ORIGINAL ARTICLE MYCOLOGY

Serum (1 → 3)-β-D-glucan measurement as an early indicator of *Pneumocystis jirovecii* pneumonia and evaluation of its prognostic value

J. Held[1], M. S. Koch[1], U. Reischl[2], T. Danner[1] and A. Serr[1]

1) *Institute of Medical Microbiology and Hygiene, University of Freiburg, 79104 Freiburg, Germany and* 2) *Institute of Microbiology and Hygiene, University Hospital Regensburg, 93042 Regensburg, Germany*

Abstract

Pneumocystis jirovecii (carinii) pneumonia (PJP) is a major cause of disease in immunocompromised individuals. However, until recently no reliable and specific serological parameters for the diagnosis of PJP have been available. (1 → 3)-β-D-Glucan (BG) is a cell wall component of *P. jirovecii* and of various other fungi. Data from the past few years have pointed to serum measurement of BG as a promising new tool for the diagnosis of PJP. We therefore conducted a retrospective study on 50 patients with PJP and 50 immunocompromised control patients to evaluate the diagnostic performance of serum BG measurement. Our results show an excellent diagnostic performance with a sensitivity of 98.0% and a specificity of 94%. While the positive predictive value was only 64.7%, the negative predictive value was 99.8% and therefore a negative BG result almost rules out PJP. BG levels were already strongly elevated in an average of 5 days and up to 21 days before microbiological diagnosis demonstrating that the diagnosis could have been confirmed earlier. BG levels at diagnosis and maximum BG levels during follow-up did not correlate with the outcome of patients or with the *P. jirovecii* burden in the lung as detected by Real-Time PCR. Therefore, absolute BG levels seem to be of no prognostic value. Altogether, BG is a reliable parameter for the diagnosis of PJP and could be used as a preliminary test for patients at risk before a bronchoalveolar lavage is performed.

Keywords: β-D-Glucan, *Pneumocystis jirovecii* pneumonia, prognosis
Original Submission: 21 March 2010; **Revised Submission:** 8 July 2010; **Accepted:** 10 July 2010
Editor: E. Roilides
Article published online: 29 July 2010
Clin Microbiol Infect 2011; **17:** 595–602
10.1111/j.1469-0691.2010.03318.x

Corresponding author: J. Held, Institute of Medical Microbiology and Hygiene, University of Freiburg, Hermann-Herder-Strasse 11, D-79104 Freiburg, Germany
E-mail: juergen.held@uniklinik-freiburg.de

Introduction

Pneumocystis jirovecii (*carinii*) pneumonia (PJP) is a major cause of mortality in immunocompromised individuals. Therefore, early diagnosis and therapy is vital.

Currently, the laboratory reference standard for the detection of *P. jirovecii* is staining of the organism with monoclonal antibodies, followed by immunofluorescence microscopy in bronchoalveolar lavage (BAL) fluid. Together with a compatible clinical presentation and confirmation by computed tomography, this allows the diagnosis to be made. However, diagnosis of PJP is hampered by the fact that the performance of BAL is not always possible, owing to the limited respiratory function of some patients. Furthermore, detection of *P. jirovecii* is not necessarily equivalent to infection, because of the existence of clinically insignificant *P. jirovecii* colonization [1,2]. Until recently, no reliable serological parameters for the diagnosis of PJP were available.

(1 → 3)-β-D-Glucan (BG) is a cell wall component of *P. jirovecii* and of various other fungi. During the course of an invasive infection, BG is released into the serum. Data collected over recent years have shown that serum BG measurement might be a powerful new tool for the diagnosis of PJP (Table 1). However, in these studies various test systems with different characteristics have been used, and direct comparison of the reported observations is not possible. The Fungitell assay is a *Limulus* amebocyte lysate-based test for the measurement of BG. So far, there have been only two retrospective studies with sizeable numbers of patients (20 and 28, respectively) and a relevant control group that have used the Fungitell assay for the diagnosis of PJP [3,4]. Furthermore, information on the prognostic benefit of BG measurement is limited, and results are inconclusive [5–8].

TABLE 1. Published studies investigating (1 → 3)-β-D-glucan (BG) levels of patients with *Pneumocystis jirovecii* pneumonia

Author, year [Reference]	Number of PJP patients	Immunosuppression of the PJP group	Median BG level (pg/mL)	Number of control patients	Immunosuppression of the control group	Sensitivity	Specificity	Reference method	Test system	Cut-off (pg/mL)
Desmet, 2009 [3]	16	HIV	1496	16	HIV	100	96.4	PCR + IFS/microscopy	Fungitell	100
	12	Non-HIV	3779	12	Non-HIV					
Watanabe, 2009 [8]	111	HIV	175	425	NS	96.4	87.8	Microscopy	Fungitec G	23.2
Del Bono, 2009 [22]	16	Various	423	15	Various	–	–	Clinical diagnosis	Fungitell	80
				11	None					
Nakamura, 2009 [7]	19	HIV	300	24	Bacterial pneumonia	100	NS	Microscopy + PCR	Fungitec G	20
	16	Non-HIV	85.4			88				
Persat, 2008 [4]	16	HIV	945	40	None	100	NS	Microscopy	Fungitell	80
	4	Non-HIV		120	Risk of IFI					
Marty, 2007 [23]	16	Various	>500	None	–	93.8	–	IFS	Fungitell	NS
Tasaka, 2007 [24]	57	Various	NS	222	NS	92.3	86.1	Microscopy	β-Glucan test (WAKO)	31.1
Fujii, 2007 [19]	28	HIV	147	None	–	96.8	–	NS	NS	5
Iikuni, 2006 [6]	21	CTD	87.9	45	Various	77.8	76.9	PCR	NS	NS
Shimizu, 2005 [5]	15	CTD	NS	None	–	86.7	–	Microscopy and/or PCR	Fungitec G	20

