

Development and validation of a *Pneumocystis jirovecii* real-time polymerase chain reaction assay for diagnosis of *Pneumocystis pneumonia*

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BACKGROUND: *Pneumocystis jirovecii* (PJ), a pathogenic fungus, causes severe interstitial *Pneumocystis pneumonia* (PCP) among immunocompromised patients. A laboratory-developed real-time polymerase chain reaction (PCR) assay was validated for PJ detection to improve diagnosis of PCP.

METHODS: Forty stored bronchoalveolar lavage (BAL) samples (20 known PJ positive [PJ+] and 20 known PJ negative [PJ-]) were initially tested using the molecular assay. Ninety-two sequentially collected BAL samples were then analyzed using an immunofluorescence assay (IFA) and secondarily tested using the PJ real-time PCR assay. Discrepant results were resolved by retesting BAL samples using another real-time PCR assay with a different target. PJ real-time PCR assay performance was compared with the existing gold standard (ie, IFA) and a modified gold standard, in which a true positive was defined as a sample that tested positive in two of three methods in a patient suspected to have PCP.

RESULTS: Ninety of 132 (68%) BAL fluid samples were collected from immunocompromised patients. Thirteen of 92 (14%) BALs collected were PJ+ when tested using IFA. A total of 40 BAL samples were PJ+ in the present study including: all IFA positive samples (n=13); all referred PJ+ BAL samples (n=20); and seven additional BAL samples that were IFA negative, but positive using the modified gold standard. Compared with IFA, the PJ real-time PCR had sensitivity, specificity, and positive and negative predictive values of 100%, 91%, 65% and 100%, respectively. Compared with the modified gold standard, PJ real-time PCR had a sensitivity, specificity, and positive and negative predictive values of 100%.

CONCLUSION: PJ real-time PCR improved detection of PJ in immunocompromised patients.

Key Words: *Diagnosis; Immunocompromised; P jirovecii; Pneumocystis pneumonia; Real-time PCR*

Pneumocystis jirovecii (PJ) is an opportunistic fungal pathogen that causes *Pneumocystis pneumonia* (PCP) in humans (1). PCP is an important cause of mortality and morbidity in patients with immunosuppression, particularly among those with HIV infection (2-4). Severe PCP pneumonia has an in-hospital mortality between 7% to 11%; however, in critically-ill patients, it can be as high as 29% to 62%, especially where an etiological diagnosis is delayed (5). Although the incidence of PCP in HIV-infected patients has decreased with the advent of anti-retroviral therapy and chemoprophylaxis (6), its incidence among

Mise au point et validation d'un test de réaction en chaîne par polymérase en temps réel du *Pneumocystis jirovecii* pour diagnostiquer une pneumonie à *Pneumocystis*

HISTORIQUE : Le *Pneumocystis jirovecii* (PJ), un champignon pathogène, provoque une grave pneumonie à *Pneumocystis* interstitielle (PPC) chez les patients immunodéprimés. Les chercheurs ont validé un test de réaction en chaîne par polymérase (PCR) en temps réel pour détecter le PJ et ainsi améliorer le diagnostic de PPC.

MÉTHODOLOGIE : Les chercheurs ont d'abord vérifié 40 prélèvements de liquide bronchoalvéolaire (LBA) entreposés (20 cas positifs connus au PJ [PJ+] et 20 cas négatifs connus au PJ [PJ-]) au moyen du test moléculaire. Ils ont ensuite analysé 92 prélèvements séquentiels de LBA au moyen d'un test par immunofluorescence (IFA), puis d'un test de PCR en temps réel du PJ. Ils ont résolu les résultats divergents au moyen d'un nouveau test par PCR en temps réel des prélèvements de LBA axée sur une autre cible. Ils ont comparé le résultat du test de PCR en temps réel du PJ à la référence absolue (l'IFA) et à une référence modifiée, dans laquelle un véritable cas positif désignait un prélèvement positif par deux méthodes sur trois chez un patient atteint d'une PPC présumée.

