

Multicenter Clinical Evaluation of the (1→3) β -D-Glucan Assay as an Aid to Diagnosis of Fungal Infections in Humans

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Background. Measurement of (1→3)- β -D-glucan (BG) has emerged as an adjunct diagnostic strategy for invasive fungal infections (IFI).

Methods. Subjects at 6 clinical sites in the United States were enrolled as either fungal infection–negative subjects ($n = 170$) or subjects with proven or probable IFI according to European Organization for the Research and Treatment of Cancer/Mycoses Study Group criteria ($n = 163$). A central laboratory and 4 sites performed assays. A single sample was obtained per patient and was evaluated using an assay to detect serum BG derived from fungal cell walls (range, 0 to >7000 pg/mL).

Results. At a cutoff of 60 pg/mL, the sensitivity and specificity of the assay were 69.9% and 87.1%, respectively, with a positive predictive value (PPV) of 83.8% and a negative predictive value (NPV) of 75.1%. At a cutoff value of 80 pg/mL, the sensitivity and specificity were 64.4% and 92.4%, respectively, with a PPV of 89% and an NPV of 73%. Of the 107 patients with proven candidiasis, 81.3% had positive results at a cutoff value of 60 pg/mL, and 77.6% had positive results at a cutoff value of 80 pg/mL. Of the 10 patients with aspergillosis, 80% had positive results at cutoff values of 60 and 80 pg/mL. The 3 subjects diagnosed with *Fusarium* species had positive results at a cutoff value of 60 pg/mL. Patients infected with *Mucor* or *Rhizopus* species (both of which lack BG) had negative results at both cutoff values, and of the 12 patients with *Cryptococcus* infection, 3 had positive results at a cutoff value of 60 pg/mL, and 2 had positive results at a cutoff value of 80 pg/mL. Of the subjects with proven positive results who were receiving antifungal therapy ($n = 118$), 72.9% had results positive for BG at a cutoff value of 60 pg/mL, and 69.5% had results positive for BG at a cutoff value of 80 pg/mL. The interlaboratory sample test r^2 was 0.93.

Conclusion. Reproducible assay results with high specificity and high PPV in a multicenter setting demonstrate that use of an assay to detect serum BG derived from fungal cell walls is a useful diagnostic adjunct for IFI.

The incidence of invasive fungal infection (IFI) has been steadily increasing as a consequence of factors such as aggressive chemotherapy for cancer, bone mar-

row and organ transplantation, AIDS, and advanced critical care [1]. These infections are associated with significant mortality and morbidity and are often not diagnosed or are diagnosed late in the course of the disease, because current diagnostic techniques are less than ideal [2, 3]. Blood culture results are positive in only ~50% of cases of invasive candidiasis and in <10% of cases of invasive aspergillosis [4]. Histopathological diagnosis and cultures of samples obtained from protected anatomical sites are often not feasible in these critically ill populations; therefore, clinicians often face these diseases with little more than clinical and radiological diagnostic clues. For these reasons, the devel-

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opment of new diagnostic methods has been a research priority in medical mycology.

Among the new diagnostic techniques is the assay for the serum (1 \rightarrow 3)- β -D-glucan (BG) derived from fungal cell walls [5]. BG produced by fungi [6] is detected by a glucan assay on the basis of its recognition by the innate immune system of horseshoe crabs, specifically *Tachypleus tridentatus* and *Limulus polyphemus* [7]. Although this system is best known because of the activation (and, thus, detection) of endotoxin from gram-negative bacteria by factor C zymogen, factor G zymogen is activated by BG [8], a substance that is present in many fungal cell walls. The *Limulus* amoebocyte lysate (LAL) assay and the BG-specific variant Glucatell (now known as Fungitell) (Associates of Cape Cod) are described in detail in Odabasi et al. [9]. In brief, this assay is manufactured by removing bacterial endotoxin-sensitive factor C from LAL, making this reagent specific for BG. This modified lysate is formulated with a synthetic chromogenic substrate and salts. The reagent is used in a quantitative assay that detects BG in the serum of patients with symptoms of or medical conditions predisposing to IFI. BG levels measured with a similar reagent prepared from *Tachypleus* have also proven to be highly sensitive and specific for BG in earlier clinical studies involving both experimental candidiasis [10] and human populations with a variety of fungal infections [5, 11–14]. A previous study established the optimal cutoff value for the Fungitell test and proved its efficacy with a multiple-sampling strategy in an immunocompromised population [9]. The purpose of the current study was to evaluate the performance of the BG assay as a diagnostic adjunct for IFI in a single-sample, multicenter validation study.

