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Diagnosis of Invasive Candidiasis by a Dot Immunobinding Assay for *Candida* Antigen Detection

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A dot immunobinding assay which uses a polyclonal rabbit anti-*Candida* immunoglobulin G as the primary antibody and colloidal gold coated with goat anti-rabbit immunoglobulin G as the secondary antibody for the detection of *Candida* cytoplasmic antigens is described. It was able to detect as little as 1 ng of total *Candida* protein per ml when a cytoplasmic extract of *Candida albicans* was seeded into buffer and 10 ng/ml when the same extract was seeded into pooled human serum. Serial serum samples from four groups of patients were assayed for *Candida* antigen: (i) 22 patients with candidemia, (ii) 16 patients at high risk for invasive candidiasis, (iii) 3 patients with other deep mycoses, and (iv) 50 hospitalized patients at low risk for serious *Candida* infection. Of the 22 candidemic patients, 19 had invasive candidiasis and 3 had transient candidemia. Antigenemia was detected in 16 of the 19 patients with invasive candidiasis (including patients with *C. albicans*, *Candida tropicalis*, *Candida glabrata*, *Candida krusei*, and *Candida parapsilosis*) and in 4 of 16 patients at high risk for invasive candidiasis. There was no detectable antigen in 12 high-risk control patients, 3 patients with transient candidemia, 3 patients with other deep mycoses, and 50 relatively low-risk patients. The sensitivity for detecting invasive disease in candidemic patients and specificity for all patients studied were 84.2 and 94.4%, respectively. The positive predictive value was 80%; the negative predictive value was 95.7%. The sensitivity for neutropenic patients with invasive disease was 85.7%. This assay is rapid and accurate and appears to be useful in identifying candidemic patients with invasive candidiasis.

The incidence of nosocomial systemic fungal infections, which are primarily due to *Candida* species, particularly *Candida albicans*, is increasing (4, 5, 18, 19, 32). Data from the National Nosocomial Infections Surveillance System (NNIS) reveal that *Candida* species accounted for 7.7% of nosocomial bloodstream infections in the United States from 1985 to 1988 (18), ranking them as the fourth most common etiologic agent of nosocomial bloodstream infection, whereas in the 1984 NNIS study, *Candida* species had ranked as the eighth most common etiologic agent (19).

Patients with central intravenous catheters, hematologic malignancies, extensive burns, recent surgery, or prosthetic heart valves and those who are receiving broad-spectrum antimicrobial agents, corticosteroids, cytotoxic chemotherapy, or parenteral hyperalimentation are at the greatest risk of developing invasive *Candida* infections (1, 20, 27, 31, 37). Clinical diagnosis can be very difficult. Other than the occasional presence of endophthalmitis or skin lesions, there is a paucity of pathognomonic signs and symptoms of disseminated candidiasis (9). Since *Candida* species frequently colonize the oral cavity and upper respiratory tract of debilitated patients and the bladder of those with indwelling urinary catheters (23), cultures from these sites are not specific for invasive disease. Even blood cultures lack sensitivity and specificity, and the mere presence of candidemia does not necessarily imply disseminated infection (10). Therapy can be very toxic, and therefore clinicians are often reluctant to give it empirically except for neutropenic patients. It is because of the problem of making a timely diagnosis of serious *Candida* infection that attention has turned to the development of serological tests as diagnostic aids.

This article describes a rapid and accurate dot immunobinding assay for the detection of cytoplasmic *Candida* protein antigens and the application of this new assay in the serodiagnosis of invasive candidiasis. This assay employs the unique technology of a gold probe as the secondary antibody (30). The gold probe consists of colloidal gold particles coated with affinity-purified antibody—in this case, goat anti-rabbit immunoglobulin G (IgG). A pink color, the intensity of which is proportional to the amount of antigen present, develops at the site of immune reaction. When silver enhancement is performed, the silver precipitates on the surface of the colloidal gold particles, increasing the assay's sensitivity. There is a permanent visual record of the reaction without the need for autoradiography or enzymatic development.