RÉSULTATS : Quatre-vingt-dix prélèvements de LBA (68 %) sur 132 provenaient de patients immunodéprimés. Treize prélèvements de LBA (14 %) sur 92 étaient PJ+ d'après l'IFA. Dans la présente étude, 40 prélèvements de LBA étaient PJ+, y compris tous les prélèvements positifs à l'IFA (n=13), tous les prélèvements de LBA PJ+ aiguillés (n=20) et sept autres prélèvements de LBA négatifs à l'IFA, mais positifs selon la référence modifiée. Par rapport à l'IFA, la PCR en temps réel du PJ avait une sensibilité, une spécificité et des valeurs prédictives positive et négative de 100 %, 91 %, 65 % et 100 %, respectivement. Par rapport à la référence modifiée, la PCR en temps réel du PJ avait une sensibilité, une spécificité et des valeurs prédictives positive et négative de 100 %.

CONCLUSION : La PCR en temps réel du PJ en améliore la détection chez les patients immunodéprimés.

the non-HIV immunocompromised population has increased (7). Early diagnosis and treatment is critical for survival of all patients diagnosed with PCP, regardless of HIV status (8). Laboratory detection of PJ in lower respiratory samples must be highly sensitive and specific, so as not to miss any potential cases of PCP, while still being able to avoid unnecessary antimicrobial treatment in patients with asymptomatic colonization.

Many clinical microbiology laboratories continue to rely on direct microscopic detection of the cyst form of *Pneumocystis* in stained lower

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respiratory tract samples (9,10). Laboratory-developed molecular methods have recently been reported, and have a higher sensitivity for the detection of PJ in lower respiratory tract samples compared with microscopy (11-14). Due to a lower cyst burden, diagnosis of PCP may be difficult using microscopic methods for immunocompromised patients without HIV infection, and in HIV-infected patients receiving anti-retroviral therapy. Conventional staining and microscopic examination of bronchoalveolar lavage (BAL) fluid ranged between 70% and 92% in non-HIV infected immunocompromised patients, while the sensitivity for sputa was even lower, between 38% and 53% (14,15). Although several laboratory-developed qualitative PJ real-time polymerase chain reaction (PCR) assays had been reported for the diagnosis of PCP (16-20), at the time of the present study, no commercial molecular assays were available (21). The present study describes the clinical and laboratory development, and validation of a unique qualitative PJ real-time PCR assay for the rapid detection of PCP.

METHODS

Clinical setting

Calgary Laboratory Services (CLS) is a large regional centralized laboratory that provides diagnostic testing for infectious diseases for a population of approximately 1.5 million in Calgary and southern Alberta. Approximately 1200 BAL fluid samples are submitted each year from immunocompromised patients for PJ detection. Immunocompromised patients in the present study included those with solid organ or hematopoietic stem cell transplants, HIV or malignancy (solid/haematological). Demographical data and brief clinical history were initially obtained for each enrolled patient through completed laboratory requisitions, which included the patient's age, sex, date of birth and reason for immune compromise including the presence of neutropenia. The CLS study microbiologist (DLC) also subsequently reviewed positive PJ real-time PCR results with the attending physician to determine whether that positive result correlated with the presence of PCP versus asymptomatic colonization.

Sample collection

A total of 132 BAL fluid samples were collected from patients in whom PCP was considered in the differential diagnosis and tested during the study. Forty BAL fluid samples collected between 2006 and 2012 were obtained (20 known PJ positive [PJ+] and 20 known PJ negative [PJ-]) from St Paul's Hospital, Vancouver, British Columbia, designated as the referral laboratory (RL). All BAL samples from the RL were initially analyzed using microscopic examination after staining, and secondarily tested using their laboratory developed PJ real-time PCR assay (see Methods). Aliquots of the BAL fluid samples were then stored frozen at -80°C until referral. Another 92 BAL fluid samples were sequentially collected retrospectively at CLS between May 2011 and September 2012. Aliquots of the BAL fluid samples were frozen at -80°C after initially being tested using an immunofluorescence assay (IFA). Storage of multiple sample aliquots allowed later use in discrepant analysis without an additional freeze-thaw. If multiple BAL fluid samples were stored from the same patient, each was tested individually and not pooled.

Microscopic examination

All BAL fluid samples from the RL were initially analyzed using Toluidine Blue O stain (22) and/or a modified Gomori's methenamine silver borate stain and microscopic examination. All of the BAL fluid samples collected at CLS were initially tested using a commercial direct IFA. The Genetic Systems Monofluor *Pneumocystis* (Meridian Bioscience Inc, USA) immunofluorescence assay was performed according to the manufacturer's instructions.

