



Semiquantitative Real-Time PCR to Distinguish *Pneumocystis* Pneumonia from Colonization in a Heterogeneous Population of HIV-Negative Immunocompromised Patients

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ABSTRACT *Pneumocystis jirovecii* is a threat to iatrogenically immunosuppressed individuals, a heterogeneous population at rapid growth. We assessed the ability of an in-house semiquantitative real-time PCR assay to discriminate *Pneumocystis* pneumonia (PCP) from colonization and identified risk factors for infection in these patients. Retrospectively, 242 PCR-positive patients were compared according to PCP status, including strata by immunosuppressive conditions, human immunodeficiency virus (HIV) infection excluded. Associations between host characteristics and cycle threshold (C_T) values, semiquantitative real-time PCR correlates of fungal loads in lower respiratory tract specimens, were investigated. C_T values differed significantly according to PCP status. Overall, a C_T value of 36 allowed differentiation between PCP and colonization with sensitivity and specificity of 71.3% and 77.1%, respectively. A C_T value of less than 31 confirmed PCP, whereas no C_T value permitted exclusion. A considerable diversity was uncovered; solid organ transplant (SOT) recipients had significantly higher fungal loads than patients with hematological malignancies. In SOT recipients, a C_T cutoff value of 36 resulted in sensitivity and specificity of 95.0% and 83.3%, respectively. In patients with hematological malignancies, a higher C_T cutoff value of 37 improved sensitivity to 88.5% but reduced specificity to 66.7%. For other conditions, assay validity appeared inferior. Corticosteroid usage was an independent predictor of PCP in a multivariable analysis and was associated with higher fungal loads at PCP expression. Semiquantitative real-time PCR improves differentiation between PCP and colonization in immunocompromised HIV-negative individuals with acute respiratory syndromes. However, heterogeneity in disease evolution requires separate cutoff values across intrinsic and iatrogenic predisposition for predicting non-HIV PCP.

IMPORTANCE *Pneumocystis jirovecii* is potentially life threatening to an increasing number of individuals with compromised immune systems. This microorganism can cause severe pneumonia in susceptible hosts, including patients with cancer and autoimmune diseases and people undergoing solid organ transplantation. Together, these patients constitute an ever-diverse population. In this paper, we demonstrate that the heterogeneity herein has important implications for how we diagnose and assess the risk of *Pneumocystis* pneumonia (PCP). Specifically, low loads of microorganisms are sufficient


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 Semiquantitative real-time PCR can improve differentiation between non-HIV PCP and colonization, but a significant heterogeneity in fungal loads at disease evolution requires separate cut-off values across non-HIV immunosuppressive predispositions.

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to cause infection in patients with blood cancer compared to those in solid organ recipients. With this new insight into host versus *P. jirovecii* biology, clinicians can manage patients at risk of PCP more accurately. As a result, we take a significant step toward offering precision medicine to a vulnerable patient population. On the one hand, these patients have propensity for adverse effects from antimicrobial treatment. On the other hand, this population is susceptible to life-threatening infections, including PCP.

KEYWORDS *Pneumocystis jirovecii*, PCP, colonization, immunosuppression, real-time PCR

P*neumocystis jirovecii* is an atypical fungus and causative agent of *Pneumocystis* pneumonia (PCP) (1). Historically, PCP reemerged with the onset of the human immunodeficiency virus (HIV) epidemic as an opportunistic infection and hallmark of AIDS in the 1980s (2). Since the introduction of antiretroviral therapy and prompt administration of PCP prophylaxis, this disease burden is declining (3). Rather, it is becoming overshadowed by PCP in non-HIV immunocompromised populations, especially in resource-rich countries with universal health care (3). Nowadays, *P. jirovecii* represents a life threat to patients with malignancies, immunological disorders, chronic lung diseases, and those undergoing solid organ transplantation (SOT) (4). Their susceptibility to PCP is largely attributed to iatrogenic immunosuppression besides intrinsic host factors (5).

The clinical characteristics of PCP vary according to the degree of immunosuppression and, more markedly, with respect to the host's HIV status (3). First, non-HIV patients typically have a more fulminant onset, rapid progression of severe pneumonitis with respiratory failure, and higher mortality (4). Second, their respiratory samples contain fewer *P. jirovecii* organisms and more neutrophils, features of both diagnostic and prognostic importance (1). Although HIV status is the principal host distinction, HIV-negative patients represent a heterogeneous population with diverse risk profiles (3). Moreover, diagnosing non-HIV PCP is notoriously difficult due to absence of pathognomonic features and a broad differential (6).

Diagnostic guidelines for PCP recommend a multimodal algorithm including detection of *P. jirovecii* (7). Microscopic visualization has been the gold standard, since culturing of *P. jirovecii* is extremely difficult, but the sensitivity of microscopy is especially poor when applied to respiratory samples from non-HIV patients (1). Since the 1990s, highly sensitive PCR-based assays have become widely utilized (8). However, difficulties with differentiating between PCP and colonization, that is, presence of *P. jirovecii* in the absence of clinical pneumonia, has proven a drawback of this technology (4). In fact, this has repercussions for antimicrobial treatment guidance. Prompt initiation is vital for the prognosis of PCP, whereas management of colonization remains debated (1). Our objective was to assess the utility of an in-house semiquantitative real-time PCR-assay for diagnosing PCP in HIV-negative immunocompromised patients and identify predictors for infection.

RESULTS

Description of study population and comparisons according to PCP status. A total of 242 HIV-negative patients (100 female, 142 male) with positive *P. jirovecii* PCR were included, representing 84.0% of 288 presumed eligible patients (Fig. 1). Patient characteristics and univariate comparison according to PCP status are presented in Table 1.

With the present case definition, the condition was classified as PCP (PCP⁺) in 196 patients and as colonization (PCP⁻) in 46 patients. Demographics were comparable apart from cardiovascular comorbidity being more common among PCP⁻ patients. Chronic lung diseases were associated with colonization. Otherwise, PCP status seemed independent of immunosuppressive condition and regimen. However, the median corticosteroid dose (first quartile [q₁] to third quartile [q₃]) at presentation was higher among PCP⁺ patients (10 [5 to 24] versus 4 [4 to 8] mg methylprednisolone/

