Multicenter, Prospective Clinical Evaluation of Respiratory Samples from Subjects at Risk for *Pneumocystis jirovecii* Infection by Use of a Commercial Real-Time PCR Assay†‡

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Received 25 November 2010/Returned for modification 21 January 2011/Accepted 17 February 2011

*Pneumocystis jirovecii* pneumonia (PCP) is a common opportunistic infection. Microscopic diagnosis, including diagnosis using the Merifluor-Pneumocystis direct fluorescent antigen (MP-DFA) test, has limitations. Real-time PCR may assist in diagnosis, but no commercially validated real-time PCR assay has been available to date. MycAssay Pneumocystis is a commercial assay that targets the *P. jirovecii* mitochondrial large subunit (analytical detection limit, ≤3.5 copies/μl of sample). A multicenter trial recruited 110 subjects: 54 with transplants (40 with lung transplants), 32 with nonmalignant conditions, 13 with leukemia, and 11 with solid tumors; 9 were HIV positive. A total of 110 respiratory samples (92% of which were bronchoalveolar lavage [BAL] specimens) were analyzed by PCR. Performance was characterized relative to investigator-determined clinical diagnosis of PCP (including local diagnostic tests), and PCR results were compared with MP-DFA test results for 83 subjects. Thirteen of 14 subjects with BAL surveillance after lung transplantation had positive PCR results; sensitivity, specificity, and positive and negative predictive values (PPV and NPV, respectively) were 93%, 91%, 59%, and 99%, respectively. Fourteen of 83 subjects for whom PCR and MP-DFA test results were available had PCP; PCR sensitivity, specificity, PPV, and NPV were 93%, 90%, 65%, and 98%, respectively, and MP-DFA test sensitivity, specificity, PPV, and NPV were 93%, 100%, 100%, and 98%. Of the 9 PCR-positive subjects without PCP, 1 later developed PCP. The PCR diagnostic assay compares well with clinical diagnosis using nonmolecular methods. Additional positive results compared with the MP-DFA test may reflect low-level infection or colonization.

*Pneumocystis* pneumonia (PCP) remains a formidable infection for both AIDS and non-AIDS patients, with mortality rates in excess of 20% (5, 9). The clinical presentations of the two groups differ, as do the principal differential diagnoses (9, 13, 30). While oxygen desaturation on exercise followed by hypoxemia are universal in PCP, these are not specific features, just as an elevated serum lactate dehydrogenase (LDH) level and pulmonary infiltrates are not (30). Therapy with high-dose corticosteroids may be given empirically, but these treatments are not without toxicity or immunosuppressive penalties. A worsening status, common early in the course of therapy, may precipitate a shift to pentamidine or a combination of primaquine and clindamycin, again with significant toxicities (3, 5, 34). Transfer to a high-dependency unit or ventilatory support may be required. Empiricism may be necessary, but ruling PCP in or out as a serious diagnostic consideration pays many management dividends for the patient and the clinician.

The diagnosis of PCP is established by microscopy of lung tissue, bronchial lavage, or other deep respiratory samples (3, 9, 15). PCP primarily affects the alveoli, and deep pulmonary samples are necessary for adequate microscopy. In the only comparative study of the diagnostic performance of different microscopic techniques in PCP, immunofluorescence (IF) was nonstatistically superior in sensitivity (91%) to staining with Calcofluor white (74%) and silver (77%), which were themselves more sensitive than Diff-Quik (48%) (26). Many fewer organisms are usually present in non-AIDS patients with PCP, which adversely affects diagnostic performance (34).

The first reports of PCR for the detection of fungal infec-
tions were for Pneumocystis carinii and Candida albicans, both in August 1990 (2, 38). There are many published papers indicating the utility of standard and, more recently, real-time PCR to diagnose Pneumocystis pneumonia (1–4, 10, 11, 14, 16, 18, 22, 38, 39). Many laboratories have developed their own assays and use them routinely. However, lack of standardization and clinical validation of the laboratory-developed assays is the primary reason why the EORTC/MSG group decided to omit all fungal PCR from the definitions of invasive fungal diseases (7).