CLS PJ real-time PCR assay

The BAL fluid samples were centrifuged before DNA extraction. DNA was extracted from stored BAL samples (180 μL aliquot) using the QuickGene 810 on the DNA Tissue Mode program according to

the manufacturer's protocol (FujiFilm Life Sciences, Japan). The QuickGene 810 extraction procedure typically yields 100 μL of eluent. A DNA purity check of the extract was performed by measuring the 260/280 nm ratio using a spectrophotometer (ND-100, NanoDrop Technologies Inc, USA), which consistently yielded 260 nm to 280 nm ratios of approximately 1.8. Aliquots (10 μL) of the DNA extract were stored frozen at -80°C .

Five microlitres of this DNA extract was used for each real-time PCR assay. The PJ real-time PCR assay was based on a previously described method with a positive cut-off cycle threshold (Ct) ≤ 37 (23). Integrated DNA Technologies (www.idtdna.com) supplied the hybridization probe and primers specific for the mitochondrial large subunit (mtLSU) ribosomal RNA gene of PJ. The forward primer (mtLSU FWD: 5'-TGG TAA GTA GTG AAA TAC AAA TCG G-3' [start = 178, stop = 203]) and the reverse primer (mtLSU REV: 5'-ACT CCC TCG AGA TAT TCA GTG C-3' [start = 277, stop = 299]) were identical to those previously published (18). However, the sequence of the hybridization probe (mtLSU PRB: 5'-/56-FAM/ ACT AGG ATA / ZEN/TAG CTG GTT TTC TGC GA/3IABkFQ/-3') and the fluorescent dyes used to tag the 5' and 3' probe ends differed from those previously published (23). PJ real-time PCR reactions contained 20 μL SmartMix HM Mastermix (5 Smart Mix beads, one QC bead, 185 μL of Sigma H₂O and 5 μL of each of the primers and the probe) and 5 μL purified DNA. During each run, a PJ DNA positive control and a template negative control were included. The SmartCycler (Cepheid, USA) program followed 50 cycles of 10 s at 95°C , 10 s at 60°C and 10 s at 72°C .

A positive patient sample that was IFA and PJ real-time PCR positive (ie, with an estimated 1×10^5 copies/mL) was used to estimate the assay's limit of detection. The BAL sample was serially diluted approximately 10,000-fold that of the original material before the PJ real-time PCR became negative (ie, no amplification signal above baseline). The dilution series of samples were also repeatedly tested (ie, up to six times) using the real-time PCR assay to establish the reproducibility of the Ct values for PJ+ samples over the range of dilutions in which PJ was detected (ie, 1:2, up to 1: approximately 10,000). Clinical BAL fluid samples were also tested in duplicate on three separate days to establish the precision of the assay, including two negative and three positive samples within the low, mid and high Ct range of the assay.

RL PJ real-time PCR assay

The St Paul's Hospital laboratory-developed PJ real-time-PCR assay was adapted from one previously published (19). Briefly, 400 μL of BAL fluid in phosphate-buffered saline was extracted with 10 μL of internal amplification control according to the total nucleic acid isolation blood protocol using a Roche Magna Pure Compact instrument (Roche Molecular Diagnostics, USA) and eluted in 100 μL . Respiratory samples containing thick mucus were pretreated with proteinase K (25 μL 1 g/mL) and sodium dodecyl sulfate then lysed using zirconium beads (150 μL 2.4 mm) and silica beads (50 μL 0.1 mm) (Biospec Products, USA) before DNA extraction. Primers and fluorescence resonance energy transfer probes were previously described, and were designed to detect a 166-base pair region of the *cdc2* gene of PJ (19). PCR was performed on 5 μL of DNA extract using the LightCycler-FastStart DNA Master Hybridization Probes kit (Roche Diagnostics, Canada) in the presence of 4 mmol/L MgCl₂ using the Light Cycler 2.0 (Roche Diagnostics, Canada). Melting curve analysis was performed at the end of the *cdc2* PCR amplification to confirm positives. All primers and probes were synthesized by Metabion (Germany).

