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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Abstract:	*Pneumocystis jirovecii* 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

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insufficient. We evaluated the performance of serum (1, 3)-β-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)-β-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)-β-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 *P. jirovecii* 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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46 diagnosing PCP in non-HIV patients; however, the results should be carefully interpreted in

47 case of MTX administration.

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49 **Lay summary**

50 The Fungitec G-test MK kit for measuring serum (1, 3)-β-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.[1] 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,[2] though PCP cysts are relatively few among

66   non-HIV patients compared to HIV patients, and detecting cysts by microscopy has poor

67   sensitivity.[3, 4] Therefore, testing for *P. jirovecii* DNA by polymerase chain reaction (*Pj*-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)-β-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.[5, 6] 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.[7, 8] However, studies[7, 8] 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,[3, 9] 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 β-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.[4,

86   [10, 11] 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.[12] 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.

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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

99 suspected PCP who underwent bronchoscopy and were tested with *Pj*-PCR using BW or

100 BAL fluid. Patients with HIV, as well as invasive fungal infections other than PCP, or those

101 who received empirical treatment for PCP lasting more than 3 days before *Pj*-PCR testing

102 were excluded. Patient characteristics (age, sex, underlying disease, and immunosuppressive

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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.[13-15] 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:

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_2 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   *P. jirovecii* DNA by PCR (probable PCP) in BW or BAL fluid.[2] *Pj*-PCR was conducted by

124   SRL Inc. (Tokyo, Japan) using previously reported methods[16] 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 β-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.[17,]

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7   133   [18]
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10   134   **Statistical analysis**
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14   135   The difference in the distribution of values between the participants' groups was
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17   136   evaluated by a Mann–Whitney U statistic. The p-values for the U statistic was calculated
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20   137   from its normal approximation. The null hypothesis of independence between two binomial
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23   138   variables was tested by a two-sided Fisher's exact test. A p-value of <0.05 was stated as
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26   139   statistically significant. The multiplicity of hypothesis testing was not considered unless
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29   140   otherwise stated. Statistical analyses were exploratorily conducted; therefore, the type 1 error
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32   141   rates were not under nominal p-values.
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36   142   The cut-off value in serum BDG levels for PCP diagnosis was determined by the
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39   143   Youden's index. The concordance between the serum BDG levels and the PCP diagnosis was
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42   144   evaluated as the area under the receiver-operating-characteristic (ROC) curve (AUC). Test
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45   145   accuracy was assessed regarding sensitivity, specificity, and both positive and negative
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48   146   likelihood ratios (positive LR and negative LR, respectively). Based on the interest to identify
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51   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.

$$The\ degree\ of\ shift\ in\ accuracy = \log \frac{OR_{sub}}{OR_{total}}\ where$$

$$OR_{sub} = \frac{\Pr(PCP=1|BDG=1,z=1)/\Pr(PCP=0|BDG=1,z=1)}{\Pr(PCP=1|BDG=0,z=1)/\Pr(PCP=0|BDG=0,z=1)},$$

$$OR_{total} = \frac{\Pr(PCP=1|BDG=1)/\Pr(PCP=0|BDG=1)}{\Pr(PCP=1|BDG=0)/\Pr(PCP=0|BDG=0)}$$

155 $\hspace{10cm}$ (*eq.1*)

156 Statistical analyses were performed using GraphPad Prism 5, JMP®□ 13 (SAS Institute Inc.,

157 Cary, NC, USA) and the R environment version 4.1.1.[19]

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159 **Results**

160 **Eligible patients**

161 $\hspace{2cm}$ Among the 219 patients who underwent bronchoscopy and were tested using *Pj*-

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162   PCR during the study period, 175 were included in the study (Fig. 1). Of the 50 patients

163   diagnosed with PCP, 12% (n = 6) were diagnosed with proven PCP by identification of *P.*

164   *jirovecii* cysts, and 88% (n = 44) had probable PCP diagnosed using *Pj*-PCR as per

165   EORTC/MSGERC case definitions.[2]

166   **Patient characteristics**

167   The baseline characteristics of the patients are shown in Table 1. The most common

168   underlying diseases were malignancies, followed by systemic autoimmune diseases. Among

