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Combined Quantification of Pulmonary *Pneumocystis jirovecii* DNA and Serum (1→3)-β-D-Glucan for Differential Diagnosis of *Pneumocystis* Pneumonia and *Pneumocystis* Colonization

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This study assessed a quantitative PCR (qPCR) assay for *Pneumocystis jirovecii* quantification in bronchoalveolar lavage (BAL) fluid samples combined with serum (1→3)-β-D-glucan (BG) level detection to distinguish *Pneumocystis* pneumonia (PCP) from pulmonary colonization with *P. jirovecii*. Forty-six patients for whom *P. jirovecii* was initially detected in BAL fluid samples were retrospectively enrolled. Based on clinical data and results of *P. jirovecii* detection, 17 and 29 patients were diagnosed with PCP and colonization, respectively. BAL fluid samples were reassayed using a qPCR assay targeting the mitochondrial large subunit rRNA gene. qPCR results and serum BG levels (from a Fungitell kit) were analyzed conjointly. *P. jirovecii* DNA copy numbers were significantly higher in the PCP group than in the colonization group (1.3×10^7 versus 3.4×10^3 copies/μl, $P < 0.05$). A lower cutoff value (1.6×10^3 copies/μl) achieving 100% sensitivity for PCP diagnosis and an upper cutoff value (2×10^4 copies/μl) achieving 100% specificity were determined. Applying these two values, 13/17 PCP patients and 19/29 colonized patients were correctly assigned to their patient groups. For the remaining 14 patients with *P. jirovecii* DNA copy numbers between the cutoff values, PCP and colonization could not be distinguished on the basis of qPCR results. Four of these patients who were initially assigned to the PCP group presented BG levels of ≥100 pg/ml. The other 10 patients, who were initially assigned to the colonization group, presented BG levels of <100 pg/ml. These results suggest that the combination of the qPCR assay, applying cutoff values of 1.6×10^3 and 2×10^4 copies/μl, and serum BG detection, applying a 100 pg/ml threshold, can differentiate PCP and colonization diagnoses.

Pneumocystis jirovecii is a transmissible fungus and the causative agent of various presentations of pulmonary infections in humans (1). *Pneumocystis* pneumonia (PCP) is the most severe presentation of disease and occurs in HIV-infected patients and other immunocompromised patients (2). PCP is mostly associated with high pulmonary *P. jirovecii* burdens, and the diagnosis is based on microscopic detection of cysts and trophic forms in lung samples. Less frequently, the pulmonary burden is low and detection of the fungus requires highly sensitive techniques such as DNA amplification in PCR assays, while microscopic findings remain negative. However, low *P. jirovecii* burdens also can be observed in patients who have an alternative diagnosis of PCP and colonization by *P. jirovecii* (3, 4). Therefore, the differential diagnosis between PCP and pulmonary colonization with *Pneumocystis* cannot be based exclusively on interpretation of negative microscopic findings combined with positive PCR results. This differential diagnosis is clinically relevant, as these two presentations of *Pneumocystis* infection are associated with different prognoses.

Quantitative PCR (qPCR) assays have been recently described as useful tools to assess *P. jirovecii* burdens in pulmonary samples. Using these assays, several authors have applied qPCR cutoff values in order to distinguish between PCP and colonization (5–8). A *P. jirovecii* DNA copy number below the cutoff value would support a diagnosis of colonization, and a copy number above the cutoff value would support a diagnosis of PCP. Applying these cutoff values, sensitivity and specificity values for PCP diagnosis ranged from 88% to 100% and from 85% to 98.6%, respectively, depending on the methods. Other teams have suggested applying two qPCR cutoff values in order to increase both sensitivity and specificity (9–12). In this context, a *P. jirovecii* DNA copy number below the lower cutoff value would support a diagnosis of colonization, whereas a DNA copy number above the upper cutoff value would support a diagnosis of PCP. The lower cutoff value and the upper cutoff value therefore provide 100% sensitivity and 100% specificity, respectively, of the qPCR assay for the diagnosis of PCP. Nonetheless, there is a zone between these two cutoff values in which the differential diagnosis of PCP versus colonization cannot be determined.

(1→3)-β-D-Glucan (BG) represents a major structural component of the cell walls of most fungi. Thought to be rare or absent in *Pneumocystis* trophic forms, it is synthesized throughout the microorganism life cycle from the sporocytic stages to the cysts, in which it is fully formed and abundant in cell walls (13). Various studies have reported high serum BG levels in patients with PCP (14–23). In contrast, we recently showed that serum BG levels determined with the Fungitell test kit (Associates of Cape Cod, Inc., Cape Cod, MA) were below the manufacturer's positive threshold of 80 pg/ml for six of eight colonized patients. The remaining two patients presented positive BG results but with values (82.7 and 84 pg/ml) close to the threshold (24). Similarly, Shimizu et al. showed that serum BG levels determined using the Wako test

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kit (Wako Pure Chemical Industries, Tokyo, Japan; manufacturer's threshold, ≥11 pg/ml) were negative for 7 of 7 colonized patients (25). Using the same assay with a threshold adjusted to 15.6 pg/ml, Matsumura et al. determined a specificity of 80% and a sensitivity of 100% for PCP diagnosis with the serum BG assay (26). Interestingly, in that study, a qPCR assay using a single cutoff value was also tested for the differential diagnosis of PCP versus colonization. However, serum BG levels and qPCR assay results were analyzed independently.

The objective of this study was to define additional laboratory criteria to distinguish PCP from colonization. We retrospectively enrolled 46 patients infected with *Pneumocystis* who underwent bronchoalveolar lavage (BAL) fluid and serum sampling. *P. jirovecii* quantification in BAL fluid samples using a qPCR assay with two cutoff values and a serum BG assay was performed. The results of *P. jirovecii* quantification and serum BG levels were analyzed conjointly.

