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A TaqMan[®] RT-PCR Assay for the Detection of *Feline calicivirus*

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Abstract: *Feline calicivirus* (FCV) is one of the most common pathogens of cats causing upper respiratory tract infections. A real time RT-PCR assay was developed for the rapid detection of FCV. A set of probe/primers was designed to amplify a region of 151 bp based on the conserved region of 7 FCV strains (FCV 255, 2280, NADC, F9, KCD, CF I and Urbana) by sequence comparison. The assay was found to be more sensitive than virus isolation and was linear over a wide range of template concentrations.

Key words: Real time RT-PCR, respiratory infection, *Feline calicivirus*

INTRODUCTION

Feline calicivirus (FCV) is a single stranded RNA virus belonging to the family *Caliciviridae* and is a common cause of upper respiratory tract infection in cats (Gaskell and Dawson, 1994). In addition, FCV may also cause conjunctivitis, stomatitis, enteritis and lameness (Baulch-Brown *et al.*, 1997). Infections caused by strains of low virulence are usually restricted to oral mucosa, nasal passages and conjunctiva while those by virulent and pneumonic strains show signs of severe respiratory infection. Mortality is high in the presence of secondary bacterial infections (Murphy *et al.*, 1999). Recovered cats can serve as carriers of the virus and are important in the maintenance and dissemination of infection in domestic and wild cat populations. Harbour *et al.* (1991) reported virus isolation rates of 20% from oropharyngeal swabs collected from domestic cats without any breed or sex variation. A similar rate of high FCV carriage was reported from a cat shelter and purebred cattery in California (Tenorio *et al.*, 1991). Although the duration of shedding is variable, approximately 50% of the infected cats cease virus shedding by 75 days post-infection although individual cats may shed virus for up to 2 years (Barlough, 1992).

Clinical diagnosis of FCV is based on history and clinical signs but definitive diagnosis requires virus isolation in cell cultures and/or serology. Since virus isolation procedures are time consuming and expensive, tests based on nucleic acid amplification (e.g., reverse transcription-polymerase chain reaction or RT-PCR) have been developed. However, these tests have been hampered by difficulty in designing oligonucleotide primers that are able to amplify nucleic acids from all different strains of FCV (Sykes *et al.*, 1998). In addition, PCR products need to be detected by gel electrophoresis or Southern blotting thus increasing the time and expense in the specific detection process. Recently, Marsilio *et al.* (2005) reported on a nested PCR (nPCR) for the detection of FCV. Although more specific than RT-PCR, nPCR requires the use of two sets of primers and two cycles of PCR followed by electrophoretic separation of bands, which again increases the time as well as cost of diagnosis.

Recently, real time-PCR has become a method of choice for pathogen detection because it allows real time quantification of PCR products, thus eliminating the need for any post-amplification steps

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(Leutenegger, 2001). Fluorogenic PCR assays have been successfully used in amplification and detection of various bacterial and viral pathogens (Batt, 1997). Gut *et al.* (1999) described a one-tube fluorogenic, RT-PCR for the quantification of feline corona viruses. Schweiger *et al.* (2000) reported the development of a TaqMan[®] RT-PCR for typing and subtyping of influenza viruses in the human clinical samples. This test was more sensitive than viral isolation as it could detect up to 0.1 TCID₅₀ of the virus. The TaqMan assay employs 5' → 3' exonuclease activity of *Thermus aquaticus* (Taq) DNA polymerase to hydrolyze an internal TaqMan probe labeled with a fluorescent reporter dye (FAM) and a quencher dye (TAMRA). The most striking advantage of TaqMan assay over conventional RT-PCR is the ability to save time as it allows real time quantification of amplification products. In this report, we describe the development of a TaqMan[®] assay for the detection of FCV in clinical samples.

