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## TaqMan-based real-time polymerase chain reaction assay for specific detection of bocavirus-1 in domestic cats

Yong Wang<sup>a,1</sup>, Jianfei Sun<sup>a,1</sup>, Xu Guo<sup>a</sup>, Da Zhang<sup>a</sup>, Yongqiu Cui<sup>a</sup>, Wei Li<sup>a</sup>, Guangqing Liu<sup>c</sup>, Yongdong Li<sup>b,\*</sup>, Shudong Jiang<sup>a,\*\*</sup>

<sup>a</sup> Anhui Province Key Laboratory of Veterinary Pathobiology and Disease Control, College of Animal Science and Technology, Anhui Agricultural University, Hefei, 230036, PR China

<sup>b</sup> Municipal Key Laboratory of Virology, Ningbo Municipal Center for Disease Control and Prevention, Ningbo, 315010, PR China

<sup>c</sup> Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai, 200241, PR China

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### ABSTRACT

Feline bocavirus-1 (FBoV-1) was first discovered in Hong Kong in 2012, and studies have indicated that the virus may cause feline hemorrhagic enteritis. Currently, there is a lack of an effective and quantitative method for FBoV-1 detection. In this study, a TaqMan-based quantitative real-time PCR (qPCR) for FBoV-1 detection was established. Primers and probes were designed to target the conserved region of the FBoV-1 NS1 gene. The sensitivity analysis indicated that the minimum detection limit was  $4.57 \times 10^1$  copies/ $\mu$ L. The specificity test revealed no cross-reaction with seven other common feline viruses, including the same species—FBoV-2 and FBoV-3. The sensitivity of this method was 100 times higher than that of conventional PCR (cPCR). The established method showed good repeatability, with the intra-assay and inter-assay coefficients of variation of 0.18%–1.00% and 0.27%–0.45%, respectively. Furthermore, the analysis of feline feces revealed that the detection rate by qPCR was 7.0% (9/128), whereas that by cPCR was 4.7% (6/128). In conclusion, the established qPCR assay can quantitatively detect FBoV-1 with a high sensitivity, high specificity, and good reproducibility, making it a promising technique for the clinical detection of and basic and epidemiological research on FBoV-1.

### 1. Introduction

Bocavirus (BoV) belongs to the subfamily Parvovirinae and is a non-enveloped, linear, single strand DNA (ssDNA) virus with a genome of 5.4 kb [1–5]. BoV has three open reading frames (ORF), namely ORF1, ORF2, and ORF3, encoding non-structural protein (NS1), structural viral capsid proteins (VP1 and VP2), and nuclear phosphoprotein (NP1). Of the three ORFs, ORF3 is a genetic structure unique to BoV compared with other viruses in the subfamily Parvovirinae [6–8].

Feline bocaviruses (FBoVs) are divided into three types, and they belong to the genus *Carnivore bocapavovirus* 3–5 [4]. FBoV-1 was discovered in 2012 in fecal, kidney, nasal, and blood samples from clinically asymptomatic cats in Hong Kong [8]. The subsequent reports indicated that FBoV-1 could be easily detected in cats with diarrhea compared with healthy cats [2]. Notably, FBoV-1 has been closely

related to severe enteritis, and it may show co-infection with feline panleukopenia virus (FPLV) [9,10]. As a human companion animal, cats infected with BoV are a cause of concern, and thus, preventive measures for BoV should be taken.

Establishing an efficient FBoV-1 clinical detection method can provide a powerful tool for the diagnosis of BoV infection in cats with and without diarrhea. However, currently, only conventional PCR (cPCR) has been established for FBoV detection. Thus, there is a need to develop a more rapid, sensitive, and specific method to detect FBoV-1. TaqMan assay has the characteristics of high sensitivity and specificity, and it can be used for quantitative analysis. Indeed, TaqMan assay is now becoming the first choice for pathogen detection in clinical samples [11, 12]. In this study, we aimed to establish a sensitive and specific TaqMan-based quantitative real-time polymerase chain reaction (qPCR) method for the rapid detection of FBoV-1 in field samples.

