MAJOR ARTICLE

Reappraisal of the Serum (1→3)-β-D-Glucan Assay for the Diagnosis of Invasive Fungal Infections—A Study Based on Autopsy Cases from 6 Years

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Background. The prevalence of invasive fungal infection is increasing. An effective diagnostic test is required to identify and treat them successfully.

Methods. All autopsy records at our hospital for the period from January 2000 through December 2004 were reviewed for cases of invasive fungal infection. The diagnostic efficacy of a serum (1→3)-β-D-glucan (β-glucan) assay was examined using only those cases in which patients had been tested for fungal infection within 2 weeks before death.

Results. Of 456 autopsies, 54 (11.8%) involved cases of invasive fungal infection. Leukemias were the most frequent underlying disease (in 52% of cases of invasive fungal infection), and *Aspergillus* species was the most frequent pathogen detected (in 70%). Of the 54 patients with invasive fungal infection, 41 had β-glucan testing performed within 2 weeks before death, as did 63 patients without invasive fungal infection; 48 of 54 patients with invasive fungal infection had a blood culture performed. The sensitivity and specificity of the β-glucan test for the detection of invasive fungal infection were 95.1% and 85.7%, respectively, with a cutoff value of 30 pg/mL; 85.4% and 95.2%, respectively, with a cutoff value of 60 pg/mL; and 78.0% and 98.4%, respectively, with a cutoff value of 80 pg/mL. The sensitivity of blood culture testing was 8.3%. With a prevalence of 11.8%, the positive and negative predictive values for the β-glucan test were 47.1% and 99.2%, respectively, with a cutoff of 30 pg/mL; 70.4% and 98.0%, respectively, with a cutoff of 60 pg/mL; and 86.7% and 97.1%, respectively, with a cutoff of 80 pg/mL. During the 6-year period studied, of 21 patients with fungus-positive blood cultures that were preceded or followed by a β-glucan test within 2 weeks, 4 had negative β-glucan test results (β-glucan level, <30 pg/mL), and 17 had positive results (β-glucan level, >60 pg/mL); the concordance between culture results and β-glucan test results was 81.0%. Contrary to the general belief, 5 of 6 cases of cryptococcemia were associated with high serum β-glucan levels.

Conclusion. The β-glucan test is an effective diagnostic tool for invasive fungal infection.

More than 10 years have passed since the introduction into clinical use in Japan of a serum (1→3)-β-D-glucan (β-glucan) assay for diagnosis of deep fungal infection [1]. The assay is now widely accepted in this country as an indispensable tool for managing febrile episodes in immunocompromised hosts [2], and it is listed in the *Guidelines for the Diagnosis and Treatment of Deep Mycosis* [3] proposed by Japanese experts. The clinical usefulness of the β-glucan assay was also documented outside of Japan [4, 5], and the test was recently approved by the US Food and Drug Administration (FDA), although the test kit that the FDA used (Fungitell; Associates of Cape Cod) is different than the test evaluated here with respect to its reactivity to β-glucan. A positive β-glucan assay result is now one of the microbiological criteria for probable invasive fungal disease in the *Consensus Revised Definitions Draft VI* [6] produced by the joint committee of the European Organization for Research and Treatment of Cancer and the United States Mycoses Study Group. As the use of the test has become common, however, different opinions have arisen about its usefulness [7, 8]. One of the reasons for this is probably the use of different com-

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mercial kits with different levels of chemical reactivity to β-glucan. In Japan, practically all β-glucan assays are conducted with either the colorimetric Fungitec G-Test MK (Seikagaku Corporation) or the turbidimetric β-Glucan Test Wako (Wako Pure Chemical Industries). These 2 tests' detection limits, however, differ widely (7.0 pg/mL for the former and 111.6 pg/mL for the latter when compared in reference to a common standard β-glucan purified from *Candida albicans*) [9]. Another reason for the different opinions concerning the usefulness of these assays would be that invasive fungal infection is often difficult to diagnose while the patient is alive, such that the diagnoses on which test evaluation was based might not always have been definite. Thus, we thought it appropriate to reappraise the effectiveness of the β-glucan test on the basis of definite cases. For this purpose, we reviewed all autopsy records for the 6-year period from 2000 through 2005 at the Tokyo Metropolitan Cancer and Infectious Disease Center, Komagome Hospital (Tokyo, Japan), and examined the efficiency of a colorimetric β-glucan assay in cases of autopsy-verified invasive fungal infection.

