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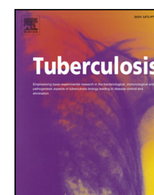

Normalised quantitative polymerase chain reaction for diagnosis of tuberculosis-associated uveitis

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ABSTRACT

Polymerase chain reaction (PCR)-based diagnosis of tuberculosis-associated uveitis (TBU) in TB-endemic countries is challenging due to likelihood of latent mycobacterial infection in both immune and non-immune cells. In this study, we investigated normalised quantitative PCR (nqPCR) in ocular fluids (aqueous/vitreous) for diagnosis of TBU in a TB-endemic population. Mycobacterial copy numbers (*mpb64* gene) were normalised to host genome copy numbers (*RNAse P* RNA component H1 [*RPPH1*] gene) in TBU (n = 16) and control (n = 13) samples (discovery cohort). The *mpb64*:*RPPH1* ratios (normalised value) from each TBU and control sample were tested against the current reference standard i.e. clinically-diagnosed TBU, to generate Receiver Operating Characteristic (ROC) curves. The optimum cut-off value of *mpb64*:*RPPH1* ratio (0.011) for diagnosing TBU was identified from the highest Youden index. This cut-off value was then tested in a different cohort of TBU and controls (validation cohort, 20 cases and 18 controls), where it yielded specificity, sensitivity and diagnostic accuracy of 94.4%, 85.0%, and 89.4% respectively. The above values for conventional quantitative PCR (≥1 copy of *mpb64* per reaction) were 61.1%, 90.0%, and 74.3% respectively. Normalisation markedly improved the specificity and diagnostic accuracy of quantitative PCR for diagnosis of TBU.

1. Introduction

Microbiological diagnosis of extrapulmonary tuberculosis (EPTB) is challenging due to paucibacillary nature of disease, inaccessibility of tissue samples, and non-uniform distribution of bacteria within the respective organs [1,2]. Nucleic acid amplification tests (NAAT), commonly performed as polymerase chain reaction (PCR), hold the promise to fill this gap in the diagnostic armamentarium of EPTB. These tests provide direct evidence of infection from the diseased tissue and are much faster than conventional culture techniques. However, PCR outcomes too can be limited by inadequate sample volumes, presence of PCR inhibitors in clinical samples, contamination during handling of samples and inability to distinguish between viable and non-viable organisms [1]. While the first three can be resolved at least partially, by increasing sample volumes, modification of DNA extraction technique, and application of real-time PCR respectively, the last one – false positive results due to non-viable organisms, has not been adequately investigated. mRNA-based reverse transcriptase PCR has been attempted to identify viable organisms [3], but the assay has low sensitivity and is difficult to perform routinely in clinical samples. Thus, there is a pressing need to evaluate alternative strategies for exclusion of false positive results due to presence of non-viable organisms in EPTB samples, especially in TB-endemic countries.

Tuberculosis-associated uveitis (TBU) probably best exemplifies the above-mentioned challenges to PCR-based diagnosis of EPTB. TBU is one of the most common causes of infectious uveitis (ocular inflammation) in TB-endemic countries [4]. Delay in diagnosis and initiation of anti-TB therapy in TBU results in chronic, recurrent ocular inflammation and moderate to severe visual impairment [5,6]. Current diagnostic criteria for TBU are based on the presence of specific clinical signs, immunological and/or radiological evidence of past TB infection and exclusion of non-TB entities [7]. Definitive evidence of *Mycobacterium tuberculosis* (*Mtb*), such as in smear or culture, is rarely found in ocular fluid (aqueous/vitreous) samples. Hence, molecular diagnostic techniques such as PCR from ocular fluids are possibly the only direct evidence of tissue infection in TBU. Initial studies reported low

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PCR positivity (~37.5%) in different clinical presentations of TBU [8]. Later, multi-target PCR using multiple primers for identification of *Mtb* DNA, resulted in significantly increased PCR positivity rates (> 70%) in TBU [9,10]. However, it was also noted that *Mtb* PCR in ocular samples is associated with high false positive results. In our earlier study, 9 of 56 (16.1%) vitreous samples from culture-proven non-TB endophthalmitis were PCR-positive for *Mtb* [10]. We attributed these false positive results to the presence of 'bystander' *Mtb* DNA within host inflammatory cells in ocular fluid samples of patients with latent TB. Subsequently, other investigators detected *Mtb* genome in the sub-retinal fluid of patients with latent TB and rhegmatogenous retinal detachment, but no evidence of uveitis [11].

We developed a hypothesis, that a high inflammatory load in ocular fluid samples increases the likelihood of presence of 'bystander' *Mtb* DNA, and thereby false positive PCR results. Therefore, normalisation of *Mtb* copy numbers (measured by quantitative PCR for *mpb64* in our study) to host cellular load in tested samples, would counterbalance the confounding influence of host cells and increase the specificity of PCR results. The host cellular load could be estimated by measuring copy numbers of a host gene (human *RNAse P* RNA component H1 gene, *RPPH1* in our study). The concept of normalisation is borrowed from RNA expression studies where RNA expression between different samples is compared by calculating the ratio of RNA copy numbers to a housekeeping gene (with constant expression) in the given sample. Our study had two components. First, we calculated the *mpb64*:*RPPH1* ratio that had the highest sensitivity and specificity for diagnosis of TBU. Since no microbiological evidence was available in these cases (discovery cohort), we used the current diagnostic criteria for TBU [7], as the reference standard. Next, we validated the diagnostic accuracy of the previously calculated cut-off value of *mpb64*:*RPPH1* ratio, in a second cohort of TBU patients (validation cohort). Thereby, the aim of our study was to not only introduce a novel approach to molecular diagnosis of TBU in endemic countries, but also validate the diagnostic utility of this approach.

