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Serum (1-3)-β-D-Glucan as a Tool for Diagnosis of *Pneumocystis jirovecii* Pneumonia in Patients with Human Immunodeficiency Virus Infection or Hematological Malignancy▽

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(1-3)-β-D-Glucan (BG) reactivity was tested in serum samples from 28 patients with human immunodeficiency virus infection or a hematological malignancy and *Pneumocystis jirovecii* pneumonia (PCP) and 28 control patients. The sensitivity and specificity of BG detection with the Fungitell assay for PCP were 100 and 96.4%, respectively, using a cutoff value of 100 pg/ml. Serum BG testing looks promising for the noninvasive diagnosis of PCP. Our data suggest that a higher cutoff value for the diagnosis of PCP than for the diagnosis of invasive aspergillosis or candidiasis could be used safely and will improve the specificity of the test.

Pneumocystis jirovecii pneumonia (PCP) remains a serious cause of morbidity and mortality in immunocompromised patients. PCP may be difficult to diagnose owing to nonspecific signs and symptoms and possible coinfection with microorganisms other than *P. jirovecii*. Moreover, *Pneumocystis* cannot be propagated in culture. Diagnosis relies on the visualization of the fungus upon microscopic examination of induced sputum samples, bronchoalveolar lavage (BAL) fluids, or biopsy specimens. The sensitivity of microscopy varies according to the staining technique (it is highest with monoclonal antibodies) and the sample type (10). PCR detection of *Pneumocystis* nucleic acids has been shown to have higher sensitivity for the diagnosis of PCP than conventional staining techniques (1). However, PCR may also give positive results for patients with *P. jirovecii* colonization, and the clinical management of the disease in patients with positive PCR results but negative microscopy findings remains challenging. Furthermore, the diagnosis of PCP generally relies on invasive diagnostic tests, such as bronchoscopy, which is not always feasible for patients with severe respiratory distress.

The measurement of serum (1-3)-β-D-glucan (BG), a cell wall component of most pathogenic fungi, including *P. jirovecii*, may be a useful aid for establishing the diagnosis of PCP. There are a number of diagnostic kits commercially available for detecting BG. The Fungitell BG assay (Associates of Cape Cod, East Falmouth, MA) is approved by the U.S. Food and Drug Administration as an adjunct for the diagnosis of invasive fungal disease, and the assay kit also carries the European CE mark. Up to now, data about the performance characteristics of the Fungitell BG test for the diagnosis of PCP have been scarce (2, 5, 8, 9). Few patients were included in the studies reported, and generally no relevant control patients were included. It is not known whether the cutoff value proposed by the manufacturer (80 pg/ml) can be used for the diagnosis of PCP. Elevated BG levels in PCP patients have been detected, but since many factors were reported to cause false-positive results, data for control groups are needed before the test can be used in routine practice.

We retrospectively measured BG concentrations in sera from PCP patients and controls in two major risk groups, namely, patients with advanced human immunodeficiency virus (HIV) infection and patients with a hematological malignancy, with the aim of determining the diagnostic potential of BG testing for both groups.

MATERIALS AND METHODS

Patients. All patients with HIV infection or a hematological malignancy diagnosed with PCP at University Hospitals Leuven, Leuven, Belgium, between March 2001 and April 2009 were included. The diagnostic criteria for PCP infection were the following: progressive dyspnea and/or fever and/or a nonproductive cough lasting no longer than 3 months and the detection of *P. jirovecii* DNA in BAL fluid by PCR. Additionally, *Pneumocystis* cysts needed to be detected in BAL fluid after staining with Grocott methenamine silver (GMS) or monoclonal antibodies, or at least four of the following criteria needed to be fulfilled: presence of bilateral interstitial infiltrates upon chest imaging, hypoxia, elevated lactate dehydrogenase levels, and appropriate response to anti-PCP therapy or a CD4 cell count less than 200 cells/mm^3. Culture of blood samples (by using the BacT/Alert system) and BAL fluids on Sabouraud agar was performed for the diagnosis of concurrent mycotic diseases. In addition, sera from the hematology patients were tested for the presence of galactomannan by using the Platelia *Aspergillus* enzyme immunoassay (Bio-Rad Laboratories, Marnes-La-Coquette, France). The control group consisted of (i) HIV-positive patients with CD4 cell counts less than 200 cells/mm^3, measurable HIV RNA levels, and no indication of PCP infection and (ii) patients from hematology wards who were at risk for developing invasive fungal infection but who had no identified invasive fungal infection according to the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group criteria (3).

