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Evaluation of Enzyme Immunoassay for *Candida* Cytoplasmic Antigens in Neutropenic Cancer Patients

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A *Candida albicans* cytoplasmic antigen enzyme immunoassay (CACP antigen EIA) was developed with antibodies raised against antigens prepared from yeast cells grown under standardized growth conditions. The *C. albicans* components reactive in the EIA were shown to be predominantly proteins with associated carbohydrates. Denaturing gel electrophoresis revealed the presence of five major CACP proteins with molecular weights between 36,000 and 44,000. The clinical usefulness of the CACP EIA was evaluated by retrospective blinded measurement of 89 serum samples from 31 granulocytopenic patient episodes. Twice-weekly surveillance cultures, sequential serum samples (approximately once per week or with change of the clinical course), and standard diagnostic criteria of fungal infection were used to categorize patients. The sensitivity and specificity of the CACP assay on the basis of serum samples were 82 and 100%, respectively (67 and 100% on the basis of patient episodes). The positive and negative predictive values were 100 and 97% for serum (100 and 93% for patient episodes). By comparison, the CANDTEC assay had low sensitivity (33%) and poor positive predictive values (50%). The CACP EIA may be a useful test suitable for further evaluation as a method for the diagnosis of invasive *Candida* infection in neutropenic cancer patients.

Detection of *Candida* antigenemia, particularly in neutropenic cancer patients, may be an important adjunct to the diagnosis and treatment of invasive fungal infection. Presently, definitive diagnosis of candidiasis is dependent upon tissue histology and cultures or positive blood cultures. Both methods, while specific, are insensitive for the diagnosis of deep fungal infection. In the absence of practical diagnostic methods, patients are often treated empirically with antifungal agents should broad-spectrum antibacterial therapy fail to resolve apparent infectious episodes. However, the response to empiric antifungal therapy is difficult to assess. Causes of persistent fever in the neutropenic host include poorly responsive bacterial infection, bacterial superinfection, viral infection, fungal infection, underlying disease, blood products, and drug fever either singly or in combination. Therefore, laboratory diagnostic support would be invaluable in deciding upon treatment for these patients.

Neutropenic and immunosuppressed patients may not mount a significant antibody response to candidal antigens despite deep tissue infection. Candidal antibody tests have been poor predictors of invasive disease. In the past decade, the detection of fungal antigens has been proposed as a better predictor of infection in these patients. A number of methods have been developed to address this issue (6, 11, 17, 18, 26, 28, 29, 40, 44, 45, 48–50). Three commercial assays are available for detection of *Candida* antigens (13, 14, 22, 24, 37, 42). One of these (LA-CADS; Immunomycologics Inc., Norman, Okla.) has not been subjected to a controlled clinical evaluation. The CANDTEC assay (Ramco Laboratories Inc., Houston, Tex.) has been shown to be relatively insensitive (30 to 50%) in comparative studies (1, 31, 41). The most recent, an enolase assay (B-D Microbiological Systems, Baltimore, Md.) has been suggested to have a sensitivity of approximately 85%, although antibody to enolase has been detected in patients negative for the antigen (10, 33, 43, 48). Most of these investigations have been done with single serum samples from individual patients. Furthermore, since fungal infection in the neutropenic host can have an insidious onset, it may be important to longitudinally analyze patients' sera for *Candida albicans* antigens and to correlate data obtained with the clinical presentation of the patient.

We report here on the development and preliminary clinical evaluation of a *C. albicans* cytoplasmic protein antigen enzyme immunoassay (CACP antigen EIA) in neutropenic patients with underlying hematologic malignancies, comparing microbiologic diagnosis, surveillance cultures, clinical course, and the contribution of serologic analysis.

MATERIALS AND METHODS

***Candida* antigen preparation.** Candidal antigens were derived from clinical strains of *C. albicans* (60553, 88114, 88117, and 12591), *Candida parapsilosis* (64538), and *Candida tropicalis* (89724), and from *C. albicans* ATCC 31353 from the American Type Culture Collection. The organisms were maintained at room temperature on Sabouraud's agar slants (Difco, Detroit, Mich.) containing 4% (wt/vol) dextrose and were subcultured when required. Clinical isolates were recovered from patients with underlying hematologic malignancies. Sabouraud's dextrose broth (50 ml in a 250-ml Ehrlenmeyer flask) was inoculated with a loopful of organism from an overnight Sabouraud's agar slant culture and inoculated in a shaking water bath at 120 rpm at 30°C for 12 h. As determined by light microscopy, approximately 99% of the cells grown were budding yeast cells. The yeast cells were counted with a hemocytometer and inoculated at 10^6 cells per ml in 800 ml of

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Sabouraud's dextrose broth contained in a 4-liter Ehrlenmeyer flask. The cultures were incubated in a shaking controlled-temperature incubator at 120 rpm at 30°C until the late-log phase of growth (11 to 12 h). The cells were collected by centrifugation for 10 min at 2,000 × *g* at 4°C and resuspended in an equal volume of deionized double-distilled H_2O. The cells were recentrifuged and, after an additional wash cycle, were resuspended in 20 ml of double-distilled H_2O. The resulting slurry was passed five times at 4°C and 69,000 kPa through a French pressure cell (American Instrument Co., Silver Spring, Md.). Cell disruption was estimated at 95% as determined by phase-contrast microscopy. Unbroken cells and cellular debris were removed by centrifugation at 12,600 × *g* at 4°C for 30 min. Antigens in this supernatant are referred to as CACP antigens. Antigens in the supernatant after ultracentrifugation at 100,000 × *g* at 4°C for 1 h are referred to as Ultra CACP antigens. Protein concentrations were determined with the Bio-Rad protein reagent (Bio-Rad Laboratories, Richmond, Calif.), and bovine serum albumin (Sigma, St. Louis, Mo.) was used as a standard. Carbohydrate content was determined by the orcinol method with D-mannose as the reference. Mannan from the cell wall was prepared by the method of Peat et al. (39).

