Association of mycobacteria with Eales’ disease


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Received January 23, 2006

Background & objectives: Eales’ disease is an idiopathic disease resulting in retinal neovascularization, recurrent haemorrhages, with or without retinal detachment predominantly affecting healthy young males (97.6%) in the Indian subcontinent. Inspite of several studies, the aetiology of Eales’ disease is not clear. The isolation of Mycobacterium fortuitum from the aqueous humour of a patient with classical Eales’ disease, led us to hypothesize that rapid growing nontuberculous mycobacteria (RGNTM), particularly M. fortuitum and M. chelonae could be associated with Eales’ disease. We therefore undertook this study to detect DNA of these RGNTM and also of M. tuberculosis in vitreous fluids (VFs) from patients with Eales’ disease and non-Eales’ disease.

Methods: We developed and optimized seminested polymerase chain reactions (SnPCRs) to detect DNAs of M. fortuitum and M. chelonae on archival ERMs (33) and VFs (19) of Eales’ and control patients along with conventional mycobacteriological investigations.

Results: In the retrospective study, 70 per cent ERM samples were positive for one or more Mycobacterium spp. tested by snPCR. M. fortuitum and M. chelonae were isolated from two VFs, which were also positive by snPCR in the prospective study. Statistical evaluation of the results of both retrospective and prospective investigations showed a statistically significant association of Mycobacterium spp. with Eales’ disease.

Interpretation & conclusion: The results of the present study suggested the involvement of Mycobacterium spp. in the aetiopathogenesis of Eales’ disease. Further studies on a larger sample will be required to confirm these findings.

Key words Eales’ disease - rapid growing nontuberculous mycobacteria (RGNTM)

Eales’ disease is an idiopathic disease with retinal perivasculitis predominantly affecting the peripheral retina during the inflammatory stage followed by sclerosis of the retinal veins and retinal ischaemia and finally, retinal or optic disk neovascularization, recurrent haemorrhages, with or without retinal detachment. The disease predominantly affects healthy young adults with a male preponderance of about 97.6 per cent and is more common in the Indian subcontinent. Although the clinical characteristic and natural course of Eales’ disease are well known, its aetiopathogenesis is not yet well understood. In recent years, clinical and basic researchers have provided significant clues to the understanding of the clinical features and the aetiology.
of Eales’ disease\textsuperscript{3-12}. Of the several aetiologies proposed, most favoured are tuberculosis and hypersensitivity to tuberculoprotein\textsuperscript{13-14}. In an earlier study, we demonstrated the presence of \textit{Mycobacterium tuberculosis} complex DNA in 5 of 12 vitreous fluids (VFs) from Eales’ disease patients compared to 1 of 45 VFs of the control group by application of polymerase chain reaction (PCR) technique using IS 6110 primers\textsuperscript{7}. Further, we reported the presence of MPB 64 gene of \textit{M. tuberculosis} in significant number of ERM of well-documented Eales’ patients as controls with well-documented non-Eales’ patients\textsuperscript{8}. Though the histopathologic demonstration of the presence of tubercle bacilli has been mentioned in two earlier studies\textsuperscript{15,16}, to the best of our knowledge culture has not been attempted so far. Since we isolated \textit{M. fortuitum} from the anterior chamber tap of a patient with classical Eales’ disease, we hypothesized that non-tuberculous mycobacteria (NTM) particularly rapid growers could be associated with Eales’ disease. Our records for the last 12 yr showed an association of \textit{M. fortuitum} and \textit{M. chelonae} with ocular infections (unpublished data). Therefore, a study on the role of these two non-tuberculous mycobacteria apart from \textit{M. tuberculosis} in the aetiopathogenesis of Eales’ disease was undertaken. For this purpose, we developed and optimized sensitive semi-nested PCRs (SnPCRs) to detect DNAs of \textit{M. fortuitum} and \textit{M. chelonae} in clinical specimens and applied them on the archival paraffin embedded ERMs of Eales’ and non-Eales’ disease patients. Further, a prospective study with application of semi-nested PCRs (SnPCRs) to detect DNA of \textit{M. fortuitum} and \textit{M. chelonae} and nested PCR for \textit{M. tuberculosis} DNA\textsuperscript{1} on fresh VFs from Eales’ and non-Eales’ disease patients was also done along with conventional mycobacteriological investigations.

\textbf{Material & Methods}

\textit{Patients:} Patients included in this study were those clinically diagnosed as Eales’ disease by the criteria described by Das \textit{et al}\textsuperscript{8} and non-Eales’ patients with non-infective diseases such as proliferative diabetic retinopathy, retinal detachment and proliferative vitreoretinopathy as controls. ERMs and VFs for both retrospective and prospective studies belonged to different patients. They were grouped into two: group I for the retrospective studies and group II for prospective studies.

