Loop-Mediated isothermal amplification in human Cytomegalovirus diagnostic

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SUMMARY

The pathogenicity and the consequences of human cytomegalovirus (HCMV) infection in immunocompromised persons are reported in the literature and, HCMV Viral DNA detection is the reliable indicator of infection especially to study its reactivation after organ transplantation in the recipient patients. Congenital infections in pregnant women causes the opportunistic diseases of the central nervous system in new-born. HCMV increases suffering and death in patients with chronic diseases like diabetes, tuberculosis, hepatitis C or patients with immunodeficient HIV/AIDS. Diagnostic methods are present in medical analysis laboratories either by immunofluorescence, Chemiluminescence, immunochromatography, western blot or by molecular biology techniques such as PCR are used to detect qualitatively and/or quantitatively IgG and IgM antibodies in the organism using serum or plasma as sample. The follow-up and treatment of patients is carried out in case the clinical data correlate with specific pathologies and symptoms, thus, the analysis of cytomegalovirus by PCR is requested to be sure of the cause of this pathology and to eliminate other doubts because this virus shares genetic and pathological similarity with Epstein-Barr virus (EBV), Varicella-Zoster virus (VZV) and Herpes Simplex virus (HSV) as they are all of the same family of the Herpesviridae. The viral load quantified by PCR is a key element for monitoring and treatment. Molecular biology researches have developed different PCR amplification techniques to answer the question of sensitivity, efficiency and speed of this technique and more recently in 2000, Notomi and the EIKEN Chemical Co. Ltd. developed a method of amplification under isothermal conditions named LAMP (Loop-mediated isothermal amplification) which combines speed and easy access for all laboratories that want to use only the less expensive equipment like those.
Introduction

Cytomegalovirus is a more widespread opportunistic adenovirus with a prevalence of 60-80% in the world (Griffiths, 2002). It remains in the latent and asymptomatic phase for immunocompetent people but it is very dangerous in patients and people with weakened immunity as in pregnant women whose congenital infection can contaminate the fetus (Griffiths, 2015), transplant patients who are under treatment with immunosuppressant’s (De Keyzer, 2011), those with chronic diseases such as diabetes (Ecochard, 2012), tuberculosis (Cheng, 2009) hepatitis C or HIV/AIDS.

The bibliography reports the different diagnostic methods developed to diagnose this virus, as a ubiquitous virus and present in most biological fluids of the body, the relationship between this virus and pathologies can be exploited by radiological data, serological and at the level of molecular biology by amplification of human cytomegalovirus genes by PCR. Different studies show that the antigens of this virus can settle in urine, cerebrospinal fluid, ascites fluid, tears, and saliva (Delforge, 2017) and cause pathologies that must be treated with effective and reliable treatments, the pathologies are cured and immunity is established. Nucleic acid amplification is a tool that has major applications in diagnosis and research in the fields of health, clinical biology to diagnose infectious diseases, nowadays, there are a multitude of different detection by PCR that have been invented as nucleic acid sequence-based amplification (NASBA) (Fakruddin, 2012) self-sustained sequence replication (3SR) (Ichihashi, 2016) and strand displacement amplification (Toley, 2015).

Each of these methods have innovative points as for example, PCR uses heat denaturation of double-stranded DNA products to promote the next round of DNA synthesis. 3SR and NASBA eliminate heat denaturation by using a set of transcription and reverse transcription reactions to amplify the target sequence. Similarly, SDA eliminates the heat denaturation step in cycling DNA synthesis by employing a set of restriction enzyme digestions and strand displacement DNA synthesis with modified nucleotides as substrate. All these methods present the progress of molecular biology, but still need a method that will be much cheaper, easier to handle and faster, so came the LAMP method in 2000 which uses four to six primers under isothermal conditions.

Human cytomegalovirus (HCMV)

Human cytomegalovirus (HCMV) or human herpesvirus 5 strain Merlin is an opportunistic double-stranded DNA adenovirus that consists of a genome, a capsid, and an envelope covered by glycoprotein’s (Ho, 2008). The genome is packaged in a protein structure called the capsid, which has a polyhedral (icosahedral) geometric conformation. It consists of a linear DNA genome of 235,646 bp. It is a member of the same family of herpesviruses that also includes herpes simplex virus (HSV), Epstein-Barr virus (EBV) and varicella-zoster virus (VZV), and its infections often go unnoticed (A Ross, 2011; Stockdale, 2018). Thus, its pathogenicity is only noticed in patients whose immune defenses have been weakened, such as those treated with immunosuppressant’s drugs, those with AIDS, and in the
fetus. It is a very fragile virus and can be acquired through all bodily secretions: saliva, blood, semen, tears, breast milk, vaginal and cervical secretions. In Europe the prevalence is 30-70% in Western Europe and North America, and 80-100% in developing countries (Morton, 2006).

