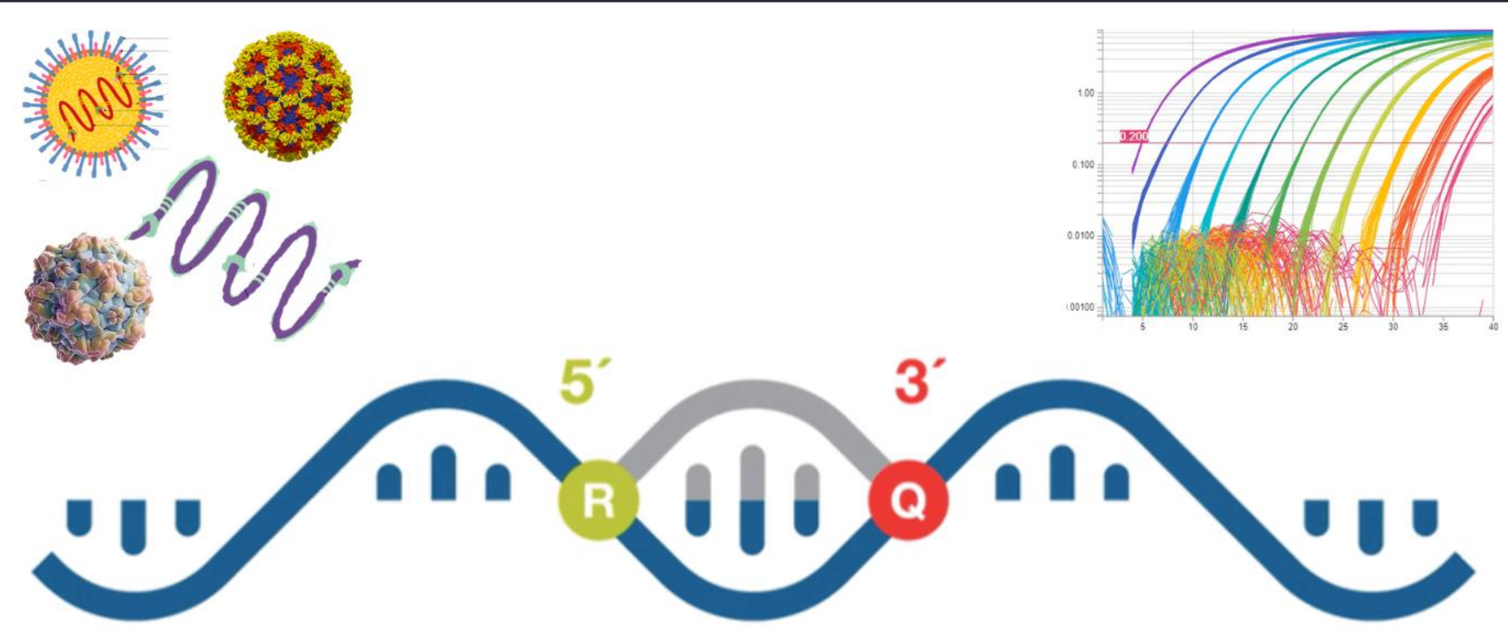
ANNUAL PLAN OF OPERATIONS 2023-24

PROJECT COMPLETION REPORT

Development of Multiplex Real Time RT-PCR assay for detection of major viral infections in captive wild animals



TAMIL NADU FOREST DEPARTMENT

ADVANCED INSTITUTE FOR WILDLIFE CONSERVATION

(Research, Training & Education)

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PROJECT COMPLETION REPORT

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BY

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G.O. (D) No.: 44, Environment, Climate Change and Forests Department dated 11.03.2024

PROJECT PERIOD: APRIL 2024 – MARCH 2025

Development of Multiplex Real Time RT-PCR assay for detection of major viral infections in captive wild animals

TAMILNADU FOREST DEPARTMENT
ADVANCED INSTITUTE FOR WILDLIFE CONSERVATION
(RESEARCH, TRAINING & EDUCATION)

Declaration:

I (we) declare that this project report has been prepared in accordance with the project completion report format of the Department of Science and Technology, Ministry of Science and Technology, Government of India (Reference: accessed on 29 May 2024).
https://dst.gov.in/sites/default/files/PROJECT%2520COMPLETION%2520REPORT.pdf

To cite this report:

Shinta Ann Jose, C. Bala Amarnath, S. Siva Ranjani, S. Senbagapriya, D. Eswaran, A. Udhayan. (2025). Development of Multiplex Real Time RT-PCR assay for detection of major viral infections in captive wild animals. AIWC – APO 2023-24/Report.

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ACKNOWLEDGEMENT

The Project Scientist and Project Associate of this project place their heartfelt gratitude and acknowledge the continuous help and support provided by:

The Principal Chief Conservator of Forest and the Director, Deputy Director (Admin), Deputy Director (Tech), Forest Veterinary Assistant Surgeons, Forest Range Officer, Forester, Forest Guard, and all administrative staff of the Advanced Institute for Wildlife Conservation for their constant and kind support.

The Professor and Head, Department of Animal Biotechnology, Madras Veterinary College, Vepery, Chennai, for providing feline and canine viral positive swab samples. The Director, Department of Clinics, Madras Veterinary College, Vepery, Chennai, for granting permission to collect clinical blood samples. The Professor and Head, Department of Clinics, Madras Veterinary College, Vepery, Chennai, for guidance in identifying suitable cases from the infectious diseases outpatient ward. The B.V.Sc. Final-year students for their help in collecting blood samples.

Dr. C. Bala Amarnath

Project Scientist-II

Ms. Shinta Ann Jose

Project Associate-II

ABBREVIATIONS

DNA	– Deoxyribonucleic Acid
RNA	– Ribonucleic Acid
PCR	– Polymerase Chain Reaction
qRT-PCR	– Quantitative Reverse Transcription Polymerase Chain Reaction
CDV	– Canine Distemper Virus
CPV	– Canine Parvo Virus
FCV	– Feline Calici Virus
FPV	– Feline Panleukopenia Virus
E. coli	– Escherichia coli
NCBI	– National Centre for Biotechnology Information
nBLAST	– Basic Local Alignment Search Tool for (n) nucleotide database
°C	– Degree Celsius
µL	– Microliter
µM	– Micro Molar
ng	– Nanogram
NC/NTC	– Negative Control/No Template Control
PC	– Positive Control
AAZP	– Arignar Anna Zoological Park
GAPDH	– Glyceraldehyde-3-Phosphate Dehydrogenase
LB	– Lysogeny Broth

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PROJECT COMPLETION REPORT

1	**G.O. No**	:	G.O. (D) No.: 44, Environment, Climate Change and Forests Department dated 11.03.2024
2	**Project Title**	:	Development of Multiplex Real Time RT-PCR assay for detection of major viral infections in captive wild animals.
3	**Duration**	:	April 2024 to March 2025
4	**Principal Investigator**		
	Name	:	The Director
	Organisation	:	Advanced Institute for Wildlife Conservation (Research, Training & Education) Tamil Nadu Forest Department Vandalur – Kelambakkam road, Vandalur, Chennai – 600 048.
5	**Co-Principal Investigator**	:	NIL
6	**Collaborating organisation's**	:	NIL

PART A – SUMMARY REPORT

1. Project objectives

Objectives as per the approved project	Fully Achieved / Partially achieved (indicate shortfall)	Reason for partial achievement
To design a viral serovar sequence-specific primer and probe for each virus.	Fully achieved	NIL
To optimise the reaction conditions in a simplex and multiplex reaction.	Fully achieved	NIL
To evaluate the specificity and sensitivity of multiplex reaction assay.	Fully achieved	NIL

2. Deliverables

Deliverables as per the approved project	Fully/Partially/Not Achieved	Reason for partial / Non-achievement
Development of viral detection qPCR multiplex assay	Fully achieved	NIL

3. Specific Benefits/Outcome

i) Patent, if any:

NIL

ii) Product/Process developed/Technology transferred:

qPCR Multiplex diagnostic assay product and Process thrived.

iii) Publications

One research article may be published after conducting some additional work.

iv) Linkages developed

NIL

v) Manpower trained

1 No. of Project Associate-II

4. **Summary of significant Science & Technology achievements:**

    The qPCR Multiplex diagnostic assay was developed indigenously for the detection of CDV, FCV, CPV, and FPV, with a detection limit of 100 copies of virus per millilitre of sample.

5. **Project Budget:** Rs. 37,20,000/-

6. **Suggestions for utilisation of Project outcome:**

    The developed qPCR Multiplex diagnostic assay may support the Arignar Anna Zoological Park's captive animal surveillance for screening viral pathogens. Wildlife managers may utilise this test as a part of wildlife health management.

PART B – COMPREHENSIVE REPORT

1. Project title

Development of Multiplex Real Time RT-PCR assay for detection of major viral infections in captive animals.

2. Outcome of project – Identify beneficiaries

The developed qPCR Multiplex diagnostic assay may support the Arignar Anna Zoological Park's captive animal surveillance for screening viral pathogens. Wildlife managers may utilise this test as part of wildlife health management. Hence, AAZP is identified as a key beneficiary. Wildlife managers may also utilise the developed assay as an extended viral disease surveillance program in free-ranging wild animals by testing non-invasive faecal samples.

3. Scientific Description

The qPCR Multiplex diagnostic assay was developed indigenously for the detection of Canine Distemper Virus (CDV), Feline Calici Virus (FCV), Canine Parvo Virus (CPV), and Feline Panleukopenia Virus (FPV), with a detection limit of 100 copies of virus per millilitre of sample. The developed assay is very robust, cost-effective, and rapid, with a turnaround time of 1 hour.

4. Scientific Formulation and Methodology

4.1. Introduction

The recurring emergence of diseases in human and animal populations is a significant concern for public health and veterinary communities. According to Brown (2004), the term 'emerging disease' generally comprises the occurrence of i) a known agent appearing in a new geographic area, ii) a known agent or a closely related one occurring in a previously unsusceptible species, or iii) an unknown agent detected for the first time. Regardless of these categories, the majority of diseases emerge from an animal source in humans. Research has consistently illustrated that approximately 75% of emerging diseases affecting people over the last two decades have originated from an animal pathogen transmitted into the human host and are hence classified as 'zoonotic'. The term 'Zoonosis' refers to any disease or infectious agent that moves into humans from an animal source, and the term was coined by the German physician and pathologist Rudolf Virchow during his studies on *Trichinella* in 1855. His notion

of linking human and veterinary medicine as' one medicine' has considerably influenced the current status of global health (Brown, 2004). Multiple research studies on recent examples of emerging zoonoses reveal the impact and unpredictability of these diseases.

Emerging zoonotic diseases have fundamentally reshaped our understanding of global health dynamics, presenting an increasingly complex and multifaceted challenge. The continued emergence of these diseases is not only inevitable but is also expected to accelerate over time. While predicting the origin and specific nature of future outbreaks remains impossible, historical patterns suggest that new diseases often emerge from unexpected sources. Given this certainty, it is imperative to enhance preparedness for both veterinary and public health communities (Brown, 2004).

WILDLIFE IN INDIA

The Indian subcontinent spans an area of approximately 3.2 million square kilometres, comprising 28 states and eight union territories. India has three biodiversity hotspots among the 34 identified globally, located in the Western Ghats, the Eastern Himalayas, and the Indo-Burma region, respectively. According to the Environmental Information System (ENVIS) Centre on Wildlife and Protected Areas' database, as of February 2025, India has a network of 1,134 wildlife Protected Areas (PAs), covering an area of 187,162 km² and comprising 574 wildlife sanctuaries, 106 national parks, 145 conservation reserves, and 309 community reserves (GoI, 2025). These Protected Areas (PAs) are home to over 96,000 species of fauna and 29,000 species of flora (Government of India, 2017). From the standpoint of zoonotic diseases, India is home to a large number of vital wildlife species. Such animals with reported cases of infection include elephants, wild pigs, bats, non-human primates, rodents, wild aquatic and terrestrial birds, canids and felids, bovids, vultures, owls, and reptiles (Altaf, 2020; Bandyopadhyay *et al.,* 2010; Michalak *et al.,* 1998; Peiris, 2009; Singh & Gajadhar, 2014; Vanak & Gompper, 2009).

Wildlife populations present unique challenges in disease detection and management due to the vast diversity of species and pathogens that they harbour. The emergence of novel diseases in wildlife populations is frequently understudied, and validation of diagnostic tests remains inadequate (Jia *et al.,* 2020). Wildlife is essential for preserving the integrity of the ecosystem, but it also poses a significant threat to emerging zoonoses. Wildlife species, including mammals, birds, reptiles, amphibians, and fish, play a crucial role in maintaining

ecological balance; however, they also serve as significant sources of emerging zoonotic diseases. In recent decades, approximately 75% of emerging diseases, including zoonoses, have been traced back to their origin in wildlife. In India, wildlife-related zoonotic pathogens have been documented, highlighting the need for surveillance and research (Rhyan & Spraker, 2010; Thompson *et al.,* 2009). Zoonotic diseases caused by viruses are becoming more common in different geographical areas among humans, domestic animals, and wild animals (including captive ones).

