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Full Length Article

Optimization of Colorimetric Loop-Mediated Isothermal Amplification for the Diagnosis of Human Cytomegalovirus in Kidney Transplant Patients

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Abstract

Human Cytomegalovirus infection is one of the acute risk factors for rejection after kidney transplantation. qPCR used for detection is an expensive method, so, molecular biology rapid diagnosis technique is essential to determine the viral load and initiate early treatment to avoid graft rejection. We therefore proposed to develop a colorimetric CMVLAMP technique by using WarmStart Colorimetric LAMP 2X Master mix; this method has the advantages of being fast, reliable, very sensitive and cost-effective. The results of the present study demonstrated that the developed method has high specificity to HCMV DNA, not cross-reacting with viruses with genetic similarity to HCMV such as herpes simplex virus type HSV1-2, varicella zoster virus, Epstein Barr Virus, this method was sensitive to viral load > 150 copies/mL as the results of qPCR the reference method. The sensitivity of this method was 100%, the specificity 100%. In conclusion, in the present study, we developed the colorimetric CMVLAMP method that is revealed to be sensitive, specific, rapid cheap and, the coloration indicator simplifies the pathogen detection, thus, in transplant patients, this method presents an economical alternative in medical diagnosis.

Keywords: Kidney transplant recipients; Molecular biology; PCR; Colorimetric CMVLAMP; Human cytomegalovirus; Medical diagnosis; WarmStart Colorimetric LAMP2X Master Mix; Bromophenol Blue

Introduction

Human cytomegalovirus is ubiquitous opportunistic adenovirus that is widespread throughout the world with a high prevalence of 60-80% (Griffiths and Reeves 2021). Diagnosing this virus is difficult because of its genetic similarity to other herpesviruses such as Hespes Simplex Virus (HSV_{1-2}), Epstein Barr Virus (EBV) or Varicella Zoster Virus (VZV) (Hollier and Grissom 2005). This viral infection also stays unremarked without clinical symptoms in people with robust immunity, therefore, the clinical symptoms are often noticed in newborns because of congenital infection (Demmler-Harrison *et al*. 2020; Uchida *et al*. 2020) in patients with chronic diseases such as diabetes (Uchida *et al*. 2020), Hepatitis C (Khalil *et al*. 2022), tuberculosis (Olbrich *et al*. 2021), HIV (Pang *et al*. 2020) and in transplant patients treated with immunosuppressant's (Rump *et al*. 2020). To diagnose the serology of this virus, medical analysis laboratories have different reagents available on the market that use principles such as chemioluminescence (Dourado Junior *et al*. 2021), immunofluorescence (Faure-Bardon *et al*. 2021), or western blot (Zheng *et al*. 2020). The treatment and follow-up of a patient infected with human cytomegalovirus is conditioned by the quantification of the viral load by the PCR method, which remains the standard method for detecting this virus, but the cost of the analysis remains expensive and sometimes less sensitive at very low viral load, and trained professionals are needed for this method (Uwiringiyeyezu *et al*. 2019, 2022).

Various studies that have been conducted to develop other PCR-derived methods to facilitate the accessibility. The LAMP is the one of the developed methods and has many advantages. These include the use of isothermal conditions and detection by colorimetric change to reveal positivity or negativity (Notomi *et al*. 2000; Nagamine *et al*. 2001; Suzuki *et al*. 2006, 2010). The consequences of HCMV infection, which belongs to the Herpesviridae family, are accompanied by chronic graft rejection if HCMV is not diagnosed before in the donor and graft recipient. This virus is also known to have a latent phase and can reactivate later as a result of weakening of the body's immunity and still have several consequences (Heald-Sargent *et al*. 2020). Research has

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shown a relation between active HCMV infection in transplant patients and graft rejection (Rump *et al*. 2020). The risk of viral infection is higher in transplant patients due to the use of immunosuppressive drugs such as tacrolimus or cyclosporine (Rump *et al*. 2020; Winstead *et al*. 2021) and leads to disruption to the graft function and rejection, which can be chronic (Rump *et al*. 2020; Winstead *et al*. 2021; Da Cunha and Wu 2021).

