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Enolase Antigen, Mannan Antigen, Cand-Tec Antigen, and β-Glucan in Patients with Candidemia

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We compared the specificities and sensitivities of four tests used for the serodiagnosis of candidemia in 39 patients with candidemia, including 10 patients with superficial *Candida* colonization, 10 patients with deep mycosis, and 20 healthy subjects. The results obtained by the dot immunoblotting assay for detecting the enolase antigen (48 kDa) were compared with those of assays for detecting mannan antigen, heat-labile antigen (a threshold titer of four times), and β-glucan (≥60 pg/ml). Enolase antigen was detected in 28 (71.8%) patients with candidemia, while 30 (76.9%), 10 (25.6%), and 27 (84.4%) patients were positive for the heat-labile antigen by the Cand-Tec assay, the mannan antigen by the Pastorex *Candida* assay, and β-glucan by the *limulus* test, respectively. Ten patients with superficial *Candida* colonization, 5 patients with invasive pulmonary aspergillosis, 5 patients with cryptococcosis, and 20 healthy subjects were negative for both enolase antigen and mannan antigen. Two patients with superficial *Candida* colonization, one patient with invasive pulmonary aspergillosis, and two patients with cryptococcosis were positive by the Cand-Tec assay. The β-glucan concentration was more than 60 pg/ml in all patients with invasive pulmonary aspergillosis; however, it was less than 10 pg/ml in all patients with cryptococcosis. The specificity of enolase antigen in the serodiagnosis of candidemia was 100%, but the sensitivity was 71.8%. The specificity and sensitivity of Cand-Tec, the assay for mannan antigen, and the assay for β-glucan were 76.9 and 87.5%, 25.6 and 100%, and 84.4 and 87.5%, respectively. Our results demonstrated that antigen detection tests are useful for the diagnosis of candidemia; however, none is satisfactory for the serodiagnosis of candidemia. We suggest that a combination of two assays may increase the accuracy of diagnosis of candidiasis.

The incidence of nosocomial systemic fungal infections is increasing, and *Candida* species are some of the most common etiologic agents of nosocomial bloodstream infections. Deep-seated candidiasis is a cause of morbidity and mortality; however, it is generally difficult to establish a clinical diagnosis of deep-seated candidiasis. The conventional diagnostic approach consists of blood culture and tissue biopsy. For such tests, including the lysis-centrifugation method, a few days are required to establish the correct diagnosis. Invasive approaches, including biopsy, are usually limited because of the serious clinical condition of the patients. Therefore, serological diagnostic tests for antibody, antigen, or metabolite detection have been used for laboratory diagnosis (1, 7).

Strockbine et al. (18) were the first to identify the immunodominant cytoplasmic 48-kDa antigen of *Candida* spp.; it was subsequently recognized as the glycolytic enzyme enolase (2, 8). It was later suggested that the presence of *Candida* enolase in the blood may represent a novel marker for the diagnosis of invasive candidiasis (19).

In the study described in this report, we compared the efficacy of the dot immunobinding assay for detecting enolase antigen in patients with candidemia with those of three other tests: an assay for mannan antigen (5), the Cand-Tec assay for the heat-labile antigen (4), and an assay for β-glucan (6, 8, 15).

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MATERIALS AND METHODS

Subjects. (i) Patients with candidemia. Serum samples were collected from 39 patients with candidemia who were admitted to The Nagasaki University Hospital and affiliated hospitals during a 4-year period extending from 1990 to 1993. The characteristics of these patients are summarized in Table 1. All patients demonstrated positive blood cultures for *Candida* species and were febrile (>38°C) at the time of blood culture. *Candida* species were identified with the API 20C identification kit (Bio Merieux, Marcy l'Etoile, France), while *Candida albicans* isolates were recognized by the germ tube test. *C. albicans* isolates were detected in 9 patients, *Candida parapsilosis* isolates were detected in 14 patients, *Candida tropicalis* isolates were detected in 5 patients, *Candida* (*Torulopsis*) *glabrata* isolates were detected in 6 patients, *Candida guilliermondii* isolates were detected in 5 patients, and a *Candida krusei* isolate was detected in one patient.

