Polymerase Chain Reaction for Detection of *Mycobacterium tuberculosis* in Epiretinal Membrane in Eales’ Disease

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**PURPOSE.** Tuberculous etiology has been suggested in Eales’ disease. Because epiretinal membrane (ERM) is formed on the inner surface of the retina in Eales’ disease, it could be the most appropriate intraocular specimen for investigation. Therefore, a nested polymerase chain reaction (nPCR), which detects MPB64 gene of *Mycobacterium tuberculosis* on the archival specimens of ERM of well-documented Eales’ and non-Eales’ patients, was applied and the results compared.

**METHODS.** nPCR technique was standardized, and the sensitivity and specificity of the primers were determined. nPCR technique was applied to tissue sections obtained from formalin-fixed and paraffin-embedded tissues of ERM from 23 patients with Eales’ disease and 27 noninfective and non-Eales’ disease patients as controls.

**RESULTS.** nPCR technique was specific for *M. tuberculosis* genome and sensitive enough to detect 0.25 fg (corresponding to the presence of a single bacillus). Eleven (47.8%) ERM of 23 Eales’ disease and 3 (11.1%) of 27 controls were positive for *M. tuberculosis* genome. The difference between the two groups was statistically significant ($P = 0.001$), indicating association of this bacterium with Eales’ disease.

**CONCLUSIONS.** The demonstration of the presence of *M. tuberculosis* DNA by nPCR technique in significant number of ERM of Eales’ disease compared with the controls further emphasizes the probable role of this bacterium in the pathogenesis of this enigmatic clinical condition. (*Invest Ophthalmol Vis Sci.* 2000;41:822-825)

Eales’ disease is an idiopathic condition with a primary retinal vasculitis predominantly affecting the peripheral retinas of young and otherwise healthy adults in the age group of 15 to 40 years. In India, Eales’ disease has been shown to occur in 1 in 135 ophthalmic patients in a referral ophthalmic center and 1 in 200 to 250 in a general eye hospital. Although the clinical characteristic and natural course of Eales’ disease are well known, its etiopathogenesis is not well understood yet. Etiologies proposed have been tuberculosis, hypersensitivity to tuberculoprotein, focal sepsis, hematologic abnormalities, worm infestations, and cell-mediated immunity. The most favored have been tuberculosis and hypersensitivity to tuberculoprotein. In reports of Eales’ disease, the association of systemic tuberculosis was found to range from 6.2% to 35% of cases. Mantoux positivity (hypersensitivity to tuberculoprotein) varied between 42.1% and 98% of Eales’ cases. We did not find any statistically significant difference of Mantoux positivity among Eales’ disease patients with age- and sex-matched controls. In a previous study, we followed polymerase chain reaction (PCR) technique using IS6110 primers and detected *Mycobacterium tuberculosis* complex DNA in 5 of 12 vitreous fluids (VFs) aspirated from the group with Eales’ disease and 1 of 45 VFs from the control group. The difference was statistically significant ($P = 0.001$). Bacteriologic examination of these VFs did not reveal the presence of acid fast bacilli (AFB). The nested polymerase chain reaction (nPCR) technique specific for MPB64 gene as described by Narita et al. was standardized in our laboratory. MPB64 is a highly immunogenic protein found in unheated culture filtrates of *M. tuberculosis* and some strains of *M. bovis* BCG. nPCR technique was found to be not only specific but also 10,000 times more sensitive than PCR using IS6110 primers. Because etiologic association of tuberculosis and Eales’ disease remained unresolved, and because of higher sensitivity of nPCR, we applied this technique on the archival tissue specimens of epiretinal membrane (ERM) obtained from patients with and without Eales’ disease. Because ERM is the fibrocellular tissue formed on the inner surface of the retina in various vitreoretinal disorders including Eales’ disease, it was considered the most appropriate intraocular specimen for this study.

**METHODS**

**Collection of Tissue Sections of ERMs**

Fifty sequential archival ERM tissue sections available in the ocular pathology department of the Vision Research Foundation, Sankara Nethralaya, from 1992 through 1998 were in-
cluded in this retrospective study. Of the 50 ERM tissue sections, 23 were from patients clinically diagnosed with Eales’ disease by the criteria described by Das et al.1 and were obtained only at the inactive stage of the disease and when there was no active periphlebitis, whereas the other 27 were from non-Eales’ patients (as controls) with noninfective disorders like proliferative diabetic retinopathy, proliferative vitreoretinopathy, retinal detachment, complicated cataract, and post-penetrating trauma. Indications for surgery were vitreous hemorrhage or tractional retinal detachment, which occurs secondary to these diseases. The clinical data on the past history of pulmonary or any other systemic tuberculosis of all these patients were obtained from the files of the patients.

Epiretinal membranes were obtained from both sets of patients by membrane peeling during vitrectomy. Membranes were fixed in 10% neutral-buffered formalin and processed into paraffin blocks. Three to four sections of 5- to 6-μm-thickness were cut and used for DNA extraction.

