**Feline calicivirus. Molecular Detection with Primers Design**

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**Abstract**

One of the infectious agents causing the Feline Viral Respiratory Complex is *Feline calicivirus* (FCV), participating in one of the main diseases that affect domestic felines, causing high morbidity rates and low mortality, mainly in kittens.

FCVV specifically affects the *Felidae* family and mainly the domestic cat, however, infection has also been described in wild cats. This virus is small in size (27 - 40 nm), uncovered, with an icosahedral capsid and a single-stranded RNA genome, positive polarity and approximately 7700 base pairs. This genome has three Open Reading Frames (ORFs) and ORF 2 contains the gene that codes for the VP1 capsid protein, a detection target in this study.

The diagnosis is made mainly based on the clinical signs of the affected cats, however, its interpretation is usually doubtful, as there is a low correlation between the symptoms and the presence of the virus.

Although the presence of FCV in Chile has already been described, its detection based on the primers design in line with the information available in the Genbank® is still a viable alternative when considering the geographical distribution of the different strains of FVC already described.

Thus, the alignment of 10 VP1 sequences according to their geographical distribution yielded a single common nucleotide zone that was used as a candidate sequence for the primers by the OligoPerfect Design software from ThermoFisher Scientific®. The RT-PCR protocol used was effective, since on the one and clear DNA band were obtained without nonspecific amplification in positive controls and no amplification in negative and reagent controls. The utility of the primers was reflected by the BLAST software.

Therefore, it is suggested to subsequently use the protocol in real samples, as a complementary diagnostic alternative in national Veterinary Medicine.

**Keywords:** Diagnosis; Primers Design; FCV; RT-PCR

**Background**

*Feline calicivirus (FCV)*

It is one of the most frequently reported pathogens causing oral and upper respiratory disease in cats. The virus has a worldwide distribution, is resistant to disinfection and can persist for up to a month in the environment. Furthermore, it is highly contagious, and the disease associated with FCV causes high morbidity and, in general, low mortality; these are only occasional cases of a more virulent
Feline calicivirus. Molecular Detection with Primers Design

Disease [1,2].

FCV is a relatively specific pathogen in its host, predominantly infecting domestic cats. There is more limited evidence supporting the infection of some non-domestic members of the Felidae family [3,4].

Although FCVs demonstrate significant antigenic variation, seroneutralization studies using various polyclonal antibodies have shown substantial cross-reactivity to various strains. Due to this antigenic overlap, all FCV isolates were considered to belong to a single serotype [5-7].

Classified in the genus Vesivirus of the Caliciviridae family [8], the FCV corresponds to a small virus (27 - 40 nm) [9] not enveloped in an icosahedral capsid, with a single RNA genome positive polarity chain, approximately 7700 base pairs with three Open Reading Frames (ORFs) [10]. ORF2 encodes a capsid protein (VP1) and contains both conserved and variable sequences.

Comparative analysis of ORF2 sequences has been used to determine phylogenetic relationships between the various FCV strains [10]. The sequences of the genes encoding the FCV capsid demonstrate some variation. On average, the identity of the ORF2 nucleotide sequence is approximately 80%, and the amino acid identity of VP1 is 88% [8]. Finally, the FCV isolates appear to belong to a single genotype [11,12].

Etiopathogenesis

Cats are infected via the oronasal and conjunctival lines by contact with contaminated nasal or oral secretions, and the primary site of replication is the oropharynx. Transient viremia is established in 3 - 4 days, with a course of approximately 2 to 3 weeks. The virus induces necrosis of epithelial cells; vesicles, located on the margin of the tongue, progress to ulcers and in other affected regions, the dermis is infiltrated with neutrophils [2,13].

FCV usually affects other tissues, causing pneumonia and lameness [14]. On the other hand, the pathogenesis of virulent systemic disease caused by FCV (FCV-SV) differs considerably from the classic picture; these strains cause generalized vasculitis, multi-organ failure, and death in up to two-thirds of infected cats [15,16].

