ESTABLISHMENT OF MULTIPLEX REAL-TIME PCR ASSAY FOR SIMULTANEOUS DETECTION OF *HERPES SIMPLEX VIRUS* AND *VARICELLA-ZOSTER VIRUS*

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ABSTRACT

The herpes simplex viruses types 1 and 2 (HSV-1 and HSV-2) and varicella-zoster virus (VZV) can cause life-threatening infections of the central nervous system and lead to severe disease in newborns and immunosuppressed patients. In these cases, rapid diagnosis is crucial. We have successfully established a process to simultaneously detect of HSV and VZV by multiplex real-time PCR using Taqman probes based on primers/probe from the DNA polymerase genes of those viruses. The performance of these techniques was assessed by analyzing serial dilutions of DNA known concentrations and the lowest limit of detection was found to be 10^2 DNA copies/ml. Of the 83 clinical samples collected from Tropical Diseases Hospital in HoChiMinh city and National Dermatology Hospital, 8/83 samples were infected with HSV, 10/83 samples were infected with VZV and 1/83 sample was co-infected with both HSV and VZV. The PCR results were confirmed by DNA sequencing showing 100% specificity. The method will be further tested on larger number of samples before being put into clinical application.

Keywords*: DNA polymerase gene, HSV, real-time PCR, Taqman, VZV.*

1. Introduction

Herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2) cause a variety of clinical symptoms in the central nervous system. On the patients with the Immunodeficiency Diseases and common virus infection are leading to several serious clinical consequences including mucosal inflammatory disease and inflammatory lung disease. HSV can be transmitted from an infected mother to newborn and cause serious infections, consequently leading to death (Weidmann et al, 2003).

Varicella-zoster virus (VSV) causes chickenpox and herpes zoster (shingles). It also causes serious central nervous system infection, especially, the infection accurs at immunodeficency patients (Weidmann *et al*, 2003).

It is necessary to have a rapid diagnosis of infected individuals, especially, on the case of the infection of high risk virus which may cause serious central nervous system infection with the unclear clinical manifestations. Moreover, it is very imporatant to get the diagnosis of the HSV and VZV infection in the newborn, for the aims to prevent many serious consequences caused by these infections (Hufert *et al*., 1995; Kessler *et al*., 2000). Nowadays, the treatment of HSV and VZV infection may have an effective achievement antiviral drug such as acyclovir. However, the treatment should have been done at the early stage as soon as the onset of infection to

reduce demange as well as to minimise the number of patients with neurological damage due to virus infection (Whitley, 2012). Many moleculer techniques have been studied to detect the herpesvirus infection, especially, the Real-time PCR (Aurelius et al., 1993; Koskiniem et al., 1996; Ryncarz et al., 1999; Epsy et al., 2000; Epsy et al., 2000; Kessler et al., 2000; Furuta et al., 2001; Weidmann et al., 2003). The purposes of our studies are establisment of the method in order to co-detection both HSV and VZV by multiplex Real-time PCR with the Taqman probe towards the convenience of future applications.

2. Materials and methods

Clinical samples

A total of 83 samples including 37 cerebrospinal fluids from Hospital of Tropical Disease, Ho Chi Minh City and 46 gential swab specimens from Hospital of Dermatolog were collected and submitted to our diagnotic laboratory for HSV and VZV detection.

Other isolates

To test for the specificity and cross-reactivity of the real-time PCR assay, other microorganisms including *Hepatitis B virus* (HBV), *Cytomegalovirus* (CMV), *Human papiloma virus* (HPV) and *Epstein-Barr virus* (EBV) obtained from our laboratory were used.

Primers, probes and amplicons

Primers, probes (Table 1) and amplicons used in this study were designed basing on *DNA polymerase* gene sequences form both viruses: HSV (Accession number: FU018121.1) and VZV (Accession number). Some soflwares were used including Primer3, Clustal X 1.81 (EMBL, Europe), Annhyb – 4.922, 2004 (Olivier Friard, USA), BLAST (NCBI, USA) http://www.ncbi.nlm.nih.gov/BLAST/ and Oligo Analyzer 3.0 (IDT, USA) http://scitools.idtdna.com/scitools/Applications/OligoAnalyzer/.