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TABLE 1. Patient demographics, clinical findings, and results of serum BG test and *Pneumocystis* PCR assay for BAL fluids[a]

Patient group	Patient	Age (yr)/sex	Microscopy result (method[s] of analysis)	BG level (pg/ml)	GM (index)[b]	PCR C_T value	Absolute CD4 cell count (cells/mm^3)	Plasma HIV load (log copies/ml)	LDH level (U/liter)[c]	Findings from chest imaging	Clinical outcome	Clinical syndrome
HIV-infected patients	1	62/M	Positive (GMS staining)	802		20.7	38	5.2	724	Bilateral patchy infiltrates	Survived	New diagnosis of advanced HIV infection
	2	35/M	Positive (GMS staining)	264		21.6	25	5.2	636	Alveolar infiltrates in left lower lobe and right upper lobe	Survived	New diagnosis of advanced HIV infection
	3	46/M	ND	1,552		25.4	ND	4	894	Bilateral moderate perihilar infiltrates	Died from primary brain lymphoma	New diagnosis of advanced HIV infection
	4	48/M	Positive (GMS staining)	1,811		22.7	1	5.1	612	Diffuse bilateral alveolar infiltrates	Survived	Known advanced HIV infection; no medical follow-up
	5	31/F	Negative (GMS staining)	411		23.4	6	5.2	945	Bilateral patchy infiltrates	Survived	New diagnosis of advanced HIV infection
	6	46/M	Positive (IFA)	2,732		26.7	4	4.9	1,440	Diffuse bilateral alveolar infiltrates	Survived	New diagnosis of advanced HIV infection
	7	36/M	Positive (IFA)	4,302		22.0	22	5.2	714	Bilateral interstitial infiltrates	Survived	New diagnosis of advanced HIV infection
	8	52/M	Positive (GMS staining)	1,510		20.3	19	5	464	Diffuse bilateral alveolar infiltrates	Survived	Known advanced HIV infection; no medical follow-up
	9	33/F	ND	1,482		20.0	21	5.6	1,026	Bilateral basal alveolar infiltrates	Survived	New diagnosis of advanced HIV infection
	10	60/M	ND	975		22.9	13	5.4	713	Interstitial infiltrates in left lung	Survived	New diagnosis of advanced HIV infection
	11	44/M	ND	2,085		18.0	159	5.6	ND	Bilateral interstitial infiltrates	Survived	New diagnosis of advanced HIV infection
	12	40/M	Positive (GMS staining)	1,083		20.0	19	5.6	926	Diffuse bilateral alveolar infiltrates	Survived	Known advanced HIV infection; no medical follow-up
	13	47/M	ND	2,082		20.6	65	5.6	689	Diffuse bilateral alveolar infiltrates	Survived	New diagnosis of advanced HIV infection
	14	49/M	ND	1,138		27.9	ND	6.2	ND	Bilateral alveolar infiltrates in lower lobes	Survived	New diagnosis of advanced HIV infection
	15	52/M	Positive (GMS staining)	4,157		21.0	103	5.3	1,375	Alveolar infiltrates in left lower lobe	Survived	New diagnosis of advanced HIV infection
	16	36/M	ND	1,036		24.4	21	4.9	508	Diffuse bilateral alveolar infiltrates	Survived	New diagnosis of advanced HIV infection
Patients with hematological malignancy	1	31/F	Positive (GMS staining, biopsy)	4,302	0.1	24.6			1,493	Bilateral pleuropulmonary consolidations in the lower lobes	Died	ALL, alloHSCT
	2	23/F	Positive (GMS staining)	111	0.1	23			465	Diffuse ground-glass opacities with parenchymal consolidation in the right middle lobe	Survived	Common-B-ALL, consolidation therapy
	3	48/F	ND	3,916	0.8	25			948	Bilateral consolidations	Died	AML, alloHSCT, IA, candidemia
	4	27/M	Positive (GMS staining)	15,620	0.3	22.9			838	Bilateral diffuse ground-glass opacities and pleural fluid	Died	ALL, alloHSCT, GvHD
	5	42/M	ND	16,109	0.1	25.3			1,961	Bilateral perihilar consolidations	Died	Cerebral NHL, IA
	6	69/F	ND	21,938	0.1	25			1,594	Bilateral perihilar consolidations	Died	AML, IA
	7	41/M	Positive (GMS staining)	14,414	0.0	18.4			763	Bilateral patchy consolidations	Died	Hodgkin lymphoma
	8	71/M	ND	3,642	0.2	26.2			1,052	Bilateral patchy consolidations	Survived	Diffuse large-cell NHL
	9	47/M	Positive (GMS staining)	3,371	0.1	25			447	Bilateral patchy and ground-glass consolidations	Survived	AML, alloHSCT
	10	24/F	Positive (GMS staining)	404	0.1	20.2			935	Ground-glass opacities in both upper lobes	Survived	Hemophagocytic syndrome
	11	47/M	ND	1,348	0.2	20.4			685	Bilateral diffuse infiltrates, patchy consolidations, and ground-glass opacities	Died	AML, alloHSCT, fungal pneumonia
	12	22/M	ND	360	0.1	27.3			1,075	Bilateral infiltrates and consolidations	Died	ALL, alloHSCT, CMV infection