Antiserum production. Male New Zealand White rabbits were immunized intramuscularly in each flank with 0.5 mg of CACP antigen protein emulsified in 0.5 ml of Freund's complete adjuvant. The immunization was repeated at day 14 with Freund's incomplete adjuvant. At day 28, the rabbits were immunized intramuscularly in a single flank with 0.1 mg of CACP antigen emulsified in 0.5 ml of Freund's incomplete adjuvant. This was repeated on days 55 and 90. The rabbits were euthanized on day 132 and terminally bled by cardiac puncture. Rabbit immunoglobulin G (IgG) was purified by chromatography on protein A-Sepharose (27). The concentration of the purified IgG was determined with an extinction coefficient of 13.8 at 280 nm. Conjugation with horseradish peroxidase (type VI; Sigma) was done according to the method of Nakane and Kawaoi (36), except that the blocking step was omitted since there is negligible binding of horseradish peroxidase to itself following the reaction with periodate.

Trap EIA. Purified rabbit anti-CACP IgG was diluted to 7.5 μg/ml in phosphate-buffered saline (PBS) containing 20 mM sodium phosphate and 140 mM NaCl (pH 7.4), and 200 μl was added to wells of Dynatec Immulon II EIA plates (Baxter, Edmonton, Canada). After overnight incubation at room temperature, PBS and unabsorbed IgG were removed from the wells by inversion on absorbent towels. Dilutions of CACP antigen or patient's serum were made in assay diluent (PBS containing 2% [vol/vol] bovine serum albumin and 2% [vol/vol] Tween 20), and 200 μl was added per well. After incubation for 1 h at 37°C and washing four times in double-distilled H_2O, 200 μl of horseradish peroxidase-conjugated anti-CACP antigen IgG diluted 1:800 in assay diluent was added (this dilution of horseradish peroxidase-conjugated antibody was predetermined to give an optical density of 2.5 to 3.0 at 492 nm in an EIA with CACP antigen-coated wells). After 1 h at 37°C, the plate was again emptied and washed. Two hundred μl of 80 mM sodium citrate-sodium phosphate (pH 5.5), containing freshly dissolved 3-mg/ml *o*-phenylenediamine and 0.02% (vol/vol) H_2O_2, was added to each well. The optical density at 492 nm was measured with a Bio-Rad 2550 EIA plate reader interfaced with a MacPlus Macintosh computer. Averaged values of test wells were corrected for the average optical densities of wells to which no CACP antigen had been added. In this test format, the CACP EIA could be completed within 24 h.

One- and two-dimensional SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions according to the method of Laemmli (30). A Hoefer SE600 vertical slab gel unit (Hoefer Scientific Instruments, San Francisco, Calif.) was used with stacking and separating gels consisting of 4 and 10% acrylamide (British Drug Houses [BDH], Vancouver, Canada), respectively. Sample lanes received 75 μg of protein. Electrophoresis was initiated at 100 V and increased to 125 V once the tracking dye had entered the separating gel. After the tracking dye had exited the separating gel, the gel was removed and stained with Coomassie blue R-250 (Sigma).

Two-dimensional SDS-PAGE was performed according to the method of O'Farrell (38). Protein samples were diluted 1:3 in sample buffer (9.5 M urea [BDH], 2% Nonidet P-40, 2% ampholytes [Sigma], 5% β-mercaptoethanol [Sigma]), and the cathode buffer was 10 mM phosphoric acid. The isoelectric gradient was prerun sequentially for 15 min each at 200, 300, and 400 V. Samples (50 μg of protein) were subjected to isoelectric focusing (4 h, 1,000 V). Afterwards, the tube gels were frozen at −20°C. Upon thawing, the gels were expelled from the glass tubes, fixed to the top of SDS gels with agarose (1% agarose [BDH], 125 mM Tris-HCl [pH 6.8], containing 0.1% SDS), and electrophoresed as described above.

Characterization of EIA-reactive CACP antigens. For each test, 2.1 mg of CACP antigens in 1.0 ml of PBS was used. Treated and untreated CACP antigens were then compared for reactivity in the CACP antigen EIA. Heat treatment was at 56 or 80°C for 30 min. Protease digestion was with 20.5 μg of either trypsin (Sigma) or proteinase K (Merck, Darmstadt, Germany) at room temperature for 18 h. $CaCl_2$ was added to 5 mM for optimal proteinase K activity. After the incubation, trypsin was inhibited by adding 20.5 μg of egg white trypsin inhibitor (Sigma), while proteinase K was inhibited by adding phenylmethylsulfonyl fluoride (Sigma) to 400 μM. Finally, carbohydrate was oxidized with 0.08 mM sodium periodate ($NaIO_4$) at room temperature for 30 min (36). Excess $NaIO_4$ was blocked by adding ethylene glycol to 160 mM.

Patient samples. The patients evaluated in this study were inpatients at the Health Sciences Centre, Winnipeg, Canada. All patients included in the evaluation had an underlying hematologic malignancy or solid tumor treated by myelotoxic agents or a severe aplastic anemia resulting in prolonged and severe neutropenia with attendant potential for development of invasive fungal infections. Samples of serum were collected from these patients on admission to the hospital, at the onset of neutropenic febrile episodes ($<1.0 \times 10^9$ granulocytes per liter), and at approximately weekly intervals thereafter while the patients remained neutropenic or when there was clinical suspicion of invasive fungal infection. Serum samples were stored at −70°C and were tested for candidal cytoplasmic antigens in a blinded fashion. Unpublished data from our laboratory have shown that there is no significant loss of antigen activity in samples that have been stored at −70°C for up to 5 years.

Cultures. Blood cultures and cultures from clinically infected foci were collected according to established clinical guidelines when required (5). Blood cultures were incubated at 35°C for up to 21 days with the BACTEC 460 system. Twice-weekly surveillance cultures from the nose, throat, rectum, and urine were collected during the course of each neutropenic episode in the hospital. Soiled swabs were vortexed in 0.5 ml of PBS, and 0.01 ml was streaked onto Sabouraud's agar, inhibitory mold agar (BBL, Baltimore, Md.), 5% sheep's blood agar, and MacConkey agar. Semiquantitative estimation of organism numbers was measured by the

highest quadrant of growth: 1+ is the first quadrant, and 4+ is the fourth quadrant. The correlation coefficient of this method, compared with that of dilutional quantitative cultures, was 0.8, with 1+, 2+, 3+, and 4+ being representative of $\leq 10^4$, 10^5, 10^6, and $\geq 10^7$ CFU/g of feces, respectively (32a). For the purpose of expressing the density of fungal colonization at each site for each neutropenic episode, the mean-plus count of fungal colonization was determined. Urine samples were cultured with 0.01- and 0.001-ml loops.