MATERIALS AND METHODS

Patients and samples. Forty-six patients (mean age, 59 years [range, 24 to 84 years]; 34 men and 12 women) for whom *P. jirovecii* had been initially detected in BAL fluid samples (46 BAL fluid samples) were retrospectively enrolled. These patients had been hospitalized in two French university hospitals (hospitals A and B) between January 2008 and January 2012 for investigation of pulmonary symptoms (abnormal chest X-ray findings or cough) or fever. *P. jirovecii* was detected in BAL fluid samples by microscopic examination with methanol-Giemsa staining and an immunofluorescence assay (Monofluokit *Pneumocystis*; Bio-Rad, Marnes-La-Coquette, France) and/or a qualitative PCR assay targeting the mitochondrial large subunit (mtLSU) rRNA gene, as described previously (27). This assay was performed on an Applied Biosystems 7300 real-time PCR system using a plus/minus assay, which determined whether or not a specific target sequence was present. *P. jirovecii* DNA quantification was not performed at that time. Forty-six serum samples from the 46 patients were contemporaneously collected over a period ranging from 8 days before to 15 days after BAL fluid retrieval (median, 0 days). Extracted BAL fluid DNA and serum samples were stored at −80°C and −20°C, respectively, until fungal quantification and BG detection.

Patients were initially assigned to either the PCP or colonization group based on positive *P. jirovecii* detection results, medical chart review, and the physician's final diagnosis. Patient characteristics are presented in Table 1.

Seventeen of the 46 patients were assigned to the PCP group (mean age, 59 years [range, 36 to 77 years]; 12 men and 5 women) because they presented clinical and radiological findings consistent with a PCP diagnosis according to the criteria described by the Centers for Disease Control and Prevention (28); specifically, they presented dyspnea or cough, chest X-ray evidence of interstitial infiltrates, hypoxia with an arterial partial O_2 pressure of <70 mm Hg, and no evidence of bacterial pneumonia. Microscopic evidence of *P. jirovecii* in BAL fluid samples was initially positive for 10 patients. Microscopic findings were negative for the other seven patients, while fungus detection findings were positive using the plus/minus PCR assay described above. Underlying conditions included HIV infections (4 patients), lymphoma (3 patients), renal transplantation (3 patients), solid malignancies (3 patients), and myelodysplasia, acute myeloid leukemia, acute alcoholic hepatitis, and sarcoidosis treated with high-dose corticosteroids (1 patient each). Physicians considered a PCP diagnosis for these 17 patients, including the seven patients for whom *Pneumocystis* microscopic detection findings were negative. All patients received anti-*Pneumocystis* treatment after PCP diagnosis. Clinical improvement was observed after 3 weeks of treatment for all patients.

Twenty-nine of the 46 patients were assigned to the *Pneumocystis* colonization group (mean age, 59 years [range, 24 to 84 years]; 22 men and 7 women). *P. jirovecii* was not detected in BAL fluid samples by microscopic examination but was detected by the plus/minus PCR assay. Underlying conditions included chronic obstructive pulmonary disease (6 patients), autoimmune diseases treated with high-dose corticosteroids (7 patients), renal transplantation (5 patients), acute myeloid leukemia (4 patients), lymphoma (3 patients), solid malignancies (2 patients), asthma (1 patient), and undetermined causes (1 patient). Alternative diagnoses of PCP were bacterial pneumonia (18 patients), chronic obstructive pulmonary disease exacerbation (4 patients), tuberculosis (2 patients), lung cancer (2 patients), and cytomegaloviral pneumonia, pulmonary sarcoidosis, and asthma exacerbation (1 patient each). Clinical improvement was observed for all patients despite the absence of any specific anti-*Pneumocystis* treatment. Patients with bacterial or viral pneumonia were successfully treated with appropriate antimicrobial therapy. The patient with pulmonary sarcoidosis was successfully treated with corticosteroids. For all colonized patients, follow-up periods with no PCP occurrence ranged from 2 to 10 months. For these reasons, the physicians ruled out a diagnosis of PCP, considering these patients to be colonized.

Forty-five *Pneumocystis*-uninfected patients (mean age, 56 years [range, 12 to 86 years]; 29 men and 16 women) were also retrospectively enrolled. The patients had been hospitalized in hospital A between May 2008 and January 2012 for investigation of pulmonary symptoms or fever. *P. jirovecii* detection was performed with BAL fluid samples (45 samples) by microscopic examination and the qualitative PCR assay targeting the mtLSU rRNA gene, as described above. *P. jirovecii* detection findings were negative for all samples, and these 45 patients constituted the control group. Forty-five serum samples from the 45 patients were contemporaneously collected over a period ranging from 21 days before to 33 days after BAL fluid retrieval (median, 0 days). Serum samples were stored at −20°C until serum BG assays.

None of the 46 patients infected with *Pneumocystis* or the 45 patients of the control group presented clinical or laboratory signs of invasive fungal infection. Detection of *Aspergillus* galactomannan and *Candida* mannan antigens in serum samples using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Platelia Candida Ag plus, Platelia Aspergillus Ag, Bio-Rad, Marnes-la-Coquette, France) and blood cultures (Bactec Mycosis; Becton, Dickinson and Company, Sparks, MD) were negative. None of the patients had received antibiotics before sampling.

