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Epidemiological investigation of feline infectious peritonitis in cats living in Harbin, Northeast China from 2017 to 2019 using a combination of an EvaGreen-based real-time RT-PCR and serum chemistry assays

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ABSTRACT

Feline infectious peritonitis (FIP) is caused by the FIP virus (FIPV), a highly virulent mutant form of feline coronavirus (FCoV). This disease is one of the most important infectious diseases in cats, and it is associated with high mortality, particularly among younger cats. In this study, we isolated a wild-type FIPV HRB-17 epidemic strain from the blood sample of household pet cat exhibiting the characteristic wet-form FIP symptoms, which has been confirmed further by animal infection. Further, we developed an EvaGreen-based real-time RT-PCR assay for the accurate detection of FCoV based on the amplification of the highly conserved FIPV N gene. Then, using a combination of the real-time RT-PCR approach and a serum chemistry assay, we performed an epidemiological survey of FIPV infection in cats living in Harbin City, Northeast China. The results indicated that the EvaGreen-based real-time RT-PCR assay can be used for screening FCoV infection in the affected cats at an analytical detection limit of 8.2×10^1 viral genome copies/μL, but could not effectively distinguish FIPVs from FECVs. Additionally, the results of the epidemiological survey investigating feline blood samples (n = 1523) collected between July 2017 to July 2019 revealed an FIPV prevalence of approximately 12% (189/1523). Maybe, the prevalence would be less than 12% due to the real-time RT-PCR assay could not accurately differentiate FIPV and FECV. Nevertheless, it still highlighted the severity of the FIP epidemic in cats and reiterated the urgent need to develop effective anti-FIP therapeutic agents and anti-FIPV vaccines. As pet cats are household animals, risk communication and continuous region-extended surveillance cat programs are recommended.

1. Introduction

Feline infectious peritonitis (FIP) is caused by the feline infectious peritonitis virus (FIPV), and is one of the most important infectious diseases in household pet cats. This disease is associated with high mortality, and this is particularly true among younger cats that are housed in breeding catteries and animal shelters [1]. This disease continues to be an elusive and frustrating problem for veterinary practitioners and cat breeders, mainly due to the challenges associated with achieving a definitive diagnosis and determining effective treatments [2]. FIP was first reported in the United States in 1963 as an important disorder in cats [3], and its causative agent was subsequently determined to be a virus [4]. FIPV, a highly contagious coronavirus, has been clearly demonstrated to belong to the genus *Alphacoronavirus* and family *Coronaviridae*, which is a virulence biotype of the feline enteric coronavirus (FECV) [5–7]. Due to the general application of the generic name feline coronavirus (FCoV) to all serotypes and biotypes of feline coronaviruses, FECV is technically an enteric biotype of FCoV, and FIPV, the mutant form is an infectious peritonitis biotype [8]. Although FIPV is similar to FECV morphologically and serologically, it possesses a highly virulent mutation that results in the development of the fatal feline infectious peritonitis disease that presents the characteristic symptom of fibrin peritonitis—the accumulation of a large number of ascites—and a high mortality rate [8–11]. Unfortunately, there are currently no effective methods to prevent or control FIPV infection.

FIP is commonly categorized into two clinical phenotypes that

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include the “wet” and “dry” forms. The wet form causes significant ascites effusion and can be effectively diagnosed following the appearance of clinical symptoms, while the dry form results in the development of granulomas within the organs of cats, making its diagnosis more challenging due to the lack of easily recognizable clinical signs [1,12,13]. Although FIPV is a mutant form of the feline enteric coronavirus FECV [14], it is rarely detected in the feces of cats presenting FIP clinical symptoms [15,16]. The clinical signs of FIP rapidly progress to a severe state, necessitating the requirement of rapid and accurate diagnostic methods. In China, type I and type II FCoV strains have been shown to co-circulate within FIP-affected cats, and the type I FCoV strains exhibit high prevalence and genetic diversity [17]. The traditional method of isolation and identification of the causative virus is not suitable for rapid clinical FIP diagnosis. Although many researchers have used RT-PCR or real-time RT-PCR to detect FCoVs in both experimentally and naturally infected cats [18–20], it remains challenging to effectively distinguish FIPVs from FECVs. In some cases, hyperglobulinemia, which is observed in approximately 90% of the FIP-infected cats, could support the clinical diagnosis of the disease [21].

In this study, we developed an EvaGreen-based real-time RT-PCR assay for the detection of FCoV using the N gene of the wild-type FIPV HRB-17 epidemic strain that was isolated from blood sample obtained from household pet cat suffering from wet form FIP as the template. This assay was used in combination with serum chemistry assays (including albumin, globulin, and γ-globulin) to perform an epidemiological survey of FIPV infection in cats in 12 counties and six districts within Harbin City, Northeast China, from July 2017 to July 2019. Blood samples (n = 1523) were collected from household pet cats and stray cats, and were screened to assess the prevalence of FIPV infection in Northeast China.

