Establishment and Application of a Taqman Reverse Transcriptase Quantitative Real Time Pcr Assay for Feline Calicivirus

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Abstract

Feline calicivirus (FCV) is an infectious pathogen that causes disease in cats. With the current emergence of FCV-associated virulent systemic disease (FCV VSD) worldwide, the establishment of a rapid, sensitive, and reproducible diagnostic assay for its detection is important to inform prevention and control strategies. In this study, specific primers and TaqMan-FAM probes were designed based on the conserved regions of the FCV genome sequence, and a TaqMan reverse transcriptase quantitative real time PCR assay was established. This assay could specifically detected the FCV genome. The assay had a wide dynamic range, with linear detection in the range of 9.6×10^9 copies/μL to 9.6×10^0 copies/μL, with a limit of detection of 9.6×10^0 copies/μL, showing high sensitivity and repeatability. In addition, we used this assay to evaluated clinical samples (n=100) taken from cats from across China for the presence/absence of FCV genetic material For samples with low virus content, the positive detection rate of TaqMan reverse transcriptase quantitative real time PCR assay (RT-qPCR) was much higher than that of conventional reverse transcriptase PCR assay (cRT-PCR). And The qRT-PCR assay was used to detect the viral load of cat swabs within 17 days after FCV infection. From days 1-9, the oral and nasal swabs generally had higher viral loads than the anal swabs. While from days 10-17, the levels in the oral and nasal swabs being generally lower than those in the anal swabs. Overall, this FCV TaqMan RT-qPCR assay assay represents a rapid and accurate.

1. Introduction

Caliciviruses are a type of RNA virus that can potentially cause serious disease in humans and animals. At present, the family *Caliciviridae* consists of *Saporovirus*, *Lagovirus*, *Vesivirus*, *Nebovirus*, and *Norovirus*. Rabbit hemorrhagic disease virus (RHDV) is a representative virus of the genus Rabbit virus. In 1984, RHDV first broke out in Wuxi and Jiangyin, Jiangsu, China[1]. Subsequently, it was reported on all continents in the world, such as North America and Europe[2, 3]. Among them, the human norovirus and Sapporo virus are the main pathogens that cause acute viral gastroenteritis in humans, collectively known as human calicivirus[4]. Epidemiological surveys show that approximately one-fifth of all acute gastroenteritis cases in the world are caused by noroviruses, with total norovirus infections reaching as high as 267 million person-times, resulting in about 200,000 deaths every year[5]. In 2017–2019, the positive detection rate of norovirus in 904 children with diarrhea under 5 years old in Hohhot, China was as high as 24%[6]. The important public health problems caused by norovirus have attracted more and more attention from the international scientific community. Since noroviruses and most other caliciviruses are difficult to culture in vitro, Feline calicivirus (FCV) of the same family can proliferate in cat kidney cells at high titers, and since animal models are relatively mature, they are often used as models for calicivirus research[7].

The FCV is a representative strain of Caliciviridae and vesicular viruses that can infect all cat species. The virus is distributed worldwide and can spread between species [8, 9]. FCV infection can cause oral ulcers, chronic stomatitis, pneumonia, and other upper respiratory diseases in cats[10]. In recent years, there have been many reports of FCV-associated virulent systemic disease (FCV VSD) strains in many

countries. FCV VSD can cause subcutaneous edema, oral ulcers, and different degrees of ulceration of the skin, such as in the auricles and foot pads[11]. It has a very high fatality rate[12]. The high infection rate and variability of FCV provide the necessary conditions for the evolution of FCV, the recombination of various strains, and even the emergence of FCV VSD. At present, vaccination is the main measure to control viral infection, but it does not provide good immune protection for cats; it cannot prevent infection or prevent infected cats from becoming virus carriers[13]. FCV is a non-enveloped RNA virus. FCV is stable in the environment, and the virus can survive for several days to several weeks in dry environment, and longer in cold and wet conditions[14, 15]. Sick cats, infected cats in the incubation period are the main sources of FCV infection. They can excrete FCV for a long time post infection and pose serious risks to public health [16]. Healthy cats usually get infected through direct contact with sick cats, their secretions, or contaminated equipment[17].

