AMERICAN ACADEMY OF OPHTHALMOLOGY
The Eye M.D. Association

Detection of *Mycobacterium tuberculosis* Genome in Vitreous Fluid of Eyes with Multifocal Serpiginoid Choroiditis

Reema Bansal, MS,[1,*] *Kusum Sharma, MD,*[2,*] *Amod Gupta, MS,*[1] *Aman Sharma, MD,*[3] *Mini P. Singh, MD,*[4] *Vishali Gupta, MS,*[1] *Samyak Mulkutkar, MS,*[1] *Mohit Dogra, MS,*[1] *Mangat R. Dogra, MS,*[1] *Shivali Kamal, PhD,*[1] *Surya Parkash Sharma, MSc,*[1] *Paul D. Fiorella, PhD*[5]

Purpose: To compare 3 different molecular techniques to detect the *Mycobacterium tuberculosis* genome in vitreous fluid of eyes with multifocal serpiginoid choroiditis (MSC).

Design: Prospective, interventional case series.

Participants: Eleven patients (11 eyes) with active MSC in at least 1 eye underwent diagnostic pars plana vitrectomy (PPV) between October 2012 and December 2013.

Methods: Vitreous fluid samples were subjected to multitargeted polymerase chain reaction (PCR) for a *M. tuberculosis* assay, the Gene Xpert MTB/RIF assay (Cepheid, Sunnyvale, CA), and a line probe assay (GenoType MTBDRplus; Hain Lifescience, GmbH, Nehren, Germany). The samples with positive results were subjected to *rpoB* gene sequencing to demonstrate rifampicin resistance. The clinical details, digital fundus imaging, and treatment details and outcomes also were noted.

Main Outcome Measures: Detection of the *M. tuberculosis* genome and rifampicin resistance in the vitreous samples.

Results: Of the 11 eyes subjected to PPV, the multitargeted PCR results for tuberculosis were positive for 10 eyes, the MTBDRplus assay results were positive in 6 eyes, and the Gene Xpert MTB/RIF assay results were positive in 4 eyes. Rifampicin resistance was detected in 3 eyes by *rpoB* gene sequencing, in 3 eyes by the MTBDRplus assay, and in 1 eye by the Gene Xpert MTB/RIF assay.

Conclusions: We detected the *M. tuberculosis* genome in the vitreous fluid of eyes with MSC using 3 different molecular techniques. Rifampicin resistance was detected for the first time in eyes with MSC. *Ophthalmology 2015;■:1−11 © 2015 by the American Academy of Ophthalmology.*

Multifocal serpiginoid choroiditis (MSC) or serpiginous-like choroiditis is presumed to be tubercular in origin in tuberculosis-endemic areas.[1−3] The diagnosis of MSC is essentially clinical, based on a characteristic morphologic presentation. It bears significant etiologic, demographic, clinical, therapeutic, and prognostic differences from classic serpiginous choroiditis.[4−7] Although several infectious or noninfectious causes of MSC have been recognized,[8] laboratory evidence for *Mycobacterium tuberculosis* or other causative agents is lacking. There are isolated reports of patients with serpiginous choroiditis with evidence of *M. tuberculosis*,[2,9,10] herpes,[11] or toxoplasmosis[12] that has been shown using molecular techniques such as polymerase chain reaction (PCR).

In tubercular MSC, 4-drug antitubercular therapy (ATT) in combination with corticosteroids prevents recurrences of inflammation.[1−3] However, poor response in some patients, despite the conventional 4-drug therapy, suggests possible drug resistance.[13]

The role of diagnostic pars plana vitrectomy (PPV) in various uveitic entities is well known[14,15]; however, it has not been described in MSC. The intraocular specimen used for PCR in serpiginous choroiditis in previous reports involved the aqueous humor[2,11,12] more often than the vitreous humor.[2,10] Although the vitreous has inflammatory cells in most eyes with tubercular MSC,[1,4] the anterior chamber has minimal or no inflammation.[1] We performed diagnostic PPV in MSC to detect the *M. tuberculosis* genome and drug resistance.

