Detection of *Pneumocystis jirovecii* by Quantitative PCR To Differentiate Colonization and Pneumonia in Immunocompromised HIV-Positive and HIV-Negative Patients

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Pneumocystis jirovecii pneumonia (PCP) is an acute and life-threatening lung disease caused by the fungus *Pneumocystis jirovecii*. The presentation of PCP in HIV-positive patients is well-known and consists of a triad of dyspnea, fever, and cough, whereas the presentation of PCP in HIV-negative patients is atypical and consists of a sudden outbreak, O₂ desaturation, and a rapid lethal outcome without therapy. Despite the availability of direct and indirect identification methods, the diagnosis of PCP remains difficult. The cycle threshold (C_T) values obtained by quantitative PCR (qPCR) allow estimation of the fungal burden. The more elevated that the fungal burden is, the higher the probability that the diagnosis is pneumonia. The purposes of the present study were to evaluate the C_T values to differentiate colonization and pneumonia in a population of immunocompromised patients overall and patients stratified on the basis of their HIV infection status. Testing of bronchoalveolar lavage (BAL) fluid samples from the whole population of qPCR-positive patients showed a mean C_T value for patients with PCP of 28 (95% confidence interval [CI], 26 to 30) and a mean C_T value for colonized patients of 35 (95% CI, 34 to 36) ($P < 10^{-3}$). For the subgroup of HIV-positive patients, we demonstrated that a C_T value below 27 excluded colonization and a C_T value above 30 excluded PCP with a specificity of 100% and a sensitivity of 80%, respectively. In the subgroup of HIV-negative patients, we demonstrated that a C_T value below 31 excluded colonization and a C_T value above 35 excluded PCP with a specificity of 80% and a sensitivity of 80%, respectively. Thus, qPCR of BAL fluid samples is an important tool for the differentiation of colonization and pneumonia in *P. jirovecii*-infected immunocompromised patients and patients stratified on the basis of HIV infection status with different C_T values.

Pneumocystis jirovecii pneumonia (PCP) is an acute and life-threatening lung disease caused by the fungus *Pneumocystis jirovecii* (1, 2). This disease mainly occurs in immunocompromised patients, such as HIV-positive (HIV⁺) patients and patients receiving corticosteroid therapy, chemotherapy, or biotherapy. PCP used to be the most common opportunistic infection among AIDS patients, occurring at an incidence of 3.9 per 1,000 patients per year in the 1980s and early 1990s (3). Highly active antiretroviral therapy (HAART) has prodigiously decreased the rate of PCP; however, it remains a cause of death among AIDS patients (4, 5). Moreover, immunosuppression due to chemotherapies for cancers and autoimmune diseases has become a new risk factor for PCP among HIV-negative (HIV⁻) patients (6, 7). The presentation of PCP in HIV-positive patients is well-known and consists of a triad of dyspnea, fever, and cough, whereas the presentation of PCP in HIV⁻ patients is atypical and consists of a sudden outbreak, O₂ desaturation, and a rapid lethal outcome without therapy (8–10).

During the 2000s, new diagnostic methods, such as molecular biology methods, allowed the detection of *P. jirovecii* in the sputum of healthy immunocompetent individuals, highlighting the fact that subjects without clinical symptoms of PCP can be colonized by the fungus (11–16). This colonization of nonimmunocompromised patients has cast doubt on its importance in sudden infant death syndrome, chronic obstructive pulmonary disease, cystic fibrosis, and other pulmonary syndromes (12, 15, 17–28). Although the clinical conditions and diseases for which *P. jirovecii* is responsible are unclear, the rate of colonization among individuals is underestimated (29).

Despite the availability of direct and indirect identification methods, the diagnosis of PCP remains difficult (30–32). Indeed, the *P. jirovecii* fungus is difficult to grow in culture, and the sensitivity of direct microscopic examination is low (26, 27, 33–35). PCR has greatly increased the sensitivity of detection of *P. jirovecii* DNA. However, the differentiation between colonization and pneumonia can be difficult (36). In quantitative PCR (qPCR), amplification and, thus, the cycle threshold (C_T) value are correlated with the fungal burden in the sample. The more elevated that the fungal burden is, the higher the probability that the diagnosis is pneumonia (37, 38). Thus, the use of qPCR could greatly increase the specificity of detection with a good sensitivity even if it could not detect all cases of PCP (39–44).