***Pneumocystis jirovecii* DNA quantification in bronchoalveolar lavage fluid samples.** *P. jirovecii* DNA was quantified in the 46 BAL fluid samples in which the fungus was previously detected using a real-time qPCR assay targeting the mtLSU rRNA gene of *P. jirovecii*, as described elsewhere (29). Specificity of the two primers and the probe for *P. jirovecii* DNA was checked using Blast software. Thermocycling and fluorescence detection were performed with an Applied Biosystems 7300 real-time PCR system, in a final volume of 25 μl, using TaqMan Gene Expression Master Mix (Applied Biosystems) with 0.2 mmol/liter of each primer, 0.1 mmol/liter of the probe, and 5 μl of extracted DNA. After 2 min at 50°C and 10 min at 95°C, amplification consisted of 40 cycles of 15 s of denaturation at 95°C and 1 min of annealing and extension at 60°C. Plasmid suspensions were used as standards for quantification and as positive-control samples. They were prepared by cloning the mtLSU rRNA insert into the plasmid vector pGEM-T (pGEM-T Easy VectorSystem II; Promega, Madison, WI). After propagation and purification of the plasmids, the mtLSU rRNA gene copy number (number of copies per microliter) was derived from the 260-nm optical density measurement and the plasmid molecular weight. Each PCR run included eight serial 10-fold dilutions of the plasmid suspension, ranging from 7×10^0 to 7×10^7 copies/μl of extracted DNA, and two negative-control samples (ultrapure water). Plasmid dilutions were used to establish a calibration curve indicating the correspondence between cycle threshold values and the numbers of copies per microliter of extracted DNA. The detection limit was estimated to be 7 copies/μl, corresponding to a cycle threshold value of 39. All BAL fluid samples, as well as plasmid dilutions and negative-control samples, were assayed in duplicate, with one tube containing an internal positive control (TaqMan exogenous internal positive control reagents; Applied Biosystems) to detect PCR inhibitors. The quantity of *P. jirovecii*

TABLE 1 Characteristics of 46 patients infected with *Pneumocystis* who underwent *Pneumocystis jirovecii* quantification in bronchoalveolar lavage fluid samples combined with serum (1→3)-β-D-glucan detection

Patient no.[a]	Age (yr)	Gender[b]	Hospital	Underlying disease (alternative diagnosis of PCP)[c]	Date of BAL fluid retrieval (mo-day-yr)	Microscopic/ PCR detection of *P. jirovecii* in BAL fluid sample	Time between serum and BAL fluid sampling (days)
P1	69	M	A	Solid malignancy	08-06-08	−/+	10
P2	53	M	A	HIV infection	09-16-08	+[d]/+	0
P3	41	M	A	Acute alcoholic hepatitis	10-06-08	−/+	0
P4	60	F	A	RT	01-07-09	+[d]/+	1
P5	71	M	A	Myelodysplasia	06-29-11	−/+	3
P6	59	F	A	Sarcoidosis	09-12-11	−/+	1
P7	72	M	A	Lymphoma	12-02-11	−/+	−1
P8	67	M	A	RT	01-06-12	−/+	−1
P9	40	F	B	Lymphoma	02-02-10	+[d]/+	−1
P10	56	M	B	AML	02-12-10	+[d]/+	−1
P11	75	M	B	Solid malignancy	07-22-10	+[d]/+	−1
P12	48	F	B	HIV infection	08-12-10	+[d]/+	−2
P13	56	F	B	RT	08-26-10	+[d]/+	0
P14	77	M	B	Lymphoma	08-31-10	−/+	−5
P15	65	M	B	Solid malignancy	11-23-10	+[d]/+	0
P16	55	M	B	HIV infection	11-25-10	+[d]/+	−7
P17	36	M	B	HIV infection	01-25-11	+[d]/+	0
C1	53	M	A	Sarcoidosis (bacterial pneumonia)	01-31-08	−/+	15
C2	51	M	A	RT (bacterial pneumonia)	04-24-08	−/+	4
C3	39	M	A	COPD (COPD exacerbation)	05-16-08	−/+	0
C4	51	F	A	Lymphoma (bacterial pneumonia)	07-15-08	−/+	2
C5	77	M	A	COPD (bacterial pneumonia)	10-21-08	−/+	2
C6	24	F	A	Lymphoma (bacterial pneumonia)	12-26-08	−/+	2
C7	66	M	A	AML (bacterial pneumonia)	03-11-11	−/+	0
C8	55	F	A	COPD (COPD exacerbation)	03-28-11	−/+	5
C9	62	F	A	RA (bacterial pneumonia)	03-28-11	−/+	3
C10	76	M	A	RT (bacterial pneumonia)	05-03-11	−/+	13
C11	57	M	A	Solid malignancy (lung cancer)	06-20-11	−/+	1
C12	76	M	A	COPD (COPD exacerbation)	07-01-11	−/+	14
C13	51	M	A	CAH (tuberculosis)	07-04-11	−/+	1
C14	55	M	A	Solid malignancy (lung cancer)	07-06-11	−/+	7
C15	43	M	A	COPD (tuberculosis)	07-13-11	−/+	−2
C16	76	M	A	PA (bacterial pneumonia)	08-19-11	−/+	−5
C17	54	M	A	RT (CMV pneumonia)	08-31-11	−/+	−8
C18	29	M	A	AML (bacterial pneumonia)	09-07-11	−/+	0
C19	70	M	A	COPD (COPD exacerbation)	09-12-11	−/+	4
C20	59	F	A	AML (bacterial pneumonia)	10-25-11	−/+	0
C21	84	M	A	Lymphoma (bacterial pneumonia)	11-09-11	−/+	0
C22	64	M	A	Sarcoidosis (pulmonary sarcoidosis)	11-24-11	−/+	4
C23	71	M	A	WD (bacterial pneumonia)	12-28-11	−/+	2
C24	57	M	A	CAH (bacterial pneumonia)	01-06-12	−/+	0
C25	54	M	A	RT (bacterial pneumonia)	01-18-12	−/+	2
C26	53	F	B	RT (bacterial pneumonia)	02-04-11	−/+	2
C27	56	M	B	AML (bacterial pneumonia)	02-24-11	−/+	0
C28	78	F	B	ND (bacterial pneumonia)	04-08-11	−/+	13
C29	77	M	B	Asthma (asthma exacerbation)	06-10-11	−/+	5

