Canine Distemper Virus in Wildlife: Knowledge and Gaps in Understanding

By

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Abstract

Canine distemper virus (CDV) is a highly contagious respiratory pathogen that infects a broad range of terrestrial carnivores with documented spillover into marine mammals. The virus is particularly prevalent in urban wildlife, but the transmission dynamics that maintain the virus in wildlife are unclear. We conducted a survey of the literature to summarize what is known about CDV in wildlife and to identify gaps and biases in our understanding of this virus. Though CDV is well characterized in dogs, gaps and biases limit our understanding about CDV in wildlife including how the virus evolves within and between hosts. We present two original contributions that aim to address these gaps in knowledge – a proposed study to evaluate a commercially available lateral flow diagnostic for use in raccoons and the results of an initial study to look at signatures of positive selection in each of the proteins CDV encodes. A sensitive and specific point of care diagnostic could be used at wildlife rehabilitation centers, not only to potentially prevent unnecessary euthanasia of an uninfected animal, but also to identify infected animals for further testing of wildtype viral genomes. The results of our positive selection study indicate that additional proteins encoded by CDV, beyond the hemagglutinin and fusion proteins, which are commonly cited in the literature, may also be under positive selection pressure. These results indicate more detailed studies may be warranted for a deeper understanding of selection pressures on the virus.

Introduction

Instead of specializing in one host, like HIV or measles, CDV routinely infects multiple species and has an expanding host range.[1] CDV was first identified in domestic dogs in the 1700s but is capable of infecting over 90 species ranging from marmots to elephants.[2] Disease caused by distemper was described in South America in 1746 and in Europe by 1770.[3] Edward Jenner described the disease in 1809, noting "it is as contagious among dogs as the smallpox, measles, or scarlet fever among the human species" and that "the animal which has once gone through it, very rarely meets with a second attack."[4] Over 200 years later, Jenner's observations have now been well established - that CDV, like other morbilliviruses, is highly contagious and infection produces what is considered lifelong immunity. Because of their high reproductive number, morbilliviruses require high levels of vaccination to prevent outbreaks.[5,6] A vaccine for CDV exists and is widely used; however, CDV remains a threat not only to dogs, but to wild carnivores as well. CDV is especially prevalent among canids, including dogs and foxes, but notable outbreaks have also occurred in endangered animals such as black-footed ferrets and Caspian seals, and in species of economic importance, such as farmed mink and laboratory macaques.

Since 1951, over 2,500 journal articles have been published on CDV (Figure 1), supporting a wealth of knowledge on the virus, its host and geographic range, and the disease it causes. However, there are still many gaps in our understanding of CDV biology, including the molecular mechanisms that underpin CDV evolution and adaptation to new hosts, and the frequency and dynamics of cross-species transmission events. Many of these gaps reflect a simple lack of research effort, while others reflect sampling and other forms of biases that may skew our interpretation of existing data.

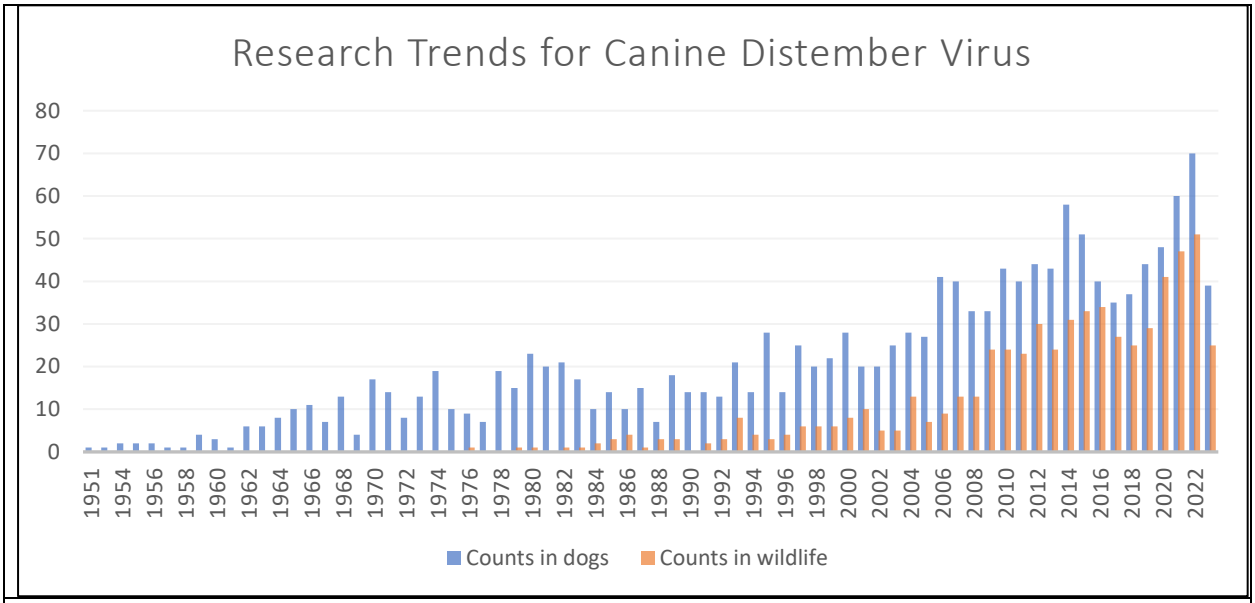


Figure 1. Histogram showing counts of journal articles indexed in PubMed pertaining to 'canine distemper virus' or 'canine morbillivirus' by host. This graph shows peer-reviewed articles about the virus have been published since at least 1951 in dogs and since the early 1990s in wildlife.

This thesis aims to summarize our understanding of canine distemper virus in wildlife. The goal of this work is to identify key gaps in our understanding of the virus in wildlife and to propose future avenues of research. Where appropriate, we draw comparisons with what is known about CDV in dogs for the purpose of illustrating gaps in our understanding of the virus in wildlife. The thesis is organized broadly into two parts: Part 1 describes how the virus is classified, how it functions and how it is diagnosed; Part 2 describes what we know about the global distribution of the virus, which animals it infects, and how it crosses species borders. Two key original contributions are also included, one for each of the two sections of this thesis. The first original contribution is a proposal to test a commercially available lateral flow assay developed for dogs in raccoons. The second is an evolutionary analysis focused on comparing signatures of positive selection in different CDV genes. In both cases, these contributions serve to catalyze new research directions and advance both clinical application and our basic understanding of the virus. Altogether, this thesis suggests we still have much to learn about the mechanisms that underpin CDV adaptation within and between hosts.

Part 1: Virology and pathogenesis

This section contains information on CDV taxonomy, virus structure and infection cycle, clinical disease and pathogenesis, and diagnostics. The key original contribution in this section is a proposal to evaluate the sensitivity and specificity of Abbexa's lateral flow assay for use in raccoons.

Taxonomy and classification

Canine distemper viruses (species *Morbillivirus canis)* are classified within the *Morbillivirus* genus and the *Paramyxoviridae* family. The International Committee on Taxonomy of Viruses (ICTV) currently lists seven morbillivirus species, including: *Morbillivirus canis* (prototype virus: canine distemper virus), *Morbillivirus caprinae* (prototype virus: peste des petits ruminants virus), *Morbillivirus ceti* (prototype virus: dolphin morbillivirus), *Morbillivirus felis* (prototype virus: feline morbillivirus), *Morbillivirus hominis* (prototype virus: measles virus), *Morbillivirus pecoris* (prototype virus: rinderpest virus), and *Morbillivirus phocae* (prototype virus: phocine distemper virus).[7] In addition to these seven established species, recent surveys of bats in South America have identified new putative species within the morbillivirus genus.[8,9] These novel bat viruses are most closely related to canine distemper virus and phocine morbillivirus and demonstrate that our knowledge of morbillivirus diversity is still far from complete.

Morbillivirus species are characterized primarily by host range and sequence divergence.[7] According to ICTV, species are defined phylogenetically based on the RNA-dependent RNA polymerase (RdRp) amino acid sequence. Viruses separated by a branch length of 0.03 or less are considered a single species within the *Morbillivirus* genus. Among terrestrial animals, measles virus primarily infects primates, rinderpest (eradicated in 2011) primarily infects cattle, and peste des petits ruminants primarily infects goats and sheep.[10,11] Among marine mammals, dolphin morbilliviruses are part of a larger group of cetacean morbilliviruses that primarily infect cetaceans (whales, dolphins, and porpoises), while phocine distemper virus primarily circulates among pinnipeds (seals, sea lions, and

walruses), which caused a large outbreak among Harbor seals in 1988.[12,13] Dolphin and phocine morbilliviruses have caused mass mortality events among marine mammals and thus pose a major conservation concern.

A review of the morbillivirus phylogeny reveals evidence of cross-species transmission events driving genetic diversification. Rinderpest and measles viruses are closely related and share a recent common ancestor (Figure 2).[14] It is thought that measles virus most likely evolved following the spillover of a rinderpest-like virus in cattle roughly 2,000 – 3,000 years ago, facilitated by close contact between humans and livestock.[15,16] Canine distemper and phocine morbilliviruses are similarly closely related (Figure 2), suggesting these two viruses may also have evolved into two discrete lineages following a cross-species transmission event. Though the origins of CDV are unclear, there is some evidence to suggest it was introduced into the Americas during colonization and then spread with the movement of dogs as the global pet trade established and grew.[17,18] Some groups suggest after evolving as a virus in dogs, CDV was then transmitted to marine mammals, where it served as the progenitor for what we now recognize as phocine morbillivirus.[19]

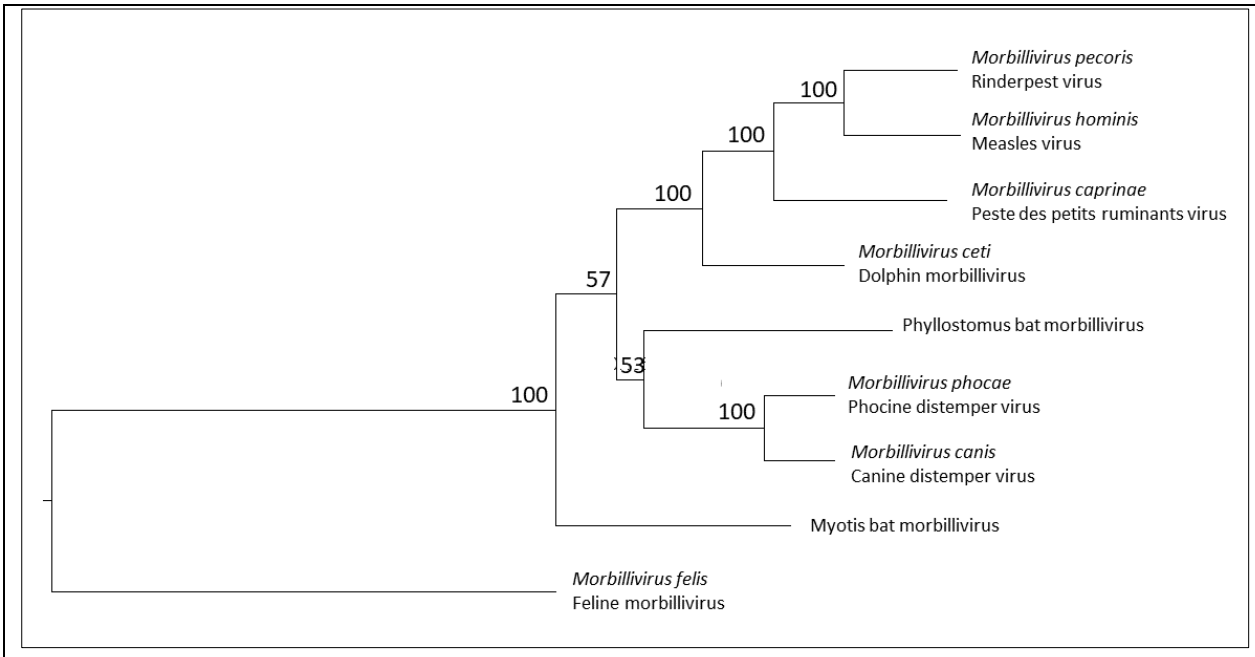


Figure 2. Evolutionary history of morbilliviruses was inferred using the Maximum Likelihood method in MEGA11. The tree is based on the complete RNA-dependent RNA polymerase sequence. Branch lengths measured in the number of substitutions per site. The tree was constructed using the General Time Reversible Model, Gamma Distributed with Invariant Sites (GTR + G + I) substitution model, which was determined to be the best fit model based on Akaike information criterion (AIC) in MEGA11. Confidence in inferred nodes was calculated using 100 bootstraps (values shown on tree nodes). The phylogeny is rooted with feline morbillivirus. Rooting the tree using a more distantly related virus, such as Sendai virus, produced the same arrangement. With the exception of Myotis bat morbillivirus, all viruses are officially recognized by the ICTV. Recognition of Myotis bat morbillivirus is pending.

Summary

Key findings: CDV is a member of the morbillivirus genus, which includes well known members such as measles virus and the now eradicated rinderpest virus.

Gaps: Recently discovered bat morbilliviruses, which are closely related to CDV based on the RdRp phylogenetic tree, indicate our knowledge of morbillivirus diversity is incomplete.

Future research directions: Additional surveillance of wildlife hosts is needed to reveal a more complete picture of the diversity of morbilliviruses circulating in nature. While surveillance efforts can be costly, large biobanks of samples already exist that could be utilized for these efforts. For instance, samples remaining from the PREDICT project, a large international surveillance project, could be used. Further, as investigators increasingly turn to next generation sequencing and submit raw data files to public SRA

databases, it may also become feasible to conduct in silico surveillance for morbillivirus sequences. Whatever the approach, describing unknown morbilliviruses in wildlife is necessary to support a deeper understanding of the ecological and evolutionary forces shaping the diversity and distribution of these important human and animal pathogens.