2. Materials and methods

2.1. Clinical sample collection

Blood samples collection from household pet cats and stray cats were performed in accordance with the international (OIE Terrestrial animal health code) and national guidelines (CNAS-CL06:2018) for the care and use of laboratory animals. This project (2017NEAU07082) was approved by the Committee on the Ethics of Animal Experiments of Northeast Agricultural University of China (Jul 11, 2017). A total of 1523 feline blood samples were collected from household pet cats and stray cats across 12 counties (ACheng, ShuangCheng, HuLan, BinXi, WuChang, BaYan, Mulan, ShangZhi, YanShou, FangZheng, TongHe, and YiLan) and six districts (DaoLi, DaoWai, NanGang, PingFang, XiangFang, and SongBei) within Harbin City, Heilongjiang Province, Northeast China, from July 2017 to July 2019. Among these blood samples, 124 samples were collected from the cats that were suspected of wet-form feline infectious peritonitis with the clinical symptoms of abdominal distension and ascites production, and other 1399 samples were collected from the cats without any obvious clinical signs of feline infectious peritonitis. Blood samples of the cats were collected by anticoagulant-free vacuum blood collection tubes, and were placed overnight at 4 °C, and were then centrifuged at 1000 × *g* for 10 min. The sera were stored at −40 °C until use.

2.2. Phylogenetic analyses of the FIPV strain HRB-17

In this study, a wild-type FIPV epidemic strain HRB-17 was isolated from the blood sample of household pet cat that exhibited typical clinical signs of wet-form feline infectious peritonitis. The total RNA of the FIPV HRB-17 strain propagated on F81 cells was extracted by TRIzol Reagent Kit (Invitrogen, Carlsbad, CA, USA) [22] in accordance with the manufacturer's instructions followed by reverse transcription using a Superscript Reverse Transcriptase Reagent Kit (Takara, Dalian, China), and the N gene and S gene of the HRB-17 strain were amplified by polymerase chain reaction (PCR) with the primers listed in Table 1. Subsequently, the PCR products were purified using a DNA Gel Extraction Kit (Qiagen, Germany) and were subjected to N and S gene sequencing. And then, the gene sequences were submitted to GenBank database, and molecular evolutionary analysis was performed targeting the S gene (accession number MK987175) and N gene (accession number MK840958) of the HRB-17 strain.

Table 1
Oligonucleotide sequences used in this study.

	ID	Primer sequence (5′ →3′)	Amplicon size
N gene	N–F	ATGGCCACACAGGGACAAC	1134 bp
	N-R	TTAGTTCGTAACCTCATCAATCAT	
S gene	S–F	ATGATTGTGCTCGTAACTTGCCTCTT	4359 bp
	S-R	TTAGTGGACATGCACTTTTTCAATTGG	
Real-time RT-PCR	qPCR-F	TGCTTCGGCTAACTTTGGTG	368 bp
	qPCR-R	CAATCATCTCAACCTGTGTGTCAT	

2.3. Total RNA extraction of blood samples

Genomic RNA was extracted from blood samples (200 μL for each) using a TRIzol Reagent Kit (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. After that, the first-strand cDNA was reverse transcribed using a Superscript Reverse Transcriptase Reagent Kit (Takara, Dalian, China) and was then stored at −80 °C until use.

2.4. Establishing the EvaGreen-based real-time RT-PCR assay targeting the N gene

An EvaGreen-based real-time RT-PCR assay specific for FCoV detection that targeted the highly conserved N gene was developed with the primer pair listed in Table 1. Briefly, the full-length N gene of the FIPV HRB-17 strain was amplified and subcloned into the plasmid, pMD-19 T [23] to create pMD-N. Subsequently, using the pMD-N with initial concentration of 4.33×10^8 copies/μL [which was calculated by the following formula: $(6.02 \times 10^{23}) \times (ng/\mu L \times 10^{-9})/(DNA\ length \times 660)$] as a plasmid standard, the EvaGreen-based real-time RT-PCR assay was developed using the primer pair qPCR-F/R. The final PCR reaction volume was 20 μL, which included 5 × Golden HS EvaGreen qPCR Mix (4 μL), ROX reference DyeII (0.4 μL), plasmid template (1 μL) or cDNA (3 μL), 10 μM primer pair (1 μL), and deionized water. PCR was performed on an Applied Biosystem 7500 real-time PCR System with the following conditions: 95 °C for 5 min, 40 cycles at 95 °C for 10 s, and 60 °C for 30s. The detection results were judged in accordance with the following rules (i) when the cycle threshold (Ct) value of samples detected by the assay was less than or equal to 35, the samples were considered FCoV positive; (ii) when the Ct value was more than 38, the samples were considered FCoV negative; (iii) when the Ct value was between 35 and 38, the samples should be evaluated repeatedly, and if the Ct value was above 38, the result was judged FCoV negative; otherwise, it was positive.

