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*International Journal of Current Research*
*Vol. 6, Issue, 02, pp.4896-4899, February, 2014*

**INTERNATIONAL JOURNAL OF CURRENT RESEARCH**

**ISSN: 0975-833X**

**RESEARCH ARTICLE**

# MULTIPLEX POLYMERASE CHAIN REACTION FOR THE DETECTION OF HERPES SIMPLEX VIRUSES (HSV1 AND HSV2)

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**ARTICLE INFO**

***Article History:***
Received 06th November, 2013
Received in revised form
10th December, 2013
Accepted 17th January, 2014
Published online 21st February, 2014

***Key words:***

HSV 1,
HSV 2,
Viruses,
Monoplex PCR,
Multiplex PCR.

**ABSTRACT**

Herpes simplex viruses (HSV) are important human pathogens causing diseases in a variety of different human tissues and animal species. HSV-1 is being most often transmitted non-sexually and HSV-2 most usually transmitted sexually. Primary HSV infections are usually symptomatic but may be sub clinical. Worldwide 65% - 90% rate of HSV infection found in different part of world and HSV1 is more common than HSV2. Few studies from India documented for HSV 1 and HSV 2 infections in different population groups. In India HSV 1 and HSV 2 seroprevalence in adults are 63% and 16.6% respectively. Due to the high impact of these viruses on our health, there should be a rapid, sensitive, specific and cost effective method for the detection of these viruses. Presently Herpes viruses are diagnosis by virus isolation, rapid commercial kits, conventional and real time PCR. Virus isolation is time consuming, rapid kit have low sensitivity and specificity while real time PCR are costly hence convention PCR are comparatively good choice. Thus this study was conceptualized for HSV1 and HSV2 detection by conventional PCR from standard strains of HSV1 and HSV2 which can on the clinical samples. First we standardized two monoplex PCR in two separate PCR tubes for HSV1 and HSV2 then Single tube Multiplex PCR for HSV 1 and HSV 2 was standardized. The specificity of the primers was checked with other related virus such as human cytomegalovirus. Multiplex PCR assay was applied on panel of clinical samples. The study will serve as rapid, accurate, specific and sensitive diagnostic assay which is crucial for HSV1 and HSV2 to help in patient management and prevention of spread of disease.



## INTRODUCTION

Herpes simplex viruses (HSV) belong to the family *Herpesviridae.* This family is divided into three subfamilies; α (alpha), β (beta) and γ (gamma) herpesviruses. At present nine herpesviruses are recognized as the natural human pathogens *(*Roizman and Pellet, 2001). Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) and Varicella-zoster virus (VZV) belong to the alphaherpesviruses, these viruses have a wide host range, a relatively short life cycle and establish latent infections preferentially in sensory ganglia. HSV are important human pathogens causing diseases in a variety of different tissues and animal species. HSV-1 is being most often transmitted non-sexually and HSV-2 most usually sexually transmitted (Umene and Kawana, 2000, Umene and Kawana, 2003). All herpes virions consist of four elements: DNA core, capsid, tegument and glycoprotein-containing envelope (Roizman and Pellet, 2001). The structure of herpes viruses consists of a relatively large double-stranded, linear DNA genome encased within an icosahedral protein cage called the capsid, which is wrapped in a lipid bilayer called the envelope. The envelope is joined to the capsid by means of a tegument (Mettenleiter *et al*., 2006). HSV-1 and HSV-2 each contain at least 74 genes within their genomes (McGeoch *et al*., 2006). These genes encode a variety of proteins involved in forming the capsid, tegument and envelope of the virus, as well as controlling the replication and infectivity of these viruses. Transcription of HSV genes is catalyzed by RNA polymerase II of the infected host (McGeoch *et al*., 2006). Immediate early genes, which encode proteins that regulate the expression of early and late viral genes, are the first to be expressed following infection. Early gene expression follows, to allow the synthesis of enzymes involved in DNA replication and the production of certain envelope glycoprotein. Expression of late genes occurs last; these groups of genes predominantly encode proteins that form the virion particle (McGeoch DJ *et al*. (2006). HSV-1 and HSV-2 are common human pathogens that can cause primary and recurrent infections of mucous membranes. Primary HSV infections are usually symptomatic but may be sub clinical. Recurrent infections are generally less severe than the primary infection. The most commonly seen clinical manifestations include are oro-facial and genital

