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1 **Prospective comparison of (1,3)-beta-D-glucan detection using**
2 **colorimetric and turbidimetric assays for diagnosing invasive**
3 **fungal disease**

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18
19 **Short Title:** Prospective comparison of colorimetric and turbidimetric (1→3)-β-D-glucan assays

20 **Keywords:** (1→3)-β-D-glucan, colorimetric assay, turbidimetric assay, invasive fungal disease

21
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26 **Abstract**

27 Serum (1→3)-β-D-glucan (BDG), an antigen that is common to several fungi, is detected in
28 some invasive fungal diseases (IFDs). Colorimetric or turbidimetric detection is the basis of
29 two different commercial kits, the Fungitell assay (FA) and the Wako assay (WA), and we
30 compared the WA to the FA.

31 We used both assays over a 4-month period to prospectively test 171 patients (sex ratio M/F,
32 60%; mean age, 48±16 years) who mainly had haematological conditions (62%) and who
33 experienced episodes (n=175) of suspected IFI. The tests were performed according to the
34 manufacturers' recommendations.

35 Twenty-three episodes due to BDG-producing fungi were diagnosed (pneumocystosis, n=12;
36 invasive aspergillosis, n=5; candidemia, n=3; invasive fusariosis, n=2; hepato-splenic
37 candidiasis, n=1). Both assays provided similar areas under the receiver operating
38 characteristic curves (AUC=0.9). Using the optimised positivity thresholds (≥120 pg/ml for FA
39 and ≥4 pg/ml for WA), the sensitivity and specificity were 81.8% (95% confidence interval CI:
40 61.5–92.7) and 94.8% (95%CI: 90.1–97.3) for FA and 81.8% (95%CI: 61.5–92.7), 95.4%
41 (95%CI: 90.9–97.8) for WA. Negative predictive value was 97.3% (95%CI: 93.3–99.0) for both
42 tests. If the manufacturer's positivity threshold (≥11 pg/ml) was applied, the WA sensitivity
43 decreased to 50%. Among 71 patients with bacterial infections, 21.1% were FA-positive (≥80
44 pg/ml) and 5.6% were WA-positive (≥11 pg/ml; $p < 10^{-2}$).

45 The WA performed similarly compared to the FA when an optimised cut-off was used to
46 diagnose IFD. The WA is a single sample test that is clinically relevant when a prompt
47 therapeutic decision is required.

48

49 **Lay summary**

50 Serum (1→3)-β-D-glucan testing is dominated by two kits including Fungitell colorimetric assay
51 (FA) and the Wako turbidimetric assay (WA). We compared them prospectively and observed
52 that they perform similarly when selecting their optimal threshold (≥120 pg/ml for FA and ≥4
53 pg/ml for WA).

54

55

56 INTRODUCTION

57 The prognosis of invasive fungal diseases (IFD) is still worrisome and one of the reasons for
58 this is the poor performance of microbiological tools, and more particularly for an early
59 diagnosis.^{1,2} Because the clinical and imaging signs of IFDs are not specific, therapy is either
60 delayed or prescribed on a prophylaxis or empirical schedule, which risks potential side effects,
61 the possibility of resistance selection, and/or increased costs.³ Thus, biomarkers of IFD are
62 required to improve the use of antifungal drugs.^{4,5}

63 Among the biomarkers, the fungal antigens galactomannan (GM) and (1→3)-β-D-
64 glucan (BDG) have shown sufficient efficacy to be included in the diagnostic criteria of IFD.⁶
65 While GM is more specific to *Aspergillus* spp. infection detection, BDG is produced by a wide
66 variety of medically important fungi, with the exception of Mucorales and *Cryptococcus* spp.⁷
67 Indeed, *Cryptococcus* spp. do produce BDG which can be detected in cerebrospinal fluid in
68 cryptococcal meningitis but in a too little quantity to be detected in serum.⁸ BDG assay
69 performance is especially good for the diagnosis of *Pneumocystis* pneumonia (PCP), with a
70 pooled sensitivity and specificity of 95%–96% and 84%–86%, respectively.⁹ Thus, specific
71 recommendations for PCP diagnosis have been proposed in patients with haematological
72 malignancies using the BDG results.¹⁰ The assay performance for the other IFDs, not including
73 PCP, is lower and has greater heterogeneity, which is between 67%–84% and 79%–90 for the
74 pooled sensitivity and specificity, respectively.⁷

