Development of a new generation vaccine for the canine distemper virus (CDV). An *in silico, in vitro* and *in vivo* approach.

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"It is the responsibility of scientists never to suppress knowledge, no matter how awkward that knowledge is, no matter how it may bother those in power; we are not smart enough to decide which pieces of knowledge are permissible and which are not."

- Carl Sagan

"Knowledge is a map that guides us while imagination is the territory where we can roam freely and search for answers and opportunities. Imagination knows no restraint and it is the power that puts knowledge to use."

- Albert Einstein

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ABSTRACT

This thesis is structured into four chapters, each one addressing a research question focused on the development of a next-generation vaccine against canine distemper virus (CDV). Each chapter covers topics that address diverse aspects to answer this question, providing the reader with not only theoretical context but also *in silico, in vitro,* and *in vivo* experimental results for the development of a new immunization strategy for domestic and wild species affected by CDV.

The first chapter delves into the theoretical framework of virus biology, discussing themes such as the replication cycle, pathogenesis, clinical presentation, and virus-host interaction. This chapter provides the reader with the biological and molecular context of CDV.

The second chapter presents a systematic literature review with a scoping approach to determine the current status of CDV vaccination in domestic and wild species. This chapter is developed in this thesis because there has been no comprehensive study summarizing the current state of vaccine development for controlling CDV spread and infection. A total of 2,321 articles identified and 68 studies were eligible based on predefined inclusion criteria focusing on CDV vaccines across various animal species including canines, ferrets, minks, mice, and others. Research efforts originated from scientific communities from the United States, Canada, France, and Denmark. Diverse vaccine formulations, a wide range of immunization routes and schedules were observed. Safety and efficacy underscored the significance of these immunogens within the evaluated animal populations. Among these findings is the inclusion of 37 studies which conducted post-immunization CDV challenge assessments, predominantly in canine subjects, pointing the survival outcomes among vaccinated populations. Nonetheless, substantial gaps in vaccine research were identified, particularly in wildlife reservoirs. Thus, collaborative endeavors are imperative for the formulation of effective vaccination strategies aimed at preserving animal welfare and attenuating the worldwide repercussions of CDV, particularly among endangered species.

The third chapter presents the development of immunogenic peptides derived from the genetic and antigenic information of all CDV variants circulating worldwide using computational tools for the development of a universal CDV vaccine, followed by safety assessment *in silico* and *in vitro*. An immunogenic CDV peptide-based candidate was developed, utilizing a peptide library derived from consensus sequences of CDV H and F proteins from circulating strains worldwide. Computational tools were employed to assess the immunogenic potential of these peptides. Molecular docking and dynamic simulations were utilized to evaluate the molecular interaction of selected peptides with canine MHC-I, -II, TLR-2, and -4. *In silico* safety was assessed through antigenicity, allergenicity, toxicity potential, and comparison with homologous canine peptides, while *in vitro* safety was evaluated through cytotoxicity assays using cell lines, canine peripheral blood mononuclear cells (cPBMCs), and hemolysis potential assays with canine red blood cells. Subsequently,

a multiepitope CDV polypeptide was synthesized based on the most promising peptides, and the *in silico* and *in vitro* evaluations were conducted to compare it with single immunogens. At this stage, five peptide candidates were identified, both individually and as a multiepitope CDV polypeptide, for evaluation as a next-generation peptide-based vaccine in an animal model.

The final chapter involves the evaluation of immunogenic peptides either individually or as a polypeptide in an *in vivo* mouse model to determine their safety and immunogenicity in a vaccine formulation with a commercial adjuvant. Twenty-four BALB/c mice were distributed into four groups and subjected to three different vaccine dosing regimens over a 28-day period. Seroconversion was determined using ELISA, while cellular immune responses were measured via flow cytometry, specifically focusing on activation-induced markers (AIMs). Both the peptide mixture, multiepitope CDV polypeptide and commercial vaccine (CV) were safe in mice, with statistically significant seroconversion observed in the groups vaccinated with the multiepitope CDV polypeptide and the CV compared to the control group. Furthermore, mice immunized with the multiepitope CDV polypeptide exhibited increased levels of antigen-specific CD4+ CD134+ and IFN-γ+ T cells, CD8+ T cells, as well as TNF-α and IL-6-producing cells compared to the placebo group. These experimental results collectively indicated seroconversion for the multiepitope CDV polypeptide lower than the CV vaccine and the establishment of cellular immune memory. The development of multiepitope polypeptide vaccines presents a promising preventive strategy against CDV, and our integrated approach represents a significant advancement in the development of new immunization candidates or alternative to enhance current vaccination efficacy for controlling CDV disease and its spread among domestic dogs and wildlife animals.

Therefore, this doctoral thesis addresses the need to update vaccination strategies against CDV through an *in silico, in vitro,* and *in vivo* approach for the development of a vaccine platform based on immunogenic peptides derived from the genetic information of relevant viral agents, with a universal character necessary for controlling the infection, spread, and transmission of highly prolific viruses such as CDV.

Key words: *Morbillivirus canis*, *in silico*, immunogenic peptides, vaccines design, *in vivo*, multiepitope polypeptide.

RESUMEN

Esta tesis está estructurada en cuatro capítulos donde se desarrolla una pregunta de investigación orientada al desarrollo de una vacuna de nueva generación contra el virus del distemper canino (CDV). En cada capítulo se muestran temáticas que permiten abordar diferentes aspectos para responder a esta pregunta, de modo que el lector encuentre todos los elementos no solo de contexto teórico, sino de resultados experimentales *in silico*, *in vitro* e *in vivo*, para el desarrollo de una estrategia nueva de inmunización para especies domésticas y silvestres, afectadas por el CDV.

El primer capítulo abordó todo el sustento teórico sobre la biología del virus, donde se tratan temáticas como el ciclo replicativo, patogénesis, presentación clínica e interacción virus-hospedero. Este capítulo permite al lector, tener un contexto biológico y molecular del CDV.

En el segundo capítulo se realizó una revisión sistemática de la literatura de enfoque amplio, con el fin de determinar el estado actual de la vacunación contra el CDV en especies domésticas y silvestres. Este capítulo fue desarrollado en esta tesis, puesto que no existía un estudio sumario donde se estableciera el estado actual de desarrollo de vacunas para el control de la diseminación e infección por el CDV. Un total de 2,321 artículos fueron identificados y 68 estudios fueron elegibles según criterios de inclusión predefinidos con un enfoque en vacunas contra el CDV en diversas especies animales, incluidos caninos, hurones, visones, ratones y otros. Los esfuerzos de investigación provinieron de comunidades científicas de los Estados Unidos, Canadá, Francia y Dinamarca. Se observaron formulaciones de vacunas diversas, así como una amplia gama de rutas y esquemas de inmunización. La seguridad y la eficacia resaltaron la importancia de estos inmunógenos dentro de las poblaciones animales evaluadas. Entre estos hallazgos se incluyen 37 estudios que realizaron evaluaciones de desafío post-inmunización contra el CDV, predominantemente en sujetos caninos, destacando los resultados de supervivencia entre las poblaciones vacunadas. Sin embargo, se identificaron brechas sustanciales en la investigación de vacunas, especialmente en los reservorios de vida silvestre. Por lo tanto, los esfuerzos colaborativos son imperativos para la formulación de estrategias de vacunación efectivas destinadas a preservar el bienestar animal y atenuar las repercusiones mundiales del CDV, especialmente entre las especies en peligro de extinción.

En el tercer capítulo, se evidenció el desarrollo de péptidos inmunogénicos derivados de la información genética y antigénica de todas las variantes del CDV circulantes alrededor del mundo a través de herramientas computacionales para el desarrollo de una vacuna universal para el CDV y su posterior evaluación de seguridad *in silico* e *in vitro*. Se desarrolló un candidato a vacuna de CDV basado en péptidos inmunogénicos, utilizando una biblioteca de péptidos derivados de secuencias de consenso de las proteínas H y F de CDV de cepas circulantes en todo el mundo. Se emplearon herramientas computacionales para

evaluar el potencial inmunogénico de estos péptidos. Se utilizaron el acoplamiento molecular y simulaciones dinámicas para evaluar la interacción molecular de los péptidos seleccionados con MHC-I, -II, TLR-2 y -4 caninos. La seguridad in silico se evaluó mediante antigenicidad, alergenicidad, potencial de toxicidad y comparación con péptidos caninos homólogos, mientras que la seguridad in vitro se evaluó mediante ensayos de citotoxicidad utilizando líneas celulares, células mononucleares de sangre periférica canina (cCMSPs) y ensayos de potencial de hemólisis con glóbulos rojos caninos. Posteriormente, se sintetizó un polipéptido multiepítopos de CDV basado en los péptidos más prometedores, y se realizaron evaluaciones in silico e in vitro para compararlo con inmunógenos individuales. En esta etapa, se identificaron cinco candidatos de péptidos, tanto individualmente como en forma de polipéptido multiepítopos del CDV, para su evaluación como vacuna basada en péptidos de próxima generación en un modelo animal.

El último capítulo contempló la evaluación de los péptidos inmunogénicos ya fuera individualmente en una única formulación o como polipéptido en un modelo *in vivo* de ratón para determinar su seguridad e inmunogenicidad en una formulación vacunal acompañada de un adyuvante comercial. Veinticuatro ratones BALB/c fueron distribuidos en cuatro grupos y sometidos a tres regímenes de dosificación de vacunas diferentes durante un período de 28 días. La seroconversión se determinó utilizando ELISA, mientras que las respuestas inmunes celulares se midieron mediante citometría de flujo, enfocándose específicamente en los marcadores inducidos por activación. Tanto la mezcla de péptidos, el polipéptido multiepítopos de CDV y la vacuna comercial (CV) fueron seguros en los ratones, con una seroconversión estadísticamente significativa observada en los grupos vacunados con el polipéptido multiepítopos de CDV y la CV en comparación con el grupo de control. Además, los ratones inmunizados con el polipéptido multiepítopos de CDV exhibieron niveles aumentados de células T específicas de antígenos CD4+ CD134+ e IFN-γ+, células T CD8+, así como células productoras de TNF-α e IL-6 en comparación con el grupo placebo. Estos resultados experimentales indicaron colectivamente la seroconversión y el establecimiento de la memoria inmunológica tanto para el polipéptido multiepítopos de CDV como para las vacunas CV. El desarrollo de vacunas de polipéptido multiepítopos presenta una estrategia preventiva prometedora contra el CDV, y nuestro enfoque integrado representa un avance significativo en el desarrollo de nuevos candidatos a vacunas o alternativas para mejorar la eficacia de la vacunación actual para controlar la enfermedad del CDV y su propagación entre perros domésticos y animales silvestres.

Por tanto, esta tesis de doctorado aborda la problemática de actualización de las estrategias de vacunación contra el CDV a través de un enfoque *in silico, in vitro* e *in vivo,* para el desarrollo de una plataforma vacunal basada en péptidos inmunogénicos derivados de la información genética de agentes virales de interés, con un carácter de universalidad, necesario para el control de la infección, diseminación y transmisión de virus altamente prolíficos como el CDV.

Palabras clave: *Morbillivirus canis*, *in silico*, péptidos inmunogénicos, diseño de vacunas, *in vivo*, polipéptido multiepítopos.

INTRODUCTION

Theoretical framework and background

Morbillivirus canis, commonly known as Canine distemper virus (CDV) is the etiologic agent of a contagious disease affecting the respiratory, gastrointestinal, integumentary, and neurological systems (MacLachlan et al., 2011) of domestic dogs and wildlife animals, which has been reported in at least 20 families within 7 to 8 orders, demonstrating the great capacity to cross species barrier (Lunardi et al., 2018; Martinez-Gutierrez & Ruiz-Saenz, 2016).

CDV belongs to the family *Paramyxoviridae*, genus *Morbillivirus*, which includes other well-known viruses such as measles virus, phocine distemper virus, rinderpest virus and small ruminant virus, which are of epidemiological importance to human and animal populations (MacLachlan et al., 2011). CDV has a negative-sense single-stranded RNA genome, consisting of 15,690 nucleotides, including 6 genes encoding for structural proteins: the nucleocapsid -N- (encapsulates the viral RNA), the phosphoprotein -P- (protein cofactor of the viral polymerase), the matrix protein -M- (inner layer of the viral envelope), the fusion protein -F- (fusion between the viral envelope and the host membrane), Hemagglutinin -H- (cellular tropism) and the large protein -L- (viral polymerase) (MacLachlan et al., 2011). In addition, through an alternative gene expression strategy, the P gene encodes two non-structural proteins: protein C and V, which inhibit type I and type II interferon responses (Chinnakannan et al., 2013; Schuhmann et al., 2011).

For the phylogenetic classification of CDV, the whole H gene sequence has been employed, which has shown great genetic diversity with a geographic distribution pattern (Martella, Elia, and Buonavoglia 2010; Espinal, Díaz, & Ruiz-Saenz 2014), thus to date, more than 18 lineages of CDV have been described worldwide: America-1 , America-2 to -5, Arctic-like, Rockborn-like, Asia-1 to -4, Africa-1 to -2, European Wildlife, Europe/South America-1,

South America-2 and -3 and South/North America-4. (Anis, Newell, et al., 2018; Blixenkrone-Moller et al., 1992; Duque-Valencia, Forero-Munoz, et al., 2019; Espinal et al., 2014; Haas et al., 1997; Harder et al., 1996; Iwatsuki et al., 2000; Nikolin et al., 2017; Panzera et al., 2012; Radtanakatikanon et al., 2013; Riley & Wilkes, 2015; Zhao et al., 2010).

Various vaccine platforms have been employed for the development of licensed vaccines, each demonstrating efficacy in eliciting robust and enduring immune responses as well as conferring protection against targeted pathogens. Furthermore, diverse adjuvants have been employed to augment the immunogenicity of vaccines (Kozak & Hu, 2023). Viral vaccine development has emerged with strategies including modified live attenuated virus (MLV), whole inactivated, split inactivated, synthetic peptides or multiepitope polypeptides, virus like particles, DNA or RNA, recombinant viral vectors, recombinant bacterial and recombinant subunits (Kozak & Hu, 2023; Marian, 2021; Singh et al., 2021).

To date, there are two MLV vaccines against CDV available, which were introduced in the 1960s (Buczkowski et al., 2014; Demeter et al., 2010) employed mostly in dogs and ferrets. The first, the Onderstepoort strain-based vaccine, was developed from a natural isolate that was adapted to ferrets, then adapted to chicken embryos, and finally being replaced with chicken cell culture (Haig, 1956). The other vaccine widely used in the world produced by adaptation of the Rockborn strain to canine kidney cell culture (Hartley, 1974; Rockborn, 1959). The canine-adapted cell culture vaccine produces immunity in almost 100% of vaccinated dogs but can cause post-vaccination encephalitis in some cases because it is a modified live virus (Hartley, 1974). Other strains employed in MLV vaccines include Snyder Hill, Convac, Rockborn, or CDV3 (Wang et al., 2024).

These two vaccines have significantly reduced CDV infection in domestic dog populations, although these vaccines are not sufficiently safe to use in wildlife species since it has not been demonstrated. For example, a CDV vaccine produced in canine cells causes disease in gray foxes (*Urocyon cinereoargenteus*) and ferrets (*Mustela nigripes*) (Carpenter et al., 1976; Halbrooks et al., 1981), while an attenuated avian vaccine can be lethal to European mink (*Mustela lutreola*) and ferrets (*Mustela putorius furo*) (Carpenter et al., 1976). In general, the avian cell-adapted vaccine is considered safer for wild species and is tolerated by gray and red foxes (*Vulpes vulpes*), bush dogs (*Speothos venaticus*), farmed wolves

(*Chrysocyon brachyurus*), and desert foxes (*Vulpes zerda*) (Halbrooks et al., 1981; McInnes et al., 1992; Thomas-Baker, 1985). In contrast, of all the species mentioned above, the vaccine adapted to dog cells was found to be safe only for red foxes (Halbrooks et al., 1981).

The existence of problems associated with MLV vaccines, particularly their unknown safety in endangered wildlife species, has led to the development of recombinant vaccines that can be safely used in all domestic and wildlife species. The generated recombinant CDV vaccine, which incorporates the F and H CDV proteins into a canary poxvirus vector, has been shown to be safe in all susceptible species tested to date, including dogs, European ferrets (*Mustela putorius furo*), giant pandas (*Ailuropoda melanoleuca*), fennec foxes, meerkats (*Suricata suricatta*), and Siberian polecats (*Mustela eversmanni*) (Bronson et al., 2007; Coke et al., 2005; Stephensen et al., 1997; Wimsatt et al., 2003). Although the recombinant canarypox vaccine is safe in many species, it induces a milder immune response than MLV vaccines due to replicative limitations (Schultz, 2006). However, this characteristic highlights its applicability for immunizing young animals in the presence of maternal antibodies (Pardo et al., 2007).

Other attempts have used reverse genetics techniques to study and develop new-generation vaccines, nevertheless, all of them are experimental candidates and have not been licensed yet. Those techniques encompass the rational attenuation concept of CDV by modifying the viral RNA polymerase to express a reporter gene from its open reading frame (ORF), resulting in viral attenuation as previously demonstrated for other morbilliviruses (Brown et al., 2005; Pardo et al., 2007). Similarly, deletion of N-glycosylation sites on the CDV H protein produced a virus with an attenuated phenotype that did not cause disease in ferrets (Sawatsky & Von Messling, 2010). Another approach focused on generating a chimeric virus that combined the Measles virus (MeV) replication complex with wild-type CDV glycoproteins, resulting in a recombinant that did not cause clinical signs or immunosuppression in vaccinated animals and induced protective immunity against lethal CDV exposure (Rouxel et al., 2009). On the other hand, DNA vaccines against CDV, as those against MeV, have been formulated to induce a strong and protective cell-mediated humoral immune response (Jensen et al., 2009; Nielsen et al., 2012).

Although DNA vaccine candidates have shown to be effective, there have been reported problems associated with the vaccination regimen and routes of administration that must be resolved to make DNA vaccines an effective alternative to MLV vaccine for wildlife populations (Wang et al., 2012). CDV has recently been used as a vector to express the rabies virus glycoprotein, constructing a chimeric virus that induced a strong rabies neutralizing antibody response and protected mice from a lethal dose of rabies (Wang et al., 2012). The virus was shown to be safe for both mice and dogs, and although challenge experiments with CDV were not performed, this vaccine candidate resulted in the production of long-lasting neutralizing antibodies against CDV and rabies, demonstrating the potential of multivalent vaccines for multiple morbilliviruses. In contrast, the use of a recombinant rabies virus expressing CDV glycoprotein H was also employed, which showed efficient generation of neutralizing immune responses in ferrets challenged with a circulating strain (da Fontoura Budaszewski et al., 2017). In addition, another vaccine, a recombinant Newcastle disease virus expressing CDV hemagglutinin, was evaluated in minks and proved to be an alternative vaccine, as efficiency was demonstrated when animals were challenged with a highly virulent CDV strain (Ge et al., 2015). Then, a recombinant chimeric virus of equine herpesvirus-1 expressing the CDV H protein was also investigated, which allowed efficient protection of dogs experimentally infected with a lethal dose of a circulating virus (Pan et al., 2017). In summary, several vaccine alternatives have been submitted for research, considering the potential vaccine failure of current commercial vaccines which are based on non-circulating CDV strains.

Other vaccines strategies could be employed for CDV control and dissemination such as peptide-based vaccines, however, there are not experimental or commercially available alternatives for CDV (Wilkes, 2022). This vaccination platform has been employed for addressing various viral infections including hepatitis B, influenza A, and hepatitis C, among others, showing promising immune responses in experimental settings (He et al., 2015; Sominskaya et al., 2010; Stanekova & Vareckova, 2010). There are numerous factors favoring the adoption of peptide-based vaccines including that those vaccines do not contain infectious agents, making them safer for use in wildlife animals. Additionally, they allow for straightforward incorporation of molecules to enhance immunogenicity. Peptide vaccines

can be prepared in lyophilized form, facilitating storage. Furthermore, there is no risk of virulence reversal, and finally, they can be customized to include multiple antigenic elements (Purcell et al., 2007).

In Colombia, the molecular and genetic characterization of CDV has been carried out by our research group, which has allowed the characterization of circulating viruses and has shown the existence of two different lineages, South America-3 and South/North America-4, and these lineages have been shown to contain an RNA genome that differs up to 10% in amino acid sequence compared to CDV vaccine strains (Duque-Valencia et al., 2019). These differences could imply changes in CDV H and F proteins, the main antigenic determinants that must influence the host immune response and neutralization of CDV in domestic and wild animals (Ke et al., 2015). Furthermore, the diverse substitutions present in CDV proteins, most of which are under positive selection, are close to or within previously reported B lymphocyte presentation epitopes (Li et al., 2018). Since infection has been reported in vaccinated animals, it is possible to postulate that changes in CDV H and F genes could also contribute to the low or no production of vaccine antibodies capable of neutralizing wild-type strains, as has been reported in the United States on CDV strains belonging to different CDV lineage, or could even be an explanation for the existence of strains that could escape the host immune response (Anis, Holford, et al., 2018; Zhao et al., 2014).

On the other hand, the CDV genome divergence presents an interesting difference between nucleotide and protein sequences, which could explain that emerging strains present a higher number of non-synonymous substitutions due to the lack of self-correction of the viral RNA polymerases (MacLachlan et al., 2011), supporting the idea that these substitutions are also related to vaccine failure, cross-species transmission, and virulence, as the presence of substitutions in sites under positive selection has been shown to be associated with these phenomena and also involved in evasion of innate and humoral immune responses (Duque-Valencia, Diaz, & Ruiz-Saenz, 2019). For example, an epitope in region 444-455 in the N protein reported by Duque et al. and described for presentation to B lymphocytes (humoral response) has also been described as highly variable and associated with highly virulent strains (Li et al., 2018), suggesting a coincidence of variation in CDV

lineages around the world, which could also potentially explain vaccine failure. Similarly, a mechanism of resistance to antivirals has been described in the F protein involving the I564C, V571C, G572C, and L575C substitutions (Kalbermatter et al., 2019), supporting the idea that CDV genetic variation is involved in differences in host immune responses to wild-type and vaccine strains.

Several events need to be related to the development, efficacy, and safety of vaccines against CDV in both domestic and wild animals worldwide, as the evolution and dynamics of CDV is leading to the need for research in new vaccine candidates that could be used in multiple regions and additionally safely used in wild animals, as it has not yet been demonstrated.

Problem statement

With this background, we proposed that the development and implementation of peptide immunogens can serve as an efficient and safe alternative for the induction of antibodies in animals; and that this vaccine can be used as an alternative in geographical regions, where there is circulation of different genetic variants (lineages), as is the case of Colombia and the Americas.

In addition, it has been seen that the use of recombinant vaccines can be very useful in the immunization of animals with the presence of passive immunity of maternal origin and is proposed as an efficient and safe alternative in the immunization of wildlife populations found at risk of developing CDV infection in natural conditions, increasing the risk of extinction and spread of CDV epidemics.

In this case, the use of peptide immunogens would favor the efficient generation of immunity in both domestic and wild animals, avoiding the drawbacks caused by genetic divergence of CDV in domestic populations from different geographical regions, reducing the risks associated with vaccination with live virus, the risk of use of MLV in wild populations and the adverse events of co-circulation of live viruses (virulence reversals, recombination, among others) in different populations, both domestic and wildlife animals.

General objective

To develop and evaluate different CDV immunogens based on sequences of strains circulating worldwide for the development of vaccines against CDV.

Specific objectives

- To understand the current state-of-the-art in CDV pathogenesis, transmission, and vaccine development.
- To construct a peptide library of CDV proteins from circulating CDV strains that have high immunogenic potential.
- To evaluate *in vitro* the safety of different peptide immunogens from CDV.
- To validate *in vivo* the safety of specific CDV immunogens that have overcome the *in vitro* safety evaluation.
- To assess the immunity generated by the highly immunogenic peptides derived from circulating CDV proteins in an *in vivo* model.
- To compare the immune response generated *in vivo* by immunogenic peptides with that induced by a commercial vaccine.

Research question.

Can the development of peptides with immunogenic potential based on the genetic and antigenic information of worldwide CDV circulating lineages be used safely in both domestic and wildlife?

To respond to this research question, in this dissertation the *Chapter 1*: *Tropism and molecular pathogenesis of canine distemper virus* **(Published in Virology Journal***)*, lays the foundation for this research question by providing a comprehensive literature review that synthesizes existing concepts about CDV and identifies gaps in the current understanding.

This chapter serves as the theoretical framework upon which subsequent analyses were built. Chapter 2: *scoping review about safety and immunogenicity of Morbillivirus canis vaccines for domestic and wildlife animals* (**Submitted to Viruses**) focuses on establishing the current state of research and advancements in CDV vaccination for both domestic and wildlife animals. employing a rigorous methodological approach to capture and show the current information about CDV vaccine development. The findings presented in *Chapter 2* contribute to the broader discourse on the necessity to update CDV vaccination, since commercially available vaccines have not controlled properly CDV dissemination and disease in domestic and wildlife animals worldwide. Moving forward, *Chapter 3*: *Single peptides vs multiepitope polypeptide "universal vaccine" against Morbillivirus canis: vaccinomic design with an integrated in silico and in vitro validation (***Submitted to Scientific Reports***),* describes the prediction, development, and *in vitro* validation of two different immunogen design strategies of potential immunogens against CDV, as a peptide mixture, composed of 5 selected immunogenic peptides or as one multiepitope polypeptide, assembled with the selected peptides linked with short amino acid sequences that played an important role in the polypeptide cleavage for proteasomes and junctional immunogenicity reduction. This work served as base for the submission of national and international claims of the patent for peptide-based immunogens, PCT/IB2022/061236 (national patent CO2021017322A1 and, international patent WO2023111728A1). Finally, *Chapter 4*: *safety and immunogenicity evaluation of multiple epitope polypeptide from Morbillivirus canis in mice* (**Submitted to Vaccine**)*,* synthesizes the experimental data obtained from an *in vivo* validation of the immunogenicity of a peptide-based immunogen in mice, drawing connections between the various strands of research question explored in the preceding chapters. This final chapter culminates in a nuanced conclusion that advances our understanding of safe and immunogenic peptide-based vaccines for CDV employing vaccinomics. Together, these chapters constitute a comprehensive and methodologically robust exploration of a new vaccine alternative for CDV employing genetic and antigenic information from CDV strains distributed worldwide, contributing to the scholarly discourse, and laying the groundwork for future research endeavors in this CDV vaccine development.

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CHAPTER 1

Tropism and molecular pathogenesis of canine distemper virus

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Abstract

Canine distemper virus (CDV), currently termed as *Morbillivirus canis*, is an extremely contagious disease that affects dogs. It is identified as a multiple cell tropism pathogen, and its host range includes a vast array of species. As a member of *Mononegavirales*, CDV has a negative, single-stranded RNA genome, which encodes eight proteins. Regarding the molecular pathogenesis, the hemagglutinin protein (H) plays a crucial role both in the antigenic recognition and the viral interaction with SLAM and nectin-4, the host cells' receptors. These cellular receptors have been studied widely as CDV receptors *in vitro* in different cellular models. The SLAM receptor is located in lymphoid cells; therefore, the infection of these cells by CDV leads to immunosuppression, the severity of which can lead to variability in the clinical disease with the potential of secondary bacterial infection, up to and including the development of neurological signs in its later stage. Improving the understanding of the CDV molecules implicated in the determination of infection, especially the H protein, can help to enhance the biochemical comprehension of the difference between a wide range of CDV variants, their tropism, and different steps in viral infection. The regions of interaction between the viral proteins and the identified host cell receptors have been elucidated to facilitate this understanding. Hence, this review describes the significant molecular and cellular characteristics of CDV that contribute to viral pathogenesis.

Keywords: Canine distemper virus, *Morbillivirus canis*, Molecular pathogenesis, Zoonosis, Tropism, Neuropathogenesis.

Introduction

Morbillivirus canis commonly known as Canine distemper virus (CDV) belongs to the *Paramyxoviridae* family, genus *Morbillivirus*, and is the etiological agent of canine distemper (Murphy et al., 2012). It is considered as a highly contagious and an acutely febrile disease in dogs that has been known since 1760 (MacLachlan et al., 2011). It is associated with multiple cell tropism (epithelial, lymphoid, and neurological), which leads to a systemic infection including respiratory, digestive, urinary, lymphatic, cutaneous, skeletal, and central nervous system (CNS) diseases (Lempp et al., 2014).

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, fishers, 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* (MacLachlan et al., 2011; Martinez-Gutierrez & Ruiz-Saenz, 2016). Considering the vast array of species affected by CDV, cross-species transmission has been studied among wildlife and domesticated species in terms of the interactions among them in order to establish phylogenetic relationships (Beineke et al., 2015).

The CDV particles are pleomorphic, frequently spherical, enveloped virions having a diameter of about 150nm which include a non-segmented single negative-stranded RNA (ssRNA), similar to other members of the order *Mononegavirales* (**Figure 1A**). The genome contains 15,690 nucleotides in throughout length and encodes for eight proteins (da Fontoura Budaszewski et al., 2016) (**Figure 1B**). The CDV genome structure includes six transcription units (N-P-M-F-H-L) organized in a linear form, which are separated by intergenic untranslated regions (UTRs) that are relatively uniform in length, with the exception of the UTR between the matrix (M) and the fusion (F) gene (Kolakofsky, 2016). Those transcription units contribute to the formation of the eight proteins mentioned above. However, the P gene encodes for the C and V proteins, using an overlapping open reading frame (ORF) and RNA editing by the insertion of a non-templated G residue during mRNA synthesis, respectively (Cattaneo et al., 1989; da Fontoura Budaszewski & von Messling,

2016). Both alternative gene expression strategies not only have functions that are related to transcription control and replication but also play an important role in the virus' evasion of its host's innate immune responses (Mahy & Van Regenmortel, 2010; von Messling et al., 2006).

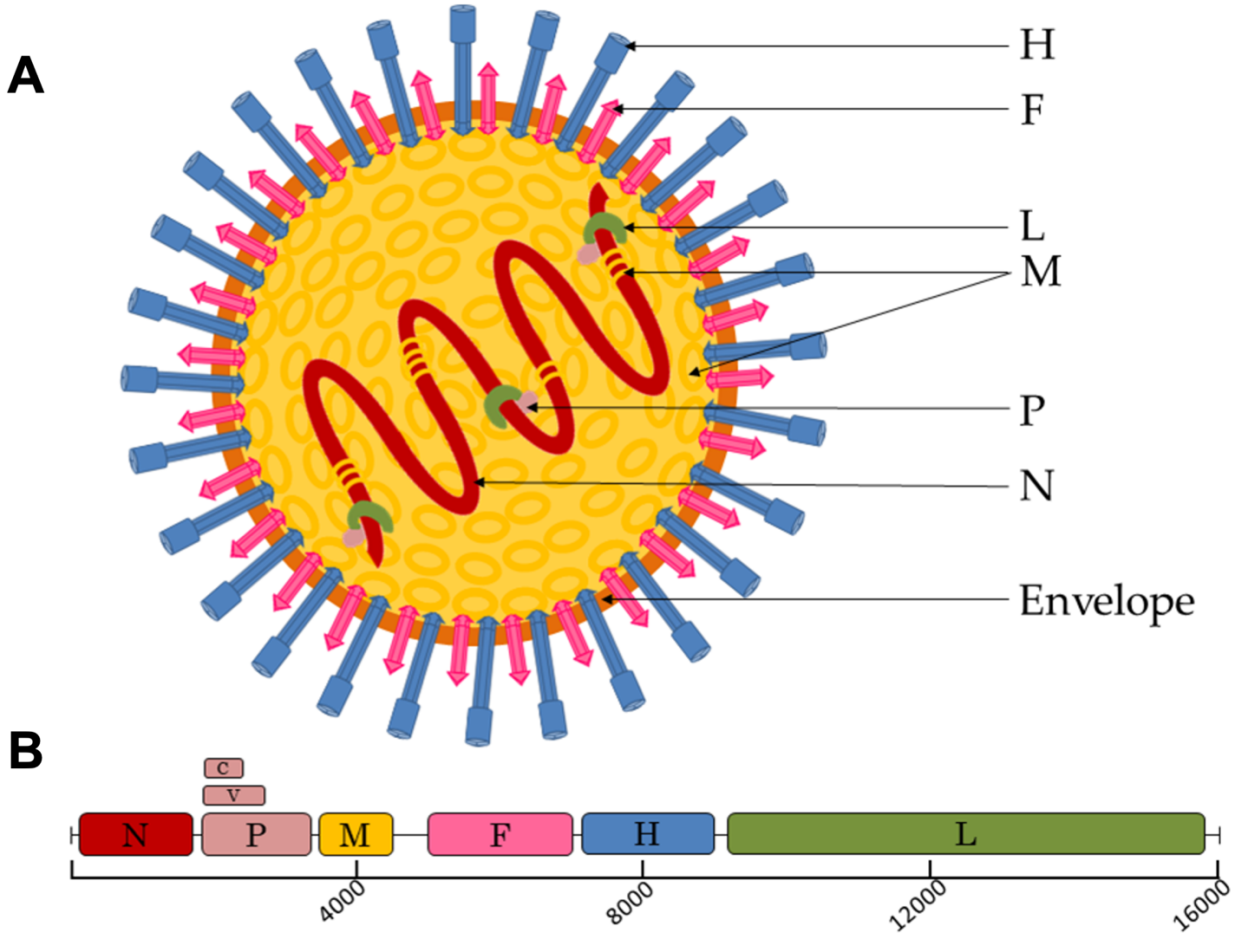

Figure 1. CDV virion and genome organization. A) Schematic diagrams of CDV particle in cross-section N: nucleocapsid, P: phosphoprotein, M: matrix protein, F: fusion protein, H: hemagglutinin, L: large polymerase protein. B) Map of genomic RNA (3' to 5') of CDV. Each box represents a separately encoded mRNA; multiple distinct ORFs within a single mRNA are indicated in overlapping boxes on P.

All the proteins have a specific function related to the viral cycle and replication, with the nucleocapsid (N) protein encapsidating the genomic RNA, and based on the CDV gene expression, N serves as a template for the transcription and replication by the viral polymerase, denoted as L, and its cofactor, the phosphoprotein (P). The N, P, and L proteins along with the viral RNA compose the ribonucleoprotein (RNP) complex (von Messling et al., 2001). The CDV envelope involves two integral membrane proteins, the fusion (F) and the hemagglutinin (H) proteins, and finally a membrane associate protein M, which contributes by mediating the contact with the RNP, encircled by the viral envelope throughout the budding process in the host cell membrane (da Fontoura Budaszewski & von Messling, 2016). This understanding has been arrived at mostly by using reverse genetics, which enables one to construct chimeric viruses that are to be employed in different fields such as pathogenesis studies, vaccine development, and gene therapy vectors (Tumpey et al., 2005).

As with other members of the *Paramyxoviridae* family, the H glycoprotein facilitates the virus binding to the host cell membrane and the F protein achieves the viral and the host membrane's fusion, enabling the viral RNP's entrance into the cytoplasm (von Messling et al., 2001). Two cellular receptors have been described regarding the CDV host's cell recognition and virus entry, which include the SLAM (Signaling Lymphocyte Activation Molecule or CD150) in the peripheral blood mononuclear cells (von Messling et al., 2005) and the nectin-4 (PVRL4) in the epithelial cells (Pratakpiriya et al., 2012). It has also been speculated that Nectin-4 can help the virus shed itself into the respiratory airways (Mühlebach et al., 2011). Based on the lack of detection of either the SLAM or the nectin-4 receptors on astrocytes, it is speculated that CDV uses an alternative receptor to invade these cells, though this potential third receptor for CDV is yet to be identified (Chen et al., 2011).

The H protein has become the most suitable target to investigate the CDV variability and evolution. It is considered the most genetically variable gene, with up to 11% nucleotide divergence among CDV strains. This fact has enabled the conduction of CDV phylogenetic and phylodynamic studies based on genetic divergence and molecular epidemiology, respectively (Ke et al., 2015; von Messling et al., 2001). Phylogenetic studies based on the

complete sequence of the H gene from several CDV strains detected in a variety of geographic locations worldwide have been conducted to infer the genetic diversity of the CDV. The genotyping classification takes into account that within each genotype the nucleotide divergence should be less than 5% (Mochizuki et al., 1999). Following this criteria, to this date 17 distinct genotypes have been described: America-1 (that includes almost all commercially available vaccine strains), America-2 to 5, Arctic, Rockborn-like, Asia-1 to 4, Africa-1 and 2, European Wildlife, Europe/South America-1, South America-2 and 3 (**Figure 2**) (Anis, Newell, et al., 2018; Blixenkrone-Møller et al., 1992; Espinal et al., 2014; Haas et al., 1997; Harder et al., 1996; Iwatsuki et al., 2000; Nikolin et al., 2017; Panzera et al., 2012; Radtanakatikanon et al., 2013; Riley & Wilkes, 2015; Zhao et zal., 2010).

