Quantitative detection of HHV-6 and HHV-7 in transbronchial biopsies from lung transplant recipients

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SUMMARY

The occurrence and significance of HHV-6 and HHV-7 were investigated in pulmonary tissue from lung transplant recipients. Eighty-seven transbronchial biopsies from 30 patients were studied by quantitative real-time PCR; the association with histopathological features was investigated. HHV-6 and HHV-7-DNA were detected in 6.9% and 9.2% transbronchial biopsies, respectively. A significant association between HHV-6 detection on transbronchial biopsies and interstitial pneumonia was found, in contrast to the lack of association between viral detection on bronchoalveolar lavage and any histopathological feature. No association was evidenced in terms of acute and chronic rejection. The finding of HHV-6 and/or HHV-7-DNA positivity in all the cases with ischemia-reperfusion injury suggests a possible role in favouring β-herpesviruses reactivation, as previously described for HCMV in renal transplantation.

KEY WORDS: Human herpesvirus 6, Human herpesvirus 7, Transbronchial biopsy, Lung transplantation

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INTRODUCTION

Human herpesviruses 6 and 7 (HHV-6 and HHV-7) are highly seroprevalent lymphotropic β-herpesviruses closely related to human cytomegalovirus (HCMV) and persistently infect the body by establishing latency in different sites, including lung, and reactivating periodically with several manifestations in immunocompromised patients (Ward *et al.*, 2005; Frenkel *et al.*, 1990; Black nad Pellett, 1999). In transplant recipients, HHV-6 and -7 are increasingly recognized as potential pathogens causing direct and indirect effects, including increased susceptibility to HCMV infection/disease (Lautenschlager *et al.*, 2000; Neurohr *et al.*, 2005; Kerschner *et al.*, 2009; Manuel *et al.*, 2009). Several studies have found an association between HHV-6 and interstitial pneumonia in transplant patients (Bauer *et al.*, 2007; Deborska-Materkowska *et al.*, 2006), while the relation with HHV-7 is less clear (Kidd *et al.*, 2000; Astegiano *et al.*, 2010).

On the other hand, one study suggested that HHV-7 plays an important role in causing interstitial pneumonia in non-transplant patients (Yamamoto *et al.*, 2005; Costa *et al.*, 2009), and Ross *et al.* found an association between bronchiolitis obliterans (BO) with organizing pneumonia and HHV-7 (Ross *et al.*, 2001).

Lung transplant (LT) recipients present specific risk factors for viral infection/reactivation, given the dysfunctional pulmonary background and impaired local immunity.

Viral infections of the graft (including those from community-acquired respiratory viruses and mostly HCMV), beside representing a major cause of morbidity and mortality, are hypothesized to be triggers for a cascade of events potentially leading to acute rejection (AR) and

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chronic graft dysfunction (histologically defined as BO), although data obtained by different studies are controversial (Estenne *et al*., 2002; Streblow *et al*., 2007; Manuel *et al*., 2009; Soccal *et al*., 2010). The role of HHV-6 and -7 reactivation in LT in relation to the development of AR, based on data obtained on bronchoalveolar lavage (BAL) specimens (Puchhammer-Stöckl, 2008), remains controversial and to our knowledge no study has investigated viral detection by molecular methods not only on BAL, but also in lung tissue, and has evaluated the relation with histopathological features.

Herein, we evaluated the prevalence of HHV-6 and -7 in transbronchial biopsies (TBB) obtained from LT recipients and investigated the association with histopathological features.

MATERIALS AND METHODS

Over an 18-month period (February 2008-August 2009), 87 TBB specimens from 30 adult patients (18M/12F; mean age±standard deviation [SD], 45.6±18.1 years) receiving a LT between January 2004 and August 2009 at the University Hospital San Giovanni Battista of Turin were evaluated.

TABLE 1 - *Main features of the study population.*

Features	*Patients N = 30*
Mean age (years ± SD)	45.6±18.1
Sex (M/F)	18/12
Underlying disease	
Cystic fibrosis	12 (40%)
Idiopathic pulmonary fibrosis	4 (13.3%)
COPD/emphysema	7 (23.3%)
Alpha-1-antitrypsin deficiency	1 (3.3%)
Bronchiectasis	2 (6.7%)
Histiocytosis X	1 (3.3%)
Hyaline membrane disease	1 (3.3%)
Extrinsic allergic alveolitis	1 (3.3%)
Progressive systemic sclerosis	1 (3.3%)
Type of lung transplant	
Single	10 (33.3%)
Double	20 (66.7%)
Retransplantation	2 (6.7%)

SD, standard deviation; COPD, chronic obstructive pulmonary disease.

