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Comparison of the SYBR Green and the hybridization probe format for real-time PCR detection of HHV-6

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Summary

A comparative study was conducted of a novel real-time quantitative PCR test (LightCycler System) with FastStart DNA Master^{PLUS} SYBR Green I dye to detect DNA of human herpes virus 6 (HHV-6). Results were compared with those of a real-time quantitative PCR with hybridization probe (HP) formats using the fluorescence resonance energy transfer method, and with those of a single qualitative PCR test. The detection limit of the test with SYBR Green I dye was 20 copies of the virus, similar to that of the other two tests. The reproducibility was satisfactory. The new test has the same advantages as real-time PCR with HP formats and offers a greater versatility at lower cost.

Introduction

The human B-lymphotropic virus was first described in 1986 (Salahuddin et al., 1986) and subsequently renamed as human herpes virus 6 (HHV-6) (Ablashi et al., 1987). Two variants have been reported to date, HHV-6A and HHV-6B, which are both highly prevalent in the general population (Roldán et al., 1995). Genome sequencing of both subtypes demonstrated >90% genetic homology (Isegawa et al., 1999). HHV-6B has been identified as an aetiological agent of *exanthema subitum* (Yamanishi et al., 1988), a childhood disease that can progress to meningitis (Huang et al., 1991); meningoencephalitis (Asano et al., 1992), hepatitis (Asano et al., 1990) and thrombocytopenic purpura (Kitamura et al., 1994). It has also been related to other clinical diseases in immunocompetent adult patients (Braun et al., 1997). In contrast, HHV-6A has yet to be implicated

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in the aetiology of human disease, and the viral infection it produces is often asymptomatic (Braun et al., 1997).

To date, there are no reliable markers of active post-primary HHV-6 infection that clearly differentiate it from other stages of the infection. The determination of IgG anti-HHV-6 in serum is of limited value because of the high prevalence of the infection (Lusso and Gallo, 1995), its variability in serum levels (Locatelli et al., 2000) and the low specificity of its detection (Berneman et al., 1992). IgM anti-HHV-6 is present in 5% of the healthy adult population and is frequently absent in individuals infected with a positive culture of the virus (Okuno et al., 1989). In contrast, determination of the viral DNA in blood may be valid to diagnose active infection. Nevertheless, because the infection persists in cells in a latent state after the primary infection, quantitative methods have been developed to determine the number of copies and differentiate between active and latent infection (Secchiero et al., 1995).

Previous studies quantitated HHV-6 DNA from the signal emitted by hybridization probes (HPs) (Gobbi et al., 1999; Locatelli et al., 2000; Ohyashiki et al., 2000; Aritaki et al., 2001; Kearns et al., 2001; Collot et al., 2002; Oster and Höllsberg, 2002). However, real-time PCRs that use labelled primers are expensive and have some drawbacks related to the increased handling and the possible loss of detection limit compared with real-time PCR using SYBR Green I dye (Aldea et al., 2002). Therefore, a comparative study was conducted between a real-time PCR with FastStart DNA Master^{PLUS} SYBR Green I dye, a real-time PCR with HP formats using fluorescence resonance energy transfer (FRET) and a conventional PCR test.

Materials and methods

Oligonucleotide primers and HP formats

A specific HHV-6 DNA gene sequence fragment was investigated with an in-house PCR test. The primers and HPs formats were from published data on the 101K region of the HHV-6 genetic sequence (Torelli et al., 1991). They belong to a region with significant genetic homology between HHV-6A and HHV-6B, permitting the simultaneous detection of both variants (Ohyashiki et al., 1999). The forward and reverse primers were, respectively, (5′-ACCCGAGAGATGATTTTGCG-3′) starting at position 20,889, and (5′-GCAGAAGACAGCAGCGCGAT-3′) starting at position 21,100. The sequences of the HPs were LCRed640-GGGTCATTTATGTTATAGACGGT, with the acceptor dye at the 5′-end (LightCycler-Red640: 6-carboxytetramethylrodamin) starting at position 21,034, and TAAGTAACCGTTTTCGTCCCA-F, with the donor dye at the 3′-end (F: 6-carboxyfluoresceín) starting at position 21,012. Primers and HPs were supplied by TIB-MOLBIOL (Germany). The specific sequences selected were analyzed at the Swiss Institute of Bioinformatics using the BLAST network service, and it was confirmed that neither the primers nor the HPs presented significant genetic homology with any known nucleotide sequences besides HHV-6. To avoid false positive amplifications, procedures recommended to prevent contamination were meticulously observed (Kwok and Higuchi, 1989).