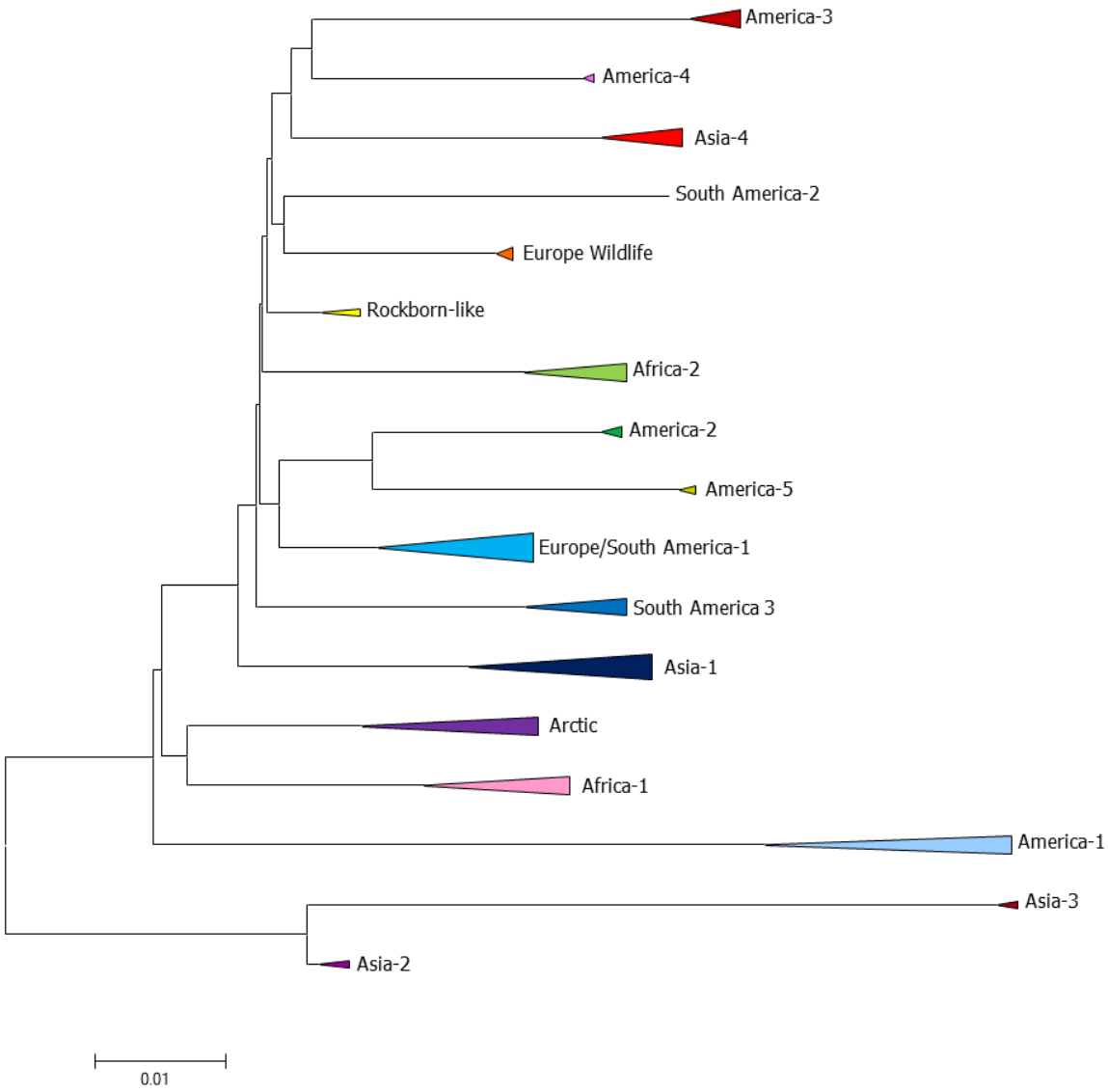

Figure 2. Phylogenetic tree constructed from the alignment of complete H gene sequences obtained from GenBank, which represents all current CDV-described genotypes. 67 H sequences representing all genotypes were retrieved and aligned with ClustalW using MEGA 6.0 software. MEGA 6.0 was also used for phylogeny inference according to the Maximum Likelihood algorithm method based on the Tamura 3-parameter model. The rate variation among sites was modeled using a gamma distribution (shape parameter = 5). The robustness of the hypothesis was examined using 1000 non-parametric bootstrap analyses. GenBank accession numbers of all isolates used to construct the tree are listed in **Table 1** as supplemental File 1.

Concerning the CDV vaccination, an attenuated CDV vaccine was released in the 1950s and its widespread usage helped to control the CDV disease in many countries (MacLachlan et al., 2011). In the last few decades, however, an increase in the canine population has resulted in sporadic cases and massive outbreaks of the CDV disease, even in animals that have been vaccinated, both domesticated animals and wildlife (Martella et al., 2011; Nagao et al., 2012). It has been suggested that antigenic differences among the vaccine strains and the circulating wild-type strains may be a causal agent (Anis, Holford, et al., 2018), as the amount of CDV genotypes have increased in the recent days.

CDV has been considered a surrogate model for Measles virus (MeV), which is a closely related morbillivirus. Both viral agents cause a similar overall pathogenesis. However, humans and non-human primates comprise the only reservoir for MeV (da Fontoura Budaszewski & von Messling, 2016). These facts enable one to believe that an extensive study concerning CDV pathogenesis and tropism based on the experimental data regarding MeV is necessary to elucidate the causes of occasional CDV outbreaks led by viral evolution and evasion of host innate immune response.

The equivalence among those viral models and the existence of methodologies such as reverse genetic studies has allowed the employing of recombinant virus to evaluate the effect of changes in viral genomes, particularly aspects regarding the viral life cycle and molecular pathogenesis. Although reverse genetics applied to viruses belonging to the *Mononegavirales* order have not been as efficient as expected, new technologies have been used to increase the rescue efficiency, turning it into an appropriate tool to investigate the basic aspects of the biology of viruses including CDV and MeV. This includes studies on the molecular determinants of virus entry and spreading between cells, besides the development of live attenuated vaccine vectors (Beaty et al., 2017).

Initially, CDV was rescued from a full-length cDNA clone based on the Onderstepoort strain, similarly to what was being previously done for MeV and *Rinderpest virus*, through which obtaining a recombinant CDV which had no differences with the Onderstepoort strain regarding the disease progression and syncytia formation, with the exception of a genetic

tag comprising two nucleotide changes that was introduced on the coding region of the L protein (Gassen et al., 2000). The introduction of the green fluorescent protein (GFP) into the cDNA clone to study the infection of the virus in a cell culture and in an animal model has been proven to be very useful in MeV studies (Duprex et al., 1999; Duprex et al., 2000). For CDV, the neurovirulent Snyder Hill strain was rescued, expressing enhanced GFP (eGFP) or red fluorescent protein (dTom), enabling a sensitive pathological assessment of the routes of virus spread *in vivo*; this showed how the virus rapidly circumvents the cerebrospinal fluid barriers and induces a dramatic viral meningoencephalitis (Ludlow et al., 2012). A wild-type strain, 5804, that is highly pathogenic for ferrets, was also rescued, expressing GFP, retaining full virulence, and illuminating the lymphocyte-based pathways through the immune system of its infected host (V. von Messling et al., 2004). The roles of morbillivirus receptors SLAM and nectin-4 in transmission have also been assessed by reverse genetics. Recombinant CDVs (rCDVs) with mutations in residues of the H gene, unable to recognize one of the receptors (SLAM-blind and nectin4-blind), were generated and inoculated in ferrets, showing that both SLAM and nectin-4 receptors are required for transmission, demonstrating the importance of sequential use of both receptors in CDV pathogenesis and transmission (Sawatsky et al., 2018a). Assessing the viral entry, intra-host dissemination and inter-host transmission, by using recombinant viruses expressing multicolor fluorescent proteins (green, red or blue) for *in vivo* competition and transmission, have exhibited that CDV enters the host competently when inoculated through the nose or lung and that infection of the host through conjunctival administration, although less efficient, is also possible (de Vries et al., 2017). However, reverse genetics is helpful not only to understand the molecular pathogenesis regarding the role of proteins in viral life cycle, spread, and transmission, but also for the development of vaccine vectors. Viral vectors expressing CDV glycoproteins, H alone or in combination with the F protein, have been tested as live attenuated vaccines, while one based on the canarypox vector expressing H and F proteins is commercially available. Recombinant NYVAC vaccinia virus and the ALVAC canarypox virus expressing CDV H/F have been tested and both protected against the development of symptomatic distemper (Stephensen et al., 1997). Rabies virus (RABV) is also an efficient and safe platform for the generation of recombinant (rRABV) bivalent vaccines, also expressing H/F CDV proteins. An attenuated rRABV-CDVH expressing only

the H protein can offer whole protection against challenge with virulent CDV in dogs (Wang et al., 2014), while a similar approach using an inactivated version generated only partial protection after the wild-type challenge of ferrets. However, the inactivated rRABV expressing both H/F proteins fully protected ferrets from lethal CDV challenges, demonstrating the critical role of immune responses directed against the F protein for the control of CDV and, likely, other morbillivirus infections (da Fontoura Budaszewski et al., 2017). Conversely, CDV has also been used as a viral vector for the expression of the G glycoprotein of RABV. Animal studies demonstrated that rCDV-RVG was safe and efficient against challenges in mice and dogs (Wang et al., 2012).

Clinical outcome

In terms of clinical characteristics, when a dog is infected with CDV, catarrhal and nervous manifestations, or a combination of both, and a chronic nervous manifestation can be observed. At the acute stage, viruses can be found in every secretion of the given animal (Avila et al., 2015). This phase is followed by various clinical signs including an onset of cutaneous rash, serious nasal and ocular discharge, conjunctivitis and anorexia, followed by gastrointestinal and respiratory signs, which are often complicated by secondary bacterial infections and neurological disorders (Beineke et al., 2009; MacLachlan et al., 2011).

The nervous signs may include myoclonus, nystagmus, ataxia, postural reaction deficits, and tetraparesis or plegia (Amude et al., 2007; Koutinas et al., 2004). However, animal recovery can be promoted by an improved immune system mostly by increasing the production of virus-specific neutralizing antibodies (Vandevelde & Zurbriggen, 2005). Albeit the fact that the virus is eliminated from different organs and peripheral blood, CDV can remain in some tissues including uvea, CNS, lymphoid organs, and footpads. Furthermore, some infected animals exhibit a retarded and diseased development and a moderate immune response with some imperceptible early clinical signs (Schobesberger et al., 2005).

In the aftermath of the viral infection of the CNS, some disturbances can be perceived. Generally, dogs with CNS pathologies do not survive. However, some may recover and exhibit lifelong neurological symptoms (Lempp et al., 2014). Demyelinating

leukoencephalitis (DL) is also commonly induced by CDV in the latest stages of the disease, and in terms of immunopathological processes, glial responses and early axonal degeneration, DL by CDV shares some characteristics with other diseases that cause demyelination, such as multiple sclerosis either in human or animal models (Ulrich et al., 2014). It has been previously studied that the chronic phase of CDV infection generates DL (Lempp et al., 2014). The decrease in viral titers, the alteration of maturation, and the plasticity of astrocytes, primary axonopathy, and a probable role of Schwann cell-mediated regeneration are crucial events in DL (Lempp et al., 2014). Virus persistence in the CNS can be observed with some CDV strains, for instance the A75/17 strain, when the virus is capable of spreading to some areas of the brain without eliciting an inflammatory response. This strain is also known to infect cell lines very inefficiently, with limited syncytia formation (Stettler & Zurbriggen, 1995).

CDV proteins and their role in pathogenesis

The CDV genome encodes eight proteins within six transcription units. CDV proteins have a specific activity regarding virus replication and in the infection cycle. The N protein not only provides the basis of the helical structure of the RNP, but it is also a requisite in some replicative viral processes (Ortin & Martin-Benito, 2015). Among its various functions, the N protein protects the genome from degradation, avoids the formation of dsRNA between viral RNAs of opposite polarity, and packs the RNA into the RNP. Moreover, as a consequence of its dynamic interaction with RNA and the L protein, it controls the L access to the RNA within the template RNP and nucleates the assembly of progeny RNPs. Consequently, the N protein due to its interaction with the genomic RNA [36] controls both the replication process and the transcription process (Koutinas et al., 2004). On the other hand, the L protein shows the polymerase activity and is carried by the virus particle. The P protein works as its cofactor, which has two fundamental functions. The first is to recognize the RNP as the polymerase template and the other is the stabilization of the nascent N protein (Ortin & Martin-Benito, 2015).

The H and F proteins' main functions are to mediate the recognition, attachment, and fusion processes of the CDV to the host cell. The attachment protein H, which lacks the neuraminidase action observed in other viruses, attaches to receptors present on the plasma membranes of host cells, such as SLAM, nectin-4, and other, in glial cells (Smith et al., 2009). Moreover, the M protein is essential in the assembly and budding of CDV particles, and acts as an intermediate between the RNP and the glycoprotein surfaces by enabling the interaction of M with the C- and N-terminal of N and the cytoplasmic tails of H and F proteins (Bringolf et al., 2017). V and C proteins are non-essential with respect to the virus replication process but critical for preventing the host immune responses. Therefore, cooperative actions between them can be critical to efficiently evade the host's immune responses and cause diseases *in vivo* (Otsuki, Nakatsu, et al., 2013).

Hence, besides the CDV protein's functions, it is important to mention that all molecular interaction depends on the nature of the molecules and the amino acidic sequences, which together define all protein functions. These factors influence not only the host cell responses but also the CDV infection cycle, and clearness in this molecular process is essential to the understanding of CDV cell tropism and pathogenesis. In the following paragraphs, we will try to address the role of each of the CDV proteins in virus replication, life cycle, and pathogenesis in further detail.

Infection cycle

Based on the presence of F and H glycoproteins, CDV can successfully overcome the plasma membrane, which is considered as the host's first cell defense barrier, through a hetero-oligomeric complex composed of a tetrameric H and a trimeric F. The H glycoprotein has been widely implicated in the interaction with specific host cell receptor, by recognition of specific amino acids, followed by multiple conformational changes in H and F. It has been recently shown that there exists a central pocket in the globular head domain of F that regulates the stability of the metastable, pre-fusion conformational state of the F trimer (Avila et al., 2014). This interaction is mediated by two hydrophobic residues located in the Ig-like domain of the F globular head domain, which contributes to the interaction between the

receptor and the membrane-proximal domain of the H stalk (Avila et al., 2015). In addition, it has been reported that the intensity of the F-triggering stimulus obtained by the H tetramers is influenced by the origin of the H protein and the molecular nature of the contacted receptor (Avila et al., 2014). This is due to the fact that critical residues are located at the front H-binding site that has been implicated in the step of inducing such fusion machinery (Khosravi et al., 2016). Such structural rearrangements in the protein complex facilitates the virus' attachment on the cell membrane surface, the formation of fusion pores, and thereafter the introduction of RNP complex into the host cell cytoplasm. Membrane fusion is also required for cell-to-cell spreading of the virus, which results in a multinucleated cell formation known as syncytia, which is a remarkable cytopathogenic characteristic of the morbillivirus (Plattet et al., 2016).

All the CDV replication and transcription strategies are similar to that of the other members of the *Mononegavirales* order, as shown in **Figure 3** (Moss & Griffin, 2006). The polymerase complex is formed by two different proteins: the subunit L, liable to the enzymatic process with its domains being involved in RNA synthesis, capping, and cap methylation, and the phosphoprotein P, an essential cofactor related to the functioning of L (Das & Arnold, 2015).

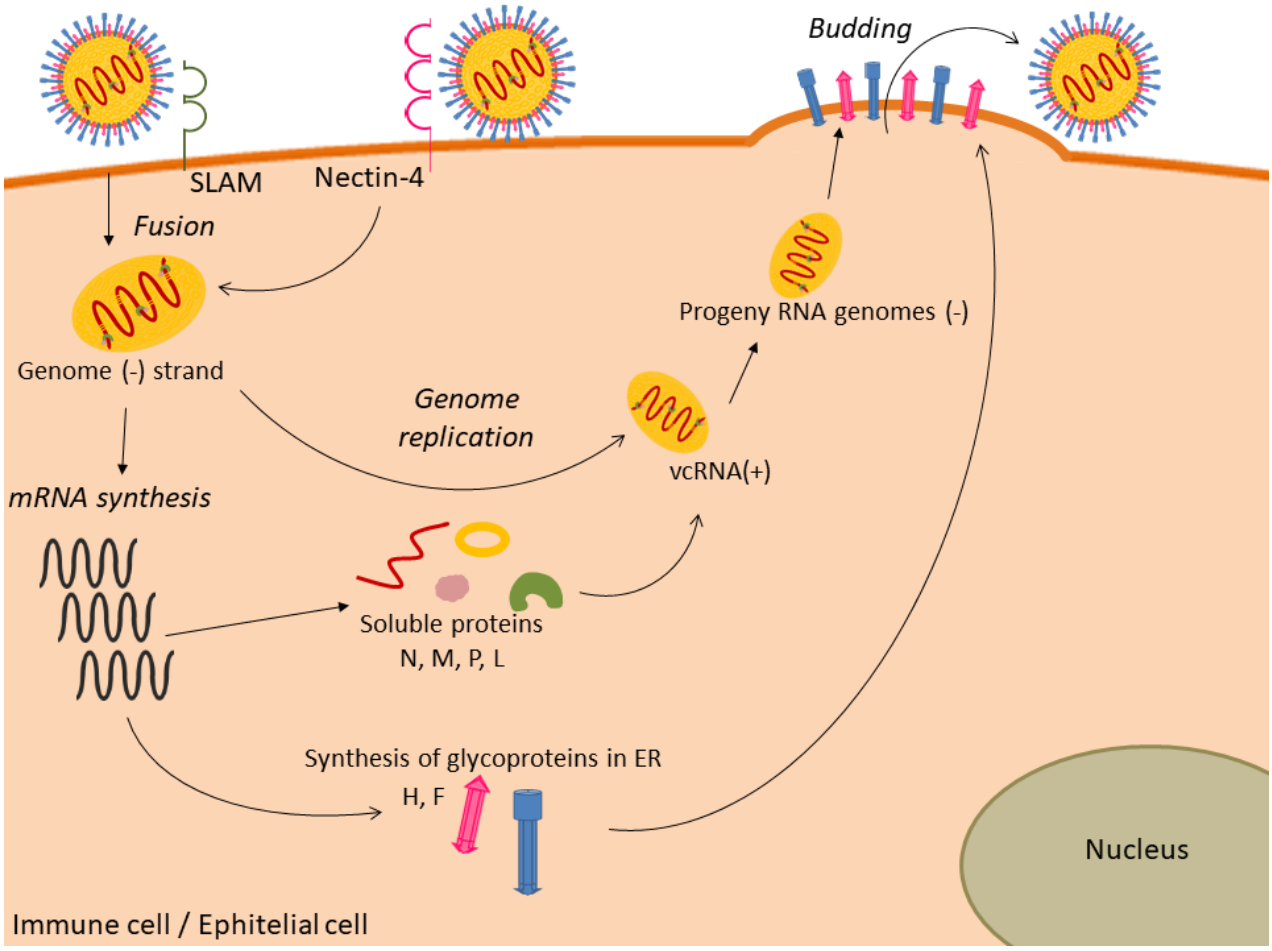

Figure 3. Replication of the CDV cycle. Virus particle recognition by host cell receptors (CD150 or nectin-4), RNP release into de cytoplasm, replication, transcription process, and virus particle budding are illustrated.

An important characteristic of the genome template in the CDV is associated with the nucleoprotein N in terms of constituting a helical nucleocapsid and forming the N-RNA structure (Lamb, 2001). While the polymerase proceeds, it recognizes the beginning and ending gene signals and generates six sub-genomic mRNA. Therefore, at a beginning gene signal, the polymerase starts the mRNA synthesis and at the ending gene signal, it releases the synthetized RNA. Afterwards, the polymerase surveys the intergenic regions to locate the next beginning gene signal. This process is done with each gene and there is evidence of a methyl cap addition and a poly A, both of which are essential for the polymerase to

change into the elongated mode (Lamb, 2001; Pfeffermann et al., 2018). Interestingly, the P ORF has also an RNA-edited form, where the RNA transcriptase hesitates on the RNA template at an RNA editing motif, leading to the addition of a pseudo-template guanine. As a result, the V protein has the same amino-terminal domain as a P protein but has a different carboxy-terminal domain. In opposition to the V protein, C mRNA transcription is initiated at an alternative start codon (Pfeffermann et al., 2018).

As mentioned before, the morbillivirus genome is composed of six transcriptional units that are separated by untranslated regions (UTRs) which are relatively uniform in length (approximately 100 to 200 nucleotides), with the exception of the UTR between the M and the F genes, which is at least three times longer and highly variable (Anderson et al., 2012). It has been documented that the F 5′ UTR of CDV is essential in translating an unusually long F signal peptide (FSP) (von Messling & Cattaneo, 2002). This signal is quite different from a classical signal sequence as this region has regulatory functions *in vitro* as *in vivo* (in ferret models of disease), indicating that the region between CDV M and F genes modulate virulence by controlling the F protein's expression (Anderson & Von Messling, 2008). On the other hand, a short putative ORF has been identified within the wild-type CDV-M 3′ UTR (Stettler et al., 1997). The proximal part of the M 3′ UTR modulates the initiation of viral genome replication and is involved in the disease's prolonged extension in ferrets, indicating that both specific sequence elements as well as the general length are required to maintain a wild type virulence (Anderson et al., 2012; Wiener et al., 2010).

Similar to the MeV replication cycle, it is crucial to assemble the M protein, which plays an important role in the assembly and budding of virions, considered as an intermediary between the RNP and the surface glycoproteins and orchestrating the viral particle assembly process (Bringolf et al., 2017; Dietzel et al., 2011). The RNP and the glycoproteins, in specific regions of the plasma membrane of the host-infected cells, form complete infectious CDV particles as the result of a coordinated interaction between viral and cellular factors (El Najjar et al., 2014). As proven, the C-terminal of the N protein is fundamental in its interaction with the M protein; mutations or deletions within it inhibit the transport of the RNP complex to the plasma membrane through infection, demonstrating the importance of the M protein in the integration of the RNP complex into the CDV particles (El Najjar et al., 2014). For the

MeV, it has been demonstrated that mutations in the M protein or deletions of the whole protein diminishes virus assembly and influences pathogenesis (Dietzel et al., 2011). Based on these facts, the M protein leads to CDV assembly and budding in spite of the deficiency of other proteins, considering that the paramyxovirus M protein is sufficient to shape virus-like particles (El Najjar et al., 2014). CDV budding is believed to be independent of the cellular Endosomal Sorting Complex Required for Transport (ESCRT) machinery for the host cell egress, which is particularly conducted by the CDV M protein (Salditt et al., 2010).

Lastly, it is believed that the F and the H proteins are assembled in intracellular milieu. The M protein attaches itself to the RNP complex in the cytoplasm and carries it to the plasma membrane, where the F and the H proteins are convened with the budding virus particle. This may be related to the observation that the CDV envelope proteins, H and F, are partitioned into cellular detergent-resistant membranes (DMRs), which may form the structural basis for membrane rafts. Consequently, the role of lipid rafts in the virus assembly as well as the release is suggested, as there is a necessity of the virus-enveloped cholesterol since the depletion of cholesterol in the cell membrane harboring the CDV envelope proteins resulted in the decrease in syncytium formation. Hence, both the incorporation of the envelope proteins into DRMs and their interaction with cholesterol may be necessary for the virus's entry and release (Imhoff et al., 2007). Furthermore, it has been shown that the first 10 residues from the CDV H cytoplasmic domain strongly influence its incorporation into virus-like particles that are formed by the CDV matrix (M) protein. In addition, this domain is required to ensure the correct translocation of nascent proteins into the endoplasmic reticulum to suffer post-translational modifications (Sawatsky et al., 2016).

Tropism and pathogenesis

CDV is considered a multi-cell pathogen that can infect three different types of host cells including epithelial, lymphoid, and neurological cells. Infections may occur not only by the inhalation of aerosol droplets or airborne virus particles but also because of direct contact with bodily fluids or through fomites (de Vries et al., 2017). Contemplated, as a systemic infection and affecting a vast array of organs and tissues, there are some CDV host cell

receptors that have been widely studied, such as the SLAM, which is expressed on activated T- and B- lymphocytes, and dendritic cells (DCs) and macrophages. These behave like the regular entry receptors for morbilliviruses. Other extensively studied receptors include nectin-4, which is recognized as an epithelial cell receptor and currently considered to function as a host exit receptor (Noyce et al., 2013; Ono et al., 2001).

Based on MeV, in the first stages of infection within the host, resident DCs and alveolar macrophages in the respiratory tract are infected along with other cells which express CD150 in the alveolar lumen (Pfeffermann et al., 2018). Similar to the MeV, CDV H protein attaches itself to the cell via the CD150 cell receptor (De Witte et al., 2008). It is believed that there is a translocation of an intracellular pool of CD150 into the cell membrane surface. Infected cells carry the virus to the draining lymph node where then the resident activated T-cells and B-cells are infected through the CD150 receptor, resulting in virus amplification and the initiation of primary viremia (**Figure 4A**) (Leonard et al., 2008). The virus gets disseminated to secondary lymphoid organs, including the spleen, the thymus, the tonsils (Veronika Von Messling et al., 2004) and subsequently a systemic spread through the entire immune system. The decline in the amount of white blood cells (WBCs) or leukocytes is remarkable (leukopenia) besides an inhibition of non-specific lymphocytes, which continue increasing while the CDV gets disseminated throughout the immune system (Delpeut et al., 2017). Due to detectable infection levels in peripheral blood mononuclear cells (PBMCs) are not significant at this point of infection; the migration of this kind of immune cells to the infection site must be an essential factor that contributes to leukopenia as well as virus-induced cell death. This immune suppression causes some opportunistic and secondary infections to arise, contributing to the morbillivirus' morbidity and mortality (Noyce & Richardson, 2012).

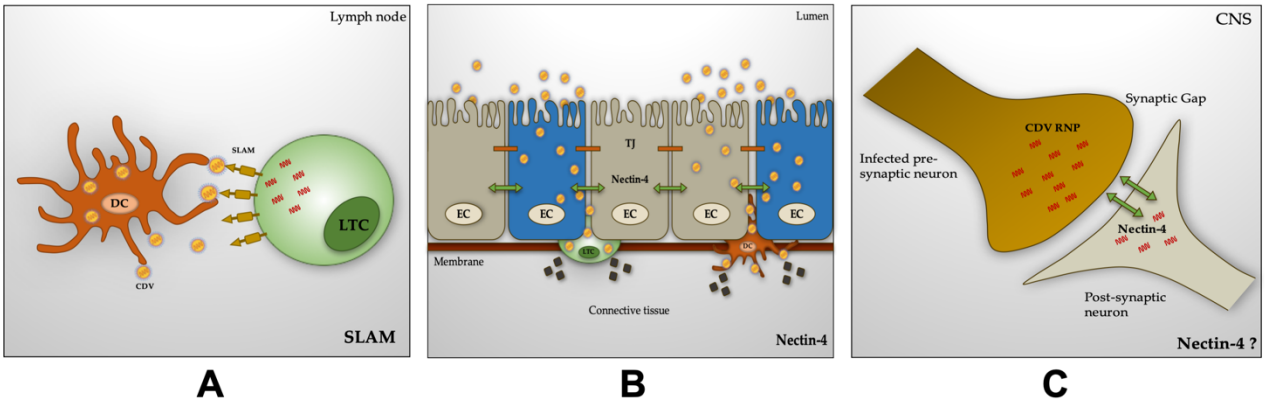

Figure 4. Principle routes of CDV infection and transmission in hosts. A) Infected DCs and alveolar macrophages progress to the local draining lymph node, where they interact with and infect T-cells and B-cells through CD150 that is also expressed on their cell surface. These infected cells further spread to secondary lymphoid organs, causing a secondary viremia. B) At the final stages of infection, shedding of infected lymphocytes to distal site of the respiratory tract. These infected lymphocytes interact with the epithelial cell receptor nectin-4, located in the adherent junctions on the basolateral surface of the epithelial cell. Infection in the airway epithelium results in the virus assembly and the release of virions into the airway lumen of the infected lung. B) CDV can infect the CNS in some instances and it has been suggested that the receptor nectin-4 has an important role in this infection.

Dissemination of the virus to distal sites including liver, skin, gastrointestinal tract, genitals, and respiratory mucosal surfaces results in the virus' spreading and subsequent transmission to uninfected individuals (**Figure 4B**) (Leonard et al., 2008). Throughout the respiratory tract, CDV infection is thought to occur via the basolateral side of the lumen epithelium via the migration of CDV-infected T, B, and DCs from the circulation (Veronika Von Messling et al., 2004). At this point, nectin-4 is located within the adherent junctions and set to interact with the viral particles that have been carried onto the surface of these infected lymphocytes, thus enabling the virus' entrance to the epithelium. CDV exits from the epithelium via its apical surface. In the absence of no nectin-4, CDV remains lymphotropic and produces primary and secondary viremia. Thus, the epithelial receptor-joined virus is rendered incapable of spreading from the respiratory route, suggesting that

nectin-4 plays an essential role in virus egress late in the infection rather than during its initial stages (Haines et al., 1999; Mühlebach et al., 2011; Veronika Von Messling et al., 2004).

A previous study reported that CDV infection through an epithelial receptor is required to have the clinical disease but not necessary for immunosuppression, deriving from the fact that after animals were inoculated with epithelial receptor-blind CDV strains (which lack the epithelial cell receptor recognition domain) they showed no clinical signs. However, there was a rapid and efficient spreading of immune cells, producing the same levels of leukopenia and inhibiting lymphocyte proliferation activity which are signals of morbillivirus immunosuppression (Sawatsky et al., 2012). Additionally, it has been confirmed through *in vivo* experimentation in ferrets that transmission was not evident in most animals infected with the SLAM- or nectin-4-blind CDV strains obtained by reverse genetics systems, although all animals infected with the nectin-4-blind virus developed continuous viremia, remarking the importance of epithelial cell infection and sequential CDV H protein interactions—at the beginning with SLAM and then with nectin-4 receptors—regarding transmission to naive hosts (Sawatsky et al., 2018b). This fact also highlights the importance of *in vivo* selection pressure on the CDV H protein interactions with SLAM receptors.

Respiratory and gastrointestinal clinical pathologies are the most common signs by the end of 6 to 10 post-infection days along with rashes (a typical symptom of CDV) in the form of erythematous patches whose diameter ranges between 3–8 mm. The neck and the face are the first body parts that are affected. An increasing number of patches appear around the mouth while the infection progresses (Delpeut et al., 2017; Pfeffermann et al., 2018). Furthermore, the skin becomes another target of the virus. Similar to other morbilliviruses, CDV can infect the epidermal cells of a wide range of species. Footpad keratinocytes are commonly infected, since the demonstration of viral antigen in the biopsies of the footpad serve as a diagnosis for CDV (Takenaka et al., 2016).

Neuropathogenesis

CDV has been also studied as a neurotropic agent; a vast number of strains are responsible for polioencephalitis and, predominantly, several of them generate DL. As is believed, the CDV must reach the brain in different ways, and some infection routes have been suggested thus far. One of the most crucial routes of neuro-invasion extend via infected PMBCs that are transported through the blood brain barrier; afterwards, there is a virus release that results in the infection of resident epithelial and endothelial cells (Lempp et al., 2014). *In vivo*, using a mouse model of infection, it has been proven that the CDV infection progresses from circulating cerebrospinal fluid into the CNS through a sequential route by infecting the neuro-ependymal cells lining the ventricular wall and the neurons of the hippocampus and the cortex that lie adjacent to the ventricle area. It then causes an extensive infection of the brain's surface, followed by the parenchyma and the cortex (Delpeut et al., 2012).

However, in ferrets, it has been proven that the CDV can enter the brain via neurons located in the olfactory mucosa, invading the olfactory nerve filaments, the olfactory glomeruli, and the deeper CNS structures. As mentioned before, the cellular receptors which contribute to neuropathology include nectin-4 (**Figure 4C**) (Leonard et al., 2008) and a third in astrocytes (Pratakpiriya et al., 2017), due to the SLAM receptor being under-expressed in the CNS (Lempp et al., 2014; Ludlow et al., 2014). Furthermore, it has been reported that viral persistence and neurological disease is related to the CDV in viral cell-to-cell spread in astrocytes, allowing the virus to avoid the immune system detection. This CDV infection is based on membrane fusion between the infected and the target astrocytes that heralds a free passage of viral nucleocapsid (Di Guardo et al., 2016). Additionally, it has been demonstrated that the functional hetero-oligomeric viral H/F complex, and thus presumably membrane fusion, are required to enable the spread of CDV in primary astrocytic cultures (Wyss-Fluehmann et al., 2010). Consequently, the CNS, the astrocytes, the microglia, the oligodendrocytes, the neurons, the ependymal cells, the choroid plexus cells and, as demonstrated by most rigorous studies on CDV, a family of growth-promoting glia including some specialized macroglia with a Schwann cell-like structure can be infected, enabling the development of neurological CDV infection (Beineke et al., 2009).

It is relevant to mention that as an implication of the neuropathology of CDV, the early stages of DL are a consequence of a direct virus-mediated damage and the invasion of CD8+

cytotoxic T cells, which are associated with an up-regulation of pro-inflammatory cytokines such as interleukin (IL)-6, IL-8, tumor necrosis factor (TNF)-α, and IL-12, and a lack of responses from immunomodulatory cytokines such as IL-10 and the transforming growth factor (TGF)-β. CD4+-mediated delayed type hypersensitivity and cytotoxic CD8+ T cells contribute to myelin loss in the chronic phase. Furthermore, an up-regulation of interferon-γ and IL-1 must occur in advanced lesions (Beineke et al., 2009). Once a dog has overcome the immunosuppression induced by the CDV infection, immune-mediated demyelination can be noticed, as MeV does in humans, along with some aspects of the inclusion of body encephalitis. Consequently, old dog encephalitis occurs a long time after one's recovery from a CDV infection and shares clinical and pathological features with the MeV subacute sclerosing panencephalitis (Pfeffermann et al., 2018).

Host-virus interaction with and evasion of the immune system

The function of V and C proteins in CDV pathogenesis still lies within the interests of researchers. Studies have shown their incidence towards CDV pathogenesis and the counteraction of the host interferon (INF) responses. The association of an innate immunity and virus-mediated immunosuppression impacts the infection development (Pfeffermann et al., 2018). The immune response is initially activated by diagnosing the pathogen-associated molecular patterns (PAMPs) through pattern-recognition receptors (PRR) including Toll-like receptors, melanoma differentiation-associated factor 5 (MDA-5), retinoic-acid inducible gene (RIG)-I-like receptors, and nucleotide-binding oligomerization domain-like receptors (NLRs). The definition of a viral infection by PRR guides us to interferon regulatory factors (IRF)-3, IRF7, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). As a result, not only are the production of type I IFN observed but the inflammatory cytokines (Pfeffermann et al., 2018) are also identified.

In general, for the *Paramyxoviridae* family, V protein interacts with RIG-I-like receptors, MDA-5, and LGP2, suggesting a positive regulator of RIG-I- and MDA-5-mediated antiviral responses meant to suppress IFN induction. Moreover, the virus escapes detection by MDA-5 by interacting with other cell factors (Pfeffermann et al., 2018). By using *in vivo* models, it

has been reported that the V protein of CDV is critical in the inhibition of IFN-α/β induction in PBMCs and of other important cytokines such as the tumor necrosis factor alpha (TNF-α), gamma IFN, IL-6, and IL-4 that controls cellular and humoral immune activation (von Messling et al., 2006). Detailed molecular analysis by using the neuropathogenic CDV A75/17 strain has demonstrated that the V protein specifically ablates the nuclear import of STAT1 and STAT2 without affecting their activated phosphorylation states (Rothlisberger et al., 2010). Furthermore, the inhibition of IFN-α/β-dependent signaling is correlated with the capacity of the V protein to efficiently interact with both STAT molecules with both the N-terminal and the C-terminal regions of V, playing a synergistic role in the IFN evasion (Rothlisberger et al., 2010). Additionally, a domain of V protein, which is shared with the P protein due to its alternative gene expression, can obstruct Type I and Type II IFN responses. This action is attributed to amino acids 110 and 130 with the tyrosine 110, which is an essential an amino acid in terms of binding to the signal transducer and the activator of the transcription 1 (STAT1) molecule. As reported, inhibition of the V protein results in a 70% loss of Type I IFN inhibitory action, since this region directly inhibits IFN-β synthesis through its interaction with MDA-5 (Svitek et al., 2013).

Through systematic mutagenesis, it has been revealed that both aspartic acid 248 and phenylalanine 246 are essential for the inhibition of STAT2 nuclear translocation. Comparatively, arginine 235 is necessary for MDA-5 interference in paramyxovirus. Thus, the importance of the V protein regarding morbillivirus virulence and particularly in relation to MeV and CDV has been demonstrated, due to its relationship with the host cell factors. This also indicates that the V protein sequence may be crucial in the molecular interaction and modulation of the host's immune responses (Svitek et al., 2013).

