Validation of a Diagnostic Multiplex Polymerase Chain Reaction Assay for Infectious Posterior Uveitis

Humeyra Dabil, MD; Michelle L. Boley, BS; Therese M. Schmitz, BS; Russell N. Van Gelder, MD, PhD

Objective: To validate a multiplex polymerase chain reaction (PCR) assay capable of simultaneously screening vitreous biopsy specimens for a panel of common pathogens in posterior uveitis.

Methods: A multiplex PCR assay using novel primer sets for cytomegalovirus (CMV), herpes simplex virus (HSV), varicella zoster virus (VZV), and Toxoplasma gondii was developed. The sensitivity of the assay was determined for purified pathogen DNA. Twenty-one vitreous specimens from patients with posterior uveitis were tested by both multiplex and monoplex PCR.

Results: Fewer than 10 genomes of VZV and fewer than 100 genomes of HSV, CMV, and T. gondii could be detected using the new primer sets. When used in multiplex, the assay lost less than 1 log of sensitivity. Monoplex PCR detected pathogen DNA in 18 of 21 patient samples; multiplex PCR detected pathogen DNA in 15 of the 18 samples positive by monoplex PCR. None of 10 negative control samples were positive for pathogen DNA.

Conclusions: Multiplex PCR has adequate sensitivity to simultaneously screen a substantial differential diagnosis for posterior uveitis in a single reaction, without loss of specificity. This assay may reduce the time and cost involved in PCR-based molecular diagnostics of infectious pathogens.

Clinical Relevance: Multiplex PCR may allow rapid diagnosis of infectious posterior uveitis.

Arch Ophthalmol. 2001;119:1315-1322

The most common identifiable causes of posterior uveitis are infectious. In immunocompetent patients, Toxoplasma gondii is the most common infectious cause of posterior uveitis,1,2 while in patients with acquired immunodeficiency syndrome, cytomegalovirus (CMV) is the major cause of retinitis.3 Other relatively common causes of posterior uveitis are infectious as well. Varicella zoster virus (VZV) and herpes simplex virus (HSV) have both been implicated as causative agents in acute retinal necrosis syndrome and progressive outer retinal necrosis. Both of these diseases are associated with poor visual prognosis.4,5

Prompt diagnosis of posterior uveitis is vital for early and proper treatment. Treatment regimens for acute retinal necrosis syndrome, CMV retinitis, and Toxoplasma retinochoroiditis have minimal overlap, and the appropriate regimen can be initiated only once the correct diagnosis has been made. The diagnosis of infectious posterior uveitis is usually based on clinical presentation and appearance. However, in a subset of patients, media opacity or atypical appearance can necessitate additional testing to support a diagnosis. Historically, dilemmas in posterior uveitis have been analyzed by means of local antibody production (ie, the Witmer coefficient of normalized intraocular to serum antibody titers)6 or direct viral cultures.7-10 Although local antibody production has utility for determining a cause of some cases of posterior uveitis, including toxoplasmosis and acute retinal necrosis syndrome,11 it is not useful for others, such as CMV retinitis. Viral cultures from the eye have poor recovery, and some organisms (such as T. gondii) are not readily cultured. In recent years, the polymerase chain reaction (PCR) has been used in the diagnosis of posterior uveitis. The PCR can directly detect RNA or DNA of the causative microorganisms, with sensitivity and specificity often greater than that of culture. Polymerase chain reaction assays have been developed for CMV, HSV, VZV, and T. gondii, and have shown clear utility in...
MATERIALS AND METHODS

PRIMER DESIGN

In designing a novel multiplex PCR, we reasoned that compatible primers would share similar annealing characteristics, sequence complexity of the amplicon, and size of the amplicon. Previous investigators have shown that use of short PCR amplicons leads to more rapid and specific amplification. We designed a protocol for performing short tandem amplification of multiple pathogens (STAMP), with the goal of producing primer sets for individual pathogens that could be combined to function in multiplex reactions. To find such primers, we designed a short computer program to scan the genomes of VZV, HSV-1 and HSV-2, CMV, and T gondii, and selected primers found in coding regions (with the presumption that these sequences would be more likely to be conserved than noncoding DNA). These are referred to as the STAMP primers (Table 1).

