

Impact of HIV Infection Status on Interpretation of Quantitative PCR for Detection of *Pneumocystis jirovecii*

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Quantitative PCR (qPCR) is now a key diagnostic tool for *Pneumocystis* pneumonia. However, cutoffs to distinguish between infected and colonized patients according to their HIV status have not yet been determined. According to clinical, radiological, and biological data, we retrospectively classified bronchoalveolar lavage (BAL) samples subjected to qPCR over a 3-year period into four categories, i.e., definite PCP, probable PCP, *Pneumocystis* colonization, and no infection. Fungal burden was then analyzed according to the HIV status of the patients. Among 1,212 episodes of pneumonia screened in immunocompromised patients, 52 and 27 HIV-positive patients were diagnosed with a definite and probable PCP, whereas 4 and 22 HIV-negative patients had definite and probable PCP, respectively. Among patients with definite or a probable PCP, HIV-negative patients had a significantly lower burden than HIV-positive patients ($P < 10^{-4}$). In both groups, the median fungal burden was significantly higher in patients with definite PCP than in colonized patients. A single cutoff at 1.5×10^4 copies/ml allowed to differentiate colonized and infected HIV-positive patients with 100% sensitivity and specificity. In HIV-negative patients, cutoff values of 2.87×10^4 and 3.39×10^3 copies/ml resulted in 100% specificity and sensitivity, respectively. Using cutoffs determined for the whole population would have led us to set aside the diagnosis of PCP in 9 HIV-negative patients with definite or probable PCP. qPCR appeared to be the most sensitive test to detect *Pneumocystis* in BAL samples. However, because of lower inocula in HIV-negative patients, different cutoffs must be used according to the HIV status to differentiate between colonized and infected patients.

Pneumocystis jirovecii is the fungal agent responsible for human *Pneumocystis* pneumonia (PCP) (1). PCP is an opportunistic infection that commonly complicates the course of human immunodeficiency virus (HIV) infection. However, PCP also occurs in HIV-negative immunocompromised patients, such as solid organ transplant patients and those receiving immunosuppressive drug therapy or cytotoxic chemotherapy (2, 3). Since the advent of highly active antiretroviral therapy (HAART), the epidemiology of PCP has changed, and at present, HIV-positive and HIV-negative patients are equally represented among patients with PCP (4).

The diagnosis of PCP relies on clinical and radiological data, with confirmation being obtained by laboratory procedures. While an *in vitro* culture system for *Pneumocystis* has recently been described (5), laboratory diagnosis of PCP currently relies on the demonstration of cysts and/or trophic forms of *P. jirovecii* directly from respiratory samples, such as bronchoalveolar lavage (BAL) fluid samples or induced sputum (6). The fungal elements can be visualized using standard staining, such as Giemsa staining, silver staining, or toluidine blue staining, but a direct immunofluorescence assay, notably one targeting the cysts, significantly enhances the sensitivity of detection (7). Nonetheless, several PCR-based techniques to detect *P. jirovecii* DNA from bronchopulmonary samples have been described (8, 9). These more sensitive techniques have also revealed the existence of a new clinical form of *Pneumocystis* infection, so-called colonization, corresponding to the detection of *P. jirovecii* DNA in bronchopulmonary samples in the absence of clinical and radiological signs of PCP. Incidences of *P. jirovecii* colonization between 9 and 69% have been reported, depending on the kind of patients investigated (10, 11). This rep-

resents a serious drawback for the diagnosis of PCP, but it can be partially overcome by the use of real-time quantitative PCR (qPCR). Indeed, in addition to avoiding false-positive results, qPCR offers the possibility of quantifying the fungal burden in the considered sample, and previous studies have shown that infected patients harbor higher fungal burdens than those who are only colonized (12, 13). However, the interpretation of qPCR results remains challenging, since precise cutoffs to distinguish between these patients have rarely been calculated (13–17). Moreover, these cutoffs are difficult to compare, since either multicopy (mtLSU or MSG) or single-copy genes (DHPS or HSP70) were targeted with different technologies (FRET or TaqMan) in those studies (13, 14, 16, 18, 19).

Several features differentiate PCP occurring in HIV-positive patients from PCP occurring in HIV-negative patients. The clin-

Received 29 July 2015 Returned for modification 17 August 2015

Accepted 8 October 2015

Accepted manuscript posted online 14 October 2015

Citation Louis M, Guitard J, Jodar M, Ancelle T, Magne D, Lascols O, Hennequin C. 2015. Impact of HIV infection status on interpretation of quantitative PCR for detection of *Pneumocystis jirovecii*. *J Clin Microbiol* 53:3870–3875. doi:10.1128/JCM.02072-15.

Editor: D. W. Warnock

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This article is dedicated to Patricia Roux, who initiated the study of *Pneumocystis* in our hospital.