The main features of study population are summarized in Table 1. Specimens were collected during routine follow-up bronchoscopy at one month post-transplantation, subsequently at three-month intervals until 24 months and then annually or, in addition, to investigate clinical signs/symptoms and/or new infiltrates on chest X-ray. Informed consent was obtained from all the patients.

Whole blood specimens concomitantly collected were available in 48 cases from 18 patients. According to our Center's practice, TBB samples are routinely investigated for HCMV-DNA, while BAL specimens, contextually collected by bronchoscopy (N=87), are tested using a molecular panel detecting 16 respiratory viruses, as previously described (Gambarino *et al*., 2009).

A standard therapeutic regimen was given to all the patients: long-term immunosuppression with cyclosporine or tacrolimus (in patients with cystic fibrosis as underlying disease), mycophenolate mofetil and prednisone (to be tapered at low dosage or discontinued); anti-viral prophylaxis with ganciclovir or valganciclovir (450 mg twice daily) from day 21 for 3 weeks associated with HCMV-IG (Cytotect Biotest) at days 1, 4, 8, 15, and 30 (1.5 ml/kg body weight) and every month until 1 year (1 ml/kg), irrespective of HCMV mismatching (Solidoro *et al*., 2009). Discharge diagnosis was made according to the International Classification of Diseases (ICD9CM; codes 480.XX-486.XX for pneumonia, 460.XX-466.XX for acute respiratory insufficiency or other acute respiratory conditions).

Lung tissue specimens were fixed in 4% neutral formalin and then paraffin-embedded. Sections of 4µm thickness were cut and stained with hematoxylin-eosin, periodic acid Schiff, Masson's trichrome, and based on pathologist's judgement further sections were used for specific stains (i.e. Elastic-Van Gieson, Giemsa, Grocott methenamine silver stain).

Acute rejection (defined by the presence of perivascular and interstitial mononuclear infiltrates, with small airway inflammation forming a lesion know as lymphocytic bronchiolitis [LB]) and BO were diagnosed and graded according to the criteria defined in the 2007 International Society for Heart and Lung Transplantation Working Formulation (Stewart *et al*., 2007). Histopathological diagnosis of pneumonia was

established with the presence of viral inclusions associated with an inflammatory reaction (Stewart, 2007).

Nucleic acid extraction from TBB, BAL, and whole blood specimens was performed using the NucliSens easyMAG platform (bioMeriéux, Marcy l'Etoile, France), as previously described (Gambarino *et al.*, 2009; Bergallo *et al.*, 2010). Real-time PCR assays were performed with the 7300 Real-Time PCR System (Applied Biosystems, Monza, Italy) by two homemade protocols of real-time TaqMan and LUX™ (Light Upon eXtension) PCR, respectively, as previously described (Costa *et al.*, 2008; Bergallo *et al.*, 2009). Amplifications were set up in a reaction volume of 25 µl, containing 5 µl of the extracted specimen, or negative control (sterile double-distilled H_2O), or standard plasmid dilutions, and 20 µl of the corresponding reaction master (master mix with ROX [Invitrogen, Carlsbad, CA], 200 nM primer sense, 200 nM primer antisense, 100 nM probe, and H_2O, for HHV-6; master mix with ROX, 400 nM primer sense LUX [Invitrogen], 400 nM primer antisense, and H_2O, for HHV-7) and processed with the following thermal profile: 50°C for 2 min (decontamination) and 95°C for 10 min (initial denaturation), followed by 45 cycles of 15s at 95°C (denaturation) and 1 min at 60°C (annealing and extension).

Amplifications data were analysed by Sequence Detection System software (Applied Biosystem). Specimens were subjected to simultaneous TaqMan PCR detection of the housekeeping gene -actin; results were considered acceptable only in the presence of -actin with a threshold cycle (Ct) <39. For quantitation on TBB, results were normalized to 10^4 cells using the housekeeping gene glycerin-aldehyde-3-phosphate-dehydrogenase. The limit of detection in BAL and whole blood specimens was 880 and 1000 copies/ml, respectively.

Statistical analysis was performed using the chi square test and t-test, as appropriate, employing a commercially available software (MedCalc; version 9.2.1.0). A p-value <0.05 was considered significant.