H protein structure as the key molecular factor in CDV tropism

As an attachment protein, the H protein is a monomer belonging to the transmembrane glycoprotein type II, consisting of a small N-terminal cytoplasmic tail, a transmembrane domain, and a large C-terminal ectodomain. This ectodomain is confirmed as a stalk and a six-blade (B1–B6) β-propeller fold lying near a central cavity. Each blade holds four-stranded

anti-parallel β-sheets (S1–S4) (Massé et al., 2004; von Messling et al., 2001). It has been postulated that the H protein, after binding to specific receptors on target cells, induces an oligomeric conformational change on the stalk domain, which in turn may translate into an F activation. In addition, the ectodomain stalk supports the membrane-distal cuboidal head region (Avila et al., 2014). Many studies have evidenced that the paramyxovirus attachment protein stalk domain physically interacts with the F trimers and forms short-range contact with the large globular head domain of the trimeric F, which causes an overlapping H-F association model in which the H heads are located above the F heads (Lee et al., 2008).

Additionally, due to H protein being considered as the most variable gene in CDV strains, analyzing the incidence of amino acidic variability in initial interactions, virulence, host range, immune system responses, and neutralization of the epitopes of CDV is completely relevant (Ke et al., 2015). Several comparative studies have demonstrated that this heterogeneity in the H proteins of diverse strains is associated with higher genetic-antigenetic variations compared other CDV genes; consequently, neutralization-related sites are affected and then one observes the disturbance of important epitopes. This genetic diversity of the H CDV gene disarranges the antigenicity of the arising CDV strains (this holds for CDV strains that are used as current vaccines), which was demonstrated by the CDV stains which were isolated in Italy (Martella et al., 2006).

Even though the SLAM receptor has been broadly studied as the CDV receptor in the determined immune cells and correlates with the immunosuppression associated with the CDV-mediated cytolytic infection of the lymphoid tissue, there is evidence behind the statement that the other types of receptors must facilitate CDV entrance to nectin-4, as the epithelial cell receptor contributes to CDV multi-tropism. Similar to MeV, 11 residues of the CDV H protein have been identified by a site-directed mutational analysis of this protein, which regulates and mediates plasma membrane recognition and posterior fusion in epithelial keratinocytes (Langedijk et al., 2011). Furthermore, the SLAM receptor binds itself to the CDV H protein at specific regions which comprise 500 to 550 amino acids (Langedijk et al., 2011).

Altogether, there are several mechanisms by which to obtain an understanding of the molecular interactions between the H protein and the host cell as the mediator of the CDV

entry. Computational tools and directed mutagenesis of the H protein are some of the useful strategies to study these types of interactions. Regarding the computational biology, a crystallized CDV H structure which enables a structural study has unfortunately been unavailable till date (Langedijk et al., 2011). Consequently, we have modelled a CDV H protein from a reference strain (GenBank code: AAG15490.1). **Figure 5** exhibits the CDV model constructed through homology modelling with the help of Modeller, based on the MeV H protein structure (PDB code: 2RKC).

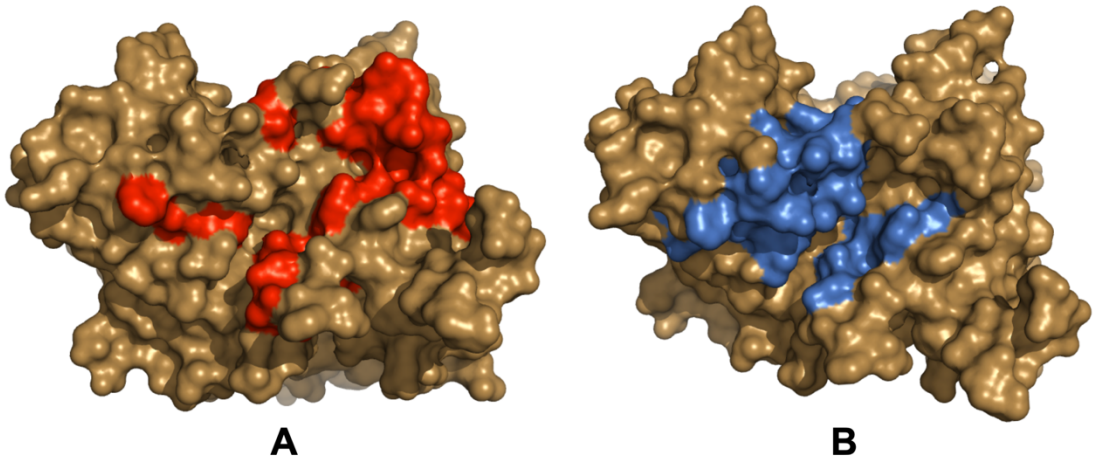

Figure 5. H protein from CDV reference strain, based on MeV crystal structure. Surface representation of the CDV H structure accomplished through homology modelling based on MeV H (PDB: 2RKC), using the software PyMOL, Molecular Graphics System, Version 2.0 Schrödinger, LLC. A) In red, residues that potentially interact with mononuclear cell receptors (SLAM); B) In blue, positions of CDV H protein that interact with the epithelial cell receptor (nectin-4). The interaction positions of CDV H protein are presented based on the interaction sites of the MeV H protein.

Structural studies on the H protein have confirmed the existence of interaction sites with cellular receptors. The SLAM receptor interacts with such a region that has multiple contacting sites (Langedijk et al., 2011), as shown in **Figure 5A** (Hashiguchi et al., 2011). Similarly, the nectin-4 receptor has established some interaction sites with the CDV H

protein (**Figure 5B**) (Hashiguchi et al., 2011). These interaction sites are reported based on MeV (Hashiguchi et al., 2011; Zhang et al., 2013). Consequently, there is structural and functional evidence that an H surface behaves as a multiple-receptor binding domain and gives an idea about high selectivity, suggesting that there might be differences between the H protein and the receptor interface, which is essential in leading the CDV entry into and infection of specific cells (Langedijk et al., 2011).

CDV as a potential cross-species agent

To date, there has been no evidence of a human infection by CDV. However, as reported, it can be isolated from human cancer cell lines such as those that are observed in breast, lung, and prostate cancer. As is already known, CDV employs dog SLAM receptors efficiently, but fails to do so with human SLAM. Therefore, in contrast to this the human nectin-4 present in cancer cell lines, as mentioned before, also operates as efficiently as CDV receptors (Otsuki, Nakatsu, et al., 2013). As an explanation behind this phenomenon, there is a small species-related variation in the nectin-4 sequences between humans, mice, and dogs to such an extent that mice's nectin-4 can function as a receptor for MeV while human nectin-4 functions as a receptor for CDV. However, the mice SLAM cannot function as a receptor for either MeV or CDV, and the attachment appears to be dictated by the amino acid sequence in the V loop of this protein (Noyce et al., 2011).

The V loop of nectin-4 is also involved in the process of virus attachment. Yet, there are just three amino acids that differ in the V domain of the dog homologue and six different amino acids in the V domain of a mouse, as compared to the human protein sequence (Noyce et al., 2013). In 2013, Otsuki *et al*. demonstrated that the Ac961 CDV strain replicates in human epithelial NCI-H358 cells, expressing nectin-4, and adapts to them. Surprisingly, no amino acid change in the H protein was required for adaptation. Therefore, the ability to use human nectin-4 is an intrinsic phenotype feature of wild-type CDV strains (Otsuki, Sekizuka, et al., 2013). In 2006, the CYN07-dV CDV strain was isolated *in vitro* in the Vero cells that expressed the dog SLAM receptor. After phylogenetic analysis, this strain was found to be similar to the one observed during a CDV outbreak in China. However, YN07-dV uses the

Macaca SLAM and Macaca nectin-4 receptors as efficiently as the dog SLAM and dog nectin4, respectively (Sakai et al., 2013).

In 2014, De Vries *et al*. through reverse genetics generated a recombinant CDV and studied its virulence and tropism by expressing an EGFP in naïve and MeV-vaccinated *Cynomolgus macaques*, finding that in naive animals CDV produced viremia and fever by infecting lymphocytes and dendritic cells that expressed SLAM receptor (de Vries et al., 2014). These facts demonstrated that CDV could infect nonhuman primates; however, partial protection was distinguished in MeV-vaccinated macaques, as demonstrated by a controlled virus replication. Moreover, neither CDV infection nor MeV vaccination induced noticeable cross-reactive neutralizing antibodies. MeV-specific neutralizing antibody levels in MeV-vaccinated macaques were increased by CDV infection, which suggests that cross-reactive epitopes do exist (de Vries et al., 2014).

In other studies, it was found that CDV isolated from monkeys (Monkey-BJ01-DV) replicates itself efficiently in Vero cells expressing the SLAM receptors and originating from dogs and monkeys. However, it does not replicate itself in cells of human origin express the SLAM receptors. In this regard, the essential cause can be the substitutions in the isolated H protein and the CDV H protein. Moreover, while the amino acid sequence identity of the dog SLAM and the monkey SLAM is only 63.6 %; the Monkey-BJ01-DV strain is able to replicate itself on the Vero cells expressing the SLAM receptors and originating from dogs as efficiently as the Vero cells which originate from monkey SLAM (Feng et al., 2016), indicating a potential cross-species event.

Even though the CDV can infect non-human primates, the human SLAM's incompatibility with the CDV attachment protein, as a consequence of its sequential difference from the dog SLAM receptor, can result in the absence of the infection in humans based on the infection cycle, since it is believed that cells expressing SLAM receptors are infected at the beginning. Furthermore, a cross-reactive immunity between MeV and CDV might be protecting humans against CDV infection (Otsuki, Nakatsu, et al., 2013; Wyss-Fluehmann et al., 2010). It has been proven that MeV and attenuated CDV have induced incomplete immune responses to canine distemper disease (Appel et al., 1984). Other studies have compared both MeV and CDV vaccines for the prevention of canine distemper in young

dogs, which were also challenged with CDV virulent strains (Snyder-Hill). All dogs were protected against this challenge with only a few clinical signs being displayed (Chalmers & Baxendale, 1994). Therefore, the idea of developing a cross-species infection in humans still poses a threat, since a punctual mutation in H protein *in vitro* allows CDV to infect cells using the human SLAM receptor (Otsuki, Nakatsu, et al., 2013).

Conclusions

This review summarizes the most important aspects of CDV tropism and pathogenesis from a molecular perspective, comprising not only the viral protein interactions with the host cell receptors but also the influence of host factors on CDV virulence and the development of different pathologies from neurological to gastric clinical signs. The use of diverse receptors delimits CDV's cellular tropism since lymphoid, epithelium, and CNS cells have different receptors that are implicated in the CDV infection at different stages, suggesting that the pathogenesis derived from a particular tropism is receptor dependent.

CDV pathogenesis is quite diverse and dynamic due to its wide tropism spectrum. Understanding the mechanism by which the CDV generates its virulence specifically in dogs, as molecular interactions between host cell receptors and viral proteins, helps in clarifying the tropism and pathogenesis of CDV more accurately and understanding the failure of vaccinations in some cases. Undoubtedly, a lack of information about CDV's molecular interactions limits the analysis of its multi-tropism. However, a valuable amount of data can be deduced using a MeV infection. Thus, the molecular process of a CDV infection cycle is essentially understood based on the involved MeV mechanisms. The reverse genetics technologies have played an important role in the construction of this understanding, specifically through the study of the CDV H protein that allows one to elucidate certain responses from a wide range of CDV strains, since the interaction between CDV and its cellular receptors depends on the H protein. This fact becomes highly relevant because the first step in all CDV infections involves this viral protein. Thus, understanding the incidence of modifications in the primary structure of the H protein in CDV variants becomes a necessity.

CDV induces multiple pathogenic effects due to the different interactions between the viral particle and the host. Its interplay with the immune system and subsequently transient immunosuppression is considered crucial in the development of different clinical signs of CDV infection. This immunosuppression has been considered the result of the interaction between viral proteins since their modifications inhibit the immunosuppression.

Morbillivirus, as CDV, are distributed among carnivores. Considering the proximity of humans to domesticated animals such as dogs, the above fact represents the need for constant treatment, considering that infections in non-human primates has already been demonstrated. Additionally, viral replication in human cell lines using human nectin-4 as the entry receptor allows one to wonder whether the CDV can initiate a cross-species event in humans because of virus adaption.

Hence, many computational studies and directed mutagenesis *in silico,* as preliminary tools, have proved that *in vitro* and *in vivo* experiments are necessary to establish a better understanding of actual vaccination problems, interspecies cross-transmission, and diverse pathological signs related to CDV, not only to develop new alternative therapeutic approaches and treat the symptoms shown by domesticated dogs but most importantly to avoid the interspecies cross-transmission of CDV to humans.

Abbreviations

CDV: Canine Distemper Virus

CNS: Central nervous system

DL: Demyelinating leukoencephalitis

dTom: red fluorescent protein

EGFP: enhanced green fluorescent protein

ESCRT: Endosomal Sorting Complex Required for Transport

MeV: Measles Virus

ORF: Open reading frame

PAMPs: Pathogen-associated molecular patterns

PRR: Pattern-recognition receptors

PBMCs: Peripheral blood mononuclear cells

RNP: Ribonucleoprotein complex

SLAM: Signaling Lymphocyte Activation Molecule

UTRs: intergenic untranslated regions

Supplementary File 1. Chapter 1. GenBank accession numbers of isolates used to Figure 2.

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CHAPTER 2

Safety and immunogenicity of canine distemper virus (CDV) vaccines for domestic and wild animals: A scoping review

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Journal classified as a Q1 for infectious diseases and Q2 for virology in Scimago.

Abstract: *Morbillivirus canis* (canine distemper virus (CDV)) is recognized as a multihost pathogen responsible for a transmissible disease affecting both domestic and wild animals. A considerable portion of wildlife populations remain unvaccinated due to a lack of safety and immunogenicity data on existing vaccines for the prevention of CDV infection in these species. This review aimed to assess the current state of CDV vaccination research for both domestic and wild animals and to explore novel vaccine candidates through in vivo studies. It also sought to synthesize the scattered information from the extensive scientific literature on CDV vaccine research, identify key researchers in the field, and highlight areas where research on CDV vaccination is lacking. A scoping review was conducted across four databases following the PRISMA-ScR protocol, with information analyzed using absolute and relative frequencies and 95% confidence intervals (CIs) for study number pro-portions. Among the 2321 articles retrieved, 68 met the inclusion criteria and focused on CDV vaccines in various animal species, such as dogs, ferrets, minks, and mice. Most of the scientific com-munity involved in this research was in the USA, Canada, France, and Denmark. Various vaccine types, including MLV CDV, recombinant virus, DNA plasmids, inactivated CDV, and MLV measles virus (MeV), were identified, along with diverse immunization routes and schedules employed in experimental and commercial vaccines. Safety and efficacy data were summarized. Notably, 37 studies reported postimmunization CDV challenge, primarily in dogs, revealing the survival rates of vaccinated animals. In summary, CDV vaccines generally demonstrate an acceptable safety profile in dogs and show promise as a means of controlling CDV. However, significant gaps in vaccine research persist, particularly concerning wildlife reservoirs, indicating the need for further investigation.

Keywords: *Morbillivirus canis*, vaccination, domestic animals, wildlife, scoping review.

Introduction

Morbillivirus canis commonly known as canine distemper virus (CDV) causes a highly contagious disease in domestic and wildlife animals, and CDV infection has been reported in more than 8 orders and 20 families worldwide (Rendon-Marin et al., 2019). The host range of CDV predominantly includes species within the order Carnivora, spanning diverse families such as *Canidae Procyonidae*, *Mustelidae*, *Ursidae, Ailuridae*, and *Felidae*, and, to a lesser extent, other notable families from different orders, namely *Artiodactyla, Primates, Rodentia,* and *Proboscidea* (MacLachlan et al., 2011; Martinez-Gutierrez & Ruiz-Saenz, 2016; Rendon-Marin et al., 2020). Given the extensive spectrum of species affected by CDV, different studies have explored its cross-species transmission among both wild and domesticated animals, investigating not only their interactions to elucidate the molecular diversity but also strategies such as vaccination to control CDV´s spread in domestic and wild animals (Beineke et al., 2015; Rendon-Marin et al., 2019).

Canine distemper disease (CDD) manifests with a range of clinical symptoms, such as fever, as well as respiratory, gastrointestinal, and neurological issues mainly in domestic dogs (Lempp et al., 2014). The clinical signs in wildlife are mainly associated with neurological disorders (Loots et al., 2017). CDV is included in the *Paramyxoviridae* family, genus *Morbillivirus,* and has a single-stranded negative-sense RNA genome with six transcription units that encode for eight proteins (Rendon-Marin et al., 2019). Among these proteins, the H and F proteins are essential, because of their function in diverse essential viral processes such as viral attachment and internalization by the host cells (von Messling et al., 2001). Moreover, they have been employed in CDV vaccines alternatives [10], helper T-cell epitopes from the F protein have been identified [11] and, finally, H and F peptides have been recovered in higher proportions than other viral proteins from dog major histocompatibility complex (MHC) molecules (Ross et al., 2018).

The H protein is considered the most variable protein among all lineages (Martella et al., 2006) and to date, based on its entire sequence, diverse genotypes have been described such as America-1, America-2 to -5, Arctic-like, Rockborn-like, Asia-1 to -4, Africa-1 to -2, European Wildlife, Europe/South America-1, South America-2 and -3 and South/North America-4 (Duque-Valencia, Diaz, & Ruiz-Saenz, 2019; Duque-Valencia, Forero-Munoz, et

al., 2019). The re-emergence of different CDV infections in domestic and wildlife animals has demonstrated the importance of updating the vaccine strains and examining the status of CDV vaccination since commercial vaccines are not fully employed in wildlife animals due to the lack of relevant evidence of their safety and efficacy (Wilkes, 2022). Diverse circulating lineages geographically distributed with amino acid changes in viral proteins involved in the host immune response and viral neutralization, could explain the vaccine failure in response to wild-type emerging strains, cross-species transmission, and the increased virulence of emerging CDV strains in domestic dogs and wildlife animals (Duque-Valencia, Sarute, et al., 2019). Additionally, case reports associated with CDV infection in wild animals have recently been published (Martinez-Gutierrez & Ruiz-Saenz, 2016), with CDV being a threat to a wide range of in-danger species (Gilbert et al., 2020; Wilkes, 2022).

The transmission dynamics of CDV between domestic and wild animals are not fully understood. Therefore, examining these transmission dynamics and identifying CDV reservoirs holds significant promise for the formulation of effective strategies to safeguard endangered species (Duque-Valencia, Sarute, et al., 2019). Some reports suggest that vaccinating wildlife may play a pivotal role in preventing the extinction of at-risk populations due to CDV (Beineke et al., 2015; Gilbert et al., 2020). Thus, immunizing animals and ensuring safety in both domestic and wildlife populations represent a field of study that warrants extensive efforts to ensure the health of companion animals and those endangered by CDV infection.

Regarding the current state of vaccination, MLV vaccines based on traditional America-1 strains (i.e. the Onderstepoort strain) and Snyder Hill, Convac, Rockborn, or CDV3 strains (Wang et al., 2024) are commercially available. However, these strains no longer circulate around the world (Buczkowski et al., 2014). Recombinant CDV vaccines have been developed by integrating the CDV F and H proteins into a canarypox virus vector, and these vaccines have demonstrated a safe profile across various susceptible species, including dogs, European ferrets (*Mustela putorius furo*), giant pandas (*Ailuropoda melanoleuca*), fennec foxes, meerkats (*Suricata suricatta*), and Siberian polecats (*Mustela eversmanni*) (Bronson et al., 2007; Coke et al., 2005; Stephensen et al., 1997; Wimsatt et al., 2003). Although recombinant canarypox vaccines exhibit safety and moderate efficacy across a

spectrum of species, they induce a less robust immune response than MLV vaccines, primarily due to replicative limitations (Schultz, 2006). However, this characteristic underscores their suitability for the immunization of young dogs in the presence of maternal antibodies (Pardo et al., 2007). Nevertheless, it has become imperative not only to develop new vaccine strategies to control CDV infection but also to assess the efficacy and safety of the currently available vaccines, since there is limited experimental evidence regarding these vaccines in wildlife animals. Other alternative strategies included experimental assays, including a recombinant bivalent vaccine, which employs a rabies virus that expresses the H and F CDV proteins, evaluated in domestic dogs and ferrets (Wang et al., 2014).

Based on the limited spectrum of current vaccines, different researchers worldwide have investigated new vaccines against CDV through *in vivo* studies involving diverse animal species, such as domestic dogs, BALB/c mice, minks, and ferrets, including recombinant viruses (Gong et al., 2020; Pujol et al., 2023; Sadler et al., 2016; Yan et al., 2020), chimeric measles virus constructs expressing CDV proteins (Rouxel et al., 2009), DNA vaccines encoding the main antigenic determinants of CDV (Nguyen et al., 2012; Nielsen et al., 2009; Zhao et al., 2023), the H and F CDV proteins isolated as antigens (Norrby et al., 1986), recombinant mouse adenovirus 1 expressing CDV antigens (Du et al., 2022), and a novel vaccine formulation based on bacterium-like particles presenting CDV antigens (Wang et al., 2024). However, the state of CDV vaccination research, development, and efficacy evaluation have yet to be explored and summarized in order to establish different vaccination schemes to control CDV´s transmission, dissemination, and disease course.

Therefore, this scoping review aims to assess the current state of research and advancements in CDV vaccination for both domestic and wild animals. This review encompasses the evaluation of existing vaccines in various species and the investigation of novel vaccine candidates through *in vivo* studies. Given the proliferation of reports on new vaccination strategies for CDV, this inquiry is warranted to target both wildlife and domestic canine populations. Furthermore, this scoping review seeks to consolidate the fragmented information scattered throughout the extensive scientific literature regarding CDV vaccine

research. Additionally, this study aims to identify key researchers in this field and pinpoint regions where research on CDV vaccination is lacking.

Materials and methods

Type of Study

Following the recommendations of the PRISMA-ScR (Tricco et al., 2018) and the Joanna Briggs Institute, a systematic scoping review on CDV vaccination and development for domestic and wildlife animals was conducted. The scoping review was registered at https://osf.io/n9sed (accessed on 22/05/2024).

Search and study selection.

The data were searched in multidisciplinary repositories such as PubMed, SciELO, ScienceDirect, and Scopus. For term selection, we used the Descriptors in Health Sciences (DeCS), Medical Subject Heading (MeSH), and both known names for CDV, with truncated term vacc* AND canine distemper virus; and vacc* AND canine morbillivirus, in all databases. A total of 8 searches were performed as follows: SciELO: (ti:(canine distemper virus)) AND (ti:(vacc*)) AND (ab:(canine distemper virus)) AND (ab:(vacc*)) and (ti:(canine morbillivirus)) AND (ti:(vacc*)) AND (ab:(canine morbillivirus)) AND (ab:(vacc*)); Scopus: (TITLE-ABS-KEY (canine AND distemper AND virus) AND TITLE-ABS-KEY (vacc*)) and (TITLE-ABS-KEY (canine AND morbillivirus) AND TITLE-ABS-KEY (vacc*)); PubMed: (vacc*[Title/Abstract]) AND (canine distemper virus[Title/Abstract]) and (vacc*[Title/Abstract]) AND (canine morbillivirus[Title/Abstract]); and finally, ScienceDirect: canine distemper virus AND vacc? and canine morbillivirus AND vacc?. The articles retrieved from each search (restricted to title, abstract and/or keywords) were saved in a common file in Zotero to eliminate duplicates.

Screening and eligibility

We screened studies published historically until 2024 (the last update was performed on January 09, 2024), including original research (eliminating reviews, editorials, protocols, and book chapters) that was conducted on CDV vaccination and development for domestic and wildlife animals *in vivo,* and the language was also considered (including English, Spanish and Portuguese). These criteria were applied by two researchers independently. During the eligibility phase, studies were excluded if they did not include safety information such as weight changes, temperature, survival, the immunization route, or efficacy, measured as either specific or neutralizing CDV antibodies.

Data extraction

The following variables were extracted from the selected studies: title; authors; year of publication; country of publication; sample size; species; immunogen type; immunization route; vaccine safety measured by temperature, weight, behavior, leukocyte count and survival; and efficacy through specific antibody (Ab) production measured either with neutralization assays or with ELISA. In studies where viral challenge was reported, the inoculation route and challenge survival were also retrieved. All information regarding the included studies was synthesized in figures and tables.

Data charting process

The data charting process was independently developed by two researchers to ensure concordance across all phases of the search, study selection, and data extraction processes. A third investigator resolved any discrepancies through consensus and further examination.

Analysis of the information

The description of different aspects of the included studies was performed with absolute and relative frequencies and 95% confidence intervals (CIs). A scientific collaboration network

in this research field was created with Gephi. The difference in survival between vaccinated and nonvaccinated animals was calculated using the Z test or confidence interval for the difference in proportions. The frequency analyses and Z tests were performed with EPIDAT version 4.2.

Results

Study selection and Prisma Flow chart

Initially, 2,321 studies were identified in PubMed, SciELO, Science Direct and Scopus. After duplicates were removed, the number of articles for screening decreased to 1,552. Then, the inclusion criteria were applied, and 154 studies were included. Finally, 68 studies were included in this scoping review after the application of the exclusion criteria (**Figure 1**).

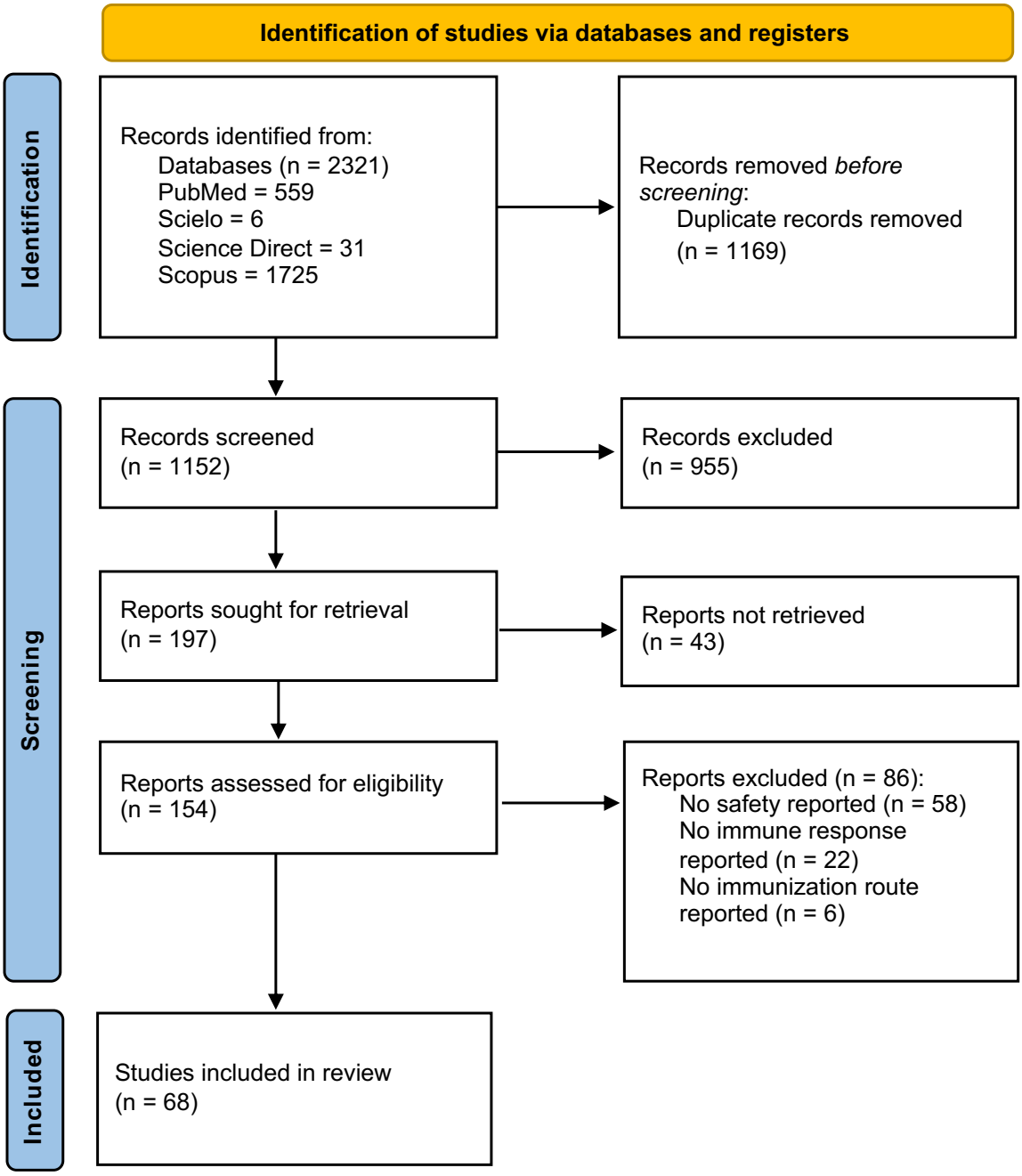

Figure 1. Study selection algorithm based on PRISMA guidelines.

International cooperation and researchers

To understand the level of international cooperation in the development of CDV vaccines, a scientific collaboration network analysis of researchers was carried out, considering their countries of affiliation. The scientific community related to CDV vaccination in domestic and wildlife animals comprised 308 researchers from diverse countries, including the United States of America, China, France, the Netherlands, Switzerland, Canada, the United Kingdom, and Denmark (**Figure 2**). Notably, there are few researchers from different countries who collaborate in CDV vaccine research, as evidenced by the presence of mostly individual nodes in the collaboration networks shown in **Figure 2**. Collaboration was evidenced only between researchers from the USA and Canada and between researchers from France and Denmark. In the figure, the size of the author's name denotes the importance of CDV vaccine development since it represents the presence of these authors in different studies. Among the authors who have contributed greatly to CDV vaccination research, in the USA, Rebecca Wilkes has performed different studies in wild animals, and Max J.G. Appel contributed to initial MLV CDV vaccines in dogs, especially those based on the Onderstepoort strain. In France, T. Fabian Wild has also contributed considerably, including studies in mice, and minks employing recombinant and DNA vaccines (**Table 1**).

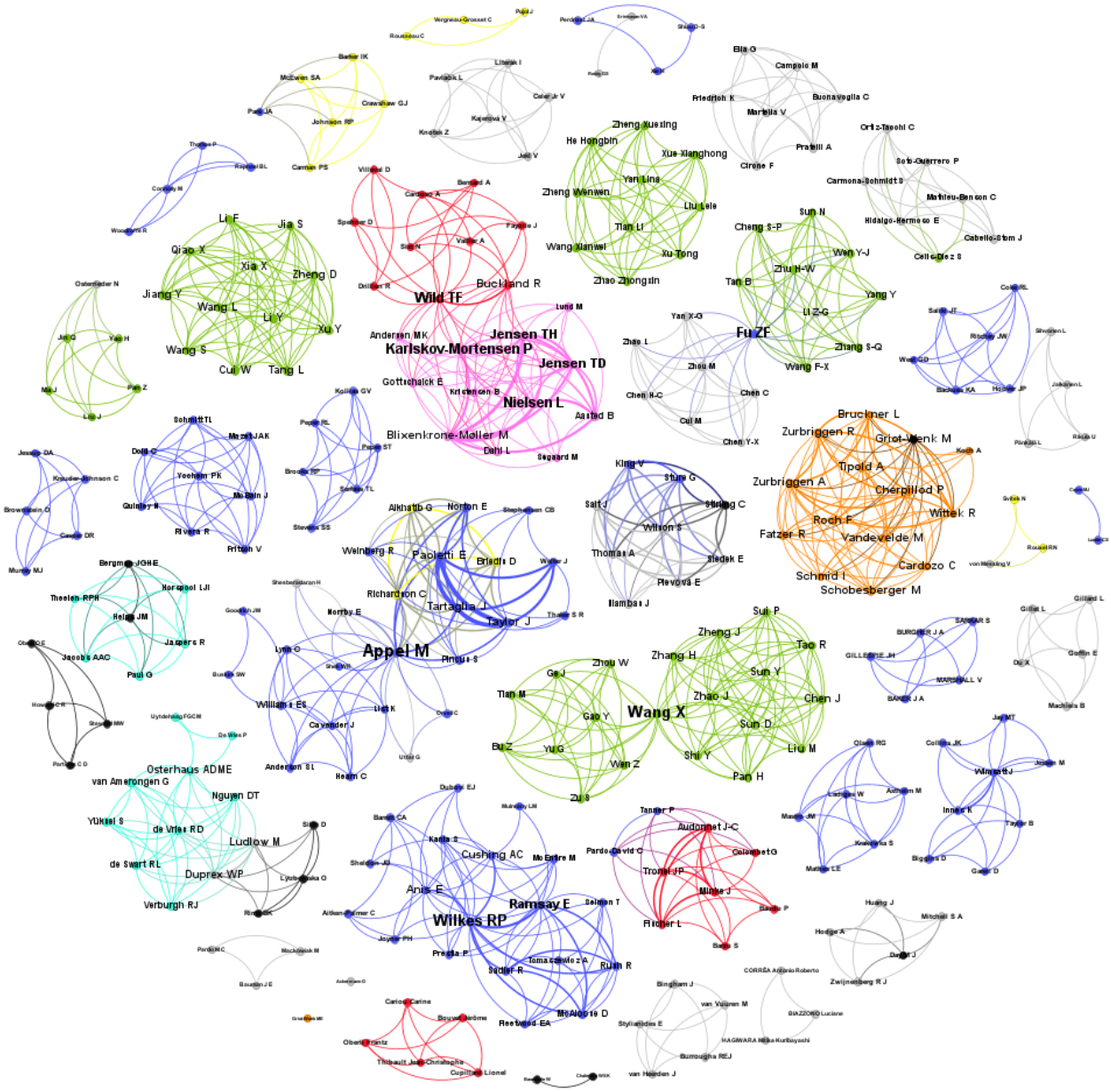

Figure 2. Scientific collaboration network related to research on CDV vaccination development. Relationships among researchers based on country of affiliation. The United States of America is marked in blue, China in green, France in red, the Netherlands in cyan, Switzerland in orange, Canada in yellow, the United Kingdom in black and Denmark in pink. Low-frequency countries are marked in gray. The diameter of each node denotes the importance of this researcher in CDV vaccine development. The graph was created with Gephi.

Included studies and countries contributing to CDV vaccine development

A total of 68 studies were included in the scoping review. The main characteristics of the included articles are summarized in **Table 1.** The geographic distribution included studies from the United States of America (*n* = 31); China (*n* = 7); France (*n* = 5); Belgium, Canada, Denmark, the Netherlands, and the United Kingdom (*n* = 3 in each country); Switzerland (*n* = 2) ; and Australia, Brazil, the Czech Republic, Finland, Germany, India, Italy, and South Africa (*n* = 1 in each country). The United States of America had the greatest number of *in vivo* CDV vaccine studies, followed by China and France, which correlates with the authors' contributions as reported in **Figure 2.**

Table 1. Studies´ main characteristics.

