Symposium

Retina and the tubercle *Bacillus*: Four decades of our journey and current understanding

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Tuberculosis continues to be a major pandemic with enormous public health implication. Involvement of ocular tissues in the form of tubercles, tuberculomas, panophthalmitis, and iris granulomas are well recognized as definitive manifestations of tuberculosis. For these lesions, confirmatory evidence is available in the form of demonstration of acid-fast *Bacillus* on Ziehl–Neelsen staining. For other retinochoroidal disorders such as central serous chorioretinopathy, retinal vasculitis, and presumed ocular tuberculosis, hard evidence about the role of *Mycobacterium tuberculosis* is lacking. In this review, work done at our center over the past four decades in the form of experimental animal studies, nucleic acid amplification assays and clinical studies regarding the above retinochoroidal pathologies and the tubercle *Bacillus* is presented. It is possible that revisiting experimental animal studies may be a way forward in the current scenario of ambiguity about the cause–effect relationship between *M. tuberculosis* and few of the retinochoroidal disorders.

Key words: Animal models, retina, tubercle bacilli, tuberculosis, uveitis, vasculitis

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It is very likely that tuberculosis has always been part of humankind from the very beginning of existence. Historical evidence for this parasitic association exists only for the past 4000 years, largely by the discovery of tubercular decay in the skeletons of Egyptian mummies.[1] In the middle ages, it was called "consumption" or "white plague" and Hippocrates notes tuberculosis as being the most widespread and fatal disease of his era.[2] The disease is believed to have its origins in Western Europe from where it spread to other regions of the world because of colonization.[3] Between 1700 and 1900 AD, about 1 billion people are recorded to have succumbed to this disease. The causative organism remained unrecognized until the evening of March 24, 1882, when Robert Koch announced his discovery of the *Bacillus*, *Mycobacterium tuberculosis*. This is what he had to say on that evening "If the importance of a disease for humankind is measured by the number of fatalities it causes, then tuberculosis must be considered much more important than those most feared infectious diseases, plague, cholera, and the like. One in seven of all human beings dies from tuberculosis." The story does not seem to have changed much even today, wherein it continues to be the number one infectious cause of human mortality globally, having overtaken human immunodeficiency virus-associated deaths. In 2015, there were 10.4 million new TB cases worldwide with 60% of the burden in six countries, India, South Africa, China, Nigeria, Pakistan, and Indonesia.[4] Tuberculosis has been difficult to eradicate worldwide owing to the abysmal attitude of people and their governments in most developing countries toward civic sense and the health benefits of maintaining a hygienic environment. The battle against tuberculosis is also made difficult by the intrinsic nature of the *Bacillus* itself: slow replication time, resistance to phagocytosis, intracellular persistence, lipophilic outer wall which makes penetration of drugs very difficult, and the ability to mutate and develop resistance to multiple drugs.

Tuberculosis is a chronic granulomatous disease that is caused by the acid-fast *Bacillus* (AFB), *M. tuberculosis*. One-third (about 2 billion) of the world population is said to be infected with this *Bacillus*, making it one of largest pandemics.[5] Infection is normally acquired by aerosol inhalation and so the primary site of the disease is the lungs. Of those infected, 90% manage to overcome the effects of the infection by a robust local immune response.

The existence of tubercular *Bacillus* in the dormant form within the tissues is known as latent tuberculosis. Nearly 5%–10% of patients with latent tuberculosis can develop reactivation tuberculosis in their lifetime.[6] The WHO identifies two categories of pulmonary and extrapulmonary tuberculosis, severe and nonsevere. Pulmonary tuberculosis is relatively easier to diagnose because of characteristic clinical and radiologic features and the availability of adequate sputum specimen to detect the *Bacillus* by microscopy and culture or identify its DNA by standard tests such as GeneXpert.

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In contrast, the diagnosis of extrapulmonary tuberculosis is extremely challenging owing to the protean manifestations and lack of distinct clinical features.[7] This has forced researchers to study and make available alternate tools with high sensitivity and specificity based on which clinicians can make a more accurate diagnosis of extrapulmonary tuberculosis. However, a diagnostic tool that can be designated as the gold standard for accurately identifying extrapulmonary tuberculosis remains elusive.

