Molecular Detection of Feline Calicivirus in clinical samples: a validation study comparing detection by RT-qPCR directly from swabs and after virus isolation

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Introduction & Aims of the Study

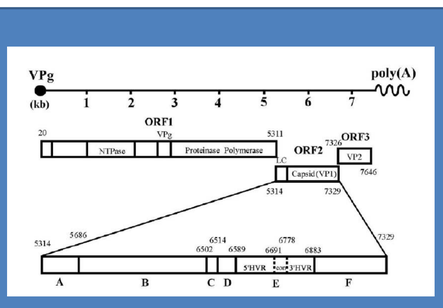
Feline Caliciviruses (FCV) are non-enveloped RNA viruses responsible for upper respiratory tract disease (Helps et al., 2005). They show a high genetic variation (Johnson, 1992). Conventional, nested and real-time reverse-transcriptase PCR (RT-qPCR) assays have been developed to detect FCV in clinical specimens. In comparison to virus isolation (VI), molecular methods are faster and more specific; the latter, however, may result in a lower diagnostic sensitivity (not all strains are recognized). In contrast, VI may fail particularly due to virus inactivation during transport (Radford et al., 2009). This study was initiated to validate and compare two RT-qPCR assays, apply the assays to samples from a FCV field study and compare them to VI. Moreover, the influence of storage conditions on the detectability of FCV was evaluated.

Feline Calicivirus

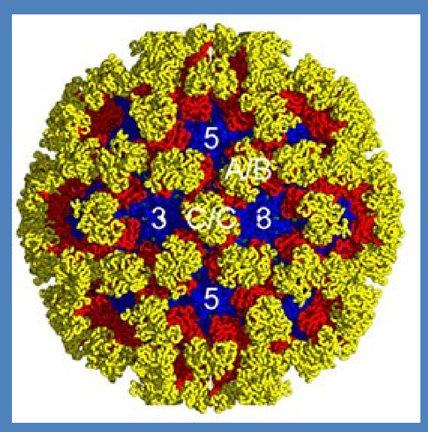
Feline Caliciviruses (FCV) are non-enveloped RNA viruses responsible for upper respiratory tract disease, oral ulcerations and limping syndrome (Radford et al., 2009). Occasional outbreaks of virulent-systemic (VS)-FCV infections, characterized by cutaneous edema, skin ulcerations of the head and feet, and occasionally jaundice have been described in the USA (Pedersen et al., 2000) and Europe and have also been observed in Switzerland during the last years (Willi et al., in preparation).

The FCV genome consists of a small, approximately 7.7 kb, positive, single-stranded RNA. It encodes for three open reading frames (ORFs) (Carter et al., 1992a). ORF 1 encodes for 6 non-structural proteins, such as the RNA polymerase (Sosnovtsev et al., 2002), whereas ORF 2 encodes the precursor protein for the major capsid protein VP1 which consists of 6 coding regions A – F (Carter et al., 1992b; Seal et al., 1993). Regions A, B, D and F are relatively well conserved, whilst regions C and E show high variability (Seal et al., 1993). Region E is known to contain several epitopes for neutralizing antibodies (Geissler et al., 2002; Radford et al., 1999; Tohya et al., 1997). ORF 3 encodes for the minor structural protein VP2, which is substantial for the assembly of infectious virus particles and viral replication (Sosnovtsev et al., 2005).

A common feature of RNA viruses is the plasticity of the genome caused by a high mutation rate during replication, due to the low efficiency or even lack of proofreading and post-replicative repair activities of the viral RNA-polymerase, as well as recombination events. These characteristics create a high variability and enable the viruses to evade the hosts' immune response. The overall identity of FCVs worldwide isolates is about 80%.



FCV genome, reproduced from Ohe et al., 2006



X-ray structure of FCV reproduced from Ossiboff et al., 2010

Materials & Methods

Real-time RT-qPCR assays for FCV

Two previously published RT-qPCR assays (Helps et al., 2002; Abd-Eldaim et al., 2009), designated S1 and S2 respectively (Figure 1 and Table 1), targeting different regions on the open reading frame 1 were evaluated. Analytical sensitivity and efficiency were optimized using ten-fold serial dilutions of synthetic RNA standards applying a primer-probe concentration matrix and different commercially available RT-qPCR mastermixes.

Validation of sample collection and transport/storage conditions

To optimize the sample collection and transport/storage conditions for field studies, the stability of FCV on dry swabs and using different transport media (Microtest™ M4RT®; in-house recipe) was assessed at different time points after collection (0h, 2d, 4d, 5d) and at different temperatures (4°C, -20°C). The in-house medium consisted of Dulbecco's MEM containing 10% heat inactivated FCS, 16 mM HEPES buffer, 0.012% Antibiotic-Antimycotic and 0.12% $NaHCO_3$. The pH was adjusted to ≈ 7 using 1 M NaOH.

