Open Forum Infectious Diseases

MAJOR ARTICLE

Pneumocystis PCR: It Is Time to Make PCR the Test of Choice

Laura Doyle, Sherilynn Vogel, and Gary W. Procop

Section of Clinical Microbiology, Department of Laboratory Medicine, Pathology and Laboratory Medicine Institute, Cleveland Clinic, Cleveland, Ohio

Background. The testing strategy for *Pneumocystis* at the Cleveland Clinic changed from toluidine blue staining to polymerase chain reaction (PCR). We studied the differences in positivity rates for these assays and compared each with the detection of *Pneumocystis* in companion specimens by cytology and surgical pathology.

Methods. We reviewed the results of all *Pneumocystis* test orders 1 year before and 1 year after the implementation of a *Pneumocystis*-specific PCR. We also reviewed the corresponding cytology and surgical pathology results, if performed. Finally, we reviewed the medical records of patients with rare *Pneumocystis* detected by PCR in an effort to differentiate colonization vs true disease.

Results. Toluidine blue staining and surgical pathology had similar sensitivities and negative predictive values, both of which were superior to cytology. There was a >4-fold increase in the annual detection of *Pneumocystis* by PCR compared with toluidine blue staining (toluidine blue staining: 11/1583 [0.69%] vs PCR: 44/1457 [3.0%]; chi-square $P < .001$). PCR detected 1 more case than surgical pathology and was far more sensitive than cytology. Chart review demonstrated that the vast majority of patients with rare *Pneumocystis* detected were immunosuppressed, had radiologic findings supportive of this infection, had no other pathogens detected, and were treated for pneumocystosis by the clinical team.

Conclusion. PCR was the most sensitive method for the detection of *Pneumocystis* and should be considered the diagnostic test of choice. Correlation with clinical and radiologic findings affords discrimination of early true disease from the far rarer instances of colonization.

Keywords. *Pneumocystis jirovecii*; PCR; surgical pathology; cytology.

Pneumocystis jirovecii is an opportunistic pathogen that was first widely recognized early in the HIV epidemic [1, 2]. In addition to HIV infection, *Pneumocystis* causes pneumonia in patients with solid organ and stem cell transplants, those receiving corticosteroids and other immune-modulating agents, and individuals with other immunocompromising conditions [3–6].

The diagnosis of pneumocystosis is hindered by the lack of a practical culture method and has traditionally relied on morphologic methods of detection [7–9]. The presence of granular, eosinophilic alveolar casts informs the surgical pathology and cytopathologist [8, 9]. Thereafter, a histochemical stain such as Gomori's methenamine silver (GMS) is commonly used to highlight the cyst forms of *Pneumocystis* and confirm the diagnosis. Calcofluor white, Giemsa, toluidine blue, and commercially available *Pneumocystis*-specific immunofluorescent preparations are other recognized staining methods for the detection of *Pneumocystis* [10]. The variations in performance of these stains has been described, but they are likely dependent, in part at least, on the experience and skill of the microscopist [10]. Another important factor is the degree of immunosuppression. Patients with only mild to moderate immunosuppression may have a lower burden of *Pneumocystis*, which may be less obvious when assessed by morphologic methods; similarly, clinical and radiologic findings are not as well developed (ie, are less typical) as in patients with profound immunosuppression [3, 6, 11].

We introduced a previously described *Pneumocystis*-specific PCR, following a thorough validation [12]. The evidence supported the replacement of the morphologic assessment of toluidine blue–stained preparations with the *Pneumocystis* PCR (data not shown). Herein, we review a year-long experience with a *Pneumocystis* PCR, which includes evaluation of pre- and postimplementation detection rates, comparing both toluidine blue–based and *Pneumocystis* PCR–based detection with the detection of *Pneumocystis* by cytology and surgical pathology in companion specimens. Companion specimens were split specimens derived from the same procedure (eg, a bronchoalveolar lavage [BAL] split between cytology and microbiology and/or a transbronchial biopsy). Finally, we reviewed the medical records of patients in whom low quantities of *Pneumocystis* were detected in an attempt to determine if detection was associated with disease or colonization.

Received 19 July 2017; editorial decision 30 August 2017; accepted 31 August 2017.