Seroconversion can be used to detect anti-HCMV antibodies in transplant patients (Zheng *et al*. 2020; Dourado Junior *et al*. 2021; Faure-Bardon *et al*. 2021), however, it is rarely effective, especially when the transplant donor has latent HCMV which would allow seronegativity to be concerned patient. Detection of virus genome by quantitative polymerase chain reaction (qPCR) has become an important laboratory tool for the diagnosis and treatment of this viral infection (Uwiringiyeyezu *et al*. 2019, 2022). Many other previous studies have reported qPCR for the detection and quantification of HCMV in various biological fluid samples. LAMPPCR is a new and evolving nucleic acid amplification method and has been recommended for the detection of HCMV viral genomic DNA. This method has been used for the rapid diagnosis of a numerous infectious diseases including herpes viruses (Miyachi *et al*. 2021), Epstein-Barr virus (Nie *et al*. 2008), hepatitis B virus (Cai *et al*. 2008) and CMV (Wang *et al*. 2015; Uwiringiyeyezu *et al*. 2019, 2022). The LAMP method is capable of amplifying specific DNA sequences under isothermal conditions and requires relatively simple and inexpensive equipment, making it suitable for use in all diagnostic and research laboratories.

As outlined in Fig. 1, our objective was to develop and evaluate a CMVLAMP method for the colorimetric detection of HCMV DNA in plasma of kidney transplant patients. This method is a potential alternative for screening and monitoring HCMV infection.

Materials and Methods

Clinical samples and HCMV DNA extraction

Whole blood samples from 135 transplant patients who were registered at AL KINDY Medical Analysis Laboratory (Casablanca, Morocco) were selected since 2017 and used in the present study. The study was conducted by comparing HCMV positivity and negativity of CMVLAMP and real-time qPCR method. Patient consent was obtained, and the study was authorized by the Human Research Ethics Committee of Hassan II University in Casablanca. The samples were first centrifuged at 4000 rpm for 10 min. The obtained plasma was used for nucleic acid extraction with the EZ1 DSP Virus kit on the EZ1 Advanced XL (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. Extracted DNA was eluted into 60 μL of elution buffer and stored at -20ºC before being used for the following steps. One part of total extracted DNA was quantified by optical density (OD) measurements at 260 nm using a spectrophotometer (Nanodrop lite; Thermo Fisher Scientific, Wilmington, DE, USA) and the other was used for real-time PCR amplification using the Artus-CMV-QS-RGQ kit (Qiagen GmbH, Hilden, Germany), the other for CMVLAMP isothermal amplification. Other samples positive for herpes simplex virus HSV (1-2), varicella zoster virus (VZV), Epstein Barr virus (EBV), BKV were extracted with the same kits to evaluate the specificity of our method.

Primer design for LAMP

The primers for CMVLAMP amplification in this study target the glycoprotein B (gB) gene from data obtained from the GenBank virus genome (accession number: M60931) https://www.ncbi.nlm.nih.gov/nuccore/M60931.1/ (Wang *et al*. 2015). The Oligonucleotide primers used in the present study were designed using Primer Explorer V5 software (Eiken Chemical Co. Ltd., Tokyo, Japan) http://primerexplorer.jp/e/ (Notomi *et al*. 2000), Primers designers http://www.premierbiosoft.com/isothermal/lamp.html and Biolabs software (New England Biolabs, Beverly, MA) https://lamp.neb.com/#!/. The designed primer sequences were verified with BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi De La Fuente *et al*. 2018) to exclude any possibility of cross-reaction with herpes simplex virus HSV $_{(1\text{-}2),}$ varicella zoster virus (VZV), Epstein Barr Virus (EBV) or BKV. Primers consisted of two outer primers (F3/B3) and two inner primers (FIP)/ (BIP) and 2 additional loop primers (LF/LB). Details of the sequence and location of each nucleotide primer in the target DNA sequences are provided in Table 1.

LAMP reaction with WarmStart colorimetric LAMP 2X master mix

Colorimetric CMVLAMP reaction is performed using WarmStart Colorimetric LAMP 2X Master Mix (New England Biolabs, Inc., Ipswich, MA, USA). The protocol is available on the link https://international.neb.com/protocols/2016/08/15/warmstart-colorimetric-lamp-2x-master-mix-typical-lamp-protocol-m1800 (Rubinfien *et al*. 2020). In a 25 μL mixture, 12.5 μL of WarmStart Colorimetric LAMP 2X Master Mix, 2.5 μL of primer mix, 5 μL of DNA and 5 μL of molecular biology grade water were used. The mixture was incubated at 65°C in a water bath for 30 min, the positive samples changed color from purple to yellow and the negative samples remained purple.