Year	Country	Species	Individuals	Safety	Efficacy	Challenge
1958	USA	Dogs	45	T, leukocyte count, and IS daily	nAb	Yes
1965	USA	Dogs	23	IS daily	nAb	Not
1965	Germany	Dogs	32	T and CS daily	nAb	Not
1980	USA	Dogs	8	T, W weekly, and CS daily	nAb	Yes
1984	USA	Dogs	9	T, W weekly, and CS daily	nAb	Yes
1986	USA	Dogs	11	T, W biweekly, and CS daily	nAb and sAb*	Yes
1986	USA	Dogs	8	T and lymphocyte count	nAb and sAb*	Not
1988	The Netherlands	Dogs	12	T, W, CS, and lymphocyte count	nAb and sAb*	Yes
1991	USA	Dogs	14	T, W, and CS daily	nAb	Yes
1992	USA	Dogs	15	T, W, and CS daily	nAb	Yes

1993	France	Mice	77	Survival	nAb	Yes
1994	UK	Dogs	36	T, W, and CS daily	nAb	Yes
1994	USA	Badgers	11	W, and CS daily	nAb	Not
1995	UK	Mice	48	Survival	sAb*	Yes
1996	USA	Hyb FxSP	24	T and CS daily	nAb	Yes
1997	USA	Ferrets	26	T, W, CS, and survival	nAb	Yes
1997	USA	Dogs	33	Inoculation site and CS daily	nAb	Yes
1998	France	Mice	58	CS and survival	nAb and sAb*	Yes
1999	Canada	Raccoons	47	CS local or systemic	nAb	Yes
1999	USA	Ferrets	37	T, W, CS, and, leukocyte count	nAb	Yes
2000	Switzerland	Dogs	7	T, CS, and leukocyte and lymphocyte count	nAb and N-sAb	Yes
2000	USA	Dogs	60	CS and survival	nAb	Not
2000	USA	Ferrets	84	T, W, CS, CDV infection daily, and survival	nAb	Yes
2001	USA	Ferrets	16	W, CS of CDV infection	nAb	Yes
2001	Brazil	Dogs	11	CS	nAb	Not
2001	Finland	Minks, Raccoon dogs, silver foxes,	Mink: 40 Raccoon dog: 39 Silver fox: 40	CS	nAb	Not

		and blue foxes	Blue fox: 42			
2001	Switzerland	Dogs	45	CS	sAb*	Not
2002	South Africa	Wild dogs	10	CS daily	nAb	Not
2002	India	Dogs	12	CS and survival	nAb	Not
2002	USA	Dogs	25	CS and survival	nAb	Yes
2003	USA	Siberian polecats	29	CS and survival	nAb	Yes
2003	France	Dogs	9	CS and survival	nAb	Yes
2004	France	Minks	22	CS and survival	nAb and sAb*	Yes
2004	Italy	African Wild dogs	9	CS	nAb	Not
2005	USA	Fennec foxes and Meerkats	11	CS	nAb	Not
2007	The Netherlands	Dogs	24	T and CS	nAb	Not
2007	UK	Ferrets	15	T daily, W weekly, and CS of CDV infection	nAb and sAb*	Yes
2007	Czech Republic	Ferrets	5	T and CS daily	nAb	Not
2009	Canada	Ferrets	9	T, W, and CS	nAb and sAb*	Yes
2009	USA	Southern Sea Otters	8	CS	nAb and sAb*	Not
2009	Denmark	Minks	13	CS	nAb	Yes

2009	Denmark	Minks	10	CS, lymphocyte count, and survival	nAb	Yes
2012	Denmark	Minks	17	CS	nAb	Yes
2012	Australia	Dogs	235	CS	nAb	Not
2012	The Netherlands	Ferrets	24	T, W, CS, and behavior	nAb	Yes
2013	USA	African wild dogs	21	CS	nAb	Not
2013	USA	Harbor seals	5	CS local and systemic	nAb	Not
2014	USA	Wild neartic river otters	22	CS	nAb	Not
2014	Belgium	Dogs	15	CS	nAb	Yes
2014	China	Dogs	24	T, W, and CS	nAb	Yes
2014	Belgium	Dogs	7	T and CS daily	nAb	Yes
2015	China	Minks	20	T, W, and CS	nAb	Yes
2016	USA	Cats	20	CS	nAb	Not
2016	USA	Tigers	102	CS	nAb	Not
2017	USA	Two-toed sloths	7	CS	nAb	Not
2017	China	Dogs	15	T, W, CS, and behavior	nAb	Yes
2019	USA	Dogs	100	W and CS	nAb	Not
2019	China	Mice	20	W and CS daily	nAb and sAb*	Not

2019	China	Mice and Dogs	Mice: 240 Dogs: 32	T, CS, and survival	nAb and sAb*	Yes
2019	USA	Red Foxes	17	CS	nAb	Not
2020	China	Mice and Foxes	Mice: 96 Foxes: 18	CS and survival	nAb and sAb*	Yes
2020	USA	Maned wolves	9	CS	nAb	Not
2020	USA	Tigers and cats	Tigers: 8 Cats: 17	IS daily	nAb	Not
2021	USA	African wild dogs	10	CS and behavior	nAb	Not
2022	Belgium	Mice	45	CS	nAb and sAb*	Not
2022	France	Dogs	25	CS	nAb	Not
2023	China	Ferrets	20	T, W, CS, and behavior	nAb	Yes
2023	Canada	Walruses	3	CS	nAb	Not

*Specific antibodies (sAb) were measured by ELISA. Hyb FxSP: hybrid ferret and Siberian polecat, T: temperature, CS: clinical signs, W: weight, IS: illness signs, nAb: neutralizing antibodies, N-sAb: N-protein specific antibodies.

Domestic and wild animals in CDV vaccine trials

As shown in **Table 2,** 37.3% of the studies were carried out in dogs, followed by ferrets, mice, minks, African wild dogs, tigers, and cats, with only one study on other species (**Table 2**), shown as a relative frequency (RF). A total of 2,363 individuals were included in all studies on diverse animal species; however, considering the vast array of species affected by CDV, there is still a lack of *in vivo* CDV vaccine studies focused on the control of CDD and its dissemination among domestic and wildlife animals, due to the lower number of studies corresponding to either domestic or laboratory animals.

Table 2. Domestic and wildlife species included CDV vaccine trials.

Specie	n[a]	RF (%)	RF CI 95%	Individuals
Dogs	28	37.3	(26.4 – 49.3)	892
Ferrets	10	13.3	(6.6 – 23.2)	236
Mice	7	9.3	(3.8 – 18.3)	584
Minks	6	8.0	(3.0 – 16.6)	122
African wild dogs	3	4.0	(0.8 – 11.3)	40
Tiger	2	2.7	(0.3 – 9.3)	110
Cat	2	2.7	(0.3 – 9.3)	37
Others*	17	23.7	(13.8 – 33.8)	342
Total	75	100		2.363

[a]Number of animal populations evaluated in included studies in the *in vivo* CDV vaccine trials (**Table 1**). *The other species include Badger, Fennec fox, Meerkat, hybrid Ferret and Siberian polecats, Harbor seals, Maned wolves, Raccoon dogs, silver foxes, blue foxes, Raccoons, Red foxes, Siberian polecats, Southern Sea Otters, Two-toed sloths, Walruses, Wild dogs, and Wild Nearctic River otters, with just one study that employed each of these animal species.

CDV Vaccine characteristics in included studies

The different immunogenic approaches employed in the included studies and their characteristics are summarized in **Table 3**. The most frequent vaccine types were MLV CDV (*n* = 25) and recombinant virus (*n* = 25), with the same number of studies, as well as DNA plasmids (*n* = 13), inactivated CDV (*n* = 6), MLV MeV (*n* = 3), and others (*n* = 4). On the other hand, different immunization schemes were employed with one (*n* = 25), two (*n* = 31), three (*n* = 16), and four (*n* = 5) doses (**Table 3**), where the two-dose scheme had the highest percentage, at 40.3% of the total number of studies. Moreover, regarding the inoculation route, most studies employed subcutaneous immunization (*n* = 36), followed by intramuscular (*n* = 30), oral (*n* = 6), and other methods (*n* = 11).

Table 3. Characteristics of vaccines used in CDV studies.

		n[a]	RF (%)	RF CI 95%
Vaccine type	MLV CDV	25	32.9	(22.5 – 44.6)
	Recombinant virus	25	32.9	(22.5 – 44.6)
	DNA plasmids	13	17.1	(9.4 – 27.5)
	Inactivated CDV	6	7.9	(3.0 – 16.4)
	MLV MeV	3	3.9	(0.8 – 11.1)
	Others*	4	5.3	(1.5 – 12.9)
	Total	76	100	
Scheme (doses)	One	25	32.5	(22.3 – 44.1)
	Two	31	40.3	(29.2 – 52.1)
	Three	16	20.8	(12.4 – 31.5)
	Four	5	6.5	(2.1 – 14.5)
	Total	77	100	
Inoculation route	SC	36	39.6	(29.5 – 50.4)
	IM	30	33.0	(23.5 – 43.6)
	IN	8	8.8	(3.9 – 16.5)
	Oral	6	6.6	(2.5 – 13.8)
	Others**	11	12.1	(6.2 – 20.6)
	Total	91	100	

[a]Number of studies that included the vaccine type, scheme, and inoculation route in the *in vivo* CDV trial. Nine studies included more than one vaccine type, eight studies included more than one vaccination scheme, and thirteen studies included two or more inoculation route trials (**Table 1**). *****Chimeric MeV expressing CDV proteins, ISCOMs, peptide-based vaccines, and purified H and F CDV proteins from only one study. **Intracranial, intraperitoneal, intradermal, and intravenous. MeV: measles virus, SC: subcutaneous, IM: intramuscular, IN: intranasal.

Safety and efficacy of CDV vaccines from included studies

The main characteristics of vaccines are safety and efficacy. Although there are diverse alternatives to evaluate safety, in the included studies, the clinical signs (*n* = 65), temperature (*n* = 26), weight loss (*n* = 21), survival (*n* = 14), animal behaviors (*n* = 6) and leucocyte counts (*n* = 5) were the primary indicators. In addition, neutralizing antibodies (*n* = 66) and specific antibodies such as IgG (*n* = 15), detected by ELISA were reported as measures of the humoral immune response to immunization with CDV vaccines (**Table 4**).

Overall, 54.4% of the included studies involved infectious virus challenge (n = 37) with different CDV lineages, and intranasal challenge was the most common route (n = 22), followed by intravenous challenge (n = 2) and other methods (n = 17), as shown in **Table 4**.

Table 4. Measures of safety, immunogenicity, and viral challenge with route in CDV vaccine studies.

		n[a]	RF (%)	RF CI 95%
Safety	Clinical signs*	65	47.4	(38.9 – 56.1)
	Temperature	26	19.0	(12.8 – 26.6)
	Weight loss	21	15.3	(9.7 – 22.5)
	Survival	14	10.2	(5.7 – 16.6)
	Behaviors	6	4.4	(1.6 – 9.3)
	Leukocyte count	5	3.6	(1.2 – 8.3)
	Total	137	100	
Immunogenicity	nAb	66	81.5	(71.3 – 89.2)
	Specific Ab ELISA	15	18.5	(10.8 – 28.7)
	Total	81	100	
Challenge	Yes	37	54.4	(41.9 – 66.6)
	No	31	45.6	(33.5 – 58.1)
	Total	68	100	
Challenge Route	IN	22	53.7	(37.4 – 69.3)
	IV	2	4.9	(0.6 – 16.5)
	Others**	17	41.5	(26.3 – 57.9)
	Total	41	100	

[a]Number of studies that included different strategies for safety and efficacy in the *in vivo* CDV trial. Most studies included more than one safety measure, thirteen studies included both nAb and specific Ab with ELISA, and only two studies included only specific Ab with ELISA. *Clinical signs include symptoms such as dysphagia, rash, apnea periods, hair and skin changes, respiration alterations, and pale conjunctivae among others, which may be detected during a physical examination. **IP: intraperitoneal, IM: intramuscular, oculonasal and oral. IV: intravenous, nAb: neutralizing antibodies for CDV, Ab: antibodies.

As mentioned before, a total of thirty-seven studies reported postimmunization virus challenge (**Table 3**) on different species. Seventeen studies included dogs, eight included ferrets, five included minks, four included mice, one study included foxes, one included raccoon, one included Siberian polecats, and one included hybrid Ferret and Siberian polecats. One study included two animal populations, mice and foxes, totaling thirty-eight animal populations.

Postimmunization challenge and efficacy for CDV vaccine trials

The efficacy of the CDV challenge is reported in **Figure 3** for different animal populations. As expected, the dog was the most common animal included in vaccine and challenge studies, followed by ferrets and mice (**Figure 3A-E**, *left panels*). Intranasal, oral, and oronasal CDV inoculation was broadly employed in dogs, ferrets, and minks (**Figure 3A-C**, *middle panels*), since this route could simulate a natural CDV infection, in contrast to other routes such as IM, IC, and IP, among others, used for mice and other animals (**Figure 3D and E**, *middle panels*).

For each animal species, the overall survival percentage was evaluated as the cumulative survival, including all vaccine platforms colored in red (**Figure 3A-E**, *right panels*). For dogs and minks, all employed vaccines exhibited a survival percentage greater than 80%, and the global survival rates of the whole population were 97.2% and 97.9%, respectively (**Figure 3A and C**, *right panels*). Similarly, for the grouped populations of foxes, raccoons, Siberian polecats, and hybrid ferrets and Siberian polecats, the survival percentage was greater than 80%, and the global percentage was 100% (**Figure 3E**, *right panel*). In contrast, for ferrets, 100% of the animals that received MLVs and chimeric MeV-expressing CDV survived, with a global survival rate of 66.7%, since DNA vaccines were not effective enough (**Figure 3B**, *right panel*). Finally, in mice, a peptide-based vaccine resulted in 75% survival; however, the global survival rate was 87.7% (**Figure 3D**, *right panel*). Overall, the employed vaccine platforms have demonstrated moderate to high protection against CDV infection, considering that, for all animals, survival in negative vaccination control individuals in populations such as mice and ferrets was 0.0%, and all differences were statistically

significant. On the other hand, the MLV CDV vaccine demonstrated approximately 100% protection in all animal populations, indicating the importance of this type of vaccination platform; however, for other alternatives such as DNA vaccines, their efficacy depends on the animal species (**Figure 3A-E**, *right panels*).

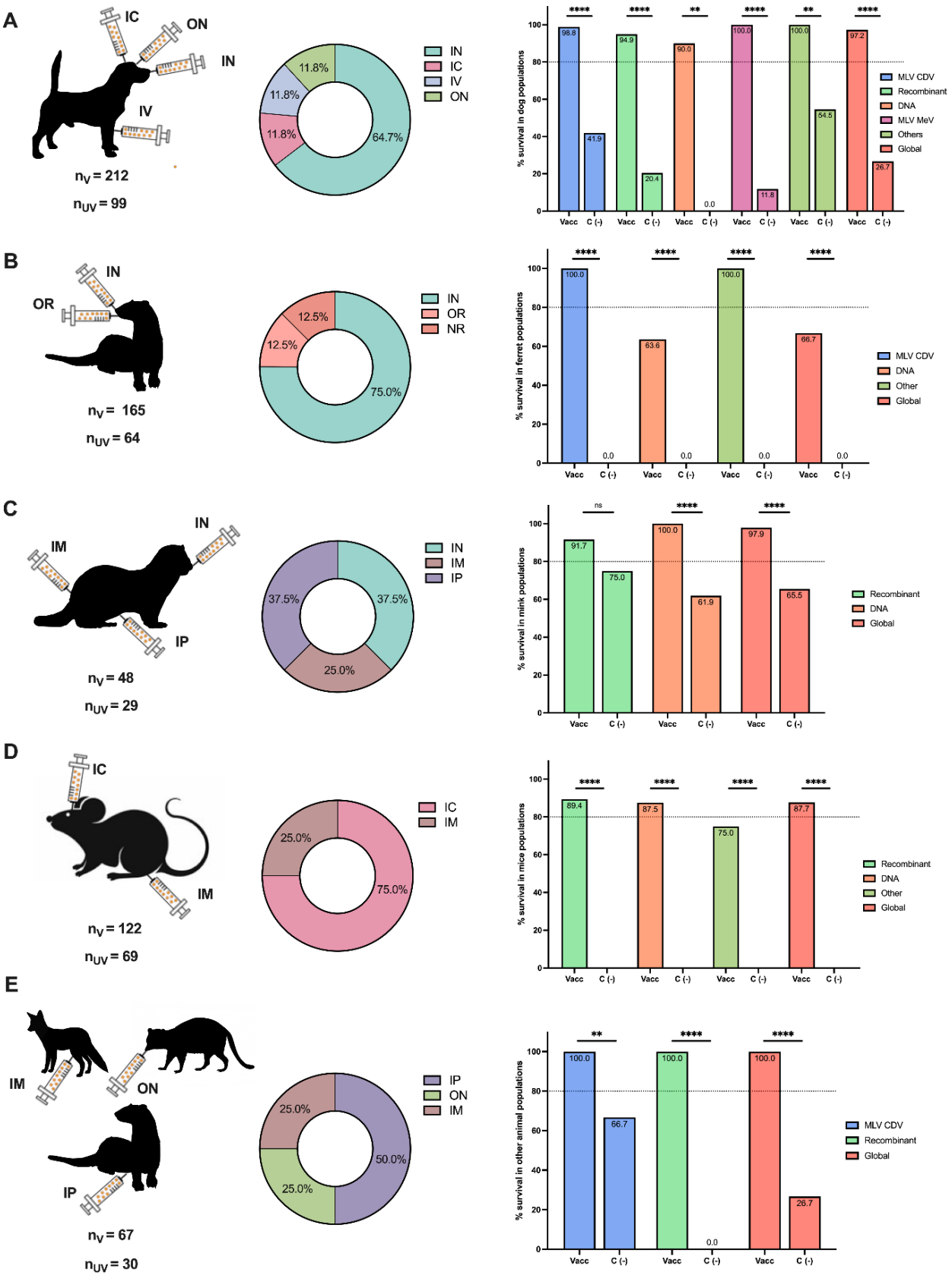
A
IC
ON
IN
IV
n_V = 212
n_{UV} = 99
IN
IC
IV
ON
11.8%
11.8%
11.8%
64.7%
% survival in dog populations
98.8
41.9
94.9
20.4
90.0
0.0
100.0
11.8
100.0
54.5
97.2
26.7
MLV CDV
Recombinant
DNA
MLV MeV
Others
Global
B
IN
OR
n_V = 165
n_{UV} = 64
IN
OR
NR
12.5%
12.5%
75.0%
% survival in ferret populations
100.0
0.0
63.6
0.0
100.0
0.0
66.7
0.0
MLV CDV
DNA
Other
Global
C
IN
IM
IP
n_V = 48
n_{UV} = 29
IN
IM
IP
37.5%
37.5%
25.0%
% survival in mink populations
ns
91.7
75.0
100.0
61.9
97.9
65.5
Recombinant
DNA
Global
D
IC
IM
n_V = 122
n_{UV} = 69
IC
IM
25.0%
75.0%
% survival in mice populations
89.4
87.5
75.0
87.7
Recombinant
DNA
Other
Global
E
IM
ON
IP
n_V = 67
n_{UV} = 30
IP
ON
IM
25.0%
25.0%
50.0%
% survival in other animal populations
100.0
66.7
100.0
0.0
100.0
26.7
MLV CDV
Recombinant
Global
Vacc
C (-)

Figure 3. Postimmunization CDV challenge in different animal populations. The numbers of included vaccinated (n_V) and negative vaccination control ($n_{c(-)}$) animals of all challenged species are reported. The inoculation routes are shown, as well as the percentage of survival based on the vaccine type for A. dog populations, B. ferret populations, C. mink populations, D. mouse populations, E. other populations, such as foxes, raccoons, Siberian polecats, and hybrid ferrets and Siberian polecats, are described. Other vaccines included MLV MeV, inactivated CDV, ISCOMs, and purified H and F CDV proteins in dogs; chimeric MeV-expressing CDV antigens in ferrets; and peptide-based vaccines in mice. IN: intranasal, IC: intracranial, SC: subcutaneous, IP: intraperitoneal, IM: intramuscular, ON: oculonasal, IV: intravenous, OR: oral, ID: intradermal, IDU: intraduodenal, NR: not reported. Z test for the difference in proportions with *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 and ns: not significance.

Discussion

Currently, there are different CDV vaccine alternatives for domestic dogs, including MLV vaccines, recombinant viruses, and multivalent vaccines (Buczkowski et al., 2014; Rendon-Marin et al., 2019; Wang et al., 2014). This scoping review provides valuable insights into the safety and efficacy of CDV vaccines *in vivo* across domestic and wildlife animal populations as reported in scientific databases. Through a systematic examination of the literature, this review synthesizes key findings, identifies knowledge gaps, and outlines implications for future research and vaccination strategies, since there are many species that could be affected by CDV, and most of them lack structured research on CDV vaccines.

In total, we included sixty-eight *in vivo* studies on CDV vaccine development (**Figure 1**) and research, where the United States of America, China, and France had the highest number of *in vivo* CDV vaccine trials in domestic and wildlife animals, which coincides with the authors affiliations (**Figure 2**). As reported, collaboration among researchers from different countries seems low in CDV vaccine development, as shown in **Figure 2.** There is a need for collaborative research in wildlife animal vaccination, new-generation effective and safe vaccines, and population-based immunization strategies to control CDV´s transmission and

dissemination. This is essential in advancing scientific knowledge and addressing complex global challenges such as CDV infection for domestic and wildlife animal conservation. By pooling diverse expertise, resources, and perspectives, international collaborations foster innovation and accelerate scientific breakthroughs. Such partnerships enable the sharing of data, methodologies, and best practices, leading to more robust and reproducible findings (Pagliusi et al., 2019). Moreover, collaboration across borders enhances the cultural understanding and promotes mutual respect among scientists, laying the foundation for long-term partnerships that transcend geopolitical boundaries (Francisco, 2015). In an increasingly interconnected world, where many scientific questions require multidisciplinary approaches, international collaboration is not only beneficial but also essential in pushing the boundaries of human knowledge and tackling the pressing issues facing human and animal health (Bregu et al., 2011). Although conducting vaccination studies within wildlife populations is re-source-intensive, both financially and logistically, and is influenced by various factors, including the vaccination policy recommendations of the respective countries, there is an urgent need for CDV vaccination in wildlife populations in several biodiverse regions of Asia, South America, and Africa.

Considering that CDV can affect a vast array of domestic and wildlife animals (MacLachlan et al., 2011; Martinez-Gutierrez & Ruiz-Saenz, 2016), there is a small number of species for which there are *in vivo* CDV vaccine studies, which are shown in **Table 2**, demonstrating the necessity of exploring CDV vaccines for the broad range of wildlife animals affected by CDV. This is crucial as controlling CDV transmission and dissemination in endangered wildlife animal species may contribute to the conservation of these threatened animals (Gilbert et al., 2015; van de Bildt et al., 2002; Wilkes, 2022). As expected, most studies were conducted on dogs, ferrets, and mice (**Table 2**). The presence of only a few studies in other species indicates an essential limitation in CDV vaccine development since there are reports about the transmission of CDV in different species (Duque-Valencia, Sarute, et al., 2019). The circulation of CDV lineages in domestic dogs that have been disseminated only to wild animals has been reported, indicating the importance of controlling CDV infection in domestic and wild animals (Duque-Valencia, Forero-Munoz, et al., 2019).

There are different commercially available vaccines for CDV infection in dogs, ferrets and minks based on MLVs and recombinant vaccines (Wilkes, 2022). Here, MLV CDV vaccines were studied more frequently than other vaccine alternatives. Notably, recombinant viruses have been widely employed for CDV infection in different animal species (**Table 3**). Nevertheless, there are some experimental vaccines for CDV based on new alternatives, such as more recombinant viruses, primarily based on the canarypox backbone (Gong et al., 2020; Pujol et al., 2023; Sadler et al., 2016; Yan et al., 2020), recombinant mouse adenovirus 1 expressing CDV antigens (Du et al., 2022), and formulations based on bacterium-like particles presenting CDV antigens (Wang et al., 2024). This review indicates that the development of CDV vaccines has become an important research field since the emergence of geographically distributed CDV lineages has led to new challenges in CDV infection control and dissemination. This is because the current vaccines are based on the Onderstepoort strain, which has not circulated for many years (Buczkowski et al., 2014) and, differs from the current circulating lineages worldwide by more than 10% (Duque-Valencia, Diaz, & Ruiz-Saenz, 2019).

On the other hand, it is well known that CDV has a remarkable ability to cross species barriers (Beineke et al., 2015), since the mutations affecting the CDV H protein, which is essential for virus attachment to host cell receptors and the humoral immune response, impair CDV control and facilitate the emergence of novel strains, for which the current vaccines are not completely effective (Rendon-Marin et al., 2019). The presence of antigenic variations among some of the examined CDV wild-type isolates, as well as disparities between these isolates and the vaccine strain Onderstepoort, which is presently employed worldwide, was demonstrated by a cross-neutralization assay, indicating the necessity of developing revised CDV vaccines based on the virus´ genetic and antigenic variations (Anis et al., 2018).

The safety profile of a vaccine emerges as a focal point when a new vaccine has been developed or tested in other species (Woodroffe, 2021). While the vaccines reported in this review generally exhibited an acceptable safety profile, varying degrees of adverse reactions, including local injection site reactions, temperature changes, weight loss, behavior changes and clinical signs, could be reported, as shown in **Table 4**. The safety and

efficacy measures underscore the importance of ongoing vaccine trials, particularly in diverse animal populations, in which the vaccine responses may differ. There are many species for which there are no data about vaccine safety and efficacy, as demonstrated in this review, based on the small number of studies in other species compared to studies in dogs, ferrets and even minks (**Tables 2 and 4**). This is a notable gap identified in this scoping review because of the limited data on CDV vaccine safety and efficacy in wildlife species. Although vaccination efforts have traditionally focused on domestic dogs, wildlife reservoirs play a significant role in CDV´s transmission dynamics (Wilkes, 2022). The lack of comprehensive data on vaccine responses in wild animals underscores the urgency of expanding the research efforts in this area. Technical limitations must be overcome, such as the application of vaccination strategies in the field, the use of drones or immunogen baits, vaccine storage and transportation in forest zones, and the monitoring of safety and efficacy, among others, because vaccine evaluation in these animal populations has been possible only in captive animals. Moreover, this scoping review highlight that the humoral immune response has been considered an essential measure of vaccine efficacy, as has been demonstrated for diverse viral infection limitations (Schmid et al., 2000), since most studies have measured nAb (**Table 4**).

Postimmunization challenge has been employed as an effective measure in vaccine development because it simulates the natural infection protection under controlled conditions with circulating virus strains (Wang et al., 2024). As reported in **Table 4**, approximately half of the included studies investigated CDV challenge in vaccinated animals such as dogs, ferrets, minks, mice, foxes, raccoons, Siberian polecats, and hybrid ferrets x Siberian polecats. Intranasal inoculation, which reproduces the natural CDV infection (Rendon-Marin et al., 2019), was the most commonly employed route (**Figure 3A-E**). Although most employed vaccines have demonstrated moderate to high efficacy, the MLV CDV vaccine is the most effective immunogen in dogs, ferrets, and other evaluated species, as shown in **Figure 3**, which is consistent with the current literature (Buczkowski et al., 2014; Woodroffe, 2021). Moreover, other vaccines, such as DNA plasmids and recombinant viruses, could be employed in animal populations. High survival rates after CDV challenge were demonstrated, in contrast to individuals that were employed as negative vaccination

controls, which developed CDD and mostly died (**Figure 3A-E**, *right panels*). As with MLV CDV vaccines, recombinant vaccines have been used in different animals, such as dogs, ferrets, minks, and red pandas, to control CDV infection and disease (Ramsay et al., 2019; Wang et al., 2012). However, commercial recombinant vaccines are based on the Onderstepoort lineage, which reduces the neutralization ability of newly emerged CDV lineages (Duque-Valencia, Diaz, & Ruiz-Saenz, 2019). Although MLVs can induce a strong immune response, attenuated viral strains may not adequately cover the spectrum of CDV variants circulating in diverse geographical regions (Wilkes, 2022).

One crucial approach to address this evolving scenario is the development of a universal CDV vaccine, as has been proposed for other viruses, such as the *Alphainfluenzavirus influenzae* (Influenza A virus) virus (Opriessnig et al., 2024; Stanekova & Vareckova, 2010), considering the emergence of diverse CDV lineages. The development of a universal vaccine against CDV holds significant importance in mitigating the global impact of this highly contagious and often fatal disease in domestic and wild animals. The current commercially available vaccines offer protection against specific strains of CDV; however, the virus exhibits considerable genetic diversity, leading to vaccine escape and outbreaks among susceptible populations (Duque-Valencia, Sarute, et al., 2019; Martella et al., 2006). A universal vaccine targeting the conserved regions of the CDV genome could provide broad-spectrum protection against diverse viral strains. Such a vaccine would not only benefit domestic dogs but also contribute to the conservation of endangered wildlife species susceptible to CDV, such as African lions and Amur tigers (Gilbert et al., 2020; Weckworth et al., 2020).

Through diverse advances in immunology and genomics, researchers are actively exploring novel vaccine candidates capable of eliciting robust and durable immune responses against CDV. The development of a universal vaccine represents a crucial step in safeguarding the health and well-being of canine populations worldwide, as well as protecting vulnerable wildlife species from this devastating viral pathogen (Martella et al., 2008). Efficacy assessments revealed notable variability in vaccine performance across different animal hosts and formulations (**Table 4 and Figure 3**). Factors such as the vaccine strain, dose, and administration route influence the level of protection conferred against CDV infection

(Gonzalez et al., 2023). Such variability underscores the complexity of vaccine host interactions and highlights the need to explore new vaccination approaches. Furthermore, the vaccine efficacy in domestic and wildlife animal species emphasizes the importance of species-specific considerations in vaccine development. Peptide-based vaccines could be a potential alternative (Tahir Ul Qamar et al., 2020) because they enable the introduction of multiple immunogenic epitopes. Moreover, their safe profile due to the absence of potential reversion has been considered a notable advantage compared to the inherent risks associated with highly effective live-attenuated vaccine formulations, especially for endangered wildlife animals (Purcell et al., 2007).

This scoping review underscores the imperative need for the design of standardized international trials in prototype species by family or species groups, enabling the validation of the safety and efficacy of the current and prototype vaccine technologies. Emphasis is placed on the necessity of public private partnerships, as these vaccines have a significantly limited market, rendering them unattractive for investment by commercial enterprises (McDowell, 2022). Additionally, alternative vaccination systems must be developed to reach target species without direct contact with humans, akin to those established for Rabies and Plague viruses (Abbott et al., 2012; Ballesteros et al., 2007); moreover, "transmissible vaccines" have emerged as a novel strategy to augment the reach and protection in wildlife populations (Smithson et al., 2019; Streicker et al., 2024).

Among the limitations of this scoping review were the language restrictions, as the article search was confined to Spanish, English, and Portuguese. Although these languages constitute an essential portion of the scientific production, they do not account for all of it, as studies in Mandarin, German, French and other languages were excluded. Similarly, the full texts of many articles could not be accessed due to their unavailability. Additionally, the heterogeneity in defining vaccine safety and efficacy and the diversity of CDV vaccine platforms prevented the inclusion of a greater number of articles, since, in some cases, the information could have been confusing. Another important limitation is that the vaccine industry is not required to publish of all its developments and clinical trials in the scientific literature. Therefore, it is plausible that the industrial developmental data of vaccines may not entirely align with the data presented in the current scoping review, despite the existence

of animal study guidelines such as GRADE. There are currently no standardized guidelines for the development and evaluation of biological agents, which would allow for the uniformity and systematization of all existing trials across different species. Consequently, significant developments and evaluations in species crucial for conservation efforts could not be included in this scoping review. This limitation arose due to the unavailability of the required information as inclusion criteria in published articles, as exemplified by studies on tigers (*Panthera tigris*) (Sadler et al., 2016), pandas (*Ailuropoda melanoleuca*) (Bronson et al., 2007; Geng et al., 2020), and red pandas (*Ailurus fulgens*) (Ramsay et al., 2019), among others. Finally, the survival results combined data from different vaccines, administration routes, and strains used in the immunological challenge. Future studies should conduct meta-analyses of the efficacy considering this variability and heterogeneity.

Conclusions

In conclusion, this scoping review provides a comprehensive synthesis of the current literature on the safety and efficacy of CDV vaccines *in vivo* in domestic and wildlife animal populations. Our analysis underscores the importance of ongoing vaccine efficacy and safety evaluation, particularly in diverse animal populations affected by CDV with insufficient vaccine alternatives or studies of commercially available vaccines. While CDV vaccines generally exhibit an acceptable safety profile in dogs, there are significant gaps in vaccine research, particularly in the context of wildlife reservoirs, since little information regarding the current vaccines and new alternatives has been described, although vaccines have shown promise as tools for the control of CDV. Addressing these knowledge gaps through targeted research efforts and collaborative initiatives is imperative for the development of effective vaccination strategies to safeguard animal health and mitigate the impact of CDV on global animal populations, especially for endangered species.

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CHAPTER 3

Universal peptide-based potential vaccine design against canine distemper virus (CDV) using a vaccinomic approach

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Abstract: Canine Distemper Virus (CDV) affects many domestic and wild animals. Variations among CDV genome linages could lead to vaccination failure. To date, there are several vaccine alternatives, such as a modified live virus and a recombinant vaccine; however, most of these alternatives are based on the ancestral strain Onderstepoort, which has not been circulating for years. Vaccine failures and the need to update vaccines have been widely discussed, and the development of new vaccine candidates is necessary to reduce circulation and mortality. Current vaccination alternatives cannot be used in wildlife animals due to the lack of safety data for most of the species, in addition to the insufficient immune response against circulating strains worldwide in domestic species. Computational tools, including peptide-based therapies, have become essential for developing new-generation vaccines for diverse models. In this work, a peptide-based vaccine candidate with a peptide library derived from CDV H and F protein consensus sequences was constructed employing computational tools. The molecular docking and dynamics of the selected peptides with canine MHC-I and MHC-II and with TLR-2 and TLR-4 were evaluated. *In silico* safety was assayed through determination of antigenicity, allergenicity, toxicity potential, and homologous canine peptides. Additionally, *in vitro* safety was also evaluated through cytotoxicity in cell lines and canine peripheral blood mononuclear cells (cPBMCs) and through a hemolysis potential assay using canine red blood cells. A multiepitope CDV polypeptide was constructed, synthetized, and evaluated *in silico* and *in vitro* by employing the most promising peptides for comparison with single CDV immunogenic peptides. Our findings suggest that predicting immunogenic CDV peptides derived from most antigenic

CDV proteins could aid in the development of new vaccine candidates, such as multiple single CDV peptides and multiepitope CDV polypeptides, that are safe *in vitro* and optimized *in silico*. *In vivo* studies are being conducted to validate potential vaccines that may be effective in preventing CDV infection in domestic and wild animals.

Keywords: vaccinomics; peptide; *in silico*; *Morbillivirus canis*; vaccine design; immunogenic.

Introduction

Morbillivirus canis, commonly known as canine distemper virus (CDV) is a single-negative stranded RNA virus belonging to the *Paramyxoviridae* family that naturally infects a vast array of carnivorous and no carnivorous species including domestic dogs, raccoons, ferrets, and other wildlife animals (Walker et al., 2021). It is the causative agent of a highly contagious disease known as canine distemper (CD), which is characterized by respiratory, digestive, skin, and neurological symptoms (Lempp et al., 2014). CDV has an RNA genome that includes six linearly organized transcription units, which give rise to eight viral proteins, including the hemagglutinin (H) and the fusion (F) protein (Rendon-Marin et al., 2019). H is a glycoprotein that is involved in adhesion and interaction with cellular receptors and has the greatest genetic variation (Martella et al., 2006). Moreover, the F protein facilitates viral and host cell membrane fusion, facilitating viral genome entry into the cytoplasm (von Messling et al., 2001). Both structural proteins are considered the main antigenic determinants of CDV since a greater number of H- and F-derived peptides are recovered from major histocompatibility complex (MHC) molecules, than from other CDV proteins (Ross et al., 2018).

To date, there are diverse CDV lineages distributed worldwide (Duque-Valencia, Diaz, & Ruiz-Saenz, 2019; Duque-Valencia, Forero-Munoz, et al., 2019). CDV has a high genomic substitution rate, where circulating variants differ by more than 10% at the amino acid level from ancestral strains used in vaccines and other circulating strains around the world (Duque-Valencia, Forero-Munoz, et al., 2019). This variation could imply consequences in the vaccine-induced immune response, and the constant occurrence of disease even in vaccinated animals (Anis et al., 2018), in addition to the worldwide re-emergence of infections in wildlife, for which commercial vaccines cannot be used due to the lack of safety and efficacy data for most susceptible species (Rendon-Marin et al., 2019). There is no specific treatment for the disease and most efforts are focused on prevention by administering two or more doses of a vaccine between the sixth or seventh week of age and up to three or four months, followed by revaccination every three years throughout the life of the animal (Day et al., 2020).

Some alternatives vaccines include a modified live virus (MLV) type vaccine based on the ancestral strain Onderstepoort. Therefore, considering that this variant no longer circulates, the immunity generated by this vaccine may decrease compared to that of other lineages distributed worldwide (Buczkowski et al., 2014). There is a recombinant vaccine that uses a backbone of the canarypox virus, which expresses the H and F proteins of CDV, which protects against the development of symptomatic distemper (Stephensen et al., 1997). In addition, a new platform, considered a recombinant bivalent vaccine, uses the rabies virus, which expresses the CDV H and F proteins, and has been tested in domestic dogs and ferrets (Wang et al., 2014). Moreover, a replication-competent adenovirus-vectored vaccine has been developed as a single oral immunization in mice (Du et al., 2022). However, they are not available commercially.

The use of computational tools has become an essential key to developing new generation vaccines for diverse models, including peptide-based therapies and mRNA vaccines (Akhtar et al., 2022; Khan, Khan, et al., 2022), considering that the development of alternative vaccines based on experimental methods is time-consuming and financially expensive (Sami et al., 2021). This field has been called vaccinomic, which refers to the integration of immunogenetics and immunogenomics with systems biology and immune profiling, as well as immunoinformatics (Poland et al., 2011). Hence, the application of “omics” technologies has advanced in the field of vaccinology through the characterization of host-vector-pathogen molecular interactions and the identification of potential protective antigens (de la Fuente & Contreras, 2021; T. Khan, A. Khan, et al., 2021). Epitope-based peptide vaccines are based on in-silico prediction of immunogenic peptides from antigenic dominant pathogen proteins (Gu et al., 2017; Yashvardhini et al., 2021). The use of peptides derived from viral antigens could allow B cells to be stimulated by helper T cells and become plasma cells to produce antibodies. In addition to neutralization by antibodies, helper CD4+ T cells and CD8+ T cytotoxic cells are required for complete virus clearance from the host (Rakib et al., 2020). In addition, T-cell-mediated immunity is dependent on MHC−peptide complexes, peptides that come from the antigen. MHC proteins are encoded by the dog leukocyte antigen (DLA). Each of the DLA alleles represents only a specific set of peptides on the surface of an infected cell and is recognized by T-cell receptors (Rakib et al., 2020). Hence,

antigenic peptides could take advantage of immunological processes involving helper CD4+ T cells and CD8+ T cytotoxic cells (Sami et al., 2021).

Peptide-based vaccines have been employed for other viral agents such as hepatitis B, influenza A, and hepatitis C, among others, which exhibit certain immunity in employed models (He et al., 2015; Sominskaya et al., 2010; Stanekova & Vareckova, 2010). There are many reasons to consider peptide-based vaccines since they are not infectious materials. In addition to the easy introduction of molecules to improve immunogenicity, they could be lyophilized preparations, which provides an advantage in storage. Moreover, there is no risk of reversal of virulence, and they can be designed for the inclusion of multiple antigenic determinants. However, no peptide-based vaccine is commercially available (Purcell et al., 2007).

