**Rapid Detection and Identification of Uveitis Pathogens by Qualitative Multiplex Real-Time PCR**

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**PURPOSE.** Infectious uveitis is a serious sight-threatening infection commonly caused by herpesviruses and *Toxoplasma gondii*. Etiologic diagnosis based on the clinical evaluation is often challenging. We developed and validated a multiplex real-time PCR assay coupled with high-resolution melting (HRM) for rapid detection and identification of herpes simplex viruses 1 and 2 (HSV-1 and HSV-2), varicella-zoster virus (VZV), cytomegalovirus (CMV), and *T. gondii*.

**METHODS.** The assay was designed to target pathogen genome regions that yield products with distinct melting temperatures. Analytical specificity, sensitivity, and precision of HRM identification were determined. Clinical validation was performed by testing 108 intraocular fluids collected from eyes suffering with infectious uveitis (*n* = 30) and controls (*n* = 78).

**RESULTS.** A nonoverlapping high-precision profile for each pathogen was generated following HRM (coefficient of variation 0%). The assay was highly sensitive, with a limit of detection of 20 genome copies for herpesviruses and 200 genome copies for *T. gondii*. The intra- and interassay variability of cycle threshold (Ct) measurement was ≤4% and ≤6%, respectively. Thirteen intraocular specimens collected from suspected cases of infectious uveitis were positive (mean Ct values varied from 19.4 to 27.7). Melting profiles of positive cases were consistent with HSV-2 (*n* = 5), VZV (*n* = 5), CMV (*n* = 2), and *T. gondii* (*n* = 1). Amplicon identities were confirmed by sequencing. Control intraocular samples from patients without a clinical diagnosis of infectious uveitis were all negative.

**CONCLUSIONS.** This assay allows rapid, sensitive, and reliable detection and identification of the most common known causes of infectious uveitis, making early pathogen information-based intervention possible.

Keywords: infectious uveitis, herpesviruses, toxoplasmosis, multiplex real-time PCR, high-resolution melting

Uveitis is the inflammation of the uvea, the pigmented vascular middle layer of the eye, which comprises the iris, ciliary body, and choroid. In the United States, uveitis has a prevalence of 57.5 to 115.3 cases per 100,000 person-years1,2 and is thought to be associated with 10% of all cases of blindness.3 The etiology of uveitis is often unclear, and only approximately 17% of uveitis cases are caused by an infectious agent that is ultimately identified.4 *Toxoplasma gondii* and herpesviruses, including herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), varicella-zoster virus (VZV), and cytomegalovirus (CMV), are the most common known causes of infectious uveitis, although bacteria, fungi, and other viruses and parasites may also be involved.4–7

Diagnosis of uveitis is frequently made on the basis of the patient’s history and findings upon clinical examination. Ascribing an etiology is complicated by overlapping clinical findings among cases caused by different infectious agents and also with observations made for noninfectious causes of uveitis, such as those caused by autoimmune diseases and trauma.4,8 This ambiguity may delay the onset of potentially effective treatment. For example, syphilitic uveitis, atypical toxoplastic retinitis, fungal endophthalmitis, and noninfectious uveitis may be misdiagnosed as viral retinitis.9,10 Furthermore, many noninflammatory ocular disorders (neoplastic and nonneoplastic) may present as intraocular inflammatory diseases (masquerade syndromes) and may be diagnosed and treated as uveitis.11 For these reasons, there is considerable interest in molecular detection of uveitis-associated pathogens directly in intraocular fluids of suspected uveitis cases.7,9,12,13 Therefore, our goal was to develop a rapid and sensitive diagnostic test for the leading known causes of infectious uveitis, with the potential to impact management of patients in a timely fashion. To achieve this we developed a multiplex assay using real-time PCR detection,14 coupled with high-resolution melting (HRM) analysis of the amplification products15 to rapidly detect and identify herpesviruses (HSV-1, HSV-2, VZV, and CMV) and *T. gondii* in a single reaction. This goal was achieved with an assay that provided both high sensitivity and specificity.

**METHODS**

**Selection of Target Sequences and Primer Design**

Conserved areas of pathogen genomes identified in herpesviruses and *T. gondii* from human specimens (Table 1;
The main variables used to optimize discrete melting profiles with respect to temperature (dF/dT) axis in the dissociation curve. This yielded theoretical PCR products with the highest predicted melting peak in the derivative of fluorescence with respect to temperature (d Helicity/d Temp)).