**Standardization of nPCR**

Two *M. tuberculosis* strains (H37Rv and H37Ra) and 6 mycobacteria other than *M. tuberculosis*—MOTT strains (*M. avium complex, M. kansasii, M. chelonae, M. smegmatis, M. xenopi, and M. fortuitum*)—were obtained from The Tuberculosis Research Center (TRC) of the Indian Council of Medical Research, Chennai, India. Other strains included were our laboratory isolates whose identification was confirmed by TRC. Genomic DNA from these strains was extracted as described by Hermans et al.5 Briefly, 2 to 3 colonies of the mycobacterium strain from Lowenstein Jensen (LJ) medium were suspended in 1.5 ml of Tris-EDTA (TE) buffer, mixed well over the 20°C incubator, and kept at 80°C for 20 minutes. The suspension was spun in a microcentrifuge at 14,000 rpm for 2 minutes until a compact pellet was formed. The supernatant was discarded, and the deposit was resuspended in 500 μl of TE buffer and mixed well. Fifty microliters of 10 mg/ml lysozyme (Bangalore Genei Pvt., Bangalore, India) was added, mixed, and incubated for an hour at 37°C, followed by the addition of 70 μl of 10% sodium dodecyl sulfate (SDS; SISCO Research laboratories Pvt., Mumbai, India) and 6 μl of 20 mg/ml proteinase K (Bangalore Genei Pvt.) and incubated at 65°C for 10 minutes. Phenol–chloroform extraction was done followed by ethanol precipitation, and the precipitated DNA was dissolved in 50 μl of sterile Milli Q water.

**Extraction of DNA from Paraffin-Embedded Tissue Sections of ERM**

Tissue sections were scraped from the deparaffinized slide with the help of a sterile blade, and DNA was extracted according to the method of Manjunath et al.10 The tissue samples were digested overnight with proteinase K (100 μg/ml) at 37°C in a solution containing 100 mM NaCl, 10 mM Tris–HCl (pH 8.0), 25 mM EDTA, 0.5% SDS, followed by phenol–chloroform extraction and ethanol precipitation. The pelleted DNA was dissolved in 40 μl of sterile Milli Q water. The amount of DNA was quantified spectrophotometrically using a Beckman DU 640 spectrophotometer.

**Primers**

Nested primers included outer sense primers for first round of amplification consisting of upstream primer 5′-TCCGCTGC- CAGTCGTTTCC-3′ and downstream primer 5′-GTCTCGCGG- GAGTCTAGGCCA-3′ and inner sense primer for second round of amplification consisting of upstream primer 5′-ATTITG- CAAGGTGAACGTG-3′ and downstream primer 5′-ACATC- GATGCATGCGGA-3′. Outer primer set coded for 240-bp region (nucleotides 460–700), whereas the inner primer set code for a 200-bp region within the 240 bp (481–661 nucleotides) from MPB64 gene.4

**Amplification of DNA**

Amplification of *M. tuberculosis*–specific 200-bp DNA was done in two rounds. First step of amplification was done in a 50-μl reaction mixture, which consisted of 5 μl of 10× buffer (500 mM potassium chloride, 100 mM Tris chloride, 15 mM magnesium chloride, gelatin 0.1%, pH 8.3), 100 ng each of primers, 200 μM each of deoxyribonucleotide triphosphate, 1 U *Taq* DNA polymerase (Bangalore Genei Pvt.), and 5 μl of DNA template. Distilled water was added to make the volume to 50 μl. The reaction mixture was overlaid with 50 μl of sterile mineral oil. PCR was performed in a Perkin–Elmer automatic thermal cycler (model 480; Cetus, Norwalk, CT). Amplification was done by using a three step profile (i.e., denaturation for 1 minute at 94°C, annealing for 1 minute at 55°C, and extension for 1 minute at 72°C for a total of 35 cycles).

Subsequently, a second step amplification was performed using 5 μl each of the first-round products. A reaction mixture was constructed in the same way, except that the outer sets of primers were replaced by the inner sets. A cycle count of 25 cycles (i.e., total of 60 cycles) was adopted. Each set of amplification was done in the presence of two negative controls—one for sample extraction and another as a reagent control—and a positive control, which consisted of 5 μl *M. tuberculosis* H37Rv DNA. The nPCR for each specimen was repeated at least twice to confirm the reproducibility of the results.

**Determination of Specificity**

Specificity of PCR for detection of *M. tuberculosis* was done using DNA from a variety of mycobacteria, listed above.

**Determination of Sensitivity**

To determine sensitivity of nPCR, 10-fold dilution of H37Rv DNA was made in sterile Milli Q water. Five microliters each of dilution of the genomic DNA was used to set up PCR according to the method described here.

**Detection of Amplified Product**

After amplification, PCR products were separated by electrophoresis in 2% agarose gel containing 0.5 μg/ml ethidium bromide and visualized on an ultraviolet transilluminator at 302-nm wavelength (Pharmacia, Uppsala, Sweden).

**Precautions**

To prevent contamination of PCR, separate rooms were used for preparation of DNA, its amplification, and analysis of the amplified product. PCR preparation was performed in a laminar flow workbench with single use aliquots of reagents and dedicated pipettes. The microfuge tubes and mineral oil aliquots were double sterilized.

