

BRIEF COMMUNICATION

OPTIMIZATION OF THE SYBR GREEN REAL TIME PCR FOR THE DETECTION OF HUMAN HERPES VIRUS TYPE 6 (HHV-6)

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SUMMARY

HHV-6 is the etiological agent of Exanthem subitum which is considered the sixth most frequent disease in infancy. In immuno-compromised hosts, reactivation of latent HHV-6 infection may cause severe acute disease. We developed a Sybr Green Real Time PCR for HHV-6 and compared the results with nested conventional PCR. A 214 pb PCR derived fragment was cloned using pGEM-T easy from Promega system. Subsequently, serial dilutions were made in a pool of negative leucocytes from 10^{-6} ng/ μ L (equivalent to 2465.8 molecules/ μ L) to 10^{-9} (equivalent to 2.46 molecules/ μ L). Dilutions of the plasmid were amplified by Sybr Green Real Time PCR, using primers HHV3 (5' TTG TGC GGG TCC GTT CCC ATC ATA 3') and HHV4 (5' TCG GGA TAG AAA AAC CTA ATC CCT 3') and by conventional nested PCR using primers HHV1 (outer): 5'CAA TGC TTT TCT AGC CGC CTC TTC 3'; HHV2 (outer): 5' ACA TCT ATA ATT TTA GAC GAT CCC 3'; HHV3 (inner) and HHV4 (inner) 3'. The detection threshold was determined by plasmid serial dilutions. Threshold for Sybr Green real time PCR was 24.6 molecules/ μ L and for the nested PCR was 2.46 molecules/ μ L. We chose the Real Time PCR for diagnosing and quantifying HHV-6 DNA from samples using the new Sybr Green chemistry due to its sensitivity and lower risk of contamination.

KEYWORDS: Human herpes virus 6 (HHV-6); PCR; Real-time PCR; Sybr Green.

HHV6 is the etiological agent of Exanthem subitum²⁰ the 6th most common disease in infancy, with seroepidemiological rates between 80 to 95%. It was first isolated in 1986 from Peripheral Blood Mononuclear Cells (PBMC) of patients with lympho proliferative disorders¹⁷. Two variants of HHV-6 with differences in molecular and antigenic characteristics are known⁴. Reactivation may occur after primary infection later in life in healthy individuals but mainly in immunodeficient patients leads to acute or even fatal disease⁵. Several conventional PCR techniques have already been described^{1,8} but processing time is long, results and number of copies are only shown at the end of the reaction and contamination risks are greater when compared to real time PCR. HHV6 quantification methods are necessary to monitor the different stages of disease such as latency, reactivation or active infection⁷. This study describes a Real Time PCR system using Sybr Green chemistry and compares its results with those obtained with conventional nested PCR.

Sample utilized for initial DNA extraction: Five microliters of CSF sample DNA from a bone marrow transplant patient from Hospital das Clínicas da Universidade de São Paulo were utilized. DNA was extracted by QIAamp[®] DNA Mini Kit (QUIAGEN, Valencia, CA, USA),

according to manufacturers instructions.

Nested and Real Time PCR assays: Primers for nested PCR⁹ which codifies the main viral capsid protein (MCP) were HHV1 (outer): 5' CAA TGC TTT TCT AGC CGC CTC TTC 3'; HHV2 (outer): 5' ACA TCT ATA ATT TTA GAC GAT CCC 3', amplicon size 480bp and HHV3 (inner): 5' TTG TGC GGG TCC GTT CCC ATC ATA 3'; HHV4 (inner): 5' TCG GGA TAG AAA AAC CTA ATC CCT, amplicon size 214bp (Invitrogen, São Paulo, SP, Brazil). For real time PCR, only primers HHV3' and HHV4 (inner) were utilized. The following amplification protocols were used: 1 cycle at 95 °C for five minutes followed by 35 cycles consisting of 30 seconds at 95 °C, 45 seconds at 55 °C and one minute at 72 °C, and a final 10 minutes extension at 72 °C in an automated thermal cycler. (Mastercycler[®] gradient - Eppendorf (HAMBURG-GERMANY)) for nested PCR and one cycle at 50 °C for two minutes, one cycle at 95 °C for 10 minutes, followed by 40 cycles consisting of 15 seconds at 95 °C, one minute for 60 °C and one cycle at 95 °C for 15 seconds, 60 °C for 30 seconds, 95 °C for 15 seconds for dissociation stage for real time PCR (ABI 7300 Real Time PCR Systems Applied Biosystems - FOSTER CITY, USA).