Currently, the methods for FCV detection mainly include virus isolation, electron microscope observation, ELISA detection of antigens or antibodies, PCR, fluorescence-based quantitative PCR, etc[18, 19]. Virus isolation is time-consuming, and coinfection often occurs clinically. Most of the other calicivirus, except FCV, cannot proliferate well in the cells, bringing inevitable problems for the virus isolation. More virions are required for electron microscope observation. Although this method is intuitive but not sensitive, normally virion numbers in the clinical sample usually cannot fulfil the requirements for electron microscope observation. The cost of ELISA detection is relatively high, and there are only a few commercial detection kits on the market with low specificity. Although traditional PCR is widely utilized, its sensitivity is low. Moreover, traditional PCR is not appropriate for detection of some early infections. Fluorescence-based quantitative PCR methods mainly consist of two types, dye-based method and probe-based method. Generally, probe-based method is more accurate and more specific than dye-based method in quantitative detection, and is widely used[20]. Therefore, it is of great significance to establish an accurate and rapid FCV detection method for the prevention and control of this disease.

In this study, 41 FCV genome sequences reported in NCBI were compared to determine the conserved regions, and a specific, sensitive, and accurate FCV TaqMan qRT-PCR detection assay was successfully established. Detection of FCV qRT-PCR will lay a foundation for the diagnosis, disease prevention, and molecular epidemiological investigation of FCV, and provide a reference for the molecular biology and epidemiology of noroviruses and other members of Caliciviridae.

2. Material And Methods

2.1 Preparation of virus and tested samples

Samples containing Feline calicivirus (FCV), feline parvovirus (FPV), feline herpesvirus (FHV-1), canine parvovirus (CPV), and canine coronavirus (CCoV) were collected from pet hospitals in China and identified in our laboratory. A total of 100 clinical samples were obtained from pet hospitals in China between 2019 and 2020. Eight 3-month-old experimental cats weighing 1.5 kg were purchased from a market in Beijing. They were tested for FCV antigen and all antibody tests were negative. 0.5ml FCV was

used to instill the eyes on the nose. 1-17 days after challenge, oral, nasal and eye swabs and anal swab were collected. FCV. All samples were kept at -80°C in our laboratory.

2.2 Design of Primers and TaqMan probes

Full-length sequences of FCV (n=41) were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/) and aligned using MEGA7.0. The most conservative regions were identified manually for the design of primers and probes. DNAMAN was used to evaluate the physical properties of the primers and TaqMan probes. FCV-BZP-F and FCV-BZP-R primers were expected to amplify a 624bp fragment. The Primer Express DNAMAN software was then used to prepare the FCV-F and FCV-R qRT-PCR primers and the FCV TaqMan-FAM probe (Table 1), which were predicted to generate a 129 bp fragment. The qRT-PCR probe, labeled at the 5' end with FAM and with a dark quencher dye at its 3' end, was designed to anneal an internal sequence of the amplified region.

2.3 RNA extraction and reverse transcription

1g stool samples were diluted with 1 mL of PBS (Gibco□CA). After three freeze-thaw cycles, the samples were then spun for 5 min at 3,000 g at 4 °C, with supernatants being collected[21]. and stored at -80°C for later use. The viral RNA was extracted using the Qiagen Viral RNA kit (Qiagen, Hilden, Germany), in accordance with to the kit manufacturer's instructions, after which the Reverse Transcription Kit (TIANGEN Beijing, China) was used to prepare cDNA based on provided guidelines. Extracted cDNA was stored at -20 °C. Every 20 μL reverse transcription reaction contained 4 μL 5×FastKing-RT SuperMix, 2 μL Total RNA, and 14 μL RNase-Free water. The PCR protocol for the reverse transcription was 15 min at 42°C, then 3 min at 95°C. The synthesized cDNA was stored at -20°C for later use.

