

# Real-Time Quantitative Polymerase Chain Reaction Diagnosis of Infectious Posterior Uveitis

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**Objective:** To validate a real-time polymerase chain reaction (PCR) assay allowing rapid and sensitive detection and quantitation of 4 common infectious posterior uveitis pathogens.

**Methods:** A real-time PCR assay using previously validated primer sets for cytomegalovirus, herpes simplex virus, varicella-zoster virus, and *Toxoplasma gondii* was developed. A standard curve for quantitation of pathogen load was generated for each pathogen using SYBR Green I fluorescence detection. Ocular samples from patients with posterior uveitis and from negative control samples were assayed and compared with standards to identify pathogens and quantify infectious load.

**Results:** Sensitivity for detection of purified pathogen DNA by PCR was not reduced by application of the real-time method. Standard curves for the quantitation of pathogen loads showed sensitivity to fewer than 10 organisms for all pathogens. The technique was applied to 2 clinical prob-

lems. First, sensitivities of existing monoplex and multiplex PCR were compared by real-time PCR. No significant difference in sensitivity was observed between multiplex and monoplex techniques. Second, pathogen loads of vitreous specimens from patients previously diagnosed as having infectious posterior uveitis were calculated. Pathogen loads were found to be generally higher for patients with disease caused by varicella-zoster virus than those caused by cytomegalovirus or herpes simplex virus.

**Conclusions:** Real-time PCR may be applied to infectious agents responsible for posterior uveitis. This technique will likely prove useful for the diagnosis of posterior uveitis as well as the linkage of pathogen to disease.

**Clinical Relevance:** Real-time PCR provides a rapid technique for quantitatively evaluating ocular samples for the presence of infectious pathogens.

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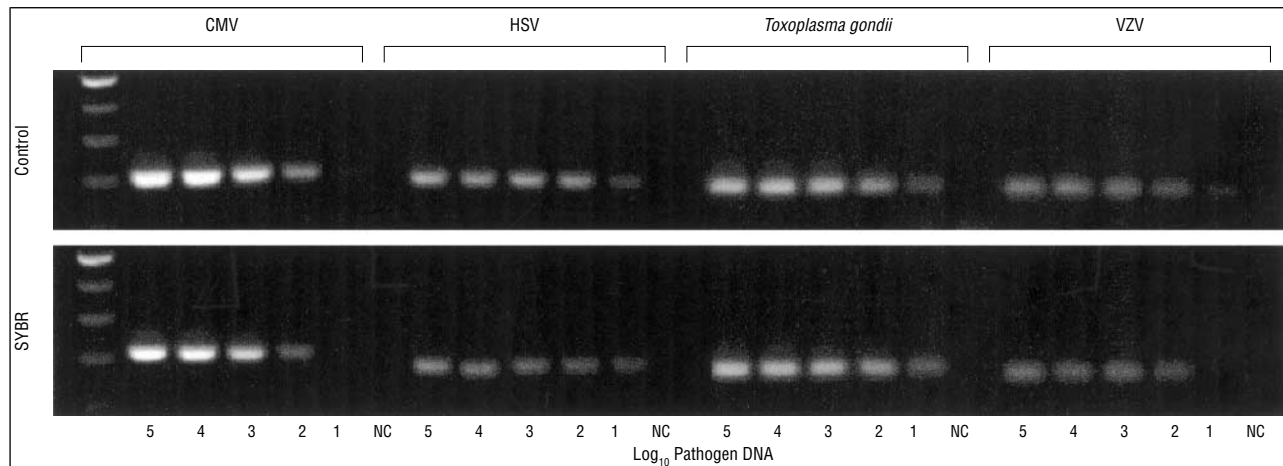
**P**OLYMERASE CHAIN reaction (PCR) analysis is a powerful molecular biological technique for the production of analytic quantities of nucleic acids from infinitesimal starting quantities. The DNA from pathogenic organisms can be detected from very small sample volumes. At present, PCR is commonly used in ophthalmology for the diagnosis of infectious external-surface disease, keratitis, and infectious anterior and posterior uveitis.<sup>1</sup>

The PCR results in exponential nucleic acid amplification. Theoretically, with each cycle of amplification, the molar quantity of DNA product doubles. Although this exponential amplification is the basis for the extreme sensitivity of the technique, it also produces 2 potential pitfalls. First, very small amounts of contaminating pathogen DNA (from unaffected adjacent tissue harboring latent organisms or normal flora, or from laboratory cross contamination) may create false-positive results. Second, PCR re-

sults are generally reported as present or absent, depending on the ability to visualize the amplified product after electrophoresis. No quantitative information regarding pathogen load is obtained from results of routine PCR.

