

β -D-Glucan as a Diagnostic Adjunct for Invasive Fungal Infections: Validation, Cutoff Development, and Performance in Patients with Acute Myelogenous Leukemia and Myelodysplastic Syndrome

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The GlucateLL (1 \rightarrow 3)- β -D-glucan (BG) detection assay (Associates of Cape Cod) was studied as a diagnostic adjunct for invasive fungal infections (IFIs). On the basis of findings from a preliminary study of 30 candidemic subjects and 30 healthy adults, a serum BG level of ≥ 60 pg/mL was chosen as the cutoff. Testing was performed with serial serum samples obtained from 283 subjects with acute myeloid leukemia or myelodysplastic syndrome who were receiving antifungal prophylaxis. At least 1 serum sample was positive for BG at a median of 10 days before the clinical diagnosis in 100% of subjects with a proven or probable IFI. IFIs included candidiasis, fusariosis, trichosporonosis, and aspergillosis. Absence of a positive BG finding had a 100% negative predictive value, and the specificity of the test was 90% for a single positive test result and $\geq 96\%$ for ≥ 2 sequential positive results. The GlucateLL serum BG detection assay is highly sensitive and specific as a diagnostic adjunct for IFI.

The mortality rate for invasive fungal infections in neutropenic subjects is 50% for subjects with *Candida* infection [1, 2] and may approach 100% for those with invasive aspergillosis [3, 4], fusariosis [5], or trichosporonosis [6]. Early diagnosis of invasive fungal infection in neutropenic subjects has the potential to increase antifungal therapeutic response, but meaningful diagnostic tests have proven to be elusive. Histopathologic demonstration of organisms in tissue specimens or growth of fungal agents in culture media is still the

“gold standard” method, but obtaining such specimens may be difficult. Blood culture results are positive in only 50% of invasive *Candida* and *Fusarium* infections [5, 7] and are positive very rarely in cases of invasive aspergillosis [8]. Cultures of bronchoalveolar lavage fluid or brushing specimens are only positive for $<50\%$ of subjects with invasive pulmonary aspergillosis [9]. Finally, positive results of cultures of specimens from nonsterile body sites may be related to either colonization or infection, and distinguishing between these can be difficult.

Nonculture-based diagnostic tests may provide a useful adjunct to these more traditional approaches. Of these, detection of circulating (1 \rightarrow 3)- β -D-glucan (BG), galactomannan, or fungal DNA has appeared quite promising [10]. BG is a component of the cell wall of a wide variety of fungi and can be detected by its ability to activate factor G of the horseshoe crab coagulation cascade [11–13]. It specifically binds to the α subunit of factor G, activating its serine protease zymogen β

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subunit [14]. When this reaction is combined with the pro-clotting enzyme of the cascade, and the chromogenic substrate Boc-Leu-Gly-Arg-p-nitroanilide, BG levels as low as 1 pg/mL can be quantified spectrophotometrically [15]. Assay systems for detection of these components have been developed: the Fungitec-G glucan detection test (Seikagaku) and GlucateLL (Associates of Cape Cod). The 2 reagents differ in that they are derived from different species of horseshoe crabs: the Fungitec-G reagent is based on amoebocyte enzymes from *Tachypleus tridentatus* [11, 12, 15], whereas the GlucateLL reagent uses enzymes from *Limulus polyphemus* amoebocytes [16]. For the Fungitec-G assay, the positive serum cutoff level for BG is defined as 20 pg/mL [15], but a corresponding cutoff value for the GlucateLL assay has not been determined.

In this study, we compared the relative abilities of the GlucateLL and Fungitec-G assays to detect different glucan compounds. We also used the GlucateLL assay to determine a BG cutoff level for the diagnosis of invasive fungal infections in a study involving nonneutropenic, candidemic subjects and healthy adults. Finally, we examined the utility of the GlucateLL assay for the diagnosis of invasive fungal infection in subjects who were undergoing induction chemotherapy for newly diagnosed acute myelogenous leukemia (AML) or myelodysplastic syndrome (MDS) and who were receiving antifungal prophylaxis.