After all, a research question has arisen. Can we predict and evaluate peptides with immunogenic potential based on the genetic and antigenic information of worldwide circulating variants? To this aim, a peptide library derived from CDV H and F protein consensus sequences from circulating strains worldwide with immunogenic potential through computational tools was built. Additionally, the molecular interactions of the selected peptides with canine MHC-I, MHC-II, and TLR-2 and TLR-4 were evaluated via molecular docking and dynamic simulations. Moreover, the *in silico* safety of the peptides was assayed through their antigenicity, allergenicity, toxicity potential, and homologous canine peptides, and the *in vitro* safety was also evaluated through cytotoxicity in cell lines and canine peripheral blood mononuclear cells (cPBMCs) and a hemolysis assay in canine red blood cells. Multiple polypeptide epitopes were constructed, synthetized, and evaluated based on the best single peptides to validate and compare the usage of multiple immunogens to that of a single antigen based on linked immunogenic predicted peptides.

Materials and methods

CDV H and F protein consensus sequence analysis

Sequences from H and F proteins of CDV variants reported worldwide were obtained from the NCBI database. Consensus sequences were generated for both the H and F CDV proteins with EMBOSS (Madeira et al., 2022), to predict peptides based on the consensus sequences of the H and F CDV proteins.

Prediction of potential immunogenic peptides based on CDV H and F protein sequences and peptide selection

Potential immunogenic peptide prediction employing H and F protein consensus sequences was carried out with different computational tools to predict helper CD4+ T cells, CD8+ T cytotoxic cells, and linear B-cell epitopes. To accomplish this goal, the online tools MHC2PRED (http://crdd.osdd.net/raghava/mhc2pred/), CTLPRED (http://crdd.osdd.net/raghava/ctlpred/), IEDB from the La Jolla Institute (https://www.iedb.org/), and SVMTRIP (http://sysbio.unl.edu/SVMTriP/) were used to predict the immunogenic peptides derived from the consensus sequences of the H and F CDV proteins for CD4+ and CD8+ T-cell and B-cell linear epitopes. All these computational tools are based on support vector machines that enable the prediction of diverse peptides based on databases that have been trained with peptides that have positive and negative desired functions.

Antigenicity, allergenicity, toxicity potential, and physicochemical property evaluation

In silico safety was determined for selected peptides derived from CDV H and F consensus sequences. Antigenicity, the allergenic profile, and toxicity prediction were characterized for individual predicted peptides using the VaxiJen v2.0 server (http://www.ddg-pharmfac.net/vaxijen/), AllerTOP v2.0 server (https://www.ddg-pharmfac.net/AllerTOP/), and ToxinPred server (http://crdd.osdd.net/raghava/toxinpred/). The VaxiJen v2.0 tool enables the determination of whether a peptide sequence has the potential to be a viral antigen (Doytchinova & Flower, 2007). The AllerTop v2.0 server uses a k-nearest neighbor (kNN) method, amino acid descriptors, and ACC transformation methods to isolate nonallergens from allergens with 85.3% prediction accuracy via fivefold cross-validation

(Dimitrov et al., 2014). The ToxinPred server estimates the properties of different peptides by employing support vector machines (SVMs), a machine learning approach with a quantitative matrix for predicting toxicity (Gupta et al., 2013; Gupta et al., 2015). The ProtParam tool from ExPASy was used to measure the physicochemical properties of the peptides by the Swiss Institute of Bioinformatics (Wilkins et al., 1999).

BLAST homology assessment of CDV-derived peptides

NCBI Protein BLAST (BLASTp) was used to determine the homology between the selected peptides and the canine-reported proteome. The purpose of this cross-checking analysis was to avoid the inclusion of self-protein peptides in the dog proteome. To determine dog homology, the BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) tool protein BLAST module was used. In this experiment, comparisons were made with the default parameters for Canis lupus familiaris (taxid: 9606), and the threshold e-value was set to 0.05. Immunogenic peptides were determined to be nonhomologous peptides when no hits under the threshold e-value were found (Altschul et al., 1990; Mount, 2007).

Homology modeling and validation of canine MHC-I or -II, TLR-2, and TLR-4

Homology models were constructed to evaluate the interactions between the selected peptides and canine MHC-I, MHC-II, TLR-2, and TLR-4; since no crystallographic structures were available, homology modeling was performed. The amino acid sequences of canine MHC-I, MHC-II, TLR-2, and TLR-4 were retrieved from the NCBI. Human proteins were used as templates (PDB Codes: 5F1N, 4FQXA, 4FQXB, 6NIG, 4G8A). MODELLER v. 10.0 was used to model the 3D structure. A total of 100 different structures with model quality scores (molpdf, DOPE, GA341) were obtained (Sali & Blundell, 1993). The homology models and the respective templates were overlapped to determine the root mean square deviation (RMSD) differences between the model and template structures by employing TM-Align, a protein structure alignment algorithm based on the TM-score (Zhang & Skolnick, 2005). The homology models were validated with bioinformatics tools such as SWISS-MODEL, which provides global and local model quality based on the Z score and QMEAN, and each residue

quality calculation from the amino acid sequence and Ramachandran plots were used to establish amino acids in energetically favorable regions, regarding dihedral angles ψ against φ of amino acid residues in the protein structure (Berger & Barnard, 1999). ProSA-Web, a Z score for the overall model quality tool, was used to check whether the Z score value of the input structure was within the range of scores typically found for native proteins of similar size, with the PDB as the reference database (Wiederstein & Sippl, 2007).

Molecular docking and dynamics of CDV peptides and canine MHC-I or MHC-II, TLR-2, and TLR-4

The interactions between the selected peptides and MHC-I, MHC-II, TLR-2, and MHC-4 were assayed by employing three online molecular docking tools. HPEPDOCK is a blind protein-peptide docking tool that functions through a hierarchical algorithm. Instead of running lengthy simulations to refine peptide conformations, this tool considers peptide flexibility through an ensemble of peptide conformations generated by the MODPEP program (Zhou et al., 2018); MDOCKPEP is a server that predicts ab initio protein–peptide complex structures starting with the protein structure and peptide sequence in three steps (Xu & Zou, 2020); and CABSDOCK is a server that provides an interface for modeling protein–peptide interactions using a highly efficient protocol for the flexible docking of peptides to proteins (Kurcinski et al., 2020). For all the molecular docking tools, the ligand and the receptor are considered rigid structures. The most likely poses were selected based on the best tool scores. Molecular dynamics simulations were carried out employing GROMACS® software (Groningen Machine for Chemical Simulation, developed at the University of Groningen, The Netherlands) (Abraham et al., 2015). A topology file of the protein–peptide complexes was generated, and the conditions of the water box were established in a neutral ionic environment. The system was equilibrated, and the simulations were conducted with a constant number of molecules, temperature, and pressure (NVT and TPN assemblies). The final molecular dynamics simulation was run for 50 ns for each complex, and the trajectories were analyzed with Xmgrace software (Oregon Graduate Institute of Science and Technology, Hillsboro, OR, USA). All 3D complex graphics were generated using the software UCSF Chimera (Pettersen et al., 2004).

Peptide synthesis and cytotoxicity in cell lines and cPBMCs

Peptides were synthesized by BIOMATIK (USA) using standard solid-phase synthesis with a purity >75% and characterized by mass spectrometry. The Vero cell line expressing dog signaling lymphocyte activation molecules (named Vero-Dog-SLAM), which was kindly donated by Yusuke Yanagi from Kyushu University, Fukuoka, Japan, and the MDCK cell line (Madin-Darby canine kidney) from the ATCC were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 1% antibiotic/antifungal at 37°C in a humidified atmosphere containing 5% CO2. Cell monolayers from the cell lines mentioned above were harvested, seeded in 96-well plates, and inoculated after 24 hours. Then, the cells were treated with each peptide diluted in DMEM supplemented with 2% FBS (Gibco, Grand Island, NY, USA) at twofold serial dilutions from 6.125 to 200 nM for 72 hours. For cPBMCs, peripheral blood from two healthy donors was diluted with 1× PBS at a 1:1 ratio; then, a density gradient was generated by adding 3 ml of Ficoll-Histopaque 1077 (Sigma-Aldrich, St. Louis, MO, USA) and centrifuging for 23 min at 2300 rpm. The interface that corresponded to the PBMCs was recovered, and the cells were washed with 1× PBS and centrifuged at 1800 rpm for 5 minutes. Then, the PBMCs were treated with each peptide diluted in RPMI supplemented with 10% FBS (Gibco, Grand Island, NY, USA) at twofold serial dilutions from 6.125 to 200 nM for 72 hours. Afterward, the cells were washed twice with PBS, and 50 µL of MTT solution (to a final concentration of 0.5 mg/mL) was added to each well and incubated for 2 hours at 37°C. The formazan precipitates were dissolved with the addition of 100 µL of DMSO, and the absorbance at 450 nm was measured using a microplate reader (Thermo Fisher Scientific, USA). Wells without peptide treatment were used as a viability negative control, while wells treated with 0.5% Triton X-100® served as a cytotoxicity positive control. Two independent experiments with 4 replicates (n = 8) were carried out for each cell line, and nontreated cells were used as negative controls. Additionally, peptides Y, B, and K1, which were kindly donated by Sergio Orduz and have reported antimicrobial activity potential (Valencia et al., 2016), were used as noncytotoxic control peptides. The percent viability

was determined based on the absorbance of the nontreated cells. Means and standard deviations are reported.

Ethical considerations

This study received approval from the Ethics Committee for Animal Experimentation at the Universidad Cooperativa de Colombia in Bucaramanga. All procedures adhered to pertinent guidelines and regulations. Written informed consent was obtained from the legal guardians of all animals participating in this research.

Hemolytic potential of the peptides in canine red blood cells

The hemolytic potential of the selected peptides was established by using canine red blood cells (cRBCs) from fresh canine blood obtained from two healthy donors and collected in sodium citrate buffer. Informed consent was obtained from the participants' legal guardians. Blood samples were centrifuged at 1000 × g for 7 min at room temperature. One milliliter of the pellet was washed three times with 3 mL of PBS, and the red blood cells were suspended in 9 mL of PBS. Afterward, cRBCs were treated with twofold serial dilutions from 6.125 to 200 nM in a U-bottom 96-well plate and incubated for 3 h at 37°C and 90 rpm. The samples were centrifuged at 1000 × g for 7 min, and 50 µL of the supernatant from each peptide treatment was placed in another flat-bottom 96-well plate. The absorbance at 620 nm was measured using a microplate reader. Two independent experiments with 4 replicates (n = 8) were performed. PBS was used as a negative hemolytic control, and 0.1% Triton X-100® was used as a positive hemolytic control. The percent hemolysis was calculated as the difference between the treatment and control absorbance.

Multiple-epitope polypeptide construction and in silico and in vitro evaluation

A multiepitope polypeptide was constructed based on the best single peptides identified via *in silico* and *in vitro* assays. We employed amino acid likers such as AYY, GPGPG, and KK

for the MHC-I, MHC-II and B-cell epitopes, respectively (Sami et al., 2021). Polypeptide was synthesized by BIOMATIK (USA) using standard solid-phase synthesis with a purity >95% and characterized by mass spectrometry. Similar *in silico* and *in vitro* validation through viral antigenicity in VaxiJen v2.0 and IEDB immunogenicity, tools, allergenicity, toxicity potential, potential homologous canine peptides, canine TLR-2 or TLR-4 polypeptide interaction, and cytotoxicity and hemolytic assays were carried out at concentrations ranging from 6.125 to 25 nM, respectively, as described above for single peptides.

Results

Construction of an immunogenic potential peptide library using computational tools

After a consensus sequence of H and F CDV proteins from all reported linages was assembled, we selected peptides that were immunogenic to B cells linearly and those predicted to induce CD4+ and CD8+ T-cell responses, all of which could be employed as vaccine formulations. A total of 1399 peptides were predicted based on the best score from the consensus sequence of the H and F CDV proteins (**Figure 1**). The helper CD4+ T-cell epitopes were more abundant for both the H and F proteins, and the linear B-cell epitopes were less abundant for both CDV proteins. Thus, a peptide library with immunogenic potential was constructed to determine the most promising antigens in a peptide-based vaccine.

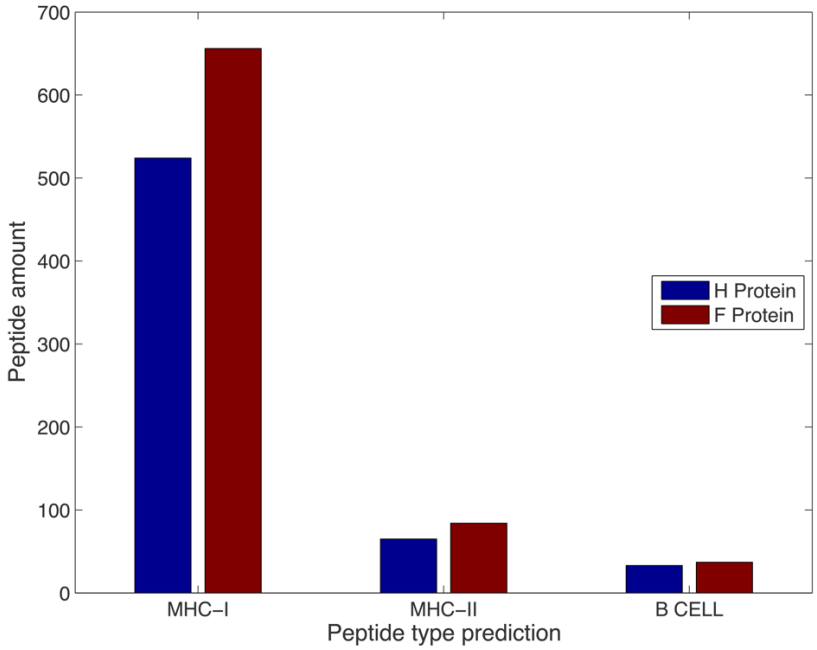


Figure 1. Total predicted peptides from H and F CDV proteins for CD8+ T cytotoxic cells (MHC-I), helper CD4+ T cells (MHC-II), and linear B cells. Computational tools such as MHC2PRED, CTLPRED, IEDB from the La Jolla Institute, and SVMTRIP were used to predict the immunogenic peptides based on the consensus sequence of the H and F CDV proteins.

Candidate peptide selection and physicochemical properties

From the total amount of predicted peptides, 12 peptides were selected based on the highest prediction score issued by the prediction tools and their physicochemical properties, such as charge, length, stability, and predicted half-life (**Table 1**). All peptides are classified as epitopes for CD8+ T cytotoxic cells (PCIs), helper CD4+ T cells (PCIIs), and linear B cells (LIBBs) from either the H or F protein. Importantly, after computational prediction, the peptides PCII-F3-012 and PCI-F3-038 had the same sequence and were predicted to have immunogenic potential for both CD8+ T cytotoxic cells and helper CD4+ T cells. Regarding the physicochemical properties, as shown in **Table 1**, the charges of the selected peptides are between 0 and +2, except LINB-IEDB-H, which has a charge of -1; most of them have a favorable instability index, indicating their stable nature, and the length of the peptides

oscillates between 9 and 13 amino acids. The half-life in hours varies between 1 and 100 hours, which is important for the use of a potential vaccine candidate. Therefore, we concluded that the peptides selected based on the best score exhibited favorable physicochemical properties and could be considered vaccine candidates.

Table 1. Selected peptides and physicochemical properties

ID	Peptide	Length (amino acids)	Position	Charge	Molecular weight (g/mol)	Stability	Half-life (hours)
P1	**PCI-H1-004**	9	36	0	1090,41	+	>20
P2	**PCI-H1-030**	11	97	0	1272,55	+	1
P3	**PCII-H1-008**	9	592	1	1145,37	+	100
P4	**PCI-F3-053**	12	651	2	1311,50	+	5.5
P5	**PCII-F3-012**	9	316	1	1198,43	+	5.5
P6	**PCI-IEDB-H1**	9	473	0	1026,24	+	7.2
P7	**LINB-IEDB-H**	11	367	-1	1321,42	+/-	100
P8	**PCI-F3-054**	12	645	1	1261,44	+	>20
P9	**PCII-IEDB-H2**	13	32	0	1512,98	+	>20
P10	**PCI-IEDB-F1**	9	552	0	1039,26	+	30
P11	**PCII-IEDB-F2**	13	488	2	1360,62	+/-	30
P12	**LINB-IEDB-F**	9	134	1	1123,28	+	1.3

Homology modeling, molecular docking, and molecular dynamics

Due to the lack of crystallographic structures from either canine MHC molecules or TLRs, homology modeling was employed to determine the 3D structure, which could allow the interaction of selected canine MHC molecule peptides with TLRs to be assayed. A 3D model of canine MHC molecules and TLRs is shown in **Figure 2A-B**. These models were carefully validated through computational tools. Ramachandran plots revealed that more than 95% of the amino acids in all the models were located in favorable regions for rotation and torsion (data not shown). The overall model quality calculated with ProSA-Web indicated that all

models are located within the distribution of all the proteins in the PDB that come from X-ray crystallography (**Supplementary Table 1**). The TM values indicated that all the models had global folding identical to that of the template, with values close to one (**Supplementary Table 1**). Structural alignment with the templates was carried out (**Figure 2C-D**). Both the models and the templated model exhibit overall folding since they can match after alignment. To observe the capacity of immunogenic peptides to interact with either canine MCH molecules or TLRs, molecular docking was employed.

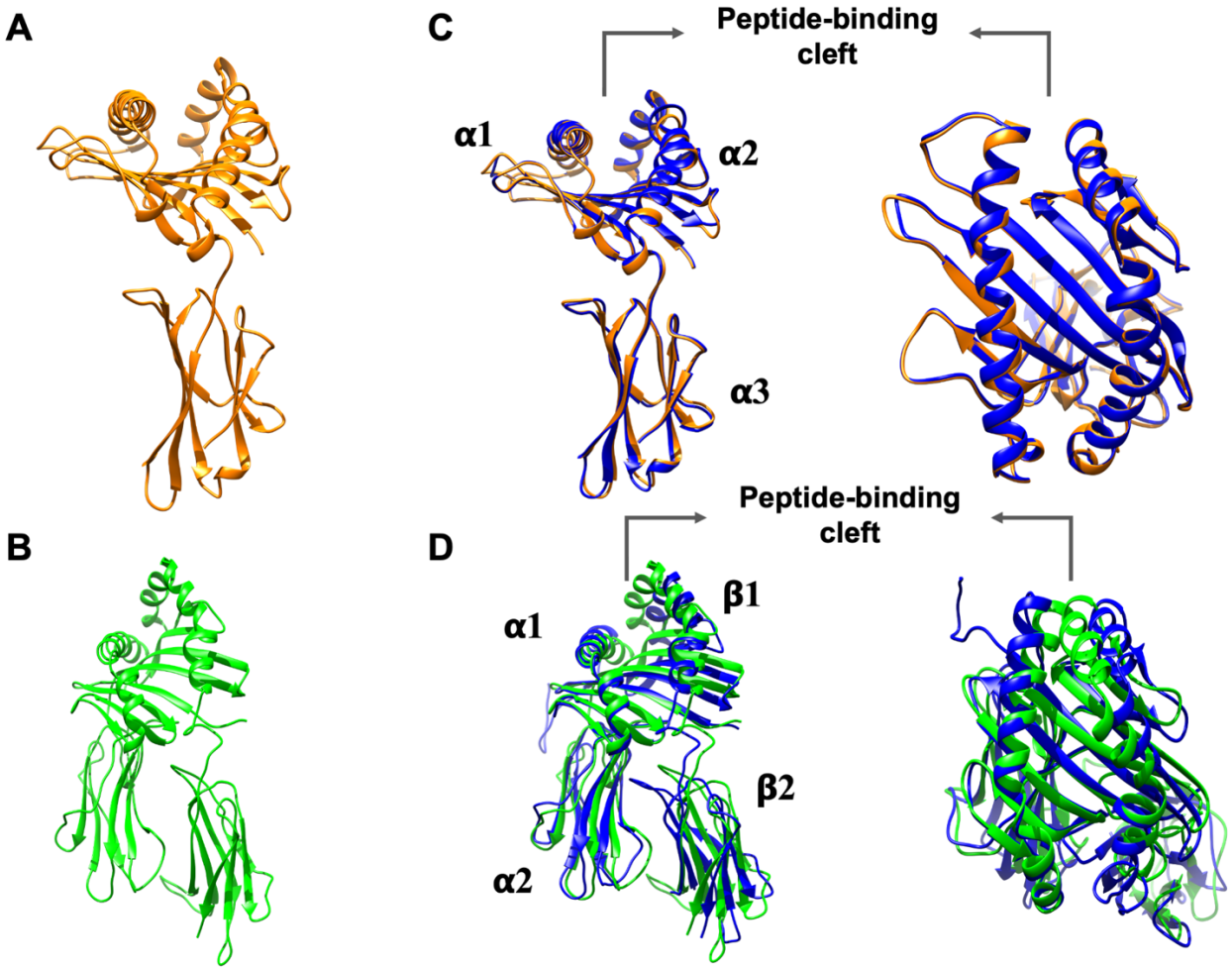


Figure 2. Canine MHC molecule and TLR homology models obtained by MODELLER v. 10.0 and structural alignments with PDB template structures. A) Canine MHC class I molecule homology model (orange). B) Canine MHC class II molecule homology model (green). C) Structural alignment of the template (PDB code: 5F1N) (blue) and canine MHC

class I molecule homology model (orange). D) Structural alignment of a template (PDB code: 4FQX) (blue) and canine MHC class I molecule homology model (green). Homology modeling was performed using MODELLER v. 10.0. The peptide-binding cleft was marked in both MHC molecules.

Three different tools were used, and the molecular docking scores of the selected peptides are reported in **Table 2**. The peptide PCI-F3-053 exhibited a favorable docking score in all the tools used, indicating a consensus prediction. Moreover, the PCII-IEDB-H2 peptide obtained the best docking score only with MDOCKPEP, and the PCI-H1-004 peptide had the best docking score with the HPEPDOCK server. Peptide PCI-F3-054 exhibited a low cluster density but the highest number of cluster elements, indicating that the 3D structure in this cluster is the most likely among the predicted poses, according to CABSDOCK. In contrast, the peptide PCI-F3-053 exhibited the highest cluster density because of the low number of elements, indicating a less probable pose among the predicted poses.

Table 2. Molecular docking scores obtained with different computational tools for selected peptides with canine MHC molecules

ID	Peptide	HPEPDOCK	MDOCKPEP	CABSDOCK		
				Cluster density	RMSD	Elements
P1	**PCI-H1-004**	-308.716	-184.3	75.4363	1.829	138
P2	**PCI-H1-030**	-221,010	-189.3	56.5631	2.333	132
P3	**PCII-H1-008**	-214,753	-167.3	86.7120	1.614	140
P4	**PCI-F3-053**	-224,458	-215.3	156.2350	0.294	46
P5	**PCII-F3-012**	-220,671	-189.3	105.4340	0.882	93
P5.1	**PCI-F3-038***	-252,723	-200.4	98.3842	1.128	111
P6	**PCI-IEDB-H1**	-249,353	-173.9	101.203	1.324	134

P7	**LINB-IEDB-H****	NA	NA	NA	NA	NA
P8	**PCI-F3-054**	-251,275	-173.2	54.0866	4.918	266
P9	**PCII-IEDB-H2**	-225,170	-218.0	96.4651	1.057	102
P10	**PCI-IEDB-F1**	-281,525	-162.1	46.7683	3.057	143
P11	**PCII-IEDB-F2**	-228,006	-186.2	54.9054	1.821	100
P12	**LINB-IEDB-F****	NA	NA	NA	NA	NA

*****This peptide has the same sequence as P5; however, it was predicted to be present in both MHC class I and II.

**There is no docking score for linear B-cell epitopes interacting with MHC molecules

For cTLR-2 and cTLR-4, the HPEPDOCK server was used to assay the interaction of the selected peptides with the innate immune receptors. The peptide PCI-H1-004 had the greatest interaction with TLR-2, and LINB-IEDB-F had the greatest interaction with TLR-4 (**Table 3**). Therefore, we conclude that all peptides obtained favorable docking scores when employing different computational tools, considering that all docking servers were blind, which means that none of them had a previous location to interact and that all peptides were in the peptide-binding cleft, which is the most likely interaction area.

Table 3. Molecular docking scores obtained with different computational tools for selected peptides containing canine TLR-2 and TLR-4.

ID	Peptide	TLR-2	TLR-4
P1	**PCI-H1-004**	-250,001	-177,975
P2	**PCI-H1-030**	-182,783	-183,632
P3	**PCII-H1-008**	192,197	-176,631
P4	**PCI-F3-053**	-215,147	-185,100
P5	**PCII-F3-012**	-211,496	-180,756

P5.1	**PCI-F3-038***		
P6	**PCI-IEDB-H1**	-219,952	-178,147
P7	**LINB-IEDB-H**	-143,050	-138,441
P8	**PCI-F3-054**	-210,443	-164,827
P9	**PCII-IEDB-H2**	-207,528	-177,352
P10	**PCI-IEDB-F1**	-213,697	-155,800
P11	**PCII-IEDB-F2**	-191,366	-163,752
P12	**LINB-IEDB-F**	-209.526	-194,692

*This peptide has the same sequence as P5; however, it was predicted to be present in both MHC class I and II.

Molecular docking complexes from PCI-F3-038 and canine MHC-I (**Figure 3A**) were employed as the starting structure for molecular dynamic simulations to determine whether interactions between the selected peptides and MHC and TLRs are stable over time. As shown in **Figure 3B**, the peptide PCI-F3-038 tended to be stable after 30 ns of simulation, with an RMSD between 0.5 and 0.8 nm. On the other hand, the complex from PCI-0H1-004 and MHC-I (**Figure 3C**) exhibited a nonstable trend, oscillating from 0 to 1 after 50 ns of simulation, indicating that the peptide from PCI-0H1-004 and the MHC-I complex could be less stable than that from PCI-F3-038 (**Figure 3D**). Notably, molecular docking complexes were obtained with blind-docking tools since both structures were submitted separately; however, in the complexes shown in **Figure 3A** and **3C**, both peptides were located in the peptide-binding cleft, allowing us to wonder about the capacity of those peptides to interact with MHC-I because the molecular docking tool indicates the most likely pose after hundreds of evaluated poses. Other molecular dynamics simulations between selected peptides in complex with canine MHC molecules are shown in **Supplementary Figure 2.**

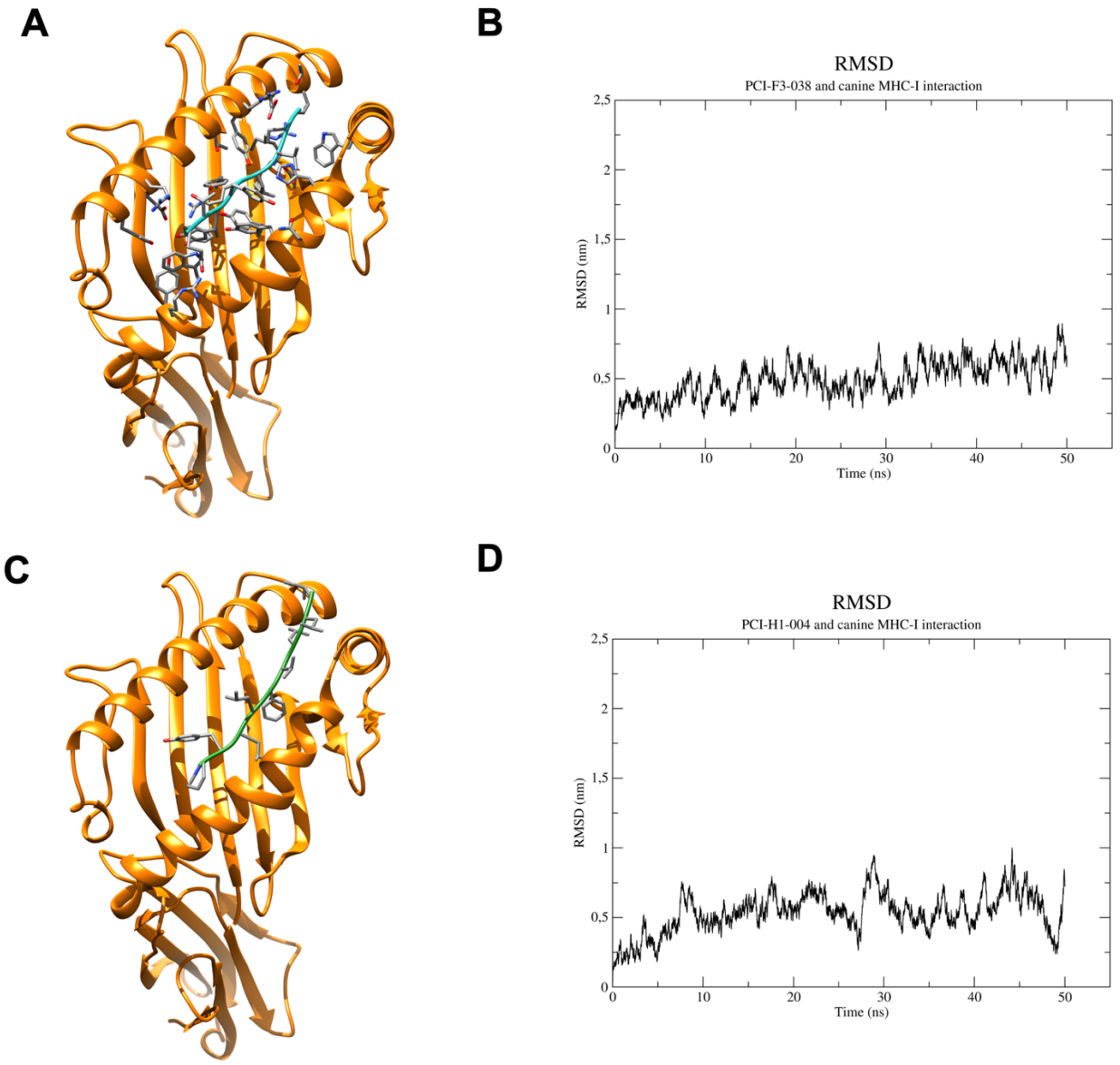


Figure 3. Molecular docking and dynamics of the predicted peptides in complex with MHC molecules. A) Molecular interaction between PCI-F3-038 and canine MHC-I molecules obtained with a molecular docking tool. B) Root mean square deviation (RMSD) plot of the interaction interface between PCI-F3-053 and MHC class I. C) Molecular interaction between PCI-H1-004 and canine MHC-I molecules obtained with a molecular docking tool. D) RMSD deviation plot of the PCI-F3-004 and MHC class I interactions. Representative peptide–MHC complex molecules are shown. All 3D graphics were generated using the software UCSF Chimera. Graphs for molecular dynamics were obtained

with Xmgrace software (Oregon Graduate Institute of Science and Technology, Hillsboro, OR, USA).

In silico safety evaluation through computational tools

To determine the potential use of the selected peptides, we conducted an *in silico* safety evaluation through multiple computational tools to assess antigenicity, allergenicity, and toxicity. As shown in **Table 4**, some peptides have the potential to be allergens (PCI-H1-004, PCI-F3-053, PCI-IEDB-H1, PCI-F3-054, PCII-IEDB-H2, and LINB-IEDB-F). Furthermore, all selected peptides were determined to be nontoxic to ToxinPred, and when antigenicity was evaluated, most peptides exhibited antigenic potential, except for PCI-H1-030, PCI-H1-030, and PCII-IEDB-F2, which had negative values (**Table 4**). Additionally, some of them were not classified as antigens; however, they were predicted to be immunogenic peptides with other computational tools (**Figure 1**). In addition, the sequence homology between the peptides and canine proteome was determined, revealing that there were no peptides with 100% coverage and that the identity was any match, indicating that no identical peptide was found in the canine proteome. BLAST homology assessment indicated that the predicted peptide vaccine did not cause autoimmune responses to the host since no peptide was identical in any canine protein (**Supplementary Table 2**). In summary, all peptides have been demonstrated to be nontoxic, and some of them have the potential to be allergenic since they have a potential prediction score.

Table 4. *In silico* safety assessment of the antigenicity, allergenicity, and toxicity of selected peptides.

ID	Peptide	ToxinPred	AllergenFP	Vaxigen
		Toxic / Non-Toxic	**Allergen / non-allergen**	**Antigen / non-antigen**
P1	**PCI-H1-004**	Non-Toxic	Allergen	Antigen
P2	**PCI-H1-030**	Non-Toxic	Non-Allergen	Non-Antigen

P3	**PCII-H1-008**	Non-Toxic	Non-Allergen	Antigen
P4	**PCI-F3-053**	Non-Toxic	Allergen	Antigen
P5*	**PCII-F3-012**	Non-Toxic	Non-Allergen	Non-Antigen
P6	**PCI-IEDB-H1**	Non-Toxic	Allergen	Antigen
P7	**LINB-IEDB-H**	Non-Toxic	Non-Allergen	Antigen
P8	**PCI-F3-054**	Non-Toxic	Allergen	Antigen
P9	**PCII-IEDB-H2**	Non-Toxic	Allergen	Antigen
P10	**PCI-IEDB-F1**	Non-Toxic	Non-Allergen	Antigen
P11	**PCII-IEDB-F2**	Non-Toxic	Non-Allergen	Non-Antigen
P12	**LINB-IEDB-F**	Non-Toxic	Allergen	Antigen

*****This peptide has the same sequence as P5; however, it was predicted to be present in both MHC class I and II.

[a]ToxinPred determines whether peptides are toxic in the 10 amino acid window, with a dichotomic result indicating toxicity or nontoxicity

[b]AllergenFP predicts the probability of allergenicity with a dichotomic result as probable allergen or probable no allergen

cVaxiJen prediction of antigens and subunit vaccines with a dichotomous result as a viral antigen or nonviral antigen

In vitro safety evaluation of selected peptides in cell lines demonstrated their low cytotoxicity.

The *in vitro* safety of the selected peptides was determined after chemical synthesis in cell lines. To establish whether the peptides were cytotoxic, different cell lines were treated with several dilutions of peptides. For Vero-Dog-SLAM, most peptides exhibited cell viability greater than 90% at low concentrations (< 50 nM) (**Figure 4A-C**). Thus, the peptides PCI-IEDB-H1 and PCII-IEDB-F2 showed cell viability lower than 90% at concentrations higher than 100 nM. For MDCK cells, similar to Vero-Dog-SLAM cells, most peptides exhibited cell viability greater than 90% at low concentrations (< 50 nM) in MDCK cells. However, the viability of cells treated with the peptides PCI-F3-054 and PCII-IEDB-F2 was lower than 90%

at concentrations higher than 100 nM (**Figure 4D-F)**. Both the Y and B control peptides had a cell viability of approximately 100% at all the evaluated concentrations (**Figure 4**). We concluded that most peptides were noncytotoxic at the evaluated concentrations when treated with two cell lines, one from a dog kidney and another expressing the dog SLAM receptor, which are important for virus recognition and entry into the cell, except for the peptides PCI-IEDB-H1, PCII-IEDB-F2 and PCI-F3-054.

