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# Development and evaluation of multiplex real-time PCR for diagnosis of HSV-1, VZV, CMV, and *Toxoplasma gondii* in patients with infectious uveitis

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ABSTRACT

Infectious uveitis is a vision threatening inflammatory ocular disease wherein early diagnosis may prevent the loss of vision. The purpose of this study was to develop a multiplex real-time PCR for the diagnosis of Herpes simplex virus-1, Varicella zoster virus, cytomegalovirus and *Toxoplasma gondii* in patients with suspected infectious uveitis. A total of 126 intraocular samples (aqueous and vitreous humor) were collected and subjected to multiplex real-time PCR. Overall 26.2% (33/126) patients were found to be positive for one or more of the pathogens tested. The overall positivity for VZV, HSV, CMV and *T. gondii* was found to be 16 (12.7%), 7 (5.6%), 5 (3.9%), and 9 (7.1%); with mean pathogen load of $5.07\times10^5$, $9.5\times10^4$, $1.08\times10^4$ and 394 (copies/μl) respectively. The development of highly sensitive and specific assay for early differentiation of pathogens is important for the early initiation of treatment thereby preventing irreversible damage to the ocular structures.



## 1. Introduction

Uveitis and its associated complications contribute to nearly 25% of the blindness in developing countries (Rao, 2013). The etiology of uveitis can be infectious and noninfectious. However, causative agents cannot be differentiated based on clinical findings alone. Among infectious agents, *Mycobacterium tuberculosis*, herpes simplex virus type-1 (HSV-1), varicella zoster virus (VZV), cytomegalovirus (CMV), and *Toxoplasma gondii* play an important role in the development of the disease. The precise diagnosis of infectious agent is important as the treatment varies depending upon the pathogen involved.

There is limited role of serology for the diagnosis of uveitis, though some studies have shown the utility of Goldmann Witmer coefficient for the diagnosis of toxoplasmosis with a sensitivity of 60–92% (Bourdin et al., 2014; De Groot-Mijnes et al., 2006; Errera et al., 2011; Rothova et al., 2008; Westeneng et al., 2007); the availability of small sample volume limits its utility. The cell culture isolation is time consuming and also lacks sensitivity due to the low pathogen load in ocular samples. The limited amount of intraocular sample and the need for early diagnosis to prevent loss of vision requires the use of highly sensitive laboratory methods. Among the molecular methods, polymerase chain reaction (PCR) has gained importance in the diagnosis as it provides the direct evidence of infection (by directly amplifying the DNA of the pathogen) and has high sensitivity and specificity. To overcome the issue of small sample volume, multiplex PCRs have been widely developed and used. Since the conventional PCR assays are time consuming and prone to contamination, the real-time PCR technology is favored and also has higher sensitivity and specificity.

In real-time PCRs, use of different fluorophore with different emission spectra makes the diagnosis more specific and also has the ability to quantify the pathogen load. Though some studies have developed multiplex assays for diagnosis of viruses causing uveitis (Fan et al., 2014; Heaton et al., 2015; Pillet et al., 2015; Sankuntaw et al., 2011; Sugita et al., 2008); considering *T. gondii* as an important pathogen causing uveitis, the present study aimed to develop and validate a quantitative multiplex real-time PCR for the diagnosis of HSV-1, VZV, CMV and *T. gondii* in ocular fluids of patients with clinically suspected infectious uveitis.

## 2. Material and methods

### 2.1. Study Subjects and samples

The present study enrolled the patients at the Advanced Eye Centre of the Post Graduate Institute of Medical Education and Research, Chandigarh during January 2013–September 2015. Patients with uveitis

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suggestive of infectious (viral or Toxoplasmic) etiology such as presence of increase intraocular pressure, decreased corneal sensation, iris atrophy, necrotizing or non-necrotizing retinitis, active retinochoroiditis, dense vitritis, etc. were included ($n = 126$). Patients with uveitis suggestive of noninfectious etiology on clinical examination were excluded from the study. The sex matched control group consisted of cataract patients ($n = 100$) with no signs of intraocular inflammation. Aqueous humor samples were collected during anterior chamber tap in patients of study group ($n = 37$), and during routine cataract surgery in control group. Additionally, vitreous humor samples were collected during diagnostic pars plana vitrectomy in the study group ($n = 89$). All the samples were collected by an experienced ophthalmic surgeon (RB, AG, JR) only after obtaining written informed consent. The study approval was obtained from Institute Ethics Committee as per the National guidelines.

### 2.2. DNA extraction

The DNA was extracted from aqueous and vitreous humor samples using commercially available kit (Qiagen, Germany) as per the manufacturer's instructions with slight modifications.

### 2.3. Conventional PCR

The conventional PCRs were performed in aqueous humor and vitreous tap samples for HSV (1 + 2), VZV, CMV and *T. gondii* targeting glycoprotein D gene, ORF 28, immediate early 1 gene and B1 gene respectively as described earlier (Goyal et al., 2013; Mewara et al., 2010; Singh et al., 2015, 2016).