MATERIALS AND METHODS

All autopsy records from January 2000 through December 2004 at Tokyo Metropolitan Komagome Hospital, an 800-bed hospital primarily concerned with the treatment of malignancies and infectious diseases, were reviewed for cases of invasive fungal infection. The prevalence of infection, underlying diseases, and causative fungi were studied. Those cases of invasive fungal infection in which the patient's blood specimen was cultured or serum samples were tested for β-glucan levels within 2 weeks before death were selected. With autopsy finding as a gold standard, the sensitivity of each test was calculated.

The specificity of the β-glucan assay was calculated on the basis of cases lacking pathological evidence of fungal infection in which the test had been performed within 2 weeks before death. For reference, serum samples of 44 healthy volunteers were subjected to the β-glucan assay, and the distribution and the upper limit of the measurements was studied.

Positive and negative predictive values were calculated, with an assumption that the general prevalence of invasive fungal infection was the same as that of the present autopsy series. The sensitivity, specificity, and positive and negative predictive values were calculated with cutoff values at 30 pg/mL, 60 pg/mL, and 80 pg/mL. The lowest cutoff was set on the basis of the results from the healthy volunteers described above, although the currently recommended cutoff is 20 pg/mL [1]. Binomial 95% CIs were computed using a Web site [10].

Finally, the records of blood specimen cultures from January 2000 through December 2005 at our hospital were reviewed for cases that included a positive culture for fungi. From those with fungi detected, cases were selected in which the serum β-glucan level was assayed within 2 weeks of inoculation of blood specimen to culture medium to allow understanding of the concordance between the 2 tests.

Serum β-glucan concentration was determined using Fungitec G-Test MK according to the manufacturer's instructions. The procedure is the same as that of Fungitell, an FDA-approved test kit, although the Fungitell test reports values that are approximately 3-fold higher than those reported by Fungitec G-Test MK [4]. Blood was cultured using the BacT/ALERT system (bioMerieux).

RESULTS

Of 456 autopsies, 54 (11.8%) revealed invasive fungal infection. Underlying diseases were leukemias (28 cases [52%]), malignant lymphomas (7 [13%]), acquired immunodeficiency syndrome (7 [13%]), solid malignancies (6 [11%]), and autoimmune diseases (6 [11%]). Of the 28 cases involving leukemias, 12 were in patients who had undergone hematopoietic stem cell transplantation. Causative fungi were *Aspergillus* species (38 cases [70%]), *Pneumocystis jiroveci* (7 [13%]), *Candida* species (4 [7%]), *Cryptococcus neoformans* (3 [6%]), *Trichosporon asahii* (1 [2%]), and Zygomycetes (1 [2%]). Of the 54 cases of invasive fungal infection, 41 were in patients whose serum β-glucan levels had been determined within 2 weeks before death. Forty (97.6%) of these 41 patients received antifungal medications; the 1 exception died within 24 h after admission to the hospital. β-glucan concentrations were above the cutoff of 30 pg/mL in 39 patients (table 1). The sensitivity of the β-glucan assay was, therefore, 95.1% (95% CI, 83%–99%; 39 of 41 cases of infection were detected). Likewise, sensitivity was 85.4% (95% CI, 71%–94%; 35 of 41 cases detected) with a cutoff at 60 pg/mL, and it was 78.0% (95% CI, 62%–89%; 32 of 41 cases detected) with a cutoff at 80 pg/mL (table 2). On the other hand, blood was cultured within 2 weeks before death in 48 of the 54 cases, and culture results were positive in only 4 cases. Thus, the sensitivity of blood specimen culture for the detection of fungal infection was 8.3% (95% CI, 2%–20%; 4 of 48 cases detected).

Of 402 cases that lacked postmortem evidence of invasive fungal infection, 63 occurred in patients whose serum β-glucan levels had been determined within 2 weeks before death. Forty-one (65.1%) of the 63 patients received a prophylactic or empirical antifungal treatment. The distribution of β-glucan measurements, with a mode between 5 and 10 pg/mL, closely resembled that in healthy volunteers ($n = 44$) (figure 1). The upper limit of normal serum β-glucan concentrations was 25 pg/mL. Of the 63 patients, 54 had a serum β-glucan concentration <30 pg/mL. Therefore, with a cutoff of 30 pg/mL, the specificity was 85.7% (95% CI, 75%–93%; negative test results in 54 of 63 cases). Likewise, specificity was 95.2% (95% CI, 87%–99%; negative test results in 60 of 63 cases) with a cutoff of 60 pg/mL and was 98.4% (95% CI, 91%–100%; negative

Table 1. Forty-one patients with autopsy-proven invasive fungal infection and their serum (1→3)-β-D-glucan (β-glucan) measurements within 2 weeks before death.