2.4 Preparation of standard plasmids

The 624 bp target gene fragments were amplified via PCR in a reaction mixture containing 1 μL each of the forward and reverse primers (FCV-F and FCV-R, 10 μmol/L), 2 μL FCV cDNA template, 21 μL RNase-free water, and 25 μL Prime STAR Max Premix (TaKaRa Biotechnology□Dalian, China). The thermocycler settings were as follows: 98°C for 10 s, 55°C for 30 s, 30 cycles of 72°C for 10 s, and finally 72°C for 5 min. The amplified PCR products were incorporated into a pEASY-Blunt cloning vector (TransGen Bitech, Beijing, China), and then transformed into Trans1-T1 competent cells (TransGen Bitech, Beijing, China) according to the manufacturer's guidelines. The bacteria containing positive recombinant plasmids were screened by PCR and sent to a sequencing facility for identification. Plasmids with correct sequences containing the FCV target fragment genes were the standard products. The plasmids were then purified using a commercial column and the plasmid concentration was quantified with a NanoDrop 1000 (ThermoFisher Scientific, USA). The number of plasmid copies in the sample was estimated based on the molecular weight of the plasmid standard and the plasmid concentration. (Calculation formula: copy number=$6.02*10^{23}$ (copies/mol) × [standard concentration (ng/μL) × 10^{-9}]/standard length (bp) × 660 (Dalton/bp)). Ten-fold serial dilutions of plasmids were prepared in RNase-free water(Solarbio, Beijing□ China), and aliquots of each dilution were frozen at -80°C. Each aliquot was used only once for qRT-PCR.

Dilutions of the standard plasmids were tested by qRT-PCR, and a standard curve was generated using Microsoft Excel(USA).

2.5 Optimization of real-time fluorescence quantitative PCR reaction conditions

Standard plasmids were amplified via qRT-PCR in an ABI QuantStuio 7 Flex Sequence Detection System (ThermoFisher Scientific, USA) using the Hieff Unicon® qPCR TaqMan Probe Master Mix (Hieff Unicon, China). Each 20 μL reaction mix contained 10 μL 2× Hieff Unicon® TaqMan multiplex qPCR master mix, 0.5 μL ROX reference dye as a passive reference, forward and reverse primers (FCV-F and FCV-R, 0.2-1.0 mol/L), probes (0.4-0.6 mol/L), standard plasmids (1-100 ng), and RNase-free water up to 20 μL. The thermocycler settings were as follows: 95°C for 1 min and 40 s, 95°C for 10 s, 30 cycles of 50°C for 35 s. The data were collected at the annealing step of each cycle and the threshold cycle (Ct) for each sample was calculated by determining the point at which the fluorescence exceeded the threshold limit.

2.6 qRT-PCR standard curve preparation

The standard plasmid was serially diluted 10-fold from 9.6×10^9 copies/μL to 9.6×10^0 copies/μL. The final standard curve was generated based on the Ct value and the logarithm of the standard copy number.

2.7 Specificity test

The specificity of the assay was evaluated using the above conditions with a range of different viruses (FCV, FPV, FHV-1, CPV, CCoV), together with the negative controls.

2.8 Sensitivity and reproducibility analyses

To determine the sensitivity of the assay, standard plasmids were serially diluted 10-fold with concentrations ranging from 9.6×10^9 to 9.6×10^0 copies/μL. The prepared standard plasmids were amplified with a qRT-PCR system to confirm the detection limit. The sensitivity of this qRT-PCR assay was assessed using a cRT-PCR assay. Intra-assay reliability was evaluated by simultaneously assessing three replicates of each of the three dilution samples. Inter-assay reliability was assessed by repeating three independent experiments. The inter- assay and intra-assay coefficients of variation (CVs) were determined by dividing the standard deviation of each tested sample by its mean prior to multiplying the result by 100.

2.9 Clinical sample analyses

In total, 100 clinical samples were collected from pet hospitals all over China and stored at -80°C in our laboratory. RT-qPCR and RT-PCR assay were performed to compare the detection rates of FCV of the two methods.