CTD, connective tissue disease; HIV, human immunodeficiency virus; IFI, invasive fungal infection; IFS, specific immunofluorescence staining; PJP, *P. jirovecii* pneumonia; NS, not specified.
Only studies with ten or more patients are shown. Three of these studies did not include a negative control group [5,19,23], and another four studies had a control group without immunosuppression or without specifications [7,8,22,24]. One study used clinical presentation only as diagnostic method, without confirming the diagnosis by IFS or PCR [22]. Both Fungitell and Fungitec G are *Limulus* amebocyte lysate-based tests. However, they use extracts from different horseshoe crab species (Fungitell, *Limulus polyphemus*; Fungitec G, *Tachypleus tridentatus*), and the results of the two tests cannot be compared directly, owing to the different reactivities of their factor G proteins.

We therefore conducted a retrospective study on 50 patients with PJP and 50 control patients to evaluate the diagnostic performance of serum BG measurement, with a special focus on its prognostic relevance.

Materials and Methods

PJP patients

During the study period (January 2002 to January 2010), 2287 BAL samples from 1488 patients were examined for *P. jirovecii* (2158 by immunostaining; 129 by nested PCR). Two hundred and sixty-five analyses on samples from 150 patients (10.1%) gave positive results. Seventy-eight of these *P. jirovecii*-positive patients had an archived serum sample around the day of the bronchoscopy (±7 days). Fifty of them (34 with positive immunostaining and positive PCR findings, 12 with positive immunostaining only and four with positive PCR findings only) showed a clinical picture typical of PJP, and were not subject to any of the exclusion criteria. Clinical presentation was considered to be typical if pulmonary infiltrates compatible with PJP were present and if at least four of the following criteria were met: existing immunosuppression, fever, dyspnoea, cough, elevated lactate dehydrogenase (LDH) level and hypoxia. Exclusion criteria were culture of any fungus from relevant materials, positive serum galactomannan assay (Platelia Aspergillus EIA; Bio-Rad Laboratories, GmbH, Munich, Germany) or positive serum *Candida* antigen assay (Cand-Tec; Ramco Laboratories, Inc., Stafford, TX, USA). Colonization with yeast or superficial candidiasis was not considered to be an exclusion criterion, because BG levels are not normally elevated in those patients [9,10].

Quantitative real-time PCR of the four patients who tested positive in PCR only showed very high copy numbers (two with $>10^6$ copies/mL; one with 10^5–10^6 copies/mL; and one with 10^4–10^5 copies/mL). Given the clinical findings, it seemed unlikely that PCR detected only colonization. These 50 patients were included in the PJP group (Table 2). Further analysis of the PJP group showed that 45 patients were admitted to the hospital with acute PJP. The remaining five patients had already been in hospital when they developed PJP. The reason for their initial admittance was intestinal graft-versus-host disease after haematopoietic stem cell transplantation, chemotherapy for B-cell non-Hodgkin lymphoma, methotrexate and corticosteroid therapy for pemphigus vulgaris, and heart transplantation (two patients). All 50 patients were hospitalized after diagnosis of PJP for intravenous antibiotic treatment.

Endpoints of the analysis were death from all causes or discharge. The median length of hospitalization was 25 days

TABLE 2. Baseline characteristics of the study populations and main results

	PJP group	Control group
No. of patients	50	50
Mean age (years) (range)	51 (4–74)	56.5 (6–83)
Sex (male/female)	33/17	25/25
Underlying disease (no. of patients)		
HIV	17	1
Haematological malignancies	10	18
OTX (KTX/HTX/LTX)	11 (6/5/0)	6 (2/0/4)
Immunological disorder	5	10
Haematological malignancies + HSCT	4	12
Solid tumours	3	3
Mean $CD4^+$ T-cells of HIV patients (cells/μL) (range)	37 (3–139)	120
Median $BG_{DIAGNOSIS}$ (pg/mL) (IQR, range)	823 (461–4870, 31–38 400)	27 (8–48, 8–273)
Median BG_{MAX} (pg/mL) (IQR, range)	1153 (461–7444, 31–38 400)	–
Interpretation of BG test		
Positive (>85 pg/mL)	49	3
Negative (≤85 pg/mL)	1	47
Sensitivity (%) (95% CI)	98.0 (89.3–99.7)	
Specificity (%) (95% CI)	94.0 (83.4–98.7)	
Positive predictive value (%) (95% CI)	64.7 (36.6–86.8)	
Negative predictive value (%) (95% CI)	99.8 (95.2–99.0)	
Likelihood ratio, positive (95% CI)	16.33 (15.1–17.7)	
Likelihood ratio, negative (95% CI)	0.021 (0.002–0.2)	

BG, (1 → 3)-β-D-glucan; HIV, human immunodeficiency virus; HSCT, haematopoietic stem cell transplantation; HTX, heart transplantation; IQR, interquartile range; KTX, kidney transplantation; LTX, lung transplantation; OTX, organ transplantation; PJP, *Pneumocystis jirovecii* pneumonia.
The PJP group contains a considerably higher number of HIV-positive patients than the control group. This is because the suspected diagnosis of PJP proved to be true in most cases, and therefore HIV patients with a negative result for *P. jirovecii*-specific immunofluorescence staining were rare.

