Research Square

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Development and evaluation of a SYBR Green I RT-qPCR assay for Feline Infection Peritonitis virus detection

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Abstract

Background Feline Infectious Peritonitis (FIP) is a fatal, systemic disease caused by a mutant form of Feline Infectious Peritonitis virus (FIPV) and has been reported to occur worldwide in domestic cats and massive wild feline species. Meanwhile a definitive diagnosis of FIPV ante mortem remains challenging. The objective was to develop a qPCR for the detection of FIPV in cats and applied the assay to detected the viral loads in different autopsied organs of a cat naturally infected with FIPV. Results: After genetic comparison, We develop a SYBR Green I based quantitative transcription PCR assay (qPCR) targeting the structural protein N of FIPV. The sensitivity of the new assay in detecting FIPV nucleic acids was approximately 1000 times higher than that of the conventional RT-PCR assay (PCR). There were no cross-reactions with other common viruses. Organ assay showed that FIPV were present in the Heart, liver, spleen, lung, kidney, duodenal and ascites of the autopsied cat. Histological lesions showed that macrophages, non-toxic neutrophils and lymphocytes predominated in different organs which confirmed that the cat was infected with FIPV. Conclusions We developed a quantitative platform for epidemiological investigations study of FIPV that was simple, sensitive, and rapid.

Background

Feline coronavirus (FCoV) are enveloped, positive-sense RNA viruses, which belong to the genus *Alphacoronavirus* of the subfamily Coronavirinae, family Coronaviridae within the order Nidovirales[1]. And FCoV has been divided into two biotypes, known as feline in fectious peritonitis virus (FIPV) and feline enteric coronavirus (FECV)[2]. FECV infection in cats is extremely common, often being asymptomatic or causing only mild enteric infections[3]. Only 10% of FCoV infections result in the fatal disease Feline Infectious Peritonitis (FIP) [4]. FIP is a fatal, systemic disease caused by a mutant form of Feline Infectious Peritonitis (FIPV) and has been reported to occur worldwide in domestic cats and numerous wild feline species[5].

FIPV contains a approximately 29k-nucleotide positive-sense single-stranded genomic RNA, which is encoding the four structural proteins (the spike, envelope,membrane, and nucleocapsid proteins), and nonstructural proteins(3a, 3b, 3c, 7a, and 7b)[6, 7]. The functions of these gene products are not fully understood. The N proteins have a molecular weight of about 50 kDa, together with the viral RNA, they form the flexible, helical nucleocapsid and seem to be critical for viral transcription[8].

The present study was aimed at observing changes in FIPV genome quantity in different tissues following natural infection with wild-type FIPV and defining the correlation between the viral quantity and histopathology development by a SYBR Green I based quantitative-PCR (q-PCR) assay for the simultaneous detection of the N gene of FIPV.

Results

2.1 standard curve, Specificity and Sensitivity analyses

The recombinant plasmid N- pET-28a concentration before dilution was 114 ng/μL. According to the formula: copies/ml = 6.02×10^{23} (copies/mL)×concentration of nucleic acid (g/μL)/average molecular weight of nucleic acid (g/mol), the plasmid N- pET-28a concentration was equivalent to 1.15×10^{9} gene copies/mL. And the standard curve of the q-PCR showed that an R^2 value (square of the correlation coefficient) of 0.9953 and reaction efficiencies of 94.69 % (Fig. 1).

The q-PCR assay informed positive results for the FIPV strains, after which the figure for the other viruses and the water control showed negative results (PEDV, PCV-2, PRRSV, PPV7, FCV, and TEGV) (Fig. 2a).

The sensitivity was tested using the N-pET-28a as a template. There was no non-specific amplification when the single melting curve was reaching the plateau (Fig.2b). And the limitation of the q- PCR assay detection was 10 copies of DNA, the figure for conventional PCR assay was 10^4 copies (Fig. 2c, 2d).