Wildlife is the primary reservoir for most emerging diseases, with pathogens capable of infecting multiple host species (Gilbert *et al.,* 2013; Haydon *et al.,* 2002; Taylor *et al.,* 2001). The role of wildlife in spreading diseases such as influenza, severe acute respiratory syndrome (SARS), and Nipah virus is well recognised. Several key and interrelated factors influence the emergence of zoonotic diseases, which have to be considered with importance, including animal movement, movement of vectors and host species, ecological disruption, climate change, the presence of uncultivatable microorganisms, expanding human populations, the persistence of chronic diseases, advancements in disease surveillance, and the potential risks associated with bioterrorism (Bengis *et al.,* 2004; De Meneghi, 2006; Kruse *et al.,* 2004). Hunting and consuming wildlife also heightens the risk of zoonotic spillover (Wolfe *et al.,* 2005). Opportunities for spillover events may pose a significant threat to the conservation of endangered species. (Singh & Gajadhar, 2014).

EFFECTS ON WILDLIFE

Captive wildlife populations face significant disease risks due to their regular interaction with human populations. The National Association of State Public Health Veterinarians (NASPHV) released reports on zoonotic disease outbreaks linked to animal displays, as well as guidelines for minimising the spread of zoonotic diseases from animals to humans in these environments. These guidelines aim to develop and distribute recommendations for preventing diseases and injuries resulting from such exhibits. Many pathogens, including *Escherichia coli, Rabies virus, Cryptosporidium spp., Salmonella*, and *Brucellosis*, have been detected in captive wild species (Bender & Shulman, 2004). Although domestic and wild animals rarely interact, numerous reports have been made of zoonotic disease transmission between species, including *Mycobacterium bovis* and RNA viruses from domestic animals to wildlife (spillover). This spillback has persisted over millennia. (Bose, 2008; Johnson *et al.,* 1980). Rare and endangered species may be threatened by viruses that

humans and cattle can transmit to wildlife, raising concerns that potential outbreaks could lead to the extinction of these species. (Singh & Gajadhar, 2014).

VIRAL DISEASES OF CANINE AND FELINE SPECIES

CANINE DISTEMPER VIRUS (CDV)

The causative agent is Canine Distemper Virus (CDV), a relatively large (150–250 nm) single-stranded RNA virus with a lipoprotein envelope, classified as a morbillivirus within the family Paramyxoviridae (Greene & Appel, 1990; Osterhaus *et al.,* 1995). Members of the Morbillivirus genus also cause three additional well-known illnesses: Peste Des Petitsin Ruminants in small ruminants, Rinderpest in artiodactyls, and measles in primates. This genus also includes three newly identified viruses: cetacean morbilliviruses in porpoises and dolphins and phocine distemper virus in seals. Although other bodily fluids and excretions, such as urine, might infect susceptible hosts if aerosolised, the primary route of CDV transmission is the aerosolisation of virus-laden respiratory secretions (Ettinger and Feldman, 2010). Canine distemper is highly contagious, and viral shedding may occur for 60 to 90 days after infection (Krakowka *et al.,* 1977; Visser *et al.,* 1993). In domestic dogs, transplacental infections have been reported. The epidemiological function of vertical transmission in CDV remains unclear, as does its potential occurrence in non-domestic species. The virus may survive at lower temperatures (for example, 48 hours at 25°C and 14 days at 5°C), despite its typically brief environmental half-life (Shen *et al.,* 1980). It can be spread by direct contact or by fomites. The identification of the infecting species, host age and immunological status, environmental factors, and the pathogenicity of the viral strain all affect the clinical manifestations of CDV. The most often impacted systems in all animals are the respiratory, gastrointestinal, integumentary, and central nervous systems. Viremia is frequently linked to diphasic fever and overall malaise. Leukopenia-related infections are frequent and can complicate the clinical course (Deen *et al.,* 2000).

CANINE PARVOVIRUS (CPV-2)

According to Murphy *et al.* (1995), every known Parvovirus that infects and damages carnivore species is a member of the genus Parvovirus, which is a member of the family Parvoridae. The feline parvovirus subgroup includes several viruses that are genetically and antigenically very closely related, including the feline panleukopenia virus (FPV), canine Parvovirus (CPV-2) and its antigenic types CPV-2a and CPV-2b, mink enteritis virus (MEV),

blue fox parvovirus (BFPV), raccoon parvovirus (RPV), and raccoon dog parvovirus (RDPV). Aleutian mink disease virus (ADV) and canine minute virus (also known as minute virus of canines, or MVC) or canine parvoviruses type-1 (CPV-1). A novel viral illness that affected canine species first appeared in 1978 (Appel *et al.,* 1979) and once more, clinical parallels to feline FPV infection were reported. The new canine virus, known as canine parvovirus type 2 (CPV-2) to differentiate it from the distantly related canine parvovirus 1 (minute virus of dogs, or MVC), which was detected years earlier, was shown to have a very close relationship with FPV, according to subsequent antigenic and genomic analyses.

With over 98% genome homology and only six coding nucleotide changes in the VP2 protein (positions 3025, 3065, 3094, 3753, 4477, 4498; Parrish *et al.,* 1988; Parrish, 1991; Truyen *et al.,* 1995), CPV-2 and FPV are closely related. These few genetic alterations had significant biological repercussions, as CPV-2 lost its capacity to reproduce in cats but gained the ability to infect dogs (Truyen *et al.,* 1994). Both FPV and CPV-2 have complicated host ranges that vary in vitro and in vivo. While FPV does not infect canine cells in vitro and only exhibits a limited tissue spectrum in vivo, it does replicate in feline cells in vitro and in cats in vivo. Although CPV-2 can replicate in both canine and feline cells in vitro, it can only replicate in canids in vivo (Truyen *et al.,* 1992). New antigenic forms of CPV, known as CPV-2a and CPV-2b, appeared and proliferated in 1979 and 1984. These viruses differ from the original CPV-2 in common ways due to additional coding and nucleotide alterations at locations 3045, 3685, and 3699. At position 4062, CPV-2b had one additional nucleotide change. The original CPV-2 virus has been replaced globally by these substitutions, which altered antigenic epitopes that monoclonal antibodies can identify. These antigenic types are the most common strains currently circulating in various dog populations (Parrish *et al.,* 1991; Truyen *et al.,* 1996; Steinel *et al.,* 1998).

FELINE CALICIVIRUS (FCV)

Domestic cats are commonly infected with the highly mutagenic RNA virus known as feline calicivirus (FCV) (Zheng *et al.,* 2021). It has little zoonotic potential and is host-specific to the Felidae family. In feline populations, FCV shows significant genetic and antigenic diversity (Huang *et al.,* 2010). FCV is a highly contagious disease belonging to the Caliciviridae family and the Vesivirus genus. In addition to animal-specific viruses, such as the European brown hare syndrome virus and the rabbit hemorrhagic disease virus, this family also includes well-known human diseases, including noroviruses. The calicivirus family gets its

name from the distinctive cup-shaped depressions on its particles (Hofmann-Lehmann *et al.,* 2022). The single-stranded RNA genome of FCV is roughly 7.5 kilobases in size and has a positive sense strand. Viral replication lacks a proofreading mechanism, resulting in a high rate of mutation and, consequently, a rapid potential for evolution (Vinjé *et al.,* 2019).

FCV has a high rate of mutation and notable evolutionary dynamics within and between populations. According to Coyne et al. (2007), the annual rate of nucleotide changes for FCV varies between 1.32×10^{-2} and 2.64×10^{-2} in individuals and between 3.84×10^{-2} and 4.56×10^{-2} in groups. Clinical signs of an FCV infection can include upper respiratory tract disease (URTD), lingual ulcerations, gingivostomatitis, limping syndrome, and, in more severe cases, alopecia, cutaneous lesions, oral lesions, pinnae lesions, and necrotising pododermatitis with serous crusts. Infected cats have a significant mortality rate due to additional symptoms such as pancreatic, hepatic, and splenic necrosis, bronchointerstitial pneumonia, and subcutaneous oedema (Spiri, 2022).

FELINE PANLEUKOPENIA VIRUS (FPV)

A highly contagious illness known by several names, including feline distemper, feline infectious enteritis, feline parvoviral enteritis, pseudomembranous enteritis, laryngoenteritis, feline agranulocytosis, and show fever, is caused by the feline panleukopenia virus (FPV). Although the illness was known to exist before 1900, the viral agent was not isolated until 1928, and the virus itself was not identified with certainty until 1962. The organism is a parvovirus with single-stranded DNA. This non-enveloped virus is exceptionally contagious and tends to rapidly replicate within cells, particularly those found in the intestinal epithelium, bone marrow, lymphoid tissue, and fetal and neonatal cerebellar tissue. It is common, can cause infections that are frequently lethal and can be quite virulent in vulnerable animals. Though closely related to canine Parvovirus, FPV has distinct biological characteristics and just one serotype, (CPV may have developed as a mutation of FPV) (Scott 1987; Sturgess 2003). Although initially incapable of infecting cats, CPV type 2 has given rise to several variations (CPV-2a, CPV-2b, and CPV-2c) that can now infect cats and cause clinical illness in those individuals (Nakamura et al. 2001). Cats have also been shown to have mixed infections with FPV and CPV-2 genotypes (Sykes 2013).

CHALLENGES IN WILDLIFE DISEASE DETECTION AND SURVEILLANCE

Enhancing knowledge of the ecology of wildlife diseases and the part that wildlife plays in their development and spread is a key element of contemporary One Health initiatives. These are acquired through ecological and epidemiological studies in the affected, suspected, and vulnerable populations, and such studies require accurate and reproducible detection tests to provide information about the disease or infection status of individuals and populations. The findings from these studies play a key role in guiding and monitoring health management policies, including control of important animal and zoonotic diseases. Existing surveillance methods include pathogen isolation, which permits a greater epidemiological understanding of the circulating pathogen diversity within and among reservoir and incidental hosts (Streicker *et al.,* 2010). Even in the most ideal laboratory settings, isolating pathogens from animals can be a challenging task. Infection burdens may be low; often, animal infectious periods are short, as seen with rabies virus and canine distemper virus. Consequently, only a small population of animals may be infected, at any given time, thereby requiring large sample sizes to reliably detect infection (Deem *et al.,* 2000; Hampson *et al.,* 2009).

Direct pathogen detection tests, other than isolation, such as antigen-detection assays and molecular diagnostic tools (e.g., the Polymerase Chain Reaction—PCR), can be used to detect evidence of active or latent infection. Serological testing, including antibody prevalence studies, provides insights into an individual's infection history at the population level but does not determine their infection status. Some pathogens evade host immune detection, complicating serological tracking. Other limiting factors of the detection tests include sequestration in organs that require lethal sampling, intermittent pathogen excretion, and the need for high-containment facilities. However, molecular technologies have revolutionised the field of zoonotic disease surveillance and response. The polymerase chain reaction (PCR) is a key molecular technology employed in zoonotic disease surveillance. It enables the detection of infections in various materials, including blood, saliva, and environmental samples, by amplifying specific DNA or RNA sequences. These cutting-edge methods provide prompt and focused responses by enabling the quick and precise detection, identification, and characterisation of infections.

An advanced type of PCR assay, namely real-time PCR (qPCR) with careful study design and interpretation, can be invaluable for understanding the disease prevalence, even in poorly understood systems such as wildlife populations. Two Taq-Man-based multiplex PCR

detection methods were developed and validated to detect and differentiate all four canine hookworm species in naturally infected dog faeces (Jia *et al.,* 2020; Massetti *et al.,* 2020). This multiplex approach offers several advantages over conventional PCR, including its rapidity, cost-effectiveness, and minimal sample requirements. Real-time PCR has significantly increased the acceptance of PCR thanks to its improved rapidity, higher sensitivity, higher specificity, reproducibility, quantitative measurement, lower contamination rate, and ease of standardisation. This technique integrates relative and competitive (end-point) RT-PCR, making it accurate, precise, high-throughput, and relatively easy to perform. This developed method has enabled the accurate quantification of target nucleic acids from the research laboratory to utilise its capabilities in diagnostic applications. This technique facilitates the early detection of zoonotic diseases, providing valuable information about the prevalence of pathogens and aiding in monitoring potential drug dosage and treatment (Espy *et al.,* 2006; Parida, 2008; Zhang *et al.,* 2024). However, sample integrity is a key factor for field studies, and maximising the probability of successful pathogen isolation or detection often requires cold-chain or laboratory capacity, which is difficult in a field setting, especially in remote geographic areas.