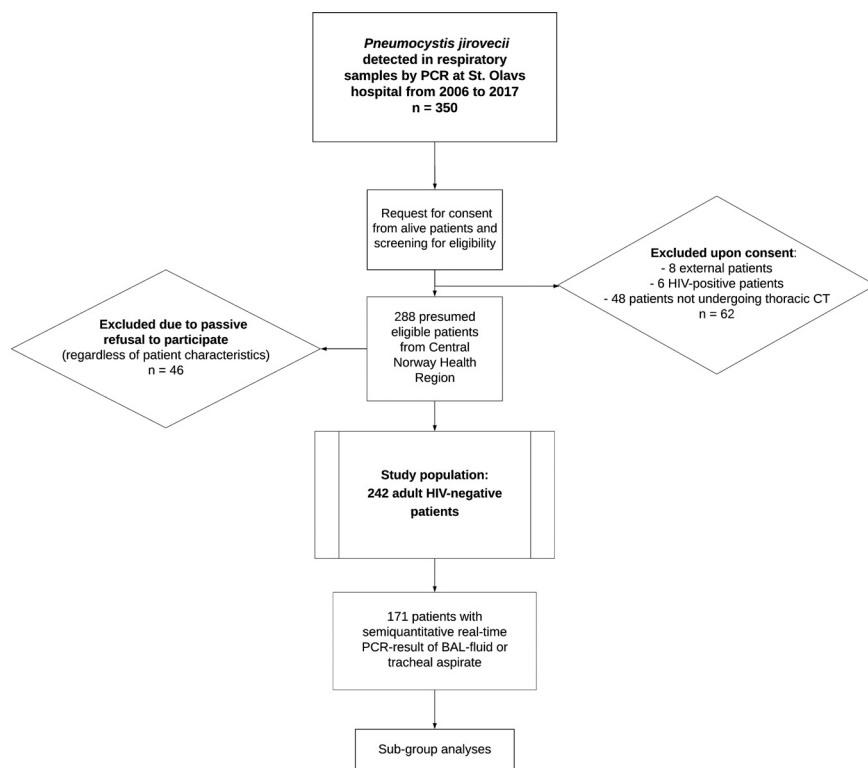


FIG 1 Flowchart of the study population. Adult patients tested in the regional referral laboratory and undergoing thoracic CT during diagnostic workup were eligible for inclusion. External referral and HIV seropositivity were exclusion criteria. All deceased patients were included, whereas recruitment of alive patients required active consent. BAL, bronchoalveolar lavage; CT, computed tomography; HIV, human immunodeficiency virus; PCP, *Pneumocystis pneumonia*.

day, $P < 0.001$). Besides, PCP⁺ patients manifested more signs and symptoms of respiratory impairment and specific laboratory and radiological abnormalities (e.g., lymphopenia and crazy paving, respectively).

Sensitivity of microscopy and diagnostic discrimination by semi-quantitative real-time PCR. Respiratory samples were mainly collected as bronchoalveolar lavage (BAL) fluid ($n = 203$, 83.9%), followed by sputum ($n = 25$, 10.3%), induced sputum ($n = 8$, 3.3%), tracheal aspirate ($n = 4$, 1.7%), respiratory biopsy sample ($n = 1$, 0.4%), and nasopharyngeal swab sample ($n = 1$, 0.4%) (see Fig. S1 in the supplemental material). Direct immunofluorescence (DIF) microscopy was performed on 99 samples, with 44 (44.4%) examinations resulting in positives. The sensitivity of DIF microscopy for *P. jirovecii* detection was positively associated with low cycle threshold (C_T) values, regardless of respiratory sample (adjusted odds ratio [OR], 0.77; 95% confidence interval [CI], 0.66 to 0.89) (Fig. S2).

C_T values from semi-quantitative real-time PCR analysis of BAL fluid or tracheal aspirate were retrievable for 171 patients (Table S5). The median (q_1 to q_3) C_T value was lower among PCP⁺ patients than among PCP⁻ patients (35 [32 to 37] versus 38 [37 to 41], $P < 0.001$) (Fig. S3), confirming higher fungal loads in individuals with clinical infection. However, it was impossible to find an optimal C_T cutoff value for discrimination between PCP and colonization due to overlaps (Fig. S4). The receiver operating characteristic (ROC) curve analysis gave an area under the curve (AUC) of 0.80 (95% CI, 0.73 to 0.88) (Fig. 2A). A C_T value of 36 came closest to maximizing sensitivity and specificity simultaneously, being 71.3% (95% CI, 63.7% to 78.9%) and 77.1% (95% CI, 63.2% to 91.1%), respectively. This corresponded to a positive predictive value of 92.4% (95% CI, 87.3% to 97.5%) and a negative predictive value of 40.9% (95% CI, 29.0 to 52.8%). The validity and percentage of correctly classified patients varied according to C_T cutoff

TABLE 1 Characteristics of study population and comparison of patients with *Pneumocystis pneumonia* and colonization^a

Characteristic	No. (%) in case of missing	Value			P value difference
		Study population (n = 242; 100%)	PCP+ (n = 196; 81.0%)	PCP- (n = 46; 19.0%)	
Demographics and comorbidity					
Median age (yrs [q ₁ -q ₃])	NA	66 (59-73)	65.5 (59-73)	68 (60-74)	0.39
Male sex (no. [%])	NA	142 (58.7)	119 (60.7)	23 (50.0)	0.18
History of smoking (no. [%])	235 (97.1)	131 (55.7)	106 (55.8)	25 (55.6)	0.98
Median Charlson comorbidity index (q ₁ -q ₃)	NA	6 (4-8)	6 (4-8)	6 (4-8)	0.97
Comorbidities (no. [%])					
Cardiovascular disease	NA	66 (27.3)	45 (23.0)	21 (45.7)	0.002
Chronic kidney disease		32 (13.2)	26 (13.3)	6 (13.0)	0.97
Chronic liver disease		2 (0.83)	2 (1.0)	0 (0.0)	1.00
Chronic pulmonary disease		43 (17.8)	32 (16.3)	11 (23.9)	0.23
Congestive heart failure		13 (5.4)	10 (5.1)	3 (6.5)	0.72
Diabetes mellitus type 1 or 2		33 (13.6)	26 (13.3)	7 (15.2)	0.73
Hematological malignancy ^b		12 (5.0)	10 (5.1)	2 (4.3)	1.00
Hypertension		75 (31.0)	60 (32.1)	15 (27.3)	0.79
Rheumatic disease		7 (2.9)	6 (3.1)	1 (2.2)	1.00
Solid tumor		28 (11.6)	24 (12.2)	4 (8.7)	0.62
Any of the above		157 (64.9)	124 (63.3)	33 (71.7)	0.28
Primary PCP prophylaxis at presentation	NA	2 (0.8)	2 (1.0)	0 (0)	1.00
Microbiology					
C _T value of semiquantitative real-time PCR-analysis (median [q ₁ -q ₃])					
Any respiratory sample ^c	202 (83.5)	36 (33 to 37)	35 (32-37)	38 (37-41)	<0.001
BAL fluid or tracheal aspirate ^c	171 (70.7)	36 (33-37)	35 (32-37)	38 (37-41)	<0.001
Immunosuppressive conditions					
Distribution across PCP groups					
Hematological malignancies	NA	89 (37.6)	75 (38.3)	14 (30.4)	0.19
Solid tumors		68 (28.7)	59 (30.1)	9 (19.6)	Ref.
Immunological disorders		38 (16.0)	28 (14.3)	10 (21.7)	0.66
Solid organ transplantation		29 (12.2)	23 (11.7)	6 (13.0)	0.17
Chronic lung diseases		13 (5.5)	8 (4.1)	5 (10.9)	0.54
Other/miscellaneous ^d		5 (2.1)	3 (1.5)	2 (4.3)	0.059
Pulmonary metastasis from solid tumor		12 (5.0)	9 (4.6)	3 (6.5)	Excluded
Premorbid iatrogenic immunosuppression, chemotherapy and corticosteroid exposure					
Any immunosuppressive regimen (no. [%])	NA				
Last 5 yrs		230 (95.0)	187 (95.4)	43 (93.5)	0.70
At presentation		205 (84.7)	168 (85.7)	37 (80.4)	0.37
Regimen at presentation (no. [%])					
Chemotherapy for hematological malignancy and adjuvant steroids	NA	54 (22.3)	47 (24.0)	7 (15.2)	0.33
Chemotherapy for solid tumor and adjuvant steroids		31 (12.8)	26 (13.3)	5 (10.9)	
Chemotherapy for hematological malignancy		10 (4.1)	10 (5.1)	0 (0)	
Chemotherapy for solid tumor		14 (5.8)	11 (5.6)	3 (6.5)	
Corticosteroids in monotherapy		35 (14.5)	29 (14.8)	6 (13.0)	