In addition, several papers attest to the fact that PCR diagnosis of PCP is more sensitive than microscopy (4, 10, 11, 13, 22, 24). The generally higher sensitivity of PCR assays than of microscopy may be disproportionately important for non-AIDS patients and may result in fewer missed clinical diagnoses.

We report here the first prospective multicenter evaluation of a commercially launched real-time PCR assay for Pneumocystis, primarily in non-AIDS patients. The four clinical centers were located in Europe and the United States. Subjects were enrolled in the study if they were at risk for infection with Pneumocystis jirovecii. Clinical diagnosis was provided for all enrolled subjects in accordance with local routine diagnostic procedures at the four institutions, and the diagnostic comparator method of choice was the Merifluor direct fluorescent antigen (DFA) immunofluorescence test. Respiratory samples were collected, and DNA was extracted at all sites using a standardized extraction system and was subsequently tested with the MycAssay Pneumocystis kit on a SmartCycler real-time PCR platform.

**MATERIALS AND METHODS**

**Materials required.** The following equipment or materials were provided or required by the clinical sites to conduct this study: MycAssay Pneumocystis kits (Myconostica Ltd., Manchester, United Kingdom), the MyxXtra fungal DNA extraction kit (Myconostica Ltd.), the BD BBL Mycoprep specimen digestion/decontamination kit (BD Diagnostic Systems, Oxford, United Kingdom) according to the manufacturer’s instructions or a local NALC procedure for acid-fast bacillus (AFB) decontamination. DNA extracts were shipped on dry ice to Myconostica for molecular testing.

**Molecular testing.** MycAssay Pneumocystis is a commercially available qualitative real-time PCR test utilizing molecular beacons (36) for the detection of *P. jirovecii*. The target sequence is the *Pneumocystis* mitochondrial ribosomal large subunit (mLSU). The primers and molecular beacon avoid the polymorphisms described in this region (35), ensuring that detection is not affected by known genetic heterogeneity in the *Pneumocystis* population (5). Human DNA is not detected. The kit also contains an internal amplification control (IAC) sequence, a DNA fragment not present in *P. jirovecii*, to confirm amplification. The *P. jirovecii* target sequence is labeled with a 6-carboxyfluorescein (FAM) beacon, and the IAC sequence is labeled with a HEX beacon. The Cepheid SmartCycler real-time PCR system was used to monitor the fluorescence emitted by each beacon within the reaction mixture. On this platform, the assay limit of blank, defined as the experimentally determined negative cutoff value used to distinguish positive from negative results, is a cycle threshold (*Ct*) of 39. A *Ct* lower than this is positive (20). The *Ct* value can be interpreted in a semiquantitative manner, since there is an approximate relationship between the *Ct* and the fungal burden.

Failure of the IAC renders a negative assay unreportable. The assay limit of detection (LoD), the lowest DNA concentration at which ≥95% of results are positive (i.e., *Ct* below the limit of blank), was calculated in accordance with NCCLS procedure EP17-A (21) and was determined to be <35 copies per 10 μl of target DNA sample.

Upon receipt from the clinical sites, DNA extracts were stored at −80°C at Myconostica Ltd. until the PCR testing was complete. Storage time for the extracts ranged from 9 months to 6 weeks. All samples were thawed, batched, and assayed on the same day. The PCR results were not returned to the clinician or patient, nor were they used for establishing a clinical diagnosis.

**Merifluor DFA immunofluorescence testing.** The comparator diagnostic test was the Merifluor DFA immunofluorescence test. The manufacturer’s instructions were followed.

**Other diagnostic tests.** Other diagnostic tests, including Calbucido white (3 sites) and Gomori methenamine silver (GMS) (1 site) microscopy, serum lactate dehydrogenase (LDH) levels, C-reactive protein (CRP), plasma viscosity (PV), and erythrocyte sedimentation rate (ESR), were conducted in accordance with local requirements. Where these results were available, the data were captured on the study forms.