The targeted regions were selected for predicted melting temperature (Tm) values differing by at least \( \pm 2°C \) for each organism and the greatest value for the negative derivative of helicity with respect to temperature (d(-Helicity)/dT). The targeted regions were selected for predicted melting temperature (Tm) values differing by at least \( \pm 2°C \) for each organism and the greatest value for the negative derivative of helicity with respect to temperature (d(-Helicity)/dT). This yielded theoretical PCR products with the highest predicted melting peak in the derivative of fluorescence with respect to temperature (dF/dT) axis in the dissociation curve. The main variables used to optimize discrete melting profiles were length of product and guanine-cytosine (GC) content.

<table>
<thead>
<tr>
<th>Target</th>
<th>Name</th>
<th>Sequence</th>
<th>Gene (Locus Tag)</th>
<th>Product Size, bp</th>
<th>Product %GC</th>
<th>Tm ( ^{\circ}C ) Prediction*</th>
<th>Practical Tm ( ^{\circ}C )</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1</td>
<td>HSV1/2Fw</td>
<td>CATGACCAAGTGCGAGCA</td>
<td>Glycoprotein (UL27)</td>
<td>235</td>
<td>64 (HSV1)</td>
<td>91.6 (HSV1)</td>
<td>90.3</td>
</tr>
<tr>
<td>HSV-1</td>
<td>HSV1/2Rv</td>
<td>CAGGAGTAGTCGCGCTG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMV</td>
<td>CMVFw</td>
<td>AAGGGGAAAGCCGCGGATG</td>
<td>Regulatory protein IE2 (UL122)</td>
<td>214</td>
<td>55</td>
<td>87.1</td>
<td>85.7</td>
</tr>
<tr>
<td>CMV</td>
<td>CMVRv</td>
<td>ACGGGCTCTTCTAAGGGTG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VZV</td>
<td>VZVFw</td>
<td>TCGTCTTCTGATTCCTTC</td>
<td>Glycoprotein B (ORF31)</td>
<td>223</td>
<td>45</td>
<td>83.0</td>
<td>81.7</td>
</tr>
<tr>
<td>VZV</td>
<td>VZVRv</td>
<td>TTTAAGCCTACGGGAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T. gondii</td>
<td>ToxFw</td>
<td>AGGGTGTCTGCTCCATT</td>
<td>Glycerol-3-phosphate dehydrogenase</td>
<td>114</td>
<td>44</td>
<td>80.5</td>
<td>79.1</td>
</tr>
<tr>
<td>T. gondii</td>
<td>ToxRv</td>
<td>AAGTAGGAGGGAGGCT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Tm, melting temperature; GC, guanine-cytosine.

Supplementary Table S1 were scrutinized for coding strategies that would yield PCR products with distinct, predicted melting curve profiles. Such amplification targets were identified in silico using the melting curve prediction software uMelt. Predictions were made based on thermodynamic parameters described by Blake and Delcourt, assuming default concentrations of monovalent ions (20 mM), free magnesium (3 mM), and a temperature step size of 0.1°C over the range of 60°C to 90°C. The targeted regions were selected for predicted melting temperature (Tm) values differing by at least \( \pm 2°C \) for each organism and the greatest value for the negative derivative of helicity with respect to temperature (d(-Helicity)/dT). This yielded theoretical PCR products with the highest predicted melting peak in the derivative of fluorescence with respect to temperature (dF/dT) axis in the dissociation curve. The main variables used to optimize discrete melting profiles were length of product and guanine-cytosine (GC) content. After identifying theoretically optimal regions for amplification, a set of target-specific primers was designed to uniquely amplify VZV, CMV, and T. gondii DNA. A set of universal primers that can amplify both HSV1 and HSV2 was also included (Table 1). The universal HSV1/2 primers were designed to amplify a variable region within the gene UL27 that results in distinct DNA melting profiles, allowing differentiation of these two herpes simplex virus subtypes by Tm. Primers were further optimized to minimize potential for primer-dimer and hairpin formation using AutoDimer.

