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MAJOR ARTICLE

Molecular and Culture-Based Bronchoalveolar Lavage Fluid Testing for the Diagnosis of Cytomegalovirus Pneumonitis

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Background. Cytomegalovirus (CMV) is a major cause of morbidity and mortality in immunocompromised patients, with CMV pneumonitis among the most severe manifestations of infection. Although bronchoalveolar lavage (BAL) samples are frequently tested for CMV, the clinical utility of such testing remains uncertain.

Methods. Retrospective analysis of adult patients undergoing BAL testing via CMV polymerase chain reaction (PCR), shell vial culture, and conventional viral culture between August 2008 and May 2011 was performed. Cytomegalovirus diagnostic methods were compared with a comprehensive definition of CMV pneumonitis that takes into account signs and symptoms, underlying host immunodeficiency, radiographic findings, and laboratory results.

Results. Seven hundred five patients underwent 1077 bronchoscopy episodes with 1090 BAL specimens sent for CMV testing. Cytomegalovirus-positive patients were more likely to be hematopoietic cell transplant recipients (26% vs 8%, $P < .0001$) and less likely to have an underlying condition not typically associated with lung disease (3% vs 20%, $P < .0001$). Histopathology was performed in only 17.3% of CMV-positive bronchoscopy episodes. When CMV diagnostic methods were evaluated against the comprehensive definition, the sensitivity and specificity of PCR, shell vial culture, and conventional culture were 91.3% and 94.6%, 54.4% and 97.4%, and 28.3% and 96.5%, respectively. Compared with culture, PCR provided significantly higher sensitivity and negative predictive value ($P \leq .001$), without significantly lower positive predictive value. Cytomegalovirus quantitation did not improve test performance, resulting in a receiver operating characteristic curve with an area under the curve of 0.53.

Conclusions. Cytomegalovirus PCR combined with a comprehensive clinical definition provides a pragmatic approach for the diagnosis of CMV pneumonitis.

Keywords. bronchoalveolar lavage; cytomegalovirus; diagnostics; pneumonitis.

Cytomegalovirus (CMV) is a common human viral pathogen with 45%–100% of adults demonstrating serologic evidence of CMV exposure [1]. Both primary infection and reactivation of latent virus may result in significant CMV-related disease in immunocompromised patients. In particular, CMV is an important cause of morbidity and mortality after hematopoietic cell transplantation (HCT) and solid organ transplantation (SOT) [2, 3].

Pneumonitis is among the most serious manifestations of CMV infection. Although the incidence of CMV pneumonitis in HCT recipients has decreased to approximately 1% to 3% due to the widespread use of CMV prevention strategies, the mortality associated with CMV pneumonitis remains high at 30%–50% [4]. In SOT, the incidence of CMV pneumonitis varies based on the type of organ transplanted [5]. Lung transplant recipients are at particularly high risk and have an overall CMV mortality estimated to be 2%–12% [6].

The definitive diagnosis of CMV pneumonitis is made via the detection of characteristic intranuclear inclusions or positive immunohistochemical staining in biopsied lung tissue [3]. This is a challenging diagnosis, however, because tissue for histopathologic evaluation is rarely obtained. Many of these patients have risk factors that preclude biopsy, such as significant thrombocytopenia or coagulopathy, leading physicians to forgo this invasive diagnostic procedure [7, 8].

Bronchoalveolar lavage (BAL) is frequently used as a less invasive means by which to access lung pathology and aid in the diagnosis of CMV pneumonitis. Compared with lung biopsy, BAL is more easily performed and results in fewer complications [8]. The lavage fluid can be sent for a variety of tests to detect CMV, including culture-based and polymerase chain reaction (PCR) methods. Several studies have investigated these diagnostic modalities in a variety of patient populations. Early

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studies evaluated the performance of conventional viral culture and rapid shell vial culture of BAL specimens from patients in which CMV pneumonitis was diagnosed based on histopathology [9–12]. Although shell vial and traditional viral BAL cultures demonstrated similar sensitivity and specificity, the number of histologically confirmed cases was low, and, overall, the sensitivity and specificity of shell vial and traditional culture compared with histopathology varied widely between studies.

