Human cytomegalovirus load in fresh and glycerolized skin grafts

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--- **SUMMARY** ---

This study evaluated the detection of Human Cytomegalovirus (HCMV)-DNA in donors' skin samples. HCMV-DNA was quantified in 100 skin specimens, including 50 fresh samples and as many corresponding glycerol-preserved specimens by a home-made Real Time PCR. HCMV-DNA was detected in 19/50 (38%) fresh specimens and 23/50 (46%) glycerol-preserved (p=n.s.). Nevertheless, the mere detection of HCMV-DNA does not imply the presence of infectious virions and therefore does not imply a risk of HCMV transmission, as treatment with glycerol is particularly efficacious in inactivating viral particles. Therefore, HCMV serology confirms its pivotal role in the setting of skin grafting.

KEY WORDS: Human cytomegalovirus, skin graft, Real Time PCR, serology

Human Cytomegalovirus (HCMV) is a DNA virus belonging to the β-herpesvirus subfamily of the *Herpesviridae* family. Following primary infection, that usually occurs early in childhood, HCMV persists latently in the host in various tissues (e.g. blood, endothelial cells, renal tubular cells and salivary glands)(Gaeta *et al.,* 2006; Tenenhaus *et al.,* 2006; Fishman *et al.,* 2007; Pérez-Sola *et al.,* 2008).

In the immunocompetent host, HCMV infection may be asymptomatic or manifests as a mononucleosis-like syndrome, whereas in immunocompromised patients, such as transplant recipients (Bergallo *et al.,* 2008; Lilleri *et al.,* 2009), viral reactivation can result in direct effects or HCMV disease, defined as a combination between specific organ dysfunction and the presence of high rates of viral replication, and indirect effects caused by virus interaction with the host immune system (Pérez-Sola *et al.,* 2008). Burn patients belong to the category of immunosuppressed individuals (Tenenhaus *et al.,* 2006) and are particularly susceptible to infections and sepsis due to the impairment in the functional capacity of T cells, as well as to the loss of the cutaneous barrier (Rennekampff and Hamprecht, 2006). Skin allografts are widely employed in the treatment of extensive burns.

The skin grafting procedure is codified employing rigorous quality and safety standards defined by the European Directive 2004/23/CE. Serology for HCMV plays a pivotal role in donor selection and according to the centre's practice IgM-positive donors are excluded.

Two preservation methods for skin allograft are currently used: cryopreservation at -80°C with either glycerol or dymethylsulfoxide (DMSO) as cryoprotectant, and preservation in 85% glycerol at 4°C (Alotto *et al.,* 2002). Several techniques are described in literature (cell culture, histomorphology and biochemistry) for the comparison between fresh and preserved skin in terms of skin viability (Castagnoli *et al.,* 2003). In this study HCMV-DNA positivity was evaluated in fresh and glycerolized skin specimens.

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A total of 100 donor skin samples obtained from 50 donors (two specimens from each donor) of the Skin Bank of CTO Hospital of Turin were analysed, including 50 fresh and 50 glycerolized samples. All the specimens were tested for the presence of HCMV by a home-made Real Time PCR. Serology data (IgG and IgM for HCMV) were abstracted from clinical charts.

Nucleic acid extraction was performed using the Nuclisens EasyMag automatic extractor (Biomérieux, Marcy France). Briefly, the samples were first cut in small pieces, then treated with 200 µl of A2x solution (containing 400 mM Tris-HCl ph 7.5, 500 mM NaCl, 50 mM EDTA and 1% SDS) and homogenized using Tissue Ruptor (Quiagen, Hilden, Germany); subsequently, 200 µl of A2x solution were added, incubated at 100°C for 5 min and then centrifuged 1 min at 13000 rpm. Afterwards, 300 µl of the supernatant was extracted using the automatic extractor. For the preparation of standard plasmid, a DNA fragment of approximately 200 bps, targeting the immediate early (IE) gene, was cloned into the TA vector and propagated in competent *Escherichia coli* TOP10 cells using the Topo TA PCR cloning kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The concentration of the plasmid DNA was quantified by using a high-resolution spectrometer. HCMV-DNA was detected by a Taqman Real Time PCR as previously reported (Tanaka *et al.,* 2000). Briefly, the reaction mixture contains 1x master mix (Invitrogen), 200 nM of primers and 100 nM of probe labeled with a 5'-carboxy-fluorescein. Five microliter of extracted DNA were added in the plate containing 15 µl of the reaction mix.

The amplification profile was optimized for the 7300 real time PCR System (Applied Biosystems, Cheshire, UK) as follows: one cycle of decontamination: 50°C for 2 min, one cycle of denaturation: 95°C for 10 min followed by 45 cycles of amplification at: 95°C for 15 s, 60°C for 60 s. Uracil-DNA glycosylase was used to eliminate PCR 'carry over' contaminations from previous PCR reactions.

Standard curves for the quantification of DNA were constructed by plotting the threshold cycle (C_t [crossing point of the amplification curve with the preset threshold of fluorescence detection]) against the logarithm of serial 10-fold dilutions of the corresponding plasmid. Amplification da-

ta were analysed by the Sequence Detection System software (Applied Biosystems).