Virus structure and infection cycle

Like all morbilliviruses, CDV is an enveloped virus with a single-stranded, negative-sense RNA genome of approximately 15,690 nucleotides. CDV virions are pleomorphic and can be both spherical and filamentous, with a diameter range from 150-350 nanometers.[20] CDV encodes six structural proteins that are sequentially transcribed: 3' – nucleocapsid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin protein (H), and RdRp, also known as the long protein (L) – 5' (Figure 3). Two non-structural proteins, C and V, are expressed through translation of alternative start sites within the P gene and are involved with immune evasion and virulence.[21,22,23,24] The V protein acts as a virulence factor by blocking the host's interferon response while the C protein acts as a polymerase cofactor to regulate viral RNA synthesis.[25] The N protein encapsidates and protects the RNA genome and together the N, P, and L proteins form the ribonucleoprotein complex within the virion.[26] Each N protein monomer interacts with exactly 6 nucleotides, leading to an interesting feature common to all paramyxoviruses known as the 'rule of six'.[27] Because each N protein interacts with six nucleotides, paramyxovirus genomes are always multiples of six nucleotides (i.e., the full genome length can always be divided perfectly by six). The viral envelope, which consists of the M, F, and H proteins, surrounds the ribonucleoprotein complex.[26] The M proteins within the envelope help stabilize the virion and the spike proteins, H and F, facilitate attachment, fusion and entry into host cells. Neutralizing antibodies are induced against the H and F proteins. Therefore, it is thought that in response to the selection pressure imposed by neutralizing antibodies against the H protein, in particular, the H protein maintains the highest degree of genetic diversity.[28]

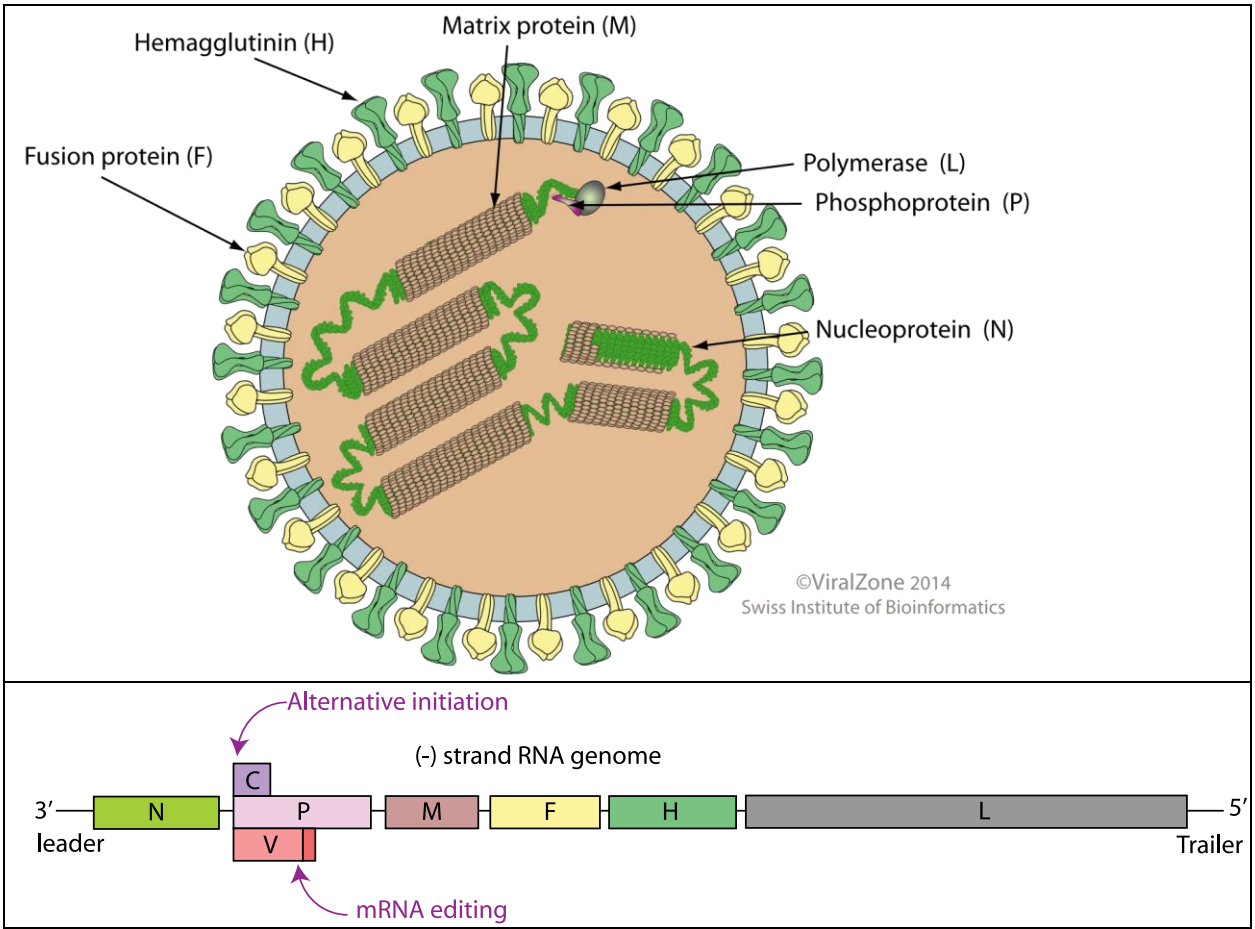


Figure 3. Schematic depiction of the morbillivirus virion (top panel) and genome (bottom panel) depicting the coding sequences 3′ – nucleocapsid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin protein (H), and RNA-dependent RNA polymerase, also known as the long protein (L) – 5′. Non-coding sequences between genes contain start, stop, and polyadenylation signals for mRNA transcripts. Image credit: ViralZone (available here).

Binding of the H protein to a host-cell receptor triggers a conformational change that drives fusion of the F protein to the cell membrane and facilitates the release of the ribonucleoprotein complex into the cell cytoplasm.[29] The RNA-dependent RNA polymerase (RdRp) then initiates sequential transcription of genomic RNA, leading to the accumulation of mRNA transcripts, which are capped and polyadenylated. Once sufficient N protein is synthesized (presumably enough to encapsidate new viral genomes) the N protein binds nascent RNA, which signals the RdRp to start replicating complete positive sense genomes (antigenomes) that serve as templates for the negative sense RNA genomes.[26] Thus, synthesis of viral proteins rapidly amplifies. The P protein binds to the RNA:N protein complex, as well as

the L (RdRP) protein, and serves as a cofactor to facilitate replication by the polymerase.[30] The M protein facilitates virus assembly by interacting with the ribonucleoprotein complex to drive transport to the plasma membrane and assembly of viral particles.[31] During assembly at the cell membrane, F and H proteins are incorporated into the virus envelope and progeny viral particles are released through budding. A summary of the replication cycle is provided below in Figure 4.

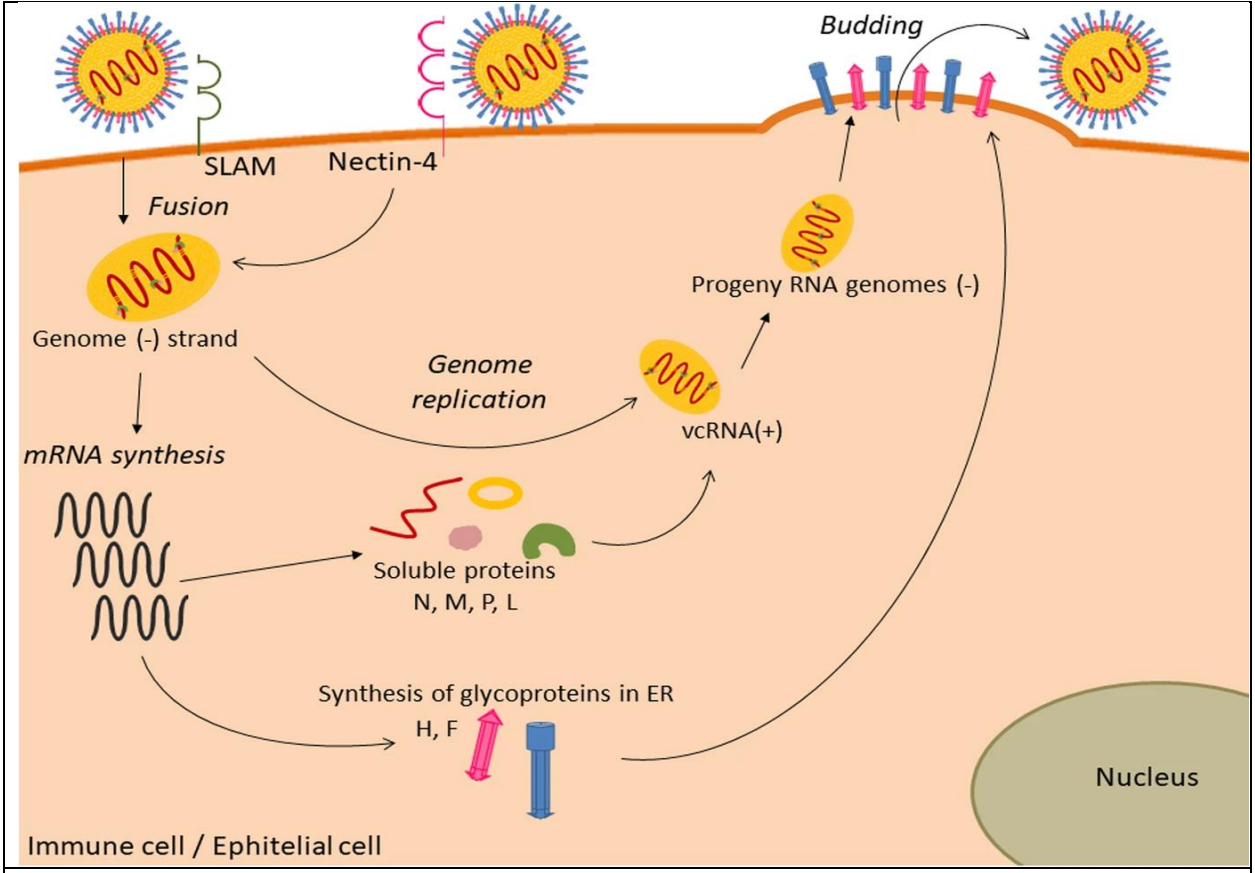


Figure 4. Replication of the CDV cycle. Virus particle recognition by host cell receptors (CD150 or nectin-4), RNP release into the cytoplasm, replication, transcription process, and virus particle budding are illustrated. Image from Rendon-Marin, S., da Fontoura Budaszewski, R., Canal, C.W. et al. Tropism and molecular pathogenesis of canine distemper virus. Virol J 16, 30 (2019).

An interesting feature of morbilliviruses is that they are dual-tropic, first using SLAM/CD150 as a receptor to enter alveolar immune cells (primarily macrophages and dendritic cells) and later using nectin-4, a cell adhesion molecule, as a receptor to enter lung epithelial cells.[32] After SLAM-mediated entry into alveolar immune cells in the lung and initial replication in regional lymph nodes,

morbilliviruses then move into the bloodstream (i.e., viremic phase) where they infect other lymphoid organs, such as the spleen, thymus, appendix, and tonsils. Accordingly, the first stage of infection with a morbillivirus is typically marked by profound immunosuppression due to the destruction of immune cells.[33] When infection in the lymphatic system reaches its peak, morbilliviruses begin to infect lung epithelial cells using nectin-4 expressed on the basolateral surface of lung epithelial cells.[34] This switch in receptor use from SLAM early in infection to nectin-4 late in infection is a hallmark of morbilliviruses. Infection in the lung epithelium results in virus assembly and release of virions into the airway, where it can then be transmitted to the next host. The late stage of infection is essential for transmission of the morbillivirus to susceptible hosts.[35] Experimental animal models show in vivo that measles virus and CDV that cannot bind nectin-4 do not shed virus in the airways, thereby preventing efficient transmission.[35,36] Therefore, nectin-4 appears to play a pivotal role in viral transmission during the late stages of infection.[34] Similarly, the recently identified Myotis bat morbillivirus also depends on nectin-4 for replication in an epithelial cell line.[37] Cyclical adaptation of MeV populations have been shown to facilitate binding first to immune cells then to epithelial cells in vitro, but it remains unknown whether CDV populations also facilitate binding to diverse tissues throughout the course of infection.[38]

Summary

***Key findings*:** CDV encodes six structural proteins and two nonstructural proteins that are involved in immune evasion and virulence. The hemagglutinin and fusion proteins are recognized by the immune system and targets of host antibodies. CDV infects first lung immune cells using SLAM, then lung epithelial cells using nectin-4.

***Gaps*:** The mechanisms by which CDV adapts to different tissues during infection is unknown. One possibility is that, similar to MeV, CDV maintains sufficient fine-scale genetic diversity (i.e., exists as a quasi-species) during infection that it can readily adapt to different tissues within a single host. If true, the switch from SLAM to nectin-4 would presumably impose significant selection pressure akin to a

bottleneck and dramatically alter the distribution of variants towards those with a nectin-4 binding phenotype. While plausible, this model does not explain how the virus then re-acquires a preference for SLAM after transmission to a new host. How CDV switches from SLAM to nectin-4 and then back to SLAM remains an open question.

***Future research directions*:** The first step to understanding the role of intra-host selection on cell tropism could be to deep sequence the virus from different tissues over the course of infection. Such a study would require an animal experiment to obtain a time course of infected tissues. Deep sequencing of CDV genomes from different tissues would facilitate the comparison of viral population variants (i.e., mutations) by tissue type and over time. A dominance of mutations from virus in the lungs early in infection that is different from the distribution of mutations from virus in other tissues later in infection would indicate a role for selection in driving changes in receptor use. Future research could also address why CDV, and paramyxoviruses more generally, switch the receptors they use. One could assume the switch provides an advantage in transmission, but empirical evidence to support this hypothesis is lacking. Studies to understand the relative fitness advantage of using SLAM vs. nectin-4 may provide further insight into CDV life history.

