

# Diagnostic evaluation of serum (1, 3)-β-D glucan levels using the Fungitec G-Test MK kit for *Pneumocystis jirovecii* pneumonia (PCP) in non-HIV patients

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Keyword:	(1, 3)-β-D glucan, Fungitec G-Test MK, methotrexate, HIV-negative patients, <i>Pneumocystis jirovecii</i> pneumonia
Abstract:	<i>Pneumocystis jirovecii</i> pneumonia (PCP) is an opportunistic and life- threatening pulmonary infection with an increasing prevalence among individuals who are human immunodeficiency virus (HIV)-negative. Evidence regarding diagnostic testing of PCP in this patient population is

insufficient. We evaluated the performance of serum (1, 3)- $\beta$ -D glucan (BDG) using the Fungitec G-test MK kit for diagnosing PCP in non-HIV patients. We retrospectively analyzed data from 219 non-HIV adult patients who underwent bronchoscopy and were tested for P. jirovecii DNA by PCR using lavage samples from the lower respiratory tract. Fifty PCP patients and 125 non-PCP patients were included. The most common underlying diseases were malignancies and systemic autoimmune diseases. Using the serum BDG Fungitec G-test MK test to diagnose PCP, the area under the receiver operating characteristic curve (AUC) was 0.924, while the modified cut-off value of 36.6 pg/mL had a sensitivity and specificity of 92.0% and 84.8%, respectively. The AUC for patients with systemic autoimmune diseases was 0.873, and the accuracy of serum BDG test declined when using methotrexate (MTX). In conclusion, the serum BDG test was useful for diagnosing PCP in non-HIV patients; however, the results should be carefully interpreted in case of MTX administration.

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1	Diagnostic evaluation of serum (1, 3)- $\beta$ -D glucan levels using the Fungitec G-Test MK kit
2	for Pneumocystis jirovecii pneumonia (PCP) in non-HIV patients
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4	Short title: BDG evaluation for PCP in non-HIV patients
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31 Abstract:

32	Pneumocystis jirovecii pneumonia (PCP) is an opportunistic and life-threatening pulmonary
33	infection with an increasing prevalence among individuals who are human
34	immunodeficiency virus (HIV)-negative. Evidence regarding diagnostic testing of PCP in
35	this patient population is insufficient. We evaluated the performance of serum $(1, 3)$ - $\beta$ -D
36	glucan (BDG) using the Fungitec G-test MK kit for diagnosing PCP in non-HIV patients.
37	We retrospectively analyzed data from 219 non-HIV adult patients who underwent
38	bronchoscopy and were tested for <i>P. jirovecii</i> DNA by PCR using lavage samples from the
39	lower respiratory tract. Fifty PCP patients and 125 non-PCP patients were included. The most
40	common underlying diseases were malignancies and systemic autoimmune diseases. Using
41	the serum BDG Fungitec G-test MK test to diagnose PCP, the area under the receiver
42	operating characteristic curve (AUC) was 0.924, while the modified cut-off value of 36.6
43	pg/mL had a sensitivity and specificity of 92.0% and 84.8%, respectively. The AUC for
44	patients with systemic autoimmune diseases was 0.873, and the accuracy of serum BDG test
45	declined when using methotrexate (MTX). In conclusion, the serum BDG test was useful for

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6 7	46	diagnosing PCP in non-HIV patients; however, the results should be carefully interpreted in
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10 11	47	case of MTX administration.
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13 14	48	
15 16 17	49	Lay summary
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19 20 21	50	The Fungitec G-test MK kit for measuring serum (1, 3)- $\beta$ -D glucan (BDG) levels had a
22 23	51	sufficient diagnostic performance for Pneumocystis jirovecii pneumonia (PCP) in human
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26	52	immunodeficiency virus-negative patients. However, the results should be carefully
27 28	02	minimulationereney virus negative parents. Hervever, the results should be easerably
29 30 31	53	interpreted in case of MTX administration.
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# 58 Introduction

59	Pneumocystis jirovecii pneumonia among patients not infected with human
60	immunodeficiency virus (non-HIV-PCP) is a life-threatening pulmonary infectious disease.
61	Its prevalence has recently increased, especially in patients with transplants, hematologic
62	diseases, malignancies, systemic autoimmune diseases, and lung diseases, and those
63	undergoing dialysis. <sup>1</sup> The definitive diagnosis of PCP includes detection of the organism
64	microscopically in tissue, bronchoalveolar lavage (BAL) fluid, expectorated sputum using
65	conventional or immunofluorescence staining, <sup>2</sup> though PCP cysts are relatively few among
66	non-HIV patients compared to HIV patients, and detecting cysts by microscopy has poor
67	sensitivity. <sup>3, 4</sup> Therefore, testing for <i>P. jirovecii</i> DNA by polymerase chain reaction ( <i>Pj</i> -PCR)
68	using bronchial washing (BW) or BAL samples has recently been used more frequently for
69	non-HIV-PCP diagnosis. Moreover, numerous patients who cannot undergo bronchoscopy
70	are clinically diagnosed with PCP based on their backgrounds, symptoms, and chest
71	radiography findings; therefore, less-invasive diagnostic testing for non-HIV-PCP is required.
72	(1, 3)- $\beta$ -D glucan (BDG) is one of the common components of the fungal cell wall,
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73	and is widely used as a biomarker for invasive fungal diseases. <sup>5, 6</sup> Serum BDG testing can be
74	useful for PCP diagnosis in HIV patients, with a pooled sensitivity and specificity of 95–96%
75	and 84–86%, respectively. <sup>7, 8</sup> However, studies <sup>7, 8</sup> using several different BDG test kits,
76	including the Fungitell assay (Associates of Cape Cod, USA), the Fungitec G-test MK
77	(Seikagaku Corp, Japan until 2012, Nissui Pharmaceutical Co., Ltd., Japan after 2012), and
78	the Wako $\beta$ -glucan test (Wako Pure Chemical Industries Ltd, Japan)—all of which have
79	independent cut-off values for diagnosis—and the information on non-HIV-PCP patients is
80	limited. As both HIV and non-HIV-PCP's clinical features and the number of detected cysts
81	by microscopy greatly differ, <sup>3, 9</sup> the respective cut-off values for serum BDG need to be
82	determined; therefore, it is necessary to evaluate the usefulness for each assay kit. The
83	Fungitell assay was approved by the U.S. Food and Drug Administration (FDA) in 2004 and
84	became CE marked in 2008; the Wako $\beta$ -glucan test was CE marked in 2018. Recently, these
85	assays have been reevaluated for the diagnosis of PCP in patients including non-HIV adults. <sup>4,</sup>
86	<sup>10, 11</sup> Conversely, to the best of our knowledge, no study has evaluated PCP diagnosis using
87	the Fungitec G-test MK in non-HIV-PCP patients. <sup>12</sup> In this study, we aimed to evaluate the

usefulness and cut-off value of serum BDG levels using the Fungitec G-test MK for the diagnosis of non-HIV-PCP. Moreover, we assessed the underlying diseases in non-HIV-PCP patients and assessed the utility of measuring serum BDG levels for PCP diagnosis in terms of each underlying disease. Finally, we determined the factors resulting in false-positive occ per serum BDG results. **Methods** Patients and study design We conducted this retrospective cohort study at the Nagasaki University Hospital, a tertiary care and teaching hospital in Japan, between April 2009 and August 2020. This study included only adult patients ( $\geq 20$  years) with unexplained lung infiltrations and clinically suspected PCP who underwent bronchoscopy and were tested with Pj-PCR using BW or BAL fluid. Patients with HIV, as well as invasive fungal infections other than PCP, or those who received empirical treatment for PCP lasting more than 3 days before Pj-PCR testing were excluded. Patient characteristics (age, sex, underlying disease, and immunosuppressive

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5 6 7	103	medications) and laboratory data— including peripheral absolute lymphocyte counts (ALC),
8 9 10	104	serum lactate dehydrogenase (LDH), Krebs von den Lungen-6, galactomannan, and BDG
11 12 13	105	levels— were extracted from electronic medical records. Serum BDG values—measured on
14 15 16		
17 18 19	106	the closest date from the date of bronchoscopy—were used. Furthermore, we investigated
20 21 22	107	patients with bacteremia in intensive care units, those treated with glucan-containing gauze,
23 24 25	108	albumin products, immunoglobulin products, and those undergoing hemodialysis with
26 27 28	109	cellulose-containing filters—all of which were previously reported as false positive factors—
29 30 31	110	to analyze potential factors contributing to false-positive serum BDG results. <sup>13-15</sup> Regarding
32 33 34 35	111	albumin and immunoglobulin products, we included patients who had been treated within 1
36 37 38	112	week of BDG measurement. This study was conducted in compliance with the Declaration
39 40 41	113	of Helsinki and approved by the Ethics Committee of the institution (Nagasaki University
42 43 44	114	Hospital, Approval Number: 19102128). Patient consent was waived due to the retrospective
45 46 47 48	115	and anonymous nature of the study.
49 50 51	116	Definition of PCP diagnosis
52 53 54	117	A PCP diagnosis was determined by the presence of all of the following:
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118	compatible immunocompromising factors (any transplant, hematologic disease, cancer,
119	immunosuppressive drug administration, and primary immunodeficiencies); acute
120	respiratory signs or symptoms (shortness of breath, dry or productive cough, and increased
121	O <sub>2</sub> requirement); newly appearing ground-glass opacities in bilateral lungs on chest
122	computed tomography; and microscopic detection of the organism (proven PCP) or positive
123	<i>P. jirovecii</i> DNA by PCR (probable PCP) in BW or BAL fluid. <sup>2</sup> <i>Pj</i> -PCR was conducted by
124	SRL Inc. (Tokyo, Japan) using previously reported methods <sup>16</sup> in which the exon 45 region
125	of the dystrophin gene was used as an internal control to evaluate PCR inhibition. Patients
126	who did not meet the above criteria were classified as non-PCP, and those whose condition
127	improved without treatment for PCP- despite meeting the above criteria- were also
128	included in the non-PCP group.
129	BDG assay kit
130	In Japan, the Fungitec G-test MK and the Wako $\beta$ -glucan test are available as BDG
131	assay kits. Here, we used a previously reported manipulation: the Fungitec G-test MK, which
132	has a manufacturer-recommended cut-off value of 20 pg/ml for invasive fungal infections. <sup>17,</sup>