75 In addition to the differences between the IFDs and the populations that are at risk for
76 IFDs, an additional variability factor is that there are several different commercial assays for
77 BDG, in contrast to GM detection which has been widely performed using a unique assay.¹¹
78 For BDG detection, at least five assays have been released for diagnostic use, as follows:
79 Fungitell (Associates of Cape Cod, Inc., East Falmouth, MA, USA), B-Glucan Test Wako
80 (FUJIFILM Wako Chemicals, Osaka, Japan), Fungitec G-Test MK (Seikagaku Corporation,
81 Kogyo, Tokyo, Japan), B-G Star (Maruha Corporation, Osaka, Japan or Maruha-Nichiro,
82 Foods Inc., Tokyo, Japan), and Dynamiker Fungus (Dynamiker Biotechnology Co., Ltd, Tianjin,
83 China).¹² Fungitell assay (FA) was approved in the United States in 2004,¹³ whereas the Wako
84 assay (WA) only recently received European marketing approval. The difference between FA
85 and WA is that they rely on the horseshoe crab as the source of the amoebocyte lysate, which
86 is from *Limulus polyphemus* (FA) or *Tachypleus tridentatus* (WA), and the mode of revelation,
87 colorimetric (FA) or turbidimetric (WA), is also different between the tests. This results in
88 different reactivity for BDG detection and cut-offs that define a positive test result, as follows:
89 ≥80 pg/ml for FA and ≥11 pg/ml for WA, according to the manufacturers.

90 The recent availability of WA has stimulated comparative studies with FA, first in 2011¹⁴
91 and in more recent years.¹⁵⁻¹⁸ These comparative studies were performed on archived serum

92 samples that were selected to diagnose PCP,¹⁵ candidaemia,¹⁶ or several IFDs,^{14,17,18} and
93 selected controls have also been used. In contrast to these retrospective studies, we
94 performed a prospective comparison of the analytical capacities of the two assays to evaluate
95 the feasibility of their routine use.

96

97 **MATERIALS AND METHODS**

98 *Patients and definitions*

99 All the BDG requests by the clinicians from December 21, 2018 to April 23, 2019 were included
100 without selection on the basis of the underlying disease. The classification as proven, probable,
101 or no-IFD was based on criteria from the European Organisation for Research and Treatment
102 of Cancer and from the Invasive Fungal Infections Cooperative Group and the Mycoses Study
103 Group Education and Research Consortium (EORTC/MSGERC).⁶ For invasive mould
104 infections, the medical files were analysed to classify the patients according to our local
105 committee, as was previously reported.¹⁹ FA results were transmitted to the clinicians as part
106 of the routine work-up for an IFD diagnosis. WA tests were performed by two of the authors
107 who were blinded to the routine FA results. Because the results were part of an evaluation,
108 WA results were not transmitted to the clinicians to avoid any interference with the clinical
109 decision. Because some patients were hospitalised several times and tested each time for
110 suspicion of IFD, we defined different episodes of BDG as being tested at least 30 days apart
111 during two different hospitalisations. The first positive serum per episode was selected for
112 analysis.

