). The (+)mRNA is 5' capped and 3' polyadenylated, and it is transcribed by the host ribosomes into viral proteins.[105,106] When a certain threshold of N protein is created, they bind to the nascent RNA chain, prompting the RdRp to ignore the message-termination signals. A full-length, N-encapsidated (+)cRNA is produced, which will be used as a template for producing the progeny viral genomes. [4,109] Some conflicting information suggests that the process may be more complex. For instance, replication is not increased relative to transcription when

N protein is increased in some but not all models.[110,111] New models propose the existence of different forms of RdRp, to either transcribe or replicate the viral genome. The appearance of these forms would depend on the interaction of not only the N protein with RdRp, but also L, P, and possibly even host factors.[11,112,113] In any case, the production of new negative sense genomic RNA will result in an exponential increase in synthesis of mRNA and the accumulation of RNP structures, which form the characteristic acidophilic cytoplasmic inclusions (for a review, see[114,115]).

Synthetized viral surface glycoproteins (H and F) are modified post-translationally (including proteolytic cleavage of F0 and glycosylation of both proteins) in the endoplasmic reticulum and the Golgi apparatus and are transported to the surface of the cell. They are not randomly distributed but are concentrated in microdomains on the cell plasma membrane, which are rich in sphingolipids and cholesterol (membrane rafts).[116,117] They also concentrate at the apical surface of epithelial cells due to the influence of the M protein, which reverts the intrinsic basolateral distribution of H and F.[35] Finally, the M protein plays a crucial role in viral assembly by mediating the association of the cytoplasmatic RNPs with the cell membrane viral glycoproteins H and F, which will result in the budding of the mature viral particles.[41,118,119] These free particles will find new cells to infect and start the cycle again. Alternatively, viral infection can be maintained in environments with low density of cell receptors by direct cell-to-cell spread as a result of the fusogenic capabilities of the fusion protein and the formation of cell-to-cell syncytia. This has been proven to be an important factor of viral spread in both CDV and measles virus when certain strains or genotypes, with decreased ability to produce cell-free particles, have been associated with increased neurovirulence and cases of canine demyelinating leukoencephalitis and subacute sclerosing panencephalitis, respectively.[120–122]

Pathogenesis

The pathogenesis of any viral disease is at least partially determined by the cells the virus is capable of infecting. For a successful viral infection, cells must be accessible, susceptible, and permissive. Susceptible cells express receptors on the cell surface that allow entry of the virus, while permissive cells can support viral replication.[123]

Canine distemper virus causes similar pathogenesis in all susceptible species, but we focus on the disease in domestic dogs to simplify the discussion and because they represent the most thoroughly studied species. As discussed previously, CDV is an immunotropic and epitheliotropic virus. This tropism is defined by the distribution of specific protein receptors. The two better characterized receptors, SLAM and Nectin-4 are key to determine the progression of the disease. SLAM is present in activated lymphocytes (B and T cells), macrophages and dendritic cell subsets. Its role is mostly centered on immune cell signaling, but it also participates in the innate immune response against Gram-negative bacteria.[124] Nectin-4 is distributed in the basolateral domain of epithelial cells, mostly in mucosal surfaces of the respiratory and digestive tract. It is important for cell adhesion and forms part of the adherens junctions, which attach polarized epithelial cells.[72]

While in the environment, the highly infectious CDV particles are aerosolized or persist in contaminating fomites for extended periods of time. The main path of infection is through inhalation of contaminated droplets, however, the possible role of arthropod vectors (e.g. fleas) is uncertain and needs further investigations.[125] Inhalation leads to infection of SLAM-expressing cells (macrophages and dendritic cells) in the respiratory tract. Epithelial cells cannot be initially infected since the expression of Nectin-4 is restricted to the inaccessible basolateral domain in these polarized cells. Cells infected by CDV then migrate to local lymph nodes, where the virus replicates extensively in resident B and T cells. These cells spread from the lymphatic tissues into the blood resulting in the initial viremia, the first phase of CDV infection. The viremia and systemic spread allows the virus to reach other lymphoid tissues and the

basolateral surface of mucosal epithelial cells of the host, where a new phase of infection will result in a second viremia. Both viremic stages are associated with clinical fever. Nectin-4 positive cells will become infected, including those of lungs, bladder, intestines, and skin. Replication in these cell types not only correlates with the majority of the clinical signs of infection but also allows the release of viral particles in the lumen of these organs, favoring transmission to new hosts. [4,72] This biphasic life cycle has been confirmed with pathological observations at different times post-infection: CDV can be rapidly detected in mucosa-associated lymphoid tissues (MALT) without epithelial lesions.[72]

Therefore, we can conclude that SLAM is responsible for the spread of the virus in the immune system after initial infection and immunosuppression while Nectin-4 is the cause of the development of clinical signs and allows the virus to be shed outside the host.

The central nervous system (CNS) presentation may occur during the acute phase of the disease or it could occur several weeks to months later.[126] Neuroinvasion of CDV occurs predominantly via the hematogenous route[127], and its spread within the brain can be tracked through the cerebrospinal fluid (CSF), affecting the ependymal and subependymal white matter.[128] Spread to other sections of the CNS (olfactory nerve, meninges) has also been the topic of research.[129] Astrocytes seem to be a preferential site of replication for some demyelinating strains[130], and these CDV strains replicate in the astrocytes in a non-cytolytic fashion with little viral production.[122] This has led to speculation about a possible host-cellular factor that promotes non-cytolytic, astrocyte-to-astrocyte transmission during neuroinvasion,[82,131] although the presence of Nectin-4 has also been reported in the brain.[95]

Lesions associated with infection are easy to identify in the distinct organs. Intranuclear and intracytoplasmic acidophilic inclusions are common in astrocytes, and epithelial cells of the lung, stomach, renal pelvis, and urinary bladder.[4,132]

In immune tissues, such as the spleen, thymus, and lymph nodes, there is marked necrosis and depletion of lymphocytes. Thymic atrophy derived from the loss of both cortical and medullar thymocytes with great reduction of the medulla is particularly common. Lymph nodes can display syncytial cells, which also commonly contain inclusion bodies. Hyperplasia of histiocytic cells can develop later in the infection, but the repopulation of the node by lymphocytes will not be complete for weeks or months.[132]

Infection of epithelial tissues will result in the classical clinical signs of distemper that result from the development of pneumonia, enteritis, tracheitis, conjunctivitis, and rhinitis. Fused epithelial cells form syncytia that can often be observed in lesions, in addition to the characteristic intracytoplasmic and intranuclear inclusion bodies.[72]

In the respiratory tract, serous, catarrhal, or mucopurulent exudate covers the nasopharynx. Histological lesions of distemper include pulmonary edema, broncho interstitial pneumonia with epithelial (bronchiolar) necrosis and thickening of alveolar walls that contain protein-rich edema fluid. Immunosuppression, and disruption of physiological protective functions of the lung commonly result in secondary bacterial infection of the lungs.[4,72,132] In large felids (genus Panthera) diffuse hyperplasia of type 2 pneumocytes is common and they typically exhibit strong immunohistochemical staining for CDV.[133] Various epithelial cells of the gastrointestinal system may develop inclusion bodies, mild degenerative changes, and mononuclear cell infiltrates. The cell types affected vary and may include the gastric surface epithelium, chief and parietal cells of the stomach, pancreatic ductular epithelium, epididymis, and testis.[132] Liver is the second most common tissue to contain histologic lesions suggestive of CDV infection. These lesions are often limited to the periportal areas with bile ducts containing prominent intra-cytoplasmic, eosinophilic, viral, inclusion bodies.[134]

Neural disease, albeit rare, is more frequent in CDV infection than in disease caused by other morbilliviruses.[135] Dogs may have prolonged encephalitis in dogs, while cats have milder lesions.

Different CDV strains vary in their neurovirulence and tropism, resulting in differential expression of the symptoms (i.e. A75 causes focal demyelinating disease[131]). Central nervous system lesions are variable, and include a combination of demyelination, neuronal necrosis, gliosis, and nonsuppurative meningo-encephalomyelitis in dogs[4], while cats have milder lesions that lack the demyelination pattern.[133]

Both white and grey matter may have lesions, although they differ histologically. Polioencephalitis is a rare manifestation of CDV infection and is predominantly located in the cerebellum and nuclei of the brain stem.[129] Affected neurons undergo necrosis, followed by mild nonsuppurative meningitis.[136] Demyelination prevails in the white matter tracts, with intranuclear inclusion bodies in astrocytes.[129,137,138] Early axonal damage and demyelination are the initiators of CDV leukoencephalitis, that progresses into trophic sclerosis of the white matter. The leukoencephalitis derived from CDV infection is a complex process which involves degeneration and possible regeneration of many cell types in the central and in the peripheral nervous system.[139] Autophagy is part of the pathogenic process, as microtubule-associated protein 1 light chain 3 (LC3), a marker related to autophagy, can be detected extensively in the white matter of infected cerebella. The localization of LC3 is particularly evident in the neurons and gemistocytic astrocytes.[140] In more chronic processes, infection will progress to gliosis and axonal degeneration with mononuclear cell infiltration.

Old dog encephalitis is a rare manifestation of CDV infection, possibly caused by persistent infection with a replication-defective virus. The lesion distribution is different than that of distemper encephalomyelitis, as the cerebral cortex is consistently affected. The lesions consist of perivascular infiltrates of lymphocytes and plasma cells, and intranuclear inclusions in astrocytes and neurons.[136,141,142] Administration of modified live CDV vaccines may cause canine distemper, with neuronal necrosis, intranuclear inclusions, and lymphocytic encephalitis.[136]

Ocular lesions are predominantly degenerative, but they also include conjunctivitis, keratitis, retinitis, and optic neuritis. Conjunctivitis is common in the early stages of the disease and can progress into ulcerative keratitis. Retinal lesions are more prevalent during systemic infection, and such lesions include degeneration of ganglion cells and retinal edema that can progress to neuronal loss and retinal scarring. Lesions in the optic nerve are not frequent and mimic those found in the rest of the nervous system, such as nonsuppurative demyelinating neuritis and the presence o eosinophilic inclusion bodies in ganglion cells or astrocytes.[143–145]

Two classical lesions of distemper in puppies are metaphyseal osteosclerosis in long bones and enamel hypoplasia (odonto-dystrophy).[4] The latter is a result of necrosis and cystic degeneration of ameloblasts in developing teeth.[132]

Finally, hyperkeratosis and parakeratosis may affect the footpad and nose, and rarely the haired skin with the presence of syncytial cells and inclusion bodies. The epidermis may contain syncytial cells and nuclear and cytoplasmic inclusion bodies.[132]

Immune reaction

Vaccination or infection induces both humoral[146] and cellular[147] immune responses. In the case of humoral immunity, a robust neutralizing antibody response is developed especially against F[148] and H[149] proteins. Morbilliviruses induce cross-protection, and measles vaccination provides protection against CDV infection.[150]

Cell-mediated immune responses are critical for overcoming morbillivirus infection and are more important than humoral immune responses. However, neutralizing antibody titers can be used to indicate a defensive response and long-term protection.[4,151] During active infection, upregulation of innate immune system cytokines[152] and production of specific cytotoxic lymphocytes[147] are associated with the survival of the infected host. In puppies, maternal antibodies can offer a certain level of protection but wane after 4 weeks of age. At that time, puppies become susceptible to CDV infection and vaccination is recommended.[153]

The most immunodominant epitope of CDV is predicted to be located between aa residues 364 and 392 of CDV-H protein.[154,155] The equivalent epitope in measles virus is known as the hemagglutinating and noose epitope (HNE) because the disulfide bond between three cysteines exposes a loop.[156] Monoclonal antibodies against HNE of measles virus block binding of the virus to the receptor (neutralization) and prevent infection.[17] Therefore, substitutions in the CDV equivalent HNE could alter the ability of the vaccine to provide adequate protection against infection.[157]

Clinical disease

Distemper is one of the most lethal infectious diseases of dogs. Mortality rates up to 50% have been recorded.[158] Clinical presentation of distemper varies through the infection phases and corresponds to the anatomic locations of viral replication. After 3 to 6 days of incubation, infected animals develop the first fever with depression and anorexia, corresponding to the first viremic phase. At this point, the disease can further progress, or it may resolve. The outcome of the infection depends on a number of factors, including the immune status of the host, amount of circulating antibody, host age, and the strain of the virus. If an adequate immune response is developed, infected dogs can clear the infection as soon as day 14 and may not even shed any virus. Puppies at 12 to 16 weeks of age are at highest risk of developing clinical disease due to waning passive immunity.[4,132]

In other cases, viremia causes systemic infection of mucosal epithelium, and virus is shed from the epithelium into respiratory secretions, feces, and urine. Infection of these tissues causes mucopurulent nasal discharge and conjunctivitis. Dogs can either develop lower respiratory signs, which are a reflection of bronchitis and interstitial pneumonia, or gastrointestinal signs which include vomiting and watery diarrhea due to infection of the intestinal cryptal epithelium.[159] Other manifestations of the disease include pustular and hyperkeratotic cutaneous lesions, dental defects, and abortion. Duration of the signs is variable and depends on complications due to secondary bacterial infections derived from the CDV-induced immunosuppression, which are sometimes the main cause of clinical symptoms. Among other agents, common agents in complicated distemper cases include *Bordetella*, *Pneumocystis, Toxoplasma,* and *Clostridium piliforme*.[4,132]

Neurological manifestations can appear 1 to 4 weeks after recovery from the disease or be the primary clinical manifestation. They depend on the anatomic location of the lesions and include seizures, cerebellar or vestibular ataxia, paraparesis or paraplegia, and myoclonus, as a result of axonopathy,

inflammation, and extensive demyelination.[4,72,132,139] Infected animals commonly develop abnormal behavior which can be reminiscent of the furious form of rabies. As described above dogs can, on rare occasions, develop old dog encephalitis long after the resolution of acute infection, similar to sub-sclerosing pan-encephalitis seen in humans during chronic measles virus infection. [72] These animals may develop circling, swaying, and weaving. old dog encephalitis will last 3-4 months and typically progresses into coma or death. Post-vaccinal canine distemper encephalitis is an acute clinical course that appears a few days after vaccinating with attenuated vaccines and can cause aggressive behavior. The causes leading to the development of post-vaccinal distemper are not well understood, but the lesions are restricted to the CNS, but sparing the white matter.[136]

Since it is derived from similar pathogenic pathways, clinical disease is similar across species, at least within individual outbreaks. Some examples are represented by the parallels in disease of raccoons and gray foxes[160], and of minks and sympatric mesocarnivores.[161]

Diagnosis

The characteristic clinical signs are helpful in making an antemortem diagnosis of distemper in dogs, particularly in non-vaccinated dogs. Inclusion bodies of CDV can be seen in the cytoplasm of lymphocytes in blood smears, but only during the acute phase of infection. Central spinal fluid (CSF) alterations include increased protein (0.25 mg/dl) and an increased cell count (0.10 cells/ml) with a predominance of lymphocytes. The presence of anti-CDV IgG is evidence of neurologic dissemination of CDV since it is not present in vaccinated dogs.[162,163] For years, multiple enzyme-linked immunosorbent assays (ELISA) have been used to detect serum IgG and IgM antibodies to CDV[164–166] and CDV antigen.[166,167] An IgM response indicates a recent infection or vaccination within 3 weeks of the test, while IgG detection can be a result of both infection or vaccination. Cytologic smears from conjunctiva, tonsil, genitalia or respiratory epithelium can be used for immunofluorescence detection of CDV with

good success. Immunocytology can also be performed on blood smears, buffy coat smears, CSF, skin, and foot pads. The virus can persistence in skin and the epithelium of foot pads, for up to 60 days.[162,168]

Findings of the characteristic postmortem gross and histological lesions and the presence of inclusion bodies described above are used to confirm infection. Samples collected for diagnosis in suspected cases should include at least lung, liver, lymph nodes, brain, and spleen. Immunohistochemistry is commonly used as the definitive tool for CDV diagnosis and strong staining can be observed in commonly affected tissues.[136,162,169–172]

The best way to identify CDV infection is to isolate live virus, viral RNA, viral antigen, or host-specific antibodies. Viral isolation can be done in cells expressing SLAM or Nectin-4. There are many established RT-PCR or RT-qPCR processes to detect viral RNA[173–176] in clinical samples such as whole blood, conjunctival swabs, nasal swabs, CSF, or urine. Urine is a particularly interesting sample, where CDV can be detected for longer than in other samples.[177] Viral antigens can be detected by agar gel immunodiffusion or immunocapture ELISA.

Serological assays are commonly used for epidemiological monitoring of CDV, especially in wildlife populations. It appears to be mono-serotypic, facilitating this kind of surveillance.[127,178–187] Various established tests are available including virus neutralization tests, indirect ELISAs, and competitive ELISAs, all of which target immune responses to the F, H, or N protein. Additionally, sequencing of different CDV isolates, often targeting the N, F, or H genes, is commonly used for phylogenetic characterization and tracking of outbreaks.[72,188–194]

Epidemiology

History

The first morbillivirus to emerge was rinderpest virus (RPV) which appeared together with the domestication of cattle in the first millennium BC, probably in Asia.[195] Rinderpest virus, or its ancestor, is thought to be the source of measles virus. This host switching may have occurred as early as the 9th century AD according to written records,[195] although modern analysis using estimates of evolutionary rate place the divergence between rinderpest virus and measles virus around the 11th to 12th centuries.[196] Measles virus caused devastating epidemics in the 16th century in North and South America. On the opposite hand, CDV is thought to have originated in the Americas, before spreading to Europe, Asia, and Africa due to colonization.[195] It was first reported in Europe in the second half of the 1700s, with hundreds of dogs dying in a single day.[197] Henri Carré identified its etiology as a filterable agent in 1905 and the English developed a vaccine in the early 1930s, introducing the ferret as a model for respiratory viruses.[198] Canine distemper virus still circulates globally, and infects many different species.[199]

Variation of CDV

Because it is an RNA virus, CDV mutations are common, especially in the H protein, leading to a high diversity of strains.[200,201] Even for a morbillivirus, CDV has increased plasticity: while measles virus cannot tolerate much genetic change in regions that interact with the receptor, CDV can compensate for deleterious mutations with additional changes in other regions that stabilize CDV.[202] There are many more complete H gene sequences than any other CDV gene sequences available in public databases for phylogenetic comparisons. Therefore, the H protein is commonly used to determine the phylogenetic relationship between different CDV isolates and propose new genotypes, which are commonly referred as lineages.

The condition for lineage assignment states that two strains belong to the same lineage if they cluster together in the phylogenetic tree, and show an H amino acid divergence of less than 4%.[203] Conversely, the common criterion for the classification of CDV lineages with the H-gene sequence is that a new strain should be considered a new lineage if it has more than 5% nucleotide difference or 4% amino acid difference from other lineages.[203–205] At least 12 well-defined CDV lineages have been identified worldwide. They are identified as America-1 (vaccine strains), America-2, America-3, America-4, Europe 1/South America-1, Europe 2/Europe-wildlife, Europe 3/Arctic-like, Asia-1, Asia-2, South Africa, South America-2 and South America-3.[189,206] However multiple recent studies describe CDV isolates diverse enough to be considered their own lineages. This would increase the number to 19, resulting in the additions of proposed America-5[189], Canada-1[173], Asia-3, Asia-4[177], Asia-5[207], Africa-1, and Africa-2.[208] There are probably even more around the world since new studies are continuously being published that identify sequences that could justify new additions to the list.[209] In the USA, raccoons are the likely source of these ‘‘new’’ genetic lineages.[210]

Most of the lineages are defined by the geographical location where they were first described, but different lineages of the virus can occur simultaneously in the same geographic areas.[200,211,212] The same strains may circulate in wildlife and dogs in the same location, but there is more variation in wildlife than in domestic dogs.[210] Analysis of the H gene sequences revealed that there are at least three main lineages circulating in the US: America-3, America-4, and America-5. None of these lineages show a close genetic relationship to the most commonly used CDV vaccine strain, the Onderstepoort strain, which belongs to the America-1 lineage.[189,213] America-1 has not been recently documented in the USA, but it was found in a dog in China in 2008 and in a seal in the Caspian sea in 2007.[189] America-2 and America-3 have both been detected in Colorado.[211] Two additional distinct and yet unnamed lineages have been identified circulating in wildlife in the Northeast and Midwest regions.[210,214] Asia-1 was found in dogs in the northeastern USA. These dogs were imported from South Korea, so it is likely that the origin of the

infection was in Asia and Asia-1 was probably not circulating in the USA at the time.[189] In the early 21st century, the European wildlife strain was regarded as the only virulent variant in the USA.[215] This strain caused outbreaks in wildlife and it was also found in dogs from California, Missouri, and Oklahoma.[213] However, the European wildlife lineage circulating in the USA might be different from the one circulating in Europe. [216] In Missouri the Arctic lineage was also present.[213]

In South America, Europe-1 was the predominant circulating lineage (prompting the suggestion of renaming it Europe/South America-1) until the recent identification of new strains in wildlife (South America-2, in Argentina) and domestic dogs (South America-3, in Colombia).[72,206,217]However, Europe/South America-1 is still commonly associated with outbreaks of distemper in South America.[218] In Europe, CDV strains from the Artic lineage are currently circulating in Italy. This lineage affects wolf populations in particular, and contributed to a spill-over event in badgers.[219,220] In Italy, the Artic lineage cocirculates with the Europe wildlife lineage, which is also found in other regions of southern[219,221,222] and central[223] Europe. Multiple lineages appear to circulate in Asia, all of them belonging to the Asia group. An epidemic of wild mammals in Japan during 2007 and 2008 was identified as part of the Asia-1 lineage,[224,225] while outbreaks in China seem to be a combination of Asia-1 and Asia-2.[226,227]

Variations in the H protein can have an effect in both spread and presentation of the disease, but not all loci have the same impact in protein-receptor interaction or vary at the same rate. Amino acid sites 519, 530, and 549, all located within the SLAM binding region of the CDV H-protein, are under positive selection.[228,229] Differences in residues at these sites have been associated with an adaptation of CDV to non-domestic dog hosts, as is shown by the amino acid substitution of Tyrosine (Y) with Histidine (H) at site 549.[75,189,228] Substitutions of either glycine (G) or glutamate (E) to arginine (R), aspartate (D) or asparagine (N) in position 530 are also involved in the interspecies transmission from domestic dogs to non-dog hosts.[228,230] In a study of the non-canid CDV strains from the Serengeti outbreak of 1993 to 1994, the substitution of the arginine (R) in position 519 with isoleucine (I) was associated with clinical

disease in hyena (*Crocuta crocuta*) and lion (*Panther leo*). Moreover, the combination of 519I and 549H was not found in the canid strain and related to fatal outcomes in the two species it affected. The original 519R, regardless of the residue in position 549 (Y or H), would cause death in both wild and domestic canids.[229] For a more comprehensive review on the impacts of amino acid substitutions in the H-protein, see Duque-Valencia *et al*. 2019.[231] A summary of these and other impacts of reported substitutions can be found in the table below.