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9 10 11	134	Statistical analysis
12 13 14 15	135	The difference in the distribution of values between the participants' groups was
16 17 18	136	evaluated by a Mann–Whitney U statistic. The p-values for the U statistic was calculated
19 20 21	137	from its normal approximation. The null hypothesis of independence between two binomial
22 23 24 25	138	variables was tested by a two-sided Fisher's exact test. A p-value of <0.05 was stated as
26 27 28	139	statistically significant. The multiplicity of hypothesis testing was not considered unless
29 30 31	140	otherwise stated. Statistical analyses were exploratorily conducted; therefore, the type 1 error
32 33 34 35	141	rates were not under nominal p-values.
36 37 38	142	The cut-off value in serum BDG levels for PCP diagnosis was determined by the
39 40 41	143	Youden's index. The concordance between the serum BDG levels and the PCP diagnosis was
42 43 44 45	144	evaluated as the area under the receiver-operating-characteristic (ROC) curve (AUC). Test
46 47 48	145	accuracy was assessed regarding sensitivity, specificity, and both positive and negative
49 50 51	146	likelihood ratios (positive LR and negative LR, respectively). Based on the interest to identify
52 53 54 55	147	the disruptor in PCP diagnosis by serum BDG level, we evaluated the "degree of shift in the
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14	8 accuracy" in the diagnosis by selecting participants based on a clinical factor— that is— a
14	9 candidate of a disruptor. The "degree of shift in the accuracy" was defined as a subtract of a
15	0 log-transformed odds ratio on the total population $(OR_{total})$ from one of the selected
15	subpopulations $(OR_{sub})$ (equivalent to the logarithm of a ratio of the odds ratios [ <i>eq.1]</i> ). The
15	2 effect on shifting the accuracy of selection of the subjects was evaluated as the rank of the
15	3 ratio calculated from the observed data, among its null distribution generated by 2000
15	4 permutations in the labels of the clinical factor used in the selection of the subjects.
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15	7 Cary, NC, USA) and the R environment version 4.1.1. <sup>19</sup>
15	8
15	9 Results
16	0 Eligible patients
16	Among the 219 patients who underwent bronchoscopy and were tested using <i>Pj</i> -
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5 6 7 8	162	PCR during the study period, 175 were included in the study (Fig. 1). Of the 50 patients
9 10 11	163	diagnosed with PCP, 12% (n = 6) were diagnosed with proven PCP by identification of $P$ .
12 13 14 15	164	<i>jirovecii</i> cysts, and 88% (n = 44) had probable PCP diagnosed using $Pj$ -PCR as per
16 17 18	165	EORTC/MSGERC case definitions. <sup>2</sup>
19 20 21	166	Patient characteristics
22 23 24 25	167	The baseline characteristics of the patients are shown in Table 1. The most common
26 27 28	168	underlying diseases were malignancies, followed by systemic autoimmune diseases. Among
29 30 31	169	chronic lung diseases, interstitial lung disease and asthma were found in 24 (14%) and 4 (2%)
32 33 34 35	170	out of 175 patients, respectively (asthma was not included in Table 1), with no case of chronic
36 37 38	171	obstructive pulmonary disease. Additionally, there was only one case of bacteremia in each
39 40 41	172	group. Patients who were administered systemic prednisolone (PSL) ≥5 mg or methotrexate
42 43 44 45	173	(MTX) were more frequent in the PCP than in the non-PCP group. PCP prophylaxis was only
46 47 48	174	administered in the non-PCP group. All prophylactic drugs were
49 50 51	175	trimethoprim/sulfamethoxazole (TMP/SMX). No patients were treated with glucan-
52 53 54 55 56	176	containing gauze or were undergoing hemodialysis with cellulose-containing filters, both of
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which could have affected the BDG results. Laboratory test results showed that the patients in the PCP group had significantly lower ALC and higher serum LDH and BDG than the those in the non-PCP group. **BDG** values The Fungitec G-test MK results of the BDG values for the PCP and non-PCP groups are shown in Fig. 2A. The median (interquartile range) serum BDG values of both PCP and non-PCP patients were 146.9 (67.7–441.1) pg/ml and 11.5 (6.0–23.6) pg/ml, respectively (p < 0.01). In the group of patients who used albumin or immunoglobulin products, the median (interquartile range) serum BDG values of both PCP and non-PCP patients were 252.2 (78.3–550.4) pg/ml and 17.9 (8.2–34.4) pg/ml, respectively (p = 0.01) (Fig. 2B). Accuracy of PCP diagnosis using the serum BDG level The concordance of the serum BDG level for PCP diagnosis is shown as the ROC curve in Fig. 2C. These two variables were highly concordant at the AUC of 0.924. The modified cut-off value by the Youden's index was 36.6 pg/ml, and the comparison of

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5 6 7 8	192	accuracy (sensitivity, specificity, etc.) with the manufacturer's defined cutoff value of 20.0
9 10 11	193	pg/ml is summarized in Table 2.
12 13 14 15	194	Moreover, to examine the characteristics of the association of the serum BDG level
16 17 18	195	with the diagnosis of PCP in patients with underlying diseases, we extracted patients with
19 20 21	196	malignancies and systemic autoimmune diseases including rheumatoid arthritis (RA),
22 23 24 25	197	respectively, which were the two most prevalent underlying diseases among those observed.
26 27 28	198	In patients with malignancies, the concordance between the serum BDG level and the
29 30 31	199	diagnosis of PCP was high (AUC = 0.940; Supplementary Figure S1a) in all cases. Sensitivity
32 33 34 35	200	and specificity were 100% and 81.2%, respectively, at the cut-off value of 25.8 pg/ml. In
36 37 38	201	patients with systemic autoimmune diseases, the AUC of BDG for diagnosing PCP was 0.873
39 40 41	202	(Supplementary Figure S1b), and the sensitivity and specificity were 81.8% and 88.1%,
42 43 44 45	203	respectively, at the cut-off value of 56.5 pg/ml.
46 47 48	204	Factors that reduce the accuracy of BDG testing
49 50 51	205	Aiming to identify disruptors in PCP diagnosis by serum BDG level using Fungitec
52 53 54 55	206	G-test MK, we explored subpopulations in which the diagnostic accuracy of the serum BDG
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207	test (cut-off value: 36.6 pg/ml) was declined compared with that of the total cases, using the
208	"degree of shift in the accuracy" (detailed in the section of statistical analysis). On some of
209	the clinical factors, the "degree of shift in the accuracy" was not finite because of the
210	complete separation in some trials of the 2000 permutations. We found that the degree of a
211	negative shift in the accuracy was seen in patients using PSL $\geq$ 5 mg or MTX, and the shift
212	was more evident in MTX-treated patients (Figure 3). We further analyzed the association of
213	the higher dose of MTX ( $\geq 10$ mg/week vs. <10 mg/week) with the more frequently erroneous
214	diagnosis based on the serum BDG test with the cut-off value (36.6 pg/ml). As a result, a
215	significant relationship was not observed (the odds ratio [the 95%CI] of the dose of MTX
216	$\geq$ 10 mg/week for the erroneous diagnosis =1.55, the 95%CI:[ 0.25 to 9.76]). The results for
217	the analysis of other factors are provided as supplemental information (Supplementary Figure
218	S2.).
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220	Discussion
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6 7 8	221	We evaluated the diagnostic utility of serum BDG levels for non-HIV-PCP using
9 10 11 12	222	the Fungitec G-test MK assay. Patients with PCP had a significantly higher BDG value
13 14 15	223	than those without PCP. The best cut-off value of serum BDG to distinguish non-HIV-PCP
16 17 18	224	was 36.6 pg/ml, showing comparable negative LR and higher positive LR compared to the
19 20 21	225	manufacturer's recommended cut-off value of 20.0 pg/ml.
22 23		
24 25 26	226	To the best of our knowledge, this is the first large-scale study aiming to evaluate
27         28         29         30         31         32         33         34         35         36         37         38         39         40         41         42         43         44         45         46         47         48         49         50         51         52         53	227	serum BDG levels in the diagnosis of PCP limited to non-HIV patients. Previous studies
	228	included a mixed sample of both HIV and non-HIV patients, and the diagnosis of PCP
	229	required confirmation by cyst visualization using microscopy; <sup>7, 8</sup> these diagnostic criteria
	230	might miss many non-HIV-PCP cases, as only 12% of PCP patients in this study the
	231	organism detected microscopically.
	232	Previous studies that evaluated the accuracy of the diagnosis of serum BDG for
	233	non-HIV-PCP included patients with malignancy in a high proportion. <sup>11, 20</sup> A large-scale
	234	study that recently evaluated the serum BDG test using the Fungitell assay for the diagnosis
54 55 56	235	of PCP in cancer patients <sup>4</sup> found that most non-HIV PCP patients either had malignancies
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236	or systemic autoimmune diseases. The number of non-HIV-PCP patients with these
237	underlying diseases has been increasing; <sup>1</sup> the colonization rate of <i>P. jirovecii</i> among
238	outpatients both during cancer chemotherapy and among patients with RA during
239	immunosuppressive therapy was reported to be high. <sup>21, 22</sup> In our study, 54% of non-HIV-
240	PCP patients had malignancies or systemic autoimmune diseases. The accuracy of serum
241	BDG test to diagnose non-HIV-PCP in each of these group was sufficient to use in clinical
242	practice by the subgroup analysis (Supplementary Figure S1), and we believe that this is the
243	first study to evaluate the accuracy of serum BDG test for PCP diagnosis focusing on
244	systemic autoimmune diseases. However, the BDG AUC for PCP diagnosis was lower,
245	with a higher cut-off value in patients with systemic autoimmune diseases compared to
246	patients with malignancies, thus suggesting that patients with systemic autoimmune
247	diseases potentially possess factors that negatively affect BDG test's diagnostic accuracy.
248	The declining effect on the accuracy of the BDG test of selecting the patients,
249	especially using MTX, was observed. In the PCP group, four patients had BDG values
250	below the modified cut-off value, of which two had extremely low BDG values (Figure
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6 7 8	251	2A). One of the two patients responded poorly to PCP treatment with TMP/SMX and was
9 10 11	252	later diagnosed with Mycobacterium avium complex lung infection, potentially ruling-out
12 13 14 15	253	PCP; the other patient showed elevated levels of BDG—above the cut-off value of 36.6
16 17 18	254	pg/ml a week after the initial test. In this study, BDG was tested multiple times within 1
19 20 21	255	week in 33 patients (66%) in the PCP group, with 12 patients showing an elevated BDG
22 23 24 25	256	value a week later. This suggested that the timing of the BDG test may have affected the
26 27 28	257	results. Moreover, two out of four patients in the PCP group whose BDG values were
29 30 31	258	below the cut-off value were using MTX. Small amounts of <i>P. jirovecii</i> may trigger lung
32 33 34 35	259	inflammation in patients with RA undergoing MTX treatment, <sup>23</sup> suggesting that patients
36 37 38	260	with PCP using MTX could have a false-negative BDG test result. Conversely, 15.2% of
39 40 41	261	patients in the non-PCP group had false-positive BDG results. Among non-PCP patients,
42 43 44	262	increased cut-off value of serum BDG (36.6 pg/ml), i.e. false-positive result of PCP
45 46 47 48	263	diagnosis, was associated with MTX use (Supplementary Table S1). MTX is a risk factor
49 50 51	264	for PCP. <sup>24, 25</sup> Further, it is difficult to clinically differentiate between RA-related interstitial
52 53 54 55 56	265	pneumonia (IP), MTX-related IP, and PCP. We found four patients who completed PCP
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266	treatment within 2 or 3 weeks, despite negative Pj-PCR results; these patients tended to
267	have high BDG values (Supplementary Table S2). A possible factor of false positives for
268	BDG was that these patients might have had undetected conditions that affected their BDG
269	values, despite the absence of any history, such as bacteremia, use of albumin or
270	immunoglobulin products, or ICU admission. These other factors could rarely affect false-
271	negative <i>Pj</i> -PCR results. <sup>26</sup> Differentiating true PCP in patients using MTX remains a future
272	challenge.
273	This study has several limitations. First, our sample of 50 PCP patients was
274	modest; this single-center study only included patients who had undergone bronchoscopy.
275	In a clinical setting, BDG is important either for the diagnosis or exclusion of PCP,
276	especially in patients who cannot undergo bronchoscopy. Second, we used conventional
277	PCR to diagnose PCP. Although amplification of <i>P. jirovecii</i> DNA by quantitative real-
278	time PCR (qPCR) is preferred to conventional qualitative PCR, <sup>2</sup> in Japan, qPCR use for
279	PCP is limited, and our outsourced manufacturer handles only conventional PCR. This may
280	have affected the diagnosis of PCP. Third, we did not fully evaluate the pre-test probability
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6 7 8	281	of PCP due to the retrospective nature of the study. Finally, BDG is found in various foods,
9 10 11	282	thus making it impossible to evaluate patient intake of potential BDG sources, <sup>27</sup> as their
12 13	283	consumption may cause BDG false-positives.
14 15 16		
17 18 19	284	Overall, our study showed that optimizing the serum BDG cut-off value using the
20 21 22 23	285	Fungitec G-test MK assay increased the positive LR without compromising the good
24 25 26	286	negative LR for diagnosing PCP in non-HIV patients. MTX could negatively affect the
27 28 29	287	accuracy of the BDG test for diagnosing PCP, and we should be aware of the BDG results
30 31 32	288	when PCP is suspected in patients using MTX. Further research is warranted to compare
33 34 35 36	289	this assay with other BDG tests and, more importantly, a prospective study is expected both
37 38 39 40	290	for calibration and validation of the cut-off value.
41 42 43 44	291	
45 46 47 48	292	Acknowledgments: We thank SRL, Inc. for providing the necessary data.
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6 7 8	294	Funding: This work was supported by the 2019 Japan Rheumatism Foundation Grant to K.
9 10 11	295	Yamamoto.
12 13 14 15 16	296	
17 18 19	297	References
20 21 22 23	298	1 Maini R, Henderson KL, Sheridan EA, et al. Increasing pneumocystis
24 25 26	299	pneumonia, England, UK, 2000-2010. Emerg Infect Dis. 2013; 19: 386–392.
27 28 29 30	300	2 Donnelly JP, Chen SC, Kauffman CA, et al. Revision and update of
31 32 33	301	the consensus definitions of invasive fungal disease from the European
34 35 36	302	Organization for Research and Treatment of Cancer and the Mycoses Study
37 38 39 40	303	Group Education and Research Consortium. Clin Infect Dis. 2020; 71: 1367–
40 41 42 43	304	1376.
44 45 46	305	3 Limper AH, Offord KP, Smith TF, Martin WJ, 2nd. Pneumocystis
47 48 49 50	306	carinii pneumonia. Differences in lung parasite number and inflammation in
50 51 52 53 54 55	307	patients with and without AIDS. <i>Am Rev Respir Dis</i> . 1989; 140: 1204–1209.
56 57 58		21
59 60		http://mc.manuscriptcentral.com/tmmy