[a] Patients P1 to P17, patients with *Pneumocystis* pneumonia; patients C1 to C29, patients with *Pneumocystis* colonization.
[b] M, male; F, female.
[c] AML, acute myeloid leukemia; BAL, bronchoalveolar lavage; CAH, chronic alcoholic hepatitis; CMV, cytomegalovirus; COPD, chronic obstructive pulmonary disease; ND, not determined; PA, psoriatic arthritis; RA, rheumatoid arthritis; RT, renal transplant; WD, Wegener's disease.
[d] Methanol-Giemsa staining and immunofluorescence assay (Bio-Rad, Marnes la Coquette, France) findings were both positive.

DNA in the 46 BAL fluid samples was determined according to a standard curve. Results were expressed as numbers of *P. jirovecii* DNA copies/μl of extracted DNA.

(1→3)-β-D-Glucan detection in serum samples. BG levels were determined for 46 serum samples from the 46 patients infected with *Pneumocystis* and 45 serum samples from the 45 patients in the control group by using the Fungitell kit (Associates of Cape Cod, Inc., Cape Cod, MA), according to the manufacturer's instructions. The results of a kinetic colorimetric assay performed at 37°C were read at 405 nm for 40 min. The BG concentrations in samples were calculated automatically by using a calibration curve established with standard solutions ranging from 6.25 to 100 pg/ml. BG levels higher than 80 pg/ml were considered to be positive,

as defined by the manufacturer. Serum assays were performed in duplicate. Samples with BG levels higher than 500 pg/ml were diluted and reassayed.

Statistical analysis. Results for *P. jirovecii* DNA copies/μl in BAL fluid samples and serum BG levels were compared between the PCP group and the colonization group using Student's test. Statistical significance was defined as $P \leq 0.05$. A receiver operating characteristic (ROC) curve was constructed using the results for *P. jirovecii* DNA copies/μl in BAL fluid samples and was analyzed by the SAS System (SASApp, X64_SRV08) to define two qPCR cutoff values, the lower value achieving 100% sensitivity for PCP diagnosis and the upper value achieving 100% specificity.

RESULTS

The results of *P. jirovecii* DNA quantification in BAL fluid samples and serum BG levels are shown in Table 2. The numbers of *P. jirovecii* DNA copies/μl in the PCP group ranged from 1.6×10^3 to 1.3×10^8 copies/μl, with a mean value of 1.3×10^7 copies/μl and a median value of 4.2×10^5 copies/μl. The numbers of *P. jirovecii* DNA copies/μl in the colonization group ranged from 7×10^0 to 2×10^4 copies/μl, with a mean value of 3.4×10^3 copies/μl and a median value of 4.4×10^2 copies/μl. The numbers of *P. jirovecii* DNA copies/μl were significantly higher in the PCP group than in the colonization group ($P < 0.05$). Results are shown in Fig. 1.

Significant differences in the *P. jirovecii* burdens in the PCP group and the colonization group allowed determination of two cutoff values for PCP diagnosis. ROC curve analysis also indicated an area under the curve of 0.96, confirming the ability of the qPCR assay to distinguish PCP from colonization in 96% of cases (Fig. 2). The lower and upper cutoff values were estimated as 1.6×10^3 and 2×10^4 copies/μl, respectively. A qPCR result lower than 1.6×10^3 copies/μl ruled out a diagnosis of PCP and indicated a diagnosis of *Pneumocystis* colonization. The sensitivity of the qPCR assay for PCP diagnosis using the lower cutoff value was therefore 100%. Using this value, the specificity was 65.5%. A qPCR result higher than 2×10^4 copies/μl indicated a diagnosis of PCP. The specificity of the qPCR assay for PCP diagnosis using the upper cutoff value was therefore 100%. Using this value, the sensitivity was 76.5%. The positive predictive value of the qPCR assay for PCP diagnosis was 100% when the *P. jirovecii* DNA copy numbers in BAL fluid samples were higher than 2×10^4 copies/μl. The negative predictive value of the assay was 100% when the *P. jirovecii* DNA copy numbers in BAL fluid samples were less than 1.6×10^3 copies/μl. Values ranging between the two cutoff values defined an indeterminate zone.

By applying these cutoff values, 13/17 patients initially assigned to the PCP group were again classified in the PCP group based on the qPCR assay results, as they all presented *P. jirovecii* DNA copy numbers above the upper cutoff value of 2×10^4 copies/μl. Similarly, 19/29 patients initially assigned to the colonization group were again classified in the colonization group, as they all presented *P. jirovecii* DNA copy numbers below the lower cutoff value of 1.6×10^3 copies/μl. The remaining 14 patients (4 patients initially assigned to the PCP group and 10 patients initially assigned to the colonization group) presented *P. jirovecii* DNA copy numbers within the indeterminate zone. For these 14 patients, therefore, the differential diagnosis between PCP and *Pneumocystis* colonization could not be established on the basis of the qPCR assay results.