2.10 Detoxification test

The viral loads of the oral, nasal swabs and the anal swabs collected before and 17 days after the FCV infection were tested, and the law of FCV external detoxification was studied.

3. Results

3.1 Preparation of standard plasmids

Using the FCV reverse transcription product cDNA as a template, the 624 bp target gene fragments were amplified. Amplified PCR products were incorporated into a pEASY-Blunt cloning vector, and the bacteria containing the positive recombinant plasmid were screened out and sent for sequence identification. After sequencing, BLAST search was performed to match the gene sequences registered in GenBank. Using spectrometry, the concentration of recombinant plasmid standard product was determined to be 480 ng/μL and the copy number was calculated to be 9.6×10^{10} copies/μL. This was used as the standard for qRT-PCR and cRT-PCR sensitivity evaluation.

3.2 Optimization of real-time fluorescence quantitative PCR reaction conditions

Through optimization of primer concentration and elimination temperature, the optimal qRT-PCR reaction conditions obtained are as follows: 2× Hieff UNICON qPCR TaqMan Probe Master Mix: 10 μL; FCV-F (10 μM) 1.2 μL; FCV-F (10 μM) 1.2 μL; TaqMan probe Needle (10 μM) 0.6 μL; 50× low Rox 0.4 μL; template DNA 2 μL; RNase-free water 4.6 μL; for a total of 20 μL per reaction. The PCR program was: 95℃, 1 min 40 s; and 45 cycles of 95℃, 10 s, 50℃, 35 s.

3.3 qRT-PCR standard curve preparation

The standard plasmids were serially diluted 10-fold from 9.6×10^{9} to 9.6×10^{0} copies/μL. Refer to the optimized qRT-PCR reaction system and conditions for fluorescent qRT-PCR amplification, and draw a standard curve with reference to the results. The qRT-PCR standard curve is shown in (Figure 1), the linear regression equation is: y = -3.327x + 43.194, R^2 = 0.9934, the slope is 3.327, with the minimum detection limit being 9.6 copies/μL. efficiencies of 90%-110% (efficiency = $(10^{(-1/\text{slope})} - 1)$), were regarded as acceptable. The standard plasmid showed a very good linearity between 9.6×10^{9} and 9.6×10^{0} copies/μL.

3.4 Specificity test

The specificity of the qRT-PCR assay was evaluated using FCV, FPV, FHV-1, CPV, and CCoV, with RNase-free water as a negative control. While strong fluorescent signals were obtained for the FCV sample, all other samples yielded negative results, emphasizing that this assay specifically detects FCV (Figure 2).

3.5 Assessment of the sensitivity and reproducibility of this real-time PCR assay

The sensitivity of the qRT-PCR assay was evaluated by testing 10-fold serial dilutions of the RNA standards (9.6×10^{9} to 9.6×10^{0} copies/μL). Quantitative analysis identified a detection limit of

approximately 9.6 copies/μL of viral RNA(Figure 3). The lowest detection limit of the cRT-PCR method was calculated to be approximately 9.6×10^2 to 9.6×10^3 copies/μL (Figure 4).

Standard plasmids with three dilutions of the same batch were used as templates (9.6×10^7 copies/μL, 9.6×10^4 copies/μL, 9.6×10^1 copies/μL) for the intra-batch reproducibility test, with three replicate wells for each concentration. Three batch-to-batch repeatability tests were performed under the same reaction conditions. The results showed that the coefficients of variation within the groups were 0.8%, 0.22%, and 0.66%; the coefficients of variation between groups were 1.9%, 1.6%, and 0.72%, all of which were less than 2% (Table 2). The results showed that the method we developed has good repeatability.

3.6 Clinical sample testing

The FCV TaqMan qRT-PCR method established in this study was used to detect 100 clinical samples, with conventional PCR detection performed in parallel. The positive detection rate of the qRT-PCR was 21%, whereas the detection rate of cRT-PCR was only 5% (Table 3). The copy number of five samples was about 1.0×10^8 to 1.2×10^9 copies/μL, and the copy number of 16 samples was about 9.6×10^0 to 3.1×10^3 copies/μL.