### Primer Validation and PCR Optimization

Purified genomic DNA from HSV1 strain MacIntyre (VR-529DQ), HSV2 strain MS (VR-540DQ), VZV strain Ellen (VR-1367DQ), CMV strain AD169 (VR-538DQ), and T. gondii strain RH (ATCC 50174D) obtained from American Type Culture Collection (ATCC; Manassas, VA, USA), were used for primer validation and PCR optimization. To confirm the specificity and potential cross-amplification of the designed primers, each primer pair was first tested against all five targets. Correct amplification was confirmed by gel electrophoresis and by analysis of Tm values. Subsequently, the multiplex reaction was optimized by testing different primer concentrations, ranging from 100 to 700 nM.

### Real-Time Multiplex PCR

Real-time PCR amplification and HRM analysis was performed in 25-μL reactions containing 12.5 μL 2X Express SYBR GreenER qPCR Supermix (ThermoFisher, Waltham, MA, USA), 2 μL purified DNA, and sterile water to complete to the final volume. PCR was performed using a real-time PCR cycler system (Rotor-Gene Q5-Plex; Qiagen, Hilden, Germany) under the following conditions: 50°C initial annealing and extension for 2 minutes, 95°C denaturation for 5 minutes, followed by 40 cycles of denaturation (95°C for 5 seconds) and annealing/extension (60°C for 10 seconds). HRM analysis was immediately performed after heating samples from 70°C to 90°C, using a temperature ramp of 0.1°C/s. Negative controls for contamination lacked added DNA but contained all PCR reagents and were included on each run. As an endogenous positive reaction control, amplification of the human β-globin gene was performed in a separate singleplex PCR format, as previously published.

### Intra- and Interassay Variation

The intra- and interassay coefficient of variation was determined by measuring the Ct values of concentrations near or at (200 copies per reaction) and above the limit of detection (2 \( \times 10^3 \) copies per reaction). The variation in the Tm values for pathogen identification was also determined. Precision calculations were based on the results of five technical replicate reactions performed on each of 4 consecutive days.

### Analytical Specificity and Sensitivity

In addition to testing each primer set for absence of cross-amplification of targets in the multiplex reaction, analytical specificity was also determined by spiking TE buffer with DNA (10 ng/μL) from other pathogens often associated with nonuveitis intraocular infections. These included Staphylococcus epidermidis, Propionibacterium acnes, Staphylococcus aureus, Candida albicans, Mycobacterium tuberculosis, and Treponema pallidium.

Analytical sensitivity of the assay was evaluated using purified control genomic (for HSV-1, HSV-2, CMV, VZV) or plasmid (carrying the targeted T. gondii genome region) DNA of known concentration. To clone the relevant portion of the T. gondii genome on a plasmid for propagation in E. coli, the T. gondii sequence of interest was amplified by PCR using high-fidelity DNA polymerase (Q5; New England Biolabs, Ipswich, MA, USA) cloned into the pCR4 Blunt TOPO vector (Zero Blunt TOPO PCR Cloning Kit; ThermoFisher), and transformed into chemically competent E. coli TOP10 (ThermoFisher). Solutions containing known amounts of purified DNA were diluted 10-fold in nuclease-free water to obtain final concentrations ranging from 1 \( \times 10^5 \) genome copies/μL to 1 copy/μL.

### Assay Validation

Protocols for collection of discarded aqueous and undiluted and diluted vitreous specimens were approved by the Massachusetts Eye and Ear Institutional Review Board. All
subjects were treated in accordance with the Declaration of Helsinki. Samples were obtained either by anterior paracentesis (aqueous samples), posterior chamber paracentesis (undiluted vitreous samples), or during pars plana vitrectomy (both undiluted vitreous and diluted vitreous washing samples). Following collection, all specimens were immediately transported to the laboratory and stored at −20°C. In total, 108 aqueous or vitreous fluid samples were included in this study. Of those, 30 were collected from 18 patients with clinically suspected viral or toxoplasma uveitis. Control specimens included those collected from patients undergoing diagnosis or treatment for conditions other than suspected uveitis (e.g., patients undergoing aqueous fluid removal as a routine part of scleral buckle surgery for retinal detachment [21 specimens]) or patients with suspected bacterial or fungal endophthalmitis (30 specimens from patients with culture-positive bacterial or fungal endophthalmitis and 27 specimens from patients with culture-negative but clinically diagnosed endophthalmitis) (Supplementary Table S2). All specimens were tested in triplicate in a masked fashion. Positive samples were sequenced (Genewiz, South Plainfield, NJ, USA) for confirmation of amplicon identities.