4.2. OBJECTIVES

1. To design a viral serovar sequence-specific primer and probe for each virus.

2. To optimise the reaction conditions in a simplex and multiplex reaction.

3. To evaluate the specificity and sensitivity of the multiplex reaction assay.

4.3. MATERIALS & METHODS

4.3.1. PRIMER – PROBE DESIGN AND SYNTHESIS

The primer pairs specific for Canine Distemper Virus (CDV) were designed from the nucleocapsid gene, Feline Calicivirus (FCV) from the structural protein VP1 gene, Canine Parvovirus (CPV) from the structural protein VP2 gene, and Feline Panleukopenia Virus (FPV) from the structural protein VP2 gene. The respective gene sequences for each virus reported from various geographical locations were downloaded from the NCBI nucleotide database (Table 1). These sequences for the respective viruses were aligned using the Multiple Sequence Alignment tool on the T–T-Coffee web Server (https://tcoffee.crg.eu/). Furthermore, the qPCR primers were designed using the Integrated DNA Technologies Primer Quest tool (https://sg.idtdna.com/PrimerQuest/Home/Index) with the qPCR 2 Primers + Probe design, and all other design parameters were set to their default options. The best primer pair (forward and reverse) with a T_m difference of less than 5°C, the probe with a T_m difference of not more than 8°C with the primers, and the set with a GC content of 45-50% were selected for synthesis. Other parameters in the oligo analyser tool, such as the self-dimer, heterodimer, and hairpin T_m, were also carefully considered before proceeding to synthesis. Similarly, the primers and probe for the Internal Control were also designed using multiple sequence alignment of canine and feline GAPDH gene sequences. The binding of each primer site was tested by simulating the primer binding sites using the PCR product tool available in the Sequence Manipulation Suite (SMS) web tool (https://www.bioinformatics.org/sms2/pcr_products.html). The primers were also analysed in silico using NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast) and the University of the Basque Country's bioinformatics in-silico tool (http://insilico.ehu.es/user_seqs/) to assess their specificity against different strains of the target viruses. The fluorophore molecules for the probes were chosen based on the available fluorophore prism in the qPCR

instrument (Bio-Rad Real-Time PCR Machine; CFX Opus 96), and the selected primer-probe sets (**Table 1**) were outsourced for chemical synthesis. The designed primers were synthesised and purified using the HPLC (Barcode Biosciences, Bangalore).

Table1. qPCR primer-probe sets designed for the detection of viral pathogens

S. No.	Target	Primer Sequence 5' to 3'
1	Canine Distemper Virus	F: CTCGGGCAAGAAATGGTTAGA
		R: TCCGTTGTCTTGGATGCTATTT
		Probe: *6-FAM* -TCTGCTGGCAAAGTAAGCTCTGCA -BHQ2
	Gen Bank ID	AF014953, MK037459, KU578257, AY386316, KJ466106, AB475099, MH496778, EU726268, AF378705
2	Feline Calici Virus	F: CCGGAATTTGGAACTGTTTGG
		R: AAGTGGATGCCCATTCATCA
		Probe: *HEX* - TTCAAGTGGTGACCGATCGCAATC - BHQ2
	Gen Bank ID	M86379, KT206207, KC835209, JX519214, AF479590, DQ424892, L40021, KU373057, KJ572401
3	Canine Parvo Virus	F: CGTCTACACAAGGGCCATTTA
		R: TGTTTCTCCTGTTGTGGTAGTT
		Probe: *6-FAM* - 5' TCAAGCAGCAGATGGTGATCCAAGA 3' - BHQ2
	Gen Bank ID	KX766013, KX766021, KU866420, KF366250, KU866402, KC713932, DQ182620, AJ698134, KR869669, JQ996155, KP893078, KF539791, KC196114, JX475234, U72695, JF414823
4	Feline Panleukopenia Virus	F: 5' GAGATCTGAGACATTGGGTTTCT 3'
		R: 5' GTGCCACTAGTTCCAGTATGAG 3'
		Probe: *HEX* - 5' TGGAAACCAACCATACCAACTCCATGG 3' - BHQ2
	Gen Bank ID	OQ266795, MH559110, KX434461, FJ231389, EF988660, KX685354, MW650831, KP769859, OM640096, MN127781
5	Internal Control (IC) - Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)	F: 5' GATGCTGGTGCTGAGTATGT 3'
		R: 5' GAGATGATGACCCTCTTGGC 3'
		Probe: *Cy5* - 5' TTCACCACCATGGAGAAGGCTGG 3' - BHQ2
	Gen Bank ID	NM_001290607, XM_042945628, KT221792, XM_027074123, XM_049624975, OP894024, XM_019464745, XM_053895449, XM_060658612, XM_043562141, XM_025995354, AB038240.

4.3.2. PLASMID INSERT PREPARATION

The synthesised primers were optimised by conventional PCR following the composition and conditions listed in Tables 2 & 3. Gradient PCR was performed at annealing temperatures of 54°C, 56°C, and 58°C for CDV, FCV, CPV, and FPV viruses using nucleic acid extracted from Nobivac DHPPi and Nobivac Tricat Trio vaccine strains as the template. The amplified PCR products were analysed using Agarose Gel Electrophoresis (AGE) with a 2% gel. The target products, expected at 119 bp for CDV, 91 bp for FCV, 143 bp for CPV, and 114 bp for FPV, were observed. These amplified PCR products were then purified using the QIAGEN PCR purification kit following the manufacturer's protocol. The purified products were then used in gene cloning to develop plasmid-positive controls for each virus.

Table 2. Composition of the PCR reaction mix

Reaction Components	Final Volume	Initial concentration	Final concentration
Nuclease-Free Water (NFW)	10.5 µL	-	-
Ampliqon Red master mix	12.5 µL	2X	1X
Forward primer	0.5 µL	10 pmol	0.5 pmol
Reverse primer	0.5 µL	10 pmol	0.5 pmol
DNA template		1 µL	vaccine
Reaction volume	**25 µL**		

Table 3. PCR conditions used for conventional PCR in primer optimisation

PCR Steps	CDV/FCV/CPV/FPV Temperature / Time
Initial Denaturation	94°C / 2 mins
Denaturation	94°C / 30 sec
Annealing	54°C, 56°C, 58°C / 30 sec
Extension	72°C / 30 sec
Final extension	72°C / 10 mins
Cycles	30

4.3.3. DEVELOPMENT OF PLASMID POSITIVE CONTROL

GENE CLONING

Gene cloning is a traditional technique in molecular biology where a gene or target of interest is located and copied (cloned) through recombinant DNA, transformation, and gene isolation. This technique has multiple downstream applications, and in this study, it is utilised in developing the positive controls for the four viruses. The positive controls for CDV, FCV, CPV, and FPV were constructed by cloning their respective viral sequences into pGEM®-T plasmid.

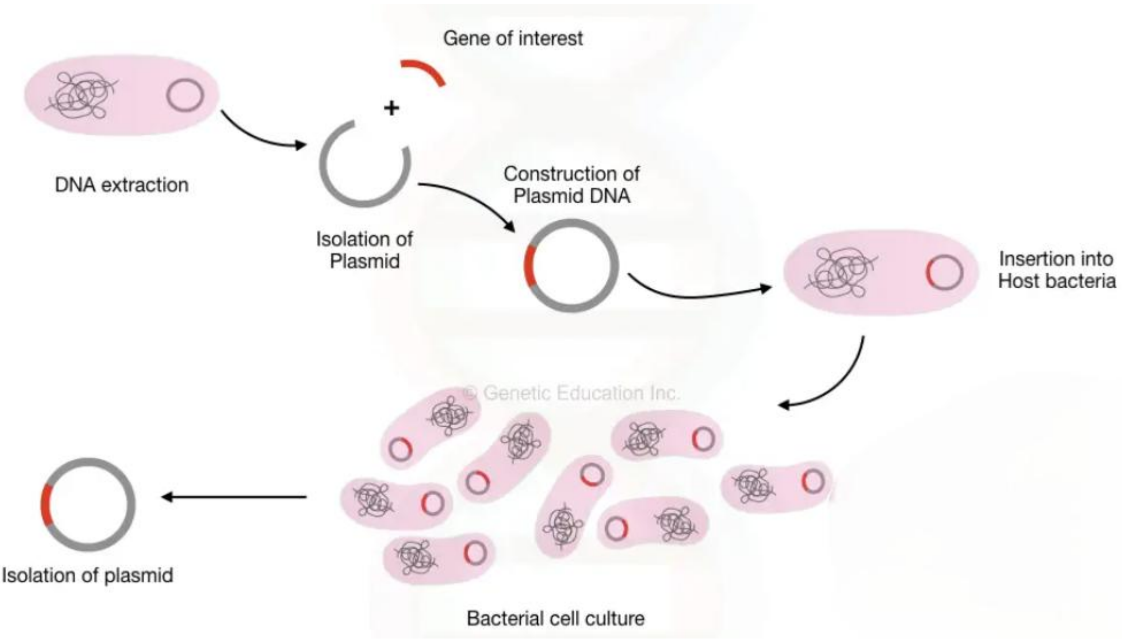

Figure 1. Pictorial representation of gene cloning. (Source: https://geneticeducation.co.in/gene-cloning-definitions-steps-procedure-applications-and-limitations/)

a) Selection of Host Organism, Cloning Vector, and Target Insert

The DH5α strain of *E. coli* was used as the host carrier for gene cloning. The cloning vector used was the pGEM-T Vector (Promega), which has a size of 3 kb. The target inserts, i.e., the viral sequences for the DNA viruses CPV and FPV and the RNA viruses CDV and FCV, were amplified and purified.

b) Preparation of recombinant DNA - Ligation

The ligation was performed using the pGEM®-T Vector System I supplied by Promega Corporation. The T vector is a linearised vector with a single 3' -terminal thymidine at both ends, and the T overhangs at the insertion site improve the ligation efficiency of the PCR products by preventing circularisation of the vector and providing a compatible overhang for the PCR products generated by thermostable polymerases. The Promega ligation kit uses T4 DNA ligase, pGEM®-T Vector, and a rapid ligation buffer.

The cloning molar ratio is a critical parameter in gene cloning. It refers to the ratio of the number of moles of insert DNA to the number of moles of vector DNA. This ratio is essential for ensuring the efficient ligation of the inserted DNA into the vector DNA. An optimal molar ratio can significantly increase the chances of successful cloning, leading to higher yields of recombinant DNA. The recommended optimal ratio, 1:3 vector to insert molar ratio, was calculated for the viral sequences, and the ligation was carried out under the recommended conditions and protocols from the supplier and stored at 4ºC (short-term storage). A negative control ligation was performed by adding a vector but no insert.

To calculate an equimolar quantity of the two fragments, the following formula was used (Bertero, Brown, and Vallier 2017):

$$\frac{\text{Size of the insert (bp)} \times \text{Concentration of the insert}}{\text{Size of the plasmid vector}} \times \frac{3}{1}$$

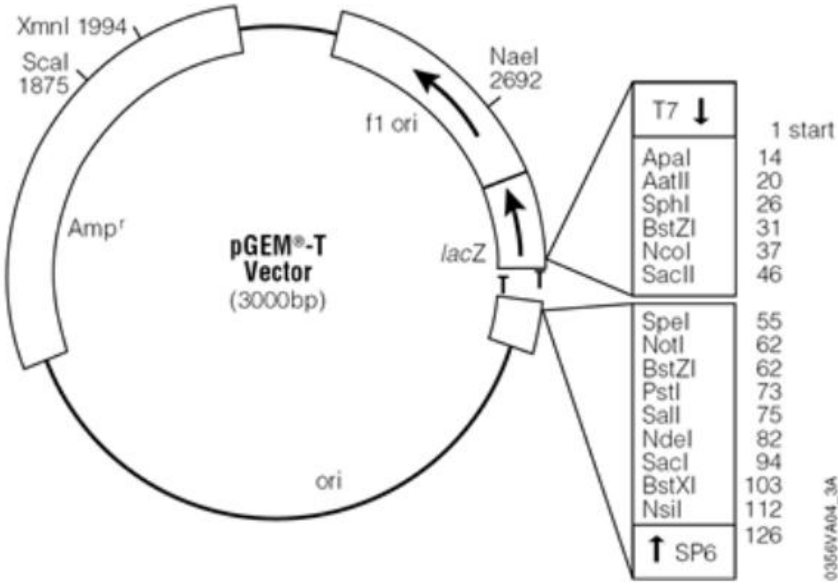

Figure 2. The pGEM®-T Vector is derived from the pGEM®-5Zf (+) Vector (GenBank® Accession No. X65308). The pGEM®-T Vector was created by linearising the pGEM®-5Zf (+) Vector with EcoRV at base 51 and adding a T to both 3´-ends. (Source: pGEM®-T Vector Systems | T Vector Cloning).

c) Preparation of Competent Cells

The *E. coli* DH5α cells were streaked on LB agar media and cultured overnight at 37ºC on an orbital shaker incubator. The fully grown culture was further inoculated in 100 mL LB broth media, and the OD_{600} was observed every subsequent hour. Once the OD_{600} reached 0.3 to 0.4, the culture was transferred to 50 mL Falcon tubes and centrifuged at 1000 × g for 10 min at 4 °C. A loose pellet was observed as a white mass, and the supernatant was discarded immediately. The tubes with the pellets were placed on ice for 5 min.

