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Protocols

Detection of cytomegalovirus in bronchoalveolar lavage fluid from immunocompromised patients with pneumonitis by viral culture and DNA quantification

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ABSTRACT

Purpose: To compare the detection of human cytomegalovirus (HCMV) in bronchoalveolar lavage (BAL) fluid by viral culture and quantitative polymerase chain reaction (qPCR), and to establish a viral load threshold that can identify cases of HCMV replication indicative of pneumonitis. There is currently no universal viral load cut-off to differentiate between patients with and without pneumonitis, and the interpretation of qPCR results is challenging.

Methods: 176 consecutive BAL samples from immunosuppressed hosts with signs and/or symptoms of respiratory infection were prospectively studied by viral culture and qPCR.

Results: Concordant results were obtained in 81.25% of the BAL samples. The rest were discordant, as only 34% of the qPCR-positive BAL samples were positive by culture. The median HCMV load was significantly higher in culture-positive than in culture-negative BAL samples (5038 vs 178 IU/mL). Using a cut-off value of 1258 IU/mL of HCMV in BAL, pneumonia was diagnosed with a sensitivity of 76%, a specificity of 100%, a VPP of 100% and VPN of 98%, and HCMV was isolated in 100% of the BAL cultures.

Conclusion: We found that a qPCR-negative was a quick and reliable way of ruling out HCMV pneumonitis, but a positive result did not always indicate clinically significant replication in the lung. However, an HCMV load in BAL fluid of $\geq$ 1258 IU/mL was always associated with disease, whereas $<$ 200 IU/mL rarely so.

1. Background

Human cytomegalovirus (HCMV) is a DNA virus belonging to the family *Herpesviridae*, subfamily *Betaherpesvirinae*. The seroprevalence in adults is between 50% and 98%, being higher in developing countries (Boeckh and Geballe, 2011). After primary infection, the virus may remain latent in different cells and tissues and reactivate throughout life. In immunocompetent hosts, primary infection very rarely causes severe disease, usually being asymptomatic, although it can present clinically as a mononucleosis-like syndrome. In immunosuppressed hosts, it can cause severe systemic or end-organ disease, such as pneumonitis, colitis, or retinitis, both after primary infection and upon reactivation of the latent virus. Despite the reduction in the incidence of HCMV disease, due to pre-emptive antiviral therapy or prophylaxis and better diagnostic tools, HCMV remains the most important and feared opportunistic pathogen associated with transplant patients, especially those with impaired T-cell-mediated immunity (Boeckh and Geballe, 2011).

HCMV pneumonitis causes significant morbidity and mortality in

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immunosuppressed patients (transplant recipients, HIV-positive, or receiving immunosuppressive drug therapy), even with the correct antiviral treatment. In allogeneic haematopoietic stem cell transplant patients, HCMV pneumonitis is one of the most dangerous infectious complications, with a mortality rate of up to 70% (Boeckh and Geballe, 2011; Ibrahim et al., 1997; Erard et al., 2015; Boivin et al., 1996). The symptoms and radiological findings of HCMV pneumonitis are non-specific, which hinders and delays diagnosis (Franquet et al., 2003).

At the Fourth International Conference on HCMV in Paris in 1993, a report was drawn up to define and unify the concepts of HCMV infection and disease, with a recent update in 2017 (Ljungman et al., 2017). Accordingly, it defines proven HCMV pneumonitis as the detection of the virus in lung tissue (by culture, histopathology, immunohistochemistry or DNA hybridisation techniques), accompanied by signs and/or symptoms of pneumonia. However, this requires a lung biopsy, which is invasive and often contraindicated, limiting the possibility of making this diagnosis. Fortunately, bronchoalveolar lavage (BAL) has become a widely accepted alternative to lung biopsy for the evaluation of lung disease (Stover et al., 1984), whereby probable HCMV pneumonitis can be diagnosed by virus detection in BAL fluid by culture or DNA quantification, accompanied by signs and/or symptoms of pneumonia.