Correspondence: G. W. Procop, MD, Molecular Microbiology, Mycology, Parasitology and Virology, Cleveland Clinic, 9500 Euclid Avenue/LL2-2, Cleveland, OH 44195 (procopg@ccf.org).

Open Forum Infectious Diseases®

DOI: 10.1093/ofid/ofx193

The assessment of new assays that have superior sensitivities compared with current test methods is challenging, as evidenced by the introduction of nucleic acid amplification tests for *Neisseria gonorrhoeae* and *Chlamydia trachomatis* [13, 14]. The specificity of the PCR assay studied here was not in question as it was determined to be high in our validation studies with no cross-reactivity demonstrated with a wide variety of microorganisms that may be present in human respiratory specimens (data not shown). Similarly, it has been our clinical experience and it has been demonstrated elsewhere that the morphologic detection of *Pneumocystis* by a variety of methods is also highly specific (ie, there are few false positives) [8–10].

METHODS

The specimens included in this study were liquid specimens (ie, BAL, sputa, or induced sputa), which were split between cytology and microbiology if both tests were ordered by the provider and/or transbronchial biopsy specimens were ordered for surgical pathology. We considered each positive result a true positive, regardless of method, given the high positive predictive value of each of the assays studied, as denoted above. Therefore, we limited our calculations to the sensitivity and negative predictive value for each test. For these calculations, the total number of positive specimens was determined for each group of tests studied (eg, positive specimens studied by both toluidine blue staining and cytology). The total number of tests performed in the studied cohort was also recorded. The sensitivity for each test within this cohort was determined based on the number of true positives detected by the test divided by the sum of the true positives plus false negatives. The negative predictive value was calculated by dividing the number of truly negative by the composite of the number of true negatives and the false negative test results. All statistical comparisons were made using EpiCalc 2000 (Brixton Health, http://www.brixtonhealth.com/epicalc.html). This study was approved by the Cleveland Clinic Institutional Review Board.

Comparison of Toluidine Blue Staining With Cytology and Surgical Pathology

We reviewed the detection of *Pneumocystis* by 3 morphologic methods in the year prior to the implementation of the *Pneumocystis* PCR assay. Tests were ordered by a physician of record and were performed as part of routine patient care. We compared the detection of *Pneumocystis* by toluidine blue staining performed in clinical microbiology with the detection of this organism by cytology and surgical pathology in companion specimens. If granular cast material was seen in either the cytology or surgical pathology preparations, then a GMS stain was performed to determine if the cyst forms of *Pneumocystis* were present. We performed pairwise comparisons, as well as a subset analysis of those specimens wherein all 3morphologic studies were performed. When *Pneumocystis* was detected by 1 method but not another, the negative result was considered a false negative.

Comparison of *Pneumocystis* Rapid-Cycle PCR With Morphologic Methods

We could not directly compare the detection of *Pneumocystis* by toluidine blue staining with PCR as PCR was a replacement technology. We did, however, compare the frequency of detection of *Pneumocystis* by toluidine blue staining 1 year prior to the implementation of the PCR assay with the frequency of detection 1 year after implementation. There was no change in the patient population served by our institution during the time of this comparison. For the year after *Pneumocystis* PCR implementation, we compared PCR detection of *Pneumocystis* with the detection rates of cytology and surgical pathology in companion specimens. As above, all positives were considered true positives in this assessment. When *Pneumocystis* was detected by 1 method but not another, the negative result was considered a false negative.

Review of Clinical and Radiologic Findings of Patients With Rare *Pneumocystis jirovecii* by PCR

In our experience, *Pneumocystis* PCR results with an early-to-mid PCR crossing threshold [Ct] have consistently correlated with pneumocystosis diagnosed by standard methods. We performed chart reviews on all patients with a "rare *Pneumocystis* detected" (ie, repeatedly positive test results with a Ct between 40–45 cycles) result in an effort to distinguish colonization from an early phase of infection.

The medical record review consisted of the following information from the clinical episode associated with the finding of rare *Pneumocystis* detected: (1) review of the surgical and cytopathology findings from companion specimens, if performed; (2) the primary diagnosis of the patient; (3) the presence of a traditionally recognized immunocompromising condition and, if present, the type of condition; (4) the presence and nature (eg, bilateral) of pulmonary infiltrates on chest roentgenogram; (5) if a CT scan was performed and, if so, if ground-glass infiltrates were present; (6) the type and results of other microbiologic studies performed, particularly noting the presence of any other potential pathogens that could be responsible for the clinical and radiologic findings of the patient; (7) the response of the clinical service to the "rare *Pneumocystis* detected" result; and (8) associated mortality.