LAMP reaction with thermopol buffer

This reaction was conducted with 10x Thermopol buffer (New England Biolabs, Inc., Ipswich, MA, USA) which is a buffer composed of 20 m*M* Tris-HCl, 10 m*M* (NH4)2SO4, 10 m*M* KCl, 2 m*M* MgSO4, 0.1% Triton® X-100, pH 8.8@25°C The LAMP reaction was performed according to the protocol available on the link:

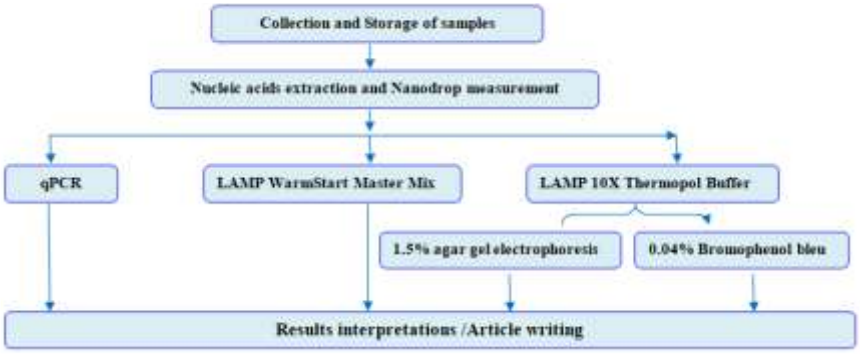

Fig. 1: Experimental design that it illustrates all the stages we have used from the start to the end of our research study

Table 1: Primers targeting glycoprotein B, gB of human cytomegalovirus (HCMV)

Primers	Primers sequences	Mm (g/mol)	Tm	Qty (µg)
CMVgB-LB	5'-GCCCTACCTCAAGGGTCTGGA-3'	6407.2	53.1°C	409
CMVgB-LF	5'-GAAGGTGGCAACGCCTTCG-3'	5853.9	62.0°C	395
CMVgB-BIP	5'- CGCCCAGGCCGCTCATGAGGTTTTTAAGGTAGTCGACCCGCTACC-3'	13773.9	69.5°C	936
CMVgB-FIB	5'- AGCCATTGGGGCCGTGGGTGTTTTACGCTCCGAAGGGGTTTTTG-3'	13672.9	67.9 °C	222
CMVgB-B3	5'- AAGCAGCGGGTAAAGTAC-5'	5581.7	54.0°C	360
CMVgB-F3	5'- GGCTATGGCCACGAGGAT-3'	5564.7	58.0°C	149

----81170
----AGTGATAATGACTAC**GGCTATGGCCACGAGGAT**GATGGTG A **ACGCTCCGA AGGGGTTTTTG A**G**GAAGGTG**
F3 F2
GCAACGCCTTCGA CCACGGA GGC **CACCGCG CCACCCACGG CCCCAATGGCT** ACGCCAACGG CCTTTCCCGCGG
LF F1
CGCCCAGGCCGCTCATG AGGT CG **TCCAGACCCTTGAG GTAGGGC** **GGTAGCGGGTCGA CTACCTT** GTCCTCCAC
B1 LB B2
GTAC TTTACCCGCT GCTT GTACGA GTTGAATTCG CGCATGATCTCTCTTCGAGGTC
B3
AAAAACGTTGCTGGAACGCAGCTCTTTCTG-------

https://international.neb.com/protocols/2014/11/21/typical-lamp-protocol-m0275 (Nagamine *et al*. 2001). In 25 µL of total volume composed of 2.5 µL of 10X Thermopol Buffer containing 2 m*M* MgSO4 ,1.5 µL of MgSO4 (100 m*M*),3.5 µL of dNTP Mix (10 m*M*) 1.4 m*M* each,1 µL of FIP/BIP Primers (25X) 1 6 µM, 1 µL F3/B3 Primers (25X) 0.2 µM, 1 µL LoopF/B Primers (25X) 0.4 µM, 1 µL Bst DNA Polymerase, Large Fragment (8,000 U/mL) 320 U/mL and 5 µL of DNA extract and 8.5 µm of molecular biology grade water, incubated for 30 to 45 min in a water bath, CMVLAMP products were checked on 1.5% agarose gel.

LAMP reaction with bromophenol blue

The LAMP reaction was performed in a 25 µL volume composed of 2.5 µL of 10x Thermopol buffer (20 m*M* Tris-HCl, 10 m*M* (NH4)2SO4, 10 m*M* KCl, 2 m*M* MgSO4, 0.1% Triton® X-100, pH 8. 8@25°C),1.5 µL MgSO4 (100 m*M*), 3.5 µL dNTP Mix (10 m*M*) 1.4 m*M* each,1 µL FIP/BIP Primers (25X) 1.6 µM, 1 µL F3/B3 Primers (25X) 0.2 µM, 1 µL LoopF/B primers (25X) 0. 4 µM, 1 µL Bst DNA Polymerase Large Fragment (8,000 U/mL) 320 U/mL, 5 µL of 0.04% Bromophenol Blue as indicator, 5 µL of DNA extract and 3.5 µL of molecular biology grade water, incubated 30–45 min in a water bath, the colorimetric CMVLAMP products were checked by color change following deprotonation during amplification. The protocol is on the link https://international.neb.com/protocols/2014/11/21/typical-lamp-protocol-m0275 Accessed on 10/08/2021 (Nagamine *et al*. 2001).