Subsequent work evaluated CMV DNA detection in the BAL fluid of HCT, SOT, and acquired immune deficiency syndrome patients using conventional PCR strategies [13–15]. In addition, numerous studies investigated the performance of CMV PCR methods on BAL fluid specimens from lung transplant recipients [16–24]. Although definitive diagnosis of CMV pneumonitis consistently relied upon review of biopsied lung tissue, the definition of CMV lung disease in the absence of histological assessment differed substantially between studies. For example, some studies provided specific diagnostic criteria [12], whereas others used general clinical features without specific criteria [25]. As a consequence, CMV PCR, even with quantitative thresholds, and culture-based diagnostics demonstrated discordant sensitivities and specificities.

To address these limitations, we developed a comprehensive definition of CMV pneumonitis that takes into account clinical features, underlying host immunodeficiency, radiographic findings, and laboratory results. In this context, we evaluated the performance of CMV diagnostic methods in BAL specimens, including conventional viral culture and shell vial culture as well as qualitative and quantitative CMV PCR.

METHODS

Ethics

This study was reviewed and approved by the institutional review board of Stanford University.

Study Population

We retrospectively reviewed all BAL specimens sent for CMV PCR, shell vial culture, and conventional viral culture in patients >18 years old who underwent bronchoscopy at our institution between August 1, 2008 and May 31, 2011. Inclusion in the study required interpretable results for all 3 methods. Data were analyzed for individual specimens, bronchoscopy episodes, and patients in the cohort. Patients were categorized as CMV positive or CMV negative. Cytomegalovirus-positive patients were required to have at least 1 BAL specimen from 1 bronchoscopy episode that was positive for CMV by PCR, shell vial culture, or conventional viral culture. Cytomegalovirus-negative patients had negative results for all 3 methods in BAL fluid for each bronchoscopy episode, if more than 1 bronchoscopy was performed.

Laboratory Methods for the Detection of Cytomegalovirus

Bronchoalveolar lavage specimens were collected in sterile containers and stored at 2–8°C before testing. Specimens were vortexed for 30 s in the original container, then vortexed again for 30 s after the addition of 7–10 sterile glass beads. For CMV PCR, 100 µL of each specimen was extracted on the MagNA Pure LC (Roche, Indianapolis, IN) using the MagNA Pure LC DNA isolation kit with the DNA I Blood Cell High Performance protocol. Amplification and detection were performed using the Cobas Amplicor CMV Monitor and was setup manually using 50 µL of the 100 µL elution [26].

For culture methods, specimens were mixed 1:1 with antimicrobial mix containing gentamicin (150 µg/mL), vancomycin (1000 µg/mL), and amphotericin B (35 µg/mL). Of the processed specimen, 0.25 mL was inoculated onto each conventional tube culture monolayer including human foreskin (HF) fibroblasts (Quidel/Diagnostic Hybrids) and human embryonic lung MRC-5 fibroblasts (Viromed) containing 1.0–1.5 mL of the manufacturer's culture medium. Conventional viral cultures were incubated for 21 days at 35°C. Cell monolayers with demonstrable cytopathic effect (CPE) were scraped, washed with phosphate-buffered saline (PBS) (Sigma-Aldrich) spotted to Cel-Line Supercured HTC slides (Thermo Fisher Scientific), and fixed for 10 minutes in acetone. The cells were then stained for the presence of CMV by indirect immunofluorescence using anti-CMV antibodies targeting the immediate early antigen (Millipore) according to the manufacturer's specifications.

For shell vial cultures, 2 human fibroblast vials (1 HF and 1 MRC-5 from the vendors described above) were each inoculated with 0.25 mL processed BAL after aspiration of the culture medium. The vials were centrifuged at 3000 rpm for 30 minutes, the monolayers were refed, and the vials were incubated at 35°C. At 24 hours, the cell monolayer from 1 vial (HF) was washed with PBS, fixed with acetone, and stained as described above with the same antibody reagent used for conventional viral culture. The other vial was processed (MRC-5) at 48 hours. Results were reported only if the monolayers were >60% confluent, the negative control showed no specific fluorescence, and the positive control, cultured CMV AD169 reference strain (American Type Culture Collection), exhibited the expected apple green nuclear fluorescence. A positive result on a patient sample was reported if at least 1 vial had 2 or more cells that demonstrated this characteristic staining. Shell vials that demonstrated intense nonspecific fluorescence or excessive destruction of the monolayer by specimen toxicity or contamination were reported as unsatisfactory.