DNA Extraction
DNA extraction and purification was performed using a DNA isolation kit (DNeasy Blood and Tissue Kit; Qiagen), following the tissue protocol with few modifications. Chemical lysis was achieved by mixing 100 μL primary aqueous or vitreous sample with proteinase K, followed by the addition of a buffer (AL; Qiagen) according to the manufacturer’s protocol. Purified DNA was eluted from the silica membrane in 50 μL EB buffer (Qiagen).

RESULTS

Assay Optimization
Each of the five amplification targets—HSV-1, HSV-2, VZV, CMV, and T. gondii—when tested individually, generated products with characteristic melting curves possessing distinct peak values by HRM analysis (Fig. 1). The identity of the amplified product was verified by gel electrophoresis (Supplementary Fig. S1) and then by DNA sequence. Experimentally determined $T_m$ values for each target were $79.1^\circ\text{C}$ for T. gondii, $81.7^\circ\text{C}$ for VZV, $85.7^\circ\text{C}$ for CMV, $90.3^\circ\text{C}$ for HSV-1, and $90.8^\circ\text{C}$ for HSV-2. This assay is able to detect and identify any of the five targeted pathogens in a single assay in 1 hour, 40 minutes.

Analytical Specificity
To ensure that each primer pair amplified only the intended product and did not cross-react in multiplex, each pair was tested in separate reactions for product generation using control DNA for HSV-1, HSV-2, VZV, CMV, and T. gondii. To determine whether the primers selected would be confounded by DNA from other causes of intraocular infection, we also tested them using DNA from rarer known causes of infectious uveitis (T. pallidum and Mycobacterium tuberculosis) and leading causes of endophthalmitis (S. epidermidis and S. aureus), including microbes associated with chronic and low-grade endophthalmitis that can mimic some uveitis symptoms (P. acnes and C. albicans). Each set of primers was verified as being specific for the intended target only.

Analytical Sensitivity
Serial 10-fold dilutions of DNA ranging from $1 \times 10^5$ genome copies per microliter to one copy per microliter were tested in order to determine the linearity and limits of detection, the lowest concentration tested yielding consistent Ct values for each triplicate sample (Fig. 2). Only dilutions showing a clear and sharp $T_m$ peak of the predicted value were considered positive. For all the targets, the Ct values and $T_m$ peaks were
clearly positive for reactions containing 20 genome copies, except for T. gondii, for which the limit of detection was estimated to be 200 genome copies per reaction.

**Precision**

To determine the random analytical error of our assay, we calculated the intra- and interassay coefficient of variation by measuring the Ct and \( T_m \) values of control DNA at or near the limit of detection (200 copies per reaction) and well above (2 \( \times 10^5 \) copies per reaction), using five technical replicates per run. Interassay variability was determined by performing the same test on 4 consecutive days (Supplementary Table S3). The intra-assay variability of Ct measurements was \( \leq 4\% \) for all the targets and concentrations, and interassay variation was \( \leq 6\% \). No \( T_m \) variability was seen. To approximate the range of \( T_m \) variation that may stem from diversity in amplification products generated from the diversity of natural isolates that may occur in the patient population, melting curve predictions were performed for DNA sequences corresponding to the regions that would be amplified from a diverse collection of strains collected across multiple countries. Sequences were retrieved from National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and aligned using software (Geneious v8; Biomatters, Inc., Newark, NJ, USA). The accession numbers as well as the predicted \( T_m \) values for each pathogen/strain amplicon are displayed in Supplementary Table S1. For most of the targets included, none or very small numbers of polymorphisms in the targeted regions were found. These mutations would not be predicted to alter \( T_m \) to an extent that would confound identification of the amplified product (predicted variations ranged from 0.1°C to 0.5°C).