SUBJECTS, MATERIALS, AND METHODS

Subjects. Subjects without IFI and subjects with proven or probable IFI (determined according to the European Organization for the Research and Treatment of Cancer/Mycoses Study Group [EORTC/MSG] criteria [15]) from 6 clinical sites in the United States consented to participate in this study, and a single blood sample was drawn from each subject. For subjects with IFI, the sample was drawn within 72 h after diagnosis of IFI (i.e., after positive results or findings of blood cultures, biopsy, imaging studies, or serological testing, as appropriate). This assured uniformity and that all subjects had active disease at the time of enrollment. Patient accrual began in March 2001 and was completed in November 2002. The presence of IFI was established with use of the EORTC/MSG criteria for diagnosis of fungal infections [15]. The investigational review board of each participating institution approved the study.

Collection and storage of samples. Blood samples were collected in sterile BG-free vacuum tubes. Serum was separated, aliquoted, and stored at 2°C–8°C for up to 48 h or frozen at

–20°C to –80°C until tested. Four cycles of freezing and thawing of the serum samples had no significant effect on the BG detected by the assay (data on file, Associates of Cape Cod). No effect of storage time was discerned when samples that had been stored at –80°C for up to 2 years were retested (data on file, Associates of Cape Cod).

BG assay. The BG assay we used (Fungitell; Associates of Cape Cod) is a BG-specific LAL product. BG activity is calibrated with pure pachyman, a linear BG. All assays were performed in triplicate, in a microtiter plate. Serum samples (5 μ L) were pretreated for 10 min at 37°C with an alkaline reagent (20 μ L; 0.125 M KOH/0.6 M KCl) to inactivate serine proteases as well as inhibitors in human serum and to enhance the reactivity to activated factor G [16, 17], according to the manufacturer's instructions. After addition of the BG assay reagent, the microtiter plate was inserted into a ThermoMax plate reader (Molecular Devices) preincubated to 37°C, and a kinetic assay [14] was run using SoftMax Pro software (Molecular Devices). A central laboratory and 4 clinical sites assayed serum samples obtained from a single blood sample obtained from each subject (range, 0 to >7000 pg of BG per mL of serum).

Performance evaluation and statistical analysis. The clinical performance of the BG assay was assessed by the calculation of the sensitivity and specificity for subjects with proven IFI who were receiving antifungal medication, subjects with proven IFI who were not receiving antifungal medication, subjects with probable IFI who were receiving antifungal medication, and subjects with probable IFI who were not receiving antifungal medication. In addition, the clinical performance of the assay was also assessed by the calculation of the sensitivity and specificity grouped according to subjects having fungemia, aspergillosis, candidiasis, fusariosis, and other deep-seated mycoses. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated using standard formulas on 4 \times 4 tables constructed for each analysis condition, including positive and negative results obtained at each proposed cutoff value. Descriptive and correlation statistics were obtained using Statistica for Windows (Statsoft).

RESULTS

One hundred ninety-six subjects were enrolled in the study as control subjects. The control group largely consisted of healthy volunteers or outpatients with minor medical problems, as well as hospitalized patients with medical problems other than IFI. Twenty-six control subjects were found to be inappropriately enrolled, critically ill subjects at high risk for IFI for whom IFI was not clearly ruled out; in addition, many of these 26 subjects were receiving empiric antifungal therapy. Thus, they were excluded from the study, leaving the control group with 170 subjects. One hundred and sixty-three subjects were enrolled in the proven or probable IFI group. The demographic char-

Table 1. Demographic and clinical characteristics of control subjects and subjects with proven or probable invasive fungal infection (IFI).