**Analysis of the Results**

For analysis, χ² statistical test was used for comparison of the results of nPCR on ERM of Eales’ disease and control patients.
Most of the patients were chosen for this retrospective study because they were obtained from clinically documented cases. The availability of an eyeball in active stage of the illness is quite unlikely for these investigations because it is a disease of young healthy persons. Therefore, we believe that ERM is the most appropriate specimen for such studies. The pathologic nature of the ERM from cases of Eales' disease is only fibrovascular because they are obtained only at the inactive stage of the disease with no active periphlebitis. Thus, the cellular infiltration in the membranes of both the experimental and control groups is considered similar. As stated earlier, M. tuberculosis complex DNA was detected in a statistically significant number of VFs obtained from Eales' disease patients compared with those from non-Eales' patients, using the IS6110 primers. However, the recent studies carried out in Chennai, South India, have shown that 40% of the local clinical isolates of M. tuberculosis have either single copy or no copy at all of IS6110. In addition, Populaire et al. also have found PCR using IS6110 to be insensitive. Bearing in mind these recent findings on IS6110, in search of a more sensitive primer for M. tuberculosis, we embarked on nPCR approach for the gene coding for MPB64. We found it to have 100% analytical specificity and also to be many-fold times more sensitive, to detect the presence of even a single bacillus in a clinical sample. Because a sufficient amount of DNA ranging from 4.5 to 27.8 μg/ml was present in all ERM specimens, and because the results of the nPCR on them were reproducible, false PCR negativity was excluded.

The pathogenesis, cellular constituents, and various factors modulating the formation of ERM are not yet precisely known. The demonstration of the presence of M. tuberculosis DNA by nPCR in significant number of ERM from those with Eales' disease compared with the controls emphasizes the probable role of this bacterium in the pathogenesis of this enigmatic clinical condition. Interestingly, our study also showed negative results of nPCR on the ERM of 3 patients with Eales' disease and 1 of the control group, despite their having had a history of pulmonary tuberculosis. This raises the possibility that transient infection with tubercle bacillus at the retinal site may only be required and not necessarily an active disease to trigger the inflammatory changes seen in Eales' disease. The mere presence of the mycobacterial DNA in the ERM is not indicative of the active infection, but it may have a role in triggering the inflammatory processes that take place in the pathogenesis of Eales' disease. Hence, we hypothesize that the presence of the sequestered mycobacterium in the eyes can be one of the triggering factors in a significant number of Eales' patients, initiating the cascade of inflammatory processes resulting in the immunopathologic changes that occur in Eales' disease.

The immunohistochemical study of ERM of Eales' disease cases from our center indicated the predominant T lymphocyte involvement in the inflammation of ERM and subretinal membrane of Eales' disease patients, indicating a cell-mediated immune reaction. However, in another study, 56 patients with Eales' disease and 50 age- and sex-matched volunteers with normal fundus findings taken as controls were subjected to

**FIGURE 1.** Ethidium bromide stained 2% agarose gel with amplification products from ERM from Eales' disease cases. N, Reagent control of the first round; N2, reagent control of the second round; lane 1, sample extraction control, negative; lane 2, H/805/97, positive; lane 3, H/506/98, positive; lane 4, H/529/97, negative; lane 5, H/120/97, negative; lane 6, H/784/97, positive; P, positive control M. tuberculosis (H37Rv); M, molecular weight marker Phi X 174 DNA/HaeIII Digest. * denotes laboratory identification number.
Mantoux testing using 2 TU/0.1 ml of purified protein derivative (PPD) and lymphocyte proliferation assay to PPD. The results of Mantoux testing and lymphocyte proliferation assay did not differ significantly in the two groups, suggesting a similar cellular immune response. Because tuberculosis is an endemic disease in India, PPD status often does not indicate the active state of the disease. This is supported by the results of a survey conducted for the prevalence of nonspecific sensitivity in different parts of India, which showed that the Mantoux sensitivity of healthy individuals ranged between 67.4% and 90.35%. When the above facts and the results of our earlier report are considered, PPD status of our study groups is not likely to be different, although data regarding the PPD status of the patients in both groups of the present study are not available for further evaluation.

In yet another study, we evaluated the possible role of HLA phenotypes and found statistically significant higher phenotype frequencies of HLA B5 (B51), DR1, and DR4 in Eales’ disease patients compared with healthy persons using peripheral blood. The results of HLA typing of the present study groups of Eales’ patients and controls, being a retrospective one, were not available for them to be correlated.

Based on the results of the present nPCR on ERMs and our previous studies, we hypothesize that individuals with the HLA predisposition may develop retinal vasculitis as a result of a cell-mediated immunologic tissue damage triggered by a sequestered M. tuberculosis antigen in an inactive form and clinically present as Eales’ disease. Because tuberculosis is an endemic disease in India, further prospective studies will be required to prove the association of HLA types and of sequestered M. tuberculosis antigen in triggering the immune system, resulting in Eales’ disease.

References