The pathogenesis of FCV-SV infection is unknown and may include viral evolution and/or immunomediated components, as well as environmental and management factors [17]. After recovery, most cats clear the infection within 30 days; a few transmit the virus for much longer, possibly for life. This carrier state persists, and viral spread can occur from the oropharynx for weeks to months, likely due to amino acid changes in the virus capsid protein, allowing FCV variants to escape the host immune response and thus persist [1,11].

Symptomatology

In FCV infection, considerable strain diversity can lead to some variation in clinical signs. The classic sign is oral ulceration, typically on the tongue; Other signs characteristic of upper respiratory tract disease such as sneezing, nasal/ocular discharge and conjunctivitis are less common than those seen in VHF-1 infection but remain frequent findings [18].

With some FCV strains, lameness and pyrexia may be characteristic, with or without respiratory/oral disease; Other strains can induce interstitial pneumonia with infection of alveolar macrophages [19], which occurs particularly in kittens, manifesting itself through dyspnea, cough, fever, and depression, even leading to death. Furthermore, FCV infection is associated with chronic stomatitis, although its exact role in the condition is unclear [1].

Thus, some hypervirulent strains of CFV-SV have emerged in North America and in several European countries [20]. In addition to upper respiratory tract disease, affected cats demonstrate varying degrees of pyrexia, anorexia and jaundice, in addition to cutaneous edema.
and ulcerative dermatitis, the latter due to deep vasculitis. The mortality rate of FCV-SV is high and usually more than half of infected cats can die because of severe vasculitis, hepatocellular necrosis, disseminated vascular coagulation, or other symptoms of the condition [21,22].

Adult cats are usually more severely affected than kittens, and on the ground, the disease is seen in both vaccinated and unvaccinated individuals. Each outbreak appears to be caused by a different strain; so far none of these FCV-SVs appear to have become widely established in the population [23,24].

**Epidemiology**

In Chile, Cerda, et al. [25] carried out a study in which FCV was isolated from samples of oropharyngeal secretions from cats with respiratory symptoms; A protocol for the development of the PCR was defined, a test that allowed molecular identification of the calicivirus and the acting strain in Chile. The identified strain was coincident with the sequences that possess the nitrogenous bases of the vaccine strain F9 and a European strain; The ordering of the latter was the model used to synthesize the first one used to recognize the native virus and the content of commercial vaccines applied in the country. This allows us to infer that the calicivirus strains that cause the disease in cats are widely spread worldwide. Currently there are no studies on the prevalence of the disease in the country.

**Immunization**

The most effective prevention measure is done through vaccination; In Chile there are four vaccines authorized by the Livestock Agricultural Service for veterinary use, which contain the live attenuated *Feline calicivirus* virus vaccine strain F9, in addition to the attenuated feline infectious rhinotracheitis and feline panleukopenia viruses [26].

**Diagnosis**

Due to the asymptomatic carrier phase, and the fact that viruses in live vaccines can occasionally be disseminated post vaccination, special caution should be used when interpreting positive FCV results due to poor correlation between the presence of the virus and clinical signology [27,28]. FCV-SV, on the other hand, is diagnosed based on clinical signs, high contagion and mortality, and based on the isolation of the same strain from the blood of several sick cats (evaluated by genomic sequencing of hypervariable regions in the capsid gene) [1,29].

The diagnostic methods are as follows.

**Viral isolation**: It is a useful method to detect FCV infection; indicates the presence of virus replication and has the advantage of being less sensitive to the effect of variation between strains than RT-PCR. The virus can be isolated from nasal, conjunctival or oropharyngeal swabs, but viral isolation may fail due to a low virion load in the sample, inactivation of the virus during its transfer, or due to the presence of antibodies in extracellular fluids that prevent virus replication *in vitro* [13,30].

**Serology**: Antibodies against FCV can be detected by viral neutralization or by ELISA Seroprevalence is generally high in the cat population due to natural infection and vaccination. Consequently, the presence of specific antibodies is not useful to diagnose the infection [13,31,32].