Electrophoresis of LAMP products

LAMP products from WarmStart Colorimetric LAMP 2X Master Mix (New England Biolabs, Inc., Ipswich, MA, USA) were detected on the agarose gel for amplification verification. On the 1.5% agarose gel in TBEX1 buffer supplemented with 2.5 µL of ethidium bromide, 10 µL of the LAMP products mixed with 5 µL of green Taq loading buffer were deposited and visualized under ultraviolet.

Statistical data analysis

All statistical analysis were performed using Microsoft Excel. Normality was tested for all datasets using the D'Agostino Pearson omnibus normality test. Kruskal–Wallis test with Dunn's correction and Mann–Whitney test were conducted to compare the yield of nucleic acids. Mean values of statistical data and standard deviation curves were calculated using Grubbs Tests. For each protocol, the samples were used with 3 times extractions. Statistically significant values were taken at $P < 0.05$.

Results

Comparison of the results of the methods used in our study

To evaluate the reproducibility of the results of the present study, the samples were repeated 3 times and the values presented in the table are the average. These results showed correlation of the methods used as shown in Table 2. We observed 100% positivity and 100% negativity of the correlation between qPCR results, WarmStart Colorimetric LAMP 2X Master Mix and 0.04% Bromophenol Blue.

By using Nanodrop lite, the DNA concentration values showed that amplification of the human Cytomegalovirus specific gene took place. The amplified DNA (A280/A260: 1.77 for amplification with WarmStart and 1.81 for ThermoPol buffer) showed a high purity in comparison with the non-amplified DNA (A280/A260: 2.41) which presents a presence of RNA. It was also noticed that the concentration of DNA is very important in the case of amplified DNA than non-amplified. (Table 3).

Positivity and negativity with WarmStart colorimetric LAMP 2X master mix

In a water bath, the reaction mixture was incubated with the negative control, the colour change to yellow (which is the proof of positivity) was observed in the samples already positive with qPCR and the negative samples remained purple as shown in (Fig. 2–3).

LAMP reaction sensitivity and specificity with WarmStart colorimetric LAMP 2X master mix

The specificity of our method was performed on the plasma of samples tested positive for different viruses such as Herpes Simplex Virus HSV (1-2) Epstein Barr Virus (EBV), Varicella Zona Virus (VZV), these 3 viruses share genetic similarities with Human Cytomegalovirus (HCMV) and BKV of the polyomavirus family and very well known as an opportunistic adenovirus in kidney transplant patients. Our method was able to detect up to 10 copies/μL of HCMV viral load which was the same sensitivity (Fig.4) as the Artus CMV-QS-RGQ kit that we used in this study for comparison. The results show that the LAMP method developed in our study was specific for the HCMV genome and does not cross-react with other viruses (Fig. 5).

Agarose gel of LAMP products/thermopol buffer/WarmStart colorimetric LAMP 2X master mix

The protocol was used like normal classic PCR, we have used 2 types of LAMP products, and results with Thermopol buffer on the agarose gel appear as conventional PCR bands (Fig. 6), therefore, LAMP products with WarmStart Colorimetric LAMP 2X Master Mix on the agarose gel appear to have the repeated bands of conventional LAMP (Fig. 7).

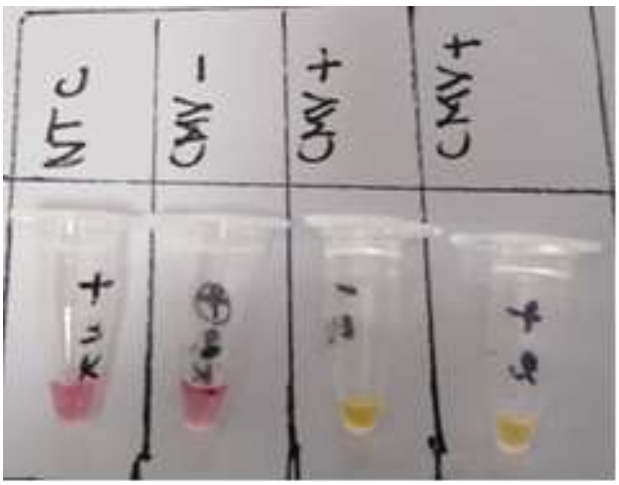

Fig. 2: CMV-negative samples remain purple in color after 30 minutes of incubation and CMV-positive samples turn yellow