Extrapulmonary tuberculosis develops in 15%–20% of patients with tubercular infection.[8] Extrapulmonary tuberculosis develops from hematogenous dissemination of the organism from the lungs to various other organs of the body, including the eye and orbit. Over the past century, published literature alludes to several forms of ocular involvement in tuberculosis with most of these being either case reports or small case series. Between 1869 and 1993, there were only forty cases of histologically confirmed intraocular tuberculosis in literature.[9] Because of this and the lack of high level of evidence, most drafts on tuberculosis, including the one from WHO, fail to mention the eye as a site of extrapulmonary tuberculosis. With the recent release of the index guidelines on extrapulmonary tuberculosis, wherein ocular tuberculosis is considered under a separate section, there is likely to be greater thrust and debate on the topic.[10]

Tubercles and tuberculoma are the most characteristic lesions of intraocular tuberculosis, and the tubercular etiology of these is beyond doubt. The majority of patients with these lesions also have unequivocal tubercular lesions elsewhere in the body, and so these lesions will not be discussed further in the manuscript. The focus of this manuscript is on the associations that have been made in the past and currently between some retinochoroidal diseases and the tubercle *Bacillus*, these include central serous chorioretinopathy (CSCR), retinal vasculitis, and uveitis. This association between *M. tuberculosis* and retinal and uveal pathologies has also been evaluated at our center for more than four decades using experimental animal models, nucleic acid amplification tests (NAATs), and clinical studies. An effort has been made to encapsulate and highlight the background and observations made during these studies. The relevance of revisiting experimental animal studies in the current era, to better understand the pathogenetic relation between the tubercle *Bacillus* and retina-choroid, is emphasized.

Animal Studies on Experimental Tuberculous Maculopathy

CSCR was first described by von Graefe as an inflammatory pathology. In 1918, Masuda strongly blamed tuberculosis as the etiology of this disorder as they found tuberculin reaction to be strongly positive in many cases.[11-13] They also considered the small yellow spots seen in this condition to be sites of exudation. There were other reports claiming definite response to antitubercular treatment in the 1980s. Owing to the popularity of antitubercular treatment in cases of CSCR despite clinching evidence, a study on a reproducible animal model was carried out. An earlier animal study using rabbit eyes conducted at our center was not carried forward as rabbit eyes were reported to be markedly different from human retina, one major difference being the absence of macula. In addition, rabbits have been found to be relatively more resistant to *M. tuberculosis* infection compared to monkeys and guinea pigs. Experiments on rhesus monkey eyes are considered ideal for the study of macular lesions as, like humans, they have a pure cone fovea and central avascular central area. The macula in monkey eyes is identified as an ill-defined, yellow, capillary-free zone located temporal and slightly below the center of the optic nerve head [Fig. 1a]. On histopathology, the rhesus monkey retina is multilayered with similar architecture [Fig. 1b]. In 1975, Hayreh reported that the end arterial nature of the choroid vessels and the lobular pattern of the choriocapillaris made the choroid particularly vulnerable to inflammatory, metastatic, and degenerative lesions.[14] Watershed zones, prone to ischemic changes, were also believed to run through the macula or within a close range. Before the study undertaken by Tewari HK (HKT) *et al*. at the center, reports of fluorescein angiographic (FA) studies on rabbit eyes and monkey eyes existed.[15,16]

Various techniques of producing experimental lesions in the fundus had also been described. Vogel described the suprachoroidal approach using which he studied injection of India ink, suspension of beryllium particles, tubercle *Bacillus*, other bacterial suspensions, and malignant cells. For producing tubercular lesions, suspensions of 200 bacilli per high-power field prepared by turbidimetric method was injected.[17] In 1968, Nozik and O'Connor used a similar approach to produce experimental toxoplasma retinochoroiditis.[18] In 1973, Mohan *et al*. from our center used a modified suprachoroidal technique to study presumptive amoebic uveitis.[19-21] In 1982, Culbertson *et al*. described producing experimental toxoplasma retinochoroiditis using the nasal transvitreal approach.[22] This technique was simple to accomplish but suffered from the higher risk of direct retinal trauma and postinoculation vitreous haze.