2.2 Reproducibility analyses

Three different laboratories performed the q- PCR test independently, after serially diluting the plasmid N-pET-28a. with the results compared, the inter-assay variation coefficients (the variation among the three testers) were below 5% (Table 1).

2.3 viral detect in clinical sample

The copy numbers obtained by *N* q-PCR in heart, liver, spleen, lung, kidney, duodenum and ascites are reported in Fig. 3. FIPV was abundant in the spleen and the maximum difference in the viral load per organ was 10^2 copies (Fig.3).

2.4 Viruses isolation and Histopathology

Upon virus inoculation, infected FCWF cells showed initial CPE following first passage at three days. Infected FCWF cells became rounded, sloughed granular and clumped forming syncytial cells which increased in size and number as the incubation period extended. Control uninfected cells remained normal (Fig.4). The virus about 100 nm in diameter enveloped and spherical in shape under the transmission electron microscope (Fig.5).

Histology showed that macrophages, non-toxic neutrophils and lymphocytes predominate in different tissues. In liver, kidney and intestines, foci of macrophages around vessels and these foci are surrounded by dense infiltrates of lymphocytes (mainly B-cells) and plasma cells that extend into surrounding tissues and edema, hyperemia, necrosis, fibrin deposition and protein exudation was common (Fig. 6).

Discussion

FCoV Infection is common in cats throughout the worldwide with causes almost no clinical signs[9]. However, infection with FCoV is associated with the development of the fatal and progressive disease manifestation of FIP in some cats[10]. Stem from its lethality, the difficulties in diagnosing FIP ante

mortem and controlling the spread of FIPV, FIP is among the most serious viral infections in cats. N protein is a important viral structure protein of FIPV. N protein is dynamically associated with sites of viral RNA replication, suggesting that N may also function to protect the genome or possibly mediate genome transport to the budding site[11]. In this study, specific primers were designed based on the conserved N gene of FIPV to build a q-PCR assay.

At present, TaqMan based real-time PCR and conventional PCR assays for detecting FCoV have been previously reported[12-14]. However, conventional PCR methods are not quantitative and can sometimes include non-specific products of the same size. Compared to TaqMan based real-time PCR, the q-PCR assay does not require probes and is therefore economical and easily adaptable compared with the conventional PCR system.[15, 16] There are possibilities of false negative results in the probe due to the mutation rate in the probe-binding site of virus genome, which would hinder the annealing of the probe and the subsequent signal detection. Further, the q-PCR assay detects the target PCR product accumulation independent of the sequence, allowing the quantifying of the viral genome with minor variations in the sequence.[17] To avoid these issues, a q-PCR assay based on SYBR Green I was developed for the detection and quantification of FIPV.

Our results showed that the standard curve of N had R^2 of 0.9953, Slope of −4.6226, and E of 94.69%.And the sensitivity of the q-PCR method is approximately 1000-fold higher than that of conventional PCR, and the q-PCR method also shows good reproducibility. The direct SYBR Green I qPCR was further applied to detect viruses in 5 different positive FIPV cats' samples from AnHui and Shanghai province of China, and the results described that there were differences among individuals and tissues. In detail, cats HF1901 and HF1903 showed higher load in the various tissues tested. Notably, Spleen and duodenum resulted the higher viral load of individual, and the maximum difference in the viral load per organ was 10^2 copies. These results were different from Pedersen which may be due to the detection in different time periods or differences in infection pathways. This is the first report of a broadly reactive SYBR Green□q-PCR assay for detecting of FIPV. The rapid and sensitive performance of this assay makes it a useful tool for diagnostic applications.