3.7 The progression of the virus infection in artificial infected cats test

The Taqman qRT-PCR assay established in this study was used to detect the viral load of cat oral, nasal, eyes and anal swabs within 17 days after FCV infection, with three replicates for each sample. Within 1-17 days after the infection, the virus excretion amount was variable at first and eventually stabilized: the 9th day had the highest excretion of viruses, with the first 8 days being the unstable stage. The results showed that the oral , nasal, eyes swabs generally had higher viral loads than the anal swabs, and that overall detoxification volume increased with time. From days 10-17, the virus levels in all swabs remained steady, with the levels in the oral and nasal swabs being generally lower than those in the anal swabs (Figure 5) .

4. Discussion

Feline calicivirus is one of the main pathogens that cause respiratory diseases in cats. The typical clinical signs of cats infected with FCV include malaise, mouth and nose ulcers, conjunctivitis, fever, and pneumonia. Some cats showed acute febrile claudication syndrome and other clinical symptoms. The differences in the symptoms was directly related to the virus strain[22]. Since FCV was first isolated from the gastrointestinal tract of sick cats in 1957, it has also been isolated from domestic and wild cats in many countries and regions around the world[8, 23]. A malignant systemic disease (VSD) caused by FCV, with extremely high mortality, was first reported in 1998[24]. Since then, reports of FCV VSD have appeared in many countries, such as Italy, France, and Germany[12, 25, 26]. This disease was associated with a high mortality rate and new clinical characteristics, hence, its discovery could signal the emergence of a highly pathogenic strain in the future. Epidemiological investigations showed that clinically mixed infections of FCV, FHV-1, and FPV often occur[27]. The clinical symptoms of FCV and FHV-1 infections

are similar, and a differential diagnosis cannot be made based on clinical symptoms alone. As one of the common pathogens in cats, FCV is distributed in all regions of China. Current vaccines on the market cannot provide a comprehensive and effective protection against an epidemic[28]. Therefore, the establishment of an accurate and rapid FCV detection method is important for the prevention and control of the disease. In 2009, Abd-Eldaim M.M. et al. first established the FCV TaqMan fluorescence quantitative detection method, with a minimum detection limit of 70 copies/μL[29]. In 2013, the sensitivity of the method established by Jiang X. et al. was further improved, compared with the method established by Abd-Eldaim M.M.. The minimum detection limit is 22.6 copies/μL[30]. However, we found that its probe region was not conserved through comparing 41 full-length FCV sequences in the NCBI database. Therefore, we compared 41 full-length genome sequences of FCV reported so far, found the conserved region on FCV ORF1, and designed FAM probes to optimize the reaction conditions to detect this sequence. This method does not detect cross-reaction signals of other common viruses of dogs and cats, and the sensitivity of this method has been greatly improved. The minimum detection limit of our method was approximately 2–7 times more sensitive than that of the previous two methods. Our method has good repeatability, with the coefficient of variation within and between groups at less than 2%. Even if different personnel operate, accurate and reliable detection results can be obtained.

After testing the clinical samples, we found that the detection rate of 100 clinical samples was 21%. Among them, five samples with higher virus content had the same results between qRT-PCR and cRT-PCR, but the remaining 16 samples had lower virus content, the detection rate of cRT-PCR was 0, and the detection rate of Taqman Real-time PCR was 100%. This indicates that the Taqman qRT-PCR is more sensitive and accurate. Epidemiological investigations showed that most of the 16 cats were from catteries and cat farm kittens, which showed fever and mild respiratory symptoms, and were diagnosed with FCV infection by this method. Early diagnosis can enable cat farms to take sufficient time for epidemic prevention, control, and diagnosis, to reduce losses.

