Detection of Herpes Viruses by Multiplex and Real-Time Polymerase Chain Reaction in Bronchoalveolar Lavage Fluid of Patients with Acute Lung Injury or Acute Respiratory Distress Syndrome

Ryo Tachikawa^a Keisuke Tomii^a Ryutaro Seo^b Kazuma Nagata^a Kyoko Otsuka^a
Atsushi Nakagawa^a Kojiro Otsuka^a Hisako Hashimoto^c Ken Watanabe^d
Norio Shimizu^d

Departments of ^aRespiratory Medicine and ^bCritical Care, Kobe City Medical Center General Hospital, and ^cDepartment of Cell Therapy, Institute of Biomedical Research and Innovation, Kobe, and ^dVirology, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan

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Key Words

Acute lung injury · Acute respiratory distress syndrome · Human herpes virus · Multiplex polymerase chain reaction · Real-time polymerase chain reaction

Abstract

Background: Human herpes viruses (HHVs) are important pathogens in acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). Rapid and efficient diagnostic tools are needed to detect HHVs in the lung in ALI/ARDS patients. **Objectives:** This study aimed to evaluate the usefulness of multiplex and real-time polymerase chain reaction (PCR) analysis of bronchoalveolar lavage fluid (BALF) for detecting HHV reactivation in ALI/ARDS patients. **Methods:** Between August 2008 and July 2012, eighty-seven BALF samples were obtained from ALI/ARDS patients with unknown etiology and analyzed for HHVs. The types of HHVs in the BALF samples were determined using qualitative multiplex PCR followed by quantitative real-time PCR. **Results:** Multiplex PCR identified herpes simplex virus type 1 (HSV-1) (n = 11), Epstein-Barr virus (EBV) (n = 16), cytomegalovirus (CMV) (n = 21), HHV type 6 (HHV-6) (n = 2),

and HHV-7 (n = 1) genomic DNA in 35 (40%) of the BALF samples, including 14 (16%) samples containing 2 or 3 HHV types. CMV and EBV reactivation was rare in immunocompetent patients, whereas reactivation of HSV-1 was predominantly observed in intubated patients regardless of their immune status. Overall, HHVs were almost exclusively found in patients with immunosuppression or endotracheal intubation. Real-time PCR detected $0.95\text{--}1.59 \times 10^6$ copies of viral DNA/ μg human genome DNA, and HSV-1 (n = 4), CMV (n = 9), and HHV-6 (n = 1) were identified as potentially pathogenic agents. **Conclusions:** The implementation of multiplex and real-time PCR of BALF was feasible in ALI/ARDS patients, which allowed efficient detection and quantification of HHV DNA.

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Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) remain critical illnesses with substantial morbidity and mortality [1]. Despite advances in understanding the pathophysiology of ALI/ARDS, the

mainstay of treatment for these diseases primarily involves supportive care and management of the underlying clinical disorder. Therefore, the pathogenic factors underlying these medical conditions need to be identified to indicate potential therapeutic interventions.

Human herpes viruses (HHVs) have been recognized as clinically important pathogens of pulmonary infections that can result in ALI/ARDS in immunocompromised patients [2–4], and they have also been recognized as potential pathogens in nonimmunocompromised critically ill patients [5–9]. However, there have been few comprehensive studies to date on the prevalence and pathogenic role of HHVs in ALI/ARDS patients, partly due to the limited diagnostic tools available for identifying different HHVs in a clinical sample.

In recent years, development of the multiplex polymerase chain reaction (PCR) assay has enabled simultaneous detection of a wide range of viruses [10]. Although a positive PCR for HHVs does not distinguish asymptomatic shedding from an active infection, quantification of the viral load in bronchoalveolar lavage fluid (BALF) could potentially differentiate between these two conditions [6, 11–13]. Therefore, rapid screening of BALF by qualitative multiplex PCR to identify virus-positive samples followed by quantitative PCR of the positive samples may be a useful diagnostic approach for evaluating lung diseases possibly caused by HHVs.

The aim of the present study was to evaluate the usefulness of multiplex and real-time PCR analysis of BALF for detecting HHV reactivation in ALI/ARDS patients.

