

APPLICATION OF POLYMERASE CHAIN REACTION TO DIFFERENTIATE HERPES SIMPLEX VIRUS 1 AND 2 SEROTYPES IN CULTURE NEGATIVE INTRAOCULAR ASPIRATES

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Abstract

Purpose: To standardize and apply a polymerase chain reaction (PCR) on the glycoprotein D gene to differentiate Herpes simplex virus (HSV) 1 & 2 serotypes in culture negative intraocular specimens. **Methods:** Twenty-one intraocular fluids collected from 19 patients were subjected to cultures for HSV and uniplex PCR (uPCR) for DNA polymerase gene. To differentiate HSV serotypes, as 1 & 2, a seminested PCR (snPCR) targeting the glycoprotein D gene was standardised and applied onto 21 intraocular fluids. The specificity of the snPCR was verified by application onto ATCC strains of HSV 1 and 2, clinical isolates and DNA sequencing of the amplified products. All specimens were also tested for the presence of cytomegalovirus (CMV) and varicella zoster virus (VZV) by nucleic acid amplification methods. **Results:** Four of the 21 intraocular fluids were positive for HSV by uPCR. snPCR detected HSV in three additional specimens (total of seven specimens), and identified three as HSV 1 and four as HSV 2. DNA sequencing of PCR products showed 100% homology with the standard strains of HSV 1 and 2 respectively. None of the samples were positive in culture. Among the other patients, CMV DNA was detected in two and VZV DNA in five others. **Conclusions:** The standardized snPCR can be applied directly onto the culture negative specimens for rapid differentiation of HSV serotypes.

Key words: HSV 1 & 2 serotypes, HSV DNA polymerase gene, HSV Glycoprotein D gene, Viral retinitis, seminested PCR

Viral retinitis is a major sight threatening disease commonly caused by any of the three herpes viruses namely – herpes simplex virus (HSV), cytomegalovirus (CMV) and varicella zoster virus (VZV). Rapid detection of the specific causative agent helps in the timely institution of the specific antiviral therapy, as they differ for each herpes group of viruses. The conventional methods followed for the aetiological diagnosis are the immunofluorescence staining on the direct smears from the lesions and viral culture.¹ These conventional methods lack sensitivity as only a minute quantity of the ocular specimen is available for such investigations, which are also time consuming. Moreover, CMV and VZV are difficult to cultivate.² Hence a more rapid method for detecting the presence of these viral agents is by nucleic acid-based molecular technique of polymerase chain reaction (PCR).³⁻⁵ Initially a uniplex PCR was standardized for the detection of HSV DNA polymerase gene, which detected both the serotypes of HSV. It is necessary now to identify the serotype causing the infection, as the serotypes of virus appear to influence the frequency of reactivation of the infection.⁶ Therefore, in this study we have standardized a seminested PCR targeting the glycoprotein D gene of HSV, which is serotype specific and was applied on intraocular specimens suspected of viral retinitis to help in the rapid identification of the serotypes as either HSV 1 or HSV 2.

Materials and Methods

Patients and clinical specimens

The intraocular clinical specimens used in the study were either collected from the eye in the out patient department or at the operation theatre of Sankara Nethralaya ophthalmic hospital, Chennai over a study period of one year from January - December 2004. The collected clinical specimens were transported to the laboratory and processed for PCR and viral culture for HSV within fifteen minutes of collection. Twenty-one intraocular fluids collected from 19 patients (16 with acute retinal necrosis (ARN), two with viral retinitis, and one each of progressive outer retinal necrosis (PORN), CMV retinitis and optic neuritis were included in the study. Seventeen aqueous humor (AH) and 4 vitreous aspirates (VA) were collected. Dual specimens of AH and VA were collected from two patients with ARN. A minimal amount of approximately 50µL of the collected clinical specimen placed in 1mL of Dulbecco's minimum essential medium (DMEM) containing 3% fetal calf serum (FCS) was used for virus isolation with the remaining part processed for PCR for the detection of HSV, CMV and VZV DNA. Since the intraocular specimens have a minimal cellular material, they were centrifuged at 10,000 RPM at 4°C for 15 minutes and the deposit was used for DNA extraction.