Patient	Autopsy diagnosis	Tissue site(s) positive for fungi	Blood culture result[a]	β-glucan level, pg/mL
1	Pulmonary aspergillosis	Lung	Negative	34
2	Pulmonary aspergillosis	Lung	Negative	73
3	Pulmonary aspergillosis	Lung	Not done	114
4	Pulmonary aspergillosis	Lung	Negative	125
5	Pulmonary aspergillosis	Lung	Not done	212
6	Pulmonary aspergillosis	Lung	*Aspergillus fumigatus*	300
7	Pulmonary aspergillosis	Lung	Negative	944
8	Pulmonary aspergillosis	Lung	Negative	1764
9	Pulmonary aspergillosis	Lung	Negative	1856
10	Pulmonary aspergillosis	Lung	Negative	4598
11	Systemic aspergillosis	Lung, myocardium, and epicardium	Negative	31
12	Systemic aspergillosis	Lung, liver, adrenal gland, and pancreas	Negative	54
13	Systemic aspergillosis	Lung and myocardium	Negative	64
14	Systemic aspergillosis	Lung, esophagus, stomach, and small intestine	Negative	76
15	Systemic aspergillosis	Lung, liver, and large intestine	Negative	106
16	Systemic aspergillosis	Lung and esophagus	Negative	190
17	Systemic aspergillosis	Lung and myocardium	Negative	232
18	Systemic aspergillosis	Lung and kidney	Negative	378
19	Systemic aspergillosis	Lung and small intestine	Negative	500
20	Systemic aspergillosis	Stomach and small intestine	Negative	635
21	Systemic aspergillosis	Lung, stomach, and cecum	Negative	1636
22	Systemic aspergillosis	Lung and brain	Negative	1801
23	Systemic aspergillosis	Lung, brain, kidney, and thyroid gland	Negative	1812
24	Systemic aspergillosis	Lung and small intestine	Negative	2181
25	Systemic aspergillosis	Lung, myocardium, and thyroid gland	Negative	2249
26	Systemic aspergillosis	Lung, myocardium, thyroid gland, kidney, liver, pancreas, muscle, and subcutaneous tissue	Negative	3137
27	Systemic aspergillosis	Brain[b]	Negative	4232
28	Systemic aspergillosis	Lung, brain, and thyroid gland	Negative	4731
29	Systemic candidiasis	Lung, kidney, and small intestine	*Candida krusei*	116
30	Systemic candidiasis	Lung, liver, spleen, bone marrow, base of the tongue, and abdominal lymphnode	Negative	3571
31	Systemic candidiasis and pulmonary aspergillosis	Lung, myocardium, kidney, spleen, stomach, and small and large intestine	*Candida tropicalis*	6083
32	Carini pneumonitis	Lung	Negative	42
33	Carini pneumonitis	Lung	Not done	353
34	Carini pneumonitis	Lung	Negative	591
35	Carini pneumonitis	Lung	Negative	1229
36	Carini pneumonitis	Lung	Negative	3611
37	Carini pneumonitis	Lung	Negative	21030
38	Systemic trichosporonosis and pulmonary aspergilloma	Lung, myocardium, liver, spleen, pancreas, gallbladder, kidney, adrenal gland, thyroid gland, stomach, small intestine, bone marrow, and spinal cord	*Trichosporon asahii*	705
39	Disseminated cryptococcosis	Lung, brain, kidney, and myocardium	Negative	411
40	Cryptococcal meningitis	Lung, meninges, and adrenal gland	Negative	7
41	Systemic zygomycosis	Lung, myocardium, kidney, spleen, stomach, jejunum, and large intestine	Negative	5

[a] Negative blood culture results indicate that no causative pathogen was identified.
[b] Autopsy consent was obtained only for the brain. Chest radiography and CT findings demonstrated pulmonary involvement.

Table 2. Serum (1→3)-β-D-glucan (β-glucan) levels in patients with culture results positive for fungi ($n = 21$) at the Cancer and Infectious Diseases Center, Komagome Hospital, January 2000–December 2005.

