Title

Improvement in quantitative detection of Human Herpesvirus 6 with a ready-to-use PCR-based Real Time PCR assay

Authors

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Background

Human Herpesvirus 6 (HHV-6) is a set of two closely related herpes viruses known as HHV-6A and HHV-6B. HHV-6B infects nearly 100% of human beings, typically before the age of three. HHV-6A can promote the progression of HIV disease toward AIDS, as both clinical and experimental evidence suggest. Like other herpesviruses, HHV-6 establishes life-long latency and can become reactivated later in life. Reactivation occurs mostly in transplant patients taking immunosuppressant drugs or individuals with immune deficiencies and it can involve brain, lungs, heart, kidney and gastrointestinal tract. HHV-6 reactivation/re-infection seems to occur before the time when other opportunistic infections usually appear. Both HHV-6 viruses are highly cell associated and can be detected in plasma briefly during the initial infection or acute reactivation. Detection of HHV-6 DNA in plasma generally means the patient has an active infection. The aim of this work was to evaluate the performance of a new assay designed to detect and quantify HHV-6 DNA in human samples.

Material and methods

The assay was developed as a ready-to-use test containing all the required elements for the amplification of both HHV-6 DNA fragment and human beta-globin gene as internal control. The two sets of primers and probes are combined in a lyophilized and ready-to-use mix, co-amplified and detected by a Real-Time PCR instrument. In the present study, several samples obtained from San Raffaele Hospital, previously tested with "*artus* HHV-6 RG PCR Kit" (Qiagen), were investigated. PCR reactions were performed on nucleic acids extracted from plasma, whole blood and cerebrospinal fluid (CSF).

Results

All tested samples were previously diagnosed as positive. The new freeze-dried ready-to-use assay demonstrated robust and accurate target amplification, according to the data obtained at San Raffaele Hospital. This detection kit proved to be specific for HHV-6. The assay did not cross-react with any of the other Human Herpesviruses tested. The test showed a LoQ of 5 genome copies/reaction and a LoD of 2 genome copies/reaction.

Conclusions

The described Real-Time PCR assay proved its effectiveness for the detection and quantitation of HHV-6 DNA in samples. The test showed a sensitivity and a specificity of 100%. The high-sensitivity and specificity of this assay, linearity and quantitation performances, associated with the ready-to-use and room temperature storage, would have a direct impact on the early and correct management of the affected patients.

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Conclusions: The described Real-Time PCR assay proved its effectiveness for the detection and quantitation of HHV-6 DNA in samples. The test showed a sensitivity and a specificity of 100%. The high-sensitivity and specificity of this assay, linearity and quantitation performances, associated with the ready-to-use and room temperature storage, would have a direct impact on the early and correct management of the affected patients.

Background

HHV-6 is a member of the β-herpesvirus subfamily of human herpesviruses. There are two subtypes of HHV-6, type A and type B, which share certain biological properties and a high level of sequence homology, but are clearly distinct, both virologically and epidemiologically. HHV-6 infects multiple cell lines and tissues and establishes latent infection in mononuclear cells. This virus infects virtually all children within the first three years of life and, like other herpesviruses, it establishes latency after primary infection. In immunocompromised patients who underwent transplantation, HHV-6 may exit the latent state and actively replicate, causing serious consequences from a clinical point of view. Real Time PCR assays are able to identify the presence of HHV6 virus in biological specimens and they allow to collect clinical indications about the efficacy of therapy, anti-viral drugs resistance, preventive treatments and disease progression.

Materials and Methods

This HHV6 detection assay is a freeze-dried and ready-to-use kit that includes all the required elements for the amplification of nucleic acid targets (Figure 1). Its intended use is the detection of HHV6 in human samples.

Figure 1: HHV6 assay components and freeze-dried mix.