MATERIALS AND METHODS

Glucan compounds (oligosaccharides and polysaccharides). The reactivity of the GlucateLL and Fungitec-G glucan assays was measured using certain glucan compounds, which were obtained as follows: (1→3)- β -D xylan (degree of polymerization [d.p.], 8–15) was obtained from Seikagaku; laminarioligosaccharide (d.p., 6–21) was obtained from Associates of Cape Cod; curdlan, carboxymethylcellulose (CMC), curdlan sodium salt, and paramylon were obtained from WakoPure Chemical Industries; baker's yeast β -glucan, laminaran (*Laminaria digitata*), barley β -glucan lichenans, CM-cellulose sodium salt, nigeran, mannan, and (1→4)- β -D-xylan were obtained from Sigma; laminaran (*Eisenia bicyclis*) was obtained from Nacalia Tesque; schizophyllan was obtained from Kaken Chemical; lentinan was obtained from Ajinomoto; and pachyman and pustulan were obtained from Calbiochem. *Candida* β -glucan was a gift from Dr. Ohno (Tokyo University of Pharmacy, Tokyo, Japan), and galactan was a gift from Dr. Y. Hashimoto (Saitama University).

Test kits. Each GlucateLL kit contains GlucateLL, a (1→3)- β -D-glucan-susceptible *L. polyphemus* amoebocyte lysate, which is nonresponsive to endotoxin as a result of the removal of factor C (an endotoxin-activated serine protease zymogen); *Limulus* amoebocyte lysate reagent water (LRW); pyrosol reconstitution buffer (Tris-HCl buffer; pH, 7.8); glucan standard (pachyman); and sample extraction reagents A (0.25 M KOH)

and B (1.2 M KCl). Fungitec-G-Test MK test kits were obtained from Seikagaku. All laboratory materials (e.g., pipette tips and syringes) were certified as being free of contaminating glucan by statistical sampling and empirical testing by Seikagaku Corporation or Associates of Cape Cod.

Glucan assay. Glucan compounds were prepared for testing by weighing and dissolving each compound in LRW or 0.3 N NaOH (or 0.6 N NaOH, for pachyman) to yield a 1-mg/mL solution. To prepare the serum samples, 5 μ L of the serum specimens to be tested were diluted 1:5 by the addition of 20 μ L of pretreatment reagent (a 1:1 mixture of sample extraction reagents A and B that inactivates interfering serum substances and alters the conformation of the BG to the single helix form [17]). The resulting mixture was incubated at 37°C for \geq 10 min before further testing was done.

Twenty-five- μ L samples of pachyman at 50, 25, 12.5, and 6.25 pg/mL were used for the standard curve. Twenty-five- μ L samples of LRW were used as reagent blanks. GlucateLL was reconstituted with 2.8 mL of LRW followed by 2.8 mL of pyrosol reconstitution buffer (0.2 M Tris HCl; pH, 7.4), and 100 μ L of this mixture was added to each sample, standard, or blank well. With use of a THERMOMax plate reader at 37°C, the rate of change of absorbance per min (Δ OD/min) at 405 nm less a background reading at 490 nm was recorded for 40 min using the instrument's kinetic reading mode and SOFTmax PRO software, version 3.1 (Molecular Devices Corporation), for instrument control, data acquisition, and processing. All samples were tested in triplicate. A kinetic standard curve correlating Δ OD/min versus pachyman concentration was constructed. For unknowns, the mean rate of change based on triplicate replicates was computed, and the glucan concentration was interpolated from the kinetic standard curve. Inter-assay and interlaboratory reproducibility of assay results has been shown to be 0.96 (data not shown). The Fungitec-G-Test MK was performed the same way except that the assay was run for 30 min.