Fungus, isolate	β-glucan level, pg/mL	Period between obtaining culture sample and serum sample, days
Candida albicans		
Isolate 1	16	2
Isolate 2	61	1
Isolate 3	456	3
Candida glabrata	2305	2
Candida krusei[a]	116	10
Candida tropicalis[a]	6038	0
Candida parapsilosis		
Isolate 1	591	0
Isolate 2	596	0
Candida guilliermondii		
Isolate 1	142	3
Isolate 2	2047	9
Candida famata	6	7
Candida species	104	2
Cryptococcus neoformans		
Isolate 1	13	2
Isolate 2	86	4
Isolate 3	124	0
Isolate 4	135	10
Isolate 5	638	0
Isolate 6	2234	0
Trichosporon asahii[a]	705	1
Rhodotorula rubra	9	8
Aspergillus fumigatus[a]	300	1

[a] Fungal infection was verified at autopsy.

test results in 62 of 63 cases) with a cutoff of 80 pg/mL. Positive and negative predictive values were 47.1% and 99.2%, respectively, with a cutoff of 30 pg/mL; 70.4% and 98.0%, respectively, with a cutoff of 60 pg/mL; and 86.7% and 97.1%, respectively, with a cutoff of 80 pg/mL.

In the 6-year period studied, there were 21 fungus-positive blood cultures from 21 patients that were preceded or followed by performance of the serum β-glucan assay within 2 weeks of the culture inoculation. Among these 21 patients, β-glucan concentrations were >30 pg/mL in 17, >60 pg/mL in 17, and >80 pg/mL in 16. Thus, the concordance of the β-glucan assay with blood specimen culture results was 76%–81%. The most frequently isolated pathogen was *Candida* species (in 11 cases), followed by *Cryptococcus neoformans* (6). Five of the 6 cases of cryptococcemia were associated with high serum β-glucan concentrations. *Aspergillus fumigatus* was detected in culture in only 1 case (table 2).

DISCUSSION

β-glucan is a major cell wall constituent characteristic of fungi other than the Zygomycetes. Other pathogenic microorganisms, from protozoa to viruses, lack this polysaccharide. Animals, including humans, do not synthesize β-glucan, although a minute amount of β-glucan of external origin does exist in their blood. Therefore, for practical purposes, the increase of β-glucan levels in the blood should signify the intrusion of fungi into the bloodstream. Actually, in certain organisms, β-glucan is known to operate as a signal of invasion by fungi. Soybeans, for example, sense an encroachment by fungi with a special protein on the plasma membrane that binds with β-glucan and, in turn, elicits the production of phytoalexin, a substance that inhibits growth of the invading fungi [11]. The horseshoe crab, likewise, senses fungal invasion by a hemocoagulation enzyme (Factor G) that is extremely sensitive to β-glucan. Activated by β-glucan, Factor G triggers a cascade reaction of the coagulation system, which seems to be important for the encapsulation and elimination of invading fungi and seems to be linked with phagocytosis [12]. This activation of the coagulation system forms the basis of the β-glucan assay that we now use for diagnosis of invasive fungal infections in humans.

As we have reported elsewhere [1], the sensitivity of the test was 90% and the specificity was 100% at a 20-pg/mL cutoff value. The high sensitivity and specificity were preserved in the present study at a cutoff value of 30 pg/mL (95.1% and 85.7%, respectively). The shift of the cutoff value appears to result from a modification in the assay procedures, which originally included the manual use of test tubes and perchloric acid for pretreatment of samples but are now automated using microtiter plates and potassium chloride and potassium hydroxide for pretreatment. The sensitivity remained high at a cutoff of 60 pg/mL, and even at a cutoff as high as 80 pg/mL, sensitivity was nearly 80%. Specificity was >95% at cutoffs of both 60 pg/mL and 80 pg/mL.

With the same kit that we used, Kami et al. [13] reported a sensitivity of 63% (cutoff, 20 pg/mL) in 16 patients with definite invasive aspergillosis, which they defined as present in patients with positive histological findings and culture results from sputum or lung specimens. However, when they recalculated using only patients with disseminated invasive aspergillosis, the sensitivity increased to 88%. If Kami et al. [13] included aspergillomas in the definition of localized invasive aspergillosis, then this likely made the sensitivity low. Kawazu et al. [14] also evaluated a β-glucan test to detect invasive aspergillosis; using a turbidimetric assay (β-Glucan Test Wako), they reported a sensitivity of 55% in 11 patients with proven or probable invasive aspergillosis (cutoff, 11 pg/mL). We should note that an 11-pg/mL value measured by the turbidimetric test is not equal to an 11-pg/mL measurement by the Fungitec G-Test MK, be-