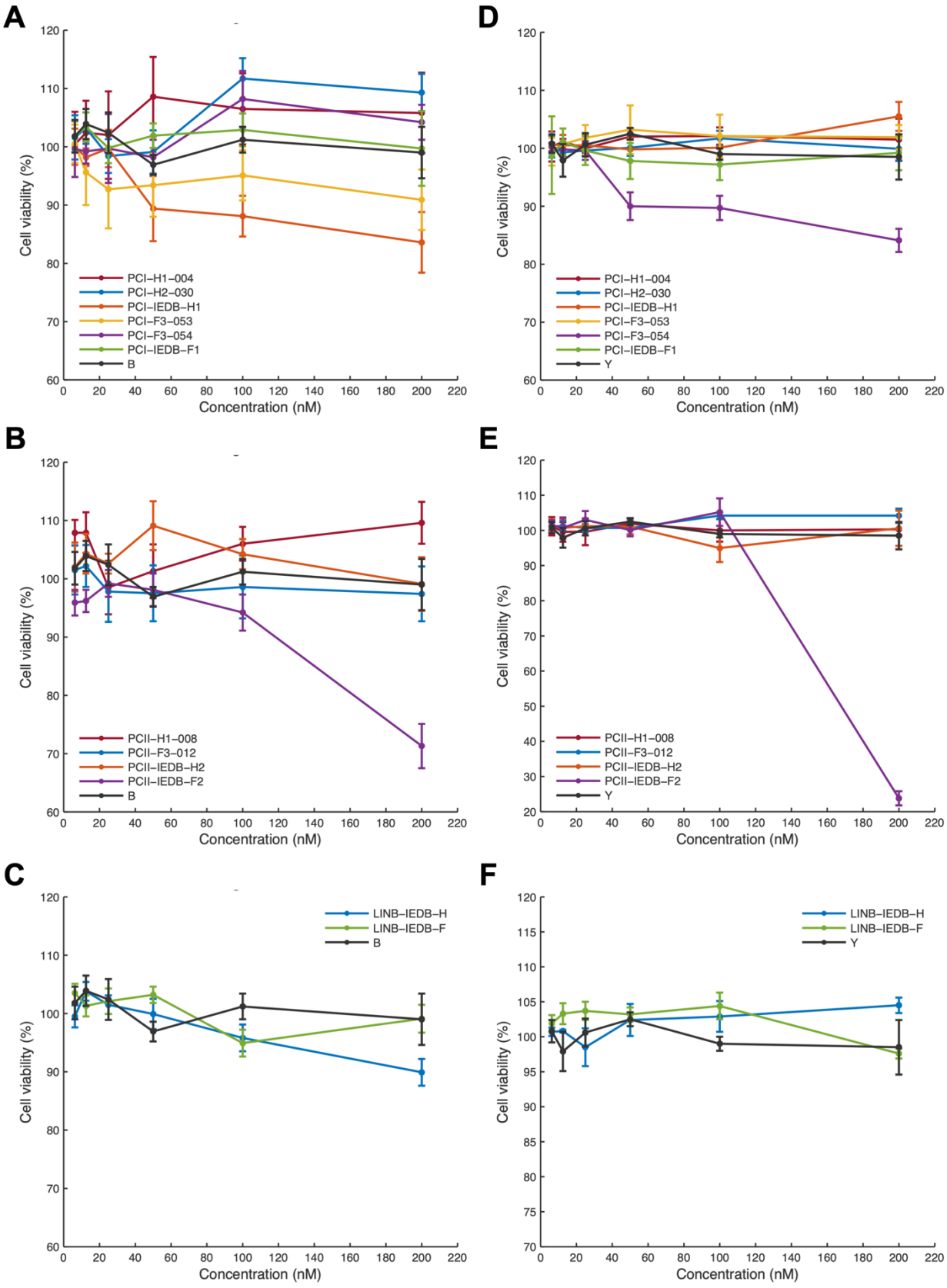

A
Cell viability (%)
Concentration (nM)
PCI–H1–004
PCI–H2–030
PCI–IEDB–H1
PCI–F3–053
PCI–F3–054
PCI–IEDB–F1
B
D
Cell viability (%)
Concentration (nM)
PCI–H1–004
PCI–H2–030
PCI–IEDB–H1
PCI–F3–053
PCI–F3–054
PCI–IEDB–F1
Y
B
Cell viability (%)
Concentration (nM)
PCII–H1–008
PCII–F3–012
PCII–IEDB–H2
PCII–IEDB–F2
B
E
Cell viability (%)
Concentration (nM)
PCII–H1–008
PCII–F3–012
PCII–IEDB–H2
PCII–IEDB–F2
Y
C
Cell viability (%)
Concentration (nM)
LINB–IEDB–H
LINB–IEDB–F
B
F
Cell viability (%)
Concentration (nM)
LINB–IEDB–H
LINB–IEDB–F
Y

Figure 4. *MTT assay for in vitro* evaluation of the viability of selected peptide-treated Vero-Dog-SLAM and MDCK cells. A) Cell viability was evaluated in the Vero-Dog-SLAM cell line after treatment with the predicted MHC class I peptides. B) Cell viability was evaluated in the Vero-Dog-SLAM cell line after treatment with the predicted MHC class II peptides. C) Cell viability was evaluated in the Vero-Dog-SLAM cell line after treatment with linear B-cell-predicted peptides. D) Cell viability was evaluated in the MDCK cell line after treatment with the predicted MHC class I peptide. E) Cell viability was evaluated in the MDCK cell line after treatment with the predicted MHC class II peptide. F) Cell viability was evaluated in the MDCK cell line after treatment with linear B-cell-predicted peptides. Cells were treated with twofold serial dilutions of selected peptides ranging from 6.125 to 200 nM for 48 hours. Cells without peptide were used as a viability negative control, and cells treated with 0.5% Triton X-100® served as a cytotoxicity positive control. Two independent experiments with 4 replicates (n = 8) were carried out for each cell line, and nontreated cells were used as negative controls. Peptides B and Y, which have reported antimicrobial activity, were used as noncytotoxic control peptides. The means and coefficients of variation are shown in the graph.

The selected peptides were safe for use in cPBMCs and cRBCs.

To confirm the results obtained in the cell lines, we performed an assay in primary cells from dogs to determine whether the selected peptides were cytotoxic when exposing cRBCs and cPBMCs to them. When cytotoxicity was evaluated in cRBCs, most peptides had a hemolysis percentage lower than 2% at all assayed concentrations, except for the peptide PCII-IEDB-F2 (**Table 5**). This peptide exhibited allowed hemolysis of less than 2% at concentrations less than or equal to 25 nM. Therefore, we concluded that most peptides exhibited a low potential cytotoxic effect on primary cells, cPBMCs, and cRBCs, except for the peptide PCII-IEDB-F2, which has high hemolytic potential. As shown in **Figure 5**, all the evaluated peptides, including the control peptides B, K1, and Y, exhibited cell viability greater than 80% in the cPBMCs. Most of the peptides had cell viability close to 100% compared to that of untreated cells. However, PCI-H1-030, PCII-H1-008, and PCII-F3-012

resulted in approximately 80% cell viability. On the other hand, the viability of the PCI-H1-004-treated cells was greater than that of the nontreated cells (**Figure 5**).

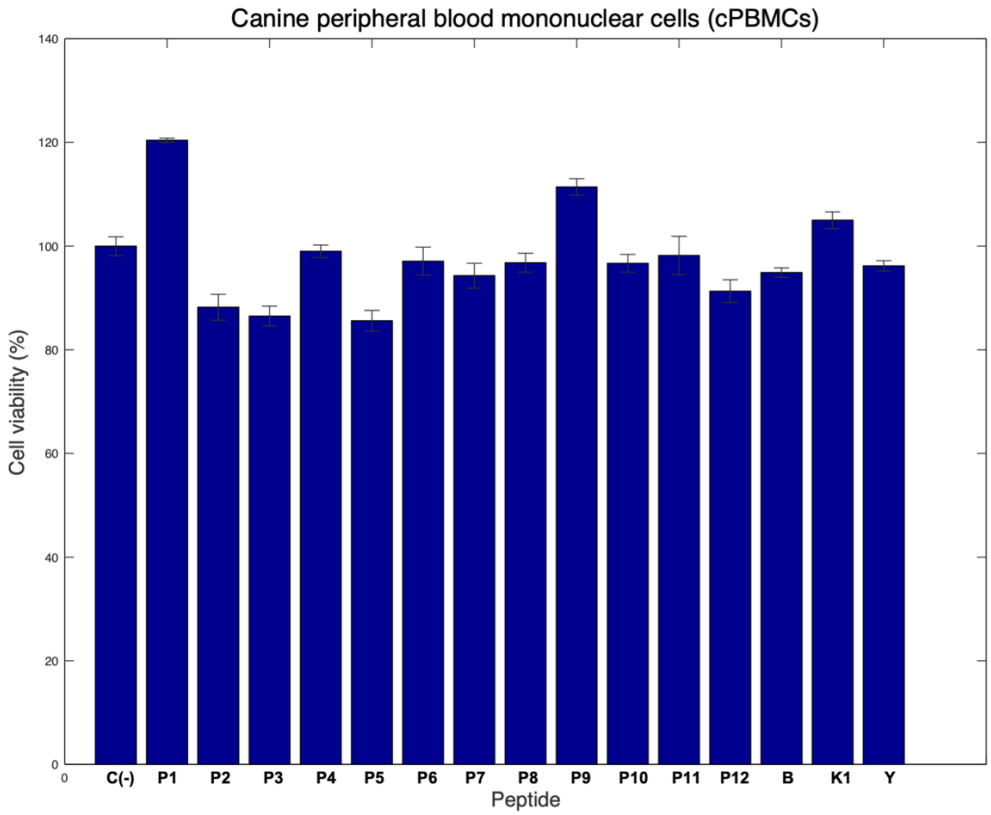


Figure 5. *In vitro* selected peptide safety evaluation in canine primary cells. Evaluation of cell viability after treatment with selected peptides in cPBMCs through the MTT assay. Cells were treated with the selected peptides at 100 nM for 48 hours. Cells without peptide were used as a viability negative control, and cells treated with 0.5% Triton X-100® served as a cytotoxicity positive control. Two independent experiments with 4 replicates (n = 8) were carried out, and nontreated cells were used as negative controls. Peptides B, K1, and Y, which have reported antimicrobial activity, were used as noncytotoxic control peptides. The means and coefficients of variation are shown in the bar graph.

Table 5. Hemolytic potential of selected peptides in canine RBCs.

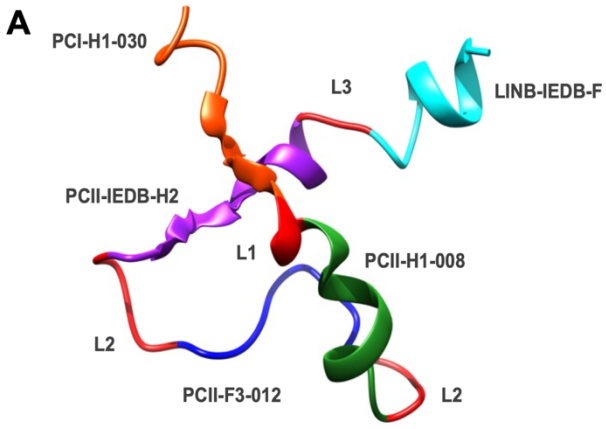
ID	Peptide	Safe concentration	Hemolytic (Yes/No)
P1	**PCI-H1-004**	< 200 nM	No
P2	**PCI-H1-030**	< 200 nM	No
P3	**PCII-H1-008**	< 200 nM	No
P4	**PCI-F3-053**	< 200 nM	No
P5	**PCII-F3-012**	< 200 nM	No
P6	**PCI-IEDB-H1**	< 200 nM	No
P7	**LINB-IEDB-H**	< 200 nM	No
P8	**PCI-F3-054**	< 200 nM	No
P9	**PCII-IEDB-H2**	< 200 nM	No
P10	**PCI-IEDB-F1**	< 200 nM	No
P11	**PCII-IEDB-F2**	< 25 nM	No
P12	**LINB-IEDB-F**	< 200 nM	No

*PBS was used as a negative hemolytic control, and 0.1% Triton X-100® was used as a positive hemolytic control. Two independent experiments with 4 replicates (n = 8) were carried out. The percent hemolysis was calculated as the relationship between the treatment and control absorbance, and a safe concentration was reported.

Multiepitope polypeptide exhibited a safe profile in silico and in vitro

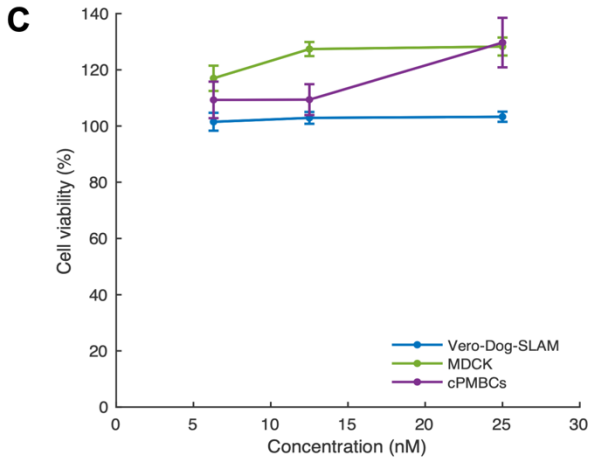
After peptide validation, a polypeptide composed of PCI-H1-030, PCII-H1-008, PCII-F3-012, PCII-IEDB-H2 and LINB-IEDB-F was constructed based on the best peptides that overcome all *in silico* and *in vitro* safety assays. L1, L2 and L3 refer to amino acid linkers such as AYY, GPGPG and KK, respectively. A threading model with I-TASSER was obtained to determine the 3-D structure (**Figure 6A**). Then, we performed an *in silico* safety evaluation through multiple computational tools to assess antigenicity, allergenicity, and toxicity. Polypeptide

was demonstrated to be safe *in silico* since all the employed validations exhibited acceptable values (**Figure 6B**). Notably, the polypeptide has an IEDB score of 0.57349, which is classified as a probable antigen from MCH-I. Additionally, no homologous proteins in the canine proteome were found, indicating that there is no risk of developing autoimmunity (**Figure 6B**). The *in vitro* safety of the peptides was assessed similarly to that of single peptides in cellular models such as Vero-Dog-SLAM, MDCK and cPBMCs to determine whether the polypeptide was cytotoxic when it was treated with several dilutions. As shown in **Figure 6C**, the polypeptide exhibited a cell viability greater than 90% at low concentrations (<25 nM) in both cell lines and primary cells as cPBMCs. On the other hand, when cytotoxicity was evaluated in cRBCs, polypeptides had a hemolysis percentage lower than 2% at all assayed concentrations, from 6.125 to 25 nM, similar to that of selected single peptides (**Figure 6D**).



B

Tool	Result
ToxinPred	Non-toxic
VaxiJen v2.0	Antigen
AllerTOP v2.0	Non-allergen
BlastP*	No homologous protein
IEDB Immunigenecity**	Immunogen
Docking score TLR-2***	-328.69
Docking score TLR-4***	-300.96



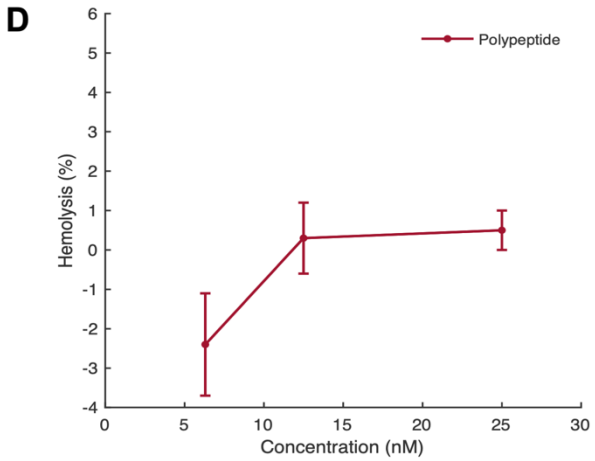


Figure 6. Multiepitope polypeptide characteristics and *in silico* and *in vitro* safety validation. A) Structural model of the multiepitope polypeptide obtained with I-TASSER. **B**) *In silico* safety validation data for the polypeptide. **C**) Evaluation of the viability of Vero-Dog-SLAM, MDCK and cPBMC cells after polypeptide treatment through the MTT assay. Cells were treated with twofold serial dilutions of polypeptide from 6.125 to 25 nM for 48 hours. Cells without polypeptide were used as a viability negative control, and cells treated with 0.5% Triton X-100® served as a cytotoxicity positive control. **D**) Hemolytic potential of the polypeptide in canine RBCs. PBS was used as a negative hemolytic control, and 0.1% Triton X-100® was used as a positive hemolytic control. Two independent experiments with 4 replicates (n = 8) were carried out, and nontreated cells were used as negative controls. The means and coefficients of variation are shown in the graph. *Evaluated with the *Canis lupus familiaris* proteome as the dataset. **Score of 0.57349, classified as a probable antigen MCH-I. ***Docking score obtained with the HPEPDOCK tool.

Discussion

Vaccine development has played an important role in human and animal health. For CDV, there are either conventional or recombinant vaccines based on the Onderstepoort strain (Rendon-Marin et al., 2019). However, neither peptides nor inactivated vaccines are available. New-generation vaccines have been employed as important development platforms since they have been used in animals and humans (A. Khan et al., 2021; T. Khan, M. Abdullah, et al., 2021). In dogs and humans, recombinant vaccines have been used, and there are different recombinant vaccines for CDV (Wang et al., 2014), Ebola (Henao-Restrepo et al., 2017), Dengue (Villar et al., 2015), and SARS-CoV-2 (Knoll & Wonodi, 2021). Diverse computational tools have been employed for vaccine design because multiple candidates can be identified reducing not only *in vitro* experiments but also, the risk of pathogens propagation in wet laboratory (Mugunthan & Harish, 2021). Vaccine alternatives for diverse viral agents include vaccinomics, which may contribute to the design of vaccine candidates (Fatima et al., 2022). Although CDV is not considered a threat to humans, in animals, it is a highly contagious disease that can affect a wide range of domestic and wild animals, including some that are endangered (Martinez-Gutierrez & Ruiz-Saenz,

2016; Rendon-Marin et al., 2020). Therefore, an effective vaccine candidate must be designed since the emergence of new variants has made CDV a threat to animal health and well-being since there is a high mortality rate among infected animals (Gilbert et al., 2014).

We generated a peptide library based on the main antigenic determinants of CDV, proteins H and F, employing a consensus sequence that considered all reported sequences from the CDV lineage via diverse computational tools (**Figure 1**). These tools are assembled with machine learning, indicating their immunogenic potential based on known peptides employed in machine training. Selection based on the best score and physicochemical data, including antigenicity, allergenicity, and toxicity profile, was subsequently performed, and the results were submitted to *in silico* safety validation (**Table 1**), since vaccines can stimulate the immune system and cause allergic reactions. A protein sequence is considered potentially allergenic if its sequence has at least six contiguous amino acid identities within the range of 80 amino acids, a sequence identity of 0.35%, with a known allergen (Hasan et al., 2019). Therefore, the most promising antigenic peptides identified in this study have nonallergenic potential.

To induce a prolonged and effective immune response, both B-cell and T-cell functions are required to promote not only humoral but also cellular immunity mediated by the cells mentioned above (Purcell et al., 2007). Here, we predicted helper CD4+ T cells, CD8+ T cytotoxic cells, and linear B-cell epitopes to obtain a great number of prospective epitopes that will have the ability to prompt a robust immune response since the B-cell epitope of a target molecule must combine with a T-cell epitope; thus, a peptide vaccine can be considerably immunogenic (Purcell et al., 2007). There is an essential step in immune response development for epitopes, which consists of the recognition of epitopes for MHC molecules (Purcell et al., 2007). In dogs, MHC alleles have diverse characteristics regarding variability, which could imply consequences for peptide immunogenic effects and presentation. The class I region contains one highly polymorphic gene, DLA-88, in addition to several other genes (Kennedy et al., 2000). The class II molecular region includes four genes, DLA-DRA1, which seems to be monomorphic, and DLA-DRB1, DQA1, and DQB1, which have been reported to be highly polymorphic (Kennedy et al., 2000). Currently, 106 DLA-DRB1, 26 DLA-DQA1, and 62 DLA-DQB1 alleles have been identified in dogs and

other related canids (Kennedy, 2007). However, there is a lack of within-breed variability in MHC alleles expressed in dogs, and it has been reported that the English springer spaniel exhibits a slightly above-average diversity of MHC alleles (Kennedy et al., 2002). Nevertheless, diverse peptides that can represent not only all CDV lineages but also the capacity to be presented in any MHC molecule context must be generated to develop new-generation vaccines that increase immunization capacity.

Homology modeling is a helpful tool for elucidating the 3D structure and interactions of proteins because the number of reported sequences in databases is greater than the number of crystallographic structures in the PDB (Khan, Muzaffar, et al., 2022; Waterhouse et al., 2018). This is the case for canine MHC molecules and TLRs since no crystallographic structures are available due to a lack of experimental data. We used homology modeling to model canine MHC molecules and TLRs based on human MHC molecules and TLRs reported in the PDB (**Figure 2 and Supplementary Table 1**). Moreover, molecular docking was employed to determine whether selected peptides could interact with modeled canine MHC molecules (**Table 2 and Figure 2**) and TLRs (**Table 3**) to predict their molecular interaction, since several *in silico* approaches have demonstrated potential vaccine candidates through techniques such as molecular docking and dynamics, employing other viral models as targets, such as Dengue, Canine Circovirus and Marburg virus (Ali et al., 2017; Jain et al., 2021; Sami et al., 2021). The docking study resulted in negative values of binding energy (**Tables 2 and 3**), which demonstrates the potential high binding affinity between peptides and canine MHC molecules and between TLR-2 and TLR-4 since the H protein from another Morbillivirus virus, such as the measles virus, has been shown to interact with these PRRs (Bieback et al., 2002; Hahm et al., 2007). Thus, these interactions with TLR-4 may elicit a protective innate immune response. Notably, peptides that can interact not only with MHC molecules but also with PRRs may have a coadjuvant function since they are dedicated to identifying PAMPS from diverse microorganisms. After TLR recognition and activation by viral ligands, cytokine production, in addition to the upregulation of MHC molecules, issues the link between the adaptive immune response and the innate immune system (Akira & Takeda, 2004). Considering the low immunogenicity of

peptides in comparison to live-modified attenuated vaccines, their coadjutant function could be an important advantage of peptide-based vaccines (Wang et al., 2020).

Molecular docking and dynamics enable us to demonstrate at least computationally the possibility of selected peptides interacting with MHC molecules (**Figure 3 and Supplementary Figure 2**) since blind-docking tools were employed to investigate the capacity of selected peptides to interact with MHC molecules. As expected, selected peptides that were predicted to have immunogenic potential could interact spontaneously in the peptide-binding cleft (**Figure 3A and C**), and some interactions must also be stable over time (**Figure 3B and Supplementary Figure 2**). Several studies have shown similar approaches to determine whether potential peptides or polypeptides could interact with molecules from the host immune system, indicating, at least from a computational approach, potential protein-based new vaccines for human and veterinary viral agents (Ali et al., 2017; Hasan et al., 2019; Jain et al., 2021).

Peptide properties and physicochemical characteristics are major concerns in active peptide development, as the half-life of peptides is considered a challenging limitation (Berger & Barnard, 1999; Mathur et al., 2016). In this work, there was a wide range of values for the half-life measured by the ProtParam tool, which led to the consideration of the stability of immunogens in a potential peptide-based vaccine (**Table 1**). Although some peptides have a low half-life, their capacity to interact with TLRs (**Table 3**) indicates their coadjuvant properties in combination with an adjuvant in a future new peptide-based vaccine, as has been proposed for other viral agents (Ali et al., 2017; Rakib et al., 2020). Additionally, the stability of all the selected peptides was measured, and the results indicated that most of the peptides are stable in biological environments (**Table 1**). The half-life, stability and even immunogenicity of peptides could be easily improved by the introduction of nonnatural amino acids and peptide-like molecules into peptides, enabling the development of vaccines based on rational drug design (Purcell et al., 2007). Peptide charge plays an important role considering that those abundant in amino acids with positive charges attaches to the negatively charged lipid bilayer of red blood cells. This attachment causes the membrane to break down, enabling water and other molecules to penetrate the cell. Consequently, the osmotic pressure within the red blood cell rises, resulting in cell enlargement and eventual

rupture (Kumar et al., 2020; Li et al., 2005). Therefore, a peptides a PCII-IEDB-F2 must be improved through charge decreasing (**Table 1**). On the other hand, homology peptides were searched in the dog proteome, there was no identical peptide in canine proteins (**Supplementary Table 2**), reducing the risk of autoimmune diseases after immunization with selected peptides (Sami et al., 2021). Thus, to guarantee some aspects, such as charge, homology with host proteins, stability, and half-life, reported in other studies to demonstrate the potential of immunogenic peptides (Sami et al., 2021), our predicted peptides exhibited potential activity, at least from a computational perspective.

After the *in silico* approach, *in vitro* studies were performed to evaluate the characteristics, such as the cytotoxicity and hemolytic potential, of the selected peptides to elucidate whether they could be used as alternative vaccines composed of multiple peptides oriented toward stimulating the main aspects of the immune response. Here, we evaluated the cytotoxicity of selected peptides in Vero-Dog-SLAM and MDCK cells (**Figure 4**) and in primary PBMCs (**Figure 5B**), indicating, from an *in vitro* perspective, the potential safety of the selected peptides. Moreover, the hemolytic potential was assayed (**Figure 5A**), and most peptides exhibited a hemolytic percentage lower than 2%, indicating their nonhemolytic profile. These results lead to questions about the safety of the selected peptides since these kinds of molecules have been reported to be highly safe (Purcell et al., 2007) because this material lacks infectious material that can support live or attenuated vaccines, and there is no risk of reversion that can lead to virulence, which is a potential issue with these kinds of vaccines. There is no risk of genetic integration or recombination, which is a problem facing regulatory authorities that are dealing with DNA vaccines (Purcell et al., 2007). However, DNA vaccines could be more cost-effective than other vaccines, such as peptide vaccines, and they are also considered next-generation vaccines (Al-Fattah Yahaya et al., 2023), in addition to some disadvantages that peptide vaccines must overcome, such as reduced immunogenicity compared to that of live attenuated vaccines (Naz & Dabir, 2007), the limited range of immune recognition and potential ineffectiveness in individuals with diverse MHC profiles (Purcell et al., 2007), the need for the incorporation of multiple epitopes to elicit robust immune responses (Moyle & Toth, 2013), and the need for the induction of short-lived immune responses (Azizi & Diaz-Mitoma, 2007). However, some interesting

characteristics of immunogenic peptides include the insertion of other chemical groups, such as lipids, carbohydrates, and phosphates, to improve their immunogenicity, stability, and solubility. Moreover, peptide formulations can be stored easily, which avoids the need for low-temperature storage and facilitates transport and distribution. Considering that peptide vaccine development has emerged as an interesting alternative for other viruses including CD4+ and CD8+ T-cell epitopes (Hamley, 2022; Malonis et al., 2020), the induction of cellular immunity may have broad advantages in defense against CDV, since cross-cellular immunity seems to offer grater chances for obtaining protection (Kundu et al., 2022; Sarvmeili et al., 2024), based on CDV biology (Rendon-Marin et al., 2019). Finally, the inclusion of multiple immunogenic peptides, as single peptides in a mixture or as multiepitope immunogen polypeptides, is an important topic of wide discussion (Purcell et al., 2007). Several studies have elucidated the importance and usage of vaccinomics, the employment of “omics”, in the field of vaccinology to develop vaccine candidates (de la Fuente & Contreras, 2021; Poland et al., 2011). New candidate peptides-based vaccines have been developed with two alternatives: multipeptide vaccines, comprising different single peptide or multiepitope polypeptide vaccines, combined with peptide linkers with potential coadjuvant activity (Saltiel & Olefsky, 2017).

In TCR–MHC structures, the TCR contacts both the peptide antigen and the MHC. The peptide, despite its small size compared to that of the MHC molecule, can contribute greatly to the buried surface area or peptide binding cleft of the MHC, indicating the contribution of potential immunogenic peptide interactions with MHC molecules in rational epitope discovery to the design of next-generation vaccines based on peptides (Szeto et al., 2020). A significant positive correlation between the TCR affinity and the TCR–MHC binding cleft has been found, even though it could be expected that an increase in the number of molecular interactions and contacts, no matter their power, must potentially contribute and accumulate toward a higher affinity (Szeto et al., 2020). This is a crucial parameter for T-cell activation, and there is a direct link between the structural parameters of the TCR–MHC complexes that impact T-cell function, in contrast to the importance of peptides that interact in the MHC binding cleft (Dash et al., 2017; Fodor et al., 2018). For CTLs, the employed method allows the prediction of epitopes using quantitative matrix and machine learning

techniques such as support vector machine and artificial neural network approaches restricted to MHC-I in predicted T-cell epitopes. Subgroup analysis can discriminate between T-cell epitopes and other MHC binders and nonepitopes (Bhasin & Raghava, 2004). The SMM-align method employed for MHC-II prediction methods enables the determination of quantitative peptide-MHC-II binding affinity values, which makes this tool suitable for rational epitope discovery since the prediction method was trained and evaluated on a publicly available dataset for nine HLA-DR supertypes (Bhasin & Raghava, 2004). For linear B-cell epitopes, we used a computational tool capable of distinguishing virus peptides, and hence, this tool has a greater chance to correctly predict with a sensitivity higher than 80%, indicating a considerable probability of reaching potential linear B-cell epitopes (Yao et al., 2012). Therefore, all the computational tools employed for the prediction of CDV immunogenic peptides, as well as those reported in the literature, are supported by robust computational and experimental methods that reliably predict potential immunogenic peptides.

To recover immunogenic peptides, defective ribosomal initiation products must be analyzed. Once MHC class I molecules are loaded with immunogenic peptides, they may be protected from proteolysis, and these complexes pass through the Golgi apparatus following their way to the cell surface, where they are sensed by CD8+ T cells (Zanker et al., 2019). Furthermore, when referring to diverse mechanisms in the context of MHC class II molecules, the process could involve intact exogenous antigens since both must be loaded into the MHC class II molecules in the antigen-binding cleft. As MHC class I-targeted epitopes, immunogenic peptides can reach MHC class II molecules and replace existing peptides via a surface-exchange mechanism that is mediated by high concentrations of these peptides (Falk et al., 2002). We predicted peptides derived from H and F CDV proteins that could bind to MHC class II molecules (**Figure 1**) to take advantage of their molecular properties in the context of MCH class II molecules (**Table 2**). Peptides that have entered outside the context of the native antigen must be subjected to a wide range of cell-surface and extracellular proteases throughout the immunization process, becoming an important limitation. Exogenous antigen and immunogenic peptides from potential vaccine candidates can enter the endosomal pathway by macropinocytosis or by receptor-mediated events,

such as B-cell-surface-immunoglobulin uptake of antigen or Fc-receptor-mediated uptake of immune response complexes (Dijkstra & Yamaguchi, 2019). Once the immunogenic peptide enters the endocytic section, protease action must be deleterious; by the cathepsin family, proteins are in charge of antigen degradation (Falk et al., 2002). Then, the immunogenic peptides must overcome this challenge by surviving this environment and being transported to the MHC class II-rich endosomal compartment, where peptides are loaded onto the MHC class II molecules, a process led by chaperones responsible for removing invariant chains (Dijkstra & Yamaguchi, 2019), which has the advantage of involving peptides predicted to interact with MHC class II molecules (**Table 2**).

Overall, several studies have discussed the usage and utility of new subunit vaccines based on either single immunogenic peptides or multiepitope polypeptides (Ali et al., 2017; He et al., 2015; Jain et al., 2021; Nielsen et al., 2007; Sami et al., 2021). In this study, we evaluated *in silico* and *in vitro* a multiepitope polypeptide that includes all potential immunogenic predicted single peptides that overcome all *in silico* and *in vitro* validations (**Figure 6A**). The constructed multiepitope CDV polypeptide exhibited several *in silico* safe characteristics, such as being nonallergenic, nontoxic and having no homologous proteins in the canine proteome, and it has immunogenic potential since both the VaxiJen and IEDB tools provided favorable results, indicating that it can trigger a potent immune response without generating any desired allergenic or toxic reactions (**Figure 6B**). On the other hand, we also evaluated the cytotoxicity of the polypeptide in Vero-Dog-SLAM and MDCK cell lines and in primary cells, PBMCs and cRBCs and obtained considerable positive results for a potential polypeptide-based vaccine (**Figure 6C-D**). Therefore, multiepitope vaccines may be recognized as a promising platform therapy against viral infections, with *in silico* safety and immunogenicity evaluations (Sami et al., 2021). One possible concern is the immune response, which is robust enough to be protective as a vaccine formulation. However, other studies on SARS-CoV-2 (Tahir Ul Qamar et al., 2020) and this study have included different epitopes as single peptides or multiepitope polypeptides to facilitate both cellular and humoral immune responses.

Recently, the development of new-generation vaccines for viral pathogens such as CDV has advanced since vaccine failure was reported (Anis et al., 2018; Martella et al., 2008). This

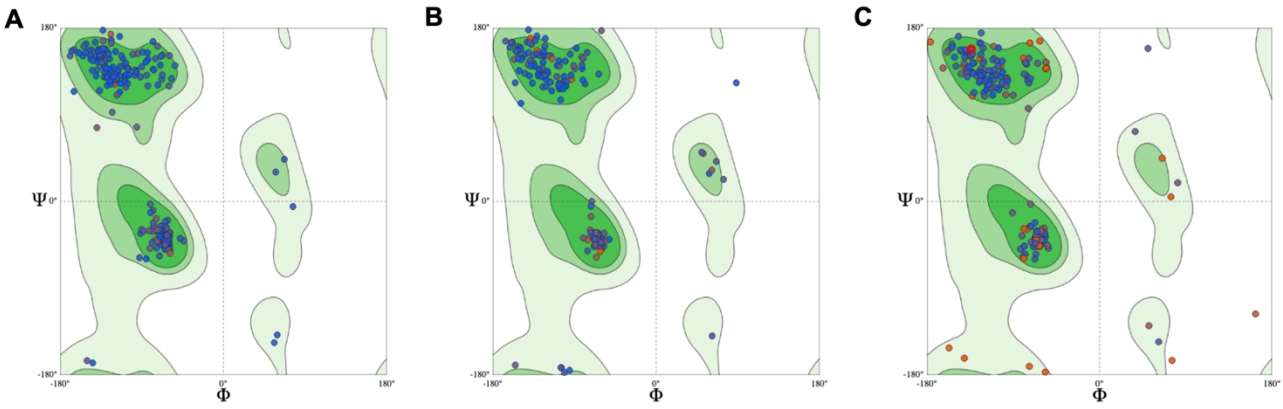
vaccine failure has been explained through the emergence of diverse CDV strains, which must also be led not only by evolution but also by vaccination, which has played an important role in CDV lineage variation (da Fontoura Budaszewski et al., 2017). Moreover, lineage-specific neutralizing epitopes from the CDV H protein of diverse lineages, different from those of vaccine-based Onderstepoort strains, have been reported (Bi et al., 2022). Understanding the genetic variation of CDV may become even more critical over time if the low protection afforded by available vaccines becomes more predominant around the world (Wilkes, 2022). Although there are some new vaccine candidates based on recombinant platforms and other nonconventional vaccine alternatives (Wright et al., 2022), there is an essential need not only to understand the transmission dynamics of CDV (Duque-Valencia, Sarute, et al., 2019) but also to improve and develop vaccines for nondomestic species (Wilkes, 2022). Some alternatives have emerged as experimental bivalent vaccines employing vectors such as replication-defective human adenoviruses that express the CDV H protein and rabies proteins or bacterium-like particles that express the CDV H and F proteins (Wang et al., 2024; Yan et al., 2020). Regardless of the effort of vaccine alternatives in animals, there are still limitations in the design of veterinary vaccines based on the available tools used to predict potential immunogenic molecules, such as peptides, in humans; however, the methodology employed in this study has been suggested (Dimitrov et al., 2016; Xu et al., 2016), indicating the importance of continuing to explore new approaches in the field of veterinary vaccines and vaccinomics (T. Khan, A. Khan, et al., 2021). These facts invite us to reflect on the necessity of finding a way to develop an ideal universal vaccine suitable for domestic and nondomestic animals threatened by CDV and other viral agents in the context of One Health.

Conclusions

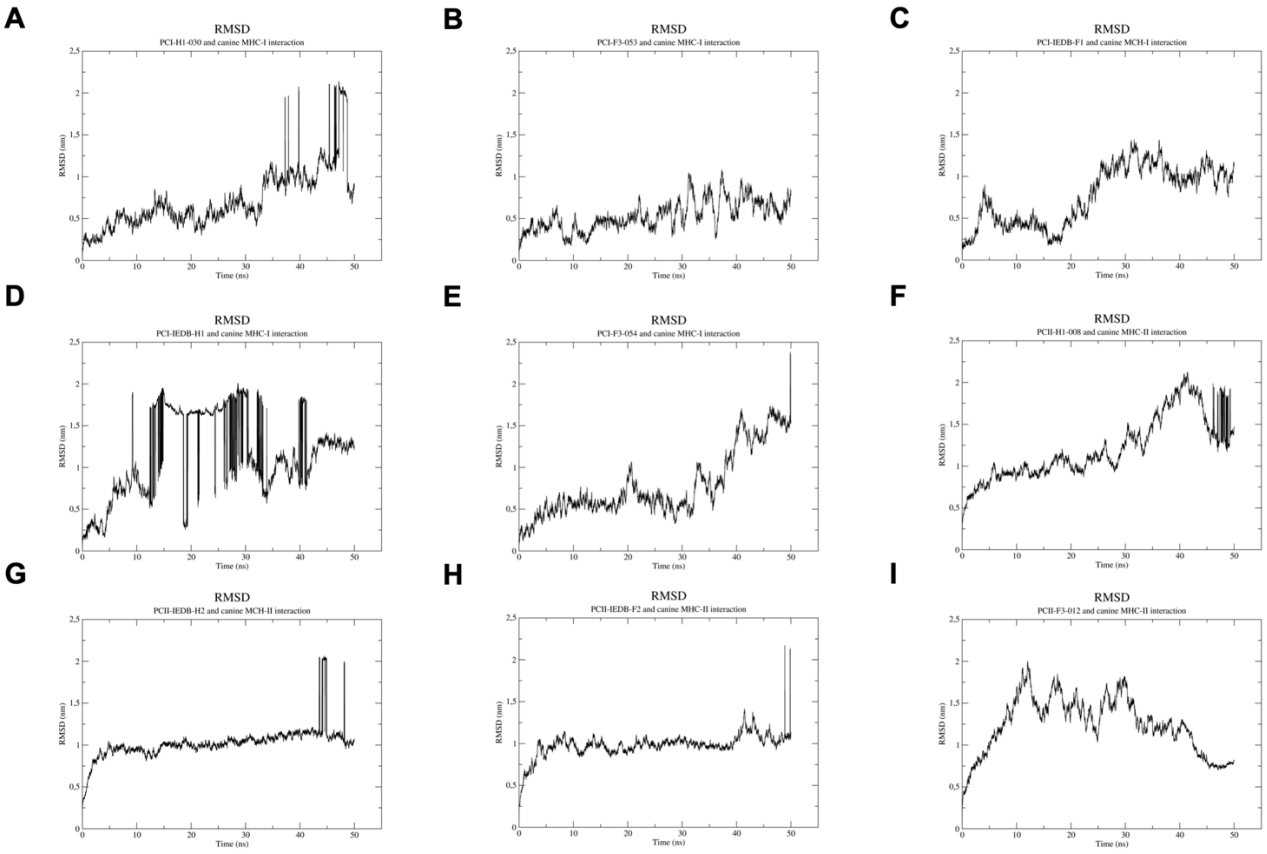
Peptide-based vaccines either single or multiepitope polypeptides have become an alternative therapy strategy to prevent not only infectious diseases but also cancer. The usage of computational tools and the immunoinformatic, integrating the vaccinomics have enabled to design and develop rational new-generation vaccines based on peptides and other molecules in a cost-effective and less time-consuming manner. In this study, we have built a peptide library and selected a group of immunogenic peptides, based on the main

antigenic determinant proteins, H and F from CDV. Since viral infections have been demonstrated to stimulate both humoral and cellular immunity, a multi-peptide vaccine may contain a combination of helper CD4+ T cells, CD8+ T cytotoxic cells, and linear B cell epitopes. Hence, in this study, we have built a peptide library with computational tools containing helper CD4+ T cells, CD8+ T cytotoxic cells, and linear B cell epitopes with immunogenic potential. Also, for selected peptides, based on the best score and physicochemical properties, a docking study was proposed, exhibiting a greater binding affinity with MHC molecules and TLR-2 and TLR-4 receptors. Although, they showed significant *in silico* results, *in silico* and *in vitro* safety protocol was carried out, reviewing the results of antigenicity, toxicity, and allergenicity analysis of these peptides and, the cytotoxicity in cell lines and primary cells, besides the hemolytic potential, all of this, when evaluated both as single peptides and multiepitope polypeptide with a representation of helper CD4+ T cells, CD8+ T cytotoxic cells, and linear B cells epitopes with linkers that carry a co-adjuvant activity. This preliminary data for a vaccine candidate requires further *in vivo* experiments to evaluate the effectiveness of an adjuvant. We are looking forward that our prediction model will exhibit positive effects *in vivo* to prevent CDV infection in domestic and wildlife animals.