Nowadays, several issues remain with PCP. First, the low fungus burden among HIV⁻ immunocompromised patients makes the etiological agent responsible for the respiratory syndrome

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FIG 1 Flowchart of the study population.

difficult to establish (6). Second, the clinical evolution is different among patients, with severe outcomes being seen among HIV[−] patients with nonspecific respiratory syndromes (2). Lastly, whereas for pathologies such as AIDS or Wegener granulomatosis there are recommendations for prevention, for other pathologies the means of prevention remain unclear (45–51). In addition, because of the possibility of drug resistance and side effects, appropriate antibiotics should be prescribed and an accurate diagnosis should be made (6, 7, 12, 52, 53).

The purposes of the study were to evaluate the use of C_T values for the differentiation of colonization and pneumonia among an overall immunocompromised patient population and among subgroups of HIV⁺ and HIV[−] patients.

MATERIALS AND METHODS

Samples and patients. Respiratory samples from Nice University Hospital and other health care facilities in the southeast of France were analyzed. qPCR and microscopy were performed in the parasitology-mycology laboratory of Nice University Hospital. This prospective study was noninterventional, monocentric, and simple blinded (the patients and physicians knew the diagnosis and the qualitative results of the qPCR, but neither the physicians nor the patients knew the C_T values). This inception cohort study was conducted from 1 April 2008 to 1 October 2013 with a minimal follow-up of 3 months. All respiratory samples (sputum, induced sputum, bronchoalveolar lavage [BAL] fluid) from patients with respiratory symptoms received in our laboratory were analyzed by qPCR and microscopic assays for the detection of *P. jirovecii*. All results positive for *P. jirovecii* by qPCR were included in the analysis.

The following clinical and biological data for each patient were re-

corded: underlying disease (cancer, leukemia, AIDS and HIV infection status, autoimmune disease), radiological signs (obtained by X-ray analysis, computed tomography scan), data from a biological workup (lymphocyte cell count, lymphocyte CD4/CD8 ratio, HIV DNA burden, results of direct physical examination), treatments (curative and prophylactic treatments, long-term treatment for the underlying disease [i.e., corticosteroids, chemotherapies, HAART]), and clinical outcome.

All samples were isolated from patients as part of routine diagnosis and treatment, and no unnecessary invasive procedures were performed.

qPCR methods. Extraction of DNA from respiratory samples was performed with a QIAamp DNA minikit (Qiagen, Courtabœuf, France) according to the manufacturer's instructions. The elution volume was 100 μ l. For all BAL fluid samples, a standardized volume of 2 ml was extracted. For the remaining types of samples, the whole sample was used. Before extraction, all samples were centrifuged at 3,000 rpm for 5 min. The qPCR described by Meliani et al. was performed (54). The amplified target was the mitochondria1 large subunit (mtLSU) rRNA gene. A TaqMan probe (FAM-AGATAGTCGAAAGGGAAC-TAMRA, where FAM is 6-carboxy-fluorescein and TAMRA is 6-carboxytetramethylrhodamine) was used with the following primers: PCW3 (CTTAAATATAATCAGACTATGTGCGATAAG) and PCW4 (GGAGCTTTAATTACTGTTCTGGGC). Negative controls (two samples without any DNA) and positive controls (*P. jirovecii* DNA) were included in each run. The C_T value was registered for positive samples and was defined as the cycle number at which the fluorescence generated within a reaction crossed the fluorescence threshold. In addition to the *Pneumocystis* qPCR, for each sample, amplification of the human albumin gene was performed to assess the correct progress