Viral culture is considered the gold standard for the diagnosis of HCMV infection and positive cultures are highly correlated with disease (Razonable et al., 2002; Kotton, 2013). In the late 1980 s, culture-centrifugation (shell vial) was introduced and became the mainstay of HCMV diagnosis, providing faster results than traditional culture, and reducing the turnaround time from several weeks to one day (Rabella and Drew, 1990). Despite being the reference techniques for the diagnosis of viral infections, in most laboratories culture-based methods have been replaced, due to their laboriousness and longer turnaround time, by molecular techniques such as qPCR. However, although qPCR analysis is more sensitive and rapid than viral culture, it is limited in that it detects viral genetic material without being able to distinguish between clinically relevant HCMV replication (disease) and viral shedding or latent HCMV infection (Leuzinger et al., 2021 Jun). Due to this lack of specificity, guidelines recommend quantification of the viral load (VL) of HCMV in BAL fluid as an aid to interpret the positive qPCRs (Ljungman et al., 2017). Nevertheless, a universal VL cut-off value that discriminates between patients with and without HCMV pneumonitis has not been established to date. This is partly due to the pathogenesis of the virus itself (potentially establishing latency or asymptomatic replication without causing disease) and also to the numerous variables that can affect DNA quantification in BAL samples (type of patient, the BAL procedure and subsequent processing, or the DNA quantification assay) (Ljungman et al., 2017; Razonable et al., 2002; Piñana et al., 2019; Chemaly et al., 2004; Lodding et al., 2018).

Several studies have sought to establish whether the HCMV load in blood (DNAemia) is useful for the diagnosis of pneumonitis, and whether the blood VL has a cut-off value indicative of clinically significant replication in the lung. Again, no unanimous conclusions have been reached. While some reports indicate that it has a high predictive value for lung disease (Meyers et al., 1990; Saksirisampant et al., 2022; Lee et al., 2017), others found that a considerable percentage of pneumonitis patients have undetectable HCMV in the blood as a consequence of local replication in the lung, concluding that a negative-DNAemia does not exclude pneumonitis (Leuzinger et al., 2021 Jun; Beam et al., 2018; Westall et al., 2004).

This lack of consensus in interpreting VL values in both BAL fluid and blood renders the diagnosis of pneumonitis a challenge. Therefore, more well-designed prospective studies, ideally based on reference microbiological techniques, are needed (Piñana et al., 2019).

2. Objective

The primary objectives were to compare HCMV detection in BAL fluid by viral culture and qPCR for the diagnosis of pneumonitis in immunosuppressed hosts, as well as to establish a cut-off value for the HCMV load in BAL samples that discriminates between cases of active virus replication and viral shedding, using culture as the reference technique. The secondary objective was to assess plasma HCMV load in patients with suspected pneumonitis and its correlation with the VL in BAL fluid.

3. Study design

3.1. Clinical samples

A prospective study was carried out between 1 January 2021 and 31 July 2022 at the Microbiology Department of a tertiary level university hospital in Barcelona.

Consecutive BAL samples (n = 176) from immunosuppressed patients with signs and/or symptoms of respiratory infection, received in our laboratory for virological study, were included.

The reference methods for HCMV detection in BAL samples in our laboratory are traditional viral culture (in tubes) and centrifugation culture (shell vial). In addition, an HCMV qPCR assay was performed on these samples.

Retrospectively, we established whether these patients had been tested for the HCMV load in plasma (DNAemia) in the week before or after BAL fluid collection.

3.2. BAL fluid collection by bronchoscopy

Bronchoscopy was performed using standard procedures in accordance with the Spanish Society of Pulmonology and Thoracic Surgery (SEPAR) protocol no. 28, "Quality plan for the collection and processing of endoscopic specimens" (Manual 28 de procedimientos SEPAR). The technique consists of inserting the bronchoscope into the subsegment of the lung pre-selected by radiological criteria until the tip is wedged in a bronchiole with visualization of the distal airway in the center of the image. Sterile normal saline (room temperature) is injected via a handheld syringe and then gradually withdrawn back into the syringe. A total of up 150 cc of sterile saline was injected in 3 aliquots of 50 cc syringes. The first fraction recovered was discarded due to risk of contamination. After collection, the sample was placed in the corresponding specific sterile microbiological recipients and sent to the Microbiology Department within minutes of collection. From collection to processing (always less than 72 h) the samples were stored at 2–6ºC.