Real-time PCR<sup>2,3</sup> is a variant of PCR in which the amplification reaction is performed using fluorescent probes or DNA intercalating dyes that increase in fluorescence with the accumulation of double-stranded PCR product. Each PCR reaction is monitored by means of fiber-optic fluorimetry during the thermal cycling process. The resulting curve of fluorescence accumulation can be used to quantify the amount of pathogen in the sample. Monitoring of the pathogen load has become standard practice in evaluating human immunodeficiency virus infection, and is readily performed by means of real-time PCR.<sup>4-6</sup> Real-time techniques have recently been implemented for the detection of a variety of pathogenic organisms.<sup>7-14</sup>

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**Figure 1.** Comparison of specificity for polymerase chain reaction (PCR) detection of posterior uveitis pathogens with and without SYBR Green I dye (SYBR) (Applied Biosystems, Foster City, Calif). For each set, serial dilutions of DNA purified from  $10^5$  to  $10^1$  pathogens were subjected to PCR for the indicated pathogen. Upper panel shows control samples (without SYBR Green I dye); first lane, molecular weight marker. CMV indicates cytomegalovirus; HSV, herpes simplex virus; VZV, varicella-zoster virus; and NC, negative control.

Very little information exists on the prognostic or diagnostic significance of pathogen load in the diagnosis of infectious posterior uveitis. We therefore sought to convert a validated PCR technique<sup>15</sup> to real-time use for the detection of herpes simplex virus (HSV), varicella-zoster virus (VZV), cytomegalovirus (CMV), and *Toxoplasma gondii*.

## METHODS

### PATIENT SAMPLES

Aqueous and vitreous samples were obtained from patients with posterior uveitis, boiled for 5 minutes, and stored at  $-80^{\circ}\text{C}$ . Samples had been obtained anonymously and underwent PCR testing as part of previous studies approved by our Institutional Review Board. Detailed clinical histories were not available for most samples. All samples were known to be positive for CMV, HSV, or VZV owing to previous standard PCR findings. Vitreous samples obtained incidentally in the course of surgery for nonuveitic retinal disease also underwent testing as negative control samples. Samples were assayed via real-time PCR against the various dilution series under experimenter-masked conditions.

### PRIMER SEQUENCES

Primers specific for CMV, HSV, *T gondii*, and VZV sequences previously designed for monoplex and multiplex standard PCR reactions were used for real-time PCR reactions. All primers were 20 base pairs (bp) long and produced a 100- to a 300-bp product. Forward and reverse sequences were as follows: CCT TTC CCT CGG CTT CTC AC and GGA CAT CCC GCG TTG TTT CG, respectively, for CMV (201-bp product); GTG TGG GAC ATA GGC CAG AG and GCG ACA TCC CCG CCT ACT AC, respectively, for HSV (190-bp product); CCC GCT GGC AAA TAC AGG TG and CGC AGT ACA CCA GGA GTT GG, respectively, for *T gondii* (155-bp product); and CGG TTG GGT TGT CTT CTG TG and GCG ACG AAC CGT AAG CGT GG, respectively, for VZV (152-bp product).