(interquartile range (IQR) 16–34). The median duration from admission to microbiological diagnosis was 5 days (IQR 2–10). The median duration from microbiological diagnosis to discharge was 21 days (IQR 12–29). Survivors (n = 35) had a median follow-up period of 29 days (IQR 22–43).

Controls

The control group consisted of all patients, between December 2008 and January 2010, whose BAL fluids were negative for *P. jirovecii* in immunostaining (n = 312) and for whom an archived serum sample taken around the day of the bronchoscopy (±7 days) existed (n = 86). Only control patients who were immunocompromised and who had a suspected infectious respiratory disease (pulmonary infiltrates, clinical symptoms, elevated leukocyte count and/or elevated C-reactive protein (CRP)) were included. Exclusion criteria were identical to those for the study group. The collection was stopped when 50 patients had been included (Table 2).

Collection of clinical data

Patient demographics and clinical characteristics were collected, including age, sex, underlying disease, type of immunosuppression, CRP level, leukocyte count, CD4 cell count, LDH level, PaO_2, albumin level, creatinine level, alanine transaminase level, aspartate transaminase level, γ-glutamyltransferase level, PJP prophylaxis and microbiological results. The therapy was reviewed for possible confounding factors for BG measurement, including intravenous immunoglobulin and albumin.

Serum collection and BG measurement

The sera retrospectively tested were drawn around the time of microbiological diagnosis and, if available, until discharge. One or more subsequent sera were available from 34 of the 50 *P. jirovecii*-positive patients. Serum samples were originally taken for various microbiological analyses other than BG, and were routinely frozen at −80°C. The use of these sera was approved by the local ethics committee (application number 105/09). Serum samples were examined for the presence of BG with the Fungitell assay (Associates of Cape Cod, Inc., East Falmouth, MA, USA). The test was performed at our own institution, according to the manufacturer's recommendations. Each serum was tested in duplicate. The persons who tested the sera were not blinded. Samples with BG levels above 500 pg/mL were diluted and retested. BG levels below 31 pg/mL (lower validation limit) were calculated by extrapolation.

Indirect immunofluorescence staining

Monoclonal antibody staining for *P. jirovecii* was performed with the DETECT IF test (Axis Shield Diagnostics Limited, Dundee, UK) according to the manufacturer's recommendations.

Touchdown PCR

DNA from BAL fluids was isolated by proteinase K digestion followed by phenol–chloroform extraction, and serum DNA was extracted with the QIAmp DNA Mini Kit (Qiagen GmbH, Hilden, Germany). Touchdown PCR was performed as previously described [11]. All amplification products were

sequenced and confirmed to be part of the *P. jirovecii* mitochondrial large-subunit rRNA gene.

Real-time PCR

On the basis of a previously published protocol [12], quantitative real-time PCR was performed on DNA preparations from serum and BAL samples. Briefly, 5-μL aliquots were used as template DNA for subsequent PCR testing on a LightCycler (Roche Diagnostics, Mannheim, Germany). Samples positive for the specific amplicons were identified by the PCR instrument at the cycle number where the individual fluorescence value exceeded that measured for background. The quantitative interpretation of the results was assisted by a set of external standards that were tested in parallel.

Statistical methods

Statistical analysis was performed using SPSS, version 17.0. Unless otherwise stated, BG levels are expressed as median concentration with IQR. For comparison of variables, Pearson's chi-square test, the Mann–Whitney *U*-test or the Kruskal–Wallis test was used. Differences were considered significant for $p < 0.05$. Receiver operating characteristic (ROC) analysis was carried out using MedCalc, version 10.0. The optimal BG cut-off was determined with the maximum Youden index.

Results

BAL samples from 1488 patients were examined between January 2002 and January 2010 for the presence of *P. jirovecii*. Fifty *P. jirovecii*-positive patients met the clinical inclusion criteria and were enrolled in the PJP group. Between December 2008 and January 2010, examination for *P. jirovecii* in BAL fluid gave negative findings in 312 patients. Of these, 50 immunosuppressed patients with pneumonia were selected as controls (Table 2).

Serum BG measurement as an indicator for *P. jirovecii* pneumonia

Because the BG cut-off recommended by the manufacturer (80 pg/mL) was originally designed for the diagnosis of invasive fungal infections other than PJP, we first performed a ROC analysis on our data. According to the ROC analysis, the optimal BG cut-off for the diagnosis of PJP, determined with the maximum Youden index, would be >85 pg/mL. The area under the ROC curve was 0.987 (95% CI 0.941–0.998; p 0.0001). With this optimized cut-off, 49 of 50 patients in the PJP group tested positive for serum BG at the time of diagnosis. The negative patient had a serum BG level of 31 pg/mL. The median BG concentration in the PJP group at the time of diagnosis was 823 pg/mL (IQR 461–4870, range 31–38 400). The median highest BG level during hospitalization was 1153 pg/mL (IQR 461–7444, range 31–38 400). In the control group, three patients tested positive (Table 3) and 47 patients were negative for serum BG. The median BG concentration in the control group was 27 pg/mL (IQR 8–48), ranging from non-detectable to 273 pg/mL. The difference in BG levels between the PJP group and the control group was highly significant ($p < 0.001$). On the basis of these results, the sensitivity of the BG assay for the diagnosis of PJP