Characteristic	Control subjects (n = 170)	Subjects with IFI ^a (n = 163)
Age, mean years ±SD	41.3 ± 13.6	50.9 ± 16.3
Sex		
Male	37.6	46
Female	62.4	54
Race		
White	61.2	52.8
Black	15.9	32.5
Hispanic	12.3	11.7
Asian	5.3	0.0
Other	5.3	0.6
Not available	0.0	2.5
Main underlying diagnosis, symptom, or risk factor		
Allergy	1.2	...
Autoimmune disorder	0.6	3.1
Cardiovascular disease	11.8	8.0
Depression	2.4	...
Diabetes	2.9	5.5
Gastrointestinal bleeding	1.8	...
Receipt of gastrointestinal surgery	...	8.6
Gastrointestinal symptoms	4.7	...
Headache	2.4	...
Hematological malignancy	0.6	20.2
HIV/AIDS	...	6.1
Liver cirrhosis	...	3.1
Pancreatitis	...	3.7
Pneumonia	1.2	0.6
Renal failure	0.6	0.6
Sepsis	1.2	1.8
Solid tumor	1.8	8.6
Receipt of surgery	1.2	0.6
Trauma	...	4.3
None/healthy	57.6	5.5
Receipt of organ transplant	...	12.3
Other endocrine disorder	2.9	...
Other infection	0.6	5.5
Other pulmonary disease	1.8	...
Other disease	2.9	1.8

NOTE. Data are percentage of patients, unless otherwise indicated.

^a Determined according to European Organization for the Research and Treatment of Cancer/Mycoses Study Group criteria.

acteristics and main underlying diagnoses of the control group and the proven or probable IFI group are shown in table 1. Age, sex, and race did not influence test results. Table 2 shows the distribution of IFIs among patients with proven IFI (142 subjects) and those with probable IFI (21 subjects). As seen in

figure 1, the interlaboratory results from sample testing had an r^2 of 0.93. The results from the central laboratory were used for analysis.

BG assay results for the control group and the proven or probable IFI group at the different cutoff values are presented in table 3. Specificity was 87.1%–97.6% at BG levels of 60–150 pg/mL. The PPV over this range was 83.8%–95.9%, and the sensitivity was 69.9%–57.1%. This analysis included all subjects with either proven or probable IFI and the 170 subjects in the control group. An analysis performed without excluding the 26 inappropriately enrolled subjects showed that the performance was not significantly affected by the exclusion of these subjects. For example, at the 80-pg/mL cutoff value, when these subjects were excluded, the sensitivity, specificity, PPV, and NPV were 64.4%, 92.4%, 89.0%, and 73.0%, respectively, as opposed to 64.4%, 87.2%, 80.8%, and 74.7%, respectively, when the subjects were not excluded.

We analyzed the data to determine whether the antifungal therapy that was administered to 83% of the subjects with proven IFI influenced the results. The sensitivity of the assay at the 60-pg/mL cutoff value for subjects with proven IFI who were not receiving antifungal therapy was numerically (but not statistically) higher (20 [83.3%] of 24) than the sensitivity for the subjects with proven IFI who were receiving antifungal therapy (86 [72.9%] of 118) ($P = .69$).

BG levels varied by group and by type of IFI. The group with negative results ($n = 170$) had an mean BG level of 47.5 pg/mL (range, 0–3742 pg/mL). One patient with a BG level of 3742 pg/mL had renal failure and was receiving dialysis, a treatment that has been associated with BG contamination of patient blood (see Discussion). Excluding this individual, the range of BG levels for the group with negative results was 0–212 pg/mL. When analyzing BG levels by organism, we found that the 2 subjects infected with *Mucor* species, 1 subject infected with *Rhizopus* species, and 12 of the 15 subjects infected with *Cryptococcus* species had serum BG levels of <60 pg/mL. The 12

Table 2. Distribution of types of invasive fungal infection (IFI) among subjects with proven or probable IFI, by pathogen.