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ical presentation of PCP in HIV-negative patients is more severe than in HIV-positive patients, leading to estimated mortality rates of 40% and 15%, respectively (20–22). This is probably due to differences in the pathophysiology of the disease, and particularly in the host immune response. Moreover, some studies have suggested that the fungal burden may be lower in HIV-negative patients (14, 18, 19). However, the determination of qPCR cutoffs to distinguish colonization from infection in each of these populations of patients has never been performed, to the best of our knowledge.

In this study, we retrospectively analyzed clinical and biological aspects of a large panel of PCP cases diagnosed in our institution. Our results confirm the lower fungal burdens in BAL fluids from HIV-negative patients with PCP and support the need to use qPCR cutoffs specifically calculated for each population of patients to distinguish between *P. jirovecii* colonization and active PCP.

MATERIALS AND METHODS

Patients and clinical samples. We retrospectively reviewed the data associated with all the BAL fluids collected from immunocompromised adult patients receiving care in our institution from January 2011 to December 2013. A composite diagnosis of PCP based on clinical and radiological data, and results of the microscopic examination and of the qPCR performed on the BAL fluids was used to classify the BAL fluids into 4 groups: (i) definite PCP, defined as demonstration of trophic forms or cysts of *Pneumocystis* in a BAL fluid sample; (ii) probable PCP, defined as clinical PCP including outcome after anti-*Pneumocystis* therapy and radiological data supporting the diagnosis of PCP and positive qPCR but negative direct examination for *Pneumocystis* forms; (iii) *P. jirovecii* colonization, defined as absence of clinical and radiologic evidence of PCP but positive qPCR and negative direct examination for *Pneumocystis* forms; (iv) lack of *P. jirovecii* infection, defined as the absence of clinical data supporting the diagnosis of PCP and absence of positive qPCR or a positive direct examination. For each BAL fluid sample, demographic data of the patients (age and sex) and underlying condition, notably HIV status, were recorded. All samples and data were collected as a part of routine diagnostic procedures.

Microscopic diagnosis. For microscopic examination, BAL fluids were cytocentrifuged at 3,000 rpm for 5 min on two spot slides. Slides were then subjected to Giemsa staining and to a direct immunofluorescence assay (DFA) targeting *Pneumocystis* cyst forms (Monofluo kit *Pneumocystis*; Bio-Rad), according to the manufacturer's recommendations.

Real-time PCR assay. We used a previously described protocol of qPCR targeting the mitochondrial large subunit DNA locus (23). Briefly, DNA was extracted from 500 μ l of BAL fluid using the Qiagen DNA blood minikit (Qiagen) and automated purification on a QiaSymphony SP system. Five microliters of DNA solution was subjected to qPCR using a TaqMan probe on a Stratagene MX3000P system. Quality controls included a negative control and the amplification of human albumin in order to detect potential PCR inhibitors. In each run, a serial 10-fold dilution calibrated from 9 to 9,000 copies of a plasmid containing the target was used to convert cycle threshold (C_T) into number of copies per ml. A run was validated only if the qPCR efficiency was between 98 and 105%. The analytic sensitivity (lower limit of *P. jirovecii* DNA detection) of our method had been calculated at 180 copies of the target/ml in a previous study (23).

Statistical analysis. The sensitivity of the qPCR as a diagnostic test was calculated as the true-positive rate, i.e., the number of BAL fluid samples diagnosed as PCP positive, according our stratification, among all the BAL fluids with a positive qPCR. The specificity was considered the true-negative rate, i.e., number of BAL fluids collected from noninfected patients among all the BAL fluids returning a negative qPCR test. Comparisons of fungal burdens between BAL fluids collected from HIV-positive and HIV-

TABLE 1 Population characteristics and diagnosis of PCP of 1211 BALF

| Characteristic | HIV ⁺ | HIV ⁻ | P ^c |
|--|------------------|-------------------------|-------------------|
| No. of BAL samples (no. of patients) | 216 (180) | 995 (823 ^b) | |
| Sex ratio (F/M) | 0.31 | 0.7 | <10 ⁻⁴ |
| Age (yr) (mean \pm SD) | 50 \pm 12 | 61 \pm 15 | <0.05 |
| No. in category ^a (incidence [%]) | | | |
| Definitive PCP | 52 (23.8) | 27 (2.7) | |
| Probable PCP | 4 (1.8) | 22 (2.2) | |
| Colonization | 8 (3.7) | 117 (11.8) | |
| Lack of <i>Pneumocystis</i> infection | 152 (69.7) | 829 (83.4) | |

^a See Materials and Methods for definitions.

^b One HIV-positive patient receiving antiretroviral combination therapy was included in the group of HIV-negative patients because she had an undetectable viral load and 600 CD4 cells/mm³ and developed Still syndrome, requiring high-dose corticosteroid, methotrexate, and etoposide treatments.