The animal model used by Tewari *et al*. (henceforth termed Hem Kumar Tewari-Rajendra Prasad Centre (HKT-RPC) model) to study experimental tuberculosis is now described in-depth.[23] The results and the relevance of this landmark study to help improve the current understanding of the still enigmatic association between tubercle *Bacillus*

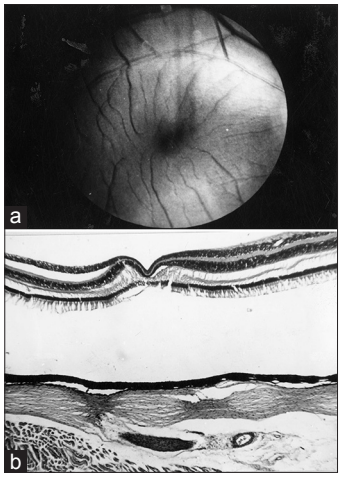

Figure 1: (a) Fundus photograph of rhesus monkey (right eye) showing foveal area located temporal to the optic nerve head (like human eye). (b) H and E stain of a section of the rhesus monkey eye passing through the macula. Foveal pit and multilayered ganglion cells are seen (×100)

and certain retinochoroidal pathologies are highlighted. As mentioned earlier, rhesus monkey (*Macaca mulatta*) was chosen as the experimental animal because of similarity in microanatomy of human and monkey macula and past description of successful production of tubercular lesions identical to that seen in humans. Rhesus monkeys of average weight (3.5 kg), no obvious systemic infection, and normal fundus on dilated examination were studied. Tuberculin test was also performed before start of the study. Injection of 0.1 ml of purified protein derivative (intraperitoneal [IP]) with 100 tuberculin units/ml was administered into the upper lid. The site was then observed for 48 h for any reaction. Interpretation was made according to guidelines set by the primate facility at the All India Institute of Medical Sciences (AIIMS) - no reaction (Grade 0), erythema with or without edema (Grade +), edema with ptosis (Grade ++), complete ptosis (Grade +++), and complete ptosis with marked edema (Grade ++++). Only monkeys with reaction below grade + were taken up for the study.

A pilot study was first undertaken in three monkey eyes to standardize the surgical technique of injection and the dose of inoculum. Injection using the nasal transvitreal route resulted in direct retinal trauma and endophthalmitis and so this technique was not considered. Instead, a modified trans-scleral, submacular suprachoroidal injection was tried and found to be satisfactory for the study. Injection paraldehyde IP (1 ml/kg) was used for anesthesia. The total calculated dose was injected at two sites (gluteal area and upper arm) to prevent tissue necrosis. As paraldehyde reacts with plastics, only glass syringes were used for giving the injection. Asepsis of the surgical site was achieved using mercurochrome paint. Lateral canthotomy was done after placing a lid speculum. Limited conjunctival peritomy was made to enable isolation and temporary disinsertion of the lateral rectus muscle. Then, the inferior oblique muscle was identified and its insertion was carefully traced. Anterior end of its insertion was found to be about 9 mm behind the midpoint of lateral rectus insertion. Then, a point just behind insertion of the inferior oblique muscle was marked on the sclera. At this point, under the operating microscope, the sclera was punctured carefully using a 27-gauge needle mounted on a tuberculin syringe. The needle was carefully advanced until the scleral resistance gave way, whence it was withdrawn. Through the same opening, a 30-gauge needle (with blunted tip) is inserted first vertically, then tangentially into the globe for about 0.5 mm. Slowly 0.075cc of saline of suspension is injected without altering the orientation of the needle. The needle was withdrawn and the site was compressed for 2 min using a cotton applicator. The globe was then brought back to primary position and ophthalmoscopy was performed to confirm creation of a dark gray elevated area in the macular region. Reinsertion of lateral rectus muscle was followed by final closure of the peritomy. By this method, a lesion primarily in the macular region was produced. Since vitreous was not disturbed, unhindered documentation of the retinochoroidal changes was possible using both ophthalmoscopy and FA.

H37Rv strain of live tubercle bacilli grown of Lowenstein–Jenson medium, sensitive to streptomycin was obtained from Department of Microbiology (AIIMS) and used in the study. Desired focal lesion at the macula could be created using a dose of 0.3 mg/ml. For dead inoculum, suspension of organisms was kept in boiling water bath for 30 min. Smear was made and stained by Ziehl–Neelsen (ZN) method to confirm the absence of any live bacilli.

FA was performed by injecting 1 ml of sodium fluorescein dye (20%) through the cannulated femoral vein. Eyelids were kept open using a lid speculum, and fundus images during the arterial, arteriovenous, and venous phase were captured using Zeiss fundus camera. FA was carried out at 48 h, day 7, day 14, and day 30.