Furthermore, 40 mL of ice-cold $CaCl_2$ was added to each tube, and the pellet was gently mixed. The sample was then kept on ice for 5 minutes of incubation, followed by centrifugation at 900×g for 5 minutes at 4 °C. Next, 30 mL of $CaCl_2$ was added to the pellet and incubated on ice for an additional 5 minutes, after which it was centrifuged at 1000×g for 10 minutes at 4 °C. The supernatant was discarded, and the pellet was maintained on ice. This pellet, comprising the competent cells, was then stored in 30% Glycerol at -80 °C.

d) Introduction of recombinant DNA into the Host and selection of clones

5 µL of the ligation mix for each virus was used to transform 50 µL of chemically competent DH5α *E. coli)* for cloning into plasmids. The mixture was incubated on ice for 5 minutes, followed immediately by a 90-second incubation at 42°C (heat shock), and then returned to the ice for an additional 5 minutes. Furthermore, 1 mL of SOC was added to each tube and incubated on an orbital shaker at 37°C for 1 hour at 160 rpm. As the plasmid vector used here carries an ampicillin resistance gene, the successfully cloned cells are expected to grow colonies on ampicillin plates. Hence, the transformed *E. coli* was plated on LB agar containing ampicillin (125µg/mL) and incubated overnight at 37°C. The ampicillin-resistant colonies grown were selected.

e) Screening for clones with the desired insert

Bacterial colonies on the plates were individually picked using a sterile tip, and conventional PCR was carried out with M13 plasmid primers to verify the presence of our desired insert.

f) Isolation of plasmid

The colonies in which successful amplification was observed in PCR were then inoculated into 10 mL of LB supplemented with 125 µg/mL ampicillin in a boiling tube and cultured for 16 hours at 37°C with agitation (160 rpm) in an orbital shaker. The plasmid from the 16-hour culture was extracted using a commercial kit (Biologix SMART Prime Plasmid DNA Extraction kit) according to the manufacturer's protocol. The extracted plasmids were then eluted in TE (10 mM Tris-Cl, 0.5 mM EDTA). The extracted plasmids were quantified using a Nano-drop spectrophotometer (Thermo Fischer Scientific, USA). The extracted plasmid was further confirmed by Sanger DNA sequencing for the presence of viral-specific fragments.

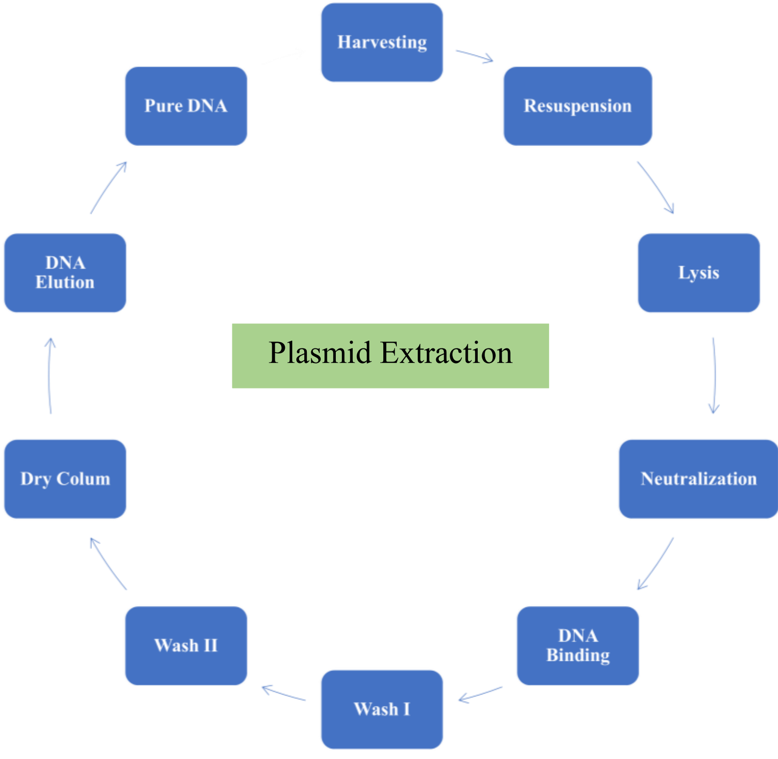

Figure 3. Steps involved in Plasmid extraction

4.3.4. Real-time PCR assay

4.3.5. Primer efficiency

The plasmid DNA quantity was used to measure the copies of viral fragments present in each microliter of the extracted plasmid. An online tool from Thermo Fischer Scientific Ltd. was used to calculate the copy numbers (https://www.thermofisher.com/in/en/home/brands/thermo-scientific/molecular-biology/molecu lar-biology-learning-centre/molecular-biology-resource-library/thermo-scientific-web-tools/dna-copy-number-calculator.html). The plasmids were further diluted 10 times starting from 10^9 to 1 copy per microliter for all four viruses (CDV, FCV, CPV & FPV). RNA viruses CDV & FCV RNAs were converted to cDNA according to the manufacturer's protocol (GoTaq® RT-qPCR, Promega, USA). The qPCR reagent ingredients and the cycling conditions are given in Tables 4 and 5. The assay was performed using the CFX Opus 96 Real-Time PCR System (Bio-Rad) in triplicates. The primer efficiency percentages for CDV, FCV, CPV, and FPV were calculated using the formula Efficiency = $-1 + 10^{(-1/\text{Slope})}$.

Table 4: qPCR reaction composition

Reagents	DNA virus (CPV or FPV)	RNA Virus (CDV or FCV)
Premix (2x)	Promega GoTaq® Probe qPCR – 5 µL (1x)	Promega GoTaq® RT-qPCR – 5 µL (1x)
Forward Primer (10 µM)	0.5 µL (0.5 µM)	0.5 µL (0.5 µM)
Reverse Primer (10 µM)	0.5 µL (0.5 µM)	0.5 µL (0.5 µM)
Probe (10 µM)	0.5 µL (0.5 µM)	0.5 µL (0.5 µM)
Nuclease Free Water (NFW)	2.5 µL	2.5 µL
Template	1 µL plasmid DNA	1 µL plasmid DNA
Total Rxn volume	10 µL	10 µL

Table 5: qPCR cycling conditions

qPCR Steps	CDV/FCV/CPV/FPV
Initial Denaturation	95°C / 2 mins
Denaturation	95°C / 15 sec
Annealing/ Extension	60°C / 30 sec
Cycles	40

4.3.6. Primer-probe optimisation of Single-plex Assay

The Singleplex assays for CDV, FCV, CPV, and FPV were optimised in the qPCR assays using the 10^7 copy plasmid DNA of the viral positive standards as templates varying primer and probe concentrations. Initially, primers of all four viruses varied from 0.1 µM to 0.5 µM while maintaining a constant probe concentration of 0.5 µM. Similarly, primer concentration was kept constant at 0.5 µM, and probe concentration was varied from 0.1 µM to 0.5 µM. The optimum CT-yielding primer and probe concentration were chosen further for multiplex assay.

4.3.7. Internal control (IC) optimisation

The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene primer was used as an internal control for PCR or as a control for extraction. Similar to viral primers and probes, IC primers and probes were also tested for their efficiency by varying concentration from 0.1 µM to 0.5 µM. DNA and cDNA templates were prepared from

DNA/RNA extracted from canines and felines, including Leopard, Lion, Wild Dog, Jackal, and Tiger, from an in-house research sample repository.

4.3.8. Optimisation of Multiplex Assay

The multiplex qPCR assays were designed to test two DNA viruses – CPV and FPV, and the Internal control (IC) in a single reaction tube. The RNA viruses – CDV, FCV, and IC are tested in a separate single reaction tube. The multiplex assay was optimised with positive standards of 10^7, 10^5, and 10^2 plasmid copies. The qPCR cycling conditions were the same as those mentioned in Table 5. Table 6 provides the composition of the multiplex reaction mix.

Table 6. Composition of qPCR Multiplex assay reaction mix for CDV, FCV, CPV, and FPV

Reagents (Stock conc.)	DNA virus (CPV, FPV & IC)	RNA Virus (CDV, FCV & IC)
Premix (2x)	Promega GoTaq® Probe qPCR – 5 µL (1x)	Promega GoTaq® RT-qPCR – 5 µL (1x)
Forward Primer (10 µM)	0.75 µL (0.75 µM) *	0.75 µL (0.75 µM) *
Reverse Primer (10 µM)	0.75 µL (0.75 µM) *	0.75 µL (0.75 µM) *
Probe (10 µM)	1.05 µL (1.05 µM) #	1.05 µL (1.05 µM) #
Nuclease Free Water (NFW)	1.45 µL	1.45 µL
Template	1 µL plasmid DNA / 20-30 ng	1 µL plasmid DNA / cDNA
Total Rxn volume	10 µL	10 µL

Note:

* - 0.75 µM includes 0.3 µM of each forward and reverse primer of the respective viruses and 0.15 µM of each forward and reverse primer of IC.

- 1.05 µM includes 0.4 µM probe from each virus and 0.25 µM probe from IC.

4.3.9. Clinical Sample Collection

The positive swab samples for CDV, FCV, CPV, and FPV were obtained from the Department of Animal Biotechnology, Madras Veterinary College (MVC), Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Chennai. Clinical blood samples, approximately 2 mL each, were collected from 20 domestic dogs (Canine sp.) and 15 domestic cats (Feline sp.) from the outpatient ward, Department of

Infectious Diseases Unit, MVC, TANUVAS, Chennai. Blood collection from AAZP captive wild animals is opportunistic and only possible during medical treatment. Hence, for the assay validation, suspected domestic animals along with positive samples were used. The blood samples were collected from the animals' veins using a butterfly needle in a blood collection vacutainer tube coated with EDTA. The commercially available feline and canine vaccines (Nobivac - DHPPi for CDV and CPV; Nobivac - Tricat Trio for FCV and FPV) were used as controls for validating the developed assay. All these samples are handled and processed in accordance with the relevant biosafety level requirements.

Table 7. Details of blood samples collected from domestic cats (Feline)

S. No.	Sample Name	Age	Sex	Remarks
1	C1	3 months	M	
2	C2	1 year	F	
3	C3	1 year	M	
4	C4	1 year	F	
5	C5	2 years	M	
6	C6	1 year	F	
7	C7	1 year	M	
8	C8	7 months	F	
9	C9	1.5 years	M	
10	C10	2.5 years	M	
11	C11	1.5 years	M	
12	C12	1 year	F	
13	C13	41 weeks	M	
14	C14	3 years	M	
15	C15	3 years	F	
M – Male, F - Female				

Table 8. Details of blood samples collected from domestic dogs (Canine)

S. No.	Sample Name	Age	Sex	Remarks
1	D1	1 year 2 months	M	CPV recovery stage
2	D2	4 months	M	CPV early stage
3	D3	2 months	M	
4	D4	50 days	M	CPV recovery stage
5	D5	3 months	F	Suspected CPV
6	D6	7 months	M	
7	D7	3 months	M	
8	D8	3 months	M	
9	D9	3 months	F	
10	D10	3 months	F	
11	D11	3 years	M	Suspected CDV
12	D12	3 months	F	
13	D13	4 months	F	
14	D14	2 years	M	Suspected CDV
15	D15	2 months	M	
16	D16	1.5 years	M	
17	D17	3 months	F	Suspected CDV
18	D18	2 months	M	
19	D19	7 years	M	
20	D20	4 months	M	
M – Male, F – Female				

4.4.0. RNA/DNA Extraction and cDNA Conversion

The RNA and DNA extractions from the vaccines, positive swabs, and blood samples were performed using commercial viral extraction kit protocols. The nucleic acid extractions from clinical blood samples were performed using two different commercial extraction kits, and the efficiency of the qPCR results was compared. The DNA extraction was performed using the Wizard Genomic DNA Purification Kit (Promega), and RNA Extraction was carried out using the SV Total RNA Isolation System (Promega). The extractions were also repeated using the Thermo Scientific™ GeneJET Viral DNA/RNA

Purification Kit. All extractions were performed according to the respective manufacturer's protocols. The extracted nucleic acids were quantified through a Nano-drop spectrophotometer (Thermo Fischer Scientific, USA). The extracted DNA was directly used as a template for the DNA virus multiplex assay. The extracted RNA was converted to cDNA using the GoScript Reverse Transcription System (Promega) according to the manufacturer's protocol and stored at -20 °C.

4.4.1. Multiplex qPCR Assay Validation

The multiplex qPCR Assay was performed for all four positive swab samples (CDV, FCV, CPV & FPV), two vaccine samples (Nobivac - DHPPi and Nobivac – Tricat) and 35 test samples (20 dogs and 15 cats) along with respective no template controls (NTC) and plasmid standards of all four viruses from 10^7 to 10^3 copies. The qPCR reaction mix composition and cycling conditions are outlined in Tables 4 and 5.

