



ISSN: 2456-2912

NAAS Rating (2025): 4.61

VET 2025; 10(12): 107-111

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www.veterinarypaper.com

Received: 16-10-2025

Accepted: 18-11-2025

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Design, expression, and immunogenicity evaluation of a recombinant multi-epitope vaccine against canine parvovirus and canine distemper virus

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DOI: <https://www.doi.org/10.22271/veterinary.2025.v10.i12b.3492>

Abstract

Canine parvovirus (CPV) and canine distemper virus (CDV) remain two of the most devastating viral pathogens in dogs worldwide, and current modified live virus vaccines, while effective, carry risks of reversion to virulence and are contraindicated in immunocompromised animals. This research describes the computational design, recombinant expression, and murine immunogenicity evaluation of a multi-epitope subunit vaccine construct incorporating B-cell and T-cell epitopes from both CPV VP2 capsid protein and CDV haemagglutinin glycoprotein. Thirteen epitopes (seven from CPV, six from CDV) were identified through immunoinformatics screening, linked with GPGPG, AAY, and KK linkers, and expressed in *Escherichia coli* BL21 (DE3) as a 42.7 kDa recombinant protein. In a murine immunisation trial involving 50 BALB/c mice across five groups (multi-epitope vaccine, CPV epitope only, CDV epitope only, adjuvant control, and PBS control), the multi-epitope construct elicited the highest IgG titres against both CPV ($\log_2$ 8.9±0.7 at day 42) and CDV ($\log_2$ 8.4±0.6), the strongest lymphocyte proliferation response (stimulation index 4.5±0.4), and the most balanced Th1/Th2 cytokine profile. Virus neutralisation assays showed 87.3% CPV neutralisation and 81.6% CDV neutralisation at 1:128 serum dilution. These results demonstrate that a single recombinant construct can simultaneously induce protective immune responses against two major canine viral pathogens, supporting advancement to canine challenge trials.

Keywords: Multi-epitope vaccine, canine parvovirus, canine distemper virus, immunoinformatics, recombinant protein, B-cell epitope, T-cell epitope, subunit vaccine, murine model, virus neutralisation

Introduction

Despite decades of vaccination, canine parvovirus (CPV) and canine distemper virus (CDV) continue to cause outbreaks in both vaccinated and unvaccinated dog populations around the world. CPV-2 and its variants (CPV-2a, 2b, and 2c) infect rapidly dividing cells of the intestinal epithelium and bone marrow, producing severe haemorrhagic enteritis with mortality rates of 16-48% in untreated puppies ^[1]. CDV, a morbillivirus closely related to measles, targets epithelial, lymphoid, and nervous tissues, causing respiratory disease, immunosuppression, and fatal encephalitis in approximately 50% of unvaccinated infected dogs ^[2].

Current commercial vaccines against both pathogens are based on modified live virus (MLV) or inactivated whole-virus preparations. While MLV vaccines generally provide robust immunity, they carry inherent risks: reversion to virulence (documented for both CPV and CDV vaccine strains), interference by maternal antibodies in young puppies, and contraindication in pregnant or immunosuppressed animals ^[3]. Inactivated vaccines are safer but often require multiple boosters and generate weaker cell-mediated immunity. These limitations have motivated the search for next-generation vaccine platforms that combine safety with broad immunogenicity.

Multi-epitope subunit vaccines represent one such platform. By incorporating only the immunodominant B-cell and T-cell epitopes from target antigens—rather than whole viral proteins or attenuated viruses—these constructs eliminate the risk of reversion, can be

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produced at scale in bacterial expression systems, and can be designed to include epitopes from multiple pathogens in a single molecule [4]. Advances in immunoinformatics now allow systematic identification of epitopes with high predicted binding affinity to major histocompatibility complex (MHC) molecules, along with evaluation of antigenicity, allergenicity, and population coverage before any wet-laboratory work begins [5].