Pathogenesis and clinical disease

CDV pathogenesis in dogs is well documented based on experimental and naturally occurring infections. CDV is highly contagious and spread through the respiratory route, primarily through contact with droplets or aerosols.[30,37] Upon inhalation into the lungs, CDV first replicates in the lymphatic tissue of the upper respiratory tract where it replicates primarily in monocytes and macrophages in the lungs. This initial phase of viral replication occurs in the first 24 hours following infection. The virus then spreads through the lymphatic system to regional lymph nodes and tonsils.[39] Initial infection is marked by fever and immunosuppression caused by apoptosis of leukocytes. At 4-6 days post infection, CDV spreads more widely and can be detected in distal lymphoid tissue, including mesenteric lymph nodes

and the spleen.[39] This second phase of infection is marked by viremia and a second fever (biphasic fevers are typical of morbillivirus infections) as the virus spreads through the blood to infect parenchymal tissues in the respiratory tract, digestive tract, skin, and, in some cases, the central nervous system.[1] At this stage, 9-14 days post infection, disease manifestations can include respiratory signs, such as conjunctivitis, coughing, and nasal discharge, as well as gastrointestinal signs, such as vomiting, diarrhea, and dehydration. Secondary bacterial infections can exacerbate clinical signs. Lethargy and loss of appetite are common. Like other morbilliviruses, CDV can also infect epidermal cells, leading to the proliferation of footpad keratinocytes in many species (Figure 5).[40]

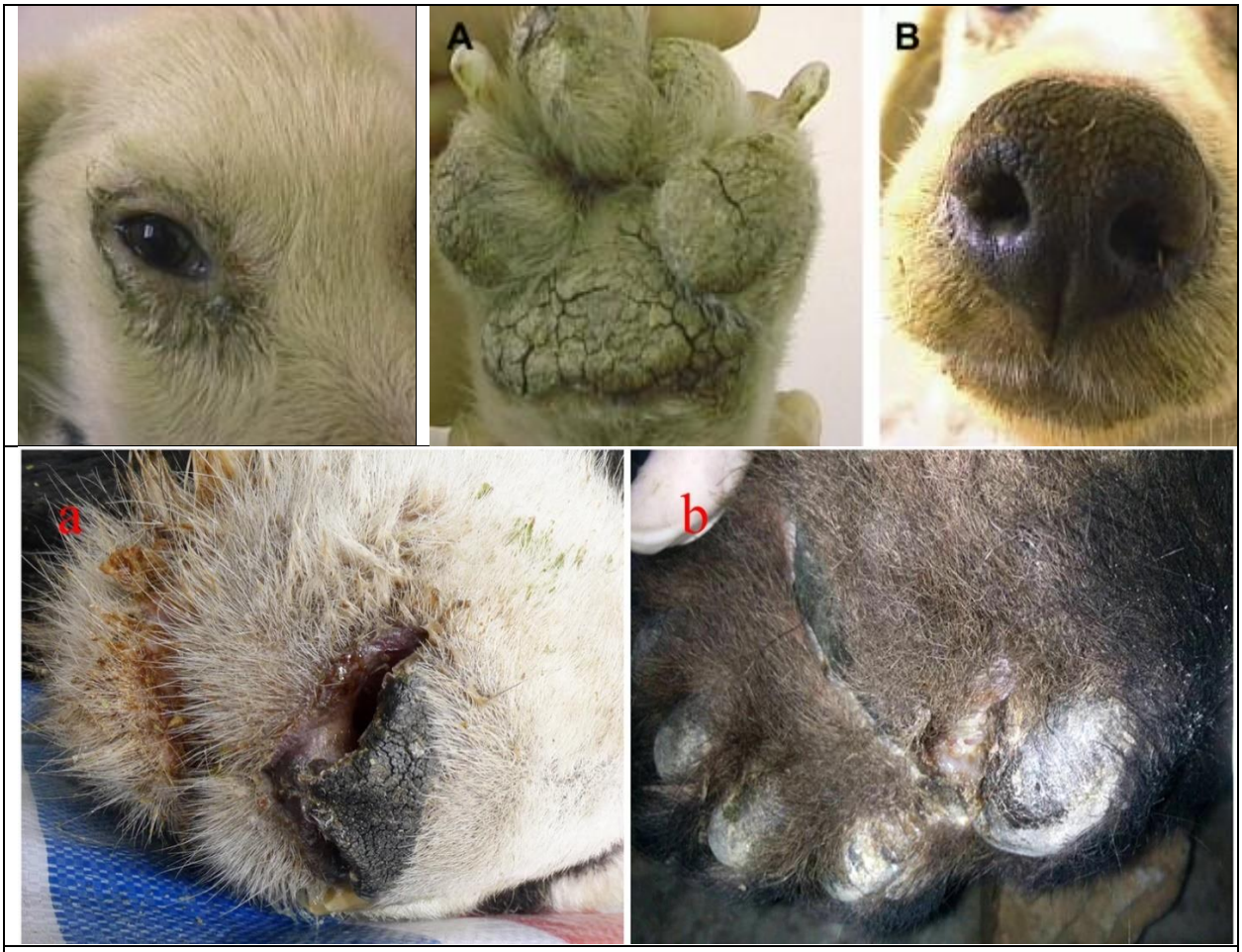


Figure 5: Top panel: Images from Martella V. et. al.: Dog with CDV infection showing conjunctivitis with periocular discharge (left) and hyperkeratosis of the foot pads (A) and nose (B). Images from: Vito Martella, Gabrielle Elia, and Canio Buonavoglia. Canine Distemper Virus, Veterinary Clinics of North America: Small Animal Practice, Volume 38, Issue 4, 2008, Pages 787-797, ISSN 0195-5616, https://doi.org/10.1016/j.cvsm.2008.02.007.

Bottom panel: Images from Feng, N. et al.: Giant panda with CDV infection showing a) nasal hyperkeratosis and (b) footpad hyperkeratosis. Images from Feng, N., Yu, Y., Wang, T. et al. Fatal canine distemper virus infection of giant pandas in China. Sci Rep 6, 27518 (2016). https://doi.org/10.1038/srep27518.

CDV strains are lymphotropic and epitheliotropic, but sometimes neurotropic as well.[41] In a ferret model, CDV can enter the central nervous system through the olfactory nerve or across the blood-brain barrier and can then be detected in neurons and glial cells 4-5 weeks post infection.[42] Although nectin-4 is expressed by neurons and may be the cell receptor for CDV entry into neurons, CDV has also been found in astrocytes, which do not express nectin-4 or SLAM; therefore, whether CDV uses a receptor to infect astrocytes and, if so, which receptor, is still unknown.[43,44] Neuroinvasion of CDV in dogs can cause fatal acute encephalitis. If the virus spreads to the central nervous system, neurological signs can include ataxia, seizures, and paralysis and is often fatal. The molecular determinants underlying CDV neuropathogenesis are largely unknown.[45]

Pathogenesis largely depends on the dog's immune response – a robust cellular and humoral immune response are required, especially the immediate generation of CDV-specific IgM antibodies, or the infection can be fatal.[1,33] Weak or delayed cellular and humoral immune responses can lead to virus spread and persistence, with sequelae that include lymphoid depletion and impaired immune function, similar to sequelae described for infection with measles virus.[33]

CDV disease is best characterized in dogs. In dogs, clinical manifestations of infection can range from subclinical to fatal, with an estimated 50% mortality rate. Clinical signs in dogs include fever, lethargy, and respiratory and gastrointestinal signs, such as coughing, nasal secretions, vomiting, inappetence, and diarrhea. Neurological signs, such as head tilt, paralysis, or trembling, can occur if the virus enters the central nervous system. CDV is a serious disease in dogs with mortality rates second only to rabies.

Clinical disease caused by CDV varies by species and can range from subclinical to fatal, depending on the virus strain, as well as host age and immune status.[46] Clinical signs in raccoons and

foxes range from fever, coughing, difficulty breathing, ocular discharge, diarrhea, and listlessness to ataxia, lethargy, convulsions, paralysis, emaciation, and coma.[47,48,49] Neurological signs in these animals are well documented, and include disorientation, a loss of fear of humans, and respiratory distress; these cases must be differentiated from rabies.[1,50,51] Humans may disproportionately see wild animals with advanced CDV disease simply because advanced CDV disease can lead to neurological and behavioral changes that render an animal unwary of humans.

Mortality rates vary widely in diverse species and could also depend on the CDV strain. For example, a CDV outbreak among terrestrial carnivores in Switzerland with particularly high mortality was attributed to genetic changes in the virus that increased its neurotropism, thereby increasing its pathogenicity.[52] In this case, there was a sudden emergence of clinical signs, including respiratory and neurologic disease, in red foxes and Eurasian lynx, compared to previous decades of surveillance indicating serologic evidence of exposure to CDV with no (observed) clinical cases.[52] Mortality rates of 50% have been estimated in raccoons and skunks, whereas CDV is considered almost 100% fatal in mustelids, such as ferrets, martens, and otters, as well as raccoon dogs (family: *Canidae*, genus: *Nyctereutes)*.[53,54,55,56,57]

Summary

***Key findings*:** CDV is lymphotropic, epitheliotropic, and often neurotropic, causing severe disease in a broad range of species. Clinical signs vary between species, but often include respiratory and gastrointestinal signs. Biphasic infections are typical of morbilliviruses and mark the transition from infection of lymphocytes to infection of lung epithelial cells.

***Gaps*:** The molecular mechanisms underlying why and how CDV sometimes breaches the central nervous system are undefined.

Future research directions: To determine whether there might be a genetic basis for CDV neurotropism, one could compare the pattern of mutations from CDV found in the central nervous system (brain and

spinal cord) with the pattern of mutations from CDV found in other tissues. Deep sequencing and genetic analysis could reveal whether specific mutations in the virus are associated with neurotropism. In addition, we would expect the patterns of mutation associated with neurotropism to be recapitulated in an in vitro model system. For example, we would expect neurotropic CDV to outcompete wildtype CDV as measured by growth curves in a nervous tissue cell line.

Diagnostics

Diagnosis of CDV in wildlife can be challenging. Generally, presumptive diagnosis of CDV is based on clinical signs, which can vary between species and include respiratory and neurologic signs that are also commonly caused by other diseases.[58,59,60] In addition, clinical signs may not be apparent in the early stages of infection.

Diagnostic tools exist to detect CDV, including virus isolation, nucleic acid detection, antigen detection, and antibody detection. Virus isolation is considered the 'gold standard' for diagnosis of CDV but is not always successful. There are numerous PCR based assays to detect viral nucleic acid, including quantitative reverse transcription PCR (qRT-PCR) to amplify portions of the viral genome, as well as deep sequencing assays (Illumina and Nanopore platforms) to determine full-length CDV genomes.[61] ELISAs have been developed to detect CDV antigens (H and F proteins) and antibodies generated against CDV. Immunohistochemistry is often used for postmortem detection of CDV in tissue samples.[62] A summary table of diagnostic assays for CDV from Karki et. al. is provided below (Table 1).[63]

Diagnostic tool	Target	Application	Application
Virus isolation	(MDCK, Vero cells-SLAM B95a, etc)	Virus	Gold standard test Highly sensitive, helpful for generating virus repository (Require live virus titre, specific cell line and cell culture facilities)
	Direct ELISA	CDV antigen	Detects antigen in serum
Antigen detection	Sandwich ELISA	H protein	High specificity Detection and quantitation
		F protein	Efficient in field application with fecal and serum samples
	Sandwich dot ELISA	Virus	Epidemiological surveillance
	LFA	F protein	Practically applicable in the field for quick diagnosis
Nucleic acid detection	RT-PCR	N gene	Standard laboratory test
	One-step nested-RT-PCR	N gene	100-fold sensitivity than RT-PCR and nested PCR
	Double step real time-RT-PCR	N gene	Highly sensitive and specific Quantitate viral load in clinical samples
	One-step real-time RT-PCR	--	To study viral replication and kinetics of viral RNA load in infection
	RT-LAMP assay	H gene	100-times sensitive than RT-PCR Only 1 hour reaction
Virus-specific antibody detection	ELISA	IgG Antibody	Detect within 6 days of infection Sensitive as SNT
	Dot blot assay	N-protein specific IgM	Detecting recent infections
	Capture sandwich ELISA	N-protein specific IgG & IgM Antibody	No Cross-reactivity with other Morbilliviruses

Table 1. Diagnostic tools for routine diagnosis of CDV in laboratory and field. Table from Karki, M., Rajak, K.K. & Singh, R.P. Canine morbillivirus (CDV): a review on current status, emergence and the diagnostics. VirusDis. 33, 309–321 (2022). https://doi.org/10.1007/s13337-022-00779-7

Molecular assays exist for detection of CDV among wildlife, including a pan-genotypic quantitative PCR (qPCR) that detects a conserved region of the P gene and can detect all globally available genotypes of CDV.[64] There are also nanopore protocols for sequencing full-length CDV genomes.[65] In this case, CDV genomes were sequenced from foxes with a pan-genotype CDV-specific amplicon-based sequencing method that was then optimized for the Nanopore platform.[65] However, qPCR and nanopore assays must be done in laboratories with appropriate equipment, which prohibits real-time clinical decisions to euthanize an animal or not. To this end, a qPCR based assay using the Biomeme platform has undergone preliminary testing for use in wildlife, but the assay is slightly less sensitive compared to lab-based methods.[66] For wildlife clinic settings, a lateral flow diagnostic that is affordable, portable, shelf-stable, easily used in-house by staff and trained volunteers without special reagents or machines, that can be used on non-invasive tissue samples, such as conjunctival swabs, and that provides a result within minutes would be ideal.[62,63]

A lateral flow diagnostic could prevent unnecessary euthanasia for animals presenting with clinical signs but not infected with CDV.[63] Multiple lateral flow tests have been developed, including a commercially available test developed by the British Manufacturer Abbexa, to detect CDV antigen in nasal fluid, serum, saliva and conjunctival secretions from domestic dogs; however, the assay has not been evaluated for use in other species.[67] Since morbilliviruses are considered monoserotypic and antibodies generated against one morbillivirus cross-react with antibodies generated against other morbilliviruses, it is likely that the canine antibody used in commercially available lateral flow assays for dogs would detect CDV antigen in wildlife, but studies to measure analytic and diagnostic sensitivity and specificity of a lateral flow assay in other species are needed.[15,16,68,69] Better diagnostic tools for detecting CDV in wildlife are needed.

Summary

***Key findings*:** There are a variety of ways to detect CDV in the laboratory.

***Gap*:** Clinical diagnostic tools for detecting CDV are needed, especially point-of-care antemortem diagnostics to facilitate clinical decisions. Such a tool could also be used to screen animals for CDV. CDV-infected animals, who would be humanely euthanized because of the infection, could also be identified to collect tissue samples for further testing, such as deep sequencing. Thus, the diagnostic tool could help facilitate generation of surveillance data, as well.

Future research directions: We propose a study to estimate the analytical and diagnostic specificity and sensitivity of Abbexa's lateral flow assay for detection of CDV antigen in conjunctival swabs from raccoons compared to a validated dual reverse transcription (RT)-qPCR assay for reference.

A lateral flow test used as a confirmatory point-of-care diagnostic in raccoons could help wildlife veterinarians confirm or refute presumptive CDV cases, thereby potentially preventing unnecessary euthanasia.