Herpes simplex virus isolation

Attempts to isolate HSV was done by inoculating 100µL of DMEM containing the intraocular clinical specimen onto a monolayer of cultured Vervet monkey kidney cells (Vero

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obtained from NCCLS, Pune, India) in duplicate test tubes which were incubated at 37°C. If at the end of 5-7 days no cytopathogenic effect (CPE) of a viral growth was visualized microscopically, three further passages were carried out to rule out the presence of cultivable virus in the clinical specimen.

DNA extraction

DNA extraction from the clinical specimens was done using the clinical genomic DNA mini prep kit, Biogene Inc., CA, USA as per the manufacturer's instructions. The extracted DNA was used for uPCR and snPCR for HSV; Nested PCR for CMV and semi nested PCR for VZV.

uniplex PCR for HSV

A uniplex PCR (uPCR) for the detection of HSV targeting the DNA polymerase gene earlier standardized by us⁷ was applied on to the 21 intraocular fluids. The custom synthesized primers and the reagents for PCR were procured from Bangalore Genei Pvt. Ltd (Bangalore, India)

seminested PCR

A seminested polymerase chain reaction (snPCR) with primers flanking the glycoprotein D gene of HSV genome⁸ (HSV1 & 2) was performed. The snPCR was performed in a 50µL reaction volume containing 1X PCR buffer (10mM Tris with 15mM MgCl₂), 200mm of each dNTPs, 2.5 units of *Taq* DNA polymerase, 1mM each primer (custom synthesized by Bangalore Genei Pvt. Ltd). The primer sequences for the I and II round of snPCR are shown in (Table 1). All the reagents used for the PCR were procured from Bangalore Genei Pvt. Ltd. HSV type specific snPCR was carried out using a common thermal profile for HSV type 1 and 2. Thermal profile for the first round was for 35 cycles with each cycle consisting of three steps denaturation at 94°C / 40 seconds,

annealing at 50°C / 40 secs and extension at 72°C / 40 secs in the thermal cycler (PE Applied Biosystems 2700, USA). The second round of amplification using the seminested primers was done for 20 cycles of incubation at 94°C, 65°C and 72°C for 40 seconds each. All the reactions were carried out using appropriate controls reagent and positive controls. DNA extracted from respective standard strains of HSV 1 ATCC 733 VR and HSV 2 SP 753167 was used as the positive controls.

Sensitivity of snPCR

The sensitivity of the snPCR was tested by serial ten fold dilutions of the extracted positive control DNA of both HSV -1 and 2 in sterile Milli Q water. snPCR was performed on the diluted samples and the sensitivity was determined.

Specificity of snPCR

The specificity of the standardized PCR was verified by testing the PCR against the several DNA samples extracted from bacteria – *Staphylococcus aureus* (ATCC 25293), *Chlamydia trachomatis* serotype A (ATCC VR 517B), *Mycobacterium tuberculosis* (H37Rv) and laboratory strains of *Propionibacterium acnes*. Fungal DNA tested included - *Candida albicans*, *Aspergillus flavus*, *Aspergillus niger*. The cross reactivity between the herpes group of viruses were also determined by testing the primers against varicella zoster virus DNA (Oka vaccine strain), cytomegalovirus DNA (AD169).

Validation of the type specific PCR with the isolates

DNAs of 10 random clinical isolates and the ATCC standard strains of HSV 1 and 2 were extracted and snPCR was performed. The results were analyzed. These clinical isolates were earlier serotyped by us using the neutralization test, PCR based restriction fragment length polymorphism (PCR-RFLP) and DNA sequencing.⁹

DNA sequencing

The 50µL amplified products of the clinical samples were run on a 2% agarose gel and electrophoresed. The 272 bp amplified product was visualized in the UV transilluminator and the amplified product was cut using a sterile blade and transferred into a 1.5mL microfuge tube. The DNA was eluted from the agarose gel using the QiAmp DNA mini gel elution kit in a total of 10µL elution volume and the whole 10µL was provided to Bangalore Genei Pvt. Ltd for sequencing. All the seven positives obtained in the snPCR were sequenced to determine the specificity of the PCR and to rule out any false positivity.

nested PCR for cytomegalovirus genome

A nested PCR targeting the morphological transforming region II of CMV standardized earlier by us was applied for the detection of the virus.¹⁰ The nested PCR was applied onto