**Clinical risk factors and final diagnosis.** At study entry, in addition to assigning the pretest probability of moderate or low risk for PCP infection, the enrolling clinician was asked to assign the clinical condition or conditions that contributed to the subject’s at-risk status for infection.

Clinical diagnosis for each subject enrolled in the study was performed by the enrolling clinician, in accordance with his or her local diagnostic procedures and paradigms, up to 10 weeks after the subject had been enrolled and the sample had been taken for analysis. Information taken into account in reaching a clinical diagnosis of PCP included radiological appearance, hypoxemia, serum LDH level, identification of other pathogens, and local microscopy results for *P. jirovecii*. Additional clinical information was requested for 3 months after the study for subjects whose PCR result was positive but whose clinical diagnosis did not confirm PCP infection.
TABLE 1. Subject enrollment, disposition, and demographics

| Characteristic                  | Valuea                                                                 |
|--------------------------------|------------------------------------------------------------------------|
|                                | Site 1 | Site 2 | Site 3 | Site 4 |
| Enrollment                     |        |        |        |        |
| Unique subjects                | 18     | 29     | 22     | 62     |
| Subjects entering into primary analysis | 18     | 22     | 22     | 48     |
| Subjects with repeat visits    | 0      | 0      | 0      | 16     |
| No. of samples assayed         | 18     | 22     | 22     | 70     |
| Gender                         |        |        |        |        |
| Male                           | 7      | 10     | 13     | 29     |
| Female                         | 11     | 12     | 9      | 19     |
| Race                           |        |        |        |        |
| Caucasian                      | 12     | 4      | 0      | 48     |
| Black                          | 6      | 13     | 0      | 0      |
| Other (nonwhite)               | 0      | 5      | 0      | 0      |
| No data                        | 0      | 0      | 22     | 0      |
| Mean (range) age (yr)          | 57 (26–78) | 54 (21–75) | 52 (26–74) | 56 (23–73) |
| Pretest probability of PCP     |        |        |        |        |
| Moderate                       | 0      | 3      | 4      | 0      |
| Low                            | 18     | 19     | 18     | 48     |
| Sample type                    |        |        |        |        |
| BAL                            | 18     | 20     | 20     | 43     |
| Sputum                         | 0      | 1      | 1      | 0      |
| Other LRT                      | 0      | 1      | 1      | 5      |
| At-risk group                  |        |        |        |        |
| HIV/AIDS                       | 0      | 3      | 6      | 0      |
| Lung transplant                | 8      | 0      | 1      | 31     |
| Other transplants              | 1      | 7      | 3      | 0      |
| Leukemia                       | 1      | 1      | 7      | 0      |
| Other solid tumors             | 2      | 1      | 1      | 7      |
| Other nonmalignant conditions  | 6      | 10     | 4      | 10     |

*The testing and enrollment sites for the study were Duke University School of Medicine, Durham, NC; Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, NY; Centre Hospitalier Universitaire Vaudois and University of Lausanne, Lausanne, Switzerland; and Medizinische Universität and Landeskrankenhaus Natters, Innsbruck, Austria.

**Except where otherwise indicated, values are numbers of patients.

*Subjects were excluded from analysis if they withdrew consent or if there was inadequate sample left for PCR testing.

Results

Subject disposition. Data from 110 enrolled subjects were eligible for analysis. The demographics of the study subjects, by site, are recorded in Table 1. The population studied was predominantly not HIV infected (92%), and by the study entry criteria, their probability of infection was considered to be low. The risk categories assigned at the time of study entry were combined into groups for analysis, based on the predisposition for PCP. The analysis groups were as follows: (i) HIV/AIDS, (ii) lung transplant (one subject with a dual organ transplant [lung/kidney] was included in this category), (iii) all other organ transplants and allogeneic hematopoietic stem cell transplants (HSCT), (iv) leukemia, other hematological disorders, and autologous HSCT, (v) other solid tumors, and (vi) other nonmalignant conditions.