169   chronic lung diseases, interstitial lung disease and asthma were found in 24 (14%) and 4 (2%)

170   out of 175 patients, respectively (asthma was not included in Table 1), with no case of chronic

171   obstructive pulmonary disease. Additionally, there was only one case of bacteremia in each

172   group. Patients who were administered systemic prednisolone (PSL) ≥5 mg or methotrexate

173   (MTX) were more frequent in the PCP than in the non-PCP group. PCP prophylaxis was only

174   administered   in   the   non-PCP   group.   All   prophylactic   drugs   were

175   trimethoprim/sulfamethoxazole (TMP/SMX). No patients were treated with glucan-

176   containing gauze or were undergoing hemodialysis with cellulose-containing filters, both of

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177   which could have affected the BDG results. Laboratory test results showed that the patients

178   in the PCP group had significantly lower ALC and higher serum LDH and BDG than the

179   those in the non-PCP group.

180   **BDG values**

181   The Fungitec G-test MK results of the BDG values for the PCP and non-PCP

182   groups are shown in Fig. 2A. The median (interquartile range) serum BDG values of both

183   PCP and non-PCP patients were 146.9 (67.7–441.1) pg/ml and 11.5 (6.0–23.6) pg/ml,

184   respectively (p <0.01). In the group of patients who used albumin or immunoglobulin

185   products, the median (interquartile range) serum BDG values of both PCP and non-PCP

186   patients were 252.2 (78.3–550.4) pg/ml and 17.9 (8.2–34.4) pg/ml, respectively (p = 0.01)

187   (Fig. 2B).

188   **Accuracy of PCP diagnosis using the serum BDG level**

189   The concordance of the serum BDG level for PCP diagnosis is shown as the ROC

190   curve in Fig. 2C. These two variables were highly concordant at the AUC of 0.924. The

191   modified cut-off value by the Youden's index was 36.6 pg/ml, and the comparison of

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192   accuracy (sensitivity, specificity, etc.) with the manufacturer's defined cutoff value of 20.0

193   pg/ml is summarized in Table 2.

194   Moreover, to examine the characteristics of the association of the serum BDG level

195   with the diagnosis of PCP in patients with underlying diseases, we extracted patients with

196   malignancies and systemic autoimmune diseases including rheumatoid arthritis (RA),

197   respectively, which were the two most prevalent underlying diseases among those observed.

198   In patients with malignancies, the concordance between the serum BDG level and the

199   diagnosis of PCP was high (AUC = 0.940; Supplementary Figure S1a) in all cases. Sensitivity

200   and specificity were 100% and 81.2%, respectively, at the cut-off value of 25.8 pg/ml. In

201   patients with systemic autoimmune diseases, the AUC of BDG for diagnosing PCP was 0.873

202   (Supplementary Figure S1b), and the sensitivity and specificity were 81.8% and 88.1%,

203   respectively, at the cut-off value of 56.5 pg/ml.

204   **Factors that reduce the accuracy of BDG testing**

205   Aiming to identify disruptors in PCP diagnosis by serum BDG level using Fungitec

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 ≥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 (≥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   ≥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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221   We evaluated the diagnostic utility of serum BDG levels for non-HIV-PCP using

222   the Fungitec G-test MK assay. Patients with PCP had a significantly higher BDG value

223   than those without PCP. The best cut-off value of serum BDG to distinguish non-HIV-PCP

224   was 36.6 pg/ml, showing comparable negative LR and higher positive LR compared to the

225   manufacturer's recommended cut-off value of 20.0 pg/ml.

226   To the best of our knowledge, this is the first large-scale study aiming to evaluate

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;[7, 8] 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.[11, 20] A large-scale

234   study that recently evaluated the serum BDG test using the Fungitell assay for the diagnosis

235   of PCP in cancer patients[4] 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;[1] the colonization rate of *P. jirovecii* among

238   outpatients both during cancer chemotherapy and among patients with RA during

239   immunosuppressive therapy was reported to be high.[21, 22] 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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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 *P. jirovecii* may trigger lung

259   inflammation in patients with RA undergoing MTX treatment,[23] 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.[24, 25] 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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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 *Pj*-PCR results.[26] 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 *P. jirovecii* DNA by quantitative real-

278   time PCR (qPCR) is preferred to conventional qualitative PCR,[2] 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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281 of PCP due to the retrospective nature of the study. Finally, BDG is found in various foods,

282 thus making it impossible to evaluate patient intake of potential BDG sources,[27] as their

283 consumption may cause BDG false-positives.