Histopathologic evaluation of formalin-fixed paraffin-embedded tissues included the assessment of hematoxylin and eosin (H&E)-stained slides for the degree and acuity of inflammation and the presence of viral-type inclusion bodies. Cytomegalovirus immunohistochemical staining was performed at the discretion of the attending pathologist, using an anti-CMV antibody blend consisting of 2 mouse monoclonal antibodies, CCH2 and DDG9 (Dako), that recognize immediate early and early antigens, respectively. Automated staining was

Table 1. Clinical Diagnostic Criteria for CMV Pneumonitis

1. At least 2 of the following (a, b, c):
    a. Signs/symptoms of pneumonia or evidence of organ dysfunction as manifested by 2 or more of the following:
        i. Fever
        ii. Cough
        iii. Dyspnea or tachypnea (RR > 20)[a]
        iv. $FEV_1 \leq 80\%$ of baseline
        v. New or increasing oxygen requirement
    b. Host factors/predisposing conditions: SOT, HSCT, chemotherapy, rituximab, leukemia/lymphoma
    c. Radiographic changes consistent with CMV pneumonia
        i. CXR: reticulonodular or interstitial infiltrates
        ii. CT: ground glass opacities (GGOs), small nodules (<1 cm), absence of large nodules, air space consolidation

AND

2. If a lung biopsy or autopsy was performed, this showed no evidence of a more likely explanation (acute rejection, GVHD, BCNU toxicity).

AND

3. Requires that no other pathogen is isolated in culture or identified by DFA/staining that represents a more likely cause of the patient's presentation and findings[b].

Abbreviations: BCNU, bis-chloroethylnitrosourea; CMV, cytomegalovirus; CT, computed tomography; CXR, chest x-ray; DFA, direct fluorescent antibody; FEV_1, forced expiratory volume in 1 second; GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; HSV, herpes simplex virus; RR, respiratory rate; SOT, solid organ transplant.

[a] Although 20 breaths/minute is not a normal respiratory rate, this value was selected because it is frequently documented when the patient is breathing comfortably on room air.

[b] Coagulase-negative *Staphylococcus*, oropharyngeal flora, *Candida* spp, *Mycobacterium gordonae*, nontyped mycobacterial species, diphtheroids, HSV-1, HSV-2, *Enterococcus*, and *Penicillium* would not be considered pathogens.

performed using the BOND-MAX instrument (Leica Biosystems), at a 1:100 dilution, with citrate-induced antigen retrieval, and the Bond Polymer Refine Detection system. Staining was visualized using diaminobenzidine as the chromogen.

Other Viral Diagnostic Methods

Respiratory viruses were evaluated by direct fluorescent antibody testing [27] at the discretion of the ordering clinician. In brief, 1 mL BAL fluid was transferred to a 15 mL conical tube, diluted with 9 mL PBS, and centrifuged at 2500 rpm to generate a cell pellet. After a second PBS wash, the well mixed sediment was spotted onto an 8-well Teflon masked slide. The cells were then fixed with acetone and stained with a respiratory virus direct fluorescent antibody panel (Quidel/Diagnostic Hybrids) that includes antibodies for the detection of influenza A, influenza B, respiratory syncytial virus (RSV), human metapneumovirus, adenovirus, and parainfluenza virus 1, 2, and 3. Samples were considered positive if 1 or more intact cells exhibited specific fluorescence. A minimum sampling of 15 ciliated columnar epithelial cells or alveolar macrophages per well was required for a negative result. All direct fluorescent antibody slides were read by at least 2 clinical laboratory scientists, which was standard protocol.

Bronchoalveolar lavage fluid for conventional viral culture was processed as described above. In addition to the fibroblast cell lines used for CMV identification, 0.25 mL of the processed specimen was inoculated onto human lung carcinoma A549 cells and Rhesus Monkey Kidney cells (both obtained from Quidel/Diagnostic Hybrids). Cell monolayers were monitored for CPE, and the presence of virus was confirmed by direct or indirect immunofluorescence, depending on the target virus (reagents from Quidel/Diagnostic or Merck/Millipore/Light Diagnostics; details available upon request). In addition to CMV, these cell lines support the isolation of a number of viruses that may be found in the lower respiratory tract, including influenza A, influenza B, RSV, adenovirus, parainfluenza virus 1, 2, and 3, herpes simplex virus (HSV)-1 and -2, and varicella-zoster virus. A laboratory-developed real-time, reverse-transcriptase PCR for influenza A was performed as described previously [27], and it was available from November 2009 through the end of the study period.