### STANDARD DILUTION SERIES

Purified pathogen DNA was obtained from Advanced Biotechnologies, Inc, Columbia, Md. A dilution series of DNA ex-

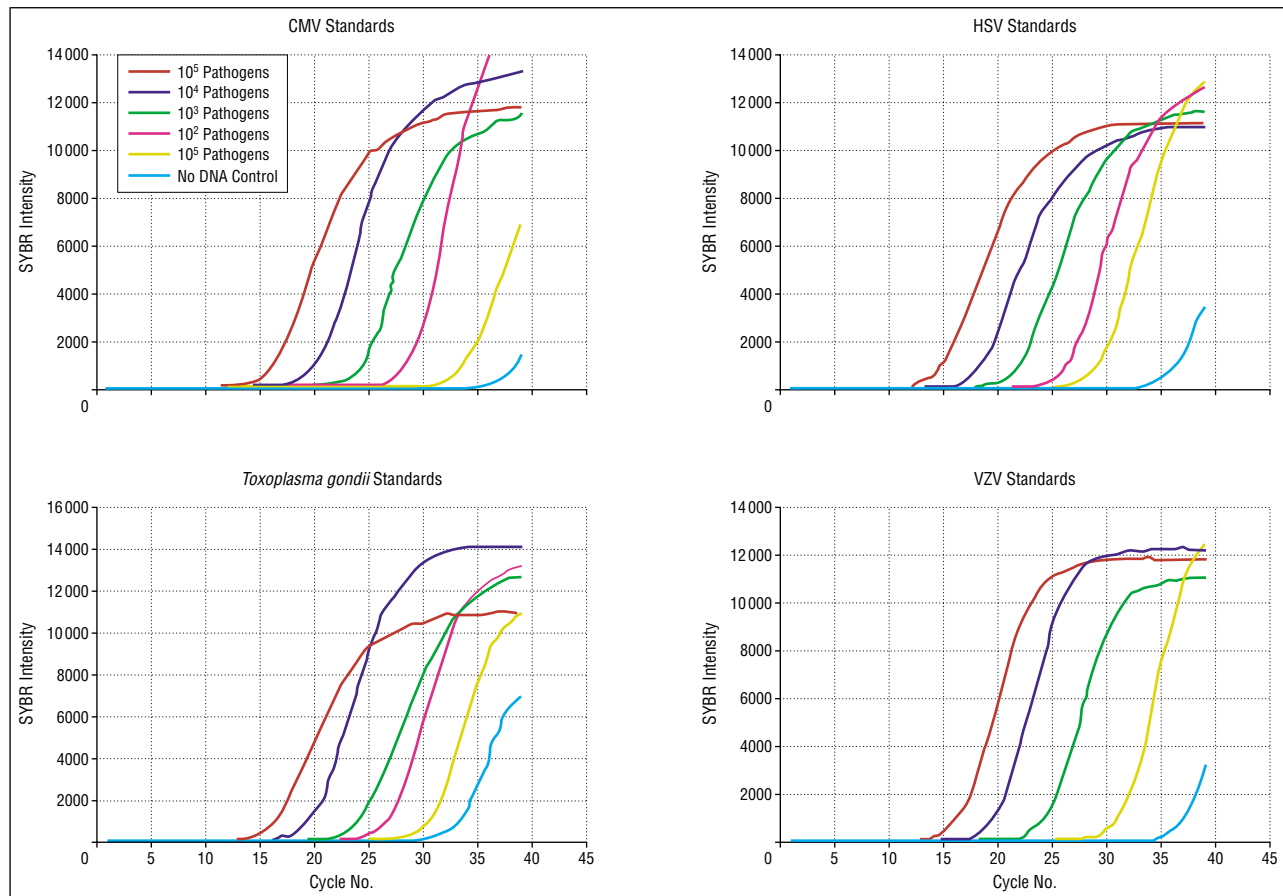
tracted from  $1 \times 10^5$  to  $1 \times 10^1$  purified pathogens was generated for CMV, HSV, *T gondii*, and VZV.

