

Study of Herpes Simplex Amplitude by PCR in Children CSF Suspicious to Cerebral Encephalitis

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HSV1 is one of the common agents recurrent aseptic meningitis and fatal endemic encephalitis with an incidence rate 7-10.5%. Routine and available methods of diagnosing Herpes viruses, can't diagnose Herpes contamination in virus culture promptly and accurately and because of limitation in frequent methods, molecular new methods such as polymerase PCR chain reaction for recognizing parts of DNA genes of Herpes simplex specially compliment fixed sequence are developed. This study for the first time with PCR technique for diagnosing Herpes simplex virus type I & II in Iran was modified and used. This technique is one of the In this study PCR reaction using a pair of designed special primer for a common area DNA polymerase coding gene of HSV 1 & HSV 2 viruses on the DNA achieved from standard species, was modified and studied due to sensitivity and specificity. Multiplying product was cloned and its sequence was determined. The people under study in this research was contained 184 CSF samples suspicious to cerebral encephalitis in children 1-10 years old admitted in Tehran Mofid hospital. After making sure of sensitivity and specificity of modified test the people under study were analyzed by PCR test. PCR modified product in this study was 454 bps. The sensitivity of test about 50 copies of target DNA gene was determined. This reaction has a very high specificity so that with DNA of many microorganism, crossed reaction was not seen. From 184 samples of CSF, 45 was positive by PCR and in this order incidence of cerebral encephalitis resulting from serotype 1 & 2 of Herpes virus was about 24% among the people under study. Achieved result in this study shows the point that this modified molecular method is an effective tool for immediate diagnose of Herpes simplex virus in affected samples.

Key words: Herpes simplex, PCR, virus culture, cerebrospinal encephalitis.

Herpes viruses are belong to Herpes Viridae class and they are infection factors nearly common in children and causes systematic disease that involving nervous system is the most important sign of this disease^{1,2}. HSV is one of the factors of recurrent aseptic (Mollaret meningitis) and sporadic fatal encephalitis. That is recognizable with the PCR in CSF³. According to control disease center [CDC] report in USA, Incidence of causes a lot of side effects in patient Using Antivirus

appropriate agent (Acyclovir) mortality rate reduces to 20%⁴. The primitive clinical diagnosis of herpetic encephalitis is not enough Regarding^{3,4}. High mortality rate of herpetic encephalitis in patients, quick and rapid diagnosis the virus in the patients suffering from herpetic cerebral encephalitis is important⁵. Herpes simplex diagnosis procedures can be divided in two groups. 1) Ways based on culture and 2) noncultural methods, that they have differences in speed, reliability, specificity and sensitivity^{1,6}. Procedure based on culture are time consuming (several weeks) with the relatively lower sensitivity and need facilities and enough experience for explanting the results. Nonculturing methods 1)

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Immunofluorescence 2) Antibody Assay 3) complement fixation 4)ELISA 5) Neutralization 6)Polymerase chain reaction or [PCR]^{2,7}.The methods based on inoculation to cellular cultures like (VERO) cellular line, because of their special needs like recurrent passage or comparing based on Elisa with special antibodies, are time consuming⁸.Methods based on neutralization and compliment fixation have low sensitivity Therefore most of the available For diagnosis of herpetic infection are not fit to quick detection^{1,9}. For overcomes these problems, methods of multiplying of nucleic acid such as PCR , in two last decades greatly have been developed¹⁰. Methods based on PCR, because of their sensitivity and more accuracy are appropriate for immediate diagnosing a wide range of infection factors, particularly herpes virus¹¹. Methods based on multiplying for recognizing some of special parts of herpes genome DNA have highly speeded and specification^{6,12}. However the rate of sensitivity in this technique greatly depends with : ‘sample and many other factors .The design primers are generally for type Δ and $\Delta\Delta$ herpes simplex polymerase DNA gene . Common sequence of polymerase DNA gene of both virus types was determined^{8,9}.The target of this study is improving PCR technique to diagnose herpes simplex virus in affected samples [CSF] that are suspicious to herpetic cerebral encephalitis with high sensitivity and repeat capability.