MycAssay Pneumocystis. Thirteen of the 14 subjects with a final clinical diagnosis of PCP were positive by the MycAssay Pneumocystis kit (Table 2), giving an assay sensitivity of 93%. Five of these 13 subjects (group 1) had HIV/AIDS, with a moderate probability of PCP infection; they recorded an average \( C_T \) of 25 (range, 19.6 to 33.5). The remaining 8 positive subjects (group 2), all with a low probability of infection, recorded an average \( C_T \) of 29.4 (range, 24.6 to 32.9) (Fig. 1). A third group, of 9 subjects who were PCP negative by clinical diagnosis and PCR positive, recorded an average \( C_T \) of 34.2 (range, 29.1 to 38.9). One-factor analysis of variance (ANOVA) showed a statistically significant difference among the three groups (\( P < 0.001 \)). Posthoc multiple-comparison tests (Scheffe’s test) adjusting for multiple testing gave a statistical difference (\( P < 0.001 \)) between groups 1 and 3, with a less significant difference (\( P = 0.031 \)) between groups 2 and 3. Comparison of the results in groups 1 and 2 demonstrated some limited evidence of a difference that did not reach significance (\( P = 0.10 \)).

One subject had an assigned clinical diagnosis of PCP; how-
ever, the PCR was negative. This subject was a 41-year-old female with HIV/AIDS, with a CD4+ cell count of $33 \times 10^6$/liter, on corticosteroids, and admitted to the ICU. The IF test result was positive, and the LDH level was 249 U/liter (normal). The sample obtained was induced sputum, with a volume of <1 ml, which is below the recommended extraction volume of 2 ml. A comment written on the relevant data collection form queried the quality of the sample.

One 63-year-old male lung transplant recipient with a clinical diagnosis of PCP was PCR positive ($C_T = 24.6$) and negative by IF. He was on corticosteroids and other immunosuppressants and had an elevated LDH level of 527 U/liter and a CRP level of 20 mg/liter. Microscopy and IF were both negative.

Nine subjects were positive by PCR, without clinical evidence of PCP, and negative by IF (Tables 3 and 4; Fig. 1). All were considered to have a low probability of PCP infection at enrollment. Five of the subjects had lung transplants, 2 had renal transplants, and 2 were enrolled with other nonmalignant clinical conditions. Eight were microscopy negative, and one was microscopy positive for yeasts. Four of the nine had normal chest X-ray or chest computed tomography (CT) results reported at the time of testing. LDH levels ranged from 154 to 423 U/liter and CRP levels from <0.3 to 150 mg/liter. Clinical follow-up data for these nine subjects were requested for a period of 3 months post-study entry; one of these subjects developed PCP infection a month after sampling.

The PCR test demonstrated a sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of 93%, 91%, 59%, and 99%, respectively; positive and negative likelihood ratios of 9.9 and 0.08; and a diagnostic odds ratio of 125.6. These sensitivity and specificity numbers are comparable to, or better than, those published for other real-time PCR methods. These sensitivity and specificity numbers are comparable to, or better than, those published for other real-time PCR methods.

TABLE 3. Comparison of Merifluor immunofluorescence test with MycAssay Pneumocystis results

| MycAssay Pneumocystis result | Clinically diagnosed with PCP ($n = 14$) | Clinically diagnosed with a condition other than PCP ($n = 69$) |
|------------------------------|------------------------------------------|---------------------------------------------------------------|
| IF+                          | 12                                       | 0                                                             |
| IF-                          | 1                                        | 0                                                             |

<sup>a</sup> A total of 83 patients were tested.

Pretest probability. Subjects with HIV/AIDS and the presence of bilateral ground glass effects on radiography or hypoxemia are considered to have a moderate probability of infection with *P. jirovecii*. Seven of the 9 subjects with HIV/AIDS presented with the criteria for moderate probability, and 6 (86%) were assigned a clinical diagnosis of PCP at study exit.