TABLE 3. Characteristics of false-negative and false-positive patients

	False-negative patient	False-positive patient 1	False-positive patient 2	False-positive patient 3
Age (years)	37	70	73	11
Sex	Female	Female	Male	Female
Underlying disease	Arthritis under corticosteroid therapy	AML with HSCT	AML with HSCT	ALL
Detection of *Pneumocystis jirovecii*	IFS + PCR	Negative	Negative	Negative
$BG_{DIAGNOSIS}$	31	273	177	145
Confounding factors	None	None	Dialysis	None
Other pathogen in BAL fluid	No	No	*Pseudomonas aeruginosa*	RSV
Bacteraemia/viraemia	CMV	No	*Pseudomonas aeruginosa*	No
Mucositis	No	No	No	Yes
Creatinine (mg/L)	0.23	2.70	2.92	0.85
$AST_{DIAGNOSIS}$ (U/L)	Not tested	47	35	554
$ALT_{DIAGNOSIS}$ (U/l)	Not tested	58	20	199
$GGT_{DIAGNOSIS}$ (U/L)	Not tested	218	Not tested	124

ALL, acute lymphoblastic leukaemia; ALT, alanine transaminase; AML, acute myeloid leukaemia; AST, aspartate transaminase; BAL, bronchoalveolar lavage; BG, (1 → 3)-β-D-glucan; CMV, cytomegalovirus; GGT, γ-glutamyltransferase; HSCT, haematopoietic stem cell transplantation; IFS, specific immunofluorescence staining; RSV, respiratory syncytial virus.

The false-negative patient had a proven PJP by standard criteria. Despite high-dose trimethoprim–sulphamethoxazole therapy, the condition of the patient deteriorated, and she died 6 days after admission to the intensive-care unit. In the post-mortem lung biopsy specimen, *P. jirovecii* could still be detected by immunostaining. The reason for the negative BG result is unclear. False-positive patient 2 had a positive blood culture for *Pseudomonas aeruginosa* 11 days after the serum for BG testing was drawn. However, blood cultures taken on the day of serum sampling and 1 day and 2 days after serum sampling were negative for *Pseudomonas aeruginosa*. Although *Pseudomonas aeruginosa* is described as a source of false-positive BG results [25], it seems unlikely that, in this patient, the delayed bacteraemia was responsible for the elevated BG level. Another possible confounding factor in this patient was dialysis. However, the dialysis membranes used at our hospital were tested, and dialysis had no significant effect on BG levels. False-positive patient 3 had a mucositis, and it is possible that BG from the gastrointestinal tract was entering the bloodstream.

TABLE 4. Mortality of *Pneumocystis jirovecii* (*carinii*) pneumonia patients stratified by median, tertile and quartile (1 → 3)-β-D-glucan (BG) levels

	1st	2nd	3rd	4th	p-value
BG measurement at microbiological diagnosis					
Mortality, median (%)	28.0	28.0	–	–	1.000
Mortality, tertiles (%)	31.3	11.8	41.2	–	0.125
Mortality, quartiles (%)	41.7	15.4	33.3	23.1	0.482
Maximum BG levels during hospitalization					
Mortality, median (%)	28.0	28.0	–	–	1.000
Mortality, tertiles (%)	31.3	23.5	29.4	–	0.874
Mortality, quartiles (%)	41.7	15.4	30.8	25.0	0.524

For determination of the prognostic value of BG levels, the patients were divided into groups based on the median, tertile and quartile BG levels. This was performed for the BG levels at diagnosis and for the maximum BG levels during hospitalization. The mortality of the respective groups was then compared with Pearson's chi-square test. There was no significant difference in mortality, and therefore absolute BG levels do not predict the outcome.

was 98.0% (95% CI 89.3–99.7%) and the specificity was 94.0% (95% CI 83.4–98.7%). The positive predictive value and negative predictive value were 64.7% (95% CI 36.6–86.8%) and 99.8% (95% CI 95.2–99.0%), respectively.

BG measurement for earlier confirmation of diagnosis

Sera from 36 patients were available in the 3 weeks prior to diagnosis. The median time of serum sampling before diagnosis was 7 days. Thirty-four of these patients (94%) already had highly elevated BG levels (792 pg/mL, IQR 316–3557, range 122–14 220) at an average of 5 days (IQR 1.8–9.3, range 1–21) before microbiological diagnosis. As expected, the shorter the time interval between sample date and diagnosis date, the higher the serum BG levels. Within the last 10 days before diagnosis, the median BG level was 831 pg/mL (n = 26, IQR 541–4202, range 122–14 220). In the period between day 10 and day 21 before diagnosis, the median BG concentration was 269 pg/mL (n = 8, IQR 154–656, range 127–6460). For the two patients who had negative results, serum samples were drawn at day 19 and day 20 before diagnosis.

Correlation of BG levels with clinical outcome

To determine the prognostic value of BG levels at diagnosis ($BG_{DIAGNOSIS}$) and of maximum BG levels during hospitalization (BG_{MAX}), the patients were divided into groups based on the median, the tertile and the quartile BG levels. The cumulative mortality of the respective groups was then compared. However, no statistically significant differences in mortality were found between the groups (Table 4).