Criteria for the Diagnosis of Cytomegalovirus Pneumonitis

Based on review of the literature, we identified signs and symptoms, predisposing host conditions, and radiographic findings consistent with CMV pneumonitis (Table 1) [4, 25, 28–30]. The diagnosis of CMV pneumonitis required a clinical syndrome compatible with CMV pneumonitis, including the absence of a more likely cause for the patient's presentation, and a positive result for CMV by at least 1 test on BAL fluid (PCR, shell vial, or conventional culture).

Patients were considered to have a clinical syndrome compatible with CMV pneumonitis if they met at least 2 of 3 criteria: signs and symptoms, predisposing host condition, or radiographic findings. Signs and symptoms were defined as having 2 or more of the following: fever (temperature $\geq$38.5°C), cough, dyspnea or tachypnea (respiratory rate >20/minute), forced expiratory volume in 1 second (FEV_1) $\leq$ 80% of baseline, and new or increasing oxygen requirement. Predisposing underlying host conditions included HCT, SOT, leukemia or lymphoma, exposure to chemotherapy, and rituximab use. Radiographic findings for CMV pneumonitis were defined as the presence of reticulonodular or interstitial infiltrates on chest x-ray or the presence of ground glass opacities, small nodules <1 cm, absence of large nodules >1 cm, and air space consolidation on computed tomography chest imaging. Microbiologic or histopathologic findings that were considered a more likely cause for the patient's presentation included acute rejection, graft-versus-host disease, bis-chloroethylnitrosourea toxicity, or identification of other pathogens in bronchoscopy specimens, as described for viruses above and via routine microbiological cultures.

Statistical Analysis

Demographics, underlying host conditions, bronchoscopy episodes, and bronchoscopy indications in CMV-positive and CMV-negative patients were analyzed using univariate statistics as appropriate (Pearson χ^2 or Fisher exact test for categorical variables and Student's *t* test for continuous variables). Differences in quantitative CMV PCR viral loads in BAL fluid in those who did and did not meet histopathologic or clinical criteria for CMV pneumonitis were evaluated by Mann–Whitney *U* test. Receiver operating characteristic (ROC) curves were generated using XLSTAT 2014 (Microsoft, Redmond, WA). Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated with comparisons between tests made with Fisher exact testing. Statistical analysis was performed using XLSTAT 2014 (Microsoft).

RESULTS

Patient Characteristics

During the study period, 705 patients underwent 1077 bronchoscopy episodes and had 1090 specimens sent for CMV PCR, shell vial culture, and conventional viral culture. Sixteen bronchoscopy specimens (1.5%) from 6 patients were excluded due to uninterpretable shell vial cultures (n = 12), failed PCR (n = 2), and contaminated conventional viral culture (n = 2). Of the excluded specimens, 93.8% (15 of 16) were negative by the other 2 CMV BAL testing methods, whereas 1 of the conventional culture specimens excluded for contamination was positive for CMV by both PCR and shell vial culture. Of the 699 patients included in the analysis, the median age was 53.2 years old. Two hundred thirty-five (33%) patients were SOT recipients, 71 (10%) were HCT recipients, 85 (12%) had hematologic malignancy, and 77 (11%) had solid malignancy.

Ninety patients tested positive for CMV in BAL fluid by PCR, shell vial culture, or conventional viral culture. One hundred seven positive specimens were obtained from these patients during 103 bronchoscopy episodes. Cytomegalovirus-positive patients were more likely to be HCT recipients (26% vs 8%, $P < .0001$), less likely to have an underlying condition not typically associated with lung disease (3% vs 20%, $P < .0001$), and had more bronchoscopy episodes per patient (1.81 vs 1.49, $P = .019$) compared with CMV-negative patients. The most frequent bronchoscopy indication was for respiratory compromise, which was also significantly higher in CMV-positive patients (69% vs 59%, $P = .031$). Table 2 outlines patient characteristics, and Table 3 summarizes bronchoscopy episodes, specimens, and indications.