113

114 *Test realisation*

115 Both assays were performed according to the manufacturers' recommendations on the same
116 day. For the FA, 5 µl of serum was added to 20 µl of pre-treatment reagent in a glucan-free
117 96-well plate. After 10 min at 37°C, 100 µl of Fungitell reagent was added to the pre-treated
118 sample and the kinetic colorimetric results were followed for 40 min at 37°C using a Multiskan
119 photometer (Thermo Fisher Scientific, Waltham, MA, USA). In parallel, a standard curve was
120 established using four 1/2 serial dilutions of a 100-pg/ml standard solution that was provided
121 in the kit. The mean change in the absorbance over time was calculated and the titres were
122 expressed based on the standard curve. The test was performed in duplicate and the result
123 was the mean of the duplicates. The results were considered to be positive when the result
124 was ≥80 pg/ml. Overflow results preventing reliable absolute quantification were assigned a
125 value of ≥500 pg/ml. When the results were discordant and associated with a coefficient of
126 variation (CV) of the duplicates above 20%, the test was repeated in duplicate, and the first
127 results were discarded. Of note, high concentrations of hyperbilirubinemia and
128 hypertriglyceridemia are known to cause false negative results.²⁰

129

130 For the WA, 900 µl of pre-treatment buffer were added to 100 µl of serum, heated at
131 70°C for 10 min, and cooled on ice. Then, 200 µl of this pre-treated sample were added to a
132 vial containing freeze-dried *Limulus* amoebocyte lysate. The speed of the increase in the
133 turbidity of the sample was then continuously measured over 90 min using a dedicated MT-
134 6500 toxinometer (FUJIFILM Wako Chemicals, Osaka, Japan). The pre-set calibration curve
135 that was provided by the manufacturer for each reagent lot was used for quantification. The
136 positivity threshold was ≥ 11 ng/ml and the manufacturer does not recommend retesting
137 positive samples. No significant interference with WA were reported as mentioned by the
138 manufacturer.

139

140 *Statistical analysis*

141 The mycological diagnosis was established using microscopy, culture, GM, and diagnostic
142 quantitative PCR results for PCP,²¹ no matter what the BDG result was that was transmitted
143 to the clinician. Therefore, the BDG performance was calculated without integrating the BDG
144 result, except for the diagnosis of hepato-splenic candidiasis where the BDG result was used
145 as a criterion for establishing the diagnosis (both FA and WA were positive with 275.2 and
146 11.89 pg/ml, respectively). Diagnostic performances of both FA and WA tests were calculated
147 for patients with invasive aspergillosis, candidiasis (including one case of hepato-splenic
148 candidiasis), fusariosis, and PCP. A receiver operating characteristic (ROC) curve was drawn
149 for each assay, and the area under the curve (AUC) was calculated. The best Youden index
150 indicated the optimal diagnostic threshold. Sensitivity and specificity of both assays were
151 compared using McNemar's chi-square test. To determine the correlation between the BDG
152 titres, we performed linear regression on samples ranging from 31 to 500 pg/ml for FA and
153 from 2.359 to 600 pg/ml for WA. All statistical analyses were performed using Prism v9.0
154 (GraphPad software). $P < 0.05$ was considered to be significant).

155

156 *Ethics Statement*

157 The study was a non-interventional evaluation of a new test with no change in the usual
158 procedures, and the clinicians were blinded to the evaluated test results. The clinical data were
159 collected and registered with the approval of our hospital review committee (reference number:
160 2018000000077). French Public Health Law (CSP Art L1121-1.1) does not require specific
161 approval from an ethics committee for this kind of study, which is exempt from the requirement
162 for informed consent. The 2015 STARD guidelines for diagnostic accuracy studies were
163 followed.²²