Results for BG detection in the control group ranged from 8 to 70 pg/ml, with a median value of 28 pg/ml. Results for BG detection in the PCP group ranged from 122 to 8,000 pg/ml, with a median value of 1,945 pg/ml. All 17 patients assigned to this group presented serum BG levels higher than 100 pg/ml. Results for BG detection in the colonization group ranged from 8 to 87 pg/ml, with a median value of 40 pg/ml. Twenty-three of the 29 patients assigned to this group had serum BG levels lower than 80 pg/ml, the positive threshold defined by the manufacturer. Serum BG levels for the remaining six patients of the colonization group

TABLE 2 Results of *Pneumocystis jirovecii* DNA quantification in bronchoalveolar lavage fluid samples and (1→3)-β-D-glucan levels in serum samples from the 17 patients who developed *Pneumocystis* pneumonia and the 29 patients colonized by *Pneumocystis*

Patient no.[a]	*P. jirovecii* quantification (DNA copies/μl)	(1→3)-β-D-Glucan level (pg/ml)
P1	1.6×10^3	184
P2	1.3×10^8	2,710
P3	1.0×10^4	1,507
P4	4.0×10^6	2,500
P5	7.0×10^4	363
P6	5.9×10^7	3,480
P7	3.3×10^4	1,945
P8	1.0×10^4	126
P9	7.5×10^5	306
P10	4.6×10^4	4,350
P11	1.2×10^5	5,049
P12	4.2×10^6	850
P13	1.5×10^7	464
P14	7.6×10^5	4,100
P15	5.6×10^3	122
P16	4.8×10^5	8,000
P17	4.2×10^5	394
C1	1.6×10^3	49
C2	4.2×10^2	70
C3	5.4×10^2	84
C4	1.6×10^2	83
C5	1.1×10^2	64
C6	7.5×10^1	40
C7	7.0×10^0	23
C8	3.5×10^2	82
C9	7.0×10^2	10
C10	1.7×10^2	10
C11	1.0×10^4	22
C12	5.0×10^1	37
C13	1.7×10^4	47
C14	3.2×10^2	22
C15	1.9×10^3	82
C16	2.8×10^2	8
C17	1.1×10^2	14
C18	6.7×10^2	61
C19	2.9×10^2	18
C20	9.9×10^3	9
C21	2.0×10^4	53
C22	1.0×10^2	13
C23	3.6×10^3	21
C24	4.4×10^2	87
C25	3.7×10^3	8
C26	3.7×10^3	60
C27	1.9×10^4	83
C28	1.4×10^2	37
C29	1.2×10^3	40

[a] Patients P1 to P17, patients with *Pneumocystis* pneumonia; patients C1 to C29, patients with *Pneumocystis* colonization.

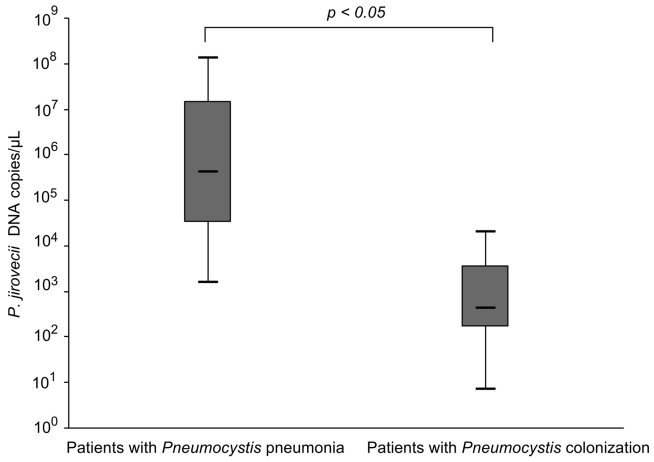

FIG 1 *Pneumocystis jirovecii* DNA copies per μl of extracted DNA in patients with *Pneumocystis* pneumonia and patients with *Pneumocystis* colonization. Gray boxes, 50% of the sample data; black horizontal bars, median values. The median value for patients with *Pneumocystis* pneumonia was 4.2×10^5 copies/μl (interquartile range, 3.3×10^4 to 1.5×10^7 copies/μl). The median value for patients with *Pneumocystis* colonization was 4.4×10^2 copies/μl (interquartile range, 1.6×10^2 to 3.7×10^3 copies/μl). *Pneumocystis jirovecii* DNA copy numbers were determined by a qPCR assay targeting the mitochondrial large subunit rRNA gene.

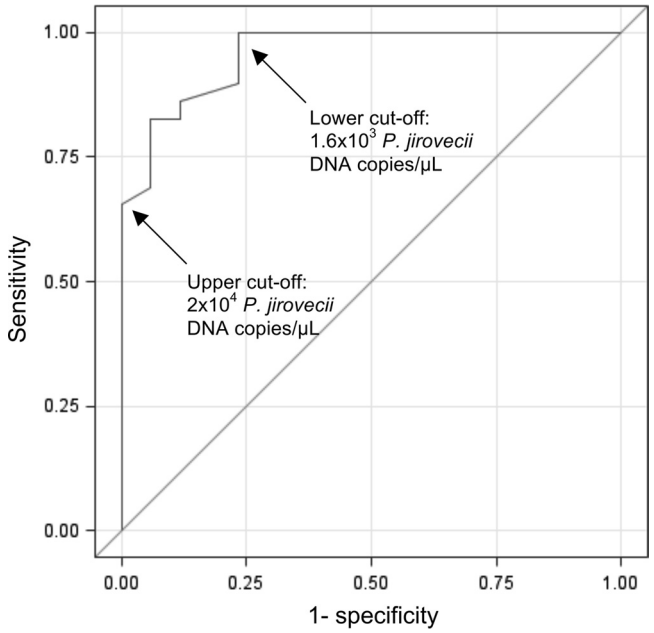

FIG 2 Receiver operating characteristic curve for *Pneumocystis jirovecii* DNA copy numbers for the diagnosis of *Pneumocystis* pneumonia. Arrows, cutoff values for the qPCR assay used for quantification of *Pneumocystis jirovecii* DNA copy numbers in bronchoalveolar lavage fluid samples from patients infected with *Pneumocystis*.

ranged between 82 and 87 pg/ml. All 29 patients in this colonization group therefore had serum BG levels lower than 100 pg/ml. Serum BG levels were significantly higher in the PCP group than in the colonization group ($P < 0.05$).