A specific set of primers and probe was designed to amplify a 118 bp DNA fragment within a conserved region of HHV6 genome, a second set of primers and probe amplifies another conserved region of 160 bp of HHV6 genome and a third set of primers and probe amplifies a 127 bp Human Beta Globin gene fragment, used as an internal amplification control (IC). In this assay the three targets are co-amplified and detected by a CFX96 Real-Time PCR instrument (BioRad) using the protocol indicated in Table A.

Step	Cycles Number	Denaturation	Annealing	Total run time
1	1	95 °C 2 min	-	90 min
2	45	95 °C 15 sec	-	
		-	60 °C 1 min	

Table A: Thermal cycler protocol.

HHV6 assay's performances were evaluated on:

- a total of 18 plasma, whole blood and CSF samples previously diagnosed as positives;
- a total of 15 plasma whole blood and CSF samples previously diagnosed as negatives.
- a quantified reference strain obtained from Vircell. (Vircell – Granada, Spain.)

The panel of 33 samples were extracted at San Raffaele Hospital (HSR) with QIAsymphony DNA extraction system (Qiagen) from human plasma, whole blood and CSF and previously diagnosed as positives and negatives with the "Artus HHV-6 RG PCR Kit" (Qiagen) (Table B).

	Positives	Negatives
HSR samples	18	15

Table B. Human samples panel obtained from HSR.

A quantified reference DNA strain (Vircell) was also tested; it was also used to determine the limit of detection (LoD) of the assay (Table C) testing 7 diluitions.

Strain	HHV6						
Copies/reaction	10^4	10^3	10^2	10	5	2	1

Table C. Dilution used to determine the LoD of the HHV6 assay.

Results

To assess the effect of freeze-drying process on the quality of the assay, five 10-fold serial dilution corresponding to 10^4, 10^3, 10^2, 10 and 1 copies/reaction, spotted with Human genomic DNA (Promega), were tested. The amplification profiles obtained before and after lyophilization showed to be very similar, preserving the same Ct and fluorescence values for all the DNA quantities tested.

The Analytical sensitivity of the assay was evaluated testing seven dilution of HHV6 strain. The genomic DNA of HHV6 was mixed with Human genomic DNA, simulating human samples (Figure 2).

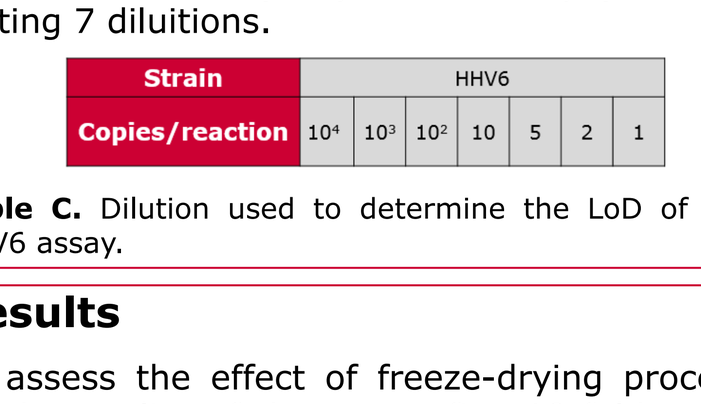

Figure 2: LoD of HHV6 Assay. Real-Time PCR amplification plot of human beta globin gene (IC, Internal Control) in blue, seven dilutions of HHV6 quantified template in green, corresponding to four serial dilutions 10^4, 10^3, 10^2, 10 and three additional dilutions: 5, 2 and 1 copies/reaction. No Template Control (NTC)

The Limit of Detection of the assay showed a final value of 2 genome copies/reaction. The Limit of quantitation of the assay showed a final value of 5 genome copies/reaction.

A panel of 33 samples was tested to evaluate the diagnostic sensitivity (Figure 3) and the diagnostic specificity (Figure 4) of the assay.