RESULTS

Comparison of Toluidine Blue Staining With Cytology and Surgical Pathology

Two hundred thirty-one specimens had both toluidine blue staining and cytology performed (Table 1). *Pneumocystis* was detected in 7 patients (7/231, 3.0%); 6/7 (85.7%) specimens were detected by toluidine blue, whereas 3/7 (42.9%) were detected by cytology. The sensitivity and negative predictive value (NPV) were 85.7% and 99.6% for toluidine blue staining and 42.9% and 98.2% for cytology, respectively.

One thousand forty-six specimens had both toluidine blue staining and a surgical pathology assessment performed.

Table 1. The Performance Characteristics of Test Methods

Tests Performed	Positive Patients/Number Tested (%)	Number (Percentage) Detected by Method (%)	Statistical Significance (Chi-Square)	Sensitivity, %	Negative Predictive Value, %
Comparison of morphologic methods					
Toluidine blue and cytology	7/231 (3.0)	TB: 6/7 (85.7) Cy: 3/7 (42.9)	Not performed [a]	TB: 85.7 Cy: 42.9	TB: 99.6 Cy: 98.2
Toluidine blue and surgical pathology	7/1046 (0.67)	TB: 5/7 (71.4) SP: 5/7 (71.4)	$P = 1.00$	TB: 71.4 SP: 71.4	TB: 99.8 SP: 99.8
Toluidine blue, cytology, and surgical pathology	7/114 (6.1)	Detected by: All 3 methods: 1/7 (14.3) TB alone: 2/7 (28.6) SP alone: 1/7 (14.3) TB and SP: 2/7 (28.6) SP and Cy: 1/7 (14.3)	Not performed[a]	TB: 71.4 Cy: 28.6 SP: 71.4	TB: 98.2 Cy: 95.5 SP: 98.2
Comparison of PCR with cytology and surgical pathology					
PCR and cytology	21/282 (7.4)	PCR: 21/21 (100) Cy: 5/21 (23.8)	$P < .001$	PCR: 100 Cy: 23.8	PCR: 100 Cy: 94.2
PCR and surgical pathology	4/874 (0.46)	PCR: 4/4 (100) SP: 3/4 (75)	Not performed[a]	PCR: 100 SP: 75	PCR: 100 SP:99.9
PCR, cytology, and surgical pathology	3/123 (2.4)	Detected by: PCR and SP: 2/3 (66.6) PCR alone: 1/3 (33.3)	Not performed[a]	PCR: 100 SP: 66.6 Cy: 0.0	PCR: 100 SP: 99.2 Cy: 0.0

Abbreviations: Cy, cytology; NPV, negative predictive value; PCR, *Pneumocystis* polymerase chain reaction–based assay; SP, surgical pathology; TB, toluidine blue staining.
[a]Chi-square tests were not performed when cell values were less than 5.

Pneumocystis was detected in 7/1046 (0.67%) of these specimens. Both methods detected 5 of the 7 positive specimens. The sensitivity and NPV for both toluidine blue staining and surgical pathology were 71.4% and 99.8%, respectively.

There were 114 specimens in the year prior to the implementation of PCR wherein all 3 morphologic methods had been performed. *Pneumocystis* was detected in only 1 of the 7 positive specimens by all 3 methods. *Pneumocystis* was detected in 2 specimens by both toluidine blue staining and surgical pathology. It was detected by toluidine blue staining alone in 2 specimens, it was detected by both surgical pathology and cytology in 1 specimen, and it was detected by surgical pathology alone in 1 specimen. In an analysis of this subset, the sensitivity and NPV were, respectively, as follows: toluidine blue, 71.4% and 98.2%%; cytology, 28.6% and 95.5%; and surgical pathology, 71.4% and 98.2%.

Comparison of *Pneumocystis* PCR With Morphologic Methods

Pneumocystis jirovecii was detected in 11 of 1583 (0.69%) specimens by toluidine blue staining in the year prior to PCR implementation, whereas 44 of 1457 (3.0%) specimens were positive for *Pneumocystis* in the year following implementation (chi-square $P < .001$) (Table 1).