Monitoring and treatment of cytomegalovirus is based on the use of antivirals such as Ganciclovir (Komatsu, 2014) which reduces or interrupts viral replication in vivo. Infectious diseases occurring during pregnancy can lead to fetal damage such as hearing impairment and the risk of neurosensory sequelae, mental retardation, and the infected fetus will have clinical and/or echographic manifestations of the infection with severe sequelae such as microcephaly, dilation of the brain ventricles and ocular damage.

Liver involvement is characterized by jaundice and bleeding disorders; digestive involvement with hyperechogenicity of the intestinal tract; thrombocytopenia; intrauterine growth retardation. The diagnosis is generally made on the basis of ultrasound (De la Calle, 2020) serological data such as IgG and IgM antibodies, pp65 viral antigen detection and gene amplification or PCR of the virus, which is a reference method for the diagnosis of fetal damage by the virus (Bhatia, 2004). The antiviral drugs Ganciclovir and the phosphonoformic acid Foscarnet. A third drug is Cidofovir for the treatment of human cytomegalovirus ((Komatsu, 2014).

**Loop-mediated isothermal amplification method (LAMP)**

Loop-mediated isothermal amplification (LAMP) is an innovative method of nucleic acid (DNA/RNA) amplification, using 4 to 6 primers targeting 6 to 8 regions of the target gene under isothermal conditions in less than one hour (Notomi, 2000) this method uses the activity of BST DNA polymerase which is an isothermal enzyme. It is a variant of PCR that combines many advantages such as speed, specificity, high sensitivity and very low equipment cost. (Reddy, 2010) The following strand displacement DNA synthesis primed by an outer primer releases a single-stranded DNA. This serves as template for DNA synthesis primed by the second inner and outer primers that hybridize to the other end of the target, which produces a stem-loop DNA structure.

In subsequent LAMP cycling one inner primer hybridizes to the loop on the product and initiates displacement DNA synthesis, yielding the original stem-loop DNA and a new stem-loop DNA with a stem twice as long. The cycling reaction continues with accumulation of 10^9 copies of target in less than an hour. The final products are stem-loop DNAs with several inverted repeats of the target and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand. Because LAMP recognizes the target by six or eight distinct sequences initially and by four to six distinct sequences afterwards, it is expected to amplify the target sequence with high selectivity.

Since its discovery in 2000, the World Health Organization has endorsed its validity as an alternative to conventional PCR in molecular diagnostics and recommends it for research and diagnostics especially in areas far from laboratories as portable LAMP kits are cheaper and do not require sophisticated equipment. LAMP data can be analyzed on agars gel, in real time or by colorimetric.
Loop-mediated isothermal amplification (LAMP) in the diagnosis of human cytomegalovirus

Diagnosing human cytomegalovirus with the Loop-mediated isothermal amplification (LAMP) method is an alternative to PCR; the samples can be urine, amniotic fluid, cerebrospinal fluid, tears, saliva etc. (Fakruddin, 2012). Various software programs can be used to identify the human cytomegalovirus genomes and choose a gene or genes to target and region-specific primers in order to obtain reliable results and avoid cross-reactions with other viruses that have genetic similarity to cytomegalovirus such as Epstein-Barr virus (EBV), varicella-zoster virus (VZV) and herpes simplex virus (HSV), (Enright, 2004).

Studies often target the glycoprotein B (gB) which is known to play the role in fusion and entry of the virus into target host cells such as endothelial cells, smooth muscle cells and glandular cells. Other genes are also targeted such as MIE (Major-immediate Early) which plays a role in regulating cytomegalovirus gene expression during replication.

This method can be applied directly to samples without DNA extraction step or after performing a nucleic acid extraction, the LAMP reaction mixture is almost similar to that of PCR, as reported in studies done by Notomi et al with EIKEN Chemical. Co.Ltd used a 25µl reaction mixture containing 0.8 µM each FIP and BIP, 0.2 µM each F3 and B3, 400 µM each dNTP, 1 M betaine (Sigma), 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH4)2SO4, 4 mM MgSO4, 0.1% Triton X-100 and the specified amounts of double-stranded target DNA. The mixture was heated at 95°C for 5 min, then chilled on ice, 8 U Bst DNA polymerase large fragment (New England Biolabs) were added, followed by incubation at 65°C for 1 h and heating at 80°C for 10 min to terminate the reaction. (Notomi, 2000). To analyze the results of LAMP, Aliquots of 5 µl of LAMP products and 1 µl of the products digested with restriction enzymes were electrophoresed in 2% agarose gels (0.5X TBE) followed by staining with SYBR Green I (Molecular Probes Inc.). Southern blot analyses were performed by transfer to Hybond N+ nylon membrane (Amersham-Pharmacia). Oligonucleotide probes end-labeled with a DIG Oligonucleotide Tailin g Kit (Roche Diagnostics) were used for detection according to the manufacturer's protocol. An aliquot of 5 µl of LAMP products was also run for 14 h in a 0.7% alkaline agars gel containing 50 mM NaOH and 1 mM EDTA, followed by neutralization with1M Tris-HCl (pH 8.0), and staining with SYBR Green I which showed similarity to conventional PCR. et al 2010 on retinitis caused by human cytomegalovirus (Reddy, 2010), on congenital human cytomegalovirus infection (Wang,2015), In 2005, Ryota S. et al 2005 performed a LAMP method on CMV (AD-169), Herpes virus (HSV-1) (KOS)-, HSV-2 (186), varicella-zoster virus (Oka-vaccine), Human herpes (HHV)-6 A (U1102)-(HHV-6 B (Z29) and (HHV-7) (RK) infected cells (Reddy, 2010). The sensitivity for LAMP-CMV was 500 copies/tube and they confirmed the results on gel electrophoresis and turbidimeter measurement. This research on 180 samples of hematopoietic stem cells from 20 children transplant recipients, for the detection of CMV in whole blood the LAMP method and real-time PCR as results > 500 copies/tube and > 5000 copies/200µl of whole blood was confirmed as positive for CMV infection.