3.3. BAL viral culture

To avoid culture contamination, each sample was first treated with a mixture of antibiotics (vancomycin 500 μL/mL and gentamycin 2.5 mg/mL) and an antifungal agent (amphotericin 0.05 mg/mL) in a 10% volume/volume ratio, for 30 min.

- Tube culture. 0.3 mL of the pre-treated sample was inoculated into a cylindrical tube containing a monolayer of human MRC5 cells (RD-Biotech, France) with maintenance medium (Minimal Essential Medium x10 with fetal bovine serum 2.5%), and incubated in a horizontal position at 36 ± 2ºC for up to 21 days. Cell monolayers were examined daily with an inverted microscope (x40). A culture was considered positive when a characteristic cytopathic effect was observed.
- Centrifugation culture (shell vial). This method is used to detect immediate early HMCV antigen and is based on the centrifugation of samples on a cell monolayer (MRC5 cells) arranged on a 12 mm diameter lens. Thus, 0.3 mL of each pre-treated sample was inoculated into two vials with the cell monolayer. To increase viral absorption, the vials were centrifuged at 700 x g for 30 min. The sample was then decanted and 1 mL of growth medium (Minimal Essential

Medium x10 with fetal bovine serum 10%) was added and incubated at 36 ± 2ºC (one vial for 16 h and the other for 40 h). Then, specific monoclonal antibodies and a fluorescein conjugate (Vircell, Spain) were added, and each culture was observed under a fluorescence microscope (x400) at 16 h or 40 h. The shell vial was considered positive if one or more fluorescent nuclei of MRC5 cells were observed.

- Samples were considered culture-positive if either the tube culture or shell vial were positive.

3.4. Quantification of HCMV DNA (VL)

ELITe MGB® Kit CMV reagent in an ELITe InGenius® instrument (ELITechGroup, France) was used for this assay. This fully automated system performs the extraction and amplification directly from the sample, and the results are interpreted by the instrument software. The primers and HCMV probe are specific for the exon 4 region of the HCMV MIEA gene (major immediate early antigen, HCMV UL123). The primers and probe for the internal control are specific for the promoter and 5′ UTR region of the human beta-globin gene to ensure that both the BAL cell number and extraction-amplification processes occur correctly.

For both BAL fluid and plasma, 0.2 mL of sample was used. HCMV DNA quantification was expressed as IU/mL in BAL and plasma. According to manufacturer's indications 1 IU/mL = 5 copies/mL ("1st WHO International Standard for HCMV for Nucleic Acid Amplification Techniques").

The system considered these samples to be positive if the HCMV load was above the detection limit of the reagent (178 IU/mL for BAL and plasma). The test was considered invalid if the internal control was not detected.

3.5. Statistical analysis

Results are presented as the number of cases and percentages for categorical data and as medians and the other two quartiles for ordinal data. Comparison of cycle threshold (Ct) values and VL between positive and negative cultures was performed with the nonparametric Mann-Whitney test. The statistical significance level was 5% (a=0.05), and two-tailed tests were used throughout. All analysis was performed using IBM-SPSS software (version 26; SPSS. Armonk, NY).

3.6. Ethical approval

The study protocol was evaluated and approved by the HSCSP Ethics Committee (IIBSP-VIR-2014–41).

4. Results

A total of 176 BAL samples from 158 immunosuppressed patients with signs and/or symptoms of respiratory infection were tested for HCMV by culture and qPCR during this period. The results of viral culture and qPCR are shown in Table 1. Concordant results were obtained in 81.25% of the BAL samples, the remaining 18.75% giving discordant results. Of the 50 BAL samples positive for HCMV by qPCR, only 17 (34%) were positive by culture. Fifteen (88.2%) of them were positive by tube culture and shell-vial, while the other two (11.8%) were only positive by shell-vial and negative by tube culture.Fig. 1.