Twelve rhesus monkeys were selected for the study and allotted to three groups: Group 1 (control group, n = 3), Group 2 (dead bacilli injection, n = 3), and Group 3 (live bacilli injection, n = 6). Each of the three groups was injected with 0.075cc of sterile normal saline alone, suspension of 0.3 mg/ml dead bacilli in 0.075cc of normal saline, and suspension of 0.3 mg/ml of live bacilli, respectively. Monkeys in Group 3 were further subgrouped into those receiving injection streptomycin (n = 2), injection dexamethasone (n = 2), and no treatment (n = 2).

All eyes were enucleated at the end of the planned experiment period and fixed in 10% formalin fixative. After 48 h, sections (to involve the macular area) were made and stained with both hematoxylin and eosin (HE) and ZN stain. In the past, obtaining histopathological sections through the macular area in enucleated specimens had been challenging for ocular pathologists. Hence, in this study, a novel modification of leaving behind the insertion stump of inferior oblique during enucleation was adopted. Tissue sectioning within 1 mm of the inferior oblique insertion helped in obtaining microscopic details through the macula.

During the follow-up period, it was found that all three groups had elevation of the macular area immediately after the injection. In Group 1 eyes, there was persisting elevation in the macular area at 48 h and rest of the vitreous and fundus appeared unremarkable. By day 7, the macular elevation had regressed. It had completely disappeared by day 14. No abnormality was observed on FA and histopathology. In the dead *Bacillus* group (Group 2), at 48 h, there was persisting macular elevation along with overlying vitreous haze. On FA, multiple, small hyperfluorescent lesions were observed [Fig. 2a]. There was no systemic change in the animal. At day 7, FA showed persistence of the lesions with more pronounced hyperfluorescence. Histopathology showed serous detachment at the macula with clumps of polymorphonuclear cells on HE staining [Fig. 2b]. No AFB was seen on ZN staining. By day 14, the media had cleared significantly. Multiple, small lesions with well-defined margins were seen above the fovea. Thirty days after injection, four small well-defined chorioretinal scars with adjacent mild pigment clumping was noticed, indicating signs of evolving regression. No AFB was seen on ZN staining.

In the live *Bacillus* injection group (Group 3), vitreous haze over the region of macular elevation was seen at 48 h. A single irregular hyperfluorescent lesion was noted on FA [Fig. 3a]. By day 7, the haze had worsened but two lesions with fluffy margins were evident. General examination of the animal was normal. One eye was subjected to histopathological examination (HPE) at this stage and AFB was seen in the retina and choroid. Massive chorioretinal reaction with lymphocytes and giant cells was noted on HPE [Fig. 3b]. Following streptomycin injection, (Group 3a), clearing

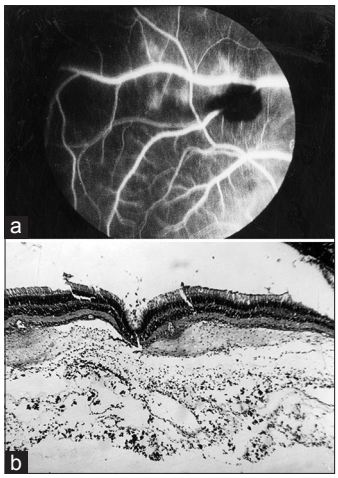

Figure 2: (a) Fluorescein angiographic image of eye following injection of dead *Bacillus* shows four hyperfluorescent lesions. (b) H and E stain of macular area 7th day after injection of dead bacilli shows presence of polymorphs and lymphocytes (x100)

