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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-years[1,2] 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 (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 toxoplasmic 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 products[15] 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;

TABLE 1. Primer Sequences Used in This Study and Characteristics of Their Respective PCR Products

Target	Name	Sequence	Gene (Locus Tag)	Product Size, bp	Product %GC	T_m Prediction*	Practical T_m
HSV-1 HSV-2	HSV1/2Fw	CATGACCAAGTGGCAGGA	Glycoprotein (UL27)	235	64 (HSV1)	91.6 (HSV1)	90.3
	HSV1/2Rv	CAGGTAGTACTGCGGCTG			66 (HSV2)	91.9 (HSV2)	90.8
CMV	CMVFw	AAGGGGAAGACGCGGTAG	Regulatory protein IE2 (UL122)	214	55	87.1	85.7
	CMVRv	ACGCGTCCTTTCAAGGTG					
VZV	VZVFw	TCCGTTCGTTTTGGCTTC	Glycoprotein B (ORF31)	223	45	83.0	81.7
	VZVRv	TTGGACTTTCCACGGGAG					
T. gondii	ToxFw	AGCGTGTTCGTCTCCATT	Glycerol-3-phosphate dehydrogenase	114	44	80.5	79.1
	ToxRv	AAGATAGGAGGGAGGCGT					

T_m, melting temperature; GC, guanine-cytosine.

* The T_m for the PCR products of each target were predicted using the melting curve prediction software uMelt.[16] Temperatures displayed represent the most frequent values from all the sequences tested.

Supplementary Table S1) were scrutinized for coding stretches 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.[16] Predictions were made based on thermodynamic parameters described by Blake and Delcourt,[17] 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 99°C. The targeted regions were selected for predicted melting temperature (T_m) values differing by at least ±2°C for each organism and the greatest value for the negative derivate of helicity with respect to temperature (−d[Helicity]/d[Temp]). 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 T_m. Primers were further optimized to minimize potential for primer-dimer and hairpin formation using AutoDimer.[18]

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 T_m 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 uL 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 afterward by heating samples from 70°C to 99°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.[19]

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 (2×10^4 copies per reaction). The variation in the T_m 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 pallidum*.

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 1×10^5 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
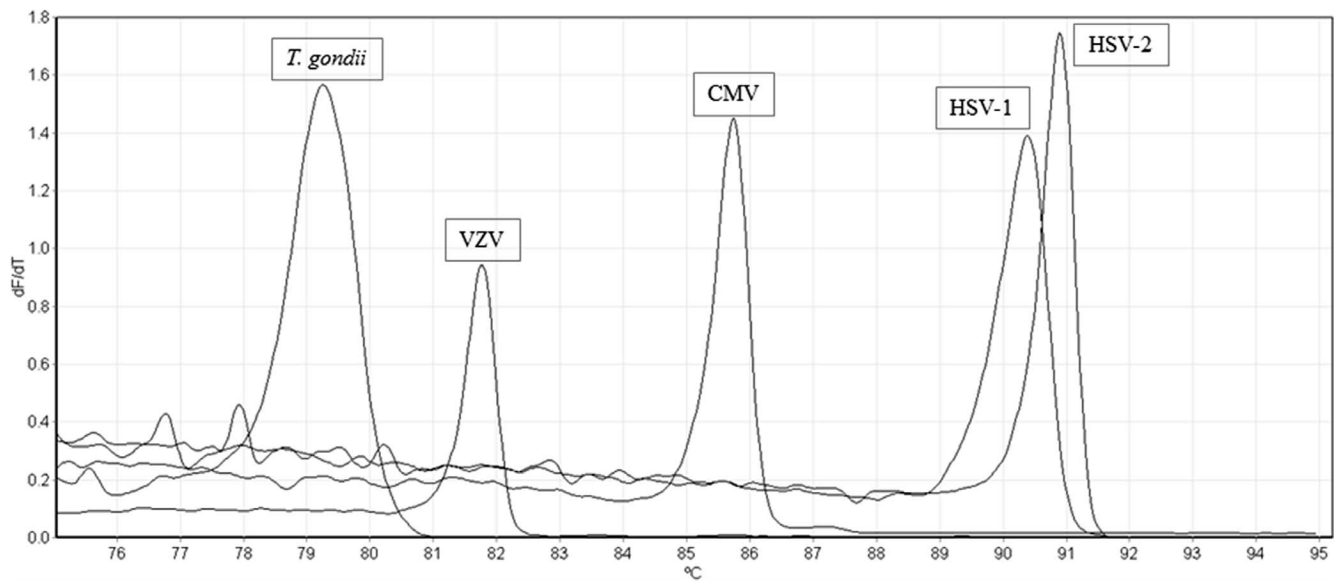
FIGURE 1. Melting curve profiles for each target included in the assay following HRM analysis using SYBR GreenER. T_m values corresponding to each peak are 79.1°C for *T. gondii*, 81.7°C for VZV, 85.7°C for CMV, 90.3°C for HSV-1, and 90.8°C for HSV-2.