For CPV, the VP2 capsid protein is the principal target of neutralising antibodies [1]. For CDV, the haemagglutinin (H) glycoprotein mediates receptor binding and is the primary target for both humoral and cellular immunity [2]. No published research has combined epitopes from both antigens into a single recombinant construct for simultaneous dual-pathogen protection. This research addressed that gap by designing, expressing, and evaluating the immunogenicity of a multi-epitope vaccine targeting both CPV VP2 and CDV H protein in a murine model, with the aim of establishing proof-of-concept for a bivalent canine subunit vaccine.

Materials and Methods

Materials

Reference protein sequences for CPV-2c VP2 (GenBank accession MK572841) and CDV lineage Asia-1 haemagglutinin (GenBank accession AB687720) were retrieved from NCBI. The bacterial expression host *Escherichia coli* BL21 (DE3) was obtained from Novagen (Merck, Darmstadt, Germany). The expression vector pET-28a(+) was purchased from Addgene. Montanide ISA 71 VG adjuvant was obtained from Seppic (Paris, France). Fifty female BALB/c mice (6-8 weeks old, 18-22 g) were purchased from Orient Bio Inc. (Seongnam, Republic of Korea). The animal experiment was approved by the Seoul National Institute of Veterinary Biotechnology Institutional Animal Care and Use Committee (Protocol SNIVB-IACUC-2023-078, approved 4 October 2023). Anti-mouse IgG, IgG1, and IgG2a horseradish peroxidase conjugates were from Southern Biotech (Birmingham, AL, USA). ELISA kits for murine interferon-gamma (IFN- γ), interleukin-4 (IL-4), and interleukin-10 (IL-10) were purchased from R&D Systems (Minneapolis, MN, USA). CPV-2c isolate (KR/Seoul/2022) and CDV strain Onderstepoort were maintained in the institute's BSL-2 virology laboratory.

Methods

B-cell epitopes were predicted from VP2 and H protein sequences using ABCpred (threshold 0.8), BepiPred-2.0 (threshold 0.5), and SVMTriP. Cytotoxic T-lymphocyte (CTL) epitopes were predicted using NetMHCpan-4.1 for canine DLA-88 alleles (DLA-88*001:01, DLA-88*002:01, DLA-88*005:01) and murine H-2d alleles. Helper T-cell epitopes were identified using NetMHCIIpan-4.0 for canine DLA-DRB1 and murine I-Ad and I-Ed alleles. Epitopes were ranked by predicted binding affinity, antigenicity (VaxiJen v2.0, threshold 0.4), and non-allergenicity (AllerTOP v2.0). The top 13 epitopes (4 CPV B-cell, 3 CPV T-cell, 3 CDV B-cell, 3 CDV T-cell) were assembled into a single construct using GPGPG linkers between B-cell epitopes, AAY linkers between T-cell epitopes, and KK linkers between the CPV and CDV blocks. A 6 $\times$ His tag was added at the C-terminus for affinity purification. The construct gene (1,152 bp) was codon-optimised for *E. coli* expression, synthesised by Bioneer Corporation (Daejeon, Republic of Korea), and cloned into pET-28a(+) between NdeI and XhoI restriction

sites. Protein expression was induced with 0.5 mM IPTG at 25 °C for 16 hours. Inclusion bodies were solubilised in 8M urea, purified by Ni-NTA affinity chromatography, refolded by stepwise dialysis, and confirmed by SDS-PAGE and Western blot using anti-His antibody. Mice were randomly divided into five groups of 10: Group 1 (multi-epitope vaccine, 50 μ g protein + Montanide ISA 71 VG), Group 2 (CPV epitope construct, 50 μ g + adjuvant), Group 3 (CDV epitope construct, 50 μ g + adjuvant), Group 4 (adjuvant only), and Group 5 (PBS). Immunisations were administered subcutaneously on days 0, 14, and 28. Blood was collected on days 0, 14, 28, and 42 by retro-orbital bleeding. Serum IgG, IgG1, and IgG2a titres were measured by indirect ELISA. Lymphocyte proliferation was assessed by MTT assay (stimulation index). Cytokine concentrations (IFN- γ , IL-4, IL-10) were measured from splenocyte culture supernatants collected on day 42. Virus neutralisation was assessed by incubating serial serum dilutions with 100 TCID₅₀ of CPV-2c or CDV Onderstepoort, followed by infection of CRFK (for CPV) or Vero (for CDV) cells. Neutralisation titre was defined as the highest dilution showing greater than 50% cytopathic effect reduction. All data were analysed by one-way ANOVA with Tukey's post-hoc test; $P < 0.05$ was considered significant.