1		22
2 3 4		
5 6 7 8	308	4 Morjaria S, Frame J, Franco-Garcia A, Geyer A, Kamboj M, Babady
9 10 11	309	NE. Clinical Performance of (1,3) Beta-D-Glucan for the diagnosis of
12 13 14	310	Pneumocystis pneumonia in cancer patients tested with PCP PCR. Clin Infect
15 16 17 18	311	<i>Dis</i> . 2019; 69: 1303–1309.
19 20 21	312	5 McCarthy MW, Petraitiene R, Walsh TJ. Translational development
22 23 24	313	and application of (1>3)-beta-d-glucan for diagnosis and therapeutic
25 26 27 28	314	monitoring of invasive mycoses. Int J Mol Sci. 2017; 18: 1124.
29 30 31 32 33 34	315	6 Farhour Z, Mehraj V, Chen J, Ramendra R, Lu H, Routy JP. Use of
	316	(1>3)-beta-d-glucan for diagnosis and management of invasive mycoses
35 36 37 38	317	in HIV-infected patients. <i>Mycoses</i> . 2018; 61: 718–722.
39 40 41	318	7 Karageorgopoulos DE, Qu JM, Korbila IP, Zhu YG, Vasileiou VA,
42 43 44	319	Falagas ME. Accuracy of beta-D-glucan for the diagnosis of Pneumocystis
45 46 47 48	320	jirovecii pneumonia: a meta-analysis. Clin Microbiol Infect. 2013; 19: 39–
49 50 51	321	49.
52 53 54	322	8 Onishi A, Sugiyama D, Kogata Y, et al. Diagnostic accuracy of serum
55 56 57 58		
59 60		22 http://mc.manuscriptcentral.com/tmmy

1 2

2 3 4		
5 6 7 8	323	1,3-beta-D-glucan for pneumocystis jiroveci pneumonia, invasive
9 10 11	324	candidiasis, and invasive aspergillosis: systematic review and meta-analysis.
12 13 14 15	325	J Clin Microbiol. 2012; 50: 7–15.
16 17 18	326	9 Kato H, Samukawa S, Takahashi H, Nakajima H. Diagnosis and
19 20 21	327	treatment of Pneumocystis jirovecii pneumonia in HIV-infected or non-HIV-
22 23 24 25	328	infected patients-difficulties in diagnosis and adverse effects of
26 27	329	trimethoprim-sulfamethoxazole. J Infect Chemother. 2019; 25: 920–924.
28 29 30 31 32 33 34 35	330	10 Dichtl K, Seybold U, Wagener J. Evaluation of a turbidimetric beta-
	331	d-glucan test for detection of Pneumocystis jirovecii pneumonia. J Clin
36 37 38	332	Microbiol. 2018; 56: e00286-18.
39 40 41	333	11 Mercier T, Guldentops E, Patteet S, Beuselinck K, Lagrou K,
42 43 44 45	334	Maertens J. Beta-d-glucan for diagnosing Pneumocystis pneumonia: a direct
46 47 48	335	comparison between the Wako beta-glucan assay and the Fungitell assay. $J$
49 50 51	336	Clin Microbiol. 2019; 57: e00322-19.
52 53 54 55 56	337	12 Watanabe T, Yasuoka A, Tanuma J, et al. Serum (1>3) beta-D-
57 58 59		23
60		http://mc.manuscriptcentral.com/tmmy

1		24
2 3 4		
5 6 7 8 9 10 11 12 13 14 15 16	338	glucan as a noninvasive adjunct marker for the diagnosis of Pneumocystis
	339	pneumonia in patients with AIDS. Clin Infect Dis. 2009; 49: 1128-1131.
13 14	340	13 Digby J, Kalbfleisch J, Glenn A, Larsen A, Browder W, Williams D.
	341	Serum glucan levels are not specific for presence of fungal infections in
16 17 18 19 20 21 22 23 24 25 26 27 28	342	intensive care unit patients. Clin Diagn Lab Immunol. 2003; 10: 882–885.
23 24	343	14 Marty FM, Koo S. Role of (1>3)-beta-D-glucan in the diagnosis of
26 27	344	invasive aspergillosis. Med Mycol. 2009; 47 Suppl 1: S233–40.
29 30 31	345	15 Acosta J, Catalan M, del Palacio-Perez-Medel A, et al. Prospective
32 33 34	346	study in critically ill non-neutropenic patients: diagnostic potential of (1,3)-
35 36 37 38	347	beta-D-glucan assay and circulating galactomannan for the diagnosis of
39 40 41	348	invasive fungal disease. Eur J Clin Microbiol Infect Dis. 2012; 31: 721–731.
42 43 44	349	16 Wakefield A, Pixley F, Banerji S, Sinclair K, Miller R, Moxon E, Hopkin
45 46 47 48	350	J. Detection of Pneumocystis carinii with DNA amplification. Lancet. 1990;
49 50 51	351	336: 451–453.
52 53 54 55	352	17 Obayashi T, Yoshida M, Mori T, et al. Plasma (1>3)-beta-D-glucan
56 57 58		24
59 60		http://mc.manuscriptcentral.com/tmmy

Page 26 of 75

1 2 3		23
4 5		
6 7 8	353	measurement in diagnosis of invasive deep mycosis and fungal febrile
9 10 11	354	episodes. <i>Lancet</i> . 1995; 345: 17–20.
12 13 14	355	18 Yasuoka A, Tachikawa N, Shimada K, Kimura S, Oka S. (1>3)
15 16 17 18 19 20 21	356	beta-D-glucan as a quantitative serological marker for Pneumocystis carinii
	357	pneumonia. Clin Diagn Lab Immunol. 1996; 3: 197–199.
22 23 24	358	19 Team RC. R: A Language and Environment for Statistical Computing.
25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41	359	R Foundation for Statistical Computing. 2020.
	360	20 de Boer MGJ, Gelinck LBS, van Zelst BD, et al. $\beta$ -d-Glucan and S-
	361	adenosylmethionine serum levels for the diagnosis of Pneumocystis
	362	pneumonia in HIV-negative patients: a prospective study. J Infect. 2011;
	363	62: 93–100.
42 43 44	364	21 Takemoto S, Ebara M, Hasebe S, Yakushijin Y. A study on the
45 46 47 48	365	colonization of Pneumocystis jirovecii among outpatients during cancer
49 50 51	366	chemotherapy and among healthy smokers. J Infect Chemother. 2017; 23:
52 53 54	367	752–756.
55 56 57		
58 59 60		25 http://mc.manuscriptcentral.com/tmmy

1		26
2 3		
4 5		
6 7 8	368	22 Mori S, Cho I, Sugimoto M. A followup study of asymptomatic
9 10 11	369	carriers of Pneumocystis jiroveci during immunosuppressive therapy for
12 13 14	370	rheumatoid arthritis. <i>J Rheumatol</i> . 2009; 36: 1600–1605.
15 16 17 18	371	23 Shimada K, Yokosuka K, Nunokawa T, Sugii S. Differences in clinical
19 20 21	372	Pneumocystis pneumonia in rheumatoid arthritis and other connective tissue
22 23 24	373	diseases suggesting a rheumatoid-specific interstitial lung injury spectrum.
25 26 27 28	374	Clin Rheumatol. 2018; 37: 2269–2274.
29 30 31	375	24 Fragoulis GE, Conway R, Nikiphorou E. Methotrexate and interstitial
32 33 34	376	lung disease: controversies and questions. A narrative review of the
35 36 37 38	377	literature. Rheumatology. 2019; 58: 1900–1906.
39 40 41	378	25 Hashimoto A, Suto S, Horie K, et al. Incidence and risk factors for
42 43 44	379	infections requiring hospitalization, including Pneumocystis pneumonia, in
45 46 47 48	380	Japanese patients with rheumatoid arthritis. Int J Rheumatol. 2017; 2017:
49 50 51	381	6730812.
52 53 54 55	382	26 Fan LC, Lu HW, Cheng KB, Li HP, Xu JF. Evaluation of PCR in
56 57 58		26
59 60		http://mc.manuscriptcentral.com/tmmy