Pathogen	Proven IFI (n = 142)	Probable IFI (n = 21)
<i>Candida</i> species	107	4
<i>Aspergillus</i> species	10	12
<i>Fusarium</i> species	3	...
<i>Mucor</i> species	2	...
<i>Rhizopus</i> species	1	...
<i>Cryptococcus</i> species	12	...
Other	7	5

NOTE. Data are no. of cases. Proven or probable IFI was determined according to European Organization for the Research and Treatment of Cancer/Mycoses Study Group criteria.

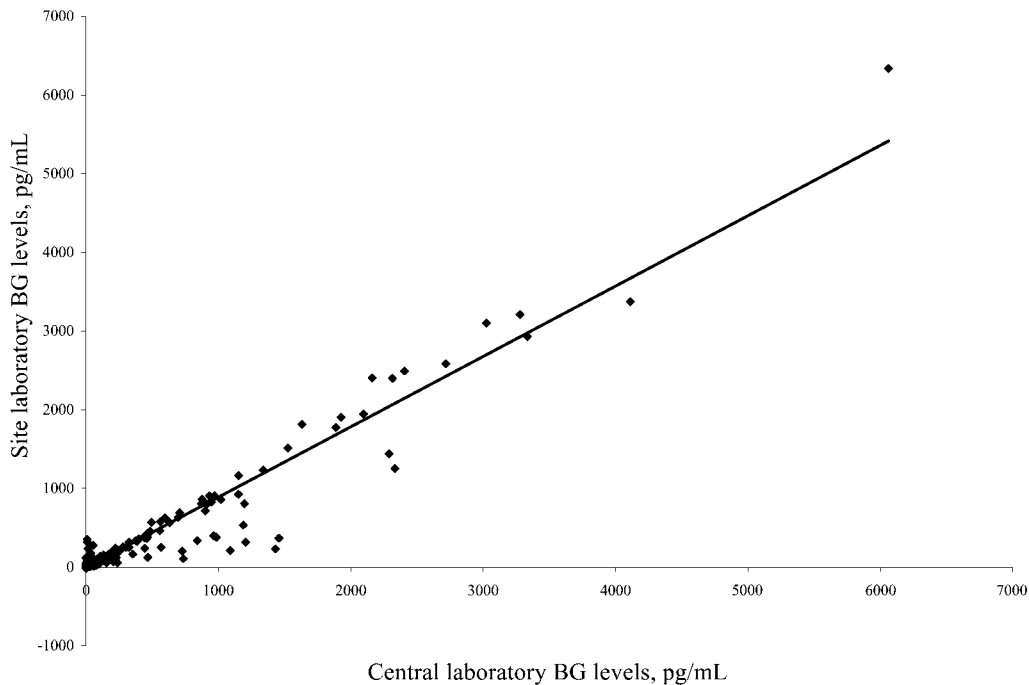


Figure 1. Interlaboratory correlation of (1→3)-β-D-glucan (BG) measurement. The BG level scattergram shows the correlation of central laboratory testing results with individual site laboratory results ($r^2 = 0.93$).

subjects infected with *Cryptococcus* species had a mean BG level of 60 pg/mL (range, 0–458 pg/mL). The zygomycetes (*Mucor* and *Rhizopus* species) produce little or no BG, whereas *Cryptococcus* species form capsules and contribute little BG to serum [5]. The mean levels of BG in serum of subjects infected with *Candida* species (755 pg/mL), *Aspergillus* species (1103 pg/mL), or *Fusarium* species (1652 pg/mL) were all elevated. Individual values for the IFI-positive group ranged from undetectable to >7000 pg/mL. Of the 107 subjects with proven candidiasis, 81.3% had positive results at a cutoff value of 60 pg/mL, and 77.6% had positive results at a cutoff value of 80 pg/mL. The sensitivity of the assay for subjects with proven candidemia ($n = 92$) is reported in table 4. Species identification is reported for isolates obtained from 91 subjects. Three subjects were found to be infected with 2 *Candida* species. Sensitivity of the assay for the detection of *Candida* species was >80% at a cutoff value of 60 pg/mL, with the exception of *Candida parapsilosis* (table 4). The distribution of the *Candida* species was as follows: *Candida albicans*, 39%; *Candida glabrata*, 28%; *C. parapsilosis*, 18%; *Candida tropicalis*, 12%; and *Candida krusei*, 3.3%.