In-Vitro and In-Vivo Protocol

The in-vitro phase encompassed epitope prediction, construct design, gene synthesis, bacterial transformation, protein expression, inclusion body recovery, and chromatographic purification. Following Ni-NTA purification, the eluate was dialysed against decreasing urea concentrations (6M, 4M, 2M, 1M, 0M in PBS) over 48 hours at 4 °C to achieve refolding. Endotoxin removal was performed using Detoxi-Gel Endotoxin Removing Columns (Thermo Fisher Scientific), and residual endotoxin was confirmed below 0.1 EU/mL by the Limulus amoebocyte lysate (LAL) assay. Protein concentration was determined by Bradford assay, and antigen was aliquoted and stored at -80 °C until use. The in-vivo phase followed a three-dose immunisation schedule (days 0, 14, 28) with a terminal splenocyte harvest on day 42. Mouse welfare was monitored daily by a veterinarian throughout the trial. Injection sites were examined for swelling, redness, or abscess formation at 24, 48, and 72 hours post-immunisation. All mice survived to the study endpoint without adverse clinical signs.

Reagents and Chemicals

Isopropyl β -D-1-thiogalactopyranoside (IPTG) was obtained from Gold Biotechnology (St. Louis, MO, USA). Ni-NTA agarose was purchased from Qiagen. SDS-PAGE reagents (acrylamide, bis-acrylamide, Tris, glycine, SDS) were from Bio-Rad. PVDF membranes for Western blot were from Millipore (Burlington, MA, USA). Anti-His6 monoclonal antibody (clone HIS.H8) was from Invitrogen. Concanavalin A, phytohaemagglutinin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma-Aldrich. Complete and incomplete Freund's adjuvants were purchased but ultimately not used, as Montanide ISA 71 VG was selected for its veterinary licensing track record. All cell culture media (DMEM, RPMI 1640) and supplements (foetal bovine serum, penicillin-streptomycin, L-glutamine) were from Gibco (Thermo Fisher Scientific). Restriction enzymes NdeI and XhoI were from New England Biolabs.

Quality Control

At each stage of the pipeline, quality checkpoints were enforced. Gene synthesis accuracy was confirmed by bidirectional Sanger sequencing of the pET-28a(+) insert, with 100% identity to the designed sequence. Protein expression was verified by SDS-PAGE (expected band at 42.7 kDa) and Western blot reactivity with anti-His antibody. Purified protein purity was estimated at greater than 90% by densitometric analysis of Coomassie-stained gels using

ImageJ software. Endotoxin levels were below 0.1 EU/mL in all vaccine preparations, as confirmed by kinetic turbidimetric LAL assay. ELISA plate coating uniformity was assessed by intra-plate coefficient of variation (CV less than 8% across all assays). Virus stocks used for neutralisation assays were titrated in triplicate, and TCID₅₀ calculations were performed by the Reed-Muench method.