Original contribution 1: Proposal to evaluate Abbexa's commercially available rapid test for specific and sensitive detection of canine distemper virus in raccoons

Introduction:

Canine distemper virus (CDV) is a globally distributed respiratory virus that causes epizootic outbreaks with high mortality in urban wildlife, as well as species of economic and conservation concern.[70,71,72,73,74] CDV infects a broad range of carnivores and can be readily found in gray foxes, raccoons, coyotes, and striped skunks.[2] A study in Michigan from 2008-2018 found 10-year average prevalence rates of CDV antigen detected by immunohistochemistry of 79.4% in gray foxes, 59.6% in raccoons, 50.0% in coyotes, and 44.4% in striped skunks.[75] Animals presented to wildlife clinics with suspected CDV have poor prognoses and are humanely euthanized (personal communication with the lead veterinarian at Lindsay Wildlife Rehabilitation Hospital). A lateral flow test used as a confirmatory point-of-care diagnostic in raccoons could help wildlife veterinarians confirm or refute presumptive CDV cases, thereby potentially preventing unnecessary euthanasia. The purpose of this study would be to

evaluate the performance of a 'Canine Distemper Virus Antigen Rapid Test Kit' developed by the British manufacturer Abbexa for confirming CDV infection in raccoons. Abbexa's lateral flow test was developed for detection of CDV antigen in serum, urine, nasal fluid, saliva, and conjunctival secretions from domestic dogs, but the assay has not been evaluated for use in other species. Our research aims would be to estimate the analytical and diagnostic specificity and sensitivity of Abbexa's lateral flow assay for detection of CDV antigen in conjunctival swabs from raccoons compared to a validated dual reverse transcription (RT)-qPCR assay for reference.

Abbexa's lateral flow assay uses a conjugated antibody to detect CDV antigen in dogs, however it is unknown whether the antibody will detect CDV antigen in raccoons. Since morbilliviruses, like CDV, are considered monoserotypic and antibodies generated against one morbillivirus cross-react with antibodies generated against other morbilliviruses, we are confident the canine antibody used in Abbexa's lateral flow assay will detect CDV antigen from infected raccoons.[68,69] At the same time, cross-reaction could cause false positive results if a raccoon is infected with another morbillivirus, but the chances of a raccoon being infected with another morbillivirus are low since the other morbilliviruses are species-specific or eradicated. If Abbexa's lateral flow assay meets diagnostic cutoffs of 100% specificity and at least 90% sensitivity (since the test will be used as a confirmatory diagnostic rather than a screening tool we will not accept false positive results, but will accept a level of false negative results), this project will lay the foundation for further assay validation, including reproducibility, with the long-term goal of adoption by wildlife clinics as a point of care confirmatory diagnostic for CDV in raccoons.

Materials and Methods:

Study population, sampling strategy, and eligibility criteria: This proposed project leverages an established collaboration with Lindsay Wildlife Rehabilitation Hospital in Contra Costa County, CA, where antemortem conjunctival swabs and blood samples from raccoons will be collected by trained

personnel. The study population will include raccoons with or without suspected distemper who are presented to the clinic during a 1-year collection time frame (e.g., January 1, 2024 - December 31, 2024). Thus, our study will be based on a convenience sample of raccoons representing a subset of the larger target population of raccoons in Contra Costa County, CA. Inclusion criteria will include any raccoon, with or without suspected CDV infection, that arrives at Lindsay Wildlife Rehabilitation Hospital during the 1-year sample collection timeframe. Exclusion criteria are species other than raccoons.

Study design and data collection: The study design is a prospective case-control study in which a 'case' is defined as a raccoon with signs that lead the veterinarian to a presumptive diagnosis of CDV, which may include ocular or nasal discharge, emaciation, and abnormal behavior, such as ataxia and disorientation. 'Controls' are defined as raccoons without signs of CDV but have arrived at the clinic for another reason, such as being hit by a car. When a raccoon arrives at the clinic and is sedated for examination (according to standard clinic procedures), trained personnel will collect two samples: a conjunctival sample with a cotton swab that is placed immediately into Abbexa's diluent for testing on the lateral flow assay and a 0.5mL blood sample using venipuncture collected into a tube containing viral transport media (Figure 6). The blood sample will be stored at 4°C and transported to UC Davis for future dual RT-qPCR testing in the laboratory. The person collecting samples and running the Abbexa lateral flow test will not be blind to the clinical presentation of the raccoon, while the person conducting the dual RT-qPCR for detection of CDV in blood samples will be blind to clinical information. The person running the PCR test will not know the results of the Abbexa

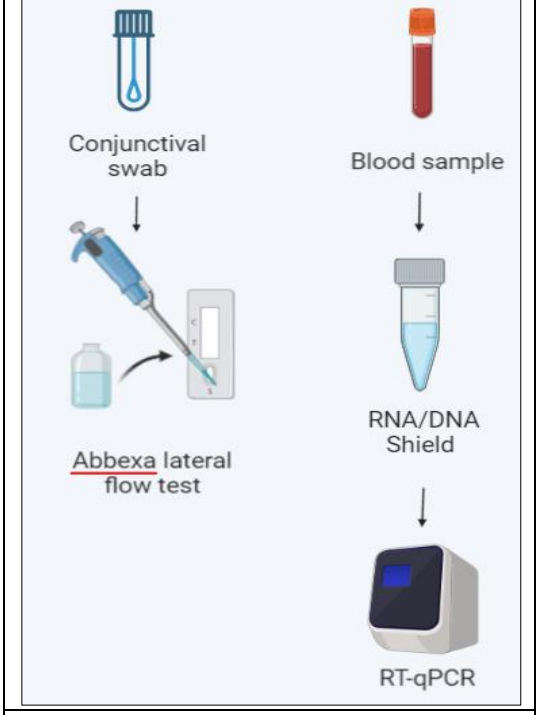


Figure 6. Sample collection scheme.

test and vice versa. Sample collection, labeling, transport, and storage protocols will be optimized and adhered to for the duration of the study to limit potential variability and sources of preanalytical error. In addition to sample collection, the study team will also record the raccoon's sex, estimated age (juvenile, subadult, or adult), reason for presentation, and clinical outcomes in an excel database. There is little evidence to suggest CDV in wildlife infects males or females disproportionately, but some evidence to suggest younger animals may be more susceptible to infection.[59] Therefore, we will analyze our data with and without stratification on sex and age to confirm whether stratification is warranted.

Test methods: The reference standard for this study is a dual RT-qPCR developed and tested on wildlife samples from diverse species, tissue types, and geographic locations.[76] Halecker et. al. estimated the dual RT-qPCR assay was 98.9% sensitive and 100% specific. We chose this reference standard because it has been validated in wildlife species, including raccoons, and because it is highly sensitive and specific in detecting diverse lineages of CDV, which is important because CDV shows geographical patterns of distribution; the CDV lineages circulating in Europe are distinct from those circulating in North America.[77] In this study, we will use the same protocol, reagents, primers, and probes described by Halecker et. al. We will replicate the analytical sensitivity and specificity testing in our laboratory and, assuming similar results, will use the cycle threshold (Ct) cutoff value of ≤ 34 described by Halecker et. al. to define a positive test result. The expected range of results for the dual RT-qPCR is 0 to 40 Ct and results will be categorized as 'positive' for Ct values of ≤ 34 or 'negative' for Ct values > 34. Results for Abbexa's lateral flow assay will be categorized as 'positive' if both the control and test lines are present and 'negative' if only the test line is present (please see appendix).

Sample size estimates and justification: We will calculate the sample size needed to estimate two proportions: one to estimate sensitivity and one to estimate specificity. Sample sizes will be calculated with EpiTools and will consider an apparent CDV prevalence among raccoons in Contra Costa County of 20%-30% (personal communication with Lindsay Wildlife Rehabilitation Hospital manager). Our sample

size will also increase if CDV prevalence is <20%.

Statistical methods for calculating test characteristics: Preliminary feasibility studies to estimate analytical test characteristics will be conducted using a vaccine strain of CDV as a positive control to establish lower limits of detection and a panel of paramyxoviruses as negative controls to determine cross-reactivity.[78] Diagnostic test characteristics will be analyzed by comparing the results of both the dual RT-qPCR reference test and Abbexa's lateral flow assay using samples collected from raccoons.

Analytical sensitivity (lower limit of detection): Onderstepoort canine distemper vaccine strain will be passaged in Madin Darby Canine Kidney cells and a six-fold serial dilution ($10^4 – 10^{-1}$) of the virus will be used as a positive control for replicates of Abbexa's lateral flow assay and the dual RT-qPCR assay. The lower limit of detection for each test will be determined as the lowest virus titer that yields a positive test result for 95% of the replicates for each test.

Analytical specificity (cross-reaction profile): Replicates of Abbexa's lateral flow assay will be evaluated against a panel of paramyxoviruses, including two closely related morbilliviruses (peste des petits ruminants virus and measles) and two more distantly related paramyxoviruses (Newcastle and mumps). Both the reference dual RT-qPCR assay and Abbexa's lateral flow assay should yield negative results for all viruses except the canine distemper vaccine strain.

Diagnostic sensitivity and specificity will be calculated with the following 2x2 table:

	Reference dual RT-qPCR test		
Abbexa test	**+**	**-**	**Total**
+	A	B	A + B
-	C	D	C + D
Total	A + C	B + D	A + B + C + D

Diagnostic sensitivity = A/(A + C) is the probability of a positive test result given the animal is truly infected with CDV and diagnostic specificity = D/(B + D) is the probability of a negative test result given the animal is truly not infected with CDV.[79] Once these proportions are determined, confidence intervals for the sensitivity and specificity point estimates will be generated in openepi.com, under 'counts', and

'proportion'. We will use the 2x2 table because we assume the two tests are independent (Abbexa's lateral flow assay detects CDV antigen whereas the dual RT-qPCR assay detects CDV nucleic acid); however, we will conduct a sensitivity analysis to account for the possibility that the tests may not actually be independent, since both tests detect acute infection. If our diagnostic targets of 100% specificity and at least 90% sensitivity are reached, Abbexa's lateral flow test could be used as an additional tool to confirm presumptive diagnosis of CDV in raccoons based on clinical signs and lay the foundation for further assay validation.

Part 2: Ecology, epidemiology, and evolution

This section contains information on CDV genetic diversity and global distribution of lineages, host range, notable outbreaks, and disease prevalence, vaccination, cross-species transmission dynamics, and signatures of adaptations. The key original contribution in this section is a preliminary comparison of selection pressures for each gene encoded by CDV.

Genetic diversity and global distribution of lineages

CDV strains are relatively conserved with little genetic diversity between strains. Beinke et. al. notes "though sequence data analysis of field isolates of CDV revealed several clusters of distemper strains, overall a considerable genetic stability of CDV even in recent outbreaks was observed."[33] Among the six structural CDV proteins, F and H contain the most diversity, while M and L amino acid sequences diverge very little (Table 2). The most diverse amino acid sequence is found in the F protein, which shares 92.8% amino acid identity across different CDV strains, whereas the most conserved protein is the M protein, which shares 99.9% amino acid identity across different CDV strains.

Coding sequence	Nucleotide		Amino acid	
	Min distance	Average	Min distance	Average
N	87.9	95.7	93.6	97.5
P	91.5	95.8	88.5	94.7
M	92.0	96.1	94.9	99.9
F	88.0	93.6	86.3	92.8
H	88.4	94.2	86.9	93.8
L	91.0	95.6	94.0	98.0

Table 2. Nucleotide and amino acid percent identity show F and H are most diverse, while M and L are most conserved. To generate this table, full-length CDV genomes were aligned in Geneious, then individual genes were extracted and re-aligned to quantify genetic diversity within each gene (nucleotide) and protein (amino acid). Pairwise sequence identities were calculated in Geneious and minimum and average distances are reported.

Though there is only one serotype of CDV, multiple genetic lineages circulate worldwide. CDV lineages vary by geography and were named after the regions where they were discovered (Figure 7).

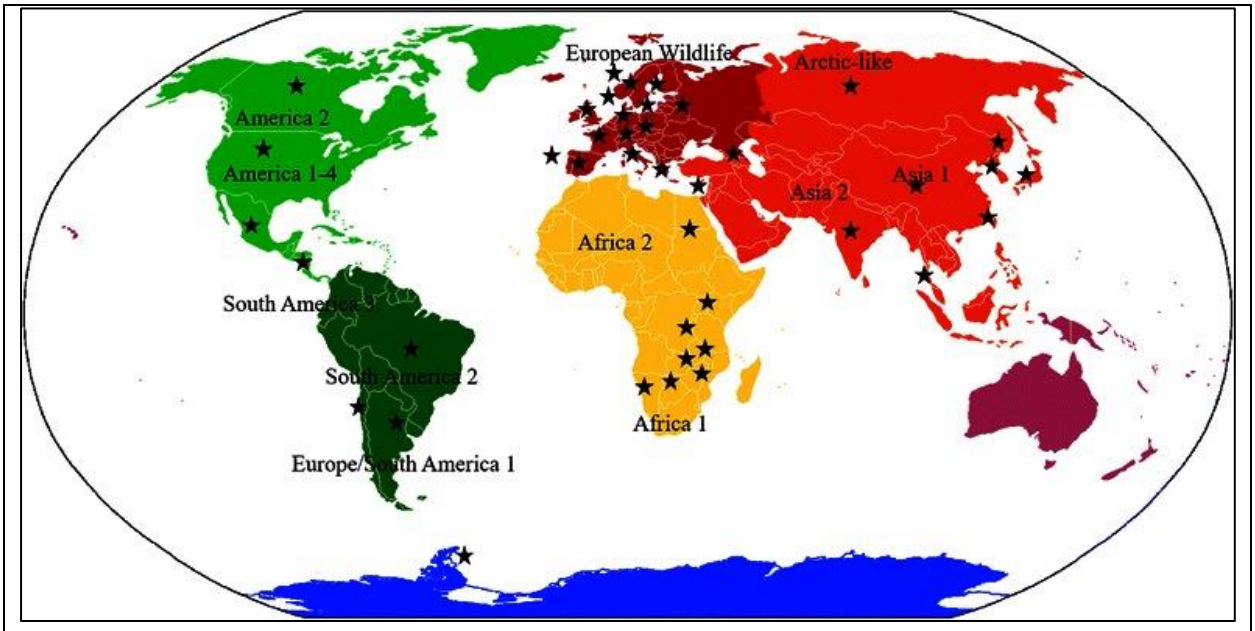


Figure 7. CDV lineages are generally distributed by geographic region. Image credit: Martinez-Gutierrez M, Ruiz-Saenz J. Diversity of susceptible hosts in canine distemper virus infection: a systematic review and data synthesis. BMC Vet Res. 2016 May 12;12:78. doi: 10.1186/s12917-016-0702-z. PMID: 27170307; PMCID: PMC4865023.