Methods

Patients and Clinical Samples

Between August 2008 and July 2012, a total of 134 diagnostic bronchoalveolar lavage (BAL) procedures were performed in new-onset ALI/ARDS patients when the etiology of ALI/ARDS was unknown and was unlikely to be caused by bacterial pneumonia based on the clinical presentation, radiological findings, or the response to antibiotics. Of these, 87 BALF samples were analyzed for HHVs using multiplex PCR as part of the diagnostic examination, and the data were retrospectively examined in this study. BAL sampling was carried out via standard techniques, usually instilling five 30-ml aliquots of normal saline, and the specimens were stored at -80°C until the PCR was performed. The BALF was routinely tested for common pathogens (bacteria, fungi, and *Mycobacterium*) and for viral inclusion bodies by cytology. Additional microbiological studies, including quantitative PCR for *Pneumocystis jirovecii* in BALF and cytomegalovirus (CMV) pp65 antigen levels in blood were performed as appropriate. Clinical and laboratory data were also collected from medical records. This study was approved by our institutional review board.

Classification of Viral Pneumonia Cases and Clinical Definitions

Classification of viral pneumonia cases was based on viral detection in BALF and on the following criteria: (1) proven viral pneumonia: specific cytopathic effects (inclusion bodies) in cells from BALF or transbronchial biopsy, (2) probable viral pneumonia: otherwise unexplained ALI/ARDS with a clinical response to specific antiviral agents, and (3) possible viral pneumonia: viral load $>10^4$ copies/ μg DNA in BALF, otherwise unexplained ALI/ARDS, and antiviral agents ineffective or not administered. In possible viral pneumonia cases, there may be a clinical response to specific antiviral agents administered concurrently with efficacious treatments for known causes of ALI/ARDS. ALI/ARDS was defined according to previously reported criteria [14]. An immunocompromised patient was defined as one receiving immunosuppressants or corticosteroid therapy for more than 1 month or having AIDS.

PCR Analysis

HHV genomic DNA was measured by two independent PCR assays: qualitative multiplex PCR followed by quantitative real-time PCR of the HHV-positive samples detected by multiplex PCR.

The multiplex PCR was designed to identify the genomic DNA of 8 HHVs: herpes simplex virus type 1 (HSV-1), HSV-2, varicella zoster virus (VZV), Epstein-Barr virus (EBV), CMV, HHV type 6 (HHV-6), HHV-7, and HHV-8. DNA extraction was performed on 200- to 400- μl BALF samples using E21 virus minikits (Qiagen Inc., Valencia, Calif., USA). The multiplex PCR amplifications were set up in 2 capillaries (capillary 1: HSV-1, HSV-2, VZV, CMV, and HHV-6; capillary 2: EBV, HHV-7, and HHV-8), each containing 5 μl DNA extract, specific primers, hybridization probe mix, and Accuprime Taq (Invitrogen, Carlsbad, Calif., USA). The primers and probes for HHVs have been described previously [15] and are shown in table 1. The reactions were performed using the LightCycler PCR System (Roche, Switzerland) with the following conditions: an initial denaturation step at 95°C for 2 min, followed by 40 cycles at 95°C for 2 s, 58°C for 15 s, and 72°C for 15 s, with a final extension at 40°C for 30 s. Hybridization probes were then mixed with the PCR products and melting curves were analyzed using the LightCycler System. The sensitivity of the multiplex PCR analysis was 50 copies/tube.

Subsequently, for HHV-positive samples, real-time PCR was performed to measure the viral load using AmpliTaq Gold and the Real-Time PCR 7300 System (ABI, Foster City, Calif., USA). The sequences of the primers and probes for the HHVs and the PCR conditions used in this study have been previously reported [16]. The BALF samples were also analyzed by PCR for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control, and the number of viral DNA copies was calculated as copies per microgram of human genome DNA. The viral DNA in the blood samples of selected patients was measured using the same multiplex PCR conditions, and the results were calculated as copies per milliliter of whole blood.

Statistics

Continuous variables are expressed as means $\pm$ SD unless stated otherwise. Categorical variables were compared using the χ^2 test or Fisher's exact test as appropriate. The CMV viral load in BALF was assessed as a diagnostic test for CMV pneumonia us-