The individual primers were designed such that each would be 20 base pairs (bp) long; would be 60% rich in guanine (G) and cytosine (C) (G+C); would produce approximately 100-300-bp-long amplicons, with a total G+C content of 50% and no stretch of 20 bp with greater than 70% G+C; and would not cause primer dimers and would not amplify homologous genomic sequences in human chromosomal DNA or other common pathogens. Results were confirmed by nested PCR of the multiplex product with internal primers. The nested primers were 20 bp long; approximately 50% G+C content; and at least 10 bp internal to the outside primers. All primers were designed by means of sequence databases at the National Center for Biotechnological Information with the Basic Local Alignment Search Tool family of programs. All primers were synthesized in 30-nmol quantities by IDT, Inc (Des Moines, Iowa). Primer sequences and their locations within the target genes are shown in Table 1 and Table 2.

PCR CONDITIONS

Purified pathogen DNA for CMV, HSV-1, T gondii, and VZV (Advanced Biotechnologies Inc, Columbia, Md) were used to optimize the sensitivity of each monoplex PCR reaction. The individual monoplex and multiplex PCR cycling conditions were extensively optimized for denaturation, annealing, and extension temperatures; magnesium chloride concentration; number of cycles; and concentration of primers for each pathogen.

For each monoplex reaction, 5 µL of sample (either purified pathogen DNA or patient vitreous sample) was combined with 5 µL of 10× PCR buffer (500mM potassium chloride; 100mM Tris hydrochloride [pH 9.0, at 25°C]; 1.0% Triton X-100); 5 µL of 25mM magnesium chloride; 1 µL of 0.2mM each dinucleotide triphosphates (dNTP), 5 pmol of each primer of HSV, T gondii, and VZV or 10 pmol of each CMV primer; and 0.25 U of recombinant Taq DNA polymerase (Promega, Madison, Wis) in a total volume of 50 µL. For multiplex PCR, 5 µL of DNA was combined with the same concentrations of 10× PCR buffer, magnesium chloride, dNTP, and recombinant Taq DNA polymerase as for monoplex. The same concentrations of all HSV, CMV, and T gondii primers were equally or more sensitive than existing primer sets. Of note, the STAMP VZV primer was as sensitive in monoplex as the conventional primer set. No comparison measurements for T gondii were performed, as the STAMP T gondii PCR is based on the highly repetitive BI gene and is similar to primer sets already in use.

RESULTS

MONOPLEX PCR SENSITIVITY OF STAMP PRIMERS

The sensitivities of the STAMP primers were tested in monoplex PCR (ie, with only 1 primer pair per reaction) against serial dilutions of purified pathogen DNA. As shown in Figure 1 (left), the sensitivities of monoplex STAMP PCR were approximately 10 genomic copies for VZV, CMV, and T gondii (Figure 2C, left), and approximately 100 virus genomes for HSV. Since viral DNA amounts were calculated by the manufacturer on the basis of the number of virions used in the DNA preparation (not accounting for recovery of the DNA), these sensitivities should be considered relative, not absolute. To determine whether these sensitivities were comparable to those achieved by current PCR diagnostic techniques, the same pathogen DNA dilutions were tested with published primers from other laboratories (Figure 1, right, and Table 3). When used in monoplex, STAMP primers were equally or more sensitive than existing primer sets. Of note, the STAMP T gondii primer was as sensitive in monoplex as the conventionally used nested PCR primer set. No comparison measurements for T gondii were performed, as the STAMP T gondii PCR is based on the highly repetitive BI gene and is similar to primer sets already in use.

MULTIPLEX PCR SENSITIVITY OF STAMP PRIMERS

We next sought to determine the sensitivity of STAMP primers when all primers were combined in a multiplex PCR reaction. Reaction conditions were identical to those for monoplex PCR, except that all primer sets were included in a single reaction. No false-positive bands were produced when multiplex PCR was performed on purified pathogen DNA. Sensitivity comparisons of monoplex and multiplex PCR are shown in Figure 2. Multiplex PCR for VZV was as sensitive as the monoplex PCR on the purified VZV DNA. The sensitivities for CMV, HSV, and T gondii decreased less than 1 log unit.