Patient management. Patients were hospitalized in single isolation rooms; diet consisted of normal hospital food with the exclusion of salads. Gut decontamination in afebrile patients included trimethoprim-sulfamethoxazole or nalidixic acid (8). Oral antifungal therapy with nystatin (10^6 U every 6 h) was added for clinically apparent mucosal infection (9). At the onset of fever (>38.5°C for more than 12 h or chills, rigors, and fever >39°C), oral antibacterial gut decontamination was discontinued, and empiric intravenous antimicrobial therapy was started. Antibiotics included ticarcillin, moxalactam, or aztreonam, plus an aminoglycoside; gram-positive coverage with vancomycin or cloxacillin was added at the discretion of the infectious disease service. Intravenous amphotericin B (0.6 to 1.0 mg/kg/day—maximum 60 mg/day) was added if patients failed to respond to 4 to 10 days of antibacterial therapy.

For the purposes of analysis, the patients were divided into four subgroups on the basis of clinical and microbiological criteria. Group I included patients who were not colonized by *Candida* species, were less likely to be at risk for disseminated candidiasis on the basis of previous epidemiologic studies (9, 15), did not develop criteria for disseminated candidiasis, or had documented nonfungal infection. Group II included patients who became colonized by *Candida* species in surveillance cultures but who had documented nonfungal infection or had autopsy evidence excluding fungal infection. Group III included patients clinically suspected to have fungal infection on the basis of their failure to respond to antibacterial therapy and their response to empiric amphotericin B and/or coincident resolution of neutropenia with or without the isolation of fungi. Group IV included patients with documented fungal infection on the basis of blood cultures, tissue biopsies, or postmortem examination.

Control serum samples were obtained from six healthy laboratory staff, 30 infant serum samples, and pooled normal human sera. None of these gave positive responses in the EIA antigen assay. Although the CACP antigen EIA was capable of detecting as little as 5 ng of *Candida* antigen, the threshold concentration for positivity of patient samples was conservatively determined to be 150 ng/ml, since the most linear portion of the standard curve began at this antigen concentration, and a more precise computerized interpolation of results from patients' sera was possible.

The CANDTEC assay was used according to the manufacturer's instructions in serial twofold dilutions beginning at ratios of 1:2 to 1:16. Positive and negative control sera were included with each test, and a positive result was taken to be a titer of $\geq$1:4 according to the manufacturer's recommendations. The sensitivity and specificity of these assays were developed with methods outlined by Galen and Gambino (23).

RESULTS

Standardized growth and preparation of CACP antigens. The same growth curve was consistently obtained with all four *C. albicans* strains and the other species tested. Similar concentrations of protein (27 ± 5 mg/ml) and carbohydrate (4.4 ± 0.5 mg/ml) were found for each of 12 CACP antigen preparations: 4 from *C. albicans* 60553 and 8 from the other three clinical strains of *C. albicans* and *C. albicans* ATCC 31353.

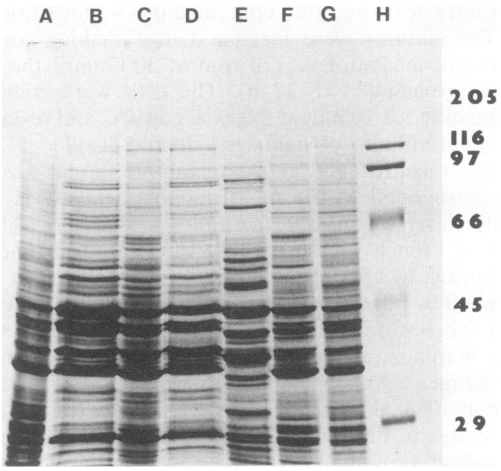

FIG. 1. SDS-PAGE of *Candida* cytoplasmic antigens. Lanes: A, CACP antigens from strain 60553; B, Ultra CACP antigens from strain 60553; C, CACP antigens from ATCC 31353; D, Ultra CACP antigens from ATCC 31353; E, cytoplasmic antigens from *C. parapsilosis* 64538; F, CACP antigens from strain 88114; G, CACP antigens from strain 88117; H, molecular weight standards (in thousands).

One- and two-dimensional SDS-PAGE of CACP antigens. Five major proteins with molecular weights of 36,000, 38,000, 41,000, 42,000, and 44,000 (Fig. 1, lanes A, C, F, and G) were observed in the *C. albicans* strains tested, including ATCC 31353. These proteins were enriched in Ultra CACP antigen preparations (Fig. 1, lanes B and D). Analysis of cytoplasmic antigens from *C. parapsilosis* 64538 revealed three major proteins in the same molecular weight range (Fig. 1, lane E). Cytoplasmic antigens of *C. tropicalis* 89724 showed only one major protein (molecular weight, 36,000) in this size range (data not shown). The five major CACP proteins of *C. albicans* 60553 are shown by two-dimensional SDS-PAGE in Fig. 2. The

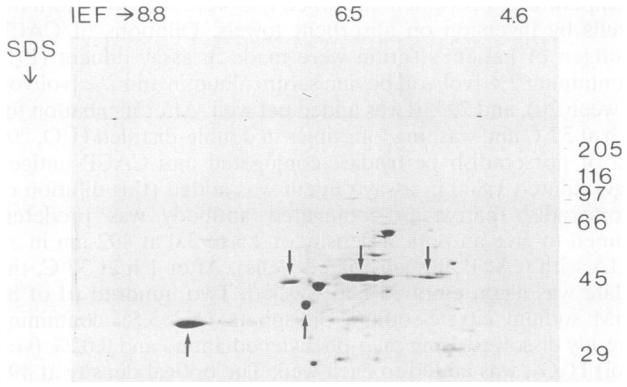

FIG. 2. Two-dimensional SDS-PAGE of CACP antigens from strain 60553. Arrows denote major antigens seen in all four strains of *C. albicans* tested. The directions of SDS-PAGE and isoelectric focusing (IEF [with the appropriate pH range]) are indicated. Molecular weight correlates (in thousands) are indicated on the right side of the figure.