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lesions. Ocular infections may include any part of the eye including the retina, conjuctiva, cornea and eyelids (Ganatra *et al*., 2000; Liesegang, 2001; Umene *et al*., 2003). Meningitis is usually benign, but the HSV encephalitis has been associated with high mortality (Tyler *et al*., 2004). Neonates are particularly at risk for serious HSV infections; early treatment appears to be an important determinant of the outcome (Kimberlin *et al*., 2001). In addition, immunocompromised patients are at risk of developing more severe HSV infections. HSV is now a major health concern, confirmed by the epidemic of genital HSV and enhanced acquisition of human immunodeficiency virus (HIV) infections in association with HSV infections (Mbopi-Keou *et al*., 2003; Celum, 2004). Worldwide 65% - 90% rate of HSV infection found in different part of world and HSV1 is more common than HSV2 (Chayavichitsilp *et al*., 2009). These rates of infection are determined by the presence of antibodies. An estimated 536 million people worldwide are infected with HSV-2 in 2003, with highest rate in sub-Saharan Africa and the lowest rate in Western Europe (Xu, F; *et al*. 2011). The prevalence of HSV-2 infection among health adult populations is higher in the USA than in Europe. Furthermore, HSV-2 seroprevalence varies widely among European countries (Malkin, 2004). In USA 57.7% population are HSV-1 infected and 16.2% are HSV-2 infected. Among these HSV-2 seropositive, only 18.9% were aware that they were infected of HSV (John Leo *et al*. 1982). During 2005-2008, prevalence of HSV-2 was 39.2%in block and 20.9% in woman (fiala *et al*., March 1973). Few studies from India have also documented seroprevalence of HSV 1 and HSV 2 infections in different population groups. HSV 1 and HSV 2 seroprevalence are in adults 16-40 year of age, are 63% and 16.6% respectively (Kaur *et al.,* 2005).

Due to the high impact of these viruses on our health, there should be a rapid, sensitive, specific and cost effective method for the detection of these viruses. Thus this study was conceptualized for HSV1 and HSV2 detection by conventional PCR from standard strains of HSV1 and HSV2 which can apply on the clinical samples. Presently Herpes viruses are diagnosis by virus isolation, rapid commercial kits, conventional and real time PCR. Virus isolation is time consuming, rapid kit have low sensitivity and specificity while real time PCR are costly hence convention PCR are comparatively good choice. There are few antiviral available in the market which appears very effective against HSV, if given in early after the infection. Hence a rapid accurate and sensitive diagnostic assay is crucial for HSV1 and HSV2 to help in patient management and prevent spread of disease.

## MATERIALS AND METHODS

### 1. DNA Extraction

Prototypes strains of Herpes Simplex Viruses (HSV1 and HSV 2) were obtained from Virology laboratory of All India Institute of Medical Sciences (AIIMS), New Delhi. Viral DNA was extracted from the aliquots of two Prototypes strains of HSV1 and HSV 2 with the help of a commercial kit (HiPurA$^{TM}$ Mammalian Genomic DNA Purification Spin Kit) following the manufacturer's instructions as well as by conventional phenol chloroform and iso-amyl alcohols method. Extracted DNA was use for standardization of the PCR for HSV1 and HSV2 viruses.