2 3 4		
4 5		
6 7 8	383	bronchoalveolar lavage fluid for diagnosis of Pneumocystis jirovecii
9 10 11	384	pneumonia: a bivariate meta-analysis and systematic review. PLoS One.
12 13 14 15	385	2013; 8: e73099.
16 17 18	386	27 Nakashima A, Yamada K, Iwata O, et al. β-Glucan in foods and its
19 20 21	387	physiological functions. J Nutr Sci Vitaminol. 2018; 64: 8–17.
22 23 24 25	388	
26 27 28	389	
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390 FIC	GURE LEGENDS
391 Fig	ure 1. Diagram outlining the selection of eligible patients.
392 PCI	P, Pneumocystis jirovecii pneumonia; Pj-PCR, Pneumocystis jirovecii DNA by
393 poly	merase chain reaction; BW, bronchial washing; BAL, bronchoalveolar lavage; CPA,
394 chro	onic pulmonary aspergillosis; IPA, invasive pulmonary aspergillosis; HIV, human
395 imn	nunodeficiency virus.
396	
397 Fig	<b>ure 2.</b> Serum BDG values in PCP and non-PCP patients for all patients (PCP: $n = 50$ ,
398 non	-PCP: $n = 125$ ) included in the study (A), and for patients who used albumin or globulin
399 prod	ducts (PCP: $n = 4$ , non-PCP: $n = 12$ ) (B). Horizontal bars represent medians. Dotted lines
400 repr	resent a modified cut-off value (36.6 pg/ml) and a manufacturer's recommended cut-off
401 valu	e (20.0 pg/ml), respectively. Statistical analysis was performed using the Mann–Whitney
402 U te	est. (C) ROC curve for the predictive performance of serum BDG level for PCP diagnosis.
403 The	figures are the cut-off values of the serum BDG level identified by the Youden's index
404 and	the figures in the parentheses are the sensitivity and the specificity at the cut-off value.
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6	405	BDG, (1, 3)-β-D glucan. PCP, <i>Pneumocystis jirovecii</i> pneumonia. ROC, receiver operating
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11	400	characteristic.
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16	408	Figure 3. The empirical cumulative distribution function of the degree of shift in the accuracy
17	408	<b>Figure 5.</b> The empirical cumulative distribution function of the degree of shift in the accuracy
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19 20	409	of PSL $\geq$ 5 mg and MTX. The figures in the parentheses denote the value of x (i.e., the degree
20 21	409	of PSL $\geq 5$ mg and MTA. The figures in the parentneses denote the value of x (i.e., the degree
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23	410	of shift in the accuracy) and the ECDE(w) calculated from the abcoming data. The actorialis
24	410	of shift in the accuracy) and the $ECDF(x)$ calculated from the observed data. The asterisks
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30	412	PSL, prednisolone. MTX, methotrexate.
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33	410	PSL, prednisolone. MTX, methotrexate.
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# 420 TABLES

## **Table 1.** Patient Characteristics

	Total	РСР	Non-PCP	<i>p</i> -value
	(n = 175)	(n = 50)	(n = 125)	
Age	67 (62–73)	66 (62–71)	67 (62–74)	0.48
Male	112 (64)	27 (54)	85 (68)	0.09
Underlying disease:				
Solid tumor	63 (36)	17 (34)	46 (37)	0.86
Hematologic malignancy	25 (14)	6 (12)	19 (15)	0.81
Rheumatoid arthritis	35 (20)	9 (18)	26 (21)	0.83
Other autoimmune diseases	33 (19)	13 (26)	20 (16)	0.14
Interstitial lung disease	24 (14)	9 (18)	15 (12)	0.33
Diabetes mellitus	25 (14)	4 (8)	21 (17)	0.16
Any transplant	6 (3)	0 (0)	6 (5)	0.18
Renal failure and dialysis	4 (2)	1 (2)	3 (2)	1.00

ICU admission	10	(6)	1	(2)	9	(7)	0.29
Medications:							
PSL ≥5 mg	69	(39)	26	(52)	43	(34)	0.04
МТХ	28	(16)	13	(26)	15	(12)	0.04
MTX ≥10 mg/week	14	(8)	3	(6)	11	(9)	0.76
Other immunosuppressants	23	(13)	6	(12)	17	(14)	1.00
Biologics	13	(7)	4	(8)	9	(7)	1.00
PCP prophylaxis	13	(7)	0	(0)	13	(10)	0.02
Albumin products	11	(6)	2	(4)	9	(7)	0.73
Immunoglobulin products	6	(3)	2	(4)	4	(3)	1.00
Laboratory tests (serum):							
ALC (/µl)	910	(520–1470)	705	(428–1178)	1010	(560–1570)	<0.01
LDH (U/l)	284	(229–365)	361	(277–445)	260	(225–340)	<0.01
KL-6 (U/ml)	515	(290–1095)	927	(341–1333)	473	(282–1002)	0.05
GM <sup>†</sup> ≥0.5 COI	42	(39)	14	(44)	28	(36)	0.52

1 2 3								3:	2
4 5 6 7 8		GM ≥1.0 COI	20	(18)	8	(25)	12	(16)	0.28
9 10 11		BDG (pg/ml)	21.8	(8.2–81.4)	146.9	(67.7–441.1)	11.5	(6.0–23.6)	< 0.01
$\begin{array}{c} 12 \\ 13 \\ 14 \\ 15 \\ 16 \\ 17 \\ 18 \\ 19 \\ 20 \\ 21 \\ 22 \\ 23 \\ 24 \\ 25 \\ 26 \\ 27 \\ 28 \\ 29 \\ 30 \\ 31 \\ 32 \\ 33 \\ 34 \\ 35 \\ 36 \\ 37 \\ 38 \\ 39 \\ 40 \\ 41 \\ 42 \\ 43 \\ 44 \\ 45 \\ 46 \\ 47 \\ 48 \\ 49 \\ 50 \\ 51 \\ 52 \\ 53 \\ 54 \\ 55 \\ 56 \\ 57 \\ 58 \\ 59 \end{array}$	422 423 424 425 426 427	Data are presented a <sup>†</sup> : GM was evaluated PCP: <i>Pneumocystis</i> J MTX: methotrexate; Krebs von den Lung	l in 31 patien <i>iirovecii</i> pneu ALC: absolu en-6; GM: ga	ts in the PCP umonia; ICU: ite lymphocy ilactomannan	group ar intensiv te count; ; COI: cu	nd 66 patients i e care unit; PS LDH: lactate o utoff index; BE	L: predi dehydro OG: (1, 1	nisolone; ogenase; KL-6 3)-β-D glucan	
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#### Table 2. Serum BDG performance for PCP diagnosis Modified cut-off with highest Manufacturer's recommended Youden's index cut-off Cut-off value (pg/ml) 36.6 20.0 92.0 Sensitivity (%) 96.0 Specificity (%) 64.8 84.8 Positive LR 6.1 2.7 0.09 0.06 Negative LR BDG: (1, 3)-β-D glucan; PCP: Pneumocystis jirovecii pneumonia; LR: likelihood ratio

1	Diagnostic evaluation of serum (1, 3)- $\beta$ -D glucan levels using the Fungitec G-Test MK kit
2	for Pneumocystis jirovecii pneumonia (PCP) in non-HIV patients
3	
4	Short title: BDG evaluation for PCP in non-HIV patients
5	
6	Authors: Shuhei Ideguchi, <sup>a,b</sup> Kazuko Yamamoto, <sup>b,c,d</sup> # Tatsuro Hirayama, <sup>b</sup> Takahiro
7	Takazono, <sup>b</sup> Yoshifumi Imamura, <sup>b</sup> Taiga Miyazaki, <sup>b</sup> Noriho Sakamoto, <sup>b</sup> Koichi Izumikawa, <sup>c</sup>
8	Katsunori Yanagihara, <sup>e</sup> Shimpei Morimoto, <sup>f</sup> Hiroshi Mukae <sup>a,b</sup>
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24	Phone: +81-95-819-7273
25	
26	Keywords: (1, 3)-β-D glucan, Fungitec G-Test MK, <i>Pneumocystis jirovecii</i> pneumonia,
27	methotrexate
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2 3 4		
5 6 7	31	Abstract:
8 9 10 11	32	Pneumocystis jirovecii pneumonia (PCP) is an opportunistic and life-threatening pulmonary
12 13 14	33	infection with an increasing prevalence among individuals who are human
15 16 17 18	34	immunodeficiency virus (HIV)-negative. Evidence regarding diagnostic testing of PCP in
19 20 21	35	this patient population is insufficient. We evaluated the performance of serum (1, 3)- $\beta$ -D
22 23 24 25	36	glucan (BDG) using the Fungitec G-test MK kit for diagnosing PCP in non-HIV patients.
25 26 27 28	37	We retrospectively analyzed data from 219 non-HIV adult patients who underwent
29 30 31	38	bronchoscopy and were tested for <i>P. jirovecii</i> DNA by PCR using lavage samples from the
32 33 34	39	lower respiratory tract. Fifty PCP patients and 125 non-PCP patients were included. The most
35 36 37 38	40	common underlying diseases were malignancies and systemic autoimmune diseases. Using
39 40 41	41	the serum BDG Fungitec G-test MK test to diagnose PCP, the area under the receiver
42 43 44	42	operating characteristic curve (AUC) was 0.924, while the modified cut-off value of 36.6
45 46 47 48	43	pg/mL had a sensitivity and specificity of 92.0% and 84.8%, respectively. The AUC for
49 50 51	44	patients with systemic autoimmune diseases was 0.873, and the accuracy of serum BDG test
52 53 54	45	declined when using methotrexate (MTX). In conclusion, the serum BDG test was useful for
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46	diagnosing PCP in non-HIV patients; however, the results should be carefully interpreted in
47	case of MTX administration.
48	
49	Lay summary
50	The Fungitec G-test MK kit for measuring serum (1, 3)- $\beta$ -D glucan (BDG) levels had a
51	sufficient diagnostic performance for Pneumocystis jirovecii pneumonia (PCP) in human
52	immunodeficiency virus-negative patients. However, the results should be carefully
53	interpreted in case of MTX administration.
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# 58 Introduction