RESULTS

Virological results according to histopathological examination are summarized in Table 2. Overall, HHV-6 and/or -7-DNA resulted positive in 13/87 (14.9%) TBB specimens (HHV-6/HHV-7 co-infection in one case) from 10/30 (33.3%) patients in comparison to 46/87 (52.9%) BAL from 24/30 (80%); in particular, HHV-6-DNA in six (6.9%) TBB vs 14 (16.1%) BAL and HHV-7 in eight (9.2%) TBB vs 39 (44.8%) BAL.

The prevalence of positivity on TBB was significantly lower than that on BAL (p<0.0001); this was attributable in particular to results for HHV-7 (p<0.0001). Viral load on TBB was 601±897 copies/10^4 cells for HHV-6 (mean±SD; median, 99; range, 6-2155) and 2927±5007 (median, 738; range, 37-14622) for HHV-7; whereas viral load on BAL was 3788±11178 copies/ml (median, 880; range, 880-47107) for HHV-6 and 190411±724153

TABLE 2 - *Detection of HHV-6 and -7 in transbronchial biopsies (TBB) from lung transplant recipients in relation to histopathological findings.*

	HHV-6 pos. N (%)	*HHV-7 pos. N (%)*
Total N = 87	6 (6.9%) 601±897 (mean viral load ± SD)	8 (9.2%) 2927±5007 (mean viral load ± SD)
Histopathological features		
Interstitial pneumonia N = 13	4* (30.8%)	2 (15.4%)
Organizing pneumonia N = 5	4 (80%)	0
Acute rejection N = 24	1 (4.2%)	3 (12.5%)
Lymphocytic bronchiolitis N = 8	1 (12.5%)	1 (12.5%)
Bronchiolitis obliterans N = 2	0	0
Ischemia-reperfusion injury N = 4	1 (25%)	4 (100%)

*p<0.002 for the association between interstitial pneumonia and HHV-6 detection on TBB.

(median, 2197; range, 880-3302803) for HHV-7. Mean viral load of HHV-7 resulted significantly higher than that of HHV-6 in both TBB ($p<0.0001$) and BAL ($p=0.017$). In four TBB (4.6%) and 26 BAL (29.9%) at least another viral pathogen was concomitantly detected (HCMV on TBB; mainly another herpesvirus or rhinovirus on BAL). HHV-6 and -7-DNA were detected in four specimens each, out of the 48 available samples (8.3%) from 18 patients (22.2%); in only one case was HHV-6 detected in both TBB and whole blood.

Interstitial pneumonia was diagnosed in 13/87 (14.9%) TBB specimens: four positive to HHV-6 (30.8%; $p<0.0001$; mean viral load±SD, 891±1002 copies/10^4 cells) and two to HHV-7 (15.4%; p=n.s.; mean viral load±SD, 5687±7827), none of the cases presented co-infection. Viral load on TBB did not differ between patients with and without interstitial pneumonia.

A significant association between the occurrence of interstitial pneumonia and DNA positivity on TBB was found also for HCMV (seven specimens from as many patients positive of 87 [8.1%], $p<0.0001$); in contrast to the lack of significance in relation to HHV-6 and HCMV positivity on BAL. A pattern of organizing pneumonia was found in 5/13 (38.5%) TBB samples, four positive to HHV-6. Acute rejection was diagnosed in 24/87 (27.6%) TBB samples (grade ≥A2 in eight): one positive to HHV-6 (4.2%; viral load 15 copies/10^4 cells) and three to HHV-7 (12.5%; mean viral load±SD, 1703±2675). No significant association between AR and HHV-6 and -7-DNA detection on TBB, as well as on BAL, was found; although prevalence on BAL tended to be higher in comparison to that on TBB (25% and 41.7% for HHV-6 and -7, respectively). Interestingly, the only case with HHV-6 positivity on both TBB and whole blood presented AR;

Lymphocytic bronchiolitis was diagnosed in eight specimens (9.2%): one positive to HHV-6 (12.5%) and one to HHV-7 (12.5%), with no significant association with viral detection on TBB, as well as on BAL. Two specimens presented BO (C1 grade): none resulted positive to HHV-6 or -7 on TBB, while both resulted positive to HCMV and/or HHV-6 and -7 on BAL. Ischemia-reperfusion injury was evidenced in four cases (4.6%): one positive to HHV-6 (p=n.s.) and all to HHV-7 ($p<0.0001$).