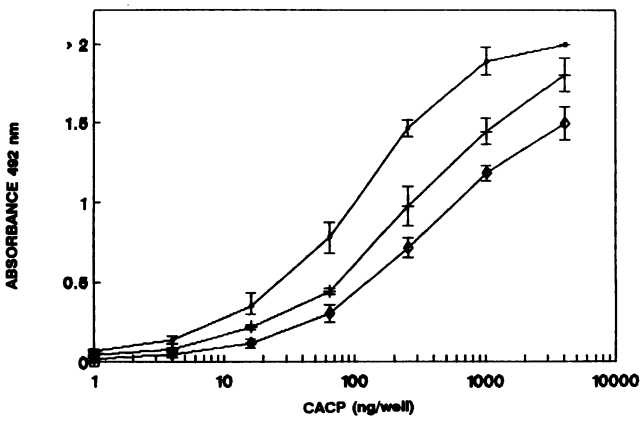

FIG. 3. Standard curve for the CACP antigen EIA. CACP antigen from strain 60553 was used. Pooled serum (not heat inactivated) from 10 adult donors was added to the assay diluent at 0 (●), 10 (+), or 20% (◇). Standard deviations are noted by error bars.

isoelectric points of the five proteins ranged from below pH 6.5 to approximately pH 8.5.

EIA standard curve. CACP antigen concentrations of 1 to 4,096 ng per well (Fig. 3) were used. If a cutoff of 3 standard deviations above the mean of wells to which no CACP antigens had been added was used, the sensitivity end point of the EIA was routinely <2 ng per well (10 ng/ml) when the CACP antigens were in assay diluent. The working range of the assay then extended from 4 to 1,024 ng per well. When CACP antigens were diluted in assay diluent containing 10 or 20% human serum, the standard curve shifted up twofold and four- to fivefold, giving sensitivity end points of approximately 4 ng per well (20 ng/ml) and 10 ng per well (50 ng/ml), respectively. Therefore, when clinical serum specimens are tested at 1:5 or 1:10 dilutions and results are compared with those for CACP antigens in assay diluent alone (i.e., the most easily established standard curve), CACP antigen concentrations in the samples will be underestimated.

Cross-reactivity. Only 30 ng of CACP antigens derived from strain 60553 per well was required to yield an assay value of 0.5 (Fig. 4). *C. tropicalis* 89724 cytoplasmic antigens did not react as well in the EIA, since 180 to 200 ng of antigen per well was required before an assay value of 0.5 was obtained. Cytoplasmic antigen from *C. parapsilosis* 64538 reacted very weakly in the CACP antigen EIA, since 4,096 ng per well did not yield an absorbance of 0.5. Purified *C. albicans* 60553 mannan-containing components also reacted in the EIA, indicating that some of the reactivity seen with the CACP antigens is likely due to cell wall mannoproteins.

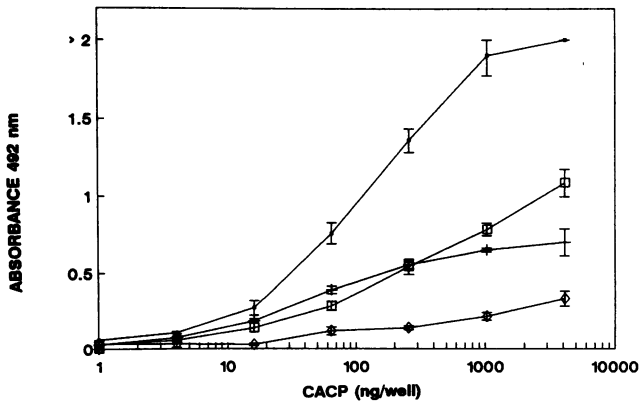

FIG. 4. Reactivity in the CACP antigen EIA of other *Candida* antigens. Results with cytoplasmic antigens from *C. tropicalis* 89724 (□) and *C. parapsilosis* 64538 (◇), cell wall mannoprotein from *C. albicans* 60553 (+), and CACP antigens from strain 60553 (●) are shown. Standard deviations are noted by error bars.

Effect of heat, proteases, and periodate on reactivity in the CACP antigen EIA. Heating CACP antigens from *C. albicans* 60553 at 56°C for 30 min caused an eightfold reduction in reactivity, while heating at 80°C for 30 min abolished reactivity. Incubation of CACP antigens with periodate caused a twofold reduction in assay reactivity, while a fivefold reduction was observed when CACP antigens were treated first with proteinase K and then with periodate. Digestion of CACP antigens with trypsin and proteinase K resulted in 2- and 2.5-fold decreases in assay reactivity, respectively. These data suggested that the EIA reactivity was predominantly protein, with carbohydrate moieties additionally responsible.

Clinical profiles. The results of assays of the four patient groups are given in Table 1 and show a correlation between invasive infection with *C. albicans* and positive CACP antigen EIA results. A total of 864 surveillance cultures were obtained from four sites, with a mean of seven cultures at each site per patient episode. The mean duration of neutropenia was 19.7 days in group I and II patients, whereas patients in groups III and IV had a mean duration of neutropenia of 30.7 days and were at increased risk of disseminated fungal infection. Mean fungal densities per site in each patient did not show a strong trend to more intense colonization by fungi before the onset of fungemia. The duration of neutropenia is likely an important variable. Nevertheless, the negative surveillance cultures separated patients into those at lower versus those at higher risk of fungal invasion.

Patients who were not colonized and had no invasive candidal infection (group I) did not have antigens in serum that reacted in the CACP antigen EIA. An analysis of group I and II patients showed that patients with *Streptococcus pneumoniae* (no. 11), *Bacillus* species (no. 6 B), and *Micrococcus* bacteremia (no. 3 A) can have low titers in the 60- to 130-ng/ml range but below the 150-μg/ml cutoff for the assay. The same was true for patients colonized with *C. albicans* who had no clinical evidence of invasive fungal infection (group II). Two of these patient episodes (no. 14 and 15) involved serum titers on single samples considered falsely positive by the CANDTEC assay. Patient 14 had pyrexia of unknown origin, organ infiltration with chronic lymphocytic leukemia, and no evidence of fungal infection at postmortem examination. Patient 15 had fever secondary to a lymphoma and had been recovered from neutropenia for 11 days at the time of the positive test.