164

165

166 **RESULTS**

167 We prospectively tested 321 serum samples from 171 patients (sex ratio M/F, 102/69 [1.48];
168 mean age, 48±16 years) who mainly had haematological conditions (62%; 106/171) using both
169 assays. Four patients were tested during two different hospitalisations that were more than 30
170 days apart (two before and after allogeneic human stem cell transplantation and two during
171 two different chemotherapy courses). Thus, the analyses were performed considering 175 IFD
172 suspicion episodes with a median of one [range, 1–11] sample per episode. There were 25
173 IFD episodes (PCP n=12; invasive aspergillosis n=5; candidemia n=3; invasive fusariosis n=2;
174 hepato-splenic candidiasis n=1; mucormycosis n=1; and cryptococcosis n=1) (Table 1). The
175 mucormycosis and cryptococcosis cases were BDG-negative with both assays. Because BDG
176 is known for not being produced at detectable levels in serum by Mucorales and *Cryptococcus*
177 spp.,⁷ these two cases were not included to calculate the performance of the BDG tests.

178 When considering one sample/episode, the AUC of the two ROC curves was similar
179 (AUC, 0.9) for both assays (Figure 1). Thus, the sensitivity, specificity, positive predictive value
180 (PPV), and negative predictive value (NPV), and positive likelihood ratio (PLR), were as follows
181 for all patients when using a cut-off for positivity of ≥120 pg/ml for FA (Youden = 76.6%) and ≥
182 4 pg/ml for WA (Youden = 77.3%): 81.8% (95% confidence interval [CI]: 61.5–92.7), 94.8%
183 (95%CI: 90.1–97.3), 69.2% (95CI%: 50.0–83.5), 97.3 (95%CI: 93.3–99.0), and 15.75 for FA
184 and 81.8% (95%CI: 61.5–92.7), 95.4% (95%CI: 90.9–97.8), 72% (95%CI: 52.4–85.7), 97.3%
185 (95%CI: IC93.4–99.0), and 18 for WA, respectively. The detailed sensitivities and specificities
186 associated with specific IFD and the optimal thresholds found in this study are presented in
187 Table 2. Results between FA and WA are not very different with WA harbouring a better
188 specificity and with IA being associated with the lowest sensitivity (Table 2).

189 When using the positivity threshold that is recommended by the manufacturers (≥80
190 pg/ml for FA and ≥11 pg/ml for WA), the specificity of FA slightly decreased to 90.9% (95%CI:
191 85.3–94.5) but the sensitivity was unchanged (Youden = 72.7%, PLR = 9), and the sensitivity
192 of WA was significantly lower at 50% (95%CI: 30.7–69.28, p=0.007) but the specificity was
193 very similar 96.7 (95%CI: 92.6–98.6) (Youden = 46.7%, PLR = 15.4) (Figure 2). Sensitivities
194 and specificities associated with the optimal thresholds found in this study in various IFD are
195 presented in Table 2.

196 Overall, among the 321 serum samples, 71 (22.12%) were from patients with a final
197 diagnosis of bacterial infection and 209 (65.1%) were obtained from patients without evidence
198 of bacterial infection. Among the 71 samples from patients with bacterial infections, 15 samples
199 (21.1%) were FA-positive (≥80 pg/ml) and four (5.6%) were WA-positive (≥11 pg/ml) (p<10⁻²).
200 The difference was not significant anymore if the 4-pg/ml WA threshold was used (p=0.11).
201 Among these 15 FA-positive samples, seven (47%) were also WA-positive (of whom four were
202 ≥11 pg/ml). Among the 209 samples from patients with no evidence of bacterial infection, ten

203 (4.8%) had a FA of ≥ 80 pg/ml (of whom seven were ≥ 120 pg/ml) and only one (0.5%) was WA-
204 positive > 11 pg/ml ($p < 10^{-2}$). This WA-positive sample was not FA-positive (76 pg/ml), and the
205 result was < 3.7 pg/ml after retesting.

206 Multiple serum samples (209 samples; median/episode, 2; range, 2–11) were obtained
207 for 63 episodes, mainly (88.9%) from haematology patients. Fifty episodes (168 samples) were
208 FA and WA-negative, corresponding to no IFD episodes except for one case of mucormycosis
209 in a haematology patient and one case of cryptococcosis in an AIDS patient. The remaining
210 41 samples corresponded to samples that were drawn to confirm a first positive result or to
211 follow-up on the effectiveness of the treatment. However, the numbers were too low and the
212 sampling over time was too irregular to allow for a useful comment.