Two hundred eighty-two specimens were submitted for both *Pneumocystis* PCR and cytology. Twenty-one specimens were positive by PCR, whereas only 5 were detected by cytology. There were no specimens wherein *Pneumocystis* was detected by cytology but not detected by PCR. The sensitivity and NPV for cytology were 23.8% and 94.2%, respectively, whereas for *Pneumocystis* PCR these were 100% each.

Eight hundred seventy-four specimens were submitted for both *Pneumocystis* PCR and surgical pathology during this time period. There were 4 PCR-positive specimens and 3 detected by surgical pathology. There were no specimens that were positive for *Pneumocystis* by surgical pathology that were negative by PCR. The sensitivity and negative predictive value for surgical pathology were 75% and 99.9%, whereas for *Pneumocystis* PCR, these were 100% each.

One hundred twenty-three patients had specimens submitted for *Pneumocystis* PCR, cytology, and surgical pathology. Three of the specimens within this subset contained *Pneumocystis*. Two were positive by both PCR and surgical pathology, whereas the third was detected by PCR alone. None of 3 specimens was detected by cytology. In the assessment of this subset, the sensitivity and NPV for PCR were 100% each, for cytology they were 0% each, and for surgical pathology they were 66.6% and 99.2%, respectively.

Medical Record Review of Patients With Rare *Pneumocystis* Detected by PCR

There were 21 patients with a low level of *Pneumocystis jirovecii* DNA detected by PCR (ie, reported as Rare *Pneumocystis* Detected) in the year following the implementation of this assay. The charts were not reviewed for 2 of these patients because 1 was unavailable and the other was a patient who had a previous strong positive (ie, Ct < 40 cycles) and had repeat testing during therapy. We reviewed salient features of the medical record for the remaining 19 patients (Table 2).

In brief, 15/19 (78.9%) patients had a recognized immunocompromising condition and/or were receiving immunosuppressive medications for an underlying condition. These patients were receiving corticosteroids (7), had a hematopoietic malignancy (5), solid organ transplant (1) or stem

Table 2. Clinical and Radiologic Findings of Patients With Rare *Pneumocystis jirovecii* Detected by PCR

Immunocompromising Condition/ Therapy, n (%)	Presence and Type of Pulmonary Infiltrates, n (%)	Pathology/Laboratory Findings, n	Clinically Recognized and Treated, n (%)	Associated Mortality, n (%)
15/19 (78.9)	Pulmonary infiltrates: 17/19 (89.5) Bilateral infiltrates: 16/17 (94.1) CT scan performed: 14/19 (73.7) Ground glass infiltrates: 14/14 (100)	Cytology: 0/9 detected Surg. path: 1/3 detected Other pathogens detected: Influenza A: 1 *S. pneumoniae*: 2 CMV: 1 MAC: 1	18/19 (94.7)	4/19 (21.5) Direct mortality: 1/4 Contributory (comorbid condition): 3/4

Abbreviations: CMV, ; CT, computerized tomography; MAI, ; PCR, polymerase chain reaction.

cell transplant (1), untreated HIV (1), and/or were receiving chemotherapy (1). Some patients with malignancies were also receiving immunosuppressive therapy. Three patients were not considered traditionally immunocompromised; these patients had bronchiectasis/tracheomalacia (1), decompensated congestive heart failure (1), and end-stage cirrhosis of the liver (1).

All but 2 of the patients in this cohort had pulmonary infiltrates detected by chest roentgenograms; the 2 patients without infiltrates were both children with bronchiectasis/tracheomalacia. All of the pulmonary infiltrates present were bilateral, with the exception of the unilateral infiltrate present in the patient with decompensated congestive heart failure and influenza A. Fourteen patients had CT scans performed, all of which demonstrated ground glass infiltrates.

Nine of the 19 patients in this cohort had companion specimens that were submitted for cytology; *Pneumocystis jirovecii* was not detected in any of these specimens. Tissue was submitted for surgical pathology for 3/19 patients; *P. jirovecii* was detected in 1 of these specimens. The histopathologic diagnoses for the specimens from the other 2 patients were nonspecific, consisting of organizing acute lung injury (1) and focal inflammation (1).

