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Tubercular DNA PCR of ocular fluids and blood in cases of presumed ocular tuberculosis: a pilot study

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Abstract

Background: The definitive diagnosing of ocular tuberculosis (TB) is difficult; therefore, there is a need of better understanding of investigating TB DNA in presumed ocular TB patients.

Objectives: The aim of this study is to correlate tubercular DNA PCR of aqueous/vitreous and blood in cases of presumed ocular TB.

Design: A prospective study.

Methods: DNA was extracted from aqueous of cases of choroidal tuberculoma (group 1) and serpiginous choroiditis (group 2) and from vitreous of cases of vasculitis (group 3) and macular hole/retinal detachment (group 4). Gel-based PCR and real-time PCR amplification were performed using IS6110 primer on ocular fluids. The same was also performed on the blood samples of cases in which tubercular DNA was detected in the ocular fluids.

Results: Overall, 31 cases were analysed in our study. Tubercular DNA was detected in ocular fluids of seven cases: group 1, two cases (67%); group 2, one case (17%); group 3, four cases (27%); and no case of group 4. Blood samples of six of these seven patients were positive for tubercular DNA. Of these six patients, four had evidence of systemic TB and were on ATT. Two cases had no evidence of active systemic TB, yet PCR was positive from blood and ocular fluids.

Conclusion: Tubercular DNA detected from ocular fluids may possibly be due to bystander DNA and may not indicate primary ocular tubercular infection. Thus, caution must be exercised prior to labelling a case of uveitis as being tubercular based on the results of molecular assays on ocular fluids alone. The results of PCR on ocular fluids should be correlated with PCR on blood and systemic findings.

Keywords: choroidal tuberculoma, Mantoux, polymerase chain reaction, serpiginous choroiditis, vasculitis

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Introduction

Mycobacterium tuberculosis can infect almost all ocular tissues. It is well accepted that choroidal granulomas can be tubercular and need to be treated with a combination of anti-tubercular therapy and steroids.[1] Many authors have also suggested a link between tuberculosis (TB) and serpiginous choroiditis[2] and retinal vasculitis.[3,4] Hutchinson described the occurrence of serpiginous like choroiditis in a case of systemic TB way back in 1900.[5] However, he also described the same in a case of syphilis and in another case with no known systemic disease. Similarly, retinal vasculitis is seen in cases of systemic TB,[6] but it is also seen in cases without any evidence of TB at all. Thus, it is debatable whether serpiginous choroiditis and retinal vasculitis are manifestations of active tubercular infection. Studies that demonstrate the presence of tubercular deoxyribonucleic Acid (DNA) in ocular fluids of cases with

serpiginous choroiditis[2] and retinal vasculitis[3] point towards a tubercular aetiology. However, it is now well known that detection of DNA alone cannot differentiate between viable and non-viable tubercular bacilli.[7] Also, tubercular DNA may still be found in macrophages even 500 days after resolution of the active infection.[8] Because of the wide spectrum of presentations and non-feasibility of uveal biopsy for culture and microscopic examination, diagnosing ocular TB is quite difficult.[9] Therefore, it is only presumptive. Recently, studies have investigated the application of polymerase chain reaction (PCR) on blood to diagnose TB.[10–12] Performing quantitative PCR (qPCR) from blood may be more easily performed and minimize ocular tissue manipulation. Therefore, it can also serve as a valuable alternative to assist the diagnosis of ocular TB.

Material and methods

Study design

This study included 37 patients, which was divided into four groups as follows – group 1: patients with established tubercular posterior uveitis in the form of a choroidal tuberculoma with evidence of systemic TB ($n = 3$); group 2: patients with presumed tubercular uveitis consisting of cases of serpiginous choroiditis, multifocal serpiginous choroiditis or serpiginous-like choroiditis ($n = 7$); group 3: patients with retinal vasculitis undergoing vitrectomy for non-resolving vitreous haemorrhage ($n = 18$); group 4: patients undergoing routine vitrectomy for retinal detachment/macular hole as controls ($n = 9$).