Clinical materials. For the determination of a provisional assay cutoff, stored serum samples obtained from 30 nonneutropenic subjects with blood cultures positive for *Candida* species and associated signs and symptoms of infection (e.g., fever or hypotension) were compared with samples obtained from 30 healthy adults.

In a separate clinical study, serum samples were obtained systematically twice per week during the period of neutropenia from 300 consecutive subjects with newly diagnosed AML or MDS who were undergoing initial induction chemotherapy. All of these adults received open-label antifungal prophylaxis with intravenous itraconazole or caspofungin in addition to their standard care. The clinical course of each subject was reviewed, and invasive fungal infection-related events were assessed using the EORTC/MSG criteria for diagnosis of invasive fungal in-

Table 1. Comparison of reactivities of Fungitec-G (Seikagaku) and GlucateLL (Associates of Cape Cod) tests against various polysaccharides.

Glycosidic linkage	Name	Molecular weight, Da	Origin	Reactivity, log g/mL ^a	
				Fungitec-G	GlucateLL
(1 → 3)-β-D	CM Curdlan Na Salt	>95,000	<i>Alcaligenes faecalis</i>	10	11
	Paramylon	>118,000	<i>Euglena gracilis</i>	10	9
	Curdlan	>136,000	<i>A. faecalis</i>	11	11
(1 → 3), (1 → 6)-β-D	Schizophyllan	76,800	<i>Schizophyllum commune</i>	11	10
	Pachyman ^b	80,000	<i>Poria cocos</i>	11	11
	Lentinan	94,700	<i>Lentinus edodes</i>	11	10
(1 → 3), (1 → 4)-β-D	Barley BG	>23,100	Barley	9	11
(1 → 4)-β-D	CM-cellulose	NR	Synthetic	6	6
(1 → 3)-β-D	Galactan	NR	Gum arabic	<6	<6
(1 → 3)-β-D	Xylan	NR	<i>Bryopsis maxima</i>	<6	<6
(1 → 2), (1 → 3), (1 → 6)-α-D	Mannan	NR	<i>Saccharomyces cerevisiae</i>	<6	<6

NOTE. NR, not reported.

^a Data are minimum concentration Fungitec-G/GlucateLL activation that produced a defined activity (1.0 mAbs/min). A value of <6 log g/mL was considered nonreactive.

^b Although the reactivity for pachyman appears to be similar, kinetic analysis showed that GlucateLL was ~2.5-fold less reactive than the Fungitec-G for this compound (data not shown).

fection in research settings [18]. All samples were stored at -70 °C for up to 1 year until tested. (Data on file at Associates of Cape Cod suggest that BG is stable in frozen serum for an indefinite time, resisting also many freeze-thaw cycles.) The glucan assays were performed in a blinded fashion before the clinical information was compiled and analyzed, thus eliminating potential for bias.

This study was approved by the local institutional review committee, and informed consent was obtained from all patients.

RESULTS

Comparison of Fungitec-G and GlucateLL assays. Both the GlucateLL and the Fungitec-G assays were found to be specific for polysaccharides composed of, or containing, BG sequences. They were largely unreactive with (1→4)-β-D-glucan, (1→6)-β-D-glucan, and nonglucans containing (1→3)-β-D-glucan linkages (table 1). These results are consistent with those reported by Tanaka et al. [19]. Kinetic analysis showed that the GlucateLL assay was ~2.5-fold less reactive than the Fungitec-G assay with the pachyman standard (data not shown), reducing the slope of its standard curve with pachyman.

Determination of the BG cut-off for the GlucateLL assay. The serum samples obtained from the 30 healthy adults contained a mean BG concentration (±SD) of 17 ± 34 pg/mL (range, 0–86 pg/mL), with only 2 serum samples containing >60 pg/mL (63 pg/mL and 86 pg/mL; figure 1). Serum samples obtained from the 30 candidemic subjects contained a mean BG concentration (±SD) 2999 ± 5190 pg/mL (range, 36–22,263 pg/mL). Of these subject samples, 29 contained a BG concentration of ≥60 pg/mL. Thus, 60 pg/mL was chosen as

the positive cutoff for the diagnosis of invasive fungal infection with the GlucateLL assay. At this cutoff value, the assay had a sensitivity of 97% and a specificity of 93% for the tested samples.