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VZV, and *T. gondii* primers were then added in combination in a total volume of 50 µL. Samples were amplified in 200-µL thin-walled tubes in an automated thermocycler with heated lid (RoboCycler Gradient 96; Stratagene, La Jolla, Calif). Cycling conditions were as follows: an initial 3-minute denaturation at 94°C followed by 35 cycles of 30-second denaturation at 94°C, 30-second annealing at 52°C, and 30-second extension at 72°C.

The specificity of positive multiplex PCR results was confirmed by dividing the primary PCR product and performing individual confirmatory PCR with individual nested primer sets. Nested primers are shown in Table 2. One microliter of a 1:100 dilution of positive multiplex PCR product was combined with 5 µL of 10× PCR buffer (composition as above); 5 µL of 25 mM magnesium chloride; 1 µL of 0.2 mM each dNTP; 5 pmol of each nested primer of HSV, *T. gondii*, or VZV; or 10 pmol of each CMV primer; and 0.25 U of recombinant Taq DNA polymerase in a total volume of 50 µL. Reaction conditions were the same as for the monoplex PCR. All nested reactions could be performed simultaneously on the gradient thermal cycler.

Monoplex sensitivities were compared with published protocols.13,17,18 Primer sequences are shown in Table 3. For HSV detection, an initial denaturation at 94°C for 10 minutes was followed by 35 cycles of denaturation at 95°C for 45 seconds, annealing at 64°C for 45 seconds, and extension at 72°C for 45 seconds. For VZV detection, after an initial denaturation at 94°C for 3 minutes, 35 PCR cycles of denaturation at 94°C for 30 seconds, annealing at 44°C for 30 seconds, and extension at 72°C for 40 seconds were performed. The VZV was detected by means of nested PCR, with an initial denaturation at 94°C for 3 minutes and 7 cycles at 94°C for 30 seconds and at 72°C for 40 seconds. The CMV was amplified with an initial 3-minute denaturation at 94°C, and 35 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 30 seconds.

All amplified DNA was detected by agarose gel electrophoresis on 2% gels stained with ethidium bromide.

**VITREOUS SAMPLES**

Anonymous primary vitrectomy specimens were obtained from our own and other practices. We obtained 16 vitreous specimens from cases of posterior uveitis that had previously been shown by other laboratories to contain viral DNA.12 Three vitreous specimens from patients with active toxoplasmosis and 2 vitreous specimens from patients with clinical acute retinal necrosis or progressive outer retinal necrosis syndrome were obtained at local institutions at the time of vitrectomy. Ten negative control vitreous samples were obtained at the time of vitrectomy from patients undergoing macular hole repair (4 patients), retinal detachment repair (3 patients), clearance of diabetic vitreous hemorrhage (2 patients), or submacular surgery for neovascular complex secondary to age-related macular degeneration (1 patient). Vitreous samples were immediately frozen at the time of acquisition and stored at −20°C or lower until assay. Vitreous specimens were thawed at room temperature, and PCR inhibitors were eliminated by boiling the samples for 15 minutes before assay.12

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**VALIDATION OF MULTIPLEX STAMP WITH VITREOUS BIOPSY SPECIMENS**

We tested the STAMP monoplex PCR assay on 16 vitreous samples collected from cases of posterior uveitis previously shown to contain viral DNA by means of established primer sets. Frozen vitreous samples for CMV (10 cases), HSV (3 cases), and VZV (3 cases) were used. The monoplex STAMP PCR assay yielded CMV in 8 of 10 CMV samples, VZV in 3 of 3 VZV samples, and HSV in 2 of 3 HSV samples (Table 4 and Figure 3).

Single, positive bands were produced after multiplex STAMP PCR of these same samples in 5 of 8 CMV cases, 3 of 3 VZV cases, and 2 of 2 HSV cases. Several methods for confirmation of positive multiplex bands were attempted, including Southern hybridization of specific probes to immobilized multiplex PCR products, reverse Southern hybridization of labeled multiplex PCR product to immobilized specific probes, and nested PCR of multiplex products for individual pathogens. We found the last technique to be most rapid (approximately 30 minutes) and specific. Multiplex PCR products were diluted by 1:100, split into individual reactions, and amplified with each nested primer set. All samples that were positive by the multiplex PCR were specifically positive with the nested primer set for the appropriate pathogen. In each case, the detected pathogen agreed with the pathogen detected by monoplex PCR (Figure 4). One of the 10 tested vitreous samples produced 2 positive bands on nested PCR, for VZV and for *T. gondii*. As only VZV was detected by monoplex PCR, it is possible that the *T. gondii* signal either was a contaminating false-positive finding or represented a very rare commensal organism. Although the patient sample was initially classified as VZV, no clinical data were available to determine which diagnosis was more consistent with the clinical presentation.