Comparison of the assays using field samples

The RT-qPCR assays were applied to 300 samples from a Swiss FCV field study (Berger et al., in preparation) and compared with VI.

Sample processing & virus isolation (VI)

From each cat oropharyngeal cytobrushes and nasal and conjunctival swabs were collected in virus transport medium; samples from the same cat were pooled and either directly processed for FCV RT-qPCR or enriched by VI (Figure 2). For VI, Crandell-Rees feline kidney cells (CRFK) were incubated at 80% confluency with sterile-filtered samples. Cultures were observed daily for cytopathic effect as a sign of virus replication. Cell culture supernatants were tested by FCV RT-qPCR.

Validation of the results

A sample was considered FCV positive if one of the tests was positive. In samples with incongruent results for RT-qPCRs S1 and S2 the target regions of the assays were sequenced.

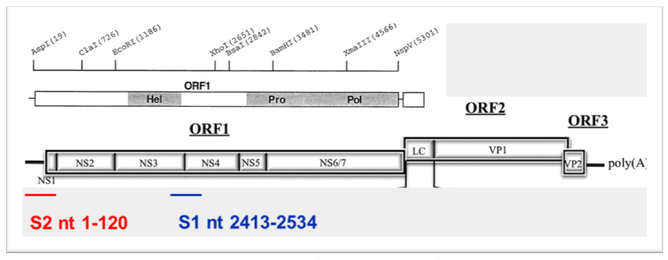


Figure 1: Schematic representation of the position of the RT-qPCR assays on ORF1. Reproduced from Sosnovtseva et al., 1999 and Sosnovtsev et al., 2002

Assay	Oligos*	Sequence (5'-3')	Reference
FCV RT-qPCR S1	FCV.forw (2413 – 2435)	GTTGGATGAACTACCCGCCAATC	Modified from Helps et al., 2002
	FCV.p (2456 – 2475)	6-FAM-TCGGTGTTTGATTTGGCCTG-TAMRA	
	FCV.rev (2507 – 2534)	CATATGCGGCTCTGATGGCTTGAAACTG	
FCV RT-qPCR S2	FCV.1f (1 – 21)	GTAAAAGAAATTTGAGACAAT	Abd-Eldaim et al., 2009
	FCV.26p (26 – 49)	YYE-CAAACTCTGAGCTTCGTGCTTAAA-BHQ-1	
	FCV.120r (104 – 120)	TACTGAAGWTCGCGYCT	

Table 1: Sequences of the primers used for the assays. *Numbers: Position in genome with respect to the FCV F9 Strain (M86379)

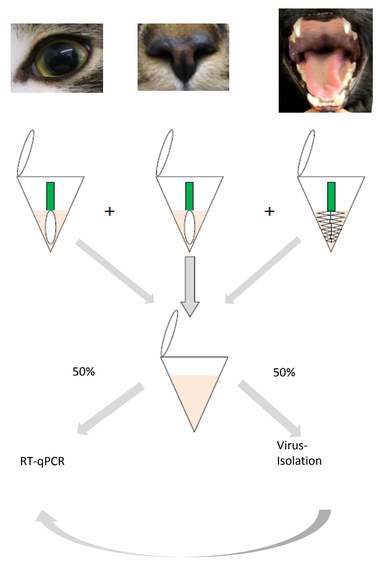


Figure 2: Schematic representation of the sample collection and processing for the study. A total of 300 cats were tested. For each animal a nasal and a conjunctival swab as well as an oral cytobrush stored in transport medium were pooled and analyzed for FCV RT-qPCR both directly or after VI. A sample was considered FCV positive if one of the tests (FCV RT-qPCR S1 or S2 from swabs or from VI cell culture supernatant) was positive.

Results

Analytical sensitivity, efficiency and dynamic range of the RT-qPCR assays

Both RT-qPCR systems reached an analytical sensitivity of 100 copies per reaction and a dynamic range of at least 6 logs with the same reaction composition (300 nM forward primer, 900 nM reverse primer, 250 nM probe) but using different mastermixes and different thermal profiles. Whereas S1 reached an almost 100% efficiency, S2 could not go beyond 81% (Table 2).