Working on viral retinitis (Reddy, 2010) noted a specificity for CMV DNA of the LAMP method that they developed they did not notice cross-reaction with
DNA of HSV-1, VZV, Adenovirus, Aspergillus flavus or with Staphylococcus aureus, the sequences of the CMV LAMP-positive results were 100% well matched to the CMV sequence found in the GENBANK databases. In their study, the sensitivity of the method was 10 copies/µl of CMV DNA. This test was performed on 40 patients suspected of having viral retinitis. In a study of this technique in pregnant women to understand the association between congenital HCMV infection and active maternal HCMV infection during pregnancy, they tested for CMV virus in 336 whole blood samples and 11 amniotic fluid samples, the sensitivity was >10 copies/tube. In all these studies, the role played by human cytomegalovirus in the target pathologies was revealed, which facilitates the follow-up and treatment of patients and also reduces the cost.

LAMP amplification results validated the structure of CMV bands on agarose gel with structure in several bands of different size, sensitivity of LAMP method confirmed by gel electrophoresis and turbidimeter, observation of LAMP results by naked eye to know positivity and negativity after adding SYBR green, as colorimetric shows yellowish green (positive) and orange-red (negative), (Wang, 2015), the studies also report the validation of optimal conditions for the best LAMP including different concentrations of Mg2+ , primer concentration, temperatures and time needed for the reaction they found that the results are well optimized at 25 µl volume consisting of 1. 6µM of FIP and BIP, 0.2µM of F3 and B3, 0.8 µM of LF and LR, 10mM of MgSO4, 1µL BST DNA polymerase and 5 µL of target DNA, amplification was carried out at 64°C for 30 minutes and the reaction was completed at 85°C for 5 minutes. The limit of detection was 10 copies/tube as positivity.

Conclusion

PCR amplification remains a standard reference method of molecular diagnosis but is expensive, LAMP as an alternative shows the progress of research in molecular biology and it is a method that does not use sophisticated equipment which reduces the costs of analysis and helps to save patients' money.

The literature shows how human cytomegalovirus is a public health issue as its infection remains associated with a higher risk of diabetes, opportunistic infections and graft rejection. Using LAMP to diagnose human cytomegalovirus has allowed us to understand the pathologies it can cause in different biological fluids, thus making the monitoring and treatment of patients reliable, rapid and effective.

Today, LAMP can be performed using pre-prepared kits such as Loopamp DNA amplification kit (Eiken chemical, Tochigi, Japan), pre-prepared buffers such as 10X BST DNA polymerase (New England, Biolabs, MA), special devices such as TERAMECS LA2 (teramecs, kyoto) for real-time LAMP, and online software that automatically generates LAMP primers. This highly sensitive, cheap, fast, specific and efficient technique is recommended as an alternative in research and molecular diagnosis especially in third world countries and for remote locations that are difficult to access as portable LAMP kits can be taken anywhere.

Disclosure

The authors declare that they do not have any financial involvement or affiliations with any organization, association, or entity directly or indirectly with the subject matter or materials presented in this article. This
also includes honoraria, expert testimony, employment, ownership of stocks or options, patents or grants received or pending, or royalties.

**Funding**

This paper was not funded.

**Authorship Contribution**

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted, and decided to be accountable for all aspects of the work.

**References**

- De la Calle, M., Baquero-Artigao, F., Rodríguez-Molino, P., Cabanes, M., Cabrera, M., Antolin, E., ... & Bartha, J. L. (2020). Combined treatment with immunoglobulin and valaciclovir in pregnant women with cytomegalovirus infection and high risk of symptomatic fetal disease. The Journal of Maternal-Fetal & Neonatal Medicine, 1-5.