Evaluation of the GlucateLL assay in patients with AML and MDS. Of the 300 enrolled neutropenic subjects, 17 subjects were excluded from the study because they refused to participate, had not received antifungal prophylaxis or chemotherapy, or had <2 serum samples available. The remaining 283 evaluable subjects provided an average of 7.3 specimens per subject over a period of 3 weeks, for a total of 2070 specimens. During the course of the study, 16 subjects had proven invasive fungal infection, 4 had probable invasive fungal infection, and 33 had possible invasive fungal infection (table 2). Using the predefined cutoff value of 60 pg/mL, all subjects with proven or probable invasive fungal infections had ≥1 serum sample that tested positive for BG (table 3). Absence of a positive sample had a

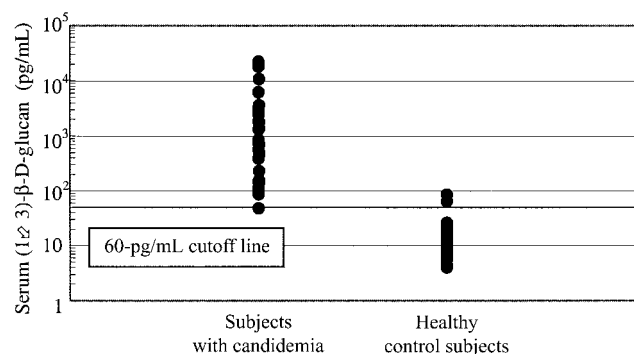


Figure 1. Serum glucan levels in 30 subjects with candidemia and 30 healthy control subjects.

Table 2. Correlation of invasive fungal infection with (1→3)-β-D-glucan (BG) levels.

Type of invasive fungal infection	Organism	Maximum BG level, pg/mL ^a	No. of positive BG test results	δ ^b
Proven fungemia (n = 16)	<i>Candida tropicalis</i>	3422	3	2
	<i>Candida albicans</i>	2415	6	10
	<i>Candida glabrata</i>	1838	5	14
	<i>Candida krusei</i>	1650	3	7
	<i>C. glabrata</i>	1438	8	10
	<i>Fusarium</i> species	1072	3	12
	<i>Trichosporon asahii</i> ^c	627	3	18
	<i>C. glabrata</i>	621	3	32
	<i>C. krusei</i>	526	3	10
	<i>T. asahii</i>	365	3	3
	<i>T. asahii</i>	252	1	1
	<i>C. glabrata</i>	225	3	4
	<i>Aspergillus terreus</i> ^d	153	1	12
	<i>C. glabrata</i>	108	2	-2
	<i>C. parapsilosis</i>	64	1	1
	<i>C. glabrata</i>	61	1	0
Probable fungal pneumonia (n = 4)	<i>Aspergillus fumigatus</i>	2105	4	18
	<i>Fusarium</i> species	207	1	4
	<i>A. terreus</i> and <i>C. albicans</i>	108	1	23
	<i>A. fumigatus</i>	81	1	-1
Possible fungal pneumonia (n = 33)	None	519	1	-7
	None	398	3	4
	None	264	2	0
	None	180	3	-4
	None	144	1	0
	None	125	1	0
	None	123	4	10
	None	96	1	15
	None	90	1	-8
	None	86	2	-4
	None	83	2	3
	<i>C. glabrata</i>	74	1	-6
	None	70	2	0
	None	69	1	0
	None	69	1	0
	None	64	1	-7
	None	63	1	5
	None	58
	None	56
	None	51
	None	50
	None	48
	None	47
	None	42
	None	42
	None	42
	None	40
	<i>Candida</i> species	35
	None	32
	None	30

(continued)

Table 2. (Continued.)