284   Overall, our study showed that optimizing the serum BDG cut-off value using the

285 Fungitec G-test MK assay increased the positive LR without compromising the good

286 negative LR for diagnosing PCP in non-HIV patients. MTX could negatively affect the

287 accuracy of the BDG test for diagnosing PCP, and we should be aware of the BDG results

288 when PCP is suspected in patients using MTX. Further research is warranted to compare

289 this assay with other BDG tests and, more importantly, a prospective study is expected both

290 for calibration and validation of the cut-off value.

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390 **FIGURE LEGENDS**

391 **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.

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397 **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

402 *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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405   BDG, (1, 3)-β-D glucan. PCP, *Pneumocystis jirovecii* pneumonia. ROC, receiver operating

406   characteristic.

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408   **Figure 3.** The empirical cumulative distribution function of the degree of shift in the accuracy

409   of PSL ≥5 mg and MTX. The figures in the parentheses denote the value of *x* (i.e., the degree

410   of shift in the accuracy) and the ECDF(*x*) calculated from the observed data. The asterisks

411   denote imputation for infinity in the *x*.

412   PSL, prednisolone. MTX, methotrexate.

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420   **TABLES**

421   **Table 1.** Patient Characteristics

	Total		PCP		Non-PCP		*p*-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

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ICU admission	10	(6)	1	(2)	9	(7)	0.29

Medications:

PSL ≥5 mg	69	(39)	26	(52)	43	(34)	0.04
MTX	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[†] ≥0.5 COI	42	(39)	14	(44)	28	(36)	0.52

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GM ≥1.0 COI	20	(18)	8	(25)	12	(16)	0.28
BDG (pg/ml)	21.8	(8.2–81.4)	146.9	(67.7–441.1)	11.5	(6.0–23.6)	<0.01

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

423 [†]: GM was evaluated in 31 patients in the PCP group and 66 patients in the non-PCP group.

424 PCP: *Pneumocystis jirovecii* pneumonia; ICU: intensive care unit; PSL: prednisolone;

425 MTX: methotrexate; ALC: absolute lymphocyte count; LDH: lactate dehydrogenase; KL-6:

426 Krebs von den Lungen-6; GM: galactomannan; COI: cutoff index; BDG: (1, 3)-β-D glucan

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428 **Table 2.** Serum BDG performance for PCP diagnosis

	Modified cut-off with highest Youden's index	Manufacturer's recommended cut-off
Cut-off value (pg/ml)	36.6	20.0
Sensitivity (%)	92.0	96.0
Specificity (%)	84.8	64.8
Positive LR	6.1	2.7
Negative LR	0.09	0.06

429 BDG: (1, 3)-β-D glucan; PCP: *Pneumocystis jirovecii* pneumonia; LR: likelihood ratio

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1   Diagnostic evaluation of serum (1, 3)-β-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)-β-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 *P. jirovecii* 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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46 diagnosing PCP in non-HIV patients; however, the results should be carefully interpreted in

47 case of MTX administration.

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49 **Lay summary**

50 The Fungitec G-test MK kit for measuring serum (1, 3)-β-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.[1] 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,[2] though PCP cysts are relatively few among

66 non-HIV patients compared to HIV patients, and detecting cysts by microscopy has poor

67 sensitivity.[3, 4] Therefore, testing for *P. jirovecii* DNA by polymerase chain reaction (*Pj*-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)-β-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.[5, 6] 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.[7, 8] However, studies[7, 8] 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,[3, 9] 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 β-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.[4,]

86 [10, 11] 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.[12] In this study, we aimed to evaluate the

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

99   suspected PCP who underwent bronchoscopy and were tested with *Pj*-PCR using BW or

100   BAL fluid. Patients with HIV, as well as invasive fungal infections other than PCP, or those

101   who received empirical treatment for PCP lasting more than 3 days before *Pj*-PCR testing