To determine specificity of multiplex PCR for posterior uveitis, vitreous samples from 10 patients undergoing vitrectomy for nonuveitic conditions were analyzed using multiplex and nested confirmatory PCR. None of the 10 samples produced visible products when tested with either multiplex PCR or on nested PCR of the multiplex products (data not shown). Positive control reactions run simultaneously showed sensitivities of at least 100 genomes for all pathogens tested, and less than 10 genomes for all pathogens following nested confirmatory testing.

Five additional patient samples from patients with active posterior uveitis were tested by multiplex PCR. These included 2 vitreous samples from patients with a clinical diagnosis of acute retinal necrosis syndrome or progressive outer retinal necrosis syndrome and 3 vitreous samples from patients with a clinical diagnosis of ocular toxoplasmosis. For the patients with herpetic retinitis, one of the samples was found to be positive for HSV and the other was positive for VZV with the monoplex PCR with the use of either STAMP or conventional primers. Although no band was seen after multiplex PCR with either sample, af-
ter the nested PCR of the (invisible) multiplex product, these vitreous samples were positive for HSV or VZV, respectively. One of the 3 vitreous samples from patients with presumed ocular toxoplasmosis was positive for \textit{T. gondii} by means of monoplex PCR. The nested PCR was positive for \textit{T. gondii} on all 3 samples (Figure 5).

The PCR is a powerful technique for detecting pathogen DNA or RNA as an indication of infection.\cite{12,19,20} It is rapid, taking only a few hours to complete, and requires only a

**COMMENT**

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*STAMP indicates short tandem amplification of multiple pathogens; bp, base pairs; CMV, cytomegalovirus; HSV, herpes simplex virus; VZV, varicella zoster virus; HHV, human herpesvirus; and ORF, open reading frame.

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few microliters of sample volume. The technique is extremely sensitive and specific; as we demonstrated in this study, sensitivities on the order of 10 to 100 pathogen DNA molecules can be routinely obtained. Knox et al.12 demonstrated the utility of PCR in establishing a diagnosis in cases of posterior uveitis that presented as a diagnostic dilemma because of media opacity, atypical appearance, or atypical response to treatment. This group detected a specific virus by PCR in 24 (65%) of 37 cases examined. The PCR diagnosis was consistent with the ultimate clinical course in each case. Knox et al did not perform PCR for toxoplasmosis in this study; as several of the cases in their study followed a clinical course consistent with \textit{T. gondii} infection, the yield of PCR diagnoses would likely have been even higher had this group been able to perform PCR for the parasite. Mitchell et al.21 similarly tested vitreous from 50 patients with retinitis and acquired immunodeficiency syndrome, determining a diagnosis in 47. Their PCR-based assays were similarly consistent with the clinical courses of these patients.

Although PCR is a rapid and useful diagnostic technique for detection of common posterior uveitis pathogens, it has not yet achieved widespread or routine use. Obstacles to the routine use of PCR include the relatively small number of laboratories performing the technique, the lack of standardization among laboratories, the lack of clinical experience in interpretation of results (particularly negative results), and the expense and time involved in performing this technique. One of the major hurdles in the performance of diagnostic PCR is the necessity of testing for individual pathogens serially. Because protocols for individual pathogens have been derived in independent laboratories, optimal buffer and cycling conditions rarely allow for simultaneous performance of assays. Serial testing becomes expensive and time-consuming for a large differential diagnosis. Sample may also become limiting if a large number of PCR reactions need to be performed. The multiplex PCR was initially described in 1988.22 This technique involves detecting multiple targets simultaneously, in a single reaction. Multiplex PCR has had limited application to ocular or systemic infectious diseases.15,16 For ocular disease, Jackson et al.16 reported a multiplex and degenerate PCR for the detection of adenovirus (subgenera B, C, D) and HSV from conjunctival swabs. This group was able to detect adenoviral DNA in 5 of 6 specimens culture-positive for adenovirus and HSV DNA in 5 of 6 specimens culture-positive for HSV.