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MATERIALS AND METHODS

Patients and controls. Sera were obtained from 22 candidemic patients; 16 noncandidemic patients defined as being at high risk for invasive candidiasis, i.e., patients who had colonization of mucosal surfaces with *Candida* spp., had indwelling intravascular catheters, and were receiving broad-spectrum intravenous antibiotics and hyperalimentation; 3 patients with other systemic fungal infections (2 with aspergillosis and 1 with cryptococcemia); and 50 hospitalized patients at relatively low risk for serious infection with *Candida* spp. Low-risk patients did not have colonization of mucosal surfaces with *Candida* spp., indwelling intravascular catheters, major surgery, broad-spectrum antibiotics, or hyperalimentation.

The charts of these patients were reviewed with special attention to risk factors for *Candida* infection, clinical diagnoses, microbiology and pathology data, and patient categorization. Patients were categorized as to transient candi-

demia, invasive candidiasis, high risk, or low risk without knowledge of the results of antigen detection.

Transient candidemia was defined as a single blood culture positive for *Candida* spp. Invasive candidiasis was defined as histologic evidence of tissue invasion by organisms morphologically consistent with *Candida* spp. with or without microbiologic evidence or a clinical syndrome suggestive of invasive *Candida* infection such as endophthalmitis, multiple blood cultures positive for *Candida* spp. in the absence of a removable focus of infection, or cultures positive for *Candida* spp. from normally sterile body sites.

Collection and handling of specimens. Serum samples were collected retrospectively from candidemic patients by obtaining extra sera from the hospital chemistry laboratory with particular attempts to retrieve specimens from the day or days on which the patient was candidemic and the days immediately preceding and following candidemia. Subsequent samples from patients and controls were collected prospectively. The samples were coded and frozen at −70°C until they were assayed. The assays were graded by a blind observer.

Identification of *Candida* species. *Candida* species were identified by the germ tube test, by formation of pseudohyphae on cornmeal-Tween 80 agar, and by the API 20 identification system (Analytab Products, Plainview, N.Y.).

Preparation of cytoplasmic antigen extract. *C. albicans* B311 (serotype A) (ATCC 32354) was grown on Sabouraud's slants for 18 to 24 h at 25°C. The organism was then inoculated into Sabouraud's broth, and flasks were placed onto a reciprocal shaker and incubated for 24 h at 150 rpm at 37°C. Yeast cells were harvested by centrifugation at 2,000 × *g* for 10 min and washed twice in 0.01 M sodium phosphate buffer (pH 7.2). The yeast cells were broken by mechanical disruption of a 1:1 mixture of the yeast cell slurry and glass beads in a Braun homogenizer for 4 min at 35,000 lb/in^2. Breakage was checked microscopically. Unbroken cells, cell walls, and debris were removed by centrifugation at 10,000 × *g* at 4°C for 30 min. The supernatant was centrifuged at 80,000 × *g* at 4°C for 90 min. The supernatant from this high-speed centrifugation was the cytoplasmic extract and contained soluble protein antigens. The protein content of this antigen preparation was 6.2 mg/ml, as determined by the method of Lowry et al. (26).

