Medical Mycology May 2006, **44**, 267–272

Taylor & Francis
Taylor & Francis Group

Differences in beta-glucan levels in culture supernatants of a variety of fungi

ZEKAVER ODABASI*, VICTOR L. PAETZNICK*, JOSE R. RODRIGUEZ*, ENUO CHEN*, MICHAEL R. MCGINNIS† & LUIS OSTROSKY-ZEICHNER*

**UT Medical School-Houston, Houston, and †UT Medical Branch, Galveston, Texas, USA*

(1 →3)-beta-d-glucan is a well known cell wall constituent of fungal isolates that can be detected by assays *in vivo* and *in vitro*. Previous studies have shown that different fungal isolates may show different levels of reactivity with an assay for beta glucan. In this study we evaluated the *in vitro* reactivity of 127 clinical fungal isolates belonging to 40 different genera, with the Glucatell™ assay. The majority of the fungal isolates released high levels of beta glucan. Beta glucan test reactivity appears to be species-specific and this may reflect the beta glucan content of the organism.

Keywords yeast, mould, beta glucan, diagnosis, supernatant

Introduction

With the increase in the number of immunocompromised patients, the incidence of invasive fungal infections has been increasing for the past two decades. *Candida* and *Aspergillus* species are the most common causes of invasive fungal infections (IFI), but there are many emerging fungal infections such as *Trichosoporon, Fusarium, Penicillium, Scedosporium*, *Bipolaris*, and *Pseudallescheria* spp. [1–6]. Despite the development of new potential antifungal agents, the morbidity and mortality of IFI remain high. An important contributing factor to high morbidity and mortality is the lack of an early and accurate diagnostic test. Early diagnosis and early treatment of IFI significantly decreases morbidity and mortality [7]. Histology or culture based diagnostic tests are still the 'gold standards', but non-culture-based diagnostic modalities are under investigation. (1 →3)-β-D-glucan (BG) is a component of the cell wall of a wide variety of fungi. Isolates having BG in their cell wall structure can be detected by an assay based on the ability of this molecule to activate Factor G of the horseshoe crab coagulation cascade [8–10]. Serum BG testing is a promising serologic test with its high sensitivity and specificity. In an our clinical study with the Glucatell assay in neutropenic leukemia patients, the test detected IFI caused by *Candida, Aspergillus, Fusarium and Trichosporon* species as early as a median of ten days before the clinical diagnosis [11]. Also from the previous *in vivo* or *in vitro* studies with other beta glucan kits, it is known that different fungal species produce a wide range of BG values [10,12–17]. Most notably, some fungal species, like zygomycetes and *Cryptococcus* spp., usually produce low test results probably due to their low cell wall BG content [14,18]. We performed an *in vitro* study to evaluate the range of clinical fungal isolates capable of producing detectable BG, and their levels of production with Glucatell™ assay.

Received 27 July 2005; Accepted 15 November 2005
Results of this study were partially presented as abstract # M-1021 at the 43rd Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, 2003.
Correspondence: Zekaver Odabasi, Marmara University School of Medicine, Infectious Diseases Marmara Universitesi Hastanesi, Tophanelioglu caddesi No:13, Istanbul 81190, Turkey. Tel/Fax: +90 2163274142; E-mail: zekaver@marmara.edu.tr

Materials and *m*ethods

Broth media culture supernatants of 127 clinical fungal isolates (85 moulds and 42 yeasts) were tested; *Candida* spp. (*C. albicans* 5, *C. krusei* 4, *C. glabrata* 5, *C. lusitaniae* 4, *C. tropicalis* 3, *C. dubliniensis* 3, *C. parapsilosis* 3), *Aspergillus* spp. (*A. fumigatus* 3, *A. flavus* 3, *A. niger* 3, *A. terreus* 3), *Fusarium* spp. (*F. solani* 4, *F. oxysporum* 2), *Scedosporium* spp. (*S. prolificans* 6, *S. apiospermum* 4), *Wangiella dermatitidis* (5), *Phialophora verrucosa* (5),