213

214 *Coefficient of correlation*

215 The correlation coefficient was calculated based on the 30 specimens with $7.8 < \text{FA} < 500$ and
216 $\text{WA} > 2.359$ pg/ml, which prevented the inclusion of specimens with a quantification that was
217 outside the linearity of both tests. The r^2 calculated from the correlation between FA and WA
218 was 0.357, and it was significantly different from zero ($p < 10^{-3}$) (Figure 3).

219

220 *Retesting*

221 According to the FA manufacturer's recommendations, we retested 38/321 (11.8%) samples
222 that showed discrepant duplicate results ($\text{CV} > 20\%$) with 33 of these turning negative (both
223 duplicates < 80 pg/ml) and five remaining positive (> 80 pg/ml). Arbitrarily, the values that were
224 retained to calculate the performance were the retested values.

225 Although the WA manufacturer does not recommend retesting, we checked the 34 WA
226 samples that were ≥ 4 pg/ml. The samples were stored at -20°C and the second test was
227 performed after thawing and with a different WA batch. Among the 16 samples that were
228 between ≥ 4 and < 11 pg/ml on the first test run, two (12.5%) became ≥ 11 pg/ml (one bacterial
229 infection, one PCP) and two (12.5%) became < 4 pg/ml (one bacterial infection, one PCP),
230 whereas 12 samples (75%) remained in the same range. Among the 16 samples that were
231 ≥ 11 pg/ml on the first test, two (12.5%) became < 4 pg/ml (one bacterial infection and one with
232 no infection), one (6.25%) became between ≥ 4 and < 11 pg/ml (tested at 12.2, retested at 9.4;
233 one bacterial infection), and 13 (81.25%) remained ≥ 11 pg/ml.

234

235 **DISCUSSION**

236 Our study is the first prospective study that compared the turbidimetric detection of BDG using
237 WA and colorimetric detection using FA for samples that were routinely sent by clinicians for
238 BDG testing without focusing on a specific IFD or a specific population at risk for IFD. Such
239 analysis had been previously done in retrospective studies that were performed on serum

240 samples from selected patients with a previous diagnosis of IFD.¹⁵⁻¹⁸ The present AUCs
241 showed overall similar results for the two assays, as already reported.^{14,15,17} When considering
242 the clinical episodes, the diagnostic performance was highly dependent on the threshold of
243 positivity. Using the manufacturers' thresholds (≥ 80 pg/ml for FA; ≥ 11 pg/ml for WA), FA was
244 more sensitive than WA (81.8% vs. 50%; $p=0.0002$) but less specific (90.9% vs. 96.7%,
245 $p=0.0002$), as was already reported.^{14,15,17} When using optimised thresholds according to the
246 ROC curves (≥ 120 pg/ml for FA and ≥ 4 pg/ml for WA), the sensitivity of both assays was similar
247 (sensitivity 81.8% vs. 81.8%; specificity 94.8% vs. 95.4%, $p>0.05$, for FA and WA,
248 respectively).

