Relative sensitivity of polymerase chain reaction assays used for detection of feline herpesvirus type 1 DNA in clinical samples and commercial vaccines

David J. Maggs, BVSc, and Heather E. Clarke, BS

Objective—To determine relative detection rates and detection limits for 6 published polymerase chain reaction (PCR) assays used for detection of feline herpesvirus type 1 (FHV-1) DNA.

Sample Population—5 vaccines licensed for use in preventing FHV-1–associated disease; 15 conjunctival biopsy specimens collected from cats with keratitis, conjunctivitis, or both; and a plaque-purified field isolate of FHV-1 cultured in vitro.

Procedure—Vaccines and clinical samples were assessed for FHV-1 DNA by use of all 6 assays. Detection rates were calculated by assuming that any sample in which FHV-1 DNA was detected was a true-positive result. Detection limits were estimated by use of serial dilutions of DNA extracted from cultured FHV-1 and 1 clinical sample.

Results—Testing by use of all 6 assays resulted in detection of FHV-1 DNA in all 5 vaccines. Testing by use of all 6 assays yielded concordant results for 9 of 15 conjunctival biopsy specimens (8 with negative results and 1 with a positive result). Calculated detection rates for clinical samples ranged from 29% to 86%. Assay sensitivity was ranked similarly by use of detection rate or detection limit.

Conclusions and Clinical Relevance—Testing by use of all assays was equally likely to detect vaccine virus. Therefore, a positive PCR result in a cat may reflect vaccine virus rather than wild-type virus. Test sensitivity as assessed by detection limits and detection rates varied greatly. Because FHV-1 can be shed in clinically normal animals, high detection rate will not necessarily correlate with high diagnostic sensitivity. (Am J Vet Res 2005;66:1550–1555)

Feline herpesvirus type 1 (FHV-1) is an alphaherpesvirus and the cause of numerous acute and chronic or recurrent disease syndromes.1 Following primary infection and destruction of nasal and ocular epithelial cells with associated rhinosinusitis and keratoconjunctivitis, the virus typically establishes lifelong latency in neurons of the trigeminal ganglia.2,3 There is episodic viral reactivation, sometimes with recrudescence or respiratory tract disease, in some carrier animals.4 Available assays for diagnosis of FHV-1–associated disease rely on detection of an immunologic response to the organism (usually in serum samples) or viral detection by virus isolation (VI), immunofluorescent antibody (IFA) assays, or polymerase chain reaction (PCR) assays.5 However, there are a number of limitations with respect to laboratory diagnosis of FHV-1–associated disease. The detection of FHV-1–specific antibodies in serum samples by use of a serum neutralization test or ELISA does not permit differentiation between responses to wild-type or vaccine virus, and titer magnitude does not differ among clinically normal, acutely ill, or chronically affected animals.6 Therefore, serologic testing has not proven useful in the diagnosis of FHV-1.

The value of viral detection methods is limited by a major diagnostic paradox related to the extent of viral shedding at various stages of disease.1 Cats with primary herpetic infection shed virus in sufficient quantities, which makes it relatively easy to detect FHV-1 by use of traditional methods (eg, VI or IFA assay).6 However, clinical signs during this acute phase of infection typically are characteristic and self-limiting, making definitive laboratory diagnosis less necessary. By contrast, during recrudescent FHV-1–associated syndromes, the chronic or recurrent nature of the disease along with the diversity and ambiguity of clinical signs often makes it desirable to perform viral identification techniques, particularly when specific antiviral treatments are considered. However, the relative paucity of virus in animals with many of these chronic syndromes makes it difficult to definitively diagnose the disease during this phase by use of VI or IFA tests.7,8 Furthermore, there is intermittent shedding of virus in cats that have no detectable signs of disease.9–12 This degree of subclinical shedding lowers the diagnostic sensitivity of IFA and VI tests, which makes them impractical for use.9 Evidence that vaccine virus may also become latent within and be reactivated from the trigeminal ganglia further confuses the interpretation of virus detected at peripheral sites.13 For all of the aforementioned reasons, the interpretation of FHV-1 tests in cats and definitive diagnosis of FHV-1 infection in specific cats pose major challenges in the management of chronic diseases suspected to be related to FHV-1 infection.

A number of PCR assays, some of which are commercially available, have been described14,15,16 for use in the detection of FHV-1 DNA. Testing by use of these PCR techniques is more sensitive than testing by use of IFA or VI techniques, but detection limits vary widely.14,15,16 Additionally, anecdotal reports suggest that results for clinical samples may vary among commer-
cially available FHV-1 PCR assays. However, it is impossible to make direct comparisons among results for tests conducted by use of the various PCR assays because of a number of variables among reports, especially with respect to the populations tested.