DOI: 10.1080/13693780500474327

Exophiala jeanselmei (5), *Bipolaris spicifera* (4), *Paecilomyces* spp. (4), *Saccharomyces cerevisiae* (4), *Trichosporon asahii* (4), *Fonsecaea. pedrosoi* (4), *Pseudallescheria boydii* (4), *Cunninghamella bertholletiae* (4), *Rhodotorula rubra* (3), *Acremonium* spp. (*Acremonium* spp. 2, *A. alabamensis* 1), *Sporothrix schenckii* (3), *Penicillium marneffei* (3), *Madurella mycetomatis* (3), *Rhizomucor pusillus* (2), *Rhizopus arrhizus* (2), *Mucor* spp. (2), *Absidia corymbifera* (2), *Cryptococcus neoformans* (2), *Blastoschizomyces capitatus* (1), *Scopulorioposis brevicaulis* (1), *Basidiobolus ranarum* (1), and *Hansenula anomala* (1). All isolates were stored in −80°C and each mould and yeast isolate was subcultured to potato dextrose agar slants (Becton, Dickinson and Company, Sparks, MD) and Sabouraud dextrose agar plates (Becton, Dickinson and Company, Sparks, MD) respectively. Isolates were subcultured at least twice to provide enough purity and viability. Mould isolates were allowed to grow on potato dextrose agar slants for a period of 7 days at 35°C while yeast isolates were grown in Sabouraud dextrose agar at 35°C for a period of 1–2 days.

Mould slants were covered with 1 ml of sterile 0.85% saline and scraped gently to harvest conidia. The turbidity of conidial spore suspensions was measured at 530 nm and adjusted to 80–82% transmittence for most of the isolates except *P. boydii* and isolates belong to zygomycetes family which were adjusted to 68–70% transmittance. Final solutions were diluted in distilled water 1:50 (*P. boydii* suspension was diluted 1:25) to obtain final (2×) suspension of 0.4×10^4 cfu/ml. Inoculum of each yeast isolate was prepared by picking five colonies of 1 mm diameter from 24 h old *Candida* species or 48 h old *Cryptococcus* species. The colonies were suspended in 5 ml of sterile 0.145-mol/l saline and density of each suspension was adjusted to 0.5 Mcfarland standard at 530 nm wavelength by spectrophotometer. Each suspension was then further diluted 1:100 and followed by 1:20 with sterile water to yield a final solution containing (2×) 5.0×10^2 to 2.5×10^3 cells/ml. One millilitre of yeast or conidia was suspended in a 1 ml (2×) RPMI 1640 medium (Sigma Chemicals Company, St. Louis, MO) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer and incubated at 35°C for 48–96 h. After enough visible growth of organisms each test tube was vortexed and centrifuged at 4°C. Supernatants of 1ml of each broth culture media were separated to another tube and stored in −80°C untill test day.

BG levels in supernatants were measured in triplicate using the Glucatell kit (currently available as Fungitell). Each Glucatell glucan assay kit (Associates of Cape Cod, Inc., Falmouth, MA) contains Glucatell®, a 1,3-β-D-glucan-sensitive *Limulus polyphemus* amebocyte lysate (LAL) that is non-responsive to endotoxin due to the removal of Factor C (an endotoxin-activated serine protease zymogen), Limulus amebocyte lysate Reagent Water (LRW), pyrosol reconstitution buffer (Tris-HCl buffer, pH 7.4), glucan standard, sample extraction reagent A (0.25 M KOH), and sample extraction reagent B (1.2 M KCl). All laboratory materials (pipette tips, syringes, tubes, etc.) were certified free of contaminating glucan by statistical sampling and empirical testing by Seikagaku Corporation or Associates of Cape Cod, Inc.