249 The issue of thresholds is well known to be the key to evaluating serological assays.
250 Mercier et al. performed a retrospective study on 116 PCP cases (both HIV-infected and HIV-
251 non-infected patients) and 114 controls.¹⁵ Due to the retrospective design of their study,
252 calculating the predictive values involved simulating the PCP prevalence. When choosing a
253 prevalence of 20% for PCP to test broncho-alveolar lavage fluids, which is close to what is
254 observed in our hospitals,²¹ the WA NPV was better with their modified cut-off value of 3.616
255 pg/ml.¹⁵ In another retrospective study that included 120 candidemia and 200 bacteraemia
256 samples as a control group, the optimal cut-off value was ≥ 3.8 pg/ml for WA.¹⁶ Very recently,
257 Zubkowicz et al. proposed a threshold at 4.1 pg/ml.¹⁸ These optimised cut-offs are close to our
258 threshold of 4 pg/ml that was observed after our AUC analysis. However, this threshold is lower
259 than the 7pg/ml cut-off that was proposed by some authors based on a retrospective analysis
260 of selected IFD patients.¹⁷ In our study, a cut-off at 7 pg/ml gives a sensitivity of 72.3% and a
261 specificity of 96.1% with a positive likelihood ratio at 18.7 (see supplemental Table 2). Some
262 of this difference between the proposed optimal threshold could be due to the selection of
263 patients with a well-defined IFD,¹⁷ whereas patients were enrolled prospectively during a
264 period studied by Friedrich et al.¹⁶ and in the present study. Selecting patients with a well-
265 defined diagnosis avoids borderline patients in whom high titres could be less frequent.
266 However, the low specificity of a test can be overlooked if the goal is to exclude the diagnosis
267 and rely on the NPV, which was excellent in the present evaluation (97.3% for both FA and
268 WA).

269 On the other hand, a better specificity can avoid useless antifungal treatments that
270 have potential side effects and microbiological pressure leading to acquired resistance of the
271 fungi. In samples from patients with bacterial infection, we observed a 21.1% and 5.6% false-
272 positivity rate for FA and WA, respectively, which is slightly higher than the 15% and 2% false-
273 positivity rate that was observed by Friedrich et al., mainly in the bacteraemia group.¹⁶
274 However, it is always difficult to ascertain that the patients with false-positive BDG results had
275 no IFD at all in the absence of a standardised work-up to look for IFD. False WA and FA
276 positivity was mainly observed simultaneously in the same serum samples, which suggests a

277 common source of false positivity that is probably due to bacterial antigens.¹⁵ However, among
278 patients with no bacterial infection, 4.8% were FA-positive but WA-negative in our study, which
279 suggests than some causes that are responsible for false FA positivity are not encountered
280 with WA, and possibly vice versa.

281 In our prospective study, BDG testing was not systematic but requested upon
282 suspicion. This potentially result in an increased pre-test probability which can result in an
283 artificial increased performance. Indeed, if a biomarker is tested in a population in which the
284 prevalence of the disease is lower that 5%, the performance of the test will statistically
285 decrease.²³ The number of IFD analysed in our study is quite small, 25 out of 175 (14.2%)
286 suspicions of IFD episode, which is a limit regarding the performance obtained from both tests.
287 However, as a prospective study on a limited time in an era where clear indication of testing
288 exists, 14% of IFD episodes in at risk patients is already a high rate of IFD.²⁴

289 The FA positivity threshold could also be discussed. A positive threshold at 120 pg/ml
290 improves the specificity of the test without much impact on the sensitivity (94.81 vs. 90.9,
291 $p>0.05$). Other authors have already proposed higher FA thresholds for the diagnosis of
292 invasive candidiasis such as 146 pg/ml,²⁵ 105 pg/ml,¹⁷ or 350 pg/ml.²⁶ For PCP, relying on a
293 single positivity threshold no matter which patient population is being tested can be misleading,
294 and this is particularly observed in HIV-negative haematological patients.²⁷ However, it is
295 difficult to propose different cut-offs according to the IFD before knowing the diagnosis.