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TABLE 1 (Continued)

Characteristic	No. (%) in case of missing	Value			P value difference
		Study population (n = 242; 100%)	PCP ⁺ (n = 196; 81.0%)	PCP ⁻ (n = 46; 19.0%)	
Graft rejection prophylaxis after SOT		28 (11.6)	23 (11.7)	5 (10.9)	
DMARDs with or without adjunctive steroids		22 (9.1)	15 (7.7)	7 (15.2)	
Other combinations ^e		11 (4.6)	7 (3.6)	4 (8.7)	
None		37 (15.3)	28 (14.3)	9 (19.6)	
Systemic corticosteroid exposure pattern 60 days preceding presentation (no. [%])	240 (99.2)				
Daily		102 (42.5)	80 (41.2)	22 (47.8)	0.31
Intermittent		74 (30.8)	64 (33.0)	10 (21.7)	0.96
None		64 (26.7)	50 (25.8)	14 (30.4)	0.20
Methylprednisolone equivalent dose (mg/day at presentation) (median [q ₁ -q ₃]) ^f	237 (97.9)	8 (4-20)	10 (5-24)	4 (4-8)	Ref.
Symptomatology (no. [%])					<0.001
Cough	NA	140 (57.9)	117 (59.7)	23 (50.0)	0.23
Dyspnea	NA	184 (76.0)	156 (79.6)	37 (60.9)	0.007
Fever	NA	180 (74.4)	151 (77.0)	29 (63.0)	0.05
Minimum two cardinal symptoms	NA	184 (76.0)	154 (78.6)	30 (65.2)	0.056
All three cardinal symptoms	NA	81 (33.5)	74 (37.8)	7 (15.2)	0.004
No cardinal symptoms	NA	3 (1.2)	0 (0)	3 (6.5)	0.007
Objective findings and biochemistry					
Abnormal lung auscultation (no. [%])	NA	144 (59.5)	123 (62.8)	21 (45.7)	0.033
Oxygen saturation (%) (median [q ₁ -q ₃]) ^g	207 (85.5)	89 (84-93)	88 (84-93)	91.5 (88-95)	0.014
Leukocyte count × 10 ⁹ /liter (median [q ₁ -q ₃])	235 (97.1)	7.0 (4.3-10)	6.9 (4.2-10.0)	7.7 (5.2-9.9)	0.36
Neutrophil count × 10 ⁹ /liter (median [q ₁ -q ₃])	186 (76.9)	4.8 (2.8-7.3)	4.8 (2.8-7.3)	4.8 (3.1-7.0)	0.99
Neutropenia (<0.5 neutrophils 10 ⁹ /liter)	186 (76.9)	3 (1.6)	2 (1.3)	1 (3.6)	0.37
Lymphocyte count × 10 ⁹ /liter (median [q ₁ -q ₃]) ^h	122 (50.4)	0.63 (0.41-1.1)	0.6 (0.4-1.1)	1.0 (0.5-1.5)	0.047
CD4 ⁺ T cell count × 10 ⁹ /liter (median [q ₁ -q ₃])	123 (50.8)	82 (66.7)	73 (70.2)	9 (47.4)	0.052
Lactate dehydrogenase (U/liter) (median [q ₁ -q ₃])	13 (5.4)	0.13 (0.07-0.25)	0.1 (0.05-0.25)	0.32 (0.22-0.41)	0.24
Albumin (g/liter) (median [q ₁ -q ₃])	142 (58.7)	293.5 (221-390)	308 (225-390)	224 (200-441)	0.082
C-reactive protein (mg/liter) (median [q ₁ -q ₃])	174 (71.9)	33 (27-36)	32.5 (27-35.5)	33.5 (27-37.5)	0.95
	235 (97.1)	76 (38-146)	81 (42-156)	53 (24.5-116.5)	0.019
Radiological features (no. [%])					
Any remarks on chest X-ray	204 (84.3)	160 (78.4)	133 (80.1)	27 (71.1)	0.22
Any remarks on thoracic CT	NA	237 (97.9)	196 (100)	41 (89.1)	<0.001
Findings on thoracic CT					
Atelectasis		41 (16.9)	29 (14.8)	12 (26.1)	0.066
Bronchiectasis		18 (7.4)	11 (5.6)	7 (15.2)	0.025
Crazy paving pattern		55 (22.3)	53 (27.0)	4 (8.7)	0.007
Consolidations		44 (18.2)	39 (19.9)	5 (10.9)	0.20
Cysts		9 (3.7)	6 (3.1)	3 (6.5)	0.38
Emphysema		26 (10.7)	20 (10.2)	6 (13.0)	0.58
Ground glass opacities ⁱ		180 (74.4)	171 (87.2)	12 (26.1)	<0.001
Infiltrates ^j		52 (21.5)	42 (21.4)	10 (21.7)	0.96
Lymphadenopathy		40 (16.5)	32 (16.3)	8 (17.4)	0.86

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TABLE 1 (Continued)

Characteristic	Value				P value difference
	No. (% in case of missing)	Study population (n = 242; 100%)	PCP ⁺ (n = 196; 81.0%)	PCP ⁻ (n = 46; 19.0%)	
Noduli		21 (8.7)	15 (7.7)	6 (13.0)	0.24
Pleural effusion		67 (27.7)	52 (26.5)	15 (32.6)	0.41
Pneumothorax		1 (0.41)	1 (0.5)	0 (0.0)	1.00
Reticular or septal thickening		63 (26.0)	55 (28.1)	8 (17.4)	0.14
"Tree-in-bud sign"		16 (6.6)	11 (5.3)	5 (10.9)	0.20

^aCriteria for PCP were multimodal and based on available patient data (see Materials and Methods and Fig. S1 in the supplemental material). Patients not fulfilling the criteria for their respective groups were considered colonized with *P. jirovecii* (i.e., PCP⁻). BAL, bronchoalveolar lavage; CT, computed tomography; C₇, cycle threshold; DMARDs, disease-modifying antirheumatic drugs; NA, not applicable; Ref, reference group in logistic regression analysis; SOT, solid organ transplantation.