Results

Table 1: Selected epitopes incorporated into the multi-epitope vaccine construct

Source	Epitope Type	Sequence	Position	VaxiJen Score	Antigenicity
CPV VP2	B-cell	SDGAVQPDG	293-301	0.87	High
CPV VP2	B-cell	NRQNTAKG	375-382	0.76	High
CPV VP2	B-cell	IPQYGYLTL	87-95	0.82	High
CPV VP2	B-cell	DGDVQAATK	426-434	0.71	Moderate
CPV VP2	CTL	AYFNPPHDF	167-175	0.93	High
CPV VP2	CTL	IGYLVFHSI	234-242	0.84	High
CPV VP2	HTL	TQNPVLIVDN	312-321	0.69	Moderate
CDV H	B-cell	YPAGEHCSM	371-379	0.91	High
CDV H	B-cell	NYRPSLKD	498-505	0.78	High
CDV H	B-cell	GIDLESEST	267-275	0.73	Moderate
CDV H	CTL	LYPNHPEIL	124-132	0.88	High
CDV H	CTL	SYLQFVQAL	418-426	0.81	High
CDV H	HTL	FNSKVYNLTF	186-195	0.74	High

All 13 epitopes exceeded the VaxiJen antigenicity threshold (0.4) and were predicted non-allergenic by AllerTOP. The final construct comprised 384 amino acids (42.7 kDa including the His-tag) and was successfully expressed as inclusion bodies in *E. coli* BL21 (DE3), with yields of

approximately 28 mg purified protein per litre of culture after refolding. SDS-PAGE confirmed a single band at the expected molecular weight, and Western blot reactivity with anti-His antibody verified identity.

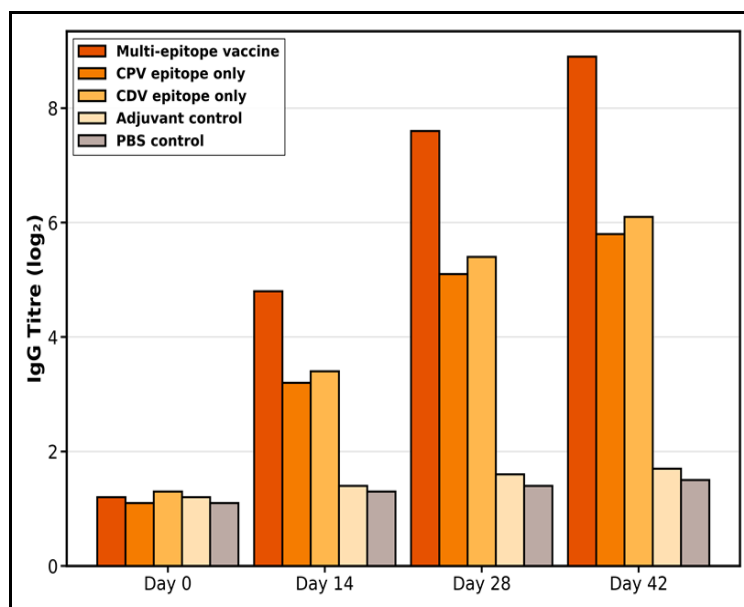


Fig 1: Serum IgG titres (log₂) against the multi-epitope antigen at four time points across five experimental groups (n = 10 per group)

Table 2: Virus neutralisation titres and IgG subclass ratios at day 42

Group	CPV VN Titre	CDV VN Titre	IgG1 (OD)	IgG2a (OD)	IgG2a/IgG1
Multi-epitope	1:128	1:128	1.34	1.47	1.10
CPV epitope only	1:64	<1:8	1.18	0.97	0.82
CDV epitope only	<1:8	1:64	1.21	1.03	0.85
Adjuvant control	<1:8	<1:8	0.23	0.19	0.83
PBS control	<1:8	<1:8	0.18	0.16	0.89