2. Materials and methods

We conducted a prospective study of TBU patients who presented to the uveitis clinic at L V Prasad Eye Institute, Bhubaneswar in eastern India, between August 1, 2013 and June 30, 2016. The study was approved by the Institutional Review Board and followed the tenets of Declaration of Helsinki. Written, informed consent was obtained from all patients.

2.1. Participants

TBU was diagnosed on the basis of currently available criteria [5], including ocular signs suggestive of TB, ancillary tests (tuberculin skin test [TST], QuantiFERON-TB Gold In-Tube test [QFT], chest radiography ± high resolution computed tomography [HRCT] of the thorax, and/or bacteriologically-proven extrapulmonary TB), and exclusion of non-TB entities. However, abnormal chest radiography was not mandatory for diagnosis of TBU. *Controls* included patients requiring therapeutic vitrectomy for non-TB uveitis or other retinal conditions, and those undergoing surgery for uncomplicated senile cataract. All controls with ocular inflammation/uveitis, were included only if they had documented negative TST ± QFT results.

To meet the objectives of the study, we divided the participants sequentially into two cohorts: discovery cohort from first 24 months, and validation cohort from last 17 months. Both followed the above inclusion and exclusion criteria. The discovery cohort was used for calculating the *mpb64*:*RPPH1* ratio with optimal sensitivity and specificity for diagnosis of TBU. The validation cohort used for estimating the diagnostic accuracy of the optimal *mpb64*:*RPPH1* ratio, in comparison to conventional quantitative PCR (qPCR).

Table 1
Primers and probe* used for *Mycobacterium tuberculosis* real-time PCR assay targeting *mpb64* gene.

Primers and probe	Sequence (5′-3′)
MTB_F	GTTCTGATAATTCACCGGGTCCAA
MTB_R	AGACCGGACAACAGGTATCGA
*MTB_P	TAGCGCCGAATGCC

2.2. Sample collection and processing

Samples up to 200 μL of aqueous or 1 mL of vitreous were collected from each patient and processed aseptically. DNA was extracted using the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) as per the manufacturer's instructions. An additional enzymatic digestion step was performed by adding lysis buffer containing lysozyme (30 mg/mL) at 37 °C for 1 h followed by adding 20 μL Proteinase K and 200 μL of lysis buffer. The samples were further incubated at 56 °C for 1 h. DNA was eluted in 50 μL elution buffer and stored at −20 °C till further analysis.

2.3. Quantitative PCR assay for Mtb

We sourced an optimized Custom TaqMan copy number assay (Applied Biosystems, USA) consisting of a set of two primers targeting *Mtb* gene*mpb64*, and a probe labelled with FAM dye at 5′ end and MGB (minor groove binder) at 3′ end of the sequence (Table 1). The quantitative PCR (qPCR) assay was performed on Applied Biosystems 7500 Fast Dx instrument in a 20 μL reaction mixture consisting of 1 μL of 20X Custom TaqMan copy number assay primers and probe, 10 μL of 2X TaqMan Fast Universal PCR Master Mix, 5 μL of template DNA and 4 μL of double autoclaved milliQ water. Amplification cycles included an initial denaturation step at 95 °C for 20 s, 40 cycles of denaturation at 95 °C for 3 s followed by an annealing and extension at 60 °C for 30 s; the run was performed in fast mode. *mpb64* copy number was calculated using the standard curve (Fig. 1) obtained from 10-fold serially diluted *Mtb* DNA (10^0–10^6 copies/μL) present in 5 μL of test sample's DNA. A sample was considered positive if it resulted in an amplification plot with cycle threshold (Ct) ≤ 34, as it corresponded to a single copy of *mpb64*.

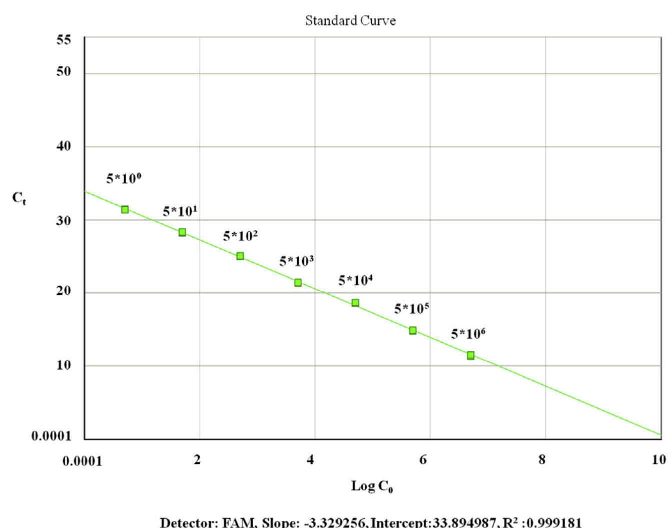

Fig. 1. Standard curve for *mpb64*. A linear standard curve was generated from 10-fold serial dilutions of *mpb64* gene of *Mycobacterium tuberculosis*, ranging from 10^6 from 10^0 copies per μL with a slope – 3.329256, R^2 = 0.999181 by plotting the threshold cycle number (Ct) on Y- axis against log of input copy number ($Log_{10}C_0$) on X-axis. Note: 5 μL of template was added to 20 μL of reaction volume, hence each of the co-ordinates is multiplied to 5.

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