### REAL-TIME PCR

For monoplex reactions, 5  $\mu\text{L}$  of DNA from a patient sample or a standard dilution series was combined with 4  $\mu\text{L}$  of 12.5mM each deoxynucleotide triphosphate mix and SYBR Green I reagents (Applied Biosystems, Foster City, Calif), which included 5  $\mu\text{L}$  of  $10\times$  SYBR Green I buffer, 6  $\mu\text{L}$  of 25mM magnesium chloride, 0.25  $\mu\text{L}$  of Taq DNA polymerase (AmpliTaq Gold; Applied Biosystems), and 0.5  $\mu\text{L}$  of deoxyuridine triphosphate (AmpErase; Applied Biosystems). Each reaction contained 1  $\mu\text{L}$  (100nM final concentration) of forward and reverse primer for CMV, HSV, *T gondii*, or VZV. For each primer set, a negative control (with 5  $\mu\text{L}$  of distilled water as a template) was assembled using the same concentrations of reagents as above. The total reaction volume was 50  $\mu\text{L}$ . Multiplex reactions were similar to monoplex reactions except that forward and reverse primers for all 4 pathogens were run simultaneously within the same reaction vessel. All samples were denatured using a thermocycler (Prism 7700; Applied Biosystems) for 3 minutes at  $50^{\circ}\text{C}$ , followed by 10 minutes at  $95^{\circ}\text{C}$ . Samples were amplified in the same machine for 40 cycles of 15 seconds at  $95^{\circ}\text{C}$  and 1 minute at  $60^{\circ}\text{C}$ . SYBR Green I fluorescence was detected and plotted using Applied Biosystems software during the  $60^{\circ}\text{C}$  extension phase for each cycle. Threshold cycles and log starting quantities for patient samples were calculated using Applied Biosystems software.

## RESULTS

To validate the conversion of our standard PCR assay to real-time use, we first performed standard PCR reactions with the fluorescent intercalating dye SYBR Green I added. To demonstrate that the addition of the SYBR Green I dye did not alter the specificity of the PCR reaction, we analyzed the products of a PCR reaction containing the SYBR Green I dye by means of gel electrophoresis. As shown in **Figure 1**, no change was seen in the specificity of PCR for the detection of HSV, VZV, *T gondii*, or CMV. Consistent with previous results,<sup>15</sup> all assays showed sensitivity at the  $10^1$ -pathogen level. As judged by ethidium bromide fluorescence, we found no



**Figure 2.** Real-time signals for standard pathogen curves. SYBR Green I (SYBR) (Applied Biosystems, Foster City, Calif) fluorescent intensity (in arbitrary units) with an increasing number of thermal cycles was monitored by the real-time polymerase chain reaction (PCR) cycle. For each standard curve, a dilution series of purified DNA of the indicated pathogen was subjected to real-time monitoring of the multiplex PCR reaction. Red indicates  $10^5$  pathogens; blue,  $10^4$  pathogens; green,  $10^3$  pathogens; purple,  $10^2$  pathogens; yellow,  $10^1$  pathogens; and teal, no DNA control. Other abbreviations are explained in the legend to Figure 1.