^bIn 12 patients, hematological malignancy was not considered the primary immunosuppressive condition or an indication for immunosuppression but rather a comorbidity.

^cRespiratory samples included bronchoalveolar lavage fluid (n = 203, 83.9%), sputum (n = 25, 10.3%), induced sputum (n = 8, 3.3%), tracheal aspirate (n = 4, 1.7%), respiratory biopsy specimen (n = 1, 0.4%) and nasopharyngeal swab sample (n = 1, 0.4%) in a total of 242 samples. C₇ values were retrievable from analysis of 202 samples, including 171 BAL fluid samples and tracheal aspirates.

^dOther/miscellaneous immunosuppressive conditions included two patients with no diagnosed condition, whereas two had received steroids for suspected autoimmune disorder and one patient with statin-induced myositis was treated with corticosteroids.

^eOther combinations include exposure to other immunosuppressants (mycophenolate, azathioprine, cyclophosphamide, calcineurin and mTOR inhibitors, and cyclosporine and hydroxychloroquine with or without adjuvant steroids) and one patient receiving both graft rejection prophylaxis for solid organ transplantation and chemotherapy for hematological malignancy with adjuvant corticosteroids.

^fMedian methylprednisolone equivalent dose per day was calculated among 117 patients having an intake the day of *P. jirovecii* detection: 95 PCP⁺ and 22 PCP⁻ patients.

^gFifty-three patients were receiving supplemental oxygen when saturation was measured: 45 (23.0%) in the PCP⁺ group and 8 (17.4%) in the PCP⁻ group (P = 0.41 for difference).

^hOne patient with chronic lymphatic leukemia was excluded from the analysis due to an abnormally high lymphocyte count (i.e., 37.9 × 10⁹/liter).

ⁱNote: Ground glass opacities and infiltrates were among the criteria for PCP⁺.

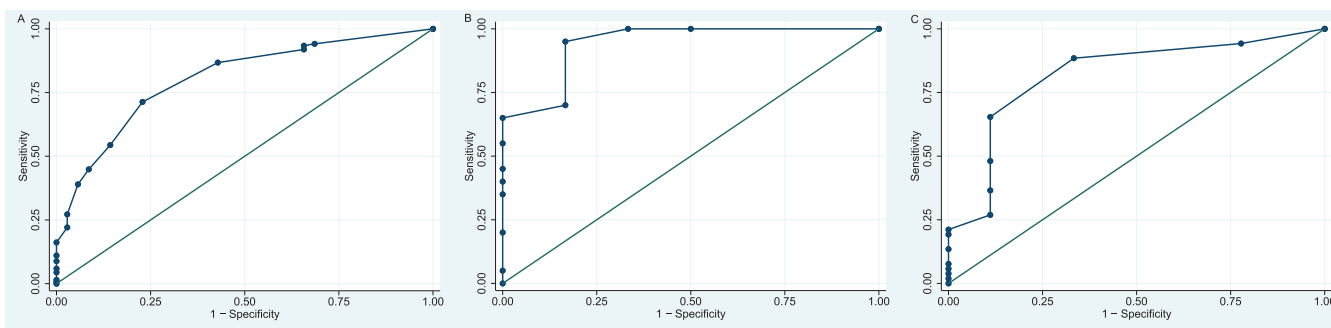


FIG 2 ROC curves of semi-quantitative real-time PCR of BAL fluid or tracheal aspirate for discrimination between *Pneumocystis* pneumonia and colonization. (A) ROC curve for population overall, based on 171 samples. (B) ROC curve for SOT recipients, based on 26 samples. (C) ROC curve for patients with hematological malignancies, based on 61 samples.

value (Fig. S5). C_T values greater than 36 defined a gray zone without definitive discrimination, comprising 39 PCP⁺ patients. Their characteristics are summarized in Table S2.

Subgroup analyses of PCP⁺ patients. C_T values of <31 corresponded to 100% specificity. To identify characteristics of this subpopulation with higher fungal loads ($n=22$), we compared it to PCP⁺ patients with C_T values of 31 and higher ($n=114$) (Table S3). Notably, fungal load appeared associated with immunosuppressive condition ($P=0.05$). SOT recipients accounted for 36.4% of the high-fungal-load population, whereas patients with hematological malignancies dominated the low-fungal-load population, constituting 40.5%. Moreover, we noted an association between corticosteroid exposure and fungal burden, with more daily users and fewer unexposed subjects in the high-fungal-load population. Median doses were comparable.

Heterogeneity in fungal loads. Successively, we further analyzed the relationships to immunosuppressive predisposition, including corticosteroid exposure and fungal burden (Fig. 3; see also Fig. S6 and S7). A linear regression model was fitted comparing C_T values in BAL fluid or tracheal aspirate across immunosuppressive conditions, with patients with hematological malignancies as a reference group, ($F[4,162]=3.03$, $P=0.019$, $R^2=0.070$). Only SOT recipients had significantly lower C_T values (Table S4). Univariate analyses confirmed this difference in medians (q_1 to q_3) compared to patients with hematological malignancies overall (34.5 [28 to 36] versus 36 [34 to 37], $P=0.072$), among PCP⁺ patients (33 [28 to 36] versus 36 [33 to 37], $P<0.01$), and to a lesser degree among PCP⁻ patients (38 [37 to 38] versus 39.5 [37 to 41], $P=0.54$).