Table 1: Primer sequences used in the seminested polymerase chain reaction (snPCR) for differentiation of HSV 1 and 2 serotypes

Virus	Primer orientation	Primer sequences
HSV 1	Outer & Inner sense	5' CGAAGACGTCCGGA AACAAC 3'
	* Outer antisense	5' CGGTGCTCCAGGAT AAA 3'
	* Inner antisense	5' TCTCCGTCCAGTCG TTTATCTTC 3'
HSV 2	Outer & Inner sense	5' GGACGAGGCCCGA AAGACA 3'
	* Outer antisense	5' CGGTGCTCCAGGA TAAA 3'
	* Inner antisense	5' TCTCCGTCCAGTCG TTTATCTTC 3'

* The outer and the inner antisense primer sequences are common for both HSV 1 and 2 serotypes.

the 21 intraocular fluids for the detection of CMV. The presence of a 128bp indicated the presence of the viral DNA.

seminested PCR for Varicella zoster virus genome

Earlier we had standardized a seminested PCR targeting the immediate early 63 gene of VZV genome and had applied onto a variety of specimens.¹¹ This well established PCR was applied onto the 21 intraocular fluids for detection of VZV DNA. The presence of an amplified product in the region of 108bp indicated the presence of VZV DNA.

Results

Herpes simplex virus isolation

Herpes simplex virus was not isolated from any of the 21 intraocular specimens.

Uniplex PCR for DNA polymerase gene of HSV

Of the 21 intraocular fluids tested, HSV DNA was detected in four. These were from AH and VA of two patients with clinical diagnosis of ARN. HSV DNA was not detected by uPCR from all other 17 intraocular clinical specimens.

Specificity and sensitivity of snPCR for HSV 1 and 2

The snPCR for glycoprotein D gene was specific for HSV DNA. It did not amplify the DNA from other organisms, which included viral, bacterial, fungal and the parasite *Toxoplasma gondii*. The results of the specificity of the primers with the clinical isolates exactly coincided with the serotyping done earlier⁹ proving their specificity. The sensitivity of the primers was determined as 0.02 attograms of DNA of both HSV 1 and 2.

Application of the standardized snPCR onto clinical specimens

This standardized snPCR was applied onto the 21

Table 2: The results of snPCR applied onto 21 intraocular fluids collected from patients with acute retinal necrosis

S. No	Clinical specimen	Results of uPCR	Results of snPCR
1*	AH	Positive	Positive / HSV 1
2*	VA	Positive	Positive / HSV 1
3•	AH	Positive	Positive / HSV 2
4•	VA	Positive	Positive / HSV 2
5	AH	Negative	Positive / HSV 2
6	AH	Negative	Positive / HSV 1
7	AH	Negative	Positive / HSV 2

AH: Aqueous humor, VA: Vitreous aspirate

* - Dual specimen collected from patient 1

• - Dual specimen collected from patient 2

intraocular fluids and HSV DNA was detected in 7 specimens (31.8%). The results of snPCR in comparison with uPCR are shown in (Table 2). The seven positives include the four of uPCR. snPCR differentiated 3 of the 7 as HSV 1 and other 4 as HSV 2. All the seven positives were from patients with clinical diagnosis of ARN. The results of the snPCR are shown in figure 1.

DNA sequencing of the snPCR amplified products

The seven positives of the snPCR were DNA sequenced (ABI prism 300,) and the results are shown in (Figure 2). The sequence that was obtained was submitted to BLAST search tool of NCBI to analyse the percentage homology with the standard strains of HSV 1 and 2 to serotype them. The results clearly showed that three positive i.e., dual specimens from a single patient of ARN and AH from another patient of ARN were identified as HSV 1 serotype. The other four positives were identified as HSV 2, which included a dual specimen of AH and VA collected from a single patient of ARN and AH collected from two patients with the same clinical

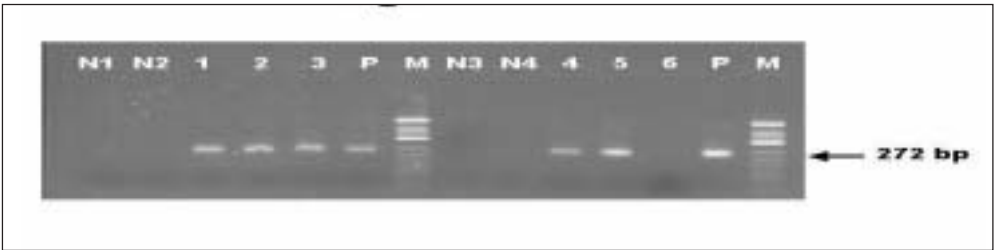


Figure 1: Agarose gel electrophoretogram showing the seminested polymerase chain reaction (snPCR) amplified products of second round for Herpes simplex virus 1 and 2 differentiated by the use of type specific primers.

