Research Article
AMERICAN JOURNAL OF PHARMACY AND HEALTH RESEARCH
www.ajphr.com
2014, Volume 2, Issue 7
ISSN:2321–3647(online)

Studying and Comparing diagnostic method to determine the abundance of type 1&2 of Herpes simplex virus in CSF in encephalitis patients

Somaieh Nasereslami*[1],Mohammad Hassan Shahhosseiny[2]
1.Department of Microbiology Science and Research Branch, Islamic Azad University, Tehran, Iran.
2.Microbiology Department, Shahr-e-Qods branch, Islamic Azad University, Tehran, Iran.

ABSTRACT

Type 1&2of Herpes simplex virus [HSV] is infection causing factor in central nervous system encephalitis in human molecular methods are the best ways to diagnose this pathogen agent. In this study new molecular technique LAMP to determine both types in virus evaluated. In an experiential study, 184 sample of CSF in Tehran Mofid hospital was provided. Sample of DNA was extracted by DNA Cinaclonekit. A bundle of 6 primers especially for DNA polymerase gene of HSV virus was designed. Reaction product LAMP by increasing saber green was confirmed. The result of LAMP technique by K square test was compared with the result of PCR technique by K square. The sensitivity of modified technique LAMP was up to 5 particle viruses and the sensitivity of PCR technique was up to 50 particle viruses. Both technique LAMP and PCR to diagnose HSV virus types 1, 2 had specificity 100%.From 184 samples CSF according to LAMP technique 60 samples were positive while in PCR technique 45 samples were reported as positive. The sensitivity of LAMP was 10 times higher than PCR technique. Comparing these two methods using K square test showed that this to methods in a meaningful level $P<0.05$ are different. Determining type 1 &2of HSV in CSF samples by LAMP technique has a high specificity and sensitivity.

Keywords : Herpes simplex virus, LAMP, PCR, CSF.

*Corresponding Author Email Somaieh_nasereslami@yahoo.com
Received 18 May 2014, Accepted 25 May 2014

Please cite this article in press as: Nasereslami S *et al.*, Studying and Comparing diagnostic method to determine the abundance of type 1&2 of Herpes simplex virus in CSF in encephalitis patients. American Journal of Pharmacy & Health Research 2014.

INTRODUCTION

Herpes simplex virus (type 1 & 2) is in group of nervous Virus. That can stay in human body for a long time in hidden, following the hidden, in sensory neuron of human ganglion restart its activity[1] .Reactivating of HSV infection can accrue automatically or under physical or excitingly pressures and weakness in immunity system[2] The method of diagnosing of the virus divides in two parts, morphologic, immune morphologic, serologic, virology and new molecular methods[3]. One of the simplest methods for diagnosing the virus is preparing sample from skin lesion base (T-zank smear) and also using electron microscope. The other diagnostic method is culturing virus that this method is gold standard [4]. Serological methods for diagnosing virus are used based on recognizing antibody gG1 or gG2 [5]. The problem with these ways is time consuming, lack of ability in diagnosing in primitive stages of disease and also there is probability that the negative and positive answers be false [6]. Therefore using molecular diagnostic methods are necessary [7.] Molecular diagnostic methods based on separation and multiplicity in virus nucleic acid containing polymerase chain reaction (PCR), isothermal amplification and hybridization that even in primitive stages of contamination can diagnose the pathogenic agent accurately [7,8]. Amplification in spite of advantages it has special problems. For example using PCR technique, that is considering a gold standard method, although with a high accuracy and a lot of usage, it needs high-tech and expensive equipment like Thermo cycles[7,9]. From molecular diagnostic methods in which multiplicity happens in form of isothermal amplification and it doesn't need term cycler, isothermal amplification technique is through the (LAMP) that for the first time was modified by Notomi et.al in 2000.In this reaction they use 6 special primers, under the title internal primers FIP (FIC, F2) and BIP(BIC, B2), external primers F3 and B3 and loop primers LB (LBP) and LF (LPF) with a high specificity is used. Internal primer FIP in DNA model joint to the complimentary area F2 (F2C) in chain model And starts compliment chain synthesis then fixing the external primers F3 and B3 with DNA enzyme of BST polymerase. This chain gets longer that it causes loop-form in DNA structure. This ring-stem structure of DNA acts as starter of LAMP reaction.FIP primer joins the loop in DNA stein-ring structure starts substitute for chain synthesis that DNA structure creates an average in ring-stem form that it has are versed copy from target sequence. That forms at the other end of gene by BIP primer .The released chain makes a structure with external ring that acts as a model for BIP primer .Finally dumbly form DNA is produced and as the substance of the stage of multiplying cycle LAMP acts with designing oligonucleotides special for these structures can use them in hybridization so that it