Table 1. Primers, probes used in this study

Name	Function	Sequence (5'-3')	Length (bp)	Size of PCR product (bp)
HSV-F	Forward primer	5'-TAC GTG CGS AAC AAC MTG GAG A-3'	22	129
HSV-R	Reverse primer	5'-GTT GTA CTT GAG GTC GGT GGT GT-3'	23	
HSV-P	Probe	5'-FAM -ACG ACC ACG AGA CCG ACA TGG AGC T-BHQ1-3'	25	-
VZV-F	Forward primer	5'-GTGCATCTGCAATTATGCGTCCA-3'	23	131
VZV-R	Reverse primer	5'-AGAACTGTTGTTATATGACGACACCGT-3'	27	
VZV-P	Probe	5'-HEX-ATTCAGCAATGGAAACACACGACGCC-BHQ2-3'	26	-

DNA extraction

Clinical samples were collected, transported to the laboratory at 4-8°C. After centrifugation at 13,000 rpm for 5 min, the pellet was dissolved in 50 µl buffer (250 mg/l proteinase K, 10 mmol/l Tris–HCl pH 7.8, 5 mmol/l EDTA, 0.45% Tween-20), followed by incubation at 56°C for 60 min and 100°C for 10 min. Centrifugation at 13,000 rpm for 5 min, supernatant liquid was collected and stored at −20°C later used for our novel multiplex Real-time PCR assay. Nucleic acids were extracted from each specimen using phenol/chloroform, according to Chomczynski

& Sacchi method (1987): 100 µl of each collected supernatant liquid was added by 900 µl Trizol, pH8. DNA was then precipitated by the same volume of Isopropanol and the helper seeDNA. DNA pellet was dissolved in TE 1X (Tris-EDTA) and stored at -20^{o}C until use.

Multiplex real-time PCR

Multiplex Realtime PCR was performed with a Mxpro-Mx3005P (Stratagene). The total volume of the Multiplex Realtime PCR was 50 µl containing 10 µl of DNA from a clinical sample or amplicons, 100 nmol/L of the primers probes, 100 nmol/L of the reverse primers, 100 nmol/L of the quencher strands, 400 µmol/L of each dNTP, 1.5 Units of hot-start *Taq* polymerase and 3 mmol/L $MgCl_2$, adding DW to 50 µl. The cycling conditions were an initial denaturation at 95 °C for 5 min, 40 cycles of 95 °C for 15 s, and 60°C for 1 min.

3. Results and discussion

Designation and the theoretical characterization of primers, probes

BLAST searches were performed to check the specificity of the DNA sequences of the primers, probes. All published sequences encoding the *DNA polymerase* genes ofHSV or VZV and no other species were aligned to the primers and probes (data not shown). In addition, we also checked some other physical characteristics of primers, probes using the analyzer tool integrated at IDT website (http://www.idtdna.com/analyzer/Applications /OligoAnalyzer). Most of the characteristics of primers, probes used in this study were suitable (table 2), including the parameters of lengths, percentages of GC, melting temperatures and energy (kcal/mol) calculated by self- hetero-dimer and hairpin-loop structures of primers and/or probes.

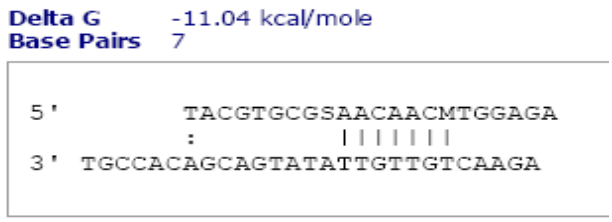
Table 2. Theoretical characterization of primers, probes