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 (Supple-

mentary Fig. S1) and then by DNA sequence. Experimentally determined T_m values for each target were 79.1°C for *T. gondii*, 81.7°C for VZV, 85.7°C for CMV, 90.3°C for HSV-1, and 90.8°C for HSV-2 (Fig. 1; Table 1), in good agreement with predicted values (consistently ~1°C higher than experimentally determined for all targets). After confirming amplification of correct products with distinct melting patterns, a single-tube multiplex assay was optimized by combining all primer pairs in concentrations ranging from 100 to 700 nM. Using purified control DNA, primer concentrations of 100 nM for HSV1/2, CMV, *T. gondii*, and 200 nM for VZV were found to be optimum for detection of fluorescent signal over noise. In the multiplex reaction, melting curve and T_m values were identical to those determined in individual reactions. Conducted in this manner, 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 1×10^5 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
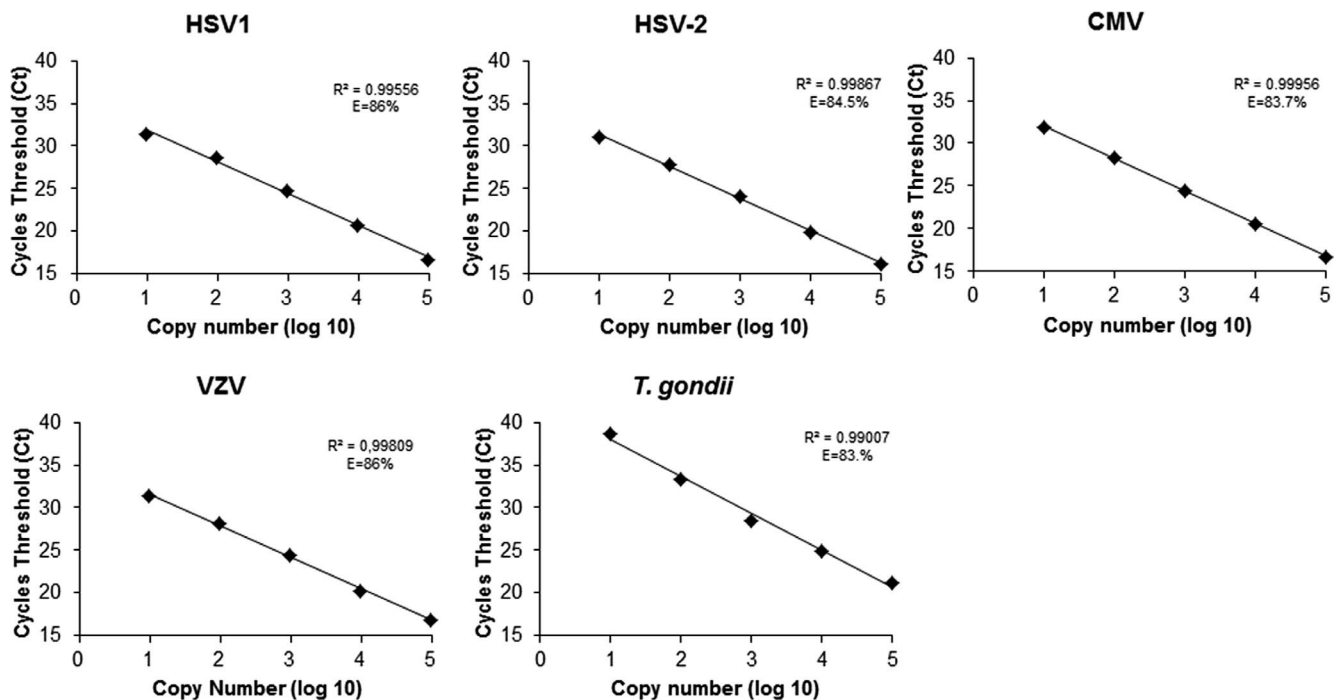
FIGURE 2. Standard curves obtained by testing serial 10-log dilutions ranging from 2×10^5 copies to 2 copies per reaction. Calculations of efficiency (E) for each reaction are shown. Efficiency was calculated from the slope given by regression analysis using the formula $E = 10(-1/-\text{slope}) - 1$. Coefficients of correlation (R^2) are also shown.

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 $\times 10^4$ 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 ≤4% for all the targets and concentrations, and interassay variation was ≤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 53 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

TABLE 2. Characteristics of Patients ($n = 18$) With Clinical Diagnosis of Infectious Uveitis Included in the Validation of the Multiplex Assay

Sex	
Male (%)	15 (83.3)
Female (%)	3 (16.7)
Age, y	
Mean (StDev)	53 (±21)
Range	14–84
Immune status	
Immunocompetent (%)	10 (55.5)
Immunosuppressed (%)	3 (16.7)
Others/unknown (%)	5 (27.8)
Initial clinical diagnosis	
ARN (%)	11 (61.1)
CMV retinitis (%)	4 (22.2)
Toxoplasmosis chorioretinitis (%)	2 (11.1)
PORN (%)	1 (5.6)

PORN, progressive outer retinal necrosis.