Histopathologic Evaluation

Biopsy was performed in 20 of the 104 (19.2%) bronchoscopy episodes that were positive for CMV by PCR, shell vial culture, or conventional viral culture. In 18 episodes from 16 patients, sufficient tissue was obtained for histopathology. One histopathology specimen demonstrated inclusions consistent with CMV. Eight of the specimens did not reveal any histopathological changes, 3 demonstrated malignancies, 2 showed mild acute rejection, and 4 showed bronchiolitis. Only 1 specimen was tested for CMV by immunochemistry (IHC). Neither CMV antigen nor histopathological changes were identified.

Of the patients with positive CMV tests on BAL who died during the study period, 6 had at least 1 BAL collected within 30 days of death (all without concurrent biopsy) and underwent autopsy that included evaluation of the lungs. Four autopsies showed no histopathologic evidence of CMV infection, whereas 2 autopsies revealed CMV pneumonitis demonstrated by

Table 2. Characteristics of Patients Who Underwent BAL With Specimens Sent for CMV Testing[a]

Characteristic	CMV Positive (n = 90)[b]	CMV Negative (n = 609)	*P* Value
Age, y, median (range)	52.9 (18.4–86.2)	55.7 (18.0–92.6)	ns
HCT (%)	24 (26)	47 (8)	<.0001
Allogeneic-MRD	14 (58)	14 (30)	.038
Allogeneic-MUD	7 (29)	21 (45)	ns
Allogeneic-Haploidentical	0 (0)	1 (2)	ns
Autologous	3 (13)	11 (22.4)	ns
SOT (%)	28 (31)	207 (33)	ns
Heart	2 (7)	12 (6)	ns
Lung	18 (65)	135 (65)	ns
Heart-lung	3 (10)	28 (14)	ns
Other SOT[c]	5 (17)	32 (15)	ns
Hematologic malignancy (%)	9 (10)	75 (12)	ns
Acute leukemia[d]	2 (22)	45 (60)	.031
Other hematologic malignancy[e]	7 (78)	30 (40)	.031
Solid malignancy (%)[f]	8 (9)	69 (11)	ns
Connective tissue disease (%)[g]	6 (6)	17 (3)	ns
Other immune deficiency (%)[h]	2 (2)	5 (1)	ns
Chronic lung disease (%)[i]	12 (13)	52 (8)	ns
Liver disease (%)[j]	0 (0)	17 (3)	ns
Other (%)[k]	3 (3)	122 (20)	<.0001

Abbreviations: BAL, bronchoalveolar lavage; CMV, cytomegalovirus; HCT, hematopoietic cell transplant; SOT, solid organ transplant; MRD, matched related donor; MUD, matched unrelated donor; PCR, polymerase chain reaction.

[a] Conventional culture, shell vial culture, and CMV PCR in BAL fluid.

[b] Defined as patients with at least 1 specimen positive for CMV by PCR, shell vial culture, or conventional culture from BAL fluid.

[c] Other SOT includes kidney, liver, heart-kidney, heart-liver-kidney, heart-lung-kidney, kidney-pancreas, liver-kidney, and lung-kidney.

[d] Acute leukemia includes acute myelogenous leukemia, acute lymphocytic leukemia, acute promyelocytic leukemia, and acute myelomonocytic leukemia.

[e] Other hematologic malignancy includes lymphoma, chronic lymphocytic leukemia, hairy cell leukemia, multiple myeloma, Waldenstrom's macroglobulinemia, myelofibrosis, and myelodysplasia.

[f] Solid malignancy includes lung, breast, esophageal, gastric, colon, pancreas, prostate, cervical, bladder, endometrial, kidney, melanoma, glioblastoma, thymoma, cholangiocarcinoma, hepatocellular carcinoma, Wilm's tumor, and sarcoma.

[g] Connective tissue disease includes lupus, scleroderma, sarcoidosis, rheumatoid arthritis, Wegner's granulomatosis, Still's disease, giant cell arteritis, Addison's disease, and myasthenia gravis.

[h] Other immune deficiency includes human immunodeficiency virus and common variable immunodeficiency.

[i] Chronic lung disease includes interstitial lung disease, chronic obstructive pulmonary disease, bronchiectasis, pulmonary hypertension, allergic bronchopulmonary aspergillosis, and bronchiolitis obliterans.

[j] Liver disease includes cirrhosis, chronic liver disease, and fulminant hepatic failure.