Methods

We prospectively enrolled 11 patients (11 eyes) with active MSC in 1 or both eyes between October 2012 and December 2013 using following inclusion criteria: (1) evidence of active MSC lesions with central healing and active edges that showed early hypofluorescence and late hyperfluorescence on fluorescein angiography in at least 1 eye; (2) presence of significant vitreous cells (2+ or more) in the affected eye with or without anterior segment inflammation; (3) documented positive (10 mm of induration or more) tuberculin skin test (TST) results at 48 to 72 hours or QuantiFERON-TB Gold In-Tube (Cellestis Limited, Carnegie, Victoria, Australia) test results, or a strong clinical suspicion of MSC in the absence of any evidence of latent tuberculosis; and (4) all known causes of infectious uveitis except tuberculosis and

http://dx.doi.org/10.1016/j.ophtha.2014.11.021
ISSN 0161-6420/14

known noninfectious uveitic syndromes excluded by clinical features or relevant tests. An induration of 10 mm or more was considered positive.[16]

Institute ethics committee approval was obtained. The study adhered to the tenets of the Declaration of Helsinki. To confirm our belief that vitrectomy has not been performed in MSC before our current study, we performed a MEDLINE search through PubMed (www.ncbi.nlm.nih.gov/pubmed) using the terms and medical subject heading terms *serpiginouslike choroiditis*, *serpiginoid choroiditis*, *serpiginous choroiditis* and *polymerase chain reaction*, or *serpiginouslike choroiditis*, *serpiginoid choroiditis*, *serpiginous choroiditis*, and *vitrectomy*.

Sample Collection

After patients provided written informed consent, the affected eye underwent 23-gauge PPV by a single surgeon (A.G.). The first vitreous sample was collected in an undiluted state by flushing air, instead of fluid, through the infusion line. Using manual aspiration with a syringe connected to the aspiration line of the cutter, approximately 750 μl undiluted and 2 to 3 ml diluted vitreous samples were collected under direct visualization.

Molecular Assays

The vitreous fluid samples were subjected to multitargeted PCR for *M. tuberculosis*,[10] the Gene Xpert MTB/RIF assay (Cepheid, Sunnyvale, CA), and a line probe assay (GenoType MTBDRplus; Hain Lifescience, GmbH, Nehren, Germany) for detecting the *M. tuberculosis* genome and rifampicin resistance (Fig 1). In multitargeted PCR, samples were considered positive if they showed 1 or more bands for the 3 genes tested. The samples with positive results for *M. tuberculosis* or rifampicin resistance by either of the techniques were subjected to *rpoB* gene sequencing to demonstrate rifampicin resistance.[17]

For the Gene Xpert MTB/RIF assay, at least 0.5 ml vitreous fluid was mixed with 2.5 ml buffer solution in a prelabeled, sterile, leak-proof Falcon (Tarsons Products Pvt Ltd, Kolkata, India) tube. After proper mixing and incubation at room temperature (as per the manufacturer's instructions), the liquefied sample was transferred to the Xpert MTB/RIF cartridge, scanned, and loaded in to the instrument module. The reports were autogenerated by measuring fluorescent signals and embedded calculation algorithms in approximately 2 hours. The results were interpreted as *M. tuberculosis* detected (low, medium, or high) or not detected, along with rifampicin resistance detected, not detected, or indeterminate.