Patients in group III included patients who failed to respond to antibacterial therapy and were treated as having possible fungal infections with the addition of intravenous amphotericin B. Patients 16, 17, and 18 were suspected to have noncandidal fungal infection and were treated empirically with amphotericin B. Postmortem examinations of these patients were refused. Patients 19 to 22 had simultaneous resolution of fever and neutropenia without documented fungal infection. Patient 23 was the only patient in group III highly suspected to have a deep fungal infection on the basis of the clinical course. This patient was colonized in the oropharynx and rectum with 3+ *C. albicans* at the time of development of a pyrexia of unknown origin. He failed to respond to antibiotics and was started on intravenous amphotericin B on day 7. The CACP antigen titer

TABLE 1. Clinical profiles and antigen results for 28 patients with 31 episodes of risk for invasive candidiasis

Group	Patient[a]	Sex/age (yr)	Disease[b]	Duration (days) of neutropenia (10^9 neutrophils/liter)	Microbiology surveillance[c]				Antigen test results[d]		Infection diagnosis (day)[e]
					Throat	Nose	Rectum	Urine	CACP (no.)	CANDTEC (no.)	
I	1	M/35[f]	AML	26	0	0	0	0	Neg (2)	Neg (2)	Viridans streptococcal bact. (day 1), *S. epidermidis* bact. (day 10)
	2	F/31	AA	42	0	0	0	0	Neg (4)	Neg (4)	*Pseudomonas aeruginosa* bact. (days 1 and 18)
	3A	M/67	AML	31	0	0, Asp. (day 20)	0	0	Neg (3)	Neg (3)	*Micrococcus* bact. (day 13), AMB for *Aspergillus* infection in nose
	4	M/61	AML	24	0	0, Asp. (day 20)	0	0	Neg (2)	Neg (2)	FUO unresponsive, CD pneumonia, query aspergillosis
	5A	M/50	AML	14	0	0	0	0	Neg (3)	Neg (3)	CD cellulitis
	6A	F/61	AML	17	0	0	0	0	Neg (3)	Neg (3)	*E. coli* bact. (day 1)
	7	M/34	CML-BC	14	0	0	0	0	Neg (3)	Neg (3)	*S. epidermidis* bact. (day 1), failed on ticarcillin-tobramycin, AMB and vancomycin added
	8	F/55	AML	21	0.2	0	0	0	Neg (3)	Neg (3)	CD pneumonia, unresponsive to antibacterial and antifungal agents, resolved with resolution of neutropenia
	9	M/29	AML	21	0	0	0	0	Neg (3)	Neg (3)	FUO unresponsive to antibiotics and antifungal agents, resolved with resolution of neutropenia.
II	5B	M/50	AML	17	1.6	0	0	0	Neg (2)	Neg (2)	FUO, responds to antibiotics (day 1)
	6B	F/61	AML	24	0	0.1	1.9	0	Neg (4)	Neg (4)	FUO, responds to antibiotics (day 1), *Bacillus* bact. (day 20)
	10	F/61	ST	8	0	0.3	1.0	10^8 (day 8)	Neg (3)	Neg (3)	*Klebsiella*, *Enterobacter*, and group B streptococcal bact. (day 1)
	11	M/71	AML	24	1.1	0	1.1	$10^5 \times 3$	Neg (2)	Neg (2)	*S. pneumoniae* bact. (day 1), *Enterococcus faecalis* and *Streptococcus mitis* bact. (day 8)
	12	F/37	AML	28	0.5	0, Alt.	0.9	0	Neg (3)	Neg (3)	CD pneumonia (day 1), viridans streptococcal bact. (day 12), *P. aeruginosa* bact. (day 17)
	13	F/52	ST	9	0.7 (Ct.)	0	1.0, 0.7 (Ct.)	0	Neg (2)	Neg (2)	*E. coli* bact. (day 1)
	14	M/56	CLL	13	2.0 (Ct.)	0	0.7 (Ct.)	0	Neg (2)	Neg (1), Pos (1 [1:4])	FUO, no fungal infection at postmortem
	15	M/63	HD	16	1.8	0	1.3	$10^{5-7} \times 2$	Neg (3)	Neg (2), Pos (1 [1:4])	Fever due to HD, neutropenia resolves in 2 days, fever persists
III	16	M/67	CLL	13	4.0	O, Pen.	0	0	Neg (1)	Neg (1)	CD pneumonia (day 1), unresponsive to antibiotics, AMB added with response, query fungal pneumonia, died, no postmortem

	3B	M/67	AML	49	0	0	0	0	Neg (3)	Neg (3)	FUO, unresponsive to antibiotics, responds to AMB
	17	F/41	AML	17	1.8	0, Asp. (day 15)	1.8	0	Neg (1)	Neg (1)	FUO, responds to antibiotics, query aspergillosis, died, no postmortem
	18	F/49	ST	12	0.4, 0.8 (Ct.)	2.2, Pen.	3.8, 0.8 (Ct.)	$10^8 \times 3$	Neg (2)	Neg (2)	FUO, responds to antibiotic, AMB added for new fever, died, no postmortem
	19	M/48	AML	49	2.6	0.1	1.6	0	Neg (6)	Neg (6)	*Staphylococcus aureus* bact. (day 7), FUO (day 15), AMB added, no response, fever resolved with resolution of neutropenia
	20	M/66	AML	24	2.5	0	0.5	10^5	Neg (2)	Neg (2)	FUO, responds to antibiotics, FUO again, responds to AMB and resolution of neutropenia
	21	F/70	CML-BC	28	0	0	1.0, 0.2 (Tg.)	0	Neg (4)	Neg (4)	*S. epidermidis* bact. (day 1), poor response to antibiotic, AMB added (day 7) and neutropenia resolved
	22	M/66	HD	19	0	0.2	0	6×10^6 (Ct.), 10^6 (Co.) $\times$ 2	Neg (4)	Neg (4)	CD cellulitis, temporary improvement, FUO (day 4), AMB added (day 9), fever resolves with resolution of neutropenia
	23	M/55	CML-BC	50	1.1, 0.2 (Ct.), 0.3 (Co.)	0.3, 0.1 (Co.)	0.8, 0.3 (Co.)	0	Neg (2), Pos (2 [200, 340])	Neg (3), Pos (1 [1:8])	FUO unresponsive to antibiotics, responds to AMB twice without resolution of neutropenia
IV	24	M/40	AML	28	0.2, 0.7 (Tg.)	0	0.3	10^6 (Ct.)	Neg (1)	Neg (1)	*C. tropicalis* fungemia (day 1), *C. tropicalis* from leg biopsy
	25	M/44	AML	19	1.0	Asp. (day 8)	0.8	0	Neg (2)	Neg (2)	*C. albicans* fungemia (day 16), viridans streptococcal bact. (day 16), died from disseminated candidiasis and pneumonitis
	26	M/45	AML	43	2.3	0.4	2.4	2×10^6 (day 23)	Neg (2), Pos (4 [520, 370, 400, 307])	Neg (6)	Sinusitis, new FUO unresponsive to antibiotics, disseminated candidiasis and *Aspergillus* pneumonia at postmortem.
	27	F/53	ASCL	37	2.0 (Ct.)	Alt. (day 11)	0.8 (Ct.)	10^6 (Ct.)	Neg (1), Pos (1 [380])	Neg (1), Pos (1 [1:8])	CD pneumonia, *E. coli* UTI unresponsive to antibiotics, *Acinetobacter* pneumonia, *S. epidermidis* bact. and disseminated *C. tropicalis* in lung and spleen at postmortem
	28	M/40	AML	34	0	0	0	0	Neg (2), Pos (2 [217, 257])	Neg (4)	FUO twice, then *C. parapsilosis* fungemia, *S. epidermidis* bact., AMB started, patient recovers with resolution of neutropenia