[k] Other includes none or unrelated conditions.

Table 3. Summary of BAL Episodes, Specimens, and Indications in Patients Who Underwent CMV Testing[a]

Variable	CMV Positive (n = 90)[b]	CMV Negative (n = 609)	*P* Value
Episodes per patient (range)	1.81 (1–8)	1.47 (1–10)	.019
Positive episodes (%)	103 of 163 (63)	0 of 898 (0)	
Positive specimens (%)	107 of 168 (64)	0 of 906 (0)	
Indication (% of total episodes)			
Respiratory symptoms[c]	112 (69)	532 (59)	.031
Transplant-related[d]	32 (19)	221 (25)	ns
Imaging abnormality[e]	16 (10)	99 (11)	ns
Other[f]	3 (2)	46 (5)	ns

Abbreviations: BAL, bronchoalveolar lavage; CMV, cytomegalovirus; ns, not significant; PCR, polymerase chain reaction.

[a] Conventional culture, shell vial culture, and CMV PCR in BAL fluid.

[b] Defined as patients having at least 1 specimen positive for CMV on bronchoalveolar lavage fluid by PCR, shell vial culture, or conventional culture.

[c] Includes hemoptysis, diffuse alveolar hemorrhage, atelectasis, airway obstruction, decreased pulmonary function with or without imaging abnormalities.

[d] Surveillance for rejection with or without respiratory symptoms or imaging abnormalities.

[e] Imaging abnormalities alone.

[f] Undefined indications or unrelated conditions such as for pulmonary toilet, lymphadenopathy, and pretransplant evaluation.

characteristic viral inclusions. One was CMV IHC positive, and although CMV IHC was not performed in the other, PCR testing of the autopsy lung tissue detected CMV DNA.

Thus, of the 24 bronchoscopy episodes from 22 patients with at least 1 positive CMV test and available histopathology, a pathology-confirmed diagnosis of CMV pneumonitis was obtained for 3 episodes from 3 patients. All 3 episodes also met the study criteria for CMV pneumonitis. Of the remaining 21 episodes from 19 patients without histopathologic evidence for CMV end-organ disease, 7 episodes from 7 patients met clinical and laboratory diagnostic criteria for CMV pneumonitis.

Cytomegalovirus Detection in Bronchoalveolar Lavage Fluid

To better understand the role of CMV PCR, shell vial culture, and conventional viral culture of BAL fluid for the diagnosis of CMV pneumonitis, we calculated the characteristics for each test compared with a composite reference that included the criteria outlined in Table 1 and a positive result for CMV by at least 1 test on BAL fluid (Table 4). The sensitivity of PCR was significantly higher than both shell vial culture (91.3% vs 54.4%, $P < .001$) and conventional viral culture (91.3% vs 28.3%, $P < .001$). The sensitivity of shell vial culture was also significantly higher compared with conventional culture (54.4% vs 28.3%, $P = .012$). In addition, the specificity of PCR was significantly lower than both shell vial culture (94.6% vs 97.4%, $P < .001$) and conventional viral culture (94.6% vs 96.5%, $P < .001$), whereas no significant difference in specificity was observed between the culture methods (97.4% vs 96.5%, $P = .085$). No significant differences in PPV were found between

Table 4. Test Characteristics of CMV Detection Methods in BAL Specimens

	CMV Pneumonitis[a]					
Test	(+)	(–)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
PCR (+)	42	56	91.3	94.6	42.9	99.6
PCR (–)	4	972				
Shell vial culture (+)	25	27	54.4	97.4	48.1	97.9
Shell vial culture (–)	21	1001				
Conventional culture (+)	13	15	28.3	96.5	46.4	96.9
Conventional culture (–)	33	1013				
PCR and shell vial (+)	21	23	45.7	97.8	47.4	97.6
PCR and/or shell vial (–)	25	1005				
PCR and culture (+)	12	13	26.1	98.7	48.0	96.8
PCR and/or culture (–)	34	1015				
Shell vial and culture (+)	10	13	21.7	98.7	43.5	96.6
Shell vial and/or culture (–)	36	1015				
PCR, shell vial, and culture (+)	9	12	19.6	98.8	42.6	96.6
PCR, shell vial, and/or culture (–)	37	1016				
PCR and/or shell vial (+)	46	60	100.0	94.2	43.4	100.0
PCR and shell vial (–)	0	968				
PCR and/or culture (+)	43	58	93.4	94.4	42.6	99.7
PCR and culture (–)	3	970				
Shell vial and/or culture (+)	28	29	60.8	97.2	49.1	98.2
Shell vial and culture (–)	18	999				
PCR, shell vial, and/or culture (+)	46	61	100.0	94.1	42.3	100.0
PCR, shell vial, and culture (–)	0	967				

Abbreviations: BAL, bronchoalveolar lavage; CMV, cytomegalovirus; NPV, negative predictive value; PCR, polymerase chain reaction; PPV, positive predictive value.