The sensitivity for detection of *Aspergillus* infection was 80% at both the 60-pg/mL and 80-pg/mL cutoff values ($n = 10$). The sensitivity for detection of *Fusarium* infection was 100% at a cutoff value of 60 pg/mL ($n = 3$). Only 3 of the 12 subjects infected with *Cryptococcus* had positive results at the 60-pg/mL cutoff value, and none of the subjects infected with *Mucor*

species ($n = 2$) or *Rhizopus* species ($n = 1$) had results positive for BG.

Twenty-one subjects with probable IFI were entered into the study. The sensitivity of the BG test for this group (positive results for 9 [42.9%] of 21 subjects at the 60-pg/mL cutoff value and for 6 [28.6%] of 21 at the 80-pg/mL cutoff value) was significantly lower than it was for the group with proven IFI. The low levels of BG in many of the samples from subjects with probable IFI may actually reflect the uncertainty of diagnosis in this group and may be caused by the absence of an IFI in at least some of these subjects.

Table 3. Performance of the (1→3)-β-D-glucan (BG) assay for detection of invasive fungal infection in 333 subjects.

BG cutoff value, pg/mL	Sensitivity, %	Specificity, %	Positive predictive value, %	Negative predictive value, %
40	79.1	79.4	78.7	79.9
50	73.0	82.9	80.4	76.2
60	69.9	87.1	83.8	75.1
80	64.4	92.4	89.0	73.0
100	62.6	94.7	91.9	72.5
125	60.1	96.5	94.2	71.6
150	57.1	97.6	95.9	70.3

NOTE. Proven or probable IFI was determined according to European Organization for the Research and Treatment of Cancer/Mycoses Study Group criteria.

Table 4. Performance of the (1→3)-β-D-glucan (BG) assay in subjects with proven candidemia, by species.

Cutoff value, pg/mL	Any <i>Candida</i> species (n = 92)		<i>Candida albicans</i> (n = 36)		<i>Candida parapsilosis</i> (n = 18)		<i>Candida tropicalis</i> (n = 11)		<i>Candida glabrata</i> (n = 26)		<i>Candida krusei</i> (n = 3)	
	No. of subjects with positive result	Sensitivity, %	No. of subjects with positive result	Sensitivity, %	No. of subjects with positive result	Sensitivity, %	No. of subjects with positive result	Sensitivity, %	No. of subjects with positive result	Sensitivity, %	No. of subjects with positive result	Sensitivity, %
40	83	90.2	33	91.7	14	77.8	11	100.0	23	88.5	3	100.0
50	78	84.8	30	83.3	13	72.2	11	100.0	22	84.6	3	100.0
60	76	82.6	30	83.3	13	72.2	10	90.9	21	80.8	3	100.0
80	72	78.3	29	80.6	11	61.1	9	81.8	21	80.8	3	100.0

DISCUSSION

The BG assay we used (Fungitell; Associates of Cape Cod) is made from *L. polyphemus* found on the Atlantic coast of North America between Florida and the Gulf of Maine. The Fungitec-G assay (Seikagaku), made from *T. tridentatus* found on the Asian coast of the Pacific Ocean, is used clinically for the detection of IFI in Japan [5, 11–14]. The reactivity of the Fungitell assay with Pachyman standard is lower than that of the Fungitec-G assay [9]. The biochemical difference between these reagents results in different cutoff values, making it necessary to perform a multicenter clinical trial to establish the performance criteria of the Fungitell assay.

In our study, which involved 163 subjects with IFI and 170 healthy control subjects, we found an overall sensitivity of 69.9% and a specificity of 87.1% at the 60 pg/mL cutoff value. The sensitivity and specificity were higher for subjects with proven IFI who were receiving antifungal therapy than they were for subjects with probable IFI. The sensitivity of the assay was significantly lower for subjects with a diagnosis of probable IFI (42.9% at a cutoff value of 60 pg/mL) than was expected, and this may suggest that some subjects in this group did not have active IFI at the time of sampling.