To help confirmed FIP in the cats, virus isolation and histopathology were using to diagnose. According to papers, it is said that activation of virulent FIPV infected and replication in monocytes was recommend as the key features to diagnose FIP [18]. In this study, after three passages, the FCWF cells showed a CPE and hematoxylin and eosin (HE) stain informed that new vessels with embedded macrophage were found in the granulation tissue. All of this evidences described that the samples in this paper were definitely infected with FIPV. And the data also provided further evidence that viral infection involving multi-organ and mainly attacking immune and intestinal organ.

Conclusion

The SYBR Green I q-PCR assay developed in this study was shown to be a sensitive, specific, high-throughput, cost-effective, and simple diagnostic tool for the rapid detection and epidemiological

surveillance of emerging FIPV infection. High levels of virus were detected terminally in grossly diseased tissues such as spleen and duodenum.

Materials And Methods

4.1Sample collection and storage

The animals used in this research were selected from of the veterinary hospital of kangnuo and chongyisheng. The five FIP positive cats(named HF1901, HF1902, HF1903, HF1904 and SH1910) which was displaying symptoms of illness including inappetence, anorexia, weight loss, lethargy, icterus, fever, diarrhea, and thoracic effusion was submitted from animal hospitals（Hefei, ANHUI and Shanghai). Collection of effusions was performed immediately following euthanasia. All the surviving cats were euthanized with intravenous injection of sodium pentobarbital (100mg/kg) with trained staff. All cats were ensured to be dead after they have no heartbeat and pupil dilation. And animal experiment was approved and performed in compliance with the guidelines of the Animal Research Ethics Board of Shanghai Veterinary Institute (Shanghai, China), CAAS (no. SHVRI-ZD-2019-012).

The sampled tissues included heart, liver, kidney, spleen, lungs and intestine, and were collected into RNAlater (Life Technologies) within 2h of death, as per manufacturer's instructions and stored in liquid nitrogen pending molecular analysis. Further samples were collected into 10% neutral-buffered formalin and fixed for 24h for histological examination. Body cavity fluid samples were collected into plain and EDTA-anticoagulant blood tubes. Where immediate storage at −80 °C was not possible, fluid was combined with RNAlater (20% v/v) upon collection and moved to long-term storage at −80 °C within 24h.

4.2 Primers and plasmid construction

Primers were selected from the conserved N gene of FIPV (Accession no. DQ010921). The target application was 156 base-pair in length (FIPV Fwd: 5'- ATTGATGGAGTCTTCTGGGTTGC -3'; FIPV Rev:5'-TGAGTTGTTCCTAGATCGGTTCG -3'). Total RNA was extracted from 200 μL of FCWF cells infected with FIPV with a QIAamp Viral RNA Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions, and used immediately for cDNA synthesis. The cDNA synthesis was performed with SuperScript II reverse transcriptase(RT) (Invitrogen, USA). Subsequently, a 1134-bp N region was cloned into the pET-28a plasmid construct the recombinant plasmid, which named N- pET-28a（(FIPV-N-F：5'-CAGGATCCATGGCCACACAGGGACAACG-3'； FIPV-N-R: 5'-CGGAATTCTTAGTTCGTAACCTCATCAATCATCTCAACCTGTGTGTC-3'). The plasmid was determined by Nano drop (Nano Drop 1000, Thermo scientific, Wilmington, DE, USA) , and used to make a standard curve.

4.3 Establishing a standard curve for q-PCR

The recombinant plasmid N- pET-28a was subjected to tenfold serial dilutions from 10^7 to 10^3 copies and used to used to build a standard curve for q-PCR. The assay was carried out in a 20 μL reaction mixture

containing 20 μg of cDNA, 0.4 μL of each primer, 10 μL of 2 × ChamQ Universal SYBR qPCR Master Mix (Vazyme), and made up to a total volume of 20 μL with RNase-free water (all the reagents provided in the kit) in a step one machine. The following thermal profile was used; 95 °C for 30 sec, 95 °C for 10s and 60 °C for 30 sec for 40 cycles.