(ii) Patients with superficial *Candida* colonization. Serum samples were obtained from six patients with hematological malignancies, two patients with tuberculosis, one patient with lung cancer, and one burn patient. All 10 patients had superficial infections, and *Candida* species were recovered from sites of oral thrush, skin lesions, or urine samples. None of these patients had a positive blood culture for *Candida* species or other organisms.

(iii) Patients with deep mycoses. Sera from 10 patients with deep mycoses were also collected. Five patients with severe neutropenia were diagnosed on autopsy as having invasive pulmonary aspergillosis. Other types of invasive mycotic infections were not detected in these five patients on autopsy. Five patients were diagnosed with cryptococcal meningitis, which was confirmed by culture of the cerebrospinal fluid. Blood cultures were negative for every patient of this group.

(iv) Healthy volunteers. Sera from 20 healthy subjects (12 males and 8 females; age range, 20 to 50 years) with no evidence of deep-seated or superficial colonization by *Candida* species served as the control group.

Dot immunoblotting assay for *Candida* enolase. Enolase isolated from *C. albicans* IFM 40009 by the method of Westhead and McLain (20) yielded a major band (48 kDa) and a relatively minor band (92 kDa) (11). The purified enolase was used to immunize New Zealand White rabbits, and the resultant antiserum was fractionated with ammonium sulfate to isolate the antienolase.

Each serum sample was diluted (1:10) with 0.9% sterile saline, and 100 μl of this diluted solution was loaded, in duplicate, onto a nitrocellulose membrane (Hybond-ECL; Amersham, Buckinghamshire, England) in the dot immunobinding plate and was allowed to drain under gravity. The membrane was blocked with 5% powdered milk in phosphate-buffered saline (PBS) for 1 h at 37°C to prevent nonspecific binding. The membrane was washed with 0.1% Tween 20–

TABLE 1. Enolase antigen assay, Cand-Tec assay, Pastorex *Candida* assay, and β-glucan assay (fungal index) results for patients with candidemia[a]

Sex	Age (yr)	Underlying disease	Organism	Enolase reaction	Cand-Tec assay titer	Pastorex assay titer	β-Glucan concn (pg/ml)
F	67	Lung cancer	A	+	8×	−	519
M	61	Hepatoma	A	+	2×	−	435
F	77	Sick sinus syndrome	A	+	2×	4×	391
M	52	Cerebral infarction	A	+	4×	1×	349
F	47	Viral encephalitis	A	−	8×	−	349
F	82	Cerebral infarction	A	+	8×	−	125
M	67	Parkinson syndrome	A	+	32×	−	NP
F	43	CRF	A	+	16×	−	NP
M	68	Parkinson syndrome	A	+	32×	−	NP
F	67	Viral encephalitis	P	+	4×	−	350
M	75	Pulmonary tuberculosis	P	+	16×	−	288
M	87	Cerebral infarction	P	−	8×	−	278
M	83	CRF	P	−	8×	−	113
M	81	Cerebral infarction	P	+	8×	−	103
M	81	Cerebral infarction	P	−	4×	−	101
M	84	Ileus	P	+	2×	−	82
F	77	Miliary tuberculosis	P	−	4×	−	71
F	93	Burn	P	−	−	−	56
F	78	Cerebral infarction	P	+	−	−	43
M	60	Cerebral bleeding	P	+	4×	−	41
M	82	Pneumonia	P	+	4×	−	17
M	74	Hepatoma	P	+	4×	−	NP
M	60	Acute pancreatitis	P and T	+	−	−	724
F	68	Cerebral bleeding	T	+	16×	2×	348
F	67	Cerebral infarction	T	−	4×	1×	227
M	57	Alcoholism	T	−	2×	1×	192
F	80	Cerebral infarction	T	−	8×	−	23
F	88	Cerebral infarction	Gl	+	4×	16×	4,486
M	55	OPCA	Gl	+	8×	4×	546
M	81	Cerebral infarction	Gl	+	2×	2×	350
F	78	Cerebral infarction	Gl	+	8×	−	62
F	94	Pneumonia	Gl	+	8×	1×	NP
F	71	Cerebral infarction	Gl	+	4×	1×	NP
M	92	Cerebral infarction	Gu	+	4×	−	901
M	66	Lung cancer	Gu	+	4×	−	147
M	74	Parkinson syndrome	Gu	+	4×	−	108
M	57	Ileus	Gu	−	4×	−	102
M	92	CHF	Gu	−	8×	−	NP
F	35	T-cell leukemia	K	+	2×	−	486