doesn't need denaturation with temperature after multiplying that indicates all stages from amplifying to diagnosing are done in the same temperature. The final products of ring-stem DNAs with several reversed sequence from target DNA and structures like cauliflower with multiple rings [10-12]. In recent years many studies in relation with usage of this technique for diagnosing HSV has been done [3-16]. This study for the first time with LAMP technique for diagnosing herpes simplex virus types 1, 2 in Iran was modified and used. This technique is one of the diagnostic methods with high speed and sensitivity and low expense. In this research CFS samples was tested with this technique and LAMP technique results compared with conclusion achieved form PCR technique.

MATERIALS AND METHODS

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 HSV1,2 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 Cinaclonekit (DN811530) and DNG plus technique from culture samples and patients CSF and PCR tests were optimized on this strain.

Synthetic primers

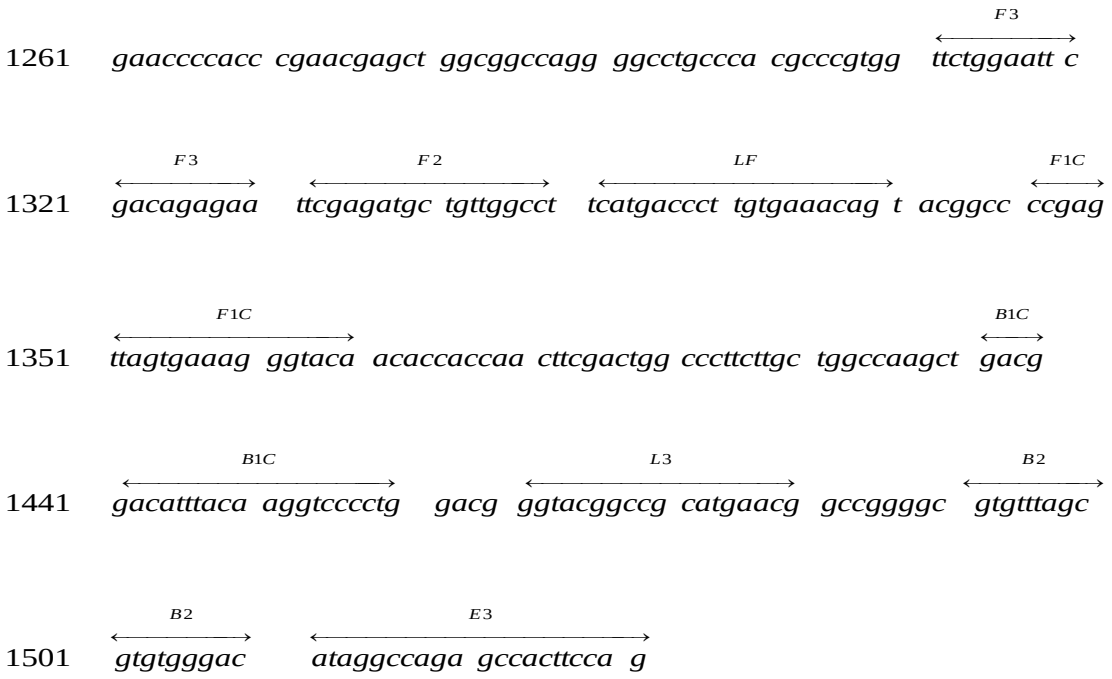

Figure 1: Specific target sequence site of LAMP primers on

LAMP primers were designed by software primer explores V4 based on DNA gene of HSV virus polymerase(number.AB231460.1Accession), that is common gene between types.(Table1-figure 1)

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 0.1g of ethidium bromide per ml in TBE0.5Xbuffer. The PCR product was visualized under UV light and photographed.