Lane N1 represents the negative (buffer) control of HSV 1, Lane N2 represents the first round negative control of HSV 1, Lane 1 represents AH specimen positive for HSV 1, Lane 2 represents VA specimen positive for HSV 1, Lane 3 represents AH specimen positive for HSV 1, Lane P represents Positive (HSV 1 DNA) control, Lane N3 represents the negative (buffer) control of HSV 2, Lane N4 represents the first round negative control of HSV 2, Lane 4 represents AH specimen positive for HSV 2, Lane 5 represents VA specimen positive for HSV 2, Lane 6 represents AH specimen negative for HSV 2, Lane P represents Positive (HSV 2 DNA) control, Lane M represents the molecular weight marker (j X 174 DNA/ *Hinf* I digest).

and ARN respectively. The PCR was sensitive enough to detect 0.002 femtograms of CMV DNA as quantitated by us earlier.^{10,12} Presence of a band in the 128bp region indicated positivity.

seminested (sn) PCR for VZV

The snPCR targeting the immediate early 63 gene of VZV

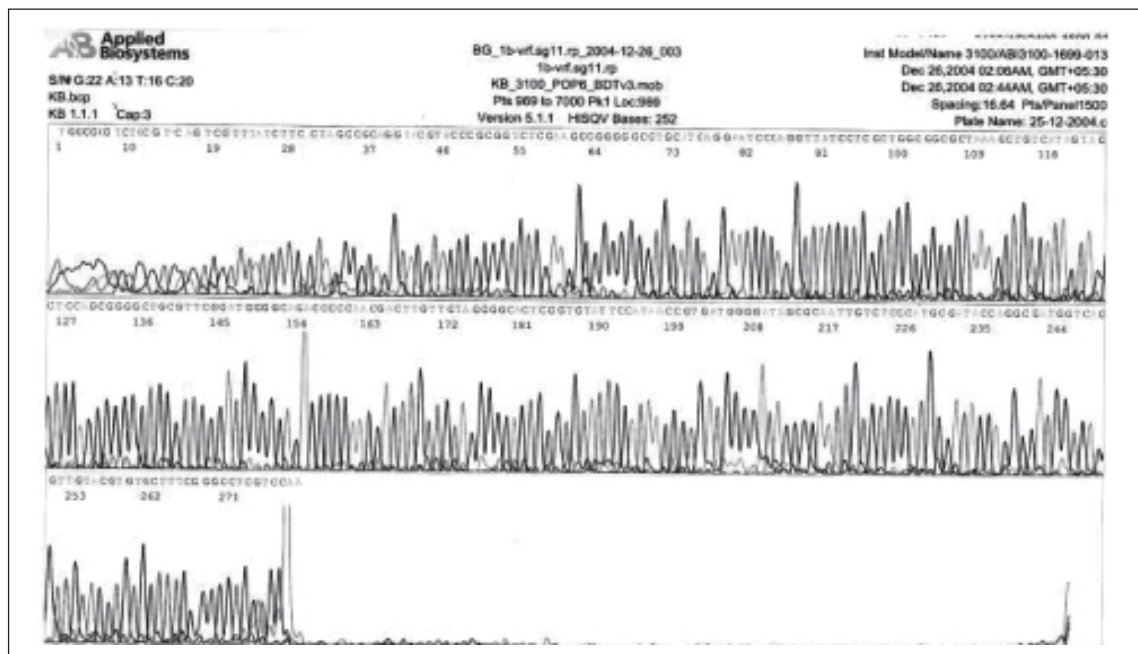


Figure 2b: Aqueous humor specimen that was positive by snPCR for HSV 2 was sequenced and the sequences have 100% homology with HSV 2 Standard strain.