**Nucleic acid detection**: PCR assays have been developed to detect FCV RNA in conjunctival and oral swabs, blood, skin scrapings, and lung tissue, depending on the clinical form and outcome of the disease. Diagnostic sensitivity depends on both the starters and the strain.
due to the high variability of the viral genome [1,33]. Therefore, molecular analyzes should be validated using a panel of different strains to minimize false negative results. RT-PCR has the advantage of identifying unique virus strains and has been shown to be useful in molecular epidemiology and outbreak investigations [11,29]. However, consistent genetic markers associated with virulence, specifically in FCV-SV strains, are not yet available [23].

In this work, the use of RT-PCR as a molecular diagnostic technique for the detection of FCV was proposed, using in silico-designed primers by determining common regions of the capsid protein gene of different virus strains. In this way, the results obtained could establish an alternative protocol to the existing ones.

**Materials and Methods**

The experimental work was carried out in the laboratories of Animal Virology and Microbiology of the Department of Preventive Animal Medicine of the Faculty of Veterinary and Livestock Sciences of the University of Chile.

**Controls**

For the implementation of RT-PCR, the attenuated *Feline calicivirus* P9 strain, present in the FELIGEN CRP® vaccine (Virbac®), was used as a positive control. As negative control, 5 RNA samples from canine distemper virus available in the laboratory were included. Nuclease-free water was used as the reagent control.

**Extraction of viral RNA**

RNA extraction was carried out using the TRIZOL® LS Extraction Kit (Invitrogen®). This procedure consisted of preparing a mixture of 0.25 mL of sample with 0.75 mL of the reagent, which has the function of breaking the viral envelope, leaving the RNA free. The mixture could incubate at 2°C for 5 minutes and thereafter, 0.2 mL of chloroform was added, stirred for 15 seconds and the incubation was repeated at 22°C for 2 to 3 minutes. Then, it was centrifuged at 12,000xg for 15 minutes at 4°C. The product was separated into two phases, where the RNA was located in the upper phase (aqueous phase). The latter was transferred to a new tube, causing the RNA to precipitate when mixing with 0.5 mL of isopropyl alcohol and allowing to incubate at 22°C for 10 minutes for its subsequent centrifugation at 12,000xg for 10 minutes at 4°C. The supernatant was removed, washing the RNA precipitate with 75% ethanol, vortexing and centrifuging at 7,500xg for 5 minutes at 4°C. Finally, the precipitate was vacuum-dried for 10 minutes, resuspended in 100 µL of nuclease-free water and stored at -18° to -20°C.

**Primer design**

The primers for the reaction were designed in silico from conserved regions that code for the capsid protein gene [11], identified by aligning nucleotide sequences of 10 FCV strains, described in different geographical locations, to have greater genomic variability.

The following sequences were selected by consulting the Genbank® database: 255 (U07130.1), UTCVM-NH12 (AY996863.1), UTCVM-NH2 (AY560114.1), USDA (AY560118.1), FCVV- U2 (AY053460.1), TIG-1 (KU373057.1), FCVV2024 (AF479590.1), F65 (AF109465.1), JOK63 (AF109466.1), GD (GU214989.1) (Annex 1). These sequences were aligned using the Clustal Ω program [34] (Annex 2), in order to determine the common regions and based on these, the partitions were designed with the OligoPerfect™ Designer [35] open access program. The selection criteria included a GC close to 50%, a ΔTm of < 3°C and a length of 20 bases. The synthesis of these primers was commissioned to the company IDT®.
Feline calicivirus. Molecular Detection with Primers Design

Annex 1: FCV VP1 sequences used, by Genbank® access number

<table>
<thead>
<tr>
<th>Genbank Access Number</th>
<th>Sequence</th>
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Feline calicivirus. Molecular Detection with Primers Design

Annex 2: Alignment according to Clustal Ω and choice of consensus sequence.
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<tr>
<th>Gene</th>
<th>Accession</th>
<th>Description</th>
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Feline calicivirus. Molecular Detection with Primers Design

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**Feline calicivirus. Molecular Detection with Primers Design**

**Reaction mixture**

To perform the RT-PCR technique, the Invitrogen® "SuperScript™ III One-Step RT-PCR System with Platinum™ Taq" kit was used following the procedure guidelines indicated by the manufacturer: 25 μL of "2X Reaction Mix" (Buffer containing 0.4 mM of each deoxyribonucleotide triphosphate (dNTP) and 3.2 mM MgSO₄), 10 μL of the RNA template, 2 μL of each primer, 2 μL of "SuperScript™ III RT/Platinum™ Taq Mix" and free water of nucleases achieving a total volume of 50 μL.