Data analysis

All patient information and PJ test results were entered into an Excel spreadsheet (Microsoft Corporation, USA) in an anonymous non-nominal fashion using the patient study number. Descriptive statistics were performed using standard methods. Performance of the CLS PJ real-time PCR assay was compared with the IFA as the existing gold

standard during the study period. Discrepancies between IFA and the CLS PJ real-time PCR assay were resolved by secondarily testing the discrepant BAL samples with the PJ real-time PCR used by the RL (see Methods). The modified gold standard (ie, a true positive result) was defined as a BAL sample that tested positive according to both molecular assays and/or IFA in a patient suspected of having PCP. The presence of PCP was not only defined by the PJ test, but also after clinical review of each patient by DLC with the attending physician. Performance of each test was calculated using standard statistical methods with the modified gold standard. All statistical analyses were performed using standard methods with Analyze-It software version 2.6.1 (Microsoft Corporation, USA).

Ethics

Once obtained, all clinical information was handled in an anonymous fashion in the working dataset at each site. BAL samples from the RL were provided in an anonymous fashion to ensure patient confidentiality. The study was granted ethics approval by the Conjoint Health Research Ethics Board at the University of Calgary/Alberta Health Services (Calgary, Alberta) and St Paul's Hospital/University of British Columbia (Vancouver, British Columbia) Ethics Review Boards.

RESULTS

Patient population

A total of 132 BAL samples from 127 patients were analyzed in the present study. Almost all of the 20 PJ+ samples sent from the RL were collected from HIV infected individuals (19 of 20 [95%]); the remaining patient was receiving corticosteroids due to a central nervous system lymphoma. Most of the 20 PJ- BAL samples from the RL were also collected from immunocompromised patients (19 of 20 [95%]), including eight patients with HIV infection, nine transplant patients, one patient with myelodysplasia and one patient with systemic vasculitis being treated with immunosuppressive agents.

A total of 92 BAL samples from 87 patients were collected by CLS; both a right and left BAL fluid sample were tested for five patients. According to the clinical history provided on the laboratory requisition, 50 of 87 (57.5%) of the study samples were collected from immunocompromised patients including: nine who had HIV (18%), 23 who were solid-organ transplant recipients (46%), two who had received previous hematopoietic stem cell transplantation (4%) and 14 with malignancies (28%). The remaining 4% of the sample order requisitions did not provide descriptions of the cause of their immunosuppression.

CLS PJ real-time PCR assay validation

The PJ real-time PCR was initially used to test the 40 clinical samples (20 known PJ+ and 20 known PJ-) from the RL, with consistent results. The developed molecular assay could detect the approximately 1:10,000 diluted sample estimated to contain 10 cysts of organism per 2 µL, yielding an estimated limit of detection of at least 10 copies per PCR. Amplification of the serially diluted BAL fluid sample (ie, estimated to contain 1×10⁵ cysts) generated Ct values of 20 for a 1:2 dilution, up to 37 for the 1:10,000 dilution. The CLS real-time PCR assay also provided excellent reproducibility. Both PJ- BAL fluid samples repeatedly tested negative including a Ct value of 0 on separate runs repeated on different days, and in two carryover runs performed back to back on the same day. The three PJ+ BAL fluid samples also repeatedly tested positive with a mean (± SD) Ct value of 20.85±0.13, 20.78±0.08, 21.2±0.12, 21.8±0.12 and 21.9±0.19, respectively, for the individual samples on two separate runs repeated on different days, and in two carryover runs performed back to back on the same day. When CLS PJ real-time PCR was performed on the 40 clinical samples (20 known PJ+ and 20 known PJ- samples) sent from the RL, similar results were found for all samples.

Figure 1 compares range and CIs for the PJ real-time assay Ct compared with the detection of PJ cysts using IFA. The 95% upper CI for PJ+ BAL fluid samples that were detected by both the IFA and