[a] Criteria for CMV pneumonitis as defined in Table 1 and Methods.

any of the methods. However, the NPV of PCR was significantly higher compared with both shell vial (99.6% vs 97.9%, $P = .001$) and routine culture (99.6% vs 96.9%, $P < .001$). The NPV of shell vial and routine culture was not statistically different ($P = .130$).

Requiring positive results for PCR and 1 or both of the culture methods resulted in significantly lower sensitivity and significantly higher specificity compared with PCR testing alone ($P < .001$). Although there were no significant differences in PPV, the NPV of PCR testing alone was significantly higher compared with combinations requiring positive results from both PCR and culture ($P < .001$). Alternatively, if positive laboratory testing was defined as a positive result in at least 1 of 2 methods including PCR, or 1 of the 3 methods, no significant differences in sensitivity, specificity, PPV, or NPV were observed compared with PCR alone. In sum, PCR testing alone offers optimal NPV and similar PPV when compared with the various combinations of tests.

When test characteristics in HCT recipients were compared with test characteristics in patients with other underlying diseases, the sensitivity, specificity, PPV, and NPV of shell vial cultures and conventional cultures were not significantly different. Likewise, PCR demonstrated no significant differences in sensitivity, specificity, and NPV. However, PCR had a significantly higher PPV in HCT recipients compared with patients with other underlying diseases (76.9% vs 30.1%, $P = .02$). Furthermore, in HCT recipients, as described above, PCR testing alone resulted in optimal test characteristics compared with the various possible combinations of CMV tests.

Cytomegalovirus quantitation in BAL fluid by PCR was also evaluated to determine whether a threshold could be identified to aid in the diagnosis of CMV pneumonitis. A ROC curve was generated, and the area under the curve (AUC) was 0.53. Furthermore, quantitative CMV DNA levels in BAL fluid were not statistically different between those who did and did not meet the study criteria for CMV pneumonitis ($P = .635$). We generated a ROC curve specifically for HCT recipients and found moderate improvement in the AUC of 0.70, with the optimal viral load threshold of 791 copies/mL yielding a sensitivity of 95.0%, specificity 50%, PPV 86.4%, and NPV 75.0%. In addition, no significant difference in DNA levels was identified between those who did and did not meet criteria for CMV pneumonitis in HCT recipients ($P = .142$).

DISCUSSION

In this report, we evaluated the performance of CMV diagnostic methods in the largest set of BAL specimens to date, collected from patients with a wide variety of underlying diseases including, but not limited to, HCT and SOT. We compared conventional viral culture and shell vial culture, as well as qualitative and quantitative CMV PCR, with a comprehensive definition of CMV pneumonitis that takes into account signs and symptoms, predisposing host conditions, radiographic findings, laboratory results, and an assessment of the most likely cause in a differential diagnosis. The intention for creating this definition was to provide a framework for the diagnosis of CMV pneumonitis that is systematic and reflective of clinical practice.

Utilizing this comprehensive definition as reference, comparison of BAL CMV diagnostic methods revealed that PCR provided significantly higher sensitivity and NPV compared with both shell vial and conventional cultures. These results suggest that CMV PCR of BAL may be most effectively used to rule out CMV pneumonitis. The high sensitivity of PCR was achieved with a moderate loss of specificity, which was significantly lower than the culture methods, although all CMV tests and combinations of tests demonstrated specificities ≥94.1%.