**Clinical Validation**

To clinically validate our assay, we first analyzed 30 samples from 18 patients with clinically diagnosed viral or toxoplasma uveitis (Table 2). Most of the patients were male (\( n = 15, 83.3\% \)), had a mean age of 55 years (±21), and were immunocompetent (\( n = 10, 55.5\% \)). On the basis of clinical presentation, the patients were initially diagnosed with acute retinal necrosis (ARN) (\( n = 11 \)), CMV retinitis (\( n = 4 \)), toxoplasmosis chorioretinitis (\( n = 2 \)), and progressive outer retinal necrosis (\( n = 1 \)). Using the newly developed multiplex assay, we were able to unambiguously identify one of the tested pathogens in 13 specimens collected from nine patients (Table 3). The assay was effective in detecting pathogen DNA in different specimen matrices, including aqueous humor (\( n = 4 \)), undiluted vitreous (\( n = 5 \)), and diluted vitreous washings (\( n = 4 \)). The mean Ct values for the positive cases, calculated from triplicates, varied from 19.4 to 27.7, with standard deviations no higher than ±0.7. Amplification of pathogen DNA from positive clinical specimens generated \( T_m \) values corresponding to HSV-2 (\( n = 5 \)), VZV (\( n = 5 \)), CMV (\( n = 2 \)), and T. gondii (\( n = 1 \)). These identities were confirmed by sequencing the PCR products and matching them to identities in GenBank using BLAST software (available in the public domain, https://blast.ncbi.nlm.nih.gov/Blast.cgi). As expected, \( T_m \) values for the positive specimens did not vary measurably from controls.

In reviewing the cases included, the initial clinical diagnosis was in agreement with PCR results for the majority of the cases (Table 4). Of the nine patients from which positive samples were collected, six had previously been diagnosed with ARN, two with CMV retinitis, and one with progressive outer retinal necrosis (PORN; which is typically caused by herpesviruses or CMV retinitis), based on clinical presentations. PCR showed specific pathogen identities consistent with all of these diagnoses, except for the one PORN patient specimen in which toxoplasma DNA was identified. T. gondii infection may cause symptoms shared by PORN. Rather than antiviral treatment, therapy specific for toxoplasma infection would be expected to be of greater benefit in such cases.

Nine other specimens derived from patients suspected of having viral retinitis or ocular toxoplasmosis yielded negative

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**Figure 2.** Standard curves obtained by testing serial 10-log dilutions ranging from 2 \( \times 10^4 \) copies to 2 copies per reaction. Calculations of efficiency (E) for each reaction are shown. Efficiency was calculated from the slope given by regression analysis using the formula \( E = 10^{(-1/slope)} - 1 \). Coefficients of correlation (\( R^2 \)) are also shown.
Table 2. Characteristics of Patients (n = 18) With Clinical Diagnosis of Infectious Uveitis Included in the Validation of the Multiplex Assay

<table>
<thead>
<tr>
<th>Sex</th>
<th>Percentage</th>
<th>Age, y (Mean ± SD)</th>
<th>Immune Status</th>
<th>Immunocompetent (n)</th>
<th>Immunosuppressed (n)</th>
<th>Others/unknown (n)</th>
<th>Initial Clinical Diagnosis</th>
<th>ARN (%)</th>
<th>CMV Retinitis (%)</th>
<th>Toxoplasmosis Chorioretinitis (%)</th>
<th>PORN (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (%)</td>
<td>15 (83.3)</td>
<td>53 ± 21</td>
<td></td>
<td>10 (55.5)</td>
<td>3 (16.7)</td>
<td>5 (27.8)</td>
<td></td>
<td>11 (61.1)</td>
<td>4 (22.2)</td>
<td>2 (11.1)</td>
<td>1 (5.6)</td>
</tr>
<tr>
<td>Female (%)</td>
<td>3 (16.7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PORN, progressive outer retinal necrosis.

PCR results. From the two patients with symptoms consistent with inflammation of toxoplastic etiology, one had a single aqueous humor sample collected and the other had already been treated with trimethoprim-sulfamethoxazole for 1 week at the time of collection. Toxoplasma DNA may be more difficult to detect in aqueous humor than that of other organisms. For the remaining suspected viral retinitis cases, two were being treated with antiviral medications at the time of sampling, and there were no known factors for the remaining five samples that may have contributed to PCR negativity (Table 5).

To determine the clinical specificity and complete the clinical validation of our assay, we collected and tested control samples with intraocular fluids collected and the other had already been treated with trimethoprim-sulfamethoxazole for 1 week at the time of collection. Toxoplasma DNA may be more difficult to detect in aqueous humor than that of other organisms. For the remaining suspected viral retinitis cases, two were being treated with antiviral medications at the time of sampling, and there were no known factors for the remaining five samples that may have contributed to PCR negativity (Table 5).