**DNA amplification**

To carry out the reaction, an Apollo thermocycler (CLP, USA) with 96 wells of 0.2 mL each was used. The amplification program was started with reverse transcription performed at 48°C for 30 minutes followed by initial denaturation and activation of the Taq Platinum™ at 95°C for 10 minutes. A sequence of 40 cycles was then performed (denaturation at 95°C for 30 seconds, alignment at 55°C * (determined by a temperature gradient thermocycler) for 30 seconds; elongation at 72°C for 30 seconds) and an elongation finish at 72°C for 7 minutes.

**Visualization of amplified products**

It was performed by electrophoresis on 2% agarose gel (Winkler®) in Tris acetate EDTA (TAE) buffer. 5 μL of the RT-PCR reaction product were mixed with 1 μL of the commercial loading product (6X Mass Ruler Loading Dye Solution, Fermentas®) to verify the progress of migration of the DNA bands. The commercial product Maestrogen®) was used as a marker of molecular size which contains DNA fragments between 100 and 3000 bp. Electrophoresis was performed at 90 volts for 45 minutes. After this, the incubation in gel with ethidium bromide (0.5 μL/mL) (Fermelo®) was carried out for 30 minutes and then it was visualized under ultraviolet light in a transilluminator (Transiluminator UVP®). Finally, the gel was digitally photographed.

**Determination of the percentage of nucleotide identity**

To determine the percentage of nucleotide identity, one of the samples that were positive to the RT-PCR reaction were sent to the Sequencing Center of the company Genytec Ltda. The resulting nucleotide sequences were aligned using the Clustal Ω program and a sequence was generated consensus. Subsequently, this sequence was entered the BLAST program [36] and the nucleotide sequences with the highest percentages of nucleotide identity were identified.

**Biosafety measures**

In order to carry out the laboratory work, security measures were followed in accordance with the biosecurity levels established for the Microbiology and Animal Virology laboratories. These included the use of clean and sterile material, the use of a closed white apron and disposable latex gloves. Because the visualization of the amplified products involved the use of Ethidium Bromide and the use of a UV light transilluminator, use was made of an acrylic plate and glasses with a UV filter in order to protect the observer’s vision. After finishing the work, the gel dipped in ethidium bromide was removed, since the latter compound has a mutagenic effect.

**Results**

**Implement an RT-PCR assay for molecular diagnosis of the FCV capsid protein gene**

**Alignment of used sequences**

Table 1 shows the common area according to the alignment using Clustal Omega.

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Table 1: Synthesis of FCV VP1 sequence alignment available from Genbank®. In red, the beginning and end of the candidate sequence for the design of partitions.

<table>
<thead>
<tr>
<th>Primer Design</th>
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<td>Table 2 shows the primers proposed by the OligoPerfect Design software.</td>
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Table 2: Features of the partitions proposed by the Invigogen® Oligoperfect Design software: sequence, size, % GC and Tm.

RT-PCR reaction

In figure 1 it is possible to observe the bands originated in the RT-PCR reaction of the controls, where clear bands were observed both in the cases of positive controls and for each one of the stool samples mixed with V1. No bands were observed in the negative controls or in the reagent control.
Figure 1: 2% Agarose gel electrophoresis.

Identify the specificity of the RT-PCR assay by the percentage of nucleotide identity of the RT-PCR product with respect to official reference sequences stored in the Genbank®.

To determine the sequence of the amplified fragment, the PCR product from lane 10 was sent to the company Genytec Ltda. The alignment of the sequences obtained (Table 3) allowed defining a consensus sequence: FACH (Table 4), which according to BLAST coincides in 92% with the VP1 gene of the *Feline calicivirus* strain JOK63 (Table 5).