\textit{Group I:} (Retrospective study) - Archival ERM and preserved VF specimens: For this part of the study, 33 sequential archival ERM tissue sections available in the ocular pathology department from the year 1992 to 2002, Vision Research Foundation, Sankara Nethralaya, Chennai, were included. Of these, 20 were from patients clinically diagnosed as Eales’ disease and 13 others were from non-Eales’ patients Group Ia. About 3-4 tissue sections of 5 - 6 µm of each ERM were used for extraction of DNA.

Nine VFs from patients clinically diagnosed as Eales’ disease and 10 VFs from non Eales’ patients with non-infectious diseases stored at - 20°C in L & T Microbiology Research Centre laboratory, Sankara Nethralaya, Chennai, during the period 1999-2003 were also included in this study Group Ib.

\textit{Group II (Prospective study) - Vitreous fluids and peripheral blood:} Ten VFs from consecutive patients clinically diagnosed as Eales’ disease and 23 VFs from non-Eales’ patients as controls were included during February 2005 to December 2005. The study was approved by the Institutional Research and Ethics Committee of the Vision Research Foundation. VF was aseptically collected at the time of vitreous surgery done as part of the management of these patients. Uncontaminated VF samples (150 - 200µl) were aspirated by tuberculin syringe connected to the suction port of the vitreous cutter at the beginning of the vitrectomy by the clinician in the operation theatre. The air was expelled carefully without causing aerosol and the needle was capped with sterile rubber bung and sent to the laboratory immediately. Approximately, 1 ml of peripheral blood was collected both from patients with Eales’ disease and control group in sterile tubes containing EDTA and stored in new sterile microfuge vials and kept frozen at - 20°C for PCR.

\textit{Mycobacteriological investigations:} Investigations for detection of \textit{Mycobacterium} spp. from VF were made as follows\textsuperscript{17}. About 100 to 150 µl of VF was processed by standard bacteriological procedures for detection of acid fast bacilli (AFB). In brief, 2-3 drops of the aspirated fluid specimens were directly inoculated onto Lowenstein Jensen (LJ) medium in duplicates along with a set of routine bacteriological media including blood agar and Mac Conkey agar. About a drop or two were cytospined (Shandon cytospin 2, UK) for smears, which were stained by Ziehl–Neelsen (ZN) method for microscopic presence of AFB. For the ERM, a crushed smear was made and stained by ZN method for AFB and inoculated into a LJ medium. The bacteriological media were incubated at 37°C for ten days and the LJ media for six weeks\textsuperscript{17}. 
Extraction of DNA from VF, blood, ERM and clinical isolates: DNA was extracted using QIAamp DNA Minikit (GmbH, Germany), according to the manufacturer’s instructions.

Extraction of DNA from paraffin sections of ERM: Tissue sections of ERMs from Eales’ disease and non-Eales’ disease patients obtained from ocular pathology laboratory were used for this purpose. Deparaffinization was accomplished with two changes of xylene and then with descending grades of alcohol following which the section was scraped from the slide using sterile Bard-Parker blade and placed in a sterile 1.5 ml microfuge vial for further extraction of DNA using QIAamp DNA Minikit.