Because the survivor group was very diverse with respect to clinical course, we subdivided them into patients with rapid improvement after the initiation of therapy (n = 18) and patients with a severe disease course (n = 17). Patients were considered to have a severe disease course if there was no clinical improvement within the first 7 days of therapy, if treatment in an intensive-care unit or mechanical ventilation was necessary, if therapy needed to be continued after 21 days, or if treatment failure was suspected and the antifungal regimen had to be changed. Again, the patients were divided on the basis of the median, tertile and quartile BG levels, and the percentages of patients in the groups with a different clinical course were compared. Subdivision of the study population on the basis of clinical course also did not result in significant differences (data not shown).

It has been reported that a number of confounding factors exist that are capable of causing false-positive BG results.

TABLE 5. Clinical characteristics of patients with *Pneumocystis jirovecii* pneumonia

	HIV	Haematological malignancies	Organ transplantation	Immunological disorders	Haematological malignancies + HSCT	Solid tumours
Number of patients	17	10	11	5	4	3
Mean age (years) (range)	41 (27–59)	58 (4–74)	62 (39–68)	71 (38–76)	65 (26–67)	55 (33–58)
Sex (male/female)	11/6	7/3	6/5	4/1	2/2	3/0
Median length of hospitalization (days)	28	25	32	20	13	16
$BG_{DIAGNOSIS}$ (pg/mL) (range)	778 (177–15 430)	554 (142–14 220)	2928 (481–38 400)	507 (31–4820)	662 (199–3300)	628 (527–7300)
BG_{MAX} (pg/mL) (range)	929 (180–31 360)	554 (146–25 600)	9730 (481–38 400)	507 (31–4820)	1349 (199–300)	628 (527–9000)
Median time $period_{BG\text{-}DIAGNOSIS}$ (days) (range)	6 (1–19)	8 (1–13)	2 (1–9)	2	10 (3–21)	6.5 (1–12)
CRP (mg/L) (range)	17 (2–227)	71 (6–331)	32 (6–228)	41 (24–425)	106 (73–188)	17 (9–36)
Leukocyte count (cells/μL) (range)	5.60 (1.7–12.9)	6.50 (3–98.9)	9.55 (3.6–18.3)	10.90 (2.2–14.4)	4.35 (1.5–12.5)	8.00 (4.3–12.0)
$CD4^+$ cells (cells/μL) (range)	37 (3–139)	151	478 (348–677)	456 (129–782)	–	–
LDH (U/L) (range)	374 (173–1186)	499 (248–1493)	555 (354–970)	285 (162–633)	240 (177–714)	582 (213–687)
PaO_2 (mmHg) (range)	49.70 (40–86)	59.10 (27–72)	69.85 (21–88)	71.40 (24–76)	54.45 (50–59)	58.00
$Creatinine_{DIAGNOSIS}$ (mg/dL) (range)	0.70 (0.46–1.10)	0.80 (0.25–1.20)	1.70 (0.50–3.00)	1.20 (0.23–2.95)	1.13 (0.57–1.70)	0.79 (0.75–0.90)
$Creatinine_{MAX}$ (mg/dL) (range)	0.90 (0.66–3.00)	1.02 (0.25–2.1)	2.22 (0.7–4.3)	1.27 (0.23–2.95)	1.39 (0.60–2.91)	0.90 (0.79–1.20)
AST (U/L) (range)	44 (22–368)	61 (18–3002)	58 (25–406)	33 (16–66)	40 (18–61)	36 –
ALT (U/L) (range)	31 (12–295)	59 (23–964)	46 (7–274)	32 (22–41)	26 (23–100)	43 (32–54)
GGT (U/L) (range)	64 (14–421)	164 (25–347)	46 (21–530)	66 (58–162)	112 (25–279)	81 (65–138)

ALT, alanine transaminase; AST, aspartate transaminase; BG, (1 → 3)-β-D-glucan; CRP, C-reactive protein; GGT, γ-glutamyltransferase; HIV, human immunodeficiency virus; HSCT, haematopoietic stem cell transplantation; LDH, lactate dehydrogenase; time $period_{BG\text{-}DIAGNOSIS}$, time period from first positive BG measurements to microbiological confirmation of diagnosis

Organ transplant recipients had significantly higher BG levels and creatinine levels than the other patients.

The major candidates are administration of fractionated blood products (e.g. immunoglobulins and albumin) and haemodialysis with certain cellulose membranes [13–18]. We reviewed the treatment protocols of every patient to identify a possible role of such confounding factors that might distort the analysis. None of our patients had been exposed to any of these confounding factors prior to the day on which the first serum sample was taken, but during the time of their treatment, 12 patients were given immunoglobulins and/or albumin. Surprisingly, median BG_{MAX} did not change when patients with possible confounding factors were excluded (n = 38; BG_{MAX} 1153 pg/mL, IQR 461–7040, range 31–38 400), and there was no significant difference in the cumulative mortality between patients of the different groups.