\* Corresponding author.

\*\* Corresponding author.

E-mail addresses: [liy0551@126.com](mailto:liy0551@126.com) (Y. Li), [jshudong@163.com](mailto:jshudong@163.com) (S. Jiang).

<sup>1</sup> Jianfei Sun and Yong Wang contributed equally to this work and considered to be the co-first authors.

## 2. Materials and methods

### 2.1. Sample collection and virus DNA extraction

A total of 128 stool samples from domestic cats with and without diarrhea were collected from January to December 2019 at veterinary hospitals in Shanghai, Nanjing, Hefei, Ma'an shan, and Bozhou, China. The number of samples from cats with and without diarrhea was 88 and 40, respectively. Nucleic acid was extracted from feline parvovirus (FPV, HF1 strain, GenBank number: MT614366), feline herpes virus (Fel-O-Vax PCT feline vaccine, Boehringer-Ingelheim), feline calicivirus (FCV, ANHF1 strain, GenBank number: MT649084), feline coronavirus (FCoV, HF1902 strain, GenBank number: MT444152), feline astrovirus (FAstV, AH-1-2020 strain, GenBank number: MN977118), FBoV-1 (AAU01 strain, GenBank number: MT577646), FBoV-2, (HFxA-6 strain, GenBank number: MT633126), and FBoV-3 (HFTH-22 strain, GenBank number: MT633128) using the TIANamp Virus DNA/RNA Kit (Tiangen, Beijing, China), according to the manufacturer's protocol. RNA from the RNA virus used in this study was extracted using the Prime Script™ RT Reagent Kit with gDNA Eraser (Takara, Dalian, China), according to the manufacturer's protocol and reverse transcribed into cDNA, which was stored at  $-80^{\circ}\text{C}$  until use.

### 2.2. Primer and probe design

Previous studies have shown that *NS1* is relatively conserved in FBoV-1 [2]. Therefore, the conserved region of FBoV-1 *NS1* was chosen after a BLAST search in the GenBank database and analysis of the conserved region (GenBank accession numbers: JQ692585.1, JQ692586.1, KM017745.1, KX228695.1, MN127776.1, MN127778.1, and NC\_017823.1) (<https://www.ncbi.nlm.nih.gov/>). According to the selected conserved region, Beacon Designer 7 was used to design an optimal pair of specific primers and probes, which were then synthesized commercially (General Biological System, Anhui, Co., Ltd.). The details of the primers and probe are provided in Table 1.

### 2.3. Construction of standard plasmids

Using the extracted DNA as a template, the target fragment of FBoV-1 was amplified using primers FBoV1-F and FBoV1-R, and the amplified product was cloned into the pMD19-T vector (Takara, Dalian, China). Subsequently, the recombinant plasmid, named pMD19T-NS1, was quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The plasmid copy number was calculated using the following formula: amount (copies/ $\mu\text{L}$ ) =  $(6.02 \times 10^{23}) \times (\text{concentration in ng} \times 10^{-9}) / (\text{plasmid length} \times 660 \text{ Da/bp})$  [13]. Plasmids were serially diluted 10-fold from  $4.57 \times 10^8$  to  $4.57 \times 10^1$  copies/ $\mu\text{L}$ , and then stored at  $-80^{\circ}\text{C}$ .

### 2.4. Optimization of qPCR and establishment of a standard curve

Based on the fluorescence and the minimum threshold period, the

**Table 1**

Primers and probe used in this study.

Name	Sequences (5'-3')	Target gene	Size (bp)
Clinical samples Detection		NP1	465
Forward	AGAACCRCRATCARTCCACT		
Reverse	TGGCRACCGCYAGCATTTC		
FBoV-1 real-time PCR		NS1	172
Forward	CTCTATGGTTGCGTCAAT		
Probe	FAM-CGTGCCGCCAAGATACA-BHQ1		
Reverse	GATGCGATTCTCTGTGT		

**Note:** The forward, reverse primers and probes in FBoV-1 real-time PCR were newly designed by this research. Conventional PCR were designed in previous research.