TABLE 1 Characteristics of the 225 qPCR⁺ patients^a

Characteristic	Value
Sample size (no. of qPCR ⁺ patients)	225
Sex ratio (no. of men, no. of women)	1.74 (143, 82)
Mean (95% CI) age (yr)	59.65 (57.39–61.91)
Biological data	
No. (%) of HIV-positive patients	45 (19.91)
Median (IQR) HIV burden (log ₁₀ no. of DNA copies/ml) for patients with a positive serology	5.11 (3.23–6.99)
Median (IQR) lymphocyte count (no. of cells/mm ³) ^b	1,000 (600–1,700)
No. (%) of patients with the following CD4/CD8 ratio ^c :	
<0.1	40 (43.38)
0.1–1	30 (32.61)
>1	22 (23.91)
No. (%) of patients with the following underlying disease or condition:	
Leukemia	47 (20.89)
Autoimmune diseases	43 (19.11)
HIV infection (main diagnosis)	44 (19.56)
Lung diseases	37 (16.44)
Cancers	30 (13.33)
Others	14 (6.22)
Transplantation	10 (4.44)
No. (%) of patients with the following symptoms:	
Fever	77 (34.22)
Cough	68 (30.22)
Dyspnea	125 (55.56)
Two symptoms	76 (33.78)
Three symptoms	27 (12)

^a CI, confidence interval; IQR, interquartile range.

^b Data were missing for 22 patients, and data for patients with aplasia were excluded.

^c Data were missing for 133 patients.

TABLE 2 Comparison of the characteristics of the PCP and PCP⁻ patients

Characteristic	Value(s) for:		P value by univariate analysis
	PCP ⁺ patients	PCP ⁻ patients	
No. (%) of patients	73 (32.44)	152 (67.56)	
No. (%) of male patients	48 (65.75)	96 (63.16)	0.63
Mean (95% CI) age (yr)	55.0 (51.21–59.8)	61.70 (59.04–64.38)	0.01
No. (%) of patients HIV positive	30 (41.10)	15 (9.87)	<0.01
Median (IQR) HIV burden (log ₁₀ no. of DNA copies/ml)	5.71 (5.31–5.91)	4.73 (3.81–5)	0.01
Biological data			
Median (IQR) lymphocyte count (no. of cells/mm ³)	800 (500–1,300)	1,100 (700–1,800)	0.03
Median (IQR) CD4 count (no. of cells/mm ³)	79 (21–258)	258 (80–794)	0.02
No. (%) of patients with the following CD4/CD8 ratio:			<0.01
<0.1	17 (35.42)	5 (11.36)	
0.1–1	17 (35.42)	13 (29.55)	
>1	14 (29.16)	26 (59.09)	
Mean (95% CI) CT value	28 (26–30)	35 (33–36)	<0.01
No. (%) of patients with the following underlying disease:			
HIV infection (main comorbidity)	30 (41.10)	14 (9.21)	<0.01
Leukemia	19 (26.03)	28 (18.42)	0.18
Autoimmune disease	11 (15.07)	32 (21.05)	0.29
Cancers	6 (8.22)	24 (15.79)	0.14
Transplantations	3 (4.11)	7 (4.61)	1
Lung diseases	3 (4.11)	34 (22.37)	<0.01
No. (%) of patients with the following symptoms:			
Fever	38 (52.05)	39 (25.66)	<0.01
Cough	35 (47.95)	33 (21.71)	<0.01
Dyspnea	55 (75.54)	70 (46.05)	<0.01
Two symptoms	42 (57.53)	34 (22.37)	<0.01
Three symptoms	23 (31.51)	4 (2.63)	<0.01
No. (%) of patients receiving the following curative treatment:			<10 ⁻⁴
Anti- <i>Pneumocystis</i> therapy	73 (100.00)	9 (14.08)	
Anti- <i>Pneumocystis</i> therapy with corticosteroids	2 (2.74)		
Antibiotics ^a	0 (0.00)	44 (61.97)	
Corticosteroids (main line)	0 (0.00)	5 (7.04)	
Antituberculosis drugs	0 (0.00)	9 (12.68)	
Other drugs ^b	0 (0.00)	9 (12.68)	
APP ^c alone	1 (2.17)	0 (0.00)	
Combination of immunosuppressive therapies and APP	2 (4.34)	4 (6.49)	
Combination of HAART and APP	2 (4.34)	0 (0.00)	
Combination of APP and other drugs ^b	1 (2.17)	1 (1.62)	
Total no. (%) of patients receiving APP	6 (13.02)	5 (8.11)	

^a Antibiotics other than anti-*Pneumocystis* therapy.^b Other drugs consisted of cardiologic drugs.^c APP, anti-*Pneumocystis* prophylaxis.

of DNA extraction and to confirm the absence of PCR inhibitors. The human albumin gene is a housekeeping gene. We used primers GCTGT CATCTCTTGTGGGCTGT (0.3 μM) and ACTCATGGGAGCTGCTG GTTC (0.3 μM) and a probe specific for the human albumin gene (VIC-GGAGAGATTTGTGTGGGCATGACA-TAMRA [0.25 μM]) (55).