Overall, determination of *P. jirovecii* DNA copy numbers in BAL fluid samples clearly assigned 32/46 patients to the PCP group or the colonization group. For the remaining 14 patients, *P. jirovecii* DNA copy numbers were in the indeterminate zone of our qPCR assay and did not allow differentiation between PCP and colonization. Four of these 14 patients had serum BG levels higher than 100 pg/ml. These four patients initially had been assigned to the PCP group on the basis of clinical criteria. Ten other patients had serum BG levels lower than 100 pg/ml and initially had been assigned to the colonization group, again on the basis of clinical criteria. Use of the qPCR assay with cutoff values of 1.6×10^3 copies/μl and 2×10^4 copies/μl to determine *P. jirovecii* DNA copy numbers in BAL fluid samples in combination with BG detection in serum samples using a threshold of 100 pg/ml allowed unambiguous classification of all patients infected with *Pneumocystis* (Fig. 3).

DISCUSSION

Since the early 2000s, about 20 studies have focused on the development of real-time PCR assays for *P. jirovecii* quantification in pulmonary samples. Some of those studies determined cutoff values for *P. jirovecii* DNA copy numbers in order to differentiate PCP from *Pneumocystis* colonization (5–12, 26). However, cutoff values differed from one study to another due to methodological differences, as various genomic targets, such as the major surface glycoprotein (MSG) gene (5, 6, 8, 9, 11), the heat shock protein 70 (HSP70) gene (7), the dihydropteroate synthase (DHPS) gene (26), and the mtLSU rRNA gene (10, 12), were amplified. The mtLSU rRNA gene was used in the present study because (i) it is a multicopy gene in the *P. jirovecii* genome that allows highly sensitive detection, (ii) it has been routinely used for *P. jirovecii* detection with a plus/minus PCR assay in our laboratory for several years, and (iii) it is the target most commonly used for *P. jirovecii* detection worldwide.

Five studies determined a single cutoff value for *P. jirovecii* DNA copy numbers to differentiate PCP from *Pneumocystis* colonization (5–8, 26). Larsen et al. determined *P. jirovecii* DNA copy numbers in oral wash samples using a quantitative touchdown PCR assay targeting the MSG gene (6). Using a cutoff value of 50 DNA copies/tube, this qPCR assay showed a specificity of 100% and a sensitivity of 70% for PCP diagnosis. With the same PCR method applied to BAL fluid samples, Chumpitazi et al. established that a cutoff value of 53.4 copies/ml for BAL fluid samples provided a specificity of 97.7% and a sensitivity of 100% for PCP diagnosis (8). Flori et al. determined *P. jirovecii* DNA copy numbers in BAL fluid samples using a qPCR assay also targeting the MSG gene (5). With a cutoff value of 10^3 DNA copies/capillary tube, this qPCR assay showed a specificity of 98.6% and a sensitivity of 100% for PCP diagnosis. Huggett et al. determined *P. jirovecii* DNA copy numbers in BAL fluid samples using a qPCR assay targeting the HSP70 gene (7). Using a cutoff value of 10 DNA copies/reaction, this qPCR assay showed a specificity of 96% and a sensitivity of 98% for PCP diagnosis. Matsumura et al. determined *P. jirovecii* DNA copy numbers in BAL fluid and induced sputum samples using a qPCR assay targeting the DHPS gene (26). Using a cutoff value of 1,300 copies/ml of sample, this qPCR assay showed a specificity of 100% and a sensitivity of 80% for PCP diagnosis.

Four other studies determined two cutoff values for *P. jirovecii* burdens in order to increase the specificity and sensitivity of qPCR assays for PCP diagnosis (9–12). Mühlethaler et al. determined *P. jirovecii* burdens in BAL fluid samples using a qPCR assay target-

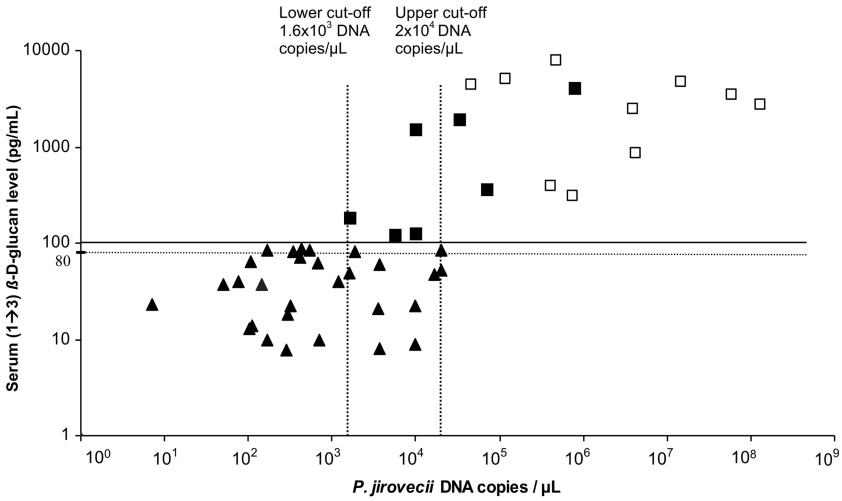

FIG 3 Results of *Pneumocystis jirovecii* DNA copies per μl of extracted DNA in bronchoalveolar lavage fluid samples combined with (1→3)-β-D-glucan levels in serum samples for patients with *Pneumocystis* pneumonia and patients with *Pneumocystis* colonization. ▲, patients initially assigned to the *Pneumocystis* colonization group; ■, patients initially assigned to the *Pneumocystis* pneumonia group with negative microscopic detection of *Pneumocystis jirovecii* in bronchoalveolar lavage fluid samples; □, patients initially assigned to the *Pneumocystis* pneumonia group with positive microscopic detection of *Pneumocystis jirovecii* in bronchoalveolar lavage fluid samples.