[a] Abbreviations: OPCA, Olivopontocerebellar atrophy; CRF, chronic renal failure; CHF, chronic heart failure; A, *C. albicans*; P, *C. parapsilosis*; T, *C. tropicalis*; Gl, *C. glabrata*; Gu, *C. guilliermondii*; K, *C. krusei*; NP, not performed; −, no reaction; M, male; F, female.

PBS and was incubated with the rabbit antienolase antibody diluted 1:1,000 in 0.1% Tween 20–PBS. After washing three times with 0.1% Tween 20–PBS over a 30-min period, the membrane was incubated for 60 min with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Amersham). The film was washed as described above and was developed by exposing it to an enhanced chemiluminescence Western blotting (immunoblotting) detection system kit (Amersham). The membrane was incubated for 60 s with the detection reagents provided by the manufacturer and was then exposed to the film (Hyperfilm; Amersham). The test detected as little as 5 ng of *Candida* enolase antigen per ml when the antigen was seeded into sterile saline or 10 ng of antigen per ml when the antigen was seeded into pooled human serum.

Latex agglutination tests. The Cand-Tec assay (Ramco Laboratories Inc., Houston, Tex.) uses latex beads coated with anti-*Candida* polyclonal antibody (4). The circulating mannan antigen was detected by using the Pastorex *Candida* assay (Diagnostics Pasteur, Marnes-la-Coquette, France), in which latex beads are coated with the anti-mannan monoclonal antibody (5). Both latex agglutination tests were performed according to the instructions provided by the manufacturer.

β-Glucan assay. (1→3)-β-D-Glucan is a major structural component of the fungal cell wall and has been reported to react with factor G, a coagulation enzyme of the *Limulus* amebocyte lysate. The content of (1→3)-β-D-glucan in the serum represented the difference in titers between two chromogenic *Limulus* assays, the conventional and the endotoxin-specific *Limulus* tests, as described previously (8, 15).

RESULTS

Enolase antigen, Cand-Tec, Pastorex *Candida*, and β-glucan assays for patients with candidemia. Enolase antigen was detected in 28 of 39 (71.8%) patients with candidemia (Table 1). In contrast, enolase antigen was not detected in any serum sample from patients with superficial *Candida* colonization, patients with other forms of deep-seated mycosis, or healthy subjects.

The Pastorex *Candida* assay for the detection of mannan antigen was positive for 10 of 39 (25.6%) patients with candidemia (Table 1). The antigen was detected in two of these patients who had positive blood cultures for *C. albicans*, three patients positive for *C. tropicalis*, and five patients positive for *C. guilliermondii*. However, mannan antigen was not detected in patients with superficial *Candida* colonization or those with other deep-seated mycoses. The mannan antigen was not detected in healthy subjects.