Mutation	**Species**	**Proposed effect**	**Source**
R519I + Y549H	Hyena and lion	Clinical disease/asymptomatic	[229]
Y549H	Dog	Statistically significant increase in lethality	[191]
519R+549H/549Y	Canids and hyena	Fatal disease in canids, asymptomatic hyaena	[229]
530 R/D/N	Carnivores	Related to transmission from canid to non-canids	[228]
519R, 530N and 549Y	Dog	Fatal outcome of nervous and respiratory disease	[206]
530K	Carnivores and primates	Infects human SLAM-expressing cells and marmoset cells.	[232]
549H	European badger	Naturally occurring in wildlife	[219]
M548T and D540G	Humans	Adaptation to human cell lines	[233,234]

The changes in CDV H sequences are not just important for lineage classification. Since they are closely related to host cell binding, amino acid changes can lead to changes in pathogenicity[235] or even outbreaks in populations where the disease was under control.[236]

Dynamics of CDV in wild species:

Canine distemper virus, like any other infectious agent, is maintained in the ecosystem by either persistence in the environment or by infecting reservoir hosts. Because it is an enveloped virus, CDV is not very environmentally persistent, but if the weather is cold enough, CDV might be preserved outside the host or in carcasses for a longer period of time.[237,238] Reservoirs are ecological systems in which an infectious agent survives indefinitely.[239] They are composed of one or more epidemiologically connected populations in which the pathogen can be permanently maintained and from which infection is transmitted to the defined target population.[240] Existence of a reservoir is confirmed when infection within the target population cannot be sustained after all transmission between target and nontarget populations has been eliminated.[240] Reservoirs are normally not the population suffering most from the disease. In the case of CDV, a sympatric carnivore species would typically be the reservoir.[241]

It is known that measles[242,243] and phocine distemper[244] require large populations of susceptible hosts for their persistence and cannot be maintained in smaller populations because they are either extirpated or develop a total resistance to the infection. This is also presumed to be the situation with CDV infections.[244,245] In the case of measles the estimated minimum human population size for persistence of the virus is between 250,000 and 500,000.[242,246,247] However, CDV has been seen to persist in small and patchy populations of carnivores.[248] The higher turnover rate of carnivores compared to humans may allow for the persistence of CDV at lower populations, but model estimates still suggest that 50,000 to 100,000 susceptible carnivores would be required for even a fifty percent chance of persisting for a period of 10 years. This is unlikely considering the density of many carnivore populations.[248] The specifics of population size thresholds and other determinants of CDV persistence are still not well understood.

CDV can circulate in wildlife in a cycle independent of domestic dogs within complex reservoir systems, which makes it even harder to determine the total susceptible population.[248–250] The existence of these reservoirs is one of the main traits threatening the conservation of species because they promote the maintenance of CDV in the ecosystem, whereas a single affected species would not be able to do so.[241,251] Mesocarnivores are often designated as the reservoirs for CDV because their population dynamics and susceptibility to infection allow maintenance of CDV.[237] The interaction between these mesocarnivores and other larger carnivores through predation, makes it feasible to be a route of transmission. Intra-species contact can also help transmit the disease. Domestic dogs can also be a relevant reservoir in areas where they are not routinely vaccinated.[252]

CDV is often associated with suburban areas, probably because they attract raccoons for easy feeding opportunities, which leads to higher densities of raccoon population than in urban and rural environments. They also represent an interphase between rural and urban environment, which can be associated with higher rates of disease circulation.[253–255] Diagnostic of CDV is also more frequent during breeding season, which can be due to the increased contact between individuals, promoting the aerosol-based spread of the virus.[255]

Interspecies transmission can easily be confirmed with molecular analysis of CDV isolates from cases associated with distemper outbreaks.[219,254,256] Genetic characterization of virus variants indicates that they easily jump between different species.[257] For example, in central Europe CDV is one of the most frequently diagnosed infections in free-ranging mustelids, which coincides with circulation of the disease in red foxes and badgers.[172,256,258] Although the same lineage may be expected during a particular epizootic event among different species, it is also true that different lineages can be cocirculating simultaneously in the same geographical region.[200,211,212] Different populations of the same species have shown different susceptibility levels to the disease, probably due to population dynamics and

environmental factors.[259,260] However, some authors claim that this differential strain susceptibility could be due to variations in virulence of the different CDV strains or to co-infections.[237]

The increased number of outbreaks in wildlife, also represents a threat for dogs to contract the disease. It stands to reason that the risk of CDV infection in domestic dogs will be greatest in areas with increased access to wildlife species that are known reservoirs of the virus (e.g. raccoons), as was demonstrated in Tennessee.[157,261]

High-density dog populations can be the most likely source of infection for wildlife,[179] although this is not always the case, and some wildlife populations show traits of persistent infection and maintain the disease independently of the dog population.[262] This great variation in the persistence mechanisms of CDV is notable; even within the same ecosystem, it can use either strategy. This is the case of distemper in the Serengeti lion populations. The first epizootic events in the lions originated from dogs, but since 1994 dogs are not the source of CDV events in lions, or at least not the only one.[249] This information is critical for wildlife management and for determining the best mitigation strategies to prevent or contain outbreaks (surveillance, wild dog population control, targeted vaccination, etc.).[181,240] A policy of core vaccination strategies against CDV in wild reservoir species could also be a feasible and more cost-effective strategy than mass dog vaccination for protecting endangered populations against extinction risks.[249]

Managing strategies depend on the epidemiologic traits of a particular disease, so understanding the epidemiological traits of an epizootic is critical for developing good responses. It has been suggested that when basic reproductive ratios (R_0, number of secondary cases produced by a primary case) are high (e.g. >3) culling is more effective than vaccination. For low R_0, the optimal response strategy is more complex and depend on multiple additional factors.[263] R_0 is not a biological constant associated with a particular pathogen; it is influenced by many factors of biological, socio-behavioral, and

environmental origin. However, certain ranges can be estimated using mathematical models.[264] While the R_0 for measles is well studied and reported to be between 12 and 18,[265] one of the highest among all infectious diseases, it is not very clear what the R_0 is for CDV. Some models have given it values from 0.73[266] to 1.26[267], but some simulations indicate the R_0 value may be as high as 10.[266]

There are several reasons to believe that vaccination of dogs alone will not be sufficient to eliminate CDV. First, vaccination in dogs might fail due to various reasons, such as the presence of maternal antibodies in puppies, failure to complete the puppy booster vaccination series, stressors in the physical environment, the animal's immune competence and specific responsiveness to CDV antigen, intercurrent exposure to other virulent viruses (e.g. canine parvovirus or coronavirus) or parasites, and improper storage and handling of the vaccine. This makes it very difficult to achieve the 95% vaccination rate needed to control distemper in dogs at a population level.[268] Furthermore, new genetic CDV variants may be associated with changes in pathogenesis or with immune evasion in dogs vaccinated with current vaccines. However, CDV infections reported in previously vaccinated dogs in Japan, Mexico, and the United States were caused by novel CDV lineages distantly related to the America-1 vaccine group.[269]

Impact of distemper

Canine distemper kills the majority of unvaccinated dogs that become infected and those that survive often suffer long-term neurological sequelae.[168] It has the second highest fatality rate among canine diseases after rabies and the virus can be shed up to 60 to 90 days post-infection.[255]

The number of described susceptible species has increased during the last few decades. Initially thought to only affect members of the Canidae family, CDV caused important outbreaks in wildlife in the 1990s, especially in large cats.[72,270] Of particular importance was the outbreak in the Serengeti National Park in Tanzania in 1994, which is considered to have a dog origin.[179,249,257] This outbreak made conservationists realize that they had be to be aware of infectious disease risks even in healthy populations. This outbreak caused high mortalities in hyenas[271] and lions, whose populations decreased by one third.[249,270,272] More than 20 years after the outbreak began, the mortality rates have decreased but the population maintains antibody titers.[187] CDV infection has been reported in virtually all families of the Carnivora order, either naturally or experimentally.[162,199] Furthermore, members of the orders Pilosa (sloths, anteaters)[273–275] and Rodentia (rodents) have demonstrated clinical, even fatal, disease. Species of Artiodactyla (even-toed ungulates) and Proboscidea (elephants) can seroconvert, although *in vitro* studies highlight the lack of adaptation of CDV to these species[276] and few of them have shown signs of clinical disease yet.[182,199,277]

Distemper can have devastating effects on wildlife, significantly undermining populations of great conservation importance.[272,273,278–280] Many species have a high fatality rate, which could tamper efforts for the conservation, recovery, or reintroduction of endangered species.[237,281–283] In normal situations, disease alone is not a great contributor to extinction, but when populations are reduced, with a high percentage of susceptible individuals and poor genetic variability, diseases such as distemper can create a critical population loss.[245,284] For example, distemper causes mortalities of more than 70% in black-footed ferrets (*Mustela nigripes*) both in captive and free-living populations, and this drove the species

to extinction in the wild in the 1980s.[282,283] CDV has been involved in population declines of African wild dog (*Lycaon pictus*),[260,285] the Santa Catalina Island fox (*Urocyon littoralis catalinae*),[286] and Ethiopian wolves (*Canis simensis*).[278,287] The threat is also significant in captive populations, particularly of felids. Even before the 1992 Serengeti outbreak, CDV events killed a number of tigers, lions, and leopards in American zoos.[288] This susceptibility of big cats is described in many other regions of the world, and mesocarnivores (e.g. raccoons, red foxes, skunks, raccoon dogs) that came into contact with captive animals are commonly thought to be the source of these outbreaks.[289] The historical evidence of the impacts of CDV on population preservation helped predict the possible outcome of CDV in Amur tigers (*Panthera tigris altaica*).[250,279,290] Population viability analysis, indicates a high risk of extinction in small populations of Amur tiger.[279] Even marine carnivores populations are threatened by infection with CDV and other morbilliviruses.[291]

In the USA, a wide range of terrestrial carnivores species can act as reservoirs for CDV, including raccoons (*Procyon lotor*), skunks (*Mephitis mephitis* and *Spilogale gracilis*), and wild canids.[292,293] Numerous species in the region can develop fatal CDV infections, including coyotes,[184,211,294,295] red foxes, grey foxes[296], peccaries (*Tayassu tajacu*),[182,277] fishers (*Martes pennanti*),[216,297] mink (*Neovison viso*),[298] and the endangered Mexican wolves (*Canis lupus baileyi).*[281] On top of that, there are a number of species that have only shown serological evidence of infection.[299–302] Raccoons seem to be the more ubiquitous reservoir for distemper with reports of CDV originating throughout the country.[293,303–306] They also seem to be the main cause of outbreaks of distemper in relatively controlled captive populations, such as zoological collections in urban areas.[133,307,308]

The CDV outbreaks reported in primates (*Macaca mulatta* and *M*. *fuscata*) in China and Japan are of significant concern, because disease in nonhuman primates might indicate a susceptibility for humans.[309–312] The close proximity of these primates to humans raises the concern of CDV becoming zoonotic in the future, especially in the post-measles eradication era. Host breadth is a good predictor of

the zoonotic potential of viruses: the higher the diversity of species a virus can infect, the higher the chances of spillover to humans.[313,314] When controlling for research effort, phylogenetic proximity to humans and overlapping geographical distribution of hosts are very good predictors of zoonotic potential, in addition to increased urbanization and incipient land conversion.[313,315] All of these factors are well represented in CDV, especially in the US. Non-human primates can be infected; mesocarnivore reservoirs (raccoon, foxes, skunk) are adapted to concentrate around human settlements; and there is a continuous increase in urbanization. These concerns are backed up by the relatively fast experimental adaptation of CDV to human cell receptors.[234,316,317] *In silico* analysis suggests the possibility that CDV interacts with ortholog human SLAM and human Nectin-4 receptors to infect human cell lines.[318] In one study, a single substitution in the H protein (P541S) was enough for it to use human SLAM, albeit poorly.[316] In another study, it was revealed that only a single amino acid exchange in the H-protein at position 540 (D540G) was required for functional adaptation to human SLAM. [234] Current evidence suggests that CDV could be a potential future threat to humans,[319] and that its zoonotic potential has only been controlled so far by cross protection from measles vaccination.[150]

Future perspectives

Many studies emphasize the importance of maintaining surveillance and phylogenetic analysis of CDV to prevent possible outbreaks and potential vaccine breakthroughs. Vaccine failures could originate from variation in the genome of the virus, especially in the H gene. We might eventually need a potent multivalent vaccine to protect against different strains of CDV.[189]

The fact that most vaccines were manufactured using CDV lineages isolated over half a century ago (America-1 in the 40s and 60s) presents a significant risk for vaccine evasion.[320,321] Genetic differences between those lineages and the ones currently circulating could deem vaccines ineffective to grant immune protection. This point has been argued by different authors.[157,261,269,322,323]

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Chapter 2: Canine distemper vaccination efficacy: a long term, retrospective and multi-institutional study.

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Abstract

Vaccines are one of the greatest medical achievements of human and veterinary medicine and are responsible for preventing millions of disease cases every year. In veterinary medicine, the vaccine for canine distemper virus (CDV) was introduced in the mid-20th century and has widespread use. The number of clinical cases is much lower than before introducing the vaccine, but there are concerns about its continuous efficacy. Several reports of disease in vaccinated dogs and the fact that most vaccines are based on lineages no longer circulating in the wild prompts the necessity of confirm that

current CDV vaccines are still useful at preventing onset of disease. We designed a multi-institutional, long term retrospective study to investigate the frequency and causes of vaccine failure. Historical data was collected from four clinical and diagnostic institutions across the USA. We analyzed the data to identify risk factors associated with cases of distemper in vaccinated animals. We identified 83 cases of vaccinated dogs that developed clinical distemper. The main predictive factor for vaccine failure was time between vaccination and disease onset. No other demographical or clinical factor was associated with higher risk. Albeit rare, vaccines can fail to prevent clinical distemper and recently vaccinated dogs should be monitored carefully for symptoms related to distemper. Something about the possibility of CDV vaccine CAUSING neuro dz?

Introduction

Vaccines are one of the greatest successes in medical history. Since their development, it is estimated that vaccines prevent two to three million human deaths each year.[1] Vaccines have been a great contributor to the eradication of smallpox in humans[2] and rinderpest in cattle[3,4] and they have regionally eliminated (absence of spread) many other important animal[5–9] and human diseases.[8–13]

Despite their invaluable contribution to human and animal health, vaccines should be monitored for safety, and surveillance systems should be put in place to assess their effectiveness at reducing disease prevalence.[14–17] Reports of vaccine failure are not new;[18] the inability of vaccines to produce an efficient immune response in certain individuals is widely documented.[19] Vaccine failure is normally defined by the inability to mount a protective antibody response, although in some cases other aspects of the immune response can protect the host in the absence of neutralizing antibodies.[20] Previous studies indicate that in humans a proper antibody response is not mounted in 2% to 10% of healthy individuals after vaccination.[19,20] In addition to failure, vaccines can cause the disease they are designed to prevent.

This reversion to virulence is associated with mutations in live vaccines that use replication competent although attenuated viruses.[21,22]

Canine distemper virus (CDV) is the causative agent of canine distemper, a highly contagious and potentially lethal disease of dogs. It was the first dog-specific disease for which a vaccine was designed.[23,24] The development of vaccines against CDV infection in the late 1950s, has considerably reduced the mortality rates, partially controlling the disease in its main reservoir host, namely domestic dogs (*Canis lupus familiaris*), and reducing spill-over of the disease into and from wildlife species.[25] However, the existence of disease reservoirs in the form of unvaccinated dogs and wildlife will probably prevent the eradication of the disease in the upcoming years.[25,26]

Control of canine distemper is dependent on immunization campaigns, and these not only protect dog populations, but also highly susceptible wildlife species such as big cats.[25,27,28] The Snyder Hill and Onderstepoort strains were the first developed modified live CDV vaccines (attenuated, MLV), and these were adapted to grow in canine kidney and chicken embryos, respectively.[29] Recombinant vaccines became the next step in distemper vaccination. The recombinant, canarypox-vectored CDV (rCDV) vaccine was licensed for use in dogs in 1997 in the United States (US)[30] and it could boost immunity of already vaccinated dogs and newly vaccinated dogs against CDV challenge.[31,32] This new vaccine incorporated the fusion and hemagglutinin (H) proteins of CDV in a strain of canarypox virus, and it was shown to be safe and effective in all susceptible species tested to date, including dogs.[29,33] However, monovalent rCDV vaccines yield lower seroneutralizing titers than MLV, but may still result in an adequate cellular response.[34] Other rCDV vaccines are currently being developed, using virus such as adenovirus or herpesvirus as the vector.[35,36] Some of these vaccines have been shown to be effective in developing an immune response even in puppies receiving maternal antibodies.[36]

Currently, 50 vaccines have been licensed against distemper in the US alone, although almost all of them are administered in combined formulations with vaccines against other common infections of dogs, such as canine parvovirus and canine adenovirus 2.[37] Newer technologies are being developed in the vaccine field, including the creation of virus like particles using CRISPR/Cas9[38] and self-assembled nanoparticles of CDV H protein tetramers.[39]

Despite the current consensus that CDV is a single serotype,[40] some studies already show differences between neutralizing titers against different strains.[41,42] As an RNA virus, CDV is characterized by great genetic diversity arising from a high mutation rate[42–45] of 4 to 11 × 10^{-4} substitutions/site/year.[46,47] There are 20 lineages of CDV described so far,[48–50] defined by variations in the gene and protein sequences of the H protein, which is the most immunogenic viral protein.[51] This high genetic variability might give rise to a new variant that could avoid the immune protection developed with vaccination, as seen in other RNA viruses such as influenza and coronaviruses.[52,53] Moreover, even if vaccine strains might be influencing wild-type strains by recombination events,[44] most of them are based on lineages no longer circulating in the wild, such as America-1, raising doubts about their ability to protect against current lineages.[41,42,46,51,54,55] Vaccine failure has already been reported in other morbilliviruses,[19] and it has been suggested or predicted in the case of CDV.[56–58] There is an ever increasing number of reports that describe clinical disease in vaccinated dogs since about 1990, and those include countries across the world[41,51,59–70] and many US states.[51,61,71–73]

Our laboratory performed a 40 year retrospective study that indicated that 30% of the canine distemper cases at the University of California-Davis Veterinary Medical Teaching Hospital (UCD-VMTH) were in dogs previously vaccinated for CDV.[74] Our goal in this study is to investigate the impact and extension of CDV vaccine failure using historical data from multiple veterinary institutions across the US. Our aim was to identify possible risk factors associated with vaccine failure and to provide evidence that vaccine breakthroughs are not necessarily incidental events.

Materials and methods:

Cases included in the study

We used the databases of 4 different institutions (3 veterinary hospitals and one diagnostic laboratory) across the USA to gather data for this study. The institutions represented were the Veterinary Teaching Hospital of the University of Georgia (UGA), the Veterinary Medical Center of the University of Tennessee (UT), Washington Animal Disease Diagnostic Lab (WADDL) and the Veterinary Medical Teaching Hospital of the University of California, Davis (UCD). Historical records of all canine distemper cases were retrieved. Only cases with a defined confirmatory test of the disease were included and those with speculative diagnoses or which diagnoses were only based on clinical signs of the disease were discarded. Confirmatory tests included histological lesions compatible with distemper; positive CDV immunohistochemistry reactions in one or more of the dogs' tissues; a positive immunofluorescence assay (IFA) of urine, conjunctive or other samples; positive IgM serology; or positive RT-PCR. From these, we discarded those that were not vaccinated or had no mention about vaccination in their records. Individuals younger than 4 months of age, which may have not developed an effective mature immune system yet were also discarded.[75] For controls, we selected dogs that died during the same period of time due to acute trauma, if they had a necropsy that excluded histological lesions compatible with a viral disease, especially distemper.

Data collected

For all cases included in this study, we collected demographic and clinical data. Demographic data included date of disease diagnosis, age at the time of diagnosis, breed, sex, geographic location and date, type of vaccine, and doses received when the information was available. For clinical data, we defined affected systems by both clinical presentation and histological lesions of canine distemper and

identified which were affected in each case. The categories considered were respiratory, neurological, gastrointestinal, lymphoid, and integumental/ocular.