Fig. 3: CMV-negative samples remain purple in color after 30 minutes of incubation and CMV-positive samples turn yellow

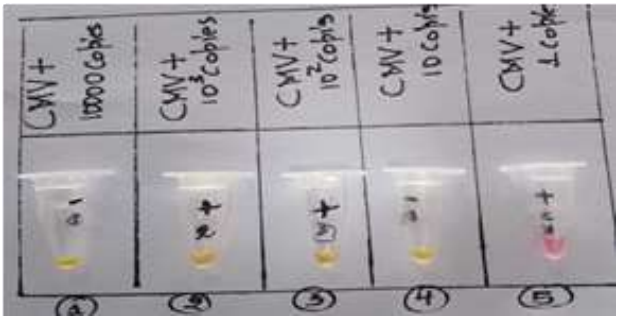

Fig. 4: The LAMP method developed in this study can detect an extract containing >10 copies/tube or >150 IU/ml

Positivity and negativity of 10x ThermoPol buffer and 0.04% bromophenol blue

In addition, to evaluate the alternative of the pH indicator, in a CMVLAMP mixture, we used 5 μL of 0.04% bromophenol blue, after 30 min, the positive samples became yellow and the negative samples remained as at the beginning, this is the proof that the amplification has taken place and that the primers are specific. This success is proof of the reliability of the reaction mixture (Fig. 8–9).

Table 2: Table summarizing the correlation of results between the methods used in our study, this method was sensitive to viral load > 150 copies/ml as the results of qPCR used as reference. The sensitivity of this method was 100%, specificity 100%. Each sample was repeated 3 times and the values presented in the table are the average of the 3 values for each part.

