Differentiation of Disease States Using Quantification of Feline Herpesvirus-1 DNA Using Real Time PCR

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ABSTRACT
Between 1988 and 1999, of 15,000 cats examined at Colorado State University, 1,573 (10.5%) had respiratory signs of disease. Feline infectious URTD has multiple causes; feline herpesvirus-1 (FHV-1) and calicivirus are the most common primary viral causes. After exposure to FHV-1, cats develop a rapid immune response, however, up to 80% of animals become latently infected. The objectives of this pilot study were to amplify FHV-1 DNA from nasal or pharyngeal swabs from cats with or without history of URTD by use of both a qPCR assay and an endpoint PCR assay to determine the optimal sampling site and to determine whether results of the qPCR could discriminate between FHV-1 carriers, suspect carriers of FHV-1, and cats clinically ill from FHV-1 infection.

INTRODUCTION
Of 15,000 cats examined at Colorado State University (CSU) between 1988 and 1999, 1,573 (10.5%) had respiratory signs of disease. Infectious feline upper respiratory tract disease (URTD) is even more common in multi-cat environments like humane shelters and catteries and can result in euthanasia rather than adoption or sale. Feline infectious URTD has multiple causes; feline herpesvirus-1 (FHV-1) and calicivirus are the most common primary viral causes (Binns et al 2000, Sykes et al 1999, Veir et al 2008). After exposure to FHV-1, cats develop a rapid immune response. However, up to 80% of animals become latently infected (Nasisse et al 1992, Ohmura et al 1993), typical of the α-herpesviruses. Prior to the widespread clinical use of polymerase chain reaction (PCR) technology, infection was documented by visualizing the organism in cells by use of fluorescent antibody assays and by detection of serum antibodies by serum neutralization (Stiles et al 1997b). However, more recently, qualitative endpoint FHV-1 PCR assays have been shown to be a sensitive method of documenting the presence of FHV-1 DNA (Burgesser et al 1999, Hara et al 1996, Reubel et al 1993, Stiles et al 1997b, Stiles et al 1997a, Sykes et al 1997, Weigler et al 1997). Because of latency and intermittent shedding or re-activation, results of these PCR assays cannot differentiate between co-incidental shedding in healthy FHV-1 carriers, shedding secondary to immunosuppression from concurrent disease, and the disease entity itself (Maggs et al 1999). In addition, FHV-1 strains used
in modified live vaccines cannot be distin-
guished from naturally occurring strains and
can be amplified from swabs collected after
vaccination (Maggis and Clarke 2005; Ruch
Gallie et al 2011).

Several studies have attempted to associ-
ate quantitative real time PCR (qPCR) for
FHV-1 DNA to clinical findings in cats. In
a study of experimentally and naturally-in-
fected cats, results of a qPCR assay targeting
the FHV-1 glycoprotein B gene (Vogtlin et
al 2002) were compared to those of a FHV-1
endpoint PCR assay using ocular fluids.
The qPCR assay had at least equal sensitiv-
ity in vitro and greater sensitivity in vivo
than endpoint PCR assay for later stages of
infection. Additionally, the authors used the
quantitative data obtained from the qPCR
assay to define “stages” of infection in the
experimentally-infected cats and applied the
data to naturally-infected cats.

In one experimental study, our labora-
tory used a FHV-1 qPCR to determine viral
load in recently vaccinated cats to demon-
strate a temporal increase in the presence
of viral DNA post-vaccination and viral
challenge (Lappin et al 2006). In a sepa-
rate study, it was shown that FHV-1 DNA
amounts were less in cidofovir treated cats
compared to control cats (Fontenelle et
al 2008). In a study of nasal tissue biopsies,
the amount of FHV-1 DNA amplified by a
qPCR appeared to be associated with spe-
cific FHV-1 histopathological lesions (Burns
et al 2010). However, the ratio of FH
-1
mean copy number of FH-1 divided by the
glyceraldehyde-3-phosphate dehydrogenase
mean copy number did not differentiate
client-owned cats with or without clinical
conjunctivitis (Low et al 2007).