concentration of the primers, probes, and template were selected. The optimized qPCR was performed with a reaction mixture of final volume of 20  $\mu\text{L}$ , comprising 10  $\mu\text{L}$  of  $2 \times$  TaqMan Fast qPCR Master Mix (probe qPCR; Sangon Biotech, Shanghai, China), 0.5  $\mu\text{L}$  of FBoV-F and FBoV-R primers (each 10  $\mu\text{mol/L}$ ), 0.6  $\mu\text{L}$  of FBoV-P probe (5  $\mu\text{mol/L}$ ), 1  $\mu\text{L}$  of template DNA, and 8.4  $\mu\text{L}$  of nuclease-free water. The qPCR conditions were as follows: initial denaturation at  $94^{\circ}\text{C}$  for 3 min, followed by 40 cycles at  $94^{\circ}\text{C}$  for 5 s, and final extension at  $60^{\circ}\text{C}$  for 30 s. The standard plasmids were serially diluted 10-fold and used to generate the final standard curve of the CT value against the logarithm of the standard number of copies. All steps in this experiment were verified three times.

### 2.5. Analysis of sensitivity, specificity, and repeatability of the qPCR assay

The optimized qPCR assay was used to detect 10-fold serially diluted recombinant plasmids, and the minimum detection limit of the method was analyzed using a standard curve. The forward and reverse primers used for the qPCR were used for the cPCR. Hence, cPCR was used to detect the template with the same concentration gradient, and agarose gel electrophoresis was carried out to observe the amplified product. Finally, the sensitivity of qPCR and cPCR was compared.

To evaluate the specificity of the qPCR established in this study, the assay was used to simultaneously detect FPV, FHV, FCV, FCoV, FAstV, FBoV-2, FBoV-3, and RNase-free  $\text{H}_2\text{O}$ .

To evaluate the reproducibility of this assay, the pMD19-NS1 plasmid of concentrations  $4.57 \times 10^8$ , 106, 104, and 102 copies/ $\mu\text{L}$  was used as a template, and the assay was repeated on three different days at three different times. The reproducibility of the method was then determined by calculating the standard deviation (SD) within and between batches.

### 2.6. Clinical sample detection

The clinical stool samples collected in this study were analyzed using the cPCR method described by Zhang (14) and the qPCR method established in this study, and the results of the two testing methods were compared. The primers used in this study were shown in Table 1 [14]. Furthermore, the positive detection rates in fecal samples from 88 cats with diarrhea and 40 cats without diarrhea were compared. In addition, all positive samples were verified by sequencing (General Biological System, Anhui, Co., Ltd.).

## 3. Results

### 3.1. Standard curves of the qPCR analysis

An FBoV-1 standard curve with the logarithm of the number of copies as the abscissa and the CT value as the ordinate was established using serially diluted DNA samples from  $4.75 \times 10^8$  to  $4.75 \times 10^1$  copies/ $\mu\text{L}$ . Each gradient was repeated three times, and the optimal curve was selected as the standard curve. The correlation coefficient ( $R^2$ ) was 0.998, slope was  $-3.225$ , and intercept was 39.970 (Fig. 1).

### 3.2. Sensitivity and specificity of the qPCR method

The minimum amount of samples for the TaqMan-based qPCR and cPCR methods was  $4.57 \times 10^1$  and  $4.57 \times 10^3$  DNA copies/ $\mu\text{L}$ , respectively (Fig. 2). The qPCR could positively detect the pMD19-NS1 standard plasmid ( $2.37 \times 10^8$  copies/ $\mu\text{L}$ ), whereas all other viral samples (FPV, FHV, FCV, FCoV, FAstV, FBoV-2, and FBoV-3) and the negative control (RNase-free  $\text{H}_2\text{O}$ ) tested negative (Fig. 3).