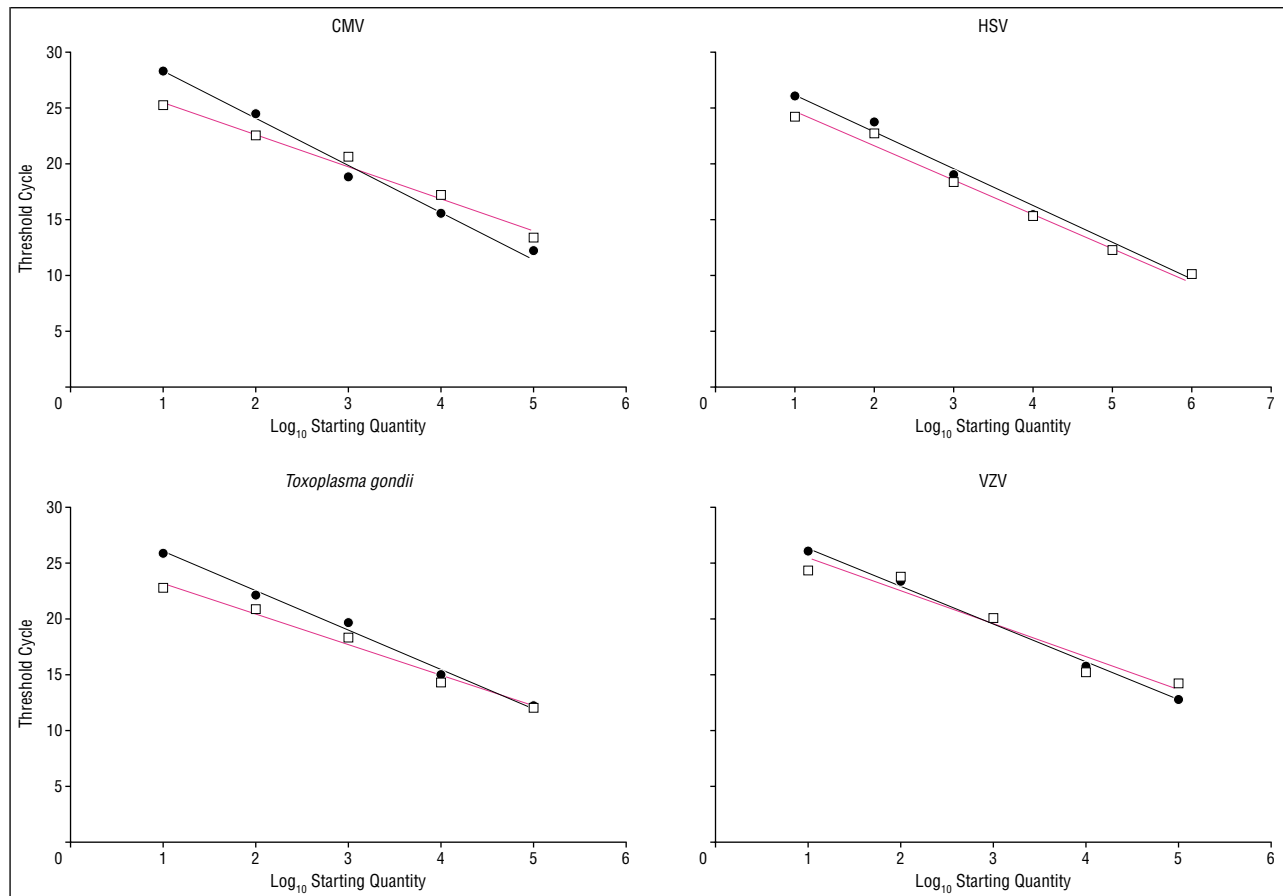
difference in sensitivity between reactions with and without SYBR Green I dye.

We next constructed standard curves for quantitation of pathogen loads by means of multiplex PCR. As shown in **Figure 2**, each PCR reaction produced a sigmoidal reaction curve. These curves were well separated by the dilution series. Conversion to log concentrations showed high log-linearity of the dilution curves (**Figure 3**, black lines). Best-fit curves for the pathogens and correlation coefficients are shown in **Table 1**.

We next applied the real-time technique to determine quantitatively whether multiplex PCR for posterior uveitis pathogens has a lower sensitivity than multiplex PCR. Multiplex PCR is a technique for the detection of multiple pathogens in a single PCR reaction, and can significantly improve the speed with which a diagnosis can be made. The relative sensitivity of multiplex PCR compared with multiplex PCR, however, is not precisely known. Multiplex PCR was performed as described<sup>15</sup> using the real-time automated analysis. Log-linear standard curves were generated. As shown in Figure 3 (magenta lines), no significant difference was found in sensitivity between multiplex and multiplex PCR. Slopes and intercepts of the standard curves were nearly equivalent for all 4 pathogens tested (Table 1), and quantitation remained linear to 10 plaque-forming units or or-

ganisms for each pathogen. Real-time monitoring thus quantitatively demonstrated that multiplex and multiplex PCR have nearly equivalent sensitivity for the detection of pathogen when these protocols are used.

As a second application of quantitative PCR, we sought to determine the relative pathogen loads in posterior uveitis in a small set of vitreous biopsy samples from patients with known posterior uveitis. These samples had previously been used for verification of our multiplex and multiplex PCR assays as described.<sup>15</sup> Unfortunately, no *T gondii*-positive samples were available for analysis in this group. Samples were subjected to real-time PCR, and threshold-crossing values were converted to pathogen genome equivalents by interpolating the observed threshold-crossing number on the standard curve of purified pathogen. Of the 10 samples undergoing testing, 9 yielded a positive signal on results of real-time testing (**Table 2**). No signal was seen in any of 5 vitreous samples from noninflamed eyes. A single sample that had previously shown a positive PCR result for CMV was not found to be positive on results of repeated real-time testing. For the 9 samples in which pathogenic DNA was detected on results of real-time testing, 8 showed a single organism (3, CMV; 2, HSV; and 3, VZV). In each case, the positive finding agreed with previous clinical and PCR findings. Calculated pathogen loads are