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

Reveres primer: 5-AAGTGGCTCTGGCCTATGTCC-3

LAMP test optimization to detect Herpes simplex

LAMP reaction mixture was prepared as following order: DDW: 5.2 micro liter, Betaine 0.8Mol, Mixture of LAMP reaction: Reaction mixture finally is 25 microliter and containing dNTPSinagen in thickness of 1.4mM, Betain in thickness of 0.8m, 8 units of BST enzyme (new England Bio labs, lot:33/110806) buffer enzyme in thickness of 1X containing Tries-Hcl(20mM),Kcl (10mM) ,(NH4)2So4 (10mM) ,MgSo4(9mM),Primers F3 and B3 each one in thickness of 0.2mM, FIP and BIP primers each one in thickness of 1.6 mM and loop primers LF and LB each one in thickness of 0.8muM

LAMP reaction evaluation

Thermal profile to glmM proliferation is in a simple heater block in 66◦C for 60 min. Positive and negative controls were used in each round of reaction. To evaluate reaction product 1 microliter cyber green 0.1% (In vitro gene cat:49753A) which was diluted 10 time more, was added to each tube and observed under trans illuminator with 302 nm wave length. Positive reaction containing tube was observed as florescent green and negative reaction as orange color.

Identification of LAMP test sensitivity

Dilution of DNA virus from 1 million to 5 particle virus by (serial-dilution) or Kochs Method prepared. By preparing the dilutions 10 ml from tube containing 1 million particles was added to

90 u/l dilute water and 100000 particle dilution was obtained. In the next stage 10 u/l from tube with 100000 particles added to 90 u/l dilute water and dilution with 10000 particles obtained. They continued it Up to 100 particles then 50 u/l from 100 particles was added to 50 u/l dilute water and obtained dilution with 50 particles. Dilution with 25 particle was prepared, finally 20 u/l from the tube containing 25 particle mixed with 80 u/l dilute water and dilution with 5 particles virus obtained. After providing serial dilution of sample and after LAMP test cyber green 0.1% added to each tube then observed under UV light.

Identification of LAMP test specificity

To LAMP test specificity extracted DNAs of Human, HSV, CMV, VZV, HBV, MTB, Saccharomyces cerevisiae, HCV and negative control were located LAMP test.

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 and LAMP methods in Erfan hospital. Comparing the results of two techniques PCR and LAMP: The obtained results from LAMP technique with the results of PCR technique by software SPSS.V10 and using K square test was compared and studied.

LAMP test optimization

LAMP test was optimized in 66◦C for 1 hour.(Figure 1) glmM gen of Herpes Simplex (After ending the reaction following adding saber green, positive pipe got light green and negative pipe got a very light orange. PCR reaction product was confirmed by electrophoresis 2% agars gel. (Figure 2)

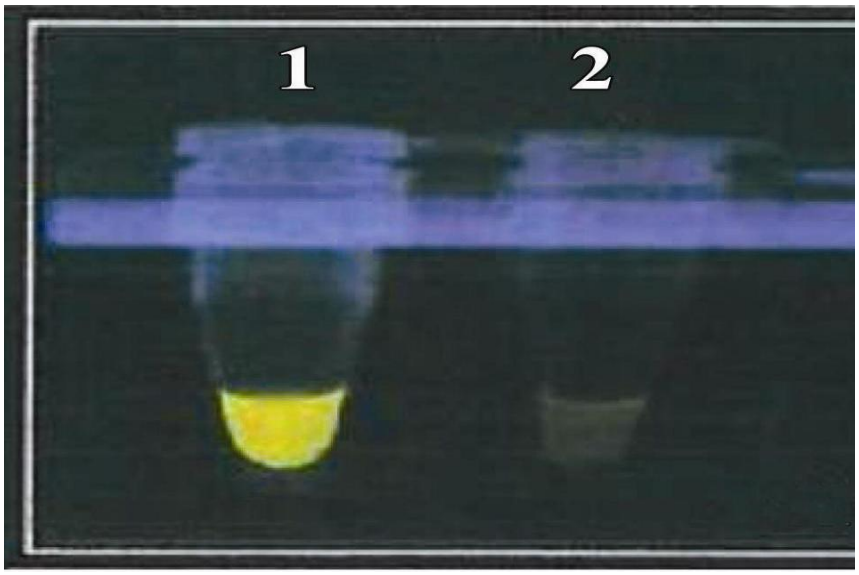

Figure 2:1-Positive Control, 2-Negative Control

LAMP test specificity and sensitivity identification

LAMP reaction was done deferent DNA dilutions of Herpes Simplex in 66◦C for 60 min. The sensitivity results of LAMP test showed that proliferation is done with only 5 copies of DNA and green color was observed which in less than 5 copies it was not seen and tube remains in light orange which shows high test sensitivity (Figure3).LAMP test had very high specificity which did not react with any other infection agents except Herpes Simplex DNA (Figure4), the sensitivity of modified technique LAMP determined up to 5 particle virus. PCR test also like LAMP technique in diagnosing this virus had specification 100% but in determining the sensitivity of PCR showed that this technique up to 50 particle virus sensitivity

