Use of uniplex polymerase chain reaction & evaluation of multiplex PCR in the rapid diagnosis of viral retinitis

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Background & objectives: Polymerase chain reaction (PCR) has been known to be a rapid and accurate diagnostic test for causative viruses of viral retinitis, but cost is the limiting factor. In the present study an attempt was made to standardize a multiplex PCR (mPCR) on intraocular specimens from patients with viral retinitis for the detection of one or more viruses [herpes simplex virus (HSV), varicella zoster virus (VZV) or cytomegalovirus (CMV)] in order to reduce the period of time required for uniplex polymerase chain reaction (uPCR).

Methods: Using the uniplex PCR (uPCR) primers, a nested mPCR was developed and standardized for the simultaneous detection of HSV, VZV and CMV. mPCR and uPCRs were applied on 9 stored specimens and 38 prospective specimens obtained from patients with viral retinitis.

Results: The specificity and sensitivity of the mPCR were concordant with that of uPCRs. Clinical specificity and sensitivity of mPCR was further confirmed by the detection of the same herpes viral DNA on the 9 stored specimens. Of the 38 specimens collected prospectively, mPCR detected HSV in 3 (7.9%), VZV in 9 (23.7%), CMV in 5 (13.2%) and both VZV and CMV in 2 (5.3%). Co-infections of two viruses were found in 7 (14.89%) of the 47 specimens.

Interpretation & conclusion: mPCR is a rapid, specific and sensitive diagnostic tool in viral retinitis. Compared to uPCR, mPCR is less time-consuming and cost effective.

Key words Cytomegalovirus - herpes simplex virus - multiplex PCR - varicella zoster virus - viral retinitis

Viral retinitis, a potentially sight threatening disease is caused by herpes simplex virus (HSV), varicella zoster virus (VZV) and cytomegalovirus (CMV). Rapid etiological diagnosis will help in timely institution of specific therapy. The conventional methods of antigen detection by fluorescent antibody test (FAT) and virus isolation (VI) in cell cultures have low sensitivity due to low antigen threshold and low viral load. PCR has been shown to be a rapid and accurate diagnostic tool in the diagnosis of ocular diseases. In diagnostic laboratories the use of PCR is limited due to its high cost. To overcome this shortcoming and also to increase the diagnostic sensitivity of PCR, a variant termed multiplex PCR (mPCR) has been developed. In mPCR more than one target sequence can be amplified by including more than one set of primers and this has been reported as a useful diagnostic method in posterior uveitis and conjunctivitis. Therefore, in this study we standardized mPCR for
the detection of HSV, VZV and CMV by combining uniplex primer pairs and evaluated it against the individual uniplex PCRs (uPCRs).

**Material & Methods**

**Patients & specimens:** The clinical specimens were collected either at the outpatient department or the operation theatres of Sankara Nethralaya ophthalmic hospital, Chennai and were received at the microbiology laboratory within 15 min from the time of collection. They were processed immediately for PCR. Thirty eight intraocular fluids [34 aqueous humor (AH) and 4 vitreous fluid (VF)] collected prospectively from 34 patients with viral retinitis during August 1999 to April 2001 were subjected to both multiplex and uniplex PCRs. Seven of these patients were serologically positive for human immunodeficiency virus (HIV) infection.

**Controls:** Stored DNAs of 9 intraocular fluids (7 AH and 2 VF) from 9 patients were also subjected to mPCR. The viral etiology (HSV, VZV and CMV) of retinitis in these patients had already been established by uPCR and 5 of them had co-infections with 2 of these viruses. Ninety intraocular fluids (30 AH collected at the beginning of cataract surgery, 20 AH and 20 VF from patients with culture proven bacterial and fungal endophthalmitis and 20 VF from patients with diabetic retinopathy and retinal detachment) were included as controls.

**Uniplex polymerase chain reaction (uPCR):** DNA extraction and PCR were carried out as described earlier\(^7\)\(^-\)\(^9\) and modified by us\(^10\)\(^-\)\(^12\). HSV primers code for the DNA polymerase gene, CMV primers code for the morphological transforming region II gene, and VZV primers code for the immediate early gene 63 were used. The custom synthesized primers and all PCR reagents were procured from Bangalore Genei Pvt. Ltd (Bangalore, India).