2.5. Sensitivity, specificity, and reproducibility of the EvaGreen-based real-time RT-PCR assay

To evaluate the detection limit of the EvaGreen real-time RT-PCR assay in clinical specimens, FIPV-positive feline blood samples were serially diluted 10-fold using PBS, and this was followed by genomic RNA extraction and reverse transcription. The EvaGreen real-time PCR assay was then performed to detect the presence of viral nucleic acids in each dilution, and the copy numbers of the viral nucleic acids were calculated using the standard curve equation. To evaluate the assay specificity, known viruses including porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), porcine rotavirus

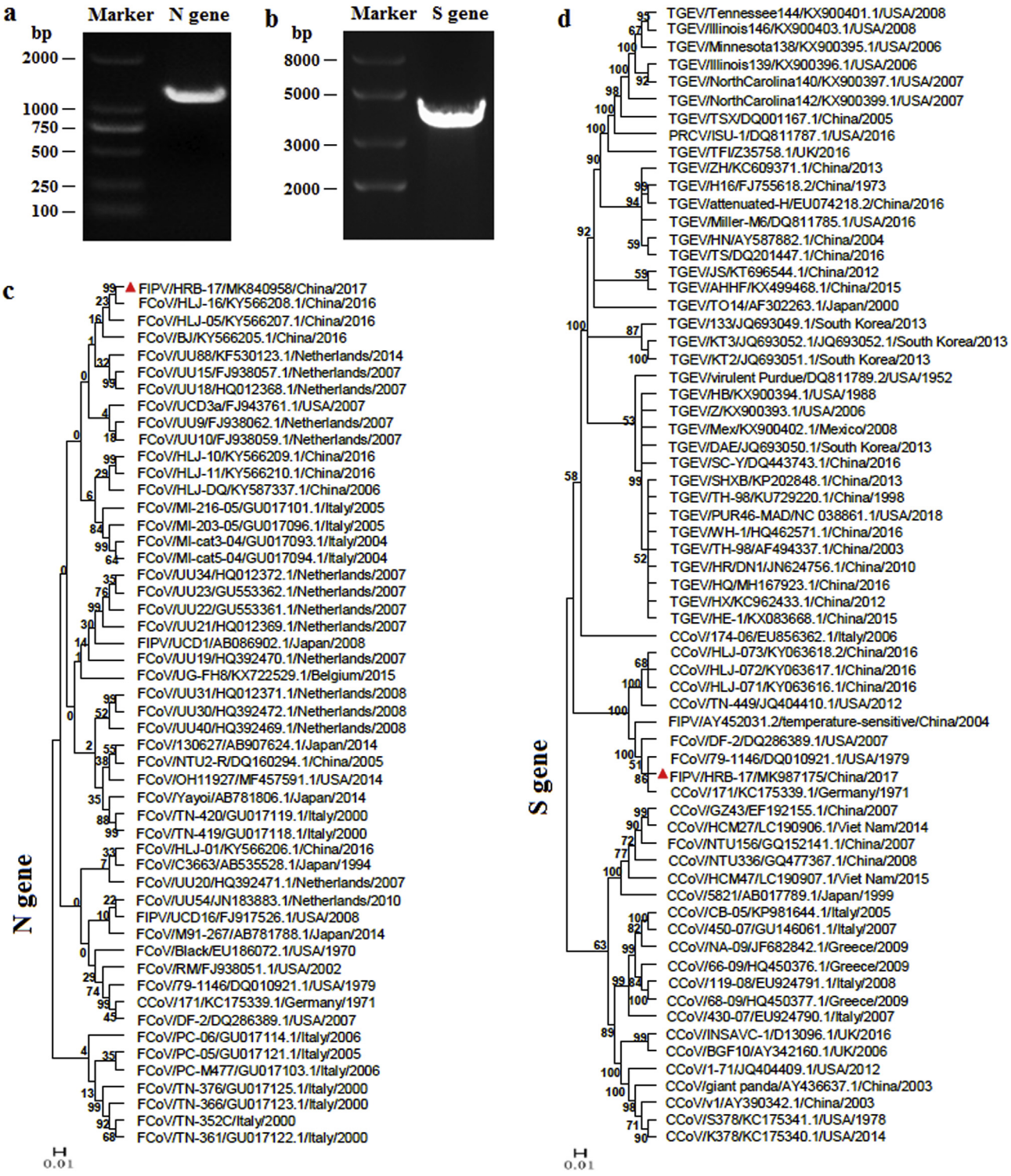

Fig. 1. Amplification of the N (a) and S genes (b) in the FIPV epidemic strain HRB-17, and phylogenetic analyses based on N (c) and S gene sequences (d) obtained in this study with reference sequences published in GenBank. The trees were constructed using MEGA version 7.0 using the Maximum Composite Likelihood model and 1000 bootstrap replicates.

(PoRV), porcine deltacoronavirus (PDCoV), bovine viral diarrhea virus (BVDV), bovine rotavirus (BRV), bovine parvovirus (BPV), bovine respiratory syncytial virus (BRSV), infectious bursal disease virus (IBDV), infectious hematopoietic necrosis virus (IHNV) that were kept in our laboratory, and FECV and FIPV that were provided by Animal Clinical Hospital of Northeast Agricultural University, Harbin, China, were tested by this assay. In parallel, the plasmid standard pMD-N was used as a positive control. To evaluate the reproducibility of assay detection, 4.33×10^8 copies/μL of the plasmid standard pMD-N was serially diluted 10-fold, and 10^1-, 10^3-, and 10^5- fold diluted plasmids were used to evaluate the intra-and inter-assay reproducibility.