296 If the area under the ROC curves was similar in the present study between FA and WA,
297 we obtained a poor coefficient of correlation ($r^2= 0.357$), which was lower than the previously
298 reported results.¹⁴⁻¹⁷ However, to obtain the coefficients of correlation, the other authors diluted
299 the samples with FA values >500 mg/ml, and retested them. We did not perform the tests in a
300 similar manner, and we excluded the overflow values. Another possible reason for the low
301 coefficient of correlation is the high CV of FA, which can impact the comparison. We retested
302 11.8% of the serum because of a FA CV of $>20\%$, which indicated discordant qualitative results
303 between the two duplicates. This figure is much higher than the 3% of sample retesting that
304 occurs for PCP diagnosis,¹⁵ but it is similar to the 10% to 15% of the samples that were retested
305 for a candidaemia diagnosis.¹⁶ Although it is not recommended by the WA manufacturer, we
306 retested every sample with a WA result >4 pg/ml, and we observed some discrepancies, which
307 were mainly in patients without IFD. The causes of these discrepancies should be investigated,
308 knowing that freeze–thaw cycles do not impact BDG values, at least using FA.²⁸ When we
309 removed for the coefficient of correlation calculation the two WA-positive (>11 pg/ml) samples
310 which turned negative (<4 pg/ml) after retesting, the coefficient of correlation considerably
311 improved (r^2 from 0.357 to 0.865), which strongly suggests that these results were true WA-
312 specific false-positives. The issue of retesting should then be resolved because of cost issues
313 and to improve the speed of the reporting to the clinicians. Another possibility to limit false-

314 positive results is to test at least two sequential serum samples. When focusing on patients
315 with haematology malignancies, which is the group with the highest prevalence of IFD, a meta-
316 analysis showed that two positive consecutive tests had better performance (sensitivity and
317 specificity between 34.0%–65.3% and 97.4%–99.5%, respectively) than one positive test.²⁹

318 Based on the implementation of routine laboratory testing, there are large differences
319 between the two assays. FA has to be performed on a large series to decrease the cost; the
320 larger the series, the less expensive are the tests. This could delay reporting to a clinician if
321 there is a wait until there are enough samples to fill the ELISA plate. FA is also performed
322 using a low volume (5 µl versus 100 µl for WA), which is associated with pipetting error when
323 different technicians perform the test in a routine laboratory. In contrast, WA is a single test
324 that is better adapted for testing on demand, especially for a rapid PCP diagnosis. A new FA
325 format allowing testing Fungitell STAT Assay (Associates of Cape Code Inc.) of one patient at
326 a time is now commercially available and FDA approved to better adapt to clinicians'
327 requirements.³⁰

328 Although this is the first prospective study, we acknowledge some limitations due to a
329 non-formalised format without regular and similar sampling between patients. Therefore, the
330 false-positive results should be interpreted with caution knowing that the extensive work-up for
331 diagnosing IFD was not homogenous between the patients, that bacterial and fungal diseases
332 are often concomitant,²⁰ and that such patients receive numerous drugs, which could be a
333 source of false positivity.^{31,32} For the same reasons, we cannot comment on the follow-up
334 evaluation of the antifungal efficacy.³³ The frequent overflow FA values are less convenient for
335 the follow-up evaluation, unless a new test is performed after dilution, whereas the WA directly
336 provides a wide range of values easy to follow. We could not assess whether performing a
337 second test on sequential samples would result in a better performance of the assays
338 either.^{25,34} However, when no IFDs were reported at the end of the hospitalisation, both assay
339 results remained negative for several samples over time in the present study, which suggests
340 a high NPV.

341 In conclusion, both assays performed similarly but only when an optimised WA cut-off
342 was used, which was at 4 pg/ml. Thus, we believe that the WA is a single-sample assay that
343 is clinically relevant when a prompt therapeutic decision is required.

344

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346 We acknowledge all the clinicians who cared for the patients with suspected fungal disease.

347

348 **Conflict of Interest**

349 The author declare that Dr Kruger (Fujifilm) provided the Wako tests at no charge for this
350 evaluation because they were not commercially available at the time of the present study.