Standardization of snPCRs for the detection of M. fortuitum and M. chelonae genomes: sn PCRs for detection of the ITS regions of the genome of M. fortuitum and M. chelonae were standardized. M. fortuitum specific semi-nested primers, the forward primer, MF1: 5’CCGTGAGGAACCGGTGCTC 3’, was common for both the first and the second round. The first round, reverse primer was MF2: 5’ CCACACGATTTGCGGTCTA 3’, which amplified a 222 bp region. The second round reverse primer MF3: 5’AGTGAGGCACACCGCCG 3’ was designed from the sequence available in the Genbank database (ACCESSION NO. AF144326). The size of the second round amplified product was 153 bp. Similarly, for M. chelonae the first round forward primer was common, MC1: 5’ GTTACTGCGCCTGTGCTG 3’, the reverse primer was designed from the sequence available in the Genbank database (ACCESSION NO. AF144327) MC2: 5’ GACAAACAAATGTGGTTCC 3’. The first round amplified product size was 132 bp. The second round reverse primer used was MC3: 5’ CAGGCTTTCATTCTATTA 3’, which amplified a 94 bp region. The first and second round reverse primers for both M. chelonae and M. fortuitum were specifically designed for this experiment. The BLAST analysis (http://www.ncbi.nlm.nih.gov) of the newly designed M. chelonae and M. fortuitum ITS primers revealed a 100 per cent identity for the ITS region of M. chelonae and M. fortuitum respectively. The first round of amplification by PCR was carried out as described by Park et al.17. Briefly, each reaction mixture contained 500 mM Potassium chloride, 100 mM Tris - HCl (pH 9.0), 1 per cent TritonX 100, 5 mM magnesium chloride, 0.01 per cent gelatin, pH 8.3, 5 µM of tetramethyl ammonium chloride (TMAC), 10 pm of each of the primers, 200 µM of each of the dNTPs, 1 unit of Taq DNA polymerase (Sanmar chemical specialities, India) and 10 µl of template DNA. The first round thermal profile was denaturation at 94°C for 5 min, followed by 1 min at 94°C, 1 min at 60°C for M. fortuitum but 52°C for M. chelonae followed by 72°C for 1 min for 30 cycles and a final extension of 72°C for 10 min. For the second round 5 µl of amplified product from the first round was transferred and the thermal profile was 94°C for 30 sec, 60°C for 1 min for M. fortuitum but 52°C for M. chelonae followed by 72°C for 30 sec for 25 cycles (PE Applied Biosystems 2700, USA). Since the first round amplification was sensitive enough, it was applied on clinical isolates of Mycobacterium spp. for their identification. For each cycle of amplification a standard strain of M. fortuitum (ATCC 1529) and M. chelonae (ATCC 1542) were used as positive controls for respective PCRs.

Sensitivity of the snPCR for ITS primers: Sensitivity of the primers was determined by amplifying serial 10 fold dilutions of template DNA from M. chelonae ATCC 1542 and M. fortuitum ATCC 1529. For the calculation of sensitivity 1 µl of template DNA was diluted to 1 ml with Milli Q Water and reading was taken at 260 nm spectrophotometrically. One OD corresponded to 50 ng /ml of DNA. The concentration of DNA was calculated from the corresponding OD value.

Specificity of the SnPCR for ITS primers: Specificity of the primers was tested by using DNA from mycobacteria including M. tuberculosis H37Rv and H37Ra, M. bovis, M. kansasii (ATCC 1201), M. intracellulare (ATCC 1403), M. xenopi (ATCC 1432), M. fortuitum (ATCC 1529), M. chelonae (ATCC 1524), M. smegmatis (ATCC 607) (all of them obtained from Tuberculosis Research Centre, Chennai, India); Gram-positive bacteria including Staphylococcus aureus (ATCC 12228), β haemolytic Streptococci (laboratory isolate), Bacillus subtilis (ATCC 6644), Diphtheroids (laboratory isolate), Propionibacterium acnes (laboratory isolate), Nocardia asteroides (laboratory isolate), Streptomyces species (laboratory isolate); Gram-negative bacteria including Klebsiella pneumoniae (ATCC 13880) and Pseudomonas aeruginosa (ATCC 7853). The fungi tested included Candida albicans (ATCC 24433) and Aspergillus flavus (laboratory isolate). The viruses tested included Cytomegalovirus (AD1659), Herpes Simplex Virus 1 (ATCC 733VR), Varicella Zoster Virus (Oka vaccine strain) and parasite including Toxoplasma gondii (RH strain). Human blood DNA from 4 blood donors of Voluntary Health Services, Chennai, India was also tested. Adequate negative controls were included for each PCR to exclude false positivity.
Detection of amplified products: The amplified products were detected by electrophoresis carried out in 2 per cent agarose with 0.5μg/ml ethidium bromide, along with a molecular weight marker and documentation was done using a Vilber Lourmat Gel documentation system (Cedex, France).

The standardized sn PCRs to detect DNA of *M. fortuitum* and *M. chelonae* and nested PCR for *M. tuberculosis* DNA were applied on the clinical specimens of both retrospective and prospective samples from Eales’ and non-Eales’ disease patients.

Statistical analysis: The statistical analysis of data was done by $\chi^2$ test for comparing the results of PCR on ERM, VF and blood of Eales’ disease and control patients of Group Ia, Ib and II individually and all the results combined together.