4.4. RESULTS

4.4.1. Plasmid insert development

Plasmid inserts for CDV, FCV, CPV, and FPV were generated by PCR amplification using the respective primers as templates and the vaccines as the source of DNA. They amplified at a wide range of annealing temperatures, and 58°C was found to be optimum. The amplified PCR products were visualised on 2% agarose gel electrophoresis (Figure 4), and the expected bands at 119 bp for CDV, 91 bp for FCV, 143 bp for CPV, and 114 bp for FPV were observed.

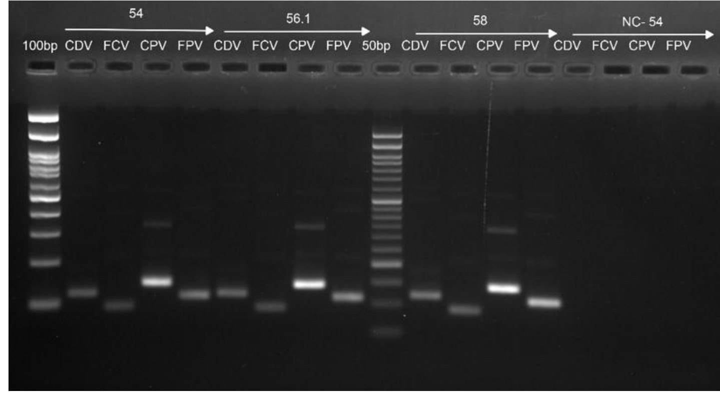

Figure 4: Agarose gel electrophoresis of plasmid inserts. Lane 1 – 100bp ladder, Lane 10 – 50bp ladder, NC – Negative control.

4.4.2. Recombinant plasmid selection

Recombinant colonies were screened through PCR amplification of M13 primers. Clones having fragment size 102 bp in addition to their respective viral fragment size are chosen and cultured further for plasmid extraction. Figure 5 shows the M13 colony PCR-amplified amplicons. The extracted plasmids were further sequenced for their DNA, and the insert fragment was identified using NCBI-BLASTn. The sequence identity percentage indicates that all four virus fragments successfully ligated into the plasmid vector.

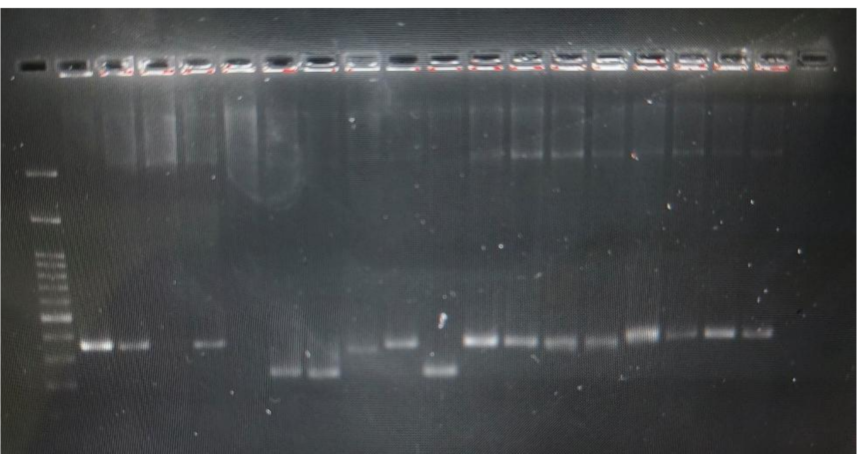

Figure 5. Representative gel electrophoresis for selection of transformed colonies on ampicillin plates. Lane 1- 100bp Ladder; Lane 2 to 5 – Canine Distemper Virus; Lane 6 to 8 – Feline Calici Virus; Lane 9 to 15 – Feline Panleukopenia Virus; Lane 16 to 19 – Canine Parvo Virus; Lane 20 - NC (Negative control).

4.4.3. qPCR efficiency

The qPCR efficiency analysis was performed for the detection of CDV, FCV, CPV, and FPV, and it was found to be 97.7%, 93.3%, 95.2%, and 92.7%, respectively (Figure 6a, 6b, 7a, 7b, 8a, 8b, 9a & 9c). The specificity of the primers was analysed in silico through the NCBI-BLASTn program, and it was found that each primer was 100% specific to its respective virus in the top 100 hits without any cross-species identification. The lower limit of detection (sensitivity) was found to be 100 copies of virus per millilitres of sample.

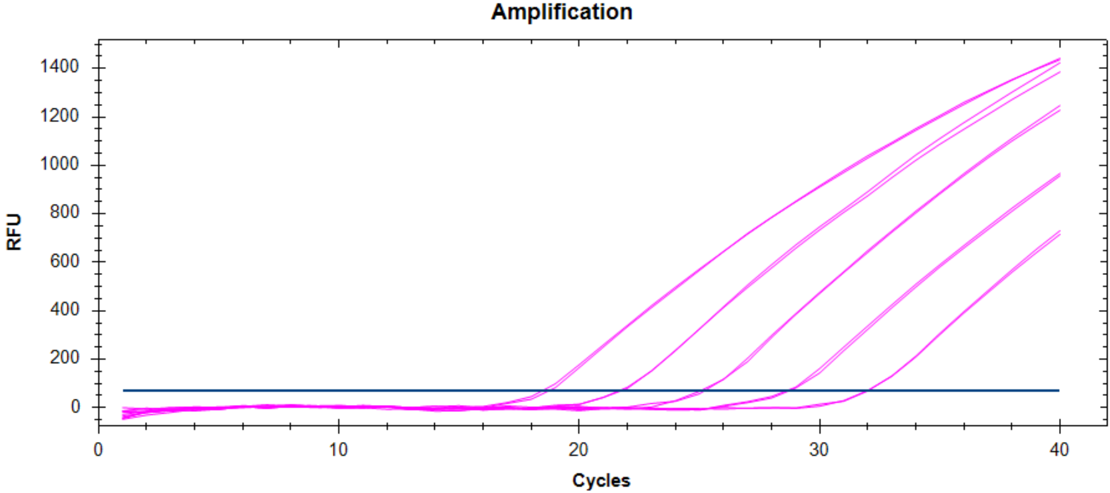

Figure 6a: Amplification plot for Canine Distemper Virus.

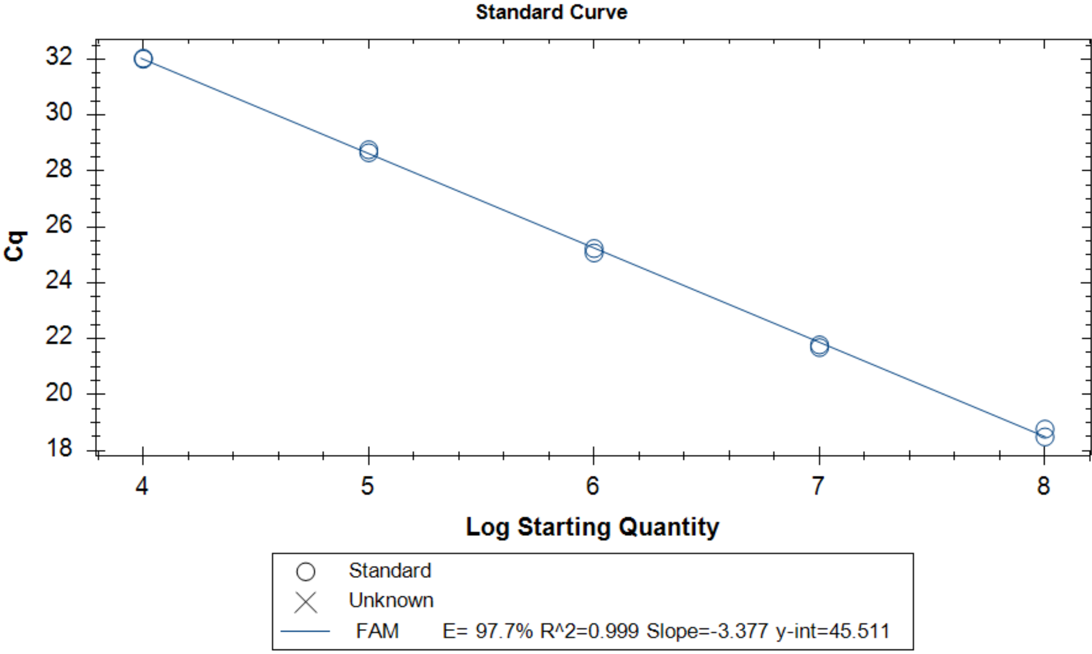

Figure 6b: Standard curve plot for Canine Distemper Virus.

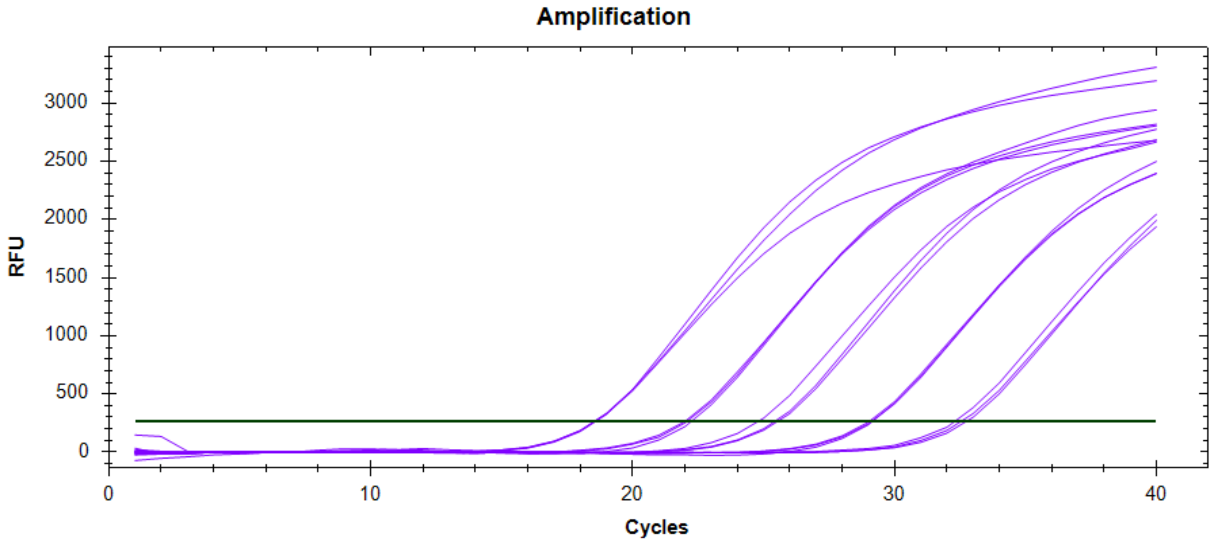

Figure 7a: Amplification plot for Feline Calici Virus.

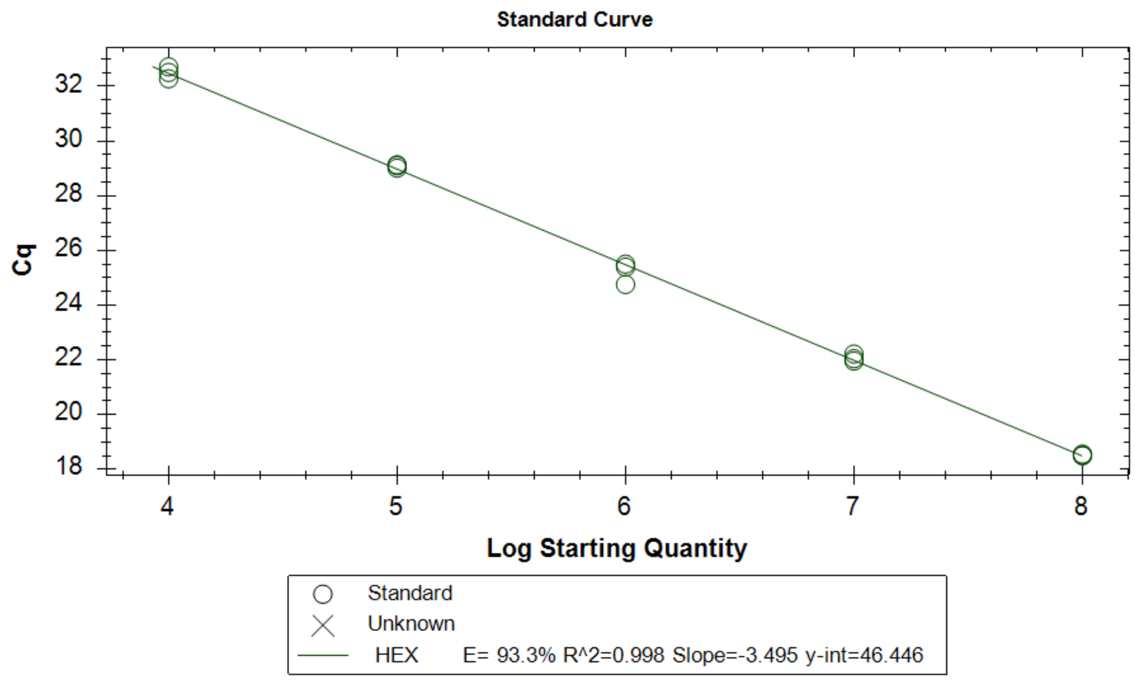

Figure 7b: Standard curve plot for Feline Calici Virus.