In clinical microbiology, the vast majority (ie, 18/19) of patients received a gram stain and bacterial culture, a fungal culture, and viral cultures and/or respiratory viral PCR. Most patients were tested for *Legionella pneumophila* by PCR (17/19), had acid-fast staining and mycobacterial cultures performed (17/19), and had cultures for *Nocardia* (15/19). *Streptococcus pneumoniae* was recovered from both the children with bronchiectasis/tracheomalacia and was considered significant by the clinical teams; only 1 of these patients, who was also receiving corticosteroids, was treated for pneumocystosis. One patient with untreated HIV also grew *Mycobacterium avium* complex (MAC) from the respiratory specimen; the clinical team believed the patient had both disseminated MAC and pneumocystosis. A patient receiving corticosteroids for Sjogren's syndrome also grew cytomegalovirus (CMV) from the BAL specimen, which may have been a copathogen. Finally, 1 nonimmunocompromised patient with decompensated congestive heart failure tested positive for influenza A, which was clinically thought to be the primary pathogen; this patient was also treated for pneumocystosis, but the degree to which *P. jirovecii* contributed to her illness was noted to be uncertain. The remainder of the patients with rare *P. jirovecii* detected by PCR had no other reason for their respiratory illness detected, although a thorough microbiologic assessment was performed.

The assessments by the clinical teams, which often included consultation by an infectious diseases specialist, concluded that the rare *Pneumocystis* detected was causing or contributing to disease in all but 1 of the patients, and treatment was given for pneumocystosis. The untreated patient was a child with bronchiectasis/tracheomalacia whose respiratory specimen also grew *S. pneumoniae.* There were 4 deaths in this cohort (4/19, 21.1%), with 1 directly attributed to pneumocystosis and 3 with pneumocystosis recognized as a contributing factor in the death of the patients.

DISCUSSION

The clinical meaningfulness of a positive *Pneumocystis* PCR result has been questioned, particularly when a low quantity of *Pneumocystis* is detected (ie, the Ct occurs late in the PCR) [11, 15, 16]. Although PCR has been shown to be a highly sensitive means of detecting *Pneumocystis,* the replacement of morphologic methods has been slow because of concerns about the proposed ubiquity of this organism and the difficulty of differentiating colonization and true infection [11, 16–22].

We reviewed the performance of PCR and traditional morphologic methods in *Pneumocystis* detection in a tertiary care medical center. The 3 morphologic methods were toluidine blue staining performed in clinical microbiology, surgical pathology, and cytology. Tests were performed at the discretion of the attending physician. The sensitivity of toluidine blue staining ranged from 71.4% to 85.7%, depending on the subset analyzed, similar to other morphologic methods [10]. The sensitivity of surgical pathology was similar to toluidine blue staining, ranging from 71.4% to 75%, whereas the sensitivity for cytology was surprisingly low, not exceeding 43%.

The poor performance of cytology is likely because the indicator for most cytologists to order the GMS stain for *Pneumocystis* is the presence of granular, acellular casts. Without these casts, GMS staining is usually not performed. It remains to be determined whether or not GMS staining of cytology specimens that lacked granular casts but were positive for *Pneumocystis* by

surgical pathology and/or toluidine blue staining would have detected *Pneumocystis*. The superior performance of surgical pathology may be secondary to bias, in that patients with more advanced disease may have been more likely to be biopsied. The superior performance of toluidine blue staining may have been because clinical microbiologists performed the staining regardless of the presence or absence of granular casts and were focused on searching only for the *Pneumocystis* forms.

There was a greater than 4-fold increase in the annual frequency of *Pneumocystis* detected after the introduction of PCR (0.69% vs 3.0%), although there were no substantial changes in our patient population. This increased detection rate appears directly related to the heightened sensitivity of this molecular assay.

There has been considerable debate regarding the use of PCR for an organism considered by some to be ubiquitous, as the detection of colonizing organisms or transient microbiota may produce clinically false-positive reactions. We challenge the concept of ubiquity as we tested 1457 respiratory specimens and detected only 44 (3.0%) positives by PCR. We propose that a higher positivity rate would have occurred if *Pneumocystis* were truly ubiquitous. Furthermore, we demonstrate below that after a thorough medical record review, only 3 patients with low quantities of *Pneumocystis* DNA detected were likely colonized; the remainder had evidence of true disease.