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- 451
- 452

453 Table 1: Repartition of the 175 episodes of suspected invasive fungal diseases (IFD) according to the underlying diseases/conditions and
 454 according to the final diagnosis of IFD, bacterial sepsis, or no IFD.
 455

Underlying disease/condition	Invasive aspergillosis n=5	Candidaemia n=3	Pneumocystosis n=12	Other IFD^a n= 5	Bacterial sepsis n=39	No IFD n=111
Haematological malignancies n=120	3	3	3	2	29	80
Solid cancer n=12	0	0	2	0	2	8
Solid organ transplantation^b n=10	1	0	0	0	1	8
Inflammatory disease n=6	0	0	3	0	0	3
AIDS n=13	0	0	4	1	1	7
ICU^c n=7	0	0	0	0	5	2
Diabetes n=2	1	0	0	1	0	0
Others^d n=5	0	0	0	1	1	3

456
 457 ^a invasive fusariosis n=2; hepato-splenic candidiasis n=1; mucormycosis n=1; cryptococcosis n=1

458 ^b kidney n= 7; kidney pancreas n= 2; liver n=1.

459 ^c out of which 6 with acute respiratory distress syndrome

460 ^d unspecified pneumonia n=3; sepsis n=1; mycetoma n=1

461

462 Table 2: Sensitivity and specificity of FA and WA with definite thresholds in various IFD.

463

Type of IFI	Test and threshold used	Sensitivity		Specificity		Likelihood ratio
		(%)	IC 95	(%)	IC 95	
Invasive aspergillosis	FA (120 pg/ml)	57.14	18.41% to 90.10%	91.52	87.64% to 94.49%	6.738
	WA (4 pg/ml)	57.14	18.41% to 90.10%	96.82	94.05% to 98.54%	17.97
PCP	FA (120 pg/ml)	78.57	49.20% to 95.34%	91.52	87.64% to 94.49%	9.265
	WA (4 pg/ml)	71.43	41.90% to 91.61%	96.82	94.05% to 98.54%	22.46
Candidiasis	FA (120 pg/ml)	72.73	39.03% to 93.98%	91.52	87.64% to 94.49%	8.576
	WA (4 pg/ml)	72.73	39.03% to 93.98%	96.82	94.05% to 98.54%	22.87
All IFI	FA (120 pg/ml)	81.82	61.48% to 92.69%	94.81	90.08% to 97.34%	15.75
	WA (4 pg/ml)	81.82	61.48% to 92.69%	95.45	90.92% to 97.78%	18

464

465

466 **Figure legends**

467 Figure 1. Receiver operating characteristic (ROC) curves and area under the curve (AUC) of
468 the Fungitell assay (FA, dashed blue line) and the Wako assay (WA, green line). The optimised
469 thresholds for positivity in FA assay (120 pg/ml in blue) and in WA (4 pg/ml in green) are
470 marked with an arrow. The manufacturers' thresholds are mentioned in black (80 pg/ml for FA
471 and 11 pg/ml for WA).

472

473 Figure 2. Violin plots of the beta-D-glucan (BDG) levels that were measured using the Fungitell
474 assay (FA, A and C) and the Wako assay (WA, B and D) for the 175 episodes of suspected
475 invasive fungal diseases (IFD). The dashed lines represent the manufacturers' thresholds (80
476 pg/ml for FA and 11 pg/ml for WA) and the dotted line represents the optimised threshold (120
477 pg/ml for FA and 4 pg/ml for WA) according to the receiver operating characteristic curves from
478 the present study.

479 IFD, invasive fungal diseases; IA, invasive aspergillosis; IC, invasive candidiasis; PCP,
480 pneumocystosis.

481

482 Figure 3. Linear regression of results for the BDG concentrations that were determined using
483 the Fungitell (FA) and Wako (WA) assays based on 28 specimens within the reliable range
484 ($7.8 < \text{FA} < 500$ pg/ml and $\text{WA} > 2.359$ pg/ml). $r^2 = 0.354$, $p < 0.001$. The dashed lines represent
485 the 95% confidence interval.