HHV-6 strains

A HHV-6 strain was used to obtain the specific sequence for selecting the primers. Dr. Fernando de Ory of the National Microbology Centre of the Carlos III Institute (Majadahonda, Madrid, Spain) donated HSB-2 cells from Type T lymphocytes of acute lymphoblastic leukaemia. The HSB-2 cells were infected with HHV-6 (GS strain) in a mixture of uninfected and infected cells (9:1). The infected HSB-2 cells were cultured in RPMI 1640 medium (BioWhittaker Europe, Belgium), inactivated by heating in 56 °C bath for 30 min and supplemented with 2% bovine foetal serum (Kraeber 6MBH&Co., Pharmazeutische Rohstoffe, Germany), 1% glutamine, 1% bicarbonate and antibiotics.

DNA extraction

DNA was extracted from the samples of HHV-6-infected cells by the High Pure PCR Template Preparation Kit (Roche Diagnostics, Germany); 200 μl sample was mixed with 200 μl Tissue Lysis Buffer (4 M urea, 200 mM Tris buffer, 200 mM NaCl, 200 mM EDTA) and 40 μl proteinase K. After incubation at 55 °C for 1 h, 100 μl of binding buffer (6 M guanidine–HCl, 10 mM urea, 10 mM Tris–HCl, 20% Triton X-100 [v/v]) was added and incubated at 72 °C for 10 min. Subsequently, 100 μl isopropanol were added, the mixture was placed in polypropylene tubes with two layers of glass fibre fleece and centrifuged at 8000 rpm for 1 min, preserving the filter; 500 μl inhibitor removal buffer (5 M guanidine–HCl, 20 mM Tris–HCl) with absolute ethanol (10:6) were added and centrifuged under the above conditions. The 500 μl of wash buffer (20 mM NaCl, 2 mM Tris–HCl) with absolute ethanol (1/5) were added and centrifuged at 8000 rpm for 1 min and at

14,000 rpm for 10 s, discarding the collection tube. Finally, 25 μl elution buffer (10 mM Tris) preheated to 70 °C were added and centrifuged at 8000 rpm for 1 min; the product of the collection tube was collected. The extract was divided into 2 μl aliquots that were stored at −20 °C until use.

Real-time and conventional PCR test conditions

Real-time PCR was performed using the LightCycler System (Roche Diagnostics) and FRET or FastStart DNA Master^{PLUS} SYBR Green I dye detection. For the FRET method, the mixture contained 2 μl of LightCycler–DNA Master HPs 1X (Roche Diagnostics), 4 mM $MgCl_2$, 500 nM of each primer, probes bound to 200 nM 6-carboxyfluoresceín and 400 nM 6-carboxytetramethylrodamin, respectively, brought to a final volume of 18 μl with water. For the FastStart DNA Master^{PLUS} SYBR Green I dye method, the mix contained 2 μl LightCycler FastStart DNA Master^{PLUS} SYBR Green I 1X (Roche Diagnostics, Germany) and 500 nM of each primer, brought to 18 μl with water. After adding 2 μl DNA extract both mixtures were transferred to reaction capillaries (LightCycler Capillaries, Roche Diagnostic GMBH). In both cases, the reaction conditions were: denaturation for 10 min at 95 °C; and 45 cycles of amplification for 0 s at 95 °C, 10 s at 55 °C and 5 s at 72 °C. Melting was done by increasing the temperature from 45 to 95 °C at 0.5 °C/s and, finally, the mixture was cooled for 30 s at 40 °C.

As reference test, a conventional PCR was performed in a thermocycler (Eppendorf® Mastercycler, Germany) as follows: denaturation for 5 min at 95 °C; 40 amplification cycles for 1 min at 95 °C, for 30 s at 56 °C and for 30 s at 72 °C; extension for 10 min at 72 °C. The mixture contained 2.5 mM $MgCl_2$, 500 nM primers, 0.2 mM dNTPs, 1U of *Taq* polymerase, brought to a final volume of 50 μl with water. The possibility of reactant contamination was tested by the inclusion of water as negative control in each test.