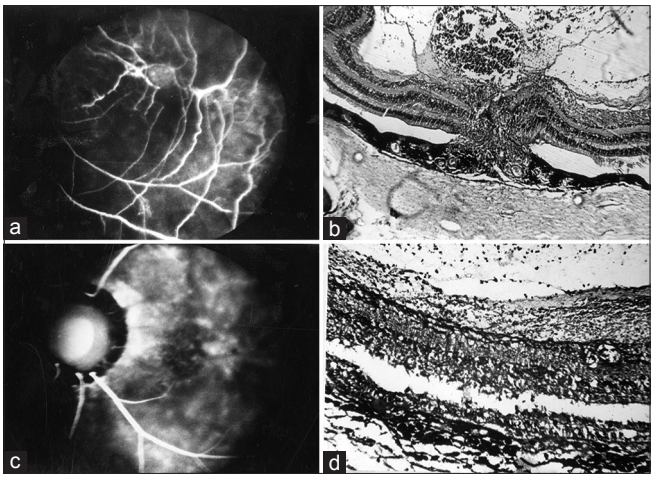

Figure 3: (a) Fluorescein angiography showing 7th day of lesion produced by live bacilli injection. Solitary hyperfluorescent lesion can be noted superior to the foveal center. (b) H and E stain of a section through the macula 7th day after injection of live bacilli. Massive chorioretinal reaction with lymphocytes and giant cells is seen (×100). (c) Fluorescein angiography following live bacilli injection and systemic dexamethasone 20th day. Hyperfluorescent areas have increased in number and intensity. (d) H and E stain showing chorioretinal scar and fibrous proliferation, with lymphocytes 45th day following live bacilli injection (x100)

of media and resolution of lesion was seen by day 30. Streptomycin injection was given intramuscularly at the dose of 30 mg/kg daily for 30 days. HPE showed few giant cells and lymphocytes. No caseation was noted and no AFB was seen. Following dexamethasone phosphate 100 mg intravenous injection (Group 3b) for 20 days, both vitreous haze and number of lesions had increased [Fig. 3c]. No worsening of general condition was seen. AFB was demonstrable on ZN staining of HPE specimen which also showed lymphocytes and giant cells. Forty-five days after injection, chorioretinal scar was noted and confirmed on HPE [Fig. 3d]. In Subgroup 3c (no treatment), lesions were most intense at day 7. Gradual resolution was observed until the end of the study period, and unlike the dexamethasone group, there was no exacerbation. The rate of lesion resolution was however slower than streptomycin group.

In summary, this study using the HKT-RPC model of experimental tuberculous maculopathy demonstrated that lesions were not related to the trauma of injection, lesions produced by injection of dead *Bacillus* was early in onset and there was early spontaneous healing. Lesions produced by injection of live bacilli had late onset and late healing if it was not treated with Streptomycin. In addition, dexamethasone injection worsened the severity and duration of the lesion [Fig. 4]. Another important observation was the demonstration of lymphocytes, giant cells, and AFB on HE and ZN staining, respectively.

Nucleic Acid Amplification Tests

One study was carried out using polymerase chain reaction (PCR) (serum ribosomal nucleic acid method for IS6110) for *M. tuberculosis*, in patients with anterior uveitis (granulomatous and nongranulomatous) and multifocal choroiditis in 1998, uniformly negative results were seen in both cases ($n = 30$) and controls ($n = 10$).[24] One of the reasons for having a negative result in all samples was the presence of PCR inhibitors in tissue fluid. More recently, it has been reported that IS6110 positivity is significantly lower in the Indian scenario compared to the immunogenic MPT64 protein. A subsequent case–control study in patients with Eales disease ($n = 31$) using vitreous samples, studied the presence of the highly immunogenic protein of *M. tuberculosis*, MPT64 using PCR.[25] This study did not reveal a statistically significant difference ($P = 0.058$). However, 50% of epiretinal membranes which were also studied during this study were positive for MPT64 protein. As with most PCR-based reports from our country, final interpretation was done using electrophoretic method.

Clinical Studies

Over a 2 year period, we analyzed patterns of uveitis presenting to our center using a prospectively enrolled database. In this study, we found 5% of our patients having uveitis in association with past or current definitive diagnosis of pulmonary or extrapulmonary tuberculosis.[26] We are yet to consider a diagnosis of presumed ocular tuberculosis as a distinct entity and do not treat patients with antitubercular drugs due to several reasons. A few of these being lack of Level 1–Level 2 evidence, suggested clinical, serological (nucleic acid amplification assays and interferon gamma release assays [IGRAs]) and tissue (aqueous and vitreous) approaches having several limitations and unanswered questions, known side effects of antitubercular drugs (14.1%) and concerns of promoting development of drug-resistant strains. In addition, 25% of patients have been reported to have relapses despite taking a full course of antitubercular treatment, and these patients responded well to an increase in the dose of corticosteroids or immunosuppressants.[27] Hence, in terms of recurrences too, these results suggest that long-term remission can be achieved with comparable efficacy using steroids (within physiological maintenance dose) and immunosuppressants alone. Reports available in literature showed that a statistical reduction in the recurrence rates with the concurrent use of anti-tuberculosis treatment (ATT) is largely confined to anterior uveitis. Whether the benefit of using ATT in anterior