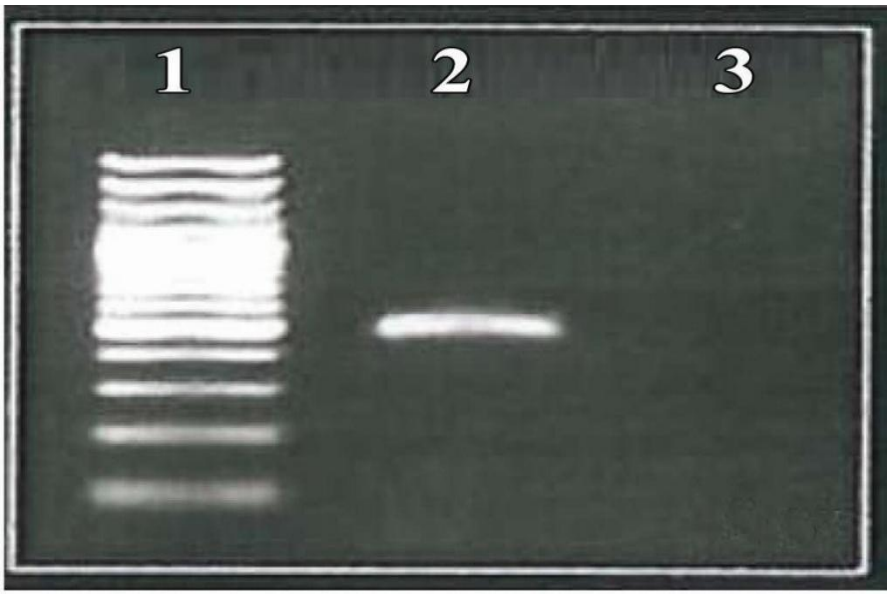

Figure 3: Size Marker 100 bps, 2-Positive Control, 3-Negative Control.

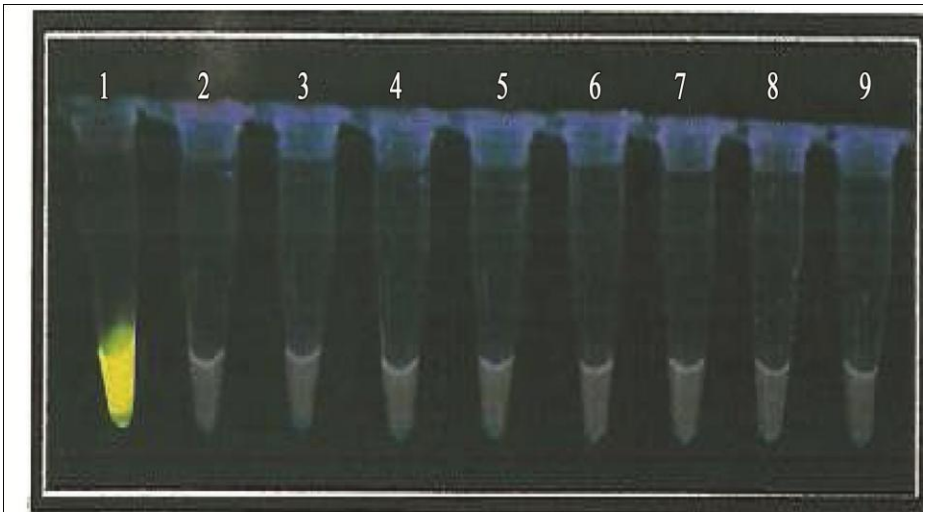

Figure 4: 1-HSV, 2-CMV, 3-VZV, 4-HBV, 5-HCV, 6-MTV, 7-Human, 8-Sccharomices, 9-Negative Control.