Type of invasive fungal infection	Organism	Maximum BG level, pg/mL ^a	No. of positive BG test results	δ^b
No invasive fungal infection ($n = 10$)	None	29
	None	28
	None	27
	None	281	2	...
	None	107	2	...
	None	101	1	...
	None	85	2	...
	None	73	1	...
	None	72	1	...
	None	71	1	...
	None	63	1	...
	None	62	1	...

NOTE. Invasive fungal infection type and certainty, by EORTC-MSG criteria [18], are shown along with the results of BG testing. Results are shown for all subjects with a diagnosis of an invasive fungal infection or a positive BG test result.

^a Maximum BG level noted at any time.

^b No. of days that the patient was BG positive before clinical diagnosis of invasive fungal infection, with a positive value indicating that a positive BG value preceded the clinical diagnosis.

^c No fungemia but disseminated invasive fungal infection with multiple nodules in the spleen and skin.

^d No fungemia; *A. terreus* was detected from autopsy of pulmonary nodules and cavities.

100% negative predictive value. BG was positive at a median of 10 days before the clinical diagnosis (range 32 days before to 2 days after) in the subjects with proven or probable invasive fungal infection. When considering all subjects with proven, probable, or possible fungal infection, BG assay results were positive in 70% at a median of 3 days before the clinical diagnosis (range, 32 days before to 7 days after clinical diagnosis). An example of the performance of the test is shown in figure 2. Once a positive test result was documented, the usual trend was for the BG levels to become increasingly and progressively elevated until an outcome was reached.

The specificity of the test for proven or probable fungal infection increased from 90% for 1 positive sample to 96% and 99% when 2 and 3 sequential positive serum samples, respectively, were required for a true positive test result (table 3). Likewise, the specificity for proven, probable, or possible fungal

infection rose to 100% with increasing required numbers of sequential positive serum samples. The results for positive predictive value were similarly improved by requiring multiple sequential positive serum samples.

DISCUSSION

In this study, we have evaluated the utility of a new BG detection system (GlucateLL) as a diagnostic adjunct for invasive fungal infection. The GlucateLL assay was compared with a similar assay, and the 2 assays were found to be specific for BG. When tested against a panel of 30 serum samples obtained from patients with proven candidemia and 30 serum samples obtained from healthy adults, the GlucateLL assay had a sensitivity of 97% and specificity of 93% at a cutoff value of 60 pg/mL. This is 3-fold higher than the cutoff value of 20 pg/mL used for the

Table 3. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of (1→3)- β -D-glucan (BG) detection in subjects with an invasive fungal infection (IFI), by EORTC-MSG diagnostic certainty category, who had 1–3 serum specimens positive for BG.

No. of BG-positive serum samples	Proven or probable IFI				Proven, probable, or possible IFI			
	Sensitivity, %	Specificity, %	PPV, %	NPV, %	Sensitivity, %	Specificity, %	PPV, %	NPV, %
1 specimen	100	90	43	100	70	96	79	93
≥2 sequential specimens	65	96	57	97	38	99	87	87
≥3 sequential specimens	60	99	80	96	28	100	100	85

NOTE. EORTC-MSG criteria are from [18].

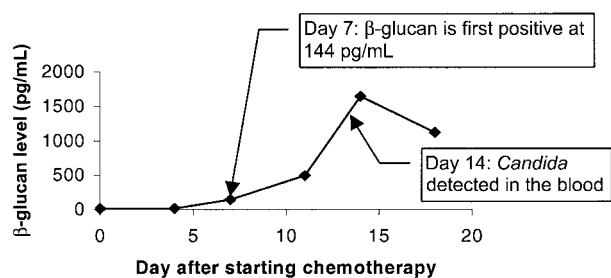


Figure 2. Detection of (1→3)-β-D-glucan in serum before clinical detection of an invasive fungal infection.