^c Determined by the Mann-Whitney statistical test.

negative patients were performed using the nonparametric Mann-Whitney test. Analyses within the groups of HIV-positive or HIV-negative patients were performed using the nonparametric Kruskal-Wallis test, completed with comparisons of the mean rank differences, using Dunn's multiple-comparison tests. All tests were performed using Prism version 6.0 (GraphPad), considering a two-tailed *P* value of <0.05 as significant. Receiver operator characteristic (ROC) curves for the diagnosis of PCP were constructed with Prism version 6.0.

RESULTS

Sample and patient characteristics. A total of 1,211 episodes of pneumonia, investigated by means of BAL performed in 1,003 patients, were included and retrospectively studied. In this panel of patients, 180 were HIV positive and 823 were HIV negative. Demographics and PCP diagnosis, according to the HIV serologic status, are summarized in Table 1. Definite PCP, probable PCP, and colonization were diagnosed for 52, 4, and 8 BAL fluid samples collected from HIV-positive patients and 27, 22, and 117 BAL fluid samples from HIV-negative patients. The incidence of definite and probable PCP was higher among the HIV-positive patients, 31.1% (56/180), than in HIV-negative patients, 6.0% (49/823). The frequency of definite diagnosis, based on the demonstration of *P. jirovecii* forms in a BAL fluid sample, was significantly higher in HIV-positive patients than in HIV-negative patients: 92.9% (52/56) versus 55.1% (27/49) (Fisher's exact test, $P < 10^{-5}$). The main underlying conditions in HIV-negative patients with probable or definite PCP were hematological malignancy ($n = 12$; 24.5%), solid organ transplantation ($n = 20$; 40.8%), malignant solid tumor ($n = 8$; 16.3%), and corticosteroid therapy, mostly for autoimmune disorders ($n = 7$; 14.3%).

qPCR results. All BAL fluids collected from patients with a definitive diagnosis of PCP had a positive PCR, whatever their HIV serologic status, i.e., the qPCR has a sensitivity of 100%. There were 4 BAL fluid samples from HIV-positive patients and 22 from HIV-negative patients corresponding to probable PCPs, i.e., with clinical symptoms suggestive of PCP and a positive qPCR assay while the DFA was negative. In both groups of patients, the fungal burden detected in BAL fluids collected in cases of definite or probable infection did not differ significantly. In contrast, the median copy number per ml in BAL fluids collected patients with definite PCP was about 100-fold higher for HIV-positive (2.5×10^7 copies/ml) than for HIV-negative patients (1.7×10^5 copies/ml) (Mann-Whitney test; $P < 10^{-4}$) (Fig. 1).