Further data evaluating the use of FH
-1
qPCR results in an attempt to discriminate
between FH-1 carriers and cats clini-
cally ill from FH-1 infection are needed.
Therefore, the objectives of this pilot study
were to amplify FH-1 DNA from nasal or
pharyngeal swabs from cats with or without
history of URTD by use of both a qPCR as-
say and an endpoint PCR assay to determine
the optimal sampling site and to determine
whether results of the qPCR could discrimi-
nate between FH-1 carriers, suspect car-
riers of FH-1, and cats clinically ill from
FH-1 infection.

MATERIALS AND METHODS

Study Groups
The study protocol was approved by the
Colorado State University Animal Care
and Use Committee. Three groups of cats
owned by staff or students of Colorado State
University Veterinary Teaching Hospital
were sampled. Owners were interrogated
regarding history of clinical signs associ-
ated with URTD (sneezing, nasal discharge,
sterorous breathing that resolved without
surgical intervention, inappetence, or ocular
discharge), and the groups were defined and
sampled as follows:

• Group 1: Healthy (no clinical signs of
URTD within the last 2 years): pharyn-
geal swabs only,

• Group 2: Suspect carriers: (clinical
signs of URTD between 1 and 3 mos
prior to sampling but normal at the time
of collection): pharyngeal swabs only

• Group 3: Clinically ill (discharge refer-
able to recurrent URTD present at time of
sampling): pharyngeal and nasal swabs.
No cats had been vaccinated within the
previous three months prior to sampling.

Sampling
Swab samples were obtained using standard
cotton tipped wooden applicators (pharynx)
or urethral culture swabs (nasal cavity)
on awake, non-sedated cats by a single
author (JKV). Swabs were immediately
placed in 1.0 mL sterile 0.01 M phosphate
buffered saline (PBS) and allowed to sit
at room temperature for 2-3 hours before
being placed into -70°C until processing
and analysis according to manufacturer’s
protocol. DNA was extracted from the PBS
using a commercial kit and assayed for
FHV-1 using previously published protocols
using both endpoint (Weigler et al 1997) and
qPCR (Vogtlin et al 2002). The qPCR was
modified for use in nasal and pharyngeal
In order to normalize cellular yield due to sampling variability between cats, quantification of feline glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was concurrently assayed using a previously published qPCR (Leutenegger et al. 1999). Products of the endpoint PCR were separated using agarose gel electrophoresis and considered positive if a single band of the appropriate size (322 bp) was visualized.

Data from the qPCR was analyzed with the instrument software. Samples were considered positive if the fluorescence intensity exceeded 10 times the standard deviation of the baseline fluorescence (threshold cycle [Ct]). All reactions were run in duplicate. A control sample consisting of DNA pooled from swabs from 10 normal cats spiked with plasmid derived FHV-1 DNA was run on each plate to ensure repeatable thermocycler conditions.

Standard curves for GAPDH and FHV-1 were generated as previously described (Veir et al. 2006). Briefly, a standard curve for GAPDH-cell equivalent was generated using RNA isolated from a feline lung epithelial cell line (Dow unpublished data) that was digested in the same manner as the test samples (cell equivalent). The standard curve for FHV-1 was generated using a 10-fold dilution series using plasmid generated DNA (pDNA). The FHV-1 pDNA was produced using a commercially available vector after purification of product obtained from a conventional PCR reaction using the primers used in the real time assay. Viral load was then defined as the calculated FHV copy number divided by the calculated GAPDH-cell equivalent copy number of each sample.

**Analysis**

Concordance rates of detection of FHV-1 DNA between the two methods (all sites grouped together) and the two sampling sites (pharyngeal compared to nasal, Clinically ill group only) were analyzed using the kappa statistic as defined by Feinstein (2002) and evaluated for significance as defined by Landis and Koch (1977). Quantitative data was compared between disease groups using the student’s unpaired t test. The level of significance was set at p < 0.05 for between disease group comparisons.