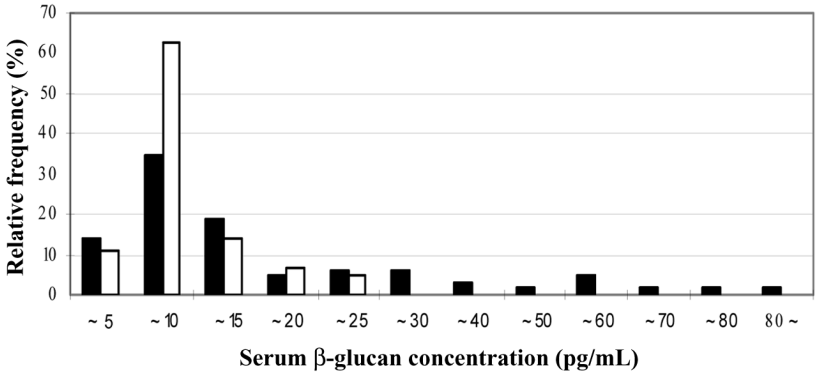

Figure 1. Distribution of serum (1→3)-β-D-glucan (β-glucan) measurements in 63 patients who had no evidence of invasive fungal infection at autopsy *(black bars)* and in 44 healthy individuals *(white bars).* Note the close resemblance between the 2 groups.

cause both the β-glucan standard used and the reactivity of reagents are different. When we compared the 2 tests against a common standard (*C. albicans*–derived soluble β-glucan [15]), there was an approximately 10-fold difference in measurement (i.e., a 1-pg/mL reading by the Fungitec G-Test MK corresponded to 1.8 pg of *C. albicans*–derived soluble β-glucan, whereas a 1-pg/mL measurement by the β-Glucan Test Wako corresponded to 18.6 pg of *C. albicans*–derived soluble β-glucan) [9]. Therefore, a reading of 11 pg/mL by β-Glucan Test Wako will correspond to a measurement of 113.7 pg/mL by the Fungitec G-Test MK. Thus, the low assay sensitivity reported by Kawazu et al. [16] could partly be ascribed to the inherent low sensitivity of the β-Glucan Test Wako kit.

The FDA-approved test kit, Fungitell, yielded an excellent sensitivity in several reports. In 1 prospective study, its sensitivity was 100% for detecting infection in a group of 20 subjects with proven or probable invasive fungal infection (cutoff, 60 pg/mL) [4]. A retrospective study showed an 87.5% sensitivity in a group of 7 patients with proven or probable invasive aspergillosis (cutoff, 120 pg/mL) [17]. In the multicenter evaluation of the Fungitell kit in the United States, the sensitivity was 81.3% in patients with proven candidiasis ($n = 107$; cutoff, 60 pg/mL) and 80% for detection of aspergillosis infection ($n = 10$; cutoff, 60 or 80 pg/mL) [5]. An additional study reported an 86.7% sensitivity in patients with positive blood culture results for yeasts ($n = 15$) [7]. Specificities were ~90% in all of the reports regarding the Fungitell kit except the most recent, which noted many false-positive reactions in blood samples that also yielded gram-positive bacteria growth [7]. This possibility of false-positive reactions needs additional investigation.

Positive and negative predictive values are heavily influenced by prevalence of infection. Using Glucatell (Associates of Cape Cod), a research-use version of the Fungitell kit, Odabasi et al. [4] reported a positive predictive value of only 43% with a single positive specimen; the prevalence of infection in their series, however, was as low as 7%. The positive predictive value increased to 80% with 3 sequential positive specimens. Kawazu et al. [14] also reported a low positive predictive value of 40% for a β-glucan test. Again, we should note that the prevalence of infection in their study was low (7.8%), and they used a less sensitive turbidimetric assay, as discussed above. Pazos et al. [17], using the Glucatell kit, presented a positive predictive value of 70% and a prevalence of infection of 21.7%, by our calculation. With a prevalence of infection of 20%, the positive predictive value in the study by Odabasi et al. [4] would be 71% even with only a single positive specimen. This is comparable with the estimate reported by Upton et al. [8] concerning the cases presented by Ostrosky-Zeichner et al. [5]. Negative predictive values were >90% in 4 of the 5 reports described above [4, 7, 14, 17].