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Table 3. Multiplex PCR and Sequencing Results for the Positive Clinical Specimens

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Specimen (Eye)</th>
<th>Multiplex PCR Assay</th>
<th>Sequencing Identification</th>
<th>Identity (%)</th>
<th>E-Value</th>
<th>Product Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vitreous (OS)</td>
<td>27.68 (0.12)</td>
<td>90.8 HSV2</td>
<td>Human herpesvirus 2 (KU310665)</td>
<td>99</td>
<td>1e-112</td>
</tr>
<tr>
<td>2</td>
<td>Vitreous (OS)</td>
<td>19.44 (0.57)</td>
<td>90.8 HSV2</td>
<td>Human herpesvirus 2 (KU310666)</td>
<td>100</td>
<td>9e-115</td>
</tr>
<tr>
<td>3</td>
<td>Aqueous (OD)</td>
<td>20.11 (0.19)</td>
<td>90.8 HSV2</td>
<td>Human herpesvirus 2 (KU310666)</td>
<td>100</td>
<td>3e-115</td>
</tr>
<tr>
<td>4</td>
<td>Aqueous (OS)</td>
<td>24.27 (0.08)</td>
<td>90.8 HSV2</td>
<td>Human herpesvirus 2 (KU310666)</td>
<td>100</td>
<td>3e-115</td>
</tr>
<tr>
<td>5</td>
<td>Vitreous (OD)</td>
<td>24.35 (0.50)</td>
<td>81.7 VZV</td>
<td>Human herpesvirus 3 (KU926322)</td>
<td>100</td>
<td>8e-109</td>
</tr>
<tr>
<td>6</td>
<td>Aqueous (OD)</td>
<td>26.76 (0.08)</td>
<td>90.8 HSV2</td>
<td>Human herpesvirus 3 (KU926322)</td>
<td>100</td>
<td>8e-109</td>
</tr>
<tr>
<td>7</td>
<td>Aqueous (OS)</td>
<td>27.50 (0.35)</td>
<td>81.7 VZV</td>
<td>Human herpesvirus 3 (KU926322)</td>
<td>100</td>
<td>8e-109</td>
</tr>
<tr>
<td>8</td>
<td>Aqueous (OS)</td>
<td>27.19 (0.68)</td>
<td>81.7 VZV</td>
<td>Human herpesvirus 3 (KU926322)</td>
<td>100</td>
<td>8e-109</td>
</tr>
<tr>
<td>9</td>
<td>Vitreous (OD)</td>
<td>26.86 (0.22)</td>
<td>79.1 T. gondii</td>
<td>T. gondii (KU926322)</td>
<td>100</td>
<td>5e-50</td>
</tr>
</tbody>
</table>

VW, vitreous washing; Ct, cycles threshold; Tm, melting temperature; OS, left eye; OD, right eye.

* T. gondii sequence was searched against the whole genome shotgun contigs database.

DISCUSSION

Uveitis is a sight-threatening inflammation of the uveal tract that is caused by a variety of infectious agents and noninfectious causes. Diagnosing an etiology for this disease based on clinical findings alone is often challenging owing to confounding and overlapping features. PCR-based assays have been used to discriminate infectious from noninfectious causes of intraocular inflammation and for identification of the causative agent.9,12,13,20–25 However, most of these tests are singleplex reactions performed by traditional PCR methods that rely on post-PCR handling for detection and identification, increasing turnaround time and risk of carryover contamination. The multiplex assay described here was designed to detect the most common uveitis pathogens using real-time PCR technology. It uses a single reaction that allows DNA amplification and fluorescence-based amplicon identification. Importantly, rapid detection of leading uveitis pathogens in a multiplex format was possible without compromising test sensitivity. The limits of detection of our multiplex assay were as high or higher than reported tests for herpesviruses or T. gondii detection from intraocular fluids and other body sites.26,27 By exploring the advantages of HRM analysis, we were able to develop a sensitive assay for simultaneous detection and precise identification of HSV-1, HSV-2, VZV, CMV, and T. gondii directly from intraocular fluids in less than 2 hours. This opens the door to rapid initiation of effective therapy.

Although TaqMan-based real-time PCR assays are extensively used for molecular diagnostics and have been described for the detection of herpesviruses from intraocular fluids,28 the multiplexing capability of this method is constrained by the availability of different filters to detect each fluorescent agent. Monitoring DNA amplification by the use of a dedicated DNA-irradiating fluorophore may allow the addition or replacement of targets according to specific clinical needs, as long as the new target displays a melting profile distinct enough from the existing targets included in the panel. Thus, the number or
nature of targets may be modified independently on the number of filters in the available PCR systems. SYBR GreenER is also more economical than most hydrolysis probes.