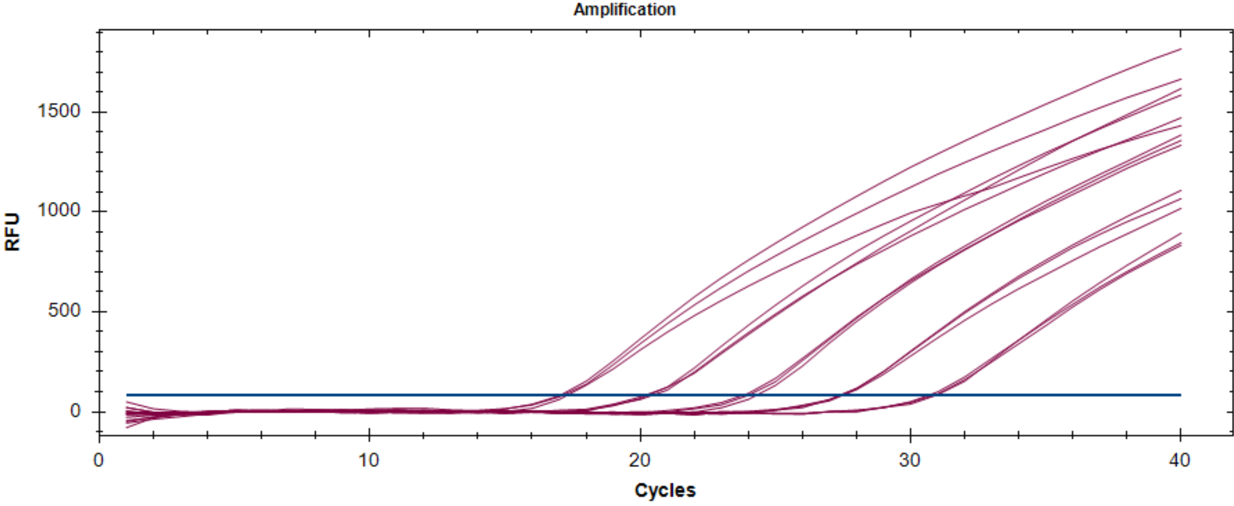

Figure 8a: Amplification plot for Canine Parvo Virus.

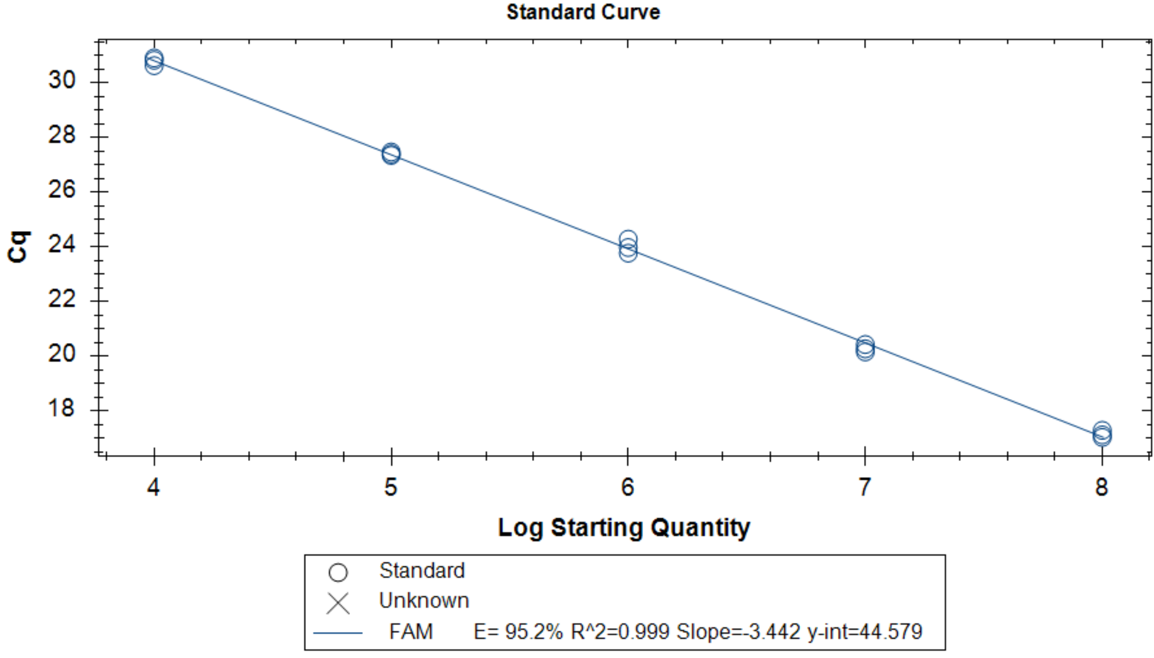

Figure 8b: Standard curve plot for Canine Parvo Virus.

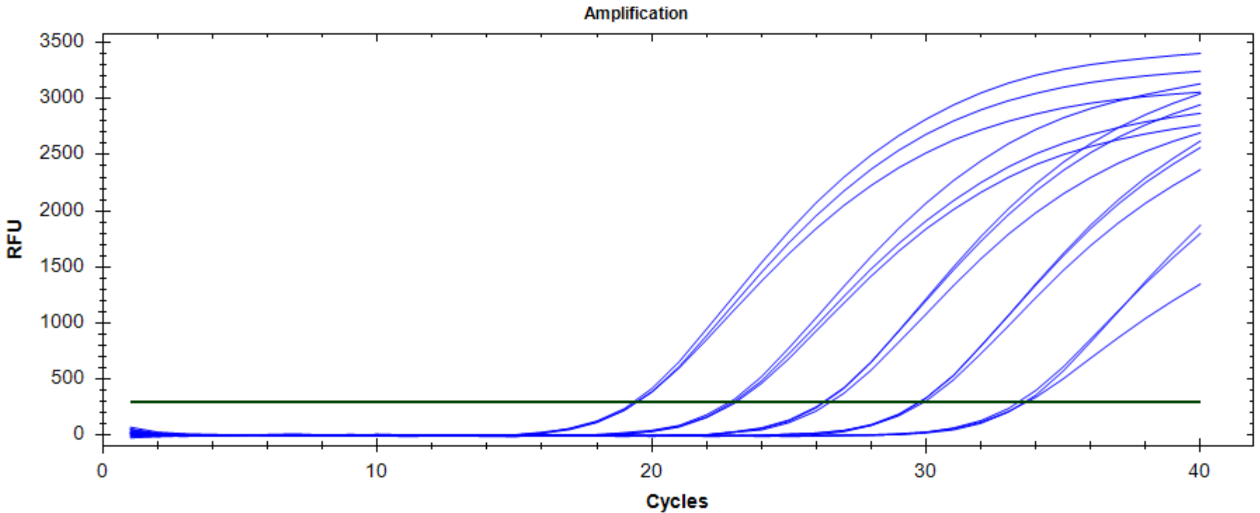

Figure 9a: Amplification plot for Feline Panleukopenia Virus.

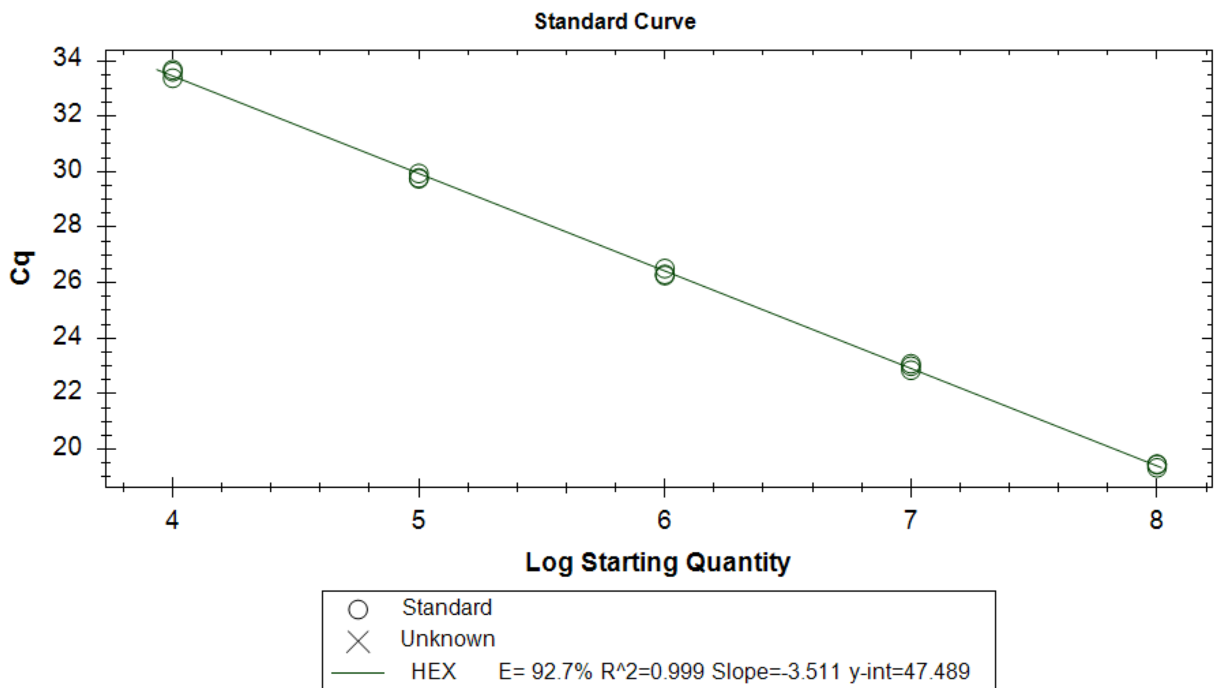

Figure 9b: Standard curve plot for Feline Panleukopenia Virus.

4.4.4. Optimisation of Singleplex and Multiplex assay

CDV, FCV, CPV, and FPV detection assays were initially optimised as a singleplex diagnostic assay. The primers of all four viruses varied from 0.1 µM to 0.5 µM while maintaining a constant probe concentration of 0.5 µM. Similarly, primer concentration was kept constant at 0.5 µM, and probe concentration was varied from 0.1 µM to 0.5 µM. The optimisation of primers and probes was performed using a 10^7 Copy plasmid DNA as a template. It was observed that 0.3 µM of primer and 0.4 µM of probe were able to detect an optimum Ct variation of 30. Similarly, the internal control (GAPDH) primer and probe were tested at concentrations ranging from 0.1 µM to 0.5 µM while maintaining a constant probe concentration of 0.5 µM. Similarly, primer concentration was kept constant at 0.5 µM, and probe concentration was varied from 0.1 µM to 0.5 µM. It was observed that the internal control primer and probe were able to detect vDNA from various canine and feline species, including leopard, Lion, Wild dog, Jackal, and Tiger, at a working concentration of 0.15 µM primer and 0.25 µM probe.

Multiplex diagnostic assays for the detection of DNA viruses (CPV+FPV+IC) and RNA viruses (CDV+FCV+IC) were optimised in a single tube. It was observed that concentrations of 0.3 µM primer and 0.4 µM probe for all four viruses and 0.15 µM primer and 0.25 µM probe for IC were efficient enough to detect 10^2 copies of the plasmid standard.

4.4.5. Clinical sample testing and assay validation

The clinical samples collected from domestic dogs and cats, including viral-positive swabs and vaccine samples, were tested using a developed qPCR multiplex assay. The amplification curve plot and standard curve plot are shown in Figures 10a, 10b, 11a, and 11b, respectively. Just like the Singleplex assay, primer efficiencies in the multiplex assay were also found to be within the range of 90% to 110%. All the tested samples were able to amplify the internal control, indicating that the nucleic acid extraction and PCR amplification had passed the expected assay's quality controls. It was observed that test samples C8, C9, and the Feline vaccine are positive for FCV; D7, D6, D8, D9, D11, and D16, as well as the Canine vaccine samples, are positive for CDV (Table 9). Similarly, test samples C1, C7, C8, the Feline vaccine, and positive

swabs were positive for FPV; D3, D6, D8, D9, D10, D12, D13, D17, D18, and the canine vaccine, as well as positive swab samples, were found to be positive for CPV, respectively (Table 10). It was observed that positive swab samples are not amplified, which might be due to the degradation of the RNA virus stored in the swab. Additionally, CPV-amplified samples are positive for FPV and vice versa. It was expected because both viruses have 98% identical genetic material, and even though primers target the hypervariable viral coat protein, gene cross-amplification was observed.