Samples	HCMV status	[C] ng/µl±SD	Ct (cycle threshold) ±SD	Viral loads (copies/ml)	Viral loads (IU/ml)	Log (IU/ml)	Rouge de phénol (WarmStart)	0.04% Bromophénol Bleu
1	Positive	18.38±0.1979	22.72±1.3410	415838.4	253560	5.404	Positive	Positive
2	Positive	17.23±0.4213	29.14±0.6782	4008.16	2444	3.388	Positive	Positive
3	Positive	20.52±1.3501	24.12±0.9007	111533.12	68008	4.832	Positive	Positive
4	Positive	15.45±1.3797	23.29±1.1617	366002.08	223172	5.348	Positive	Positive
5	Positive	14.35±1.9719	25.78±0.3785	48375.08	29497	4.469	Positive	Positive
6	Positive	18.08±0.0363	23.32±1.1523	209080.156	127487.9	5.105	Positive	Positive
7	Positive	17.08±0.5021	28.35±0.4298	22553.28	13752	4.138	Positive	Positive
8	Positive	19.63±0.8709	26.61±0.1175	34362.92	20953	4.321	Positive	Positive
9	Positive	20.47±1.3232	31.50±1.4205	1064.36	649	2.812	Positive	Positive
10	Positive	19.18±0.6286	28.85±0.5870	77949.2	47530	4.676	Positive	Positive
11	Positive	17.95±0.0337	28.54±0.4895	9065.92	5528	3.742	Positive	Positive
12	Positive	17.83±0.0983	31.85±1.5306	718.894	438,35	2.641	Positive	Positive
13	Positive	18.23±1.1096	23.70±1.0328	242959.44	148146	5.170	Positive	Positive
14	Positive	17.25±0.7439	30.00±0.9487	3989.07	2432.36	3.386	Positive	Positive
15	Négative	12.11±1.1743	N.A	< 150	< 100	N.A	Négative	Négative
16	Négative	15.65±0.1468	N.A	< 150	< 100	N.A	Négative	Négative
17	Négative	16.45±0.4453	N.A	< 150	< 100	N.A	Négative	Négative
18	Négative	11.85±1.2714	N.A	< 150	< 100	N.A	Négative	Négative
19	Positive	14.23±0.4269	23,64±0.3318	378191.757	230604,730	5.362	Positive	Positive
20	Positive	24.12±1.9999	30,20±1.1748	3592.524	2190,564	3.341	Positive	Positive
21	Positive	15.21±0.1864	31,36±1.4412	1266.129	772,030	2.887	Positive	Positive
22	Positive	11.21±1.1679	25,61±0.1206	52648.408	32102,688	4.506	Positive	Positive
23	Positive	10.56±1.3274	29,81±1.0852	2673.382	1630,111	3.212	Positive	Positive
24	Positive	18.12±0.5276	23,16±0.4420	300137.897	183010,913	5.262	Positive	Positive
25	Positive	15.22±0.1839	31,30±1.4274	926.217	564,767	2.752	Positive	Positive
26	Positive	14.65±0.3238	30,61±1.2689	2159.291	1316,641	3.119	Positive	Positive
27	Positive	17.81±0.4516	25,42±0.0769	107154.579	65338,158	4.815	Positive	Positive
28	Positive	12.11±0.9470	30,73±1.2965	2472.388	1507,554	3.178	Positive	Positive
29	Positive	13.21±0.6771	25,42±0.0769	106746.781	65089,501	4.813	Positive	Positive
30	Positive	19.22±0.7975	26,56±0.3388	47565.925	29003,613	4.462	Positive	Positive
31	Positive	20.11±1.0159	23,41±0.3846	357491.949	217982,896	5.338	Positive	Positive
32	Positive	23.14±1.7594	25,07±0.0034	110679.057	67487,230	4.829	Positive	Positive
34	Positive	18.88±0.7141	21.86±0.7406	140226.21	85503.79	4.931	Positive	Positive
35	Positive	15.55±0.1030	19.03±1.3906	1047440.3	638683.12	5.805	Positive	Positive
36	Positive	12.34±0.8906	18.97±1.4044	10871129.9	662873.76	5.821	Positive	Positive
37	Positive	11.11±1.1924	25.70±0.1412	9162.33	5586.79	3.747	Positive	Positive
38	Positive	21.21±1.2858	13.53±2.6538	51877780.8972	31632793.23	7.500	Positive	Positive
39	Positive	13.54±0.5962	22.27±0.6464	104948.91	63993.24	4.806	Positive	Positive
40	Positive	14.55±0.3483	25.07±0.0034	14395.756	8777.90	3.943	Positive	Positive
41	Positive	12.66±0.8121	23.41±0.3846	46498.1	28352.50	4.452	Positive	Positive
42	Positive	10.30±1.3912	19.33±1.3217	844440.03	514902.46	5.711	Positive	Positive
43	Positive	20.12±1.0184	25.42±0.0769	112142.25	6837.96	3.834	Positive	Positive
44	Positive	16.8±0.2037	23.64±0.3318	39579.64	24133.93	4.382	Positive	Positive
45	Positive	22.64±1.6367	30.20±1.1748	375.97	229.25	2.360	Positive	Positive
46	Positive	12.56±0.8366	26.56±0.3388	49780.01	3035.37	3.482	Positive	Positive

Table 3: DNA purity and concentration before and after amplification. S1 and S2 were negative samples while S3 to S7 were positive

Samples	without amplification		Amplification with WarmStart		Amplification with 10x Thermopol buffer	
	A280/A260	[C] ng/µl±SD	A280/A260	[C] ng/µl ±SD	A280/A260	[C] ng/µl ±SD
S1	2.17	47.7 ± 0.212	1.42	45.9 ± 1.427	1.78	57.3 ± 1.468
S2	2.46	75.0 ± 1.905	1.57	60.14 ± 1.416	1.76	72.4 ± 1.457
S3	2.69	46.7 ±0.290	1.78	2484.2 ± 0.466	1.80	2747.3 ± 0.545
S4	2.33	52.4 ± 0.153	1.83	3075 ± 0.925	1.82	2844.7 ± 0.618
S5	2.55	41.2 ± 0.716	1.95	2946.7 ± 0.826	1.89	2820.3 ± 0.599
S6	2.44	39.6 ± 0.840	1.94	2250 ± 0.284	1.84	2715.6 ± 0.521
S7	2.28	41.8 ± 0.606	1.93	2322.4 ± 0.341	1.79	2878.3 ±0.643

Discussion

Loop-mediated isothermal amplification (LAMP) has been reported to be very efficient, fast and specific (Uwiringiyeyezu *et al*. 2019, 2022 ; Notomi *et al*. 2000; Nagamine *et al*. 2001), these advantages have been improved over time to make this method even more efficient, thus we find studies with the use of 4 to 6 primers, different

Fig. 5: Using the DNA extracts of other viruses of the same family as Cytomegalovirus such as HSV, VZV, BKV and EBV, we concluded the absence of cross-reactions with other viruses which show the specificity of our method