Considering a titer of four times or more to be a positive Cand-Tec assay result (5), 30 of 39 (76.9%) patients with can-

TABLE 2. Specificity and sensitivity of serodiagnosis of candidemia

Clinical diagnosis	No. of patients	No. of patients positive					
		Enolase reaction	Cand-Tec assay with the following titer:			Pastorex	β-Glucan assay
			≥8×	4×	≥2×		
Candidemia	39	28	16	30	36	10	27[a]
Superficial *Candida* colonization	10	0	1	2	4	0	0
Invasive pulmonary aspergillosis	5	0	0	1	2	0	5
Cryptococcosis	5	0	1	2	3	0	0
Healthy subjects	20	0	0	0	0	0	0
Sensitivity		28/39 (71.8)[b]	16/39 (41.0)	30/39 (76.9)	36/39 (92.3)	10/39 (25.6)	27/32 (84.4)[a]
Specificity		40/40 (100)	38/40 (95.0)	35/40 (87.5)	31/40 (75.0)	40/40 (100)	35/40 (87.5)

[a] β-Glucan was measured in only 32 of 39 patients with candidemia.
[b] Values represent number of patients positive/total number of patients (percent).

didemia were positive by that assay (Table 1). Two of 10 patients with superficial *Candida* colonization and 3 of 10 patients with other deep-seated mycoses (1 patient with invasive pulmonary aspergillosis and 2 patients with cryptococcosis) were positive for heat-labile antigen by the Cand-Tec assay. All healthy subjects were negative by the Cand-Tec assay. Sixteen of 39 (41.0%) patients with candidemia and 2 of 40 negative controls (1 patient with superficial *Candida* colonization and 1 patient with cryptococcosis) had Cand-Tec assay results with titers of eight times or more. By using a threshold titer of two times or more, 36 of 39 (92.3%) patients with candidemia and 9 of 40 negative controls (4 patients with superficial *Candida* colonization, 2 patients with invasive pulmonary aspergillosis, and 3 patients with cryptococcosis) had positive test results.

For both healthy subjects and patients with superficial *Candida* colonization, the concentration of β-glucan was less than 10 pg/ml. By using 60 pg of β-glucan per ml as the cutoff value for a positive test result (8), 27 of 32 (84.4%) patients with candidemia had positive results (Table 1). The concentration of β-glucan in 5 of 32 (15.6%) patients with candidemia ranged from 10 to 60 pg/ml. All five patients with invasive pulmonary aspergillosis were positive for β-glucan (>60 pg/ml); however, the concentration of β-glucan was less than 10 pg/ml in all patients with cryptococcosis.

The results of these assays, including their specificities and sensitivities, are summarized in Table 2. While the specificity of the dot immunoblotting assay for *Candida* enolase antigen was 100%, the sensitivity was only 71.8%. The specificity of the Cand-Tec assay was 87.5%, with a sensitivity of 76.9%, when a positive threshold titer of four times or greater was used. When the threshold titer for a positive test was increased to eight times or greater, the specificity increased to 95.0% but the sensitivity fell to 41.0%. When the threshold titer for a positive test was set at two times, the specificity and sensitivity were 75.0 and 92.3%, respectively. The specificity for mannan antigen detection by the Pastorex *Candida* assay was 100%, but the sensitivity was low (25.6%). The specificity of the assay for β-glucan was 87.5%, with a sensitivity of 84.4%.

DISCUSSION

The detection of antibodies in patients with *Candida* infections is of limited use. The major problem with tests for antibodies is that in healthy individuals, false-positive tests may result from exposure to *Candida* species present in the intestinal flora or at other sites. Thus, the antibody test does not usually distinguish between active and past infections. If blood is collected in the early stages of infection, before the development of the humoral response, then the antibody test may yield negative results. Furthermore, there are other problems with tests for antibody, particularly in patients with opportunistic mycoses, because the antibodies may not be detectable in immunocompromised patients.

Several antigen detection tests have been reported to be improved methods for the serodiagnosis of *Candida* infections (1, 7). For example, Matthews and Burnie (10) developed a method for detecting the 47-kDa cytoplasmic protein antigen in patients with systemic candidiasis in which the dot immunobinding method was used. The rate of positivity for the 47-kDa antigen by their test was 77% for patients with neutropenia, while it was 29% by the latex agglutination reaction. A 48-kDa antigen of *Candida* species, which was subsequently recognized as enolase (3, 9), is a novel marker for the diagnosis of invasive candidiasis (20). The present study demonstrated that enolase antigenemia is present in patients with candidemia, while the antigen was not present in patients with superficial *Candida* colonization or those who had no evidence of candidiasis. Thus, the specificity of the enolase antigen for the serodiagnosis of candidemia in the present study was 100%. However, the sensitivity of the test was only 71.8%.