**Multiplex PCR (mPCR):** The same primer sets used for the individual amplification of HSV, VZV and CMV were combined and used in a nested mPCR reaction. For the first round, primers for HSV, VZV and CMV were used together. For amplification a 25µl reaction was set with 240µM each dNTPs, 1xPCR buffer [10 mM Tris-HCl (pH 8.3), 50mM KCl, 0.01% gelatin, 1.5mM MgCl\(_2\)] and 2 units of Taq DNA polymerase. One micromole of each primer for HSV, 20µM of each primer for VZV and 0.1µM of each primer for CMV were added as used in the uPCR. Then 5 µl of extracted DNA was added as template. The first round thermal profile consisted of an initial denaturation at 94°C for 2 min for 1 cycle followed by 35 cycles each consisting of denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec and extension at 72°C for 45 sec.

For the nested amplification only VZV and CMV primers were used. A 25 µl reaction was set with 160µM each dNTPs, 1x buffer [10mM Tris-HCl (pH 8.3), 50mM KCl, 0.01% gelatin, 1.5mM MgCl\(_2\)] and 1 unit of Taq DNA polymerase. Twenty micromoles of each primer for VZV and 0.1 µM of each primer for CMV were added as used in the uPCR. From the first round products, 1.5 µl was added as template for the second round. The thermal profile consisted of an initial denaturation at 94°C for 2 min for 1 cycle followed by 15 cycles each consisting of denaturation at 94°C for 45 sec, annealing at 57°C for 45 sec and extension at 72°C for 45 sec.

The amplified products were analyzed by gel electrophoresis using 2 per cent agarose (Sisco Research Laboratories, Mumbai, India) for uPCRs and 4 per cent agarose for mPCR both containing 0.5 µg/ml of ethidium bromide (Sigma, USA). The specificity of mPCR was determined with viral, bacterial, fungal and human DNA. The sensitivity of mPCR was determined using serial 10-fold dilutions of HSV (20,000 pfu/ml initial concentration). The infectivity titration of VZV and CMV was not determined for this purpose because of constraints on the free availability of diploid cell lines. VZV (1 µg/ml approximate initial concentration) and CMV (1.5µg/ml approximate initial concentration) DNAs extracted from respective virus infected diploid cell lines (WI 38 cell line supplied by National Centre for Cell Science, Pune) culture harvests were used. The end dilution of these DNA samples showing the amplified products of VZV and CMV were determined for comparing sensitivity of uPCR and mPCR.
Precautions for PCR: To prevent amplicon contamination of PCR, separate rooms were used for preparation of DNA, its amplification and analysis of the amplified products. PCR preparation was performed in a laminar flow workbench with single use aliquots of reagents, sterile guarded tips and dedicated pipettes. Being an in-house protocol, a water control was used along with the extraction reagents and a buffer control was used along with the PCR reagents. The amplification of the specimens was considered, as true positive only if both these controls were negative. True negatives were validated by confirming the absence of inhibitors upon spiking with a known quantity of target DNAs. True positives and true negatives of mPCR were also validated by the concordant results with uPCRs.

The study had the approval of the Research Sub-committee and Ethics Sub-committee (Internal Review Board-IRB) of Vision Research Foundation, Chennai, India.

Results

Results of the standardization of mPCR for the detection of HSV, VZV and CMV and comparative results of the same with those of uPCR: The primers targeting HSV, VZV and CMV were specific to the corresponding viruses by uPCRs and mPCR, as they did not amplify any of the other viruses, bacteria, fungi or human leukocyte DNA. The primers were able to amplify up to 0.2pfu/ml,10^-8 and 10^-7 dilutions with HSV, VZV and CMV DNAs respectively, both by the uPCRs and mPCR. The time taken by uPCRs for detection of one or more of the three viruses varied between 48 to 72 h, while mPCR did not exceed 24 h as the amplification time has been reduced to two rounds from five rounds. The quantity of Taq DNA polymerase used was reduced by half in the mPCR compared to uPCR.