Clinical evaluation

All the patients underwent a detailed anterior and posterior segment evaluation. The patients were screened for systemic tubercular infection with a chest X-ray [contrast-enhanced computed tomography (CECT) of the chest was performed based on chest X-ray report or systemic symptoms] and Mantoux test (1TU Intradermal, taking a reading of $\geqslant$10 mm in duration as positive for latent/active TB). The diagnosis of active pulmonary TB was established by pulmonologist based on imaging sputum or bronchoscopic biopsy/fine needle aspiration cytology (FNAC) analysis. These were patients on anti-tubercular therapy (ATT) started by pulmonologists following a confirmed diagnosis of TB.[13] Aqueous sample was drawn from cases of groups 1 and 2, and undiluted vitreous sample was drawn from cases of groups 3 and 4 in the operation theatre. Under all aseptic precautions, the anterior chamber was punctures at the limbus with a 30G needle attached to a 2-ml syringe. Gentle suction was given to collect 50–100 µl of aqueous. Care was taken to avoid the needle touching the lens. These cases were only subjected to PCR.

Sample collection

Ocular fluids such as aqueous and vitreous samples were collected and subjected to DNA extraction in the Department of Ocular Pathology, Dr. R. P. Centre for Ophthalmic Sciences, AIIMS. Samples were usually sent immediately to the laboratory after collection and kept refrigerated at 4°C during the DNA extraction process. Mean for sample processing was 15.65 ± 5.5 h. Blood samples were also collected from patients in whom tubercular DNA was detected in ocular fluids.

DNA extraction

DNA was extracted from 100 µl of aqueous, 200 µl vitreous and 200 µl of whole blood samples using the QIAmp mini kit (Qiagen, Chicago, IL, USA), following manufacturer's instructions. Genomic DNA isolated from *M. tuberculosis* was used as a positive control. All PCR reactions were carried out in designated sterile laminar hood to minimize the possibility of cross DNA contamination within the samples. A vial containing 200 µl of ultrapure autoclaved water was used as a negative control. Purified nucleic acids were eluted in a 20-µl elution buffer and stored at –20°C until nucleic acid amplification. The quantity and purity of DNA was assessed using Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). A260/A280 ratio of 1.8:2.0 was taken as an indicator of high purity.

DNA PCR amplification (gel-based assay)

DNA PCR amplification was performed using primer IS6110 (123 bp product). The primer sequence used was 5′-CCTGCGAGCGTAG GCGTCGG-3′ (forward primer) and 5′-CTC GTCCAGCGCCGCTTCGG-3′ (reverse primer).[14,15] The 10 pmol primer concentration was used for this reaction. A 25-µl PCR reaction mixture for DNA amplification was made using Platinium™ PCR SuperMix (Invitrogen, California, USA), and 3 µl of purified DNA extracted from the

samples was added to the PCR mixture. PCR amplification was carried out in an Applied biosystem 2720 thermocycler (Invitrogen, CA) as follows: 94°C for 2 min, 40 cycles of 94°C for 2 min, 68°C for 1 min, 72°C for 1 min and a final extension at 72°C for 10 min. Amplicons were resolved by electrophoresis in 2% agarose gels stained with 0.1 ml of 10 g/l ethidium bromide per millilitre. Amplified DNA bands were photographed with the Gel Doc XR system (Biorad, Cambridge, MA, USA). As a positive control, 1 ng of purified DNA extracted from *M. tuberculosis* was added to the PCR mixture. Sterile ultra-purified water with no template was used as negative control. Negative control (NTC) containing all components except template DNA was included in each PCR reaction. PCR reaction was repeated thrice for every sample to confirm the reproducibility of our results.