Results

The sensitivity of the snPCR was 10 pg of *M. chelonae* DNA and 1.5 pg of *M. fortuitum* DNA and these were specific to detect DNAs of *M. fortuitum* and the *M. chelonae* only in their respective sn PCR reactions.
Retrospective study

**Group I a:** Of the 20 Eales’ disease ERMs, 14 (70%) were positive by PCR for one or more of the mycobacteria tested. Of these, 5 each were positive for *M. fortuitum* and *M. tuberculosis* and 1 for *M. chelonae* and 2 for both *M. fortuitum* and *M. tuberculosis* and 1 for both *M. fortuitum* and *M. chelonae*. Of the 13 control ERMs, 4 (30.76%) were positive by PCR for one or more of the mycobacteria tested and of these, 2 for *M. fortuitum* and 2 for *M. tuberculosis* (Table I, Figs. 1, 2). A statistically significant (P<0.05) association of *Mycobacterium* spp. with Eales’ disease was found.

**Group I b:** Of the 9 VFs from Eales’ patients, 4 (44.4%) were positive by PCR for one or more of the mycobacteria tested. Of these, 2 were positive for *M. tuberculosis* and 2 others for both *M. tuberculosis* and *M. fortuitum*. Of the 10 VFs from control patients, only 2 (20%) were positive for *M. tuberculosis* (Table II).

**Group II - Prospective study:** All VFs from 10 Eales’ disease patients were negative by smear, two were positive by PCR, one each for *M. fortuitum* (patients 7) and *M. chelonae* (patient 2), and subsequently cultures of both these specimens also showed growth of the *Mycobacterium* spp. respectively. Both isolates were identified by conventional mycobacterium identification tests and sn PCR techniques as *M. fortuitum* and *M. chelonae*. The amplified product from the vitreous fluid of patient 7 was confirmed by DNA sequencing and showed 99 per cent identity with the 16 S – 23 S ITS spacer sequence of *M. fortuitum* when a nucleotide – nucleotide blast analysis (BLASTN 2.2.14) was done in the NCBI database. However, DNA sequencing could not be done for the patient 2 vitreous fluid due to insufficient quantity of specimen but was carried out on the mycobacterial isolate using Microseq® 500 kit targeting the 16 S rDNA (Alcon Research Laboratories, Fort Worth, Texas, USA) and the identification was confirmed as *M. chelonae*. The remaining 8 VFs were negative for all the three *Mycobacterium* spp. both by PCR and conventional mycobacterium investigations. All 23 VFs of the control group were negative by smear, culture and PCR for all three mycobacteria and peripheral blood specimens from these 23 controls were also negative for the three *Mycobacterium* spp. (Table III).

Statistical evaluation of combined results of both retrospective and prospective investigations for the three *Mycobacterium* spp. tested showed a statistically significant (P<0.001) association of *Mycobacterium* spp. with Eales’ disease.

**Discussion**

The aetiology of Eales’ disease is not clearly understood because of lack of appropriate specimens for microbiologic and histopathologic study. The vitreous also plays an important role in ERM formation, not only by providing a scaffold for cell growth but also by its modulating effects on cell behaviour. Hence, ERMs obtained from Eales’ patients, who need vitrectomy for nonresolving vitreous haemorrhage or other sequel of proliferative stages, are the most appropriate samples for studies regarding its aetiology.

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**Table I.** Results of sn PCR of *M. fortuitum* and *M. chelonae* and n PCR of *M. tuberculosis* on ERM paraffin sections of Eales’ disease patients and controls - Retrospective study

<table>
<thead>
<tr>
<th>Patients (n)</th>
<th><em>M. f</em></th>
<th><em>M. c</em></th>
<th><em>M. t</em></th>
<th><em>M. f &amp; M. t</em></th>
<th><em>M. f &amp; M. c</em></th>
<th><em>M. c &amp; M. t</em></th>
<th>Total (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eales’ disease (20)</td>
<td>5</td>
<td>1</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>14/20 (70)</td>
</tr>
<tr>
<td>Controls (13)</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4/13 (30.8)</td>
</tr>
</tbody>
</table>

*Association of Mycobacteria with Eales’ disease was statistically significant P<0.05

**Table II.** Results of sn PCR of *M. fortuitum* and *M. chelonae* and n PCR of *M. tuberculosis* on VFs of Eales’ disease patients and controls - Retrospective study

<table>
<thead>
<tr>
<th>Patients (n)</th>
<th><em>M. f</em></th>
<th><em>M. c</em></th>
<th><em>M. t</em></th>
<th><em>M. f &amp; M. t</em></th>
<th><em>M. f &amp; M. c</em></th>
<th><em>M. c &amp; M. t</em></th>
<th>Total (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eales’ disease (9)</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>4/9 (44.4)</td>
</tr>
<tr>
<td>Controls (10)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2/10 (20)</td>
</tr>
</tbody>
</table>
We have earlier shown a significant association of *M. tuberculosis* complex with Eales’ disease patients compared with those of non-Eales’ patients and this was further re-emphasized using PCR based DNA hybridization specific for *M. tuberculosis* on VF specimens from Eales’ disease patients.