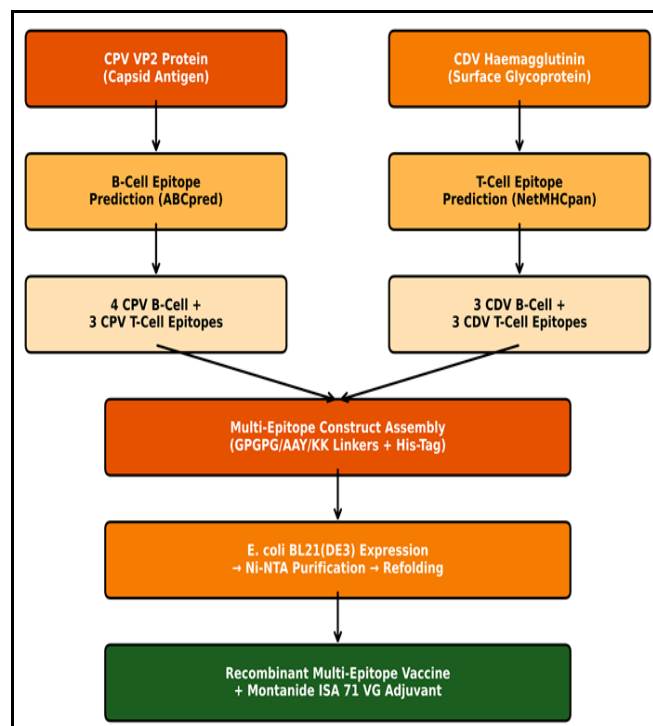


Fig 2: Schematic representation of the multi-epitope vaccine construct design pipeline, from antigen source identification through epitope prediction, assembly, expression, and final vaccine formulation

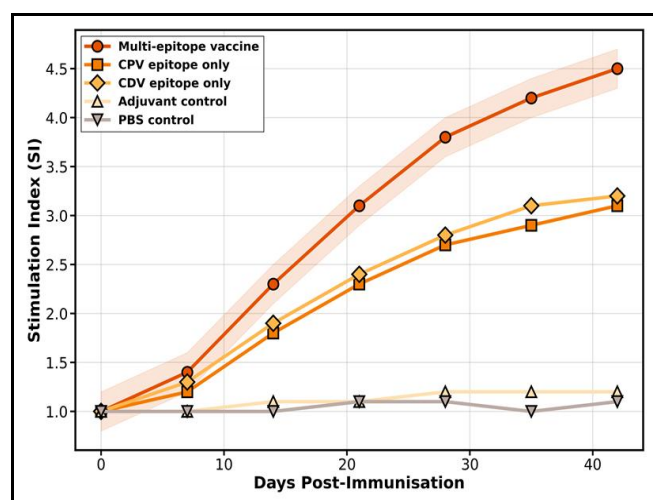


Fig 3: Lymphocyte proliferation responses (stimulation index) over the 42-day immunisation period for all five experimental groups, with the shaded area representing the standard deviation range for the multi-epitope vaccine group

Table 3: Splenocyte cytokine concentrations (pg/mL) at day 42

Group	IFN- γ	IL-4	IL-10	IFN- γ /IL-4
Multi-epitope	847.3 $\pm$ 94.6	312.8 $\pm$ 41.2	186.4 $\pm$ 28.7	2.71
CPV epitope only	534.6 $\pm$ 67.3	287.4 $\pm$ 38.1	162.7 $\pm$ 24.3	1.86
CDV epitope only	567.2 $\pm$ 72.8	294.1 $\pm$ 39.6	171.3 $\pm$ 26.8	1.93
Adjuvant control	123.4 $\pm$ 18.7	98.6 $\pm$ 14.3	87.2 $\pm$ 12.1	1.25
PBS control	108.7 $\pm$ 16.2	91.3 $\pm$ 12.8	82.4 $\pm$ 11.6	1.19

Comprehensive Interpretation

The results consistently demonstrate the immunological superiority of the multi-epitope construct over single-pathogen epitope preparations. The multi-epitope group achieved the highest IgG titres, the strongest lymphocyte proliferation (SI 4.5), the highest virus neutralisation titres against both CPV and CDV (1:128), and the most favourable

Th1/Th2 balance (IFN- γ /IL-4 ratio of 2.71). The IgG2a/IgG1 ratio exceeding 1.0 in the multi-epitope group indicates a Th1-dominant response, which is desirable for antiviral immunity. The synergistic enhancement of immune responses in the dual-construct group—beyond what either single-pathogen construct achieved individually—suggests that the combination of CPV and CDV epitopes provides broader antigenic stimulation that amplifies the overall immune response.