### 3.3. Reproducibility analysis

The results showed that the intra-assay and inter-assay SDs ranged

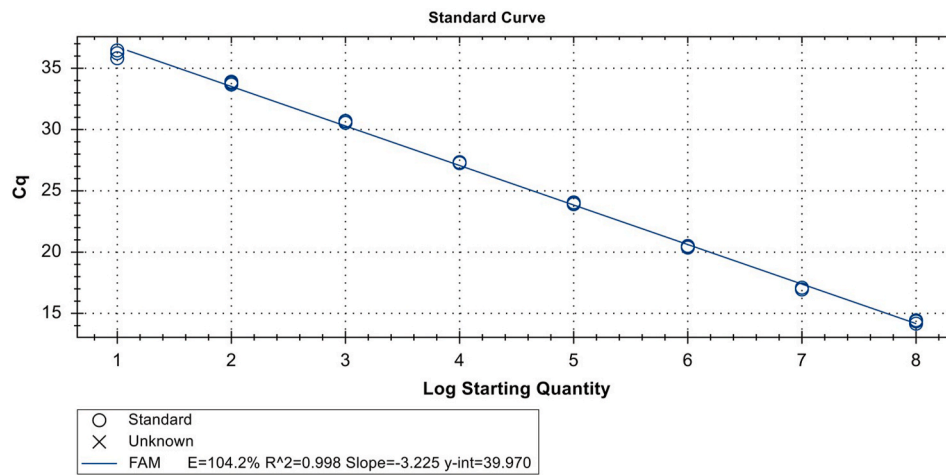


Fig. 1. Standard curve for the TaqMan-based real-time PCR assay for FBoV-1 detection. The curve was developed using duplicated 10-fold serially diluted samples ( $4.57 \times 10^8$ – $4.57 \times 10^1$  copies/ $\mu$ L); the correlation coefficient was 0.998 and slope was  $-3.217$ .

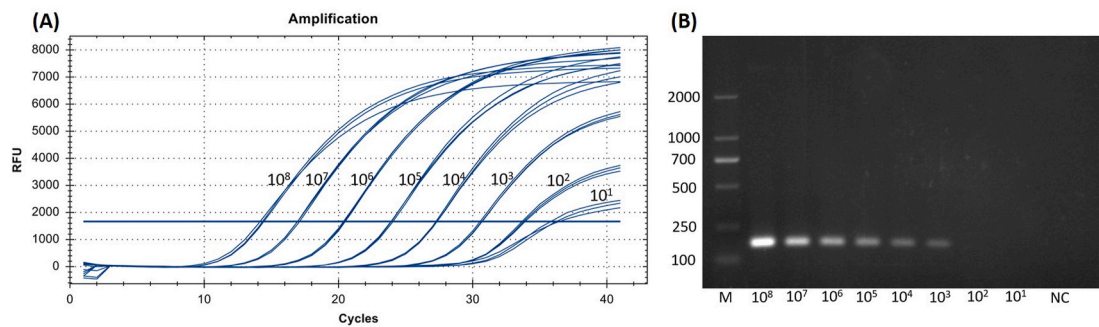


Fig. 2. A. Sensitivity of the TaqMan-based real-time PCR assay for FBoV-1 detection. B. Sensitivity of the conventional PCR for FBoV-1 detection. 1–8: The concentration of pMD19-NS1 ranged from  $4.57 \times 10^1$  to  $4.57 \times 10^8$  copies/ $\mu$ L. NC: negative control (nuclease-Free Water). M: DL 2000 marker.