<table>
<thead>
<tr>
<th>lane</th>
<th>Content</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>MSM</td>
</tr>
<tr>
<td>2</td>
<td>Negative control: ARN CDV1</td>
</tr>
<tr>
<td>3</td>
<td>Reagent control</td>
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<tr>
<td>4</td>
<td>Positive control; V1</td>
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<tr>
<td>5</td>
<td>Positive control; V1</td>
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<td>6</td>
<td>Positive control; V1</td>
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<tr>
<td>7</td>
<td>Negative control: ARN VDC2</td>
</tr>
<tr>
<td>8</td>
<td>Positive sample: V1+ feces</td>
</tr>
<tr>
<td>9</td>
<td>Positive sample: V1+ feces</td>
</tr>
<tr>
<td>10</td>
<td>Positive sample: V1+ feces</td>
</tr>
</tbody>
</table>

MSM: Molecular Size marker (Maestro®: 100-3000 pb; CDV1: canine virus distemper I; V1: Vaccina FELGEN CRP®; VDC2: Canine distemper virus).

Table 3: Alignment according to Clustal Omega. Sequences obtained from Genytec Ltda.
Table 4: FACH consensus sequence. Lab result for use in BLAST software.

Table 5: Significant alignments with respect to the FACH consensus sequence, according to BLAST.

Discussion

The development of this work raised the use of the PCR technique prior to reverse transcription as a diagnostic alternative for the detection of FCV, using in silico primers design considering at least 10 FCV sequences according to their geographical distribution.

In this sense, although the current diagnosis of FCV by means of RT-PCR may involve starters used from the literature [37-39] the strategy used seems be suitable for the detection of FCV present in a commercial vaccine and additionally in a mixture of the vaccine and feces from a healthy cat, with the idea of verifying the effect that a different matrix or inhibitors could involve that could interfere with the values of sensitivity and specificity attributable to Polymerase Chain Reaction (Erlich, 1991; Harris, 1997).

Thus, the results obtained suggest that the use of at least 10 official FCV VP1 nucleotide sequences stored in the Genbank® was a successful initial strategy, since the consensus sequence obtained - around 2700 nucleotides - turned out to be a possible candidate to use as a template for the design of the partitions, in which all those areas that did not present common spaces or that did not allow finding 20 nucleotide primers or primers were excluded.

Subsequently, it was possible to obtain primer sequences according to the Oligoperfect Design software from ThermoFisher Scientific®, which predicted the obtaining of 840 base pair DNA fragments, which was corroborated by the visualization of amplicons with a size greater than 800 bp.

The implementation of an RT-PCR technique that only generates specific bands in positive controls and no presence of DNA fragments or amplicons when using negative controls or nuclease-free water in the 2% agarose gel, corroborates an effective choice of primers.

Additionally, the sample sent to be sequenced in triplicate indicated a high percentage of nucleotide identity (PIN > 92) with respect to the official Genbank® sequence for VP1 of the JOK63 strain of Feline calicivirus and a PIN range between 76 and 83 for the others. 99 FCV sequences compared, which also suggests an excellent choice of primers.

A not less important aspect to consider in the implemented technique is the low interference, almost null, of the matrix effect in the detection of FCV, since the samples mixed with feces give positive results to such an extent that one of them (well 10 of the electrophoresis) was sent to sequence.

Although for virological diagnosis, the sampling should consider the use of lingual, nasal, conjunctival and pharyngeal swabs [40], including a sample consisting of a mixture of vaccine and feces of a healthy cat, represented an opportunity to verify the matrix effect, since FCV has been detected in a dog feces sample [41].

Finally, the results obtained in this study would suggest that the strategy of choosing a common area -present in 10 nucleotide sequences of the VP1 gene of FCV-as a possible candidate for the design of in silico starters was successful, corroborated both by sharp detection and specific as per the sequencing of the DNA fragment obtained.

Conclusion

The developed PCR protocol can be suggested as a basis for the complementary and alternative molecular diagnosis of FCV.

Bibliography


34. ClustalΩ. Multiple Sequence Alignment (2020).


36. BLAST. Basic Local Alignment Search Tool (2020).


