The Pharma Innovation Journal 2023; 12(7): 1411-1416

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; 12(7): 1411-1416

www.thepharmajournal.com
Received: 01-04-2023
Accepted: 05-05-2023

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Standardization and validation of TaqMan real-time qPCR targeting VP2 gene of feline parvovirus

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Abstract
Feline panleukopenia is one of the deadly enteric viral diseases affecting cats caused by the feline parvo virus. Early diagnosis is the key factor which decide the survivability of the infected cat. This study was designed to standardize and validate TaqMan Real-Time PCR to detect FPV from faecal samples of infected cats. The R^2 value and % E of TaqMan Real-Time qPCR were 0.9707 and 90.8058 percent respectively. A total of 50 samples, which included 25 conventional PCR positive and 25 conventional PCR negative samples were tested to validate the Real Time PCR. The sensitivity, specificity, and kappa statistics value of TaqMan Real-Time qPCR where, 100%, 92.59%, and 0.913 respectively. As conclusion, the TaqMan qPCR protocol is highly sensitive, specific, and perfect agreement with conventional PCR.

Keywords: Feline panleukopenia, feline parvovirus enteritis, FPV, TaqMan real-time qPCR, diarrhoea and vomiting in cats, phenol chloroform isoamyl alcohol

Introduction
Feline parvovirus is one of the deadly enteric viruses which causes feline viral enteritis and feline panleukopenia in cats (Rehme *et al*., 2022) [12]. FPV is clinically a lethal virus which could cause Anorexia, diarrhoea, vomiting, and severe dehydration and in most conditions mortality rate will reach up to 95 to 100% (Jacobson *et al*., 2021) [7] and majority of the casualties are because of delayed diagnosis and treatment. Early diagnosis of FPV is most crucial part for effective treatment and survivability of cats.
Point of care Lateral flow assays, ELISA based diagnostic assays, PCR assays etc has been already available for detection of FPV (Wang *et al*., 2019; Abd-Eldaim *et al*., 2009; Schunck *et al*., 1995) [16, 1, 13], but to detect the survivability and response to treatment there exist a requirement of cost-effective quantitative tests with higher sensitivity. LFAs are usually used as point of care diagnostic tool in clinical practice but sensitivity and specificity of the LFAs are mostly up to 80% each (Hasan *et al*., 2016) [6]. Moreover, LFAs are qualitative tests which cannot give viral loads, which is the most important parameter, determining the duration of isolation protocol for infected cats. ELISA on the other hand could give the viral load but most of the ELISA kits come 1with a higher price in comparison with other tests.
FPV are small ssDNA viruses (20 nm) with around 5000 bp length. Two open reading frames are the major parts of FPV genome, these ORFs express NS1, NS2, VP1, VP2 and the capsid. NS1 is the most important protein which regulates nicking of the viral DNA to genomic 5' end during replication and the virus replication using host cell DNA polymerase. The formation of capsid protein is by VP1 (90 percent) and VP2 (10%) proteins. The VP1 holds major functions (phospholipase A2 enzyme activity) necessary for cell infection (Za´dori *et al*., 2001) [18]. VP2 protein is the main part of the viral capsid which act as an important protective antigen which determines hemagglutination, species tropism, cellular tropism, and induction of neutralizing antibodies.
Genetic detection of pathogen makes PCR a unique highly sensitive and specific laboratory test to perform confirmatory diagnosis of pathogens (Yang *et al*., (2004) [17]. There are many types of PCR such as Standard PCR- Variants, Reverse Transcription-PCR (RT- PCR), Real time-PCR or quantitative PCR (qPCR) RT-PCR/qPCR combined etc and out of them qPCR is useful to estimate viral load in clinical samples (Singh *et al*., 2014) [14]. There are many PCR based diagnostic techniques to diagnose FPV infection in cats such as standard conventional PCR (Oğuzoğlu *et al*., 2013; Awad *et al*., 2018; Steinel *et al*., 2000; Zhang *et al*., 2019) [11, 2, 15,

[19] and qPCR (Jacobson *et al*., 2021; Cao *et al*., 2022) [7, 3]. In most PCR techniques VP2 gene was used as target gene for genetic identification of FPV.