In our study, the prevalence was set at 11.8%, the rate of invasive fungal infection in the present series, because the figure appeared to be reasonable in light of the 2001 annual report of Japan's nation-wide autopsy survey [18], in which invasive fungal infection was reported in 4.6% of the total number of autopsies ($n = 25{,}459$) and in 25.1% of hematological malignancies, including myelodysplastic syndrome ($n = 1037$). The selected prevalence of 11.8% would probably represent an average or even modest prevalence of infection at larger hospitals where the majority of the patients were being treated for malignancies. The positive predictive value for the assay was too low to be useful at a cutoff of 30 pg/mL. At a cutoff of 60 pg/mL, it was 70%, and at a cutoff of 80 pg/mL, it was close to 90%. Negative predictive values were >97% across the 3 cutoff levels. Consequently, at a cutoff of 80 pg/mL, invasive fungal infection could be ruled in or out with a 90% probability. If the serum level of β-glucan is <30 pg/mL, invasive fungal infection can almost always be ruled out, unless zygomycosis is involved. The close resemblance of the distribution of serum β-glucan measurements in severely ill patients who had no evidence of invasive fungal infection at autopsy to that in

Table 3. Comparison of 4 commercial kits for the serum (1→3)-β-D-glucan (β-glucan) assay.

Variable	Fungitec G-Test MK	β-glucan Test Wako	B-G Star	Fungitell
Manufacturer	Seikagaku Corporation	Wako Pure Chemical	Maruha Corporation	Associates of Cape Cod
Country	Japan	Japan	Japan	USA
Approval year	1995	1996	2001	2004
Assay method	Kinetic chromogenic	Kinetic turbidimetry	Endopoint chromogenic	Kinetic chromogenic
Sample	Serum or plasma	Serum or plasma	Serum or plasma	Serum
Pretreatment	Alkali	Dilution and heating	Dilution and heating	Alkali
Standard β-glucan	Pachyman	Carboxymethyl-curdlan	Lentinan	Pachyman
Origin of lysate	*Tachypleus tridentatus*	*Limulus polyphemus*	*Tachypleus tridentatus*	*Limulus polyphemus*
Cutoff value, pg/mL	20	11	11	60 or 80
Measurable range, pg/mL	3.9–500	6–600	1.2–120	31.25–500
Turn-around time, min	30	90	30	40

healthy volunteers is another indication of the high discriminative power of the β-glucan assay. This discriminative power should help to avoid unnecessary antifungal treatment and allow the start of such treatment when it is really needed.

Blood culture, on the other hand, was of little use for the detection of invasive fungal infection, although not all infections are hematogenous. If blood culture results were positive, it was almost always *Candida* species or other yeasts that grew in culture. The ineffectiveness of blood culture was probably attributable to the fact that most of the causative fungi were *Aspergillus* species, which are known to be difficult to culture from blood. Therefore, we need some ancillary test to deal with the surging rate of aspergillosis, and the serum β-glucan assay will serve that purpose. Besides being highly predictive, both positively and negatively, the test is able to nearly cover the entire range of fungi at once [19, 20]. Even in cases of cryptococcosis, which has been believed to not be associated with high serum β-glucan levels (described in the *Consensus Revised Definitions Draft VI* [6]), β-glucan levels are elevated in serum. This is not a surprise, because *Cryptococcus* species has β-glucan in its cell walls, although in smaller amounts than those found in *Candida* or *Aspergillus* species [21]. We reported elsewhere a case of cryptococcal meningitis in which serum β-glucan levels increased before the growth of the yeast in blood culture, and levels decreased with treatment [22]. It is conceivable, however, that if a nest of cryptococci is surrounded by a thick layer of granulation tissue in addition to a thick mucinous capsule, as in pulmonary cryptococcosis in immunocompetent hosts, β-glucan may not readily seep out into the bloodstream in detectable amounts.

Today, 4 commercial kits are available for the serum β-glucan assay (table 3). Unfortunately, however, their measurement values are totally incommutable, because they use different standard β-glucans and different species of horseshoe crab as a source of reagent, which have different reactivity to β-glucan. Thus, it should be noted that the cutoff values that we propose here apply only to Fungitec G-Test MK and not to other β-glucan test kits; the cutoff values need to be determined for each reagent. However, once appropriate cutoff values are established, the tests will bring benefits for patients who are at risk for developing opportunistic fungal infections, because the tests will encourage physicians to take prompt action against infection.

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