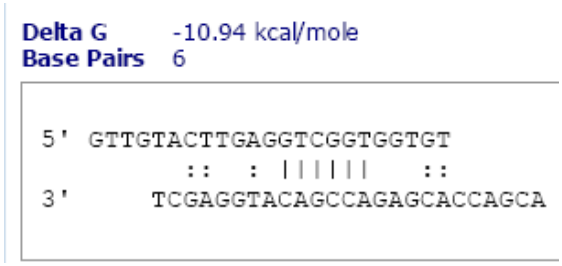
Oligonucleotide	% GC	Tm (^{o}C)	(1)	(2)	(3)					
					HSV-F	HSV-R	HSV-P	VZV-F	VZV-R	VZV-P
HSV-F	52.3	58.6-61.1	-0.03	-6.3		-6.58	-6.91			
HSV-R	52.2	59	0.04	-3.65			-10.94			
HSV-1P	60	65.4	-3.25	-6.34						
VZV-F	47.8	58.3	-1.64	-7.05	-8.48	-5.02	-6.6		-6.53	-5.24
VZV-R	40.7	57.9	-3.05	-4.95	-11.04	-9.98	-4.95			-9.67
VZV-P	50	62	-1.27	-3.61	-6.91	-6.53	-5.02			

Tm: melting temperature, (1): ΔG of hairpin-loop structure (kcal.mole^{-1}), (2): ΔG of self-dimer structure (kcal.mole^{-1}), (3): ΔG of hetero-dimer structure (kcal.mole^{-1})

According to the results showed in table 2, some of ΔG values (hetero-dimer structure beyween HSV-R and HSV-P, HSV-F and VZV-R, HSV-R and VZV-R, VZV-R and VZV-P) are less than -9 kcal.mole-1. We therefore checked the way of formation of those hetero-dimer structures. Figure 1 are displayed two kinds of secondary structure formation between HSV-F and VZV-R, HSV-R and HSV-P which showed lowest energy as -11.04 and -10.94 kcal.mole-1 (Fig 1).

Figure 1. Hetero-dimer structure formation with ΔG less than -9.0 Kcal/mol



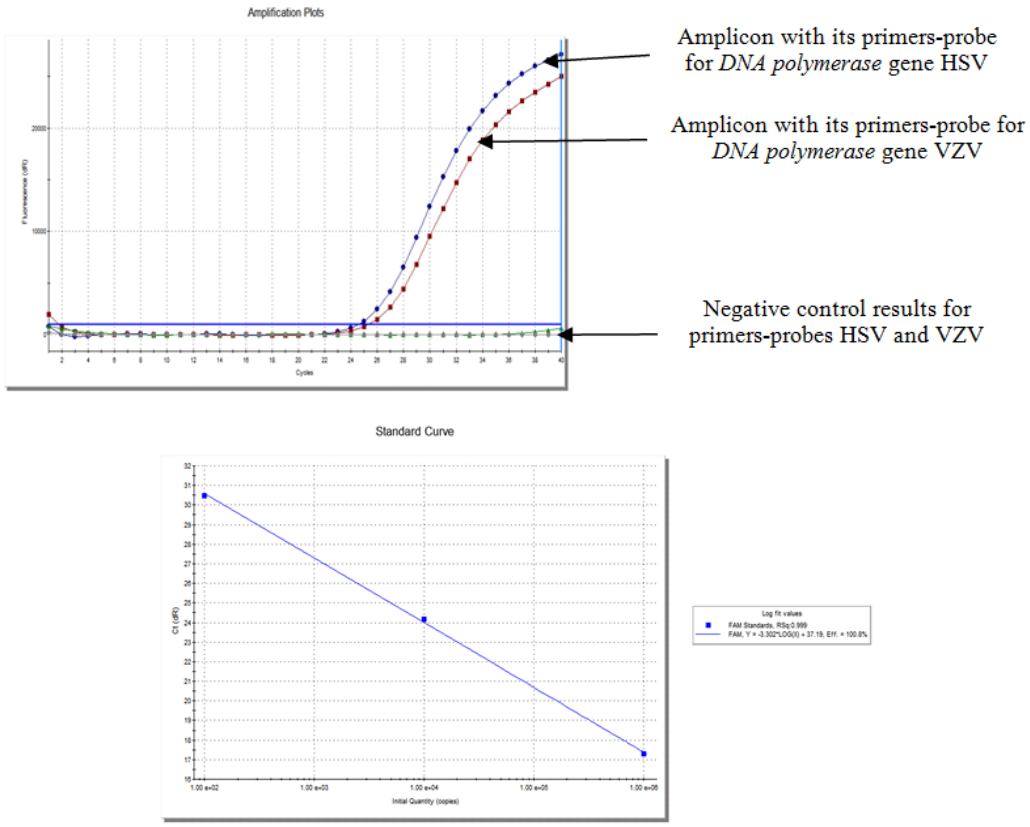


As our experience, with the way of secondary structure formation between above primers and probes, the complementary between the nucleotide that are not located at 3' terminal of primers and probes, by using a suitable hybridization temperature (expected temperature is 60°C), we tentatively predicted that above secondary structures would be easy break.