### 2. Standardization of Monoplex PCR for HSV 1 & HSV 2 using reference strains

Two monoplex PCR were standardized in two separate PCR tubes for HSV1 and HSV2 using their respective forward and reverse primers and DNA from the reference strains of HSV1 and HSV2 respectively. Different concentration of Primer, dNTP, $MgCl_2$ and Taq DNA polymerase were used for standardization of the assays. The best combination of concentration of Primer, dNTP, $MgCl_2$ and Taq DNA polymerase were selected after watching the band of HSV1 and HSV2 in the 2% agarose gel after electrophoresis.

### 3 Standardize single tube Multiplex PCR for Herpes Simplex Viruses (HSV 1& HSV2)

Single tube Multiplex PCR for HSV 1 and HSV 2 was standardized along with the reagents optimized as well as using the different conditions of initial denaturation, annealing and extension temperature. Basically both the Herpes Simplex Viruses (HSV1 and HSV2) monoplex polymerase chains reaction (PCR) were combine in a single tube with same condition of amplification. Standardized single tube Multiplex PCR assay applies on panel of clinical sample.

### 4. Specificity of primers

The specificity of the primers was checked with other related virus such as human cytomegalovirus (CMV). HSV 1 primer only amplified only HSV1 while HSV 2 primer amplified only HSV2.

### 5. Agarose gel electrophoresis

Amplicons were visualized on the 2.0% agarose gel. The agarose gel was prepared in 1X Tris, acetic acid and EDTA (TAE) and the amplicons were loaded with the help of 2µl of 1X loading dye buffer in the wells. Electrophoresis was performed for about 1hr at a constant voltage of 80V in running buffer containing 1X TAE and ethidium bromide (1µg/ml). After electrophoresis gels were visualized in a gel documentation system or the gel trans-illuminator (Gel doc™ XR, BioRad, USA).

## RESULTS

Viral DNA was extracted from the aliquots of two prototype strains HSV1 and HSV 2 by using the commercial kit as well as manual method of phenyl chloroform and iso-amyal alcohols. DNA extracted by commercial kit and manual method gives the similar results. Commercial DNA extraction method is very fast as compared with the Manual DNA extraction. Specificity of primer pairs was checked and none of the heterologus viruses got amplified in assay. Hence primers are very specific for their target and cannot amplify the other DNA in the samples. The HSV 1 primer only amplified only HSV1 while HSV 2 primer amplified only HSV 2. The primers were selected from the conserved region of the DNA

dependent DNA polymerase gene of the viruses. The optimum condition for the assays were initial denaturation at 94°C for 1 minute while denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute, Extension at 72°C for 1 minute and final extension was optimized at 72°C for 10 min. Forty (40) cycles were optimized for multiplex PCR. Amplicons were visualized on 2.0% agarose gel. Electrophoresis was performed for about 1hr at a constant voltage of 80V in running buffer containing 1X Tris, acetic acid, EDTA (TAE) and ethidium bromide (1µg/ml) after electrophoresis gels were visualized in a gel documentation system or gel trans-illuminator (Fig 1). Standardize single tube Multiplex PCR for Herpes Simplex Viruses (HSV1& HSV2) after the checking specificity was applied on the panel of ten clinical samples. The panel contains the five positive and five negative samples. All five positive were positive and all negative were negative by assay. In these five positive samples three were HSV1 and two were HSV2.

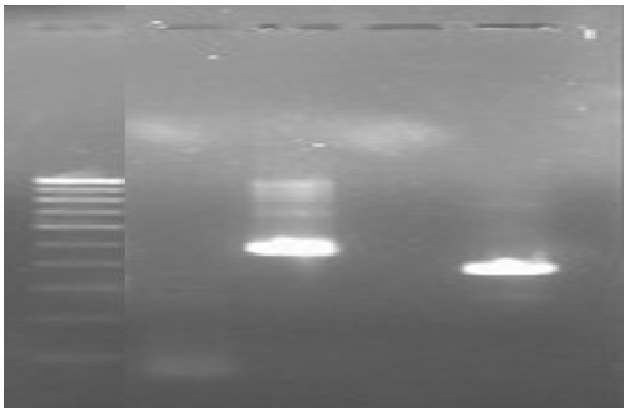

**Figure 1. Herpes Simplex Viruses (HSV 1& HSV 2) the Amplicons were visualized on agarose gel**

1st line contain marker
2nd line contain Negative control for HSV1
3rd line contain Negative control for HSV1
4th line contain Negative control for HSV2
5th line contain Negative control for HSV2