Eight of 103 (8%) subjects considered to have a low probability of infection were assigned a clinical diagnosis of PCP at study exit. Of these 8, all had been on corticosteroids or another immunosuppressive drug, and none were receiving prophylactic trimethoprim-sulfamethoxazole. They were evenly distributed across all 5 of the “at-risk categories” detailed above, excluding HIV/AIDS. Seven had evidence of bilateral ground glass effects on radiography. One patient, without HIV/AIDS, had a CD4+ count of <100 $\times 10^6$/liter. Of the remaining 95 subjects, half were not on steroids or immunosuppressive agents. Of the 48 who were receiving steroids or similar drugs, none had evidence of bilateral ground glass effects and 18 were receiving prophylactic trimethoprim-sulfamethoxazole.

**Assay robustness.** Two of the 132 (1.5%) samples tested in the study were reported “Negative/Fail” and therefore invalid because the IAC was out of range, suggesting PCR inhibition. In both cases, the sample matrix was BAL fluid, and the final diagnoses were not PCP. Under these circumstances, the results were reported as “inhibitor present, negative test result cannot be reported.”

**Repeat sample analysis.** One site enrolled 16 subjects in the study multiple times, for a total of 42 samples. Although the repeat samples were not part of the primary analysis, other analyses were done to look at the pattern of PCR positivity with sequential visits, with respect to date of diagnosis and therapy regimens. The visit schedules covered a 3- to 4-month window. Nine of the 16 patients had a single follow-up visit (where a respiratory sample was available for testing).

Two of the 16 subjects were diagnosed with PCP. Both subjects tested positive by PCR and have been included in the primary study analysis. For one subject, for whom data from three samples were available, the first PCR sample was positive 1 month before the clinical diagnosis of PCP was made (Table 4, patient 1). The third sample was negative by PCR and had been collected during a course of therapy. For the other subject, for whom three samples were available for analysis, both PCP diagnosis and PCR were positive at the first visit. Subsequent samples were negative by PCR and were collected during the course of therapy.

Nine of the 16 subjects, contributing 24 samples to the study, had a final clinical diagnosis other than PCP at the time of the initial visit and on all subsequent visits. All 24 samples tested negative by PCR.

Five of the 16 subjects, contributing 12 samples to the study, had a final clinical diagnosis other than PCP at the time of the initial visit and all subsequent visits; however, 6 samples tested positive by PCR. For one individual, a post-lung transplant surveillance subject, 2 consecutive samples taken in a 14-day window tested positive by PCR. These subjects were not treated for PCP infection, and none developed PCP during the 3-month follow-up period.
TABLE 4. Details for 9 PCR-positive patients with assigned clinical diagnoses other than PCP at study exit.