102   were excluded. Patient characteristics (age, sex, underlying disease, and immunosuppressive

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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.[13-15] 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_2 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 *P. jirovecii* DNA by PCR (probable PCP) in BW or BAL fluid.[2] *Pj*-PCR was conducted by

124 SRL Inc. (Tokyo, Japan) using previously reported methods[16] 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 β-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.[17,]

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134   **Statistical analysis**

135   The difference in the distribution of values between the participants' groups was

136   evaluated by a Mann–Whitney U statistic. The p-values for the U statistic was calculated

137   from its normal approximation. The null hypothesis of independence between two binomial

138   variables was tested by a two-sided Fisher's exact test. A p-value of <0.05 was stated as

139   statistically significant. The multiplicity of hypothesis testing was not considered unless

140   otherwise stated. Statistical analyses were exploratorily conducted; therefore, the type 1 error

141   rates were not under nominal p-values.

142   The cut-off value in serum BDG levels for PCP diagnosis was determined by the

143   Youden's index. The concordance between the serum BDG levels and the PCP diagnosis was

144   evaluated as the area under the receiver-operating-characteristic (ROC) curve (AUC). Test

145   accuracy was assessed regarding sensitivity, specificity, and both positive and negative

146   likelihood ratios (positive LR and negative LR, respectively). Based on the interest to identify

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.

$$The\ degree\ of\ shift\ in\ accuracy = \log \frac{OR_{sub}}{OR_{total}}\ where$$

$$OR_{sub} = \frac{\Pr(PCP=1|BDG=1, z=1)/\Pr(PCP=0|BDG=1, z=1)}{\Pr(PCP=1|BDG=0, z=1)/\Pr(PCP=0|BDG=0, z=1)},$$

$$OR_{total} = \frac{\Pr(PCP=1|BDG=1)/\Pr(PCP=0|BDG=1)}{\Pr(PCP=1|BDG=0)/\Pr(PCP=0|BDG=0)}$$

155   (*eq.1*)

156 Statistical analyses were performed using GraphPad Prism 5, JMP®□ 13 (SAS Institute Inc.,

157 Cary, NC, USA) and the R environment version 4.1.1.[19]

158

159 **Results**

160 **Eligible patients**

161   Among the 219 patients who underwent bronchoscopy and were tested using *Pj-*

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162 PCR during the study period, 175 were included in the study (Fig. 1). Of the 50 patients

163 diagnosed with PCP, 12% (n = 6) were diagnosed with proven PCP by identification of *P.*

164 *jirovecii* cysts, and 88% (n = 44) had probable PCP diagnosed using *Pj*-PCR as per

165 EORTC/MSGERC case definitions.[2]

166 **Patient characteristics**

167 The baseline characteristics of the patients are shown in Table 1. The most common

168 underlying diseases were malignancies, followed by systemic autoimmune diseases. Among

169 chronic lung diseases, interstitial lung disease and asthma were found in 24 (14%) and 4 (2%)

170 out of 175 patients, respectively (asthma was not included in Table 1), with no case of chronic

171 obstructive pulmonary disease. Additionally, there was only one case of bacteremia in each

172 group. Patients who were administered systemic prednisolone (PSL) ≥5 mg or methotrexate

173 (MTX) were more frequent in the PCP than in the non-PCP group. PCP prophylaxis was only

174 administered in the non-PCP group. All prophylactic drugs were

175 trimethoprim/sulfamethoxazole (TMP/SMX). No patients were treated with glucan-

176 containing gauze or were undergoing hemodialysis with cellulose-containing filters, both of

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177 which could have affected the BDG results. Laboratory test results showed that the patients

178 in the PCP group had significantly lower ALC and higher serum LDH and BDG than the

179 those in the non-PCP group.

180 **BDG values**

181   The Fungitec G-test MK results of the BDG values for the PCP and non-PCP

182 groups are shown in Fig. 2A. The median (interquartile range) serum BDG values of both

183 PCP and non-PCP patients were 146.9 (67.7–441.1) pg/ml and 11.5 (6.0–23.6) pg/ml,

184 respectively (p <0.01). In the group of patients who used albumin or immunoglobulin

185 products, the median (interquartile range) serum BDG values of both PCP and non-PCP

186 patients were 252.2 (78.3–550.4) pg/ml and 17.9 (8.2–34.4) pg/ml, respectively ($p = 0.01$)

187 (Fig. 2B).