At the same time, we used this method to monitor changes in the amount of virus excreted in the 17 days oral, nasal, eyes and anal swabs of four FCV-infected cats. The qRT-PCR Ct value of the oral, nose and eye swabs was > 40.00 on 1–2 days, so we determined them to be FCV-negative, that is, the cat did not start detoxification yet. The qRT-PCR Ct value on the 3rd day was 38.61-40.00, which we determined to be FCV-positive. In the results of an FCV pathogenicity test, Wang H. et al. showed that FCV can be detected through nose and eye swabs three days after viral infection, consistent with our conclusions[31]. On the 10th day, the Ct value dropped to 26.96, that is, the detoxification amount reached the maximum; after that, the Ct value became stable. The results showed that the amount of toxin found in the oral and nasal swabs in FCV-infected cats reached a maximum about 10 days after infection, then they entered a state of continuous detoxification. At 1–17 days, two sets swabs had the similarity changes. At 1–10 days, the oral, nose, and eye swabs for detoxification testing were generally higher than fecal swabs. This might indicate the stage of disease development. Since FCV first replicates in the oral and respiratory tissues, the body might not have completed immune clearance of the virus particles. During this time, the main detoxification method happens in the upper respiratory tract. From the 10–17 days, the virus levels in the oral, nose, eyes and anal swabs remained basically stable, but the virus levels in the oral, nose, and eye

swabs were generally lower than those in the stool swabs. At this time, the disease may be at a plateau stage. The amount of virus in oral, nose, and eye swabs and anal swabs were not notably different, but the amount of virus in oral, nose, and eye swabs were lesser than that in anal swabs.

The above results indicate that FCV can be transmitted through a variety of ways. The respiratory tract was found to be the main route of FCV transmission within 10 days of infection. After that, it was mainly excreted through fecal waste. The establishment of a specific method to study the external detoxification of FCV is of great significance to reveal the pathogenesis and to help prevent and control the spread of its disease.

Overall, this study established a highly sensitive, specific, and reproducible qRT-PCR detection method for FCV. This method overcomes the limitations of existing detection methods, and can accurately detect and quantify FCV content in samples with low and high viral load. It also provides a new method for pathogen diagnosis, disease prevention, and molecular epidemiological investigation of FCV in cats.

Declarations

Ethics statement

All animal experiments were conducted under protocols by Science Research Department (in charge of animal welfare issue) of the Institute of Animal Science, Chinese Academy of Agricultural Sciences (No.IAS2020-95, 3 August 2020). Research was conducted incompliance with the principles stated in the guide for the Care and Useof Laboratory Animals, National Research Council, 1996.

Informed consent

The author unanimously agreed to submit the manuscript to Archives of Virology.

Conflicts of Interest

The authors declare that they have no competing interests.

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Tables

Table 1. Primer and probe sequence

Primer Probe	names	Primer sequences(5’→3’)	Size/bp
Forward	FCV-F	5’-GGRAARATTGTCAATGAHARBGT-3’	129
Reverse	FCV-R	5’-ACATCATATGCGGCTCTGA-3’	
Probe	FCV-P	5’-FAM-CCGCCAATCAACATGTGGTAA-BHQ-3’	
Forward	FCV-BZP-F	AAATATTGACTCCTTGGCCCAAAC	624
Reverse	FCV-BZP-R	AATAAACTGCGTCACCACATGGTC	

Table 2. Results of the repeatability test of the qRT- PCR assay (n=3).

Concentration of standard plasmids (copies/μL)	Intra-assay variability		Inter-assay variability	
	±SD	CV (%)	*±SD*	CV (%)
9.6×10^{7}	14.93±0.12	0.80	15.33±0.30	1.90
9.6×10^{4}	26.04±0.06	0.22	26.51±0.42	1.60
9.6×10^{1}	35.22±0.23	0.66	35.31±0.25	0.72

Table 3. qRT-PCR and cRT-PCR clinical sample detection.

Detection	Positiive samples	Total samples	Positiive rate
cRT-PCR	5	100	5%
qRT-PCR	21	100	21%