DNA purification and DNA sequencing

DNA purification was performed using GeneJET Gel Extraction Kit (Thermo Scientific, K0691 and K0692) as per manufacturer's protocol. PCR products were run on 0.8% agarose gel in either TAE or TBE buffers and visualized under ultraviolet illumination. The DNA fragment of interest was excised as close to the DNA as possible to minimize the gel volume using a clean scalpel and placed into a 1.5-ml centrifuge tube. Binding Buffer and agarose gel slice were used in 1:1 volume ratio and incubated at 50–60°C for 10 min or until the gel slice was completely dissolved. After the incubation period, solubilized gel solution (800 µl) was transferred to the GeneJET purification column and centrifuged for 1 min. Additional 100 µl of Binding Buffer was added to the column to use the purified DNA for DNA sequencing. The flow through was discarded, and the column was placed back into the same collection tube after 1 min centrifugation. Wash buffer (700 µl) diluted with ethanol was added to the GeneJET purification column and centrifuged for 1 min. Again, the flow through was discarded and an empty GeneJET purification column was centrifuged for 1 min to remove residual ethanol in the purified DNA solution. Finally, 50 µl of the elution buffer was used to elute out the purified DNA into a clean 1.5 microcentrifuge tube and stored at –20°C for further experiments.

DNA sequencing of PCR-purified products of the IS6110 sequence were processed to avoid any false positivity in the DNA PCR-positive cases. This was performed using Big Dye Terminator V.3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and loaded on a DNA sequencer (ABI 3730 XL; Applied Biosystems). The chromatogram graph and sequences were obtained and analysed using the search algorithm BLAST from the National Center for Biotechnology Information (NCBI) server (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Quantitative real-time PCR (qRT-PCR)

RT-PCR was performed to analyse the ocular fluids and blood in all samples, and was performed on Applied Biosystem Step ONE plus RT-PCR system. Reaction mixture contained 0.5 µl of 10 pmol of forward primer, an equal volume of 10 pmol of reverse primer, 2 µl of DNA, 5 µl of SYBR green master mix (Thermo Scientific, USA) and 13 µl of nuclease-free water in a total reaction mixture of 20 µl. Amplification reaction included an initial denaturation step of 10 min incubation at 95°C, 40 cycles of denaturation at 95°C for 15 s, and annealing at 68°C each for 1 min. *M. tuberculosis* DNA-positive sample as control was used for the generation of the standard curve and positive controls.

Results

Amplification and melting curve analysis by qRT-PCR

Amplification signals were observed in seven ocular fluids and six blood samples. Melting curve analysis showed a single peak in the respective ocular fluid and blood samples (Figure 1(a) and (b)). A standard curve was generated under the above-mentioned amplification condition by plotting increasing dilutions of input *M. tuberculosis* DNA (ranging from 2.4 to 1.13×10^{10} genome equivalents) *versus* cycle quantification (Cq) and was used to determine the copy number (bacterial DNA load) in all samples (Figure 1(c)). In our study, a higher number of copies of bacterial genome were observed in ocular fluids in comparison with their respective blood samples. The details are described in Table 1.

Group 1 (choroidal tuberculoma with known systemic tuberculosis). We collected aqueous samples of three patients of choroidal tuberculomas with definite systemic TB. The mean age of the patients of this group was 24 years. Purity of DNA extracted in all these cases was high. Two of these