MATERIALS AND METHODS

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Preparing the standard Herpes simplex strain and culturing method

In this study 184 samples of patients CSF suspicious to cerebral encephalitis (prepared at pathology laboratory in Tehran Mofid hospital)were analyzed .Standard strains of HSV Δ & $\Delta\Delta$ viruses were provided from pasture institute virology sector and in the same sector in RPMI area and (VERO) cellular specious (kidney cells of green African monkey) cultured .

DNA extraction from standard strain

DNA was extracted using DNP (Cinaclon) kit from culture samples and patients CSF and PCR tests were optimized on this strain.

Synthetic primers

Single primer pair was used to amplify HSV gene target fragment based on Gen Bank .The primers are following.

Forward primer: 5-ACCTACCGGCATACAAGCTCA-3

Reverses primer: 5-AAGTGGCTCTGGCCTATGTCC-3

PCR test optimization to detect Herpes simplex

PCR was performed in standard enzyme concentration 5 U/50ul reaction, Mg 75mM, dNTP 500uM each, primer 10uM, template DNA 0.1-250 ng. The mixtures were incubated for 2 min at 94c for primary denaturation, 20 sec at 93c for secondary denaturation of the target DNA and then, annealing at 70 for 20 sec, and extension at 72c for 5 min that 40 cycles was performed. The amplified products were analyzed by electrophoresis on 2% agars gel containing 0.1 g of ethidium bromide per ml in TBE buffer. The PCR product was visualized under UV light and photographed.

PCR cloning product as a positive control

PCR product was cloned in pTZ57R plasmid after purification using T/A cloning Thermo scientific (cat: K1214) kit and the multiplied part using (Termination Dideoxy chain). The resulting plasmids were extracted using plasmid Mini Extraction kit from (Korean Macrogen Company)(Fig.1).Then PCR product containing plasmids were confirmed using PCR technique and used as positive control in PCR tests.

Identification of PCR test sensitivity and specificity

A suspension of serotype I & II of Herpes simplex culture was provided which its concentration is 1×10^6 CFU/ml, and its DNA was extracted using DNG plus. Extracted DNA was diluted to 1 copy using dilution method (serial dilution). To specificity evaluation Herpes Simplex Virus, VZV, CMV, HBV, HCV, Saccharomyces cerevisiae, Rat and Human DNAs were extracted and located beside the PCR test positive control.

Sample preparation

In this partial study, of 184 patients who were children 1-10 years old referred to Mofid hospital with clinical symptoms take CSF, these samples culture on the VERO cellular in institute Pasteur and then detected by PCR method in Erfan hospital.

RESULTS

Culturing HSV in cellular

Two days after; HSV culture on the VERO cellular levels, CPE resulting from virus multiplying appeared, this CPE was observed in turgid, balloon and cluster form in cell(Fig.2).

PCR test optimization

Amplicon of Herpes simplex (454 bps) observed in optimized PCR test on Agarose gel 2%, positive and negative controls.(Fig.3)

PCR specificity and sensitivity tests

PCR specificity test was done using DNAs of Herpes Simplex Virus, VZV, CMV, HBV, HCV, Saccharomyces cerevisiae, Rat and Human and unwanted bound or product was not seen, which means PCR test has very high specificity and reacted Herpes simplex gen and test specificity was reported 100%(Fig.4a).PCR sensitivity

identification test was done by preparing deferent serial dilution of Herpes simplex DNA, which results showed that proliferation is done with only 50 DNA copies. No bound was seen in less than 50 copies of DNA which means high sensitivity (Fig.4b).