To the authors' knowledge, there have been no direct comparisons of results for identical clinical samples tested by use of multiple FHV-1 PCR assays conducted in a single laboratory. Furthermore, only 1 assay has been assessed for its ability to detect vaccine virus, and only 1 vaccine type was tested. Therefore, the objectives of the study reported here were to compare directly the performance of 6 published PCR assays in a number of settings. First, we wanted to assess relative detection rates for 5 commercially available vaccines and 15 samples collected from cats believed to be infected with FHV-1. Second, we wanted to estimate relative detection limits for each PCR assay when used to detect FHV-1 DNA extracted from virus cultured in vitro or from a clinical sample.

**Materials and Methods**

**Sample population**—Five commercially available vaccines licensed as an aid to prevent FHV-1–associated disease were purchased from retail outlets or were donated by the manufacturers. Vaccines tested were killed or modified-live vaccine vaccines licensed for SC injection or mucosal (nasal or ocular) application. Vaccines were reconstituted in accordance with the recommendation of each manufacturer, and 1 µL was used for each PCR assay. One reconstituted vaccine was diluted 1:100 in Tris-EDTA because FHV-1 DNA was not detected in undiluted samples or samples diluted 1:10, presumably because of interference attributable to a component in the adjuvant; however, this premise was not tested directly.

Clinical samples were collected from 15 cats examined by a board-certified veterinary ophthalmologist or resident in training. All cats had keratitis, conjunctivitis, or both, which was suspected by the examining clinician to be associated with FHV-1 infection. Conjunctival biopsy specimens were collected from each affected eye following application of topical anesthetic. Samples were obtained from clinical cases in the course of routine diagnostic testing to determine the cause of each cat's ophthalmic condition; no additional samples were obtained specifically for this study. Immediately after collection, specimens were placed in 1 mL of sterile PBS solution and then frozen at –20°C until processing.

**PCR assays**—A commercially available kit was used to extract DNA from all biopsy specimens. The DNA concentr...
tration of each extracted sample was estimated by use of a biophotometer, and approximately 230 ng of DNA was used in each PCR assay. Although the exact quantity of DNA tested likely varied slightly among samples, the volume of DNA (equivalent to approx 230 ng of DNA) from any given sample remained constant for all 6 PCR assays. All PCR assays were single-phase or nested techniques targeting various regions of the FHV-1 thymidine kinase gene (Figure 1). All assays were performed as described by the original authors, with 2 exceptions. For 1 assay, the 10-minute hot start was omitted after experiments revealed no difference in sensitivity for detection of FHV-1 DNA in identical samples with or without the hot start (data not shown). In our laboratory, another method was routinely unsuccessful in detecting FHV-1 DNA when performed exactly as described. Therefore, we conducted a series of experiments to optimize results (data not shown). On the basis of these experiments, the technique was performed by use of 15 pmol of each primer in the first PCR step and 7.5 pmol of each primer in the nested PCR step. In addition, we used 3 mM magnesium chloride and 200 μM of each deoxynucleoside triphosphate for each reaction. Reaction conditions used for that method included an initial denaturation step of 94°C for 3 minutes, followed by 30 cycles (94°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute), and a final 3-minute elongation period at 72°C.

To minimize variation among methods, all reactions were performed by use of a total reaction volume of 50 μL and the same Taq polymerase and thermocycler. Positive and negative control samples were included in all PCR assays. Negative control samples were not subjected to DNA extraction; however, no single group of samples processed together yielded only positive results (data not shown), which suggested that there was no contamination at the DNA extraction level. For negative control samples, DNase-RNase–free water was substituted for sample DNA in the PCR mixture. For positive control samples, DNA extracted from FHV-1 strain 727 (passage 8) cultured in Crandell-Rees feline kidney cells was substituted for sample DNA in the PCR mixture. This is a plaque-purified field isolate of FHV-1 that has been verified as FHV-1 by results of immunofluorescence with FHV-1–specific antiserum. After cycling was completed, 10 μL of reaction mixture was subjected to electrophoresis on ethidium bromide–stained 1.5% agarose gels. The PCR products were identified by visual examination and digitally photographed.

Relative PCR detection limits were estimated for each method by use of 4 to 9 serial 10-fold dilutions of 1 conjunctival biopsy sample that had positive results for all assays and DNA from FHV-1 strain 727 (passage 8) cultured in Crandell-Rees feline kidney cells. Sample DNA was serially diluted in Tris-EDTA solution. Extraction of DNA, PCR assays, and identification of PCR products were performed as described for clinical samples. The lowest sample concentration at which a band could be visually identified was used to estimate the relative detection limits of the 6 PCR assays.