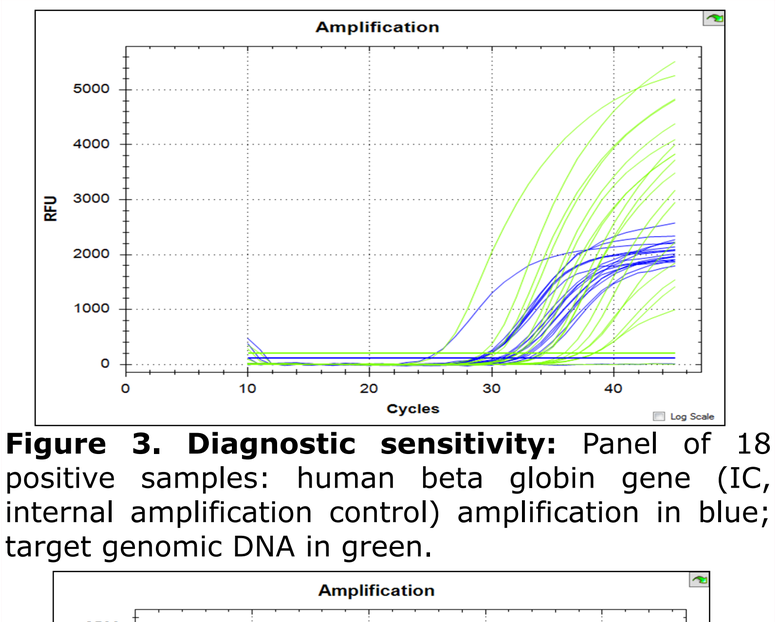

Figure 3. Diagnostic sensitivity: Panel of 18 positive samples: human beta globin gene (IC, internal amplification control) amplification in blue; target genomic DNA in green.

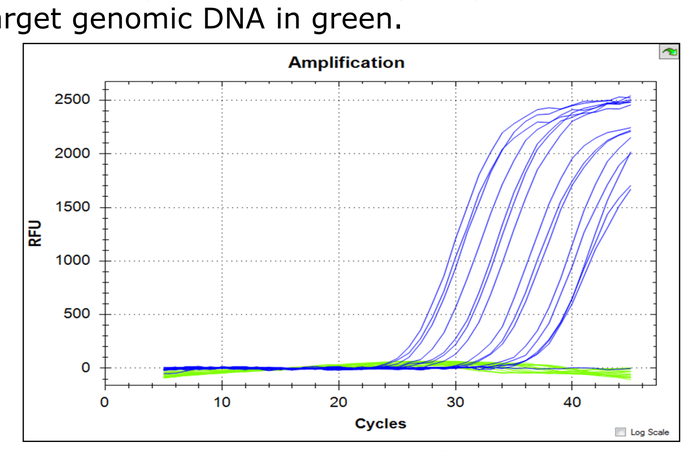

Figure 4. Diagnostic specificity. Panel of 15 negative samples: human beta globin gene (IC, internal amplification control) amplification in blue; target genomic DNA in green.

All the samples, extracted with QIA symphony DNA extraction system (Qiagen), were previously confirmed as positives and negatives with the commercial HH6 test "Artus HHV-6 RG PCR Kit" (Qiagen).The panel of samples considered in the present study includes strong positives as well as weak positives and negatives. Obtained data totally agreed with HSR results and confirmed 18 positive samples and 15 negative samples. Even in presence of very low levels of infection, developed test was able to detect the presence of HHV6 in sample, with a high level of agreement of quantitation (CT) with the reference system.

Conclusions

Lyophilization is a widely applicable technology that can be used for stabilizing at room temperature different amplification mixes with a variety of applications. The ready-to-use format avoids problems related to cross-contamination due to multi-step preparation of common PCR master mixes. The presented assay is a quick and effective new diagnostic kit for rapid detection and quantitation of HHV6 in human samples. Its characteristics of room temperature storage and ready-to-use assay, together with a high sensitivity and specificity, make this kit a rapid and easy tool to diagnose and monitor HHV6 infections in human patients.