**RESULTS**

One hundred one cats were enrolled in the study between November 2002 and January 2008. Thirty eight were classified as Healthy (mean age 6.1 years), 21 were classified as Suspect carriers (mean age 5.8 years), and 49 were classified as Clinically ill (mean age 4.4 years). Breeds represented were domestic shorthair (n = 87) and domestic longhair (n = 10) and other (n=4:...
Siamese 2, Himalayan, Persian). There was no statistical difference in mean age among groups (p = 0.75).

Other than transient sneezing associated with nasal swab sample collection, no hemorrhage or other adverse effects from either sampling technique were observed. The two assays showed similar detection rates (Table 1) in all disease groups, with the most disagreement in the Suspect carrier group. There was a low rate of detection of FHV-1 in all groups; therefore, indices of specific agreement were calculated to determine the effects of the skewed distribution of challenge. In all disease groups with a kappa between the assays of less than one, the specific proportionate agreement for negative results was higher than that for positive results, as can be expected in a group with such low rates of positive results (Table 1). In the single disease group in which sampling sites were compared (Clinically ill), correlation between results for the assays was substantial or almost perfect as defined by parameters set forth by Koch and Landis (1977) (Table 2).

After normalization to starting sample size with GAPDH, there was a significant difference between the copy number of FHV-1 per cell equivalent in pharyngeal samples between the Healthy (mean = 0.20 ± 0.01) and Clinically ill groups (mean = 19.92 ± 9.08), but no difference between the Suspect carrier group (mean 0.93 ± 0.09) and any other disease group.

DISCUSSION

All pharyngeal samples except one taken from the pharynx of a healthy cat were adequate for amplification (with no evidence of PCR inhibitors) as evidenced by GAPDH values of at least 16 cell equivalents, the lower level of detection of the assay. The sample with inadequate DNA for amplification was taken from a fractious cat which may have decreased sample size as opposed to the presence of inhibitors of PCR. In support of this, a Ct value was obtained, but it was not within the linear range of the standard curve, making quantification inaccurate. All nasal samples had adequate DNA for amplification of feline GAPDH, indicating that inhibitors of PCR should not confound sampling from this area.

Sensitivity of the qPCR assay was at least as sensitive as the endpoint PCR assay across all groups, with one additional positive in the suspect carrier group. Without a third assay such as virus isolation to be used as a gold standard, it is impossible to say whether this is a false positive or increased sensitivity of the qPCR assay. However, the detection limit for the qPCR assay has been shown to be lower than the endpoint assay in vitro (Vogtlin et al 2002), lending support

<table>
<thead>
<tr>
<th>URTD Disease Group</th>
<th>Endpoint PCR: detection rate</th>
<th>qPCR: detection rate</th>
<th>Concordance (kappa)</th>
<th>ppos</th>
<th>pneg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy (n=38, pharyngeal only)</td>
<td>12 (31.5%)</td>
<td>14 (36.8%)</td>
<td>0.88</td>
<td>0.92</td>
<td>0.96</td>
</tr>
<tr>
<td>Suspect carriers (n=21, pharyngeal only)</td>
<td>4 (19.0%)</td>
<td>7 (33.3%)</td>
<td>0.64</td>
<td>0.73</td>
<td>0.90</td>
</tr>
<tr>
<td>Clinically ill (n=49, pharyngeal)</td>
<td>11 (22.4%)</td>
<td>11 (22.4%)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Clinically ill (n=49, nasal)</td>
<td>14 (28.6%)</td>
<td>15 (30.6%)</td>
<td>0.95</td>
<td>0.97</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Table 1. Comparison of two methods of detection by PCR of FHV-1 DNA in respiratory swabs from privately owned adult cats. Samples were obtained using standard cotton tipped applicators (pharyngeal) or urethral culture swabs (nasal) from privately owned cats. Endpoint and qPCR targeting FHV-1 DNA were performed on each sample in duplicate. Mean ages at time of sampling were statistically similar between disease groups. Disease group definitions are defined in text. Indices of specific agreement (ppos and pneg) were calculated according to Feinstein (2002).
that this is truly a difference in sensitivity, not specificity. Regardless, there was a high correlation between the two assays indicating qPCR is a valid assay for detection of FHV-1 from the pharynx and the nasal cavity of cats.