Supplementary material



Supplementary Figure 1. **Ramachandran plot of canine protein models. A**) DLA-I-88. B) DLA-II-Alpha. C) DLA-II-Beta. These plots were created with SWISS-MODEL (https://swissmodel.expasy.org/).



Supplementary Figure 2. Molecular dynamic simulations of selected peptides docked with canine MHC molecules. Root mean square deviation (RMSD) plot of: A) PCI-H1-030 and MHC-I. B) PCI-F3-053 and MHC-I. C) PCI-IEDB-F1. D) PCI-IEDB-H1 and MHC-I. E) PCI-F3-054 and MHC-I. F) PCII-H1-008 and MHC-II G). PCII-IEDB-H2 and MHC-II. H) PCII-IEDB-F2 and MHC-II. I) PCII-F3-012 and MHC-II. Graphs for molecular dynamics were obtained with Xmgrace software (Oregon Graduate Institute of Science and Technology, Hillsboro, OR, USA).

Supplementary Table 1. Validation data of all models obtained by homology modelling.

Protein	Z Value	Favorable region (%)	TM Value	Align AA
DLA-I-88	-9.4	98.16	0.99066	274
DLA-II-Alpha	-5.34	96.00	0.88193	177
DLA-II-Beta	-5.27	94.33	0.89979	194
5F1N	-9.19	95.60	-	-
4FQX-Alpha	-5.47	99.44	-	-
4FQX-Beta	-5.33	96.17	-	-

*5F1N, 4FQX alpha and beta are the MHC molecules templates reported in the PDB

Supplementary Table 2. Protein BLAST homology assessment of selected peptides with *Canis lupus familiaris* proteome.

ID	Peptide	Length	Protein	Coverage (%)	Identity (%)
P1	**PCI-H1-004**	9	Sodium-dependent noradrenaline transporter	100	80
P2	**PCI-H1-030**	11	N-terminal kinase-like protein	63	100

P3	**PCII-H1-008**	9	DNA polymerase epsilon catalytic subunit A	100	75
P4	**PCI-F3-053**	12	Prosalusin	91	81,8
P5	**PCII-F3-012***	9	Terminal uridylyltransferase 4	100	85,7
P6	**PCI-IEDB-H1**	9	Pecanex-like protein 1	77	85,7
P7	**LINB-IEDB-H**	11	Cat eye syndrome critical region protein 2	81	100
P8	**PCI-F3-054**	12	Cornifin-B-like	75	56.3
P9	**PCII-IEDB-H2**	13	FERM domain-containing protein 5	76	80
P10	**PCI-IEDB-F1**	9	BCLAF1 and THRAP3 family member 3	55	100
P11	**PCII-IEDB-F2**	13	Carboxypeptidase D	92	53
P12	**LINB-IEDB-F**	9	Vacuolar protein sorting-associated protein 13D	100	75

***This peptide has the same sequence than P5.1, however, it was predicted to be presented in both MHC class I and II.

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CHAPTER 4

Evaluation of the safety and immunogenicity of a multiple epitope polypeptide from canine distemper virus (CDV) in mice

Abstract: *Morbillivirus canis* is the etiological agent of a highly contagious disease that affects diverse domestic and wild animals. Vaccination is considered the most suitable strategy for controlling CDV dissemination, transmission, and distemper disease. However, the emergence of new CDV strains has led to the need to update the current vaccine strategies employed to prevent CDV infection in domestic and wild animals. Currently, there is a lack of effective alternatives for wild animals. Diverse computational tools, especially peptide-based therapies, enable the development of new universal vaccines. Hence, the aim of this study was to evaluate the safety and humoral and cellular immune response of a new generation of vaccines based on CDV peptides as single peptide mixtures or multiepitope CDV polypeptides in mice. Twenty-four BALB/c mice were subjected to a three-dose regimen for 28 days. Seroconversion was evaluated via ELISA, and cellular immune responses were evaluated via flow cytometry through activation-induced markers (AIMs). Compared with the placebo, the peptide mixture and multiepitope CDV polypeptide were safe, and seroconversion was statistically significant in the multiepitope CDV polypeptide and commercial vaccine (CV) groups. The numbers of antigen-specific CD4+CD134+ and IFN-γ+ T cells, CD8+ T cells and TNF-α- and IL-6-producing cells were greater in the mice immunized with the multiepitope CDV polypeptide than in the control mice. This combined approach represents a potential step forward in developing new immunization candidates or enhancing current commercial vaccines to control CDV disease in domestic dogs and wild animals.

Keywords: Multiepitope, vaccination, domestic dogs, canine distemper virus, immune response, safety.

Introduction

Morbillivirus canis, also commonly known as canine distemper virus (CDV), is a member of the *Paramyxoviridae* family and *Morbillivirus* genus. It is the causative agent for canine distemper, a disease that is highly contagious and induces multiorgan disease in dogs and other carnivores (Rendon-Marin et al., 2019). It exhibits a broad cell tropism affecting epithelial, lymphoid, and neurological cells, resulting in a systemic infection encompassing respiratory, digestive, urinary, lymphatic, cutaneous, skeletal, and central nervous system (CNS) manifestations (Lempp et al., 2014). CDV particles are often spherical, enveloped virions with a nonsegmented single negative-stranded RNA (ssRNA), similar to other members of the *Mononegavirales* order. The genome, spanning 15,690 nucleotides, encodes eight proteins, including H and F proteins (Kolakofsky, 2016), the main antigenic determinants of CDV (Iwatsuki et al., 2000).

The host range of CDV primarily encompasses species within the order *Carnivora*, belonging to diverse families and, in lower proportions, other significant families from different orders, including *Artiodactyla, Primates, Rodentia,* and *Proboscidea* (MacLachlan et al., 2011; Martinez-Gutierrez & Ruiz-Saenz, 2016). Considering the wide range of species infected by CDV, research has explored cross-species transmission among wild and domestic animals since lethal disease has spread to endangered species worldwide (Beineke et al., 2015).

Vaccination is one of the most important strategy for preventing viral infections by eliciting both humoral and cellular immune responses. The common commercially available for CDV are MLV vaccines based on strains such as Onderstepoort, Snyder Hill, Convac, Rockborn, or CDV3 (Wang et al., 2024). Nonetheless, these live attenuated vaccines have the potential to induce symptomatic disease and, in some cases, lead to mortality in certain susceptible species due to their retained replicative capacity within vaccinated animals (Wilkes, 2022). Moreover, the immunization of puppies possessing maternal CDV-neutralizing antibodies may prove inefficient, as these antibodies can reduce the efficacy of live attenuated CDV vaccines (Pardo et al., 2007). Although, some subunit or innovative epitope-based vaccines have demonstrated the ability to elicit an adequate immune response into other viral agents in both *in vitro* and *in vivo* models (Ali et al., 2017; Jain et al., 2021; Sami et al., 2021), it

remains imperative to explore alternative CDV vaccines for the wide range of natural CDV hosts. The prevalence of neutralizing antibodies has been studied in some wildlife animals (Kimpston et al., 2022; Oleaga et al., 2022). There is significant concern about CDV transmission and dissemination control strategies for wild animals because some endangered animals can disappear without any intervention for CDV infection, such as vaccination (Gilbert et al., 2020; Wilkes, 2022).

The vaccine development process involves intricate, labor-intensive, and expensive *in vivo* and *in vitro* protocols during both preclinical and clinical study phases. Recently, advancements in computational biology and immunoinformatics have alleviated the reliance on *in vitro* experiments, facilitating the design of effective *in silico* vaccines based on multiepitope polypeptides (Samimi Hashjin et al., 2023). The application of vaccinomics in designing multiepitope-based vaccine models has shown promise for several viruses, including Chikungunya, Ebola, SARS-CoV-2, MERS-CoV, HIV, Lassa, Oropouche, Cytomegalovirus, Dengue, Hepatitis C, Zika, Flavivirus and, Norovirus, among others (Kar et al., 2020; Sami et al., 2021; Tahir Ul Qamar et al., 2020). These approaches allow the identification and development of vaccines against multiple pathogens, demonstrating their potential in advancing vaccine research and development. It has been reported that viral peptides enable the stimulation of B cells by helper T cells, transforming them into plasma cells that produce antibodies, as exhibited for other viruses (Pedersen et al., 2016). Adequate clearance of morbillivirus or even coronavirus requires the participation of helper CD4+ T cells and CD8+ T cytotoxic cells after immunization with antigenic peptides (Pfeffermann et al., 2018; Rakib et al., 2020). Peptide-based vaccines have been reported as promising immunization alternatives for comprehensive safety and immunogenicity (Sami et al., 2021).

Diverse computational tools, especially peptide-based therapies, have emerged as crucial components in the development of next-generation vaccines (Akhtar et al., 2022). This fact is particularly relevant considering the time and financial constraints associated with traditional experimental approaches for vaccine development (Sami et al., 2021). The focus on immunogenetics, immunogenomics, systems biology, immune profiling, and immunoinformatics has given rise the vaccinomics (Poland et al., 2011). This

interdisciplinary approach involves the comprehensive study of host-vector-pathogen molecular interactions and the identification of potential protective antigens, such as peptides derived from pathogen proteins (de la Fuente & Contreras, 2021). One specific application within vaccinomics is epitope-based peptide vaccines, which rely on *in silico* prediction of immunogenic peptides from antigenically dominant pathogen proteins (Gu et al., 2017; Yashvardhini et al., 2021). While peptide-based vaccines have demonstrated efficacy against various viral agents such as hepatitis B, influenza A, and hepatitis C, among others, their appeal lies in several distinctive features. Notably, they do not involve infectious material, enable practical insertion of different molecules to enhance immunogenicity, can be prepared in lyophilized form for advantageous storage, pose no risk of virulence reversal, and can be designed to incorporate multiple antigenic determinants (He et al., 2015; Purcell et al., 2007; Sominskaya et al., 2010; Stanekova & Vareckova, 2010). The versatility and safety profile of peptide-based vaccines make them a compelling choice for vaccine development, aligning with the evolving landscape of modern vaccinology.

To develop a new generation safe vaccine based on genetic and antigenic information on CDV linages circulating in domestic and wild animals, immunogenic peptides such as the single peptides or multiepitope CDV polypeptides that were previously assessed *in silico* and *in vitro* (Rendon-Marin & Ruiz-Saenz, 2024), were evaluated in mice by assessing their safety and humoral and cellular immune responses. This approach enables the validation of the use of multiple immunogens, such as peptide mixture or multiepitope polypeptides as a potential CDV vaccine, which could be safe and highly immunogenic for domestic dogs and wildlife animals since there is a lack of new approved alternatives for protecting a wide range of animals threatened by CDV.

Materials and methods

Ethical approval

This study was approved by the ethics committee of the Universidad de Antioquia (Act No.154, August 8th, 2023). The authors also applied the Three Rs principle and employed the ARRIVE guidelines.

Peptides and reagents

BIOMATIK (USA) synthesized single peptides and polypeptides using standard solid-phase synthesis with a purity >98% and characterized them by mass spectrometry (**Table 1**). Imject® Alum (Thermo Scientific, Wilmington, DE, USA) was used as an adjuvant in the single peptide mixture and polypeptide preparation to immunize the mice. A recombinant vaccine (Recombitek c3) was used as a positive control.

Table 1. CDV single peptides and multiepitope polypeptides.

Peptide	Sequence	Length (AA)	Purity (%)	Concentration (nM)**
P1	QVIDVLTPLFK	11	98.21	20
P2	VENLVRIRF	9	98.27	20
P3	LKLLRYYTE	9	98.45	20
P4	PPYLLFVLLILLV	13	98.12	20
P5	KAQIHWNN	9	98.72	20
Poly*	QVIDVLTPLFK**AAY**LKLLRYYTE**GPGPG**VENLVRIRF **GPGPGP**PYLLFVLLILLV**KK**KAQIHWNNL	66	98.65	25

*Polypeptides were constructed with single peptides (P1, P2, P3, P4 and P5) linked with small amino acid sequences or linkers marked in bold (Sami et al., 2021). **The CDV peptide mixture was made into an equimolar solution. Each peptide was 20 nM concentrated

Animals, clinical signs, and safety

A total of 24 8-week-old wild-type (WT) BALB/c female mice (Charles River, Portage, MI, USA) were used for this study. The mice were randomly divided into four experimental groups of 6 mice each: single peptide mixture, multiepitope polypeptide, recombinant vaccine, and placebo. Clinical signs and the GRIMACE scale were evaluated daily. Body weight was measured weekly as a clinical sign to determine potential endpoints. The inoculation site was evaluated daily to evaluate any indication of local reactions attributable

to the vaccine through heat, pain, and swelling. Daily observation of vital signs, symptoms, behavior, and mortality was conducted to detect any adverse reaction to the vaccines.

Mice immunization and sacrifice

The *in vivo* experiments were conducted within controlled, pathogen-free settings at the animal facility of the Universidad de Antioquia (Medellín, Colombia. Mice from all groups were immunized subcutaneously, with a first dose on day 1 and two boosts on days 14 and 21. A total of 100 μL of a 1:1 mixture of either single peptide mixture or multiepitope CDV polypeptide diluted in 0.9% saline solution and Imject® Alum (Thermo Scientific, Wilmington, DE, USA) was used for the single peptide mixture and multiepitope CDV polypeptide groups; 100 μL of recombinant vaccine or 0.9% saline solution was used for the recombinant vaccine and placebo groups, respectively. On day 28, all mice were euthanized via intraperitoneal overdose of ketamine/xylazine (100/10 mg/kg).

Splenocyte isolation and stimulation

After the mice had been sacrificed, the whole spleen was extracted in transport medium (RPMI supplemented with 5% penicillin-streptomycin) for splenocyte isolation. Briefly, spleens were passed through a cell strainer (BD Biosciences, San Jose, CA, USA) and suspended in RPMI supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY). The cell suspensions were washed three times and centrifuged for 5 minutes at 1800 rpm. Red blood cell (RBC) lysis buffer was employed (eBioscience, San Diego, CA, USA) for five minutes, after which the cells were washed with 1X PBS and centrifuged for five minutes at 1800 rpm. Then, the obtained cells were counted, and $1x10^6$ cells were stimulated for 24 hours with either 25 nM polypeptide or 8 μg/mL of phytohemagglutinin-PHA (Sigma-Aldrich, St. Louis, MO, USA). Nonstimulated cells were also used as a negative control for each mouse.

Evaluation of splenocyte population by flow cytometry

Splenocyte populations and activation were assessed by flow cytometry after 24 hours of polypeptide stimulation. Prior to staining, after 8 hours of culture, 6 μg/mL of brefeldin A and 2 mM of monensin (both from Thermo Scientific, Wilmington, DE, USA) were added to the cell culture and incubated at 37°C and 5% CO_2. Then, the cells were washed and stained for 30 minutes in the dark with a cocktail of the following antibodies: FITC-conjugated monoclonal antibody against CD3 (clone: 145-2C11), V500-conjugated monoclonal antibody against CD4 (clone: RM4.5), PerCP-Cy5.5-conjugated monoclonal antibody CD8 (clone: 53-6.7), BV650-conjugated monoclonal antibody against CD25 (clone: PC61), BV421-conjugated monoclonal antibody against CD134 (clone: OX-86) (all antibodies from BD Biosciences, San Jose, CA, USA). Cells were fixed with mouse Foxp3 buffer (BD Biosciences, San Jose, CA, USA) according to the manufacturer's guidelines and labeled with the PE-Cy-7-conjugated monoclonal antibody against IFN-γ (clone: XMG1.2). A final wash was carried out, and cell populations were acquired using an LS Fortessa (BD Biosciences, San Jose, CA, USA). The data were analyzed using FlowJo version 10.5.3 (FlowJo, LLC, Oregon, USA) and, were normalized to those of nonstimulated cells as a negative control for each mouse.

Cytokine quantification by cytometric beads assay (CBA)

The splenocyte culture supernatants were collected from independent wells and stored at -80°C until use. Then, the supernatants were thawed at 4°C before the CBA was applied. A Mouse Th1/Th2/Th17 Cytokine Kit, which allows interleukin (IL)-2, IL-4, IL-6, IFN-g, TNF, IL-17A, and IL-10 protein determination, was used (BD Biosciences, San Jose, CA, USA). The CBA assay was carried out according to the manufacturer's instructions. The data was acquired using a CytoFLEX (BC Life Biosciences, Brea, CA, USA). Cytokine standards were serially diluted to construct calibration curves, which were necessary to determine the protein concentrations of the mouse samples. Individual cytokine concentrations are indicated by their fluorescence intensities converted to concentrations (pg/mL) using FlowJo version 10.5.3 (FlowJo, LLC, Oregon, USA).

ELISA assay

Flat bottom ELISA plates (Thermo Fisher Scientific, USA) were coated overnight at 4°C with 100 ng per well of CDV polypeptide diluted in 0.5 M carbonate-bicarbonate buffer. The plates were washed five times with PBS containing 0.05% Tween (PBST), blocked in 1% bovine serum albumin (BSA) solution for 1 hour at 37°C, and then washed five times with PBST. Mouse serum samples were prepared at a 1:200 dilution in PBS supplemented with 10% fetal bovine serum (FBS), added at 100 μL per well, and incubated for 2 hours at 37°C. The wells were washed five times with PBST. Then, 100 μL of conjugated goat anti-mouse IgG (H + L)-HRP (CAT1706516-Biorad) at a dilution of 1:2000 in PBS supplemented with 10% FBS was to each well, and the mixture was incubated for 1 hour at 37°C. After seven washes with PBST, 100 μL of 3,3',5,5'-Tetramethylbenzidine (TMB) was added to each well for 15 minutes for color development, and the reaction was stopped with 50 μL of 2 N HCL. The absorbance was measured at 450 nm using a microplate reader (ThermoFisher Scientific, USA). The optical density (OD) values were calculated by subtracting the negative control values from all samples. The average specific IgG was determined by 2 independent experiments for each mouse serum sample for each experimental group.

Statistical analysis.

Descriptive statistics are shown as the mean and standard deviation or median and interquartile range (IQR), depending on normality. The data were analyzed with unpaired t-Student test or Mann-Whitney U test, depending on the normality assumption according to the Shapiro–Wilk normality test. Statistical significance was considered as *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 and performed using GraphPad Prism Software (La Jolla, California, USA, version 10.1).

Results

Clinical signs, weight loss and mice following.

To determine the safety of the CDV peptides mixture and multiepitope CDV polypeptide, the immunized mice were observed daily after the complete immunization scheme (**Figure 1A**); none of the mice exhibited abnormal behavior or clinical symptoms of toxicity based on the GRIMACE scale (facial actions such as orbital tightening, nose bulge, cheek budge, ear position and whisker change), which was 0 in the whole study. Weekly weight was measured as an adverse effect of the CDV peptide mixture, the multiepitope CDV polypeptide and commercial vaccine immunization. For all groups, the mice showed an increase in weight. None of them presented a weight loss greater than 10% (**Figure 1B-E**). Therefore, immunization with the CDV peptide mixture, multiepitope CDV polypeptide and commercial vaccine (CV) was safe in mice.

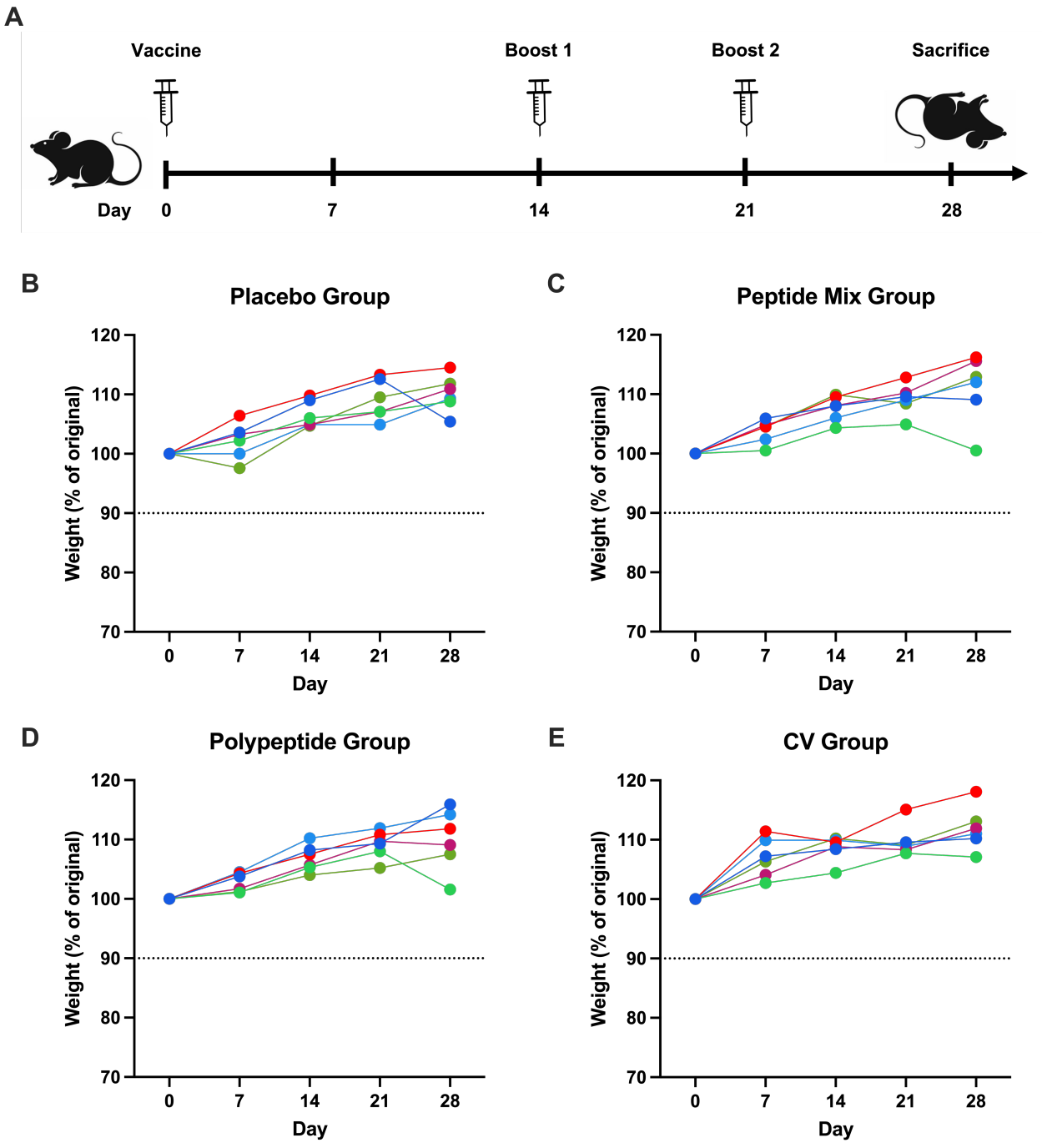


Figure 1. Immunization scheme and clinical signs. A) Mouse immunization scheme with one vaccination and two boosts. Mice were followed for weight loss for all assay days until they reached the sacrifice for the B) placebo, C) peptide mixture, D) polypeptide immunization and E) commercial vaccine (CV) groups (90% in the dotted line was the threshold of body loss).

Mice immunized with multiepitope CDV polypeptide showed increased antigen-specific IgG.

An in-house ELISA was performed to assess antibody production in all mice groups. After the euthanasia, total blood was collected, and the serum was separated to measure the presence of serum antigen-specific IgG with the CDV polypeptide as an antigen for coating. As shown in **Figure 2**, immunization with the CDV peptide mixture did not significant increase antibodies levels, as did immunization with the placebo. However, when polypeptide was used, there was a significant increase in antibody production compared to that in the placebo group (P = 0.0018). Moreover, there was a significant polypeptide-specific IgG production in the CV group compared to placebo group (P < 0.0001). Therefore, compared the immunization with the placebo, immunization with either the multiepitope CDV polypeptide or CV increased antibody production and induced more IgG than immunization with the CDV peptide mixture. CV-immunized mice had the highest multiepitope CDV polypeptide-specific IgG production.

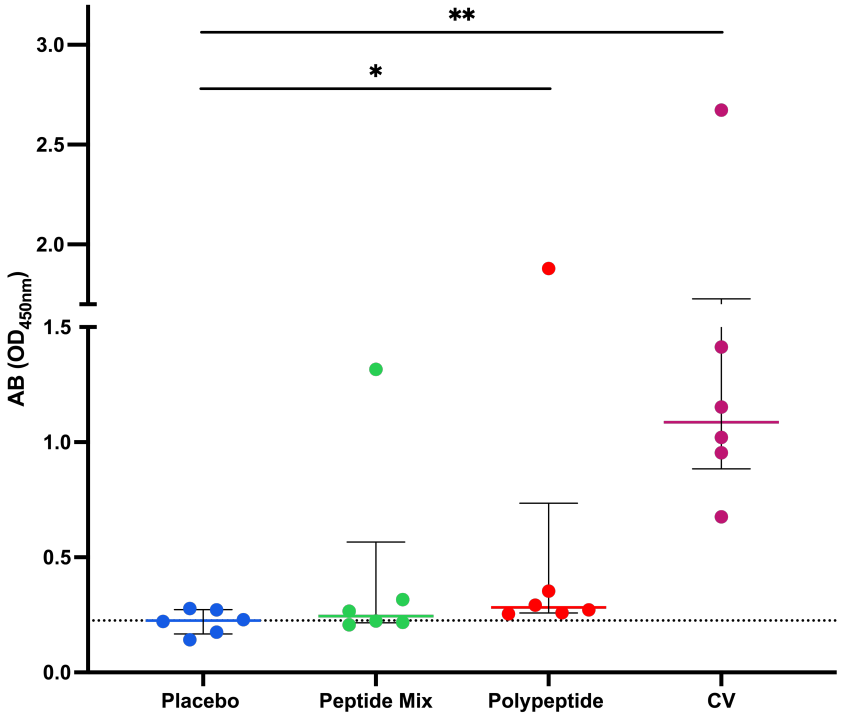
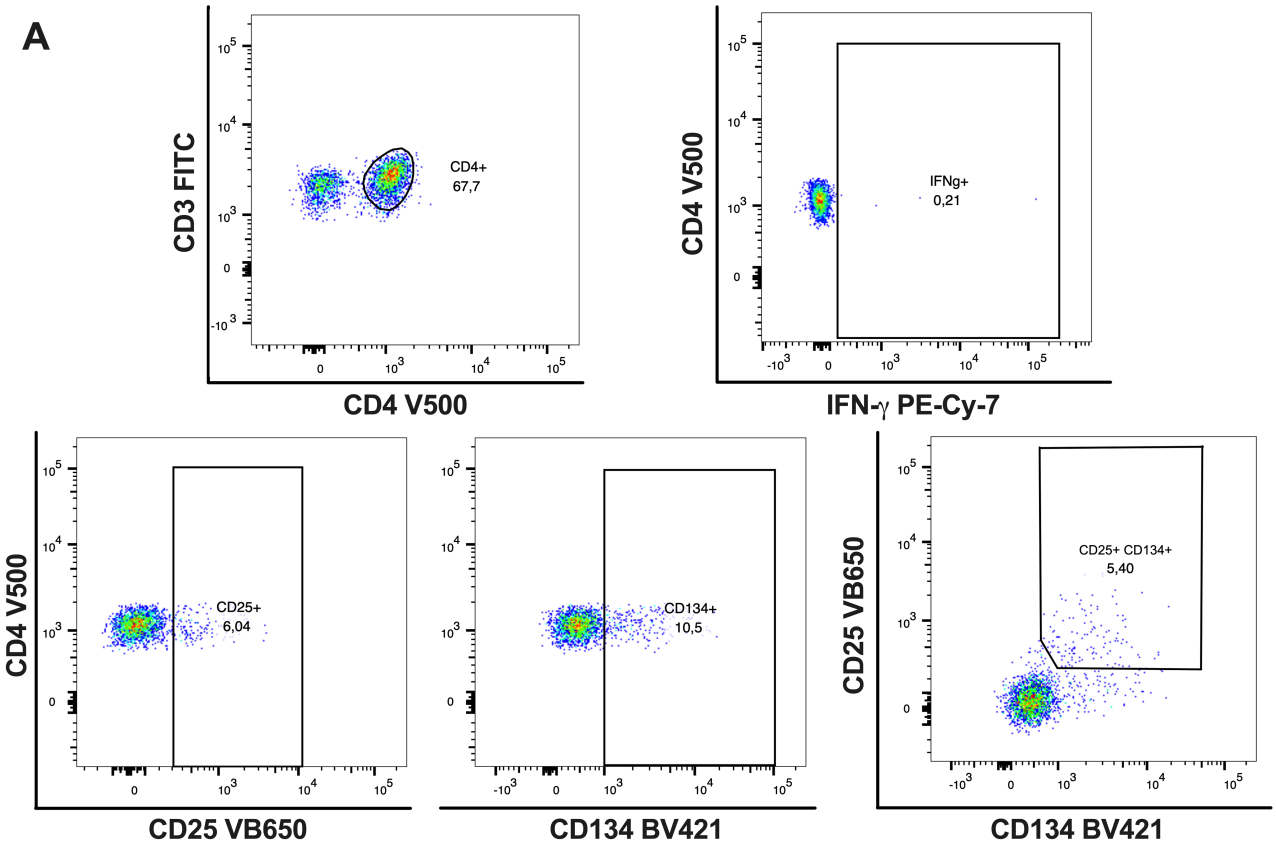


Figure 2. Immunization with the CDV polypeptide induced an increase in antigen-specific IgG. Mice were immunized with peptide mixture or polypeptide. Antibodies were detected by ELISA employing the polypeptide as a coating antigen. The data are expressed

as the means of two independent experiments after the background absorbance was subtracted, and the medians ± IQRs of all the mice are also reported. Statistical evaluations were performed with an unpaired Student's t test or the Mann–Whitney U test. *$P < 0.05$ and **$P < 0.01$.

Multiepitope CDV polypeptide induces a cellular immune response.

To establish whether immunization with either peptide mixture or polypeptide induces a cellular immune response, flow cytometry assays were carried out to measure vaccine-responsive CD4+ and CD8+ T cells in splenocytes. A representative gating strategy for CD4+ T cells and activation-induced markers assay (AIM) is shown in **Figure 3A.** These results revealed a trend toward an increase in the percentage of antigen-specific CD25+ CD4+ T cells in the CDV polypeptide group compared to that in the placebo, peptide mixture and CV groups (**Figure 3B**). On the other hand, the percentage of antigen-specific CD134+ (Ox40) CD4+ T cells was greater than that in the placebo group ($P = 0.0306$, **Figure 3C**). Although the percentage of double-positive antigen-specific CD25+ CD134+ CD4+ T cells tended to be greater in the multiepitope CDV polypeptide group than in the placebo group, the difference re was not statistically significant (**Figure 3D**). The percentage of CD4+ T cells that were IFN-γ+ was significant different ($P = 0.0398$) between the multiepitope CDV polypeptide group and the placebo group, as shown in **Figure 3E**. Consequently, the cell-mediated immune response induced by specific CD4+ T cells increased when the multiepitope CDV polypeptide was used for immunization in mice compared to that in the placebo group, and the CV group did not exhibit a considerable CD4+ T-cell immune response.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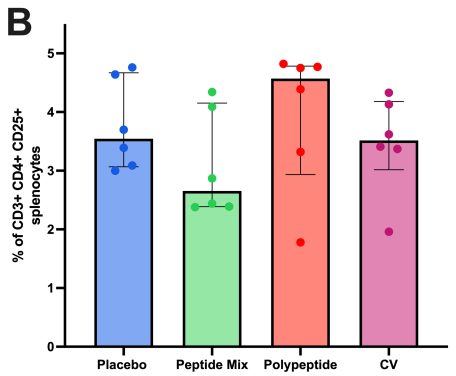
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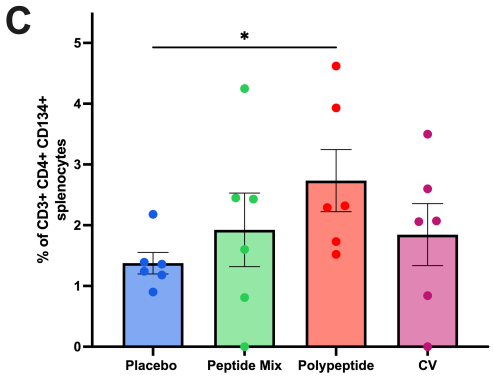
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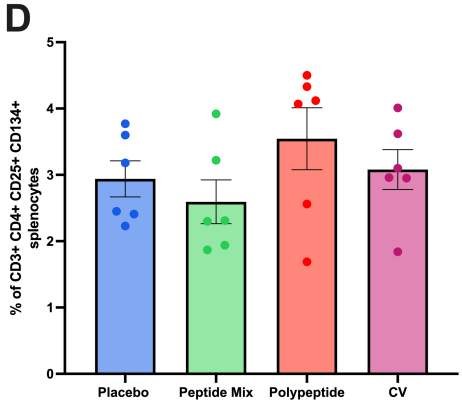
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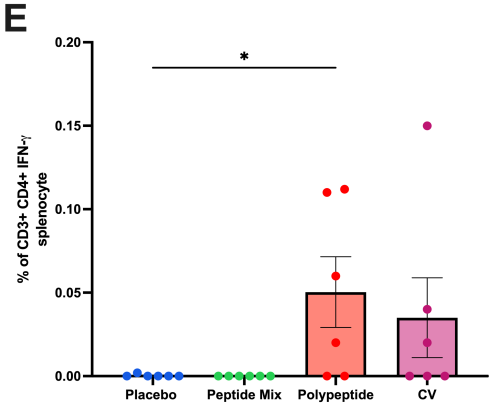
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Polypeptide
CV

Figure 3. Specific cellular immune response induced in immunized BALB/c mice for CD4+ T-cell populations in splenocytes by flow cytometry. A) A representative gating strategy for antigen-specific CD4+ T cells was evaluated in splenocytes for AIM (CD25+ CD134+ and double positive), and IFN-γ producing cells were selected in CD3+ cell population. B) Percentage of antigen-specific CD4+ CD25+ T cells. C) Percentage of antigen-specific CD4+ CD134+ T cells. D) Percentage of antigen-specific double-positive CD4+ CD25+ CD134+ T cells. E) Antigen-specific CD4+ T cells producing IFN-γ in each mice group. All cell cultures were evaluated for 24 hours and stimulated with the multiepitope CDV polypeptide. The percentages were normalized to those of nonstimulated cells for each condition. The data are expressed as the median ± IQR. Statistical evaluations were performed with unpaired t-Student or Mann-Whitney U test. $*P < 0.05$.

For CD8+ T cells, a representative gating strategy for antigen-specific CD3+ CD8+ T cells identified by flow cytometry analysis is shown in **Figure 4A**. BALB/c mice immunized with the multiepitope CDV polypeptide produced more antigen-specific CD8+ T cells than did those in the placebo, peptide mixture and CV groups, but the difference were not statistically significant (**Figure 4B**). Therefore, immunization with multiepitope CDV polypeptide could induce a slight increase in specific CD8+ T cells, indicating the potential for the development of an essential aspect of the cell-mediated immune response and no appreciable increase in antigen-specific CD8+ T cells in mice immunized with CV.