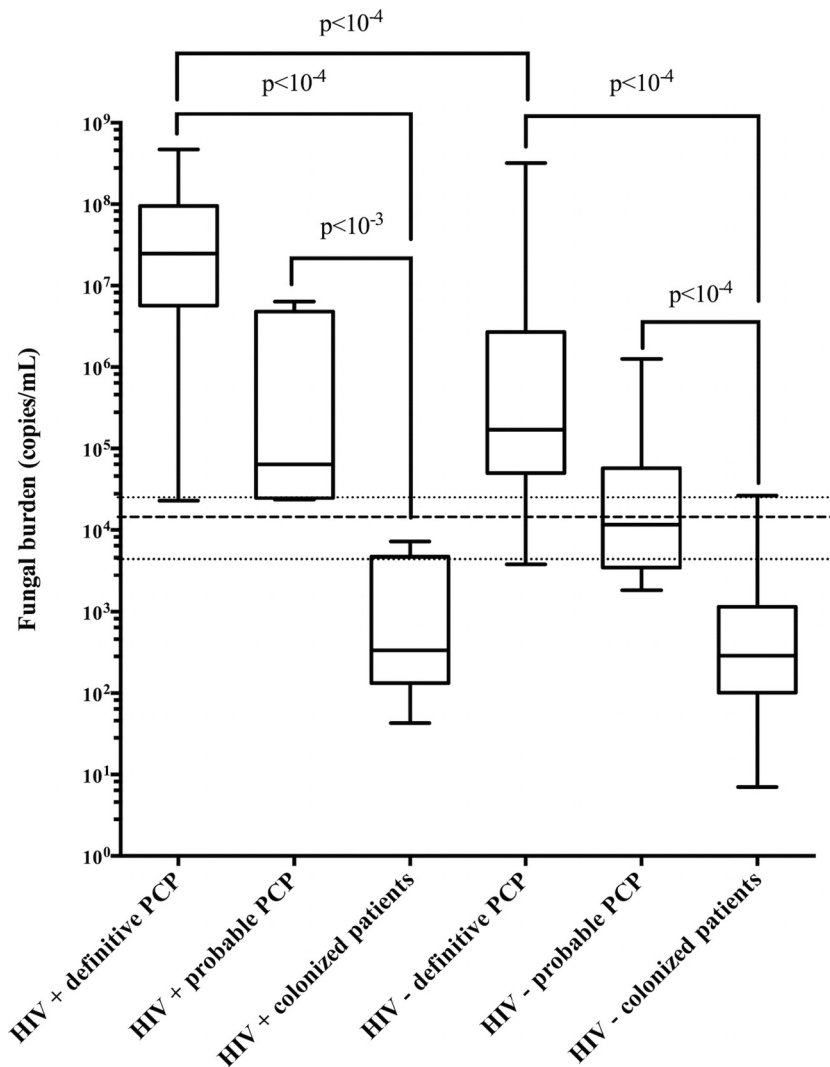


FIG 1 Box-and-whisker plots of fungal burdens according to the clinical and HIV status of the patients. Fungal burdens were calculated by determining copy numbers of the mtLSU gene detected by qPCR. *P* values were determined using the nonparametric Kruskal-Wallis test followed by comparisons of the mean rank differences using Dunn's multiple-comparison tests. Dashed and dotted lines denote the cutoff values determined using the ROC curve analysis in HIV-positive patients and HIV-negative patients, respectively.

Colonization, i.e., the presence of *P. jirovecii* DNA without clinical signs suggestive of PCP, was diagnosed for 3.7% and 11.8% of the BAL fluid samples collected from patients with and without an HIV infection, respectively. Based on these results, the specificity of the tests was calculated to be 96.3% and 88.2%, for HIV-positive and HIV-negative patients, respectively. The median fungal burden did not significantly differ between BAL fluids collected from HIV-positive and HIV-negative colonized patients (3.4×10^2 versus 2.9×10^2 , respectively; $P = 0.63$). However, in both groups of patients, the number of copies per milliliter was significantly lower than that in the BAL fluids of patients with a definitive diagnosis of PCP ($P < 10^{-4}$ in both cases) (Fig. 1).

Determination of cutoffs. We used ROC curve analysis to optimize the cutoffs in order to discriminate between colonization and infection, according to the patient's HIV status. In HIV-infected patients, a cutoff value of 1.50×10^4 copies/ml ensured a positive qPCR result for all patients with a definite or probable

diagnosis of PCP and a negative result for the others. Specificity was thus calculated to be 100% (95% confidence interval [CI], 93.1% to 100%), and sensitivity was also 100% (95% CI, 63.1% to 100%) (Fig. 2A). Considering the probable infections as true infections, this threshold allowed the corrected classification of all the 64 BAL fluids collected from HIV-positive patients with a positive qPCR (Table 2).

In HIV-negative patients, a cutoff value of 3.39×10^3 copies/ml offered the best sensitivity (100% [95% CI, 87.2% to 100%]) to differentiate between definite infection and colonization, with a specificity of 88.1% (95% CI, 80.9% to 93.6%) (Fig. 2B). Conversely, a specificity of 100% (95% CI, 96.9% to 100%) was obtained with a cutoff at 2.87×10^4 copies/ml, giving a sensitivity of 81.5% (95% CI, 61.9% to 93.7%) (Fig. 2B). Using these values to define a gray zone, 82.5% ($n = 137/166$) of the BAL fluids could be unambiguously classified as corresponding to a true infection or a colonization state.

Using the same strategy after data for BAL fluids collected from

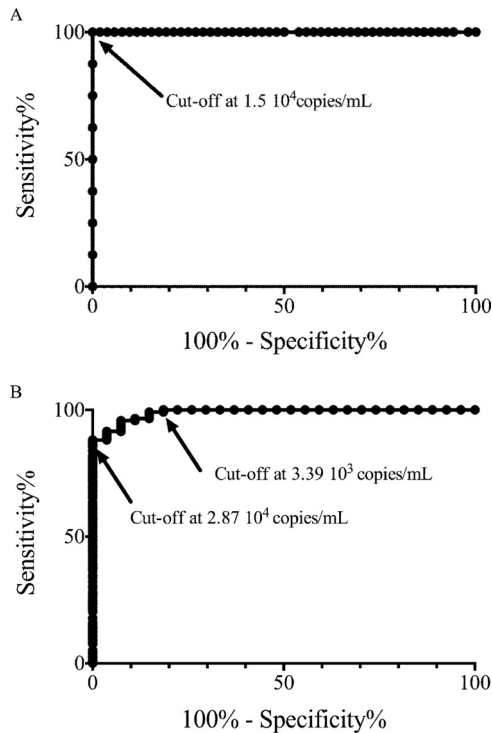


FIG 2 ROC curves of qPCR for the diagnosis of PCP in HIV-positive patients (A) and HIV-negative patients (B).

HIV-positive and HIV-negative patients were merged would have led to a lower cutoff of 6.55×10^3 copies/ml, for a sensitivity and specificity of 98.7% and 80.6%, respectively, and a higher cutoff of 1.54×10^4 copies/ml, with a sensitivity and specificity of 82.3% and 98.6%, respectively. Using these cutoffs, two and three BAL fluid samples collected from HIV-positive patients with definite and probable PCP, respectively, would have been considered to have an uncertain diagnosis, because of a fungal burden in the gray zone (Table 3). Much more worrisome are the nine cases of BAL fluids collected from HIV-negative patients, including one with a definite infection and eight with probable infections, for which the diagnosis of PCP might have been excluded, since the fungal burden was below the lower cutoff.