An unexplored theoretical challenge for the detection method described here would be in deconvoluting the signal that could result from amplification of two different targets in the same sample. The occurrence of multiple known uveitis pathogens in a sample is rare, and an uninterpretable result could be further analyzed in demultiplexed reactions using the same primers.

Validation of the assay on intraocular fluids collected from suspected cases of viral or toxoplastic uveitis demonstrated an overall clinical sensitivity (9 patients out of 18, 50%).

The etiology of infectious uveitis is not completely understood, and rapid positive identification of patients with known common causes will facilitate the discovery of new pathogens in others.

Most of the PCR-positive cases presented with ARN, a rapidly progressive, fulminant, necrotizing viral retinitis that mainly affects immunocompetent patients, which is more often caused by VZV than by HSV-1 and -2 and is rarely caused by CMV. The distribution of causative agents for the PCR-positive cases analyzed here broadly reflected this trend. HSV-2 and VZV were the most common pathogens identified, followed by CMV. A predominance of HSV-2 over HSV-1 in ARN cases has been reported and appears to be age dependent. Uveitis patients 25 years old or younger are more frequently infected by HSV-2, even without a clearly defined history of neonatal infection, whereas HSV-1 is more commonly found in uveitis specimens from older patients. All HSV-2-positive patient specimens (n = 3) analyzed here were from males: two young, immunocompetent adults (23 and 26 years old) and one elderly adult (80 years old). HSV-2 primary infection usually affects the genital tract and perigenital and anal skin areas. Thus, maternal transmission may occur congenitally or at the time of delivery.

The parasite T. gondii was detected in only one case in our series, from a patient initially suspected of having PORN, a disease commonly caused by herpesviruses. This atypical ocular toxoplastic presentation in an immunosuppressed patient highlights the potential clinical impact of molecular testing for optimal management of infectious uveitis.

The parasite T. gondii infection was negative by PCR. For one patient only an aqueous humor specimen was available. The other was under treatment (1 week of trimethoprim-sulfamethoxazole) for toxoplasmosis at the time of collection, which may have impaired our ability to detect toxoplasma DNA from the specimens.

Laboratory diagnosis of posterior toxoplastic uveitis is often challenging and may require the combination of PCR testing with intraocular antibody measurement (Goldmann-Witmer coefficient [GWC] analysis) as complementary tests. Multiple existing tests have been found to improve the rate of patients that have a final etiologic diagnosis of toxoplastic retinitis, and both traditional PCR and GWC are challenged by

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age</th>
<th>Sex</th>
<th>Immune Status</th>
<th>Therapy*</th>
<th>Clinical Diagnosis</th>
<th>PCR Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23</td>
<td>M</td>
<td>Immunocompetent</td>
<td>Intravenous acyclovir and antibiotics</td>
<td>ARN</td>
<td>HSV2</td>
</tr>
<tr>
<td>2</td>
<td>26</td>
<td>M</td>
<td>Immunocompetent</td>
<td>Intravenous acyclovir</td>
<td>ARN</td>
<td>HSV2</td>
</tr>
<tr>
<td>3</td>
<td>80</td>
<td>M</td>
<td>Unknown</td>
<td>Intravenous acyclovir</td>
<td>ARN</td>
<td>HSV2</td>
</tr>
<tr>
<td>4</td>
<td>61</td>
<td>M</td>
<td>Immunocompetent</td>
<td>Intravitreal foscarnet</td>
<td>ARN</td>
<td>VZV</td>
</tr>
<tr>
<td>5</td>
<td>64</td>
<td>M</td>
<td>Immunocompetent</td>
<td>Intravitreal foscarnet and valganciclovir</td>
<td>ARN</td>
<td>VZV</td>
</tr>
<tr>
<td>6</td>
<td>82</td>
<td>M</td>
<td>Immunocompetent</td>
<td>Unknown</td>
<td>ARN</td>
<td>VZV</td>
</tr>
<tr>
<td>7</td>
<td>49</td>
<td>M</td>
<td>Immunocompetence</td>
<td>Valganciclovir</td>
<td>CMV retinitis</td>
<td>CMV</td>
</tr>
<tr>
<td>8</td>
<td>76</td>
<td>M</td>
<td>Unknown</td>
<td>Unknown</td>
<td>CMV retinitis</td>
<td>CMV</td>
</tr>
<tr>
<td>9</td>
<td>44</td>
<td>M</td>
<td>Immunocompetent</td>
<td>Antiviral therapy</td>
<td>PORN</td>
<td>T. gondii</td>
</tr>
</tbody>
</table>

M, male; ARN, acute retinal necrosis; PORN, progressive outer retinal necrosis.