Table 1. Sequences of primers and probes used in the multiplex PCR

Virus	Target gene	Primer sequence	Probe sequence
HSV-1, HSV-2 ^a	polymerase	F: GCTCGAGTGCAGAAAAACGTTC R: TGCAGTTGATAAACGCGCAGT	3'FITC: GCGCACCAGATCCACGCCCTTGATGAGC LcRed604-5': CTTGCCCCCGCAGATGACGCC
VZV	gene 29	F: TGTCTAGAGGAGGTTTATCTG R: CATCGTCTGTAAAGACTTAACCAG	3'FITC: GGGAAATCGAGAAACCACCTATCCGAC LcRed604-5': AAGTTCGCGGTATAATTGTCAGT
EBV	BamH1	F: CGCATAATGGCGGACCTAG R: CAAACAAGCCCACTCCCC	3'FITC: AAAGATAGCAGCAGCGCAGC LcRed604-5': AACCATAGACCCGCTTCCTG
CMV	CMV glycoprotein	F: TACCCCTATCGCGTGTGTTTC R: ATAGGAGGCGCCACGTATTC	3'FITC: TCGTCGTAGCTACGCTTACAT LcRed705-5': ACACCACTTATCTGCTGGGCAGC
HHV-6	101k gene region	F: ACCCGAGAGATGATTTTGCG R: GCAGAAGACAGCAGCGAGTA	3'FITC: TAAGTAACCGTTTTCGTCCCA LcRed705-5': GGGTCATTATGTTATAGA
HHV-7	U57	F: GAAAAATCCGCCATAATAGC R: ATGGAACACCTATTAACGGC	3'FITC: GCCATAAGAAACAGGTACAGACATTGTCA LcRed705-5': TTGTGAAATGTGTTGCG
HHV-8	EB BDLF1 ORF26	F: AGCCGAAAGGATTCACCAT R: TCCGTGTTGTCTACGTCCAG	3'FITC: CCGGATGATGTAAATATGGCGGAAC LcRed705-5': TGATCTATATACCACCAATGTGTCATTTATG

F = Forward primer; R = reverse primer.

^a The primer sequences were the same for both HSV-1 and HSV-2, while the probe sequences were a complete match with the HSV-2 genome sequence but had a two-base mismatch with the HSV-1 genome sequence. Therefore, there was a difference in melting temperature between HSV-1 and HSV-2, enabling discrimination of these two viruses.

ing a receiver operating characteristic plot. $p < 0.05$ was considered statistically significant. All statistical analyses were performed using JMP 7.0.2 software (SAS Institute Inc., Cary, N.C., USA).

Results

Study Population

The clinical characteristics of the patients in this study are summarized in table 2. The underlying conditions were diverse and 49 (56%) patients were immunocompromised. ALI/ARDS developed in a nosocomial setting in 31 (35%) patients. Predominant ground-glass opacity mixed with focal air space consolidation was the most frequent radiological finding.

Viral Detection in BALF

The HHV virology results are summarized in table 3. Multiplex and real-time PCR detected HSV-1 in 11 (13%) BALF samples (2.92×10^2 – 6.22×10^5 copies/ μ g DNA), EBV in 16 (18%) samples (9.50×10^{-1} – 6.62×10^4 copies/ μ g DNA), CMV in 21 (24%) samples (2.55 – 1.59×10^6 copies/ μ g DNA), HHV-6 in 2 (2%) samples (6.90×10^4 –

6.90×10^6 copies/ μ g DNA), and HHV-7 in 1 (1%) sample (3.12×10^3 copies/ μ g DNA). HSV-2, VZV, and HHV-8 were not detected. Overall, 35 (40%) patients were positive for at least one type of HHV, and multiple HHV types were detected in 14 (16%) patients.

The prevalence of HSV-1 was higher in intubated patients than in nonintubated patients (41 vs. 3%, $p < 0.0001$), but it was not significantly different between immunocompromised and immunocompetent patients (12 vs. 13%, $p = 0.90$) (fig. 1). In contrast, in CMV- and EBV-positive patients, the prevalence of CMV (fig. 2) and EBV (fig. 3) was not significantly different between intubated patients (31 and 18%, respectively) and nonintubated patients (22 and 18%, respectively) ($p = 0.33$ and $p = 1.00$, respectively), but it was significantly higher in immunocompromised patients (39 and 29%, respectively) than in immunocompetent patients (5 and 5%, respectively) ($p = 0.0003$ and $p = 0.006$, respectively). HHV-6- and HHV-7-positive BALF was obtained from immunocompromised patients. Overall, the prevalence of HHVs was higher in immunocompromised and/or intubated patients (55%; 32/58) than in immunocompetent patients without endotracheal intubation (10%; 3/29) ($p < 0.0001$).