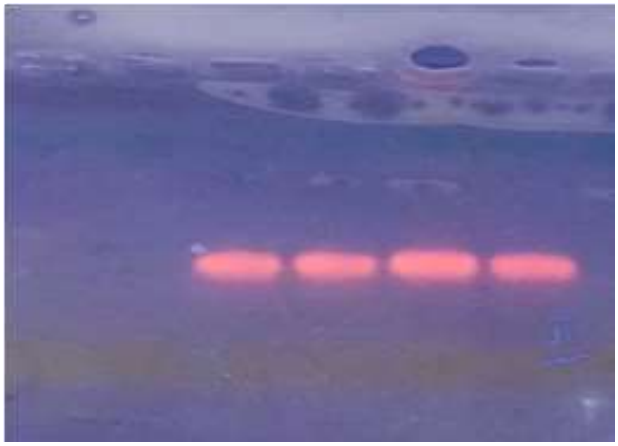

Fig. 6: LAMP products with Thermopol buffer on the agarose gel appear as conventional PCR bands. 1: CNT, 2: CMV negative and 3 to 6 CMV+ samples

Fig. 7: LAMP products with WarmStart Colorimetric LAMP 2X Master Mix on the agarose gel appear to have the bands of conventional LAMP. 1: CNT, 2 and 3 CMV negative and 4 and 5 CMV+ samples

isothermal enzymes and different types of master mixes, without forgetting the different methods of detection of LAMPPCR products (Nagamine *et al*. 2001; Nie *et al*. 2008;

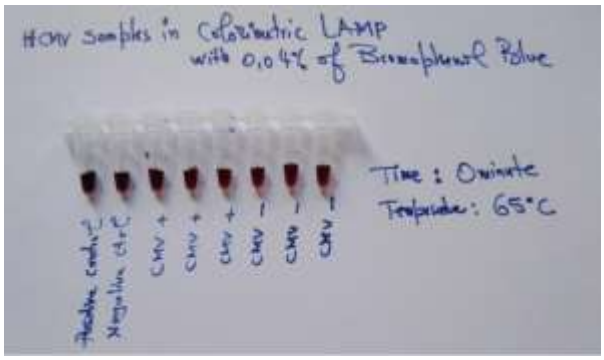

Fig. 8: Colorimetric LAMP with 0.04 % bromophenol blue before the reaction

Fig. 9: Colorimetric LAMP with 0.04% bromophenol blue 30 minutes after the reaction

Cai *et al*. 2008; Suzuki *et al*. 2006, 2010; Wang *et al*. 2015; Miyachi *et al*. 2021). Our study evaluated the viral load of human cytomegalovirus in plasma; the extraction of viral DNA was performed using the EZ1 DSP Virus kit following our validation among other extraction methods that were performed and its availability in our region (Uwiringiyeyezu *et al*. 2019, 2022).

The purity and concentration of DNA on Nanodrop lite allowed us to validate our choice of sample type; because the quantity is well important and amplifiable by qPCR which is the standard method recommended by the World Health Organization (Lamia *et al*. 2021). The set of CMVLAMP colorimetric results is evidence of an alternative and potential method in molecular biology. The WarmStart colorimetric LAMP2X Master mix has been shown to be effective in revealing positivity with phenol red, in less than 30 min, this rapidity confirms the role of staining indicator and we obtained the same results using Bromophenol blue (0.04%) in 10x Thermopol buffer (NEB) with a great consistency of the results in the literature reported using other staining indicators such as NeuRed dye (Yuan *et al*. 2018; Wang *et al*. 2021), hydroxynaphthol blue (Goto *et al*. 2009). Cresol red (Gou *et al*. 2020) Calcein (Suebsing *et al*. 2015) with the advantage of visualizing the results with the naked eye. Other biological fluids are reported to detect human cytomegalovirus, but their

Table 4: By performing a comparison of the different parameters, CMVLAMP is an alternative method for the detection of HCMV.

Comparisons	Real-time PCR	LAMP	Comments
Duration	> 2hours	30 minutes	LAMP is rapid
Automats	EZ1 Advanced XL, Rotor Q Gene	Heat bloc	
Thermocycler	Yes	No	
Kits	EZ1 DSP Virus kits	No	LAMP is cost-effective
Specificity	Specific	Specific	LAMP is also specific than qPCR
Sensitivity	>150 copies/ml	>150 copies/ml	LAMP has same sensitivity than qPCR
Primers	2	6	LAMP is very selective.
Multiplex	Yes	No	LAMP multiplex is very difficult

viral load is very low compared to the viral load in plasma, such as in urine (Nijman *et al*. 2012), amniotic fluid (Gouarin *et al*. 2002), ocular fluid (Reddy *et al*. 2010) or cerebrospinal fluid and despite the low viral load, these fluids are important to diagnose the pathologies related to them. Blood remains the best medium for viral growth, which explains the high viral loads obtained. Results of qPCR are used for monitoring and treatment of patients, but this method is still expensive due to the use of sophisticated equipment. Our CMVLAMP was cheaper due to the use of routine equipment such as water bath.