Detection by microscopy. Samples were cytocentrifuged, and then the slides were stained with May-Grünwald-Giemsa (MGG) and toluidine blue O stains (56). Experienced personnel examined the slides for cyst and vegetative forms. The results were given qualitatively.

Comparison of the C_T value with the physician's final diagnosis. In order to compare the C_T values among standardized samples, only the C_T values for the BAL fluid samples were included in the analysis of sensitivity and specificity and for definition of the C_T cutoff values.

The physician's decision to consider a diagnosis of PCP for the patient was first made according to the clinical, biological (microscopy findings, lymphocyte CD4/CD8 ratio), and radiological data together with the patient's history (presence of a chronic disease, HIV infection status, corticosteroid use). Before the end of the study, all cases were reviewed by a collegial multidisciplinary team of pneumologists and infectious disease specialists for determination of a final diagnosis of PCP or not. This final diagnosis was used as the reference, and the C_T values obtained by qPCR were compared with this final diagnosis (PCP versus colonization, i.e., no PCP [PCP⁻]). Patients considered to have PCP were treated with curative sulfadiazine-trimethoprim or curative atovaquone. Some patients were also treated with an anti-*Pneumocystis* drug to treat a pulmonary disease other than PCP. These patients were considered not to have PCP. Patients

FIG 2 ROC curves of C_T values obtained by qPCR for different populations. (A) ROC for the whole population of qPCR⁺ patients (HIV⁺ and HIV[−] patients); (B) ROC curve for the population of qPCR⁺ and HIV⁺ patients; (C) ROC curve for the population of qPCR⁺ and HIV[−] patients.

with a positive qPCR result without a final diagnosis of PCP were considered colonized (PCP[−]). If the qPCR result was negative, the patient was considered to be not infected by *P. jirovecii*.

To our knowledge, no postmortem study was performed, and a cause of death was found for all patients who died.

Statistical analysis. Statistical analyses were performed with SAS software (version 9.4). All the results were verified with an alpha risk threshold of 0.05. The Student and Mann-Whitney tests were used for qualitative variables, and the chi-square or Fisher test was used for categorical variables. Univariate survival analyses were performed by a log rank test. C_T value thresholds were estimated with receiver operating characteristic (ROC) curves, which were used to compare the sensitivity and specificity of the C_T values obtained by qPCR with the clinical diagnosis (39). C_T values were obtained with 80% sensitivity and 80% specificity.

Analysis of the risk factors for PCP was performed by logistic regression, with an adjustment for age being used for multivariate analysis. For the survival analysis, we used the Cox model with an adjustment for sex and age. The collinearities between the lymphocyte CD4/CD8 ratio and the clinical diagnosis or death were studied by use of the Spearman correlation.

On the basis of the findings of the study of Hughes et al., the prevalence of the risk of development of PCP in the population of HIV⁺ and HIV[−] patients was 35% (57). Taking this number into account, we estimated that 70 PCP patients and 130 PCP[−] patients were the minimal numbers of patients required to obtain an expected sensitivity of 90% with a lower 95% confidence limit of greater than 0.75 with a 0.95 probability (58).

RESULTS

From 1 April 2008 to 1 October 2013, we performed 1,964 qPCRs for 986 patients, with the results for 226 (11.5%) of the qPCRs being positive (qPCR⁺). Seventy-three patients (33.77%) were diagnosed with PCP. No patient with PCP had a negative qPCR result. This whole population was separated into several subgroups, as shown in Fig. 1.