DISCUSSION

Despite the advent of HAART, *Pneumocystis pneumonia* remains a frequent opportunistic fungal infection (24). Interestingly, while HIV infection was the underlying disease in about 90% of patients in the 1990s, other kinds of immunosuppression are currently detected equally often as predisposing factors (4). During our

TABLE 3 Distribution of patients according to the diagnosis of PCP and their fungal burden compared to cutoff values

| HIV status and PCP category | No. of patients | | | | | |
|-----------------------------|---------------------|-------|--------------|-------|--------------------|-------|
| | Above higher cutoff | | In gray zone | | Below lower cutoff | |
| | Distinct | Whole | Distinct | Whole | Distinct | Whole |
| Positive | | | | | | |
| Definitive | 52 | 50 | 0 | 2 | 0 | 0 |
| Probable | 4 | 1 | 0 | 3 | 0 | 0 |
| Colonization | 0 | 0 | 0 | 1 | 8 | 7 |
| Negative | | | | | | |
| Definitive | 22 | 23 | 5 | 3 | 0 | 1 |
| Probable | 6 | 9 | 11 | 5 | 5 | 8 |
| Colonization | 0 | 3 | 14 | 7 | 109 | 107 |

Values were determined for HIV-positive and HIV-negative patients separately ("distinct") or for the whole population ("whole").

3-year study, we found 56 cases of definite or probable PCP in HIV-positive and 49 in HIV-negative patients.

The direct fluorescent assay, which is frequently considered the gold standard for the diagnosis of PCP, had a sensitivity of 92.8% (52/56) in HIV-positive patients in our study. In contrast, the sensitivity dropped to 55.1% (27/49) in HIV-negative patients. This is probably due to the lower fungal burdens that we, like others (14, 18, 25), found in BAL fluids of HIV-negative patients with PCP. Physicians must be aware of this serious limitation of DFA when it is used for BAL fluids obtained from HIV-negative patients. In addition, while the clinical presentation of PCP in HIV-positive patients is usually very typical, different clinical forms may occur in HIV-negative patients, making clinical diagnosis more difficult. Thus, while in the case of HIV-positive patients, diagnostic tools are used to confirm the diagnosis, with a higher specificity, a better sensitivity is required for the detection of PCP in HIV-negative patients with a low fungal burden. In our study, the failure to use qPCR would have led to misdiagnosis of 22 cases of probable PCP in HIV-negative patients. Similarly, Cerón et al. found that the diagnosis of PCP in 45 HIV-negative patients was based on qPCR in 93% of the cases (26). Another example is the study of Mühlethaler et al., which would have missed 16.6% of patients with probable PCP if they had not used qPCR (16). Thus, it is now clear from the results of our study and the literature that qPCR must be included in the diagnostic armamentarium for PCP in HIV-negative patients.

Nevertheless, qPCR results may be difficult to interpret in the case of *P. jirovecii* colonization, found in our study in 4.4 and 13.7% of the samples from HIV-positive and HIV-negative patients, respectively, values in accordance with previous studies

TABLE 2 Classification of BALF according to the HIV status of the patients, the composite diagnosis of PCP (see definitions in the text), and the fungal burden as determined by qPCR

| Category | No. (%) of patients with diagnosis using indicated cutoff (copies/ml) | | | | |
|----------------|---|------------------------|------------------------|--|-------------------------|
| | HIV positive (n = 64) | | HIV negative (n = 166) | | |
| | <1.5 × 10 ⁴ | >1.5 × 10 ⁴ | <3.4 × 10 ³ | 3.39 × 10 ³ to 2.87 × 10 ⁴ | >2.87 × 10 ⁴ |
| Definitive PCP | 0 | 52 (100) | 0 | 5 (18.5) | 22 (81) |
| Probable PCP | 0 | 4 (100) | 5 (22.7) | 11 (50) | 6 (27.3) |
| Colonization | 8 (100) | 0 | 103 (88) | 14 (12) | 0 |

(14, 16). The distinction between active infection and colonization is crucial because the decision to treat colonized patients remains a source of debate (1). Indeed, the reference treatment is full-dose co-trimoxazole, whose hematological and dermatological toxicity is well established. Some authors support the initiation of a specific therapy when immunosuppression is persistent, considering that colonization could represent an early stage of the disease (27, 28). However, this risk was found to be low in HIV-infected patients (29) and has only occasionally been reported in other immunocompromised patients (28, 30, 31). In our study, there was one case of an HIV-positive patient, diagnosed as colonized based on a first BAL fluid analysis, who was not treated with anti-*Pneumocystis* therapy, and whose second BAL fluid sample, 10 days later, was negative for *P. jirovecii* by qPCR. In contrast, one out of 15 HIV-negative colonized patients developed definite PCP (positive DFA). This patient suffered from end-stage renal insufficiency following microscopic polyangiitis, treated for months with high doses of corticosteroids and mycophenolate mofetil. The initial fungal burden was at 674 copies/ml. He did not receive any anti-*Pneumocystis* therapy and developed PCP with a fungal burden at 1.45×10^5 copies/ml 41 days later.