[a] M, male; F, female; ND, not determined; IFA, immunofluorescence assay; GM, galactomannan; LDH, lactate dehydrogenase; alloHSCT, allogeneic hematopoietic stem cell transplantation; ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; NHL, non-Hodgkin lymphoma; IA, invasive aspergillosis; GvHD, graft-versus-host disease; CMV, cytomegalovirus.

[b] The GM index was calculated as follows: the optical density value of the specimen was divided by the mean optical density for the wells containing cutoff control serum.

[c] Normal value for LDH, 240 to 480 U/liter.

TABLE 2. Demographics and clinical data for patients with PCP and control patients

Patient group	Characteristic[a]	Value for:	
		PCP patients	Controls
HIV-infected patients	No. of patients	16	16
	Males/females	14/2	14/2
	Mean age (range) in yrs	45 (31–62)	41 (17–68)
	Mean absolute CD4 cell count (range) in cells/mm^3	37 (1–159)	54 (11–77)
	Mean plasma HIV load (range) in log copies/ml	5.2 (4–6.2)	4.0 (2.2–5.9)
	No. of newly or recently diagnosed patients with advanced HIV infection	13	15
Patients with hematological malignancy	No. of patients	12	12
	Males/females	7/5	7/5
	Mean age (range) in yrs	41 (22–71)	44 (22–67)
	No. with underlying condition:		
	Any	6	7
	HSCT	1	5
	AML	1	0
	ALL	4	0
	Other		

[a] HSCT, hematopoietic stem cell transplantation; AML, acute myelogenous leukemia; ALL, acute lymphoblastic leukemia.

BG assay. BG levels were measured with the Fungitell kit as recommended by the manufacturer. Samples with BG levels higher than 500 pg/ml were diluted and retested. The sera tested were obtained on the same day that, or 1 day before, the BAL fluid sample tested positive by PCR and before therapy was started. All sera were stored at −20°C before BG testing.

PCR assay. BAL samples from all patients were tested with an in-house real-time PCR assay targeting mitochondrial rRNA from *P. jirovecii* (11). The extraction of *Pneumocystis* DNA from BAL fluid was carried out with the Qiagen QIAamp DNA extraction kit (before May 2007) or with the easyMAG instrument (bioMérieux, Marcy l'Etoile, France) between May 2007 and April 2009. An internal control (6×10^9 copies) was added to the sample before initiation of the protocol. An in-house real-time PCR assay for the detection of *Pneumocystis* DNA was performed on an ABI 7900 real-time thermocycler (Applied Biosystems). Real-time PCR was carried out with a reaction volume of 40 μl containing 10 μl of the DNA extract, 20 μl 2× Universal mastermix (Applied Biosystems), and primers and a probe targeting the mitochondrial large-subunit rRNA gene: 0.25 μM primer PC102F (5′-GCACTGAATATCTCGAGGGAGTATG-3′), 0.25 μM primer PC249R (5′-TTGGGAGCTTTAATTACTGTTCTGG-3′), and 0.20 μM probe P190P (6-carboxyfluorescein–5′-TGTTTCCCTTTCGACTATC TACCTTATCGCACA-3′–6-carboxytetramethylrhodamine). PCR analyses were considered negative for *P. jirovecii* DNA if the threshold cycle (C_T) values exceeded 40 cycles.

RESULTS

Twenty-eight patients, 16 with HIV infection and 12 with a hematological malignancy, fulfilled the diagnostic criteria for PCP infection. The demographic characteristics, clinical findings, and results of BG, galactomannan, and PCR tests are summarized in Table 1. In Table 2, demographics and clinical findings for both patients and controls are given. *Pneumocystis* cysts were detected in BAL fluids from 12 patients by GMS staining and in BAL fluids from 2 other patients by using monoclonal antibodies. None of the PCP patients were receiving prophylactic therapy for PCP at the time of diagnosis of the disease. No concurrent mycotic disease in the HIV-infected patients was diagnosed, whereas four hematology patients (numbers 3, 5, 6, and 11) also suffered from invasive disease due to other fungal pathogens, namely, *Aspergillus fumigatus* and/or *Candida glabrata*.