Specificity of primers, probes

The specificity of primers and probes was 100% since the assay was able to detect amplicon (Fig 2) as well as from other two clinical samples (Fig 3). The linear regression coefficient is close to 1, and efficiency of the PCR is 100.8%. The specificity was 100% since the amplicon binding to its primers-probe. No fluorescence signal was found on negative control tests in which amplicons were changed by DW (Fig. 2, top). Meanwhile the standard curve (in Fig. 2, bottom) was C (copies/ml) = 1000, R= 0.999.

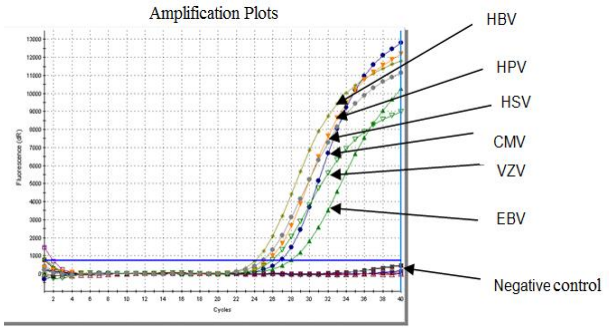
Figure 2. (Top) Fluorescence curves of each amplicon with its primers-probe.



From the left to the right, these four fluorescent curves from the amplicons with their primers/probe and the negative controls are shown. (Bottom) Standard curve used to calculate the concentration of amplicon DNAs in this experiment

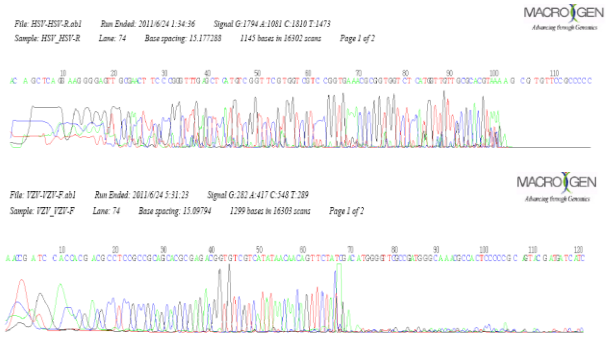
No any positive result was found after testing those primers-probes on other microorganisms mentioned in materials and methods (Fig 3), especially, no cross-reactivity of the primers and the probes was observed for the pathogenic conmensal organisms of the swab or cerebrospinal fluid tracts.

Figure 3. The results of evaluating the amplied efficiency of HSV, VSV and other sources of microorganism.



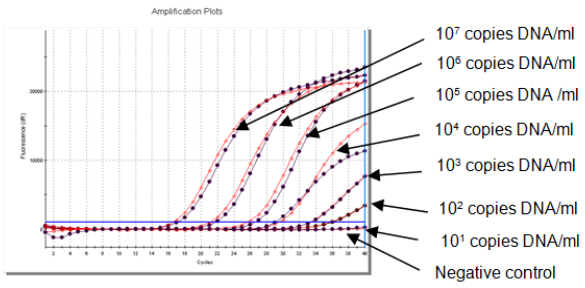
Simultaneously, the monoplex real-time PCR is carried out and its product is sequencing at Macrogen. The Fig. 4A indicates the sequence is totally specific and similar when using this sequence to find the similarity by BLAST (NCBI) and comparing to several DNA polymerase gene of HSV and VZV which sequences are downloaded from Genbank (Data not shown)

Figure 4. The results of PCR product of HSV and VZV with forward primer.