[a] A and B indicate separate episodes of neutropenia and infection in the patient indicated.

[b] Abbreviations: AML, acute myeloblastic leukemia; ALL, acute lymphoblastic leukemia; AA, aplastic anemia; HD, Hodgkin's disease; ST, solid tumor; CML-BC, chronic myelocytic leukemia in accelerated phase (blast crisis); ASCL, acute stem cell leukemia; CLL, chronic lymphocytic leukemia.

[c] Twice-weekly cultures at four sites. Cultures are expressed as total-plus counts (1+ to 4+) divided by total number of cultures; urine cultures are expressed as CFU of *C. albicans* per liter unless otherwise specified. Abbreviations: Asp., *Aspergillus* sp.; Alt. *Alternaria* sp.; Pen., *Penicillium* sp.; Ct., *C. tropicalis*; Tg., *Torulopsis glabrata*; Co., other *Candida* species.

[d] Antigen test results: CACP Neg, <150 ng/ml; CANDTEC Neg, <1:2; CACP Pos, titer recorded as nanograms per milliliter; CANDTEC Pos, titer recorded as positive dilution end point.

[e] Day refers to number of days following hospitalization for neutropenia. Abbreviations: bact., bacteremia; FUO, pyrexia of unknown origin; CD, clinically documented infection; AMB, amphotericin B; UTI, urinary tract infection.

[f] M, male; F, female.

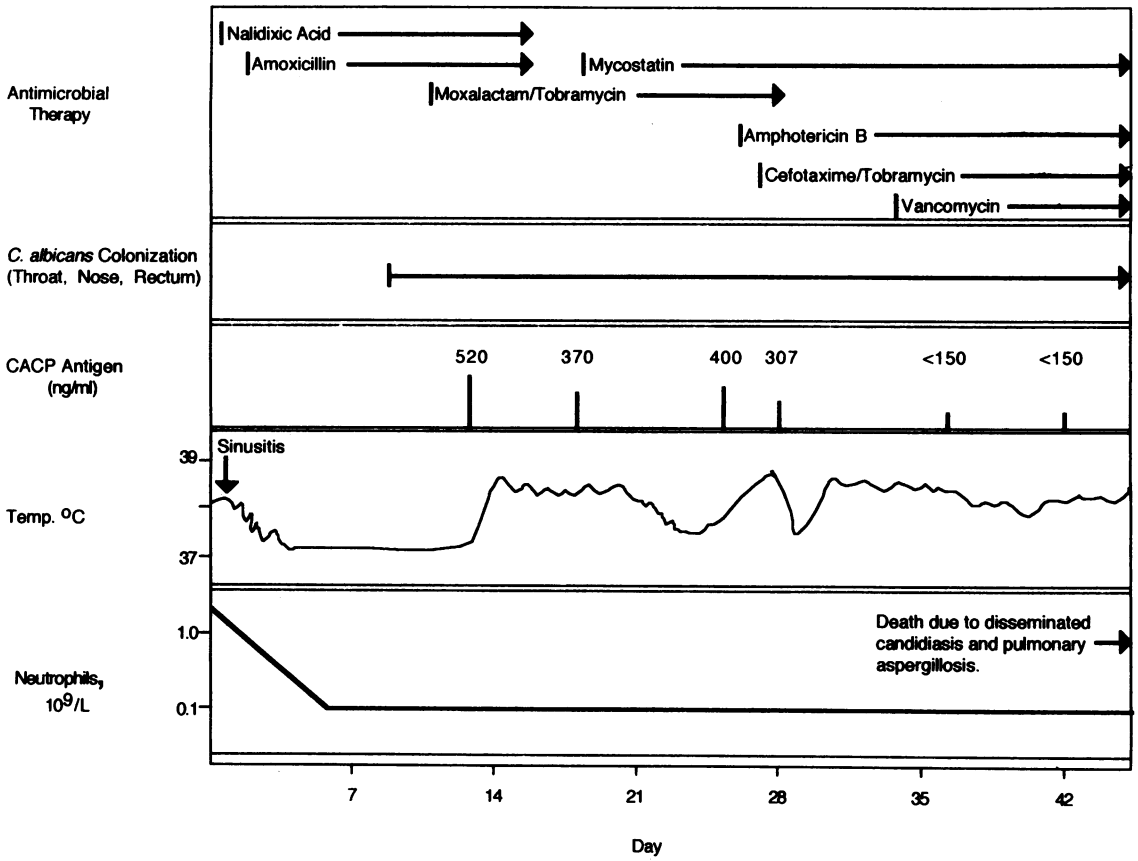

FIG. 5. Clinical course and CACP antigen results for patient 26. Mycostatin is a trade name for nystatin.

was at 110 ng/ml, and the CANDTEC assay was positive at 1:4. He responded after 7 days of a 13-day course of amphotericin B therapy. At the end of daily amphotericin B therapy, the serum CACP titer was 200 ng/ml and the CANDTEC assay was negative. With an every-other-day dosage of amphotericin B, fever recurred within a week, and the CACP titer rose to 340 ng/ml. The patient was therefore restarted on daily amphotericin B therapy with resolution of the febrile illness. The CANDTEC titer remained negative. Further serum samples following resolution of neutropenia were not available for analysis.