Table 2. Baseline characteristics of the 87 patients in this study

Age, years	64.3±14.3
Male gender, n (%)	59 (68)
Underlying disease, n (%)	
Connective tissue disease	19 (22)
Posttransplant	9 (10)
Hematologic malignancy	8 (9)
Solid cancer	8 (9)
Postoperative	3 (3)
Hemodialysis	3 (3)
AIDS	2 (2)
Underlying pulmonary disease, n (%)	
IIP	21 (24)
CTD-IP	6 (7)
Immunocompromised, n (%)	49 (56)
Nosocomial onset, n (%)	31 (35)
Mechanical ventilation, n (%)	
At the time of BAL	27 (31)
Over the course of hospitalization	61 (70)
PaO ₂ /FIO ₂ , mm Hg	163±68.7
ALI, n (%)	28 (32)
ARDS, n (%)	59 (68)
Duration of symptoms, days	9.7±9.6
LDH, IU/l	543±363
CRP, mg/dl	10.3±3.3
CT findings (%)	
GGO only	12 (14)
GGO predominant	62 (71)
Consolidation predominant	13 (15)
BALF samples ^a	
Median total cell count (range), n × 10 ⁵ /mm ³	2.35 (0.1–88.8)
Median neutrophils (range), %	28 (0–93)
Median lymphocytes (range), %	15 (1–82)
Median eosinophils (range), %	1 (0–46)
Median macrophages (range), %	32 (0–84)

IIP = Idiopathic interstitial pneumonia; CRP = C-reactive protein; LDH = lactate dehydrogenase; CTD-IP = connective tissue disease-associated interstitial pneumonia; GGO = ground-glass opacity.

^a Fiberoptic bronchoscopy was performed with supplemental oxygen (n = 23), under noninvasive ventilation (n = 42), or under endotracheal intubation (n = 22).

Diagnosis

Fourteen patients in this study were diagnosed with viral pneumonia: 9 with CMV pneumonia (5 proven, 3 probable, 1 possible viral pneumonia), 4 with HSV-1 pneumonia (all possible viral pneumonia), and 1 with HHV-6 pneumonia (probable viral pneumonia) (table 4). Diagnoses of the other patients included acute exacerbation of interstitial pneumonia (n = 18), pneumocystis pneumonia (n = 13), other pulmonary infections (n = 7), diffuse alveolar hemorrhage (n = 7), septic ARDS

(n = 3), drug-induced pneumonia (n = 3), other causes of ALI/ARDS (n = 11), and unidentified etiology (n = 12). HHVs were not detected in the BALF of patients with acute exacerbation of interstitial pneumonia, except in one BALF that had a low EBV viral load (278 copies/μg DNA). For a diagnosis of proven or probable CMV pneumonia, a cutoff value for the viral load in BALF of 1.39×10^4 copies/μg DNA had 87.5% sensitivity and 84.6% specificity.

Treatment and Outcome

Antiviral agents were administered to 13 patients with a diagnosis of viral pneumonia. Although respiratory improvement was seen, at least temporally, in 9 of the patients diagnosed with CMV or HHV-6 pneumonia, 10 (77%) patients eventually died due to a newly acquired infection, sustained respiratory failure, or multiorgan dysfunction (table 4). The overall in-hospital mortality in this cohort was 51%.

Discussion

This study showed that multiplex PCR and real-time PCR analysis of HHVs in BALF provided informative data regarding the epidemiologic characteristics of HHVs in ALI/ARDS patients with unknown etiology. To our knowledge, this is the first study to conduct comprehensive multiplex and real-time PCR analyses of HHVs in BALF of ALI/ARDS patients.

Viruses in the Herpesviridae family are the most common viral pathogens causing pulmonary infection in immunocompromised patients. Although CMV is best known for its propensity to cause pneumonia [2], other herpes viruses (e.g. HSV-1 [3, 17], VZV [18], EBV [19], and HHV-6 [20, 21]) have also been implicated as etiologic agents. Moreover, recent studies have documented frequent pulmonary reactivation and/or infection of HSV-1 [6, 7] and CMV [8, 9] in critically ill patients with no known immunocompromise. Therefore, ALI/ARDS patients with unknown etiology should be examined for possible viral pneumonia caused by HHVs, which often presents with bilateral ground-glass opacification and/or consolidation [17, 21, 22]. In this setting, the diagnosis must be broadened to a wide range of HHVs that can be efficiently examined by multiplex PCR instead of conventional microbiological tests for specific pathogens. In fact, this study found reactivation of 5 types of herpes viruses in 40% of ALI/ARDS patients, including 16% of patients with multiple types of HHVs in their BALF.