All PCP patients showed elevated BG concentrations ranging from 110 to 21,938 pg/ml, with a median concentration of 1,682 pg/ml and an interquartile range (the difference between the third and first quartiles) of 2,955 pg/ml. The BG levels measured in control patients ranged from 0 to 129 pg/ml, with a median concentration of 16 pg/ml (interquartile range of 19 pg/ml). When a cutoff value of 60 pg/ml was applied, sensitivity was 100% and specificity was 85.7%. The sensitivity remained at 100% but the specificity increased to 96.4% with an increase in the cutoff value to 100 pg/ml. At a cutoff level of 80 pg/ml, sensitivity was 100% and specificity was 89.3%.

HIV-infected patients with PCP had a median BG concentration of 1,496 pg/ml (range, 264 to 2,732 pg/ml), compared to 22 pg/ml (range, 0 to 129 pg/ml) in the HIV-infected control group ($P < 0.0001$; Mann-Whitney test using Analyze-it software).

Among subjects with hematological disease, BG levels in PCP patients were significantly higher than those in control patients ($P < 0.0001$). Median serum BG concentrations in this patient population were 3,779 pg/ml (range, 111 to 21,938 pg/ml) for the PCP-infected patients and 11 pg/ml (range, 0 to 33 pg/ml) for the control patients.

The difference in BG levels between PCP patients from the two risk groups was not statistically significant ($P = 0.11$). There was no correlation between the fungal load in the lungs estimated by the PCR analysis of BAL fluid and the BG concentration measured in serum ($P = 0.56$; Spearman correlation test). No difference in PCR results between the two risk groups was seen. The mean C_T value, which is inversely proportional to the amount of target DNA present in the original sample, was 23.0 for HIV-infected patients, compared to 23.6 for hematology patients.

DISCUSSION

In this retrospective study, the performance characteristics of BG detection in sera from HIV-infected patients and patients with a hematological malignancy were excellent for the diagnosis of PCP, with a sensitivity of 100% and a specificity of 96.4% at a cutoff value of 100 pg/ml.

According to the interpretation criteria of the manufacturer, a BG concentration higher than 80 pg/ml is considered to be a positive result, whereas a concentration between 60 and 79 pg/ml remains indeterminate. Odabasi et al. proposed a cutoff value of 60 pg/ml for the diagnosis of invasive fungal infections based on a large prospective study of patients undergoing initial induction chemotherapy for acute myelogenous leukemia or myelodysplastic syndrome (7). No patients with PCP were diagnosed in that study. A few studies indicate that BG may be a useful adjunct for the diagnosis of PCP, but none of them have been designed to determine the optimal cutoff value (2, 5, 6, 8, 9). Our results confirm that elevated BG levels are detected in sera from patients with PCP and also illustrate that generally very high levels are measured. Our data are too limited to validate a cutoff value but suggest that a higher cutoff value for the diagnosis of PCP than for the diagnosis of invasive aspergillosis or candidiasis could be used safely.

Interestingly, we found a higher mean BG level in the population with hematological malignancy than in the HIV-infected population, although the difference was not statistically significant, probably because of the very high degree of variation in BG levels among patients. This is in contrast to recent observations with the Fungitec-G test, where higher BG concentrations in patients with HIV-related PCP were seen (6). Moreover, Limper et al. observed that AIDS patients had a significantly higher number of *P. jirovecii* cysts per ml of BAL fluid than other immunocompromised PCP patients (4). Four hematology patients were coinfected with other fungal pathogens, and this coinfection may be the reason for the high BG levels measured. However, in two other hematology patients, very high (>10,000-pg/ml) BG levels were detected and no evidence of fungal infection except for PCP was found, even upon autopsy. Thus, it appears that coinfection with other fungal pathogens cannot entirely explain the very high BG levels seen in some hematology patients. We could not find a correlation between the BAL PCR results, which are a measure of the fungal burdens in the lungs, and the BG serum levels. A better understanding of the release of BG from the infected sites, as well as the clearance of BG, is certainly needed to understand the high interindividual variability of serum BG concentrations.

A limitation of the BG test is its panfungal character. Because different therapeutic approaches are necessary for different fungal infections, combination with other diagnostics tests such as galactomannan detection in serum and BAL fluid samples and culture of respiratory samples is certainly needed.

The development of PCR techniques for the diagnosis of PCP improved the reproducibility and the sensitivity of the laboratory diagnosis of PCP, but differentiation between colonization and infection by these techniques remains challenging. Furthermore, a prerequisite for PCR techniques is obtaining good-quality respiratory samples, e.g., fluids from BAL, which is invasive and not without risk for patients with respiratory distress. A limitation of our study is the relatively small number of patients with PCP that could be included. Further studies are needed to better delineate the role of BG determination in the diagnosis of PCP, but the excellent performance characteristics of serum BG testing look promising.

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