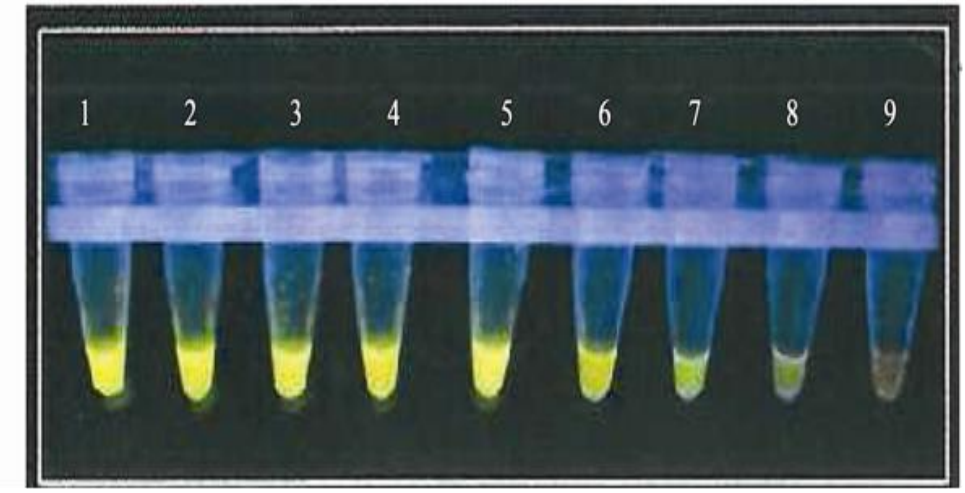

Figure 5: Serum Particles Containing From Left to Right: 1)1,000.000, 2)100,000, 3)10,000, 4)1,000, 5)100, 6)50, 7)25, 8)5, 9)Fewer than 5 Particles.

Resulting outcomes of PCR and LAMP of CSF samples

DNA of 184 CSF samples were extract using DNG plus and were by PCR under optimized condition. 24% showed absolute positive results, which after samples loading on 2% Agars gel and electrophoresis, the samples containing Herpes Simplex glmM gen showed 454 bp Fermentas size marker, aligned exactly with positive control of standard strain (Figure 3), which 454 bps fragment of standard strain was sequenced using sequencing method. Comparing of resulting sequences by BLAST program showed about 100% alignment 87% of samples showed positive reaction with LAMP test. After adding 1 microliter cyber green 0.1% and placing under UV light their color changed from orange to phosphoric green color. After using two techniques of CSF samples it was determined that from 184 CSF sample, 45 samples using PCR technique were reported positive (24%). From this number of samples, 60 samples were determined positive (32%) by LAMP technique.(Figure3Comparing the result of these two techniques using K square test showed that these two techniques are different in a meaningful level ($p< 0.05$)**.**

Table1: Primers of LAMP

F3	TTC-TGG-AAT-TCG-ACA-GCG-A-
B3	GGA-AGT-GGC-TCT-GGC-CTA-T
FIP	F2: CGA-GAT-GCT-GTT-GGC-CTT-C
	F1C: TGT-ACC-CGG-TCA-CGA-ACT-CGG
BIP	B2: GTC-CCA-CAC-GCG-AAA-CAC
	B1C: -ACG-GAC-ATT-TAC-AAG-GTC
LF	CCG-TAC-TGT-TTC-ACA-AGG-GTC
LB	GTA-CGG-CCG-CAT-GAA-CG