Further efforts to apply multiplex PCR to diagnostic applications have been limited by declining sensitivity and
specificity with increasing complexity in the primer mix. As the number of primer pairs increases, the complexity of the DNA in the annealing reactions also increases, which decreases sensitivity. The likelihood of primer-dimer pairs also increases, causing nonspecific amplification and decreasing specificity. In designing a multiplex PCR for posterior uveitis, we sought to establish a generalizable method for generating primer sets and reaction conditions that would provide high sensitivity and ready compatibility with other primer sets. To that end, we wrote a short computer searching algorithm to find compatible amplicons.

Figure 3. Monoplex detection of frozen patient samples with STAMP primers. (STAMP indicates short tandem amplification of multiple pathogens.) First lanes are the 100–base pair molecular size markers followed by 10^3 to 10^5 dilutions of the positive controls, 10 cytomegalovirus-positive samples (A) (eighth patient who was cytomegalovirus positive with the short tandem amplification of multiple pathogens is not shown), 3 herpes simplex virus–positive samples (B), and 3 varicella zoster virus–positive samples (C). L indicates DNA marker ladder. The last lane in each row is the no-DNA negative control.

Figure 4. Nested polymerase chain reaction (PCR) on patient samples with each nested primer set (nested primer sets: A, cytomegalovirus [CMV]; B, herpes simplex virus [HSV]; C, varicella zoster virus [VZV]). First lanes are the 100–base pair molecular size marker followed by 10-fold serial dilutions of a 1:100 dilution of “first-round” PCR product generated from purified pathogen DNA (extracted from 10^3 to 10^5 pathogens). Row A is CMV-positive controls; row B, HSV-positive controls; and row C, VZV-positive controls. A 1:100 dilution of each positive PCR product from Figure 3 was tested with each nested primer pair. A fifth CMV-positive sample was also observed (not shown). All nested PCR products were also tested with Toxoplasma gondii primers; one VZV-positive sample was also positive for T gondii (not shown). L indicates DNA marker ladder. The last lane in each row is the negative control.

Each primer was to have a fixed length (20 bp), have a fixed (G+C)/(A+T) composition (60%), and flank a small intervening sequence (100–250 bp) with comparable (G+C)/(A+T) composition. We were able to find such sequences in all of the viral and protozoal genomes searched. Final choice of primer sequences was directed by location in the genome; coding sequences were favored over noncoding sequences. Genes with known mutational hotspots (ie, UL97 of CMV) were avoided. Using these criteria, we developed new primer sets for each of the major pathogens of posterior uveitis. When used in monoplex, these primer sets showed sensitivity equal to or greater than that of established primer conditions, averaging between 10 and 100 genomes in sensitivity. Reliable detection of less than 10 genomes by any technique is problematic, as serial viral DNA dilutions become subject to Poisson distribution variability. The primers were compatible in multiplex. Sensitivity of the multiplex assay was approximately 5- to 10-fold lower than that for monoplex. This is probably because of the greater total complexity of nucleic acids introduced by the additional primer sequences. It is possible that further optimization of the multiplex assay could yield improved sensitivity. For samples with limited volume, however, there is minimal loss of sensitivity for the multiplex assay when compared with monoplex. Given a 5-µL sample, for example, one could split the sample into four 1.25-µL samples and test each individually, or test the 5 µL in a single multiplex reaction. Because of the 4-fold dilution of sample in the multiplex reactions, the final sensitivities of each technique would be nearly equivalent. Although limiting sample volume is rarely limiting for vitreous or aqueous biopsy, it may be limiting for conjunctival swabs or fine-needle aspiration applications of PCR diagnostics.

Our primer sets were able to detect the appropriate pathogen DNA in 10 of 13 frozen archival samples and...
5 of 5 freshly obtained samples. Most of the detection failures were from the CMV group. Several possibilities exist for the low recovery on these samples. Clinical isolates of CMV have been reported to exhibit a large degree of genomic polymorphism. For this reason, McCann and associates used 2 different primer sets for CMV to test their samples. Our initial attempt at STAMP primers for CMV showed a sensitivity equal to that of existing primer sets on reference strain AD169, but was unable to detect CMV from a number of patient samples detectable by conventional primer sets. These primers may have been from a polymorphic site and thus did not amplify any product in the PCR-negative CMV samples. We did not have enough vitreous sample in these cases to repeat the PCR using established primers. It is also possible that the DNA specimens from the outside laboratory had undergone DNA degradation, as these samples had been frozen and thawed multiple times and were in some cases many years old (Todd Margolis, MD, PhD, oral communication, April 27, 2000).