To perform a pilot study to identify FPV prevalence and quantification of FPV viral load in clinical samples in limited economic conditions there exists a requirement of cost effective standardized qPCR protocol. So, the objective of this study to standardise and validate probe-based qPCR targeting VP2 gene of FPV through a cost-effective protocol for the quantitative diagnosis of feline panleukopenia.

Material and Methods

Materials used in this study

Positive control

DNA extraction was carried out from Feligen ® CRP vaccine using PCI (phenol: chloroform: isoamyl alcohol (25:24:1)) protocol (Green and Sambrook, 2017) [4] and subjected to conventional PCR using custom designed primers (Table 1). The conditions of the PCR were initial denaturation of 95 °C for 5 minutes, then 35 cycles of 95 °C for 20 seconds, 58 °C for 30 seconds and 72 °C for 30 seconds, final extension was done at 72 °C for 10 minutes. Reaction mix composition were 400 nM primers, 25 µl of Ampliqon red master mix and nuclease free water up to 50µl.

The generated conventional PCR product (Fig 1) contains the sequence of TaqMan Real-Time PCR product. Then the gel electrophoresis was done using agarose gel with a combination of high melting temperature agarose (two %) and low melting temperature agarose (0.2%) (fig2) at 50V for 1.5 hours then PCR product were transferred to a sterile 1.5 ml Eppendorf tube using 1ml pipette (Ma and Difazio, 2008) [10]. The gel purification was done using PCI protocol (Green and Sambrook, 2017) [5]. The DNA precipitate was dissolved in 100 µl NFW and quantified using nanodrop spectrophotometer and the concentration was 23.5 ng/µl.

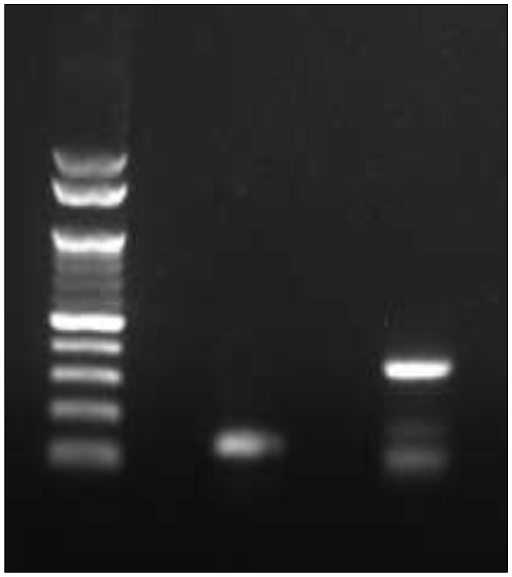

Fig 1: Gel electrophoresis with inner and outer primers; from left, 100 bp ladder, TaqMan RT-qPCR product and outer PCR product