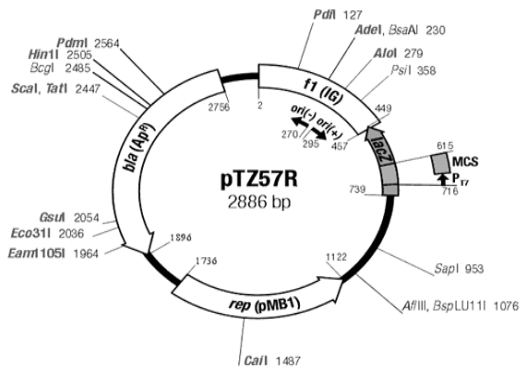


Fig. 1. pTZ57R plasmid

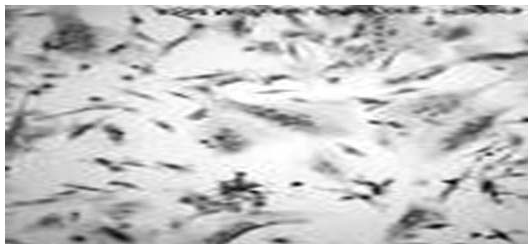


Fig. 2. Balloon and cluster form CPE of HSV on the VERO

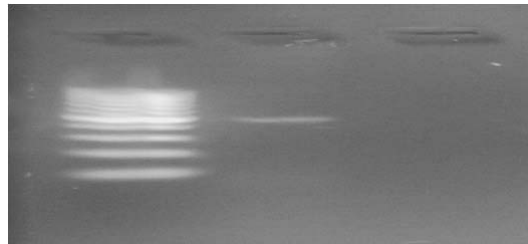


Fig. 3. Size marker Fermentase, 100 bps DNA Ladder plus 2- Multiplied part (454 bps), 3-Negative control

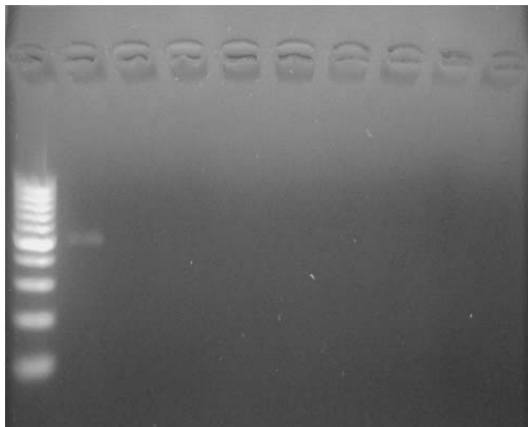


Fig. 4(a). Column 1: Sizemarker 100 bps DNA Ladder plus, 2- Positive control HSV,3- DNA of VZV,4- DNA of VZV, 5- DNA of sacaromices,6-DNA of HBV,7- DNA of HCV, 8- Human DNA, 9- Rat DNA,10- Negative control

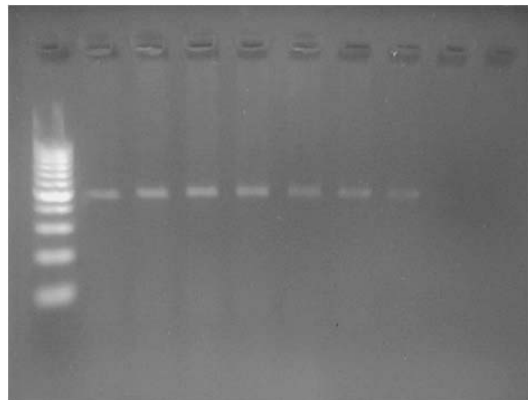


Fig. 4(b). Column1-Sizemarker 100 bps DNA Ladder, 2-Positive control (herpes simplex), 3-1/000/000 virus particles, 4-100/00 particles, 5- 10/000 particles, 6-1000 particles, 7-100 particles, 8- 50 particles, 9- 5 ones and 10-Negative control, respectively

PCR results of CSF samples

From 184 CSF samples of patient under study who were children 1- 10 years old were admitted in Tehran Mofid hospital ,45 of them had positive PCR and cerebral encephalitis resulting from serotype 1 and 2 of herpes virus in 24% of people under study was confirm. (Fig. 5)