The STAMP technique yielded a single apparent false-positive result, as 1 patient with a presumptive diagnosis of VZV-caused retinitis was also positive for T gondii. We believe this represents detection of actual pathogen DNA, as none of 10 tested nonuveitic samples were positive for any pathogen. Although early PCR assays for T gondii had relatively low sensitivity (approximately 30%), more recent primer sets using highly repetitive T gondii genes such as the B1 gene have yielded sensitivities from vitreous samples in the 60% to 70% range. Recent primer sets have had sensitivity approaching 1 tachyzoite. The prevalence of anti–T gondii antibodies in healthy adults in the United States is 40% or even higher, and T gondii cysts have been isolated from clinically normal-appearing retinal sites. Our false-positive result may represent a remote latent ocular T gondii infection, or it may have resulted from parasitemia from a nonocular site due to breakdown of the blood vitreous barrier, caused by the ocular VZV infection. In our use of PCR we have encountered several other apparent false-positive results for herpesvirus families. In particular, in cases with dense vitritis, a weak CMV-positive signal can be seen that may represent episomal virus latent in white blood cells. Short et al similarly found false-positive results for VZV when their nested PCR assay was fully optimized, possibly because of detection of rare copies of latent virus (perhaps even in the corneal and scleral nerves sampled during specimen acquisition). Performing a dilution series of positive control samples may help distinguish commensal from pathogenic infection for some infectious uveitides. In the present series of patients, for example, all patients with VZV-caused disease had semiquantitative PCR signals comparable to approximately 1000 viral genomes per 5 µL of vitreous. Commensal or carryover contamination would be expected to have lower viral loads.

Although the STAMP multiplex PCR technique is presently useful for diagnosis of posterior uveitis, the true utility of the technique will likely emerge as the differential diagnosis for PCR-detectable organisms grows. The PCR detection of less common causes of posterior uveitis, including Lyme disease, syphilis, mycobacte-ria, lymphoma, and even Whipple bacillus, has now been reported. Serial examination for all of these diagnoses would tax available sample volumes and would likely be prohibitive in terms of time and expense. Suites of STAMP primers to evaluate classes of infectious posterior uveitis could be synthesized. Similarly, PCR-based diagnosis of infectious endophthalmitis requires the ability to detect any of a large number of potentially causative microorganisms. Although the use of “universal” ribosomal DNA primers allows detection of the presence of bacteria, actual diagnosis requires precise typing. This is presently performed by sequencing, hybridizing, or restriction digest fingerprinting the universal ribosomal DNA product, but it could be performed with greater speed and high specificity through a STAMP-based multiplex PCR with the use of nested primer sets. Other future uses of multiplex PCR include the rapid diagnosis of infectious conjunctivitis (where multiple strain types of pathogens make serial PCR difficult), diagnosis of delayed-onset endophthalmitis, and multiplex strain typing of pathogens with variable antibiotic responses, such as CMV and T gondii.

Accepted for publication March 8, 2001.

Dr Van Gelder is supported by the Research to Prevent Blindness Career Development Award (New York, NY), the Becker Clinician-Scientist Award of the Horncrest Foundation (Ossining, NY), and the Thomas D. and Ruth Byers Heed Fellowship (Cleveland, Ohio). This work was supported by an unrestricted departmental grant from Research to Prevent Blindness, and a departmental core grant from the National Eye Institute, Bethesda, Md.

We thank Todd P. Margolis, MD, PhD, and Daniel F. Martin, MD, for kindly providing the reference vitreous samples for this study; Travis Meredith, MD, Henry Kaplan, MD, and Levent Akduman, MD, for providing additional patient samples; and Henry Kaplan, MD, and Michael Kass, MD, for support and encouragement.

Corresponding author and reprints: Russell N. Van Gelder, MD, PhD, Department of Ophthalmology and Visual Sciences, Washington University Medical School, Campus Box 8096, 660 S Euclid Ave, St Louis, MO 63110-1093 (e-mail: VanGelder@vision.wustl.edu).

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