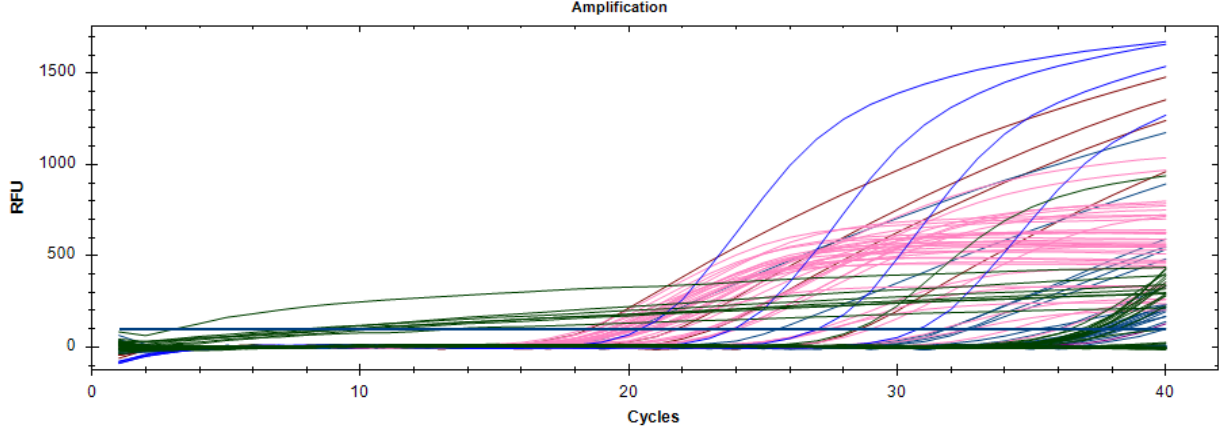

Figure 10a: Amplification plot for RNA virus multiplex detection. Blue lines – Canine Distemper Virus, Brown lines – Feline Calici Virus, Pink lines – IC, and green lines – test samples.

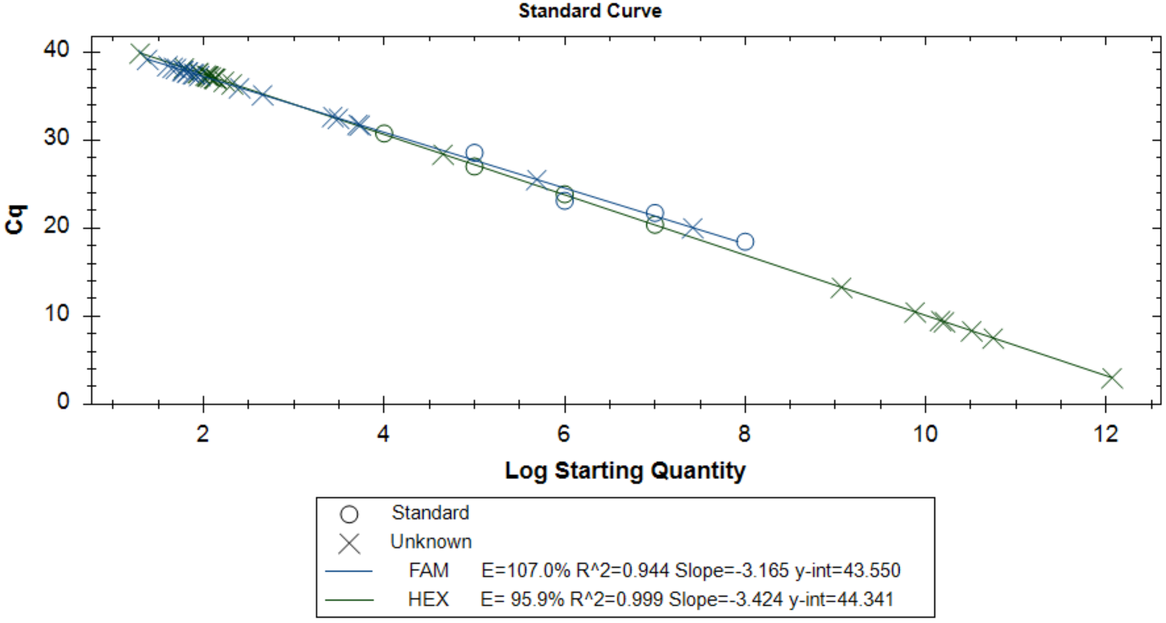

Figure 10b: Standard curve plot for RNA virus multiplex detection. FAM tagged is Canine Distemper Virus, and HEX tagged is Feline Calici Virus.

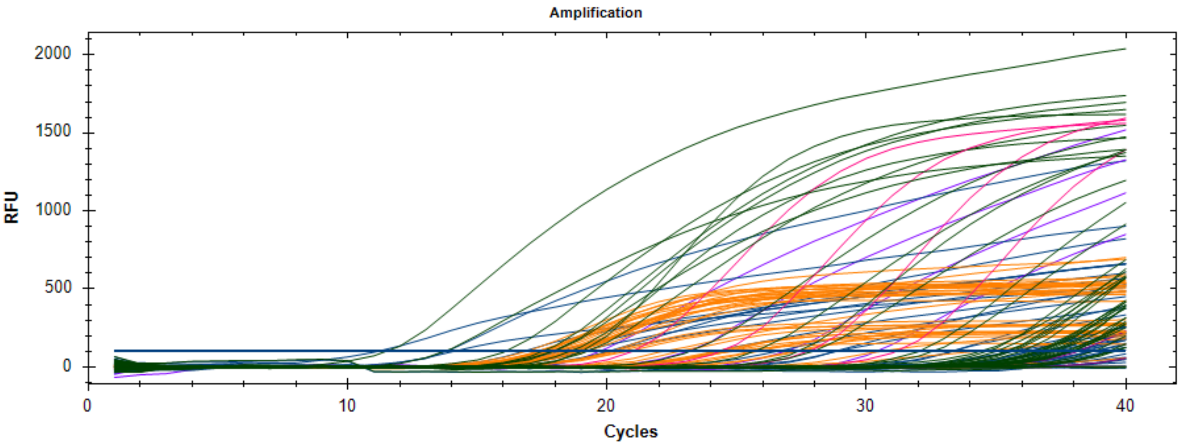

Figure 11a: Amplification plot for DNA virus multiplex detection. Violet lines – Canine Parvo Virus, Magenta lines – Feline Panleukopenia Virus, Orange lines – IC, and green lines – test samples.

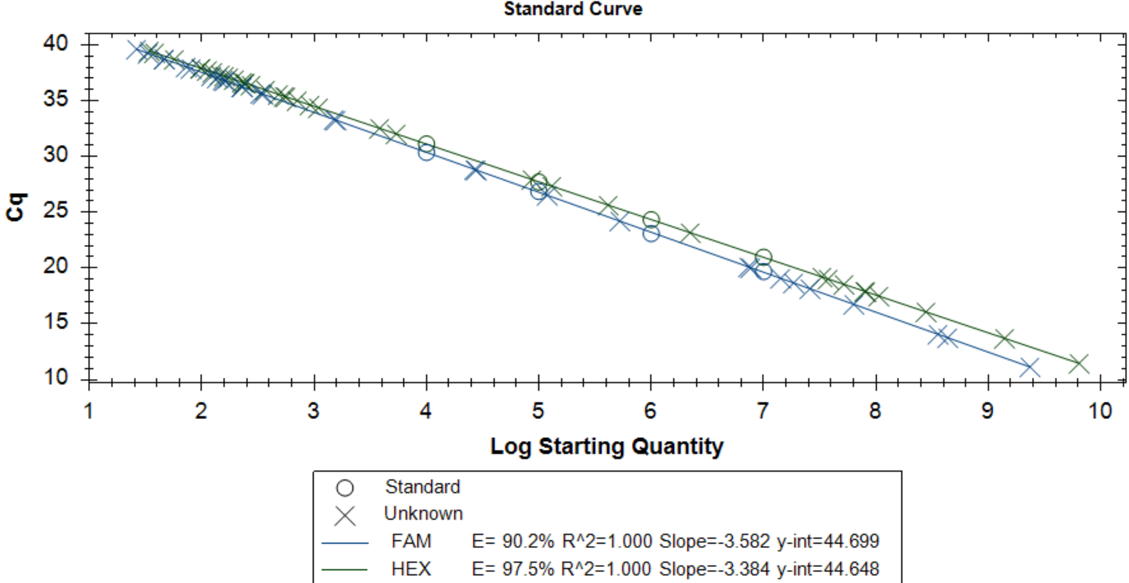

Figure 11b: Standard curve plot for DNA virus multiplex detection. FAM tagged is Canine Parvo Virus, and HEX tagged is Feline Panleukopenia Virus.

Table 9: Summary of diagnostic results for RNA virus multiplex assay

S. No.	Sample ID	Target	Ct value#	Copy number
1	C1	CDV	N.A.	Negative
		FCV	N.A.	
		IC	32.67	
2	C2	CDV	N.A.	Negative
		FCV	N.A.	
		IC	27.07	
3	C3	CDV	37.54	Negative
		FCV	38.22	
		IC	27.39	
4	C4	CDV	N.A.	Negative
		FCV	37.34	
		IC	27.48	
5	C5	CDV	N.A.	Negative
		FCV	N.A.	
		IC	21.86	
6	C6	CDV	N.A.	Negative
		FCV	37.08	
		IC	20.25	
7	C7	CDV	37.90	Negative
		FCV	N.A.	
		IC	21.42	
8	C8	CDV	N.A.	**1.61×10^{10}**
		FCV	**9.39**	
		IC	36.02	
9	C9	CDV	37.58	**1.16×10^{9}**
		FCV	**13.29**	
		IC	29.00	
10	C10	CDV	38.13	Negative
		FCV	N.A.	
		IC	23.81	

11	C11	CDV FCV IC	N.A. 37.66 22.76	Negative
12	C12	CDV FCV IC	37.94 36.70 21.02	Negative
13	C13	CDV FCV IC	N.A. N.A. 23.77	Negative
14	C14	CDV FCV IC	N.A. N.A. 23.01	Negative
15	C15	CDV FCV IC	N.A. 37.39 24.04	Negative
16	D1	CDV FCV IC	35.95 36.40 19.22	Negative
17	D2	CDV FCV IC	38.46 37.69 20.03	Negative
18	D3	CDV FCV IC	N.A. 37.05 19.27	Negative
19	D4	CDV FCV IC	37.77 N.A. 18.29	Negative
20	D5	CDV FCV IC	N.A. N.A. 20.14	Negative
21	D6	CDV FCV IC	37.75 37.28 20.78	Negative

22	D7	**CDV** FCV IC	**32.69** N.A. 20.00	**2.7×10^3**
23	D8	**CDV** FCV IC	**31.73** 39.90 19.69	**5.44×10^3**
24	D9	**CDV** FCV IC	**25.53** N.A. 20.72	**4.9×10^5**
25	D10	**CDV** FCV IC	**31.80** N.A. 20.22	**5.13×10^3**
26	D11	**CDV** FCV IC	**32.49** N.A. 19.69	**3.12×10^3**
27	D12	CDV FCV IC	N.A. 37.20 18.69	Negative
28	D13	CDV FCV IC	38.28 N.A. 19.10	Negative
29	D14	CDV FCV IC	39.18 N.A. 19.31	Negative
30	D15	CDV FCV IC	N.A. N.A. 20.98	Negative
31	D16	**CDV** FCV IC	**20.06** N.A. 19.57	**2.62×10^7**
32	D17	CDV FCV IC	N.A. 37.02 22.86	Negative

33	D18	CDV FCV IC	N.A. N.A. 19.59	Negative
34	D19	CDV FCV IC	37.35 37.30 18.72	Negative
35	D20	CDV FCV IC	N.A. N.A. 20.45	Negative
36	Canine vaccine	**CDV** FCV IC	**35.15** N.A. 31.61	**4.5 x 10^2**
37	Feline vaccine	CDV **FCV** IC	N.A. **28.41** 34.23	**4.49 x 10^4**
38	CDV Swab	CDV FCV IC	37.83 N.A. 38.60	Negative
39	FCV Swab	CDV FCV IC	N.A. 37.28 32.69	Negative
40	NTC	CDV FCV IC	N.A. N.A. N.A.	Negative

Ct value ≥ 35 is negative | N.A. – No amplification

Table 10: Summary of diagnostic results for DNA virus multiplex assay

S. No.	**Sample ID**	**Target**	**Ct value[#]**	**Viral Copies / µL**
1	C1	CPV **FPV** IC	N.A. **18.54** 34.32	**5.2 x 10^7**
2	C2	CPV FPV	N.A. 39.30	Negative