PCR results. From the two patients with symptoms consistent with inflammation of toxoplasmic etiology, one had a single aqueous humor sample collected and the other had already been treated with trimethroprim-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.[13] 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 intraocular fluids from noninfected eyes ($n = 21$) and bacterial and fungal endophthalmitis cases that were either positive ($n = 30$) or negative by culture ($n = 27$). Eight yielded a Ct value that crossed the threshold for positivity, but only one generated a well-defined T_m peak (86.7°C), which was not close (within 0.5°C) to any of the pathogens for which the test was designed. Sequencing of this sample resulted in a mixed trace file that did not give an interpretable sequence, suggesting it was a mixture of yet unknown amplification products. As this atypical T_m value fell outside the values used for pathogen identification or predicted for any known variants, it was dismissed as an artifact of amplification. These artifacts were not seen when the samples were tested individually using each primer in singleplex reactions, excluding the possibility of polymicrobial infection. None of the control samples yielded a positive result for any of the uveitis pathogens included in our assay (Supplementary Table S2).

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-intercalating fluorophore may permit 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

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

Patient No.	Specimen (Eye)	Multiplex PCR Assay			Sequencing Identification				
		Mean Ct Value (StDev)	T_m Value	T_m Identity	Best Hit (Accession Numbers)	Identity, %	Query Coverage, %	E-Value	Product Size, bp
1	Vitreous (OS)	27.68 (0.12)	90.8	HSV2	Human herpesvirus 2 (KU310665)	99	100	1e-112	235
2	Vitreous (OS)	19.44 (0.57)	90.8	HSV2	Human herpesvirus 2 (KU310666)	100	99	9e-115	235
	VW (OS)	20.11 (0.19)	90.8	HSV2	Human herpesvirus 2 (KU310666)	100	100	3e-115	235
3	Aqueous (OD)	24.27 (018)	90.8	HSV2	Human herpesvirus 2 (KU310665)	100	100	3e-115	235
	Aqueous (OS)	26.76 (0.08)	90.8	HSV2	Human herpesvirus 2 (KU310665)	100	100	3e-114	233
4	Vitreous (OD)	24.35 (0.50)	81.7	VZV	Human herpesvirus 3 (KU926322)	100	100	8e-109	223
	VW (OD)	26.2 (0.71)	81.7	VZV	Human herpesvirus 3 (KU926322)	100	100	8e-109	223
5	Vitreous (OS)	27.50 (0.33)	81.7	VZV	Human herpesvirus 3 (KX262866)	100	100	8e-109	223
	VW (OS)	27.19 (0.68)	81.7	VZV	Human herpesvirus 3 (KX262866)	100	100	8e-109	223
6	Aqueous (OD)	23.20 (0.15)	81.7	VZV	Human herpesvirus 3 (KX262866)	100	99	4e-107	222
7	VW (OS)	27.37 (0.29)	85.7	CMV	Human herpesvirus 5 (AH002793)	100	100	6e-104	214
8	Aqueous (OD)	22.38 (0.23)	85.7	CMV	Human herpesvirus 5 (AH002793)	100	100	6e-104	214
9	Vitreous (OD)	26.86 (0.22)	79.1	*T. gondii*	*T. gondii* (LN714499)*	100	100	5e-50	114

VW, vitreous washing; Ct, cycles threshold; T_m, melting temperature; OS, left eye; OD, right eye.
* *T. gondii* sequence was searched against the whole genome shot gun contigs database.

TABLE 4. Characteristics of the Nine Patients With Clinical Diagnosis of Infectious Uveitis and Positive PCR Results

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

M, male; ARN, acute retinal necrosis; PORN, progressive outer retinal necrosis.
* Therapy the patient was receiving at the sampling time.

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,[9] 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 toxoplasmic uveitis demonstrated an overall clinical sensitivity (9 patients out of 18, 50%), comparable to that of previous studies.[5,9,12] 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.[5,7,29] 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[29,30] 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.[31] It has been hypothesized that the enrichment of HSV-2 retinitis in young patients without history of neonatal infection could be associated to the reactivation of a well-established latent infection in the cranial nerve (with subsequent spreading to the retina) that was perinatally acquired, and not previously diagnosed.[32]

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 toxoplasmosis presentation in an immunosuppressed patient highlights the potential clinical impact of molecular testing for optimal management of infectious uveitis. Two other patients presenting with symptoms consistent with *T. gondii* infection were 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 toxoplasmic 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 toxoplasmic retinitis, and both traditional PCR and GWC are challenged by

TABLE 5. Characteristics of the Nine Patients With Clinical Diagnosis of Infectious Uveitis and Negative PCR Results

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

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.[22,33–35] 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.

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