Dot immunobinding assay. A 100-μl-per-well portion of the sample to be tested for the presence of *Candida* antigen was applied in duplicate to an Immobilon membrane (Millipore, Bedford, Mass.) with a 96-well microfiltration apparatus with an adjustable vacuum (Bio-Rad, Richmond, Calif.). The sample was allowed to filter through the membrane by gravity to enhance binding. The membrane was washed with 100 μl of Tris-buffered saline (TBS) (20 mM Tris-HCl and 500 mM NaCl) (pH 7.5) per well. Blocking with 200 μl of 1% bovine serum albumin (BSA)–TBS per well was performed to prevent nonspecific binding. The membrane was washed with 200 μl of 0.05% Tween 20–TBS (TTBS) per well, and the primary antibody, rabbit anti-*Candida* IgM and IgG (DAKO, Santa Barbara, Calif.), diluted 1:100 in 1% BSA-TTBS was applied and allowed to filter through by gravity. The membrane was washed again, and 100 μl of a 1:25 dilution of secondary antibody, colloidal gold particles coated with affinity-purified goat anti-rabbit IgG in 1% BSA–TTBS (Bio-Rad), was applied per well, allowed to filter by gravity, and then washed. Each gravity filtration step took 45 to 60 min. After each wash step, a vacuum was applied for a few seconds until all of the wash solution drained from the wells. Each time an assay was performed, both positive and negative controls were included. Positive controls consisted of pooled human sera (GIBCO, Grand Island, N.Y.) seeded with known quantities of the *Candida* cytoplasmic antigen preparation. Three negative controls were included per assay to control for possible nonspecific binding. They were (i) duplicate wells to which both primary and secondary antibodies, blocking, and wash steps were applied, but seeded, pooled human sera (i.e., antigen) were not applied; (ii) duplicate wells to which seeded, pooled human sera were applied along with secondary antibody, blocking, and wash steps but not primary antibody; and (iii) duplicate wells to which seeded, pooled human sera, primary antibody, blocking, and wash steps, but not secondary antibody, were applied. Antigen-antibody binding was indicated by the deposition of a pink dot. Silver enhancement was performed according to the instructions provided by the manufacturer (Bio-Rad). The membrane to be enhanced was rinsed twice in distilled, deionized water to remove chloride ions that would affect the enhancement staining. The membrane was then washed in 0.2 M citrate buffer (pH 3.7) for 5 min. A solution of 0.85 g of hydroquinone in 90 ml of 0.2 M citrate buffer (pH 3.7) and 0.11 g of silver lactate in 10 ml of distilled, deionized water was added to the blotted membrane. Development was performed for 15 min in a vessel protected from light. The reaction was stopped by decanting the silver lactate-hydroquinone solution and adding a 1:10 dilution of fixing solution. After 5 min in the fixing solution, the membrane was washed twice with water and air dried. A black dot, the intensity of which was proportional to the amount of antigen present, was formed at the site of silver enhancement of the previously deposited colloidal gold. The dots were graded visually by a blind observer. The assay, including optimum dilutions of antibodies, incubation times, pH conditions, and buffers, was developed by first seeding buffer with known quantities of the cytoplasmic antigen preparation and then seeding pooled human sera with known quantities of the cytoplasmic antigen preparation.

Statistical analysis. Statistical analysis consisted of determination of the sensitivity, the specificity, the positive predictive value, and the negative predictive value of the assay by standardized formulae (12).

RESULTS

Calibration of the dot immunobinding assay. When known quantities of the *C. albicans* cytoplasmic antigen preparation were seeded into TBS or pooled human sera and assayed, the intensity of the dot was proportional to the amount of antigen present. The lower limit of detection for the colloidal gold precipitate was 12.5 ng of total *Candida* protein per ml when the *C. albicans* cytoplasmic antigen preparation was seeded into TBS buffer and 100 ng/ml when the antigen preparation was seeded into pooled human sera. After silver enhancement, as little as 1 ng of total *Candida* protein per ml was detected when the cytoplasmic *C. albicans* antigen preparation was seeded into TBS buffer, and as little as 10 ng of total *Candida* protein per ml was detected when the same antigen preparation was seeded into pooled human sera.

Patients with invasive candidiasis. Of 22 candidemic patients, 19 were found to have invasive candidiasis. Characteristics of those patients with invasive candidiasis are shown in Table 1. Nine cases were proved by histopathologic examination and culture of a tissue specimen, and 10 cases were proved by multiple positive blood cultures in the absence of a removable focus of infection. Three of the 22 candidemic patients were found to have transient candi-

TABLE 1. Characterization of patients with invasive candidiasis

Patient no.	Underlying condition(s)[a]	Species	Isolation site(s)	Antigenemia	No. of positive samples/ total no. of samples
1	AML, N	*C. albicans*	Blood, skin	−	0/1
2	AML, BMT, N	*C. tropicalis*	Blood, skin	+	5/8
3	AML, N	*C. krusei*	Blood	+	6/6
4	AML, N	*C. krusei*	Blood, skin	+	2/3
5	AML, BMT, N	*C. tropicalis*	Blood, skin	+	7/8
6	AML, N	*C. glabrata*	Blood	+	3/3
7	Lymphoma, BMT	*C. albicans*	Blood	+	5/6
8	Uterine carcinoma	*C. albicans*	Blood	+	4/6
9	AML, N	*C. albicans*	Blood	+	3/4
10	Huntington's chorea, pneumonia	*C. parapsilosis*	Blood	+	1/3
11	Vasculitis	*C. albicans*	Blood, peritoneum	+	7/8
12	PVE	*C. albicans*	Blood, heart valve	+	1/4
13	Endocarditis	*C. albicans*	Blood, heart valve	+	1/3
14	CAD, surgery	*C. albicans*	Blood, synovium	+	2/3
15	Squamous cell carcinoma	*C. albicans*	Blood	−	0/3
16	Ischemic bowel, surgery	*C. albicans*	Blood	+	4/6
17	Endocarditis	*C. albicans*	Blood, heart valve	+	4/6
18	PVE	*C. parapsilosis*	Blood, heart valve	+	5/6
19	Pneumonia, malnutrition	*C. albicans*	Blood	−	0/2