ing the MSG gene (11). Using an upper cutoff value of 1,450 organisms/ml, this qPCR assay showed a specificity of 99.4% and a sensitivity of 66% for PCP diagnosis. However, the results of those authors did not support the definition of a lower cutoff value to exclude PCP reliably. Alanio et al. determined *P. jirovecii* burdens in BAL fluid or induced sputum samples using a qPCR assay targeting the mtLSU rRNA gene (10). Using an upper cutoff value of 1,900 trophic form equivalents/ml and a lower cutoff value of 120 trophic form equivalents/ml, this qPCR assay showed specificity and sensitivity of 100% for PCP diagnosis. Botterel et al. determined *P. jirovecii* DNA copy numbers in BAL fluid samples using a qPCR assay also targeting the mtLSU rRNA gene (12). Using an upper cutoff value of 4 $\log_{10}$ copies/μl and a lower cutoff value of 2.6 $\log_{10}$ copies/μl, this qPCR assay showed specificity and sensitivity of 100% for PCP diagnosis. Fillaux et al. also determined two cutoff values for a qPCR assay targeting the MSG gene that resulted in specificity and sensitivity of 100% for PCP diagnosis (9). However, those authors did not provide absolute quantification of *P. jirovecii*, as their cutoff values were expressed as cycle thresholds of 22 and 28 for the lower and upper values, respectively. In these studies, the lower cutoff value represents the value below which the diagnosis of PCP can be ruled out and the diagnosis of *Pneumocystis* colonization can be established. The upper cutoff value represents the value above which the diagnosis of PCP can be confirmed. However, the range between these two cutoff values represents an indeterminate zone. When *P. jirovecii* quantities determined by qPCR assays fall in this indeterminate zone, the differential diagnosis between PCP and colonization cannot be established. Similarly, the differential diagnosis between PCP and colonization could not be established on the basis of qPCR results for 14 of 46 patients enrolled in the present study.

All patients assigned to the PCP group presented serum BG levels higher than 100 pg/ml. None of those patients presented any factors known to affect serum BG levels, particularly concurrent invasive fungal infections. Positive BG detection results can therefore be correlated with the presence of *P. jirovecii* cysts in the lungs of patients, including the six patients for whom microscopic findings for detection of the fungus were negative. In contrast, 23/29 colonized patients had serum BG levels lower than 80 pg/ml, i.e., the positive threshold defined by the manufacturer. These BG detection results, combined with positive qPCR assay results and negative microscopic detection results, can be explained by low burdens of *P. jirovecii* trophic forms and the absence of cysts in the patients' lungs. The hypothesis of a small number of *P. jirovecii* trophic forms to explain positive PCR results in the absence of cyst detection has been proposed previously (30). The other six colonized patients presented positive BG detection results but with values lower than 100 pg/ml, ranging between 82 and 87 pg/ml. The use of a threshold of 100 pg/ml in order to increase the specificity of the BG assay for PCP diagnosis has been proposed by Desmet et al. (18). Our results are consistent with this proposal by Desmet et al. (18), as all patients assigned to the colonization group had negative BG results on the basis of this threshold value. Differentiation between PCP and colonization on the basis of qPCR assay results was achieved for 32/46 patients enrolled in the present study. The PCP/colonization diagnosis for the remaining 14 patients could be determined on the basis of serum BG detection. Use of the qPCR assay with two cutoff values to determine *P. jirovecii* DNA copies/μl in BAL fluid samples combined with BG detection in serum samples therefore allowed unambiguous classification of all patients included in this study (Fig. 4). A flow diagram for the diagnosis of *Pneumocystis* infection in patients with clinically suspected PCP is proposed on the basis of the results of this study (Fig. 5). This diagram may be included in the context of a global approach to the diagnosis of invasive pulmonary fungal infections.

Pneumocystis colonization can occur in a wide variety of mammals. It has been observed in free-living and laboratory animals. *Pneumocystis* colonization duration was estimated as <1 month to 6 months in free-living immunocompetent macaques (31). Studies in rodents and primates showed that *Pneumocystis* colonization induced chronic obstructive pulmonary disease develop-