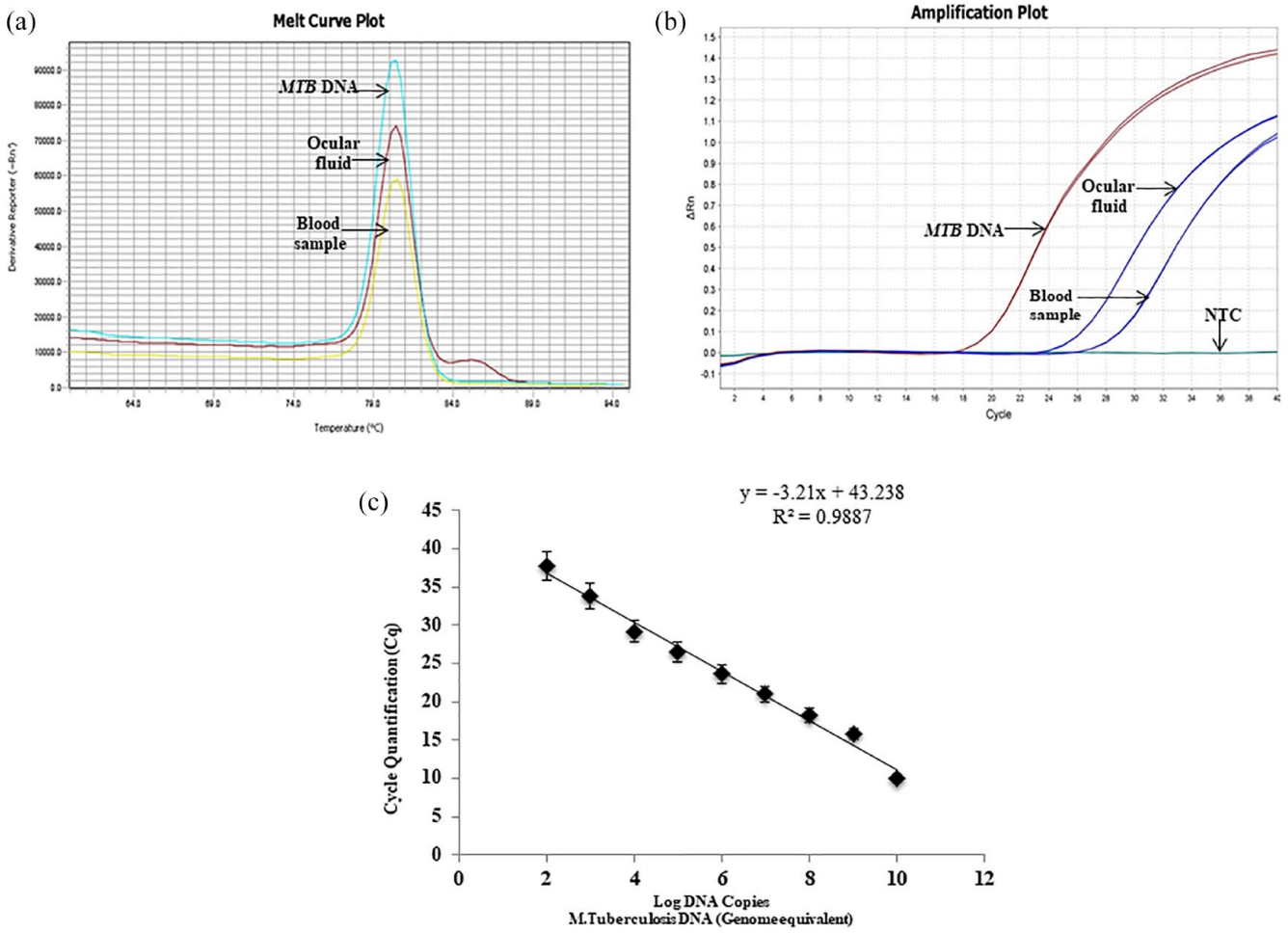

Figure 1. (a) Melting curve analysis of the ocular fluids and blood samples including MTB standard DNA as positive control indicating the specificity of the reaction product. (b) Amplification curve analysis of the ocular fluids and blood samples including MTB standard DNA as positive control. (c) qRT-PCR standard curve was generated using *M. tuberculosis* DNA over a range of 2.4–1.13×10^{10} genome equivalents. The standard curve data from nine independent experiments are shown. Cq (Cycle quantification) is plotted *versus* logarithm of the number of *M. tuberculosis* genome equivalents added to each tube at the start of the reaction.

patients had miliary TB with multiorgan involvement and one patient had pulmonary TB. One of these patients who had miliary TB had a Mantoux of zero, whereas the other two were Mantoux positive (66.67%). IS6110 DNA was detected in the aqueous of two of these patients (67%). IS6110 DNA could also be detected in the blood samples of the above two patients (Figure 2(a)).

Group 2 (serpiginous-like choroiditis). Aqueous samples of seven patients were collected in this group. Mean age of the patients of this group was 39 years. DNA of high purity was extracted from six of these samples. One of these patients had fibrotic bands on chest X-ray and gave a history of taking a full course of ATT 25 years back. The other five patients had normal chest X-ray and no history of past or present systemic TB. Four (67%) out of these six patients were Mantoux positive. IS6110 DNA was detected in the aqueous of only one (17%) of these patients. This patient was Mantoux positive but had a normal chest X-ray and did not give any history of past or present TB. IS6110 DNA was also detected from the blood of this patient despite there being no evidence of active systemic TB. The testing of blood and aqueous of this patient were performed at a different time altogether, taking all precautions to prevent cross-contamination.