MATERIALS AND METHODS

Viruses and Cells

This study was done at the Veterinary Diagnostic Laboratory, University of Minnesota, Saint Paul, MN. All seven FCV strains (FCV 255, 2280, NADC, F9, KCD, CF I and Urbana) used in this study were obtained from Dr. John Neill, National Animal Disease Center, Ames, Iowa. Monolayers of Crandell-Reese feline kidney cells (CRFK) were used to propagate and titrate FCVs. Briefly, the cells were grown in Eagle's minimal essential medium (MEM; Celox, St. Paul, MN) supplemented with 8% fetal bovine serum, penicillin (100 U mL⁻¹), streptomycin (100 µg mL⁻¹), fungizone (1 µg mL⁻¹), 15 mM HEPES and 5 mg mL⁻¹ lactalbumin hydrolysate. Confluent monolayers of CRFK cells were inoculated with the virus and after adsorption for 1 h, the cells were incubated in the maintenance medium (MEM without fetal bovine serum) at 37°C until the appearance of cytopathic effects (CPE), usually within 2-3 days post-infection. The viruses were harvested when CPE was observed in 60-80% cells. For virus harvesting, the cultures were frozen and thawed twice followed by centrifugation at 2500 x g for 10 min at 4°C. Clarified supernatant was aliquoted in 1 mL amounts and stored at -70°C until further use. For virus titration, serial 10 fold dilutions of viruses were prepared in maintenance medium and inoculated in 96 well plates containing monolayers of CRFK cells. Virus titers were calculated by the method of Reed and Muench (1938).

RNA Extraction

Viral RNA was prepared from 140 µL of cell-free extract using the Viral RNA extraction kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA was eluted from silica columns in 60 µL of nuclease-free water and stored at -70°C until further use.

Primer and Probe Design

Oligonucleotide primers were derived from conserved regions identified by aligning the sequences of capsid protein gene of 7 FCV strains. Details of strains used and the conserved regions are given in Table 1. Nucleotide sequences were retrieved from GenBank (www.ncbi.nlm.nih.gov) and aligned using ClustalX method to determine the conserved region. Primers and probe were designed using Primer Express software and were specific to FCV 255 (Table 2). Specificity of both primers and probe for other FCV strains was determined by BLAST analysis.

Real Time RT-PCR

Real time RT-PCR (rRT-PCR) was done using the TaqMan one step RT-PCR Master Mix kit (Applied Biosystems, CA). Each reaction mixture consisted of 12.5 µL of 2X Master Mix; 0.625 µL of 40X Multiscribe Mix; 0.4 µM of each primer; 0.8 µM of TaqMan probe; 5 µL of template RNA; and 2.875 µL of water to make a final volume of 25 µL. rRT-PCR was performed in 96 well plates

Table 1: Sequence alignment of different *Feline calicivirus* (FCV) strains showing homologous region

| Strain | Nucleotide accession No. | Sequence (5'-3') |
|----------|--------------------------|---|
| FCV 255 | U07130 | 5' 46- CAACCTGCGGCTAACCGTGGCTTAAATACTACGANTGGGACCCGCCACATTAAGGTTAGATGACCCCAADAGGTTTCT |
| FCV 2280 | X99445 | 5' 8- CAACCTGCGGCTAACCGTGGCTTAAATACTATGGCTGGGATCCCACTTTAGANTTAGTTGTTCAACCCCAACAAATTCCT |
| NADC | L09718 | 5' 34- CAACCTGCGGCTAACCGTGGCTTAAATACTATGATGGGACCCGCCACTTTAAGGCTTGTATTAAACCCCAACAAATTCCT |
| F 9 | M86379 | 5' 5321- CAACCTGCGGCTAACCGTGGCTTAAATAATGATGGGACCCGCCACTTTCAATGGTAAATCAACCCCAACAAATTCCT |
| Urbana | I40021 | 5' 5321- CAACCTGCGGCTAACCGTGGCTTAAATAATGATGGGACCCGCCACTTTCAATGGTAAATCAACCCCAACAAATTCCT |
| FCV CEI | U13992 | 5' 5318- CAACCTGCGGCTAACCGTGGCTTAAATACTATGATGGGATCCCTGACATCAAATTGGTAAATCAACCCCAACAAATTCCT |
| FCV KGD | L09719 | 5' 34- CAACCTGCGGCTAACCGTGGCTTAAATAATGATGGGATCCGCCACTTTCAATGGTAAATCAACCCCAACAAATTCCT |
| FCV 255 | U07130 | TTCTGTAGTTTCTGTGATPAAAGCCACTTCTTTGTGGTACCCAGACCTACCTCCCAAGAAATTTGGAACCGGTGTGGGA - 196 3' |
| FCV 2280 | X99445 | TTCTGTGGCTTTTGTGATPAAACCTCTCANGTGTGGCTATCCAGACTTGGCTTGTGAATTTGGAACCCCTGTGGGA - 158 33 |
| NADC | L09718 | ATCTGTGGCTTTTGTGATPAAACCCCTTAAATGTTGTACCCCTGAAATTACTCCAGAAATTTGGAACTGTGTGGGA - 184 3' |
| F 9 | M86379 | CTCTGTGGCTTTTGTGATPAAACCCCTTAAATGTTGTACCCCTGAAATTACTCCAGAAATTTGGAACTGTGTGGGA - 5471 3' |
| Urbana | I40021 | GTCCAAAGCTTTCGTGTGATPAAACCCCTTAAATGTTGTACCCCTGAAATTACTCCAGAAATTTGGAACTGTGTGGGA - 5471 3' |
| FCV CEI | U13992 | ACATGTTGGCTTCTGTGATPAAACCCCTTAAATGTTGTATTCGTGAATTACTGAAATTTGGCAACCATGTGGGA - 5468 3' |
| FCV KGD | L09719 | TTCTGTGGCTTTCTGTGATPAAATCCCTTAAATGTTGTATTCGTGAATTACTGAAATTTGGCAACCATGTGGGA - 184 3' |