The dogs affected by canine distemper and the controls participating in the study were both aggregated by breed group according to phylogenetic and functional groups as described before.[76]

Geographical and temporal analysis

Additional spatial, temporal and space-time analyses were performed using SatScan v10.1. For temporal analyses, only data after 1995 was included to avoid bias due to lack of controls before that year. Scanning for clusters with high rates was done using the Bernoulli model. Time periods were aggregated in periods of 4 months and maximum cluster size was limited to 5 years. Maximum cluster size for spatial analyses was limited to 100 km and 50 km radius. For spatio-temporal analyses, inference was set to 999 replications. Geographical distribution was represented using google earth maps with distribution and cluster files generated in SaTScan.

Statistical analysis

Statistical analyses were carried out using RStudio 2022.07.2+576 "Spotted Wakerobin"; p-values (*p*) less than 0.05 were considered statistically significant. Power analysis was performed using Gpower 3.1 seeking a power of 0.8 and an α of 0.05. Graphs were created using GraphPad Prism version 9.4.

Fisher/Chi-square test and Mann-Whitney/Wilcoxon rank sum test to compare median values were used to analyze categorical and numerical data, respectively. A logistic regression model was constructed using CDV infection as an outcome including statistically relevant variables.

Results:

A total of 83 cases met our criteria of canine patients developing distemper after vaccination. Most data were available for all cases and controls, except type/brand of vaccine which was inconsistently recorded. The age range was 0.3 to 15 years, with a median age of 1.95 (Fig. 1), an age distribution significantly different than the control population (Wilcoxon rank sum test W = 2725.5, p<0.001).

There was no sex bias on the affected dogs (41 males, 43 females); and the sex distribution was not significantly different than that of the control population (61 males, 63 females, Chi square, df=1, p = 1 0.957, fig. 2).

The predominant breed amongst CDV affected dogs was mixed breed (n = 31, 37.35%), followed by herding and retrievers (n = 9, 10.84% respectively), but they were not overrepresented when compared with controls (Chi square, df=11, p = 0.123, fig. 3). Institutions had a differently distributed proportion of cases and controls (Chi square, df=3, p < 0.001, fig. 4). Over time, the number of cases was regularly distributed (range of 7-13 cases every 5 years), whereas the distribution of controls during the period of study was irregular (range of 0 to 45 cases every 5 years). Cases were reported between 1976 and 2022, a distribution significantly different than controls (Chi square, df=39, p <0.001, fig. 5). There was no season (p = 0.28) or month (p = 0.106) of the year when cases were more commonly diagnosed (fig. 6).

The most common clinical presentation was neuronal (63.86%), followed by respiratory (56.63%). Multiple system presentation was common (46.99%). The time from vaccination to CDV diagnosis (median of 24.77 days) was shorter than vaccinated control dogs attending veterinary care for acute trauma (median of 156.95 days, Wilcoxon rank sum test W = 387.5 p<0.001)

When analyzing the distribution of cases, a spatio-temporal cluster was detected using a Bernoulli model (p=0.016). The cluster was 21.44 km in radius and located east of Sacramento (38.56 N, 121.32 W, red circle). The temporal distribution of the cluster was defined between March 1999 and June 2003 (Fig. 7).

A logistic regression model including all the previously determined statistically relevant factors (age, institution of origin, year of presentation and time between vaccination and diagnosis) predicted that the only influential factor was time after vaccination. The odds ratio was of 1/1200 per year passed after completed vaccination (z value=-2.11, p=0.035), indicating a protective effect of time after vaccination.

Discussion

In this study, we described the clinical and demographic characteristics of cases of canine distemper in vaccinated dogs. This was a multi-institutional study designed to elucidate the factors that could lead to vaccine failure, through evaluation of a large dataset; this approach contrasts with the case reports or incidents typically described in the literature, which cannot account for a scientifically strong basis for defending vaccine failure.[50,62–65,68] We were able to gather a significant number of patients diagnosed with CDV after vaccination (83), but they still represent a relatively small number of the total vaccinated population.

Factors that did not seem to influence vaccine failure included sex, breed and yearly distribution, despite previous authors' concerns about raising cases of vaccine failure.[41,42] Some dog breed groups were more common among cases (mixed breeds, herding dogs and retrievers), but this is likely due to the local abundance of these breeds, since they were not statistically overrepresented when compared to the control group (p = 0.11). Distemper cases were not associated with any season of the year.

Age was an important factor for vaccine failure, as young animals that have been recently vaccinated were more commonly found to have been diagnosed with distemper (p<0.001). This may be a reflection of a delayed immune response, or it could indicate animals that never develop a proper response, and which get infected as soon as they are exposed. Alternatively, this could also be a consequence of the reversion to virulence of the MLV vaccine strains. Reversion to virulence of CDV has been proven in

experimental conditions[77] and occasionally observed in vaccinated dogs.[78,79] However, when the sequence of CDV is analyzed in diseased vaccinated animals, the virus belongs to a different lineage than the vaccine lineage in most of the cases.[41,62,63,65,67] All these data together emphasize the importance of disease surveillance in young animals for infectious diseases, even if they are completely vaccinated. In distemper, cellular response rather than humoral response is the best protective reaction against infection, although antibodies' titer is normally used as a proxy for immunization and could be used to confirm proper protection against distemper.[80–82]

The distribution of cases and controls differed significantly among institutions (p <0.001), a result that was particularly influenced by the imbalance between controls (n=57) and cases (n=11) at UGA, whereas other universities had a more balanced numbers of distemper cases and controls. The level of detail when collecting data varied greatly between cases and clinicians, but it is fair to assume that a particular institution has standardized guidelines that might allow for collection of specific data in more cases, allowing to easily gather more controls than their counterparts.

Yearly distribution was also significant (p<0.001); while the incidence of canine distemper was maintained throughout the span of this study (1979-2022), the number of controls was greatly concentrated in the most recent time periods. A constant incidence of distemper was unexpected, and we expected it to decrease over time, due to advancements in preventive medicine, including greater vaccination coverage. The lack of controls in the earlier years, especially in the 1970s and 1980s, might reflect a deficiency in the medical records that would hinder a complete collection of data and make it difficult to identify controls.

Many factors can account for lack of adequate protection after immunization, including aspects related to the individual receiving the vaccine and the vaccine itself. It has been theorized that some vaccines can confer incomplete protection due to adaption to *in vitro* replication in a cell culture system.[83]

Pathogen evolution might be another factor that contributes to some vaccines becoming obsolete.[84–86] The majority of pathogens have large population sizes, a high reproduction rate and short replication times.[87] All of these are factors that result in high mutation rates and encourage evolutionary changes, especially when compared to their hosts.[88] Mutation rates are even more remarkable among RNA viruses, which exploit this characteristic as an evolutionary survival mechanism.[89–91]

Clinical presentation, as expected, were mainly associated with respiratory and neurological signs. The clinical signs that were reported corresponded to the distribution of lesions reported after gross and histopathological examination of infected animals. Despite being a common and widely discussed disease of dogs, there are few recent epidemiological reports detailing disease presentation in dogs. This contribution might dissipate doubts about changes in the common presentation of distemper, which remains similar, with few, but not negligible cases including ocular, cutaneous or digestive disease. We initially intended to collect information about the type of vaccine (brand or design), since that information could be crucial at identifying deficient, suboptimal or even potentially virulent commercialized products. However, clinical records did not reliably record this information and as a result, we were not able to draw such conclusions. More complete data could provide evidence for a link between a particular type of vaccine with protective failure.

CDV vaccine failures have often been attributed to incorrect administration and protocols or inappropriate storage of the vaccine,[92] as well as host factors such as immunodeficiency, maternal antibody interference, and vaccination during the incubation period of the pathogen.[59] The nature of retrospective studies does not allow to control the conditions in which the vaccines were administered. Therefore, we cannot eliminate the possibility that the vaccine failures were a consequence of deficient handling or application of the vaccines. However, vaccine escape as a result of pathogen diversity should not be discounted.[63,64,93] This argument is supported by the antigenic variation between circulating and vaccine strains of CDV,[54] which have proven to be significant enough to result in differences in cross-

neutralization assays.[41,42] There is also an increasing number of reports of disease in other species susceptible to distemper that were classically protected by vaccination, such as mink (*Neovison vison*), foxes (*Vulpes vulpes*) and raccoon dogs (*Nyctereutes procyonoides*).[57,94]

In the case of measles, a closely related morbillivirus with a vaccine history of outstanding success, several studies have pointed out the shortcomings of the immunity after vaccination. The prevalence of specific IgG is lower when compared to the one resulting from natural infection (6% vs 20%, respectively),[95] and their titers are lower.[96] On top of that, natural immunity is expected to be life-long, while vaccine response declines overtime and it is estimated to reach around 5% within 10–15 years after immunization.[97] Vaccine escape has also been confirmed in measles and its occurrence is carefully surveilled due to the dire consequences that any outbreak of the disease could have.[19,98,99]

Most of the CDV vaccines used in the United States are MLV, and they are based on the CDV lineage America-1, despite this lineage being practically absent from natural environments. Currently circulating lineages in the USA include America-3, America-5, and the European wildlife lineage.[50,61,69] An interesting approach would be to investigate whether or not cases of vaccines breaks are related to specific lineages of CDV, but this fell outside of the scope of the current study. It is important to keep gathering data to determine the exact CDV lineages circulating in the field and how they are genetically and antigenically related to the available vaccines.[42]

In conclusion, despite reasonable generalized concern about the effectiveness of CDV vaccines, our study did not identify an increasing number of vaccine failures during the last few decades. The concentration of vaccine failures in younger animals and shortly after completing their vaccination series or doses suggests that the lack of a protective immune response is a consequence of individual factors rather than the emergence of strains that can overcome the defenses of immunized animals. However, further research into the impact of new and emerging lineages of CDV and their relationship with the

canine immune system are needed. There are anecdotal reports of well vaccinated dogs developing canine distemper with newly emerging strains, but they are uncommon, and the causality is uncertain.

Tables

	Cases	Median age and range at presentation (years)	Sex (F/M)	Predominant breed	Median interval of vaccination to disease and range (years)	Median interval of vaccination to disease and range (days)
UCD	34	1.90 (0.30-11.00 years)	13/21	Mixed breed (n=15)	0.06 (0.01-0.94)	21.9 (3.7-343.1)
UGA	11	2.00 (0.33-15.00 years)	4/7	Mixed breed (n=3)	0.07 (0.03-5.56)	25.6 (11.0-2,029.4)
UT	35	2.06 (0.33-12.00 years)	23/12	Mixed breed (n=12)	0.11 (0.02-2.25)	40.15 (7.3-821.2)
WADDL	3	0.40 (0.40-0.40 years)	3/0	Mixed breed (n=2)	0.05 (0.04-0.06)	18.25 (14.6-21.9)
Total	83	1.95 (0.30-15.00 years)	43/40	Mixed breed (n=32)	*	*

Table 1. Summary of basic information from cases. (*) no available data.

Figures

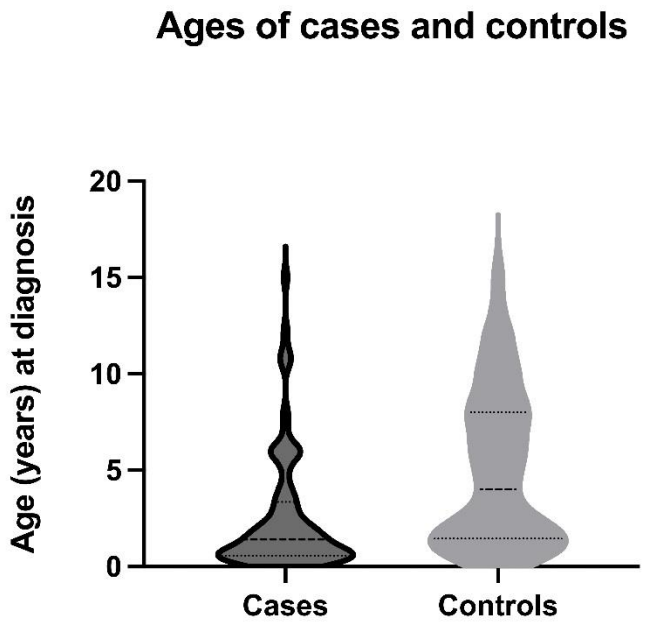


Figure 1. Age distribution. Vaccinated animals developing distemper were significantly younger ($p < 0.001$) than their vaccinated, non-infected counterparts.

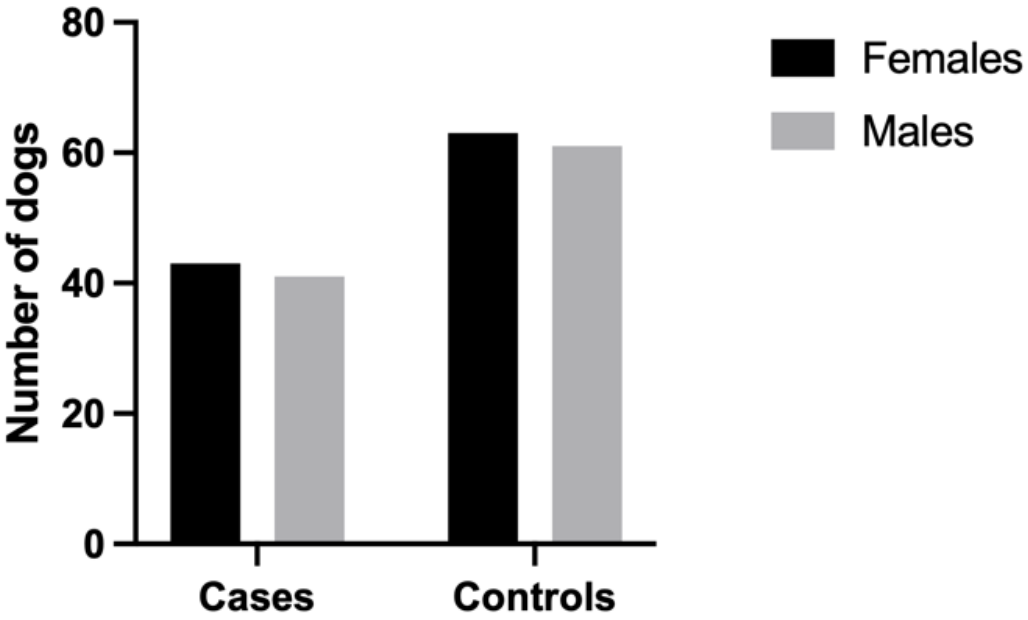


Figure 2. Sex distribution of distemper vaccine breaks. No gender bias was observed in CDV-diagnosed patients when compared with controls (p = 0.957).

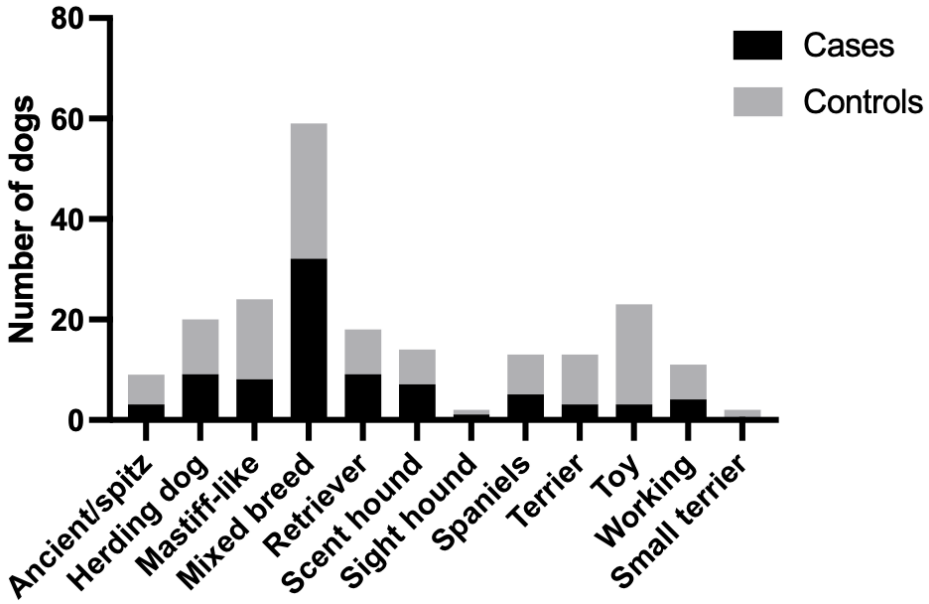


Figure 3. Breed distribution of distemper vaccine breaks. None of the breed groups was more prevalent in CDV-diagnosed patients when compared with controls (p = 0.106).

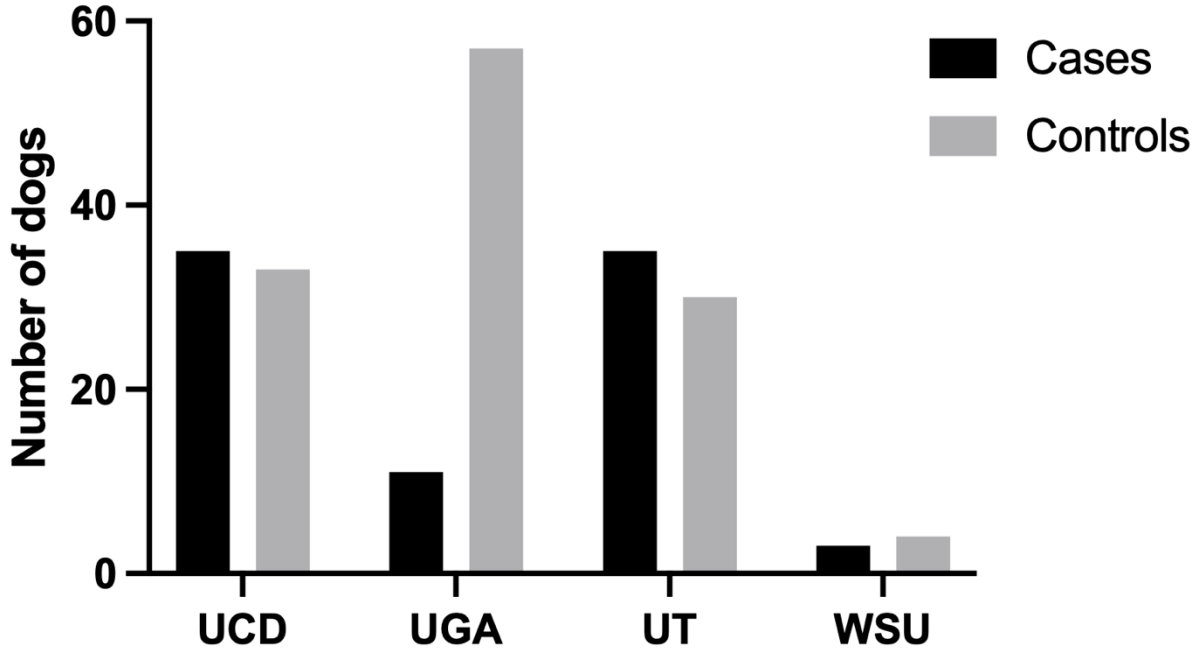


Figure 4. Institution distribution of distemper vaccine breaks. The number and proportion of cases and controls identified in each institution was variable (p-value<0.001), but these were biased by a single institution with a significant difference between the number of cases and the number of controls (UGA).