[a] AML, acute myelogenous leukemia; N, neutropenia; BMT, bone marrow transplant, PVE, prosthetic valve endocarditis; CAD, coronary artery disease.

demia. None of these patients with transient candidemia had detectable antigen. Table 2 summarizes (according to patient category) the results of antigen detection by the dot immunobinding assay. Eighty-nine serum samples from the 19 patients with invasive candidiasis (average, 4.7 samples per patient) and 19 samples from the 3 patients with transient candidemia (average, 6.3 samples per patient) were assayed. *Candida* cytoplasmic antigen was detected in the sera of 16 of the patients with invasive disease. The sensitivity, specificity, positive predictive value, and negative predictive value of the assay and the data used for their calculation are shown in Table 3. The overall sensitivity of the assay was 84.2%, the specificity was 94.4%, the positive predictive value was 80%, and the negative predictive value was 95.7%. For those patients with invasive disease and antigenemia, nine had infection with *C. albicans*, two each had infection with *Candida tropicalis*, *Candida parapsilosis*, and *Candida krusei*, and one had infection with *Candida glabrata*. Of the nine tissue-proved cases, eight had antigenemia (sensitivity, 88.8%). For those patients diagnosed with invasive candidiasis on the basis of multiple positive blood cultures, 8 of 10 were antigenemic (sensitivity, 80%).

Six of 16 patients with invasive candidiasis and antigenemia were neutropenic (absolute neutrophil count, less than 1,000). When data for these patients were analyzed separately, the sensitivity of the assay was 85.7%, the specificity was 78.5%, the positive predictive value was 66.6%, and the negative predictive value was 91.6%. Ten antigenemic patients with invasive disease were nonneutropenic. The sensitivity of the assay for this group was 83.3%, the specificity was 98.2%, the positive predictive value was 90.9%, and the negative predictive value was 96.6%.

When the detection of *Candida* cytoplasmic antigen was analyzed according to individual serum samples, the sensitivity of the assay was reduced to 67.4% for all patient groups with invasive candidiasis. Table 4 summarizes the sensitivity, specificity, positive predictive value, and negative predictive value for each category of patients with invasive candidiasis according to the number of serum samples tested.

Patients with *Candida* colonization. Sixteen patients who were at high risk for invasive candidiasis but who were not candidemic were studied as a control group. These patients all had *Candida* colonization of mucosal surfaces and indwelling intravascular catheters and were receiving broad-

TABLE 2. Results of antigen detection

Group	No. of patients (no. of serum samples)	
	Total	Positive for antigen
Invasive candidiasis	19 (89)	16 (60)
Tissue proved	9 (42)	8 (27)
Multiple blood cultures	10 (47)	8 (33)
Transient candidemia	3 (19)	0 (0)
Controls		
Colonized with *Candida* spp.	16 (85)	4 (6)
Not colonized	50 (100)	0 (0)
Other deep mycoses	3 (9)	0 (0)
Total	69 (194)	4 (6)

TABLE 3. *Candida* antigenemia in patients with or without invasive candidiasis on the basis of number of patients[a]

Group	No. of patients			Sensitivity (%)	PPV[b] (%)	NPV[c] (%)
	Antigen positive	Antigen negative	Total			
Invasive candidiasis	16	3	19	84.2	80	95.7
Tissue proved	8	1	9	88.8	66.6	98.5
Multiple blood cultures	8	2	10	80	66.6	97.1
Neutropenic	6	1	7	85.7	66.6	91.6
Nonneutropenic	10	2	12	83.3	90.9	96.6
No invasive candidiasis	4	68	72			

[a] Specificity was 94.4% for all patients studied, 78.5% for the neutropenic group, and 98.2% for the nonneutropenic group.
[b] PPV, positive predictive value.
[c] NPV, negative predictive value.