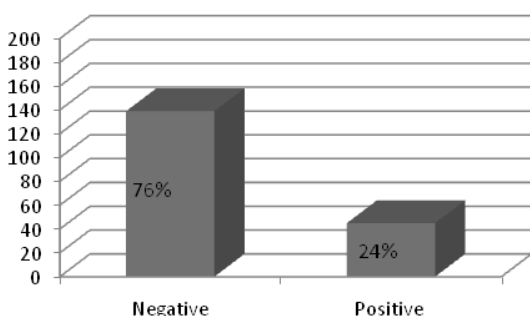


Fig. 5. PCR results of CSF samples

DISCUSSION

Herpetic cerebral encephalitis is one of the most dangerous and fatal disease in the world yet. The specified clinical symptoms of sporadic encephalitis that is high epidemic, sudden high fever, focal neurologic signs, especially signs that indicate temporal lobe involved. This herpetic infection can be diagnosed in different ways ,antibody assay , ELISA , culture area and molecular methods¹³ .To combine the serologic and culture results is usually a useful way for diagnosing herpes virus. Nevertheless these mentioned methods often are not enough for accurate diagnosis. Time limitation and low reaction , always are one of the important obstacles for using serologic methods^{1,5}. Due to importance of disease type, time and expense and sensitivity in the test , having a prompt recognition for immediate effective diagnosis in herpetic cerebral infections , have priority. Ideally this method must be able to diagnose typically encephalitis (HSV I & II)^{1,2}.The methods of culturing this virus takes 4-5 days in laboratory .since cultural method sensitivity is reported 65-70% and serologic methods is about 80% , using new methods like molecular methods such as PCR ,that their sensitivity is about 1-10 particular virus ,is necessary. One of the ways for increasing PCR sensitivity is using its changed

method named Nested PCR Following the wide researches by many researchers like Mitchel¹⁴, Koeing¹⁵, Schutzhard¹⁶ who used Nested PCR method for diagnosing herpetic encephalitis more sensitivity created to this study. But it is important to note that , this method takes longer time and needs more materials and there is more probability of contaminating.(the most important problem for multiplying methods like PCR ..In other studies , researchers like Madhavan¹⁷ ,Raymond¹⁸ and Burrows¹⁹ at the end of decade 90 and lately Kaufman *et al.*,²⁰ in 2005 , used real time PCR technique for diagnosing herpes simplex virus that different sensitivities were reported but in 2-3 cases, the sensitivity of studies , was higher than this research and for the rest it was lower than these studies.

For Multiplex PCR technique has been used a lot . Some of the scientists who used this technique are Chichili²¹ in 2005, Sugita²² in 2008 and Toshio in 2009 that they presented more higher sensitivity from this research. The other studied method is PCR microarray technique that it was the base of Jianrong²⁴ research in 2010 on diagnosing children encephalitis. This method needs very expensive equipment .In another study in 2009 by Eric *et al.*,²⁵ in diagnosing HSV using mentioned technique find that sensitivity of present study is much higher than Eric's test. Clavario *et al.*,²⁶ used [HC-PCR] Herpes consensus PCR in 2002 for study about herpetic encephalitis that sensitivity of 100 particles of virus in his test was lower than present study . In several studies by Robinson *et al.*,²⁷ in 1992 and the following a lot of similar studies like Aldea's²⁸ in 2002 and Xuelian¹² in 2009 , from random PCR was used in research of herpetic disease that usually the sensitivity was lower than modified test. Many Iranian scientists like Noorbakhsh *et al.*,²⁹ in 2004 studied about diagnosing of herpetic encephalitis and also khodadust³⁰ studied the herpetic keratitis using Nested PCR in the same year, of courses the sensitivity of modified test was much higher than their test . Finally Ashraf *et al.*, in 2009³¹ existence of Herpes in anterior lens capsule of patients eye that affected to gradually blindness syndrome with PCR technique diagnosed that sensitivity of their study with present study was completely adjusted .In this study PCR technique for immediate diagnosis of cerebral encephalitis resulting from

Herpes simplex virus, was modified using primers were proper to common sequence of DNA polymerase gene of virus serotype I and II. Specificity 100% and sensitivity 50 virus particles were obtained and sequencing, Homology 100% between both serotype virus about amplicon was confirmed. Other presented protocols by researchers have between 1-100 Herpes virus that the difference depends on: 1) Gene under research 2) Designing and type of primer 3) PCR condition 4) The method of DNA virus extraction from clinical samples and the other specificities

CONCLUSION

In general, classic diagnostic methods, because of their limitation (time consuming, low sensitivity, high expensive and skill and also hard work) are not an ideal method for recognizing a variety of infections, that help to diagnose factors like Herpes simplex. Result from this study clearly shows that PCR technique for diagnosing fixed and common sequences exist in virus DNA polymerase, is a useful, valuable and reliable technique with a sensitivity, specificity and high accuracy.