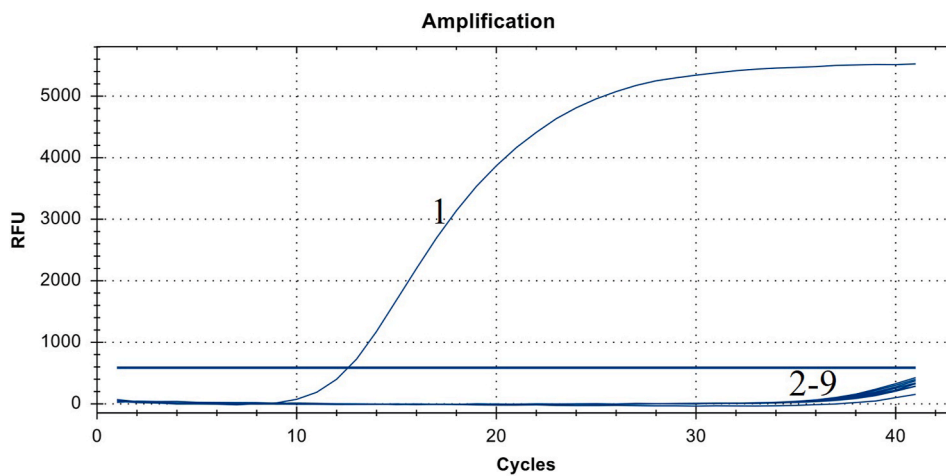


Fig. 3. Specificity of the TaqMan-based real-time PCR assay for FBoV-1 detection. 1: FBoV-1. 2–9: FPV, FHV, FCoV, FCoV, FCoV, FCoV, FCoV, and negative control.

from 0.05 to 0.14 and 0.02 to 0.09, respectively. Furthermore, the intra-assay and inter-assay coefficients of variation (CV) ranged from 0.18% to 1.00% and 0.08%–0.45%, respectively (Table 2).

### 3.4. Screening of clinical specimens by qPCR

The qPCR method established in this study and the cPCR method

reported previously were used to detect the 128 collected samples. The results showed that the detection rate using the qPCR method was 7.0% (9/128) and that using the cPCR method was 4.7% (6/128). Furthermore, the positive rate of detection of samples from cats with diarrhea using the qPCR method was 10.2% (9/88); FBoV-1 was not detected in cats without diarrhea. All test samples positively detected by cPCR could also be detected by qPCR (Table 3), and the results of all positive

**Table 2**  
The repeatability of the established qPCR.

Standard Copies/ $\mu$ L	Intra-assay variability			Inter-assay variability		
	Mean	SD	CV(%)	Mean	SD	CV(%)
$1 \times 10^8$	14.31	0.14	1.00	14.27	0.06	0.45
$1 \times 10^6$	20.43	0.07	0.32	20.42	0.06	0.30
$1 \times 10^4$	27.31	0.05	0.18	27.32	0.02	0.08
$1 \times 10^2$	33.78	0.11	0.33	33.76	0.09	0.27

samples were confirmed by sequencing.

#### 4. Discussion

Previous studies have indicated that FeAstV, FBoV, and FPLV are the common viruses that cause diarrhea in cat, and FBoV may cause co-infection with other viruses to cause diarrhea [4,15–18]. Importantly, viral diarrhea in cats can seriously affect their health and even lead to death. Recently, it was reported in northeastern China that the detection rate of FBoV in cats with diarrhea is as high as 33.3%, and the most common FBoV is type 1 [2]. However, in the diagnosis of diarrhea in cats, the virus that is first considered is FPLV [14–16], whereas FBoV-1 is easily overlooked. FBoV-1 has no specific vaccine for prevention, and only the cPCR detection method has been established. However, cPCR cannot quantitatively analyze samples, and it is not suitable for rapid quantitative detection of virus in clinical samples [19].

During recent years, with the widespread use of molecular diagnostic techniques in the clinical setting, some of the well-recognized detection methods, such as loop-mediated isothermal amplification (LAMP), SYBR Green, and recombinase-assisted amplification (RAA), have been developed. These methods have the advantages of being cheap, rapid, and specific. However, LAMP can easily cause aerosol pollution, and the experimental results can show false positives, affecting the test results. The concentration of the SYBR Green dye used is too high, and it can inhibit the amplification reaction. The RAA assay cannot be used for quantitative detection. Compared with these methods, qPCR has a high specificity and sensitivity as well as the ability to quantitatively analyze samples based on copy number. This can not only help with the data analysis in clinical diagnosis and provide assistance for future research, but also assist in research related to pathogenesis, early detection, surveillance, prevention, and epidemiology.