A key factor affecting the clinical performance of the test may have been the sampling protocol. The results reported here were based on a single sample obtained from the patient within 72 h after entry into the study. Results for subjects with acute myelogenous leukemia (AML) suggest that the sensitivity, specificity, and PPV of the assay increase significantly if samples are obtained twice weekly. Obtaining multiple samples increased the sensitivity, PPV, and NPV of the BG assay to >98% for subjects with leukemia who were receiving antifungal prophylaxis [9]. Nevertheless, we felt that it was important to explore the performance of the assay with a single-sample protocol, because this protocol is potentially closer to the way in which the assay will be used in clinical settings. In contrast to the results reported here and those reported by Odabasi et al. [9], Digby et al. [18] observed low specificity for fungal infections in a study involving 46 subjects in intensive care units. This may be because of the very low serum BG cutoff level (20 pg/mL) that was used in that study. In addition, it is unclear

whether the more stringent, standardized, and widely accepted EORTC-MSG criteria [15] were used to establish fungal infection status. Finally, the study design used by Digby et al. [18] used only a single sample per patient, which, as is reported here, is likely to be less useful than serial sample analysis in identification of both absolute levels and trends in serum BG (especially for patients in the intensive care unit).

The BG assay had a high PPV for subjects infected with *Candida*, *Aspergillus*, or *Fusarium* species. PPV in artificially constructed studies such as ours should be approached with caution, because the prevalence of the disease in the study may not reflect the prevalence of disease in other specific settings. The assay had a high sensitivity for all species of *Candida*; however, differences between species were observed. Most notably, the assay seemed to be less sensitive for *C. parapsilosis*. This fact is interesting in view of the known fact that the echinocandins (agents that act by blocking BG synthesis) have higher MICs for *C. parapsilosis* [19]. Therefore, 2 independent pieces of information point to differences in the BG content of the fungal cell wall of *C. parapsilosis*, compared with other *Candida* species. These observations warrant further investigation. The assay did not detect elevated levels of BG in subjects infected with *Mucor*, *Rhizopus*, or *Cryptococcus* species. The explanation for this is that Zygomycetes do not produce BG, and the BG produced by encapsulated *Cryptococcus* species is at low levels in infection [20]. These results support observations made using the Fungitec-G assay for BG [11–14]. Nevertheless, because of the limited number of subjects in our study infected with these species, results for organisms other than *Candida* species should be interpreted with caution.

Medical sources of BG can lead to a positive assay result in the absence of IFI. Dialysis membranes and filters made from cellulose are reported to contain BG that may be solubilized during use. The use of cellulose membranes (but not of cellulose triacetate or polymethyl-methacrylate membranes) during hemodialysis resulted in significant increases in serum BG after dialysis [21, 22]. Specific immunoglobulin products [23], cotton gauze and sponges used in surgeries [24], and some drugs (including lentinan, crestin, scleroglucan, and schizophyllan) are reported to contain BG. Medical use of any of these prod-

ucts may lead to a false-positive BG assay result. This may explain the unusually high level of BG we found in 1 patient without IFI who was receiving hemodialysis, and this may also be a factor in the observations involving subjects from intensive care units [18].

With due consideration of the concerns mentioned above, these multicenter clinical trial results demonstrate that the BG assay can be used to measure serum BG in clinical specimens with a high specificity and PPV for subjects with proven or probable IFI when compared with control subjects. This test appears to be useful both as a single-point assay for patients hospitalized with suspicion of a fungal infection or as a part of a surveillance strategy in high-risk hosts. A cutoff value of 60 or 80 pg/mL appears to be optimal for this test. The performance of the assay does not appear to be significantly affected by antifungal therapy. Despite the limited number of subjects in our study, this assay appears to be less useful for the diagnosis of IFI caused by organisms that have lower BG content (such as the *Zygomycetes* and *Cryptococcus* species). These data demonstrate that the BG assay is a useful, highly reproducible, diagnostic adjunct for IFI. Future studies should focus on head-to-head comparison of surveillance versus single-point testing strategies and should explore the relationship of this marker to clinical outcomes and prognosis.

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