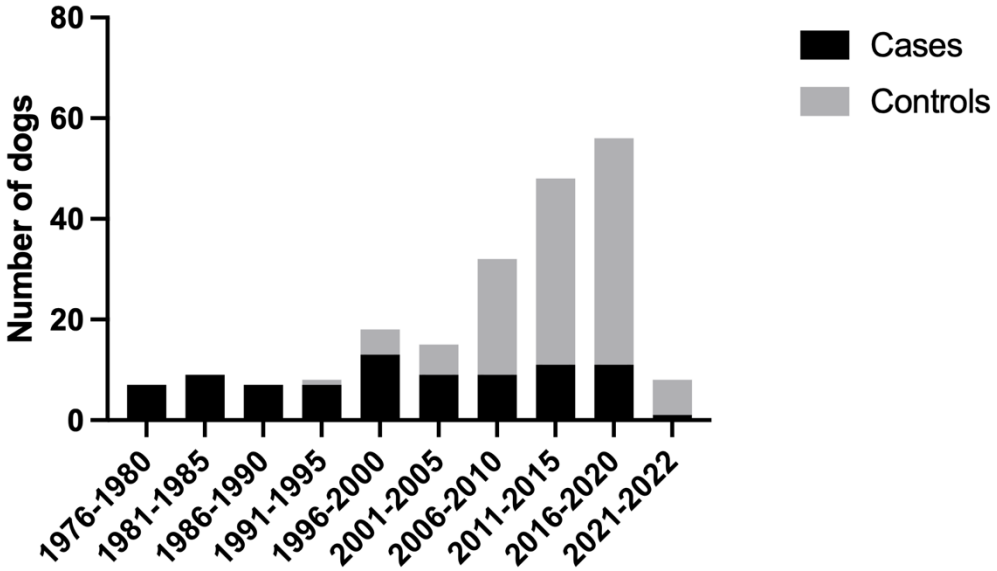
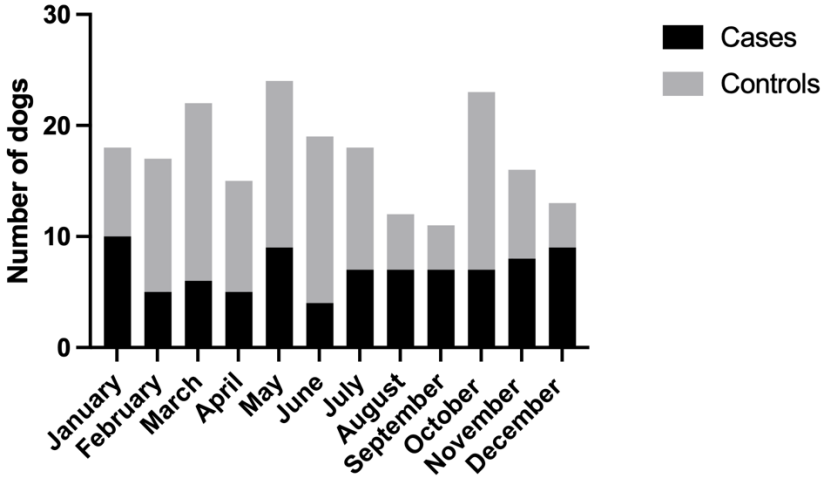


Figure 5. Year distribution of distemper vaccine breaks. The frequency of cases was consistent over time. In comparison, most of the controls were available from the last 20 years. (p-value <0.001)



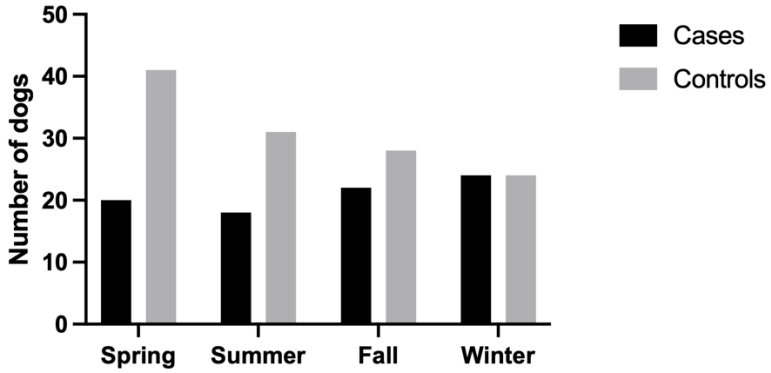
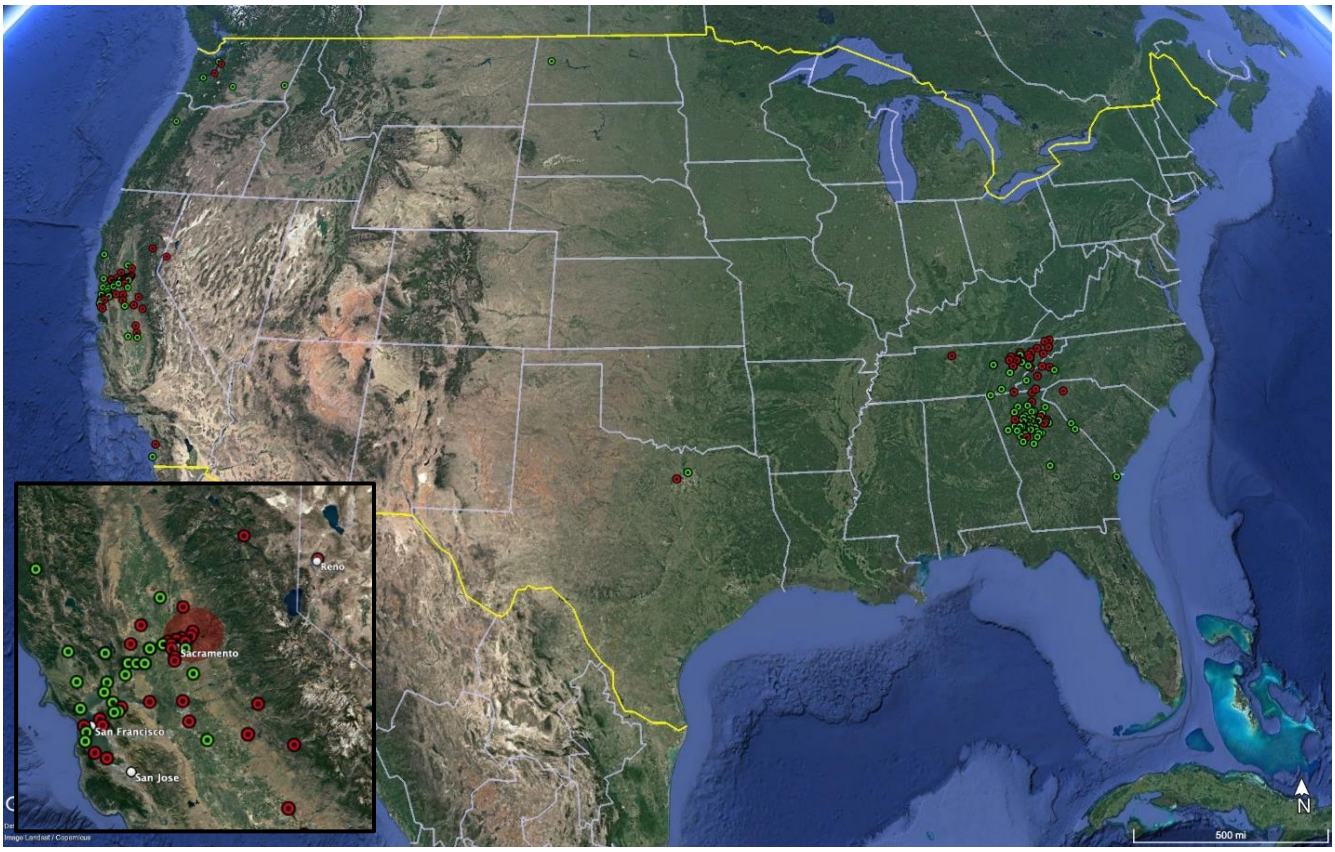


Figure 6. Monthly (top) and season (bottom) distribution of distemper vaccine breaks. The frequency of cases was consistent over both the months and seasons. When compared with the distribution of controls, the difference in number of patients was not statistically significant (p = 0.09 for month distribution, p = 0.28 for season distribution).



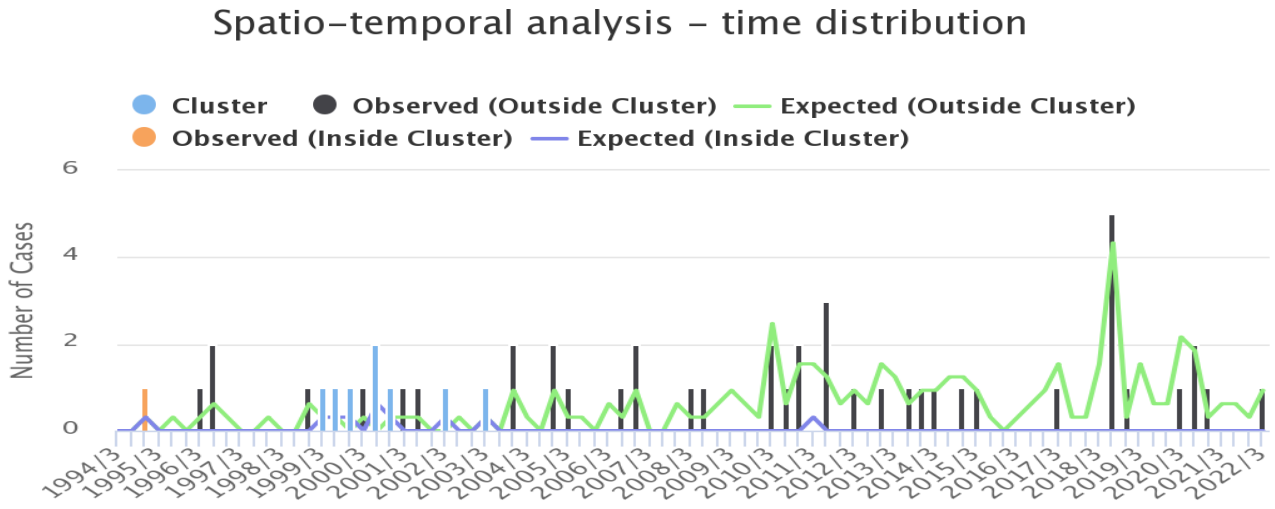


Figure 7. Spatio-temporal distribution of distemper cases in the continental US. A cluster of cases was detected using a Bernoulli model (p=0.016). Top image: Geographic distribution of distemper cases. Vaccinated animals with distemper (cases) are represented as red dots. Vaccinated animals that suffered from a trauma event (controls) are represented as green dots. Insert: a spatio-temporal 21.44

km in radius cluster was detected east of Sacramento (38.56 N, 121.32 W, red circle). Bottom image: temporal distribution of distemper cases. A spatio-temporal cluster was detected between March 1999 and June 2003 (blue bars).

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Chapter 3: Development of *in vitro* and *ex vivo* models for the study of canine distemper pathogenesis

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Abstract:

Canine distemper virus (CDV) is the cause of severe disease in dogs and other mammals. The pathogenesis of this disease is partially driven by the viral Hemagglutinin protein which mediates cellular attachment of the virus. The distinct lineages of CDV are currently defined by changes in the viral hemagglutinin sequence. However, the impacts of the mutations in the gene encoding this highly variable protein are not well defined. We developed a series of *in vitro* and *ex vivo* experiments to more effectively model the difference in pathogenesis among currently circulating CDV isolates. Full genome sequencing defined our CDV isolates as belonging to the America-3, America-4 and Rhode Island-like lineages. The replication efficiencies of these three viral bioisolates in a susceptible cell line were determined to be higher among America-4 isolates. We discuss how these newly described isolates compare to previously reported CDV sequences and establish their phylogenetic relationship. We also analyze relevant amino acid residue changes in these isolates.

Introduction:

Distemper is a highly contagious disease of various carnivores, including domestic dogs. The etiologic agent is canine distemper virus (CDV), an enveloped, negative-stranded RNA virus, which is a paramyxovirus of the genus *Morbillivirus*.[1,2] The infection in dogs is associated with a wide range of

clinical signs that can include immunosuppression, pneumonia, encephalitis and gastrointestinal signs, and it results in high morbidity and mortality rates.[2–4] Since the development of an effective vaccine in the mid-20th century, and with the increased medical care of dogs, the incidence of the disease has dropped in developed countries.[5] However, the disease stills spreads with ease in unvaccinated dog populations and is maintained in wildlife populations[6–8]. Canine distemper virus sometimes causes severe outbreaks of disease that threaten the populations of highly susceptible and endangered species of felids[9,10], mustelids[11], pinniped and canids.[12–14]

A sophisticated understanding of the pathogenesis of a disease is critical to develop adequate management, prevention and treatment strategies. Due to its impact in dog population, distemper pathogenesis was already investigated in the mid-20th century[15–17] and much of the information we have about the disease process has not been revisited through the years. However, as an RNA virus, the CDV genome is highly variable and to date 19 lineages have been described.[18,19] The most variable protein of CDV is the surface glycoprotein hemagglutinin (H protein), which is involved in viral attachment, the first step of cell infection.[20–22] The impact of this genetic diversity in the pathogenesis of CDV is not completely understood, but changes in the H protein have been attributed to spillover to other species, and possibly, to increased virulence.[23–29]

Several investigators have identified variations in the clinical presentation of distemper, ranging from digestive, neurologic, respiratory or ocular lesions, to any combinations of these.[23,30] This is inconsistent with current dogma that lesions are simply a function of the distribution of host-cell receptors or temporal changes in the infection.[31] Therefore there may be as of yet unidentified factors contributing to the pathogenesis of CDV infections.

In order to understand the characteristics and pathobiology of viral infections it is important to develop good models that replicate the process of viral infections in the host. *In vivo* models utilize animal hosts

to study the processes of viral infection. This involves infecting live animals with the pathogen of interest to evaluate different outcomes of the infection. *In vivo* studies are excellent models of disease, since they account for the whole biological interactions that occur during infection.[32] This is especially true when it is feasible to use the target host species directly for the model, making the results even more translatable. However, *in vivo* models require specialized training, complex logistics, and approval from institutional animal use committees. They also raise ethical concerns and are expensive and time-consuming when compared to *in vitro* models.

In vitro studies focus on simpler models of infection. In most cases, this results in the use of cell lines or combinations of cell lines that are both susceptible (allow viral entry) and permissive (can support viral replication) for the pathogen of interest. By design, *in vitro* studies lack the complexity of interactions involving complete organs and multi-organ systems, including the susceptible host immune reaction to natural infection. In exchange, this experimental simplification allows for an easier experimental design that enables the isolation of specific components of the process, facilitating the study of the effect of specific changes. Additionally, they have reduced costs and times, since the generational time and maturation of cells is much shorter than that of live animals.

A third experimental option involves the use of *ex vivo* models. These studies involve the isolation of tissues from a living organism and maintain them in an artificial environment for experimentation. This convenient design with components of both *in vivo* and *in vitro* models gives them a complexity and translatability akin to that of *in vivo* models while maintaining the relative design flexibility of *in vitro* experiments.[33]

In this Chapter, we investigated the development of both *in vitro* and *ex vivo* models to investigate the pathogenesis of CDV. We hypothesized that amino acid variations in the H-protein of CDV are responsible for changes in tissue tropism and the initial steps of the viral-cell interaction. We anticipate

that these variations will translate in changes in cell injury (cytopathic effect), viral replication and viral protein expression.

Materials and methods:

Cell lines

Cell lines used in this study included MDCK cells and Vero cells transfected to express the CDV receptor, dogSLAM, donated by Leslie Woods at UC Davis. Transitional cell carcinoma (TCC) cells were generously donated by Deborah W. Knapp at Purdue University. Vero dogSLAM and MDCK cells were grown on Dulbecco's Modified Eagle Medium (DMEM, Gibco) while TCC were grown in DMEM/F-12 (Gibco). In all cases, media was supplemented with 5% of fetal bovine serum (FBS, GEnClone) and 1% of antibiotic-antimycotic combination (anti-anti 100X, Gibco). Cells were incubated at 37˚C and 5% CO_2.

Viral isolates

For this study, a total of 10 CDV viral isolates were employed, originating from the 3 different regions (Table 1). All viruses were isolated from tissues of animals suffering from clinical distemper infection or part of surveillance efforts. Distemper was confirmed in the origin institutions by fluorescent antibody test or PCR. Infected brain and lung tissues from two gray foxes (*Urocyon cinereoargenteus*), and one striped skunk (*Mephitis mephitis*) were collected from cases presented at the University of California, Davis. Infected brain and lymph node tissues from four raccoons (*Procyon lotor*) and one gray fox were generously donated by Nicole Nemeth from the University of Georgia. Infected brain and lung tissues from one gray fox and one raccoon were generously donated by David Needle at the University of New Hampshire. All tissues were stored at -80 ˚C prior to viral isolation.

For viral isolation, 50 mg of selected tissues were aseptically homogenized and ground using 5mm glass beads (Fisher Scientific) and then filtered using 0.45 µm polyvinylidene fluoride membranes (Millex-HV). Filtered tissue homogenates were incubated with Vero dogSLAM cells in the same conditions as described previously until viral cytopathic effect (cell detachment, syncytia formation and cell death) was observed visually. At this time, the culture supernatant was collected, and stored at -80 ˚C.

Total RNA was extracted from it using QIAamp viral RNA Mini kit (QIAGEN) according to manufacturer instructions. The presence of CDV was confirmed using standard one step RT-PCR (One step RT-PCR, QIAGEN) in a C1000 Touch Thermal Cycler (Bio-Rad). Previous CDV isolates were used as positive controls and the master mix with nuclease-free water. All primers were custom made. The forward primer was 5'-ATGAAACGATCCCCAGGG-3' and the reverse primer was 5'-ACTGATGTAACACTGGTCT-3' and they targeted an 880 nucleotide-long segment in the CDV nucleocapsid gene. Conditions were 30 min at 50˚C, 15 min at 95˚C, 40 cycles of 30 s at 94˚C, 30 s at 50˚C and 1:30 min at 72˚C; and a final step of 10 min at 77˚C. Amplicons were run in 1% agarose electrophoresis gel and presence of CDV was confirmed by identification of a band of expected size.

Viral sequencing

Viral RNA was extracted from cell supernatant as described previously. The extracted RNA was purified using RNA clean & concentrator kit (Zymo) according to manufacturer instructions. RNA-seq libraries were generated from 7ng input RNA using the Kapa RNA Hyper kit (Kapa Biosystems, Cape Town, South Africa) following the manufacturer's instructions. The fragment size distribution of the libraries was verified by micro-capillary gel electrophoresis on a Bioanalyzer 2100 (Agilent, Santa Clara, CA). The libraries were quantified by fluorometry on a Qubit fluorometer (LifeTechnologies, Carlsbad, CA), and they were pooled in equimolar ratios. The pool was quantified by qPCR with a Kapa Library Quant kit

(Kapa Biosystems) and sequenced on partial lane of an Illumina NovaSeq 6000 sequencer (Illumina, San Diego, CA) with paired-end 150 bp reads.

The trimmed reads were mapped to a reference CDV genome (GenBank accession number KJ747372). A second round of reference-based assembly was carried out using the consensus sequence obtained in the first round as a reference. All analyses were performed using Geneious prime 2022.2.2.

Viral titration

Viral titration was performed through a viral plaque bioassay. Vero-dogSLAM cells were grown in 6-well tissue culture plates in the same conditions as described previously until 90% cell confluency was achieved. Supernatant was removed and cell monolayers were washed with 1 ml of DPBS (DPBS, Gibco, Life Technologies). Wells were incubated with 500 µl of six 10 fold dilution of viral aliquots for 1 hour at 37˚C and 5% CO_2 in duplicate. Then, 4 ml of a 0.5% agar solution diluted in equal amounts of growth media and sterile water was added in each well. Plates were incubated for 3 days at 37˚C and 5% CO_2. The agar overlay was removed and cells were stained with 0.5% crystal violet (Fisher chemical) and the number of viral plaques for each dilution was enumerated to calculate the amount of PFU/ml in the original aliquot.

Viral particles labeling

In order to develop an antibody independent detection method, the envelope of viral particles was labeled with biotin. Vero dogSLAM cells were incubated with a self-assembling biotin-lipid conjugate (DSPE-PEG2000-biotin, Avantis polar lipids) at 25 µg/ml. Incorporation of biotin into cell membrane was demonstrated with flow cytometry by using Alexa-488 conjugated Streptavidin (Invitrogen) and analyzed on aBecton Dickison LSRII flow cytometer, utilizing blue (488nm) lasers. Biotin labeled Vero dogSLAM cells were infected with CDV for one hour and then incubated for 48 h at 37˚C and 5% CO_2. Supernatant was collected and labeling of viral particles was demonstrated by infecting unlabeled Vero

dogSLAM cells with biotinylated viral particles and using flow cytometry to quantify labeling. Flow data were analyzed by Flowjo v10. Biotinylated viral particles were incubated with formalin fixed paraffin embedded (FFPE) canine kidney and lung sections from CDV-free dogs for two hours at room temperature. The presence of biotin was revealed using Alexa-488 conjugated Streptavidin (Invitrogen) and by visualizing the slides in an EVOS M5000 fluorescent microscope (Thermo Fisher).

Virus immunohistochemistry

In order to evaluate viral infection, immunohistochemistry (IHC) was performed in 5 µm thick FFPE tissue sections. Sections were deparaffinized, rehydrated and pretreated with a commercial, heat-induced, target-antigen, retrieval solution (Agilent Dako). Endogenous avidin and biotin were blocked with a commercial blocking system (Avidin/Biotin blocking kit, Life technologies). Non-specific antigens were blocked with a commercial blocking solution (Universal Blocker™ in Tris Buffered Saline (TBS), Thermo Scientific). Tissue slides were incubated at 4˚C with 10^3 plaque forming units (PFUs) of CDV in PBS overnight. Slides were then washed and incubated with a primary anti-CDV antibody (MCA1893, Bio-Rad; 1:400 dilution) overnight at 4˚C. After washing with Dulbecco's phosphate buffer saline (DPBS, Gibco, Life Technologies), slides were incubated with a 1:400 diluted secondary anti-mouse IgG1 antibody conjugated with biotin (rabbit, polyclonal, Thermo Fisher) and ultra-streptavidin-horseradish peroxidase (Thermo Fisher). A colorimetric reveal reaction was performed using 3, 3' diaminobenzidine (ImmPACT™ DAB, Vector Lab), and the slides were counter-stained with modified Mayer's hematoxylin (Thermo Scientific). Slides were dehydrated and mounted with Shandon-Mount (Thermo scientific) before microscopical examination. For positive control, a tissue section of a canine brain infected with CDV was run in parallel under the same conditions. For negative control, the primary anti-CDV antibody was substituted by incubated with an irrelevant antibody of the same species.

Ex vivo canine tissue explants

Trachea, lung, lymph nodes and spleen tissues were collected from healthy dogs whose owners consented to unrestricted *postmortem* use of their animal's tissues. Only recently (<4 hours *postmortem* interval) deceased dogs with no systemic or respiratory infection and no lesions in those tissues were included in this study. Tissue samples were transported in DPBS (Gibco) and antibiotics (anti-anti 100X, Gibco). Tissues were confirmed negative for CDV using RT-PCR as described in the viral isolates section. Tissues were washed thoroughly in transport media and sectioned in sufficient 1 x 1 x 0.3 cm portions to allow for duplicates for histology and RNA extraction. Explants were maintained in explant culture medium (ECM) at 37˚C and 5% CO_2. ECM was composed of 450 ml of Roswell Park Memorial Institute (RPMI, Gibco) with L-glutamine (Corning, Cellgro), 50 ml of fetal bovine serum (Gibco, Life Technologies), 5 ml of 100X MEM non-essential amino acids (Gibco, Life Technologies), 5 ml of 100X MEM sodium pyruvate (Gibco, Life Technologies) and a combination of antibiotics that included amikacin to 20 µg/ml (Research Products International, RPI); kanamycin to 100 µg/ml (Bioworld); vancomycin to 50 µg/ml (Bioworld); and amphotericin B to 2.5 µg/ml (Bioworld).