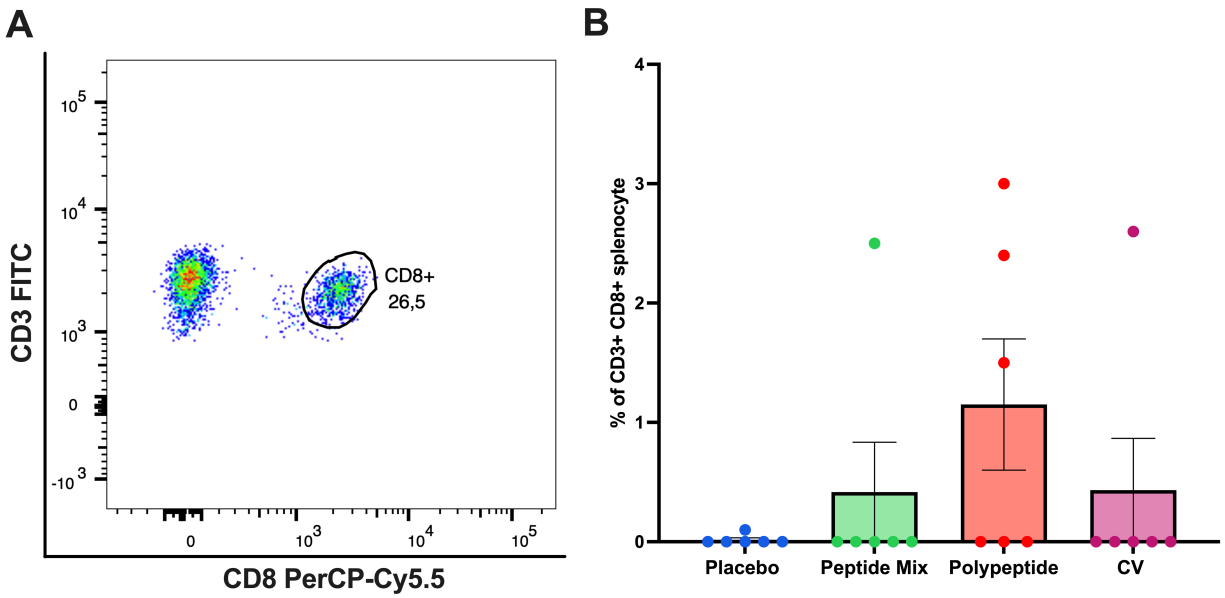

Figure 4. Specific cellular immune response induced in immunized BALB/c mice for CD8+ T-cell populations in splenocytes by flow cytometry. A) A representative gating strategy for antigen-specific CD8+ T cells evaluated in splenocytes selected from the CD3+ cell population. B) Percentage of antigen-specific CD3+ CD8+ T cells. All the cell cultures were evaluated after 24 hours of culture and stimulation with the multiepitope CDV polypeptide. The percentages were normalized to those of nonstimulated cells for each condition. The data are expressed as the median ± IQR.

Cytokine production in splenocytes stimulated with the multiepitope CDV polypeptide.

To measure cytokine production in splenocyte cultures, a cytokine bead array was perfromed to determine cytokine-producing Th1/Th2/Th17 CD4+ populations as activation and differentiation markers. As shown in **Figure 5**, for CDV peptide mixture immunization in mice, no statistically significant differences were observed in the amounts of any cytokine compared to those in the placebo group. On the other hand, TNF-α (P = 0.0281) and IL-6 (P = 0.0152) significantly increased in the multiepitope CDV polypeptide group (**Figure 5 D and G**). Moreover, there were significant differences in the concentration of INF-γ, IL-2, IL-6, IL-17 (P < 0.01), and TNF-α (P < 0.05) after CV immunization in the polypeptide group, placebo group or both groups of mice. Notably, no significant differences in the IL-4 or IL-10

concentrations were detected among the evaluated groups. Taken together, these results regarding cytokine expression in splenocytes indicate that multiepitope CDV polypeptide immunization induces the proinflammatory cytokines IL-6 and TNF-α which are important for CD4+ T cells; however, CV can induce cytokine profiles different from those of Th1/Th2/Th17 CD4+ cells.

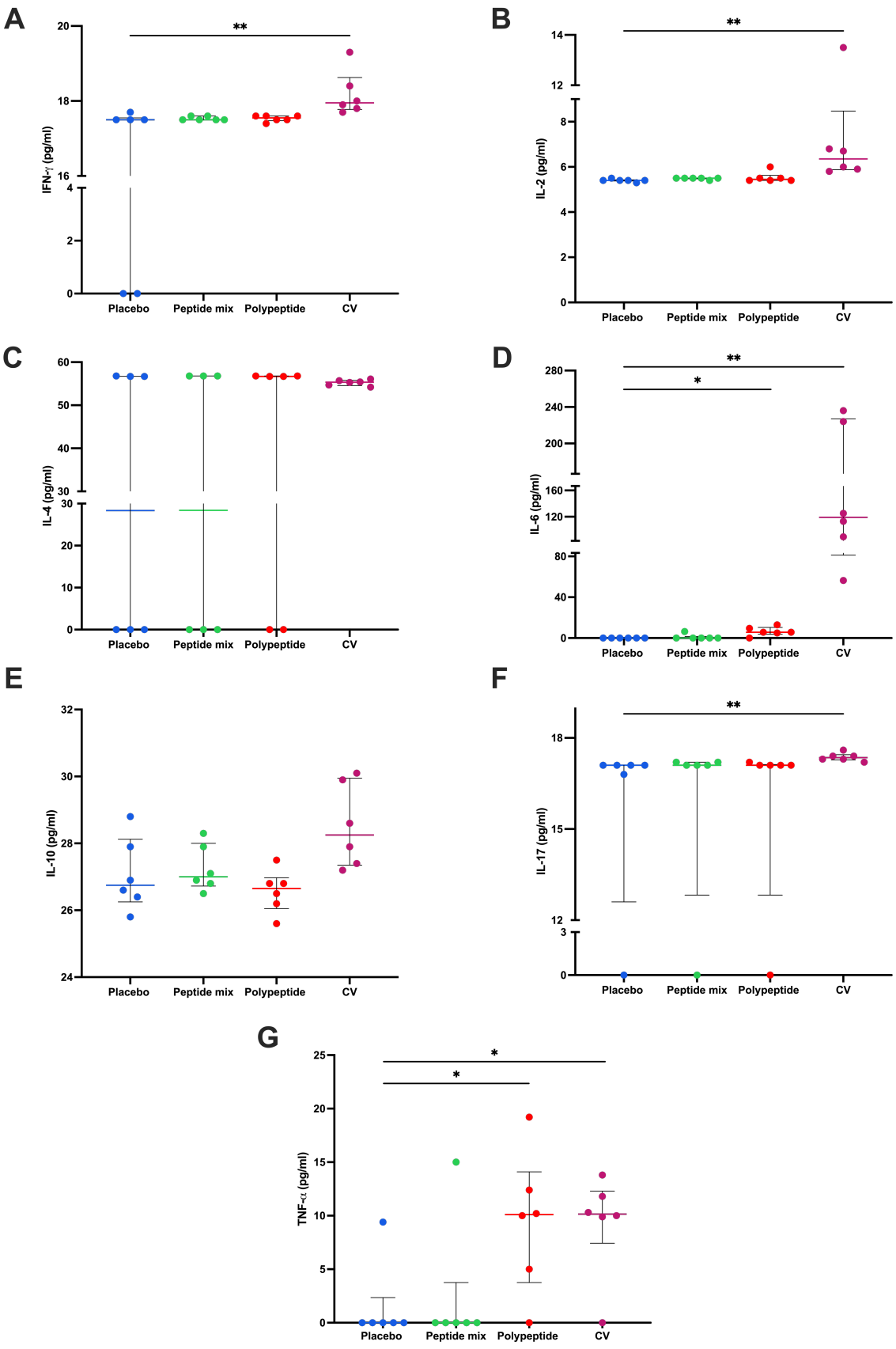
A
IFN-γ (pg/ml)
**
Placebo
Peptide mix
Polypeptide
CV
B
IL-2 (pg/ml)
**
C
IL-4 (pg/ml)
D
IL-6 (pg/ml)
**
*
E
IL-10 (pg/ml)
F
IL-17 (pg/ml)
G
TNF-α (pg/ml)
*
*

Figure 5. Splenocyte cytokine production in all immunized mice. Cytokine concentration in CD4+ Th1/Th2/Th17 cells in the supernatants of 8-hour splenocyte cultures stimulated with the multiepitope CDV polypeptide (pg/mL) measured by CBA. A) IFN-γ. B) IL-2. C) IL-4. D) IL-6. E) IL-10. F) IL-17 and G) TNF-α. The data are expressed as the mean ± standard error of the mean (SEM) or median and interquartile range. Statistical evaluations were performed with unpaired t-Student or Mann-Whitney U test. $^{*}P < 0.05$ and $^{**}P < 0.01$.

Discussion

CDV vaccine development has been explored by diverse researchers worldwide *in vivo*, in different species, such as domestic dogs, BALB/c mice, minks, and ferrets, among others, based on recombinant viruses in the backbone of adenoviruses and canarypox viruses (Gong et al., 2020; Pujol et al., 2023; Sadler et al., 2016; Yan et al., 2020), chimeric measles virus expressing CDV proteins (Rouxel et al., 2009), DNA vaccines based on the expression of CDV antigenic determinants (Nguyen et al., 2012; Nielsen et al., 2009; Zhao et al., 2023), pure H and F CDV proteins as antigens (Norrby et al., 1986), recombinant mouse adenovirus 1 (MAV-1) expressing CDV antigens (Du et al., 2022), and novel bacterium-like particle-based vaccines displaying canine distemper virus antigens (Wang et al., 2024). Nevertheless, CDV peptide-based vaccines have not yet been investigated.

In this study, we employed a three-dose CDV peptide-based vaccine evaluated in silico and in vitro previously (Rendon-Marin & Ruiz-Saenz, 2024) in mice to demonstrate safety and immunogenicity (**Figure 1A**). Common approaches in vaccine development typically employ entire microorganisms, which could lead to inadvertent exposure in susceptible wildlife animals (Durchfeld et al., 1990). Peptide vaccines, comprising short immunogenic peptide fragments, could present a viable solution to this issue by eliciting potent and targeted immune responses while mitigating the risk of unsafety (Purcell et al., 2007). Accordingly, immunization of mice with CDV peptide mixture and the multiepitope polypeptide resulted in an excellent safety profile, as measured through weight percentage changes and the GRIMACE scale (**Figure 1B-E**).

To generate a robust immune reaction, any vaccine alternative must possess the capacity to stimulate innate and adaptative immune responses. The humoral immune response has been considered the gold standard for evaluating vaccine candidate efficacy against viral pathogens (Black & Thaw, 2023). In mice, several approaches have demonstrated the importance of measuring specific IgG production as an accumulative effect of immunization for viral pathogens such as CDV (Wang et al., 2024) and considering the importance of universal vaccines for CDV. Our results indicated that antigen-specific IgG levels measured after multiepitope CDV polypeptide immunization were greater than those in the control group, but lower than those in the CV group as shown in **Figure 2**. This finding is consistent with previous studies on CDV vaccine development in which specific IgG antibodies increased after immunization with a new recombinant or DNA-based vaccines (Gong et al., 2020; Zhao et al., 2023), demonstrating the importance of the humoral immune response in effective vaccine candidates.

For agents belonging to the *Morbillivirus* genus, such as CDV, both cytotoxic T lymphocyte activity specific to the H protein (Hirama et al., 2003) and helper T-cell epitopes from the F protein (Ghosh et al., 2001) have been widely described. Cellular immunity mediated by CD4+ and CD8+ T cells is indispensable for protecting against CDV and disease (da Fontoura Budaszewski et al., 2017). The AIM assay enables the identification of antigen-specific T cells based on the upregulated expression of activation markers after antigen restimulation (Reiss et al., 2017). Although this technique has not been widely employed in mice, some studies have used this approach in lymphocytic choriomeningitis virus studies to identify T cells that upregulate AIMs, such as CD134 and CD25 after cell culture with specific antigens, allowing the quantification of murine antigen-specific CD4+ T-cells (Nguyen et al., 2023). Moreover, AIMs are considered especially advantageous for discerning antigen-specific T follicular helper (Tfh) cells, which constitutes a CD4+ T-cell subset crucial for supporting B cells (Havenar-Daughton et al., 2016). For example, this technique has been employed to detect hemagglutinin-specific Tfh cells by observing the increased expression of CD25, CD134, and CD154 after IAV infection or IAV hemagglutinin immunization in C57BL/6 mice (Jiang et al., 2019). Here, we demonstrated an increase in antigen-specific CD4+ T-cells with an increase in the AIMs, such as CD25, CD134 or double-

positive cells, after stimulation with the multiepitope CDV polypeptide (**Figure 3**)and after immunization with three doses. Hence, AIMs have emerged as an important strategy for preclinical investigations into murine vaccines, facilitating the assessment of the proportional abundance of vaccine antigen-specific cells (Nguyen et al., 2023). On the other hand, antigen-specific CD8+ T cells were also detected (**Figure 4**), consistent with recent studies based on bacterium-like particle-based vaccines displaying CDV antigens, where specific CD8+ T cells were also increased in mice and dogs (Wang et al., 2024). There were more specific IFN-γ secreting CD4+ T-cells (**Figure 3E**), indicating the potential stimulation of cytotoxic T-cell activity through the Th1 CD4+ T cells subset after multiepitope CDV polypeptide stimulation of mouse splenocytes.

Cytokine production is associated with the polarization and production of specific CD4+ T-cell subsets (Liu et al., 2001). Our results demonstrated a statistically significant difference in TNF-α and IL-6 production between splenocytes form the multiepitope CDV polypeptide mice group compared to and those from the placebo group (**Figure 5D and E**). TNF-α is a proinflammatory cytokine that is important for naïve T-cell activation and proliferation (Mehta et al., 2018). Moreover, IL-6 enhances vaccine responses by promoting Tfh cells (Parvathaneni et al., 2023) and subsequent antibody production (Dienz et al., 2009). IL-6 triggers the activation of transcription factors, specifically STAT3, via Janus kinases (JAKs) and CCAAT/enhancer-binding proteins (C/EBP) through the ras-ERK mitogen-activated protein kinase (MAPK) cascade. Activation of STAT3 leads to the upregulation of c-maf expression, while C/EBP facilitates the upregulation of NFATc2. The transcription factors c-maf and NFATc2 may collaboratively facilitate the differentiation of CD4+ Th2 or Tfh cells (Diehl et al., 2002; Dienz et al., 2007; Dienz & Rincon, 2009). Thus, the production of IL-6 after stimulation with the multiepitope CDV polypeptide could assist the relevant signaling interplay of Tfh cells, a subset of CD4+ cells to produce antibodies by B cells. When CV was used, different cytokines from Th1/Th2/Th17 CD4+ were detected, indicating the potential proinflammatory profile of recombinant vaccines, as previously reported (Gong et al., 2020).

Therefore, the production of cytokine that help antigen-specific Tfh cells, a CD4+ T-cell subset, and antibodies increased in the multiepitope CDV polypeptide group and increase in the CV mice group, but peptide mixture group exhibited no humoral response (**Figures 2,**

3 and 5). These biological events for a protective adaptative immune response against CDV epitopes orientated toward a specific antigen have been reported for other vaccine candidates with different immunogens, such as bacteria-like particles (Wang et al., 2024). Although an adjuvant that potentiates the humoral immune response was employed, the CV vaccine induced more specific IgG than both the peptide mixture and the multiepitope CDV polypeptide. Moreover, increased numbers of CDV specific IFN-γ-secreting CD4+ T cells were observed in multiepitope CDV polypeptide-immunized mice, indicating that Th1 CD4+ T cells are also important mediating cytotoxic T lymphocyte activity since the number of CD8+ T cells trended to increase in the multiepitope CDV polypeptide group compared to the placebo (**Figure 4**). Several reasons enable the investigation of peptide-based vaccines, such as their absence of infectious agents, convenient of practical integration of molecules to increase immunogenicity, easy storage, and the incorporation of multiple antigenic elements (Purcell et al., 2007). However, there are some limitations that may contribute to the low immunogenicity of peptide-based vaccines that must be overcome, including the diversity of MHC molecules in antigen-presenting cells, proper entry of peptide vaccines into the MHC pathways, bioavailability, and immunogen concentrations (Purcell et al., 2007), as demonstrated in this research.

MLV and recombinant vaccines employing canarypox vectors against CDV have been utilized in commercial applications for carnivore protection worldwide (Ramsay et al., 2019). Although the canarypox-vectored CDV vaccine was endorsed for all susceptible species by the American Association of Zoo Veterinarians' Distemper Vaccine Subcommittee, there are still some issues to overcome, such as safety and availability in several countries, in addition to the notable individual and interspecies variability in response to this vaccine for each species. On the other hand, MLVs could represent a risk of severe disease and fatality in highly susceptible species since safety has been demonstrated only in domestic dogs and some animals, such as ferrets and African wild dogs (Wilkes, 2022; Woodroffe, 2021), but there is still a vast array of species that could be at risk with commercially available vaccines. As a perspective, not only studies in affected animals but also comparisons with other commercial vaccines, such as MLV, must be performed since several studies of CDV

vaccine safety and immunogenicity in domestic and wild animals are just in experimental phases (Rendon-Marin et al., 2024)

The canine distemper virus has demonstrated the capacity to overwhelm a widening spectrum of hosts, which could be considered a considerable obstacle to control and eradication efforts for this disease. CDV can have severe effects on numerous endangered species, such as black-footed ferret (*Mustela nigripes*), Santa Catalina Island fox (*Urocyon littoralis catalinae*), African wild dog (*Lycaon pictus*), and Caspian seal (*Pusa caspica*) and can contribute to its decline or near extinction (Gilbert et al., 2015; Jo et al., 2019; Kennedy et al., 2019; Timm et al., 2009; Williams et al., 1988). Additionally, outbreaks have been reported within captive breeding facilities housing endangered African wild dogs and threatened giant pandas (*Ailuropoda melanoleuca*) (Feng et al., 2016; Kennedy et al., 2019; van de Bildt et al., 2002). Moreover, large felids have led to CDV disease outbreaks and mortalities across various species of the *Panthera* and *Lynx* genera (Gilbert et al., 2015; Wang et al., 2022). Currently, CDV remains a formidable threat to the Amur leopard (*Panthera pardus orientalis*), Javan leopard (*Panthera pardus melas*), Amur tiger (*Panthera tigris altaica*), and Asiatic lion (*Panthera leo persica*), all of which are endangered subspecies (Mourya et al., 2019; Rahman et al., 2022).

Considering that the objective of vaccination is to elicit immune-activating antigen-specific cytotoxic T lymphocytes, B cells that produce neutralizing antibodies, and T helper cells (Purcell et al., 2007), universal vaccines based on noninfectious therapies and new-generation vaccines have arisen as safe alternatives in wild animals. In CDV-susceptible animals for which the safety and efficay of current vaccines has not been demonstrated, peptide-based vaccines, especially multiepitope CDV polypeptide immunogens (Bartsch et al., 2024; Opriessnig et al., 2024), as evaluated in this study through a combined *in silico, in vitro* and *in vivo* approach could be a safe and effective alternative for CDV disease control and prevention, even as a booster in vaccinated animals with commercially available recombinant vaccines.

Conclusions

A CDV peptide-based vaccine was constructed, and either a single peptide mixture or a multiepitope CDV polypeptide was evaluated. One initial immunization with 2 boosts in an immunization scheme within 28 days in mice induced both humoral and cellular immune responses when the multiepitope CDV polypeptide was employed. The immunogenic multiepitope polypeptide was formulated on the basis of linear B cells, cytotoxic T lymphocytes, and helper T lymphocyte epitopes previously reported (Rendon-Marin & Ruiz-Saenz, 2024). After multiepitope CDV polypeptide splenocyte stimulation, antigen-specific CD4+ T cells were identified, indicating a specific immune response to the multiepitope CDV polypeptide. The development of a multiepitope CDV polypeptide has become a promising strategy against viral infections, such as CDV or a potential booster for current commercially available recombinant vaccines, considering the response of the CV group to multiepitope CDV polypeptides. Advances in computational biology and immunoinformatic tools have enabled the efficient design and construction of a multiepitope vaccine for CDVs, reducing time and costs. Improving peptide immunogens, using new generation adjuvants, and exploring higher concentrations of peptides while considering their safe profile are imperative. Additionally, it is essential to acknowledge that this preliminary-designed vaccine is not exhaustive and requires further *in vivo* experiments in target species, such as domestic dogs and endangered wildlife animals, to comprehensively assess its effectiveness, including virus challenge. Nevertheless, our approach represents a considerable step forward in developing a new immunization candidate or alternative for controlling CDV disease and dissemination in domestic dogs and wildlife.

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GENERAL DISCUSSION

In this DSc thesis, we described all the experimental innovative findings and interpretations about a particular research question: can the development of peptides with immunogenic potential based on the genetic and antigenic information of worldwide canine distemper virus (CDV) circulating lineages be used safely in both domestic and wildlife? To answer this question, six specific objectives were proposed: to understand the current state-of-the-art in CDV pathogenesis, transmission, and vaccine development; to construct a peptide library of CDV proteins from circulating CDV strains that have high immunogenic potential; to evaluate *in vitro* the safety of different peptide immunogens from CDV; to validate *in vivo* the safety of specific CDV immunogens that have overcome the *in vitro* safety evaluation; to assess the immunity generated by the highly immunogenic peptides derived from circulating CDV proteins in an *in vivo* model; and to compare the immune response generated *in vivo* by immunogenic peptides with that induced by a commercial vaccine. A literature review and different *in silico*, *in vitro* and *in vivo* strategies were employed to answer the main research question.

CDV can infect a wide range of hosts, constituting a significant challenge for disease control and eradication. CDV infection can result in severe repercussions in endangered species, potentially contributing to their decline or even extinction of diverse wildlife species (Gilbert et al., 2015; Jo et al., 2019; Kennedy et al., 2019; Timm et al., 2009; Williams et al., 1988). Moreover, CDV outbreaks have been documented within captive breeding facilities housing endangered animals (Gilbert et al., 2015; Wang et al., 2022), representing a continuous threat to species conservation (Mourya et al., 2019; Rahman et al., 2022) due to cross-species transmission as has been widely described (Beineke et al., 2015) and presented in *Chapter 1*.

Considering the wide range of domestic and wildlife species that can be affected by the CDV, in *Chapter 2*, we summarized CDV vaccination studies in domestic and wildlife animals through a scoping review demonstrating the necessity of not only developing and evaluating new vaccines for wildlife animals, but also, to improving the safety and

effectiveness of the current CDV vaccines for domestic dogs to control CDV dissemination and disease (Bronson et al., 2007; Gilbert et al., 2020). The insufficient data regarding vaccine safety and efficacy in wildlife emphasize the importance of enhancing research in this domain since a few countries have contributed to this topic, and scientific collaboration, efforts and research carried out have been insufficient to reduce the negative impact of CDV infection in wild populations. A scoping review revealed that there is a lack of new-generation vaccine alternatives because one of the most common vaccines, MLV CDV and canarypox-based vaccines were developed years ago (Wang et al., 2014). The current vaccines must be updated with a universal approach to impact CDV transmission dynamics and disease in domestic and wild animals. Notably, there is limited collaboration among researchers from different countries for CDV vaccines. Noteworthy collaborations were observed only between researchers affiliated with the USA and Canada, as well as France and Denmark. Among the authors who made substantial contributions to CDV vaccination research, Rebecca Wilkes from the USA conducted several studies focusing on wildlife animals, while Max J.G. Appel pioneered initial modified live virus CDV vaccines in dogs, particularly those derived from the Onderstepoort strain. Additionally, T. Fabian Wild from France made significant contributions, including research on mice and minks employing recombinant and DNA vaccines.

Genetic variation in the CDV H and F proteins has been reported (Duque-Valencia, Diaz, & Ruiz-Saenz, 2019). This variability has enabled the emergence of diverse CDV lineages that differ from those of current vaccines strain, such as the Onderstepoort CDV strain, which has not circulated for many years (Wang et al., 2014). Since it has been demonstrated that there are antigenic differences among wild-type CDV isolates and vaccine strains when comparing cross-neutralization potential (Anis et al., 2018), this could help to explain the vaccine failures reported in domestic dogs due to the emergence of new CDV lineages through the ongoing process of CDV evolution (Duque-Valencia, Sarute, et al., 2019).

Based on this need, the utilization of computational tools has emerged as a crucial component in advancing next-generation vaccines across various modalities, including recombinant (Chen et al., 2019), DNA plasmid (Zhao et al., 2023), bacterium-like particle (Wang et al., 2024), and peptide-based or multiepitope polypeptides vaccines (Akhtar et al.,

2022). This trend stems from the recognition that conventional vaccine development methodologies entail significant investments of time and resources (Sami et al., 2021). The interdisciplinary field, referred as “vaccinomics”, this interdisciplinary field integrates immunogenetics, immunogenomics, systems biology, immune profiling, and immunoinformatics (Poland et al., 2011). Consequently, the integration of “omics” technologies has propelled advancements in vaccinology by facilitating the characterization of molecular interactions among hosts, vectors, and pathogens, as well as the identification of potential protective antigens, such as peptides derived from pathogen proteins (de la Fuente & Contreras, 2021). Among these advancements is the use of epitope-based peptide vaccines, which rely on the *in silico* prediction of immunogenic peptides derived from dominant antigenic proteins (Gu et al., 2017; Yashvardhini et al., 2021). Peptide-based vaccines have been explored for various viral agents, including hepatitis B, influenza A, and hepatitis C, and have demonstrated considerable efficacy in experimental models (He et al., 2015; Sominskaya et al., 2010; Stanekova & Vareckova, 2010). Several advantages support the consideration of peptide-based vaccines, including their noninfectious nature, facile incorporation of molecules to enhance immunogenicity, suitability for lyophilized preparations, thereby facilitating storage, absence of virulence reversion risk, and capacity for designing with multiple antigenic determinants (Purcell et al., 2007).

In this thesis, we designed a new-generation vaccine based on genetic and antigenic information on CDV lineages circulating worldwide, as reported in *Chapter 3*, following an integrative *in silico* approach that has been employed for other viral agents, such as canine circovirus, Marburg virus and SARS-CoV-2 (Jain et al., 2021; Sami et al., 2021; Samimi Hashjin et al., 2023; Yashvardhini et al., 2021). A consensus sequence was generated for the H and F proteins, and immunogenic peptides were predicted *in silico* to develop a universal vaccine that could be employed for all CDV lineages in domestic and wild animals affected by CDV. After approximately one thousand and four hundred peptide were predicted with different computational tools, twelve peptides were selected based on their prediction scores and physicochemical properties. *In silico* and *in vitro* safety evaluations ware performed. A safe *in silico* profile employing different computational tools and *in vitro* profile in cell lines such as Vero-Dog-SLAM and MDCK cells, and canine PBMCs and red

blood cells, demonstrated not only the broad safety spectrum of *in silico* predicted peptides but also the reduced time require for traditional safe vaccine development (Samimi Hashjin et al., 2023). A national and international patents for CDV immunogenic peptides (Colombian No. CO2021017322A1 and international No. WO2023111728A1) were obtained. The peptides that fulfilled the set safety criteria were assembled in a multiepitope polypeptide with aminoacidic linkers. These peptides encompassed a combination of helper CD4+ T-cell, cytotoxic CD8+ T-cell, and linear B-cell epitopes, and were evaluated in mice either as a peptide mixture or polypeptide to further determine their *in vivo* safety and immunogenicity.

In recent years, some studies have employed mice to determine not only safety and immunogenicity but also CDV vaccine efficacy after challenge with recombinant viruses via diverse immunization routes and challenges (Chen et al., 2019; Du et al., 2022; Jiang et al., 2019; Wang et al., 2024; Yan et al., 2020). In this thesis, in *Chapter 4*, we demonstrated the safety of a CDV-derived peptide mixture or multiepitope CDV polypeptide in a murine model in a 28-day vaccine scheme. Moreover, seroconversion of mice against multiepitope CDV polypeptide by ELISA was demonstrated in contrast to that in the placebo mice group, but this response was lower than that in the commercial vaccine mice group. Additionally, cellular immunity was also assayed through polypeptide stimulated splenocytes that produce cytokines and activate of antigen-specific memory CD4+ and CD8+ T cells, which are essential for the immune response to CDV infection (Pfeffermann et al., 2018; Rendon-Marin et al., 2019). Since multiepitope CDV polypeptide-specific IgG production was greater in the multiepitope CDV polypeptide group and commercial vaccine (recombinant virus) group than in the placebo group, the multiepitope CDV polypeptide proposed in this thesis could serve as a potential booster for recombinant vaccination considering the important specific CDV polypeptide humoral response exhibited in this work.

As described in *Chapter 4*, this approach could be a valuable platform for evaluating immunogenicity and efficacy before further testing in target species such as domestic dogs and wildlife populations, which is essential for controlling the infection and CDV dissemination and transmission (Wilkes, 2022). Considering the limited response of peptide-based vaccines evaluated in this work, future research should focus on refining vaccine

formulations, for example, exploring other immunization routes, potent new generation adjuvants, higher peptide concentrations, and molecular modifications to improve adjuvants, to conduct efficacy trials in susceptible animal populations and addressing safety concerns to advance CDV vaccine development and mitigate its impact on animal health and welfare since no peptide-based vaccine has reached commercial distribution (Purcell et al., 2007).

Undeniably, there are some disadvantages regarding peptide-based vaccination that must be overcome. The reduced immunogenicity compared to that of live attenuated vaccines can lead to weaker immune responses, requiring the incorporation of modern adjuvants or delivery systems to enhance their efficacy (Naz & Dabir, 2007); the limited range of immune recognition and potential ineffectiveness in individuals with diverse MHC profiles (Purcell et al., 2007); the incorporation of multiple epitopes to elicit robust immune responses, complicating vaccine design and manufacturing processes (Moyle & Toth, 2013); and finally, the induction of short-lived immune responses, implicating frequent booster doses to maintain protective immunity in time (Azizi & Diaz-Mitoma, 2007). Therefore, *in silico, in vitro* and *in vivo* combined approaches as presented in this thesis, may help to not only overcome the abovementioned limitations but also develop a new vaccine alternatives with a rational approach.

CDV infection transmission and dissemination must be considered within the framework of the One Health concept, which connects human, animal, and environmental health (Pitt & Gunn, 2024), because controlling CDV disease will impact not only endangered species but also domestic animal health as a milestone in veterinary medicine, with the potential to significantly mitigate the impact of this devastating disease on canine populations worldwide (Duque-Valencia, Sarute, et al., 2019). The significance of CDV vaccination extends beyond the welfare of individual canines to circumscribe broader implications for public health. By preventing CDV transmission in domestic and wild canine populations, such vaccines could safeguard the health of companion animals and reduce the risk of spillover to susceptible wildlife populations and potentially to humans. Vaccination of wildlife animals could be an important challenge and different field immunization strategies, such as drones and baits, must be considered to reach susceptible animals.

The presence of metareservoirs implicates interconnected animal populations consisting of multiple species, highlighting the necessity not only to vaccinate domestic dogs against CDV infection to control transmission and dissemination but also to vaccinate endangered wildlife species to prevent catastrophic consequences such as species extinction (Wilkes, 2022). The absence of intervention in CDV infection could increase the 50-year probability extinction for Amur tiger populations, as demonstrated by simulations of infection through the predation of infected domestic dogs, wild carnivores, and direct tiger-to-tiger transmission (Gilbert et al., 2014). Indeed, evidence suggests that CDV transmission and dissemination can occur even in the absence of domestic dog populations (Duque-Valencia, Sarute, et al., 2019). Given the constant potential of CDV for cross-species transmission and its broad host range within wildlife populations, disease eradication presents a formidable challenge (Beineke et al., 2015), ultimately impacting the health of domestic dogs. The collaborative efforts of veterinarians, immunologists, epidemiologists, and policymakers in vaccine development and implementation exemplify the interdisciplinary nature of viral pathogen control. Further research, surveillance, and new vaccination alternative efforts are essential to enhance the efficacy and accessibility of CDV vaccines, ultimately impacting both animal and human health.

In summary, this thesis constitutes an original and significant contribution to veterinary virology and vaccinology. It investigates CDV infection dynamics, transmission patterns, and dissemination mechanisms, particularly in light of the emergence of novel viral lineages. Of particular interest is the examination of the potential efficacy of a peptide-based vaccine, whether formulated as a peptide mixture or a multiepitope polypeptide. This research underscores the urgent necessity for novel vaccine modalities that can be safely administered to both domestic and wild animal populations, either as a new vaccine or booster for current recombinant vaccines. This study offers novel perspectives, methodologies, and implications for the prediction, development, and application of vaccines through *in silico, in vitro,* and *in vivo* approaches. This thesis is also within the framework of One Health. Its novelty lies in the integration of diverse disciplines such as veterinary medicine, public health, and ecology to comprehensively address challenges related to the prevention of viral diseases and the promotion of health in both animals and humans.

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GENERAL CONCLUSIONS

The development of vaccines against canine distemper virus (CDV) represents a crucial area of research with significant implications for both domestic dogs and wildlife populations. Advances in computational biology and immunoinformatic tools have enabled the efficient design of a multiepitope vaccine, considering that the vaccine development process could be time-consuming. This thesis contributes to the understanding of CDV vaccine development by investigating not only the current scenario of vaccine development worldwide but also the efficacy and immunogenicity of peptide-based vaccines, including both single peptide mixtures and multiepitope polypeptides that include linear B cells, cytotoxic T lymphocytes, and helper T lymphocyte epitopes. *Using an in vivo* murine model, our findings demonstrated that vaccination with a multiepitope CDV polypeptide elicits humoral and cellular immune responses, indicating the potential of this vaccine to confer protection against CDV infection. However, it is essential to acknowledge that the vaccine candidates explored in this thesis are in development and require further evaluation in target species, including domestic dogs and wildlife animals, to comprehensively assess their effectiveness under real-world conditions, including virus challenge scenarios. Thus, this research represents a significant step forward in developing CDV vaccines, comprising an *in silico, in vitro,* and *in vivo* approach that offers promising preventive strategies for controlling CDV disease and its dissemination in both domestic and wildlife populations. Future studies should focus on refining vaccine formulations, conducting extensive efficacy trials, and addressing any challenges associated with vaccine deployment in diverse populations to ultimately mitigate the impact of CDV on animal health and welfare.

ORIGINALITY STATEMENT

I hereby attest that within the creative works referenced in this doctoral dissertation, due acknowledgment of copyright ownership to external parties has been duly provided. In compliance with the standards and conventions of the scientific community, the citation format employed for this purpose adheres to the guidelines outlined by the American Psychological Association (APA).

This doctoral thesis presents a unique and substantial contribution to the field of veterinary virology and vaccinology. It offers a comprehensive exploration of canine distemper virus (CDV) infection, transmission, and dissemination, particularly in the context of the emergence of new lineages. This research underscores the need for new vaccine alternatives that can be safely employed in domestic and wild animals. Notably, this study delves into the potential of a peptide-based vaccine, whether a new multiepitope polypeptide vaccine or a booster for current recombinant commercial vaccines, providing fresh insights, methodologies, and implications for prediction, development, and practice *in silico, in vitro* and *in vivo.*

In dog virology, the study of CDV infection and control has garnered considerable attention due to its relevance in addressing the high risk of new lineage emergence. However, much of the literature tends to describe molecular characteristics and phylogenetics. Nevertheless, vaccine failure and development have been poor, considering the threat that CDV poses to many wildlife animals, including dangerous ones. Thus, this thesis seeks to depart from conventional paradigms by introducing a new perspective and methodology integrating *in silico, in vitro* and *in vivo* approaches that shed light on unexplored facets of the CDV vaccine since a review of the literature was carried out; it became apparent that a significant gap exists in our understanding of virus genetic variation to establish potential new generation peptide-based vaccines that could be safe and effective in comparison to current commercially available alternatives. While prior studies have touched upon recombinant vaccines, subunits, or even bacteria-like particles, a comprehensive analysis of peptide-based vaccines remained conspicuously lacking until the development of this thesis for CDV.

At the heart of this research's originality is its innovative methodology, which diverges from traditional approaches in several key aspects. It integrates protein sequence analysis to predict immunogenic peptides with various computational tools. The selection process is based on the best prediction score and physicochemical properties, followed by an evaluation of *in vitro* and *in vivo* safety and efficacy in mice. This thesis, therefore, offers a fresh perspective on CDV vaccine development and opens new avenues for inquiry and exploration. Moreover, the adoption of peptide-based vaccine development through *in silico, in vitro* and *in vivo* approaches enhances the rigor and validity of the findings, thereby contributing to the credibility and reliability of the study.

In conclusion, this doctoral thesis represents a pioneering endeavor that advances the frontiers of knowledge in the field of CDV antigenic diversity to develop a new generation of vaccines focused on universal vaccines for CDV considering the genetic information of all circulating lineages worldwide. Offering a novel perspective on vaccine development within the context of CDV transmission dynamics and control fills a significant gap in the literature and lays the groundwork for future research and practice. Through its originality, methodological rigor, and practical relevance, this study contributes to the ongoing dialog and evolution of CDV knowledge.