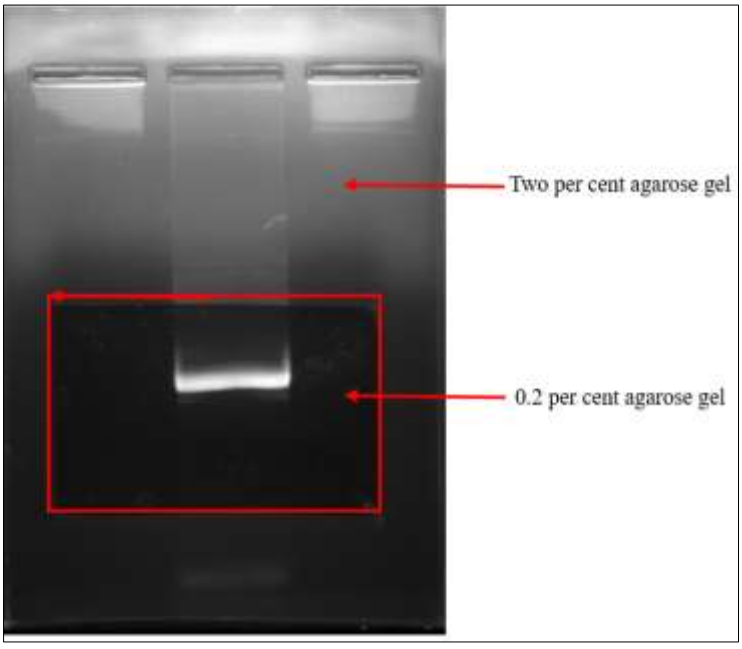

Fig 2: Agarose gel purification of positive control DNA.

Clinical samples

A total of 100 faecal swabs were collected from clinically suspected cats with clinical signs of vomiting, diarrhoea, and dehydration from cases presented to clinical complex, Madras Veterinary College, Chennai-07 during January 2022 to June 2022. DNA extraction was performed from all the samples using PCI protocol. Then all the samples were screened for FPV using custom designed primers targeting VP2 gene of FPV. Then 25 PCR positive and 25 PCR negative samples (Fig 3) were selected from the 100 samples to validate TaqMan RT-qPCR.

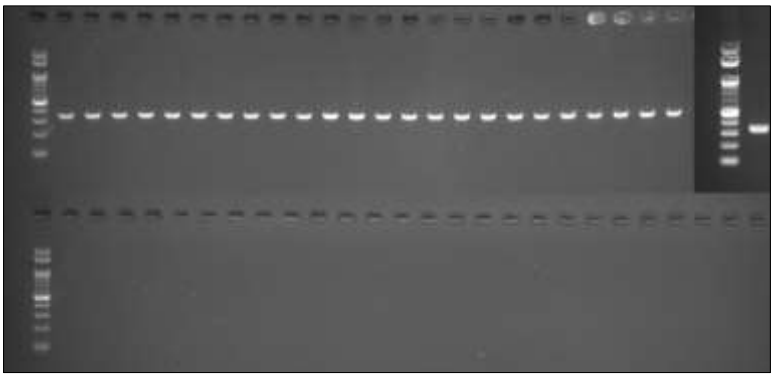

Fig 3: Gel electrophoresis images of conventional PCR positive and negative samples used in this study 100 bp ladder was used and all the samples that gave 345bp product size considered as PCR positive samples and samples without any band were considered as PCR negative samples.

Master Mixes used in this study

For conventional PCR, Taq DNA Polymerase 2x Master Mix RED (1.5 mM MgCl2 final concentration, Cat. No: A180301) was used and for TaqMan RT-qPCR, TaqMan™ Fast Advanced Master Mix for qPCR (Cat. No: 4444556) was used.

Primers and probe sequences

The primers and probe sequences were used in the study and their positions were mentioned in table 2. For TaqMan RT-qPCR, primers (rt-fpv-f and rt-fpv-r) and probe (fpv probe) sequences were adopted from Cao *et al*., (2022) [3]. For the positive control DNA purification custom designed primers (o-fpv-f and o-fpv-r) were used and for screening of FPV by conventional PCR also custom designed primers (c-fpv-f and c-fpv-r) were used.

Table 1: Primers and probe sequences used in this study and their positions in FPV VP2 gene based on GenBank Accession number OM918773.1 data

Primer name	Sequence	Position	Product size
o-fpv-f	AGCAGTTCAACCAGACGGTG	12-31	329 bp
o-fpv-r	CATCAACCAATGACCAAGGTGT	319-341	
rt-fpv-f	CGGGGGTGGTGGTGGTT	87-103	111bp
rt-fpv-r	GCTTGAGTTTGCTGTGATTTCC	177-198	
fpv probe	6-FAM- CTGGGGGTGTGGGGATTTCTACG - TAMARA	104-126	
c-fpv-f	$^{5'}$TGCCAGAAAGTGAAAATTA$^{3'}$	217-236	345bp
c-fpv-r	$^{5'}$CTGGAGTAAATGGCATAGTAT$^{3'}$	542-562	

Conventional PCR

The final primer concentrations were 300 nM each for forward and reverse primers, the template concentration was between 50 ng/µl to 100 ng/µl with 25 µl final volume of PCR mix and 12.5 µl Ampliqon red master mix. The conditions used for PCR reaction were, primary denaturation at 95 °C for five minutes, denaturation at 95 °C for 30 seconds, annealing at 56.5 °C for 20 seconds, extension at 72 °C for 30 seconds, and final extension at 72 °C for five minutes.