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detected five positives (23.8%) from 21 intraocular fluids. Of the five positives, four patients had ARN and one was with PORN in which VZV DNA was detected. The snPCR was sensitive enough to detect 20 femtograms of VZV DNA as described and quantitated by us earlier.¹²

Discussion

The association of ARN, PORN and viral retinitis with herpes viral etiology has been well documented by many authors.^{1-2,5,12-13} Virus isolation until recently was considered the gold standard for establishing the etiology of viral retinitis or any viral infection. This requires the facility of specialized tissue culture laboratory, which is not widely available. As described by us earlier, the conventional method of viral isolations is time consuming and has low sensitivity when compared with the molecular methods.⁷ Although theoretically a single viral particle is enough for virus isolation, the infectious titre is markedly reduced during transportation and storage resulting in false negativity by the conventional diagnosis.¹² PCR is a rapid and reliable tool for the diagnosis of ocular infections. Many studies undertaken to prove the usefulness of PCR have concluded that PCR techniques are useful as rapid and sensitive adjuncts to clinical diagnosis.⁵ As seen clearly from our study there were no virus isolations from all the 21 intraocular fluids tested in spite of the immediate processing of the clinical samples. This failure to isolate the virus could be attributed to the minimal amount of the clinical sample available. The results of uPCR targeting the DNA polymerase gene of HSV detected only 19% positivity in the 21 tested. We had earlier shown that the sensitivity of the uPCR is only 0.5 femtograms for HSV 1 and 0.2 femtograms for HSV 2 which is very low for the detection of the viral DNA and therefore negative results are likely.⁷

Since the uPCR detected both HSV 1 and 2 serotypes the amplified product needs to be further processed to type them. Serotyping the HSV strains is needed because of the frequency of reactivation of the disease which varied between HSV-1 and 2.⁴ Emmett Cunningham *et al* studied two cases of HSV associated retinitis who had earlier HSV associated encephalitis and later had a reactivation of HSV and developed PORN. They explained that HSV 1 affected primarily the retinal arterioles, whereas the serotype 2 affected the retinal veins more than the arterioles, which later leads to retinal detachment.⁴ Other conventional method like the neutralization test necessitates the virus isolation. PCR based restriction fragment length polymorphism (PCR-RFLP) requires trained skilled personnel.

DNA sequencing used for serotyping is a costly procedure. Hence, in this study, to aid the rapid detection of serotypes a seminested PCR for glycoprotein D gene was standardized and applied onto the intraocular clinical specimens. The usefulness of the glycoprotein D gene for HSV detection has been well established by many authors.^{14,15} The sensitivity of

the snPCR was very high requiring only 0.02 attograms of the genome and this was further indicated by the seven positives picked up by the snPCR, including the three missed by the uPCR. The specificity of the snPCR tested on the clinical isolates concordantly differentiated the HSV 1 and HSV 2 respectively with our earlier typing results indicating 100% specificity of the snPCR. The DNA sequencing also proved 100% homology with the standard strains of HSV 1 and 2. The results of the sequencing data was in concordance with that of the snPCR, indicating that the PCR can be used for serotyping. The dual specimens obtained from 2 cases of ARN also proved the specificity of the PCR as the same serotype which was detected in AH was later detected in VA of the same patient. Out of the two cases with dual specimens of AH and VA, HSV 1 DNA was detected in 1 case and HSV 2 detected in other case indicating that either of serotypes can cause the infection. Serotyping along with detection could be completed in 6-8 hours as against the 24-72 hours required for other conventional methods.

As viral retinitis is caused by any of the three herpes viruses the intraocular fluids were also tested for the presence of CMV and VZV DNA. HSV is followed by VZV with a high positivity of 23.8% in cases of ARN, as VZV shares several biological features with well characterized HSV.¹³ CMV DNA was detected in only 9.5 % i.e., only in 2 patients one in accordance with CMV retinitis as diagnosed clinically and another case of ARN. Isolated cases of ARN being caused by CMV have been reported and thus it is possible that CMV was the aetiological agent in this case also. Hence all the three herpes viruses have been detected indicating that any of the three viruses can cause the infection leading to ARN, PORN, Viral retinitis. It is concluded, that the standardized snPCR can be applied directly onto the clinical specimens for rapid detection and serotyping of HSV.

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