Discrimination across immunosuppressive conditions. With caution regarding the number of patients and observations, we investigated the validity of semi-quantitative real-time PCR across immunosuppressive conditions. Based on 26 samples from SOT recipients, the discrimination between PCP and colonization appeared outstanding and superior to the population overall (AUC, 0.94; 95% CI, 0.82 to 1.00) (Fig. 2B). A C_T value of 36 corresponded to a sensitivity of 95.0% (95% CI, 85.4% to 100.0%) and a specificity of 83.3% (95% CI, 53.5% to 100.0%). In spite of lower fungal loads, the validity was excellent for patients with hematological malignancies (AUC, 0.82; 95% CI, 0.66 to 0.98) based on 61 observations (Fig. 2C). Yet, a higher C_T cutoff value was needed to achieve a sensitivity of >75%. Here, a C_T value of 37 corresponded to a sensitivity of 88.5% (95% CI, 79.8% to 97.1%) and a specificity of 66.7% (95% CI, 35.9% to 97.5%). The validity of the PCR assay appeared inferior for the remaining conditions (Fig. S8A to C; Table S5).

Independent risk factors for PCP. Based on univariate comparisons, we performed multivariable analyses to identify independent risk factors for PCP (Table 2). Only chronic lung diseases were associated with markedly lower odds of PCP (OR, 0.30; 95% CI, 0.09 to 1.05). Presence of all three cardinal symptoms and abnormal lung auscultation were independent predictors for PCP. Moreover, corticosteroid dose at presentation was positively associated with PCP, while C_T value and oxygen saturation were negative predictors. The presence of crazy paving on computed tomography (CT) imaging was strongly associated with PCP.

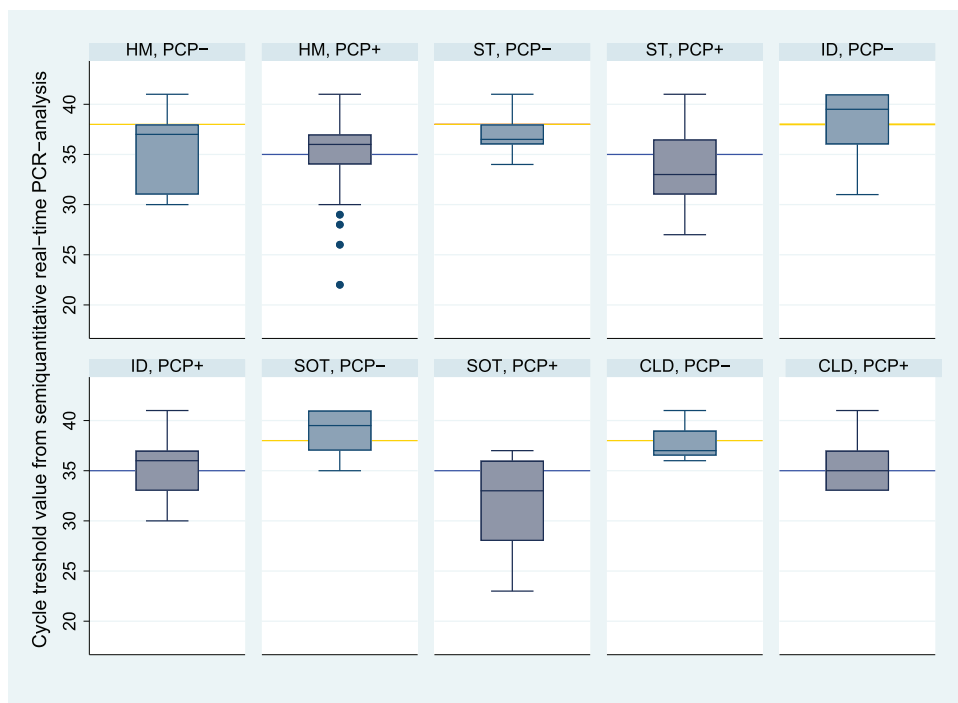


FIG 3 Relationship between semiquantitative real-time PCR-result, immunosuppressive conditions, and PCP status. C_T values from of BAL fluid or tracheal aspirate differed significantly according to PCP status ($P < 0.01$) with medians being 35 (blue line) and 38 (yellow line), respectively. Retrospectively, 196 patients were diagnosed with PCP (i.e., PCP⁺) while 46 were presumed colonized (i.e., PCP⁻). C_T , cycle threshold; CLD, chronic lung disease; HM, hematological malignancy; ID, immunological disorder; PCP, *Pneumocystis pneumonia*; PCR, polymerase chain reaction, SOT, solid organ transplant; ST, solid tumor.

DISCUSSION

This study demonstrates that semiquantitative real-time PCR can improve differentiation between PCP and colonization in immunocompromised HIV-negative patients. However, a significant heterogeneity in fungal loads across immunosuppressive predispositions implicates that universal cutoff values for predicting non-HIV PCP are inadequate.

Non-HIV populations at risk of opportunistic infections, including PCP, are growing rapidly because of prolonged survival and escalating use of immunosuppressants (3, 5). Diagnostic algorithms with high specificity are needed to avoid unnecessary treatment, especially among multimorbid patients with propensity for adverse effects and drug interactions (9). On the other hand, delayed diagnosis is associated with increased mortality risk, potentially exceeding 50% (1).

Semiquantitative real-time PCR gradually substituted microscopy for *P. jirovecii* detection in our regional referral laboratory during the last decades, but whether C_T values should be emphasized for treatment guidance remained unestablished. Here, the study subjects represented a selected population, and they had high pretest probability of PCP. Accordingly, the majority were classified as PCP⁺ in retrospect. Although C_T values were significantly lower among PCP⁺ patients, it was impossible to determine a cutoff with a 100% negative predictive value.

Several studies have assessed real-time PCR strategies to distinguish PCP from colonization. Extrapolation is limited by heterogeneity in PCR targets, PCP definitions, host characteristics, types of respiratory samples, sample volumes, DNA extraction, and quantification methods (C_T values or copies per milliliter) (10). Anyhow, the majority have found real-time PCR assays potentially useful (11–25), though gray zones are common and stratification by HIV status is of utmost importance. Inability to

TABLE 2 Uni- and multivariable analyses of risk factors for *Pneumocystis* pneumonia versus colonization^a