2.6. Epidemiological survey

We collected a total of 1523 feline blood samples from household pet cats and stray cats living in 12 counties and six districts of Harbin

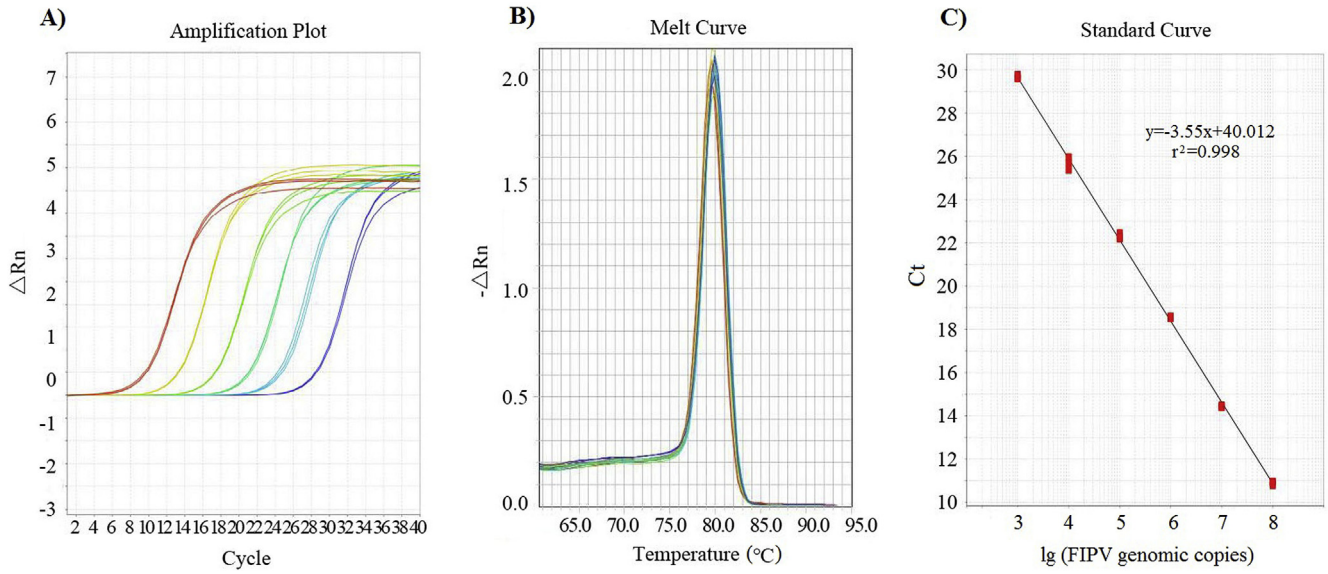

Fig. 2. Establishing a standard curve for the EvaGreen real-time RT-PCR assay. The plasmid standard pMD-N at an initial concentration of 4.33×10^8 copies/μL was serially diluted 10-fold to obtain 4.33×10^1 copies/μL, and the 10^1–10^6-fold diluted pMD-N plasmid was detected using the EvaGreen-based real-time RT-PCR assay to prepare the standard curve.