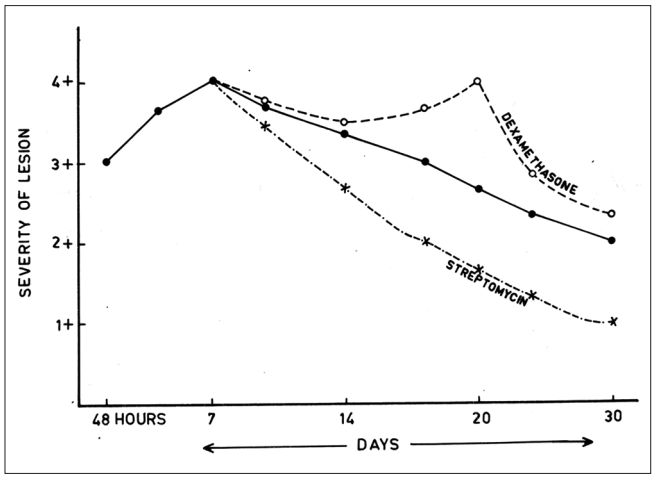

Figure 4: Graph comparing the clinical course of three subgroups of lesions produced by live bacilli injection

uveitis outweighs the other concerns needs a more detailed and independent evaluation.

In most studies on presumed ocular tuberculosis including serpiginous choroidopathy, tuberculin skin test (TST) and IGRAs have been recommended as important tools for diagnosis.[28-32] Hence, in the second study in fifty patients with varied forms of serpiginous choroidopathy in whom results of TST and IGRA were available, we looked at the results retrospectively. IGRA and TST positivity was seen in 60% and 56% respectively. However, only 38% of patients showed positive results for both TST and IGRA and the agreement between the two tests was found to be poor (0.2) (manuscript accepted for publication in NMJI, September 2016). Similar discordance has been reported in literature.[33] Hence, how should one manage a patient with positive TST and negative IGRA and vice versa? This becomes a clinical dilemma particularly when there are no facilities for undertaking NAAT-based tests and the guidelines on when to perform aqueous and vitreous tap in such patients is not clearly laid out.

To see if commonly performed tests for tuberculosis, TST, and chest radiography (CXR) was different in patients with serpiginous choroidopathy, we conducted another study evaluating the results of these tests in three groups of patients-serpiginous choroidopathy (n = 40), nonserpiginous, nonpresumed ocular tuberculosis uveitis (n = 40) and a noninflammatory retinal pathology, CSCR (n = 40).[34] The percentage TST positivity in the three groups was 58%, 40%, and 43%, respectively (P = 0.237), and the percentage of patients showing lesions on CXR was 10%, 12.5%, and 7.5%, respectively (P = 0.727). So again, these observations suggest that TST and CXR cannot be used as evidence for making a diagnosis of presumed ocular tuberculosis.

Current Understanding

M. tuberculosis is known to disseminate hematogenously and so, theoretically, it can produce retinal and choroidal diseases. However, beyond the presence of choroidal tubercles and tuberculomas, is their unflinching evidence that it produces the more frequently associated conditions like uveitis and vasculitis? The answer is no. Finding an answer has been difficult owing to a difficult gold standard (culture) with which to compare results, widespread presence of latent and active tuberculosis in endemic countries, slow-growing and paucibacillary nature of *M. tuberculosis*, inability to safely obtain adequate tissue sample, lack of tests to identify active from latent disease, lack of concordance between available tests, high reliability on commercial PCR and in-house PCR-based results which are highly prone to contamination and poor positive results when using the WHO recommended fully automated, rapid PCR (GeneXpert).[35-38] The solution may be to go back to using animal models like the HKT-RPC model described herein and try to understand the actual tissue and immune interaction between *M. tuberculosis* and inner layers of the eye. Transfection experiment is another option that needs to be explored. Using these methods, it may also be possible to fulfill Koch's postulates for cause–effect relationship with respect to an infectious etiology or an identical *M. tuberculosis* immunity-effect relationship. Hopefully, in the coming decade, revisiting experimental studies using appropriate animal models will help finally solve the enigma of uveitis and retinal vasculitis associated with tuberculosis.

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Conflicts of interest

There are no conflicts of interest.

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