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Short communication

Evaluation of UL99 transcript as a target for antiviral treatment efficacy

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A B S T R A C T

Human cytomegalovirus (HCMV) is a virus belonging to the Beta Herpes virus family. Its genome contains many different genes clustered in immediate early, early and late genes. This last cluster includes UL99, a late gene that encodes for a tegument protein called pp28. In immunocompetent patients, HCMV infection occurs asymptomatically, while its reactivation in immunocompromised patients can be a cause of pneumonia, retinitis and gastrointestinal diseases. To prevent or to contrast HCMV infection, several drugs (such as Ganciclovir, Acyclovir, Foscarnet) are available, and their efficiency is evaluated by HCMV DNA load monitoring, as also for antiviral resistance onset that may occur after the therapy. In this study is described the development of a Real Time PCR for the detection and quantification of UL99 transcript and the clearance of this target compared to HCMV DNA, both in vitro and in vivo on bronchoalveolar lavage samples.

Human cytomegalovirus (HCMV) is a virus belonging to the Beta Herpes virus family. Its genome, ~235 kb long, is organized in two regions flanked by inverted repeats that define a unique long (UL) and a unique short (US) region. Based on the region and the position in which the ORFs are located, HCMV's genes are named with abbreviations, such as UL54 DNA polymerase or UL97 protein kinase. UL99 ORF encodes for a 190 amino acids long protein, which is expressed in the late phase of viral replication and belongs to the tegument proteins (Britt and Boppana, 2004). Studies on this protein, called pp28, described its importance in the production of infectious virus, as deletion of the UL99 ORF resulted in the production of non enveloped and non infectious cytoplasmic particles (Silva et al., 2003; Jones and Lee, 2004). HCMV infects world's human population in a range that varies from 60% to 90% depending on the socio-economic class and geographic location (Pass, 2001). Primary infection in immunocompetent patients is usually asymptomatic, with few cases of mononucleosis like syndrome. Once infected the human host, HCMV persists in the organism in latent form with the possibility of a rapid reactivation in particular conditions, such as critical illness and immune system deficit (Cook and Trgovcich, 2011). Immunocompromised patients, and in particular transplanted patients, can be subjected to HCMV reactivation with risk of pneumonia, retinitis and gastrointestinal disease development (Griffiths and Walter, 2005). The therapy is based on the administration of antiviral drugs, which exert their activity on UL54 DNA polymerase (Acyclovir, Ganciclovir, Valganciclovir, Cidofovir, Foscarnet) or UL97 protein kinase (Maribavir) inhibiting the viral DNA replication (Mercorelli et al., 2008; De Clercq, 2013). Despite antiviral treatment is usually able to block the HCMV replication, the prolonged use of antiviral drugs, such as Ganciclovir, by critically ill or immunosuppressed patients can act as a promoting factor for antiviral drugs resistance onset (Erice et al., 1989). In order to evaluate therapy efficacy and antiviral drugs resistance onset, HCMV DNA load is periodically monitored after antiviral and preemptive therapy (Yoshida et al., 2001; Gerna et al., 2011). In this work, UL99 viral transcript has been evaluated as a rapid marker of therapy effectiveness and a quantitative RT Real Time PCR has been developed as a tool for antiviral therapy control. The RT Real Time PCR was tested both in vitro and in vivo on 18 bronchoalveolar lavage samples belonging to patients who were or were not subjected to an antiviral treatment.

The in vitro test for antiviral drugs susceptibility was performed as described as follows. Two T150 flasks were plated with Helf cells in order to obtain about 4×10^6 cells after three days of incubation. The flasks were then infected respectively with AD169 CMV virus strain (ATCC VR-538) and with VR5438 HCMV Ganciclovir resistant virus (both at MOI 0.01) and left for 1 h at room temperature and 1 h at 37 °C. After this incubation period, the medium from both

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the flasks was removed, MEM 10% FCS added and the flasks were incubated at 37 °C 5% CO_2 for several days until cytopathic effects were observable in most of the cells. The medium was then collected, kept separately, and the plate scraped to harvest cells. Three rapid cycles of freezing and thawing with liquid nitrogen, followed by a centrifugation at 1500 rpm for 5 min at 4 °C, were performed on cells collected. The supernatant and medium previously stored were gathered together to obtain a suspension of infectious viral particles.