TABLE 4. *Candida* antigenemia in patients with or without invasive candidiasis on the basis of number of serum samples[a]

Group	No. of serum samples			Sensitivity (%)	PPV[b] (%)	NPV[c] (%)
	Antigen positive	Antigen negative	Total			
Invasive candidiasis	60	29	89	67.4	90.9	87.7
Tissue proved	27	15	42	64.2	81.8	93.2
Multiple blood cultures	33	14	47	70.2	84.6	93.6
Neutropenic	26	7	33	78.7	83.8	90.6
Nonneutropenic	34	22	56	60.7	97.1	86.3
No invasive candidiasis	6	207	213			

[a] Specificity was 97.2% for all samples studied, 93% for the neutropenic group, and 99.2% for the nonneutropenic group.
[b] PPV, positive predictive value.
[c] NPV, negative predictive value.

spectrum intravenous antibiotics. Ten patients were neutropenic; six were nonneutropenic. Among these 16 patients, four (25%) tested antigen positive. Of 85 serum samples from these patients, 6 (7%) were positive. Two of the antigen-positive patients had acute leukemia, were profoundly neutropenic, were colonized with *C. albicans* at multiple sites, and received amphotericin B empirically. These patients may have had invasive disease. If these two patients were no longer considered falsely positive and eliminated from analysis, the assay's specificity for all patients improved to 97.1% and the specificity for neutropenic patients improved to 91.6%. A third patient had recurrent *Candida* urinary tract infection. One of two serum samples assayed from this patient was positive. The patient died before upper urinary tract disease could be excluded.

Patients without candidiasis. One hundred serum samples from 50 patients at relatively low risk for *Candida* infection were assayed as a control group. All were negative for antigen. Three patients with other deep mycoses (two neutropenic patients with aspergillosis and one patient with cryptococcosis) were also antigen negative.

DISCUSSION

Despite increasing incidence, the serodiagnosis of serious *Candida* infection remains difficult. Efforts have focused on the detection of circulating *Candida* antigens, both cell wall and cytoplasmic, but the emphasis has been on detection of the cell wall antigen, mannan, by counterimmunoelectrophoresis, radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), and latex agglutination (2, 3, 6–8, 11, 13, 15–17, 21, 22, 24, 25, 29, 34, 35, 38, 41–43). For ELISAs, sensitivities of 23 to 100% and specificities of 92 to 100% have been reported (8). Although early studies showed good sensitivity and specificity for latex agglutination tests (16), recent studies have been less favorable (6, 7, 24), especially for patients with malignancies (11). Three monoclonal antibodies to cytoplasmic protein antigens of *C. albicans* with molecular masses of 35 to 38, 44 to 52, and 120 to 135 kDa have been produced and characterized (39). These monoclonal antibodies recognize various antigens that are apparently released during deep infection, including a 44- to 52-kDa protein (39). A 48-kDa antigen has been identified as the enzyme enolase (14). A dual-antibody liposome-based assay which recognizes the 48-kDa *Candida* cytoplasmic antigen has been developed (40). The results of a multicenter prospective clinical study of patients with cancer reveal the assay to be very specific (96% overall) when data for patients with cancer were measured against data for control groups including patients with mucosal colonization, bacteremia, and other deep mycoses. The sensitivity of this assay was lower than the specificity. In patients with proved deep-tissue invasion, the sensitivity per samples tested was 54%. Testing of multiple samples improved the sensitivity for antigen detection to 85% for patients with proved deep-tissue infection and to 64% in proved cases of candidemia (40). In another study whose results have appeared in abstract form, the sensitivity was lower (33%), but the specificity remained greater than 90% (36).