Table 2: Nucleotide sequence of probe and primers used in real time RT-PCR

| Primer/probe | Sequence (5'-3') |
|---------------------------|---|
| Forward primer, FCV 1 (+) | CAA CCT GCG CTA ACG |
| Reverse primer, FCV 1 (-) | TCC CAC ACA GTT CCA AAT T |
| Probe | 6FAMCTT AAA TAY TAT GAT TGG GAY CCC CATAMRA |

(Applied Biosystems, CA) using the ABI PRISM 7900 sequence detection system. Thermocycling conditions used were: reverse transcription (RT step) at 48°C for 30 min; AmpliTaq Gold activation at 95°C for 10 min and 50 cycles of PCR consisting of denaturation and annealing at 95°C for 15 sec and 50°C for 1 min, respectively.

Specificity and Sensitivity of Assay

The sensitivity of the TaqMan assay was determined by testing RNA extracts from serial 10 fold dilutions of FCV 255 (initial titer 1.45×10^9 TCID₅₀ mL⁻¹) as a template. For standard curve, serial 10 fold dilutions of RNA extracted from undiluted virus were made in DNase and RNase-free water and then used as a template in the TaqMan assay. The detection threshold was based on the lowest concentration of viral RNA which could be detected and remained within the linearity of the standard curve. Specificity of the primers and probe was evaluated using CHV (*Canine herpes virus*) and FPV (*Feline panleukopenia virus*) isolates.

Field Isolates

Samples of trachea, spleen, lung and regional lymph nodes are routinely submitted to the Minnesota Veterinary Diagnostic Laboratory for the detection of FCV. For isolation of FCV, tissue homogenates from these samples are inoculated into CRFK cells and observed for development of CPE for up to 7 days. Samples positive for CPE are examined by negative contrast electron microscopy (Goyal *et al.*, 1987). A total of 20 such samples were tested with rRT-PCR and the results were compared with conventional virus isolation method.

RESULTS

Analytical Specificity of rRT-PCR

The designed probe/primer set was able to amplify target viral RNAs from all 7 FCV strains used in the present study. Maximum C_T (threshold cycle) value was obtained for F9 strain of FCV while the minimum value was for NADC strain. The PCR products were separated on 1.5% agarose gel and a single band at 151 bp position was observed for all seven strains (Fig. 1). The primer/probe set failed to amplify this region in CHV and FPV.

Field Samples

When 20 field samples were tested, 8 were found to be positive for FCV by the conventional virus isolation method. Of these, 7 were found to be positive by rRT-PCR.

Amplification Efficiency of rRT-PCR

Amplification efficiency of the assay was determined by using serial 10 fold dilutions of viral RNA from FCV 255 (85 µg mL⁻¹) as the PCR template. The assay was found to detect viral RNA up to 10⁻⁷ dilution (8.5 pg mL⁻¹) over the range of linearity with slope value of -3.29 and R² (coefficient of correlation) 0.993 (Fig. 2). When RNA extracts of serial 10 fold dilutions of FCV 255 (initial titer 1.45×10^9 mL⁻¹) were used as PCR template, the assay was able to detect RNA from virus suspension diluted to the equivalent of 0.01 TCID₅₀.