The sensitivity of method

Figure 5. The results of sensitivity assay of primer and prode on HSV and SZV



Regarding to the sensitivity assay, Fig. 5. indicates that the flourescent signal exceeds the basal signal from the sample DNA concentration of 10^7 copies DNA/ml with cycle threshold (Ct) descending to the sample DNA concentration of 10^2 copies DNA/ml. The sample DNA concentration of 10 copies DNA/ml has a flourescent signal lower than the basal signal. Therefore, the sensitivity of multiplex real-time PRC is 10^2 copies DNA/ml.

The results of multiplex real-time PCR on medical specimens

Initially, the multiplex real-time PCR is applied on total of 83 specimens including 37 cerebrospinal fluid (CSF) samples and 46 gential swab specimen. As the results, 8 of 83 samples were infected with HSV, 10 of 83 samples were infected with VZV and 1 of 83 sample was co-infected with both HSV and VZV.

4. Conclusion

In conclusion, we successfully established the method to co-detection of HSV and VZV by multiplex real-time PCR by using the Taqman probe. In this study, the sensitivity of this method is reported as 10^2 DNA copies/ml. Additionally, this method show the complete specific to HSV and VZV. Experimentally, the detection is carried out on total of 83 clinical samples collected from Tropical Diseases Hospital in HoChiMinh city and National Dermatology Hospital. As the results, 8/83 samples were infected with HSV, 10/83 samples were infected with VZV and 1/83 sample was co-infected with both HSV and VZV. For further study, this research will be continuously on the larger size of samples and the sampling will be conducted multiple times on the same patient during clinical monitoring and treatment in order to have more data to help evaluate the accuracy and efficiency of the multiplex real-ttime PCR in diagnosis and monitoring treatment of HSV, VSV.

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REFERENCES

Aurelius E, Johansson B, Sköldenberg B, Forsgren M. (1993). Encephalitis in immunocompetent patients due to herpes simplex virus type 1 or 2 as determined by type-specific polymerase chain reaction and antibody assays of cerebrospinal fluid. *J Med Virol., 39(3),* 179-186.

Chomczynski P, Sacchi N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem., 162,* 156-159.

Espy MJ, Teo R, Ross TK, et al. (2000). Diagnosis of varicella-zoster virus infections in the clinical laboratory by LightCycler PCR. *J Clin Microbiol., 38(9),* 3187-3189.

Espy MJ, Uhl JR, Mitchell PS, et al. (2000). Diagnosis of herpes simplex virus infections in the clinical laboratory by LightCycler PCR. *J Clin Microbiol., 38(2),* 795-799.

Hufert FT, Diebold T, Ermisch B, et al. (1995). "Liver failure due to disseminated HSV-1 infection in a newborn twin". *Scand J Infect Dis., 27(6),* 627-629.

Kessler HH, Muhlbauer G, Rinner B, et al. (2000). Detection of herpes simplex virus DNA by real-time PCR. *J. Clin. Microbiol., 38,* 2638-2642.

Koskiniemi M, Piiparinen H, Mannonen L, et al. (1996). Herpes encephalitis is a disease of middle aged and elderly people: polymerase chain reaction for detection of herpes simplex virus in the CSF of 516 patients with encephalitis. *J Neurol. Neurosurg. Psychiatry, 60,* 174-178.

Ryncarz AJ, Goddard J, Wald A, et al. (1999). Development of a high-throughput quantitative assay for detecting herpes simplex virus DNA in clinical samples. *J. Clin. Microbiol., 7,* 1941-1947.

Weidmann M, Meyer-König U, Hufert FT. (2003). Rapid Detection of Herpes Simplex Virus and Varicella-Zoster Virus Infections by Real-Time PCR. *J. Clin. Microbiol. 41(4),* 1565-1568.

Whitley RJ. (2001). Herpes simplex viruses. *In* D. N. Knipe and P. M. Howley (ed.), Fields Virology, 4th ed., vol. 2. Lippincott Williams & Wilkins, Baltimore, Md. 2461-2509.

Yasushi F, Fumio O, Hirofumi S, Satoshi F, Yukio I. (2001). Quantitation of Varicella-Zoster Virus DNA in Patients with Ramsay Hunt Syndrome and Zoster Sine Herpete. *J Clin Microbiol. 39(8),* 2856-2859.