Risk factor and covariate(s) ^b	No. of observations	OR ^d	95% CI	P value
Cardiovascular comorbidity	NA	0.35	0.18–0.69	0.002
Age and sex	NA	0.27	0.13–0.57	0.002
Any other comorbidity and sex	NA	0.29	0.14–0.60	0.001
Daily methylprednisolone equivalent dose at presentation among exposed/mg increase	117	0.47	0.17–1.26	0.13
C_T value of semiquantitative real-time PCR-analysis of BAL fluid or tracheal aspirate/unit increase	171	0.68	0.58–0.80	<0.001
Daily methylprednisolone equivalent dose at presentation among exposed/mg increase	82	0.54	0.38–0.80	<0.001
Immunosuppressive condition^c	237			
Hepatological malignancy	89	1	Ref.	Ref.
Solid tumor	68	1.22	0.50–3.02	0.66
Immunological disorder	38	0.52	0.21–1.31	0.17
Solid organ transplantation	29	0.72	0.25–2.07	0.54
Chronic lung disease	13	0.30	0.09–1.05	0.059
Daily methylprednisolone equivalent dose at presentation/mg increase	237	1.05	1.00–1.10	0.035
Daily methylprednisolone equivalent dose at presentation among exposed/mg increase	117	1.11	1.02–1.20	0.011
Dyspnea	242	2.51	1.26–4.98	0.009
Cardiovascular comorbidity	242	2.87	1.30–5.88	0.004
Immunosuppressive condition	237	2.83	1.36–5.89	0.005
Systemic corticosteroid exposure pattern 60 days preceding presentation	240	2.84	1.40–5.46	0.004
Fever	242	1.97	0.99–3.90	0.053
Daily methylprednisolone equivalent dose at presentation/mg increase	237	2.33	1.14–4.75	0.020
Daily methylprednisolone equivalent dose at presentation among exposed/mg increase	117	2.68	0.96–7.45	0.059
At least two cardinal symptoms (cough, dyspnea, fever)	242	1.96	0.97–3.92	0.059
Immunosuppressive condition	237	1.70	0.81–3.55	0.159
Daily methylprednisolone equivalent dose at presentation among exposed/mg increase	117	2.52	0.92–6.93	0.073
All three cardinal symptoms (cough, dyspnea, and fever)	242	3.38	1.44–7.94	0.005
Daily methylprednisolone equivalent dose at presentation/mg increase	237	4.28	1.71–10.7	0.002
Daily methylprednisolone equivalent dose at presentation among exposed/mg increase	117	6.23	1.30–29.7	0.022
Abnormal lung auscultation	242	2.01	1.05–3.84	0.035
Daily methylprednisolone equivalent dose at presentation/mg increase	237	1.81	0.93–3.51	0.080
Daily methylprednisolone equivalent dose at presentation among exposed/mg increase	117	3.35	1.22–9.21	0.019
Immunosuppressive regimen at presentation	242	2.17	1.10–4.28	0.026
Oxygen saturation in %/unit increase	207	0.93	0.87–0.99	0.016
Lymphocyte count × 10⁹/liter/unit increase	122	0.71	0.50–1.00	0.050
Daily methylprednisolone equivalent dose at presentation among exposed/mg increase	59	1.13	0.55–2.32	0.745
Immunosuppressive condition ^c	119	0.64	0.43–0.94	0.024
Lymphopenia (<1.0 × 10⁹/liter)	123	2.62	0.97–7.07	0.058
Charlson comorbidity index/unit increase	123	2.97	1.06–8.32	0.039
Daily methylprednisolone equivalent dose at presentation/mg increase	120	2.87	1.04–7.92	0.042

(Continued on next page)

TABLE 2 (Continued)

Risk factor and covariate(s) ^b	No. of observations	OR ^d	95% CI	P value
Daily methylprednisolone equivalent dose at presentation among exposed/mg increase	60	2.35	0.56–9.94	0.244
C-reactive protein in mg/liter/unit increase	235	1.00	1.00–1.01	0.057
Lactate dehydrogenase in U/liter/unit increase	142	1.00	1.00–1.00	0.89
Atelectasis	242	0.49	0.23–1.06	0.070
Daily methylprednisolone equivalent dose at presentation among exposed/mg increase	117	0.70	0.22–2.21	0.54
Lymphocyte count $\times 10^9$ /liter/unit increase	122	2.86	0.35–23.2	0.33
Immunosuppressive regimen at presentation	242	0.57	0.26–1.25	0.16
Bronchiectasis	242	0.33	0.12–0.91	0.032
Age, sex	242	0.37	0.13–1.05	0.063
Immunosuppressive condition ^c	237	0.43	0.15–1.27	0.13
Systemic corticosteroid exposure pattern 60 days preceding presentation	240	0.37	0.13–1.02	0.054
Daily methylprednisolone equivalent dose at presentation among exposed/mg increase	237	0.52	0.11–2.41	0.40
Immunosuppressive regimen at presentation	242	0.37	0.13–1.1	0.073
Crazy paving pattern on thoracic CT	242	3.89	1.33–11.4	0.013
Age and sex	242	4.28	1.45–12.7	0.009
C _T value of semiquantitative real-time PCR analysis of BAL fluid or tracheal aspirate	171	6.09	1.58–23.4	0.009
Immunosuppressive condition ^c	237	4.38	1.45–13.3	0.009
Immunosuppressive regimen at presentation	242	4.29	1.44–12.8	0.009
Daily methylprednisolone equivalent dose at presentation among exposed/mg increase	117	5.26	1.12–24.8	0.036
Lymphocyte count $\times 10^9$ /liter/unit increase	122	3.07	0.65–14.4	0.16

^aCriteria for PCP were multimodal and based on available patient data (see Materials and Methods and Fig. S1). Patients not fulfilling the criteria for their respective groups were considered colonized with *P. jirovecii* (i.e., PCP⁻).

BAL, bronchoalveolar lavage; CT, computed tomography; C_T, cycle threshold; NA, not applicable; OR, odds ratio.

^bRisk factors are in boldface. Plausible confounders were identified *a priori* and included in multivariable analyses. Covariates with $\geq 10\%$ effect on OR are included in the table. For complete list of covariates, refer to Table S1.

^cFive patients had immunosuppressive conditions classified as miscellaneous and were excluded from the comparative analysis. Adjustment for age and sex did not cause significant changes to odds ratios overall or P values and are not reported.

^dUnivariate analysis results are in boldface; adjusted ORs are in lightface.

discriminate the two entities has also been described (26, 27), perhaps due to a continuous progression from carriage to active infection (7).

Upon exposure, *P. jirovecii* adheres to type 1 pneumocytes, which in turn induces organism activation and multiplication (1). The passage from colonization to PCP and complications is ill defined in non-HIV patients (5), and CD4 counts fail in predicting disease (6). Paradoxically, the associated lung injury is proposed to result from an inappropriate inflammatory host response (5). Marked bronchoalveolar neutrophilia observed in HIV-negative patients likely reflects this reaction and aggravates the prognosis (1).

Since the fungus lives and thrives in the alveoli, an increasing density gradient from the upper to the lower respiratory tract is expected (7). In the attempt to avoid invasive sampling, researchers have assessed the validity of upper respiratory tract specimens compared to the gold standard of BAL fluid, with various results (10). Overall, the sensitivity appears too low to exclude PCP, while positive results support the diagnosis (7). Asymptomatic carriage in the upper respiratory tract due to recent exposure is a differential diagnosis (4), and a theoretical source of contamination unless protective invasive sampling is applied (28).