Although clinicians frequently interpret culture as providing an improved PPV, we found that shell vial culture and conventional culture individually and in combination with PCR demonstrated consistently low PPV, ranging from 42.3% to 49.1%. Although these values, in part, reflect the relatively low prevalence of CMV pneumonitis in the overall study population, no significant differences in PPV were identified compared with PCR testing alone. Furthermore, requiring positive results from either shell vial or conventional culture and PCR resulted in high false-negative rates, suggesting that phasing out culture methods in favor of molecular diagnostics, as is occurring in many clinical microbiology and virology laboratories, is unlikely to impact the diagnosis of CMV pneumonitis. It is notable that a subset analysis of HCT recipients revealed a higher PPV of PCR in HCT recipients compared with the other groups, which was likely a reflection of the higher prevalence of patients who met clinical criteria in HCT recipients in our cohort. This finding suggests improved utility of a positive PCR result for the diagnosis of CMV pneumonitis in patients that had undergone HCT.

We also investigated CMV quantitation in BAL fluid as a means by which to further improve test performance. Results from previous studies in lung transplant recipients have been mixed, with 1 study identifying a threshold for CMV pneumonitis of 500 000 copies/mL [22] and others showing no correlation of CMV levels with pneumonitis or bronchiolitis obliterans, respectively [18, 24]. Similar to the latter studies, we found CMV levels to be highly variable overall, as well as in the subset of HCT recipients, providing limited improvement in discriminating between those cases that met the criteria for CMV pneumonitis and those that did not. These observations may be due, in part, to the variation in fluid volume and cells collected from each lavage and the focal nature of CMV end-organ disease.

Although the identification of characteristic inclusions by H&E and CMV antigens by IHC remain the reference methods for the diagnosis of CMV pneumonitis, lung biopsy tissue was infrequently obtained in this study during episodes in which

CMV was detected by any laboratory method in BAL specimens. Furthermore, although H&E staining has been shown to be insensitive compared with IHC for the detection of CMV-infected cells in lung biopsy tissue [21, 31], CMV IHC was performed in only 5.6% (1 of 18) of total episodes and in none of the subset of 7 episodes that met clinical and laboratory criteria for CMV pneumonitis. This limited and suboptimal use of lung biopsy tissue brings into question the benefit of this invasive procedure, and it suggests that the comprehensive definition developed in this manuscript may provide a more practical approach to the diagnosis of CMV pneumonitis.

Further limitations include the observational design of the study and the absence of data regarding the clinical progression of patients that tested positive for CMV in BAL fluid and met clinical criteria but did not receive anti-CMV therapy. These patients comprise 34.8% (16 of 46) of the CMV-positive patients that met our clinical definition of CMV pneumonitis; future prospective studies will be required to assess disease progression in such patients. Although the clinical criteria coupled with a positive CMV test in BAL fluid was used for the diagnosis of CMV pneumonitis, including the exclusion of a more likely explanation for the patient's presentation, it is possible that other diseases could have accounted for the patient's presentation. This possibility would decrease decrease the false-positive rate and yield inflated test performance characteristics, particularly with specificity. This study is also limited by the lack of consistent, concurrently performed plasma viral load testing, which precluded a detailed analysis of the concordance between CMV detection and quantitation in plasma and BAL specimens. In addition, the CMV PCR method utilized in this study, the Roche COBAS Amplicor CMV Monitor, is analytically less sensitive than more recently developed CMV PCR assays, such as the Roche COBAS Ampliprep/COBAS TaqMan CMV and Qiagen *artus* CMV [26, 32–35]. The adaptation of such CMV DNA assays to BAL fluids may further increase clinical sensitivity at the expense of clinical specificity. Finally, the CMV PCR in this study was not calibrated to the international standard; however, the wide range of CMV levels detected in BAL samples would remain unchanged whether reported in international units or copies.

CONCLUSIONS

In conclusion, this study confirms the imperfections of CMV diagnostics in BAL specimens, but, in so doing, it introduces a systematic, clinically oriented definition of CMV pneumonitis. The development of comprehensive criteria is one strategy for disease diagnosis that is particularly well suited for target conditions that are difficult to unequivocally define [36]. In the case of CMV pneumonitis, the definitive diagnostic procedure, tissue biopsy with subsequent histopathology, is infrequently performed in routine practice and itself may lack sufficient sensitivity. Based on our findings, we suggest a practical approach to this diagnostic dilemma that combines the comprehensive clinical definition with highly sensitive CMV nucleic acid amplification testing. Future studies will be required to evaluate this approach and to identify additional biomarkers that will be able to improve the diagnosis of CMV pneumonitis.

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