In both groups of patients, the fungal burden of colonized patients was significantly lower than that observed in patients with confirmed or probable PCP. However, cutoffs determined from ROC analyses differed according to the HIV serological status of these patients. Indeed, if we had used cutoffs determined for the whole population, the diagnosis of PCP would have been ruled out for 9 HIV-negative patients with definite (1 case) and probable (8 cases) PCP.

Nevertheless, classification of HIV-negative patients based solely on qPCR results is more hazardous. Indeed, 17.5% of BAL fluids collected from those patients had a fungal burden in the gray zone, i.e., between the lower and higher cutoffs. This limitation may also be due to the fact that the population of HIV-negative patients is heterogeneous, and a single cutoff value for all these patients may be not sufficient for a clear characterization. In these cases, a concomitant β -D-glucan assay may be recommended (32, 33). In the period following the study, we identified four such HIV-negative patients, presenting a negative DFA and a number of copies in the gray zone. Three had a negative β -D-glucan assay, and one had a highly positive one (>492 pg/ml). These results were found to be fully concordant with the clinical diagnosis made by physicians.

In conclusion, our study confirms that HIV-negative patients developed PCP with a lower fungal burden. qPCR appears to be the most sensitive method to detect PCP and is mandatory in HIV-negative patients to bypass the false-negative results of the DFA. Determination of cutoffs according to the patient's HIV serological status is important for a better interpretation. However, distinction between infected and colonized patients is more difficult in HIV-negative patients than in HIV-positive patients. Since, in the former population, a better sensitivity is looked for, a cutoff value lower than that used for HIV-positive patients must be used. Further studies should determine whether the relative loss of specificity could be corrected by the use of a β -D-glucan serological assay.

ACKNOWLEDGMENTS

This study was possible only with the technical expertise of the members of the Laboratoire Commun de Biologie et Génétique Moléculaires of our

institution. We thank all the physicians of our institution who shared their expertise in the management of patients and A. Retbi at the Direction de l'Information Médicale for secretarial assistance. We are indebted to Cécile Fairhead for reviewing the English language.