Subsequently, ten fold viral dilutions till 10^{-7} were produced from both the virus propagated. Helf cells, dispensed in a 96-well microplate, were then infected with 50 μl of each viral dilution and incubated at 37 °C for 2 h. Cell medium was removed, substituted with 100 μl of Methyl-Cellulose 1% (Sigma Aldrich, St. Louis, MO, USA) and left at 37 °C for 8 days. The Methyl-Cellulose was then removed and 50 μl of Crystal violet 0.1% (Sigma Aldrich, St. Louis, MO, USA) added. The microplate was incubated 30 min in dark room, removed the crystal violet and unstained the plate with fresh water. The plaque forming unit (PFU) was then calculated in order to evaluate the viral titre.

Nucleic acids extraction was performed using automatic extractor NucliSens EasyMag (Biomérieux, Marcy L'Etoile France), according to manufacturer's instructions, to obtain 50 μl of eluate containing both DNA and RNA from 200 μl of a suspension containing Helf cells infected with HCMV AD169. A first DNAse step was carried out to eliminate the genomic DNA using Deoxyribonuclease I Amplification Grade (Life Technologies, Carlsbad, CA, USA), following manufacturer's instructions. The 11 μl of DNAse product were then purified by a second extraction in a 25 μl final volume. A subsequent retrotranscription was performed using a first mix composed by random primers (600 ng/μl) and dNTPs (10 mM). After 5 min of incubation at 70 °C with 10 μl of sample RNA, a second mix composed by $MgCl_2$ (25 mM), ImProm Buffer 5× [200 mM Tris–HCl (pH 8.4), 500 mM KCl], ImProm-II Reverse Transcriptase™ (1 U/μl) and recombinant Rnasin Ribonuclease Inhibitor (40 U/μl) (Promega, Madison, WI, USA) were added to obtain a 20 μl final volume. The total volume mixture was then incubated for 5 min at 25 °C, 60 min at 42 °C and 15 min at 70 °C using 9800 Fast Thermal Cycler for retrotranscription (Life Technologies, Carlsbad, CA, USA).

UL99 cloning primers, Real Time primers and probes were designed using Primer express 3.0 (Life Technologies, Carlsbad, CA, USA). A subsequent analysis with BLAST (www.ncbi.nlm.nih.gov/blast/) was carried out in order to confirm the design and to exclude cross recognitions with other targets.

An initial Fast PCR was performed in order to amplify UL99 target region using cDNA obtained previously. The amplification mix was prepared including: 20 pmol of cloning primers (UL99 clonF 5′-CGGGGGAAACGACAGTAGTA-3′ and UL99 clonR 5′-AGTCGCTTAGCCACCACCT-3′), 6 μl of GoTaq® HotStart Polymerase buffer 5× (Promega, Madison, WI, USA), 200 μM of each dNTP, 6 mM of $MgCl_2$ and 1 unit of GoTaq® HotStart Polymerase (Promega, Madison, WI, USA). The amplification thermal profile was: 94 °C for 3 min, followed by 35 cycles of 95 °C for 0 s, 60 °C for 15 s and 72 °C for 10 s, with a final step at 72 °C for 7 min. The Fast PCR was performed on 9800 Fast Thermal Cycler (Life Technologies, Carlsbad, CA, USA). The 260 bp DNA fragment was cloned into a pCR 2.1-TOPO plasmid vector, using pTOPO-TA cloning technology (Life Technologies, Carlsbad, CA, USA). Once produced the plasmid, it was transfected in competent *Escherichia coli* TOP10 cells, which were subsequently propagated overnight. The selected transformed clones were amplified through culture in liquid LB medium (10 g Bacto Tryptone, 5 g yeast extract, 171 mM NaCl, 15 g/l agar, pH 7.5) containing 50 μg/ml of ampicillin. Plasmid DNA was extracted and purified using the PureLink™ HiPure Plasmid Midiprep Kit (Life Technologies, Carlsbad, CA, USA). The plasmid concentration was estimated on spectrophotometric reading at OD260.

The UL99 cRNA production was performed as described in Terlizzi et al. (2011) in order to obtain scalar cDNA standard dilutions for a RT Real Time PCR absolute quantification.