**Figure 3.** Standard curves for monplex and multiplex real-time polymerase chain reaction (PCR) pathogen detection. Threshold cycle crossing for monoplex reactions in Figure 2 were calculated using Applied Biosystems software (Foster City, Calif), and plotted as log-linear (black lines). Best-fit curves were found by linear regression; equations of resultant curves are given in Table 1. Identical dilution series were prepared and tested with multiplex PCR. Multiplex threshold crossing curves were derived (magenta lines). Other abbreviations are explained in the legend to Figure 1.

shown in Table 2. Although detailed clinical information was not available for all cases, the 2 cases known to be progressive outer retinal necrosis syndrome showed the highest levels of VZV infection.

Results in a single patient were positive for both HSV and VZV. However, this patient showed nearly 20-fold higher levels of infection for VZV than for HSV. The initial PCR analysis for this patient had shown only VZV. We suspect that the positivity for HSV in this patient represents a low-level signal (eg, from sampled neural tissue harboring latent virus) rather than active disease caused by 2 viruses simultaneously.

#### COMMENT

Polymerase chain reaction has substantially improved the ability to detect infectious pathogens responsible for posterior uveitis. The technique has found widespread use<sup>1,16,17</sup> and has proved to be of great utility in the diagnosis of posterior uveitis.<sup>18</sup>

The widespread use of PCR has been in part limited by technical issues that influence performance and interpretation of results. One difficulty—the problem of having to run sequential PCR reactions to test a differential diagnosis—has been partially addressed by the development of multiplex PCR reactions in which mul-

**Table 1. Standard Curve Regressions for Quantitation of Posterior Uveitis Pathogens\***

Pathogen	Equation of Linear Regression	R <sup>2</sup> Value
CMV		
Monoplex	Pathogen Load = 10 <sup>(Threshold Cycle -32.28)/-4.15</sup>	0.993
Multiplex	Pathogen Load = 10 <sup>(Threshold Cycle -28.38)/-2.89</sup>	0.982
HSV		
Monoplex	Pathogen Load = 10 <sup>(Threshold Cycle -29.41)/-3.34</sup>	0.990
Multiplex	Pathogen Load = 10 <sup>(Threshold Cycle -27.71)/-3.34</sup>	0.987
<i>Toxoplasma gondii</i>		
Monoplex	Pathogen Load = 10 <sup>(Threshold Cycle -29.33)/-3.47</sup>	0.993
Multiplex	Pathogen Load = 10 <sup>(Threshold Cycle -25.99)/-2.79</sup>	0.986
VZV		
Monoplex	Pathogen Load = 10 <sup>(Threshold Cycle -29.73)/-3.37</sup>	0.995
Multiplex	Pathogen Load = 10 <sup>(Threshold Cycle -28.22)/-2.89</sup>	0.948

\*CMV indicates cytomegalovirus; HSV, herpes simplex virus; and VZV, varicella-zoster virus.

iple pathogens can be tested simultaneously.<sup>15</sup> In previous work, it was suggested that minimal loss in sensitivity resulted with this technique. Through the use of real-time PCR, we demonstrated that with our protocols, no substantial decrease in sensitivity for the detection of pathogens occurs with the use of the multiplex assay.

**Table 2. Pathogen Loads in Vitreous Samples With Active Posterior Segment Uveitis\***

Patient Sample No.	CMV	HSV	<i>Toxoplasma gondii</i>	VZV
1045	<b>0.41 (2.5 Genomes)</b>	0	-0.25	-0.87
1069	<b>1.10 (12.5 Genomes)</b>	0	-0.40	-0.73
1079	-0.97	0	-0.60	<b>1.55 (35.5 Genomes)</b>
1089	<b>1.89 (78.3 Genomes)</b>	0	-0.63	-0.97
1090A	-0.13	<b>1.32 (20.8 Genomes)</b>	-0.64	<b>2.60 (397 Genomes)</b>
2071A	-0.69	-1.74	-1.28	<b>3.32 (2108 Genomes)</b>
2085A	-0.42	<b>2.09 (122 Genomes)</b>	-1.13	-1.02
2090	0.09	-1.41	-1.55	<b>3.68 (4808 Genomes)</b>
2091A	-1.12	-1.26	-1.13	-1.28
2099	-0.56	-2.01	-0.57	<b>2.52 (334 Genomes)</b>