### 2.4. Primers and probes

The amplicons of representative positive sample of HSV, VZV, CMV and *T. gondii* were subjected to Sanger sequencing by using BigDye Terminator cycle sequencing kit as per manufacturer's instruction (ABI, USA). The sequences obtained were edited manually by using BioEdit 7.2.5 software and then used for the synthesis of primers and probes. In this study the HSV-1 was chosen for multiplexing as sequencing of patient's representative HSV (1 + 2) conventional PCR positive samples showed the presence of HSV-1. The primers and probes used were:

1. HSV-1 [Forward = 5′CCGAACAACATGGGCCTGAT3′, Reverse = 5′ GGCGGTGCATCCAGTACACAAT3′, Probe = VIC-5′CCGCAAATGACC AGGGCTGCCAG3′],
2. VZV [Forward = 5′TGAGACGGTTAAACGTTTGAATCCA3′, Reverse = 5′CGAACGTTAGAGCGCACAAAA3′, Probe = JUN-5′CCACCTTTACAG TTGGAGGAAAACGTCT3′],
3. CMV [Forward = 5′TCATCTTTCTCTTAAGTTCATCCTTCTTAG3′, Reverse = 5′AAGGGCGCCGCTAACAAGTTA3′, Probe = FAM-5′ CACGGGCCTTAGCCTTCAGTGCACCC3′] and
4. *T. gondii* [Forward = 5′GAGCTCGTCAGTGACTGCAACCTA3′, Reverse = 5′TCCGTTCATGAGTATAAGAAAAAAATG3′, Probe = ABY-5′TGGGAATGAAAGAGACGCTAATGTGTTTGCA3′].

### 2.5. In-house multiplex real-time PCR

The concentrations of primers and probes were standardized by using different concentrations of primers at 200 nM, 300 nM and 500 nM and probes at 250 nM. The in-house multiplex real-time was performed up to 40 cycles using specific primers and probes, 1× Taqman multiplex master mix (ABI, USA) and 4 μl of DNA in 20 μl of the final reaction. The reactions were performed in ABI 7500 real-time PCR machine (ABI, USA).

For the quantification of pathogens, the amplicons of the positive samples were PCR purified and ligated into pGEMT vector as per manufacturer's instructions (Promega, USA). The clones obtained were subjected to plasmid isolation and then quantified. These plasmids were then used as standards and further 10 fold serially diluted for the quantification of unknown samples.

### 2.6. Analytical sensitivity and specificity

The sensitivity of the real-time PCRs was checked using 10 folds serial dilution of the standards. The specificity of each primer and probes was checked to exclude the cross reactivity of each target by using DNA of HSV-1, VZV, CMV, Adenovirus, *M. tuberculosis* and *T. gondii*. The uniplex real-time PCRs were also performed to compare lower limit of detection with in-house multiplex real-time PCR for HSV-1, VZV, CMV and *T. gondii* by using 1× TaqMan Universal master mix II (ABI, USA).

### 2.7. In-house multiplex real-time PCR inhibition assay

The inhibition within the in-house multiplex real-time PCR was checked by spiking low copy number of one target with high copy number of other targets. The $10^2$ copy numbers of HSV-1, VZV, CMV and *T. gondii* was co-amplified with or without $10^6$ copy numbers of other targets by using multiplex real-time PCR.

### 2.8. Intra- and inter-assay reproducibility and correlation with in vitro diagnostic kit

The intra and inter assay reproducibility for the multiplex PCR was measured for the different dilutions in quadruple. The results of in- house developed multiplex real-time PCR were compared with in-vitro diagnostics kit (IVD) for the diagnosis of HSV-1, VZV and CMV based on TaqMan real-time PCR (Biomeurix, France) as per manufacturer's instructions in representative clinical samples (cerebrospinal fluid, vesicular swabs and bronchoalveolar lavage samples).

### 2.9. Statistical analysis

The covariance was measured to check the reproducibility of assay. The unpaired Student's t test was used to check the inhibition in the presence of high copy numbers of spiked standards. Spearman correlation test was performed for correlating in-house multiplex real-time PCR with IVD kit by using Graphpad Prism 6. Since there is no gold standard test available for the diagnosis of infectious uveitis, the sensitivity, specificity, positive predictive value, negative predictive value was calculated based on the clinical diagnosis and patient's response to treatment (Scheepers et al., 2013). The Cohen's kappa was calculated to check the agreement with gold standard by using SPSS version 22.0. The difference between the positivity in aqueous humor and vitreous tap samples was evaluated using chi square test. To find out the significant difference of clinical signs between various positive patients chi square test was performed (VZV versus HSV-1, VZV versus CMV, VZV versus *T. gondii*, HSV-1 versus CMV, HSV-1 versus *T. gondii* and CMV versus *T. gondii*) by using SPSS version 22.0.

## 3. Results

### 3.1. Demographic details

The mean age of the patients was found to be 39.6 ± 15.3 years with male:female ratio of 1.6:1. However, the mean age of controls was found to be higher 62.3 ± 11 years with male:female ratio of 1.3:1. Majority (81; 64.3%) of the patients had unilateral involvement.

### 3.2. Primer optimization

The optimal concentration of primers for HSV-1, VZV, CMV and *T. gondii* was found to be 500 nM. All the reactions of uniplex and multiplex real-time PCRs were performed at same concentration of primers.