By convention, CDV lineages are defined as clusters of CDV that share >95% amino acid sequence identity within the H protein.[34,80] This means if the amino acid sequence within the H protein is 94% or

lower, the two strains would be considered separate lineages. In 2018, there were twelve distinct CDV lineages distributed globally.[81] As of 2022, there were at least 20 distinct lineages: Africa-1, Africa-2, Arctic-like, America-1 (which includes commercially available vaccine strains), America-2, America-3, America-4, America-5, Asia-1, Asia-2, Asia-3, Asia-4, Asia-5, Asia-6, Caspian, Europe-1/South America-1, European wildlife, Rockborn-like, South America-2, and South-America-3 (Table 3).[64,77,81,82] The increase in the number of CDV lineages over the last decade could be due to the emergence of novel viruses, but is more likely explained by increased surveillance efforts.

Virus and genotype	Strain	GenBank accession	Country	Species	Year
CDV					
Africa-1	WT02SA	KY971532	South Africa	Spotted hyena	2017
Africa-2	SNP/1994	KU578256	Tanzania	Lion	1994
Arctic-like	PS88-428	MN267063	Russia	Baikal seal	1988
America-1	Onderstepoort	AF378705	USA	Fox	1939
America-2	98-2646	AY542312	USA	Raccoon	1998
America-3	Fisher1	JN836734	USA	Fisher	2009
America-4	13_2262	KJ747372	USA	Dog	2013
America-5	96 America-5	MK487379	USA	Raccoon	2016
Asia-1	CYN07dV	AB687720	Japan	Macaque	2008
Asia-2	M25CR	AB475097	Japan	Dog	<2009
Asia-3	HLJ2	EU743935	China	Dog	2005
Asia-4	CDV4_TH/2014	MH496775	Thailand	Dog	2014
Asia-5	MCL-18-Li-1/1	MK037459	India	Lion	2018
Asia-6	SC18	MW535267	China	Red panda	2018
Caspian	PC00_36	MN267064	Kazakhstan	Caspian seal	2000
Europe-1/South America-1	S460/15	MN267060	Germany	Raccoon	2015
European wildlife	599/2016	KX545421	Italy	Fox	2016
Rockborn-like	HN19	MT448054	China	Masked palm civet	2019
South America-2	Arg24	MN365662	Argentina	Dog	2005
South-America-3	MDE-44/12	MT012802	Colombia	Dog	2012
PDV					
1988	NLD/1988	KC802221	The Netherlands	Harbor seal	1988
2002	DV/25002/DK-KAT/EU-2/2002	OK104956	Denmark	Harbor seal	2002
2006	PDV_USA_2006	KY629928	USA	Harbor seal	2006

Table 3. Globally recognized CDV strains as of 2022 and described by Geiselhardt et. al.[64]

A key characteristic of CDV is its global distribution of lineages and clustering by geography.[18,83] The phylogenetic tree below shows how CDV clusters generally by geographic region, however, not exclusively (Figure 8). For example, South America-2, North America-3, and Asia-1 lineages are closely related, but the other Asian lineages are spread out (Asia-2 is most closely related with Africa-1 rather than Asia-1). If geography was the sole driver of genetic diversity, we would expect clear distinctions between the African lineages, the Asian lineages, etc. with little intermingling.

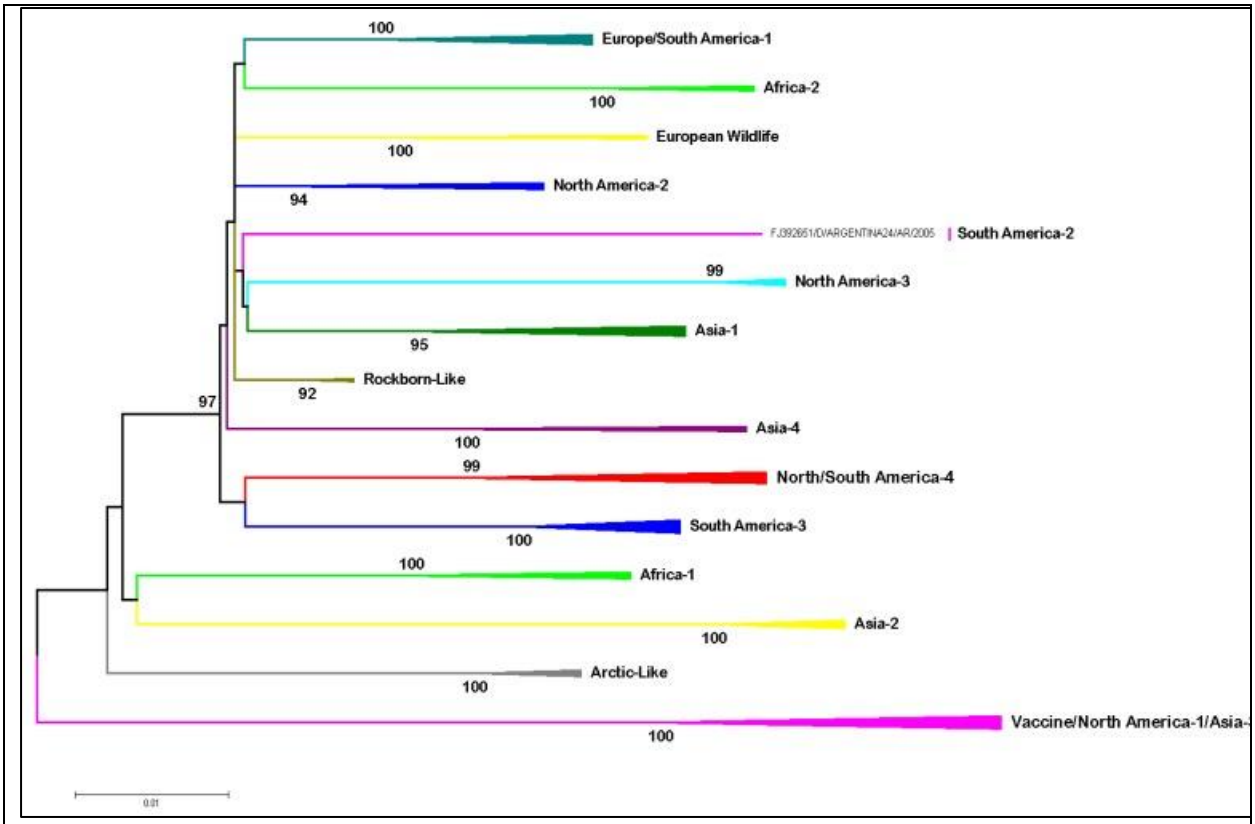


Figure 8. Phylogeny from Duque-Valencia et. al. based on the H gene sequences that shows CDV lineages generally cluster by geography, but other factors are influencing the global distribution. Image credit: Duque-Valencia J, Sarute N, Olarte-Castillo XA, Ruíz-Sáenz J. Evolution and Interspecies Transmission of Canine Distemper Virus-An Outlook of the Diverse Evolutionary Landscapes of a Multi-Host Virus. Viruses. 2019 Jun 26;11(7):582. doi: 10.3390/v11070582. PMID: 31247987; PMCID: PMC6669529.

There is likely much to be discovered regarding the diversity and distribution of CDV lineages. Surveillance efforts are not evenly distributed across the globe, with a dearth of full-length CDV sequences from Canada, South America, Africa, Europe, Russia and Australia (Figure 9). The uneven

global distribution of CDV genomes highlights the need for more surveillance and CDV sequencing from areas where they are currently lacking to gain a deeper understanding of CDV ecology and epidemiology. Increased sampling in Canada, South America, Africa, Europe, Russia, and Australia may lead to the discovery of more genetic diversity among CDV strains and show patterns in geographic distribution that are not currently apparent.

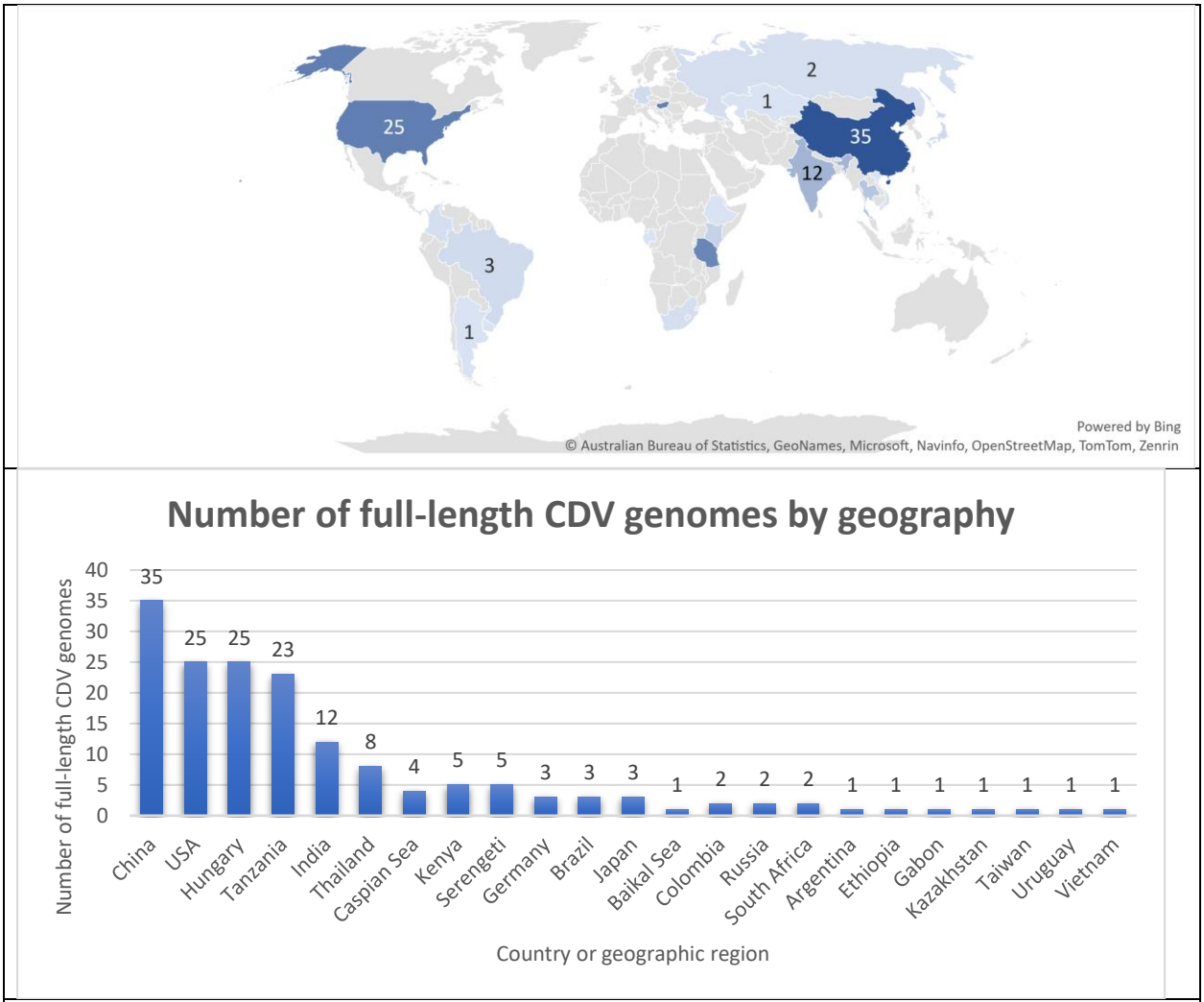


Figure 9. Map and bar graph showing the number of full-length CDV genomes in GenBank by country.

Though geography is a major factor in shaping the evolution of CDV strains, it is not the only factor. At a global scale, there is mixing of strains, such that some clusters of CDV genotypes represent multiple geographic origins. Some research contends geography is not the primary driver of CDV

distribution. For example, Ke et. al. found “no distinct geographical patterns among lineages” but rather that “wild-type isolates clustered into lineages with a spatiotemporal structure and short terminal branches.”[28] More thorough sampling from diverse locations is needed to better understand the true contribution of geography to overarching patterns of CDV distribution.

Summary

***Key findings*:** CDV strains represent one serotype and are relatively well conserved. Lineages are defined by >95% amino acid sequence identity within the H protein and lineages cluster geographically.

***Gaps*:** Though CDV exists globally, sampling biases exist with the majority of full-length CDV genomes from four regions – China, USA, Hungary, and Tanzania. However, the data from these countries are skewed toward particular species, which could preclude meaningful comparisons. For example, the sequences from China are mostly from dogs, raccoon dogs, and giant pandas; the sequences from USA are mostly from dogs and raccoons; the sequences from Tanzania are mostly from lions. Thus, comparisons of CDV genomes across countries are highly confounded by the fact that the host species represented are highly inconsistent. Increased sampling effort in other areas of the globe could yield more nuanced patterns in distribution of CDV lineages and reveal more genetic diversity.

Future research directions: Increase sampling effort to characterize CDV genomes from diverse geographic regions, especially from underrepresented areas, such as Canada, African countries, and Australia. There are likely already many existing viral genome sequences that have been generated by individual researchers that have not yet been submitted to public databases but would support future studies on the epidemiology, ecology, and evolution of this virus. By identifying key research questions, such as those outlined in this review, it is hoped that we will encourage both targeted surveillance of under-studied areas and encourage researchers to publish existing data. One under-utilized resource for obtaining a deeper understanding of CDV diversity is local wildlife rehabilitation centers. Organizations

like the Lindsay Wildlife Rehabilitation Hospital in California, receive diverse wildlife patients with suspected CDV. However, they may lack the time and resources to confirm CDV by PCR and to sequence the virus genome. Building collaborations with research labs interested in CDV would be a productive way to generate these essential data.