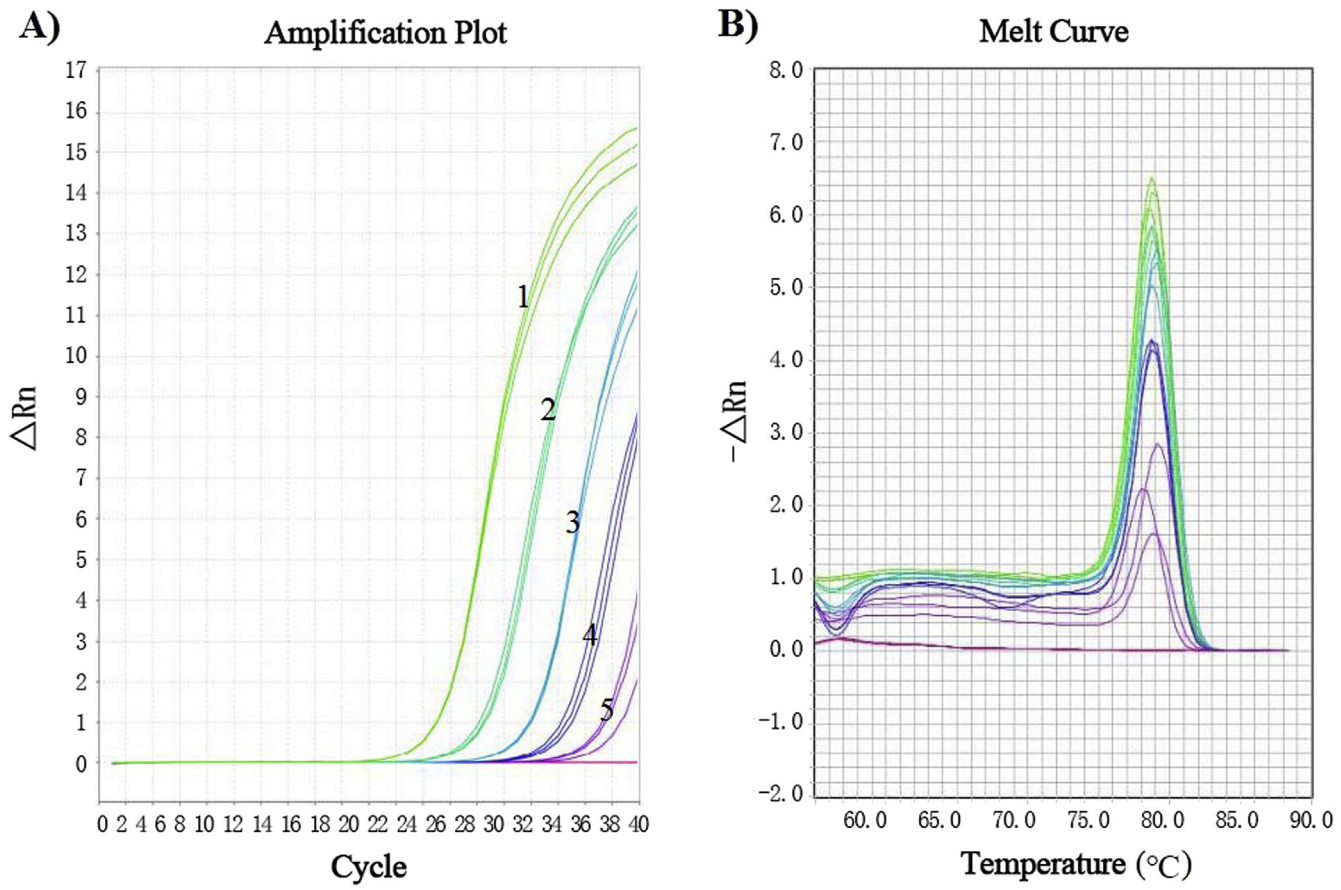

Fig. 3. Detection limit of the EvaGreen real-time RT-PCR assay. FIPV-positive feline blood samples were serially diluted 10-fold, and following total RNA extraction and detection by the EvaGreen assay, the assay detection limit for the clinical samples was 8.2×10^1 viral genome copies/μL as calculated from the standard curve equation. Panel A: 1–5 is the amplification curve of viral gene with the concentration from 8.2×10^5 to 8.2×10^1 copies/μL performed in triplicate, respectively. Panel B: corresponding melt curve of each amplification product in panel A.

City located within Heilongjiang Province, Northeast China from July 2017 to July 2019, in order to investigate the prevalence of FIPV infection in cats. These samples were subjected to FIPV and hyperglobulinemia (albumin: globulin and γ-globulin) detection using the EvaGreen real-time RT-PCR assay and serum chemistry assays, respectively.