\*Data are given as log (pathogen) based on interpolation of threshold-crossing values with standard curves. Quantities in bold represent presumptive diagnosis and are given with conversion to the number of pathogen genomes per microliter of starting vitreous. CMV indicates cytomegalovirus; HSV, herpes simplex virus; and VZV, varicella-zoster virus.

False-positive results remain another marked pitfall to the routine use of PCR. As PCR assays increase in sensitivity, the possibility of laboratory contamination or of detection of latent DNA or DNA of normal flora becomes limiting. Although PCR results must always be evaluated in a clinical context, interpretation of positive results has been limited by the possibility of false-positive results. For example, Short et al<sup>19</sup> found their initial nested PCR assay for VZV to be so sensitive that they could detect less than 1 molecule; the sensitivity of the assay was empirically reduced to minimize these false-positive results. Particularly for pathogens that may reside in latency in host tissue (which includes all of the pathogens discussed in this work), the possibility of detection of latent DNA complicates analysis. Through the use of real-time PCR, one may be able to distinguish false-positive from true-positive results by comparison of viral load. The present study suggests that, for cases of suspected VZV or HSV infection, viral titers of less than 10 pathogens per microliter may be false positive. Conversely, very high pathogen loads are more likely to be associated with active disease.

Despite the marked improvement in quantitation offered by the real-time technique, a true gold standard for pathogen quantitation remains elusive. The standard curves for viral pathogen load in the present study were derived from DNA purified from given numbers of pathogen as assayed by viral plaque counting. This introduces 2 potential confounders to precise quantification. First, because not every virion in a plaque assay for virus will produce a visible plaque, viral PCR is generally more sensitive than plaque-based assays by at least 1 order of magnitude.<sup>20</sup> Conversely, however, recovery of purified viral DNA after ultracentrifugation of the viral culture is not perfect, so some losses will occur at this step, partially countering the inherently higher sensitivity of PCR compared with culture. The slight separation of the negative control curves from the 10<sup>1</sup> pathogen curves for viral pathogens (Figure 2) suggests that the real-time assay may be able to detect less than 1 plaque-forming unit of virus. However, precise measurement of the pathogen load may require performance of standard curve calibration using more highly purified and quantified viral DNA.

Monitoring of the pathogen load has become a standard means for monitoring response to therapy in human immunodeficiency virus infection. To date, no data on pathogen load in posterior uveitis have been reported. It is unclear, for example, whether the pathogen load will affect the natural history of the disease or its response to treatment. One potential use for such monitoring may be in the management of CMV infection treated with a ganciclovir implant (Vitrasert; Bausch & Lomb Surgical, Claremont, Calif).<sup>21,22</sup> Treatment of CMV retinitis with intravenous ganciclovir leads to resistance to the medication in more than 25% of cases.<sup>23</sup> Replacement of the ganciclovir implant at 6- to 9-month intervals allows ready access to the vitreous, which could then undergo sampling for quantitative PCR to determine CMV load. Such information may allow earlier detection of resistance and more timely conversion to alternate therapy.

Finally, quantitative PCR may be invaluable in the quest to link particular pathogens to ophthalmic disease. Several studies have purported to link specific pathogens to ocular inflammatory syndromes using PCR. For example, HSV was found in the anterior chamber of 3 patients with active granulomatocyclitic crisis,<sup>24</sup> whereas *Mycobacterium tuberculosis* has been found in epiretinal membranes associated with Eales disease.<sup>25</sup> Since Koch's postulates cannot be readily fulfilled for conditions that lack animal models; it becomes important to determine whether these positive PCR results represent pathologic levels of infection or detection of nonpathogenic contaminating flora.

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