DISCUSSION

This study evaluated the prevalence of HHV-6 and -7-DNA in TBB specimens from LT recipients and investigated the association with histopathological features. The interpretation of results on specimens obtained from the lower respiratory tract may be challenging, as HHV-6 and -7 may be harboured in healthy lung tissue, as evidenced in previous studies (Puchhammer-Stöckl, 2008; Bauer *et al*., 2007; Cone *et al*., 1996; Tang *et al*., 2003). In particular, considering HHV-6 and -7 on lung specimens, some limitations should be considered: evaluation on TBB may be affected by sampling errors due to focality, thus limiting its sensitivity in comparison to BAL (Jacobs *et al*., 2003; Neurohr *et al*., 2005); few studies have employed quantitative molecular methods on non-transplant tissue specimens (Yamamoto *et al*., 2005); finally, to our knowledge, none has evaluated the association with histopathological features in LT, thus determining the lack of comparable results. Overall, the prevalence of HHV-6-DNA-positivity (6.9%) in pulmonary graft specimens did not differ from that described in samples from healthy individuals or non-transplant subjects with or without organ disease or underlying collagen disease and ranging from 0% (Tang *et al*., 2003) up to 15.8% (Yamamoto *et al*., 2005). However, when considering LT, the prevalence of HHV-6 rose to 30.8% in the presence of a histological diagnosis of interstitial pneumonia with a significant association to viral detection on TBB ($p<0.002$) in comparison to the lack of significance of detection on BAL.

The power of the association appears to be further increased when considering that no co-infection was found in these specimens, although interstitial pneumonia due to reactivation of two or more persistently infecting viruses, such as HHV-6 and HCMV, can occur. On the other hand, the prevalence of tissue HHV-7-DNA-positivity in LT in our series (9.2% of 87) resulted significantly lower than that reported by Yamamoto *et al.* (2005) in non-transplant subjects (79.2% of 24 cases with interstitial pneumonia and 57.9% of 19 controls) and by Tang *et al*. (2003) (42% of 33 cases with idiopathic pulmonary fibrosis and 24% of controls) ($p<0.0001$ at the comparison between non-transplant and LT).

Considering quantitation on TBB, mean viral

load was approximately 10^3 to $5x10^3$ copies/10^4 cells for both HHV-6 and -7 without differences in relation to histopathological findings. Similarly, no significant difference of viral load on TBB was found between LT specimens and tissue samples from non-transplant subjects described in other studies (Yamamoto *et al*., 2005; Tang *et al*., 2003), although load tended to be higher (at least one log) in non-transplant specimens. In particular, considering samples with a histopathological diagnosis of interstitial pneumonia, viral load was approximately $10^3/10^4$ cells and $6x10^3$ for HHV-6 and -7, respectively, in specimens from LT herein described in comparison to $3.7x10^3$ and $2x10^5$ in non-transplant patients. Overall, both prevalence and quantitation data seem to argue against the concept of LT as a viral replication-favouring environment in comparison to the native lung. Given the limited number of specimens evaluated in previous studies on non-transplant patients and the lack of comparable data on LT, further studies should be recommended.

As regards association between HHV-6 and -7 and histopathological features of AR, LB, BO, previous studies have reported controversial results (Streblow *et al*., 2007; Manuel *et al*., 2009); by evaluating viral detection on TBB, no significant association was found, although further confirmation is needed. More information could be obtained also by evaluating viral DNA on whole blood. Unfortunately, this was available only for 48 of our cases, with positivity for HHV-6 and -7 in four specimens each. Interestingly, the only case with HHV-6 positivity on both TBB and whole blood presented AR; nevertheless, this should be considered taking into account the number limitation.

Interestingly, all the cases with ischemia-reperfusion injury resulted positive to HHV-7 and one also to HHV-6; although this observation should be confirmed on larger series, it could be hypothesized that reperfusion injury favours -herpesviruses reactivation as described in renal transplantation in which a TNF-independent signalling induced by ischemia-reperfusion injury can contribute to the activation of the HCMV major immediate-early promoter/enhancer leading to transcriptional activation of HCMV immediate-early gene expression (Kin *et al*., 2005).

In conclusion, two main issues emerged from this preliminary study on LT as requiring more detailed evaluation. First, as prevalence data suggested the possible usefulness of HHV-6 detection on TBB in relation to a histopathological diagnosis of interstitial pneumonia in comparison to viral detection on BAL, although with no association with viral load, further studies should be recommended to evaluate the opportunity to consider molecular viral testing on TBB in the diagnostic work-up of LT recipients. Second, from an experimental point of view, the association between ischemia-reperfusion injury in transplant recipients and lymphotropic -herpesviruses replication should be specifically addressed.

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