Table 1
HCMV culture and qPCR results obtained from BAL samples (PPV: positive predictive value; NPV: negative predictive value).

n = 176	Positive culture	Negative culture	
Positive qPCR	17 (9.7%)	33 (18.7%)	PPV 34%
Negative qPCR	0	126 (71.6%)	NPV 100%
Total	S 100%	E 79.2%	

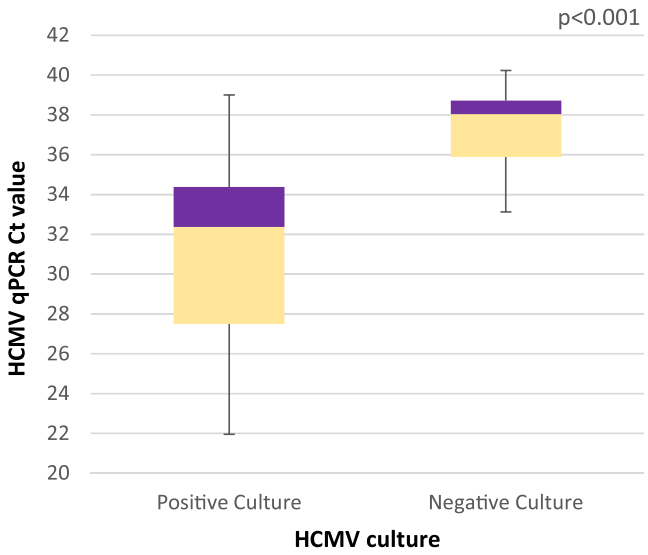


Fig. 1. Boxplot analysis of HCMV qPCR Ct values showing differences in distribution and median values between BAL samples with positive and negative culture results.

The median HCMV load and qPCR Ct values of culture-positive and -negative BAL samples were compared (Table 2, Picture 1). In culture-positive samples, the median VL was 4860 IU/mL (24,298 copies/mL) higher and the Ct value lower by 6.17 units compared to those culture-negative, both differences being statistically significant ($p < 0.005$). When the HCMV load in BAL samples was < 200 IU/mL (<1000 copies/mL), the probability of virus isolation in culture was 4.3%, as only one positive culture was obtained from the 24 BAL samples with this VL value. However, with > 900 IU/mL (>4500 copies/mL), the probability increased to 100%, as all 13 BAL samples with this HCMV load tested positive by culture (Fig. 2). Using a qPCR cut-off value of 1258 IU/mL (6290 copies/mL) HCMV in BAL, pneumonia was diagnosed with a sensitivity of 76%, a specificity of 100%, a VPP of 100% and VPN of 98% (AUC of 0.987) (Fig. 3).

In all qPCR assays, the internal control (human beta-globin gene) was amplified to ensure that both DNA extraction and amplification were performed correctly.

Retrospective analysis of DNAemia values, obtained in 58 patients in the week before or after BAL fluid collection (Table 3), revealed that 100% of patients whose BAL samples yielded positive results by both qPCR and culture analysis also tested positive by qPCR in plasma. This percentage dropped to 93% in patients with BAL samples positive by qPCR but negative by culture, and to 33% in those with negative results by both techniques. The median value of HCMV load in plasma of the first group of patients (4805 IU/mL) was more than 4000 IU/mL higher compared to the other two groups (222.5 IU/mL and 178 IU/mL respectively).

Table 2
Median viral load (VL) and qPCR cycle threshold (Ct) value in BAL of HCMV qPCR-positive samples according to the culture result.

	Positive culture (17)	Negative culture (33)	p value
Median VL (Q1; Q3) BAL (IU/mL)	5038 (1008; 39,594)	178 (178; 248)	p < 0.001
Median VL (Q1; Q3) BAL (copies/mL)	25,188 (5042; 197,974.5)	890 (890; 1239)	p < 0.001
Median qPCR Ct value (Q1; Q3) BAL	32.10 (27.22; 34.11)	38.27 (36.11; 38.97)	p < 0.001