Description of the entire qPCR⁺ population. The characteristics of the population of qPCR⁺ patients are described in Table 1. All qPCR⁺ patients suffered from chronic diseases (Table 1). Seventy-four percent of the patients were receiving immunosuppressive therapies, and 13.82% were receiving HAART. All patients were immunocompromised and had low lymphocyte counts. The diagnosis for two patients was later corrected and they were considered colonized (i.e., the diagnosis of PCP for two patients became PCP[−]).

The long-term treatment for the chronic disease was unknown for 102 patients. Among these 102 patients, 26 were HIV positive; 12 of these patients were not receiving HAART because HIV infection was newly diagnosed or the patients were noncompliant. Nine of the 12 patients were PCP positive, and 2 died. Six patients had a positive qPCR result, despite anti-*Pneumocystis* preventive therapy, and among them, 4 were diagnosed with PCP.

Direct examinations were positive for 30 (13.33%) patients, and they were all diagnosed with PCP. Interestingly, at least two different qPCR results (negative to positive or positive to negative without a statistically significant difference) were obtained for 13 (5.78%) patients who were confirmed to have a positive qPCR result. They were all PCP[−].

Comparison of the PCP and PCP[−] patients among the 225 qPCR-positive patients. The characteristics of the PCP and PCP[−] patients among the 225 qPCR-positive patients are compared in Table 2. The distribution of the underlying disease and the long-term treatments were different between the PCP and PCP[−] patients ($P < 10^{-4}$), with the cause of immunosuppression in the majority of PCP patients being a disease or condition other than HIV infection. There was no difference between the PCP and PCP[−] patients in the long-term treatments used to treat the chronic disease (PCP prophylaxis, immunosuppressive therapies, HAART, and others drugs) ($P = 0.06$), but there were differences in the curative treatments used. All patients with PCP received anti-*Pneumocystis* therapy. Two HIV⁺ patients with PCP received adjuvant corticosteroid therapy. Among the nine (14.08%) PCP[−] patients who received anti-*Pneumocystis* therapy, five were first suspected of having PCP but the diagnosis was later changed to PCP[−], and for the other four patients, either the patient had a bacterial infection that was treated with anti-*Pneumocystis* therapy or the decision was made to consolidate *P. jirovecii* prophylaxis. The rate of occurrence of PCP was 41.1% among the HIV⁺ patients and 58.9% among the HIV[−] patients ($P < 0.01$). The evolution of symptoms was favorable in 82.09% of PCP patients, whereas it was favorable in 69.08% of PCP[−] patients ($P = 0.03$). The vital status at the end of the study was unknown for two PCP[−] patients. There was no statistically significant difference between the times to death between the PCP and PCP[−] patients (487 days [interquartile range {IQR}, 377 to 597 days] and 492 days [IQR,

FIG 3 Graphical representation of C_T values (indicated on the gray bar at the top) according to the population studied with the associated exclusion diagnostics. Blue arrows and dashed blue arrows, mean C_T values and 95% CIs, respectively, for the population of PCP and PCP⁻ patients; green, orange, and yellow squares, C_T values for the different populations used to exclude PCP and colonization with 80% sensitivity and 80% specificity, respectively.

423 to 562 days] [$P = 0.15$], respectively) and the rates of survival between PCP and PCP⁻ patients (23.29% and 28.95%, respectively; $P = 0.37$). Comparison of the results of microscopic analysis and the clinical decision of whether a patient had a diagnosis of PCP or was colonized with PCP confirmed the high specificity (100%) but the low sensitivity (41%) of this method. The completeness of the 5-year follow-up in the area studied allowed us to investigate the seasonality issue, and we did not find a statistically significant result.

Cycle threshold values: a clue to differentiate *Pneumocystis jirovecii* pneumonia and colonization. The C_T values obtained for the different subgroups were compared by taking into account only the values obtained with the BAL fluid specimens (Fig. 1). The mean C_T value for PCP patients was 28 (95% confidence interval [CI], 26 to 30), whereas it was 35 (95% CI, 34 to 36) for colonized patients ($P < 10^{-3}$), confirming the low fungus burden among colonized patients. The ROC curve showed that a C_T value of 32 had an ability to discriminate between colonization and PCP with a 72% sensitivity (95% CI, 57 to 81%) and a 75% specificity (95% CI, 67 to 83%) (area under the curve [AUC], 0.88; 95% CI, 0.80 to 0.96) (Fig. 2A).