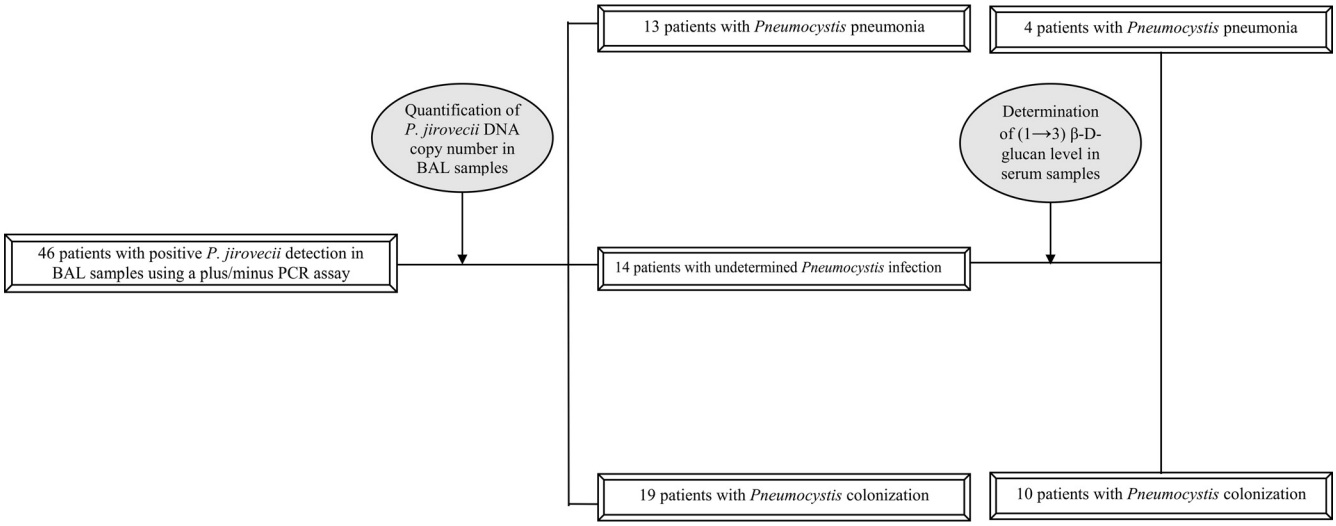

FIG 4 Characterization of patients infected with *Pneumocystis* using pulmonary quantification of *Pneumocystis jirovecii* DNA copy numbers combined with serum (1→3)-β-D-glucan levels. BAL, bronchoalveolar lavage fluid.

ment (32, 33). Moreover, using the experimental SCID-BALB/c mouse transmission model, it was shown that *Pneumocystis*-carrying BALB/c mice were able to transmit the infection to susceptible SCID mice and to naive healthy mice. The secondarily exposed hosts were able to transmit the fungus to susceptible hosts, which developed PCP (34–36). In humans, *Pneumocystis* colonization occurs in persons without signs of acute pneumonia or in persons for whom signs can be related to an alternative diagnosis to PCP. Several populations have been identified as being colonized by the fungus, such as patients with various levels of immunodeficiency, patients with acute or chronic pulmonary diseases, pregnant women with immunity changes, and health care workers in contact with patients with PCP (reviewed in reference 4). It has been shown that the fungus may play a role in the progression of underlying lung diseases or in the development of PCP in colonized patients (4). It also has been recently shown that colonized patients may act as potential infectious sources (37).

PCP is a life-threatening infection in the absence of specific treatment, whereas pulmonary colonization is a less-severe presentation of *Pneumocystis* infection. It is therefore essential to distinguish between these two clinical presentations of *Pneumocystis* infection. The differential diagnosis of PCP versus colonization can be achieved by qPCR assays targeting the mtLSU rRNA gene with two cutoff values combined with serum BG

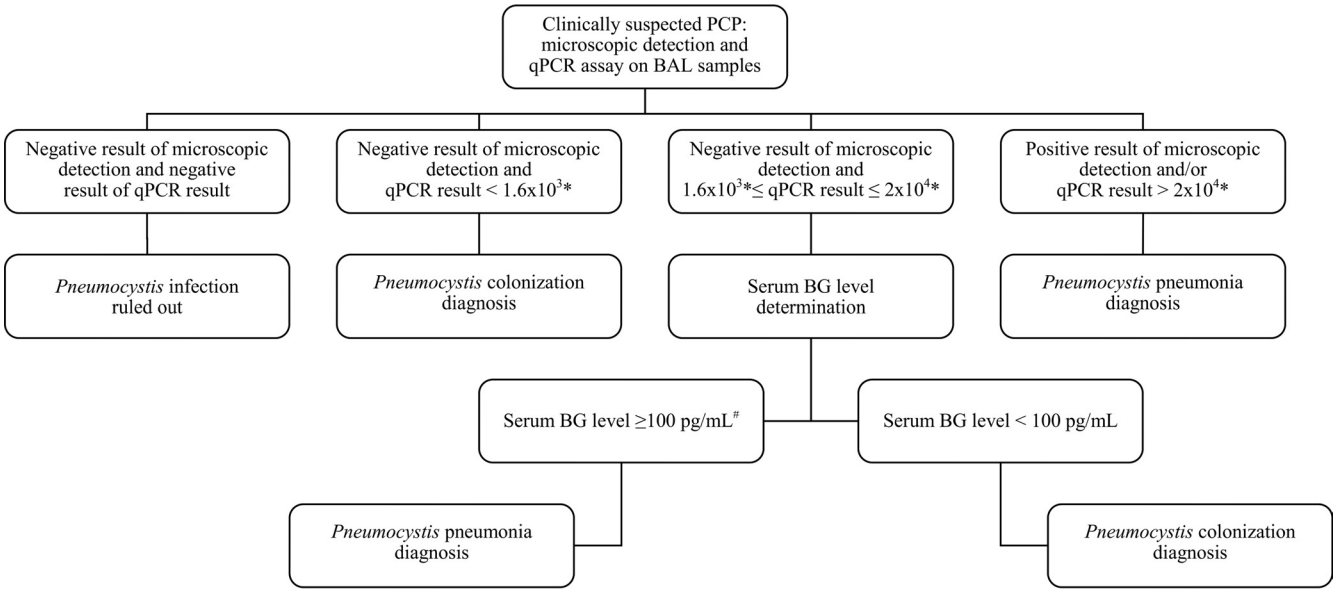

FIG 5 Flow diagram for the diagnosis of *Pneumocystis* infections in patients with clinically suspected *Pneumocystis* pneumonia. Microscopic examination using methanol-Giemsa staining and an immunofluorescence assay (Bio-Rad, Marnes la Coquette, France) and a qPCR assay targeting the mitochondrial large subunit rRNA gene of *P. jirovecii* were used. BAL, bronchoalveolar lavage fluid; BG, (1→3)-β-D-glucan; PCP, *Pneumocystis* pneumonia; *, results expressed in DNA copies/μl; #, without factors that interfere with (1→3)-β-D-glucan level determinations, particularly concurrent invasive fungal infections.

detection using the Fungitell kit and applying the threshold of 100 pg/ml.

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