Assay	Mastermix	Forw. primer (nM)	Rev. primer (nM)	Probe (nM)	Thermal conditions	Efficiency	Analytical sensitivity
S1	RT-qPCR MasterMix (Eurogentec)	300	900	250	30' 48°C 10' 95°C 45 cycles of 15'' 95°C, 1' 60°C	100% (slope = -3.32)	100 copies per reaction
S2	SuperScript® III Platinum® One-Step qRT-PCR Kit (Invitrogen)	300	900	250	30' 50°C 2' 95°C 50 cycles of 20'' 95°C, 45'' 60°C	81% (slope = -3.89)	100 copies per reaction

Table 2: Optimal conditions reached with the two RT-qPCR assays S1 and S2 using 10-fold dilutions of *in vitro* synthetized RNA-standards. Efficiency was calculated as follows: $E = 10^{-1/slope} - 1$. Analytical sensitivity was determined by end-point dilutions experiments.

FCV stability over time and at different temperatures

FCV stability on oropharyngeal dry swabs collected from infected animals was similar at both temperatures (4°C or -20°C) but viral burdens rapidly decreased to undetectable RT-PCR values after 4 days (data not shown). When the same samples were put in cell culture for virus enrichment, FCV was still detectable from the swabs stored for four days at either storage temperature but not after 7 days of storage.

Influence of transport medium on FCV stability

FCV stability was further assessed on swabs spiked with infected cell culture supernatant using two different transport media (a commercially available medium M4RT and an in-house medium) and dry swabs. Viral burdens were more stable when using the in-house viral transport medium than on dry swabs. After four days, the CT-value was increased in average by 3.6 times, indicating that the FCV load was lowered by approximately 10 times. (Figure 3).

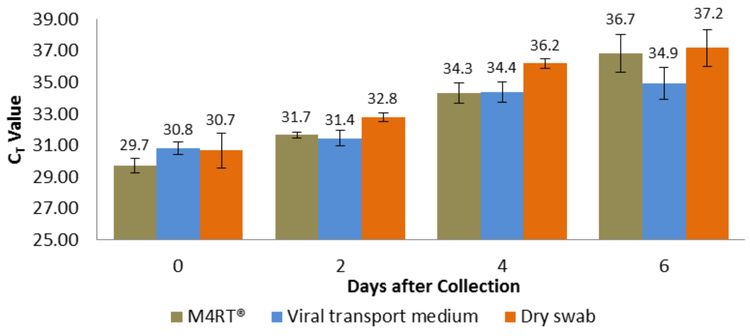


Figure 3: Comparison of mean CT-values and standard deviation of FCV in two different transport media and on dry swabs. A lower CT-value corresponds to a higher viral load. The numbers listed in the figure above the columns represent the mean CT-values

Comparison using field samples: Sensitivity of the assays and detection method

A total of 300 cats were tested. For each animal a nasal and a conjunctival swab as well as an oropharyngeal cytobrush stored in transport medium were pooled and analyzed for FCV RT-qPCR both directly or after VI. A sample was considered FCV positive if one of the tests (FCV RT-qPCR S1 or S2 from swabs or VI cell culture supernatant) was positive. A total of 97 (32%) were positive for FCV. None of the tests detected all FCV-positive samples. When comparing the different assays and detection methods the best sensitivity (96%) was reached using S1 both directly on swabs combined with VI, followed by (in decreasing order) S2 both directly on swabs combined with VI (93%), both systems in swabs (91%), both systems after VI (90%); S1 after VI (84%); S2 after VI and S1 in swabs (both 81%); S2 in swabs (77%) (Table 3).

System	Material	Positive	Total positive	Sensitivity
S1	Swab	79	97	81.4
S2	Swab	75	97	77.3
S1	VI	81	97	83.5
S1	VI	79	97	81.4
S1+S2	Swab	88	97	90.7
S1+S2	VI	87	97	89.7
S1	Swab + VI	93	97	95.9
S2	Swab + VI	90	97	92.8

Table 3: Sensitivity of the assays and detection method. FCV RT-qPCR S1 was more sensitive than FCV RT-qPCR S2 and virus isolation was more sensitive than direct testing from swabs. The combination of both detection methods, in swabs and after virus isolation, yielded the best sensitivity.

Specificity of the assays

Strains that were positive in one assay and negative in the other were sequenced and always showed mismatches in the binding regions of primer and probes.

Conclusions

- **To detect infected animals samples have to be processed within 3 days after collection and a proper transport medium and storage temperature of 4°C or colder should be guaranteed.**
- **None of the tests was able to detect all FCV-positive samples. FCV RT-qPCR S1 was more sensitive than FCV RT-qPCR S2 and virus isolation was more sensitive than direct RT-qPCR from swabs.**
- **Due to the genetic variability of caliciviruses, FCV RT-qPCR assays will suffer from lack of sensitivity, there will be hardly any assay able to detect all strains. Therefore a combination of RT-qPCR directly from swabs and after virus isolation is needed to increase the sensitivity of detection. But for routine diagnostics purposes this approach is time-consuming and expensive.**

Literature

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