For infection, explants were next incubated with $5x10^3$ plaque forming units (PFUs) of CDV for one hour at 37˚C in an atmosphere of 5% CO_2. Afterwards, ECM was added, and explants were further incubated at 37˚C with 5% CO_2.

Explants were harvested at days 1, 2 and 3 post infection. Tissue homogenates were used to extract viral RNA using QIAamp viral RNA Mini kit (QIAGEN) according to manufacturer instructions for qRT-PCR. Parallel replicates were fixed with formalin and processed for histological examination and IHC as described above.

Tissue viability was examined using TUNEL assay (Click-iT Plus TUNEL assay, Invitrogen) in histological sections and ciliary beating in tracheal explants. For the ciliary beat assay, activity of ciliated epithelial cells is measured to assess tissue viability with a simplified version of Nunes et al. procedure.[34] Briefly, 1

drop of polystyrene beads (Polybead, Polysciences, Inc) was deposited in the mucosal surface of tracheal sections and displacement of beads was examined after 1 hour under a dissecting microscope.

Real time Reverse Transcriptase-PCR

One step real time Reverse Transcriptase-PCR (qRT-PCR) was performed using AgPath-ID One-step RT-PCR Kit (Applied Biosystems) in a 7500 Fast & 7500 Real-Time PCR System (Applied Biosystems). PCR conditions were as follows: 10 min at 42˚C, 6 min at 95˚C and 40 cycles of 3 s at 95˚C and 30 s at 60˚C. For CDV quantification, primers targeting an 88 nucleotide long fragment of the nucleocapsid gene were used. The forward primer was 5'-AGCTAGTTTCATCTTAACTATCAAATT-3', the reverse primer was 5'-TTAACTCTCCAGAAAACTCATGC-3' and the probe was 5'-/5-FAM/ACCCAAGAGCCGGATACATAGTTTCAATGC/36-TAMSp/-3'.

For the *ex vivo* explants, canine GAPDH was used as a house keeping gene for normalization and it was quantified using a commercial combination of primers and probes (Cf04419463_gH, TaqMan Gene Expression Assays, ThermoFisher).

Viral replication assay

96-well tissue culture plates (Gibco) were seeded with Vero dogSLAm, MDCK or TCC cells. Wells were infected at confluency with 200 µl CDV at a Multiplicity of infection (MOI) of 0.04 and incubated at 37˚C and 5% CO_2 for 3 days. Supernatant was collected, and RNA extracted for qRT-PCR. Wells were fixed with buffered 10% formalin and stained with 0.5% crystal violet (Fisher chemical). Cell death was evaluated by loss of monolayer compared to mock infected control cells. Absorbance for each well was measured at 620 nm using an ELISA plate reader using FilterMax F3 (Molecular Devices) and processed in Softmax Pro (Molecular Devices). Average absorbance and standard error of the measurements of the six-well replicates were used for statistical analysis.

Viral phylogenetics

All analyses were carried out using Geneious Prime® 2022.2.2. For phylogenetic analyses, full sequence of the H-genes (1,824 nucleotides and deduced 607 amino acids) were used. Isolates sequenced in this study were aligned to sequenced registered in the NCBI database. After an initial screening, representatives of American lineages (America-1, America-2, America-3, America-4, America-5 and Rhode Island-like and new America lineage) and the Europe Arctic and Europe Wildlife were selected for further analyses. Sequences were aligned using MUSCLE *3.8.425 by Robert C. Edgar*.[35] After discarding short segments of the H-gene, 47 sequences from the database were aligned with our samples. Sequences were trimmed to 1,284 nucleotides to allow inclusion of representatives of several lineages while comparing fragments of the same length. All sequences were tested for recombinant events using RDP4.[36] Two sequences (MK577461 and KT266736) were excluded from further analyses after recombination events were detected. Two sequences (MK577461 and KT266736) were excluded from further analyses after recombination events were detected. Model selection was omitted in favor of using the most parameter-rich model, GTR+I+G, which has been proven to lead to similar inferences.[37] Phylogenetic trees for the H-gene were built using Geneious PhyML with 1,000 bootstrap replicates.[38] For the amino acid analysis, MEGA11 was used for the selection of model to build the tree[39] which was using Geneious PhyML with 1,000 bootstrap replicates in a JTT+G model.[38]

Statistical analysis

All statistical analyses were performed in GraphPad Prism version 9.4. The qPCR results and absorbance values were evaluated by ANOVA followed by a paired t-tested or their non-parametric equivalent. Pairwise comparison was performed using Tukey's test for normally distributed data and Dunn's test for non-normally distributed data. Normality of data distribution was confirmed with Shapiro Wilcoxon. Values under 0.05 were considered statistically significant.

Results:

Viral biotin labeling

The CDV susceptible cells were effectively labeled with biotin, as demonstrated by flow cytometry. Viral particles were able to transfer biotin labeling to unbiotinylated cells (Fig. 1). However, no fluorescence signal was detected in histological sections after being incubated with biotinylated virus. We did not detect any staining through viral immunohistochemistry. Lack of viral binding was further confirmed by lack of amplification of viral RNA by RT-PCR in histological slides after viral incubation (data not shown).

Explant assay

Ex vivo explant infection was attempted in tissues from 8 different dogs that met our criteria for tissue collection. Explants were viable for a few hours after collection, but they degraded quickly and started to show signs of marked autolysis after 48 hours. There were no lesions associated with CDV infection in any of the explants, which included samples of trachea, lung, lymph node and spleen. No positive staining was detected using IHC for CDV. Viral loads were low over time, and detection was inconsistent over time: in some cases, CDV RNA was not detected at day 2, but there were positive titers at day 3 (Table 2).

In vitro CDV kinetics

All viral isolates replicated in Vero dogSLAM cells, as demonstrated by plaque assay staining and increasing viral RNA levels (qRT-PCR). Despite being infected with the same amount of PFUs, the viral loads were significantly different at the end of the infection assay. Isolates originated from the Southeast USA had higher quantities of viral RNA at day 3 when compared with those from New Hampshire, and California had lower viral RNA (Fig. 2).

Viral RNA loads of GA-92 were significantly lower than the rest of Georgia viral isolates ($p<0.005$) and SK ($p=0.016$). The rest of the Georgia isolates had the highest viral load and GA-95 viral RNA loads were higher than those of NH3 and 1313 ($p=0.019$ and $p=0.029$). GA-299 had higher loads than NH3 and 1313 as well ($p=0.019$ and 0.028).

The viral load of TCC and MDCK at day 3 did not differ significantly when compared to cell-free wells. They also had similar levels of crystal violet staining (1.41±0.13 and 0.94±0.11, respectively) to those of uninfected cells (1.37±0.14 and 0.92±0.11, t-test p-value>0.05), but significantly higher than those of infected Vero dog SLAM cells (0.23±0.20, Dunn's multiple comparison test, p-value<0.0001).

Viral sequencing and phylogenetics

Ten full viral genomes, each consisting of 15,669 nucleotides, were sequenced. The isolates sequenced were isolated from five raccoons, four gray foxes and a striped skunk. Based on H gene nucleotide sequences and previously classification distributions,[19,40] the isolates were grouped in the America-3, America-4 and Rhode Island-like lineages. All previously described lineages formed concise and discrete clusters except for America-2 when analyzed for both nucleotide sequence (Fig. 3) and amino acid sequence (Fig. 4).

Alignment of Hemagglutinin amino acid sequences from CA-914, CA-1313 and CA-SK (all originating in California) with other America-3 sequences showed a nucleotide identity between 97.03% and 98.68% (Table 3). Hemagglutinin residues from all isolates originating in the Southeastern USA (GA-92, GA-94, GA-95, GA-299 and GA-404), showed a nucleotide identity between 98.68% and 99.67% with other America-4 sequences (Table 3). Finally, isolates from New Hampshire (NH1 and NH3) had a nucleotide identity of 97.03% to 99.6% for the hemagglutinin protein (Table 3) when compared to the recently reported Rhode Island-like lineage.

All cases exhibit hemagglutinin amino acid residues associated with wild type rather than vaccine strains (367V, 376N and 386S versus 367A, 376G and 386T). Amino acid residues 364 to 392 are considered the major neutralizing antigenic site of morbillivirus H protein.[19] All analyzed sequences matched their respective lineages for the amino acid residues in this region with two exceptions. First, NH1 and NH2 had a glutamine in position 374, in contrast with the histidine of their cognate from the Rhode Island lineage (KU666057), but in accordance to all other analyzed lineages. Additionally, MF953470 from the America-3 clade, had a methionine in position 378, while all other isolates, including the rest of America-3, with our newly described sequences, had a leucine. Most positions described as key for interaction with SLAM were conserved among all isolates (519, 525, 526, 529). One exception was position 530, which was occupied by aspartic acid in the America-3 and America-4 lineages, and by a glycine in the rest. The other one was position 549Y, which was replaced by histidine in a single case in America-4 (GA-95) and varied among isolates in America-5 and Rhode Island like (table 4).

America-4 lineages, both previously reported and described in this study, presented a series of unique variations, including 20L, 78L, 189N, 193I, 197K, 198I, 235M, 315V, 339D, 341L, 353I, 430I, 522V and 598M.

Discussion:

In this chapter we use diverse models to investigate CDV variants pathogenesis. While we were not able to establish the biotinylated viral attachment to FFPE tissues or explants models of CDV infection, our *in vitro* infections yielded interesting results that helps clarify CDV pathogenesis.

Biotin labeling has been achieved in previous studies for both bacteria[41] and viruses.[42,43] Targeted viral budding in lipid raft regions[44,45] was a concern regarding the ability of the viral particles to uptake the biotinylated lipids. However, we were able to demonstrate the biotinylation of viral particles, proving

that DSPE-PEG2000-biotin can concentrate in lipid rafts or that they can at least distribute as effectively as in other cell membrane sites. Unfortunately we were not able to achieve viral binding to FFPE tissue, as reported by other authors working with influenza virus[46,47] or other morbilliviruses.[48] There are multiple reasons that could have caused failure of cell-virus attachment. Since this step requires a good affinity between the cell receptor and the viral H protein, any process that affects the quality of the tissue at the molecular level could play a role. One such process is the time from patient death to tissue collection. In some cases, it is difficult to know this period with precision, especially when using archived samples that were originally collected for diagnostic purposes only. Additionally, despite performing antigen retrieval, samples stored in formalin are modified over time. Formalin fixation causes cross-linkages between peptides that can reduce or block immunoreactivity.[49,50] It is easy to assume that such changes can also affect the interaction between viral proteins and cellular receptors. It is recommended that researchers limit the time between fixation and paraffin embedding as much as possible to reduce changes derived from formalin fixation. To the authors' knowledge, the attachment of CDV to FFPE tissues has not been previously described in the literature, so it is still possible that CDV particles are not able to attach to cell receptors under these conditions. We recommend confirming the ability of viral particles to bind to FFPE tissue before interpreting virus histochemistry results. A suitable control to test for a fixation effect is to perform the virus histochemistry on analogous frozen tissue sections. Alternatively, distribution and tropism could be studied in samples of naturally or experimentally infected individuals through IHC or FISH, but this approach makes it more difficult to have a large and varied number of replicates in the experiment and requires characterization of the virus causing infection *in vivo*, which is not always feasible or convenient. This approach would also not allow the investigators to assess the ability of the virus to bind to receptors of cells in particular organs.

Developing *ex vivo* explant models is challenging, as tissues extracted from live animals are sensitive and manipulation can have important negative impacts on the tissue. In our experiment, explant viability

decreased sufficiently by day 3 to compromise attempts to study pathogenesis beyond that point. Tissue explants are deprived of the vascular supply that provided nutrients while they were in the host. These nutrients need to be supplied by the explant media added in the lab. In our experiment, we chose to use a system in which the explants are mostly covered in media, while other authors opted to use agar to provide a solid support and an air interface for the explant.[51] It is possible that the size of the explants or the type of tissues comprising the explants could have played a role in the explant's viability. Regarding the source of tissues collected for explant development, our experimental design emphasized the use of opportunistic samples in contrast to a previous study that use experimental animals. While these reduce the negative aspects of animal experimentation such as complex protocols, high cost, and ethical concerns, it made tissue availability and quality unpredictable. One of our requirements for specimens to be accepted in the experiment was a short *postmortem* interval (<4 hours), but the tissues were collected during routine necropsies, not in an experimental setting. Viral RNA loads decreased over time, indicating insignificant viral replication in the cells. Additionally, the lack of lesions and immunoreactivity associated with CDV infection could be derived from loss of tissue viability or the inability of the virus to infect tissue explants. An alternative could be that viral dose was low and viral replication was taking place, but not at high enough levels to translate into lesions and antigen presence before tissue decay or the end of the experiment. This is unlikely as the viral loads we used for infection, 5×10^3 PFUs, are on par to that of previous authors.[52]

In vitro studies are the most commonly used approach when starting to study a pathogen. The ability to finely control the cell lines used makes them a very attractive design to understand the basic behavior of infectious agents. Some common manipulation of cells done *in vitro* include single point mutations,[53] cell transfection,[54]gene suppression,[55] and gene editing (CRISPR).[56] In our case, we were able to show the effects of genetic differences among viral isolates. Results should be interpreted with caution, since infection was normalized by PFUs and not viral RNA. It is possible that some of the isolates had slightly

different replication efficiency (vRNA/PFU ratios). The CDV isolates belonging to the America-4 lineage, with their corresponding mutations described above, had the highest viral RNA yields. Interestingly, those isolates corresponded to cases with the least severe lesions associated with CDV infection, when compared with the rest of the isolates. It is possible that these two phenomena are related, but further research into the pathogenesis of the isolates in more complex models (*in vivo*) infections is needed to address this question. Although the main sequence differences between isolates are found in the H gene, it is possible that other genes had an impact on the differences in pathogenesis between lineages. Further comparative analysis of the genomes of CDV and study under controlled conditions would be needed to fully elucidate the impacts of every naturally occurring mutation in the pathogenesis of distemper.

Despite having limited capacity to allow viral replication in cell culture, in the conditions of our experiment, we were not able to detect cytopathic effect or viral RNA increase in TCC and MDCK cells. Put together these data indicate poor to nonexistent viral replication in these cell types. There are some possible explanations as to why TCC and MDCK cells were not infected. Both renal epithelia[57] and urotheliuml[58] are susceptible to CDV infection during natural disease, which makes it even more intriguing why the conditions for *in vitro* replication were not met. Although Nectin-4, the epithelial cell receptor for CDV, has been described in bladder cells[59], there have not been specific studies about canine nectin-4 expression in those cells. On the other hand, Nectin-4 is expressed in MDCK cells,[60–62] but they are commonly not regarded as a good model for CDV, and they need to be modified to allow efficient replication of CDV.[63,64] In both cases, it is safe to assume that they essentially not represent good candidates for CDV infection and replication *in vitro*.

Viral isolates with similarities of 95% or more in the nucleotide sequence (or 96% of the amino acid residues sequence) of the H gene are considered part of the same lineage.[65] There seems to be multiple lineages circulating in the US, including America-2, America-3, America-4, America-5, Rhode Island like

and a new cluster circulating in the Midwest.[19,66–70] In our case and following general classification rules, we were able to classify the isolates analyzed in this study in three pre-existing lineages.

Isolates originated from Californian wildlife belonged to America-3. This lineage is widespread in north America and has been isolated previously not only in California wildlife (JN836736)[71] but also in wildlife from Wyoming (MK456390) and dogs from Texas (MH665353)[18] and Mexico (HM771718).[72] Dogs affected by this lineage presented with diverse clinical signs, including neurologic, respiratory and digestive signs; while wildlife had only respiratory and skin lesions.[71,72] This lineage is thought to have originated in dogs, since it took years until it was found in wildlife. In our cases it was difficult to isolate the lesions caused by distemper, since in all cases multiple process were occurring simultaneously. Foxes from cases CA-1313 and CA-914 had concomitant *Listeria monocytogenes* infection and intestinal parasites; anticoagulants were detected in the liver of both. The skunk from case SK was also infected with adenovirus. Foxes had mostly multifocal suppurative and interstitial pneumonia and did not seem to have neurological involvement, while the skunk presented with bronchointerstitial pneumonia and necrotizing conjunctivitis and dermatitis with inclusion bodies in epithelial cells of the gastrointestinal, respiratory and urinary systems. CDV was detected in lungs (CA-1313 and CA-914) and brain (CA-1313 and SK).

America-4 has been previously described in the state of Tennessee in a dog (KJ747372), a fox - (KJ747371), and a captive sloth (MK577461).[18,69,73] Multiple raccoons and foxes of the same state have also been infected with America-4 lineages.[74] In all cases, lesions were described as consistent with distemper and included respiratory and neurological involvement, except in the cases of the sloths that had predominant digestive signs and lesions. It is thought to have originated in wildlife and then infected the dog population.[19] In our cases, the most common lesion was bronchointerstitial pneumonia (GA-95, GA-299 and GA-404). Despite all of them being initially reported for neurological signs, only one of them (GA-404) had neurological lesions (multifocal lymphoplasmacytic meningoencephalitis), but in

all of them CDV was detected in brain tissue. Inclusion bodies compatible with CDV were found in lung, liver and pancreas of cases GA-94, GA-95, GA-299 and GA-404. There were no lesions related to distemper in GA-92 and GA-92.

Unsurprisingly, samples originating from the Northeastern United States belonged to the recently described Rhode Island-like lineage (KU666057). In those cases, the most common lesions were found in the lungs (8 of 8 animals), urothelium, biliary tract, gastrointestinal tract, and brain.[68] The isolates used in this study, NH1 and NH3, belonged to animals that were reported to be sick or displaying abnormal behavior.

Previous authors have reported the mutation Y549H as an adaptation to non-dog hosts. Despite all our cases originating from wildlife, only one (GA-95) had this mutation. Within the American isolates of CDV, the only others that exhibit this particular mutation were the new America lineage (MK577458 and MK577460). There was similar variability with the mutations in position 530; G and E are considered dog variants, while R, D and N are a result of adaptation to non-dog hosts. However, the two cases from the Rhode Island lineage retained the G in position 530. The fact that this particular change is shared between the America-5 and Rhode Island strains suggests it is a trait that was recently acquired or became established in the population. The implications of this changes in the amino acids residues, including their effects in the interaction with the cell receptor are not well understood yet, but they are probably not irrelevant, since they are missense non-conservative mutations; Y549H substitutes an hydrophobic aromatic residue for a positive charged one and G530D switches from a nonpolar to a negatively charged one.

When analyzing the major neutralizing antigenic site of morbillivirus H protein (amino acid sequences 364 to 392, extrapolated from other morbilliviruses),[19,75,76] none of the analyzed sequences differed with their lineage counterparts. One exception was found in the Rhode Island cluster in position 374.

However, considering that the previously reported sequence in this cluster (KU666057) is the only one across all analyzed sequences to have the substitution Q374H, it is safe to assume that this difference reflects a change in the Rhode Island sequence after diverging from the rest of the lineages. Another exception was a mutation exchanging two nonpolar amino acids (methionine instead of leucine) in position 378 for a previously reported sequence (MF953470.1). The conservative nature of this substitution probably carries little biological impact.

While all lineages share some number of mutations, several mutations are exclusive to America-4. Although they are apparently conserved in this lineage, it is difficult to expect a substantial effect, since the amino acid residues in those positions in other isolates, albeit different, carry out similar chemical properties (hydrophobic side chains).

Further studies would be needed to determine the impact of the identified mutations in the H gene.

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Tables

Isolate	Accession number	Lineage	Origin species	Organ	Geographical origin
GA-92		America-4	Raccoon	Brain	Georgia
GA-94		America-4	Gray fox	Brain	North Carolina
GA-95		America-4	Raccoon	Brain	North Carolina
GA-299		America-4	Raccoon	Brain	Louisiana
GA-404		America-4	Raccoon	Brain	Kansas
NH1		Rhode Island	Gray fox	Lung	New Hampshire
NH3		Rhode Island	Raccoon	Lung	New Hampshire
CA-1313742		America-3	Gray fox	Lung	California
CA-1312914		America-3	Gray fox	Brain	California
SK		America-3	Striped skunk	Brain	California

Table 1. Canine distemper virus isolates were employed in this study. Isolates were isolated from three different species commonly affected by distemper in three separated geographical areas. They were demonstrated to belong to 3 previously described CDV lineages.

	Lung			Spleen		
	NH-1	CA-1313	GA-94	NH-1	CA-1313	GA-94
Day 1	4.81	12.01	11.84	2.64	2.84	0.00
Day 2	6.32	9.60	0.00	0.00	3.88	3.34
Day 3	4.99	5.50	8.61	0.00	2.94	3.68

Table 2. Viral RNA loads of a representative explant case (22k001). Viral loads of each of the virus used for infection (NH-1, CA-1313 and GA-94) in this case are expressed as 40-Ct. Viral loads did not consistently increase or decrease over time for any of the viral isolates and tissues.