In this population, the qPCR assay was able to discriminate between the Healthy and Clinically ill group. However, the assay was not able to discriminate between those animals that had recently recovered from an episode of URTD and those with current clinical signs, negating the usefulness of the assay in some cases. However, the outliers and large standard deviations as were present in the Clinically ill group will negatively affect significance.

Studies of larger populations are needed to determine if quantification of the targets used in this assay will be able to discriminate between these two disease states. As previously demonstrated, stress from other diseases can also induce shedding. Addition of a fourth group with animals suffering from a concurrent systemic illness such as neoplasia or endocrine disorders, but not showing clinical signs of URTD, as well as a group of cats recently given a modified live vaccine should be included in future studies as well. Additionally, the Clinically ill group had varied lengths of illness. Therefore, sensitivity may be blunted in chronic cases in which the immune response lessens viral shedding and therefore decreases detection of viral particles.

Finally, as stated above, a large proportion of FHV-1 infected cats are in the latent state of disease and, therefore, samples from these animals may be negative when assayed for target genes generally expressed during active infection. Other authors (Townsend et al 2004) have investigated the use of assays targeting latency associated genes in order to improve detection. Studying these targets in a quantitative manner may help further discriminate those cats in which FHV-1 is inducing disease as compared to co-incidental shedding or latency.

Results of this study suggest that detection of FHV-1 in nasal or pharyngeal swabs by qPCR is a valid method of diagnosis and quantitation can discriminate between healthy and clinically ill animals but not between animals recently recovered from a suspicious episode and any other disease group. Studies with larger populations, the addition of animals suffering from concurrent systemic illness associated with immunosuppression but not URTD signs, and targets associated with latency are needed prior to determining if quantification of FHV-1 can be a useful clinical diagnostic tool in the diagnosis and ultimately, guide the treatment decision tree of upper respiratory tract infection in cats.

ENDNOTES

i Ultrafine aluminum applicator swab, Fisher Scientific, Pittsburgh, PA
ii QIAamp DNA minikit, Qiagen, Inc, Valencia, CA
iii ABI Prism 7000 SDS Software Version 1.0 (build 81 rev 3): Applied Biosystems, Foster City, CA
iv TA Cloning Vector: Invitrogen Corporation, Carlsbad, CA
v QIAquick PCR Purification kit, Qiagen, Inc, Valencia, CA

Table 2. Comparison of sampling sites for detection of FHV-1 DNA in respiratory swabs from privately owned adult cats. Two samples of the respiratory tract from each of nine cats (Clinically ill group, Table 1) were obtained using standard cotton tipped applicators (pharyngeal) or urethral culture swabs (nasal). All cats were currently showing clinical signs consistent with URTD at the time of sampling. Indices of specific agreement (ppos and pneg) were calculated according to Feinstein (2002).

<table>
<thead>
<tr>
<th>Assay</th>
<th>Assay Pharyngeal: detection rate</th>
<th>Assay Nasal: detection rate</th>
<th>Concordance (kappa)</th>
<th>p_pos</th>
<th>p_neg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endpoint PCR</td>
<td>11 (22.4%)</td>
<td>14 (28.6%)</td>
<td>0.84</td>
<td>0.88</td>
<td>0.96</td>
</tr>
<tr>
<td>qPCR</td>
<td>11 (22.4%)</td>
<td>15 (30.6%)</td>
<td>0.79</td>
<td>0.85</td>
<td>0.94</td>
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