REFERENCES

1. Thomas CF, Jr, Limper AH. 2004. Pneumocystis pneumonia. *N Engl J Med* 350:2487–2498. <http://dx.doi.org/10.1056/NEJMra032588>.
2. Sepkowitz KA. 2002. Opportunistic infections in patients with and patients without acquired immunodeficiency syndrome. *Clin Infect Dis* 34: 1098–1107. <http://dx.doi.org/10.1086/339548>.
3. Bollee G, Sarfati C, Thierry G, Bergeron A, de Miranda S, Menotti J, de Castro N, Tazi A, Schlemmer B, Azoulay E. 2007. Clinical picture of *Pneumocystis jirovecii* pneumonia in cancer patients. *Chest* 132:1305–1310. <http://dx.doi.org/10.1378/chest.07-0223>.
4. Magne D, Angoulvant A, Botterel F, Bouges-Michel C, Bournoux ME, Bouree P, Chochillon C, Cornet M, Dannaoui E, Fekkar A, Galeazzi G, Yera H, Sarfati C, Roux P. 2011. Pneumocystosis: a network survey in the Paris area 2003–2008. *Eur J Clin Microbiol Infect Dis* 30:673–675. <http://dx.doi.org/10.1007/s10096-010-1139-0>.
5. Schildgen V, Mai S, Khalfouli S, Lusebrink J, Pieper M, Tillmann RL, Brockmann M, Schildgen O. 2014. *Pneumocystis jirovecii* can be productively cultured in differentiated CuFi-8 airway cells. *mBio* 5:e01186-14. <http://dx.doi.org/10.1128/mBio.01186-14>.
6. Tasaka S, Tokuda H. 2013. Recent advances in the diagnosis of *Pneumocystis jirovecii* pneumonia in HIV-infected adults. *Expert Opin Med Diagn* 7:85–97. <http://dx.doi.org/10.1517/17530059.2012.722080>.
7. Wolfson JS, Waldron MA, Sierra LS. 1990. Blinded comparison of a direct immunofluorescent monoclonal antibody staining method and a Giemsa staining method for identification of *Pneumocystis carinii* in induced sputum and bronchoalveolar lavage specimens of patients infected with human immunodeficiency virus. *J Clin Microbiol* 28:2136–2138.
8. Flori P, Bellele B, Durand F, Raberin H, Cazorla C, Hafid J, Lucht F, Sung RT. 2004. Comparison between real-time PCR, conventional PCR and different staining techniques for diagnosing *Pneumocystis jirovecii* pneumonia from bronchoalveolar lavage specimens. *J Med Microbiol* 53: 603–607. <http://dx.doi.org/10.1099/jmm.0.45528-0>.
9. Durand-Joly I, Chabe M, Soula F, Delhaes L, Camus D, Dei-Cas E. 2005. Molecular diagnosis of *Pneumocystis* pneumonia. *FEMS Immunol Med Microbiol* 45:405–410. <http://dx.doi.org/10.1016/j.femsim.2005.06.006>.
10. Takahashi T, Goto M, Endo T, Nakamura T, Yusa N, Sato N, Iwamoto A. 2002. *Pneumocystis carinii* carriage in immunocompromised patients with and without human immunodeficiency virus infection. *J Med Microbiol* 51:611–614. <http://dx.doi.org/10.1099/0022-1317-51-7-611>.
11. Morris A, Kingsley LA, Groner G, Lebedeva IP, Beard CB, Norris KA. 2004. Prevalence and clinical predictors of *Pneumocystis* colonization among HIV-infected men. *AIDS* 18:793–798. <http://dx.doi.org/10.1097/00002030-200403260-00011>.
12. Linsen CF, Jacobs JA, Beckers P, Templeton KE, Bakkers J, Kuijper EJ, Melchers WJ, Drent M, Vink C. 2006. Inter-laboratory comparison of three different real-time PCR assays for the detection of *Pneumocystis jirovecii* in bronchoalveolar lavage fluid samples. *J Med Microbiol* 55: 1229–1235. <http://dx.doi.org/10.1099/jmm.0.46552-0>.
13. Matsumura Y, Ito Y, Iinuma Y, Yasuma K, Yamamoto M, Matsushima A, Nagao M, Takakura S, Ichiyama S. 2012. Quantitative real-time PCR and the (1 \rightarrow 3)- β -D-glucan assay for differentiation between *Pneumocystis jirovecii* pneumonia and colonization. *Clin Microbiol Infect* 18:591–597. <http://dx.doi.org/10.1111/j.1469-0691.2011.03605.x>.
14. Alanio A, Desoubreux G, Sarfati C, Hamane S, Bergeron A, Azoulay E, Molina JM, Derouin F, Menotti J. 2011. Real-time PCR assay-based strategy for differentiation between active *Pneumocystis jirovecii* pneumonia and colonization in immunocompromised patients. *Clin Microbiol Infect* 17:1531–1537. <http://dx.doi.org/10.1111/j.1469-0691.2010.03400.x>.
15. Botterel F, Cabaret O, Foulet F, Cordonnier C, Costa JM, Bretagne S. 2012. Clinical significance of quantifying *Pneumocystis jirovecii* DNA by using real-time PCR in bronchoalveolar lavage fluid from immunocompromised patients. *J Clin Microbiol* 50:227–231. <http://dx.doi.org/10.1128/JCM.06036-11>.
16. Mühlethaler K, Bogli-Stuber K, Wasmer S, von Garnier C, Dumont P, Rauch A, Muhlemann K, Garzoni C. 2012. Quantitative PCR to diagnose

