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

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

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

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Abstract

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

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

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

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

Lay summary

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

INTRODUCTION

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

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

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

The recent availability of WA has stimulated comparative studies with FA, first in 2011[14] and in more recent years.[15-18] These comparative studies were performed on archived serum

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

MATERIALS AND METHODS

Patients and definitions

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

Test realisation

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

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

Statistical analysis

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

Ethics Statement

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

RESULTS

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

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

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

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

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

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

Coefficient of correlation

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

Retesting

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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Conflict of Interest

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

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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 (%)	IC 95	Specificity (%)	IC 95	Likelihood ratio
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

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Figure legends

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

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

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