When we stratified the patients on the basis of their HIV infection status, the mean C_T values were 24.5 (95% CI, 21.8 to 27.5) and 30.5 (95% CI, 28.0 to 32.8) ($P = 0.004$) for HIV⁺ and HIV⁻ patients, respectively. These data confirmed the high fungus burden in HIV⁺ patients.

Among the HIV⁺ patients, the ROC curve showed that a C_T value of 27 was able to exclude colonization with a sensitivity of 74% (95% CI, 46 to 84%) and a specificity of 100% (95% CI, 72 to 100%) (AUC, 0.90; 95% CI, 0.79 to 1.00) (Fig. 2B).

For the HIV⁻ patients, the ROC curve showed that a C_T value of 35 excluded colonization with a sensitivity of 80% (95% CI, 63 to 88%) and a specificity of 60% (95% CI, 50 to 69%) (AUC, 0.72; 95% CI, 0.62 to 0.82) (Fig. 2C).

For all the populations described above, we propose that the C_T values are able to exclude colonization and pneumonia with a specificity of 80% and a sensitivity of 80%, respectively (Fig. 3; Table 3). A gray zone between these C_T values remained, and for patients with these values, physicians will have to decide by the use of other parameters whether the patient is colonized or should be considered to have PCP (Fig. 3).

Interestingly, 2 PCP patients with C_T values of 35 and 36 were retrospectively considered to be PCP⁻ by the physicians. These data confirmed the good exclusion diagnostic of a C_T value above 35 in HIV⁻ patients.

In our study, all the C_T values for the subgroups described in Table 3 matched the clinical decision to consider a patient to have a diagnosis of PCP or to be colonized with PCP. The negative predictive values (NPVs) were higher for HIV⁺ patients than for HIV⁻ patients (90% and 80%, respectively). The characteristics of the patients with C_T values in the gray zone are described in Table 4.

TABLE 3 C_T values for exclusion of a diagnosis and the corresponding values of sensitivity and specificity according to the population studied

Patient group	Exclusion of PCP			Exclusion of colonization		
	C_T value	Sensitivity	Specificity	C_T value	Sensitivity	Specificity
Whole qPCR ⁺ population	>35	0.80	0.63	<31	0.66	0.80
HIV ⁺ patients	>30	0.80	0.79	<27	0.73	1.00
HIV ⁻ patients	>35	0.80	0.60	<31	0.59	0.80

TABLE 4 Clinical data for patients with qPCR⁺ result and C_T values in gray zone^a