The quantitation of *Pneumocystis* DNA with an established cutoff value and an intermittent gray zone has been used by Alanio et al. in an attempt to differentiate true disease from colonization [23]. Determining the actual quantity of *Pneumocystis* present in situ is challenging, as there are varying degrees of dilution in bronchoscopy depending on the extent of the lavage. Furthermore, the quantity of *Pneumocystis* DNA present is expected to vary with the stage of disease, with lower quantities expected early in disease and with resolution of disease. In this study, we simply categorized specimens as "rare" based on the crossing threshold, in a manner similar but not identical to that of Fauchier et al, who used Ct values as a surrogate for organism load [16].

Our review of the 19 patients with a lower quantity of *Pneumocystis* DNA present in their specimens showed that 15/19 (78.9%) patients were immunocompromised to some degree and at risk for opportunistic infections. Pulmonary infiltrates were present in 17/19 (89.5%) patients, with 16/19 (84.2%) patients having bilateral pulmonary infiltrates. Fourteen of the 19 patients received a CT scan, all of which (100%) demonstrated a ground glass appearance—consistent with pneumocystosis. All 19 patients received a thorough microbiologic assessment. These studies demonstrated that only 3 patients had a more likely pathogen detected (ie, 1 patient with influenza A and 2 children with bronchiectasis/tracheomalacia, both of which grew *S. pneumoniae,* which was considered significant). Treatment directed against *Pneumocystis* was given to 2 of these 3 patients. The cultures from 2 patients disclosed likely copathogens (ie, MAC in a patient with AIDS and CMV in a patient receiving corticosteroids); treatment was given for *Pneumocystis* for both these patients. If 3/44 results were false positives, then *Pneumocystis* PCR is estimated to have a positive predictive value of approximately 93.1%.

Fan et al. undertook a systematic review of *Pneumocystis* PCR assays, performed a bivariate metaanalysis, and concluded that PCR-based assays for *Pneumocystis* showed high sensitivity and specificity [18]. They denoted, however, that clinical, radiologic, and laboratory findings should also be considered together for diagnosis, with which we completely agree. The patients with rare *Pneumocystis* detected in this review who were thought to be colonized were either not immunocompromised or were minimally immunocompromised, had other likely pathogens detected, and/or did not have clinical and/or radiologic features supportive of pneumocystosis.

In conclusion, *Pneumocystis* PCR proved more sensitive than commonly used morphologic methods. Specimens with a low quantity of *Pneumocystis* DNA present were usually associated with pneumocystosis, but rare instances of apparent colonization did occur. We recommend some means (eg, late Cts) to designate specimens with a low quantity of *Pneumocystis*, so that, as recommended by Fan et al., correlation with clinical, radiologic, and other laboratory findings can be undertaken. The findings presented here support *Pneumocystis* PCR as the test of choice for the diagnosis of pneumocystosis.

Acknowledgement

Potential conflicts of interest. All authors: no reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Centers for Disease Control and Prevention. Pneumocystis pneumonia—Los Angeles. MMWR Morb Mortal Wkly Rep **1981**; 30:250–2.
2. Centers for Disease Control and Prevention. A cluster of Kaposi's sarcoma and *Pneumocystis carinii* pneumonia among homosexual male residents of Los Angeles and Orange Counties, California. MMWR Morb Mortal Wkly Rep **1982**; 31:305–7.
3. Calero-Bernal ML, Martin-Garrido I, Donazar-Ezcurra M, et al. Intermittent courses of corticosteroids also present a risk for pneumocystis pneumonia in non-HIV patients. Can Respir J **2016**; 2016:2464791.
4. De Castro N, Neuville S, Sarfati C, et al. Occurrence of *Pneumocystis jiroveci* pneumonia after allogeneic stem cell transplantation: a 6-year retrospective study. Bone Marrow Transplant **2005**; 36:879–83.
5. Kaur N, Mahl TC. *Pneumocystis jiroveci* (*carinii*) pneumonia after infliximab therapy: a review of 84 cases. Dig Dis Sci **2007**; 52:1481–4.
6. Wang EH, Partovi N, Levy RD, Shapiro RJ, Yoshida EM, Greanya ED. Pneumocystis pneumonia in solid organ transplant recipients: not yet an infection of the past. Transpl Infect Dis **2012**; 14: 519–25.
7. Minielly JA, McDuffie FC, Holley KE. Immunofluorescent identification of *Pneumocystis carinii*. Arch Pathol **1970**; 90:561–6.
8. Pintozzi RL, Blecka LJ, Nanos S. The morphologic identification of *Pneumocystis carinii*. Acta Cytol **1979**; 23:35–9.
9. Sun T, Chess Q, Tanenbaum B. Morphologic criteria for the identification of *Pneumocystis carinii* in Papanicolaou-stained preparations. Acta Cytol **1986**; 30:80–2.
10. Procop GW, Haddad S, Quinn J, et al. Detection of *Pneumocystis jiroveci* in respiratory specimens by four staining methods. J Clin Microbiol **2004**; 42:3333–5.
11. Maillet M, Maubon D, Brion JP, et al. *Pneumocystis jirovecii* (Pj) quantitative PCR to differentiate Pj pneumonia from Pj colonization in immunocompromised patients. Eur J Clin Microbiol Infect Dis **2014**; 33:331–6.