Fig. 1. HSV-1 prevalence and semi-log plot of the HSV-1 DNA load in BALF as a function of patient intubation or immune status. Negative PCR results were set to 1 ($\log_{10} 1 = 0$).

Fig. 2. CMV prevalence and semi-log plot of the CMV DNA load in BALF as a function of patient intubation or immune status. Negative PCR results were set to 1 ($\log_{10} 1 = 0$). The bar represents the cutoff value indicating CMV pneumonia (1.39×10^4 copies/ μ g DNA).

Table 3. HHV prevalence and viral load in BALF samples from the 87 patients in this study

	HSV-1	HSV-2	VZV	EBV	CMV	HHV-6	HHV-7	HHV-8
Positive BALF ^a , n (%)	11 (13)	0	0	16 (18)	21 (24)	2 (2)	1 (1)	0
Viral load, copies/ μ g DNA	2.92×10^2 – 6.22×10^5	–	–	9.50×10^{-1} – 6.62×10^4	2.55 – 1.59×10^6	6.90×10^4 – 6.90×10^6	3.12×10^3	–
BALF samples with a viral load, n								
≥0	0	–	–	2	3	0	0	–
≥10	0	–	–	2	4	0	0	–
≥10 ²	3	–	–	5	2	0	0	–
≥10 ³	3	–	–	3	3	0	1	–
≥10 ⁴	0	–	–	4	3	1	0	–
≥10 ⁵	5	–	–	0	5	0	0	–
≥10 ⁶	0	–	–	0	1	1	0	–

^a Two types of HHVs were detected in 12 samples (EBV and CMV in 6, HSV-1 and EBV in 3, HSV-1 and CMV in 2, and CMV and HHV-6 in 1). Three types of HHVs were detected in 2 samples (EBV, CMV, and HHV-7 in 1 and HSV-1, EBV, and CMV in 1).

Considering the diversity of HHVs detected in this study, multiplex PCR has the additional advantage of being able to identify unexpected agents that might otherwise be overlooked, thereby enabling early therapeutic intervention.

This study provided insights into the epidemiologic features of herpes viruses in ALI/ARDS. Of note, reactivation of HSV-1 was predominantly observed in intubated patients regardless of their immune status, and a high HSV-1 DNA load in BALF was not associated with high-

Table 4. Laboratory and clinical findings of 14 patients diagnosed with or suspected of having viral pneumonia

Patient No.	Underlying disease	Virus	Diagnosis	DNA load		pp65 (+) cells	Other HHVs in BALF	Dominant CT finding	Antiviral agent	Outcome
				BAL copies/ μ g DNA	copies/ml blood					
1	SLE	CMV	proven	1.59×10^6	NA	82	none	GGO	ganciclovir	dead
2	posttransplant	CMV	proven	4.03×10^5	NA	206	EBV	GGO	ganciclovir	dead
3	SLE	CMV	proven	3.12×10^5	NA	34	EBV, HHV-7	GGO	ganciclovir	dead
4	MPA	CMV	proven	2.22×10^4	NA	0	none	GGO	ganciclovir	alive
5	DIHS	CMV	proven	1.39×10^4	NA	31	none	GGO	ganciclovir	dead
6	posttransplant	CMV	probable	5.65×10^5	NA	2	none	consolidation	ganciclovir	alive
7	SLE	CMV	probable	2.26×10^5	NA	30	none	GGO	ganciclovir	alive
8	DM-ILD	CMV	probable	5.70×10^3	NA	1	none	GGO	ganciclovir	dead
9	HIV-PCP	CMV	possible	1.70×10^5	NA	74	none	GGO	ganciclovir	alive
10	sepsis	HSV-1	possible	6.22×10^5	ND	NA	none	GGO	aciclovir	dead
11	posttransplant	HSV-1	possible	4.30×10^5	ND	0	none	GGO	none	dead
12	trauma	HSV-1	possible	2.00×10^5	NA	NA	none	GGO	aciclovir	dead
13	ML	HSV-1	possible	1.72×10^5	3.30×10^2	0	CMV	GGO	aciclovir	dead
14	posttransplant	HHV-6	probable	6.90×10^6	1.39×10^5	NA	none	GGO	foscarnet	dead

SLE = Systemic lupus erythematosus; MPA = microscopic polyangiitis; DIHS = drug-induced hypersensitivity syndrome; DM-ILD = dermatomyositis-associated interstitial lung disease; PCP = pneumocystis pneumonia; ML = malignant lymphoma; NA = not assessed; ND = not detected; GGO = ground-glass opacity.