Group 3 (retinal vasculitis). We collected vitreous samples of 18 patients of vasculitis. The mean age

Table 1. Clinical details of all the patients with their copy number of *M. tuberculosis* DNA in the aqueous, vitreous and blood samples in different diagnostic groups.

Diagnostic group	S. no.	Age/ Sex	Systemic disease	Mantoux (mm)	Aqueous (A)		Vitreous (V)		Blood (B)		DNA copies		DNA sequencing	
					IS6110 gene		IS6110 gene		IS6110 gene		Ocular fluid (A/V)	Blood		
					PCR (IS6110)	qPCR (Mean C_{TJ})	PCR	qPCR (Mean C_{TJ})	PCR	qPCR (Mean C_{TJ})			A/V	B
Group 1 (choroidal tuberculoma)	1	34/F	MTB (currently on ATT)	0	P	20.5	NA	NA	P	26.2	1.69×10^9	3.78×10^7	100%	100%
	2	14/F	MTB (currently on ATT)	25	N	ND	NA	NA	NA	NA	–	–	–	–
	3	24/F	PTB (currently on ATT)	30	P	21.4	NA	NA	P	26	9.30×10^8	4.32×10^7	100%	100%
Group 2 (serpiginous-like choroiditis)	4	35/F	No	18	N	ND	NA	NA	NA	NA	–	–	–	–
	5	27/F	No	15	P	24.3	NA	NA	P	27.5	1.34×10^8	1.58×10^7	100%	100%
	6	44/F	PTB (25 years ago)	38	N	ND	NA	NA	NA	NA	–	–	–	–
	7	45/F	No	4	N	ND	NA	NA	NA	NA	–	–	–	–
	8	50/M	No	0	N	ND	NA	NA	NA	NA	–	–	–	–
	9	35/F	No	14	N	ND	NA	NA	NA	NA	–	–	–	–
Group 3 (retinal vasculitis)	10	40/M	No	12	NA	NA	P	22.4	P	26.8	4.77×10^8	2.53×10^7	100%	100%
	11	30/M	No	13	NA	NA	N	ND	NA	NA	–	–	–	–
	12	25/M	LNTB (2 years ago)	12	NA	NA	N	ND	NA	NA	–	–	–	–
	13	20/M	PTB (currently on ATT)	16	NA	NA	P	20.7	P	23.5	1.48×10^9	2.29×10^8	100%	100%
	14	48/M	No	16	NA	NA	P	24.6	NA	ND	1.09×10^8	–	100%	–
	15	32/M	No	18	NA	NA	N	ND	NA	NA	–	–	–	–
	16	26/M	No	21	NA	NA	N	ND	NA	NA	–	–	–	–
	17	38/M	No	5	NA	NA	N	ND	NA	NA	–	–	–	–
	18	22/M	No	10	NA	NA	N	ND	NA	NA	–	–	–	–

(Continued)

Table 1. (Continued)

Diagnostic group	S. no.	Age/ Sex	Systemic disease	Mantoux (mm)	Aqueous (A)		Vitreous (V)		Blood (B)		DNA copies		DNA sequencing	
					IS6110 gene		IS6110 gene		IS6110 gene		Ocular fluid (A/V)	Blood		
					PCR (IS6110)	qPCR (Mean C_T)	PCR	qPCR (Mean C_T)	PCR	qPCR (Mean C_T)			A/V	B
	19	19/M	No	8	NA	NA	N	ND	NA	NA	–	–	–	–
	20	30/M	No	12	NA	NA	N	ND	NA	NA	–	–	–	–
	21	35/M	No	5	NA	NA	N	ND	NA	NA	–	–	–	–
	22	15/M	PTB, LNTB (currently on ATT)	14	NA	NA	P	20.8	P	25.2	1.38×10^9	7.36×10^7	100%	100%
	23	26/M	No	17	NA	NA	N	ND	NA	NA	–	–	–	–
	24	48/M	No	18	NA	NA	N	ND	NA	NA	–	–	–	–
Group 4 (retinal detachment/ macular hole as control)	25	64/F	No	12	NA	NA	N	ND	NA	NA	–	–	–	–
	26	60/M	No	19	NA	NA	N	ND	NA	NA	–	–	–	–
	27	71/M	No	18	NA	NA	N	ND	NA	NA	–	–	–	–
	28	50/M	No	17	NA	NA	N	ND	NA	NA	–	–	–	–
	29	55/M	No	11	NA	NA	N	ND	NA	NA	–	–	–	–
	30	46/F	No	10	NA	NA	N	ND	NA	NA	–	–	–	–
	31	28/F	No	13	NA	NA	N	ND	NA	NA	–	–	–	–