In light of the current knowledge gaps and diagnostic challenges, a major strength of this study is the large number of high-risk cases and high-yield respiratory specimens permitting subgroup analyses. Interestingly, SOT recipients and patients with hematological malignancies distinguished themselves at different ends of a spectrum, harboring high and low fungal loads, respectively. However, an R^2 of 7.0% suggests that endogenous host predisposition explains little of the diversity. Indeed, our results indicate that immunosuppression, including corticosteroid exposure, also influences the precise intersection of host response and *P. jirovecii* concentration that results in clinical infection.

Cancer patients are primarily subject to cycles of chemotherapy regimens, for instance, rituximab, cyclophosphamide, vincristine, and prednisolone (R-CHOP) and fludarabine, cyclosporine, and rituximab (FCR), both involving significant risk of PCP (5). Moreover, corticosteroids have vast supportive care indications in oncology, increasing exposure (2). In comparison, SOT recipients are prescribed daily multidrug regimens with explicit lymphocytotoxic effects to prevent allograft rejection (29). Although SOT regimens are pleiotropic and not CD4 specific, perhaps they come closest to mimicking the lymphocyte depletion occurring during the natural course of HIV infection considering their continuity and intensity (29).

Notably, Montesinos et al. found that *P. jirovecii* concentrations were markedly heterogeneous in samples from HIV-negative PCP patients (23). Relatedly, Robert-Gangneux et al. highlighted hematological malignancies particularly for the tendency of negative microscopy examinations, *per se*, to be associated with low fungal loads (26). Altogether, we hypothesize that intrinsic and iatrogenic host factors affect *P. jirovecii* multiplication and non-HIV PCP expression. Regardless of the pathogenesis, our findings have important implications. Foremost, the validity of real-time PCR strategies may vary across immunosuppressive predispositions, and optimal cutoff values for discrimination should be validated according to these strata.

Acknowledging the importance of the recent multicenter study from the Fungal PCR Initiative comparing the performance of several commercial and noncommercial *P. jirovecii* quantitative real-time PCR assays with emphasis on standardization, our in-house assay harbors certain shortcomings (30). Specifically, the protocol only tests the efficacy of the amplification step. Ideally, one should add a negative control prior to extraction to monitor the entire real-time process. Use of an alien negative control is preferable to avoid bias from human factors (e.g., unknown quantity of human DNA in eluate). Moreover, inherent variability of biologic systems is an important bottleneck in real-time PCR studies such as ours. To limit confounding from differences in sample volumes, relative quantification (e.g., the comparative $[\Delta\Delta] C_T$ method) involving normalization of *P. jirovecii* to one or more reference genes with near constant expression should prevail over absolute quantification. Importantly, the genes must be amplified

with comparable efficacy for this method to be accurate (31). Owing to higher feasibility, easier clinical interpretation, and determination of cutoff values, diagnostic microbiology departments may still prefer absolute quantification.

The last concern regards the target gene for amplification. Beta-tubulin is a highly conserved single-copy nuclear gene (10). Single-copy genes are favorable to avoid bias in quantification and accurately reflect the quantity of organisms (30). This allows inter-strain comparisons and direct determination of cutoff values, since varied copy numbers is a nonissue. However, compared to multicopy gene targets such as the major surface glycoprotein and mitochondrial genes, inferior analytical sensitivity is a drawback (10, 30). Extraction of whole nucleic acids demonstrates an even wider detection range for *P. jirovecii* compared to that with DNA only (30). In fact, to target the mitochondrial small subunit with whole nucleic acid as a starting material appears to yield the best sensitivity (30). The rationale for using assays with the highest sensitivity obtainable is vast. Principally, even low-amount *P. jirovecii* inoculums can be associated with non-HIV PCP. With the distinct exception of SOT patients, our study underscored this characteristic, particularly among patients with hematological malignancies. Hence, the nature of this disease strongly argues for high negative predictive values, including the lower spectrum of *P. jirovecii* inoculums. The growing implications of colonization are equally important. Molecular genotyping reports involving colonized patients in nosocomial transmission networks are worrisome and emphasize the urgency for strategies to reduce circulation of *P. jirovecii* (32). Furthermore, the possible risk of developing full-blown PCP from colonization in case of deteriorated immunity favors preemptive treatment (30).

Despite the above-described issues, we believe that the main findings of our study withstand. Considering the ever-diverse population susceptible to *P. jirovecii*, these indications warrant further investigations with emphasis on appropriate study design and stratified analyses.

Besides real-time PCR, this study underlines readily available clinical characteristics to emphasize for treatment guidance. In line with previous reports (12, 14, 16, 26, 33), the sensitivity of DIF microscopy appeared associated with *P. jirovecii* loads. Concerning noninvasive investigations, history of all three cardinal symptoms and decreased oxygen saturation were independent predictors of PCP in our PCR-positive cohort. Also, lymphopenia, an established risk factor for PCP (5), was associated with PCP, based on 123 observations. In our experience, a common pitfall is declaring patients immunocompetent if their neutrophil count is normal in spite of lymphopenia. In relation to this, cumulative corticosteroid dose is worth stressing due to lymphocytotoxic effects. Although we found a positive association, dose tapering, low doses, or no preceding intake does not exclude PCP (2). Lastly, both corticosteroids and lymphopenia are risk factors for colonization too, complicating clinical discrimination (8).

Cardiovascular comorbidity favored colonization in the univariate analysis. We hypothesize that shared clinical characteristics, particularly in cardiac patients, contributed to this. However, a multivariable analysis confirmed a positive confound by corticosteroids, moderating this relationship. A reluctance toward corticosteroid therapy to these patients because of adverse circulatory and metabolic effects may explain this finding.

This study has several limitations. First, we were unable to include all alive patients. Also, to strive for diagnostic homogeneity, validation of the semiquantitative real-time PCR was primarily performed on lower-respiratory-tract specimens. These limitations represent selection bias. Second, this was a retrospective analysis, challenging data collection and reliability. Third, the lack of a gold standard for diagnosing PCP might have resulted in information bias. Fourth, an increase in familywise error rate across reported statistical analyses was not controlled for. Finally, the comparison of fungal loads is challenged by variability in respiratory specimens, host pathogen biology, and procedural and analytical factors discussed above.

In conclusion, semiquantitative real-time PCR offers high objectivity and sensitivity

for *P. jirovecii* detection in HIV-negative immunocompromised individuals. However, heterogeneity across host predispositions requires multivariable models to optimize discrimination between life-threatening PCP and colonization. Prospective studies are needed to assess the external validity of our results while reducing the risk of bias and confounding.