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REFERENCES

1. Franzenröhl.E, Tiveljunglindell.A, Grillner.E, Increased detection rate in diagnosis of herpes simplex virus type 2 meningitis by real-time PCR using cerebrospinal fluid samples, *American Society for Microbiology*, 2007; **45**: 2516-2520.
2. Ziayi.M, Herpes simplex infection, *J. Clin. Virol*, 2009; **62**: 1-12.
3. Graclabardacia.PD, Jose'pana.M, et al. Value of the Polymerase Chain Reaction in the diagnosis of herpes infections of the nervous system in the diagnosis of herpes infections of the nervous system, *Enfermedades Infecciosas y Microbiología Clínica*, 2004; **22**: 150-155.
4. Sacks.SL, Griffiths.PD, Corey.L, Cahen.C, et al, HSV Shedding, *Antiviral Research*, 2004; **63**: 19-26.
5. Thomas.FS, James.RU, Mark.JE, Lynne.MS, Emily.AV, Development, implementation, and trend analysis of real-time PCR tests for the Clinical microbiology laboratory, *Clinical Microbiology Newslette*, 2004; **26**: 145-153.
6. Nicoll.S, Brass.A, Cubie.HA, Detection of herpes viruses in clinical samples using real-time PCR, *J. Virological Methods*, 2001; **96**: 25-31.
7. Ratman.S, Severini.A, Zahariadis.G, Romanowski.PB, The diagnosis of genital herpes-beyond culture: An evidence-based guide for the utilization of polymerase chain reaction and herpes simplex virus type-specific serology, *Con J. Infect Dis Med Microbial*, 2007; **18**: 233-240.
8. Junrong.S, Yidong.W, Meiting.C, Shiqiang.S, Rapid diagnosis of herpetic encephalitis in Children by PCR- microarray technology for Simultaneous detection of seven human herpes viruses, *Springer- Virology*, 2010; **169**: 421-425.
9. Faulkner.S, Smith.A, A Prospective diary study of the role of psychological stress and negative mood in the recurrence of Herpes simplex virus [HSV1], *Stress Health*, 2009; **25**: 179-187.
10. Kaenig.M, Koch.SR, Aldons.W, Hickman.M, Comparison of light-cycler PCR, enzyme immunoassay and tissue culture for detection of HSV, *Diagnostic Microbiology and Infectious Disease*, 2001; **40**: 107-110.
11. Madhavan.HN, Priya.K, Anand.AR, Therese. KL, Detection of Herpes simplex virus [HSV] genome using polymerase chain reaction [PCR] in clinical samples Comparison of PCR with Standard laboratory methods for the detection of HSV, *J. Clin. Virology*, 1999; **14**: 145-157.
12. Xuelian.Y, Bisheng.S, Yan.G, Xianan.Z, A random PCR Screening system for the *J. Virol. Methods*, 2009; **161**: 91-97.
13. Yi-Wei.T, Mitchell.PS, Espy.JE, Thomas.FS, Persing.DH, Molecular diagnosis of Herpes simplex virus infections in the central nervous system, *J. Clin. Microbiology*, 1999; **37**: 2127-2136.
14. Mitchell .PS, Espy.MJ, Simth.TF, Toal.DR, Rys.PN, Berbari.EF, Osmon.DR, Persing.DH, Laboratory diagnosis of central nervous system infection with Herpes Simplex virus by PCR Performed with cerebrospinal fluid Specimens, *J. Clin. Microbiology*, 1997; **35**: 2873-287.
15. Koenig. M, et al, Comparison of cell culture and PCR in diagnosis of Herpes virus diseases, *Clin. Microbiol. News letter*, 2001; **26**: 19.