Twenty-five microlitre samples of pachyman at 50, 25, 12.5, and 6.25 pg/ml were used for the standard curve. Twenty-five microlitre samples of LRW were used as reagent blanks. After thawing the supernatant specimens in room temperature, 5 µl of specimens to be tested were diluted 1:5 by the addition of 20 µl pre-treatment reagent (a 1:1 mixture of sample extraction reagents A and B that inactivates interfering serum substances and alters the conformation of the (1→3)-β-D-glucan to the single helix form. The resulting mixture was incubated at 37°C for at least 10 min prior to further testing. For the Glucatell assay, 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 50 µl of this mixture were added to each sample, standard, or blank well. Using a THERMOmax plate reader at 37°C, the mAbs/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 (v 3.1, Molecular Devices Corporation) software for instrument control, data acquisition and processing. All samples were tested in triplicate. The glucan concentration was determined from the mean of the three readings, as interpolated from the kinetic standard curve.

Results

All yeast isolates produced enough visible growth at 48 h and mould isolates had a growth range of 48–96 h depending on the species, except *Madurella mycetomatis* which had visible growth around 120 h. The ranges of BG contents and mean values are summarized in Tables 1 and 2. Also by accepting the reactivity levels of *Candida* and *Aspergillus* species as 100%, reactivity of other fungal isolates were determined by calculating the ratio of mean BG content of each yeast or mould isolate to mean BG values of *Candida* or *Aspergillus* species respectively. A wide range of BG levels were seen within the different fungal species (Table 3). Among the *Candida* species, three of the four

Table 1 Beta glucan concentrations detected in broth media culture supernatants of clinical mould isolates

Species (*n*)	BG range (pg/ml)	Geometric mean BG (pg/ml)	Mean BG (pg/ml)	Reactivity** (%)
Moulds				
Bipolaris spicifera (4)	(2747–4389)	3378	3452	180
Sporothrix schenckii (3)	(2891–3633)	3119	3118	163
Wangiella dermatitidis (5)	(1320–2774)	1941	2025	106
Penicillium marneffei (3)	(1685–2297)	2015	2032	106
Aspergillus spp. (12)	(1311–2480)	1769	1915	100
Paecilomyces sp. (4)	(1199–2539)	1665	1729	90
Scopulariopsis brevicaulis (1)	1446	NA*	1446	76
Fusarium spp. (6)	(952–1860)	1383	1415	74
Basidiobolus ranarum (1)	1418	NA	1418	74
Phialophora verrucosa (5)	(1062–1281)	1138	1140	60
Exophiala jeanselmei (5)	(593–1650)	928	993	52
Acremonium spp. (3)	(625–1402)	817	885	46
Madurella mycetomatis (3)	(594–655)	633	634	33
Fonsecaea pedrosoi (4)	(423–727)	537	551	29
Rhizomucor pusillus (2)	(191–412)	NA	302	16
Scedosporium spp. (10)	(158–652)	255	292	15
Rhizopus arrhizus (2)	(89–209)	NA	149	8
C. bertholletiae (4)	(91–127)	110	111	6
Pseudallescheria boydii (4)	(139–174)	148	124	6
Mucor sp. (2)	(111–131)	NA	121	6
Absidia corymbifera (2)	(43–40)	NA	47	2
Control (sterile test medium)	40	NA	40	2

*NA, not applicable. **Percent reactivity of yeast and mould isolates compared by reactivity of *Aspergillus* species, which were accepted as having 100% reactivity.

C. lusitaniae isolates released a lower amount of BG (414– 753 pg/ml) when compared to other *Candida* species, one *C. lusitaniae* isolate released 3022 pg/ml of BG. Other *Candida* species had BG values within the same ranges. Among the other yeast isolates; *S. cerevisiae*, *T. asahii* and *R. rubra* released BG levels comparable to *Candida* spp. *Cryptococcus* isolates produced generally lower levels of BG (153–265 pg/ml) when compared to other yeasts.