4.4 Specificity、Sensitivity and Reproducibility of the q- PCR

The specificity of the q-PCR assay was determined by comparing results when different PEDV, PCV-2, PRRSV, PPV7, FCV, and TEGV were used as templates.

To determine the sensitivity of the q-PCR assay, The standard plasmid was subjected to tenfold serial dilutions from 10^8 to 10^1 copies and used to used to determine the conventional PCR and q-PCR lowest detection limit. Each reaction tube contained 1 μg templates and was tested using q-PCR and PCR. Standard plasmid and the negative control samples were amplified three times each. The primers for PCR and q-PCR were the same. PCR products were analyzed using agarose gel electrophoresis.

To determine the reproducibility of the q-PCR assay, the standard plasmid was diluted to 1×10^7-1×10^3 copies/mL and tested by three different people at three times (the same three people were used each time). The variation among the three testers at each test was analyzed.

4.5 viral detect in clinical sample

The five FIP positive cats' heart, liver, spleen, lung, kidney, duodenum and ascites were obtained. Then, those total RNA was extracted and reverse transcribed into cDNA according to the manufacture's instruction from the 200mg tissues, respectively. And the q-PCR method was applied to detect the cDNA. Nuclease-free water was used as a negative control, and three replicate reactions were set for each sample.

4.6 Viruses isolation and Histopathology

The sample was adapted and propagated in FCWF cell culture which were grown in Dulbecco's modified Eagle's Medium DMEM (Thermo Scientific, USA) supplemented with 10% Fetal Bovine Calf Seru、Penicillin (100 U mL^{-1}) and Streptomycin (100 U mL^{-1}) in 5% CO_2 at 37 °C for propagation of these viruses. Until characteristic cytopathic effect (CPE) was observed, the cells were stored at −80 °C and further cells was centrifuged by ultracentrifugation for observation under a transmission electron microscope (Hitachi, Japan). The formalin-fixed tissue samples were subjected to standard processing for histopathology. They were embedded in paraffin wax and sections prepared and stained by Haematoxylin-Eosin. Tissues that were examined histologically included liver, kidney, duodenum, jejunum and ileum.

Abbreviations

FCoV、Feline coronavirus

FIPV：Feline Infectious Peritonitis virus

q-PCR：quantitative-PCR

PEDV: porcine epidemic diarrhea virus

PCV-2: porcine circovirus-2

PRRSV: Porcine Reproductive and Respiratory Syndrome

PPV7: porcine parvovirus

FCV: feline calicivirus

TEGV: Transmissible gastroenteritis virus

FCWF: *Felis catus* whole fetus

CPE: cytopathic effect

Declarations

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Competing Interest The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Consent for publication

Not Applicable.

Availability of data and material

All data are fully available without restriction.

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Ethical Approval Animal experiment was approved and performed in compliance with the guidelines of the Animal Research Ethics Board of Shanghai Veterinary Institute (Shanghai, China), CAAS (no. SHVRI-ZD-2019-012). I obtained written informed consent to use the animals in my study from the owners of the animals.

Authors' Contributions Conceived and designed the experiments: YW, GL, YL. Performed the experiments: YHW, JW, LZ. Analyzed the data: YHW, JZ. Contributed materials/analysis tools: YHW, JZ, LZ, CC, CL, SJ. Wrote the paper: YHW, JZ, GL, CL. All authors contributed to the writing of the manuscript, read and approved the final version.