16. Schmutzhard.J, Riedel.HM, Wirgart.BZ, Grillner.L, Detection herpes simplex virus type 1,2 and Varicella-Zoster Virus in skin lesions, Comparison of real-time PCR, nested PCR and virus isolation, *J.Clin.Virology*,2004; **29**:120-126.
17. Madhavan.NH, Priya.K, Anan.AR, Therese.K, Detection of herpes simplex virus genome using polymerase chain reaction in clinical samples Comparison of PCR with standard laboratory methods for The detection of HSV, *J.Clin.Virology*, 1999; **14**:1450-1510.
18. Raymond.P, Podzorski. Jame'Baker, Joseph.R, Merline.RehanaQureshi, Joan.E, Holsinger, Utility of restriction fragment analysis for typing herpes simplex virus ampelicons following PCR of targets in the DNA polymerase gene, *Diagnotic Microbiology and Infections Disease*, 2000; **37**: 289-291.
19. Burrows.J, Vandoomum.GJJ, etal, Comparison of cell culture and PCR in diagnosis of Herpes infections, *Clin.Microbiol.News lette*, 2002; **26**: 19-23.
20. Kaufman.HE, Azcny.AM, Vamell.ED, Sloop.GD, HSV-1 DNA in tears and Saliva of normal adults, NIH.Public.Access,2005; **46**: 241-247.
21. Chichili.GR, Athmanathan.S, Farhatullah.S, Gangopadhyay.N, Jalali.S, Pasricha.G, Sharma.S, Multiplex polymerase chain reaction for the detection of herpes simplex virus, Varicella-zoster virus and Cytomegalo virus in ocular specimens, *Curr Eye Res*, 2003; **27**: 85-90.
22. Sugita.S, Shimizu.N, Watanabe.K, Mizukami.M, Use of multiplex PCR and real time PCR to detect human herpes virus genome in ocular fluids of patients with verities, *J. BMJ*, 2008; **92**: 928-932.
23. Toshi.T, Kuzuhira.K, Hidenon.S, Rapid and simultaneous detection of 6 types of herpes virus, *Biomedical Research*, 2009; **30**:279-285.
24. Jianrong. S, Yidong.W, Meiting.C, Shiqiang.S, Rapid diagnosis of herpetic encephalitis in Children by PCR-microarray technology for Simultaneous detection of seven human herpes viruses, *Springer-Virlology*,2010; **169**:421-425.
25. Eric.C, Sung.GK, Edward.JD, Rodrigo.CBC, Experimental reactivation of latent canine Herpes virus-1 and induction of recurrent ocular disease in adult dogs, *J.Veterinary Microbiology*, 2009; **138**: 98-105.
26. Calvario.A, Bozzi.A, Scavasciuli.M, Ventola.C, Seccia.R, Stomatic.D, Brancasi.B, Herpes Consensus PCR test: a useful diagnostic approach to the screening of Viral diseases infections of the central nervous system, *J. Clin. Viral*, 2002; **1**:71-78.
27. Robinson.PA, High.AS, Hume.WJ, Rapid detection of human Herpes simplex virus type 1 in saliva, *Archives of Oral Biology*, 1992; **37**: 797-806.
28. Alde.C, etal, Comparison of cell culture and PCR in diagnosis of Herpes virus infections, *Clin. Microbiol. News letter*, 2002; **26**:19-23.
29. Nourbakhsh.S, Tonkaboni.H, Asghaee.M, Hosseini.F, Detection of herpes virus infection frequency in aseptic meningitis in children admitted to Rasoul-e-Akram and Mofid hospitals, *SID*, 2004; **42**: 666-670.
30. Khodadoost. MA, Sabahi.F, Jabbarvand.B.M, Rousai.MH, Saderi.H, Bavili.A, Olyaaee.S and Karimi.A.M, Study of a Polymerase Chain reaction based Method for Detection of Herpes Simplex Virus Type 1 DNA among Iranian Patients with Ocular Herpetic Keratitis Infection, *Japanese Journal of Ophthalmology*, 2004; **48**(4):126-135.
31. Ashraf.H, Roozitalab.MH, Akbari.M, Eghtedari.M, Alborzi.A, Detection of Herpes Simplex Virus DNA in Pseudo exfoliation Syndrome, *IJMS*, 2009; **34**(2):507-513.