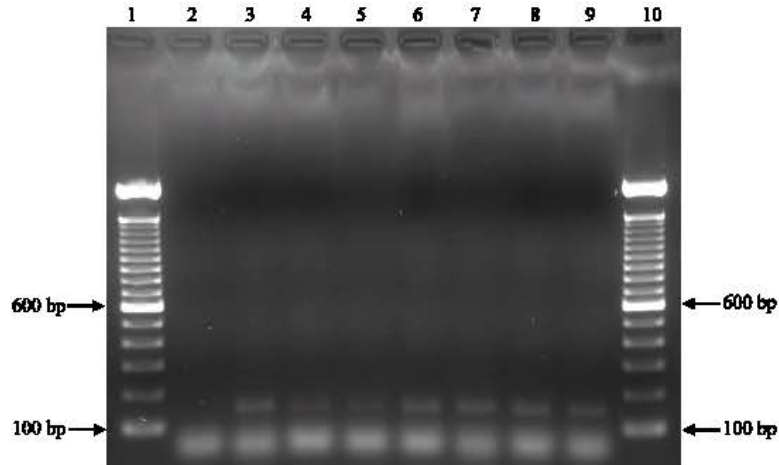


Fig. 1: Agarose gel separation of amplification products of different FCV strains after real time RT-PCR. Lane 1 and 10: Molecular marker; Lane 2: *Feline parvleukopenia virus*; Lane 3: FCV 255; Lane 4: F9; Lane 5: Urbana; Lane 6: FCV 2280; Lane 7: NADC; Lane 8: FCV KCD; Lane 9: FCV CFI

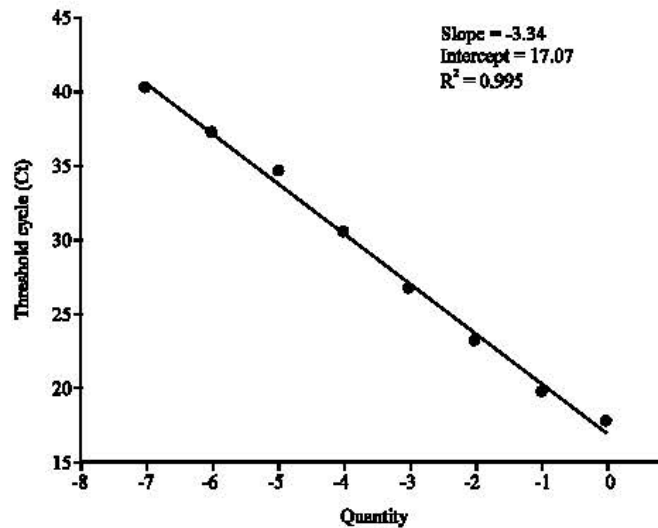


Fig. 2: Standard curve obtained with serial 10 fold dilutions of FCV 255 RNA. Ct values are plotted against different dilutions of viral RNA, when used as template for real time RT-PCR.

DISCUSSION

As with many other RNA viruses, FCV exhibits considerable antigenic heterogeneity during replication in its host. Amino acid substitutions have been shown to occur between residues 426 and 458 of FCV capsid protein E region indicating that serotypic determinants of FCV important for antigenic variation are contained in this region (Kreutz *et al.*, 1998). Thus it is important to develop a reliable, inexpensive and rapid system for the detection of all different FCV strains.

We describe the development of an rRT-PCR assay for rapid detection of FCV with high sensitivity and specificity. The assay was able to detect as little as 0.01 TCID₅₀ of FCV. The sensitivity of nPCR protocol described by Marsilio *et al.* (2005) was 1000 fold greater than virus isolation in cell culture (nPCR was positive at 10⁻¹¹ dilution of stock virus whereas virus isolation was positive to 10⁻⁸ dilution). However, nPCR requires the use of two sets of primers for specific amplification followed by separation of PCR products on agarose gel thus increasing time of detection and risk of contamination. In our protocol, the rRT-PCR is completed within 3 h without any need for the analysis of PCR products, thus reducing the time of diagnosis. Helps *et al.* (2002) reported an rRT-PCR for detection of FCV by melting curve analysis using SYBR green I dye which was more sensitive than conventional PCR as this could detect wide range of field isolates. However, this method is not as sensitive as TaqMan assay since SYBR green dye binds to any double stranded DNA produced by reverse transcription resulting in non-specific products (Helps *et al.*, 2002). The set of probe/primers used in this study did not detect CHV and FPV suggesting that the described rRT-PCR protocol is specific for the detection of FCV.

The newly developed rRT-PCR was able to detect 7 of 8 field samples this assay may not detect all FCV isolates. Helps *et al.* (2002) noted that FCV isolates are highly variable and that no single probe is able to detect all the isolates. Although a major step forward in the rapid detection of FCV, we believe that studies need to be continued to develop single unified tests that can detect all field strains of FCV. Since the detection limit of rRT-PCR is 0.01 TCID₅₀, this test should be useful in detecting carrier animals that are known to shed small quantities of virus.

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