188 **Accuracy of PCP diagnosis using the serum BDG level**

189   The concordance of the serum BDG level for PCP diagnosis is shown as the ROC

190 curve in Fig. 2C. These two variables were highly concordant at the AUC of 0.924. The

191 modified cut-off value by the Youden's index was 36.6 pg/ml, and the comparison of

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192 accuracy (sensitivity, specificity, etc.) with the manufacturer's defined cutoff value of 20.0

193 pg/ml is summarized in Table 2.

194   Moreover, to examine the characteristics of the association of the serum BDG level

195 with the diagnosis of PCP in patients with underlying diseases, we extracted patients with

196 malignancies and systemic autoimmune diseases including rheumatoid arthritis (RA),

197 respectively, which were the two most prevalent underlying diseases among those observed.

198 In patients with malignancies, the concordance between the serum BDG level and the

199 diagnosis of PCP was high (AUC = 0.940; Supplementary Figure S1a) in all cases. Sensitivity

200 and specificity were 100% and 81.2%, respectively, at the cut-off value of 25.8 pg/ml. In

201 patients with systemic autoimmune diseases, the AUC of BDG for diagnosing PCP was 0.873

202 (Supplementary Figure S1b), and the sensitivity and specificity were 81.8% and 88.1%,

203 respectively, at the cut-off value of 56.5 pg/ml.

204 **Factors that reduce the accuracy of BDG testing**

205   Aiming to identify disruptors in PCP diagnosis by serum BDG level using Fungitec

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.).

219

220   **Discussion**

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221   We evaluated the diagnostic utility of serum BDG levels for non-HIV-PCP using

222   the Fungitec G-test MK assay. Patients with PCP had a significantly higher BDG value

223   than those without PCP. The best cut-off value of serum BDG to distinguish non-HIV-PCP

224   was 36.6 pg/ml, showing comparable negative LR and higher positive LR compared to the

225   manufacturer's recommended cut-off value of 20.0 pg/ml.

226   To the best of our knowledge, this is the first large-scale study aiming to evaluate

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;[7, 8] 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.[11, 20] A large-scale

234   study that recently evaluated the serum BDG test using the Fungitell assay for the diagnosis

235   of PCP in cancer patients[4] 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;[1] the colonization rate of *P. jirovecii* among

238   outpatients both during cancer chemotherapy and among patients with RA during

239   immunosuppressive therapy was reported to be high.[21, 22] 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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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 *P. jirovecii* may trigger lung

259 inflammation in patients with RA undergoing MTX treatment,[23] 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.[24, 25] 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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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 *Pj*-PCR results.[26] 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 *P. jirovecii* DNA by quantitative real-

278 time PCR (qPCR) is preferred to conventional qualitative PCR,[2] 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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281 of PCP due to the retrospective nature of the study. Finally, BDG is found in various foods,

282 thus making it impossible to evaluate patient intake of potential BDG sources,[27] as their

283 consumption may cause BDG false-positives.

284   Overall, our study showed that optimizing the serum BDG cut-off value using the

285 Fungitec G-test MK assay increased the positive LR without compromising the good

286 negative LR for diagnosing PCP in non-HIV patients. MTX could negatively affect the

287 accuracy of the BDG test for diagnosing PCP, and we should be aware of the BDG results

288 when PCP is suspected in patients using MTX. Further research is warranted to compare

289 this assay with other BDG tests and, more importantly, a prospective study is expected both

290 for calibration and validation of the cut-off value.

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297   **References**

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390   **FIGURE LEGENDS**

391   **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.

396

397   **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

402   *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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405   BDG, (1, 3)-β-D glucan. PCP, *Pneumocystis jirovecii* pneumonia. ROC, receiver operating

406   characteristic.

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408   **Figure 3.** The empirical cumulative distribution function of the degree of shift in the accuracy

409   of PSL ≥5 mg and MTX. The figures in the parentheses denote the value of x (i.e., the degree

410   of shift in the accuracy) and the ECDF(x) calculated from the observed data. The asterisks

411   denote imputation for infinity in the x.