All *Aspergillus* species produced comparable BG levels. *Bipolaris spicifera* isolates had the highest BG levels while *Sporothrix schenckii*, *Wangiella dermatitidis* and *Penicillum marneffei* isolates showed higher BG concentrations than the *Aspergillus* isolates. *Paecilomyces* spp, *S. brevicaulis*, *Fusarium* spp, *B. ranarum and P. verrucosa* isolates released comparable levels of BG to *Aspergillus* spp. Among the *Fusarium* isolates, four *F. solani* isolates released larger concentrations of BG compared to the two *F. oysporum* isolates (mean BG of 1575 vs 1096 pg/ml respectively). *Rhizopus* spp., *Mucor* spp., *Rhizomucor* spp., *P. boydii*, *Scedosporium* spp., *Absidia corymbifera* and *Cunninghamella* spp. showed very low reactivity compared to other mould isolates. *A. corymbifera* had the lowest BG concentrations

Table 2 Beta glucan concentrations detected in broth media culture supernatants of clinical yeast isolates

Species (*n*)	BG range (pg/ml)	Geometric mean BG (pg/ml)	Mean BG (pg/ml)	Reactivity** (%)
Candida spp. (27)	(414–3765)	1839	2119	100
B. capitatus (1)	1859	NA*	1859	88
S. cerevisiae (4)	(1468–2336)	1765	1794	85
Rhodotorula rubra (3)	(1174–1815)	1363	1392	66
Trichosporon asahii (4)	(1130–1660)	1300	1316	62
Hansenula anomala (1)	670	NA	670	32
Cryptococcus neoformans (2)	(153–265)	NA	209	10
Control (sterile test medium)	40	NA	40	2

*NA, not applicable. **Percent reactivity of yeast and mould isolates compared by reactivity of *Candida* species, which were accepted as having 100% reactivity.

Table 3 Differences of beta levels among each strains

Species (*n*)	BG*	Species (*n*)	BG	Species (*n*)	BG
Bipolaris spicifera (4)	4389	*C. krusei* (4)	3438	*Exophiala jeanselmei* (cont.)	1650
	2765		3666		1160
	3908		3167		830
	2747		2858	*Hansenula anomala* (1)	670
Sporothrix schenckii (3)	2889	*C. lusitaniae* (4)	3022	*Madurella mycetomatis* (3)	594
	2891		753		653
	3633		616		655
Wangiella dermatitidis (5)	2232		414	*Acremonium* spp. (2)	625
	2774	*C. parapsilosis* (3)	1621		629
	1407		2222	*Fonsecaea pedrosoi* (4)	442
	1320		1996		423
	2393	*B. dermatitidis* (1)	1859		727
Penicillium marneffei (3)	2113	*Paecilomyces* sp. (4)	1199		611
	2297		2539	*Scedosporium prolificans* (6)	338
	1685		1595		283
A. flavus (3)	1615		1582		519
	2191	*Saccharomyces cerevisiae* (4)	1468		652
	1311		2336		262
A. fumigatus (3)	2480		1795		231
	2191		1577	*Rhizomucor pusillus* (2)	191
	2383	*Scopulariopsis brevicaulis* (1)	1446		412
A. niger (3)	1569	*Basidiobolus ranarum* (1)	1418	*Cryptococcus neoformans* (2)	265
	1843	*F. oxysporum* (2)	1240		153
	1334		952	*Scedosporium apiospermum* (4)	188
A. terreus (3)	1931	*F. solani* (4)	1550		180
	2476		1262		147
	1660		1626		118
C. albicans (5)	1454		1860	*Rhizopus arrhizus* (2)	209
	1213	*Acremonium alabamensis* (1)	1402		89
	1141	*Trichosporon asahii* (4)	1142	*Mucor* sp. (2)	131
	1992		1130		111
	1791		1332	*Pseudallescheria boydii* (4)	139
C. dubliniensis (3)	3258		1660		174
	849	*Rhodotorula rubra* (3)	1188		144
	1919		1174		139
C. tropicalis (3)	1093		1815	*Cunninghamella bertholletiae* (4)	127
	1749	*Phialophora verrucosa* (5)	1281		91
	2283		1093		105
C. glabrata (5)	2966		1062		119
	2934		1095	*Absidia corymbifera* (2)	50
	3765		1171		43
	1544	*Exophiala jeanselmei* (5)	731		
	3486		593		

(*n*), number of isolates. *BG: beta glucan level as pg/ml.

among all isolates. Two *Acremonium* spp. released 625 and 629 pg/ml of BG to the media and one *Acremonium alabamensis* released 1402 pg/ml.