| Patient no. | C<sub>T</sub> | Age (yr) | Sex | Risk factor(s) | Episode diagnosis | Test result | PCP medication | Follow-up |
|-------------|-------------|----------|-----|----------------|-------------------|-------------|----------------|-----------|
|             |             |          |     |                |                   |             |                |           |
| 1           | 32.4        | 64       | F   | Lung Tx, corticosteroids | Lung infection | −           | −              | 220       | 68   | 21 | Normal | None at time of testing | Developed PCP within 3 mo of study exit | No relevant development |
| 2           | 35.6        | 62       | M   | Renal Tx, corticosteroids | Candida colonization and presumptive BOOP | −           | −              | 423       | 50, 75 | 20, 60 | Air space opacities Patchy air space opacities and small mediastinal lymph nodes | Posttesting: cotrimoxazole (Septin or Bactrim) | None at time of testing | No relevant development |
| 3           | 34          | 55       | M   | Renal Tx, corticosteroids | Pneumonia | −           | −              | 251       | 93%  | RA(21) | Bilateral infiltrates | None at time of testing | No relevant development |
| 4           | 38.9        | 53       | F   | Lung Tx, corticosteroids | P. aeruginosa/A. fumigatus colonization and infections | −           | −              | No data   | No data | No data | Bilateral lung infiltrate and single nodule | None at time of testing; voriconazole, >30 days | No relevant development |
| 5           | 33.1        | 69       | M   | Lung Tx, corticosteroids | PLTS | −           | −              | 204       | 70   | 21 | Normal | None at time of testing | No relevant development |
| 6           | 34.1        | 67       | M   | Lung Tx, corticosteroids | PLTS | −           | −              | 187       | 81   | 21 | Normal | None at time of testing | No relevant development |
| 7           | 36.1        | 56       | F   | Lung Tx, corticosteroids | Viral pneumonia/ pneumonitis | −           | −              | 312       | 90   | 21 | Multiple consolidations | None at time of testing; posaconazole, −4 days | No relevant development |
| 8           | 29.1        | 62       | M   | Other (NMC) | Lung carcinoma | No data | −           | 154       | 59   | 21 | Infiltrates, right upper lobe | None at time of testing | No relevant development |
| 9           | 34.5        | 47       | F   | Other (NMC) | Hemoptysis | No data | −           | 167       | No data | No data | Right middle, massive infiltrates | None at time of testing | No relevant development |

*CT<sub>T</sub>, cycle threshold; IF, immunofluorescence; Tx, transplant; BOOP, bronchiolitis obliterans organizing pneumonia; LDH, lactate dehydrogenase; NMC, nonmalignant conditions; PLTS, post-lung transplant surveillance; PO2, partial pressure of oxygen; FIO2, fractional inspired oxygen; CxR, chest X ray; CT, computed tomography; RA, room air.

b, negative.

c, Voriconazole and posaconazole have no effect on P. jirovecii.
DISCUSSION

The timely diagnosis and treatment of *P. jirovecii* infection remain a challenge to the clinician and mycology laboratories, where the gold standard has been visualization of characteristic *P. jirovecii* cysts and/or trophozoites in lung tissue biopsy specimens. The advent of the AIDS epidemic led to the use of less stringent diagnostic criteria, since many patients were too ill to undergo biopsy. Microscopy of BAL and other respiratory samples became the norm for laboratory diagnosis. The comparative study of the diagnostic performance of microscopy demonstrated that immunofluorescence was nonstatistically superior to Calcofluor white and Gomori methenamine silver (GMS) staining, with a sensitivity of about 90% (26).

Twenty years on, the literature still debates different PCR protocols: standard versus real-time versus touchdown PCR and the diagnostic benefits of quantitative versus qualitative PCR. Standardization of the approach, and clinical validation, for a PCR protocol and diagnostic algorithm would facilitate the use and acceptance of PCR in patient management. The results reported in this study show the diagnostic value of a validated commercial real-time PCR assay when used for patients at risk for PCP infection. PCR was 100% concordant with a positive clinical diagnosis. In this study, the sensitivity of PCR was at least equivalent to that of immunofluorescence. If the small-volume induced-sputum sample of questionable quality were excluded, PCR would outperform immunofluorescence. The sensitivity, specificity, and likelihood ratios reported here are comparable to other reports in the literature for real-time PCR assays.

The negative IF result for the subject who was PCP positive by diagnosis and strongly positive by PCR is interesting. The $C_T$ value was very low at 24.6, suggesting a severe infection. The subject, who underwent lung transplantation in 2006 and PCP prophylaxis for 1 year posttransplantation, suffered late-onset PCP at the time of testing for this study. The failure of microscopy and immunofluorescence to detect PCP infection with a majority of trophozoites and few cysts has been discussed in the literature (28, 31, 32). An imbalance in the cyst-to-trophozoite ratio has been reported for patients studied while receiving prophylaxis (32).