AY548110.1		90.44	91.48	91.1	91.1	90.94	91.27	91.85	91.85	91.85	91.92	91.6	90.61	90.94	90.94	90.28	90.61	91.1	91.1	89.52	89.62	89.13	89.13	88.91	89.31	90.28	90.77	91.93	92.75
SK	90.44		97.53	97.03	97.03	97.53	97.86	98	98	98	98.5	97.86	92.59	93.57	93.57	93.25	92.92	93.41	93.41	92.94	92.42	91.93	91.93	91.94	92.34	92.42	92.92	93.57	94.42
OK666918.1	91.48	97.53		97.98	97.98	98.43	98.65	98.88	98.88	98.88	98.85	98.88	93.72	94.39	94.39	94.17	94.17	94.62	94.39	94.68	93.95	93.95	93.95	93.75	94.21	93.95	94.62	95.29	95.46
CA1313	91.1	97.03	97.98		100	97.53	97.86	98.5	98.5	98.5	98.68	98.35	93.08	93.74	93.74	93.41	93.57	93.9	93.9	93.35	92.92	92.75	92.75	92.74	93.15	93.08	93.57	94.56	94.8
GA299	91.1	97.03	97.98	100		97.53	97.86	98.5	98.5	98.5	98.68	98.35	93.08	93.74	93.74	93.41	93.57	93.9	93.9	93.35	92.92	92.75	92.75	92.74	93.15	93.08	93.57	94.56	94.8
MF953470.1	90.94	97.53	98.43	97.53	97.53		99.67	98.67	98.67	98.67	99.25	98.52	92.92	93.9	93.9	93.57	93.25	93.74	93.74	93.55	92.75	92.26	92.26	92.54	92.94	93.08	93.41	94.07	94.61
MF953472.1	91.27	97.86	98.65	97.86	97.86	99.67		99	99	99	99.62	98.85	93.25	94.23	94.23	93.9	93.57	94.07	94.07	93.75	93.08	92.59	92.59	92.74	93.15	93.41	93.74	94.4	94.98
JN836736.1	91.85	98	98.88	98.5	98.5	98.67	99		100	100	99.62	99.67	94.18	94.68	94.68	94.34	94.34	94.84	94.84	94.49	93.84	93.34	93.34	93.47	93.88	94.01	94.51	95.17	95.35
JN836734.1	91.85	98	98.88	98.5	98.5	98.67	99	100		100	99.62	99.67	94.18	94.68	94.68	94.34	94.34	94.84	94.84	94.49	93.84	93.34	93.34	93.47	93.88	94.01	94.51	95.17	95.35
JN836735.1	91.85	98	98.88	98.5	98.5	98.67	99	100	100		99.62	99.67	94.18	94.68	94.68	94.34	94.34	94.84	94.84	94.49	93.84	93.34	93.34	93.47	93.88	94.01	94.51	95.17	95.35
MK456390.1	91.92	98.5	98.85	98.68	98.68	99.25	99.62	99.62	99.62	99.62		99.62	93.8	94.36	94.36	94.17	93.98	94.36	94.36	95.01	93.98	93.98	93.98	94.3	94.77	93.61	94.55	95.11	95.3
AY964110.1	91.6	97.86	98.88	98.35	98.35	98.52	98.85	99.67	99.67	99.67	99.62		93.74	94.4	94.4	94.07	94.07	94.56	94.56	94.15	93.57	93.08	93.08	93.15	93.55	93.74	94.23	94.89	95.35
CA914	90.61	92.59	93.72	93.08	93.08	92.92	93.25	94.18	94.18	94.18	93.8	93.74		98.52	98.52	97.69	98.19	98.68	98.68	92.74	91.93	91.43	91.43	91.53	91.73	92.75	93.25	94.07	94.61
GA94	90.94	93.57	94.39	93.74	93.74	93.9	94.23	94.68	94.68	94.68	94.36	94.4	98.52		100	98.85	99.01	99.51	99.51	93.55	92.59	92.09	92.09	92.34	92.54	93.41	93.9	94.73	94.98
GA404	90.94	93.57	94.39	93.74	93.74	93.9	94.23	94.68	94.68	94.68	94.36	94.4	98.52	100		98.85	99.01	99.51	99.51	93.55	92.59	92.09	92.09	92.34	92.54	93.41	93.9	94.73	94.98
GA92	90.28	93.25	94.17	93.41	93.41	93.57	93.9	94.34	94.34	94.34	94.17	94.07	97.69	98.85	98.85		98.19	98.68	98.68	93.15	92.09	91.6	91.6	91.94	92.14	92.92	93.41	94.23	94.61
GA95	90.61	92.92	94.17	93.57	93.57	93.25	93.57	94.34	94.34	94.34	93.98	94.07	98.19	99.01	99.01	98.19		99.18	99.18	93.35	92.26	91.76	91.76	92.14	92.34	93.08	93.9	94.89	94.61
KJ747371.1	91.1	93.41	94.62	93.9	93.9	93.74	94.07	94.84	94.84	94.84	94.36	94.56	98.68	99.51	99.51	98.68	99.18		99.67	93.95	92.75	92.26	92.26	92.74	92.94	93.57	94.07	94.89	94.98
KJ747372.1	91.1	93.41	94.39	93.9	93.9	93.74	94.07	94.84	94.84	94.84	94.36	94.56	98.68	99.51	99.51	98.68	99.18	99.67		93.75	92.75	92.26	92.26	92.54	92.74	93.57	94.07	94.89	94.98
MH644852.1	89.52	92.94	94.68	93.35	93.35	93.55	93.75	94.49	94.49	94.49	95.01	94.15	92.74	93.55	93.55	93.15	93.35	93.95	93.75		96.98	97.38	97.38	97.78	97.78	93.95	93.95	94.56	95.32
KU666057	89.62	92.42	93.95	92.92	92.92	92.75	93.08	93.84	93.84	93.84	93.98	93.57	91.93	92.59	92.59	92.09	92.26	92.75	92.75	96.98		97.03	97.03	96.98	97.18	93.74	94.23	94.56	94.98
NH1	89.13	91.93	93.95	92.75	92.75	92.26	92.59	93.34	93.34	93.34	93.98	93.08	91.43	92.09	92.09	91.6	91.76	92.26	92.26	97.38	97.03		100	99.6	99.6	93.25	93.41	94.07	94.98
NH3	89.13	91.93	93.95	92.75	92.75	92.26	92.59	93.34	93.34	93.34	93.98	93.08	91.43	92.09	92.09	91.6	91.76	92.26	92.26	97.38	97.03	100		99.6	99.6	93.25	93.41	94.07	94.98
MH644849.1	88.91	91.94	93.75	92.74	92.74	92.54	92.74	93.47	93.47	93.47	94.3	93.15	91.53	92.34	92.34	91.94	92.14	92.74	92.54	97.78	96.98	99.6	99.6		99.6	93.35	93.35	93.95	95.08
MH644851.1	89.31	92.34	94.21	93.15	93.15	92.94	93.15	93.88	93.88	93.88	94.77	93.55	91.73	92.54	92.54	92.14	92.34	92.94	92.74	97.78	97.18	99.6	99.6	99.6		93.75	93.75	94.35	95.55
MK577457.1	90.28	92.42	93.95	93.08	93.08	93.08	93.41	94.01	94.01	94.01	93.61	93.74	92.75	93.41	93.41	92.92	93.08	93.57	93.57	93.95	93.74	93.25	93.25	93.35	93.75		95.06	96.21	96.84
MF953421.1	90.77	92.92	94.62	93.57	93.57	93.41	93.74	94.51	94.51	94.51	94.55	94.23	93.25	93.9	93.9	93.41	93.9	94.07	94.07	93.95	94.23	93.41	93.41	93.35	93.75	95.06		96.87	97.21
Z54156.1	91.93	93.57	95.29	94.56	94.56	94.07	94.4	95.17	95.17	95.17	95.11	94.89	94.07	94.73	94.73	94.23	94.89	94.89	94.89	94.56	94.56	94.07	94.07	93.95	94.35	96.21	96.87		97.96
JF283477.1	92.75	94.42	95.46	94.8	94.8	94.61	94.98	95.35	95.35	95.35	95.3	95.35	94.61	94.98	94.98	94.61	94.61	94.98	94.98	95.32	94.98	94.98	94.98	95.08	95.55	96.84	97.21	97.96	

Table 3. Mean distance matrix of the Hemagglutinin among CDV lineages. Distance is expressed as percentage of similarity in aminoacidic residues. Isolates of similarities of 96% and above are considered part of the same lineage.

		367	376	386	519	525	526	529	530	549
America-1	AY548110.1	A*	G*	T*	R	Y	D	R	N	Y
Europe wildlife	FJ011005.1	V	N	T*	R	Y	D	R	D	Y
	JN153023.1	I	N	S	R	Y	D	R	D	H
	Z47759.1	V	N	S	R	N	D	R	D	Y
	DQ889189.1	V	N	S	R	Y	D	R	D	Y
	OK666942.1	V	N	S	I	Y	D	R	V	-
	OK666957.1	V	N	S	I	Y	D	R	V	-
America-3	**SK**	V	N	S	R	Y	D	R	D	Y
	OK666918.1	V	N	S	R	Y	D	R	E	-
	CA1313	V	N	S	R	Y	D	R	D	Y
	GA299	V	N	S	R	Y	D	R	D	Y
	MF953470.1	V	N	S	R	Y	D	R	D	Y
	MF953472.1	V	N	S	R	Y	D	R	D	Y
	JN836736.1	V	N	S	R	Y	D	R	D	Y
	JN836734.1	V	N	S	R	Y	D	R	D	Y
	JN836735.1	V	N	S	R	Y	D	R	D	Y
	MK456390.1	V	N	S	R	Y	D	R	D	-
	AY964110.1	V	N	S	R	Y	D	R	D	Y
America-4	**CA914**	V	N	S	R	Y	D	R	D	Y
	GA94	V	N	S	R	Y	D	R	D	Y
	GA404	V	N	S	R	Y	D	R	D	Y
	GA92	V	N	S	R	Y	D	R	D	Y
	GA95	V	N	S	R	Y	D	R	D	H
	KJ747371.1	V	N	S	R	Y	D	R	D	Y
	KJ747372.1	V	N	S	R	Y	D	R	D	Y
Rhode Island	MH644852.1	V	N	S	R	Y	D	R	G	Y
	KU666057	V	N	S	R	Y	D	R	G	Y
	NH1	V	N	S	R	Y	D	R	G	Y
	NH3	V	N	S	R	Y	D	R	G	Y
	MH644849.1	V	N	S	R	Y	D	R	G	Y
	MH644851.1	V	N	S	R	Y	D	R	G	Y
	MK577460.1	V	S	L	R	Y	D	R	G	H
	MK577458.1	V	S	F	R	Y	D	R	G	H
America-2	Z54166.1	V	S	S	R	Y	D	R	G	H
	Z47765.1	V	N	S	R	Y	D	R	G	H
	Z47764.1	V	N	S	I	Y	D	R	G	Y
America-5	MK577457.1	V	N	S	I	Y	D	R	G	Y
	MF953431.1	V	N	S	R	Y	D	R	G	H
	MF953436.1	V	N	S	R	Y	D	R	G	H
	MF953421.1	V	N	S	R	Y	D	R	G	H
	MK577463.1	V	N	S	R	Y	D	R	G	Y
	Z54156.1	V	N	S	I	Y	D	R	G	H
	JF283477.1	V	N	S	R	Y	D	R	G	-
	MW984528.1	V	N	S	R	Y	D	R	G	H
	MW984530.1	V	N	S	R	Y	D	R	G	H

MK473366.1	V	N	S	R	Y	D	R	G	Y
MK487379.1 1	V	N	S	R	Y	D	R	G	Y
MK473365.1	V	N	S	R	Y	D	R	G	Y
MK473364.1	V	N	S	R	Y	D	R	G	Y
MK456389.1	V	N	S	R	Y	D	R	G	Y

Table 4. Hemagglutinin amino acid alignment of the major neutralizing antigenic sites. Isolates maintained relatively consistent sequences within their lineage. Notice AY548109.1 (in pink, America-1), which contains the exclusive vaccine variations 367A, 376G and 386T (indicated with an asterisk).

Figures

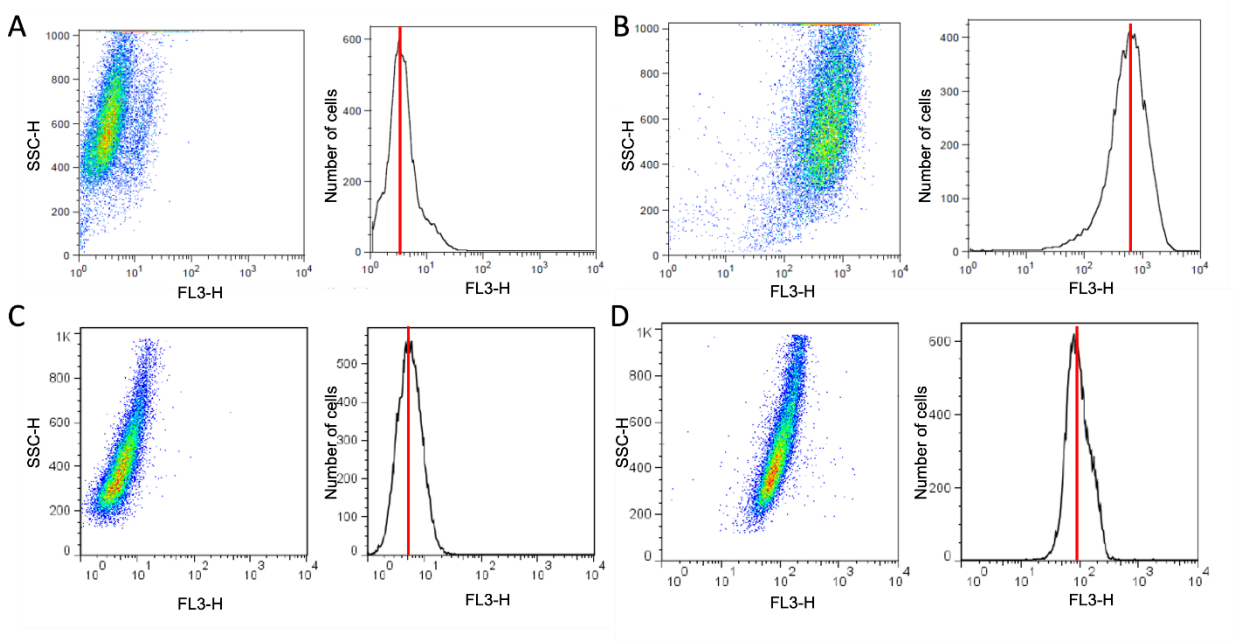

Figure 1. Flow cytometry of biotinylatated virus. Vero dogSLAM cells were incubated with a self-assembling biotin-lipid conjugate (DSPE-PEG2000-biotin, Avantis polar lipids) or CDV particles which had incorporated the biotin-lipid conjugate. Signal was detected using Alexa-488 conjugated Streptavidin. Red lines mark absorbance signal with the greatest number of events. A: unlabeled Vero dogSLAM cells. B: Vero dogSLAM cells incubated with biotin-lipid conjugate. C: Vero dog SLAM cells incubated with wild type CDV. D: Vero dogSLAM cells infected with CDV particles product of biotinylated cells.

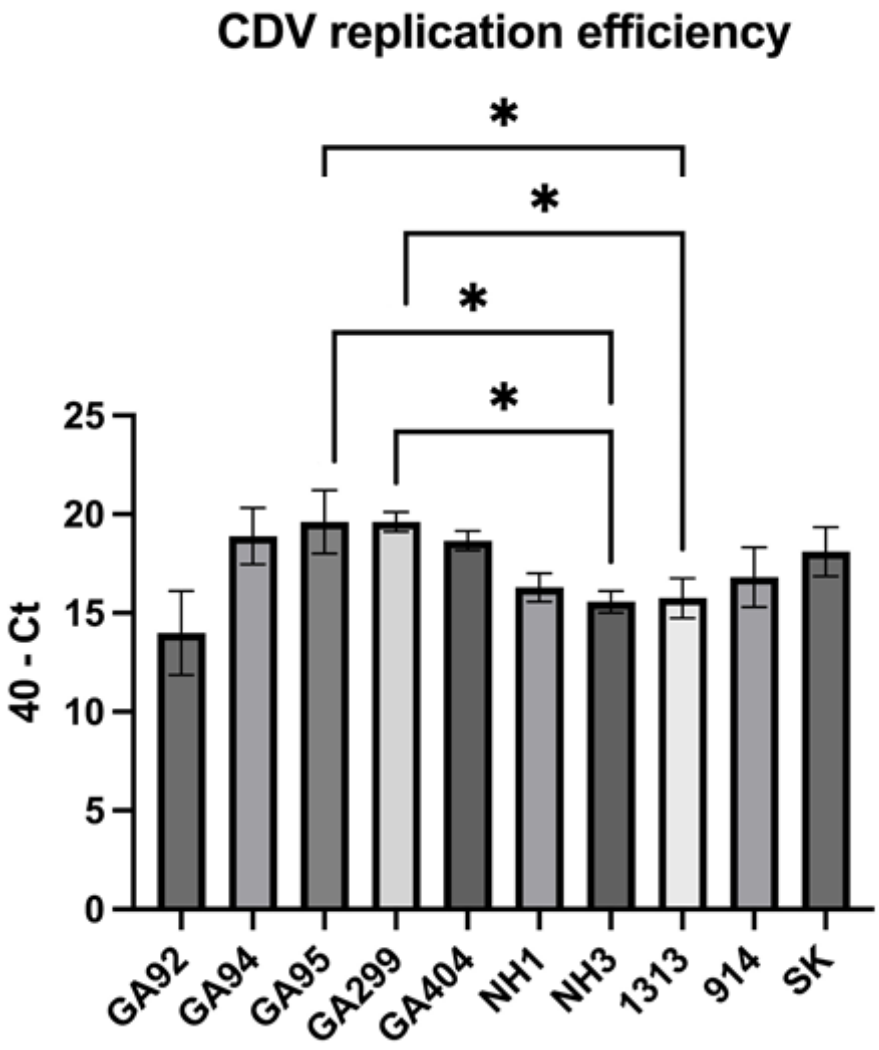

Figure 2. Viral replication efficiency across different isolates. Isolates originating from the southeastern US (labeled as GA) had higher viral loads than those of New Hampshire (NH) and California (CA). Statistical significance (p-value<0.05) is indicated with asterisk (*). Viral loads were quantified by Ct count using qRT-PCR after 3 day infection of Vero dogSLAM cells.

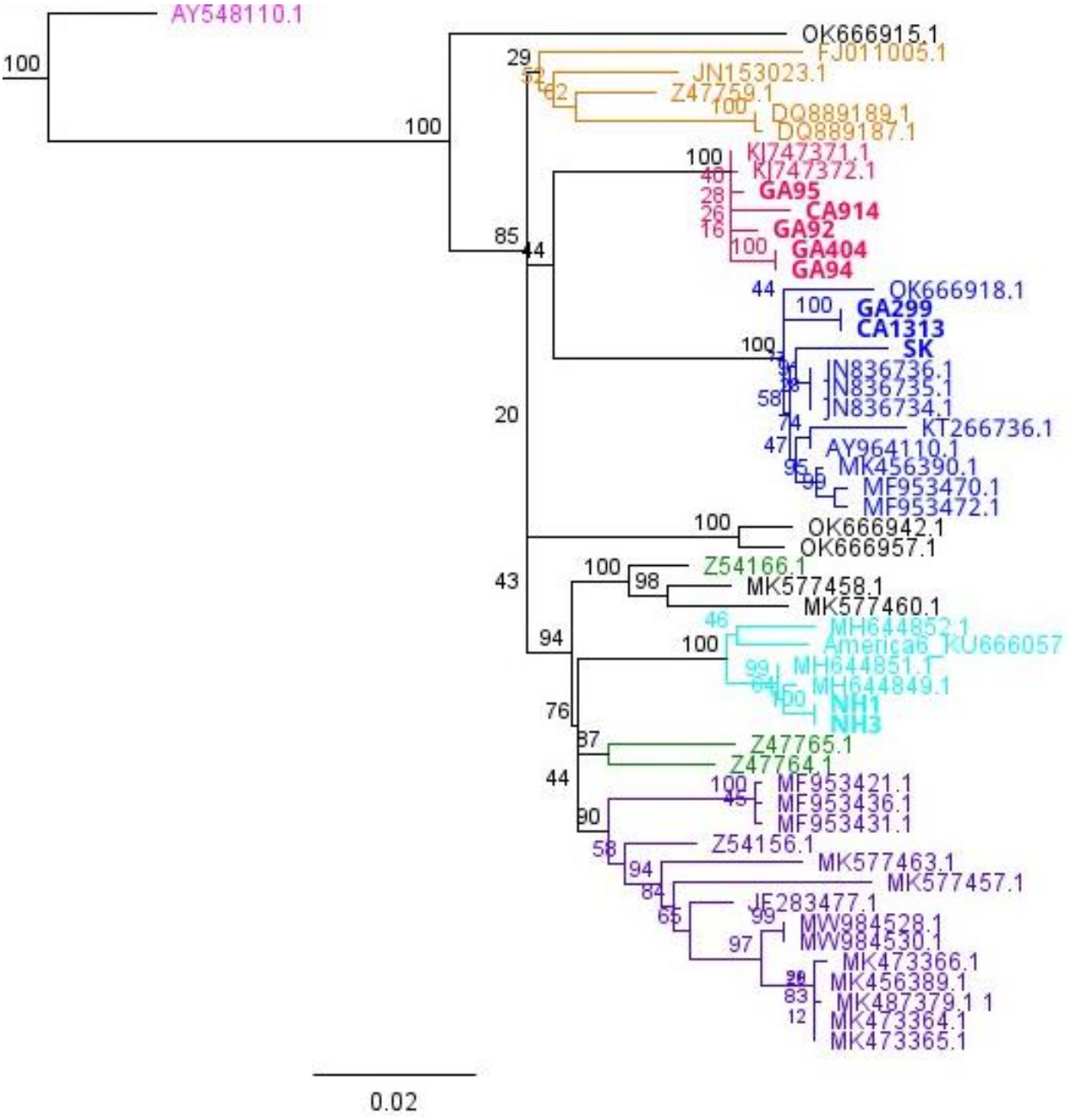

Fig 3. Phylogenetic tree of selected hemagglutinin nucleotide sequences. Colors of sequence corresponds as follows: pink: America-1, orange: Europe wildlife, red: America-4, dark blue: America-3, green: America-2, light blue: Rhode Island, purple: America-5. All previously described lineages formed concise and discrete clusters except for America-2.