TaqMan RealTime PCR

The standardised components for TaqMan Real Time PCR were 400 nM each of sense and antisense primers, 200 nM of probe, TaqMan Fast Advanced Master Mix, and DNA sample (50 ng/µl- 100 ng/µl). qPCR reaction conditions were polymerase activation at 95 °C for 20 seconds then 40 cycles of denaturation at 95 °C for 1 second and 60 °C for 20 seconds. The standard curve was prepared based on dilution of PCR product (Green and Sambrook, 2018) [5] after that initial DNA copy number quantification was carried out.

Preparation of standard curve

PCR product (23.5 ng/µl) was then serially diluted according to DNA copy number (dilution factors were calculated from Thermo-scientific online software (DNA Copy Number Calculator | Thermo Fisher Scientific - IN) as follows; $5x10^{10}$, $5x10^{9}$, $5x10^{8}$, $5x10^{7}$, $5x10^{6}$, $5x10^{5}$ and $5x10^{4}$. Then subjected to TaqMan RT-qPCR and recorded Cq values then prepared standard curve using Graphpad prism version 7based on recorded Cq values and copy number.

Quantification of initial DNA copy numbers

The DNA copy number was calculated using the formula Xo = E_{amp}(b-Cq) where X0 is the starting DNA copy number, Eamp is exponential amplification value for the qPCR assay, b is intercept, and m is slope of the standard curve.

Statistical analysis

Regression value (R^2) value and PCR efficiency were calculated using standard curve constructed using Graphpad prism version 7. Then the agreement between the conventional PCR and TaqMan RT-qPCR were analysed using Cohen's kappa statistics.

Results

Regression value (R^2) value and PCR efficiency were found to be 0.9707 and 90.81% respectively (Fig 4) and amplification curve of the same positive samples were represented as Fig 5. Cq value ranges, Mean Cq value, standard deviation, and copy number were represented in Table 2.