59	Pneumocystis jirovecii pneumonia among patients not infected with human
60	immunodeficiency virus (non-HIV-PCP) is a life-threatening pulmonary infectious disease.
61	Its prevalence has recently increased, especially in patients with transplants, hematologic
62	diseases, malignancies, systemic autoimmune diseases, and lung diseases, and those
63	undergoing dialysis. <sup>1</sup> The definitive diagnosis of PCP includes detection of the organism
64	microscopically in tissue, bronchoalveolar lavage (BAL) fluid, expectorated sputum using
65	conventional or immunofluorescence staining, <sup>2</sup> though PCP cysts are relatively few among
66	non-HIV patients compared to HIV patients, and detecting cysts by microscopy has poor
67	sensitivity. <sup>3,4</sup> Therefore, testing for <i>P. jirovecii</i> DNA by polymerase chain reaction ( <i>Pj</i> -PCR)
68	using bronchial washing (BW) or BAL samples has recently been used more frequently for
69	non-HIV-PCP diagnosis. Moreover, numerous patients who cannot undergo bronchoscopy
70	are clinically diagnosed with PCP based on their backgrounds, symptoms, and chest
71	radiography findings; therefore, less-invasive diagnostic testing for non-HIV-PCP is required.
72	(1, 3)- $\beta$ -D glucan (BDG) is one of the common components of the fungal cell wall,

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73	and is widely used as a biomarker for invasive fungal diseases. <sup>5, 6</sup> Serum BDG testing can be
74	useful for PCP diagnosis in HIV patients, with a pooled sensitivity and specificity of 95–96%
75	and 84–86%, respectively. <sup>7, 8</sup> However, studies <sup>7, 8</sup> using several different BDG test kits,
76	including the Fungitell assay (Associates of Cape Cod, USA), the Fungitec G-test MK
77	(Seikagaku Corp, Japan until 2012, Nissui Pharmaceutical Co., Ltd., Japan after 2012), and
78	the Wako β-glucan test (Wako Pure Chemical Industries Ltd, Japan)-all of which have
79	independent cut-off values for diagnosis—and the information on non-HIV-PCP patients is
80	limited. As both HIV and non-HIV-PCP's clinical features and the number of detected cysts
81	by microscopy greatly differ, <sup>3, 9</sup> the respective cut-off values for serum BDG need to be
82	determined; therefore, it is necessary to evaluate the usefulness for each assay kit. The
83	Fungitell assay was approved by the U.S. Food and Drug Administration (FDA) in 2004 and
84	became CE marked in 2008; the Wako $\beta$ -glucan test was CE marked in 2018. Recently, these
85	assays have been reevaluated for the diagnosis of PCP in patients including non-HIV adults. <sup>4,</sup>
86	<sup>10, 11</sup> Conversely, to the best of our knowledge, no study has evaluated PCP diagnosis using
87	the Fungitec G-test MK in non-HIV-PCP patients. <sup>12</sup> In this study, we aimed to evaluate the

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88	usefulness and cut-off value of serum BDG levels using the Fungitec G-test MK for the
89	diagnosis of non-HIV-PCP. Moreover, we assessed the underlying diseases in non-HIV-PCP
90	patients and assessed the utility of measuring serum BDG levels for PCP diagnosis in terms
91	of each underlying disease. Finally, we determined the factors resulting in false-positive
92	serum BDG results.
93	
94	Methods
95	Patients and study design
96	We conducted this retrospective cohort study at the Nagasaki University Hospital, a
97	tertiary care and teaching hospital in Japan, between April 2009 and August 2020. This study
98	included only adult patients (≥20 years) with unexplained lung infiltrations and clinically
98 99	included only adult patients ( $\geq$ 20 years) with unexplained lung infiltrations and clinically suspected PCP who underwent bronchoscopy and were tested with <i>Pj</i> -PCR using BW or
99	suspected PCP who underwent bronchoscopy and were tested with <i>Pj</i> -PCR using BW or
99 00	suspected PCP who underwent bronchoscopy and were tested with <i>Pj</i> -PCR using BW or BAL fluid. Patients with HIV, as well as invasive fungal infections other than PCP, or those

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103	medications) and laboratory data— including peripheral absolute lymphocyte counts (ALC),
104	serum lactate dehydrogenase (LDH), Krebs von den Lungen-6, galactomannan, and BDG
105	levels— were extracted from electronic medical records. Serum BDG values—measured on
106	the closest date from the date of bronchoscopy-were used. Furthermore, we investigated
107	patients with bacteremia in intensive care units, those treated with glucan-containing gauze,
108	albumin products, immunoglobulin products, and those undergoing hemodialysis with
109	cellulose-containing filters—all of which were previously reported as false positive factors—
110	to analyze potential factors contributing to false-positive serum BDG results. <sup>13-15</sup> Regarding
111	albumin and immunoglobulin products, we included patients who had been treated within 1
112	week of BDG measurement. This study was conducted in compliance with the Declaration
113	of Helsinki and approved by the Ethics Committee of the institution (Nagasaki University
114	Hospital, Approval Number: 19102128). Patient consent was waived due to the retrospective
115	and anonymous nature of the study.
116	Definition of PCP diagnosis
117	A PCP diagnosis was determined by the presence of all of the following:
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118	compatible immunocompromising factors (any transplant, hematologic disease, cancer,
119	immunosuppressive drug administration, and primary immunodeficiencies); acute
120	respiratory signs or symptoms (shortness of breath, dry or productive cough, and increased
121	O <sub>2</sub> requirement); newly appearing ground-glass opacities in bilateral lungs on chest
122	computed tomography; and microscopic detection of the organism (proven PCP) or positive
123	<i>P. jirovecii</i> DNA by PCR (probable PCP) in BW or BAL fluid. <sup>2</sup> <i>Pj</i> -PCR was conducted by
124	SRL Inc. (Tokyo, Japan) using previously reported methods <sup>16</sup> in which the exon 45 region
125	of the dystrophin gene was used as an internal control to evaluate PCR inhibition. Patients
126	who did not meet the above criteria were classified as non-PCP, and those whose condition
127	improved without treatment for PCP- despite meeting the above criteria- were also
128	included in the non-PCP group.
129	BDG assay kit
130	In Japan, the Fungitec G-test MK and the Wako $\beta$ -glucan test are available as BDG
131	assay kits. Here, we used a previously reported manipulation: the Fungitec G-test MK, which
132	has a manufacturer-recommended cut-off value of 20 pg/ml for invasive fungal infections. <sup>17,</sup>

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5 6 7 8	133	18
9 10 11	134	Statistical analysis
12 13 14	135	The difference in the distribution of values between the participants' groups was
15 16 17 18	136	evaluated by a Mann–Whitney U statistic. The p-values for the U statistic was calculated
19 20 21	137	from its normal approximation. The null hypothesis of independence between two binomial
22 23 24 25	138	variables was tested by a two-sided Fisher's exact test. A p-value of <0.05 was stated as
26 27 28	139	statistically significant. The multiplicity of hypothesis testing was not considered unless
29 30 31	140	otherwise stated. Statistical analyses were exploratorily conducted; therefore, the type 1 error
32 33 34	141	rates were not under nominal p-values.
35 36 37 38	142	The cut-off value in serum BDG levels for PCP diagnosis was determined by the
39 40 41	143	Youden's index. The concordance between the serum BDG levels and the PCP diagnosis was
42 43 44	144	evaluated as the area under the receiver-operating-characteristic (ROC) curve (AUC). Test
45 46 47 48	145	accuracy was assessed regarding sensitivity, specificity, and both positive and negative
49 50 51	146	likelihood ratios (positive LR and negative LR, respectively). Based on the interest to identify
52 53 54	147	the disruptor in PCP diagnosis by serum BDG level, we evaluated the "degree of shift in the
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148	accuracy" in the diagnosis by selecting participants based on a clinical factor- that is- a
149	candidate of a disruptor. The "degree of shift in the accuracy" was defined as a subtract of a
150	log-transformed odds ratio on the total population $(OR_{total})$ from one of the selected
151	subpopulations $(OR_{sub})$ (equivalent to the logarithm of a ratio of the odds ratios [eq. 1]). The
152	effect on shifting the accuracy of selection of the subjects was evaluated as the rank of the
153	ratio calculated from the observed data, among its null distribution generated by 2000
154	permutations in the labels of the clinical factor used in the selection of the subjects.
155	The degree of shift in accuracy = $\log \frac{OR_{sub}}{OR_{total}}$ where $OR_{sub} = \frac{\Pr(PCP = 1 BDG = 1, z = 1)/\Pr(PCP = 0 BDG = 1, z = 1)}{\Pr(PCP = 1 BDG = 0, z = 1)/\Pr(PCP = 0 BDG = 0, z = 1)},$ $OR_{total} = \frac{\Pr(PCP = 1 BDG = 1)/\Pr(PCP = 0 BDG = 1)}{\Pr(PCP = 1 BDG = 0)/\Pr(PCP = 0 BDG = 0)}$ (eq.1)
156	Statistical analyses were performed using GraphPad Prism 5, JMP <sup>®</sup> 13 (SAS Institute Inc.,
157	Cary, NC, USA) and the R environment version 4.1.1. <sup>19</sup>
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159	Results
160	Eligible patients
161	Among the 219 patients who underwent bronchoscopy and were tested using Pj-
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6 7 8	162	PCR during the study period, 175 were included in the study (Fig. 1). Of the 50 patients
9 10 11	163	diagnosed with PCP, 12% (n = 6) were diagnosed with proven PCP by identification of $P$ .
12 13 14	164	<i>jirovecii</i> cysts, and 88% (n = 44) had probable PCP diagnosed using $Pj$ -PCR as per
15 16 17 18	165	EORTC/MSGERC case definitions. <sup>2</sup>
19 20 21	166	Patient characteristics
22 23 24 25	167	The baseline characteristics of the patients are shown in Table 1. The most common
26 27 28	168	underlying diseases were malignancies, followed by systemic autoimmune diseases. Among
29 30 31	169	chronic lung diseases, interstitial lung disease and asthma were found in 24 (14%) and 4 (2%)
32 33 34	170	out of 175 patients, respectively (asthma was not included in Table 1), with no case of chronic
35 36 37 38	171	obstructive pulmonary disease. Additionally, there was only one case of bacteremia in each
39 40 41	172	group. Patients who were administered systemic prednisolone (PSL) $\geq$ 5 mg or methotrexate
42 43 44	173	(MTX) were more frequent in the PCP than in the non-PCP group. PCP prophylaxis was only
45 46 47 48	174	administered in the non-PCP group. All prophylactic drugs were
48 49 50 51	175	trimethoprim/sulfamethoxazole (TMP/SMX). No patients were treated with glucan-
52 53 54 55	176	containing gauze or were undergoing hemodialysis with cellulose-containing filters, both of
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5 6 7 8	177	which could have affected the BDG results. Laboratory test results showed that the patients
9 10 11 12	178	in the PCP group had significantly lower ALC and higher serum LDH and BDG than the
13 14 15	179	those in the non-PCP group.
16 17 18	180	BDG values
19 20 21	181	The Fungitec G-test MK results of the BDG values for the PCP and non-PCP
22 23 24 25	182	groups are shown in Fig. 2A. The median (interquartile range) serum BDG values of both
26 27 28	183	PCP and non-PCP patients were 146.9 (67.7-441.1) pg/ml and 11.5 (6.0-23.6) pg/ml,
29 30 31	184	respectively ( $p < 0.01$ ). In the group of patients who used albumin or immunoglobulin
32 33 34 35	185	products, the median (interquartile range) serum BDG values of both PCP and non-PCP
36 37 38	186	patients were 252.2 (78.3–550.4) pg/ml and 17.9 (8.2–34.4) pg/ml, respectively ( $p = 0.01$ )
39 40 41	187	(Fig. 2B).
42 43 44	188	Accuracy of PCP diagnosis using the serum BDG level
45 46 47 48	189	The concordance of the serum BDG level for PCP diagnosis is shown as the ROC
49 50 51	190	curve in Fig. 2C. These two variables were highly concordant at the AUC of 0.924. The
52 53 54	191	modified cut-off value by the Youden's index was 36.6 pg/ml, and the comparison of
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accuracy (sensitivity, specificity, etc.) with the manufacturer's defined cutoff value of 20.0 pg/ml is summarized in Table 2. Moreover, to examine the characteristics of the association of the serum BDG level with the diagnosis of PCP in patients with underlying diseases, we extracted patients with malignancies and systemic autoimmune diseases including rheumatoid arthritis (RA), respectively, which were the two most prevalent underlying diseases among those observed. In patients with malignancies, the concordance between the serum BDG level and the diagnosis of PCP was high (AUC = 0.940; Supplementary Figure S1a) in all cases. Sensitivity and specificity were 100% and 81.2%, respectively, at the cut-off value of 25.8 pg/ml. In patients with systemic autoimmune diseases, the AUC of BDG for diagnosing PCP was 0.873 (Supplementary Figure S1b), and the sensitivity and specificity were 81.8% and 88.1%, respectively, at the cut-off value of 56.5 pg/ml. Factors that reduce the accuracy of BDG testing Aiming to identify disruptors in PCP diagnosis by serum BDG level using Fungitec G-test MK, we explored subpopulations in which the diagnostic accuracy of the serum BDG