UL99 Real Time PCR mix was produced adding UL99 primers (UL99 F 5′-CGACGTTTCGCGAGGACAA-3′, UL99 R 5′-GTGGTGATGTTTTGAGGGTTTCTTT-3′), UL99 Taqman probe (5′-FAM-CGAAACCGAGCAAGC-MGB-3′) and 2 μl of cDNA to 1× Master Mix (Platinum qPCR supermix – UDG with ROX, Life Technologies, Carlsbad, CA, USA), obtaining a total volume of 20 μl. Primers and Taqman probe were tested in four different concentrations (respectively 900/250 nM, 900/100 nM, 250/250 nM, 250/100 nM primers/probe concentrations) to find the best amplification conditions. To confirm negative results in the bronchoalveolar lavage samples amplification and in antiviral drug susceptibility in vitro test, RPL27 ribosomal gene was used as internal control (RPL27 F 5′-GGGTGGTTGCTGCCGAAATG-3′, RPL27 R 5′-CTTTGCGTCCGGAGTAGCG-3′, RPL27 Taqman probe 5′-VIC-GGAAGGTGGTGCTTGTCCTGGCT-TAMRA-3′) in a 60/50 nM primers/probe concentration. The dynamic range, defined as the range of dilutions in which a linear regression curve can be constructed, and the sensitivity, expressed as the lowest target concentration detectable at a frequency of 100%, were obtained amplifying 10-fold dilutions of UL99 plasmid, ranging from 10^{10} to 10^{0} copies/reaction.

cDNA standards produced from UL99 cRNA and ranging from 10^{5} to 10^{2} copies per reaction were used to create a standard curve for the absolute quantification.

In order to develop an antiviral susceptibility in vitro test, Helf cells were propagated on a 24-well plate and then infected with the two virus strains AD169 and VR5438. Briefly, 1 ml of Helf cells suspension in a medium composed by MEM 10% FCS (PAA Laboratories GmbH, Pasching, Austria), 1% glutamine (L-Glutamine, Life Technologies, Carlsbad, CA, USA), 0.15% Pen-Strep solution (Sigma Aldrich, St. Louis, MO, USA), 0.2% Fungizone (Bristol-Myers Squibb, New York, NY, USA) were added in each well and the plate incubated at 37 °C 5% CO_2 until the cell confluence reached 80–90%. The Helf cells were then infected adding 10 PFU/ml of HCMV AD169 strain and HCMV drug resistant virus VR5438 and incubating the plate at 37 °C 5% CO_2 for 3 h. One well was not infected as negative control. The medium was then removed and 100 μl of Ganciclovir and Cidofovir were added in different concentrations and separately in each well. Ganciclovir concentrations tested were 5, 50 and 500 μM, while Cidofovir concentrations were 0.5, 5 and 50 μM, according to what described in previous works (Drew et al., 1993, 2006; Landry et al., 2000). The final volume was brought up to 1 ml with cell medium MEM 2% FCS, 1% glutamine, 0.15% Pen-Strep solution, 0.2% Fungizone. Ganciclovir or Cidofovir were not added in two wells, one for each virus, to obtain positive controls wells. The plate was then incubated for 10 days at 37 °C 5% CO_2. After the incubation period, the cell medium suspension in each well was collected and the Helf cells were subjected to a trypsin-EDTA digestion with 200 μl (trypsin 1%, 0.05 mM EDTA pH 8.0) for 5 min at 37 °C and then collected. The digested material was centrifuged at 13,000 rpm for 2 min and pooled with the medium collected previously. 100 μl of this mixture plus 900 μl of Lysis buffer (Biomérieux, Marcy L'Etoile, France) were extracted with NucliSens EasyMag (Biomérieux, Marcy L'Etoile, France) in a 50 μl final volume elute. Subsequently, DNAse and retrotranscription steps described before were carried out.

HCMV-DNA was quantified using Q-CMV Real Time Complete Kit (ELITech Group, Puteaux, France), targeting the exon 4 region of the HCMV MIEA gene (major immediate early antigen, HCMVUL123). The amplification was carried out on 7500 Real Time PCR System (Life Technologies, Carlsbad, CA, USA) adding 5 μl of