		IC	27.42	
3	C3	CPV FPV IC	38.73 37.91 25.56	Negative
4	C4	CPV FPV IC	N.A. 38.70 27.22	Negative
5	C5	CPV FPV IC	39.24 37.07 24.76	Negative
6	C6	CPV FPV IC	38.73 36.90 24.20	Negative
7	C7	CPV **FPV** IC	33.26 **32.02** 24.97	**5.37 x 10^3**
8	C8	CPV **FPV** IC	28.78 **27.34** 30.62	**1.31 x 10^5**
9	C9	CPV FPV IC	N.A. 37.90 25.85	Negative
10	C10	CPV FPV IC	N.A. N.A. 24.36	Negative
11	C11	CPV FPV IC	N.A. 37.71 24.64	Negative
12	C12	CPV FPV IC	36.25 35.40 20.30	Negative
13	C13	CPV FPV	37.04 35.34	Negative

		IC	20.51	
14	C14	CPV FPV IC	N.A. 35.96 20.82	Negative
15	C15	CPV FPV IC	N.A. 37.20 22.79	Negative
16	D1	CPV FPV IC	37.22 34.62 17.31	Negative
17	D2	CPV FPV IC	N.A. 39.46 18.08	Negative
18	D3	**CPV** FPV IC	**20.10** 19.20 17.74	**7.36×10^6**
19	D4	CPV FPV IC	39.62 36.59 16.91	Negative
20	D5	CPV FPV IC	36.85 35.57 18.92	Negative
21	D6	**CPV** FPV IC	**11.14** 11.46 17.85	**2.34×10^9**
22	D7	CPV FPV IC	35.67 34.37 17.84	Negative
23	D8	**CPV** FPV IC	**28.83** 27.93 18.47	**2.69×10^4**
24	D9	**CPV** FPV	**26.52** 25.64	**1.19×10^9**

		IC	19.23	
25	D10	**CPV** FPV IC	**24.21** 23.17 18.88	**5.25×10^5**
26	D11	CPV FPV IC	36.79 36.39 18.31	Negative
27	D12	**CPV** FPV IC	**33.31** 32.52 17.43	**1.51×10^3**
28	D13	**CPV** FPV IC	**16.75** 16.07 17.32	**6.35×10^7**
29	D14	CPV FPV IC	38.03 36.57 18.67	Negative
30	D15	CPV FPV IC	35.58 35.00 20.17	Negative
31	D16	CPV FPV IC	N.A. 37.33 18.81	Negative
32	D17	**CPV** FPV IC	**18.67** 17.88 22.21	**1.85×10^7**
33	D18	**CPV** FPV IC	**18.15** 17.49 17.64	**2.59×10^7**
34	D19	CPV FPV IC	37.88 37.55 17.48	Negative
35	D20	CPV FPV	36.24 36.65	Negative

		IC	18.55	
36	Canine vaccine	**CPV** FPV IC	**14.07** 13.70 N.A.	**6.35 x 10^8**
37	Feline vaccine	CPV **FPV** IC	19.08 **17.91** N.A.	**7.93 x 10^7**
38	CPV Swab	**CPV** FPV IC	**20.16** 17.91 22.13	**6.85 x 10^7**
39	FPV Swab	CPV **FPV** IC	26.45 **20.30** 28.16	**7.19 x 10^7**
40	NTC	CPV FPV IC	N.A. N.A. N.A.	
# Ct value ≥ 35 is negative | N.A. – No amplification				

4.5. DISCUSSION

The multiplex or triplex Quantitative Real-Time PCR (qPCR) assay was developed for the detection of RNA (CDV and FCV) and DNA (CPV and FPV) viruses in an indigenous setting. Initially, the project objective was to develop a qualitative assay that detects the presence or absence of the virus alone. Indeed, an attempt has been made, and a quantitative assay was developed capable of measuring the copies of the virus present in the samples. The primers for all four viruses were designed from multiple sequence alignments of the different geographical isolates of viruses around the world, including isolates from northern and southern India. The primer-amplified products were cloned into a suitable plasmid vector, and this recombinant vector was used as a standard viral nucleic acid template throughout the assay for detecting viral copy numbers. The qPCR efficiency was found to be within the range of 90% to 110%. The multiplex assay was optimised preciously to use minimal quantities of PCR ingredients, yet yielding a reasonable detection limit of 100 copies of virus per millilitres of the sample.

The tested vaccine samples of Canine and feline origin were amplified successfully in the multiplex assay, allowing for the determination of viral copies. In the case of positive control samples, DNA virus was detected only in swabs samples in the multiplex assay, and RNA virus was not detected. It was also observed that virus-positive swabs were not amplifiable when run independently as singleplex. Hence, we assume that the RNA virus in the swab was degraded due to long-term storage. A total of 15 cats (Feline) and 20 domestic dogs (Canine) were tested in our multiplex assay for RNA viruses. Six domestic dogs were found to be positive for CDV, out of which one sample was initially suspected of being positive for CDV and subsequently tested positive in the multiplex assay for RNA viruses. The unusual death of 28 Asiatic lions in Gir Wildlife Sanctuary was reported due to CDV infections (Mourya *et al.,* 2019). Wild canine species, such as the Silver fox, Jackal, Ethiopian wolf, and Coyotes, were reported to be positive for CDV. Notable feline species, including the lion, Leopard, and Siberian tiger, were also reported to be positive for CDV (Review by Karki, Rajak, & Singh, 2022).

Out of fifteen domestic cats, only two were found to be positive for FCV in our multiplex assay for RNA virus. Harrison *et al.* (2007) reported a systemic feline calicivirus epidemic in captive exotic felids, such as African lions and Amur tigers, at the Potter Park Zoo in South Pennsylvania, which caused the death of one male lion and one male tiger. Still, surprisingly, the snow leopards housed in the same captivity premises were found to be negative for FCV. Nine domestic dogs were found to be positive for CPV in the multiplex assay for DNA virus. CPV is believed to have evolved from a variant of FPV by a mechanism of host shift. Mech *et al.* (2012) reported that free-ranging wolves tested positive for CPV in the Superior National Forest (SNF), Minnesota, USA, and in Yellowstone National Park (YNP), USA. African lions in the Serengeti ecosystem, specifically in the Serengeti National Park of Africa, were reported to be positive for CPV, and the primary reservoir of the virus was found to be free-ranging dogs in the surrounding ecosystem (Behdenna *et al.,* 2019).

In our multiplex assay for DNA virus diagnosis, three domestic cats were detected positive for FPV. Two cases of fatal infection caused by FPV were reported in a white tiger and an African lion at the Lisbon Zoo in Portugal (Duarte *et al.,* 2009). A three-month-old leopard rescued from the Wadsa forest division in Gadchiroli district, Maharashtra, India, was shifted to the Wildlife Rescue Centre, Gorewada, Nagpur. The cub was diagnosed as positive for FPV and recovered after rigorous treatment efforts (Kolangath *et al.,* 2023). Similarly, captive

Siberian tigers have been reported to be infected with FPV (Yeo *et al.,* 2023). The first outbreak of FPV infection in a captive Pallas's cat in Xining Wildlife Park in China (Wei *et al.,* 2024).

Free-ranging wild tigers were reported to be more susceptible to FPV, CDV and FCV in Russia (Naidenko *et al.,* 2018; Gilbert *et al.,* 2023,) while there were significantly fewer studies available for Indian free-ranging wild tigers. In our validation of a multiplex assay for DNA and RNA viruses in domestic cats and dogs, it was observed that one sample (C8) from a cat was positive for both FCV and FPV. In contrast, three samples (D8, D9, and D10) from domestic dogs were positive for both CPV and CDV infections. A similar pattern was reported in free-ranging dogs in the Serengeti ecosystem of the Serengeti National Park in Africa, with a mixed infection of both CPV and CDV, which serves as a significant reservoir of virus infecting African lions in that ecosystem (Behdenna *et al.,* 2019). In another study, Giant Pandas in China were found to be infected with both CDV and CPV (Liu *et al.,* 2025).

4.6. CONCLUSION

NEED FOR THE DEVELOPMENT OF INDIGENOUS ASSAY

Considering all these aspects of emerging diseases in wildlife, and as most viral diseases are zoonotic, the importance of surveillance and early detection is well established. Such a monitoring system for prevalence is necessary, especially in captive animals in Zoos. Some viral infections are lethal to the Host and can become complicated during treatment or become untreatable in their chronic stages. The actual onset of viral infection varies from Host to Host and among different viral serovar types. The increasing emergence of zoonotic diseases underscores the need for a proactive approach to disease surveillance and control. The interconnected nature of human, animal, and environmental health calls for a multidisciplinary One Health approach to mitigate the risks associated with zoonotic pathogens. Improved diagnostic testing, surveillance, and international cooperation are essential to addressing these emerging threats. Without a concerted effort to bridge the gaps between human and veterinary medicine, the world remains vulnerable to future zoonotic disease outbreaks. The detection system developed in this study is a Real-time PCR assay, a modern, high-performance molecular tool for detecting two Canine and two feline viruses: Canine Distemper, Canine Parvovirus, Feline Panleukopenia, and Feline Calicivirus, using a Two-step real-time RT-PCR amplification protocol. The SOP may be referred in Annexure 1 of this report.

5. Further work

The present assay was validated for domestic animals; unfortunately, captive wild animal samples were not received during the study period as it was mainly opportunistic and hence, it is recommended to test the assay with samples obtained from captive and free-ranging wild animals of Canidae and Felidae that are suspected to be infected with the above four viruses.

6. Recommendations

The developed qPCR Multiplex diagnostic assay may support the Arignar Anna Zoological Park's captive animal surveillance for the screening of viral pathogens. Wildlife managers may utilise this test as part of their wildlife health management. Wildlife managers may also utilise the developed assay as an extended viral disease surveillance program in the free-ranging wild animals by testing their non-invasive faecal samples.

7. Output:

A. Technology development

- **i) Technology developed at lab scale/pilot scale/commercial scale:** LAB SCALE
- **ii) Technology demonstration in field setup –** NO
- **iii) Technology transfer to industry –** NO
- **iv) Buyers/ end users:** NIL
- v) **Creation of improved product:** REFER TO FURTHER WORK.
- **vi) Affordability:** MOST ECONOMICAL

B. Knowledge creation

- **i) Publications:** REQUIRE TESTING WITH CAPTIVE WILD ANIMALS – INADEQUATE
- **ii) Patent filings:** NIL
- **iii) Conference/workshops attended:** NIL

C. Capacity building

- **i) Officers/faculty:** AWARENESS INCLUDED IN REGULAR TRAININGS.
- **ii) Temporary manpower recruited/trained:** ONE – PROJECT ASSOCIATE-II.

D. Added value of project outcomes: AAZP CAPTIVE WILD ANIMAL SCREENING.

E. Achievements of the project: DETECTION LIMIT AS LESS AS 100 VIRAL COPIES.

F. Shortfalls/constraints faced: WILD ANIMAL SAMPLES NOT AVAILABLE TO TEST.

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ANNEXURE – 1

STANDARD OPERATING PROCEDURE

1. Thaw all reagents on ice and prepare a plate map template for the addition of test samples, standards, and control samples.

2. Prepare a premix by addition of the following: 1 μL of probe master mix (2x), 0.3 μL of each forward and reverse primer of CDV & FCV for RNA multiplex assay and CPV & FPV for DNA multiplex assay, 0.4 μL of the respective viral probe, 0.15 μL of each forward and reverse primers of IC and 0.25 μL of IC probe, 1.45 μL of nuclease-free water.

3. 9 μL of prepared premix was added aliquoted to all test wells or strips.

4. No template control (NC) was also included in duplicates.

5. 1 μL of standard plasmid control of respective viruses was added as known viral copy standard (10^8 to 10^4 copy).

6. 1 μL of DNA or cDNA (for RNA virus test) was added as a template for the test sample.

7. The CFX Maestro software was used to create the PCR conditions. Initial denaturation was 95°C for 2 minutes, and 40 cycles of denaturation at 95°C for 15 seconds, followed by annealing/extension at 60°C for 30 seconds. The fluorescent emitted was captured for every cycle.

8. After completing qPCR, analyse the NC quality and cycle baseline threshold and adjust the threshold line if required.

9. The data can be exported in Excel format for manual analysis.

10. The CFX Maestro software has the advantage of automatically calculating the viral copies present in test samples that are tested positive, which can be further exported as a report.

Established in October 2017, Advanced Institute for Wildlife Conservation (AIWC) is one of its kind research institution set up by the forest department of Tamil Nadu primarily to provide scientific solutions to conservation problems. The institute conducts multidisciplinary wildlife research, capacity building in wildlife conservation, training programs and advisories in wildlife research by partnering with leading research institutions in India. The AIWC conducts captive wildlife research in close coordination with Arignar Anna Zoological Park and other zoological parks of Tamil Nadu. The Institute is actively engaged in research across the length and breadth of Tamil Nadu on biodiversity related issues.