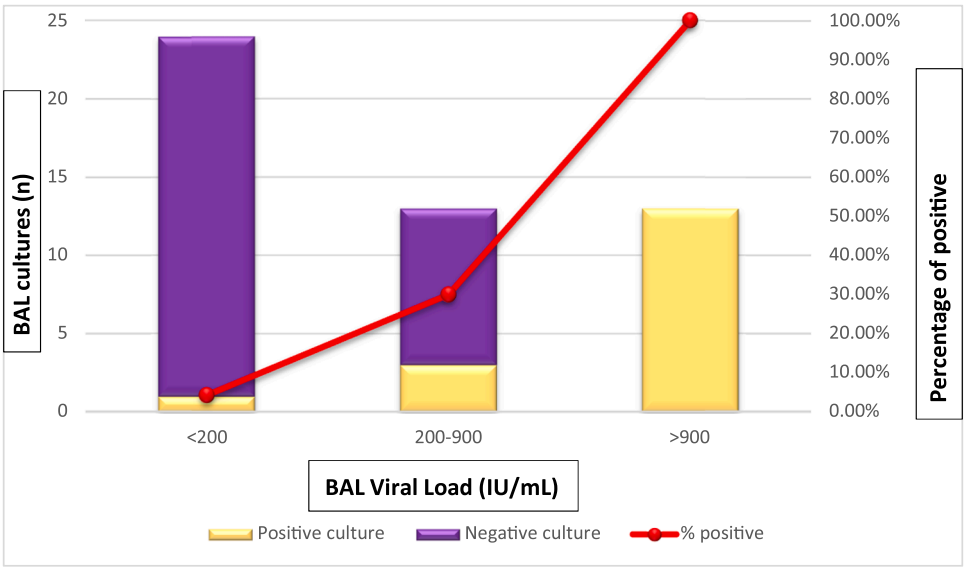
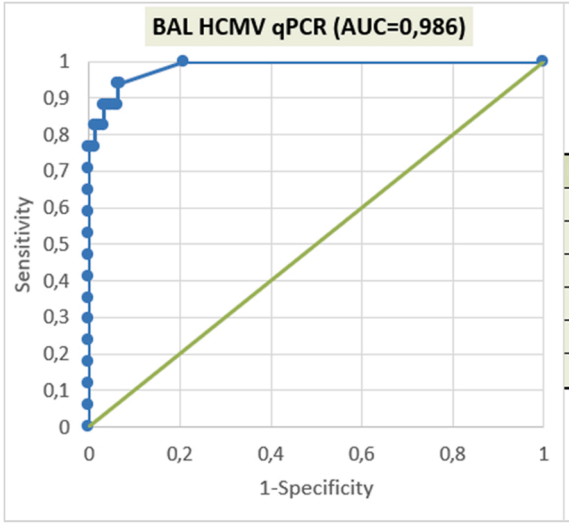


Fig. 2. HCMV culture results in BAL samples as a function of viral load value (IU/mL), and percentage of culture positivity as a function of viral load value.



UI/mL HCMV	Sensitivity	Specificity	PPV	NPV
178	100%	79%	34%	100%
201	94%	94%	62%	99%
356	88%	96%	71%	99%
623	82%	97%	78%	98%
890	76%	99%	93%	98%
1,258	76%	100%	100%	98%

Fig. 3. ROC curve for BAL HCMV qPCR for diagnosis of HCMV pneumonia. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) values as a function of HCMV load in BAL.

Table 3
Results of the HCMV DNAemia study and median HCMV viral load (IU/mL) as a function of BAL culture and qPCR result.

DNAemia study	BAL Positive culture and positive qPCR (17)	BAL Negative culture and positive qPCR (33)	BAL Negative culture and negative qPCR (126)
Not done (n)	23.5% (4)	54.5% (18)	76.2% (96)
Negative (n)	0	3% (1)	15.9% (20)
Positive (n)	76.5% (13)	42.5% (14)	7.9% (10)
• Median VL (Q1; Q3) (IU/mL)	• 4805 (461; 35,998)	• 222.5 (178; 982)	• 178 (178; 314.25)

5. Discussion

In the last years there have been major advances in the diagnosis and management of HCMV infection, which has led to a reduction in its incidence and mortality. Despite this, HCMV pneumonitis continues to be a highly feared disease in immunosuppressed patients (Erard et al., 2015). Currently, the diagnosis of proven HCMV pneumonitis is much less common than that of probable pneumonitis. Performing lung biopsies is contraindicated in many cases, and less invasive methods such as BAL are preferred. Likewise, reference techniques for the diagnosis of HCMV infection such as culture have been displaced in most laboratories by simpler, faster, and more sensitive methods such as qPCR. Despite these improvements, diagnosis of pneumonitis and the interpretation of results remains challenging (Ljungman et al., 2017).