Fungitec-G assay and is the result of the differences in affinity/reactivity of Fungitec-G and Glucatell reagents, which are extracted from different horseshoe crab genera, to the pachyman standard. Fungitec-G is more reactive, but this is unlikely to have any clinical significance with the adjusted cutoff point.

With use of the cutoff value of 60 pg/mL, the performance of the Glucatell assay was determined in a study of adults undergoing induction chemotherapy for AML or MDS. Although the 60-pg/mL cutoff value was developed in a nonleukemic population, it appeared to be applicable to this population, as evidenced by the high sensitivity and specificity. These patients are classically known to be at high risk of acquiring invasive fungal infections. All subjects with a proven or probable invasive fungal infection had ≥ 1 serum sample positive for BG (range, 61–3422 pg/mL), and the absence of positive serum samples in this group of subjects yielded a negative predictive value of 100%. The specificity of the test was increased from 90% to $>96\%$ if 2 sequential positive serum samples were required for a true-positive result. It is noteworthy that all patients were receiving antifungal prophylaxis, so although the incidence of fungal infection was low, this intervention did not seem to affect the performance of the test. The relatively high rate of cases of candidemia seen in this study may be related to the use of itraconazole prophylaxis by some of the patients.

A single serum sample positive for BG was detected in 10 subjects with no known invasive fungal infections, and 3 of these subjects had 2 sequential positive samples. None of those patients had documented fungal colonization. In contrast, of the patients without invasive fungal infection who had colonization, 2 with oral and urinary catheter colonization showed no increase in serum BG levels. False-positive reactions are known to occur in some patients. These include subjects who had experienced renal failure and who were undergoing hemodialysis with cellulose membranes [20], subjects treated with certain immunoglobulin products [21], and specimens (or subjects) exposed to glucan-containing gauze or related materials [22]. This underscores the need to combine serological BG findings with other clinical signs and symptoms and diagnostic

modalities, such as high-resolution CT scan, when making a diagnosis.

The lack of positive BG results in some of the patients with possible fungal pneumonia likely reflects the limitations of the EORTC/MSG criteria. Any subject with a symptomatic but unexplained pulmonary infiltration will be scored as having possible fungal pneumonia, but such pulmonary processes could also be due to undiagnosed viral or bacterial pneumonia, leukemia, or even cardiac failure. Seventeen of the 33 patients with possible invasive fungal infection had possible fungal pneumonia, which as discussed above, is a poorly defined syndrome which may comprise a mix of several etiologies.

The clinical performance results of the Glucatell assay were comparable to those that have been reported with the Fungitec-G assay. In a series of 188 subjects with a variety of fungal infections, the sensitivity and specificity of Fungitec-G were 95% and 100%, respectively [15]. The likelihood of any significant clinical difference between the 2 tests is low, because the only difference between the tests is the affinity to the glucan compounds, and that has been compensated with the adjusted cutoff value.

Infections detected by the BG assay in our subjects included candidiasis, fusariosis, trichosporonosis, and aspergillosis. Detection of BG also has the potential to detect invasive fungal infections due to *P. neumocystis jiroveci*, *Acremonium* species, and *Saccharomyces* species [23, 24]. However, BG concentrations will usually be low or absent in patients with cryptococcal infections, and BG is usually absent in patients with zygomycosis [25–27]. The effects of antifungal therapy on BG levels remain to be studied.

In conclusion, the presence of BG levels of ≥ 60 pg/mL, as detected by the Glucatell assay, may be a useful diagnostic adjunct for the diagnosis of invasive fungal infection, particularly in high-risk populations. The positivity of this test, particularly when used in a serial fashion, often precedes the microbiological or clinical diagnosis of invasive fungal infection. This cell wall component has the advantage of being present and detectable in a variety of fungal infections.

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Conflict of interest. F.S., R.J.R., P.A.K., and M.A.F. are employed by Associates of Cape Cod. L.O.-Z. and J.H.R. have received research grants from Associates of Cape Cod. All other authors: No conflict.

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