Host range, notable outbreaks, and disease prevalence

CDV has a broad and expanding host range.[1] According to Rendon-Marin et. al., "the host range of CDV mainly includes species from the order *Carnivora* which belongs to the families *Canidae* (dog, dingo, fox, coyote, jackal, wolf), *Procyonidae* (raccoon, coatimundi), *Mustelidae* (weasel, ferret, fisher, mink, skunk, badger, marten, otter), *Ursidae* (giant panda), *Ailuridae* (red panda), a wide range of members of the family *Felidae* (lions, leopards, cheetahs, tigers), and in a minor extension other important families belonging to different orders such as *Artiodactyla*, *Primates*, *Rodentia*, and *Proboscidea*."[34] A systematic review from 2016 noted CDV is capable of infecting 98 different species, including primates, but these results are based largely on reports from captive zoo animals and therefore not representative of naturally occurring infections among free-ranging wildlife, especially since zoo animals in close proximity to one another are at increased risk of transmission.[2,84,85]

The underlying genetic determinants of host range are largely unknown. Many studies have aimed to address the host range expansion of CDV by looking at the CDV H protein and its interaction with SLAM.[86,87] Some work has been done to compare the homology of SLAM receptors from different species to better understand whether binding affinity to SLAM contributes to CDV host range. For example, 3D protein modeling of binding interfaces suggest felid SLAM has a lower affinity for CDV H protein compared to canid SLAM. [88] Another group found raccoon and skunk SLAM may have higher affinity for CDV H compared to coyote SLAM.[89] Though the interaction between CDV and its receptors are likely a critical piece of the puzzle, these results suggest there is more research needed to understand mechanisms of host specificity.

Notable outbreaks of CDV have occurred not only in zoo animals, but also in wild endangered species. For example, CDV is, in part, attributed to the demise of the last few remaining black-footed ferrets in Wyoming during the 1980s, leading to effective extinction of the species in the wild.[74,90] In 1994, an outbreak of CDV occurred in Serengeti lions, causing fatal neurologic disease, and spreading to hyenas, leopards, and bat-eared foxes with high morbidity and mortality.[73,91,92] It was estimated that the outbreak resulted in the death of 30% of the lion population in the Serengeti National Park.[93] In 1999, CDV caused a precipitous decline in the population of endangered Santa Catalina Island foxes.[94] Then in 2000, a mass mortality event caused by CDV occurred in endangered Caspian Seals.[95] Spillover of CDV into marine mammals has already happened in the past, leading to the persistence and evolution of what is now called phocine distemper virus.[96] Thus, any outbreak of CDV in seals, or perhaps any wildlife species, is worrying because of the potential for short-term epizootics but also the potential for sustained transmission and long-term population impacts.

CDV has also caused serious outbreaks of disease among captive species of economic importance. For example, outbreaks have occurred with high mortality among farmed mink in Denmark, laboratory Cynomolgus monkeys in Japan, laboratory Rhesus macaques in China, and laboratory ferrets in Australia.[70,71,97,98] These outbreaks led to the culling of animals and economic losses.[2,84] In addition, the ability of CDV to infect primates raises concerns about the zoonotic potential of the virus.[3,68,99]

CDV is especially prevalent among urban carnivores. A 1997 serological survey in Luxembourg found 9-13% of red foxes were positive for CDV antibodies.[100] As noted above, a study in Michigan from 2008-2018 found 10-year average prevalence rates of CDV antigen detected by immunohistochemistry of 79.4% in gray foxes, 59.6% in raccoons, 50.0% in coyotes, and 44.4% in striped skunks.[75] These animals included mostly dead animals, either found by citizens or collected by hunters, trappers, or animal control, “in response to the animal's exhibiting abnormal behavior, signs of general sickness, or neurologic signs.”[75] A 2016 study in eastern Tennessee reported a high percentage of CDV in raccoons

and gray foxes, including 86% of animals with clinical signs and 55% of animals without clinical signs."[101] These data suggest roughly half of healthy gray foxes, raccoons, coyotes, and striped skunks in parts of the United States have been exposed to CDV. Among diseased animals, a study from the 1980s in Illinois found 47.2% of skunks with neurological disease but who tested negative for rabies were positive for CDV antibodies.[102] Taken together, these studies demonstrate that CDV is a significant problem for urban carnivores.

CDV is also prevalent among free-roaming dogs. Two studies focused on dogs near national parks, supposing they may act as a source of CDV among protected wildlife. A 2011 study found 100% (95% CI: 95.9–100) of the 92 dogs sampled near national parks in Uganda showed seroprevalence of CDV, compared to seroprevalence of 19.8% for rabies virus.[103] A 2019 study of 100 free-roaming dogs near a national park in Nepal found overall CDV seroprevalence of 80.0% (95% CI: 70.8–87.3).[104] These results indicate that the majority of free-roaming populations of dogs have been exposed to CDV.

Vaccination

The history of CDV vaccination in dogs began 100 years ago, starting with inactivated vaccines and live attenuated vaccines to recombinant vaccines. In the 1920s, an inactivated CDV vaccine was developed, but it was not effective at preventing disease. In 1945, a live attenuated vaccine was developed by passaging CDV in ferrets, but this vaccine led to clinical signs and death in some dogs. In 1952 and 1956 the Lederle and Onderstepoort strains were attenuated in embryonate chicken eggs (ECEs) and were successfully used to control distemper disease. In 1960, the Rockborn strain of CDV was grown in a canine kidney cell line, and this modified live attenuated vaccine became the basis of CDV vaccines used today.[105] There were tradeoffs between the inactivated and live attenuated vaccines. Though the live attenuated strains induced protective immunity, they also caused adverse reactions in some cases, such as post-vaccine encephalitis. Conversely, the inactivated vaccines did not cause encephalitis, but did not always induce protective immunity. Inactivated CDV vaccines are no longer

used in the United States. Then, in 1997, a recombinant CDV vaccine was developed using a canarypox virus vector.[106] Although recombinant vaccines do not carry the risk of causing vaccine-induced disease, like the live attenuated vaccines, the duration of immunity generated by recombinant vaccines is often shorter than the duration of immunity generated by live attenuated vaccines. Today, there are roughly 50 commercially available vaccines against distemper in dogs, often formulated in combination with other antigens and dogs are routinely vaccinated.[107]

There is no safe and effective vaccine for wildlife.[108] Two vaccines approved for use in dogs are most often studied for use in wildlife species: a live attenuated CDV vaccine (Nobivac® series, Merck) and the recombinant canarypox-vectored vaccine (Recombitek series, Merial), but with mixed results.[109] For example, the Recombitek vaccine does not appear to prevent disease in captive maned wolves or captive snow leopards.[110,111] However, the same vaccine appears to be safe and effective in captive African Wild Dogs and tigers.[109,112] Vaccine efficacy data is lacking for wildlife species because free-ranging animals are not easily recaptured and monitored following vaccination and because challenge studies in endangered captive animals may not be ethical. Therefore, vaccine data in species at risk of CDV is lacking.

Some vaccine studies in wildlife have been performed on a population level. For example, one study demonstrated field vaccination of raccoons could reduce the prevalence of CDV during an outbreak at the population level.[53] This study found that vaccination of raccoons could reduce the prevalence of CDV among raccoons, but that vaccination of domestic dogs did not seem to prevent outbreaks of CDV among wildlife. For example, a recent study of CDV in sea lions in the Galapagos Islands suggests CDV is still a threat to sea lions, despite vaccination of dogs.[113] The authors suspect the increased number of dogs due to the growing human population increases the number of direct interactions with sea lions while they rest on the beach. Another study from 2015 suggested that "widespread mass vaccination of domestic dogs reduced the probability of infection in dogs and the size

of outbreaks but did not prevent transmission to or peaks of infection in lions."[114] Prager et. al. came to the same conclusion in the case of African wild dogs, that "control of disease in domestic dogs may be insufficient to reduce transmission risks, and vaccination of wild dogs themselves may be the optimal strategy."[115]

Despite widespread use of vaccines in dogs, CDV remains an emerging pathogen with a global distribution, not only among dogs but also among wild carnivores.[116] CDV is recognized by some as "the most important worldwide infectious disease of domestic dogs."[39] Given the logistic challenges of conducting vaccine studies in free-ranging wildlife and the ethical challenges of conducting vaccine studies in collection animals, developing a safe and effective vaccine for CDV in wildlife might not be feasible. Instead, a better approach could be to understand how new CDV strains evolve and spillover into new hosts, thus guiding strategies to mitigate the risk CDV poses to wildlife.

Cross-species transmission dynamics

Historically, domestic dogs were considered a primary reservoir host for CDV. Today, CDV is considered a multi-host generalist pathogen that seems to be maintained in a network of smaller patches of susceptible hosts connected as a metapopulation.[117] As Almberg et. al. explains, CDV among wolves in Yellowstone cannot be maintained without other reservoir hosts and that "multi-host transmission is likely an essential evolutionary strategy for the long term persistence of CDV among its low-density carnivore hosts."[117] In some cases, the metapopulation may consist of both wildlife and domestic dogs. For example, Prager et. al. notes that "CDV in Northern Kenya may be maintained through the interaction of multiple subpopulations, each experiencing intermittent but non-simultaneous CDV epizootics composed of a single species, such as domestic dogs or some wild carnivore species such as jackals, or a combination of wild and/or domestic species."[118] Transmission may occur at kills, for example, where CDV infected individuals congregate and interact with diverse species.[119] In other cases, raccoons seem to be the reservoir host that drives outbreaks, including

spillback into dogs. For example, Rentería-Solís et. al. implicated raccoons as the origin of a 2013 outbreak in Berlin, Germany that drove interspecies transmission of CDV and Riley et. al. suggest that raccoons are the stable reservoir of CDV driving emergence of a novel strain of CDV among domestic dogs in the United States.[77,120] Still others contend domestic dogs continue to play a leading role in CDV transmission dynamics, especially in parts of the world with large populations of unvaccinated stray dogs, such as Mexico and Brazil.[121,122] Kapil et. al. note, "the spread and incidences of CDV epidemics in dogs and wildlife here and worldwide are increasing due to the rise in dog populations associated with growing human populations and widespread urbanization.[60] It is likely that all of these scenarios are true, and highlight the complex and variable ecology underlying the epidemiology of this disease.

More virus genome sequences are required to better understand the ecological dynamics of CDV.[123,124] However, while CDV has a broad host range, is prevalent among urban carnivores and free-roaming dogs, and has caused numerous outbreaks in diverse species, the available sequence data (e.g., in GenBank) is skewed heavily towards short fragments (rather than full genomes) and relatively few species. Snippets of CDV sequences exist for diverse species, but the majority of full-length CDV sequences, which are essential for understanding CDV evolution and cross-transmission, are comprised of only three species - domestic dogs, red foxes, and raccoons (Figure 10).[123,124] Indeed, we looked at the number of full-length, wildtype CDV genomes in GenBank to determine which host species were represented. Of the 164 full-length, wildtype CDV genomes we found in GenBank, 46 came from dogs, 26 came from red foxes, 15 came from raccoons. An additional 11 CDV genome sequences came from Asiatic lions and 11 came from African lions. The rest of the 55 CDV genomes came from animals representing 31 species. Overall, the skewed distribution of full-length CDV genomes in GenBank could reflect ease of sampling (domestic dogs, especially, are readily sampled, and in urban areas red foxes and raccoons may also be relatively accessible), increased sampling effort during outbreaks of charismatic endangered species (Asiatic and African lions), or species of economic importance (mink). A

more systematic approach to sampling of other species, especially urban carnivores such as coyotes, gray foxes, and striped skunks, which have shown high CDV seroprevalence rates in some areas and may be relatively accessible, could enable a more complete understanding of CDV transmission dynamics among wildlife in distinct ecosystems.

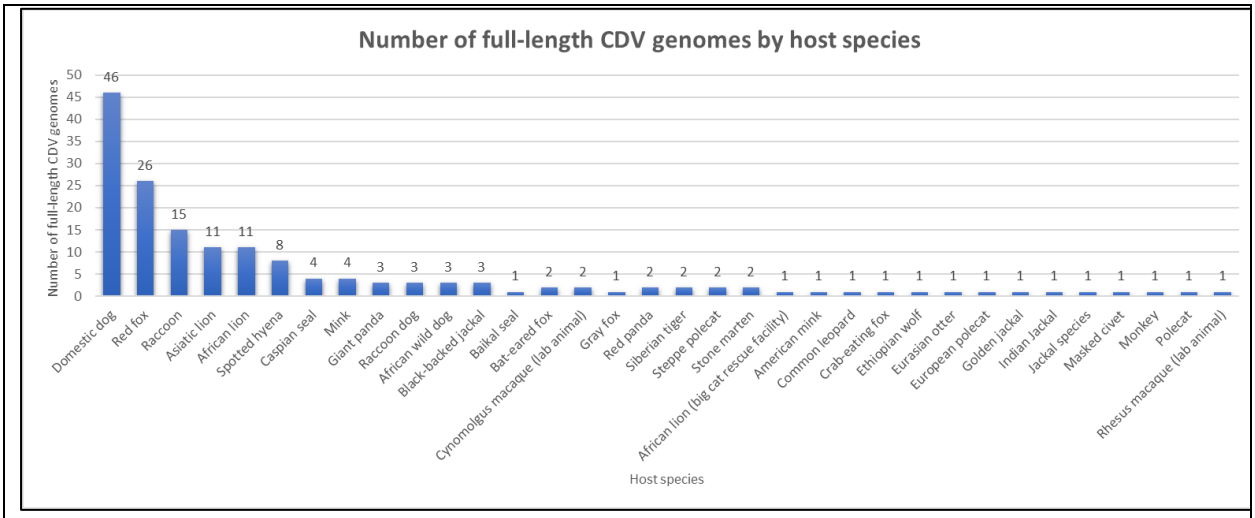


Figure 10. Of the full-length wildtype CDV genomes found in GenBank, the distribution of host species is skewed towards domestic dogs, red foxes, and raccoons. See also Figure 7 for distribution of sequences by country.

Summary

***Key finding*:** CDV is readily shared by different host species and likely maintained in a metapopulation.

***Gap*:** CDV genomes in GenBank are biased toward domestic dogs, red foxes, and raccoons, which limits our understanding of the ecology and evolution of CDV, including the viral, host, and environmental factors shaping CDV distribution and the molecular mechanisms underlying cross-species transmission.