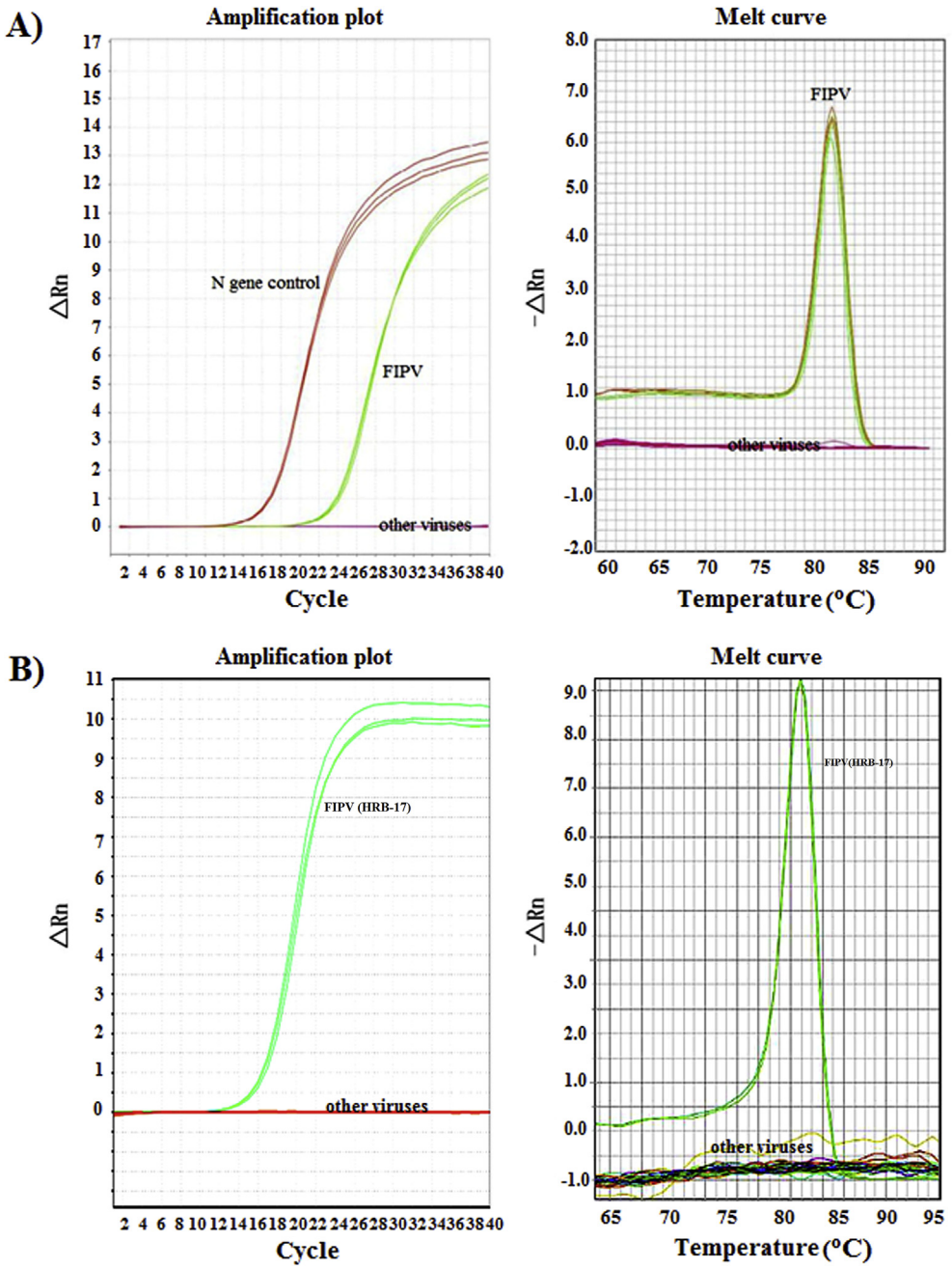

Fig. 4. The specificity of the EvaGreen real-time RT-PCR assay.

Table 2
The detection reproducibility of the EvaGreen real-time RT-PCR assay.

	Plasmid standard	Ct ± SD	CV (%)
Intra-assay	4.33 × 10^7 copies/μL	14.68 ± 0.08	0.45%
	4.33 × 10^5 copies/μL	22.43 ± 0.05	0.22%
	4.33 × 10^3 copies/μL	26.36 ± 0.07	0.26%
Inter-assay	4.33 × 10^7 copies/μL	14.52 ± 0.06	0.34%
	4.33 × 10^5 copies/μL	22.35 ± 0.09	0.40%
	4.33 × 10^3 copies/μL	26.48 ± 0.02	0.07%

3. Results and discussion

3.1. Phylogenetic analyses of the FIPV epidemic strain HRB-17

In this study, the FIPV epidemic strain HRB-17 was isolated from the blood sample collected from cat with FIP. Subsequently, the N gene (Fig. 1a) and S gene (Fig. 1b) from the FIPV HRB-17 strain were sequenced and analyzed for further phylogenetic analyses. The results revealed that the N gene and S gene from the FIPV HRB-17 strain respectively shared 91.2–98.87% and 89.38–96.70% sequence identities with reference strains that are currently available in the NCBI database. Additionally, close evolutionary relationships were observed between the FIPV HRB-17 strain and canine coronavirus (CCoV) strain 171 isolated in Germany in 1971, and between the FCoV strain 79-1146 isolated in UK in 1979 and the FCoV strain HLJ-16 isolated in China in 2016 (Fig. 1c and d). Based on the evolutionary relationship analyses, we speculated that the FIPV HRB-17 epidemic strain isolated in this study could be a mutant form of FCoV that arose following genetic sorting with CCoV.