In group IV, patients 24 and 25 had documented deep fungal infection with *C. tropicalis* and *C. albicans* but had negative test results by either method. Patient 24 had *C. tropicalis* fungemia at study entry and had negative CACP antigen and CANDTEC serology on a single sample at the time of fungemia. No further samples were obtained from this patient, and it is uncertain whether additional samples would have been antigen positive. Patient 25 developed candidemia and streptococcal bacteremia at day 16 and died with *Candida* pneumonitis on day 19. Serum from day 16 was CACP antigen negative, a false-negative result.

Figure 5 shows the clinical course of patient 26 and how this correlated with results obtained with the CACP antigen EIA. He was admitted with maxillary sinusitis and fever, responded to amoxicillin therapy, but became heavily colonized in the oropharynx and feces with *C. albicans* (Table 1). He developed pyrexia on day 10, failed to respond to broad-spectrum antibiotic therapy, and was subsequently treated with amphotericin B. He temporarily improved but developed a persisting febrile illness unresponsive to either modified antibacterial or antifungal therapy. The amphotericin B therapy was associated with a reduction in CACP antigen concentrations to <50 ng/ml, but the patient subsequently died without resolution of his fever. *C. albicans* was identified in postmortem samples from the liver and spleen. *Aspergillus fumigatus* was isolated from the lungs.

Patient 27 had acute stem cell leukemia and severe persistent marrow aplasia with granulocyte counts of less than 0.1×10^9/liter until the time of death at day 38. She presented with fever, a pulmonary infiltrate, and an *Escherichia coli* urinary tract infection. Both the CACP and CANDTEC assay were negative on presentation. She was not colonized with fungi during the initial 10 days of hospitalization. However, on moxalactam-ticarcillin therapy, she developed multiple-site colonization with *C. tropicalis*. She was treated with high-dose co-trimoxazole, ticarcillin, and gentamicin for an *Acinetobacter calcoaceticus* var. *lwoffi* pneumonitis. However, with progressive deterioration in her pulmonary status, a transbronchial brush biopsy was performed. This showed no fungi, but low counts of *Acinetobacter* sp. were recovered. The serum CACP was positive at 380 ng/ml at that time, and the CANDTEC assay was positive at 1:8. She subsequently succumbed despite added empiric amphotericin B and further antibiotics for a terminal *Staphylococcus epidermidis* bacteremia. Autopsy showed *C. tropicalis* in the lung and spleen and *Acinetobacter* sp. in the lung.

Patient 28 had a fever of unknown origin treated with moxalactam-ticarcillin and showed a temporary and partial reduction in fever from 39°C to 37.6°C between days 5 and 8 of

TABLE 2. Sensitivity and specificity of CACP EIA and CANDTEC assays according to analysis of 89 serum samples from 31 patient episodes

Result according to:	True positive (no.)	True negative (no.)	False positive (no.)	False negative (no.)	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Serum samples								
CACP EIA	9	78	0	2	82	100	100	97
CANDTEC	2	76	2	9	18	97	50	89
Patient episode(s)								
CACP EIA	4[a]	25	0	2	67	100	100	93
CANDTEC	2	23	2	4	33	92	50	85

[a] Two confirmed positive episodes and two episodes presumed positive on the basis of clinical features.

antibiotic therapy. The CACP titer was 217 ng/ml at presentation, with a negative CANDTEC assay. Fever recurred on day 9 and persisted despite a change in antibiotics to cefazolin-tobramycin and vancomycin. Isoniazid was also added because of possible reactivation of *Mycobacterium tuberculosis* infection. The CACP titer was 257 ng/ml, and the CANDTEC assay was negative on day 11. Fever temporarily improved over a week, but the patient developed high-grade fever (39 to 40°C) and developed *C. parapsilosis* fungemia on days 25 (one of two sets) and 28 (one of two sets with *S. epidermidis* also). On day 26, the CACP titer was negative at 65 ng/ml, and the CANDTEC assay was negative. Amphotericin B therapy was begun on day 28, and he survived the fungal infection with resolution of the granulocytopenia by day 33, at which time the CACP assay was nonreactive (<50 ng/ml). For all sera, CANDTEC assay results were negative or ≥1:4, with no samples reactive at a 1:2 dilution.

The sensitivity, specificity, and predictive values of the CACP antigen EIA and CANDTEC assay on the basis of the clinical parameters in these patients are shown in Table 2. The sensitivity of the CACP antigen EIA was 67%, with a specificity of 100% and a positive predictive value of 100%. The CANDTEC assay had a sensitivity of 33% and a specificity of 92%, with a positive predictive value of 50%. If individual serum samples were used to calculate the data, the sensitivity of the CACP antigen EIA rose to 82% without affecting specificity or positive predictive values.

DISCUSSION

The laboratory diagnosis of invasive candidiasis remains a problem (16). Many patients at high risk for developing invasive candidiasis are severely immunosuppressed and do not mount a significant humoral response (19, 21). Assays detecting antibodies to *Candida*-specific antigens are of little diagnostic value. Because of this, laboratory diagnosis of invasive candidiasis has shifted to detecting *Candida* antigens. Antigen assays have concentrated on mannan, since mannan antigenemia was thought to be specific for invasive candidiasis (32). However, it has also been suggested that detection of *Candida* cytoplasmic antigens may also be useful in diagnosing invasive candidiasis (19, 20).

Microbiologic tests for diagnosing invasive candidiasis show a poor positive predictive value of surface colonization data, infrequent documentation of fungemia, and lack of clinical parameters suggesting fungal infection. Furthermore, the difficulty of obtaining specimens from various tissues to recover fungi during life complicates diagnosis. The response or lack thereof to empiric amphotericin B is an uncertain therapeutic trial for diagnosing fungal infection. Decreasing postmortem rates used to verify diagnoses further obfuscate evaluation of clinical studies.