Correlation of BG levels with clinical characteristics (Table 5)

There was no correlation between age, CRP level, leukocyte count, LDH level, P_{O_2}, albumin, alanine transaminase level, aspartate transaminase level, γ-glutamyltransferase level and BG level. However, high BG levels correlated with high creatinine levels (p 0.012). In contrast to a previous study [7], our data showed no significant difference in BG levels between human immunodeficiency virus (HIV)-positive patients and non-HIV patients. The group of organ transplant recipients, however, showed significantly higher BG_{MAX} levels than the remainder of the patients ($p_{DIAGNOSIS}$ 0.063; p_{MAX} 0.022). We further stratified this group according to the organ that had been transplanted. The group consisted of six kidney and five heart transplant recipients. There was a tendency for there to be higher BG levels in kidney transplant recipients than in heart transplant recipients ($BG_{DIAGNOSIS}$ 2928 pg/mL, IQR 736–13 208 for kidney transplant recipients vs. 897 pg/mL, IQR 669–21 710 for heart transplant recipients; BG_{MAX} 13 415 pg/mL, IQR 1543–19 455 for kidney transplant recipients vs. 5020 pg/mL, IQR 689–22 430 for heart transplant recipients), but the difference was not statistically significant ($p_{DIAGNOSIS}$ 0.93; p_{MAX} 0.54). Inclusion of other clinical parameters in the analysis showed that organ transplant recipients had higher creatinine levels (p 0.003) and LDH levels (p 0.021) than the other patients.

Correlation of BG levels with DNA levels in BAL fluid and sera as detected by PCR

To evaluate whether the *P. jirovecii* burden in the lung correlated with the BG levels measured in the serum, available BAL fluids (n = 38) were tested for *P. jirovecii* DNA by real-time PCR. *P. jirovecii* DNA could be detected in all BAL samples (>10^6 copies/mL in 21 patients, 10^5–10^6 copies/mL in six patients, 10^4–10^5 copies/mL in five patients, 10^3–10^4 copies/mL in four patients, and 10^2–10^3 copies/mL in two patients). However, there was no significant correlation between the quantitative PCR results and serum BG levels ($p_{RealTimePCR}$ 0.766).

Furthermore, sera of all patients (n = 50) at the time of diagnosis as well as at the time of maximum BG levels were tested for *P. jirovecii* DNA by touchdown PCR, and selected sera were additionally tested by quantitative real-time PCR. Each PCR system could detect *P. jirovecii* DNA in a total of three sera (sensitivity of 6%). Two of these sera were positive in both PCR systems, whereas the third serum sample tested positive in one system but not in the other. Neither the levels of BG nor the outcome correlated with serum PCR positivity.

Discussion

We have conducted a retrospective study with the Fungitell assay to evaluate the usefulness of serum BG measurement for the diagnosis of PJP. Our results show an excellent diagnostic performance, with a sensitivity of 98% and a specificity of 94%, and thereby confirm the data of previous studies. The positive predictive value was only 64.7%, possibly because BG is influenced by a number of as yet unknown confounding factors, and because BG is elevated not only in PJP but also in most other invasive fungal infections. As a consequence, BG levels alone cannot prove the existence of PJP, and they must not be interpreted without considering the clinical findings. However, with a negative predictive value of 99.8%, a BG level ≤85 pg/mL almost rules out PJP, and this could be extremely helpful in patients who cannot undergo bronchoscopy or in patients where the clinical suspicion of PJP is low.

Usually, there is a significant delay between the onset of symptoms and the diagnosis of PJP. Median intervals are reported to be 21–28 days in HIV-positive patients [19]. In our study, BG levels were already strongly elevated at an average of 5 days before microbiological diagnosis. For the majority of patients, there was only one pre-existing serum available, and the median of serum sampling was at day 7. Therefore, it is unclear exactly when BG levels exceeded the cut-off. The average time-span of 5 days between BG positivity and microbiological diagnosis is most likely an underestimation caused by a lack of samples dating further back. So far, our data suggest that BG levels are elevated up to 21 days before microbiological diagnosis, and timely BG measurement could allow for the earlier initiation of effective treatment (Fig. 1).

Data on the correlation between BG levels and the outcomes of patients are limited, and the results are inconclu-