412   PSL, prednisolone. MTX, methotrexate.

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420   **TABLES**

421   **Table 1.** Patient Characteristics

	Total		PCP		Non-PCP		*p*-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

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ICU admission	10	(6)	1	(2)	9	(7)	0.29
Medications:							
PSL ≥5 mg	69	(39)	26	(52)	43	(34)	0.04
MTX	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[†] ≥0.5 COI	42	(39)	14	(44)	28	(36)	0.52

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GM ≥1.0 COI	20	(18)	8	(25)	12	(16)	0.28
BDG (pg/ml)	21.8	(8.2–81.4)	146.9	(67.7–441.1)	11.5	(6.0–23.6)	<0.01

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

423 †: GM was evaluated in 31 patients in the PCP group and 66 patients in the non-PCP group.

424 PCP: *Pneumocystis jirovecii* pneumonia; ICU: intensive care unit; PSL: prednisolone;

425 MTX: methotrexate; ALC: absolute lymphocyte count; LDH: lactate dehydrogenase; KL-6:

426 Krebs von den Lungen-6; GM: galactomannan; COI: cutoff index; BDG: (1, 3)-β-D glucan

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428 **Table 2.** Serum BDG performance for PCP diagnosis

	Modified cut-off with highest Youden's index	Manufacturer's recommended cut-off
Cut-off value (pg/ml)	36.6	20.0
Sensitivity (%)	92.0	96.0
Specificity (%)	84.8	64.8
Positive LR	6.1	2.7
Negative LR	0.09	0.06

429 BDG: (1, 3)-β-D glucan; PCP: *Pneumocystis jirovecii* pneumonia; LR: likelihood ratio

430

Fig. 1

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

Excluded: (n = 44)
- Empiric therapy for PCP more than 3days before *Pj*-PCR test (n = 15)
- Invasive fungal infections (n = 15)
  - CPA (n = 6)
  - IPA (n = 4)
  - Candidemia (n = 3)
  - Pulmonary cryptococcosis (n = 1)
  - Pulmonary *Lomentospora prolificans* infection (n = 1)
- HIV infection (n = 12)
- No available data (n = 2)

175 patients were enrolled

PCP patients
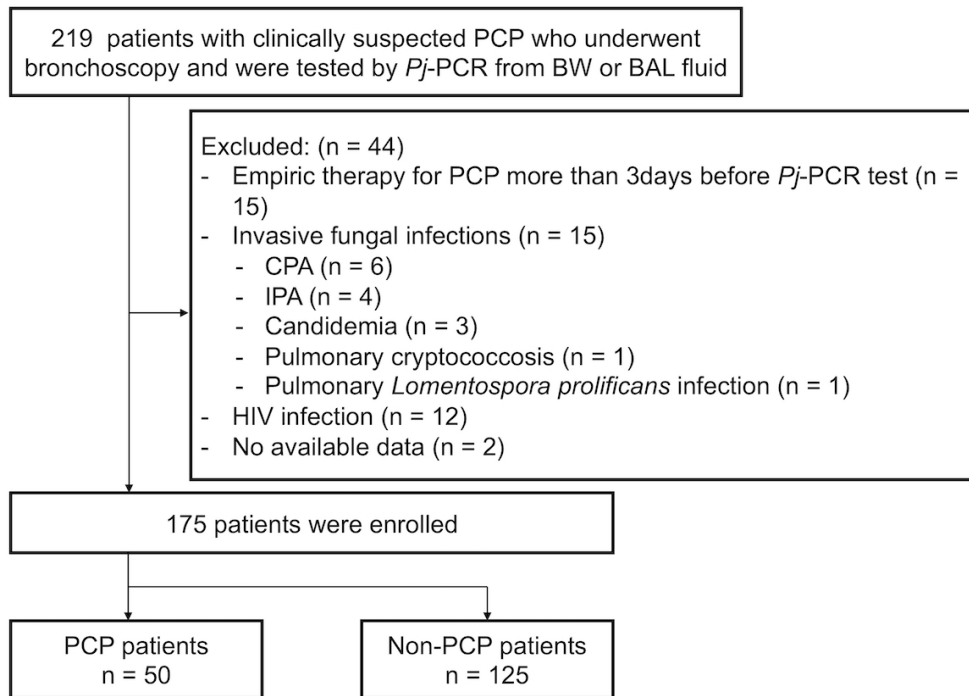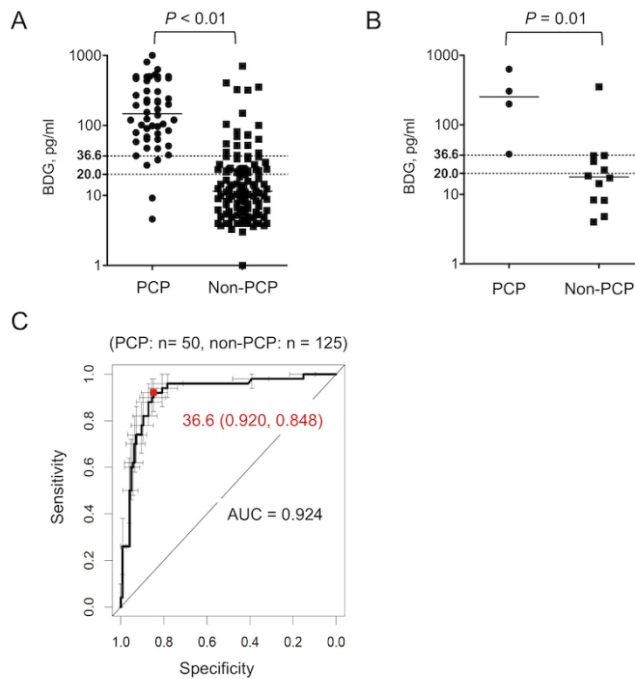n = 50