Future research directions: The transmission dynamics of CDV among wildlife are not always fully understood and could include a complex interplay of viral, host, and environmental factors.[83] Viral factors such as genetic diversity of CDV strains or molecular adaptations to particular species could enable outbreaks among domestic and wild populations.[1] Host factors could include differences in cellular receptors, or weakened immune systems caused by stress or poor nutrition that render an

animal more susceptible to disease. Environmental factors could include habitat fragmentation and climate change that work synergistically to alter patterns of animal movement, for instance, by limiting water sources and driving animals into greater contact with each other where pathogens are more easily exchanged. More comprehensive sampling of diverse wildlife species is needed to better understand CDV transmission dynamics in different ecosystems. Surveillance efforts could focus on animals that tend to live in high densities at interfaces that may facilitate cross-species transmission of CDV, such as urban wildlife (raccoons, coyotes, and foxes) and domestic animals. For example, surveillance programs could be established at wildlife rehabilitation centers and local animal shelters to screen urban carnivores and domestic dogs, respectively, for CDV. By sampling diverse hosts that are connected by space and time, we could reveal epidemiologically relevant patterns that may contribute to CDV transmission dynamics. Ongoing surveillance could reveal changes in viral genomes over time and how the virus is moving between domestic animals and wildlife, thereby potentially guiding strategies to mitigate CDV outbreaks in wildlife.

Signatures of adaptation

Given its role in host cell tropism, the H protein has largely been the focus of studies into molecular adaptations of CDV to new host species. One notable signature of adaptation is amino acid position 549 of the H protein, which is located within the SLAM receptor binding site.[125] There are patterns of particular amino acids at position 549 that seem to align with particular species. For example, tyrosine (Y) at residue 549 is often, but not always, associated with CDV isolates from domestic dogs, whereas histidine (H) at residue 549 is often, but not always, associated with CDV isolates from wild species. This has led some to hypothesize that the amino acid substitution Y549H may have driven adaptation of CDV from dogs to wildlife.[126,127] In this scenario, H at position 549 represents the virus in a domestic cycle among dogs and T at position 549 represents the virus in a sylvatic cycle among wildlife (Figure 11).

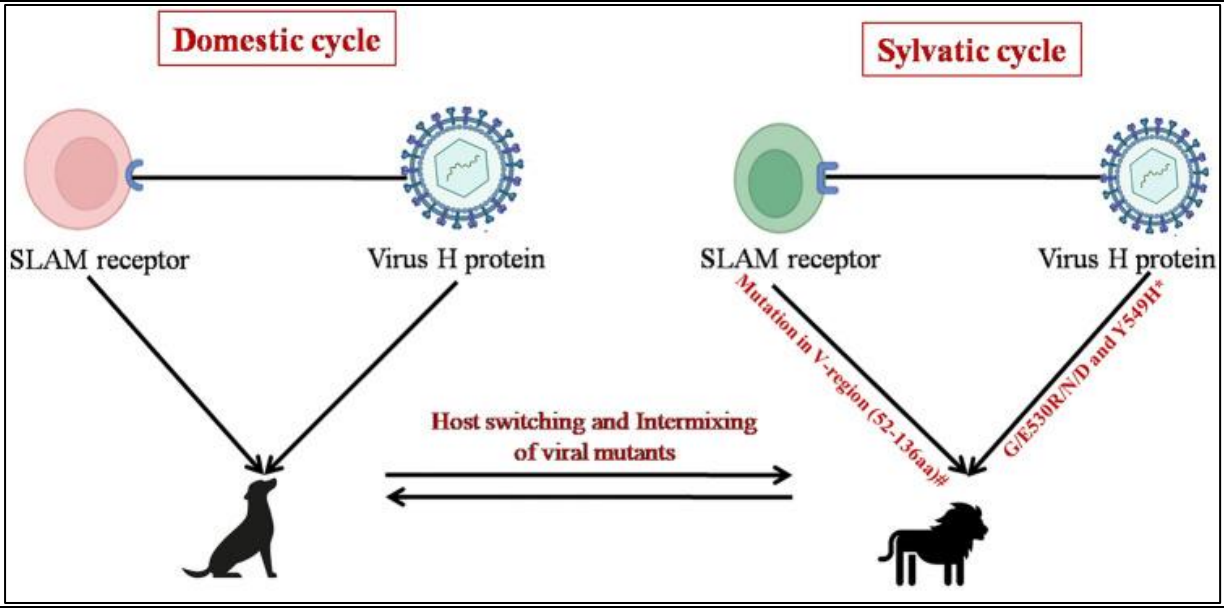


Figure 11. A proposed model in which Y549H in the H protein may have enabled CDV to switch from domestic dogs to wild animal hosts. Image credit: Karki, M., Rajak, K.K. & Singh, R.P. Canine morbillivirus (CDV): a review on current status, emergence and the diagnostics. VirusDis. 33, 309–321 (2022). https://doi.org/10.1007/s13337-022-00779-7.

Nikolin et. al. suggest Y549H underlies a functional change that enables CDV to be a specialist pathogen in dogs and a generalist pathogen in wildlife.[128] Nikolin et. al. showed in vitro that CDV expressing tyrosine at position 549 in the H protein efficiently infected cells expressing SLAM from domestic dogs but was less efficient at infecting cells expressing SLAM from lions. Conversely, CDV expressing a histidine at position 549 in the H protein could infect both cells expressing SLAM from lions and dogs marginally well, but was unable to infect either as efficiently as CDV strains with tyrosine.[128] The authors conclude that a histidine at position 549 seemed to produce a generalist virus that decently infected cells expressing dog SLAM and cells expressing cat SLAM, whereas a tyrosine at position 549 seemed to produce a specialist virus that was very good at infecting cells expressing dog SLAM but poor at infecting cells expressing cat SLAM. Perhaps this is a fitness tradeoff in which Y549H represents a generalist strain of CDV that can infect diverse wildlife hosts reasonably well compared to CDV strains that specialize one host - domestic dogs. More research is needed to build on these results and determine if antagonistic pleiotropy plays a role in CDV biology among wildlife.

Summary

***Key finding*:** Given its role in recognizing host cell receptors, researchers have focused on residues within the H protein that may be more often associated with certain host species. A tyrosine at residue 549 is often associated with CDV isolates from domestic dogs and may represent a specialist pathogen, whereas histidine at residue 549 is often associated with wildlife and may represent a generalist pathogen.

***Gaps*:** The H protein plays a key role in host cell tropism, but focusing entirely on H is a biased approach. Assessing mutations across the entire CDV genome may provide deeper insight into potential patterns of adaptation to diverse hosts.

Future research directions: We suggest all CDV proteins be considered for studies looking at signatures of selection. It would be interesting to compare patterns of selection between genes but also between host species and geographic locations. It would be important to look at individual sites (amino acids under selection) but also to consider branch tests for selection to see whether a site under selection belongs to a particular lineage. Identifying sites under positive selection can be informative for multiple reasons, including identifying amino acid residues of functional significance for virus host interactions. We would expect to see strong signatures of positive selection in sites that define particular virus host interactions, such as H binding to host cell receptors.

Original contribution 2: Evaluation of all sites under positive selection

To stimulate new research directions, we conducted an initial study to compare patterns of selection across CDV genes. Many groups have looked for molecular signatures of CDV adaptation.[28,129] A common approach to identifying signatures of viral adaptation is to compare selection pressure at each site within a sequence alignment by estimating rates of nonsynonymous and synonymous changes. Sites with a ratio of nonsynonymous to synonymous changes > 1 are considered sites under positive selection, meaning natural selection tends to favor genetic variants. Genetic diversity could be

advantageous for proteins recognized by the immune system, for example. Most sites within the CDV genome are under negative selection, as expected, because negative selection acts to preserve the amino acid sequence.[83] Preserving protein structure and function is critical and most mutations will be deleterious. However, certain changes at certain sites can induce strong phenotypic shifts that confer a particular fitness advantage.

One finding from the literature review is that key residues in H protein and F protein are under positive selection. However, the selective pressure shaping other CDV proteins are less well understood. Given its role in binding host cell receptors and thus determining host cell tropism, the H protein has largely been the focus of molecular adaptations to new host species, particularly at amino acid position 549 which is located within the SLAM receptor binding site.[125] In 2007, McCarthy et. al. first identified positions 530 and 549, both within the SLAM binding region of the H protein, as sites under positive selection.[125] Since then, other groups have looked for evidence of positive selection in CDV as well. In 2019, Duque-Valencia et. al. found evidence of positive selection at sites 522, 549, and 582 in the H protein and nine sites within the F protein.[82] In 2021, da Costa et. al. found evidence of positive selection at 14 sites within the H protein and 18 sites within the F protein.[129] Though detecting selection is heavily influenced by sequence and method choice, the H and F proteins are consistently identified. Positive selection within the H and F proteins is expected, since these proteins are recognized by the host immune response and are the target of neutralizing antibodies. Despite the focus on H and F, little is known about signatures of positive selection in other genes. While these other genes may not be the target of neutralizing antibodies, they nonetheless interact with host-cell proteins during viral replication and may experience different selective pressures in different hosts or cells. Thus, identifying sites under positive selection in all genes can help reveal more about the basic biology of this virus and the forces shaping their structure and function.

To determine whether other genes besides H and F were also under positive selection, we used

a set of full-length, wildtype CDV sequences from GenBank to enable cross-gene comparisons. Using two models of selection, we found evidence of positive at the H, F, and P genes. In addition, selective pressures may differ between host species – this is an area that has not been deeply researched in the literature. Therefore, we also evaluated selection pressure in CDV from different species. Our results suggest selection pressure differs by host species. We recommend future studies include full-length CDV genomes and comparison of selective pressure across all genes and different host species.

Materials and Methods

Dataset: We searched GenBank (NCBI) taxonomy browser for sequences assigned to the species *Morbillivirus canis*, which yielded 3,255 entries (March 2023). We then limited the search to entries with 15,000 – 16,000 base pairs to include only full or nearly full length CDV genomes, which provided 232 total sequences. Because we are interested in wildtype CDV genomes under selective pressure in nature, any sequences that had been passaged in a laboratory setting or used in a vaccine strain of CDV were excluded. Sequences without host data were also excluded. Our final set of full-length, wildtype CDV genomes was 119 sequences from wildlife species and 46 sequences from domestic dogs (Appendices 1 and 2). We limited our analyses to full-length, wildtype CDV as the majority of studies to date have focused on single genes and we wanted to compare variation in genetic patterns across genes for a consistent set of sequences.

Genetic analyses: Sequences were aligned by each coding region in Geneious and exported to Datamonkey for analysis of sites under positive episodic selection. We tested two models of selection – MEME and FUBAR, both of which look for evidence of selection by site. MEME (Mixed Effects Model of Evolution) employs a mixed-effects maximum likelihood approach to test the hypothesis that individual sites have been subject to episodic positive or diversifying selection.[130] MEME aims to detect sites evolving under positive selection under a proportion of branches. FUBAR (Fast, Unconstrained Bayesian AppRoximation) uses a Bayesian approach to infer nonsynoymous (dN)

and synonymous (dS) substitution rates on a per-site basis for a given coding alignment and corresponding phylogeny.[131] This method assumes that the selection pressure for each site is constant along the entire phylogeny.

Results

Among all host species (wildlife species and domestic dogs combined), MEME identified H, F, and P as the genes with the highest proportion of residues under positive selection, whereas FUBAR is more conservative and identified F and P with the highest proportion of residues under positive selection (Tables 1 and 2). While previous studies have highlighted the selection for H and F, our study also shows the importance of sites within P as targets of selection.

All hosts – MEME			
Coding sequence	**# of sites under positive selection**	**Total number of sites**	**Percent of sites under positive selection**
N	4	523	0.8
P	17	507	3.4
M	5	335	1.5
F	28	662	4.2
H	12	608	2.0
L	15	2184	0.7
Table 1.			

All hosts – FUBAR			
Coding sequence	**# of sites under positive selection**	**Total number of sites**	**Percent of sites under positive selection**
N	0	523	0.0
P	14	507	2.8
M	0	335	0.0
F	9	662	1.4
H	1	608	0.2
L	0	2184	0.0
Table 2.			

Both models identified 13 residues (48, 72, 86, 90, 101, 102, 106, 143, 148, 201, 237, 249, 296) within the P amino acid sequence, 9 residues (3, 12, 51, 59, 64, 72, 87, 93, 105) within the F amino acid

sequence, and 1 residue (549) within the H amino acid sequence as sites under positive selection. As P overlaps with both C and V, we mapped the sites under positive selection to assess how these sites were distributed. Interestingly, all positively selected sites within the P fall within the V or C coding region (Figure 12); they are not evenly distributed along the full-length of P.

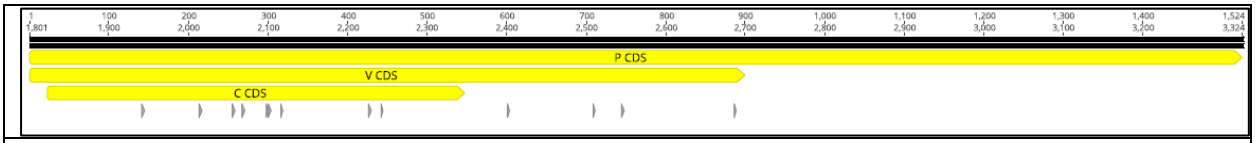


Figure 12. Image showing the positions of 13 amino acid residues within the P gene identified by both MEME and FUBAR. Interestingly, each of these 13 amino acid residues fall within either V coding region (899 base pairs) or C coding region (525 base pairs). They are not evenly distributed across the length of P, suggesting selection may be driven by C or V, rather than P.

Discussion

Evolutionary studies may provide insight into viral adaptations and differences in the forces shaping viral genomes. By limiting our analyses to the H and F gene only, we may be limiting our understanding of selection pressures on CDV. We analyzed full-length, wildtype CDV genomes and found evidence of positive selection in the H, F, and P proteins. Since partitioning by domestic dogs, raccoons, and foxes yields different results compared to the sites identified when all species are group together, this could suggest there are species-specific patterns of selection. Future studies should analyze positive selection data with and without partitioning by species to note any differences.

Most studies to date have focused on the H and F proteins. The H gene encodes hemagglutinin, which is responsible for attachment to host cell receptors. The F gene encodes the fusion protein, which is responsible for fusing the virion to the host cell following attachment to enable entry. Both the H protein and the F protein generate neutralizing antibodies, so it is possible that the H and F proteins are under diversifying positive selection given pressure from the host's immune system. We also found evidence of positive selection at residues within the P protein. The P gene encodes the phosphoprotein that helps stabilize the polymerase during transcription, but it also is the location where two nun-

structural proteins, C and V, are encoded sub-genomically and expressed through translation of alternative start sites. The C and V proteins are involved with immune evasion and virulence. Therefore, it is plausible that the P protein is also under diversifying positive selection by the immune system, since it produces two proteins that are involved with immune evasion. The sites we identified fall within either the C or V coding regions (our analysis could not resolve between them).