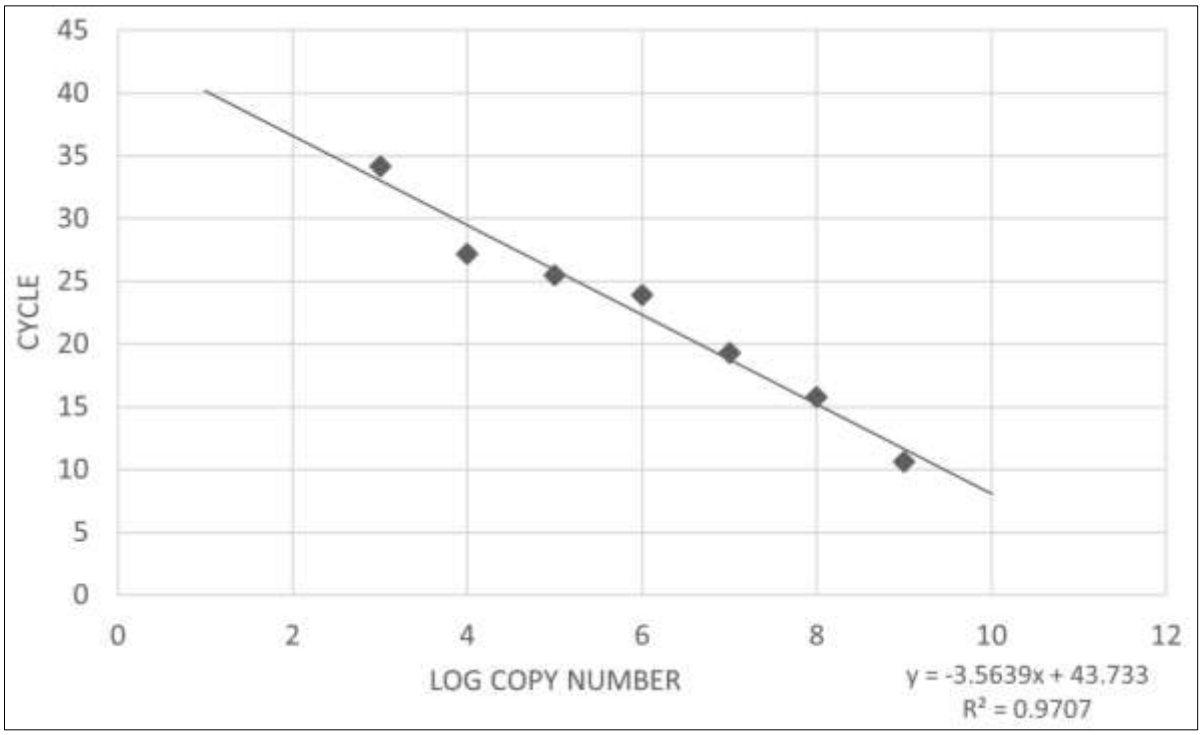

Fig 4: Standard curve

Table 2: Copy number, Cq values, Mean Cq and standard deviation of Cq values of the serially diluted samples

Copy number	Cq value	Cq value of replicate	Mean Cq	Standard deviation
$5x10^{10}$	10.65	10.58	10.615	0.049497
$5x\ 10^{9}$	15.67	15.82	15.745	0.106066
$5x10^{8}$	18.24	20.38	19.31	1.513209
$5x10^{7}$	23.28	23.51	23.395	0.162635
$5x10^{6}$	24.13	26.89	25.51	1.951615
$5x10^{5}$	27.02	27.41	27.215	0.275772
$5x10^{4}$	33.12	36.25	34.685	2.213244