Non-PCP patients
n = 125

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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Fig. 2

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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Fig. 3

PSL ≥ 5 mg

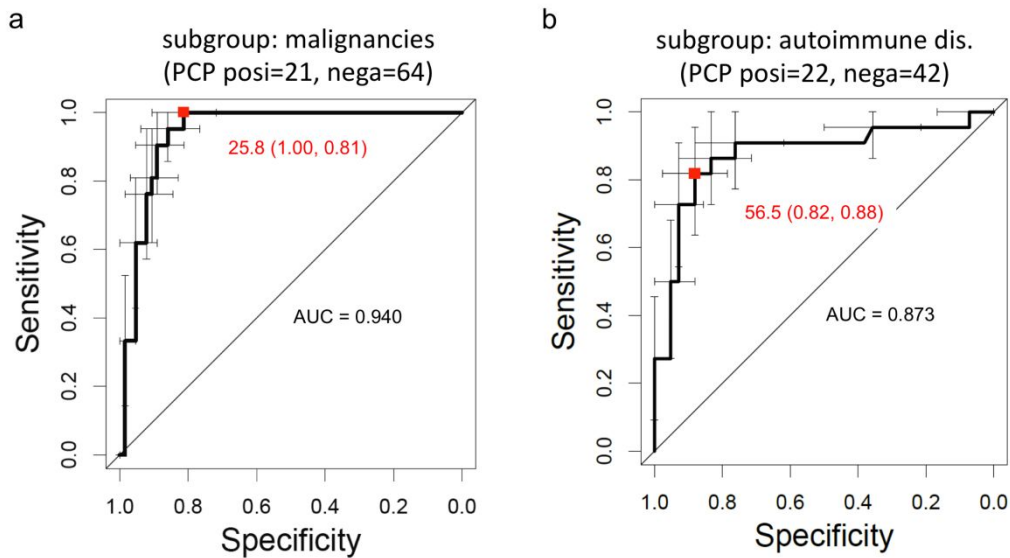(-0.602, 0.134)