The present retrospective study on the archival ERM specimens of Eales’ patients and the appropriate control patients showed statistically significant association of tuberculous and nontuberculous mycobacteria with disease in Eales’ disease patients whereas the study on the VF did not show this significance. These results indicated a need to apply the nucleic acid-based molecular techniques in parallel with conventional techniques on the prospective specimens. In the present prospective study, two of 10 (20%) VFs from Eales’ disease patients showed growth of RGNTM *M. chelonae* and *M. fortuitum*. PCR tests rapidly detected these mycobacteria correctly within 8 h of receipt of the clinical specimen. The isolated mycobacteria were identified by conventional methods and by sn PCR as *M. chelonae* and *M. fortuitum* respectively. Since isolation of an atypical (rapid growing) mycobacterium from a clinical sample should be often related to as a contaminant, the present isolations were critically reviewed and were not considered so because of absolute sterile precautions taken during vitrectomy surgery, transportation and further processing of the same in the laboratory. All specimens were processed within 1 h of their collection. Moreover, their growth was present in both the inoculated LJ bottles.

It is a proven fact that inflammation contributes to ERM formation. Further evidence was provided by the detection of inflammatory mediators in eyes containing ERMs and by the *in vitro* effects these substances have on cell types found in ERMs. Lymphocytes were observed predominantly in the ERMs obtained from Eales’ disease patients, suggestive of chronic disease and immunohistochemical studies suggested the presence of predominantly T lymphocytes.

We had earlier hypothesized that statistically significant higher phenotype frequency of HLA B5 (B51), DR1 and DR 4 occurs in patients with Eales disease compared to healthy individuals. Probably, this HLA predisposition could be responsible for the presence of sequestered mycobacterium DNA in some of the control individuals for whom the HLA typing was not done. It is also known that some individuals develop retinal vasculitis as a result of a cell-mediated immunologic tissue damage triggered by a sequestered *M. tuberculosis* antigen in an inactive form, which may occur in quite a number of individuals as in the case of tuberculosis.

The absence of DNA of both RGNTM in the blood of the two patients in whom these were isolations from VFs, suggest their localization in the eye without a systemic involvement. These patients did not show any systemic tuberculous lesions. The presence of RGNTM antigen could have also been in a sequestered form in the macrophages of Eales’ disease and control patients who might have a predisposition based on their HLA.

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Table III. Results of conventional method and PCR applied on Eales’ disease patients and control VFs and blood - Prospective study

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Clinical specimens</th>
<th>Smear +ve</th>
<th>Culture +ve</th>
<th><em>M. fortuitum</em> sn PCR +ve</th>
<th><em>M. chelonae</em> sn PCR +ve</th>
<th><em>M. tb</em> n PCR +ve</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Eales’ Vitreous fluids (N=10)</td>
<td>0</td>
<td>2 (<em>M.f</em> <em>I M,c</em>)</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Controls (N=23)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Eales’ Blood (N=10)</td>
<td>ND</td>
<td>ND</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Controls (N=23)</td>
<td>ND</td>
<td>ND</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

ND-Not done. Peripheral blood was collected from both patients and controls at the time of surgery. None of the blood specimens showed the presence of DNA of mycobacteria tested revealing that DNA detected in the 2 vitreous specimens was not due to hematogenous spread from circulation at the time of surgery.
type. It should be emphasized that 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. This is also supported by the results of a survey conducted for the prevalence of nonspecific sensitivity in different parts of India\textsuperscript{14}, which showed that not all individuals who are exposed to \textit{M. tuberculosis} antigen develop tuberculosis.

Further prospective studies will be required to prove the association of HLA types and of sequestered mycobacterium antigen in triggering the immune system, resulting in Eales’ disease. It is not known whether \textit{M. fortuitum} and \textit{M. chelonae} have a similar role like that of \textit{M. tuberculosis}, or have a complementary role when associated with \textit{M. tuberculosis}. The isolation of RGNTM from VFs in this study is suggestive of a probable role involving \textit{M. fortuitum} and \textit{M. chelonae} in the aetiopathogenesis of Eales’ disease. Further studies with a larger sample size are necessary to establish the true association of \textit{Mycobacterium} spp in Eales’ disease.

**Acknowledgment**

Authors acknowledge the Indian Council of Medical Research (ICMR), New Delhi, for providing financial support and thank Alcon Research Laboratories, Fort Worth, Texas, USA for DNA sequencing of mycobacterial isolate.

**References**


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