To correct for the variable amount of DNA in tissue samples, each sample was subjected to simultaneous TaqMan PCR for the housekeeping gene Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, Accession No. J04038), targeting the region between exon 6 and 8 (Costa *et al.,* 2009); primers and probe were designed using the Primer Express software (version 3.0, Applied Biosystems). Results were considered acceptable only in the presence of GAPDH-positivity. Standard curves for the GAPDH gene quantitation were constructed by plotting the Ct against the logarithm of serial dilutions of DNA extracted from peripheral blood leukocytes. The Ct values and number of HCMV-DNA copies and diploid sets of GAPDH-gene were calculated from the standard curve.

The assay was linear in the range 10^1-10^6 copies per reaction. In order to quantify the presence of HCMV in tissue specimens, the amplifications of HCMV and GAPDH were performed separately; the quantity of housekeeping gene reflected the number of cells in the samples and the HCMV-DNA were quantified based on the number of cells. For statistical analysis, the chi square test and T student's test were performed using a commercially available software (MedCalc; version 9.2.1.0). A p-value <0.05 was considered statistically significant.

Results are summarized in the Table 1. Overall, PCR assay for HCMV-DNA resulted positive in 42/100 (42%), in particular 19/50 (38%) fresh biopsies and 23/50 (46%) glycerol-preserved (p=n.s), with 14 donors negative on both fresh and glycerol-preserved specimens, 20 donors positive on both specimens, five donors positive on fresh specimen and negative on the glycerol-preserved specimen, and 11 fresh specimens negative and the glycerol-preserved specimens positive. Viral load ranged from 10 copies/10^4 cells to 1148 copies/10^4 cells for fresh skin (median 112/10^4 cells) and from 15 copies/10^4 cells to 6810 copies/10^4 cells for glycerolized skin (median 111/10^4 cells), without significant differences between the two groups.

Serology for IgG of donors were positive in 38/50 (76%), in particular 11 (28.9%) negative for HCMV-DNA in both fresh and glycerolized specimens, 15 (39.5%) positive for HCMV-DNA in

TABLE 1 - *Prevalence and mean viral load of HCMV in fresh and glycerol-preserved skin specimens.*
P = not significant.

Skin biopsiesTotal N = 100	HCMV-DNA positive N (%)	Mean viral load (copies/10^4 cells)
Fresh N = 50	19 (38%)	174.58 (range 10-1148)
Glycerol-preserved N = 50	23 (46%)	415.35 (range 15-6810)

both fresh and glycerolized samples, eight (21.1%) positive for HCMV-DNA only in glycerolized skin specimens, and four (10.5%) positive for HCMV-DNA only in fresh biopsies; IgM HCMV antibodies were negative in all the cases. Burns are one of the most harmful physical and psychological traumas.

Infection is the major cause of morbidity and mortality in burn patients, because of their immunocompromised state (Munster, 1996; Mousa, 2005) and the loss of cutaneous and mucosal integrity (Hayden *et al.,* 1994), as well as the use of invasive catheters, endotracheal tubes and other devices. Allogenic skin procedure follows the rigorous qualitative and safety standard defined by international (EATB) and national (in Italy: Linee Guida 19/6/07) guidelines, that include serological (HBsAg, HBcAb IgG, HBcAb IgM, anti-HBsAg, anti-HCV antibody, CMV IgM and IgG antibody, anti-HIV 1 e 2 antibody, TPHA or other tests for the detection of antibody to *T. pallidum*) and molecular tests (HBV-DNA, HIV-RNA and HCV-RNA)(Kealey *et al.,* 1996; Barnett *et al.,* 2001; Grossman and Arbel, 2001; Stramer *et al.,* 2004; Zou *et al.,* 2004; Pianigiani *et al.,* 2006). Pneumonia, wound infections and sepsis represent >50% of the mortality in patients suffering severe burn injuries. Moreover, burn patients are at risk of HCMV disease.

Although few reports on HCMV infection and disease in burn patients are available (Kealey *et al.*, 1996; Kealey, 1997), according to some studies the rate would appear to be nearly 20% (Deepe *et al.,* 1982; Kealey *et al.,* 1987; Rennekampff and Hamprecht, 2006; Tenenhaus *et al.,* 2006). The treatment of fresh skin and preservation in 85% glycerol at 4°C is currently used and glycerol has been documented to have an effect on extracellular viral particles (Hoekstra *et al.,* 1990; van Baare *et al.,* 1994; Marshall *et al.,* 1995). The present study evaluated the presence of HCMV-DNA in matched skin biopsies from the same donors comparing the fresh and the glycerol-treated specimens.

The prevalence of HCMV-DNA in skin biopsies was 38% and 46% for fresh and glycerolized specimens, respectively, without significant statistical differences. Similarly, also considering the viral load in both fresh and glycerolized skin biopsies, the p-value was not statistically significant. Nevertheless, these results have to be considered with great caution, in particular as regards discrepancies between fresh and glycerolized specimens, as negative results can be due to focal sampling while, on the other hand, the mere detection of HCMV-DNA does not imply the presence of infectious virions and therefore does not imply a risk of HCMV transmission. At this regard, the exclusion of IgM positive donors remains the milestone in donors' selection.

In conclusion, although HCMV-DNA may be detected in both fresh and glycerolized specimens, this does not imply the transmission of infectious virions by skin graft, as treatment with glycerol is particularly efficacious in inactivating viral particles; therefore, the pivotal role of serology in the setting of skin grafting is confirmed.

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