The average $C_T$ of 25 for the PCP-with-HIV/AIDS group (group 1) was lower than that (29.4) for the non-HIV/AIDS PCP group (group 2). This finding was expected, since the PCP burden in the HIV/AIDS population is generally found to be higher. The average $C_T$ (34.2) for the non-PCP PCR-positive group (group 3) demonstrated overlap with group 2, with weak significance ($P = 0.031$) confirming the difficulty of setting a clinical cutoff for non-HIV/AIDS populations who may have low-level PCP infection or colonization. Consequently, the PPV and NPV will differ in different patient populations.

The probability of PCP infection is well documented for the immunocompromised HIV/AIDS population and was used in this study to distinguish between moderate and low pretest probabilities of disease. Increasingly, PCP infection is found in other immunocompromised populations, without HIV/AIDS. The numerical risk of PCP infection in these groups is less well documented. A subset (17/48) of the non-HIV/AIDS patients enrolled in this study at one site were considered at moderate risk by their enrolling physicians. All were lung transplant patients who had stopped their prophylaxis treatment 1 year after the transplant. None progressed to PCP infection during the time course of this study. Chronic steroid use is associated with a higher rate of colonization and mortality in the non-HIV population (5), as is the presence of underlying lung disorders, including chronic obstructive pulmonary disorders (13).

Many papers have compared PCR diagnosis of PCP to microscopy (10, 17, 28, 29). The definition of the cutoff in each paper has usually been that most consistent with the microscopy results, and the different molecular targets and methods prevent direct comparison between many of the papers. The generally higher sensitivity of PCR assays than of microscopy could be of most value for non-AIDS patients, in whom the fungal load is generally lower. In this study, 1 of the 9 (11%) patients who were PCR positive and PCP negative at study exit developed PCP within 6 weeks of the initial diagnosis despite normal chest X-ray and CT results and negative microscopy results at the time of the study. The remaining 8 patients, for whom details are given in Table 4, did not progress to PCP infection during the 3 months of follow-up, and it is likely that the *P. jirovecii* amplified from the clinical samples of these patients is the result of colonization (6, 12, 19, 25, 37). Since microscopy and immunofluorescence are part of the diagnostic algorithm, a negative test is diagnostic for the absence of infection but may also be due to sampling error, low fungal burdens, or few *P. jirovecii* cysts. A positive PCR result, if microscopy is negative, could be confirmed by the β-1,3-glucan test, which is commonly positive in the blood of patients with PCP (23, 25, 33) but lacks specificity for the disease. In clinical settings where a positive PCR result is not supported by other clinical signs and symptoms, clinicians will have to decide whether to treat; with a highly sensitive PCR assay, this may lead to overtreatment. The very high NPV is helpful for excluding the diagnosis.

Of the 16 subjects studied more than once, 2 were diagnosed with PCP infection. For both these subjects, once the clinical diagnosis was made and PCP therapy started, the PCR results were negative. Although the sample size was too small to allow one to draw conclusions, this does suggest that a PCR assay loses sensitivity once the infection is being treated and is therefore unlikely to be of diagnostic use during prolonged periods of treatment. It may be possible to monitor the response to therapy with real-time PCR assays, as others have reported (8, 35). Five patients with lung transplants contributed 12 samples, of which at least 1 sample was PCR positive and negative by microscopy. One subject was receiving PCP prophylactic therapy. The most likely explanation of these PCR-positive test results is low-level colonization with *P. jirovecii* and carriage of the organism in the patient with the lung transplant. Transmission of infection between individuals, via environmental contamination, is a known risk (5, 15, 27). If colonization can be confirmed for an asymptomatic subject, this opens the possibility of treatment and targeted reduction of the reservoir of *P. jirovecii*.

ACKNOWLEDGMENTS

This study was funded by Myconostica Ltd.
We are indebted to Julie Morris for the statistical analyses.
The following authors are or were employees and/or shareholders of Myconostica at the time the study was conducted: M. Hughes, S. A.
Follett, X. Cui, F. Leung, G. Morgan, and A. Moody. D. S. Perlin is a shareholder. D. W. Denning is a Board director of Myconostica Ltd.

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