MATERIALS AND METHODS

METHODOLOGY

Methodology of this study will describe the study design, subject selection, sample collection and will outline the protocol adopted to perform the work.

The study was approved by the Institutional Ethics committee in Sri Ramachandra University and in SankaraNethralaya. Study population comprised of 126 patients and 27 control subjects attending a tertiary eye care institute in Chennai, South India over a period of four years. It is a prospective study conducted on a cohort of patients who presented with symptoms and signs of posterior uveitis. Inclusion criteria were defined as active serpiginous choroiditis and multifocal choroiditis with or without anterior uveitis. Exclusion criteria were all patients in whom diagnosis was unequivocal and the aetiology established. An informed consent was obtained from all the study subjects following which they were analysed in stages. Study patients who had serpiginous choroiditis or multifocal choroiditis were recruited for this study and the sample collection was done at SankaraNethralaya, Chennai in the outpatient uvea clinic from 2009 to 2012. Patients belonged to age groups ranging from 11 to 68 years (mean age
The duration of symptoms was less than one month in all patients. The patients were examined clinically by an ophthalmologist. In addition the patient answered questions to assess severity of vision loss, systemic status, previous or present treatment and other ocular symptoms. A follow up for a minimum period of 1 year was done on all participants.

**Statistical analysis methods**

SPSS 11.0 statistical software (Chicago IL, United States of America) was used for all statistical analyses, K-related samples and Kendall’s (coefficient of concordance) non parametric tests were selected for comparison of the various investigative procedures. The true and false, positive and negative cases were recorded. Calculations were performed to determine the sensitivity and specificity of selected tests.

The set of parameters used for statistical analysis were as follows

1) False –negative rate is defined as the probability that the classification result indicates a normal while the true diagnosis is indeed the disease (i.e positive). This case should be completely avoided as it represents a danger to the patient.
2) False-positive rate is defined as the probability that the classification result indicates a disease while the true diagnosis is indeed a normal (i.e. negative). This case can be present but should be as infrequent as possible.

3) Sensitivity is defined as the conditional probability of detecting a disease while there is in fact a disease. By definition, sensitivity = 1 − false-negative rate.

4) Specificity is defined as the conditional probability of detecting a normal choroid when the choroid is indeed normal. By definition, specificity = 1 − false-positive rate.

Between-group differences in categorical variables were tested for significance with Fisher exact tests.

The positive and negative predictive values (PPV and NPV respectively) are the proportions of positive and negative results in statistics and diagnostic tests that are true positive and true negative results. The PPV and NPV describe the performance of a diagnostic test or other statistical measure. A high result can be interpreted as indicating the accuracy of such a statistic. The PPV and NPV are not intrinsic to the test; they depend also on the prevalence.

**Materials and methods**
A complete ophthalmic evaluation was performed on all patients which included slit lamp examination, fundus biomicroscopy, indirect ophthalmoscopy, fundus autofluorescence, FFA, ICGA and optical coherence tomography (OCT). Complete blood count, purified protein derivative test, QuantiFERON- TB Gold test and high resolution chest tomography (HRCT) were performed in all 126 patients. The study was approved by the Institutional Ethics committee in Sri Ramachandra University and in SankaraNethralaya. Study population comprised of 126 patients and 27 control subjects attending a tertiary eye care institute in Chennai, South India over a period of four years. RT- PCR was done on 28 eyes and nested PCR performed on 3 eyes. It is a prospective study conducted on a cohort of patients who presented with symptoms and signs of posterior uveitis. Inclusion criteria were defined as active serpiginous choroiditis and multifocal choroiditis with or without anterior uveitis. Exclusion criteria were all patients in whom diagnosis was unequivocal and the aetiology established. During the first visit a structured questionnaire was provided to all patients .The questionnaire had queries related to clinical findings and the family history. The time for answering the questionnaire and completing examination required approximately forty five minutes time. Both blood samples and aqueous humour samples were collected from each patient.
Criteria used to continue, confirm participation and to analyse data in the study were (1) availability of complete clinical records and digital fundus images at baseline and follow-up visits, (2) positive tuberculin skin test or QuantiFERON-TB Gold (3) active serpiginous-like choroiditis in at least 1 eye and a (4) a minimum of 1 year of follow-up from initiation of treatment that included antitubercular treatment (ATT) with oral corticosteroids or corticosteroids alone.

Control group consisted of 27 healthy patients who were undergoing cataract surgery. None of the patients had any form of ocular disease. 15 patients belonged to the 7th decade, 7 patients to 6th decade, and 5 patients to 5th decade of life. There were 12 males and 15 females. 0.1 ml of aqueous humour was obtained after anterior chamber paracentesis. The sample was sent for RT-PCR. However it was not age matched as the sample was from elderly patients with senile cataract. Nested PCR was not performed on control samples as this procedure was not used in all patients.

**Purified protein derivative test (PPD test)**

5 TU of tuberculin PPD was used on all patients. It was injected strictly intradermally, using 28 or 26-gauge needle and tuberculin syringe from which 0.1 ml was delivered accurately. The reaction to intracutaneously injected tuberculin
is the classic example of a delayed (cellular) hypersensitivity reaction. T-cells sensitized by prior infection are recruited to the skin site where they release lymphokines. These lymphokines induce induration through local vasodilatation, oedema, fibrin deposition, and recruitment of other inflammatory cells to the area. Features of the reaction include (1) erythema, reaching a peak more than 24 h after injection of the antigen; (2) its indurated character; and (3) its occasional vesiculation and necrosis. The Mantoux skin test was read between 48 and 72 hours after administration. Erythema and induration was measured and documented. It was classified as 5mm and more, 10mm and more and 15mm and more.