A, aqueous samples; ATT, anti-tubercular therapy; B, blood samples; LNTB, lymph node tuberculosis; MTB, miliary tuberculosis; N, negative; NA, sample not available; ND, not determinant; P, present; PCR, polymerase chain reaction; PTB, pulmonary tuberculosis; V, vitreous samples.

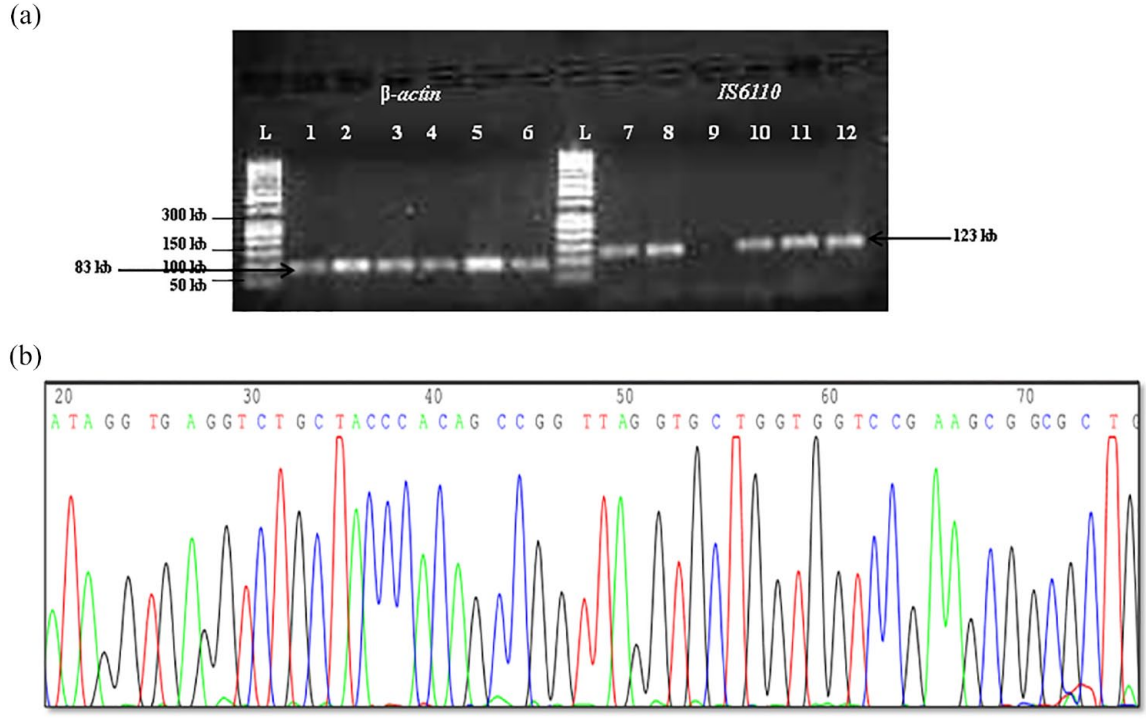

Figure 2. (a) Profile generated by β-actin and IS6110 PCR assay. (b) Sanger sequencing of IS6110 with MTB strain to confirm the specificity of PCR assay.
L, ladder; Lanes 1–6, ocular fluid cases with β-actin for quality check; Lanes 8, 10–12, positive result with IS6110 sequence in presumed tubercular uveitis cases; Lane 7, positive control; Lane 9, negative control.