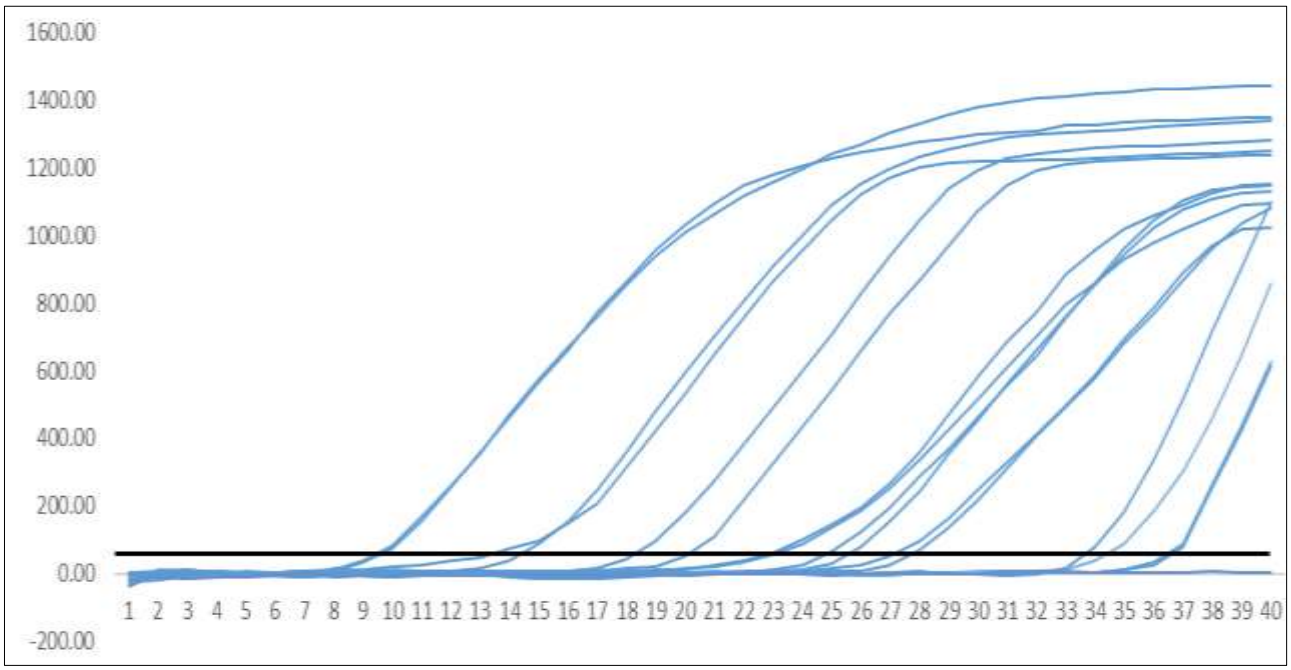

Fig 5: Amplification curve

Out of 50 samples tested, 27 samples were positive out of 50 samples tested by TaqMan Real-Time qPCR and the amplification curve was represented in fig 5. The calculate initial DNA copy number was represented in fig 6.