Results

All 6 PCR assays detected FHV-1 DNA in all 5 vaccines tested (Figure 2). Detection rate then was determined for the 15 clinical samples (Table 1). The 6 PCR assays yielded concordant results for 9 of 15 samples (8 samples had negative results for all assays and 1 sample had positive results for all assays) and discordant results for the remaining 6 samples. Assuming that any sample in which FHV-1 DNA was detected by at least 1 assay was a true-positive result, calculated detection rates for the 6 PCR assays varied between 29% and 86%.

Detection limits for each assay then were estimated by use of serial 10-fold dilutions of viral DNA extracted from FHV-1 cultured in vitro or a clinical sample that yielded positive results for all assays (Figure 3). The lowest dilution at which each method detected viral DNA extracted from these 2 sources was determined (Table 2). The ranking of assay sensitivity when estimated by use of the detection limit was similar to the ranking estimated by use of the detection rate (Table 1).

Discussion

The 6 PCR assays tested in the study reported here had striking differences in detection limits and detection rates for clinical samples that contained FHV-1 DNA and for virus cultured in vitro. By defining samples with true-positive results as those in which FHV-1 DNA was detected by any of the assays, detection rates varied between 29% and 86%. This

Table 2—Detection limits* for 6 PCR assays used to detect FHV-1 DNA.

<table>
<thead>
<tr>
<th>Reference for PCR technique</th>
<th>Cultured virus</th>
<th>Clinical sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-2&lt;/sup&gt;</td>
</tr>
<tr>
<td>17</td>
<td>10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-2&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-2&lt;/sup&gt;</td>
</tr>
<tr>
<td>14</td>
<td>10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-2&lt;/sup&gt;</td>
</tr>
<tr>
<td>16</td>
<td>10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>11</td>
<td>10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Detection limits were estimated on the basis of the lowest dilutions of DNA extracted from FHV-1 cultured in vitro or a clinical sample that yielded a visible band on an ethidium bromide–stained 1.5% agarose gel.
assumption ignores the possibility of false-positive PCR outcomes that, despite our efforts, may have resulted, especially with the nested techniques. The assumption also does not allow for the possibility that some of the negative results were falsely negative. However, when the 6 methods were ranked in order of decreasing detection rate by use of this definition, results for any specific sample tended to reflect test detection rate in a fairly predictable manner (Table 1). For example, sample No. 11 had positive results when tested by use of all methods except for the method with the lowest detection rate; and sample No. 12 had positive results when tested by use of all methods except the 2 methods with the lowest detection rates. However, the assay described by Hara et al17 yielded 2 results that did not follow this pattern (sample No. 10 yielded negative results for this assay but positive results for all other assays, including some with lower detection rates, and sample No. 15 yielded a positive result when tested by this assay but negative results when tested by all other assays). The reason that results for these 2 samples tested by use of this assay did not adhere to the pattern for other assays is not clear. It is possible that sample No. 10 yielded false-positive results when tested by use of all methods other than that described by Hara et al17; however, it seems far more likely that it represents a false-negative result arising from use of this assay. Because this assay is a nested technique, it is possible that sample No. 15 yielded false-positive results when assessed by this technique,2 even though great care was taken to avoid such an inaccuracy. When detection rates were reassessed for samples with positive results after excluding data from this technique,17 1 assay15 detected FHV-1 DNA in all 6 samples, 2 assays16 detected FHV-1 DNA in 4 of 6 samples, 1 assay14 detected FHV-1 DNA in 3 of 6 samples, and 1 assay15 detected FHV-1 DNA in 2 of 6 samples defined as true-positive samples.

Although detection rates represent one way of comparing test sensitivity, minimum dilutions at which each assay generated a visible band on the gel (detection limits) also were assessed. Test sensitivity calculated in this manner for 1 clinical sample in which FHV-1 DNA was detected by all 6 assays broadly mirrored clinical detection rates, although 2 PCR methods14,16 exchanged positions in the rankings. Rankings determined on the basis of dilutional cutoff points for DNA extracted from cultured virus yielded identical detection limits for these 2 PCR methods14,16 and therefore confirmed the ranked order of assay sensitivity suggested by detection rate estimates. Because the cutoff points we used for detection limit determination represented dilutional and not absolute quantification of viral DNA, detection limits of each assay for viral DNA extracted from cell culture and from clinical samples cannot be compared directly.