Though CDV is an ideal candidate for studying viral adaptation to new host species, biases in the data may be limiting our ability to make broad interpretations, thus obfuscating larger patterns in CDV distribution and adaptation. The literature focuses on the H and F proteins as targets of natural selection; however, we also present evidence that suggests C and V are also under positive selection. Looking beyond the H and F proteins for molecular signatures of natural selection could provide better insight into the dynamics of CDV evolution. We also recommend partitioning by species to determine if sites under positive selection show differences by species.

Conclusion

CDV has been extensively studied in dogs, but gaps and biases remain in our understanding of CDV in wildlife. Based on a phylogenetic tree of morbilliviruses, it is clear more viruses have yet to be identified and our understanding of genetic diversity is incomplete. Sampling biases exist, as well, with full-length wildtype CDV genomes in GenBank from a few species - dogs, red foxes, and raccoons – and from a few locations - China, USA, Hungary, and Tanzania. Studies on selection pressure are also skewed towards the hemagglutinin protein, which is undoubtedly important in determining host specificity, but likely not the only protein under selective pressure or contributing to complex transmission dynamics. More research is needed to understand CDV evolution within a host, including its transition from SLAM to nectin-4, as well as its ability to invade the central nervous system, and CDV evolution between hosts, including how the virus readily crosses species barriers. As with all good questions in biology, it seems

the more we learn the more we do not know.

We recommend future research should include sampling from diverse species and diverse locations, as well as analyzing all CDV genes and the proteins they encode. Such a holistic approach could provide greater insight into CDV biology. The lessons we learn about CDV may extend to morbilliviruses more broadly. CDV should be studied not only to protect wildlife but also to understand its potential as a zoonotic pathogen.

Appendices

Appendix 1: Abbexa's lateral flow diagnostic instructions for use.

Instructions for Use
Version: 2.0.1
Revision date: 28-Jan-22

Canine Distemper Virus Antigen Rapid Test Kit

Catalog No.: abx092034

Size: 40 tests

Storage: Store all reagents at 2-30 °C. Keep dry.

Application: For qualitative detection of Canine Distemper Virus Antigen in Dog serum, urine, nasal fluid, saliva and conjunctival secretions.

Introduction and assay principle

Abbexa's Canine Distemper Virus Antigen Rapid Test Kit is a qualitative lateral flow immunochromatographic assay for the detection of Canine Distemper Virus (CDV) Antigen in dog (canine) samples. The cassette contains a colloidal gold conjugated antibody against CDV, which combines with any CDV antigen present in the samples. The complex binds with the antibody coated on the test region of the cassette. A colored band develops within 15 minutes in the test region if the concentration of CDV antigen in the sample is higher than the detection limit. A control region on the upper end of the cassette confirms if the test has been successful.

Kit Components

- Test cassettes with pipettes: 40
- Sample diluent: 40 vials
- Cotton swabs: 1 pack

Material Required But Not Provided

- Timer

Sample preparation

- **Nasal fluid, saliva and conjunctival secretions:** Collect the sample using a cotton swab. Immediately insert the cotton swab into a Sample Diluent vial. Stir until the sample has completely dissolved. Wipe the swab against the inside of the tube, then discard the swab. Allow the mixture to stand.
- **Serum and urine:** Add 4-5 drops (approximately 100 µl) into a Sample Diluent vial. Mix fully, then allow the mixture to stand.

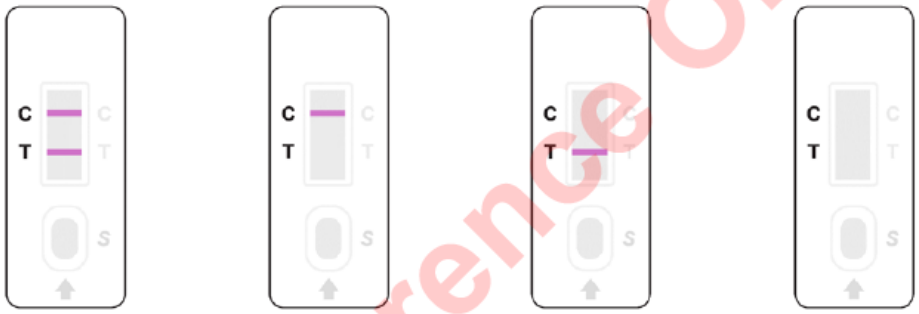
  Note: In the initial stage of CDV infection, there are no symptoms such as conjunctivitis, dry cough, and diarrhea. It is therefore recommended to test blood (serum) samples if the animal is not displaying any CDV infection symptoms. If symptoms typical of CDV infection are present, it is more convenient to take conjunctival epithelial cells or urine as the sample.

Assay procedure

1. Take a test cassette and lay it flat on a clean table. Using the provided pipette, add 2-3 drops (approximately 60 µl) of prepared sample to the sample well on the test cassette. Avoid touching the membrane in the middle of the sample well, and avoid foaming.
2. Leave at room temperature for 5-10 min, then analyze the result immediately.

Results analysis

- **Negative result:** A line is observed in the control (C) section but not the test (T) section.
- **Positive result**: A line is observed in both the control (C) section and the test (T) section.
- **Invalid result**: No line is observed in the control (C) section.



Positive **Negative** **Invalid** **Invalid**

Notes

1. The test cassettes and reagents should be brought to room temperature for at least 30 minutes before use.
2. After opening the aluminum foil, use the test cassette as soon as possible.
3. Samples should be clear with no visible particles, turbidity or bacterial pollution.
4. Do not mix or re-use the disposable pipettes to avoid cross-contamination.
5. Do not use water, PBS or serum from other animals as the negative control.
6. Avoid touching the cassette membrane through the sample well or test result window.
7. This kit is for qualitative detection of CDV antigen in dog (canine) samples.
8. Samples from animals taken 3-10 days after being vaccinated against CDV may show a positive result, as attenuated CDV vaccines can cause CDV antigen concentrations to increase in animals.
9. Samples from animals treated with monoclonal antibodies may exhibit false-positive results, as these animals generate anti-monoclonal antibodies which may interfere with the assay.
10. This kit is for research use only and the results are for reference only. It is recommended to use this kit in conjunction with another detection method.

Appendix 2: List of full-length CDV genomes from wildlife species.

Accession	Country	Host (common name)	Collection date
AB687720	Japan	Cynomolgus macaque (lab animal from China)	2008
AB687721	Japan	Cynomolgus macaque (lab animal from China)	2008
AY443350	USA	Raccoon	2000
AY445077	USA	Raccoon	1998
AY466011	USA	Raccoon	1998
AY542312	USA	Raccoon	1998
AY649446	USA	Raccoon	2001
HM046486	Caspian Sea	Caspian seal	2007
HM063009	Kazakhstan	Mink	1989
HM852904	China	Rhesus macaque (lab animal)	2008
HQ540293	China	Fox	2006
JX681125	China	Fox	2006
KC427278	China	Mink	2008
KF856711	China	Monkey	2006
KJ466106	China	Raccoon dog	2012
KJ747371	USA	Red fox	2011-2013
KJ848781	China	Raccoon dog	2014
KM926612	China	Polecat	1992
KP677502	China	Giant panda	2015
KP738610	China	Raccoon dog	2014
KP765763	China	Red fox	2014
KP769803	China	Giant panda	2014
KP793921	China	Giant panda	2014
KU578253	Serengeti	African wild dog	2007
KU578254	Serengeti	Golden jackal	2011
KU578255	Serengeti	Spotted hyena	1994
KU578256	Serengeti	African lion	1994
KU666057	USA	Raccoon	2012
KX499865	China	Red fox	2015
KX774415	Russia	Siberian tiger	2004
KY971528	South Africa	African wild dog	2016
KY971532	South Africa	Spotted hyena	2017
LC342298	Japan	Raccoon	2016
MF041963	Ethiopia	Ethiopian wolf	2016
MH316137	Russia	Siberian tiger	2004
MH337872	China	Fox	2016
MH426739	Brazil	Crab-eating fox	2014
MK037459	India	Asiatic lion	2018
MK037460	India	Asiatic lion	2018
MK037461	India	Asiatic lion	2018
MK037462	India	Asiatic lion (cub)	2018

MK037463	India	Asiatic lion (cub)	2018
MK037464	India	Asiatic lion	2018
MK037465	India	Asiatic lion	2018
MK037466	India	Asiatic lion	2018
MK037467	India	Asiatic lion	2018
MK037468	India	Asiatic lion	2018
MK037469	India	Asiatic lion	2018
MK408453	China	Mink	2017
MK408454	China	Mink	2017
MN267060	Germany	Raccoon	2015
MN267061	Germany	Red fox	2016
MN267062	Germany	Raccoon	2015
MN267063	Baikal Sea	Baikal seal	1988
MN267064	Caspian Sea	Caspian seal	2000
MN267065	Caspian Sea	Caspian seal	2000
MN267066	Caspian Sea	Caspian seal	2000
MN326827	China	American mink	2013
MN824467	USA	Raccoon	2018
MT136705	Tanzania	Spotted hyena (subadult)	1993
MT136706	Tanzania	Spotted hyena (subadult)	1993
MT136707	Tanzania	Spotted hyena (cub)	1994
MT136708	Tanzania	Spotted hyena (cub)	1994
MT136709	Tanzania	Spotted hyena (cub)	1994
MT136710	Tanzania	Spotted hyena (fetus)	1994
MT136714	Tanzania	Bat-eared fox	1994
MT136715	Tanzania	Bat-eared fox	1994
MT136716	Tanzania	African lion (subadult)	1994
MT136717	Tanzania	African lion (adult)	1994
MT136718	Tanzania	African lion (adult)	1994
MT136719	Tanzania	African lion (subadult)	1994
MT136720	Tanzania	African lion (cub)	1994
MT136721	Tanzania	African lion (cub)	1994
MT136722	Tanzania	African lion (subadult)	1994
MT136723	Tanzania	African lion (cub)	1994
MT136724	Tanzania	African lion (subadult)	1994
MT136725	Tanzania	African lion (adult)	1994
MT448054	China	Masked civet	2019
MT932492	Tanzania	Jackal species	2006
MT932493	Kenya	Black-backed jackal	2000
MT932501	Kenya	Black-backed jackal	2000
MT932502	Kenya	Black-backed jackal	2000
MT932503	Tanzania	African wild dog	2007
MT932504	USA	African lion (big cat rescue facility)	1992

MT932505	USA	Raccoon	1992
MT932506	USA	Raccoon	2013
MT932507	USA	Gray fox	2013
MT932508	USA	Raccoon	2013
MT932509	USA	Raccoon	2013
MT932510	USA	Raccoon	2013
MT932511	USA	Common leopard	1992
MW535267	China	Red panda	2018
MW535268	China	Red panda	2019
MW876862	India	Indian Jackal	2019
OK557779	Hungary	Red fox	2021
OK557780	Hungary	Red fox	2021
OK557781	Hungary	Red fox	2021
OK557782	Hungary	Red fox	2021
OK557783	Hungary	Red fox	2021
OK557784	Hungary	Red fox	2021
OK557785	Hungary	Red fox	2021
OK557786	Hungary	Red fox	2021
OK557787	Hungary	Red fox	2021
OK557788	Hungary	Red fox	2021
OK557789	Hungary	Red fox	2021
OK557790	Hungary	Red fox	2021
OK557791	Hungary	Red fox	2021
OK557792	Hungary	Red fox	2021
OK557793	Hungary	Red fox	2021
OK557794	Hungary	Red fox	2021
OK557795	Hungary	Red fox	2021
OK557796	Hungary	Red fox	2021
OK557797	Hungary	Red fox	2021
OM811640	Hungary	Eurasian otter	2006
OP209185	Hungary	Steppe polecat	2021
OP209186	Hungary	Steppe polecat	2018
OP209187	Hungary	European polecat	2019
OP209188	Hungary	Stone marten	2020
OP209189	Hungary	Stone marten	2007

Appendix 3: List of full-length CDV genomes from domestic dogs.

Accession	Country	Host (common name)	Collection date
MF437053	Gabon	Domestic dog	2015
MH496772	Thailand	Domestic dog	2014
MH496773	Thailand	Domestic dog	2014
MH496774	Thailand	Domestic dog	2014
MH496775	Thailand	Domestic dog	2014
MH496776	Thailand	Domestic dog	2014
MH496777	Thailand	Domestic dog	2014
MH496778	Thailand	Domestic dog	2014
MH496779	Thailand	Domestic dog	2014
MH484613	Brazil	Domestic dog	2017
MT012803	Colombia	Domestic dog	2017
MT012802	Colombia	Domestic dog	2012
OL675426	China	Domestic dog	2020
MW713449	China	Domestic dog	2019
MW980440	China	Domestic dog	2020
MW600730	China	Domestic dog	2020
MW460905	Brazil	Domestic dog	2019
MT932500	Kenya	Domestic dog	2000
MT932499	Kenya	Domestic dog	2000
MT932498	USA	Domestic dog	2012
MT932497	USA	Domestic dog	2013
MT932496	USA	Domestic dog	2013
MT932495	USA	Domestic dog	2013
MT932494	USA	Domestic dog	2013
MT932491	USA	Domestic dog	2013
MN296413	China	Domestic dog	2011
MN365662	Argentina	Domestic dog	2005
MT136713	Tanzania	Domestic dog	1994
MT136712	Tanzania	Domestic dog	1994
MT136711	Tanzania	Domestic dog	1994
LC159587	Vietnam	Domestic dog	2014
MK431532	Taiwan	Domestic dog	2005
MF926604	China	Domestic dog	2017
MF926603	China	Domestic dog	2017
MF926602	China	Domestic dog	2016
MF926601	China	Domestic dog	2016
MF926600	China	Domestic dog	2016
MF926599	China	Domestic dog	2016
MF926597	China	Domestic dog	2016
KU578257	Serengeti	Domestic dog	1994
KX347928	China	Domestic dog	2015

KJ747372	USA	Domestic dog	2011 - 2013
KM280689	Uruguay	Domestic dog	2012
KJ123771	USA	Domestic dog	2004
HQ540292	China	Domestic dog	2007
EU716337	USA	Domestic dog	2004

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