Patient group and no.	PCP status	Sex	Microscopy result	Underlying disease or condition ^b	Treatment	Death
qPCR ⁺ HIV ⁺ patients with gray zone C _T values between 27 and 30						
167	Neg	M	Neg	HIV infection (5.11)	HAART	No
168	Neg	F	Neg	HIV infection (4.49)	Combination of APP and other drugs ^d	No
193	Pos	M	Neg	HIV infection (6.34)	Anti- <i>Pneumocystis</i> therapy	No
qPCR ⁺ HIV ⁻ patients with gray zone C _T values between 31 and 35						
22	Neg	M	Neg	Lung disease	Other drugs ^d	No
26	Neg	F	Neg	Lung disease	Antituberculosis drugs	No
36	Neg	M	Neg	Cancer	Antibiotics	No
48	Neg	F	Neg	Leukemia	Missing data	Yes
52	Neg	M	Neg	Autoimmune disease	Antibiotics	No
55	Neg	F	Neg	Cancer	Other drugs ^d	No
63	Neg	M	Neg	Other	Missing data	Yes
69	Neg	M	Neg	Leukemia	Missing data	Yes
74 ^c	Neg	F	Neg	Autoimmune disease	Anti- <i>Pneumocystis</i> therapy	Yes
75	Neg	M	Neg	Transplantation	Missing data	Yes
77	Neg	F	Neg	Lung disease	Missing data	No
78	Neg	M	Neg	Leukemia	Antibiotics	Yes
81	Pos	M	Neg	Leukemia	Anti- <i>Pneumocystis</i> therapy	No
91	Pos	M	Neg	Leukemia	Anti- <i>Pneumocystis</i> therapy	No
94	Neg	F	Neg	Autoimmune disease	Corticosteroid	No
104	Pos	F	Neg	Cancer	Anti- <i>Pneumocystis</i> therapy	Yes
107	Neg	F	Neg	Leukemia	Corticosteroid	Yes
121	Neg	F	Neg	Autoimmune disease	Antibiotics	No
131	Pos	M	Neg	Autoimmune disease	Anti- <i>Pneumocystis</i> therapy	No
134	Neg	F	Neg	Lung disease	Other drugs ^d	No
135	Pos	M	Neg	Leukemia	Anti- <i>Pneumocystis</i> therapy	No
151	Neg	M	Neg	Autoimmune disease	Antibiotics	No
152	Neg	F	Neg	Lung disease	Antituberculosis drugs	No
174	Pos	M	Pos	Leukemia	Anti- <i>Pneumocystis</i> therapy	Yes
183	Pos	F	Neg	Leukemia	Anti- <i>Pneumocystis</i> therapy	Yes
185	Neg	F	Neg	Lung disease	Corticosteroid	Yes
197	Neg	M	Neg	Other	Antibiotics	No
198	Pos	M	Neg	Leukemia	Anti- <i>Pneumocystis</i> therapy	No
201	Neg	F	Neg	Leukemia	Antibiotics	Yes
202	Neg	M	Neg	Transplantation	Missing data	No
206	Neg	M	Neg	Autoimmune disease	Antibiotics	No
210	Pos	M	Neg	Leukemia	Anti- <i>Pneumocystis</i> therapy	Yes
211	Neg	M	Neg	Leukemia	Missing data	No
213	Neg	F	Neg	Autoimmune disease	Antibiotics	Yes
226	Neg	F	Neg	Leukemia	Missing data	No

^a C_T values in the gray zone are defined in Fig. 3. Neg, negative; Pos, positive; M, male; F, female; APP, anti-*Pneumocystis* prophylaxis.

^b The values in parentheses represent the HIV burden (log₁₀ number of DNA copies per milliliter) in HIV⁺ patients.

^c Patient 74 was first diagnosed with PCP, but the final diagnosis was an infection by influenza virus.

^d Other drugs consisted of cardiologic drugs.

Risk factors for *Pneumocystis jirovecii* pneumonia and survival analysis. In order to study the risk factors for PCP, we analyzed the underlying disease and biological parameters by the use of age-adjusted *P* values (Table 5). Because of non-log linearity, C_T values were classified into the following groups: those below 25, those between 25 and 35, and those above 35. After multivariate block analysis, C_T values, hemopathy, and HIV-positive status were found to be significant risk factors for PCP (*P* = 0.05).

Collinearity showed an inverse association between the CD4/CD8 ratio and patient death. The lower that the ratio was, the higher that the risk of death was (*F* < 0.05). The rate of survival of

TABLE 5 Final analysis of risk factors for PCP

Variable	Adjusted <i>P</i> value	Hazard ratio (95% CI)
Age	0.82	1.00 (0.97–1.02)
C _T value		
<25	<0.01	22.20 (6.53–75.41)
25–35	0.03	4.69 (1.49–14.78)
>35 ^a		
HIV	<0.01	4.84 (1.82–12.91)
Hemopathy	<0.01	3.32 (1.42–7.76)

^a The reference cutoff value.

HIV⁺ patients was better than that of HIV[−] patients (log rank test $P = 0.02$). Nevertheless, regression analysis by use of the Cox model did not find any risk factor for mortality.