Viral culture is recognized as a reliable technique capable of detecting clinically relevant HCMV involvement in the lower respiratory tract, as HCMV isolation is highly correlated with the disease (Razonable et al., 2002; Kotton, 2013). Since the introduction of molecular techniques in the 1990 s, several studies have compared the results of HCMV DNA quantification in BAL fluid with those obtained by culture-based techniques, and have attempted to establish an HCMV load in BAL fluid indicative of clinically significant virus replication (pneumonitis). Most have concluded that qPCR in BAL fluid is more sensitive and has a higher negative predictive value compared to culture but has less

specificity and positive predictive value (Razonable et al., 2002; Leuzinger et al., 2021 Jun; Boeckh et al., 2017; Liesnard et al., 1994; Riise et al., 2000; Chemaly et al., 2005; Tan et al., 2016).

To date, defining a universal VL threshold in BAL fluid that allows differentiation between patients with or without pneumonitis has proved elusive (Ljungman et al., 2017; Piñana et al., 2019). Several factors are responsible, such as the pathogenesis of HCMV itself (its ability to establish latency or asymptomatic replication without causing disease), or the lack of standardized procedures for qPCR analysis in BAL samples (BAL fluid extraction and processing or the qPCR assay used). The inherent variability of the bronchoscopy technique and the dilutional effect also make it difficult to obtain conclusive results. To collect BAL fluid, the instilled serum is distributed heterogeneously in the lung, and both the bathed lung area and the amount of sample recovered varies greatly between patients. Additionally, the abandonment of culture techniques by most laboratories means that many studies do not compare qPCR results with those of gold standard techniques and base their conclusions on statistical analysis and clinical interpretation.

Despite attempts by the WHO to standardize HCMV qPCR results, establishing HCMV threshold values to facilitate clinical decisions has also proved difficult for plasma or blood samples. In this case, standardization is hindered by the variability of HCMV qPCR protocols, which differ in assay performance (detection and quantification limits), DNA extraction methods, target genes, and amplicon size (Razonable et al., 2020).

The results of our study show that shell-vial was more sensitive than tube culture, as 11.8% of positive cultures were only detected by shell-vial. Furthermore, qPCR analysis of BAL fluid was shown to have a sensitivity and negative predictive value of 100% for the diagnosis of pneumonitis, using culture as the reference method, which indicates that qPCR is an efficient technique to rule out this disease. However, due to its low specificity (79.2%) and positive predictive value (34%), a positive result by qPCR in BAL fluid should be treated with caution, and other values should be considered to avoid overdiagnosis and unnecessary treatment. The HCMV load and the qPCR Ct value in BAL samples may be helpful to interpret the results. In our study, in line with published literature, significantly higher median VL and significantly lower qPCR Ct values were obtained in culture-positive compared to culture-negative BAL samples. In addition, the probability of virus isolation in culture increased with higher loads.

Based on our results, an HCMV load of 1258 IU/mL (6290 copies/mL) or higher in BAL fluid may be considered as indicative of clinically relevant replication in the lower respiratory tract, with high sensitivity and NPV, and full specificity and PPV. Virus was isolated in culture in 100% of these cases. In the literature, the VL values in BAL fluid reported to be diagnostic of pneumonitis vary widely from 500 IU/mL to $>$ 500.000 copies/mL (Leuzinger et al., 2021 Jun; Piñana et al., 2019; Lodding et al., 2018; Saksirisampant et al., 2022; Lee et al., 2017; Beam et al., 2018; Westall et al., 2004; Boeckh et al., 2017; Riise et al., 2000; Chemaly et al., 2005; Tan et al., 2016).

Three studies published in recent years also compare the results of viral culture and qPCR for HCMV detection in BAL fluid. Based on an analysis of 1109 BAL samples from immunosuppressed patients, Leuzinger et al. (2020) determined that $>$ 10,000 copies/mL was an indicator of active replication. A limitation compared with the present study is that only shell vial culture was used, and not traditional culture; also, some of the samples (88%) were frozen and processed for qPCR analysis retrospectively, and the qPCR assay used was not fully automated (it required prior DNA extraction), which can reduce the reproducibility of the results. After analyzing 565 BAL samples, Young et al. (2017) reported that 28,774 copies/mL was diagnostic of HCMV pneumonitis, whereas Boeckh et al. (2017), in a study of 271 BAL samples, proposed a much lower cut-off point of 500 IU/mL. The latter two studies only included patients with hematologic malignancies, so their conclusions are limited to this type of patient. They also used less automated qPCR assays than here, and did this assay on frozen samples (−80%).