12. Larsen HH, Masur H, Kovacs JA, et al. Development and evaluation of a quantitative, touch-down, real-time PCR assay for diagnosing *Pneumocystis carinii* pneumonia. J Clin Microbiol **2002**; 40:490–4.
13. Carroll KC, Aldeen WE, Morrison M, et al. Evaluation of the Abbott LCx ligase chain reaction assay for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in urine and genital swab specimens from a sexually transmitted disease clinic population. J Clin Microbiol **1998**; 36:1630–3.
14. Kehl SC, Georgakas K, Swain GR, et al. Evaluation of the abbott LCx assay for detection of *Neisseria gonorrhoeae* in endocervical swab specimens from females. J Clin Microbiol **1998**; 36:3549–51.
15. Blumenfeld W, McCook O, Holodniy M, Katzenstein DA. Correlation of morphologic diagnosis of *Pneumocystis carinii* with the presence of pneumocystis DNA amplified by the polymerase chain reaction. Mod Pathol **1992**; 5:103–6.
16. Fauchier T, Hasseine L, Gari-Toussaint M, et al. Detection of *Pneumocystis jirovecii* by quantitative PCR to differentiate colonization and pneumonia in immunocompromised HIV-positive and HIV-negative patients. J Clin Microbiol **2016**; 54:1487–95.
17. Chien JY, Liu CJ, Chuang PC, et al. Evaluation of the automated Becton Dickinson MAX real-time PCR platform for detection of *Pneumocystis jirovecii*. Future Microbiol **2017**; 12:29–37.
18. Fan LC, Lu HW, Cheng KB, et al. Evaluation of PCR in bronchoalveolar lavage fluid for diagnosis of *Pneumocystis jirovecii* pneumonia: a bivariate meta-analysis and systematic review. PLoS One **2013**; 8:e73099.
19. Fillaux J, Berry A. Real-time PCR assay for the diagnosis of *Pneumocystis jirovecii* pneumonia. Methods Mol Biol **2013**; 943:159–70.
20. Guillaud-Saumur T, Nevez G, Bazire A, et al. Comparison of a commercial real-time PCR assay, RealCycler® PJIR kit, progenie molecular, to an in-house real-time PCR assay for the diagnosis of *Pneumocystis jirovecii* infections. Diagn Microbiol Infect Dis **2017**; 87:335–7.
21. Montesinos I, Brancart F, Schepers K, et al. Comparison of 2 real-time PCR assays for diagnosis of *Pneumocystis jirovecii* pneumonia in human immunodeficiency virus (HIV) and non-HIV immunocompromised patients. Diagn Microbiol Infect Dis **2015**; 82:143–7.
22. Robert-Gangneux F, Belaz S, Revest M, et al. Diagnosis of *Pneumocystis jirovecii* pneumonia in immunocompromised patients by real-time PCR: a 4-year prospective study. J Clin Microbiol **2014**; 52:3370–6.
23. Alanio A, Desoubeaux G, Sarfati C, et al. Real-time PCR assay-based strategy for differentiation between active *Pneumocystis jirovecii* pneumonia and colonization in immunocompromised patients. Clin Microbiol Infect **2011**; 17:1531–7.