According to previous studies, *NS1* is a conserved gene in FBoV. Therefore, the probe was designed for the conserved region of *NS1* in this study. The results showed that the standard curve correlation coefficient ( $R^2$ ) of qPCR was 0.998 and the standard formula was  $y = -3.225x + 39.970$ . In addition, the minimum number of copies detected by qPCR was  $4.57 \times 10^1$  copies/ $\mu$ L, which is 100 times higher than that detected by cPCR. Furthermore, no cross signal was observed when the assay was performed with seven other common cat viruses, including FBoV-2 and FBoV-3. The clinical sample test also indicated that the sensitivity of qPCR was considerably higher than that of cPCR. In this experiment, FBoV-1 was detected in samples from cats with diarrhea, whereas it was not detected in stool samples from cats without diarrhea. This might be because FBoV-1 is more likely to infect cats with diarrhea, which is consistent with the findings of previous studies [4]. Repeatability analysis showed that the intra-assay and inter-assay CVs were  $\leq 1.00\%$  and  $\leq 0.45\%$ , respectively, indicating that the method showed good reproducibility.

In summary, a TaqMan-based real-time PCR for the detection of FBoV-1 was successfully established in this study. It is an efficient detection method with high sensitivity and specificity. Moreover, it is suitable for clinical quantitative detection and epidemiological investigation of FBoV-1, pathogenesis research, and clinical study of feline diarrhea virus co-infection.

**Table 3**  
Clinical samples detection for FBoV-1 infection by qPCR assay.

	Amount	Region	Number of positive samples	
			Conventional PCR	qPCR
<b>Total</b>	<b>128</b>		<b>6/128 (4.7%)</b>	<b>9/128 (7.0%)</b>
	23	Shanghai	0	1/23 (4.3%)
	20	Nanjing	1/20 (5.0%)	2/20 (10.0%)
	51	Hefei	3/51 (5.9%)	4/51 (7.8%)
	19	Ma'anshan	1/19 (5.3%)	1/19 (5.3%)
	15	Bozhou	1/15 (6.7%)	1/15 (6.7%)

#### Compliance with ethical standards

All experiments were compliant with the ethical standards of Anhui Agricultural University (Permit number: SYXK 2016–007).

#### Authors' contributions

Jianfei Sun, Xu Guo, Da Zhang, and Yongqiu Cui were involved in performing experiments. Wei Li and Guangqing Liu were involved in data analysis, experimental design. Yong Wang, Shudong Jiang and Yongdong Li wrote the manuscript. All authors read and approved the final manuscript.

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#### Declaration of competing interest

All authors have declared that no competing interests exist.

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#### References

- [1] A. Kapoor, N. Mehta, E.J. Dubovi, P. Simmonds, L. Govindasamy, J.L. Medina, C. Street, S. Shields, W.I. Lipkin, Characterization of novel canine bocaviruses and their association with respiratory disease, *J. Gen. Virol.* 93 (Pt 2) (2012) 341–346.
- [2] S. Yi, J. Niu, H. Wang, G. Dong, Y. Zhao, H. Dong, Y. Guo, K. Wang, G. Hu, Detection and genetic characterization of feline bocavirus in Northeast China, *Virol. J.* 15 (1) (2018) 125.
- [3] S.F. Cotmore, M. Agbandje-McKenna, J.A. Chiorini, D.V. Mukha, D.J. Pintel, J. Qiu, M. Soderlund-Venermo, P. Tattersall, P. Tijssen, D. Gatherer, A.J. Davison, The family Parvoviridae, *Arch. Virol.* 159 (5) (2014) 1239–1247.
- [4] C. Piewbang, T. Kasantikul, K. Pringproa, S. Techangamsuwan, Feline bocavirus-1 associated with outbreaks of hemorrhagic enteritis in household cats: potential first evidence of a pathological role, viral tropism and natural genetic recombination, *Sci. Rep.* 9 (1) (2019) 16367.
- [5] I.V. Babkin, A.I. Tyumentsev, A.Y. Tikunov, E.V. Zhirakovskaia, S.V. Netesov, N. V. Tikunova, A study of the human bocavirus replicative genome structures, *Virus Res.* 195 (2015) 196–202.
- [6] L.N. Binn, E.C. Lazar, G.A. Eddy, M. Kajima, Recovery and characterization of a minute virus of canines, *Infect. Immun.* 1 (5) (1970) 503–508.
- [7] M. Mochizuki, M. Hashimoto, T. Hajima, M. Takiguchi, A. Hashimoto, Y. Une, F. Roerink, T. Ohshima, C.R. Parrish, L.E. Carmichael, Virologic and serologic identification of minute virus of canines (canine parvovirus type 1) from dogs in Japan, *J. Clin. Microbiol.* 40 (11) (2002) 3993–3998.
- [8] S.K.P. Lau, P.C.Y. Woo, H.C. Yeung, J.L.L. Teng, Y. Wu, R. Bai, R.Y.Y. Fan, K. H. Chan, K.Y. Yuen, Identification and characterization of bocaviruses in cats and dogs reveals a novel feline bocavirus and a novel genetic group of canine bocavirus, *J. Gen. Virol.* 93 (Pt 7) (2012) 1573–1582.
- [9] M. Garigliany, G. Gilliaux, S. Jolly, T. Casanova, C. Bayrou, K. Gommeren, T. Fetti, A. Mauroy, E. Levy, D. Cassart, D. Peeters, L. Poncelet, D. Desmecht, Feline