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Table

Table.1 Inter-Reproducibility assay

Reproducibility	No. of copies/ul	Ct(mean±SD)	CV(%)
Inter-assay	1×107	8.62±0.41	4.76
	1×106	12.32±0.36	2.92
	1×105	16.66±0.29	1.74
	1×104	21.92±0.44	2.01
	1×103	26.93±0.24	0.89

Figures

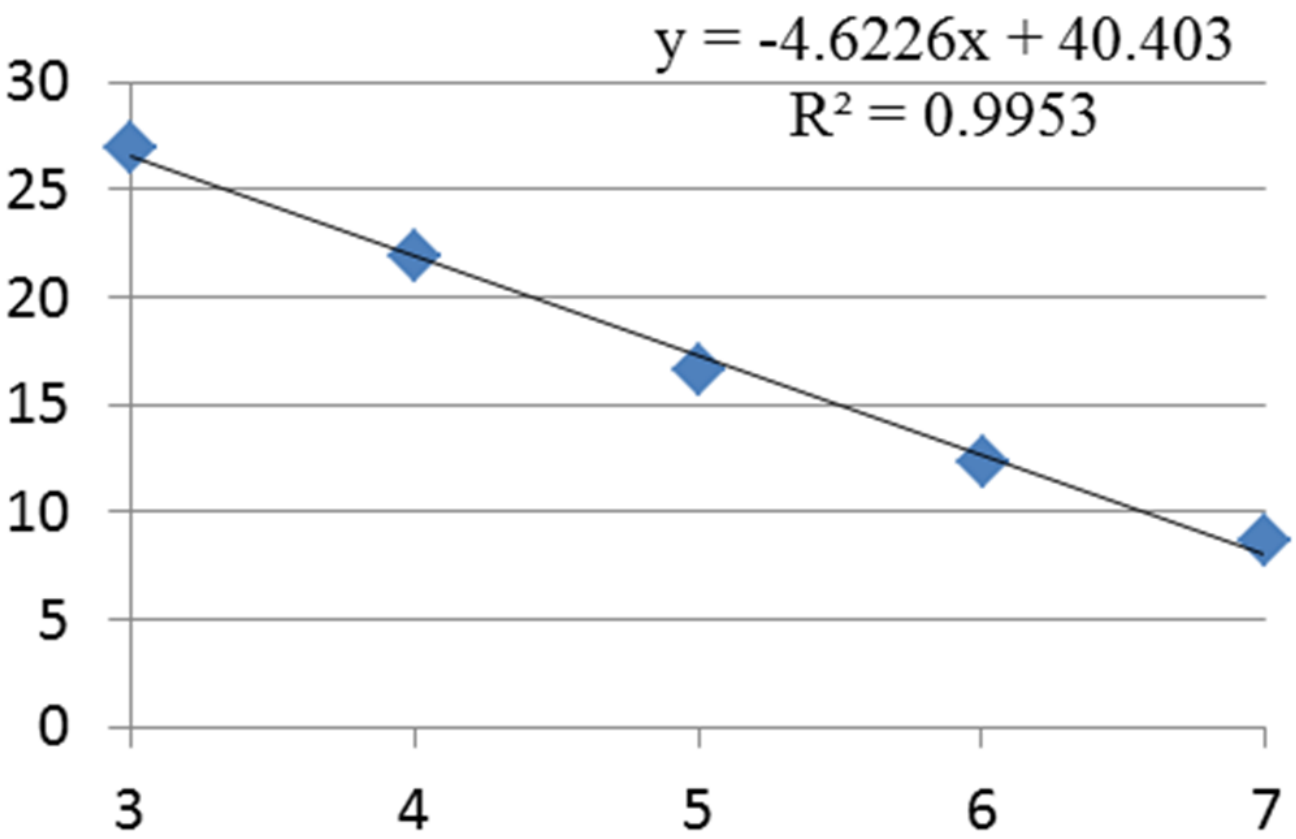

Figure 1

The standard curve for the q-PCR detection of FIPV infection the X-axis indicates the number of copies of N-pET-28a in 10-fold dilutions, and the Y-axis indicates the fluorescence data used for cycle threshold determinations.