MATERIALS AND METHODS

Setting and inclusion. St. Olavs hospital, Trondheim University Hospital, is the only tertiary referral hospital in the central Norway health region, covering approximately 700,000 inhabitants. Adult patients with respiratory samples testing positive for *P. jirovecii* by PCR at the Department of Medical Microbiology from 2006 to 2017 were identified. For inclusion, respiratory samples included BAL fluids, induced sputa, sputa, tracheal aspirates, respiratory biopsy specimens, and nasopharyngeal swab samples. Patients who were HIV negative, had been followed up regionally, and had undergone thoracic CT were eligible. Inclusion of alive patients required active consent, while all deceased patients were included.

Data collection. Comprehensive biological, clinical, and demographic data were collected retrospectively from patient records. Ongoing corticosteroid intake on the date of *P. jirovecii* detection was registered and converted into the equivalent in methylprednisolone expressed as milligrams per day. Degree of comorbidity was assessed according to the Charlson weighted comorbidity index (34). Cardiovascular comorbidities comprised coronary heart disease, stroke, and peripheral artery disease, whereas congestive heart failure and hypertension were registered separately. Epi Info (version 7.2.2.6; Centers for Disease Control and Prevention, Atlanta, GA, USA) was used for data recording.

Microbiological detection of *P. jirovecii*. DIF microscopy was performed with MONOFLUO *Pneumocystis jirovecii* IFA test kit number 32515 (Bio-Rad). Lack of positive controls from “definite” PCP patients was a challenge during the study period. For this reason and concerns regarding sensitivity and specificity, the laboratory used DIF as a complementary method in line with the guidelines (7), mainly on PCR-positive samples. In 2017, semi-quantitative real-time PCR replaced DIF definitively. The in-house assay targeting the beta-tubulin gene of *P. jirovecii* was adapted from Brancart et al. (33) with some modifications as described in detail below (11, 33).

Semi-quantitative real-time PCR-protocol. Respiratory tract samples that were viscous were pre-treated with Sputolysin (dithiothreitol, volume 1:2) for 10 min for liquefaction of mucoid fluids before DNA extraction. Next, if the sample volume was >10 ml, 3 to 5 ml was subjected to centrifugation at $3,000 \times g$ for 30 min. Thereafter, 500 μ l of the supernatant was mixed with 50 μ l proteinase K and incubated for 15 min at 65°C. If the sample volume was <10 μ l, the centrifugation step was omitted, and 1 ml of sample was mixed with 100 μ l proteinase K and incubated as described above. Then, the mixture was spun down, the supernatant was removed, and 500 μ l of precipitate was used for DNA extraction on a NucliSENS easyMAG instrument (bioMérieux) with an eluate volume of 55 μ l.

Reagents and PCR instruments used varied during the study period, but all changes were validated to ensure equal quality. During the main part of the study period, the following procedure and reagents were used: 5 μ l of eluate was added to 10 μ l of PerfeCTa multiplex qPCR supermix with uracil-*N*-glycosylase, 0.5 μ l of each primer (12 μ M) and probe (8 μ M), and 3.5 μ l molecular-grade water. BAL fluids, considered critical patient samples, were extracted and amplified in duplicates. Amplification reactions were carried out on either a CFX96 real-time system (Bio-Rad), Chromo4 system (Bio-Rad), or LightCycler 2.0 instrument (Roche) with the following cycling conditions: 45°C for 5 min, 95°C for 3 min, and then 40 cycles of 95°C, 60°C, and 72°C for 10 s each. Results were reported to clinicians as negative/positive, with a comment about low concentration of *P. jirovecii* if the cycle threshold (C_T) value was high. A cloned PCR product was used as an external positive control, and molecular-grade water was used as a negative control in all PCR runs. To control for inhibition, a separate real-time PCR targeting a human 237-bp intergenic region of chromosome 20 (position 104006 to 104242, sequence [AL133466](#)) was run, as previously described (35). All samples were positive, indicating absence of PCR inhibitors, and no samples were excluded due to nonamplification during the study period. The laboratory participated in a *Pneumocystis jirovecii* pneumonia (PCP) DNA EQA Program (QCMD) during the study period.

Retrieval of C_T values. C_T values were not reported in the laboratory information system during the study period. Therefore, C_T values were collected from the log of the PCR instruments in retrospect. Since some of the PCR instruments were replaced and discarded during the study period, C_T values for samples run on those instruments were lost. These were registered as “missing” during data collection. The retrievability of C_T values depended on which instrument the analyses were run, and the missing pattern was considered random and unrelated to patient characteristics.

Case definition. To separate infection from colonization in PCR-positive patients, multimodal criteria based on current clinical practice, previous reports (36–38), and existing diagnostic guidelines emphasizing biological detection were imposed *a posteriori* (7) (see Fig. S1 in the supplemental material). We identified three patient groups and applied the following criteria for PCP: group 1, (i) immunosuppressive state and (ii) positive DIF; group 2 (characterized by missing or negative DIF microscopy-result), (i) immunosuppressive state, (ii) at least one cardinal symptom of PCP (cough, dyspnea, and fever), (iii) typical findings on thoracic CT (ground glass opacities and/or infiltrates), and (iv) presumptive diagnosis at time of diagnosis, i.e., receiving anti-PCP treatment; group 3, patients who died in hospital within 30 days of detection without receiving anti-PCP treatment. We evaluated these patients individually with respect to cause of death and PCP status to exclude abrupt death from PCP without time to receive

anti-PCP treatment. The alternative diagnosis was colonization and PCP-unrelated death (i.e., terminal patients dying from underlying conditions). Patients not fulfilling the criteria for their respective groups were considered colonized with *P. jirovecii*. C_T values were compared to the retrospective PCP status, infection (PCP⁺) or colonization (PCP⁻).

Statistics. Continuous and categorical variables are presented as medians with second (q_2) and third (q_3) quartiles and proportions with percentages, respectively. Simple linear regression was used to compare C_T values across immunosuppressive conditions. Otherwise, univariate analyses were performed with the Wilcoxon rank sum, chi-square, or Fisher's exact test as appropriate, except for polychotomous independent variables, for which logistic regression was applied. Subsequently, multivariable logistic regression analyses were performed for variables having P values of <0.10 with covariates identified *a priori* (Table S1), with PCP versus colonization as outcomes. ROC curves were used to assess the validity of semiquantitative real-time PCR and determine sensitivity and specificity according to C_T cutoff values. Results are expressed as proportions, ORs, or AUC with 95% confidence intervals. All P values were two sided. Values of <0.05 were considered statistically significant.

Analyses were performed using Microsoft Excel (version 16.4; Microsoft Corporation, Redmond, WA, USA), STATA/MP (version 15.1; StataCorp, College Station, TX, USA), and IBM SPSS statistics for Macintosh (version 27.0; IBM Corp., Armonk NY, USA).

Ethics. This study was approved by the Regional Committee for Medical and Health Research Ethics (REC-North, reference number 2017/2419).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE S1, PDF file, 0.7 MB.

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We declare no conflicts of interest.

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