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5 6 7 8	207	test (cut-off value: 36.6 pg/ml) was declined compared with that of the total cases, using the
9 10 11	208	"degree of shift in the accuracy" (detailed in the section of statistical analysis). On some of
12 13 14	209	the clinical factors, the "degree of shift in the accuracy" was not finite because of the
15 16 17 18	210	complete separation in some trials of the 2000 permutations. We found that the degree of a
19 20 21	211	negative shift in the accuracy was seen in patients using PSL $\geq$ 5 mg or MTX, and the shift
22 23 24	212	was more evident in MTX-treated patients (Figure 3). We further analyzed the association of
25 26 27	213	the higher dose of MTX ( $\geq 10$ mg/week vs. <10 mg/week) with the more frequently erroneous
28 29 30 31	214	diagnosis based on the serum BDG test with the cut-off value (36.6 pg/ml). As a result, a
32 33 34	215	significant relationship was not observed (the odds ratio [the 95%CI] of the dose of MTX
35 36 37	216	$\geq 10$ mg/week for the erroneous diagnosis =1.55, the 95%CI:[ 0.25 to 9.76]). The results for
38 39 40	217	the analysis of other factors are provided as supplemental information (Supplementary Figure
41 42 43 44	218	S2.).
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48 49 50	220	Discussion
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We evaluated the diagnostic utility of serum BDG levels for non-HIV-PCP using the Fungitec G-test MK assay. Patients with PCP had a significantly higher BDG value than those without PCP. The best cut-off value of serum BDG to distinguish non-HIV-PCP was 36.6 pg/ml, showing comparable negative LR and higher positive LR compared to the manufacturer's recommended cut-off value of 20.0 pg/ml. To the best of our knowledge, this is the first large-scale study aiming to evaluate serum BDG levels in the diagnosis of PCP limited to non-HIV patients. Previous studies included a mixed sample of both HIV and non-HIV patients, and the diagnosis of PCP required confirmation by cyst visualization using microscopy;<sup>7,8</sup> these diagnostic criteria might miss many non-HIV-PCP cases, as only 12% of PCP patients in this study the organism detected microscopically. Previous studies that evaluated the accuracy of the diagnosis of serum BDG for non-HIV-PCP included patients with malignancy in a high proportion.<sup>11, 20</sup> A large-scale study that recently evaluated the serum BDG test using the Fungitell assay for the diagnosis of PCP in cancer patients<sup>4</sup> found that most non-HIV PCP patients either had malignancies

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5 6 7 230 8	or systemic autoimmune diseases. The number of non-HIV-PCP patients with these
9 10 237 11	underlying diseases has been increasing; <sup>1</sup> the colonization rate of <i>P. jirovecii</i> among
12 13 14 15	outpatients both during cancer chemotherapy and among patients with RA during
15 16 17 239 18	immunosuppressive therapy was reported to be high. <sup>21, 22</sup> In our study, 54% of non-HIV-
19 20 24( 21	PCP patients had malignancies or systemic autoimmune diseases. The accuracy of serum
22 23 24 25	BDG test to diagnose non-HIV-PCP in each of these group was sufficient to use in clinical
25 26 27 242 28	practice by the subgroup analysis (Supplementary Figure S1), and we believe that this is the
29 30 243 31	first study to evaluate the accuracy of serum BDG test for PCP diagnosis focusing on
32 33 244 34	systemic autoimmune diseases. However, the BDG AUC for PCP diagnosis was lower,
35 36 37 24: 38	with a higher cut-off value in patients with systemic autoimmune diseases compared to
39 40 240 41	patients with malignancies, thus suggesting that patients with systemic autoimmune
42 43 44 45	diseases potentially possess factors that negatively affect BDG test's diagnostic accuracy.
45 46 47 48 248	The declining effect on the accuracy of the BDG test of selecting the patients,
49 50 51 249	especially using MTX, was observed. In the PCP group, four patients had BDG values
52 53 54 250 55	below the modified cut-off value, of which two had extremely low BDG values (Figure
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251	2A). One of the two patients responded poorly to PCP treatment with TMP/SMX and was
252	later diagnosed with Mycobacterium avium complex lung infection, potentially ruling-out
253	PCP; the other patient showed elevated levels of BDG—above the cut-off value of 36.6
254	pg/ml a week after the initial test. In this study, BDG was tested multiple times within 1
255	week in 33 patients (66%) in the PCP group, with 12 patients showing an elevated BDG
256	value a week later. This suggested that the timing of the BDG test may have affected the
257	results. Moreover, two out of four patients in the PCP group whose BDG values were
258	below the cut-off value were using MTX. Small amounts of <i>P. jirovecii</i> may trigger lung
259	inflammation in patients with RA undergoing MTX treatment, <sup>23</sup> suggesting that patients
260	with PCP using MTX could have a false-negative BDG test result. Conversely, 15.2% of
261	patients in the non-PCP group had false-positive BDG results. Among non-PCP patients,
262	increased cut-off value of serum BDG (36.6 pg/ml), i.e. false-positive result of PCP
263	diagnosis, was associated with MTX use (Supplementary Table S1). MTX is a risk factor
264	for PCP. <sup>24, 25</sup> Further, it is difficult to clinically differentiate between RA-related interstitial
265	pneumonia (IP), MTX-related IP, and PCP. We found four patients who completed PCP
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5 6 7 8	266	treatment within 2 or 3 weeks, despite negative Pj-PCR results; these patients tended to
9 10 11	267	have high BDG values (Supplementary Table S2). A possible factor of false positives for
12 13 14 15	268	BDG was that these patients might have had undetected conditions that affected their BDG
16 17 18	269	values, despite the absence of any history, such as bacteremia, use of albumin or
19 20 21	270	immunoglobulin products, or ICU admission. These other factors could rarely affect false-
22 23 24 25	271	negative <i>Pj</i> -PCR results. <sup>26</sup> Differentiating true PCP in patients using MTX remains a future
26 27 28	272	challenge.
29 30 31 32	273	This study has several limitations. First, our sample of 50 PCP patients was
33 34 35	274	modest; this single-center study only included patients who had undergone bronchoscopy.
36 37 38 39	275	In a clinical setting, BDG is important either for the diagnosis or exclusion of PCP,
40 41 42	276	especially in patients who cannot undergo bronchoscopy. Second, we used conventional
43 44 45	277	PCR to diagnose PCP. Although amplification of <i>P. jirovecii</i> DNA by quantitative real-
46 47 48 49	278	time PCR (qPCR) is preferred to conventional qualitative PCR, <sup>2</sup> in Japan, qPCR use for
50 51 52	279	PCP is limited, and our outsourced manufacturer handles only conventional PCR. This may
53 54 55 56	280	have affected the diagnosis of PCP. Third, we did not fully evaluate the pre-test probability
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28	81	of PCP due to the retrospective nature of the study. Finally, BDG is found in various foods,
28	82	thus making it impossible to evaluate patient intake of potential BDG sources, <sup>27</sup> as their
28	83	consumption may cause BDG false-positives.
28	84	Overall, our study showed that optimizing the serum BDG cut-off value using the
28	85	Fungitec G-test MK assay increased the positive LR without compromising the good
28	86	negative LR for diagnosing PCP in non-HIV patients. MTX could negatively affect the
28	87	accuracy of the BDG test for diagnosing PCP, and we should be aware of the BDG results
28	88	when PCP is suspected in patients using MTX. Further research is warranted to compare
28	89	this assay with other BDG tests and, more importantly, a prospective study is expected both
29	90	for calibration and validation of the cut-off value.
29	91	
29	92	Acknowledgments: We thank SRL, Inc. for providing the necessary data.
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3 4 5		
6 7 8	308	4 Morjaria S, Frame J, Franco-Garcia A, Geyer A, Kamboj M, Babady
9 10 11	309	NE. Clinical Performance of (1,3) Beta-D-Glucan for the diagnosis of
12 13 14 15	310	Pneumocystis pneumonia in cancer patients tested with PCP PCR. Clin Infect
16 17 18	311	<i>Dis</i> . 2019; 69: 1303–1309.
19 20 21	312	5 McCarthy MW, Petraitiene R, Walsh TJ. Translational development
22 23 24 25	313	and application of (1>3)-beta-d-glucan for diagnosis and therapeutic
26 27 28	314	monitoring of invasive mycoses. Int J Mol Sci. 2017; 18: 1124.
29 30 31	315	6 Farhour Z, Mehraj V, Chen J, Ramendra R, Lu H, Routy JP. Use of
32 33 34 35	316	(1>3)-beta-d-glucan for diagnosis and management of invasive mycoses
36 37 38	317	in HIV-infected patients. Mycoses. 2018; 61: 718–722.
39 40 41	318	7 Karageorgopoulos DE, Qu JM, Korbila IP, Zhu YG, Vasileiou VA,
42 43 44	319	Falagas ME. Accuracy of beta-D-glucan for the diagnosis of Pneumocystis
45 46 47 48	320	jirovecii pneumonia: a meta-analysis. Clin Microbiol Infect. 2013; 19: 39-
49 50 51	321	49.
52 53 54 55 56	322	8 Onishi A, Sugiyama D, Kogata Y, et al. Diagnostic accuracy of serum
57 58 59		22
60		http://mc.manuscriptcentral.com/tmmy