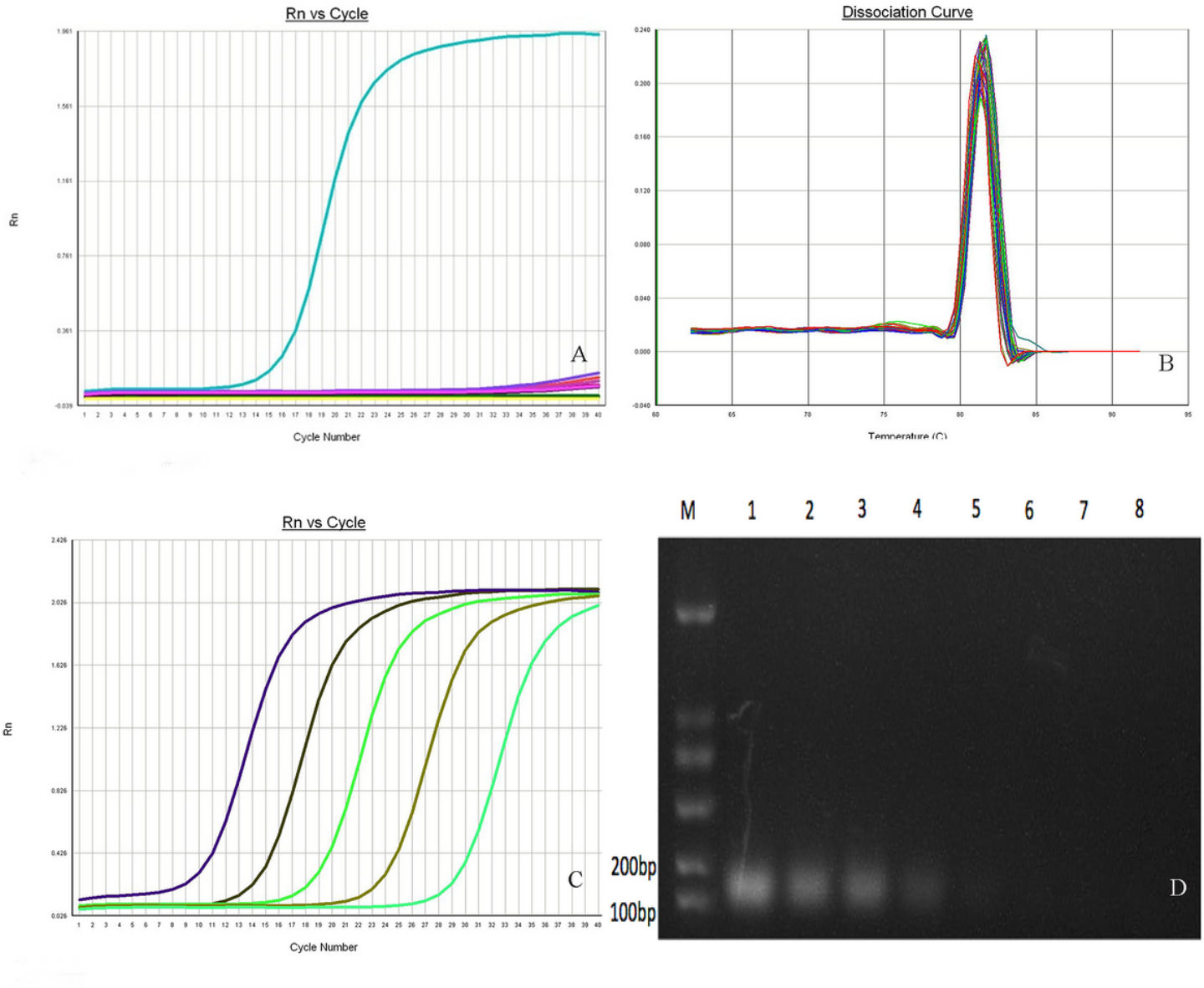

Figure 2

(A) sensitivity of the assay. (B) Melting curve of the assay. Note that the Tm of the PCR products was 82.5 °C. (C) Amplification curves of the assay. (D) conventional PCR using N-pET-28a in 10-fold dilutions of standard DNA ranged from 1 × 101 to 1 × 108 copies/mL. lanes 1-8 standard DNA (1 × 108-1 × 101copies per reaction); M DL 2000 marker.