Sequencing and cloning of HHV 6: Amplicon was purified, quantified and submitted to sequencing reaction using primers described above and the Kit Big Dye Terminator (Applied Biosystems Incorporation, Foster City, California, USA). This method uses different fluorescent labels for each dideoxynucleotide (ddA, ddC, ddT, ddG), allowing simultaneous analysis by the sequencing sensor (ABI PRISM 377 - Applied Biosystems Incorporation, Foster City, California, USA). Results obtained were compared to data on Genbank using BLAST program, available at the National Center for Biotechnological Information (<http://www.ncbi.nlm.nih.gov>). Sequencing confirmed the amplicon as a fragment derived from Human Herpesvirus 6 subtype B strand Z 29 (GenBank accession EF422414).

After confirmation, a fragment of this sample (214 bp) of HHV6 was cloned in pGEM-T easy from Promega (Promega Corporation - Madison, WI, USA).

Primer concentration optimization: In order to optimize our quantification techniques, we built a standard curve through serial dilutions of the PCR fragment in a pool of negative leucocytes, ranging from 10^5 ng/ μ L (equivalent to 2465.8 molecules/ μ L) to 10^8 (equivalent to 2.46 molecules/ μ L). Extraction was performed using QIAamp[®] DNA Mini Kit.

To achieve optimal primer concentration, we have tested in duplicates four different concentrations: 0.2 p/mol, 0.4 p/mol 0.6 p/mol 0.8 p/mol and the HHV6-containing plasmid described above and the optimal primer concentrations was selected (0.4 p/mol) due to its lowest C_T among the different duplicates.

Subsequently, a standard curve was built with the plasmid and amplified it with both Syber Green Real Time PCR and conventional nested PCR. The reproducibility of the Sybr Green assay test for HHV-6 was verified using duplicates of clone dilutions at 2.46; 24.6, 246 and 2465.8 molecules/ μ L respectively using the absolute quantification method. Threshold for this assay was of 24.6 molecules/ μ L. As for the conventional method, the Nested PCR reproducibility was tested using a single sample of the same dilutions mentioned above and the threshold was 2.4 molecules/ μ L.

Nested PCR was more sensitive than real time PCR. Indeed, using nested PCR we might be detecting HHV-latency¹². Previous data showed that a minimum of 20 copies/mL are found in cases with clinical disease¹³. The number of copies detected by real time PCR, although higher than those detected by nested PCR, but real time PCR results are reported as more reproducible¹⁰ and clinically significant^{2,11}. Furthermore, real time PCR using Sybr Green to detect HHV-6 DNA is also faster⁶. Three major advantages are found with the use of this method: 1) follow up of disease evolution or regression can be rapidly achieved^{3,16,19}; 2) it is less laborious than conventional PCR⁷; 3) results can be attained in approximately two hours¹⁵. Primer concentrations were chosen to allow a lower C_T for positive samples with maximum ΔR_n and less primer dimers^{14,18}. We must emphasize that Real Time PCR was carried out in one step reaction significantly reducing the risk of cross- contamination.

RESUMO

Otimização da PCR em tempo real - Sybr Green para detecção do Herpes Vírus Humano tipo 6 (HHV-6)

HHV-6 é o agente etiológico do Exantema Súbito e considerado a sexta doença mais comum na infância. Em indivíduos imunocomprometidos, a reativação da infecção latente pode causar doença aguda ou morte. Padronizamos PCR em Tempo Real utilizando a química Sybr Green na detecção do HHV-6 e comparamos os resultados com a PCR convencional. Um fragmento de 214 pb foi clonado através do kit pGEM-T do sistema Promega. Com este clone, foram feitas diluições seriadas em um pool de leucócitos negativos a partir de 10^6 ng/ μ L (equivalente a 2465,8 moléculas/ μ L) até 10^9 (equivalente a 2,46 moléculas/ μ L). As diluições foram amplificadas por PCR em Tempo Real utilizando Sybr Green, com primers HHV3 5' TTG TGC GGG TCC GTT CCC ATC ATA 3' e HHV4 5' TCG GGA TAG AAA AAC CTA ATC CCT 3' e pelo método convencional, PCR nested usando primers HHV1 (externo): 5' CAA TGC TTT TCT AGC CGC CTC TTC 3'; HHV2 (externo): 5' ACA TCT ATA ATT TTA GAC GAT CCC 3', HHV3 (interno) e HHV4 (interno): 5' TCG GGA TAG AAA AAC CTA ATC CCT 3'. O limite de detecção foi determinado pelas diluições seriadas do plasmídeo contendo um fragmento de HHV6: para o ensaio com Sybr Green, foi de 24,6 moléculas/ μ L e para a PCR nested, 2,46 moléculas/ μ L. Elegemos o PCR em Tempo Real - Sybr Green como método diagnóstico e quantitativo do HHV-6 devido a sua boa sensibilidade e menor risco de contaminação.

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