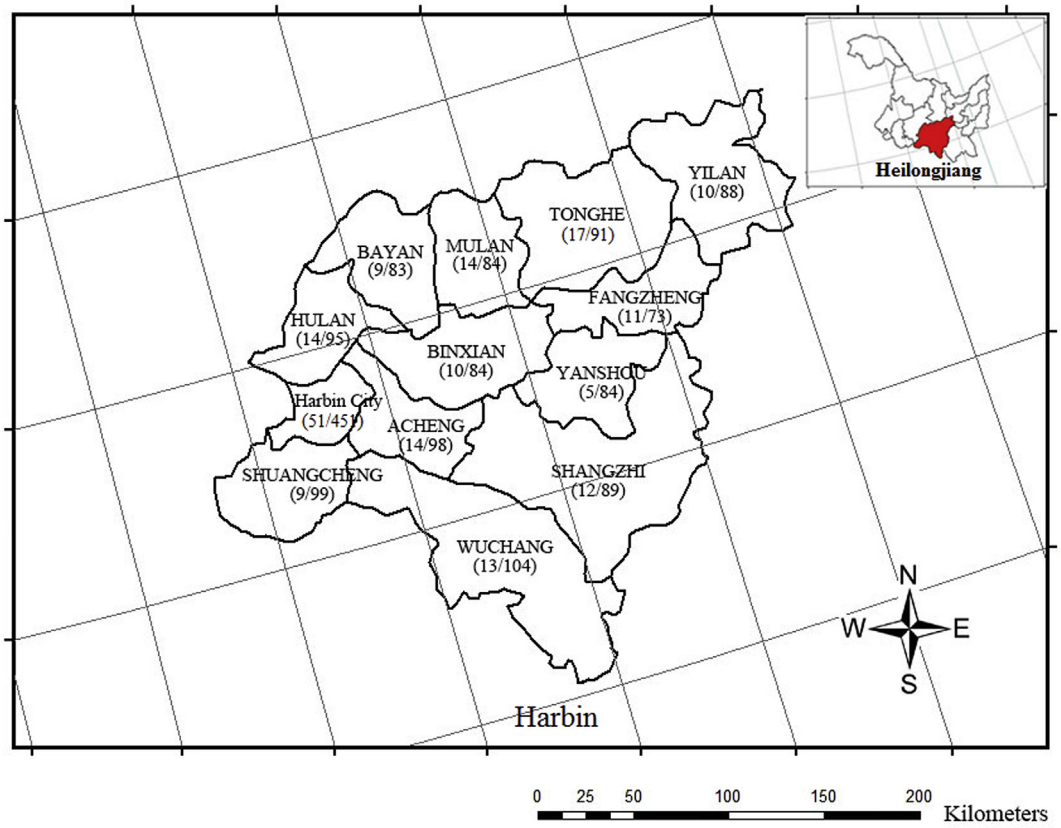

Fig. 5. A two-year epidemiological assessment of FIPV infection in cats was performed across 12 counties (ACheng, ShuangCheng, HuLan, BinXi, WuChang, BaYan, Mulan, ShangZhi, YanShou, FangZheng, TongHe, and YiLan) and six districts (DaoLi, DaoWai, NanGang, PingFang, XiangFang, and SongBei) within Harbin City, Heilongjiang Province, Northeast China.

Table 3
Epidemiological assessment of FIPV infection in cats living in Harbin (2017–2019).

Region	Numbers	EvaGreen-based real-time RT-PCR assay		Serum chemistry assay (Hyperglobulinemia)		Coincidence
		Positive[a]	Ratio	Positive[b]	Ratio	
YILAN	88 (7)[c]	10 (7)[c]	11.36%	8 (7)[c]	9.09%	80%[d] (100%)[e]
TONGHE	91 (13)	17 (13)	18.68%	13 (13)	14.29%	76.47% (100%)
MULAN	84 (7)	14 (7)	16.67%	13 (7)	15.48%	92.86% (100%)
BAYAN	83 (5)	9 (5)	10.84%	7 (5)	8.43%	77.78% (100%)
HULAN	95 (8)	14 (8)	14.73%	11 (8)	11.58%	78.57% (100%)
FANGZHENG	73 (5)	11 (5)	15.07%	8 (5)	10.96%	72.73% (100%)
YANSHOU	84 (4)	5 (4)	5.95%	5 (4)	3.57%	100% (100%)
SHANGZHI	89 (7)	12 (7)	13.48%	10 (7)	11.24%	83.33% (100%)
WUCHANG	104 (10)	13 (10)	12.50%	11 (10)	10.58%	84.62% (100%)
BINXIAN	84 (8)	10 (8)	11.90%	8 (8)	9.52%	80% (100%)
DAOWAI	81 (6)	9 (6)	11.11%	7 (6)	8.64%	77.78% (100%)
SONGBEI	78 (5)	5 (5)	6.41%	5 (5)	5.13%	100% (100%)
DAOLI	52 (4)	4 (4)	7.69%	4 (4)	7.69%	100% (100%)
NANGANG	81 (7)	12 (7)	14.81%	9 (7)	11.11%	75% (100%)
SHUANGCHENG	99 (6)	9 (6)	9.09%	9 (6)	7.07%	100% (100%)
ACHENG	98 (9)	14 (9)	14.28%	10 (9)	10.20%	71.43% (100%)
PINFANG	74 (7)	10 (7)	13.51%	8 (7)	10.81%	80% (100%)
XIANGFANG	85 (6)	11 (6)	12.94%	9 (6)	10.59%	81.82% (100%)
TOTAL	1523 (124)	189 (124)	12.41%	155 (124)	10.18%	82.01% (100%)

[a] The cycle threshold (Ct) value of samples detected by the assay was less than or equal to 35, which were considered FIPV positive.
[b] Criteria for determination of positive were ratio of albumin: globulin < 0.8 and γ-globulin > 2.5 g/dL.
[c] The cats that were suspected of wet-form feline infectious peritonitis with the clinical symptoms of abdominal distension and ascites production.
[d] The percentage of serum chemistry assay result to real-time RT-PCR assay.
[e] The coincidence of serum chemistry assay and real-time RT-PCR assay for detecting the cats with the clinical symptoms of wet-form FIP.