of the patients of this group was 30 years. DNA of high purity was extracted from 15 of these samples. Moreover, 12 (80%) out of these 15 patients were Mantoux positive. Evidence of tubercular DNA was found in 4/15 (27%) of vitreous samples of these vasculitis patients. We could only assess the blood samples of three out of these four patients. IS6110 DNA was detected in the blood of all these three samples. Again, testing of blood and vitreous of these patients were performed at different times altogether, taking all precautions to prevent cross-contamination. Two of these three patients had active systemic TB and were on ATT. The third patient was Mantoux positive but did not have any current evidence of active systemic TB.

Group 4 (normal controls). Out of the nine patients, we extracted DNA of high purity from seven vitreous samples. The mean age of the group was 50 years. All these patients were Mantoux positive (100%). None of them had a history of active or past TB. The chest X-ray of all of these patients was normal. None of these samples were positive for IS6110 DNA. Details of all the data are mentioned in Table 1.

DNA sequencing. All the DNA PCR products of IS6110-positive clinical samples (both blood and ocular fluid) were further confirmed by Sanger sequencing to exclude the false-positive results. The PCR product was then purified and sequenced to confirm the mycobacterium TB strain. BLASTn search was used to assess for similarity and alignment score on http://blast.ncbi.nlm.nih.gov/Blast.cgi against a variety of available sequence database. This BLAST search showed 100% similarities with the specific strain of *M. tuberculosis* that had the IS6110 sequence in positive samples (Figure 2(b)). Cohen's kappa agreement (κ) was used to interpret our results of DNA detection from ocular fluids and blood of patients of presumed ocular TB, and found to be almost perfect agreement with κ value of 0.903 (Table 2).

Discussion

Establishing a definite diagnosis of ocular TB is difficult as the volume of ocular fluids that can be extracted for analysis is usually very minimal. In addition, the disease is said to be paucibacillary.[16] Application of mycobacterial DNA detection techniques thus appeals to ophthalmologists suspecting

Table 2. Cohen's kappa agreement (κ) between ocular fluids and blood PCR results.

Ocular fluid PCR	Blood PCR		kappa value	Standard error (SE) of kappa	95% confidence interval
	Positive (6)	Negative (25)			
Positive (7)	6	1	.903	0.095	0.716–1.000
Negative (24)	0	24			

PCR, polymerase chain reaction.

intraocular TB. There have been various studies evaluating the role of PCR for detection of TB DNA from ocular fluids in various uveitis entities such as retinal vasculitis[3] and serpiginous choroiditis.[2] The presence of viable tubercular bacilli has still not been conclusively demonstrated in these entities. Issues of false-positive PCR also remain unresolved, and different studies have used different primers. The most commonly used DNA primer is IS6110,[17] while some studies have also used *MPT64*[18] and *Protein b*.[19] However, based on the evidence from these studies, there is a trend towards treating TB DNA PCR-positive cases with ATT. Most of these patients are also concomitantly treated with steroids, and thus it is difficult to establish whether disease resolution is due to ATT or steroids.

We did not find TB DNA in any of our controls. TB DNA was found positive in 67% cases of choroidal granuloma (group 1) patients. TB DNA could also be detected from the blood of these patients. All these cases also had active systemic TB. In these patients, the concomitant positivity from ocular fluids and blood seems to correlate with the systemic and ocular findings. Only one patient of the serpiginous choroiditis group and four of the vasculitis group had evidence of IS6110 in their ocular fluids. We could test the blood of four of these five patients (one of group 2 and three of group 3), and we found that tubercular DNA could be detected from the blood of all these four patients as well. Out of these four cases, two had systemic TB. The remaining two cases were Mantoux positive, but did not have evidence of systemic TB. The absence of active systemic TB in these patients suggests that these patients may have had subclinical or latent TB in the past and the tubercular DNA fingerprint persists in their white blood cells.