Results of the application of uPCRs and mPCR on controls and 38 prospective intraocular specimens: In 89 of the 90 intraocular fluid control samples tested, HSV-DNA, VZV-DNA and CMV-DNA were not detected. VZV-DNA was detected in one VF obtained from a patient of proven bacterial endophthalmitis. Of the 9 stored specimens from patients with viral retinitis, HSV was detected in 1, VZV in 2, CMV in 1, both HSV and VZV in 1, both HSV and CMV in 1 and both VZV and CMV in 3 by both the uPCRs and mPCR. The results of mPCR on the 38 intraocular fluids collected prospectively were also concordant with the uPCRs carried out simultaneously as shown in the Table. Of the 38 specimens, HSV was detected in 3 (7.9%), VZV in 9 (23.7%), CMV in 5 (13.2%) and both VZV and CMV in 2 (5.3%). Herpesviral etiology was detected in 19 of 38 (50.0%) intraocular fluids collected from 19 of 34 (55.9%) patients. The mPCR results of intraocular specimens are shown in Figs 1 and 2.

Co-infections of more than one virus were detected in 7 of 47 (14.9%) specimens from 7 of 43 (16.3%) patients. HSV and VZV were detected in 1 of 43 (2.3%), HSV and CMV in 1 of 43 (2.3%) and VZV and CMV in 5 of 43 (11.6%) patients. The mPCR on specimens was completed within 24 h after the receipt of the specimens, but for completion of uPCR for all the three viruses needed a varying time of 48 to 72 h depending on the availability of thermal cycler time.

Discussion

PCR is a rapid and reliable diagnostic tool for viral retinitis, and other ocular and non-ocular infections. The uPCRs for the detection of one or more of the three viruses - HSV, VZV and CMV, in a single intraocular specimen have the

<table>
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<tr>
<th>Specimen</th>
<th>Total no.</th>
<th>Positive by multiplex PCR*</th>
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<tbody>
<tr>
<td></td>
<td>HSV</td>
<td>VZV</td>
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<tr>
<td>AH</td>
<td>34</td>
<td>3</td>
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<td>VF</td>
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<tr>
<td>Total</td>
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AH, aqueous humor; VF, vitreous fluid. * The mPCR results were concordant with that of the individual uPCRs for each of the three viruses.
Fig. 1. Ethidium bromide stained agarose gel electrophoretogram showing the mPCR amplified products of herpes simle virus, varicella zoster and cytomegalovirus obtained after initial mPCR amplification. Lanes N represents the negative (buffer) control, 1 represents negative extraction control, 2 represents negative aqueous humor (AH), 3 represents HSV positive AH, 4 represents VZV positive vitreous fluid (VF), 5 represents CMV positive VF, 6 represents HSV and VZV positive AH, 7 represents HSV and CMV positive AH, 8 represents VZV and CMV positive AH, P represents positive (HSV-DNA, VZV-DNA and CMV-DNA) control, M represents molecular weight standard (φ X 174 DNA/Hinf I digest). A -234 bp (CMV); B -179 bp (HSV); C -126 bp (VZV).

Fig. 2. Ethidium bromide stained agarose gel electrophoretogram showing the mPCR amplified products of varicella zoster virus and cytomegalovirus obtained after the second round of amplification. Lanes N1 represents the negative (buffer) control, N2 represents the negative control (using I round negative control as template), 1 represents negative extraction control, 2 represents negative aqueous humor (AH), 3 represents negative AH, 4 represents VZV positive vitreous fluid (VF), 5 represents VZV and CMV positive VF, 6 represents VZV positive AH, 7 represents CMV positive AH, 8 represents VZV and CMV positive AH, P represents positive (VZV-DNA and CMV-DNA) control, M represents molecular weight standard (φ X 174 DNA/Hinf I digest). Please note that the band representing HSV is not visualized as it is not a nested PCR. A -168 bp (CMV); B -108 bp (VZV).

constraints of time and cost. Therefore, we combined the same set of primers in a nested multiplex reaction and evaluated them against the uPCRs. mPCR has reduced the time of amplification from 5 to 2 rounds and the cost of Taq DNA polymerase to half, when compared to the uPCRs. mPCR reproduced both the analytical and clinical specificity and sensitivity of the individual uPCRs. The mPCR results were concordant with that of the uPCRs on all the 9 stored and 38 prospective specimens.