* Therapy the patient was receiving at the sampling time.

<table>
<thead>
<tr>
<th>No.</th>
<th>Age</th>
<th>Sex</th>
<th>Immune Status</th>
<th>Clinical Diagnosis</th>
<th>Therapy at Time of Sampling</th>
<th>Specimen (Eye)</th>
<th>PCR Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>65</td>
<td>M</td>
<td>Immunocompetent</td>
<td>Toxoplasmosis chorioretinitis</td>
<td>Trimethoprim-sulfamethoxazole</td>
<td>Vitreous (OD)</td>
<td>Negative</td>
</tr>
<tr>
<td>11</td>
<td>28</td>
<td>M</td>
<td>Immunocompetent</td>
<td>Toxoplasmosis chorioretinitis</td>
<td>None</td>
<td>Aqueous (OD)</td>
<td>Negative</td>
</tr>
<tr>
<td>12</td>
<td>84</td>
<td>F</td>
<td>Unknown</td>
<td>ARN</td>
<td>None</td>
<td>Aqueous (OD)</td>
<td>Negative</td>
</tr>
<tr>
<td>13</td>
<td>36</td>
<td>M</td>
<td>Immunocompetent</td>
<td>ARN</td>
<td>None</td>
<td>Aqueous (OD)</td>
<td>Negative</td>
</tr>
<tr>
<td>14</td>
<td>57</td>
<td>F</td>
<td>History of lung transplant</td>
<td>CMV retinitis</td>
<td>None</td>
<td>Vitreous (OS)</td>
<td>Negative</td>
</tr>
<tr>
<td>15</td>
<td>67</td>
<td>M</td>
<td>Immunocompetent</td>
<td>ARN</td>
<td>Valganciclovir</td>
<td>Vitreous (OD)</td>
<td>Negative</td>
</tr>
<tr>
<td>16</td>
<td>61</td>
<td>M</td>
<td>Immunocompetent</td>
<td>ARN</td>
<td>None</td>
<td>Vitreous (OD)</td>
<td>Negative</td>
</tr>
<tr>
<td>17</td>
<td>51</td>
<td>F</td>
<td>Immunocompressed</td>
<td>CMV retinitis</td>
<td>Valganciclovir</td>
<td>Aqueous (OS)</td>
<td>Negative</td>
</tr>
<tr>
<td>18</td>
<td>14</td>
<td>M</td>
<td>History of lymphoma</td>
<td>ARN</td>
<td>None</td>
<td>Vitreous (OD)</td>
<td>Negative</td>
</tr>
</tbody>
</table>

VW, vitreous washing; M, male; F, female; ARN, acute retinal necrosis; OS, left eye; OD, right eye.
sensitivity issues associated with the timing of specimen collection in the course of the disease. Historically, the use of PCR by itself for the diagnosis of ocular toxoplasmosis has lower or similar sensitivity to that of local antibody detection. This may account for the relatively low number of patients with uveitis found positive for T. gondii (n = 1) among our sample set.

Although we validated this assay with clinical samples positive for most of the targets included, a specimen positive for HSV-1 uveitis was not found in our population. Nevertheless, the assay developed here detects HSV-1 control DNA with limits of detection similar to the other targets, and we would predict finding such samples in a larger set.

In summary, the multiplex assay developed here provides sensitive and reliable qualitative detection and identification of the common uveitis-associated herpesviruses and T. gondii, directly from intraocular fluids in a single closed-tube reaction. Moreover, with all reactions being performed in a single tube in a single instrument in under 2 hours, results can be generated while a patient is still available. Thus, this qualitative assay may represent a useful laboratory test for identification of pathogens in clinically suspected cases of uveitis, with the potential for reducing vision lost due to delays in the onset of effective therapy.

Acknowledgments

Note: A recent report appeared describing multiplex amplification of some of the pathogens, without the high resolution melting detection and validation used here, and showed consistent findings, providing further support for this approach. The authors thank Jenna Wurster for technical support throughout the course of this work. They also thank the physicians of the Retina and Uveitis Services of the Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, MA, USA.

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