1 2		23
2 3 4		
5 6 7 8	323	1,3-beta-D-glucan for pneumocystis jiroveci pneumonia, invasive
9 10 11	324	candidiasis, and invasive aspergillosis: systematic review and meta-analysis.
12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41	325	J Clin Microbiol. 2012; 50: 7–15.
	326	9 Kato H, Samukawa S, Takahashi H, Nakajima H. Diagnosis and
	327	treatment of Pneumocystis jirovecii pneumonia in HIV-infected or non-HIV-
	328	infected patients-difficulties in diagnosis and adverse effects of
	329	trimethoprim-sulfamethoxazole. J Infect Chemother. 2019; 25: 920–924.
	330	10 Dichtl K, Seybold U, Wagener J. Evaluation of a turbidimetric beta-
	331	d-glucan test for detection of Pneumocystis jirovecii pneumonia. J Clin
	332	Microbiol. 2018; 56: e00286-18.
	333	11 Mercier T, Guldentops E, Patteet S, Beuselinck K, Lagrou K,
42 43 44	334	Maertens J. Beta-d-glucan for diagnosing Pneumocystis pneumonia: a direct
45 46 47	335	comparison between the Wako beta-glucan assay and the Fungitell assay. $J$
48 49 50 51	336	Clin Microbiol. 2019; 57: e00322-19.
52 53 54 55	337	12 Watanabe T, Yasuoka A, Tanuma J, et al. Serum (1>3) beta-D-
56 57 58		23
59 60		http://mc.manuscriptcentral.com/tmmy

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338	glucan as a noninvasive adjunct marker for the diagnosis of Pneumocystis
339	pneumonia in patients with AIDS. Clin Infect Dis. 2009; 49: 1128-1131.
340	13 Digby J, Kalbfleisch J, Glenn A, Larsen A, Browder W, Williams D.
341	Serum glucan levels are not specific for presence of fungal infections in
342	intensive care unit patients. Clin Diagn Lab Immunol. 2003; 10: 882–885.
343	14 Marty FM, Koo S. Role of (1>3)-beta-D-glucan in the diagnosis of
344	invasive aspergillosis. <i>Med Mycol</i> . 2009; 47 Suppl 1: S233–40.
345	15 Acosta J, Catalan M, del Palacio-Perez-Medel A, et al. Prospective
346	study in critically ill non-neutropenic patients: diagnostic potential of (1,3)-
347	beta-D-glucan assay and circulating galactomannan for the diagnosis of
348	invasive fungal disease. Eur J Clin Microbiol Infect Dis. 2012; 31: 721–731.
349	16 Wakefield A, Pixley F, Banerji S, Sinclair K, Miller R, Moxon E, Hopkin
350	J. Detection of Pneumocystis carinii with DNA amplification. Lancet. 1990;
351	336: 451–453.
352	17 Obayashi T, Yoshida M, Mori T, et al. Plasma (1>3)-beta-D-glucan
	24

Medical Mycology

1		25
2 3 4		
5 6 7 8	353	measurement in diagnosis of invasive deep mycosis and fungal febrile
9 10 11	354	episodes. Lancet. 1995; 345: 17–20.
12 13 14	355	18 Yasuoka A, Tachikawa N, Shimada K, Kimura S, Oka S. (1>3)
15 16 17 18 19 20 21 22	356	beta-D-glucan as a quantitative serological marker for Pneumocystis carinii
	357	pneumonia. Clin Diagn Lab Immunol. 1996; 3: 197–199.
22 23 24	358	19 Team RC. R: A Language and Environment for Statistical Computing.
25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44	359	R Foundation for Statistical Computing. 2020.
	360	20 de Boer MGJ, Gelinck LBS, van Zelst BD, et al. $\beta$ -d-Glucan and S-
	361	adenosylmethionine serum levels for the diagnosis of Pneumocystis
	362	pneumonia in HIV-negative patients: a prospective study. J Infect. 2011;
	363	62: 93–100.
	364	21 Takemoto S, Ebara M, Hasebe S, Yakushijin Y. A study on the
45 46 47	365	colonization of Pneumocystis jirovecii among outpatients during cancer
48 49 50	366	chemotherapy and among healthy smokers. J Infect Chemother. 2017; 23:
51 52 53 54 55	367	752–756.
55 56 57 58		25
59 60		http://mc.manuscriptcentral.com/tmmy

2 3 4		
5 6 7 8	368	22 Mori S, Cho I, Sugimoto M. A followup study of asymptomatic
9 10 11	369	carriers of Pneumocystis jiroveci during immunosuppressive therapy for
12 13 14	370	rheumatoid arthritis. <i>J Rheumatol</i> . 2009; 36: 1600–1605.
15 16 17 18	371	23 Shimada K, Yokosuka K, Nunokawa T, Sugii S. Differences in clinical
19 20 21	372	Pneumocystis pneumonia in rheumatoid arthritis and other connective tissue
22 23 24 25	373	diseases suggesting a rheumatoid-specific interstitial lung injury spectrum.
25 26 27 28	374	<i>Clin Rheumatol</i> . 2018; 37: 2269–2274.
29 30 31	375	24 Fragoulis GE, Conway R, Nikiphorou E. Methotrexate and interstitial
32 33 34	376	lung disease: controversies and questions. A narrative review of the
35 36 37 38	377	literature. Rheumatology. 2019; 58: 1900–1906.
39 40 41	378	25 Hashimoto A, Suto S, Horie K, et al. Incidence and risk factors for
42 43 44	379	infections requiring hospitalization, including Pneumocystis pneumonia, in
45 46 47 48	380	Japanese patients with rheumatoid arthritis. Int J Rheumatol. 2017; 2017:
49 50 51	381	6730812.
52 53 54 55	382	26 Fan LC, Lu HW, Cheng KB, Li HP, Xu JF. Evaluation of PCR in
56 57 58		26
59 60		http://mc.manuscriptcentral.com/tmmy

2 3 4		
5 6 7	383	bronchoalveolar lavage fluid for diagnosis of Pneumocystis jirovecii
8 9 10 11	384	pneumonia: a bivariate meta-analysis and systematic review. PLoS One.
12 13 14 15	385	2013; 8: e73099.
16 17 18	386	27 Nakashima A, Yamada K, Iwata O, et al. β-Glucan in foods and its
19 20 21	387	physiological functions. J Nutr Sci Vitaminol. 2018; 64: 8–17.
22 23 24	388	
25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53	389	
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## **390 FIGURE LEGENDS**

**Figure 1.** Diagram outlining the selection of eligible patients.

392 PCP, *Pneumocystis jirovecii* pneumonia; *Pj*-PCR, *Pneumocystis jirovecii* DNA by
393 polymerase chain reaction; BW, bronchial washing; BAL, bronchoalveolar lavage; CPA,
394 chronic pulmonary aspergillosis; IPA, invasive pulmonary aspergillosis; HIV, human

395 immunodeficiency virus.

**Figure 2.** Serum BDG values in PCP and non-PCP patients for all patients (PCP: n = 50,

398 non-PCP: n = 125) included in the study (A), and for patients who used albumin or globulin

399 products (PCP: n = 4, non-PCP: n = 12) (B). Horizontal bars represent medians. Dotted lines

400 represent a modified cut-off value (36.6 pg/ml) and a manufacturer's recommended cut-off

401 value (20.0 pg/ml), respectively. Statistical analysis was performed using the Mann–Whitney

*U* test. (C) ROC curve for the predictive performance of serum BDG level for PCP diagnosis.

- 403 The figures are the cut-off values of the serum BDG level identified by the Youden's index
- 404 and the figures in the parentheses are the sensitivity and the specificity at the cut-off value.

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6 7 8	405	BDG, (1, 3)-β-D glucan. PCP, <i>Pneumocystis jirovecii</i> pneumonia. ROC, receiver operating
9 10 11	406	characteristic.
12 13 14	407	
15 16 17 18	408	<b>Figure 3.</b> The empirical cumulative distribution function of the degree of shift in the accuracy
19 20 21	409	of PSL $\geq$ 5 mg and MTX. The figures in the parentheses denote the value of x (i.e., the degree
22 23 24 25	410	of shift in the accuracy) and the ECDF( $x$ ) calculated from the observed data. The asterisks
25 26 27 28	411	denote imputation for infinity in the <i>x</i> .
29 30 31	412	PSL, prednisolone. MTX, methotrexate.
32 33 34	413	PSL, prednisolone. MTX, methotrexate.
35 36 37 38	414	
39 40 41	415	
42 43 44 45	416	
45 46 47 48	417	
49	418	
50 51 52 53 54 55	419	
56 57		
58 59		29
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## 420 TABLES

### **Table 1.** Patient Characteristics

	,	Total		РСР		Non-PCP	<i>p</i> -value
	(n	= 175)		(n = 50)		(n = 125)	
Age	67	(62–73)	66	(62–71)	67	(62–74)	0.48
Male	112	(64)	27	(54)	85	(68)	0.09
Underlying disease:							
Solid tumor	63	(36)	17	(34)	46	(37)	0.86
Hematologic malignancy	25	(14)	6	(12)	19	(15)	0.81
Rheumatoid arthritis	35	(20)	9	(18)	26	(21)	0.83
Other autoimmune diseases	33	(19)	13	(26)	20	(16)	0.14
Interstitial lung disease	24	(14)	9	(18)	15	(12)	0.33
Diabetes mellitus	25	(14)	4	(8)	21	(17)	0.16
Any transplant	6	(3)	0	(0)	6	(5)	0.18
Renal failure and dialysis	4	(2)	1	(2)	3	(2)	1.00

ICU admission	10	(6)	1	(2)	9	(7)	0.29
Medications:							
PSL ≥5 mg	69	(39)	26	(52)	43	(34)	0.04
мтх	28	(16)	13	(26)	15	(12)	0.04
MTX ≥10 mg/week	14	(8)	3	(6)	11	(9)	0.76
Other immunosuppressants	23	(13)	6	(12)	17	(14)	1.00
Biologics	13	(7)	4	(8)	9	(7)	1.00
PCP prophylaxis	13	(7)	0	(0)	13	(10)	0.02
Albumin products	11	(6)	2	(4)	9	(7)	0.73
Immunoglobulin products	6	(3)	2	(4)	4	(3)	1.00
Laboratory tests (serum):							
ALC (/µl)	910	(520–1470)	705	(428–1178)	1010	(560–1570)	<0.01
LDH (U/l)	284	(229–365)	361	(277–445)	260	(225–340)	<0.01
KL-6 (U/ml)	515	(290–1095)	927	(341–1333)	473	(282–1002)	0.05
GM <sup>†</sup> ≥0.5 COI	42	(39)	14	(44)	28	(36)	0.52