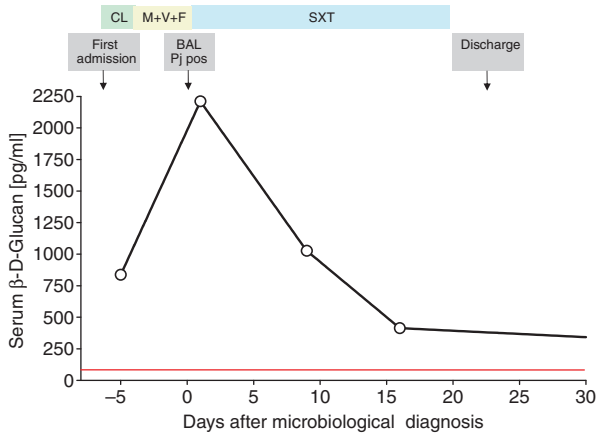

FIG. 1. (1 → 3)-β-D-Glucan (BG) kinetics of a patient with *Pneumocystis jirovecii* (*carinii*) pneumonia in whom timely measurement of BG could have prevented a significant delay in initiation of effective therapy. A 26-year-old female with acute lymphoblastic leukaemia underwent haematopoietic stem cell transplantation in December 2005. On 29 May 2006 (day −5), she presented with fever, dyspnoea and non-productive cough. The patient was on trimethoprim–sulphamethoxazole (SXT) prophylaxis. Chest X-ray showed bilateral infiltrates suggestive of atypical pneumonia. Serum *Aspergillus* galactomannan testing gave negative findings. Therapy with clarithromycin was initiated but showed no effect. The patient was re-admitted to the hospital on day −3. A computed tomography scan showed progression of the infiltrates, and therapy was changed to meropenem, voriconazole and foscavir. Bronchoscopy was performed on day 0, and *P. jirovecii* cysts were detected by specific immunofluorescence and PCR. Intravenous trimethoprim–sulphamethoxazole was started, with good treatment response. The subsequent clinical course was uneventful, and the patient was discharged on day 23. Retrospectively, BG levels were already highly elevated (836 pg/mL) on day −5, and a steady decrease in BG levels reflected a good clinical response. The red line indicates the cut-off (85 pg/mL). BAL, bronchoalveolar lavage; CL, clarithromycin; F, foscavir; M, meropenem; Pj, *P. jirovecii*; V, voriconazole.

sive. Two studies reported that high levels of BG are associated with poor prognosis [5,6], whereas two other studies could not find a significant difference in BG levels between survivors and non-survivors [7,8]. The analysis of our data showed no significant difference in the mortality or severity of PJP between patients with high and those with low BG levels. These findings are supported by our observation that there was no correlation between pulmonary *P. jirovecii* burden, as detected by quantitative real-time PCR, and serum BG levels. However, the results of real-time PCR on BAL fluids are strongly dependent on the quality of the material, and we cannot rule out the possibility that suboptimal sampling affected the data quality.

Although the absolute BG level at diagnosis and the maximum BG level during hospitalization do not seem to have prognostic relevance, this might be different for BG kinetics. It has been shown in individual cases that a favourable outcome can be paralleled by decreasing BG levels [20], an observation that is supported by our own experience. However, further work is clearly necessary to confirm these reports in larger series.

The severity and mortality of PJP, as well as BG levels, have been reported to be lower in HIV-positive patients than in non-HIV patients [19]. We did not observe a significant difference in the BG levels between those two groups of patients. However, subgroup analysis of patients with different underlying diseases showed significantly higher BG levels in organ transplant recipients. At the same time, organ transplant recipients and patients with high BG levels in general showed higher creatinine levels, whereas liver enzyme levels were not elevated. The route of BG elimination is still unknown. In rabbit models, 80% of the organ-associated BG was found in the liver and 10% in the kidney. BG was detected in organs, blood and urine at 23%, 18% and 10%, respectively [21]. Together with the observation that elimination of BG in dialysis patients takes much longer than in healthy subjects, this indicates that renal clearance seems to play some role [15]. However, another study found that the median plasma half-life of BG in dialysis patients was approximately 20 h (range 3.1–181.3 h) and was not affected by renal or hepatic impairment [14]. Therefore, a direct correlation between high BG levels and impaired renal function is hypothetical, and the reason for our observation remains unclear.

In accordance with the findings of previous studies, the diagnostic potential of serum PCR—irrespective of whether touchdown PCR or real-time PCR was used—is poor, and it cannot be recommended for the diagnosis of PJP.

In summary, serum BG levels in patients with PJP are strongly elevated and the negative predictive value is high. Therefore, measurement of BG could be used as a preliminary test for patients with suspected PJP before BAL is performed. Neither the outcome nor the pulmonary *P. jirovecii* burden seems to correlate with the serum BG level at diagnosis or with the peak BG level. Accordingly, the prognostic value of single BG measurements in PJP is low. BG levels start to increase up to 21 days before microbiological diagnosis, and this could be helpful in reducing the time until adequate treatment is initiated.

Acknowledgements

We would like to thank M. Olschewski and D. Huzly for assistance with the statistical analysis, the Department of

Virology, University of Freiburg for providing some of the serum samples, E. Rappolt for extensively searching our serum archive, and G. Häcker, V. Bui and F. von Loewenich for critically reviewing the manuscript. Some of the results were presented at the 61st Annual Meeting of the German Society for Hygiene and Microbiology (DGHM), 22 September 2009, in Göttingen, Germany.

Transparency Declaration

This work was not specially funded. Some of the results were generated during routine diagnostic activities. No commercial relationships or potential conflicts of interest exist.