Two recent studies of lung transplant recipients, which did not use culture, propose 4545 IU/mL (Lodding et al., 2018) and 46,000 copies/mL (Westall et al., 2004) as VL threshold values. In both cases, HCMV DNA quantification was carried out by manual qPCR, which requires prior DNA extraction and, as mentioned, provides less reproducible results. In contrast, several studies have concluded that it is not possible to establish a cut-off point that allows patients with pneumonitis to be differentiated from those without (Piñana et al., 2019; Riise et al., 2000; Tan et al., 2016).

In the present study, DNAaemia was not measured in all patients, which limits the conclusions that can be drawn. All patients with HCMV-positive BAL fluid (by culture and qPCR) also had HCMV-positive DNAaemia, and their plasma VL was significantly higher than in patients with culture-negative BAL samples. These results agree with other reports (Ibrahim et al., 1997; Chemaly et al., 2004; Meyers et al., 1990; Saksirisampant et al., 2022; Lee et al., 2017; Beam et al., 2018) of a significant positive correlation between HCMV DNAaemia and pneumonitis. However, it should be noted that the absence of HCMV DNA in plasma does not exclude pneumonitis. Recently, two studies found that approximately one third of patients with HCMV pneumonitis had an undetectable plasma VL, demonstrating that local replication of the virus can occur in the lower respiratory tract without systemic involvement (Leuzinger et al., 2021 Jun; Lodding et al., 2018; Westall et al., 2004).

One of the strengths of this study is its use of both traditional viral and shell vial culture as reference techniques, both of which are fully established in our laboratory routine, to compare the results of qPCR analysis in BAL samples. Furthermore, the samples were selected consecutively and prospectively, and submitted to culture and qPCR analysis as soon as they arrived at the laboratory. Finally, for optimum reproducibility and accuracy, each step, from bronchoscopy procedure to sample processing for culture and qPCR, was protocolized and standardised as much as possible. Moreover, a fully automated assay for HCMV DNA quantification was used, including DNA extraction and amplification and interpretation of the results. Additionally, the inclusion of human beta-globin as the internal control target in the qPCR assay ensured that BAL samples tested contained a sufficient number of cells. The intern control was detected in all qPCR, indicating that samples were of sufficient quality (and reaction occurred correctly), while if it was not detected (the number of cells was deemed insufficient or an incorrect reaction) the test was declared invalid to avoid false negatives. Finally, our study reports the results of VL in BAL in IU/mL and copies/mL, according to “1st WHO International Standard for HCMV for Nucleic Acid Amplification Techniques”, allowing comparison with other studies.

The main limitation of the study was that various types of immunosuppressed patients were included, which could limit the generalizability of the VL threshold values obtained. Additionally, although we have controlled that all BAL fluids had an adequate number of cells to consider the results valid (using B-globin as an internal control), this does not eliminate the impact that the number of cells contained in each BAL fluid may have on the VL detected. For this, the number of cells in each sample should be quantified to obtain normalised VL, as has been done for other viral infections (Santos Bravo et al., 2021). Lastly, the number of culture-positive BAL samples was low, and not all patients had undergone a DNAemia study, which limits the conclusions that can be drawn on this subject.

6. Conclusion

A negative qPCR for HCMV in BAL fluid quickly rules out pneumonitis, but a positive qPCR in BAL is not always an indicator of the disease. An HCMV load in BAL fluid of $\geq$ 1258 IU/mL (6290 copies/mL) would be indicative of clinically relevant viral replication in the lung, whereas with $<$ 200 IU/mL ($<$1000 copies/mL) the probability that the virus is replicating in the lungs is less than 5%. Even though this is a single-

center experience, and the data cannot be directly extrapolated to other scenarios, the consistency of our results could be a guide for other centers that do not apply viral culture.

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Declaration of Competing Interest

None of the authors report any conflict of interest.

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