RESULTS AND DISCUSSION

Type 1 & 2 of Herpes simplex virus causes different disease in human. Although nowadays diagnostic methods of serologic HSV have had advanced a lot but because of defects in this kind of methods in diagnosing infections they can't be standard for diagnosing. So it is necessary to choose molecular methods as substitution for serologic methods [7] LAMP technique is one of the molecular diagnostic methods that for the first time it was modified by Notomi et al in 2000, from that time some studies about the usage of this technique has been done. For example using this technique makes it possible to diagnose pathogens like human Herpes virus, Hinfluvirus Vibrio parahemolyticus,Trypanosomabrucei[17-21] .In recent years several studies has been done about the usage of LAMP technique for diagnosing HSV virus , such as , Enomoto et al in 2005 chose gp virus from HSV as target sequence and with modifying LAMP technique in diagnosing HSV-1 , HSV-2 virus in order of insensitivity equal with 500 and 1000 particle virus obtained . Enomoto et al use direct virus test (without DNA extraction). They believed LAMP technique in diagnosing the samples without extracting DNA has high sensitivity as well [22]. In the same year Kaneko et al chose UL gene as target sequence , after modifying LAMP technique they found that the sensitivity in this technique is 10 particle virus [23]. In 2010 a researcher in the name of Reddy, with choosing gG as target sequence, the sensitivity of his modified technique in diagnosing HSV virus, was 10 particle virus. He stated that LAMP and PCR techniques in a real time have the same sensitivity [24] .In our study DNA gene of polymerase of HSV virus for the first time was used as target in diagnosing this technique primers in other studies. As it is shown in this study, this technique has a higher sensitivity and accuracy in compare with PCR. So that from 184 CSF sample 45 with using PCR were reported positive , while from this number 60 samples using LAMP technique were reported positive . In the other word , 15 samples using PCR were reported negative , the samples using LAMP , do to types 1,2 of HSV virus were reported positive .Considering the sensitivity of LAMP technique that is 10 times more than PCR sensitivity so the difference in two results is explicable. Comparing the results of two techniques using K square test showed that these two methods in a meaningful level $p<0.05$ are different According to our study new method of amplification nucleic acid LAMP based on DNA gene of polymerase HSV virus for the first time in the world modified and then was used .Regarding to the reports, in this study LAMP technique for diagnosing HSV virus, in IRAN, for the first time was modified and used. Comparing our study at present and the studies that have been done in relation with LAMP method in diagnosing in HSV virus, can say that modified LAMP method in

this study in compare with past studies have higher sensitivity (5 particles virus) that this difference have different reasons, such as DNA extraction method, choosing different target sequence and nucleic sequence used in this study. Therefore considering the past studies and achieved results from present study, can say that LAMP technique in compare with PCR in diagnosing HSV has a higher sensitivity and in spite of high accuracy and more sensitivity it doesn't need expensive equipment such as thermo cycler and reaction will be achieved just with a simple dry plate.

CONCLUSION

Due to PCR test in based on using much more Thermo cycles, expensive thermo cycler, time consuming , hard detection and manifestation of product, therefore a more rapid and simple technique such as Isothermal loop amplification [LAMP] technique which is one stage amplification technique could be used in this study with more absolute positive results than PCR and more sensitivity and accuracy.

ACKNOWLEDGEMENTS

The authors would like appreciate the science based that it is necessary to thank management of pathology ward in Tehran Erfan hospital Dr. Zare , management of pathology ward in Tehran Mofid hospital Dr. Farzanejadali, virology department of institute Pasteur and Dr. DavoodEsmaeili.

REFERENCES

1. Kimura.K, Ihira.M, Enomoto.Y, Kawada .J, Ito.Y, Morishirna.T, et.al, Rapid detection of Herpes simplex virus DNA in cerebrospinal fluid: comparison between loop-mediated isothermal amplification and real-time PCR, Med. Microbioly. Immunology,2005; 194:181-185.
2. Whitley.R, Roizman.B,Herpes simplex virus infections Lancet, Can J Infect Dis 2001; 357: 1513- 1518.
3. Solomon.AR, New diagnosis tests for Herpes simplex and VZV infections, J Am Acad Dermatoly 1988; 18:218-21.
4. Singh. A, Frcpc.BM, Preiksaitis. S, Frcpc.MD, Ferenczy. A, Romanowski. B, et.al, The laboratory diagnosis of Herpes simplex virus infections, CanJInfect Dis,2005; 22: 92-98.
5. Burbelo. PD, Hoshino.Y, Leahy. H, Karogrnann.T, Hornung.RL, Iadarola. MJ, et.al, Serological diagnosis of human HSVI and HSVII infection luciferase immuno precipitation systems [LIPS] assay, Clin Vaccine Immunology, 2009; 21:1054-59.