Discussion

The utility of the BG assays has been evaluated in a number of laboratory and clinical studies in the past decade. The Fungitec G test (formerly known as G test) utilizes the amebocyte enzymes from *Tachypleus tridentatus,* whereas the Glucatell (now Fungitell) assay uses enzymes from *Limulus polyphemus* amebocytes [19]. In previous studies both tests effectively detected IFI caused by *Candida, Aspergillus, Fusarium and Trichosporon* species [11,12,15]. Furthermore, in a number of studies with G test, *Saccharomyces cerevisiae*, *Pneumocystis jiroveci*, *Rhodotorula rubra* and *Acremonium* spp. were shown to react with BG test [10,15,20]. *Cryptococcus* species and zygomycetes have been historically found to have low reactivity with the BG test, presumably because of their low cell wall BG concentrations [10,14,18,21].

In our study, all yeast isolates showed similar reactivity compared to previous studies. *Cryptococcus* spp. produced the lowest level of BG while *Candida* species had consistently higher levels. Among the *Candida* species, it appears that *C. lusitaniae* isolates may produce lower concentrations of BG compared to other *Candida* species. *Blastoschizomyces capitatus*, *Saccharomyces cerevisiae*, *Rhodotorula rubra* and *Trichosporon asahii* isolates released comparable BG levels to *Candida* species. A single *Hansenula anumala* isolate was found to have a comparatively lower BG level.

Aspergillus spp. isolates had high reactivity with the BG test. All four different *Aspergillus* species produced comparable BG levels to each other. However, isolates of *Bipolaris spicifera*, *Sporothrix schenckii*, *Wangiella dermatitidis* and *Penicillium marneffei* released much higher concentrations than the *Aspergillus* isolates. *Paecilomyces* spp., *Scopulariopsis brevicaulis*, *Fusarium* spp., *Basidiobolus ranarum*, and *Phialophora verrucosa* isolates also released BG to the culture media in levels comparable to *Aspergillus* isolates. As a member of the zygomycetes, *Absidia corymbifera* had the lowest BG levels. The other zygomycetes (*Mucor* sp., *Rhizopus arrhizus, Rhizomucor pusillus*, and *Cunninghamella bertholletiae*) also released lower concentrations of BG. Interestingly, *Scedosporium* spp. and *Pseudallescheria boydii* (the sexual form of *S. apiospermum*), produced low BG levels.

Our results are similar to the experiences found in previous clinical trials and *in vitro* studies of BG reactivity and content, however we describe BG levels in a wide range of isolates whose reactivity with the BG assay was previously unknown. One of the limitations of this preliminary study is the small number of some of the yeast and mould species. However, we felt it was important to present these data since it comprises new information, particularly for the more rare or infrequent organisms. Another limitation of our study is the absence of biomass-normalized data. We need further *in vivo* or clinical studies to put these *in vitro* findings in the appropriate context and explore the reason for the differences in BG levels. As a conclusion: culture supernatants of fungi appear to have species-specific BG levels. This may represent the BG content of the organism, and may in turn explain the variations found in clinical testing.

References

1 Fleming RV, Walsh TJ, Anaissie EJ. Emerging and less common fungal pathogens. *Infect Dis Clin North Am* 2002; **16**: 915–933.

2 Marr KA, Carter RA, Crippa F, Wald A, Corey L. Epidemiology and outcome of mould infections in hematopoietic stem cell transplant recipients. *Clin Infect Dis* 2002; **34**: 909–917.

3 Morrison VA, Haake RJ, Weisdorf DJ. The spectrum of non-*Candida* fungal infections following bone marrow transplantation. *Medicine (Baltimore)* 1993; **72**: 78–89.

4 Ponton J, Ruchel R, Clemons KV, *et al*. Emerging pathogens. *Med Mycol* 2000; **38**: 225–236.

5 Silveira F, Nucci M. Emergence of black moulds in fungal disease: epidemiology and therapy. *Curr Opin Infect Dis* 2001; **14**: 679–684.