The literature reports that LAMP works well with denatured and non-denatured samples (Notomi *et al*. 2000; Nagamine *et al*. 2001). It is reported that as LAMP amplifies, the magnesium pyrophosphate ions form a white precipitate and create a turbidity that can be measured and used as evidence of a positive reaction (Mori *et al*. 2001). The contribution of the LAMP technique on the diagnosis of Human Cytomegalovirus using the original reaction mixture (Suzuki *et al*. 2006, 2010; Wang *et al*. 2015; Roumani *et al*. 2021) and the results are promising, today, the mixes are pre-prepared and optimized for direct application, so, we tested the WarmStart Colorimetric LAMP 2X Master Mix (DNA & RNA) (NEB, Massachusetts USA, M1800L), thanks to its integrated indicator phenol red and the results confirm those of qPCR. By performing the reaction mixture according to the suppliers, use of 0.04% Bromophenol Blue as a pH indicator also gave interesting results. WarmStart Colorimetric LAMP 2X Master Mix (DNA & RNA) (NEB, Massachusetts USA, M1800L). The success of our study is proof of effectiveness of the WarmStart Colorimetric LAMP 2X Master Mix (DNA & RNA) (NEB, Massachusetts USA, M1800L). The mixing of this master mix is optimized to give results in just 30 min which is the advantage over most other amplification methods. This method encompasses and expands the range of application by its technical simplicity than qPCR and other derivatives. This method has to be evaluated and tested in other types of samples and other methods; it is still difficult to perform multiplex LAMP unlike qPCR. Further research is recommended on this subject to prove that LAMPPCR is an alternative and equivalent method to qPCR with more advantages and simplicity and economic (Table 4).

The literature reports interesting results with the WarmStart Colorimetric LAMP 2X Master Mix on viral infections such as the detection of HPV-16 and HPV-18 DNA with 100% specificity (Daskou *et al*. 2019), the detection of SARS-COV-2 RNA with the same reliability as RT-qPCR kits (Zhang *et al*. 2020). Pathogenic bacteria such as *Tannerella forsythia* and *Porphyromonas gingivalis* (Al-Hamdoni and Al-Rawi 2020), pathogenic parasites such as *Schistosoma japonicum* DNA (Rubinfien *et al*. 2020). Different types of samples such as serum, CSF, urine or nasopharyngeal swabs for the detection of SARS-COV-2.

Conclusion

LAMP is an alternative method of PCR that encompasses the advantages of speed, reliability, sensitivity and lower cost, this method is performed under isothermal conditions, the use of 4 to 6 primers makes the method faster and very selective. This method has come as a potential answer in different fields of biology and medicine in research and diagnosis. It has minimized the steps of other amplification methods and the reaction mixture is optimized for maximum yield, this makes LAMP, the best method when compared with other invented methods. The overall results of our study prove the advantages of the colorimetric CMVLAMP method in medical diagnosis. To use this method, epidemiological data of pandemics around the world could be recorded directly on site and contribute to the immediate control of the pandemic situation. It is a method that is suitable for any type of samples, type of pathogens or areas remote from laboratories such as forests and does not require the installation of sophisticated equipment. We recommend the use of this method in research on diseases that colonize our daily lives such as HIV, Hepatitis C, tuberculosis, malaria or COVID-19 to standardize it and make it contribute to stop pandemic invasions. This method will provide an economical alternative for medical diagnosis in transplant patients.

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Author Contributions

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.

Conflicts of Interest

The authors declare no conflict of interest.

Data Avaiability

The authors confirm that the data supporting the findings of this study are available from the corresponding author on reasonable request.

Ethics Approvals

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of Hassan II University of Casablanca (2018-321).

Informed Consent Statement

All subjects gave their informed consent for inclusion before they participated in the study.

Consent for Publication

All authors reviewed and approved the final version and have agreed to be accountable for all aspects of the work, including any issues related to accuracy or integrity.

Limitation of our Study

In future studies it is recommended to increase the number of samples, the number of protocols and to vary the different biological fluids and sample volumes.

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.

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