MTX

(-1.463, 0.06)

degree of shift in accuracy
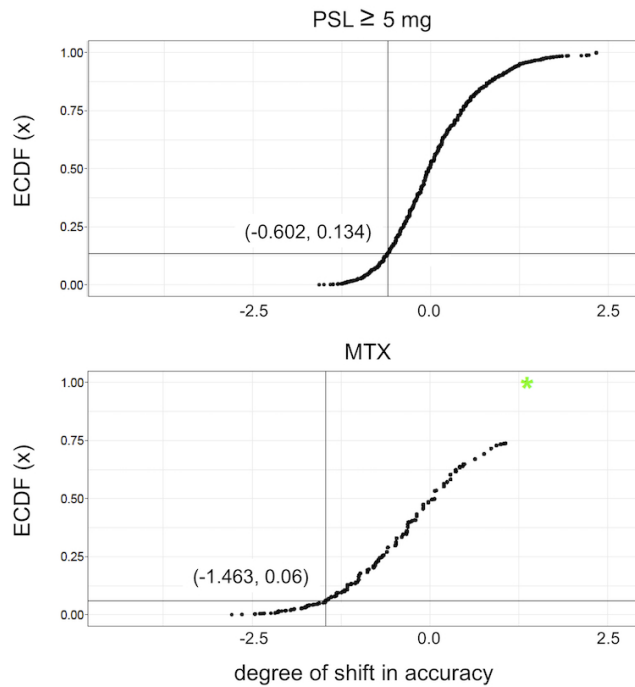
The empirical cumulative distribution function of the degree of shift in the accuracy of PSL ≥5 mg and MTX.
The figures in the parentheses denote the value of x (i.e., the degree of shift in the accuracy) and the
ECDF(x) calculated from the observed data. The asterisks denote imputation for infinity 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.

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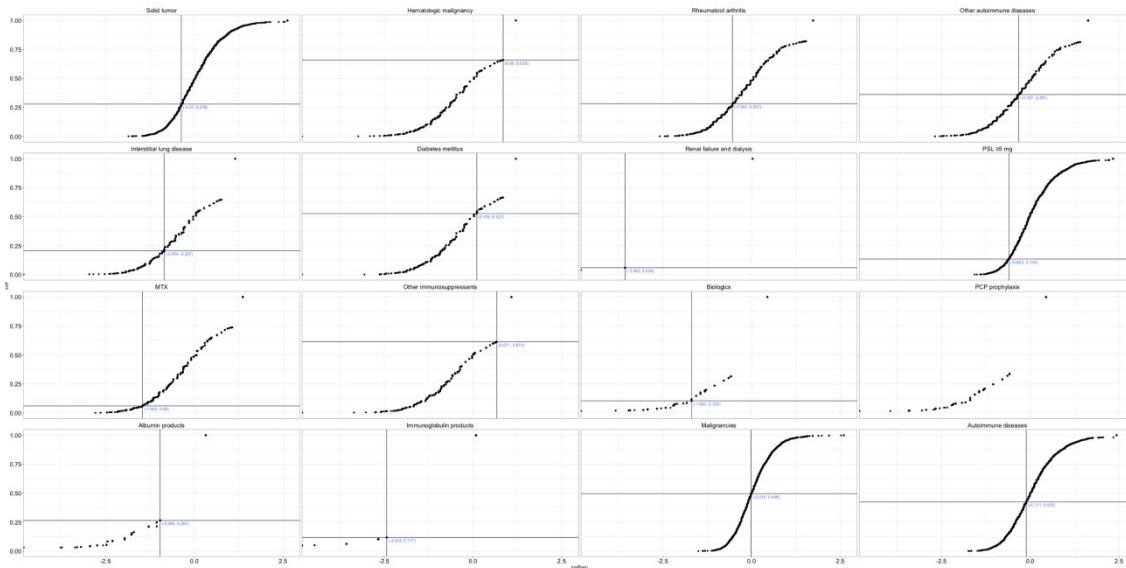
Figure S2. The empirical cumulative distribution function of the degree of shift in accuracy. The figures in the parentheses denote the value of *x* (i.e., the degree of shift in accuracy) and the ECDF(*x*) calculated from the observed data. The asterisks denote imputation for infinity in the *x*.

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

	False positive		Negative		*P* value
	(n = 19)		(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 ≥ 5 mg	8	(42)	35	(33)	0.44

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	1	(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‡ ≥ 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.

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

	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 *Pj*-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 *Pj*-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

| Case 15 | 404.9 | No treatment |

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