References

1. Ponce CA, Gallo M, Bustamante R, Vargas SL. *Pneumocystis* colonization is highly prevalent in the autopsied lungs of the general population. *Clin Infect Dis* 2010; 50: 347–353.
2. Morris A, Wei K, Afshar K, Huang L. Epidemiology and clinical significance of *pneumocystis* colonization. *J Infect Dis* 2008; 197: 10–17.
3. Desmet S, Van Wijngaerden E, Maertens J *et al.* Serum (1-3)-beta-D-glucan as a tool for diagnosis of *Pneumocystis jirovecii* pneumonia in patients with human immunodeficiency virus infection or hematological malignancy. *J Clin Microbiol* 2009; 47: 3871–3874.
4. Persat F, Ranque S, Derouin F, Michel-Nguyen A, Picot S, Sulahian A. Contribution of the (1 → 3)-beta-D-glucan assay for diagnosis of invasive fungal infections. *J Clin Microbiol* 2008; 46: 1009–1013.
5. Shimizu A, Oka H, Matsuda T, Ozaki S. (1 → 3)-beta-D glucan is a diagnostic and negative prognostic marker for *Pneumocystis carinii* pneumonia in patients with connective tissue disease. *Clin Exp Rheumatol* 2005; 23: 678–680.
6. Iikuni N, Kitahama M, Ohta S, Okamoto H, Kamatani N, Nishinarita M. Evaluation of *Pneumocystis* pneumonia infection risk factors in patients with connective tissue disease. *Mod Rheumatol* 2006; 16: 282–288.
7. Nakamura H, Tateyama M, Tasato D *et al.* Clinical utility of serum beta-D-glucan and KL-6 levels in *Pneumocystis jirovecii* pneumonia. *Intern Med* 2009; 48: 195–202.
8. Watanabe T, Yasuoka A, Tanuma J *et al.* Serum (1 → 3) beta-D-glucan as a noninvasive adjunct marker for the diagnosis of *Pneumocystis* pneumonia in patients with AIDS. *Clin Infect Dis* 2009; 49: 1128–1131.
9. Yasuoka A, Tachikawa N, Shimada K, Kimura S, Oka S. (1 → 3) beta-D-glucan as a quantitative serological marker for *Pneumocystis carinii* pneumonia. *Clin Diagn Lab Immunol* 1996; 3: 197–199.
10. Obayashi T, Yoshida M, Mori T *et al.* Plasma (1 → 3)-beta-D-glucan measurement in diagnosis of invasive deep mycosis and fungal febrile episodes. *Lancet* 1995; 345: 17–20.
11. Probst M, Ries H, Schmidt-Wieland T, Serr A. Detection of *Pneumocystis carinii* DNA in patients with chronic lung diseases. *Eur J Clin Microbiol Infect Dis* 2000; 19: 644–645.
12. Larsen HH, Masur H, Kovacs JA *et al.* Development and evaluation of a quantitative, touch-down, real-time PCR assay for diagnosing *Pneumocystis carinii* pneumonia. *J Clin Microbiol* 2002; 40: 490–494.
13. Ikemura K, Ikegami K, Shimazu T, Yoshioka T, Sugimoto T. False-positive result in *Limulus* test caused by *Limulus* amebocyte lysate-reactive material in immunoglobulin products. *J Clin Microbiol* 1989; 27: 1965–1968.
14. Kanda H, Kubo K, Hamasaki K *et al.* Influence of various hemodialysis membranes on the plasma (1 → 3)-β-D-glucan level. *Kidney Int* 2001; 60: 319–323.
15. Kato A, Takita T, Furuhashi M, Takahashi T, Maruyama Y, Hishida A. Elevation of blood (1 → 3)-beta-D-glucan concentrations in hemodialysis patients. *Nephron* 2001; 89: 15–19.
16. Ogawa M, Hori H, Niiguchi S, Azuma E, Komada Y. False positive plasma (1 → 3)-β-D-glucan following immunoglobulin product replacement in adult bone marrow recipient. *Int J Hematol* 2004; 80: 97–98.
17. Usami M, Ohata A, Horiuchi T, Nagasawa K, Wakabayashi T, Tanaka S. Positive (1 → 3)-beta-D-glucan in blood components and release of (1 → 3)-beta-D-glucan from depth-type membrane filters for blood processing. *Transfusion* 2002; 42: 1189–1195.
18. Ohata A, Usami M, Horiuchi T, Nagasawa K, Kinoshita K. Release of (1 → 3)-beta-D-glucan from depth-type membrane filters and their *in vitro* effects on proinflammatory cytokine production. *Artif Organs* 2003; 27: 728–735.
19. Fujii T, Nakamura T, Iwamoto A. *Pneumocystis* pneumonia in patients with HIV infection: clinical manifestations, laboratory findings, and radiological features. *J Infect Chemother* 2007; 13: 1–7.
20. Cuétara MS, Alhambra A, Chaves F, Moragues MD, Pontón J, del Palacio A. Use of a serum (1 → 3)-beta-D-glucan assay for diagnosis and follow-up of *Pneumocystis jiroveci* pneumonia. *Clin Infect Dis* 2008; 47: 1364–1366.
21. Yoshida M, Roth RI, Grunfeld C, Feingold KR, Levin J. Soluble (1 → 3)-beta-D-glucan purified from *Candida albicans*: biologic effects and distribution in blood and organs in rabbits. *J Lab Clin Med* 1996; 128: 103–114.
22. Del Bono V, Mularoni A, Furfaro E *et al.* Clinical evaluation of a (1 → 3)-beta-D-glucan assay for presumptive diagnosis of *Pneumocystis jiroveci* pneumonia in immunocompromised patients. *Clin Vaccine Immunol* 2009; 16: 1524–1526.
23. Marty FM, Koo S, Bryar J, Baden LR. (1 → 3)-beta-D-glucan assay positivity in patients with *Pneumocystis (carinii) jiroveci* pneumonia. *Ann Intern Med* 2007; 147: 70–72.
24. Tasaka S, Hasegawa N, Kobayashi S *et al.* Serum indicators for the diagnosis of pneumocystis pneumonia. *Chest* 2007; 131: 1173–1180.
25. Mennink-Kersten MA, Ruegebrink D, Verweij PE. *Pseudomonas aeruginosa* as a cause of 1,3-beta-D-glucan assay reactivity. *Clin Infect Dis* 2008; 46: 1930–1931.