1 2 3							32	
4 5 6 7 8	GM ≥1.0 COI	20	(18)	8	(25)	12 (16)		0.28
9 10	BDG (pg/ml)	21.8	(8.2–81.4)	146.9	(67.7–441.1)	11.5 (6.0	-23.6)	< 0.01
11         12         13       422         14       423         15       424         17       425         19       426         20       427         22       23         24       25         26       27         28       29         30       31         32       33         34       35         36       37         38       39         40       41         42       43         44       45         46       47         48       49         50       51         52       53	Data are presented <sup>†</sup> : GM was evaluate PCP: <i>Pneumocystis</i> MTX: methotrexat Krebs von den Lun	ed in 31 patients s <i>jirovecii</i> pneur e; ALC: absolut	s in the PCP monia; ICU: te lymphocyt lactomannan;	group and intensive ce count; I ; COI: cut	d 66 patients care unit; PS LDH: lactate toff index; B	SL: prednisolo dehydrogena DG: (1, 3)-β-	one; se; KL-6:	
54 55 56 57 58 59 60		http://	mc.manuscript	central.cor	n/tmmy		32	

#### Table 2. Serum BDG performance for PCP diagnosis Modified cut-off with highest Manufacturer's recommended Youden's index cut-off Cut-off value (pg/ml) 36.6 20.0 92.0 Sensitivity (%) 96.0 Specificity (%) 64.8 84.8 Positive LR 2.7 6.1 0.09 0.06 Negative LR BDG: (1, 3)-β-D glucan; PCP: *Pneumocystis jirovecii* pneumonia; LR: likelihood ratio http://mc.manuscriptcentral.com/tmmy



219 patients with clinically suspected PCP who underwent bronchoscopy and were tested by *Pj*-PCR from BW or BAL fluid

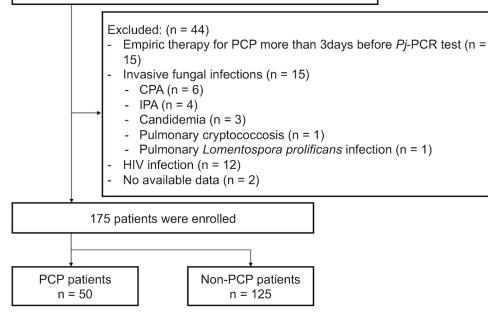
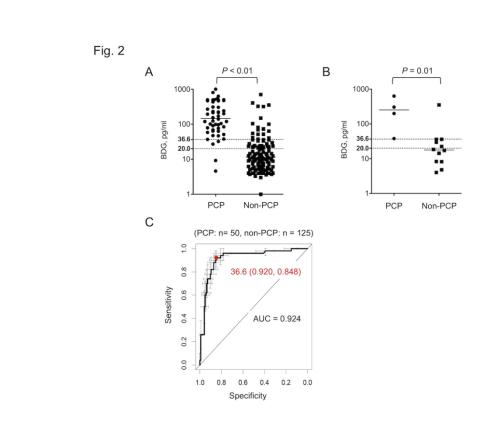


Diagram outlining the selection of eligible patients.

PCP, *Pneumocystis jirovecii* pneumonia; Pj-PCR, *Pneumocystis jirovecii* DNA by polymerase chain reaction; BW, bronchial washing; BAL, bronchoalveolar lavage; CPA, chronic pulmonary aspergillosis; IPA, invasive pulmonary aspergillosis; HIV, human immunodeficiency virus.

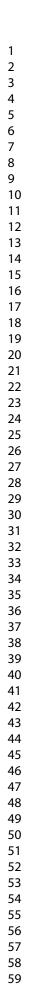
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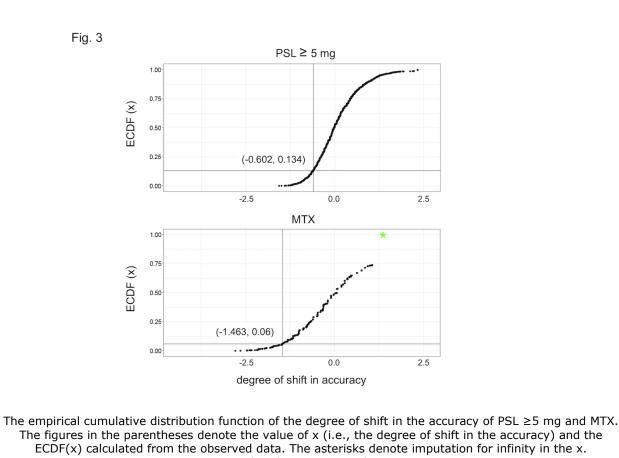


Serum BDG values in PCP and non-PCP patients for all patients (PCP: n = 50, non-PCP: n = 125) included in the study (A), and for patients who used albumin or globulin products (PCP: n = 4, non-PCP: n = 12) (B). Horizontal bars represent medians. Dotted lines represent a modified cut-off value (36.6 pg/ml) and a manufacturer's recommended cut-off value (20.0 pg/ml), respectively. Statistical analysis was performed using the Mann–Whitney U test. (C) ROC curve for the predictive performance of serum BDG level for PCP diagnosis. The figures are the cut-off values of the serum BDG level identified by the Youden's index and the figures in the parentheses are the sensitivity and the specificity at the cut-off value.

BDG, (1, 3)-β-D glucan. PCP, *Pneumocystis jirovecii* pneumonia. ROC, receiver operating characteristic.

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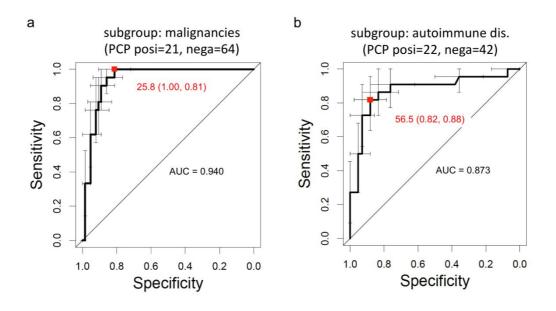




ECDF(x) calculated from the observed data. The asterisks denote imputation for infinity in the x. PSL, prednisolone. MTX, methotrexate.

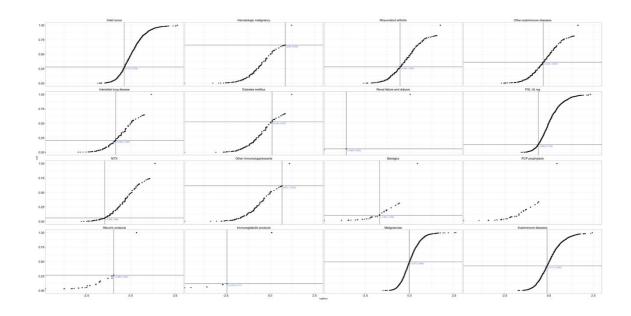
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Fig. S1



**Figure S1.** The ROC curve for the diagnosis of PCP by the BDG test in the patients with malignancy (a), and autoimmune disease (B). The bootstrap 95% confidence intervals of sensitivity and specificity are depicted as whiskers. The red figures are the cut-off point of the serum BDG level identified by the Youden's index and the (sensitivity, specificity)

at the cut-off point.



**Figure S2.** The empirical cumulative distribution function of the degree of shift in accuracy. The figures in the parentheses denote the value of x (i.e., the degree of shift in accuracy) and the ECDF(x) calculated from the observed data. The asterisks denote imputation for infinity in the x.

	False positive (n = 19)		Negative		P value
			(n	= 106)	
Age	67	(62–74)	67	(61–73)	0.84
Male	16	(84)	69	(65)	0.12
Underlying disease:					
Solid tumor	8	(42)	38	(36)	0.61
Hematologic malignancy	2	(11)	17	(16)	0.74
Diabetes mellitus	2	(11)	19	(18)	0.53
Rheumatoid arthritis	4	(21)	22	(21)	1.00
Other systemic	4	(21)	16	(15)	0.51
autoimmune diseases					
Interstitial lung diseases	2	(11)	13	(12)	1.00
ICU admission	1	(5)	8	(8)	1.00
Medications:					
$PSL \ge 5 mg$	8	(42)	35	(33)	0.44

### Table S1. Analysis of false positive serum BDG results in the non-PCP group

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MTX	5	(26)	10	(9)	0.05
MTX ≥10 mg/week	2	(11)	9	(8)	0.67
Other immunosuppressants	0	(0)	17	(16)	0.07
Biologics	1	(5)	8	(8)	1.00
PCP prophylaxis	2	(11)	11	(10)	1.00
Albumin products		(5)	8	(8)	1.00
Immunoglobulin products	0	(0)	4	(4)	1.00
Laboratory tests (serum):					
ALC (/µL)	1130	(530–1560)	970	(565–1585)	0.64
LDH (U/L)	272	(216–344)	258	(226–337)	0.95
KL-6 (U/mL)	476	(266–851)	453	(289–1044)	0.91
$GM^* \ge 0.5 COI$	8	(50)	20	(33)	0.25
GM ≥ 1.0 COI	6	(37)	6	(10)	0.01

Data are presented as frequency (%) or median (interquartile range).

\*: GM was evaluated in 16 patients in the False positive group and 61 patients in the Negative group.

BDG, (1, 3)-β-D glucan; PCP, *Pneumocystis* pneumonia; ICU, intensive care unit; PSL, prednisolone; MTX, methotrexate; ALC, absolute lymphocyte count; LDH, lactate dehydrogenase; KL-6, Krebs von den Lungen-6; GM, galactomannan; COI, cut-off index.

	Serum BDG (pg/mL)	PCP treatment
Case 1	4.2	No treatment
Case 2	8.2	No treatment
Case 3	8.8	No treatment
Case 4	14.0	No treatment
Case 5	21.2	No treatment
Case 6	21.5	5 days of empirical therapy until <i>Pj</i> -PCR result
Case 7	22.4	21 days of PCP treatment
Case 8	23.2	No treatment
Case 9	24.2	7 days of empirical therapy until <i>Pj</i> -PCR result
Case 10	35.4	21 days of PCP treatment
Case 11	51.5	No treatment
Case 12	73.6	14 days of PCP treatment
Case 13	150.0	21 days of PCP treatment
Case 14	320.1	No treatment

## Table S2. Patient Characteristics treated with MTX in non-PCP group

Case 15 4

404.9

No treatment

MTX, methotrexate; PCP, *Pneumocystis* pneumonia; BDG, (1, 3)  $\beta$ -D glucan; *Pj*-PCR, *Pneumocystis jirovecii* DNA by polymerase chain reaction.

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