6. Conrady.CD, Drevets. DA, Carr.DJ, Herpes simplex type I [HSVI] infection of the nervous system is an immune response a good thing. J Neuroimmonology,2009; 220:1-9.
7. Nototni. T, Okayama .H, Masubuchi. H, Yonekawa.T, Watanabe. K, Amino. N, et.al, Loop mediated isothermal amplification of DNA, Nucleic Acids Research,2000; 28:63-66.
8. Shahhosseiny.MH, Basic molecular diagnosis Tehran: Islamic Azad University,2005; pp 12-35.
9. Zhang. D, Zhang. W, Li. X, Konomi. Y, Detection of rare DNA targets by isothermal amplification, amplification Gene,2001; 274: 209-216.
10. Nagamine.K,Kuzuhara.Y, Notoun.T, Accelerated reaction by loop-mediated isothermal amplification using loop primers, Molecular and Cellular Probes,2002;16: 223-229.
11. Nagarnin. K, Hase. T, Notomi. T, Loop-mediated isothermal amplification reaction using a non denatured template, Clinical Chemistry,2001;47:1742-1743.
12. Mori. Y, Hirano. T, Notomi. T, Sequence specific visual detection of LAMP reactions by addition of cationic polymerase, BMC Biotechnology,2006; 6:3-11.
13. Kimura. K, Ihira. M. Enomoto. Y, Kawada. J, Ito.Y, Morishima.T, et.al, Rapid detection of Herpes simplex virus DNA in cerebrospinal fluid, Companion between loop-mediated isothermal amplification and real-time PCR, Moo Microbiology Immnno,2005; 194:181-185.
14. Enomoto. Y, Yoshikava.T. Ihira. M, Aklmoto. M, Miyak. F, Usui. C, et.al, Rapid Diagnosis of Herpes simplex virus infection by a loop-mediated isothermal amplification method Journal of Clinical Microbiology, 2005; 26: 951-955.
15. Sugiyama. H, Yoshikawa. T, Ihira. M, Enomoto.Y, Kawana. I, Asano. Y, Comparison of Loop Mediated isothermal amplification, Real-time PCR, and virus isolation for the detection of Herpes simplex virus in genital lesions, Journal of Medical Virology,2005; 75: 583-587.
16. Zhi-xinng.Z, Hua. J,Ji-ping.W, Wallg.Liu, Wang.L, Zhang.L, et.al, Detection of Herpes Simplex virus type II DNA with loop-mediated isothermal amplification assay, Journal of University of South China, 2010; 2:28-37.
17. Ihira. M, Yoshikawa. T, Enomoto.Y, Rapid diagnosis of human herpes virus VI infection by a novel DNA amplification method, loop-mediated isothermal amplification, J Clin Microbiology, 2004; 42:140145.

18. Moslemi. E, Shahhosseiny.MH, Javadi.GR, Sanari.T, Amini.HK, Loop mediated isothermal amplification [LAMP] for rapid detection of HBV in Iran, African Journal of Microbiology Research, 2009; 3:439-445.
19. Postel. A, Letzel. T, Frischmanu. S, Grund. C, Beer. M, Harder. T, Evaluation of two commercial loop-mediated isothermal amplification assays for detection of avian influenza H5 and H7 hemaglutenin genes, Journal of Veterinary Diagnostic Investigation, 2010; 22: 61-66.
20. Chen.S , Ge. B, Development of a technique of based loop-mediated isothermal amplification assay for detecting Vibrio parahaemolyticus , BMC Microbiology,2010; 10: 4147-4152.
21. Njiru.ZK, Nikusza.AS, Armstrong.T, Eyaru.JC, Ndung'u.JM, Thompson.AR, Loop - mediated isothermal amplification [LAMP] method for rapid detection of Trypanosomabruceirhodesiense, PLoS.Negl.Trop. Dis, 2008; 2:147·151.
22. Enomoto.Y, Yoshikava.T, Ihira.M, Akirnoto.M, Miyak.F,Usui.C, et.al, Rapid diagnosis of Herpes simplex virus infection by a loop-mediated isothermal amplification method, Journal of Clinical Microbiology, 2005; 26: 951-955.
23. Kaneko.H, Iida.T, Aoki.K, Ohno.S, Suzutani.T, Sensitive and rapid detection of Herpes simplex virus and varicella-zoster virus DNA by loop-mediated isothermal amplification, Journal of Clinical Microbiology,2005; 43:3290·-3296.
24. Reddy.AK, Balne.PK, Reddy.RK, Mathai.A, Kaur.I, Loop-mediated isothermal amplification assay for the diagnosis of retinitis caused by Herpes simplex virus type I, Clin Microbiology Infect, 2010; 34: 434-440.

AJPHR is
Peer-reviewed
monthly
Rapid publication
Submit your next manuscript at
editor@ajphr.com / editor.ajphr@gmail.com