To reduce the time and cost of performing five rounds (a single and two nested rounds) of PCR for every intraocular specimen, we analyzed the design of the primers used and the uPCR conditions, in order to combine them in a multiplex reaction. The length of these primers ranged between 20 and 24 bp and their GC content between 40 and 58 per cent. The concentrations of the primers were initially optimized in the uniplex reactions to 1, 20 and 0.1 µM for HSV, VZV and CMV respectively in order to achieve absolute specificity and high sensitivity to the corresponding viruses. As there were no wide variations between the primers based on the length, GC content and the concentration, we tested them in a multiplex reaction. In uniplex
amplification, single round primers were used for HSV, semi-nested for VZV and nested for CMV. The same sets of primers were applied in the multiplex amplification also, whereby the initial amplification comprised primers for all 3 viruses, while the nested amplification comprised only the VZV and CMV primers. The same analytical specificity and sensitivity of the uPCRs were reproduced by mPCR for the corresponding viruses.

In the mPCR for the detection of one or more of the three viruses per specimen, the quantity of Taq DNA polymerase was reduced by 41 per cent (22 units for each sample by uPCR, while only 13 units by mPCR - this includes the controls for every PCR reaction), the number of runs was reduced from five (a single and two nested rounds) to two (a single nested reaction) and the time spent was reduced from 48-72 h to only 24 h. Thus, the reduction of Taq DNA polymerase utilized, thermal cycler time and personnel time greatly increases the cost-effectiveness of mPCR over the uPCRs per specimen. We believe that this was the first time a mPCR has been designed using a cocktail of these three specific sets of primers for application as short tandem amplification of multiple pathogens (STAMP) primers in the detection of HSV, VZV and CMV in intraocular virus infections, though Dabil et al5 have used a different set of STAMP primers for the same.

VZV-DNA was detected in one patient (control) with post-operative bacterial endophthalmitis. It is likely that in this patient activation of VZV could have been triggered during the episode of bacterial endophthalmitis with a spill over of the virus-laden leucocytes into the vitreous cavity. The absence of HSV, VZV and CMV genomes in the intraocular fluids of patients undergoing cataract extraction and patients presenting with non-viral retinal inflammations further emphasizes the specificity of these primers to the corresponding viral genomes and indicates that the detection of herpes virus DNA by PCR implies active infection. Mitchell et al5,5 have also shown similar findings, while Fox et al6 detected CMV-DNA in one of eight vitreous fluids from patients with diabetic retinopathy. In two other reports17,18, ocular fluids obtained from patients undergoing vitreo-retinal surgery for non-viral inflammatory conditions did not show the presence of herpes virus DNA by PCR though high proportion of these patients were tested positive for herpes virus antibodies.

Herpes virus co-infections were detected in seven patients. Co-infection of herpes viruses has been reported in the central nervous system (CNS) infections19,20. Dabil et al5 have shown the coexistence of VZV and Toxoplasma gondii in case of posterior uveitis. Our results indicated that co-infection of VZV and CMV appeared to be common. Of the seven patients with co-infections, six were immunosuppressed (five with HIV infection and one with non-Hodgkin’s lymphoma). Of the five HIV positive patients, four were diagnosed to have CMV retinitis, one had acute retinal necrosis (ARN) and the non-Hodgkin’s lymphoma patient had choroiditis. The immunosuppressed status probably supported the coexistence of more than one virus in these patients. The seventh patient with coinfection had CMV retinitis and was apparently immunocompetent.

Thus, in this study PCR had attributed herpes viral etiology in 21/47 (44.7%) specimens from patients with viral retinitis, while coinfection of two virus was detected in 7/47 (14.9%) specimens. mPCR described in this study was a useful and reliable diagnostic tool for the rapid detection of herpes viral etiology in viral retinitis and was more cost-effective and less time-consuming than the conventional uPCRs.

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References


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