- panleukopenia virus in cerebral neurons of young and adult cats, *BMC Vet. Res.* 12 (2016) 28.
- [10] C. Liu, F. Liu, Z. Li, L. Qu, D. Liu, First report of feline bocavirus associated with severe enteritis of cat in Northeast China, 2015, *J. Vet. Med. Sci.* 80 (4) (2018) 731–735.
- [11] Y. Zhou, J. Xu, W.L. Wang, S.W. Song, S.K. Zhu, Q.F. Meng, F. Yu, C.P. Li, N. Liu, W.M. Luan, A TaqMan-based real-time PCR assay for the detection of Ungulate bocaparvovirus 2, *J. Virol. Methods* 261 (2018) 17–21.
- [12] C. Wan, C. Chen, L. Cheng, G. Fu, S. Shi, R. Liu, H. Chen, Q. Fu, Y. Huang, A TaqMan-based real-time PCR for detection and quantification of newly identified novel pigeon adenovirus, *J. Virol. Methods* 261 (2018) 6–9.
- [13] Z. Yu, D. Zhang, K. Yang, C. Bai, Y. Li, J. Li, S. Jiang, Y. Wang, A simple and rapid diagnostic method to detect new goose astrovirus using reverse-transcription loop-mediated isothermal amplification, *3 Biotech* 10 (1) (2020) 20.
- [14] Q. Zhang, J. Niu, S. Yi, G. Dong, D. Yu, Y. Guo, H. Huang, G. Hu, Development and application of a multiplex PCR method for the simultaneous detection and differentiation of feline panleukopenia virus, feline bocavirus, and feline astrovirus, *Arch. Virol.* 164 (11) (2019) 2761–2768.
- [15] B. Schunck, W. Kraft, U. Truyen, A simple touch-down polymerase chain reaction for the detection of canine parvovirus and feline panleukopenia virus in feces, *J. Virol. Methods* 55 (3) (1995) 427–433.
- [16] A.F. Streck, D. Ruster, U. Truyen, T. Homeier, An updated TaqMan real-time PCR for canine and feline parvoviruses, *J. Virol. Methods* 193 (1) (2013) 6–8.
- [17] S. Yi, J. Niu, H. Wang, G. Dong, Y. Guo, H. Dong, K. Wang, G. Hu, Molecular characterization of feline astrovirus in domestic cats from Northeast China, *PLoS One* 13 (10) (2018), e0205441.
- [18] G. Lu, X. Zhang, J. Luo, Y. Sun, H. Xu, J. Huang, J. Ou, S. Li, First report and genetic characterization of feline kobuvirus in diarrhoeic cats in China, *Transbound. Emerg. Dis.* 65 (5) (2018) 1357–1363.
- [19] I.M. Mackay, K.E. Arden, A. Nitsche, Real-time PCR in virology, *Nucleic Acids Res.* 30 (6) (2002) 1292–1305.