An enzyme-linked dot immunobinding assay based on the detection of a 47-kDa antigen of *C. albicans* by a monoclonal antibody has been described (28). This 47-kDa antigen is probably the same as the 48-kDa antigen (enolase). As little as 0.4 μg of total *Candida* protein per ml was detectable. The rate of detection of systemic *Candida* infections in neutropenic patients was 77%. The monoclonal antibody used in this particular assay was relatively specific to *C. albicans* and poor at detecting systemic candidiasis caused by *Candida* species other than *C. albicans*. Another problem with this particular assay is that it takes 24 h to give a result.

The assay described in the present article is also a dot immunobinding assay. The use of a gold probe as the secondary antibody to increase the sensitivity of this immunobinding assay is unique. The gold probe consists of colloidal gold particles coated with affinity-purified antibody—in this case, goat anti-rabbit IgG. Colloidal gold probes concentrating at the sites of immune reaction develop a pink color during incubation. This color can be graded visually (30). The gold deposit is then amplified by silver enhancement. Silver is precipitated on the surface of the colloidal gold particles. At least a 10-fold increase in sensitivity can be achieved. This technique serves to increase the sensitivity of the assay, allows color visualization of the reaction without the need for autoradiography or enzymatic development, and provides a permanent record. When the dot immunobinding assay and the gold probe with silver enhancement are used, as little as 1 ng of cytoplasmic antigen extract in buffer can be detected. When the dot immunobinding assay and the gold probe with silver enhancement are used to detect cytoplasmic *Candida* antigen extract seeded into pooled human sera, the lower limit of antigen detection is 10 ng.

The sensitivity of this assay improved detection of antigenemia in patients with invasive candidiasis. With a sensitivity for all patients with invasive candidiasis of 84.2%, the sensitivity of this assay is comparable to that of an improved avidin-biotin ELISA (33) and better than those of the dual-antibody liposome-based enolase assay (overall sensitivity = 75%) (40) and the enzyme-linked dot immunobinding assay (overall sensitivity = 76%) (28). The sensitivity for detection of antigenemia in tissue-proved cases of invasive disease (88.8%) was slightly better than that in cases diagnosed by multiple positive blood cultures (80%). In this study, sensitivities for neutropenic (85.7%) and nonneutropenic (83.3%) patients were comparable. Because of the polyclonal nature of the antibodies employed, antigenemia caused by other *Candida* species, including *C. tropicalis*, *C. krusei*, *C. parapsilosis*, and *C. glabrata*, was detectable. This is an important feature, since *Candida* species other than *C. albicans* are emerging nosocomial pathogens.

The specificity of this assay was very high (94.4% overall for invasive candidiasis). The assay was equally specific for tissue-proved cases and for those diagnosed by multiple blood cultures. The specificity for nonneutropenic patients

was very high (98.2%). The assay was less specific (78.5%) for neutropenic patients, however. The nature of the high-risk control group may have been responsible for the diminished specificity for the neutropenic group. Two antigen-positive patients had acute leukemia, were profoundly neutropenic, were heavily colonized with *C. albicans* at multiple sites, and received courses of amphotericin B empirically. These patients probably had invasive candidiasis with negative blood cultures. If they were eliminated from analysis, assay specificity for all patients improved to 97.1% and specificity for neutropenic patients improved to 91.6%. Although the number of patients tested was small, there was no cross-reactivity with *Aspergillus* or *Cryptococcus* spp.

The sensitivity for all patient groups improved by assaying multiple serum samples from each patient. For the detection of antigenemia in all patients with invasive candidiasis, the sensitivity was 67.4%. The sensitivity was 78.7% for the neutropenic group. The sensitivity per sample was higher with this assay than with the enolase assay and the enzyme-linked dot immunobinding assay (28, 40). When multiple serum samples were assayed, specificity decreased slightly from 97.1 to 94.4% for all patients with invasive candidiasis. For serum samples from the neutropenic group, specificity decreased from 93 to 78.5%. This is probably a reflection of the small number of false-positive samples (5 of 56 [8.9%]) compared with the number of false-positive patients (3 of 10 [30%]) in the neutropenic control group.

In summary, a rapid and sensitive dot immunobinding assay which uses relatively inexpensive, commercially available antibodies for the detection of *Candida* antigen in patients with invasive candidiasis has been described. This assay appears to be useful in the diagnosis of serious *Candida* infections. It merits further testing in a prospective manner with larger numbers of patients.

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