6 Walsh TJ, Groll AH. Emerging fungal pathogens: evolving challenges to immunocompromised patients for the twenty-first century. *Transpl Infect Dis* 1999; **1**: 247–261.

7 Aisner J, Wiernik PH, Schimpff SC. Treatment of invasive aspergillosis: relation of early diagnosis and treatment to response. *Ann Int Med* 1977; **86**: 539–543.

8 Ohki M, Nakamura T, Morita T, Iwanaga S. A new endotoxin sensitive factor associated with hemolymph coagulation system of horseshoe crab (Limulidae). *FEBS Lett* 1980; **120**: 217–220.

9 Morita T, Tanaka S, Nakamura T, Iwanaga S. A new (1-3)-ß-D-glucan-mediated coagulation pathway found in limulus amebocytes. *FEBS Lett* 1981; **129**: 318–321.

10 Miyazaki T, Kohno S, Mitsutake K, *et al*. (1→3)-beta-D-glucan in culture fluid of fungi activates factor G, a limulus coagulation factor. *J Clin Lab Anal* 1995; **9**: 334–339.

11 Odabasi Z, Mattiuzzi G, Estey E, *et al*. Beta-D-glucan as a diagnostic adjunct for invasive fungal infections: validation, cutoff development, and performance in patients with acute myelogenous leukemia and myelodysplastic syndrome. *Clin Infect Dis* 2004; **39**: 199–205.

12 Obayashi T, Yoshida M, Mori T, *et al*. Plasma (1→3)-beta-D-glucan measurement in diagnosis of invasive deep mycosis and fungal febrile episodes. *Lancet* 1995; **345**: 17–20.

13 Mitsutake K, Kohno S, Miyazaki T, *et al*. Detection of (1-3)-beta-D-glucan in a rat model of aspergillosis. *J Clin Lab Anal* 1995; **9**: 119–122.

14 Miyazaki T, Kohno S, Mitsutake K, *et al*. Plasma (1-3)-b-D-glucan and fungal antigenemia in patients with candidemia, aspergillosis, and cryptococcosis. *J Clin Microbiol* 1995; **33**: 3115–3118.

15 Yoshida M, Obayashi T, Iwama A, *et al*. Detection of plasma (1-3)-beta-D-glucan in patients with *Fusarium*, *Trichosporon*, *Saccharomyces*, and *Acremonium* fungaemias. *J Med Vet Mycol* 1997; **35**: 371–374.

16 Yuasa K, Goto H, Iguchi M, Okamura T, Ieki R. Evaluation of the diagnostic value of the measurement of (1-3)-beta-D-glucan in patients with pulmonary aspergillosis. *Respiration* 1996; **63**: 78–83.

17 Morimoto S, Shimazaki C, Goto H, *et al*. *Trichosporon cutaneum* fungemia in patients with acute myeloblastic leukemia and measurement of serum D-arabinitol, *Candida* antigen (CAND-TEC), and beta-D-glucan. *Ann Hematol* 1994; **68**: 159–161.

18 Jojima H. Early diagnosis and treatment of pulmonary opportunistic infection by using polymerase chain reaction and beta-glucan in patients with hematological neoplasms. *Kurume Med J* 2001; **48**: 117–127.

19 Saeki F, Ridge RJ, Finkelman MA, Ketchum PA, Rex JH. Application of a glucan-specific *Limulus* amebocyte lysate serum assay for diagnosis of invasive fungal infections. In: 41st Interscience Conference on Antimicrobial Agents and Chemotherapy; 2001; abstract 841

20 Mori T, Matsumura M. Clinical evaluation of diagnostic methods using plasma and/or serum for three mycoses: aspergillosis, candidosis, and pneumocystosis. *Nippon Ishinkin Gakkai Zasshi* 1999; **40**: 223–230.

21 Mori T, Ikemoto H, Matsumura M, *et al*. Evaluation of plasma (1→3)-beta-D-glucan measurement by the kinetic turbidimetric Limulus test, for the clinical diagnosis of mycotic infections. *Eur J Clinl Chem Clin Biochem* 1997; **35**: 553–560.