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 Colarado 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 distinguished from naturally occurring strains and can be amplified from swabs collected after vaccination (Maggs and Clarke 2005; Ruch Gallie *et al* 2011).

Several studies have attempted to associate quantitative real time PCR (qPCR) for FHV-1 DNA to clinical findings in cats. In a study of experimentally and naturally-infected 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 sensitivity 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 laboratory used a FHV-1 qPCR to determine viral load in recently vaccinated cats to demonstrate a temporal increase in the presence of viral DNA post-vaccination and viral challenge (Lappin *et al* 2006). In a separate 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 specific FHV-1 histopathological lesions (Burns *et al* 2010). However, the ratio of FHV-1 mean copy number of FHV-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 FHV-1 qPCR results in an attempt to discriminate between FHV-1 carriers and cats clinically ill from FHV-1 infection are needed. Therefore, 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.

MATERIALS AND METHODS

Study Groups

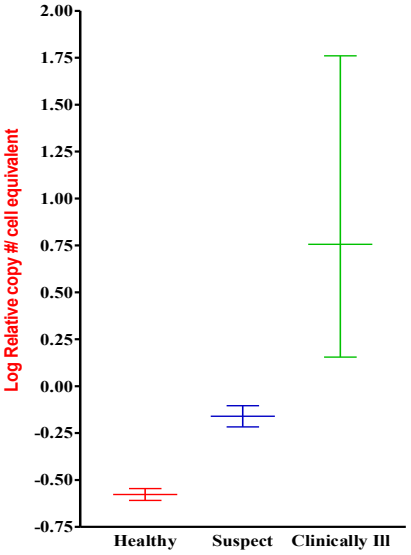
The study protocol was approved by the Colorado State University Animal Care and Use Committee. Three groups of cats owned by staff or students of Colorado State University Veterinary Teaching Hospital were sampled. Owners were interrogated regarding history of clinical signs associated with URTD (sneezing, nasal discharge, stertorous 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): pharyngeal 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 referable 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^{o}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

Figure 1. *Comparison of copy number of FHV-1 per cell equivalent in pharyngeal swabs from three groups of privately owned cats Samples were obtained using standard cotton tipped applicators from privately owned cats. Endpoint and qPCR targeting FHV-1 DNA were performed on each sample in duplicate. Mean copy number FHV-1/cell equivalent with interquartile ranges for each group are graphed. Mean ages at time of sampling were statistically similar between disease groups. Disease group definitions are defined in text. Groups labeled with different letters are statistically different (p<0.05).*



sampling as previously described (Veir *et al* 2006).

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:

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).*

URTD Disease Group	Endpoint PCR: detection rate	qPCR: detection rate	Concordance (kappa)	p_{pos}	p_{neg}
Healthy (n=38, pharyngeal only)	12 (31.5%)	14 (36.8%)	0.88	0.92	0.96
Suspect carriers (n=21, pharyngeal only)	4 (19.0%)	7 (33.3%)	0.64	0.73	0.90
Clinically ill (n=49, pharyngeal)	11 (22.4%)	11 (22.4%)	1	1	1
Clinically ill (n=49, nasal)	14 (28.6%)	15 (30.6%)	0.95	0.97	0.99

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.

DISCUSSSION

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 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).*

Assay	Pharyngeal: detection rate	Nasal: detection rate	Concordance (kappa)	p_{pos}	p_{neg}
Endpoint PCR	11 (22.4%)	14 (28.6%)	0.84	0.88	0.96
qPCR	11 (22.4%)	15 (30.6%)	0.79	0.85	0.94

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

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