**Anterior chamber tap**

Anterior chamber tap was done under aseptic precautions after using topical anaesthesia. A 26 G needle on an insulin syringe was used and 0.1ml of aqueous withdrawn. The procedure was performed with the patient in the supine position. Topical antibiotic was administered and the eye patched after the procedure. The procedure was performed after ensuring that the pupil was constricted as performing paracentesis in a patient with dilated pupil could injure the lens. Local research ethics committee approval and informed consent were obtained.
0.5% paracaine eye drops were instilled three times over a 3 minute period, followed by instillation of betadine 5% antiseptic solution. The patient was positioned at the slit lamp, the upper lid and eyelashes held out of the way by an assistant. No lid speculum was used.

The patient was examined again using a slit lamp to look for complications such as hyphaema, shallow anterior chamber and infection though it is safe in the hands of an experienced ophthalmologist. Following anterior chamber paracentesis the sample of aqueous humour was transported to the laboratory where it was stored at -20 C till DNA extraction and polymerase chain reaction. A few aqueous samples were also sent for smear and culture.

**QuantiFERON- TB Gold test**

The kit used was QuantiFERON- TB gold (QFT-G, manufactured by Cellestis Limited, Carnegie, Victoria, Australia). Aliquots of heparinised whole blood were incubated with the test antigens for 16-24 h. The blood was incubated with the test antigens ≤ 12 h after collection. Test kits included two mixtures of synthetic peptides representing ESAT-6 and CFP-10 as test antigens, phytohemagglutinin (a mitogen used as a positive assay control) and saline (used as a nil sample to measure the background level of IFN-γ). The amount of IFN-γ
released was determined by subtracting the amount in the nil from the amount in the ESAT-6 and CFP-10, or mitogen-stimulated plasma. QFT-G test results were calculated by using the software provided by the manufacturer.

Principle of Polymerase chain reaction

Figure 6: Principles of PCR
Characterization and detection of infectious disease organisms have been revolutionized by PCR. It is a technique involving enzymatic amplification of nucleic acid sequences in repeated cycles of denaturation, oligonucleotide annealing, and DNA polymerase extension (figure 6).

The PCR uses in vitro enzymatic synthesis to amplify specific DNA sequences within a few hours. Polymerase chain reaction consists of repetitive cycles of specific DNA synthesis, defined by short stretches of preselected DNA. With each cycle, there is a doubling of the final, desired DNA product such that a million-fold amplification is possible.

**Nested PCR**

PCR by nested primers which detects MPB64 gene and IS 6110 region was done on aqueous humour sample. Samples from aqueous were cultured and analysed by nested PCR for presence of mycobacterium tuberculosis nucleic acids. Two sets of primers corresponding to IS6110 region coding for 219 bp and 123 bp DNA sequences were used. Direct smear and culture for mycobacteria were performed only when the patient was symptomatic for pulmonary tuberculosis.

**Real time PCR**
Figure 7: Real time PCR kit

Real time polymerase chain reaction was performed on a subset of 28 eyes of 27 patients and 27 controls whose aqueous aspirate was obtained during cataract surgery. DNA extraction from the aqueous humour was carried out using QIAMP DNA extraction kit (QIAGEN, Germany). Real time Polymerase Chain reaction (RT-PCR) for mycobacterium tuberculosis (MTB) was carried out using Genosen’s MTB complex (Netherlands) quantitative real time PCR kit (figure 7). RT-PCR for quantitation of MTB DNA was carried out as a 25 µl reaction, using 12 µl of MTB complex super mix R1, 2.5 µl of Magnesium solution R2 and 0.5 µl of Internal control IC 1 R3 and 10 µl of aqueous humour DNA. The amplification was carried out at an initial denaturation at 95 °C for 10 minutes, followed by 45 cycles of 95 °C for 15 seconds, 60 °C for 20 seconds, 72 °C for 15 seconds. The
quantitation analysis for the internal control and M. tuberculosis was carried out using JOE (yellow) and FAM (green) channel. The copy number of mycobacterium tuberculosis was expressed in copies per ml of DNA.

**Description of research work**

Stage 1: A detailed history was obtained and ophthalmic evaluation done. The subtype of serpiginous choroiditis was confirmed. Ancillary investigations were performed.

Stage 2: After diagnosis laboratory investigations on the blood and aqueous were performed.

Stage 3: Standard preparation for obtaining 0.1ml of aqueous humour required topical anaesthesia, 5% povidone iodine, sterile lid speculum, insulin syringe and a 26G needle. The procedure was performed with the patient in the supine position. After obtaining informed consent, anterior chamber paracentesis was performed and the sample sent for polymerase chain reaction. Real time PCR was performed on a subset of 28 eyes as economic constraints did not permit this test for all
patients. Nested PCR was performed on 3 eyes in whom PCR results were negative although clinical suspicion was very high.

No complications occurred after the procedure in any of the patients.

**Research design-experimental (control groups are used):** It is a longitudinal follow-up study design where all serpiginous choroiditis cases were followed up for a period of at least 1 year.

**Sampling procedure:** Patients with serpiginous choroiditis and multifocal choroiditis suspicious of TB attending the outpatient clinic at SankaraNethralaya from August 2009 to June 2013 were analysed.

**Sample size:** 126 patients and 27 controls