Estimates of the sensitivity of specific PCR assays have compared the number of samples with positive results when tested by PCR assays versus VI and, sometimes, IFA tests.8,11,14,17 Alternatively, detection limits of purified viral DNA have been quantified, sometimes against a background of feline DNA, and the results quoted as the mass of DNA (in fg) or number of genomic copies detected.8,11,14,17 By use of a reported estimate of FHV-1 genome size (134 kbp or 0.13 fg), and assuming that 1 bp weighs approximately 1 attogram, it is possible to compare detection limits reported for the 6 techniques. Conversion of detection limits (when necessary) to the number of FHV-1 genomic copies detected allowed the assays to be ranked on the basis of increasing detection limits (decreasing test sensitivity) as follows: 0.6 genomic copies,15 19 genomic copies,16 240 genomic copies,8 384 genomic copies,17 3,000 genomic copies,11 and 10,000 to 1,000,000 genomic copies.14 Reasons for differences between this ranking and the rankings in the study reported here are not clear but perhaps reinforce the importance of directly comparing assays in a single laboratory and by use of clinical samples with background (ie, non–FHV-1) DNA that could lower test sensitivity.

The PCR techniques examined in our study represented relatively standard assays, yet they resulted in differing sensitivities. The reasons for these differences are not known and were not the focus of the study; however, major factors affecting PCR detection limits include number of cycles (especially when comparing nested and single-round techniques), primer selection, PCR buffer composition, and cycling temperatures. Of these, number of cycles is likely to exert the most impact. The assays tested in the study used 80 cycles,16 70 cycles,11 60 cycles,14 40 cycles,8 or 35 cycles.11,12 With the exception of 1 method,11 ranking on the basis of number of cycles approximated our rankings determined on the basis of relative detection rates and detection limits.

In the study reported here, we examined detection rates or detection limits as estimates of test sensitivity. However, sensitivity of an assay (ie, the probability that an FHV-1 PCR assay will detect FHV-1 DNA in a sample that contains the virus) does not necessarily equate to diagnostic sensitivity (ie, the probability that an FHV-1 PCR assay will detect FHV-1 DNA in subjects with disease attributable to FHV-1).21 Indeed, as the lower limit of viral DNA that a test can detect decreases (ie, test sensitivity increases), the number of clinically normal animals in which subclinical shedding of low amounts of virus is detected would be expected to increase. This would be associated with decreased diagnostic sensitivity of that assay.

Numerous scenarios exist in which FHV-1 DNA may be detected in a clinically normal animal. First, FHV-1 can be intermittently shed by up to 50% of cats without clinical evidence of disease.14,16,21-25 Second, at least 1 vaccinal form of FHV-1 can become latent within the trigeminal ganglia, reactivate from latency, and be shed and detected at peripheral sites.13 Additionally, vaccine administration has been associated with transient disease in some cats.26

In the study reported here, we documented that all 6 assays detected all vaccines tested and that they were indistinguishable from wild-type virus by use of these PCR methods. Although it is not known whether or how widely the 5 tested vaccines are distributed to tissue sites in vaccinated cats, analysis of our data suggests that the problem of detection of vaccinal virus
could be more widespread than originally indicated.13 Finally, PCR assays do not discriminate between viable, cultivable virions and immature, immunologically inactivated or otherwise noncultivable virus or fragments of DNA suggested to be found during some stages of infection.9,11,12 Therefore, viral DNA detected by PCR assays in samples obtained from a cat without disease may represent avirulent virus or potentially even viral DNA fragments remaining after resolution of infection.

Interpretation of viral detection in the diagnosis of affected animals is also problematic because virus may be found as the cause of the disease process being investigated, coincidentally as a result of intermittent shedding unrelated to the disease process, or potentially as a result of the stress of another concurrent, primary disease process.9 The closely related alphaherpesvirus of human beings (ie, herpes simplex virus type 1) can be reactivated by irritation of peripheral sensory neurons.17–20 Whether there is similar reactivation of FHV-1 has not been investigated to our knowledge. Therefore, when virus is detected in a cat with disease, detection of virus may indicate coincidence, consequential infection, or true causation with respect to the primary disease process. Only in the last case would diagnostic sensitivity be enhanced by detection of viral DNA by use of a PCR technique with the highest detection rate. Assessment of the epidemiologic sensitivity of each assay would require establishment of a diagnostically criterion-referenced standard against which results of these assays could be assessed. Until then, clinical studies that involve the use of PCR assays should include a carefully selected control group against which results for the test group can be compared. Similarly, results of studies that use different PCR assays should be compared cautiously. Finally, it remains difficult to assess PCR results for a specific cat, especially when that cat has been vaccinated with a vaccine that becomes latent within and reactivates from the trigeminal ganglia.

References

1554

AJVR, Vol 66, No. 9, September 2005