3.2. Standard curve

The plasmid pMD-N (initial concentration of 4.33×10^8 copies/μL) containing the full-length N gene of the FIPV HRB-17 strain was serially diluted 10-fold to a concentration of 4.33×10^1 copies/μL. The plasmid pMD-N diluted at 10^1–10^6 fold was then detected using the EvaGreen-based real-time RT-PCR assay, and a standard curve for quantifying the genomic copies of the virus was established (Fig. 2).

3.3. Sensitivity, specificity, and reproducibility of the EvaGreen-based real-time RT-PCR assay

As shown in Fig. 3, FIPV-positive feline blood samples were subjected to 10-fold serial dilution, total RNA extraction, and detection by the EvaGreen real-time RT-PCR assay. According to the standard curve calculations, the detection limit of the assay for FIPV in the clinical samples was 8.2×10^1 virus genomic copies/μL. The assay was only positive for FIPV and not for other viruses (PEDV, TGEV, PoRV, PDCoV, BVDV, BRV, BPV, BRSV, and IBDV) (Fig. 4A), indicating a good specificity. Generally, FCoV comprises two biotypes, FECVs that inhabit the feline intestine and are associated with asymptomatic, persistent enteric infections, and FIPVs, which arise from FECVs by mutation causing feline infectious peritonitis in cats and can only be detected in the blood. Although the EvaGreen real-time RT-PCR assay developed in this study could not accurately distinguish FIPVs from FECVs (Fig. 4B), it can be used for screening FCoV infection in the affected cats. Additionally, the intra- and inter-assay reproducibility results exhibited a $< 0.5\%$ coefficient of variation (Table 2), indicating improved reproducibility.

3.4. Epidemiological survey of FIPV infection in cats

Following the identification of the FIPV HRB-17 strain and the development of EvaGreen-based real-time RT-PCR assay, a two-year epidemiological assessment of FIPV infection in household pet cats and stray cats was performed in Harbin City, Heilongjiang Province, Northeast China, using 1523 feline blood samples collected from 12 counties and six districts within Harbin City from July 2017 to July 2019 (Fig. 5). As hyperglobulinemia is observed in approximately 90% of the cats afflicted with FIP [21], serum chemistry assays (including albumin: globulin and γ-globulin) were introduced to increase the accuracy of clinical diagnosis. As shown in Table 3, a total of 189 FIPV-positive feline blood samples were detected by the real-time RT-PCR assay, and the rate of FIPV infection in cats was approximately 12%, indicating a serious epidemic. Maybe, the prevalence would be less than 12% due to the real-time RT-PCR assay developed in this study could not accurately differentiate FIPV and FECV. Additionally, in accordance with established criteria that allow for the determination of FIP-associated hyperglobulinemia (albumin: globulin < 0.8 and γ-globulin > 2.5 g/dL), we tested all feline blood samples and observed a positive rate of approximately 10%. Among these blood samples, 124 samples collected from the diseased cats suspected of wet-form feline infectious peritonitis with the clinical symptoms of abdominal distension and ascites production were all positive detected by the real-time RT-PCR assay and the serum chemistry assays, showing a 100% coincidence rate. However, the total positive rate of these 1523 blood samples detected by the serum chemistry assay was lower than that of the EvaGreen-based real-time RT-PCR assay. This may be attributed to the entry of the FCoV mutant into the blood circulation that ultimately resulted in FIPV viremia during the early stage of viral infection, as at this stage, hyperglobulinemia had not yet developed [8,24,25]. Thus, at this stage the FIPV infection in cats could only be detected by the real-time RT-PCR assay and not by the serum chemistry assay. However, FIP-mediated hyperglobulinemia can be detected as the disease progressed. Therefore, combining the real-time RT-PCR assay with the serum chemistry assay could be helpful to screen FCoV infection in cats at different stages of infection, while the definitive diagnosis of FIPV infection should rely on the immunohistochemical staining of viral antigen in the macrophages or monocytes of the affected cats [26].

In conclusion, following the isolation of wild-type FIPV HRB-17 strain, an EvaGreen-based real-time RT-PCR assay for detecting FCoV was developed that targeted the highly conserved N gene. This is a sensitive and specific assay capable of helpfully screening FCoV infection in cats. The results of two-year epidemiological surveillance of FIPV infection in cats living in Harbin City, Northeast China, reiterated the urgent need to develop effective anti-FIP therapeutic agents and anti-FIPV vaccines. Additionally, as pet cats are household animals, risk communication and continuous region-extended FCoV surveillance programs are highly recommended.

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CRediT authorship contribution statement

Xueting Guan: Conceptualization, Methodology. **Hua Li:** Conceptualization, Methodology, Writing - review & editing. **Meijing Han:** Resources, Investigation. **Shuo Jia:** Resources, Investigation. **Baohua Feng:** Resources, Investigation. **Xuwen Gao:** Resources, Investigation. **Zhuo Wang:** Resources, Investigation. **Yanping Jiang:** Resources, Investigation. **Wen Cui:** Formal analysis. **Li Wang:** Formal analysis. **Yigang Xu:** Writing - review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no conflict of interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mcp.2019.101495.

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