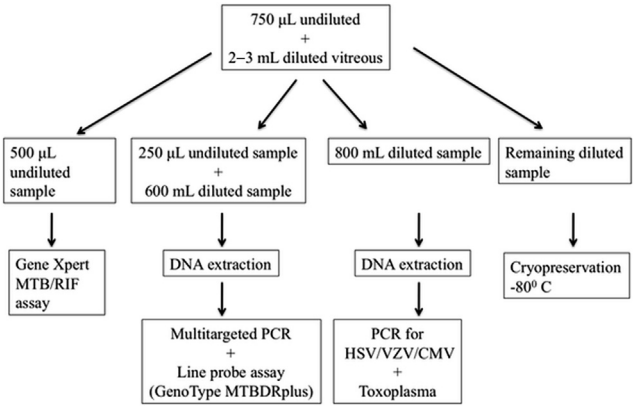

Figure 1. Schematic diagram showing how the vitreous sample was split between assays and what different assays were performed. CMV = cytomegalovirus; HSV = herpes simplex virus; PCR = polymerase chain reaction; VZV = varicella zoster virus.

We performed the line probe assay using Genotype MTBDR plus version 2.0 kit. The procedure involved DNA isolation followed by multiplex amplification of targets with biotinylated primers, which underwent reverse hybridization with specific probes. The PCR amplification mix (45 μl) was prepared in a contamination-free room and DNA samples were added in a separate area. All the reagents required for multiplex PCR were included in the amplification mixes A and B and were optimized for the given test. It comprised a 2-step PCR analysis. The first step included 20 cycles of denaturation at 95°C for 30 seconds and annealing at 65°C for 2 minutes. The second step included 30 cycles of 95°C for 25 seconds, 50°C for 40 seconds, and extension at 70°C for 40 seconds. In addition, a 15-minute initial denaturation at 95°C and 8 minutes of extension at 72°C also were applied. The hybridization procedure was carried out manually using Twin Cubator (Hain Life Sciences) with a preinstalled hybridization protocol.

Among the 21 test zones and 6 control zones in the MTBDRplus assay strip, the probes covered the *rpoB* gene for detecting rifampicin resistance and *katG* and *inhA* genes for detecting high-level and low-level isoniazid resistance, respectively. Positive staining results of all wild-type probes of a gene indicated no detectable mutation within the examined region. Absence of a signal for at least 1 wild-type probe indicated resistance of the tested strain to the respective drug.

The samples from each eye also were subjected to multiplex PCR for virus and toxoplasmosis. The specific primers targeting the open reading frame 28 region of varicella zoster virus and the B1 gene of toxoplasma were used.[18–20] Nested PCR for herpes simplex virus and cytomegalovirus was performed by amplifying the glycoprotein D gene and immediate early gene, respectively. The 5 μl eluted DNA was used for PCR amplification with 1X PCR buffer (10 mM Tris with 15 mM $MgCl_2$), 200 μM deoxynucleotide, 1 μM of each primer, and 1 unit of Taq polymerase (Bangalore Genei, Bangalore, India). For the amplification of the second round for nested PCR, 5 μl amplified product from the first round was used. Appropriate positive and negative controls were included in each run. All necessary precautions were taken to prevent any carryover contamination. The amplicons were visualized by agarose gel electrophoresis after ethidium bromide staining.

Treatment

The treatment was initiated with oral corticosteroids in all patients (prednisolone 0.5–1 mg/kg daily). The first-line 4-drug ATT included isoniazid (5 mg/kg daily), rifampicin (450 mg daily if body weight was less than 50 kg or 600 mg daily if body weight was 50 kg or more), ethambutol (15 mg/kg daily), and pyrazinamide (25–30 mg/kg daily) initially for 2 to 3 months under the supervision of the internist (A.S.). Thereafter, rifampicin and isoniazid were continued for another 9 to 10 months with pyridoxine supplementation. The treatment for multidrug-resistant (MDR) tuberculosis comprised pyrazinamide (1500 mg daily), levofloxacin (750 mg daily), ethionamide (750 mg daily), cycloserine (750 mg daily), and intramuscular injection of streptomycin 1 g daily for the first 5 months, followed by levofloxacin, ethionamide, and cycloserine for another 18 months.

Liver function tests were carried out at baseline and follow-up to monitor for any drug toxicity to ATT. Topical corticosteroids and cycloplegics were added to patients with anterior segment inflammation. Immunosuppressive therapy consisting of azathioprine (2–2.5 mg/kg daily) was given to some patients showing