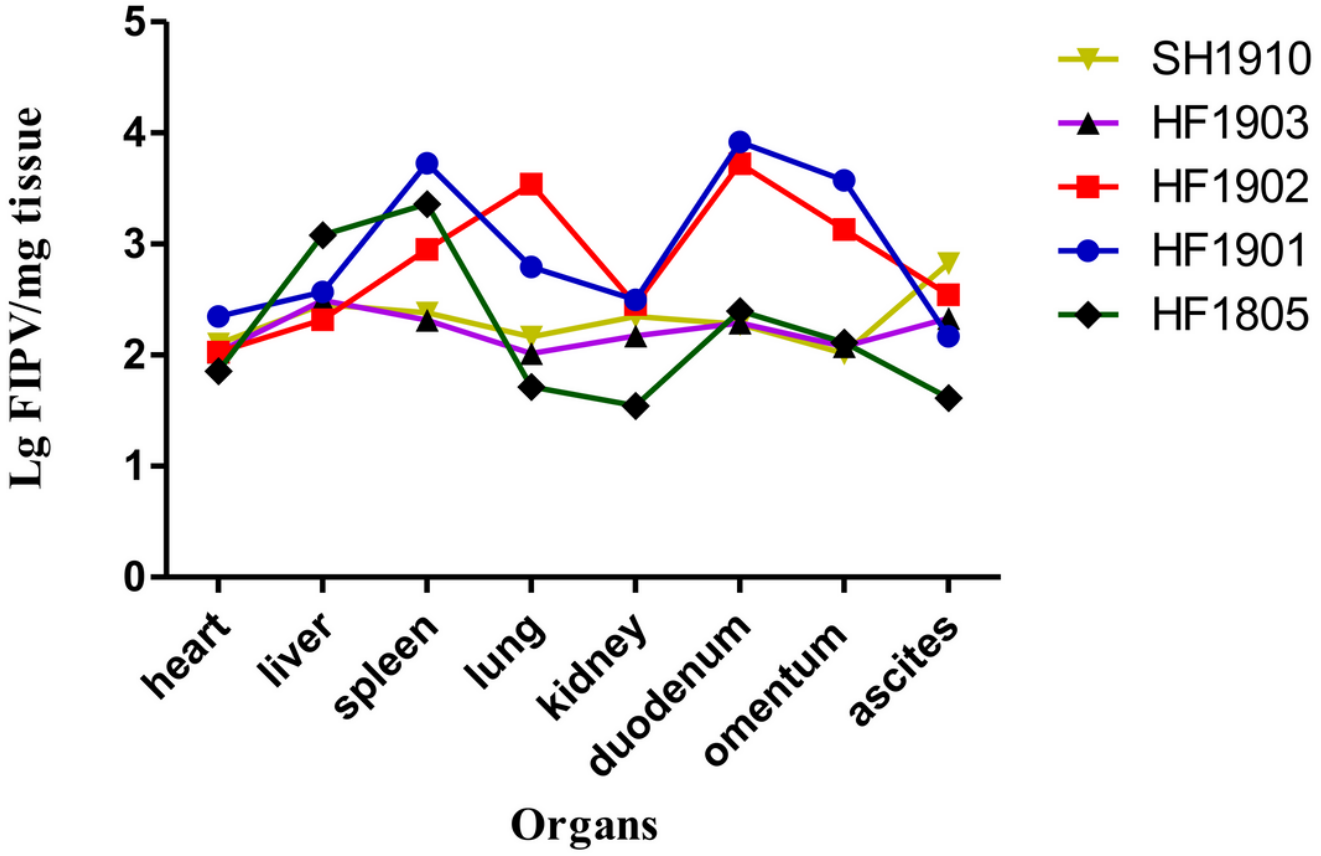

Figure 3

FCoV loads according to the q-PCR assay in the tissues of naturally infected FIPV Cats The organs examined are indicated along the X-axis. The Y-axis indicates viral loads as the logarithm of number of N gene per organ.