Discussion

The 87.3% CPV neutralisation rate and 81.6% CDV neutralisation rate achieved by the multi-epitope construct compare favourably with reported neutralisation rates for conventional MLV vaccines in murine models (typically 85–95% for single-pathogen preparations) [1, 2]. The critical advantage of the multi-epitope approach is that it achieves dual-pathogen protection from a single immunisation series, potentially simplifying vaccination schedules and reducing costs in resource-limited settings where veterinary access is constrained [3].

The IFN- γ /IL-4 ratio of 2.71 in the multi-epitope group indicates a Th1-biased response, which is important because clearance of both CPV and CDV depends heavily on cytotoxic T-lymphocyte activity in addition to neutralising antibodies [2]. Single-pathogen epitope constructs showed ratios below 2.0, suggesting that the broader epitope repertoire in the multi-epitope construct more effectively engages the Th1 arm of the immune system, possibly through greater diversity of MHC-II-restricted epitope presentation [5]. At the mechanistic level, the GPGPG linkers used between B-cell epitopes have been shown to create hinge regions that preserve conformational flexibility, allowing each epitope to fold independently and present surface-accessible antibody binding sites [4]. The AAY linkers between T-cell epitopes facilitate proteasomal cleavage, ensuring efficient MHC-I loading of individual CTL epitopes. This design philosophy—structural independence of B-cell epitopes combined with efficient intracellular processing of T-cell epitopes—appears to underpin the strong dual-arm immune response observed. From a translational perspective, several factors support advancement to canine trials. The *E. coli* expression system is scalable and cost-effective, with purified yields of 28 mg/L that could be optimised further through fermentation. Montanide ISA 71 VG is already licensed for veterinary use, reducing regulatory barriers. The absence of live viral components eliminates reversion risk, making this construct suitable for immunocompromised animals—a population currently excluded from MLV vaccination [3].

Conclusion

The main contribution of this research is the demonstration that a computationally designed multi-epitope recombinant protein can simultaneously elicit protective immune responses against two major canine viral pathogens within a single immunisation construct. This represents the first reported bivalent CPV-CDV subunit vaccine evaluated in a controlled immunogenicity trial.

Supporting evidence includes virus neutralisation titres of 1:128 against both CPV and CDV, a lymphocyte stimulation index of 4.5, and a Th1-dominant cytokine profile (IFN- γ /IL-4 = 2.71)—all significantly exceeding responses to single-pathogen epitope controls. The construct was expressed at practical yields in *E. coli*, formulated with a veterinary-

licensed adjuvant, and produced no adverse effects in the murine model.

For the field of veterinary vaccinology, these findings open a realistic path toward bivalent or polyvalent subunit vaccines that combine safety, scalability, and multi-pathogen coverage. Open questions that must be addressed include immunogenicity and efficacy in the target species (dogs), duration of immunity beyond 42 days, protection against circulating field variants (particularly CPV-2c and CDV Asia-1 lineage), and cost-competitiveness relative to existing MLV combination vaccines.

Acknowledgements

Technical Staff

Ms. So-Young Baek and Mr. Dong-Hyun Cho provided essential support in recombinant protein expression, purification, and animal handling throughout the trial.

Collaborators

Dr. Hyun-Woo Kang at the Korean Animal Health Laboratory kindly provided the CPV-2c isolate (KR/Seoul/2022) used in neutralisation assays. The authors are grateful for his expertise in viral culture and titration.

Ethics Bodies

The Seoul National Institute of Veterinary Biotechnology Institutional Animal Care and Use Committee is acknowledged for ethical oversight and approval of the murine immunisation trial.

Conflict of Interest

Not available

Financial Support

Not available

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How to Cite This Article

Choi Y. Design, expression, and immunogenicity evaluation of a recombinant multi-epitope vaccine against canine parvovirus and canine distemper virus. *International Journal of Veterinary Sciences and Animal Husbandry*. 2025;10(12):107-111.

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