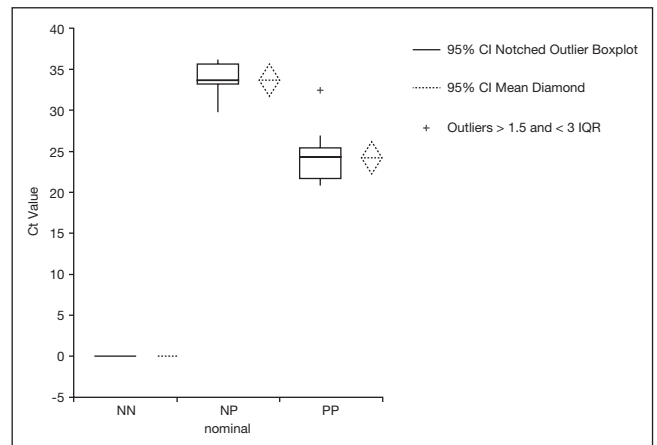


Figure 1 Comparison of the *Pneumocystis jirovecii* (PJ) real-time polymerase chain reaction (PCR) assay to that of immunofluorescence assay (IFA) to confirm the diagnostic cut-off of the assay. Ct Cycle threshold; IQR Interquartile range; NN Negative according to both IFA and PJ real-time PCR; NP Negative according to IFA and positive according to PJ real-time PCR; PP Positive according to both IFA and PJ real-time PCR

molecular assays was 26 cycles. The lower and upper 95% CIs for BAL fluid samples that were PJ- according to IFA, but PJ+ according to molecular assay, was 30 and 37 cycles, respectively. Therefore, the reportable range for the PJ real-time assay was established as follows: Ct ≤30 cycles = true positive PJ result; Ct ≥30 but <37 = low positive result that may be indicative of early infection or colonization in which clinical correlation is required to establish the presence of disease; and >37 = negative result.

The clinical performance of the PJ real-time PCR assay was compared with both the IFA and the modified gold standard. PJ cysts were detected in a total of 13 of 92 (15%) BAL fluid samples. Twelve of 13 (92.3%) of these PJ+ samples were derived from immunocompromised patients, while the clinical history for the last sample only mentioned pneumonia. Eight patients had HIV infection, three had a malignancy, and one had immunosuppression for an unspecified reason. All of the BAL fluid samples that were PJ+ according to IFA were also positive according to the molecular assay with Ct values ≤30 cycles. Another seven samples from six patients that were PJ- according to IFA, were PJ+ according to the PJ real-time PCR assay. The Ct values for these discrepant samples were all >30 but <37 (ie, 30.9, 33.9, 35.9, 33.6, 36.1, 33.2 and 34.9). Clinical review regarding these results with the attending physician enabled these results to be correlated with the presence of PCP in all cases. Two of these samples were taken from a patient (ie, right lower lobe and left lower lobe samples) with interstitial lung disease receiving high-dose steroids who was considered to have early infection. The remaining five patients had clinical PCP, and included one patient with HIV infection, one with a brain tumour receiving high-dose steroids, one hematopoietic stem cell transplantation patient with severe hypoxemia, one patient with interstitial lung disease and one patient with new-onset fever and pneumonia in whom HIV infection was suspected but not yet confirmed. One other BAL fluid sample from a patient receiving renal dialysis was PJ- according to IFA, but provided a very late Ct value (42.8) according to molecular testing. Because there was no evidence of PCP on clinical review, this result was called a true negative according to the PJ real-time PCR assay.

All of the seven BAL fluid samples that provided discrepant results between IFA (negative) and the CLS PJ real-time PCR assay (positive) were also PJ+ according to the RL real-time PCR assay. Table 1 summarizes the performance of the IFA and PJ real-time PCR assays for diagnosis of PCP. Although the IFA assay was highly specific, it was less sensitive than the molecular assay at diagnosing PCP. The IFA

Table 1
Performance of the immunofluorescence and *Pneumocystis jirovecii* (PJ) real-time polymerase chain reaction (PCR) assays for diagnosis of pneumonia*

Detection method	Positive	Negative	Total
IFA†			
Positive	13	0	13
Negative	7	72	79
Total	20	72	92
PJ real-time PCR‡			
Positive	20§	0	20
Negative	0	72	72
Total	20	72	92

Data presented as n. *True positive = bronchoalveolar lavage (BAL) positive according to immunofluorescence assay (IFA) and the PJ real-time PCR assay or IFA negative but both PJ real-time PCR assays positive in a patient with *Pneumocystis pneumonia*. †Sensitivity=13/20 (65%), specificity=72/72 (100%), positive predictive value (PPV)=13/13 (100%), negative predictive value (NPV)=72/79 (91.1%). ‡Sensitivity=20/20 (100%), specificity=72/72 (100%), PPV=20/20 (100%), NPV=72/72 (100%). §Two BAL fluid samples were tested from the same patient who was considered to have early PJ infection

also produced a lower negative predictive value because it was false negative in six patients with PCP and one who was suspected of having early PJ infection.