Reference curve construction

A reference curve for the extrapolation of results was constructed using standard values obtained by serial dilution of a plasmid harbouring a 231-bp insert. For this purpose, a 231-bp fragment of the HHV-6 101K region was cloned by using the selected primers. This was done by amplifying the target sequence present in the extract using the conventional PCR conditions described above. The DNA was purified by the MICROCON method (Millipore Corporation, USA) following the manufacturer's instructions (Krowczynska et al., 1995). The presence of the specific amplificate band was confirmed by electrophoresis in 2% agarose gels with ethidium bromide. The DNA was inserted in the PCR 4-TOPO vector of the TOPO TA Cloning Kit for Sequencing (Invitrogen, The Netherlands) using chemically competent *Escherichia coli* colonies under the conditions recommended by the manufacturer. The amount of plasmid DNA was determined by a spectrophotometer at 260 nm (GeneQuant proRNA/DNA Calculator, Amersham Biosciences, USA). Finally, serial dilutions of the extract were prepared, ranging from 10^{-6} ng/μl (equivalent to 2.2×10^2 copies/mixture) to 10^{-8} ng/μl (equivalent to 2.2 copies/mixture). The plasmid dilutions were amplified in the LightCycler with the SYBR Green I dye. Quantitative interpretation was done by the LightCycler software using the second derivate maximum method.

Results

Reference curve

The log-linear regression plot demonstrated a significant linear relationship between the logarithm of the initial copy concentration and the threshold cycles (C_t; $r = 0.98$), including, in one case, dilution of 2 copies (Fig. 1).

Optimization of FastStart DNA Master^{PLUS} SYBR Green I real-time PCR conditions

To test the reproducibility of the test three dilutions of the reference plasmid were used (2, 20 and 200 copies/mixture). Several PCRs with SYBR Green I dye were performed, modifying temperature and time. It was then established whether the C_t sequence was linear or not by using the method described above for FRET under previously published conditions (Ohyashiki et al., 2001). For the SYBR Green I dye PCR, annealing was done for 10 s at 55 °C, at 59 °C with reductions of 0.5 °C every five cycles to 55 °C, and at 61 °C with reductions of 0.5 °C every five cycles to 57 °C. Table 1 lists the C_t results under the different PCR conditions. When repeating three times, C_t's of the first and third assays showed a highly significant linearity, and were comparable with the FRET test.

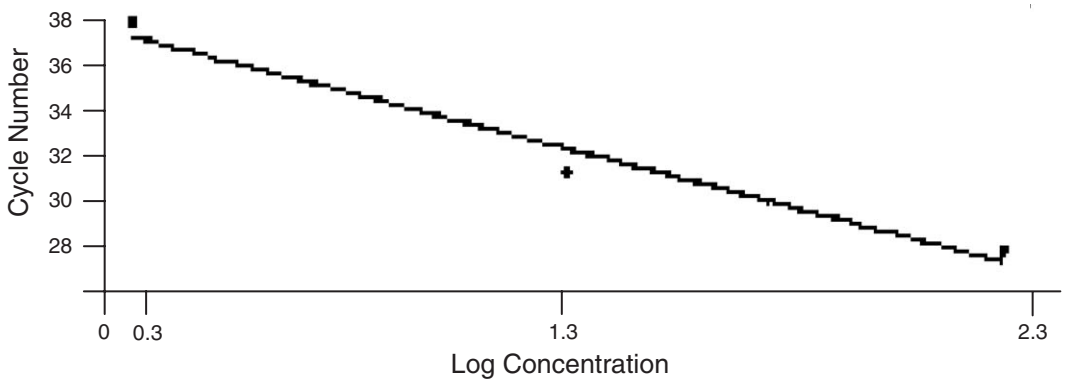

Figure 1. Standard curve obtained for HHV-6 quantitation with FastStart DNA Master^{PLUS} SYBR Green I dye. The values of three plasmid dilutions were determined, containing from 2.19×10^2 copies to 2.19 copies.

Table 1. Threshold cycle results of the PCR tests with FastStart DNA Master^{PLUS} SYBR Green I under the different conditions assayed

Plasmid dilution	C_t[a]			
	FRET	SYBR Green I dye		
	Annealing temperature/time			
	55 °C/10 s	55 °C/10 s	59–55 °C/8 s	60–57 °C/8 s
10^3	32.12	26.76	27.69	28.18
10^2	36.56	29.14	30.33	30.56
10^1	39.57	32.53	31.14	32.30

[a]Cycle threshold.