Fig. 3. EBV prevalence and semi-log plot of the EBV DNA load in BALF as a function of patient intubation or immune status. Negative PCR results were set to 1 ($\log_{10} 1 = 0$).

level viremia. These findings are in agreement with previous reports that HSV-1 pneumonia frequently presents as late-onset ventilator-associated pneumonia due to aspiration from the upper respiratory tract rather than pulmonary dissemination secondary to systemic viral infection [5, 6]. We also found that reactivation of HHVs other than HSV-1 mostly occurred in classically high-risk immunocompromised patients. Although recent studies have shown that CMV reactivation is common in nonimmunosuppressed, critically ill patients [8, 9], our results showed that CMV reactivation of the lung was rare in this patient population. Overall, clinically significant pulmonary reactivation of HHVs was almost exclusively observed in patients with endotracheal intubation or known immunocompromised status, indicating that patients with this clinical picture merit careful investigation for HHVs in the lung.

Viral pneumonia caused by HHVs still represents a diagnostic challenge. Demonstration of cytopathic effects by HHVs, indicating viral pathogenicity, is not a sensitive diagnostic tool and is often hard to obtain [23], while detection of HHVs via viral culture or PCR does not differentiate active infection from asymptomatic shedding. Therefore, combined use of real-time PCR with multiplex PCR is an integral part of the evaluation of HHV patho-

genicity. Since the amount of recovered epithelial lining fluid in BALF can vary considerably, the quantitative methodology in this study used data normalization to GAPDH, a housekeeping gene, to correct for this variation [24]. In agreement with previous studies [11–13], we found that quantification of the viral load in BALF may be useful in diagnosing CMV pneumonia. We noted that 3 patients with proven or probable CMV pneumonia had negative or clinically insignificant pp65 antigenemia, highlighting the importance of obtaining samples from the site of infection. Meanwhile, the pathogenicity of HSV-1 was unclear in this study because antiviral therapy did not lead to clinical improvement in 3 of 4 patients diagnosed with possible HSV-1 pneumonia. Previous studies also showed that HSV-1 in BALF may be a marker for underlying clinical conditions rather than a cause of mortality [25, 26]. In clinical practice, however, when there is a high viral load in BALF samples from ALI/ARDS of otherwise unexplained etiology, administration of antiviral agents against HHVs with possible lung pathogenicity (e.g. CMV, HSV-1, and HHV-6) would be an option for treatment.

We must acknowledge that it is difficult to extrapolate our results to all ALI/ARDS patients or to elucidate the etiologic role of HHVs in ALI/ARDS due to the selected group of patients and the retrospective nature of our study without a standardized diagnostic procedure. In addition, we must note that the pathogenic significance of HHVs is often difficult to determine via quantification of the viral DNA in BALF because of the substantial overlap between viral loads in symptomatic and asymptomatic patients [6, 11, 13]. Accordingly, although we have demonstrated that our diagnostic approach was technically feasible and potentially useful in ALI/ARDS patients to detect HHVs in the lung, further studies prospectively collecting all de novo ALI/ARDS patients in order to assess the exact prevalence of HHVs in ALI/ARDS or to

investigate the possible impact of common bacterial infections on the reactivation of HHVs are needed. Also, a controlled antiviral treatment trial is warranted to draw conclusions regarding the etiologic role of the detected viruses in the development or worsening of ALI/ARDS.

Our study has some more limitations. First, there was a large heterogeneity in the studied population that precluded assessment of the impact of HHVs on the prognosis. Second, we may have underestimated the prevalence of HHVs because an HHV type with a low viral load in BALF could have been missed in the multiplex PCR assays if the BALF also contained a high viral load of another HHV type. Third, the impact of virus-bacteria coinfections could not be assessed because two thirds of our patients had received antimicrobial agents at the time of the BAL. Fourth, oral contamination cannot be excluded because BALF samples obtained without a tracheal tube were possibly contaminated with oropharyngeal secretions by virtue of the technical procedure.

In conclusion, the implementation of multiplex and real-time PCR for HHVs allowed efficient detection and quantification of viral genomic DNA in BALF in selected ALI/ARDS patients of unknown etiology, especially in patients with immunosuppression or endotracheal intubation. In this setting, where clinicians must consider a wide differential diagnosis, the combination of multiplex and real-time PCR for HHVs may represent a useful diagnostic tool for the management of ALI/ARDS.

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