Performance of the PJ real-time PCR assay was similar in immunocompromised patients with HIV and those who were immune suppressed for other reasons. For the IFA, the sensitivity among non-HIV infected patients was slightly higher at 70.59% with the specificity preserved at 100%. Of the nine HIV positive patients, eight had a positive IFA test, and the ninth sample was positive based on the modified gold standard and a clinical diagnosis of PCP. The IFA test resulted in higher sensitivity (88.89%) for HIV patients, with 100% specificity.

DISCUSSION

We described a laboratory-developed real-time assay for the highly reliable detection of PJ from BAL fluid. Overall, the method can be performed the same day the sample is received and has a turnaround time of 2.5 h including DNA extraction, and has an overall increase in sensitivity of 35% over our existing IFA method. The present study confirms that PCR is more sensitive than staining and microscopic examination of BAL samples to diagnose PCP (17,24-28). The PJ real-time PCR developed and validated during the present study was highly sensitive and specific at diagnosing PCP in immunocompromised patients, particularly those without HIV infection. Real-time PCR detection of PJ is more rapid than conventional PCR, and reduces the potential for carry-over contamination run-to-run because the reaction is performed in a closed system. Real-time PCR also offers a high degree of objectivity that cannot be obtained by staining and microscopic examination of a BAL sample. In our laboratory, even highly experienced technologists would have difficulty calling the IFA positive when few cysts were present. This was also reflected in the performance of the IFA compared with the PJ real-time assay in our study. The sensitivity of IFA in our study was highest among HIV infected individuals (88.89%), which was similar to the molecular assay, although IFA was falsely negative in one patient with PCP. The sensitivity of IFA was lower when analyzing BAL samples from non-HIV immunocompromised patients (70.6%), and considerably less than the molecular assay when all patient samples were considered (65%).

The higher sensitivity of molecular detection of PJ in BAL fluid samples is consistent with the results of other studies (17,24-28). A systematic review that evaluated PJ PCR assays testing BAL fluid samples revealed an overall sensitivity of 98.3% and specificity of 91% for all of the studies included in their analysis (27), and highlighted that

one can expect false-positive PCR tests to occur in patients with no clinical evidence of PCP. All positive PCR results should, therefore, be correlated with the patient's clinical symptoms and radiological evidence of PCP; however, a negative PCR result essentially allows the diagnosis to be ruled out if controls perform to specification and PCR inhibition has not occurred (27,29). A more recent meta-analysis of reported molecular assays that included real-time PCR for PJ detection from BAL fluid samples produced a similar overall sensitivity of 98% but a higher specificity of 93% (29). Our study also confirmed the high inter-laboratory agreement among real-time PCR assays previously used for the detection of PJ (30).

Although there were no false-positive PJ real-time PCR tests in our study, previously reported validations of other laboratory developed qualitative molecular assays for PJ detection have found false-positive tests due to the higher sensitivity of molecular methods (23,31,32). Therefore, a diagnostic cut-off should be established, such as what occurred in our study, for the reportable range of the real-time PCR assay, that includes true positive, a 'grey-zone' for low-level positives, which may represent early infection or asymptomatic colonization and negative results. Clinical correlation of the results of our PJ real-time PCR assay with the presence of PCP validated that the established cut-offs for a true positive (ie, Ct ≤30) and low positive (ie, Ct >30 but <37) accurately diagnosed the presence of PCP. All of the patients with low positive PJ real-time PCR results who were negative according to IFA were confirmed to have definite PCP or suspected early infection (one patient) on clinical review. Confirmation that these seven discrepant BAL results were true positives was also documented by testing them using a second *Pneumocystis* gene target, the *cdc2* gene, by the RL. The second positive real-time PCR result, using another independent molecular method, further supported the presence of this fungal organism in the patient's sample.

Although the use of qualitative real-time PCR for PJ detection would improve our ability to rapidly and accurately diagnose PCP in our jurisdiction and our molecular assay demonstrated complete agreement with the RL's assay, standardized diagnostic performance across laboratories awaits the development of a quantitative PJ real-time PCR assay (33). A commercial quantitative PJ real-time PCR assay used in combination with serum (1,3)-β-D-glucan may enable improved differentiation of patients with PJ colonization and those with early or suppressed PCP infection (34,35), and also enhance therapeutic monitoring of PCP resolution while receiving treatment.

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