DISCUSSION

In this study, we demonstrated that the use of qPCR could greatly improve the differential diagnosis of colonization and pneumonia in patients infected with *P. jirovecii*. Contrary to the findings of some studies, no patient with a negative qPCR result was observed among patients with PCP in the area of the Nice University Hospital between April 2008 and October 2013 (39–44). All patients with positive microscopy results were diagnosed with PCP and had C_T values below the threshold for exclusion of a diagnosis of PCP. Thus, if microscopy had been chosen as the reference, there would not have been false-negative qPCR results in our study. Whereas other studies used microscopy examination as the reference method, we chose to use clinical presumption to consider whether the patient had PCP or not (37, 38, 59, 60). A rapid diagnosis of PCP is required, as any delay in the initiation of the treatment increases the risk of mortality. Once the pulmonary sample is collected, PCR can rapidly be performed; however, a positive qPCR result for immunocompromised patients could be misleading in the determination of whether the patient had PCP or was colonized. This highlights the need for an accurate diagnosis in order to avoid unnecessary treatment. It is thus important to determine C_T threshold values to give physicians information that is as accurate as possible. In our study, the C_T values obtained by qPCR showed a high power to differentiate colonization and pneumonia. Moreover, we were able to determine different C_T values according to the population of immunocompromised patients: HIV⁺ versus HIV[−] patients. As already described, we found a correlation between the fungal burden and C_T values: the higher that the fungal burden was, the lower that the C_T value was (37, 38, 61). The mean C_T value was significantly higher for HIV[−] immunocompromised patients with PCP than HIV⁺ patients with PCP. These data confirm that expression of the disease occurs with a lower fungal burden in HIV[−] immunocompromised patients than HIV⁺ patients. In our study, the C_T values were defined to obtain a sensitivity of 80% and a specificity of 80%, allowing the C_T values to provide physicians with useful insight. Some studies determined various PCR cutoff values that may be used to discriminate PCP from colonization, but very few of them gave different values according to HIV infection status (62, 63). The C_T values given in our study could be used by other laboratories, provided that extraction and qPCR are performed as described in our Materials and Methods.

Since the use of the HAART and prophylaxis, the number of HIV⁺ patients affected by PCP has decreased, but for some patients, *P. jirovecii* pneumonia still precedes a diagnosis of HIV infection and is still a cause of mortality (1, 2, 64). PCP occurs in patients with a lack of access to medical care or unaware of their HIV infection status, as was the case for 12 of 14 HIV⁺ patients in the present study. In our study, the proportion of HIV[−] patients with PCP was greater than the proportion of HIV⁺ patients with PCP. We also found a higher rate of mortality among HIV[−] patients than HIV⁺ patients (10, 39, 49, 65).

P. jirovecii pneumonia represents a serious public health care problem not only because of the severity of the disease but also because *P. jirovecii* pneumonia is often a concomitant pathology aggravating a life-threatening disease in HIV[−] patients (2).

Whereas some studies estimated the rate of mortality to be 7 to 10% among HIV⁺ PCP patients, the rate of mortality increased from 10% in renal transplantation patients to 50% in cancer patients (66–68). These rates of mortality raise the question of the condition of prophylaxis and how it should be implemented in HIV[−] patients. Another public health problem is the number of colonized patients in hospital units where other patients may be at high risk for infection or colonization. In our study, 67.6% ($n = 152$) of patients were colonized, which can potentially lead to nosocomial infections (23, 69, 70).

The analysis of the C_T value was performed by taking into account only BAL fluid samples, for which a standardized protocol of extraction and PCR allowed comparison of the data obtained for the different patients. This represents a selection bias, as not all the patients were able to undergo BAL because of their lung function; however, it was necessary to use a standard sample.

The diagnosis of PCP or colonization depends on a complex algorithm based on patient history, biological and radiological data, treatment, and patient clinical evolution. If a positive microscopic examination leads to a high probability of PCP, a negative qPCR result cannot exclude the diagnosis, especially in HIV[−] patients. In most cases, qPCR is sensitive enough to allow a diagnosis of PCP to be made in HIV[−] patients; however, the presence of a gray zone of C_T values prevents this assay from becoming the reference method (44, 71). In the case of a C_T value in the gray zone, the physician will have to choose between prophylactic or curative treatment according to the findings for the other parameters described above.

Conclusion. qPCR of BAL fluid samples is an important tool for the differentiation of colonization and pneumonia in patients with *P. jirovecii* infections. The C_T values are different after stratification on the basis of the patient's HIV infection status. When C_T values are in the gray zone, the qPCR result, in addition to the findings for other clinical and paraclinical parameters, will help to determine whether the patient should be considered to have PCP or to be colonized.

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