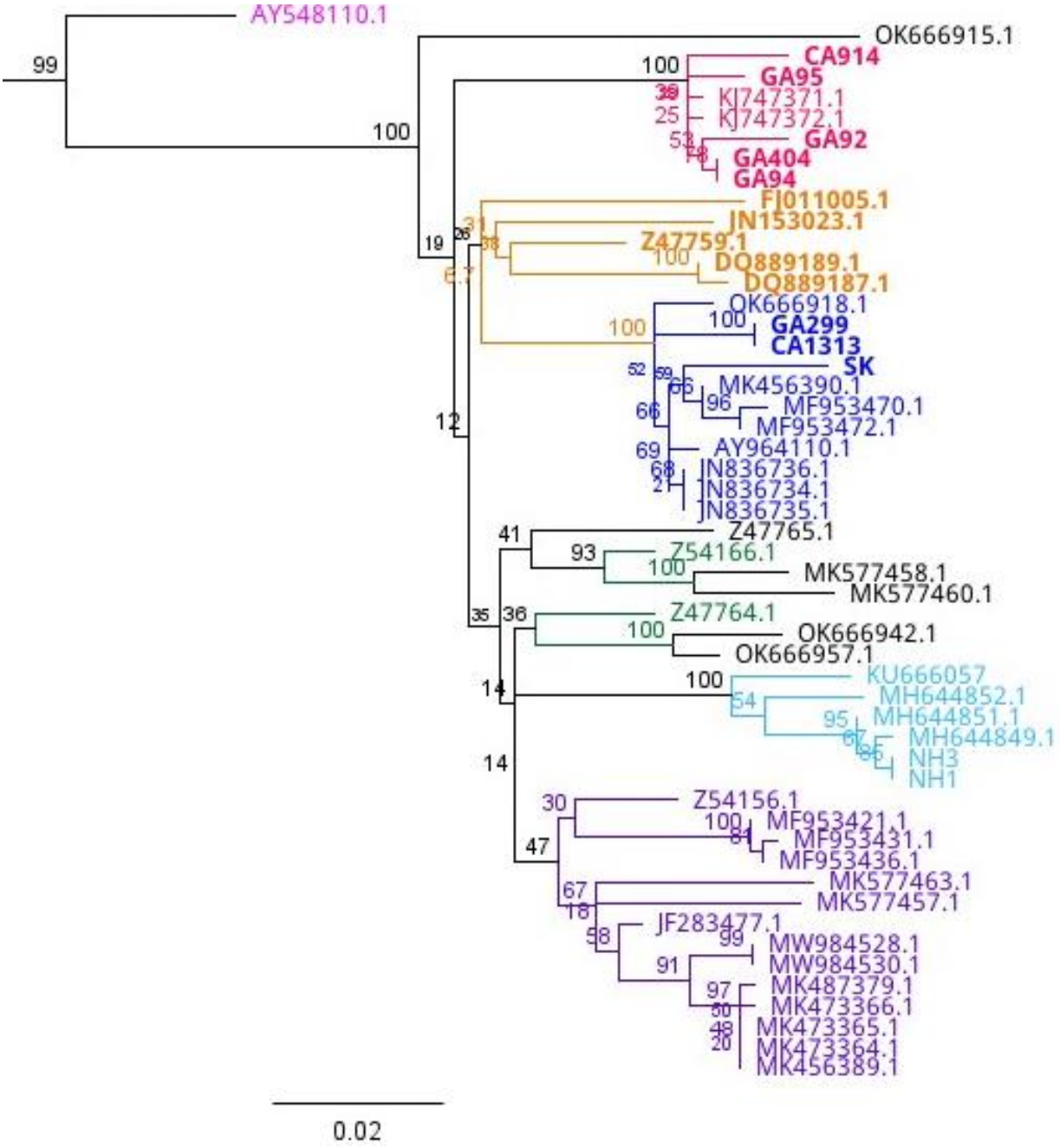

Fig 4. Phylogenetic tree of selected hemagglutinin amino acid sequences. Colors of sequence corresponds as follows: pink: America-1, orange: Europe wildlife, red: America-4, dark blue: America-3, green: America-2, light blue: Rhode Island, purple: America-5. All previously described lineages formed concise and discrete clusters except for America-2.

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Pending publication

Chapter 4: The nucleoside analog GS-441524 effectively attenuates the *in vitro* replication of multiple lineages of circulating canine distemper viruses isolated from wild North American carnivores

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Abstract:

Canine distemper is a viral disease of dogs and wild carnivores with a potentially severe clinical presentation and high mortality rate. Currently, the only available treatment for distemper is supportive care, and there is an urgent need for the identification of antiviral agents that effectively block canine distemper virus (CDV) replication and prevent systemic disease. We assessed multiple antiviral agents with different mechanisms of action for their ability to block the replication of three different lineages of CDV isolated from wild carnivores in California, Louisiana and New Hampshire, United States. Preliminary efficacy experiments excluded several agents, including ribavirin, hesperidin and rutin. Six antiviral compounds were selected for further screening including a protease inhibitor (nirmatrelvir), a polymerase inhibitor (favipiravir) and four nucleoside analogs (remdesivir, GS-441524, EIDD2801, and EIDD1931). Antiviral efficacy was determined by the attenuation of virus-induced cytopathic effect (CPE) in a tissue culture bioassay in a CDV-susceptible Vero cell line and through the inhibition of viral RNA replication in the cell culture supernatant. We determined that the nucleoside analog GS-441524 effectively blocked replication of CDV at pharmacologically relevant concentrations. Four other antiviral agents inhibited CDV replication to a lesser degree (remdesivir, nirmatrelvir, EIDD2801 and EIDD1931).

The replication of different viral lineages was differentially inhibited by the antivirals. Several of the nucleoside analogs have been safely used previously in carnivore species for the treatment of other viral diseases suggesting that they may be promising candidates for the treatment of canine distemper in dogs. We did not identify any antiviral efficacy for favipiravir, ribavirin, hesperidin and rutin, in contrast to what has been reported previously for CDV vaccine lineages. We emphasize the need to consider different viral lineages in the screening of antiviral compounds.

Keywords: antivirals, canine distemper, viral disease, dogs, vRNA, nucleoside analog

Introduction:

Canine distemper is a highly contagious, severe, and potentially lethal infectious disease of dogs and other carnivores caused by canine distemper virus (CDV).[1] Despite the name, CDV does not exclusively infect dogs and has been determined to cause disease in a wide range of carnivores including members of the *Felidae*, *Canidae*, *Mustelidae* and *Procyonidae*.[2] Although there are established vaccine protocols for dogs in the United States (US), CDV is still a relatively common disease due to its persistence in non-vaccinated dog populations and in wildlife, and possibly as a result of CDV vaccine failures.[3] Globally and in the US, viral eradication is not a feasible short term goal due to unvaccinated dog populations and susceptible wildlife species acting as reservoirs for CDV.[4–6]

Canine distemper virus has a remarkably broad cell tropism and as a result, can infect, replicate in and injure a large number of tissues, resulting in a diversity of lesions and clinical disease manifestations. The viral infection generally starts in lymphatic tissues, resulting in lymphoid necrosis and depletion of lymphocytes followed by immunosuppression. The virus then replicates in multiple epithelial tissues, potentially damaging the respiratory tract, alimentary tract, pancreas, liver, epididymis, and testis, amongst other tissues.[1,7,8] Distemper can also infect and be associated with neurological, bone and ocular lesions.[9,10]

Canine distemper virus is a paramyxovirus of the genus *Morbillivirus*. As an RNA virus, CDV has a relatively high mutation rate. The gene encoding the surface glycoprotein Hemagglutinin (H) is one of the most variable CDV genes, and as a result, the sequence of hemagglutinin is typically utilized to determine and define viral lineages or genotypes (defined by sequence divergence of 5% or more).[11–13] At least twenty different CDV lineages have been reported,[14–16] with at least 5 of them (America-2, America-3, America-4, America-5 and Rhode island-like) currently circulating in the United States.[16–18] The effects of viral sequence variation on the clinical signs and tissue lesions associated with CDV infection are presently unknown, but specific viral mutations have been associated with changes in host range and pathogenicity.[19,20] At present, there is no currently effective Food and Drug Administration-approved treatment for canine distemper in the US or elsewhere, and management of infected individuals is focused on supportive care.[3,21] Therefore, the identification of a safe and effective antiviral therapy would be highly beneficial for the canines as well as other susceptible mammalian species.

A variety of nucleoside analog drugs have been demonstrated to be effective antiviral compounds for the treatment of several RNA infections. These compounds bind to the conserved active site of the viral RNA dependent RNA polymerase (RdRp) in their triphosphated form, competing with the actual nucleoside substrates and resulting in early chain termination of the nascent viral RNA strand.[22,23] The viral RdRp is required for the replication of RNA viruses, since this enzymatic activity is absent in the mammalian cells they infect. Nucleoside analog drugs block this common molecular pathway of RNA viruses, so they are often regarded as broad-spectrum antiviral agents. Three analogues stand out for their broad-spectrum action against RNA viruses: remdesivir, ribavirin and favipiravir.[22] Remdesivir, a phosphoramidate prodrug that is metabolized into an adenosine analogue,[24,25] has demonstrated antiviral activity against several families of RNA viruses including *Filoviridae*, *Coronaviridae*, *Paramyxoviridae* and *Pneumoviridae*.[26,27] The active metabolite of remdesivir, GS-441524 has been successfully used to treat feline infectious peritonitis *in vivo*, a disease with no previous therapeutic

options.[28,29] Ribavirin, a broad spectrum purine nucleoside analog, is effective against filoviruses, paramyxoviruses,[30] and hepatitis E virus.[31] Favipiravir, which also has demonstrated direct inhibitory activity of the RNA-dependent RNA polymerase, is considered an effective antiviral compound against rabies, influenza, Ebola and several paramyxoviruses.[32–34]

Another group of antivirals, the flavonoids, which are plant metabolites including compounds like rutin and hesperidin, have also been reported to have antiviral activity for many viruses, including influenza and dengue viruses.[35–37] Although the specific mechanism of action is not completely understood, these agents are suspected to inhibit the viral polymerase[38] or to interact with viral surface glycoproteins (such as hemagglutinin) to prevent cell-virus attachment interactions.[39,40]

Several antiviral compounds are known to inhibit the replication of other paramyxoviruses or even morbilliviruses.[41–43] Although only a few of these agents have been tested against CDV, the similarity in protein composition and replication strategies of CDV with the other members of their family suggests that they might be good candidates for distemper treatment as well. The nucleoside analogs remdesivir and GS-441524 have been shown to effectively restrict the replication of another morbillivirus, measles virus.[41,44] Ribavirin can reduce the cytopathic effect of measles virus,[45] and favipiravir has an antiviral effect against the morbillivirus *peste des petits ruminants* virus.[46] EIDD1931 and its prodrug EIDD2801 are efficient inhibitors respiratory syncytial virus.[47,48] Some flavonoids and nucleoside analogues have also decreased viral replication in cells that were infected with CDV.[40,44,49,50]

Because some of these compounds have been utilized for the treatment of other animal diseases, including other canine diseases, their toxicity and pharmacodynamic properties have previously been defined. Therefore, repurposing these compounds as distemper treatments may be feasible. Favipiravir has been used as an effective treatment for influenza in mouse models,[32,51] and the toxic dose in dogs has been determined to be very high.[52] The flavonoid rutin is the recommended treatment for

chylothorax in dogs,[53,54] and an additional flavonoid, hesperidin, is a recommended pulp capping agent used to seal dental pulp cavities.[55]

Clinical trials for new drugs are necessary to establish the efficacy and safety of new drugs, however, they are expensive, and require the use of live animals, which requires complex logistics and specialized training.[56,57] To maximize the success of clinical trials, it is crucial to identify excellent candidates that can minimize the number of animals needed and the overall cost for drug testing.[58,59] Additionally, drugs become more robust candidates for treatment of specific diseases when multiple independent sources support their efficacy.

In this study, we determined the efficacy of a slate of antiviral agents for the *in vitro* treatment of canine distemper virus using a multi-modal screening assay.

Materials and methods:

Cell lines and CDV isolates

Vero cells that were transfected to express dogSLAM, donated by Leslie Woods at UC Davis, were grown on Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 5% of fetal bovine serum (FBS, GenClone) and 1% antibiotic-antimycotic combination (anti-anti 100X, Gibco). They were incubated at 37˚C in an atmosphere with 5% CO_2.

The CDV isolates for this study were obtained from three clinically affected wild carnivores in the USA, including two gray foxes and one raccoon. Canine distemper was suspected due to the presence of neurological signs and infection by CDV was confirmed by fluorescent antibody test or PCR. The isolates were named GA-299 (from Louisiana, donated by Nicole Nemeth from the University of Georgia), CA-914 (from California) and NH1 (from New Hampshire, donated by David Needle from at the University of

New Hampshire). Their lineages and other details are indicated in Table 1. To isolate the viruses, filtered tissue homogenates from these animals were incubated with Vero dogSLAM cells until cytopathic effect (cell detachment, syncytia formation and cell death) was observed. At this time, the culture supernatant was collected, and stored at -80 ˚C.

Total RNA was extracted from it using QIAamp viral RNA Mini kit (QIAGEN) according to manufacturer instructions. The presence of CDV was confirmed using standard one step RT-PCR (One step RT-PCR, QIAGEN) in a C1000 Touch Thermal Cycler (Bio-Rad). Previous CDV isolates were used as positive controls and the master mix with nuclease-free water. All primers were custom made. The forward primer was 5'-ATGAAACGATCCCCAGGG-3' and the reverse primer was 5'-ACTGATGTAACACTGGTCT-3' and they targeted an 880 nucleotide-long segment in the CDV nucleocapsid gene. Conditions were 30 min at 50˚C, 15 min at 95˚C, 40 cycles of 30 s at 94˚C, 30 s at 50˚C and 1:30 min at 72˚C; and a final step of 10 min at 77˚C. Amplicons were run in 1% agarose electrophoresis gel and presence of CDV was confirmed by identification of a band of expected size.

Antivirals

All antivirals used in this study were tested at concentrations of 5, 1.5 and 1.25 µM, unless stated otherwise. Drugs were diluted in dimethyl sulfoxide to concentrations of 100 µM and then in cell culture media to working concentrations. The list of drugs tested includes two flavonoids, rutin (Rutin hydrate, Sigma) and hesperidin (Sigma Aldrich); favipiravir; nirmatrelvir; and the nucleoside analogs remdesivir, ribavirin, GS-441524, EIDD2801, and EIDD1931 (Natural Micron Pharm Tech).

Antiviral screening

The viral plaque assay was used as described previously.[27] Briefly, 96-well plates (Corning) were seeded with 10^4 Vero dogSLAM cells and infected with CDV at confluency. Six-well replicates of infected cells were treated with antiviral compounds and compared to controls run in parallel. The controls included untreated infected cells, uninfected treated cells and wells with virus but no cells. Wells were infected at the point of cell confluency, with 200 µl of media containing CDV at a multiplicity of infection (MOI) of 0.04 for one hour. Antiviral treatments were then added at a defined final concentration for each well. Tissue culture plates were incubated at 37 ˚C in an atmosphere of 5% CO_2 for 3 additional days. 100 µl of supernatant was collected for RNA extraction. The adherent cells were then fixed with buffered 10% formalin and stained with 0.5% crystal violet (Fisher chemical). Cell death was evaluated by loss of the monolayer compared to mock-infected control cells as quantified by light absorbance. The absorbance for each well was measured at 620 nm using an ELISA plate reader (FilterMax F3 [Molecular Devices] and Softmax Pro [Molecular Devices]). The mean absorbance and standard deviation of the mean measurements of the six-well replicates were used for statistical analyses. For agents that demonstrated antiviral efficacy (reduction in virus-associated cell death), the half-maximal effective concentration (EC_{50}) was determined by plotting a nonlinear regression equation (i.e. a dose–response curve) using GraphPad Prism version 9.4.1 for MacOS (GraphPad Software, San Diego, California, USA).

Quantitative RT-PCR:

One step qRT-PCR was performed using AgPath-ID One-step RT-PCR Kit (Applied Biosystems) in a 7500 Fast & 7500 Real-Time PCR System (Applied Biosystems). PCR conditions were as follows: 10 min at 42˚C, 6 min at 95˚C and 40 cycles of 3 s at 95˚C and 30 s at 60˚C. The forward primer was 5'-AGCTAGTTTCATCTTAACTATCAAATT-3', the reverse primer was 5'-TTAACTCTCCAGAAAACTCATGC-3' and the probe was 5'-/5-FAM/ACCCAAGAGCCGGATACATAGTTTCAATGC/36-TAMSp/-3'. Efficacy of treatments was calculated as Δ(40-Ct), representing the 40-Ct difference between each measurement and the average Ct for untreated cells for the same viral isolate. For statistical analysis, Δ(40-Ct) values

were compared, as well as $2^{\Delta(40-Ct)}$ to accurately represent viral RNA copy reduction. Therefore, a value of 1 (Δ=0) indicated no difference between treated and untreated cells.

Statistical analysis

Normality of data was tested using Wilcoxon-Shapiro test. A Two-way ANOVA, or its non-parametric equivalent, was used to detect differences in absorbance and Ct (viral RNA loads) between different qRT-PCR products. A pairwise comparison was performed using Tukey's test. To calculate antiviral efficacy for viral replication, one sample t-test was used to compare Δ(40-Ct) with 0 (no effect). All p values ≤ 0.05 were considered statistically significant. The analyses were performed using GraphPad Prism version 9.4.1 for MacOS (GraphPad Software, San Diego, California, USA).

Results

Cell culture monolayers infected with CDV developed visually apparent viral plaques and attenuation of the cellular monolayer (Fig. 1). Treated, mock-infected cells did not demonstrate plaques even at the highest concentration tested for every compound. The only exception was a slight decrease in cell density with 5 µM favipiravir treatment (t-test, p-value<0.05).

The cytopathic effect of the America-4 isolate, GA-299, was reduced relative to the other viral isolates, even at the same concentrations of virus. Since cell death was not visually quantifiable even in the absence of treatment, protective effects of antiviral drugs could not be tested for this isolate, although changes in viral RNA production were quantified since they were measurable with qRT-PCR.

Five µM GS-441524 was the most effective treatment at preventing virus-associated cell death (p<0.0001, Tukey's test) for both CA-914 and NH1. At this concentration, GS-441524 was able to reduce cell death enough that absorbance was comparable to levels comparable to those of mock-infected cells

(Fig. 3). Remdesivir was also associated with a degree of protection relative to untreated infected cells ($p<0.001$, Tukey's test), and this protection was higher than all the other drugs except GS-441524 ($p<0.01$, Tukey's test). Both GS-441524 and remdesivir were had significantly higher absorbance than untreated wells ($p<0.01$, Tukey's test). All of the other antiviral agents had little to no effect at preventing visual evidence of cell death (figs. 2 and 3). We determined the EC_{50} for GS-441524 in CA-914 to be 3.95 μM (Fig. 2).

However, 5 μM nirmatrelvir, remdesivir, GS-441524, EIDD2801, and EIDD1931 all significantly reduced the amount of viral RNA produced for two viral strains, GA-299 ($p<0.01$, one sample t-test) and CA-914 ($p<0.001$, one sample t-test). For NH1, the only effective treatment was GS-441524 when compared to untreated infected cells ($p<0.001$, one sample t-test). The treatment with GS-441524 had the largest effect at reducing viral RNA production in all viral strains with up to 4 logarithmic reductions in viral RNA loads for all viral strains ($p<0.0001$, two way ANOVA, figs. 4, 5 and 6). There were no differences in viral RNA reduction when comparing among nirmatrelvir, remdesivir, EIDD2801, and EIDD1931.

Hesperidin and rutin did not demonstrate any efficacy in preventing cell death or viral replication at any tested concentration up to 100 μM. The same was true for ribavirin at concentrations up to 20 μM (data not shown).

Discussion:

In this study we determined the efficacy of several antiviral compounds with a reported ability to prevent viral replication and cell death. At least one candidate drug, the nucleoside analog GS-441524, showed promising antiviral results, suggesting that this agent is a promising candidate for antiviral assessment in CDV-infected dogs or other mammals.