A possibility of bystander tubercular DNA being detected from the ocular fluids arises if we look at the concomitant positivity of tubercular DNA from the blood and ocular fluids of our cases. The white blood cells of these patients might be carrying tubercular DNA which we were able to detect from blood and the ocular fluids. The white blood cells may have gained access to the ocular fluids, as these were eyes with either retinal vasculitis or choroiditis in which the blood ocular barrier is compromised. *M. tuberculosis* is known to readily invade macrophages.[20] *M. tuberculosis* DNA has also been detected from normal lung tissue[20] without any histological evidence of TB and from extrapulmonary locations without evidence of tubercular pathology in these sites.[21] Thus, latent organisms need not only exist in the classical primary foci of TB but also can spread widely and lie dormant without causing any clinical or subclinical disease. There are reports in literature where tubercular DNA has been detected years after therapy[22] and even after death as in Egyptian mummies.[23]

Looking at our findings, we recommend caution in analysing results of ocular fluid DNA analysis for making a diagnosis of ocular TB. These results taken in isolation can lead to over diagnosing ocular TB. Bajgai P *et al.*[24] have previously shown presence of TB DNA in subretinal fluid of cases of rhegmatogenous retinal detachment. These eyes did not have any uveitis or evidence of intraocular TB. This also suggests that mere detection of TB DNA from ocular fluids does not establish a causal relationship between the active ocular pathology and TB. Interpretation of the results of molecular assays to detect miliary tuberculosis (MTB) DNA from ocular fluids is thus challenging. Barik *et al.*[25] have offered a novel solution to rule out cases with bystander MTB DNA by normalizing the MTB DNA copy numbers to the host genome copy numbers. They propose a cut-off value of this ratio and suggest that cases having a higher ratio only have significant MTB DNA in ocular fluids to cause tubercular uveitis and cases with a lower cut-off value might be only having bystander MTB DNA. This appears to be a novel approach; however, it needs to be further tested in cases of

well-established ocular TB, and there is a possibility of the copy numbers in blood (5 litres) being disproportionately low compared with the copy numbers in the confined volume of aqueous (0.25 ml) and vitreous (4 ml).

Matos *et al.*[26] were the first group to report positive PCR results for *Mycobacterial* DNA in both vitreous and blood. Another group performed RT-PCR on aqueous, vitreous and blood samples of patients with infectious uveitis. They found that patients with presumed ocular TB, which had a negative PCR from ocular fluids, also had a negative PCR from blood. The authors attributed this to either a wrong clinical impression or low number of *Mycobacterial* DNA in ocular fluids and blood.[27] In our pilot study, we only collected blood samples of patients who showed positive PCR in their aqueous/vitreous sample. This is a limitation of our study, and in future larger studies PCR can perhaps be performed on blood samples of all cases.

Conclusion

To conclude, this study demonstrates concurrent detection of TB DNA by PCR in ocular fluids and blood samples of cases of choroidal tuberculomas, serpiginous choroiditis and vasculitis. Presence of TB DNA from blood of these cases in absence of any systemic evidence of active TB raises the possibility of bystander DNA. Further studies with a larger sample size on correlation of ocular fluid DNA analysis for TB, blood DNA analysis for TB, and systemic parameters may provide conclusive evidence on the clinical relevance of these molecular assays.

Declarations

Ethics approval and consent to participate

This study was approved by the Institutional Ethics Committee (IEC) of All India Institute of Medical Sciences (AIIMS), New Delhi, India (ethical clearance ref no: IEC-427/03/08/2018). All patients provided written informed consent.

Consent for publication

Not applicable.

Author contributions

Rohan Chawla: Conceptualization; Funding acquisition; Supervision; Validation; Writing – review & editing.

Mithalesh K. Singh: Investigation; Methodology; Validation; Writing – original draft; Writing – review & editing.

Lata Singh: Investigation; Methodology; Writing – review & editing.

Pooja Shah: Investigation; Supervision.

Seema Kashyap: Project administration; Supervision; Writing – review & editing.

Shorya Azad: Project administration.

Pradeep Venkatesh: Investigation; Methodology.

Seema Sen: Formal analysis; Visualization.

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Competing interests

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Availability of data and materials

Not applicable.

Disclosure

The author(s) have no proprietary or commercial interest in any materials discussed in this article.

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