- Pneumocystis* pneumonia in immunocompromised non-HIV patients. Eur Respir J 39:971–978. <http://dx.doi.org/10.1183/09031936.00095811>.
17. Chumpitazi BF, Flori P, Kern JB, Brenier-Pinchart MP, Hincky-Vitrat V, Brion JP, Thiebaut-Bertrand A, Minet C, Maubon D, Pelloux H. 2011. Characteristics and clinical relevance of the quantitative touch-down major surface glycoprotein polymerase chain reaction in the diagnosis of *Pneumocystis* pneumonia. Med Mycol 49:704–713. <http://dx.doi.org/10.3109/13693786.2011.566894>.
 18. Maillet M, Maubon D, Brion JP, Francois P, Molina L, Stahl JP, Epaulard O, Bosseray A, Pavese P. 2014. *Pneumocystis jirovecii* (Pj) quantitative PCR to differentiate Pj pneumonia from Pj colonization in immunocompromised patients. Eur J Clin Microbiol Infect Dis 33:331–336. <http://dx.doi.org/10.1007/s10096-013-1960-3>.
 19. Robert-Gangneux F, Belaz S, Revest M, Tattevin P, Jouneau S, Decaux O, Chevrier S, Le Tulzo Y, Gangneux JP. 2014. Diagnosis of *Pneumocystis jirovecii* pneumonia in immunocompromised patients by real-time PCR: a 4-year prospective study. J Clin Microbiol 52:3370–3376. <http://dx.doi.org/10.1128/JCM.01480-14>.
 20. Roblot F, Godet C, Le Moal G, Garo B, Faouzi Souala M, Dary M, De Gentile L, Gandji JA, Guimard Y, Lacroix C, Roblot P, Becq-Giraudon B. 2002. Analysis of underlying diseases and prognosis factors associated with *Pneumocystis carinii* pneumonia in immunocompromised HIV-negative patients. Eur J Clin Microbiol Infect Dis 21:523–531. <http://dx.doi.org/10.1007/s10096-002-0758-5>.
 21. Roux A, Canet E, Valade S, Gangneux-Robert F, Hamane S, Lafabrie A, Maubon D, Debourgogne A, Le Gal S, Dalle F, Leterrier M, Toubas D, Pomares C, Bellanger AP, Bonhomme J, Berry A, Durand-Joly I, Magne D, Pons D, Hennequin C, Maury E, Roux P, Azoulay E. 2014. *Pneumocystis jirovecii* pneumonia in patients with or without AIDS, France. Emerg Infect Dis 20:1490–1497. <http://dx.doi.org/10.3201/eid2009.131668>.
 22. Boonsarngsuk V, Sirilak S, Kiatboonsri S. 2009. Acute respiratory failure due to *Pneumocystis* pneumonia: outcome and prognostic factors. Int J Infect Dis 13:59–66. <http://dx.doi.org/10.1016/j.ijid.2008.03.027>.
 23. Meliani L, Develoux M, Marteau-Miltgen M, Magne D, Barbu V, Poirot JL, Roux P. 2003. Real time quantitative PCR assay for *Pneumocystis jirovecii* detection. J Eukaryot Microbiol 50(Suppl):651. <http://dx.doi.org/10.1111/j.1550-7408.2003.tb00670.x>.
 24. Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG, White TC. 2012. Hidden killers: human fungal infections. Sci Transl Med 4:165rv113. <http://dx.doi.org/10.1126/scitranslmed.3004404>.
 25. Fillaux J, Malvy S, Alvarez M, Fabre R, Cassaing S, Marchou B, Linas MD, Berry A. 2008. Accuracy of a routine real-time PCR assay for the diagnosis of *Pneumocystis jirovecii* pneumonia. J Microbiol Methods 75: 258–261. <http://dx.doi.org/10.1016/j.mimet.2008.06.009>.
 26. Cerón I, Rabagliati R, Langhaus J, Silva F, Guzman AM, Lagos M. 2014. Características clínicas, diagnósticas y pronósticas de pacientes con neumonía por *Pneumocystis jirovecii* en individuos infectados por virus de inmunodeficiencia humana e individuos inmunocomprometidos por otra etiología. Rev Chilena Infectol 31:417–424. <http://dx.doi.org/10.4067/S0716-10182014000400007>.
 27. Morris A, Wei K, Afshar K, Huang L. 2008. Epidemiology and clinical significance of *Pneumocystis* colonization. J Infect Dis 197:10–17. <http://dx.doi.org/10.1086/523814>.
 28. Leigh TR, Kangro HO, Gazzard BG, Jeffries DJ, Collins JV. 1993. DNA amplification by the polymerase chain reaction to detect sub-clinical *Pneumocystis carinii* colonization in HIV-positive and HIV-negative male homosexuals with and without respiratory symptoms. Respir Med 87: 525–529. [http://dx.doi.org/10.1016/0954-6111\(93\)90008-N](http://dx.doi.org/10.1016/0954-6111(93)90008-N).
 29. Davis JL, Welsh DA, Beard CB, Jones JL, Lawrence GG, Fox MR, Crothers K, Morris A, Charbonnet D, Swartzman A, Huang L. 2008. *Pneumocystis* colonisation is common among hospitalised HIV infected patients with non-*Pneumocystis* pneumonia. Thorax 63:329–334. <http://dx.doi.org/10.1136/thx.2007.088104>.
 30. Reid AB, Chen SC, Worth LJ. 2011. *Pneumocystis jirovecii* pneumonia in non-HIV-infected patients: new risks and diagnostic tools. Curr Opin Infect Dis 24:534–544. <http://dx.doi.org/10.1097/QCO.0b013e32834cac17>.
 31. Lipschik GY, Gill VJ, Lundgren JD, Andrawis VA, Nelson NA, Nielsen JO, Ognibene FP, Kovacs JA. 1992. Improved diagnosis of *Pneumocystis carinii* infection by polymerase chain reaction on induced sputum and blood. Lancet 340:203–206. [http://dx.doi.org/10.1016/0140-6736\(92\)90469-J](http://dx.doi.org/10.1016/0140-6736(92)90469-J).
 32. Desmet S, Van Wijngaerden E, Maertens J, Verhaegen J, Verbeken E, De Munter P, Meersseman W, Van Meensel B, Van Eldere J, Lagrou K. 2009. Serum (1-3)-beta-D-glucan as a tool for diagnosis of *Pneumocystis jirovecii* pneumonia in patients with human immunodeficiency virus infection or hematological malignancy. J Clin Microbiol 47:3871–3874. <http://dx.doi.org/10.1128/JCM.01756-09>.
 33. Tasaka S, Hasegawa N, Kobayashi S, Yamada W, Nishimura T, Takeuchi T, Ishizaka A. 2007. Serum indicators for the diagnosis of pneumocystis pneumonia. Chest 131:1173–1180. <http://dx.doi.org/10.1378/chest.06-1467>.