## DISCUSSION

Herpes virus super family contains eight different types of viruses which causes human infection. Herpes simplex virus (HSV-1 and HSV-2) can potentially infect any site of body, but infections of the skin, genital, oral mucosa, eyes, anus, rectum and the central nervous system are more common. Both HSV-1 (which produces most cold sores) and HSV-2 (which produces most genital herpes) are ubiquitous and contagious. They can be spread when an infected person is producing and shedding the virus. About 65% - 90% is the worldwide rates of HSV infection are found. HSV-1 is more common than HSV-2 with rate of both in increasing people age. In India 33.3% of individuals are seropositive for HSV-1 and 16.6% are seropositive for HSV-2. Those with both HSV-1 and HSV-2 antibodies are estimated at 13.3% of the population. (Kaur *et al.*, 2005). Due high impact of these viruses on our health, there should be a rapid, sensitive, specific and cost effective method for the detection of these viruses. Presently Herpes viruses are diagnosis by virus isolation, rapid kits, conventional and real time PCR. Virus isolation is time consuming, rapid kit have low sensitivity and specificity while real time PCR are costly hence convention multiplex PCR are good choice. Multiplicity reduced the cost of PCR assay as compare with monoplex PCR. In summary, this multiplex real-time PCR allows the rapid and specific detection of HSV1 and HSV 2, as well as co-infection with both, in clinical samples. Although virus isolation is gold standard but when samples need to transport, they may be freeze and though, which reduced the chance for virus isolation but it will not affected the PCR. Rate of virus isolation may be decreases due to damage of virus receptor, so result of PCR always better than virus isolation. Hence this study will serve as rapid, accurate, specific and sensitive diagnostic assay is crucial for HSV1 and HSV2 to help in patient management and prevent spread of disease.

### Conclusion

In the present title "To Standardize Multiplex Polymerase Chain Reaction (PCR) for Detection of Herpes Simplex Viruses (HSV1 and HSV2)" was standardised for detection of HSV1 and HSV2 viruses. First monoplex PCR was standardized for HSV1 and HSV2 then multiplex PCR was standardized for HSV1 and HSV2 in a single tube. These assays were standardised using reference strain of HSV1 and HSV2 viruses. The specificity of the primers was checked with other related virus such as human cytomegalovirus (CMV). HSV1 primer only amplified only HSV 1while HSV 2 primer amplified only HSV 2. Herpes Simplex Viruses (HSV1 and HSV2) have very high impact on our health. In India 33.3% of individuals are seropositive for HSV-1 and 16.6% are seropositive for HSV-2. Those with both HSV-1 and HSV-2 antibodies are estimated at 13.3% of the population. Indian men are more likely to be infected with HSV-2 than women and increasing seroprevalence of this virus is associated with an increasing age. Herpes Simplex Viruses (HSV1 and HSV2) can be diagnosis by rapid antigen test, virus isolation in VERO cell line and modern molecular techniques. These are may be either less sensitive, specific, time consuming or high cost of test. The present study has been conceptualization for the detection of Herpes Simplex Viruses (HSV1 and HSV2). As there are few antiviral available in the market which will appears very effective against HSV, if given in after early the infection. Hence this study will serve as rapid, accurate, specific and sensitive diagnostic assay is crucial for HSV1 and HSV2 to help in patient management and prevent spread of disease.

### Acknowledgement

We are highly acknowledged the Dr Lalit Dar, Professor, Department of Microbiology, All India Institute of Medical Sciences (AIIMS), New Delhi for providing standard strains of the herpes viruses.

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