Mannan is the most widely studied antigen in patients with candidiasis (1, 7). Various serological methods have been used to detect the level of circulating mannan antigen. In the present study we included a widely used commercial test, the Pastorex *Candida* assay, a monoclonal antibody-based latex agglutination assay (5). The Pastorex *Candida* assay reacts with mannans from *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. guilliermondii*, *C. parapsilosis*, and *C. pseudotropicalis*. However, it does not react with mannan from *C. krusei* (5) and is therefore not useful in the serological diagnosis of *C. krusei* infections. In the present study of patients with positive blood cultures, two of nine (22.2%) patients with *C. albicans* infections, three of five (60.0%) patients with *C. tropicalis* infections, and five of six (83.3%) patients with *C. guilliermondii* infections were positive by the Pastorex *Candida* assay. However, mannan antigen was not detected in any of the 20 patients with positive blood cultures, (14 patients with *C. parapsilosis* infections, 5 patients with *C. guilliermondii* infections, and 1 patient with a *C. krusei* infection).

The specificity and sensitivity of the Cand-Tec assay vary among reports (17). Many investigators suggested that the test should be considered positive only when the titer is four times

or greater. (17). In the present study, the specificity and sensitivity of the Cand-Tec assay were 87.5 and 76.5%, respectively, when the test titer threshold of four times or greater was used (Table 2). On the other hand, when the threshold titer for a positive result was increased to eight times or greater, the specificity increased to 95.0%, although the sensitivity fell to 41.0%. The Cand-Tec assay has some limitations; for example, the character of the antigen detected by the Cand-Tec assay is not known, and false-positive results due to rheumatoid factor have been observed (2). Therefore, it was difficult to confirm the diagnosis of candidiasis by the Cand-Tec assay alone.

Recent studies from our laboratory revealed that the (1→3)-β-D-glucan concentration is increased in an experimental model of fungal infection (12, 13, 15) as well as in the plasma of patients with mycoses (8, 14, 16). In the present study, we demonstrated that the difference in titers between the conventional *Limulus* test and the endotoxin-specific test, termed the fungal index (6), was high in patients with candidemia. At present, the Fungitec G test, which uses factor G fractionated from the *Limulus* amebocyte lysate and which was previously referred to as the G test, is also useful for the direct detection of (1→3)-β-D-glucan (13). The Fungitec G test is more sensitive than the fungal index, and the correlation between both methods is exponential (13). The cutoff value is lower for the fungal index (60 pg/ml) (8) compared with a cutoff of 20 pg/ml for the Fungitec G test (16). Recent studies demonstrated excellent results with the Fungitec G test, with a specificity of 100% and a sensitivity of 90% when the test was used to examine 202 febrile episodes (16). The concentration of (1→3)-β-D-glucan in all patients with candidemia was also demonstrated to be significantly elevated (14). The high degrees of specificity and sensitivity of the Fungitec G test suggest that the test may be useful for the early and rapid diagnosis of deep-seated mycoses in humans (14, 16).

The present study demonstrated that antigen detection tests are useful for the diagnosis of candidemia; however, all assays had certain limitations, reflected by either the sensitivity or the specificity, or both. Although none of the antigen detection assays was satisfactory for the serodiagnosis of candidemia, as indicated by their specificities and sensitivities, a combination of two assays, for example, the enolase assay with the β-glucan assay or the mannan assay with the β-glucan assay, may increase the accuracy of diagnosis of candidiasis. Repeated serum sampling may also improve the reliability of antigen detection tests for the diagnosis of candidiasis.

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