The importance of developing an efficient treatment for canine distemper is clear from the number of studies attempting to identify effective compounds at reducing the viral activity of CDV *in vitro*.[60–62] In spite of this, there is no current effective antiviral treatment for distemper. The identification of multiple effective antiviral treatments with different mechanisms of action is important to ensure the availability of therapeutic options under the emergence of resistant viral strains, as it can happen in RNA viruses such as SARS-CoV-2.[63]

We identified no drug-associated cytotoxicity at 5 µM for all of the compounds except for favipiravir. This concentration is higher than the EC_{50} for any related viruses in all the drugs. The most effective drug in our study was the nucleoside analog GS-441524, a metabolite of the prodrug remdesivir.[41,64] GS-441524 has good oral bioavailability, and it seems to lack adverse reactions even at high doses, with a 50% cytotoxic concentration that is consistently above 50 µM.[65] This medication is well tolerated in dogs orally, at maximum feasible doses of 2,000 mg/kg.[66] In comparison the equivalent dose of GS-441524 to potentially treat humans for COVID-19 has been calculated to be less than 4 mg/kg, and concentrations of 13 mg/kg have been administered to a healthy human without major adverse reactions.[65–67] In cats, GS-441524 has been proven effective experimentally in subcutaneous doses of 4 mg/kg,[28,29] and it has been used in an unlicensed manner with subcutaneous concentrations of up to 25 mg/kg without major complications.[68] In measles virus a morbillivirus related to CDV, the EC_{50} for GS-441524 has been calculated to be 0.58 µM in Vero cells.[41] In our study, GS-441524 EC_{50} was 3.95 µM, but was determined using cell protection rather than a fluorescent reporter.

Remdesivir, the prodrug of which GS-441524, also demonstrated a protective effect on cells infected with CDV, although at a smaller magnitude. The EC_{50} of remdesivir for MeV has been determined to be 1 to 4.97 µM, depending on the study.[41,44] Despite the noticeable protective effect of remdesivir in our

study (Fig. 3), higher concentrations than the ones tested in this study would be needed to calculate an accurate EC_{50} for remdesivir (Fig. 2). EIDD1931 and its prodrug EIDD2801 have previously been demonstrated to be efficacious for the treatment of RNA viruses such as coronaviruses, orthomyxovirus and paramyxoviruses and their EC_{50} values range from 0.006 µM to 3.7 µM. However, these agents did not have a clear protective effect in our study.[27,47,48]

Our qRT-PCR assay targeted the viral nucleocapsid gene, which is the first gene to be transcribed and replicated during CDV infection. In the case of GA-299, viral RNA production was measurable, despite not causing cytopathic effect. It is possible that some level of viral RNA replication was taking place, but damage to infected cells was limited by defects in completion of the viral infection cycle, which requires replication, translation and assembly of all the viral genes downstream of the nucleocapsid gene. The measurement of viral RNA reduction proved to be a more sensitive method of detecting antiviral efficacy relative to visual evidence of cell protection. It is possible that their antiviral effects were not enough to prevent cell death or that they just reduced viral RNA production, but not infective particle production. In accordance to absorbance values, GS-441524 was the most effective treatment in all viral isolates. Nirmatrelvir, remdesivir, GS-441524, EIDD2801, and EIDD1931 all had similar inhibitory effect except for the viral isolate NH1, for which they did not reduce viral RNA production. The isolate CA-914 was more sensitive to antiviral treatments. These results highlight the importance of using diverse viral isolates to test the efficacy of antiviral treatments against variations of the virus.

Nirmatrelvir is an effective and approved treatment for COVID-19, and it acts by inhibiting a specific protease of coronaviruses.[69,70] As such, it was not expected to have an antiviral effect on other RNA viruses that lack this protease, such as CDV. However, it did reduce viral RNA production in our experiments. It is possible that nirmatrelvir operates in different, still undescribed ways to block viral replication. The agents EIDD1931 and EIDD2801 were also not effective at preventing cell death, but they considerably reduced viral RNA loads. These broad spectrum ribonucleoside inhibitors have been

tested in RNA viruses, but the closest relative of CDV for which they have shown an antiviral effect is respiratory syncytial virus. Respiratory syncytial virus belongs to the same order as CDV, but it is in a different family, *Pneumoviridae*.[71]

Despite previous reports of the effectiveness of rutin,[40] hesperidin,[40] favipiravir,[50] or ribavirin[49,72] in the reduction of CDV replication *in vitro,* we did not see any protective effects. There are many instances in which our model differs from that of previous studies. Most of the previous studies focused on viral replication, rather than pathogenic effects. Additionally, these antiviral drugs were tested in Vero cell cultures, that were not modified to express CDV specific receptors. In the case of rutin and hesperidin, cells were infected with Rockborn CDV, an ancient lineage that is only distantly related to the current circulating lineages.[73] Similarly, investigations of the efficacy of ribavirin for limiting CDV infection, used the vaccine strain Onderstepoort, which belongs to the America-1 lineage.[73] For favipiravir, the specific lineage of the virus used was not disclosed, but it probably belonged to the Asian clade. The CDV isolates used in our study belong to America-3 (CA-914), America-4 (GA-299) and Rhode island-like (NH-1), all of which currently cause disease in wildlife and dogs in the USA.[18] It is difficult to attribute the differences in outcomes between studies only to the use of different lineages of CDV. However, lineages differ mostly in the surface glycoprotein hemagglutinin, and one of the proposed antiviral mechanisms for flavonoids involves interaction with viral envelope glycoproteins, which would provide a reasonable explanation at least in this group of drugs .[39]

It is also noteworthy that differences between the pathogenesis of viral strains pathogenesis was evident in our study. Despite equivalent initial infectious doses, GA-299 had a markedly lower cytopathic effect that made the effects of antiviral compounds less evident. This, once again, underscores the importance of using several, diverse viral isolates when testing the effectiveness of antiviral treatments.

Our study showed that several nucleoside analogs have a strong effect on reducing CDV pathogenesis and replication *in vitro*, even in different CDV isolates, demonstrating that they are great candidates to advance into preclinical studies for the treatment of distemper. Further research of their capacity to treat distemper *in vivo* is warranted.

Isolate	Lineage	Origin species	Organ	Geographical origin
GA-299	America-4	(Raccoon, *Procyon lotor*)	Brain	Louisiana
CA-914	America-3	(gray fox, *Urocyon cinereoargenteus*),	Brain	California
NH1	Rhode Island	(gray fox, *Urocyon cinereoargenteus*),	Lung	New Hampshire

Table 1. Summary of isolates used in this study. CA-914 was collected in our institution while GA-299 and NH1 were donated by Nicole Nemeth from the University of Georgia and David Needle from the University of New Hampshire, respectively.

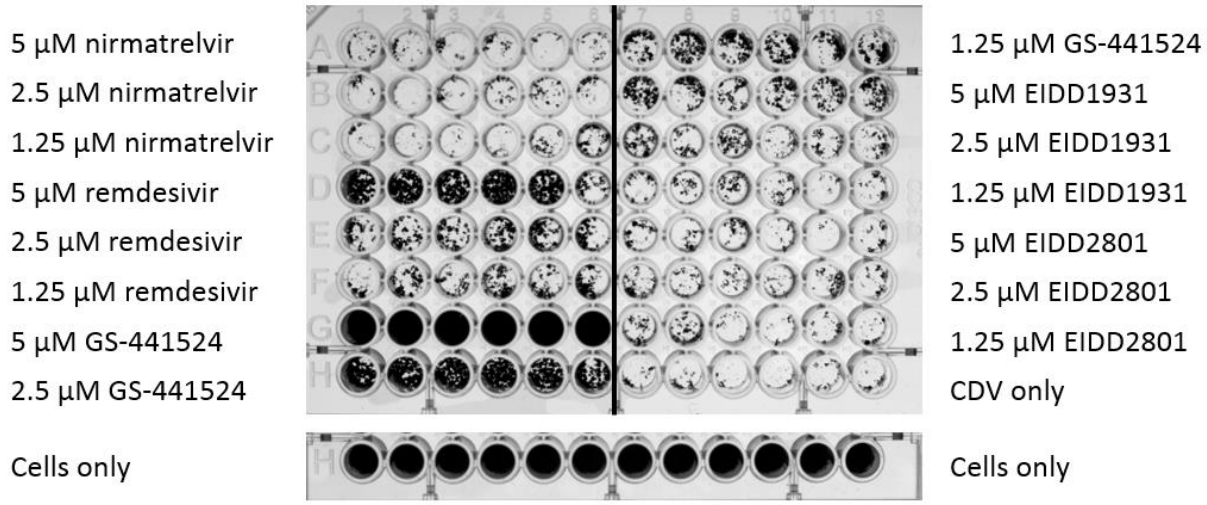

Figure 1. Example plate of antiviral screening. Vero dog-SLAM cells were infected with canine distemper virus (CDV) and different concentrations of the antiviral candidates indicated. Plaques and monolayer confluency were measured by staining the monolayers with crystal violet and measuring the absorbance at 620 nm. GS-441524 and remdesivir demonstarted antiviral effects, but there was no significant difference between wells treated with nirmatrelvir, EIDD1931 and EIDD2801 in comparison to the untreated wells.

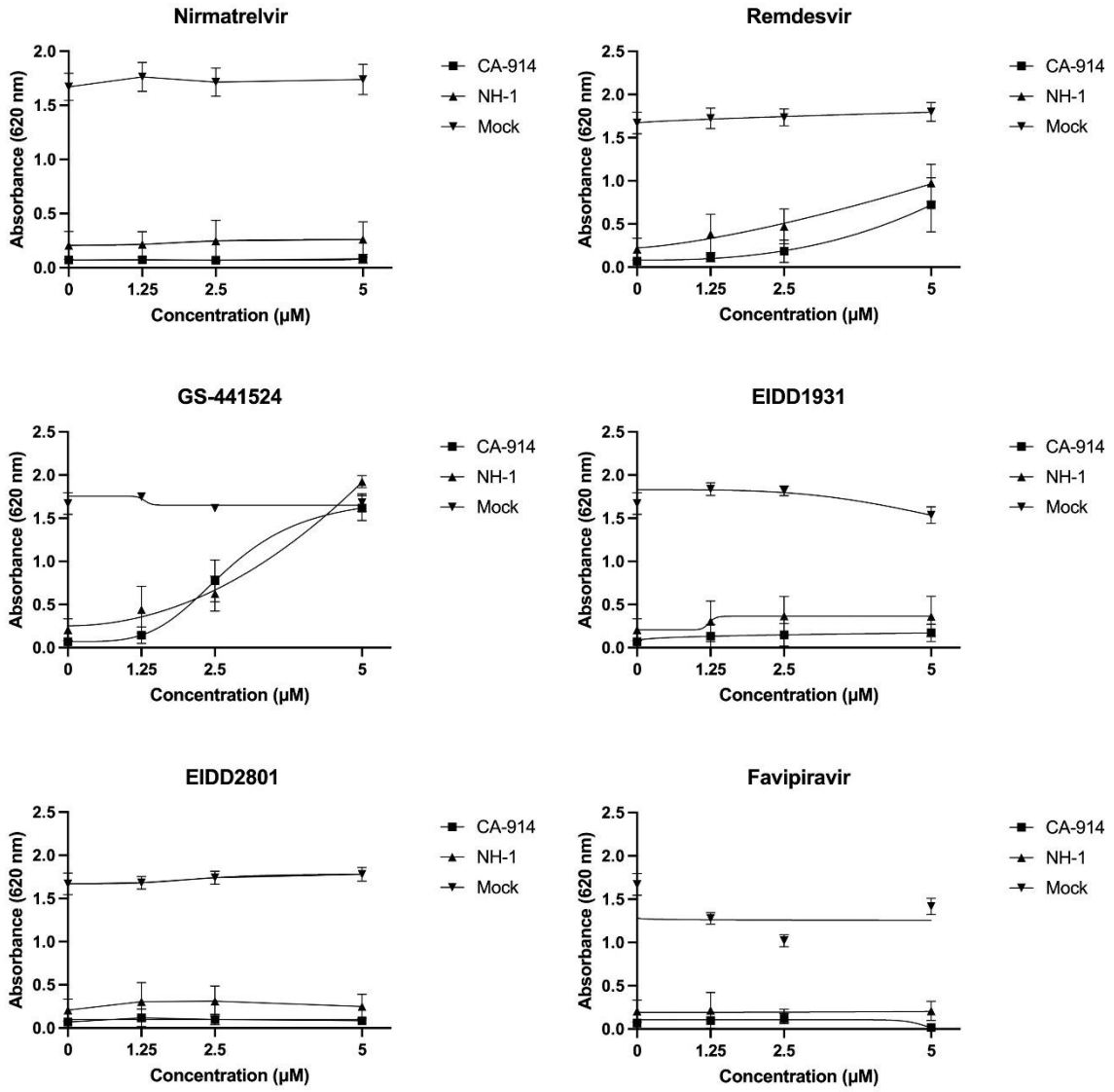

Figure 2. Effectiveness of antivirals at reducing cell death by concentration of antiviral treatment. Monolayer integrity was measure by absorbance at 620 nm after staining with crystal violet. Remdesivir and GS-441524 had a significant increase in absorbance that correlated with higher concentrations of the antiviral drug (p-value<0.05).

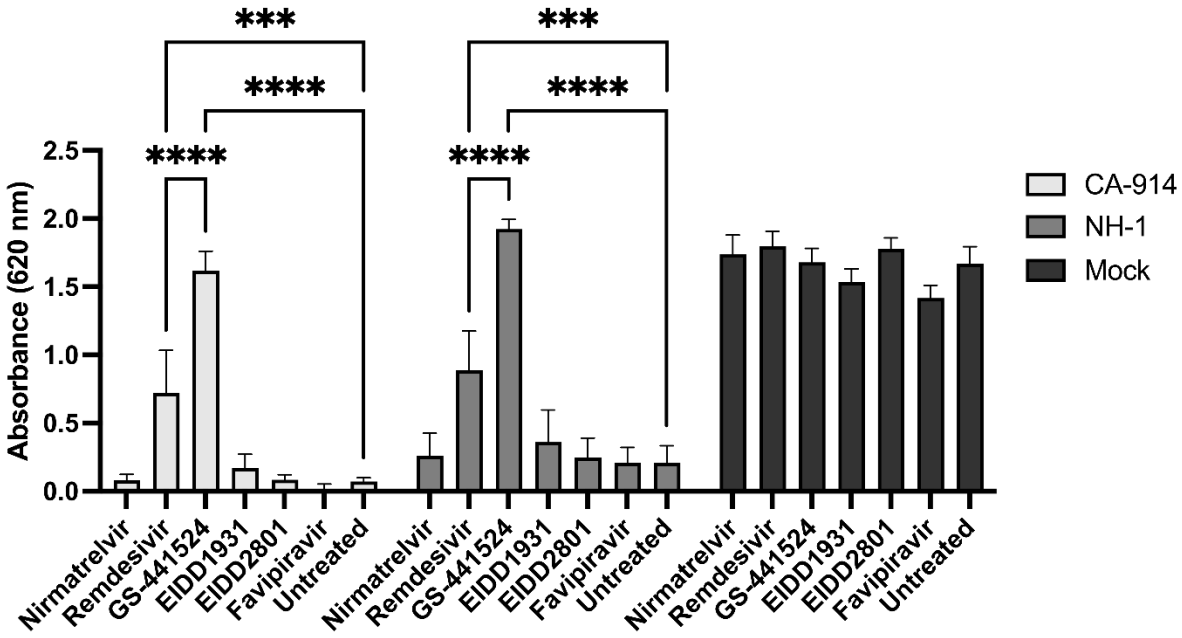

Figure 3. Effectiveness of antivirals at reducing cell death at 5 µM. Monolayer integrity was measured by absorbance at 620 nm after staining with crystal violet. GS-441524 and remdesivir, had a significant increase in absorbance that correlated with higher cell survival than any of the other treatments (statistical significance not shown, p-value<0.01). GS-441524 had increased absorbance compared to remdesivir for both NH-1 and CA-914 (p-value<0.001).

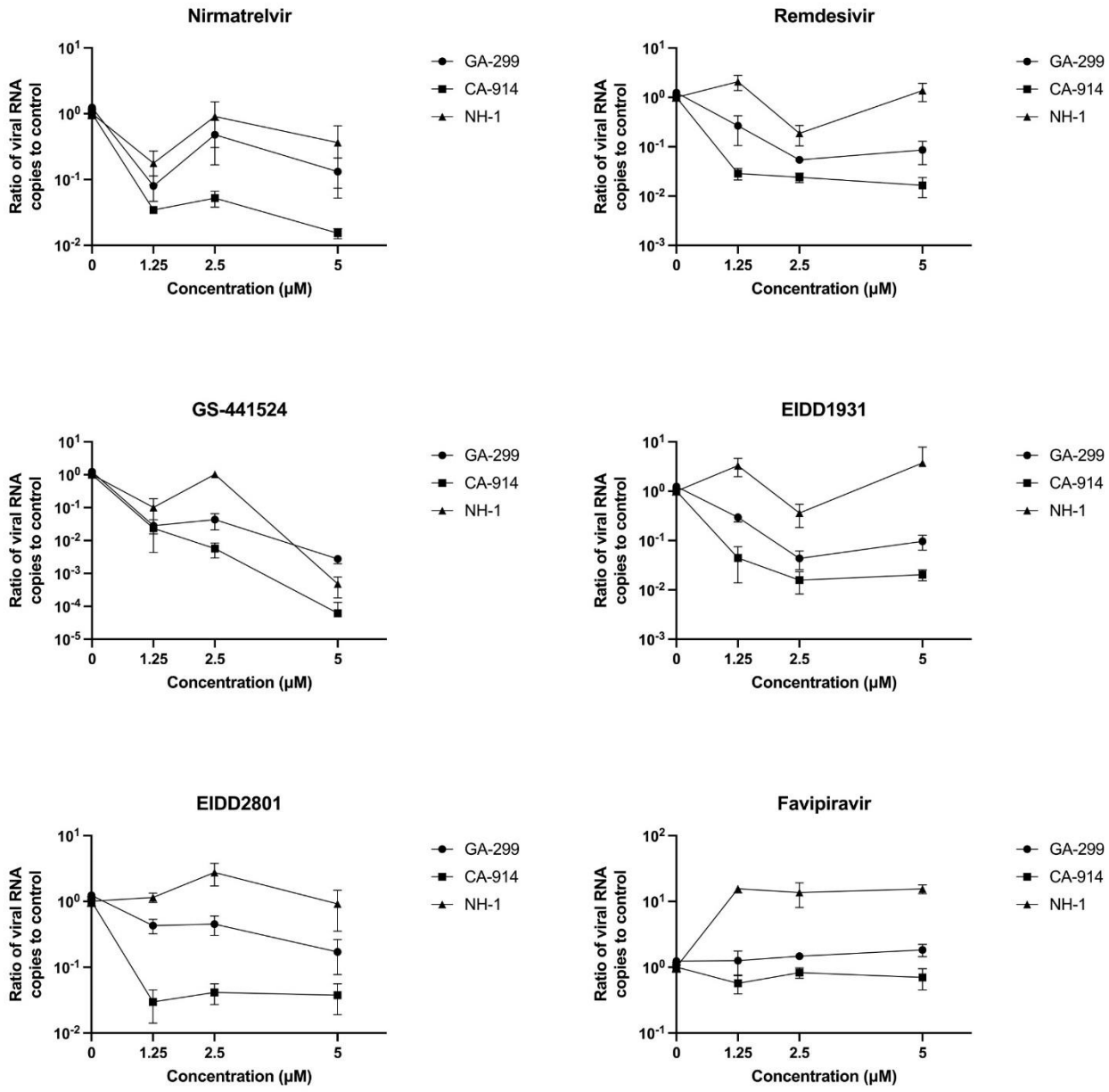

Figure 4. Effectiveness of antivirals at reducing viral RNA load by concentration of antiviral treatment. RNA reduction was calculated as RNA load reduction compared to untreated infected controls.

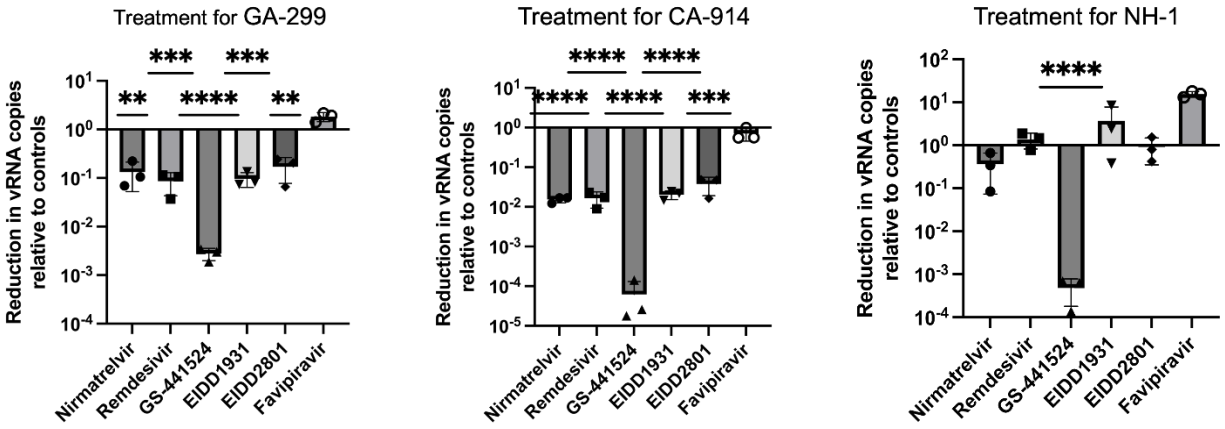

Figure 5. Effectiveness of antivirals at reducing viral RNA load at 5 µM. RNA reduction was calculated as $2^{\Delta(40\text{-}Ct)}$ for reduction respect to untreated infected controls. All treatments but faviparavir, especially GS-441524, significantly reduced viral load in GA-299 (p-value<0.01), and CA-914 (p-value<0.001), while only GS-441524 reduced viral RNA load in cells infected with NH1 (p-value<0.001).

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