In this study, candidal cytoplasmic antigens were produced under standardized growth conditions and with a simplified preparative procedure in order to minimize antigen variability. Analysis of the CACP antigens by one- and two-dimensional SDS-PAGE revealed the presence of five major proteins ranging in size from 36,000 to 44,000, with pIs ranging from approximately 6.5 to 8.5. Whether any of these five proteins equates to the *C. albicans* proteins described by others is unclear. A *C. albicans* protein with a molecular weight of approximately 45,000 has been described (4, 35, 47), but molecular weights have varied considerably between reports. This variability may be due to differences in the growth phase of the organism and differences in how the cytoplasmic antigens were prepared (35, 46). Strockbine et al. (46) described a *C. albicans* cytoplasmic protein having a molecular weight of approximately 36,000 and reported that limited proteolysis of three proteins (including a protein with a molecular weight of 36,000) immunoprecipitated by a monoclonal antibody indicated a similarity in primary sequence. Whether any of our CACP antigens are similar remains to be determined. The CACP antigen preparations consist of proteins with associated carbohydrate, as shown by the effects of heat, protease, and periodate upon reactivity in the CACP antigen EIA. Heating at 56°C partially abolished CACP antigen reactivity in the EIA, and heating at 80°C completely abolished it, suggesting protein is responsible for much of the EIA reactivity. The partial loss of CACP antigen reactivity following periodate treatment indicated that carbohydrate antigens are also present. Our CACP antigen preparations were not subjected to concanavalin A column chromatography, which others have done in the preparation of cytoplasmic antigen (25, 47). Chromatography on concanavalin A would have resulted in the removal not only of cell wall debris but also of cytoplasmic glycoproteins, some of which may be important diagnostically.

Despite the same conditions being used to prepare cytoplasmic antigens from *C. parapsilosis*, these antigens did not react appreciably in the CACP antigen EIA. Analysis of additional strains would have been helpful, since there may be strain variation in cytosol antigens. However, since invasive disease caused by *C. parapsilosis* is uncommon, the diagnostic utility of the assay may not be severely impaired (7). Burnie et al. (12) found by immunoblotting with both *C. albicans* yeast- and mycelium-specific antiserum that the cross-reactivity with *C. parapsilosis* was greater than that with *C. tropicalis*, which we found to be reversed in the present study. Burnie et al. (12) attributed the greater cross-reactivity to mannan, but they speculated that proteins with molecular weights greater than 33,000 were also involved. This suggestion appears to be

supported by our experiments in which a complex of cytoplasmic antigens, including antigens in the 36,000 to 44,000 range, prepared from different *Candida* species was detected in clinical cases of disseminated fungal infection in the neutropenic host. Additional studies of the nature and types of candidal proteins found in *Candida* species would be important for further assay development. Immunization with purified antigens would also be important.

Since both protein and carbohydrate epitopes are apparently detected in our CACP antigen EIA, this assay differs from an earlier EIA developed by Araj et al. (2) for detection of CACP antigens. The methods Araj et al. and we used to prepare CACP antigens and anti-CACP antigen antibodies appear comparable. Even so, Araj et al. reported that the antiserum used did not react with mannan, although no attempt was made to remove mannan with lectins from the antigen preparation. Araj et al. also indicated their assay detected a heat-stable antigen that did not contain carbohydrate and that a single protein, as opposed to a group of proteins, was involved. These last two findings are difficult to reconcile with the results presented here.

Although the nature and individual importance of the CACP antigens detected by the CACP antigen EIA are presently undefined, a potential strength of the assay is the ability of polyclonal antibodies to detect a spectrum of unspecified CACP antigens. Preparation of monoclonal and monospecific polyclonal antibodies for each of the five major CACP antigens and analysis of the types of antigens found in sera from patients with invasive candidiasis, in animal models of candidiasis, and in medium incubating human phagocytic cells and yeast cells would be crucial for further test development.

Antigen detection in the neutropenic host may depend on multiple variables. While it is presumed that fungal cells may be processed in these patients by the remaining tissue histiocytes, it is not known how different cell types may process fungi and whether different fungal antigens are produced. The portal of entry may also present difficulties in measuring the amount of fungal antigens. For example, intravascular catheter-associated sepsis may not allow sufficient time for host responses to generate fungal antigens.

Examining the pattern of responses in these patients, several factors or strategies may be important for the diagnosis of fungal infection. Since deep fungal infection is less common early on in the neutropenic episode, a baseline serum specimen may be helpful. Sequential serum sampling for CACP antigens on a regular basis also appears to aid diagnosis. It is not known whether sequential serum sampling would have changed the diagnosis of patient 25, who was CACP antigen negative at study entry but who did not have any subsequent serum samples collected until the diagnosis of fungemia. On the basis of the results from the group III and IV patients, specimens should be collected every 3 or 4 days during the periods of greatest risk of developing an invasive candidal infection.

Lack of sensitivity has also been a problem with all antigen assays. Studies currently in progress in our laboratory suggest that actively growing yeast cells in the absence of neutrophils do not release significant amounts of either mannoproteins or other cell-associated antigens into the surrounding fluid (3). This also may account for the variability of results from mannan-based or protein assays when the appropriate conditions are not present. Furthermore, this highlights the need to verify test results from different patient populations. Results from the nonneutropenic host may differ. In this study, antifungal therapy with amphotericin B appeared to reduce the concentration of fungal antigens in the blood. It is possible that fungal antigens could be released with a cell wall-active agent such as amphotericin B. However, we did not conduct intensive serial sampling before and after amphotericin B therapy. Both patients 26 and 27 showed reduced or negative levels of CACP antigens following initiation of amphotericin B therapy. The findings suggest either that the antifungal therapy effectively inhibited growth of the yeast, resulting in disappearance of CACP antigens in the blood, or that the yeast cells were not being processed by host inflammatory cells. We hope to shed light on this by studying CACP antigen release when *C. albicans* interacts with human neutrophils and macrophages in tissue culture.

The sensitivity and specificity of the CACP assay indicate that patients who are consistently negative for CACP antigen in blood, whether or not they are colonized with *C. albicans*, are at low risk of development of invasive infection during their periods of neutropenia. On the basis of sequential sampling of patients at high risk for developing invasive candidiasis, the sensitivity and specificity of the CACP antigen EIA were 67% (patient episodes) and 82% (serum samples), respectively. The positive predictive value of the CACP antigen EIA was 99 to 100%, compared with 50% for the CANDTEC assay. The observations with patient 27 (Table 1) show that *C. tropicalis* cytoplasmic antigens may also be detected by this assay (34).

In summary, the clinical utility of a *Candida* cytoplasmic antigen assay was evaluated blindly with 31 neutropenic patient episodes. The results are clearly superior to those with the CANDTEC assay, but due to the limited number of patients tested, it will be important to further evaluate this assay in a prospective fashion. Several variables of importance in the future evaluation of EIA fungal antigen assays were identified during this study.

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