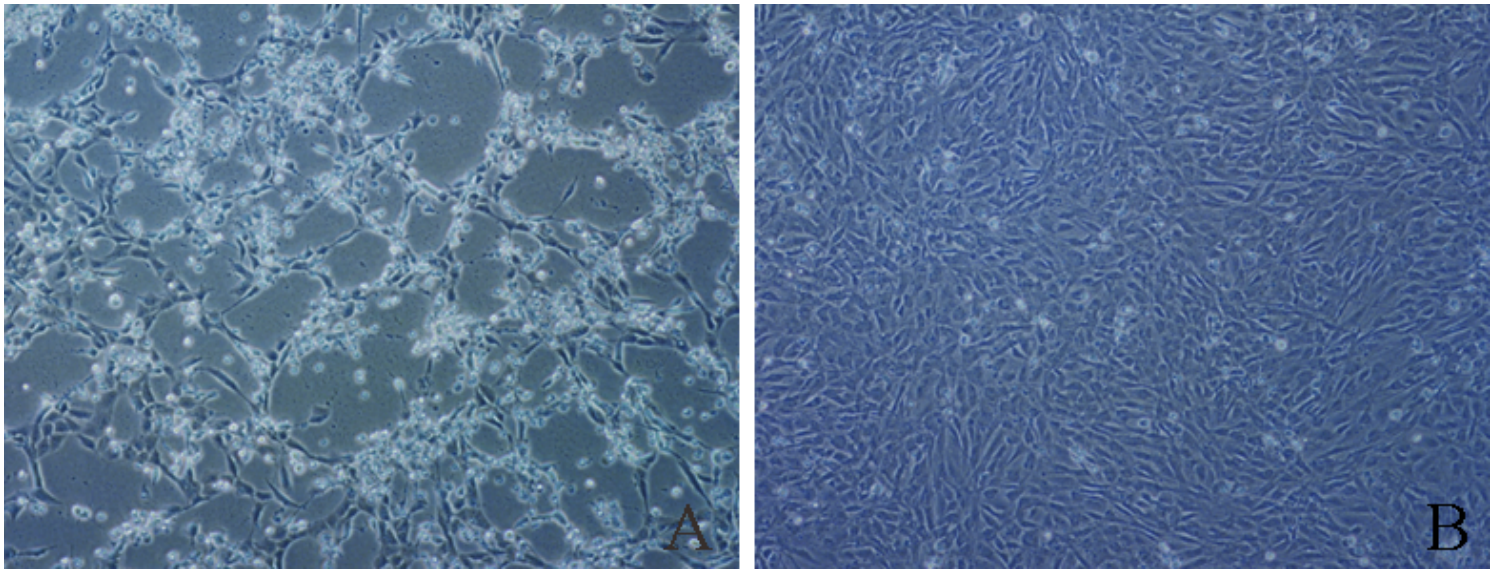

Figure 4

FCWF cell cultures showing cytopathic changes following infection with HF1902 effusion The infected cells show CPE characterized by cells rounding, clumping and detaching (A).Uninfected(B).

Figure 5

Transmission electron microscope observation of FIPV

Figure 6

Histology of the liver, kidney, duodenum, jejunum and ileum Foci of macrophages around vessels and these foci were surrounded by dense infiltrates of lymphocytes (mainly B-cells) and plasma cells that extended into surrounding tissues(A,B).In the intestinal villus epithelial cell the vacuole denaturated and the intestinal mucous membrane showed congestion, edema and leucocyte infiltration(C,D,E).

Supplementary Files

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- Checklist.pdf