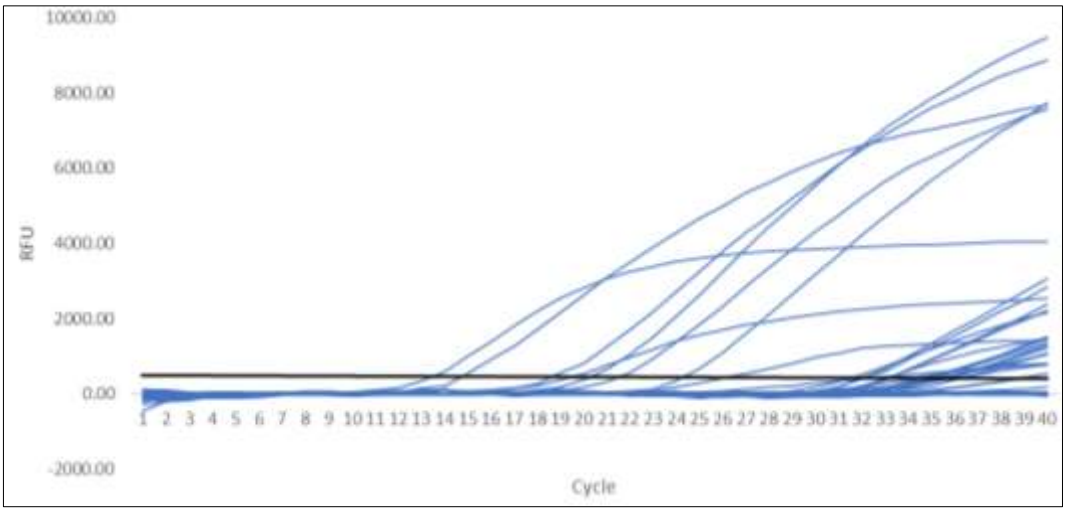

Fig 6: Amplification curves

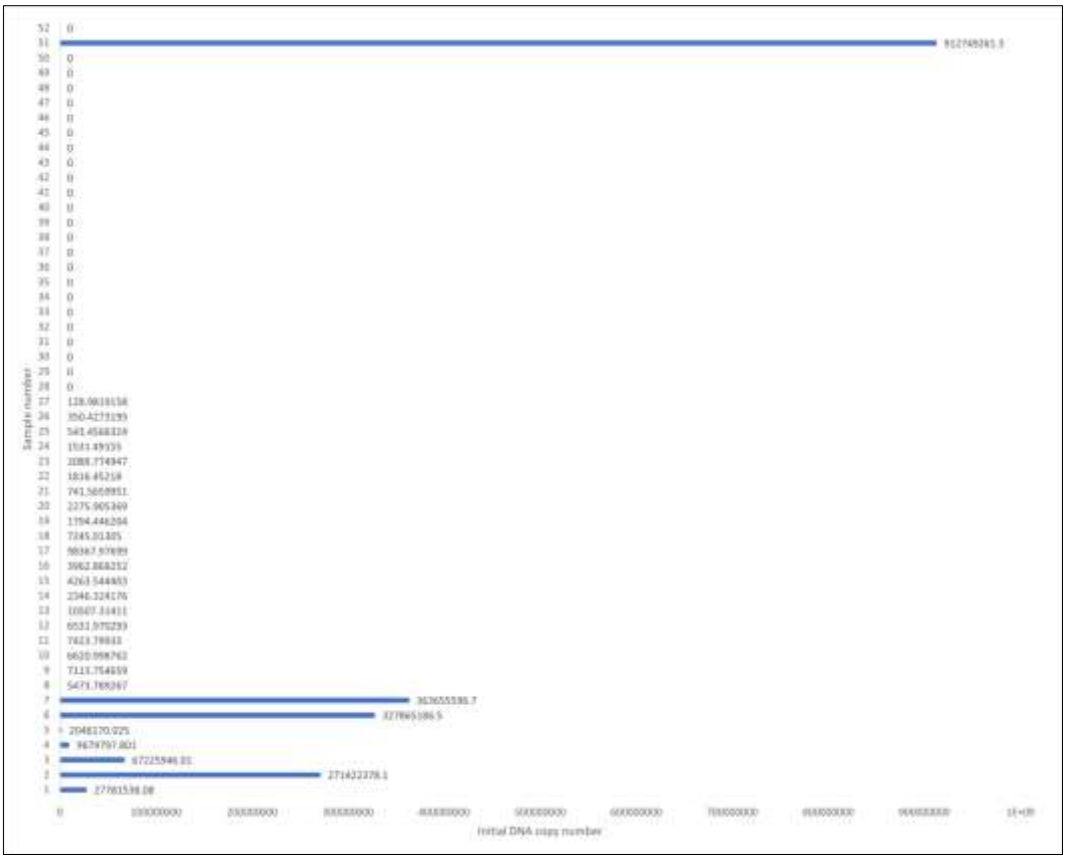

Fig 7: Initial viral DNA copy numbers based on TaqMan RT-qPCR.

The results of TaqMan Real-Time qPCR were compared with conventional PCR using Cohen's kappa statistic k = $(p_0-p_e)/(1-p_e)$, ie., k = 0.913 which means near perfect agreement is observed between conventional PCR and TaqMan Real-Time qPCR.

Discussion

Molecular detection of pathogens especially PCR is one of the greatest diagnostic methods to detect various infectious agents in animals. TaqMan Real-Time qPCR is more sensitive than conventional PCR (Lin *et al*., 2017) [9] because the former is working based on probe based fluorescent detection which could effectively identify copy number of starting DNA concentration.

To construct standard curve, cloning of target sequence is being used everywhere, this procedure makes the assay more costly. In this study we employed nested PCR principle to create pure positive control DNA and avoided the cloning step. The assay created by Cao *et al*., 2022 [3] using the same primers and probe with BHQ quencher had more R^2 value (R^2 = 0.999) and more efficiency (99%) because of the inclusion of cloning step but in the present study we tried with TAMARA quencher dye to reduce the price for probe.

There result of the present study was the standardized assay with R^2 (0.9707), efficiency more than 90% (90.8058) and the assay was strongly in agreement with conventional PCR (k = 0.913). Even though the standardized assay was inferior to the reference assay, it can be employed in limited economic

conditions as it have good R^2 value and PCR efficiency.

Conclusions

FPV is one of the most virulent enteric viruses which affects Felidae, primarily domestic cats. Early diagnosis is most crucial regarding survivability of cats. Even though many commercial kits are available to diagnose this disease, cat owners in developing countries are facing problem with the expense of these kits. For the solution of the above said problems, in this study a standardised TaqMan qPCR with R^2 value of the 0.9707 and PCR efficiency was 90.8058 percent were designed and tested without any use of commercial kits. It's Cohen's Kappa statistic value, 0.913 shows the strong agreement between TaqMan Real-Time qPCR and conventional PCR. The assay is cost effective and reliable**,** which could be employed in field level in developing countries.

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