Detection limit

The detection limit was calculated by comparing the SYBR Green I dye PCR results with those of the PCR using HPs and those of conventional PCR. The LightCycler software related the C_t results of the three reference plasmid dilutions (2, 20 and 200 copies/mixture) to the logarithm of the initial number of copies and determined the corresponding regression lines. This test was performed in triplicate. In the two real-time PCRs, the plasmid DNA was detected in a reproducible manner at a minimum concentration of 20 copies. The PCR with SYBR Green I dye detected the dilution of 2 copies in one of the three assays. These results were correlated with those of the conventional PCR, given that the reference plasmid dilutions, producing detectable bands in agarose gel electrophoreses, presented proportional C_t's values (Fig. 2).

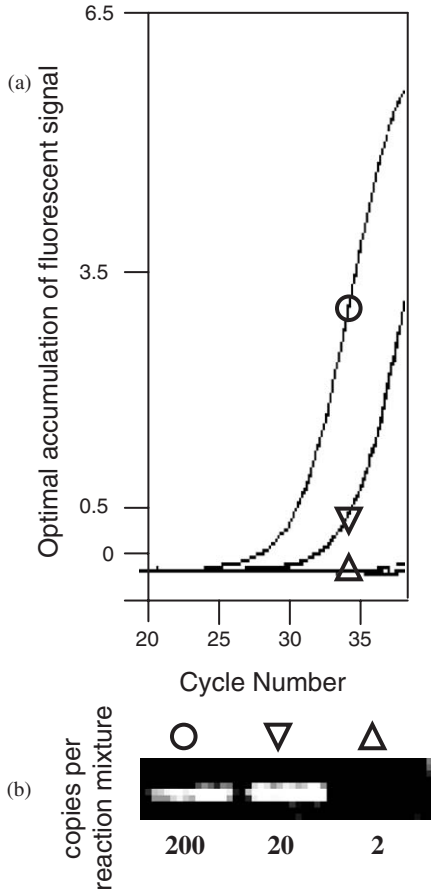

Figure 2. Comparison of assay detection limit for HHV-6 DNA detection between FastStart DNA Master^{PLUS} SYBR Green I dye (a) and single step PCR (b).

Discussion

In this study, the FastStart DNA Master^{PLUS} SYBR Green I dye PCR test detected HHV-6 DNA rapidly with adequate reproducibility and a detection limit of 20 copies, which can be considered satisfactory.

Although a detection limit of 1 copy has been reported using a real-time PCR (Locatelli et al., 2000; Alvarez-Lafuente et al., 2002), higher replication levels and more copies are required to establish the appearance of disease by this virus, as with other viruses (Nitsche et al., 2001).

The PCR test with SYBR Green I dye and that with HPs both show a direct proportionality between the initial concentration of the target sequence and the fluorescence emission detected by the system. Moreover, the PCR tests with SYBR Green I dye and FRET share major advantages: (i) a short performance time of around 35 min (excluding extraction time); (ii) a comparable reproducibility in samples containing 20 or more copies (the sample with 2 copies was not reproducibly amplified with either method or detected in gel after the conventional PCR); (iii) a lower contamination risk, because the reaction takes only one step and the capillaries are closed, involving less handling. In contrast, the use of HPs requires additional handling and a more complex preparation of the reaction mixture, increasing the risk of artifacts, after the 30th cycle (Espy et al., 2000). These authors state that any positive PCR result in probe-based assay which has a PCR crossing point above 30 cycles needs confirmation by a second assay before being reported. Finally, PCR tests with SYBR Green I dye are significantly cheaper than those using HPs and are more versatile, because they can be applied for the detection of different sequences.

We used low dilutions of the reference plasmid to assess the detection limit of the method, because HHV-6 DNA determinations usually present poor detection limits and reproducibility with 10 or less copies, as demonstrated by most studies on quantitative detection of DNA.

The main limitation of the SYBR Green I dye method is the possibility of false positive results. An erroneous signal can be present because the marker binds to double-strand products other than the target, such as primer dimers, producing a lower specificity for the emitted signal. This usually occurs when sub-optimal concentrations of primers are used, especially when the target is scarce or absent. The present study used primer concentrations demonstrated to produce no false positives due to dimers (Ohyashiki et al., 2001), and the plasmids used contained a specific sequence, ensuring the presence of the target in all cases. Furthermore, the fusion temperature corresponding to the melting curve permitted differentiation between the specific amplificate and possible non-specific products, and, because the detection limit was 20 copies per mixture, any interference at a lower level would not affect the specificity of the test.

In conclusion, real-time PCR with FastStart DNA Master^{PLUS} SYBR Green I dye appears to combine the advantages of real-time PCR with FRET because it offers adequate reproducibility, precision and detection limit, while being less expensive and more versatile.

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