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Tracking the Emerging Human Pathogen *Pseudallescheria boydii* by Using Highly Specific Monoclonal Antibodies[▽]

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***Pseudallescheria boydii* has long been known to cause white grain mycetoma in immunocompetent humans, but it has recently emerged as an opportunistic pathogen of humans, causing potentially fatal invasive infections in immunocompromised individuals and evacuees of natural disasters, such as tsunamis and hurricanes. The diagnosis of *P. boydii* is problematic since it exhibits morphological characteristics similar to those of other hyaline fungi that cause infectious diseases, such as *Aspergillus fumigatus* and *Scedosporium prolificans*. This paper describes the development of immunoglobulin M (IgM) and IgG1 κ-light chain monoclonal antibodies (MAbs) specific to *P. boydii* and certain closely related fungi. The MAbs bind to an immunodominant carbohydrate epitope on an extracellular 120-kDa antigen present in the spore and hyphal cell walls of *P. boydii* and *Scedosporium apiospermum*. The MAbs do not react with *S. prolificans*, *Scedosporium dehoogii*, or a large number of clinically relevant fungi, including *A. fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, *Fusarium solani*, and *Rhizopus oryzae*. The MAbs were used in immunofluorescence and double-antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISAs) to accurately differentiate *P. boydii* from other infectious fungi and to track the pathogen in environmental samples. Specificity of the DAS-ELISA was confirmed by sequencing of the internally transcribed spacer 1 (ITS1)-5.8S-ITS2 rRNA-encoding regions of environmental isolates.**

Pseudallescheria boydii is an infectious fungal pathogen of humans (7, 16, 40, 58, 59). It is the etiologic agent of white grain mycetoma in immunocompetent humans (7) and has emerged over recent years as the cause of fatal disseminated infections in individuals with neutropenia, AIDS, diabetes, renal failure, bone marrow or solid organ transplants, systemic lupus erythematous, and Crohn's disease; in those undergoing corticosteroid treatment; and in leukemia and lymphoma patients (1, 2, 3, 18, 27, 31, 32, 34, 36, 37, 38, 47, 49, 52). The fungus is the most prevalent species after *Aspergillus fumigatus* in the lungs of cystic fibrosis patients (8), where it causes allergic bronchopulmonary disease (5) and chronic lung lesions simulating aspergillosis (24). Near-drowning incidents and recent natural disasters, such as the Indonesian tsunami in 2004, have shown *P. boydii* and the related species *Scedosporium apiospermum* and *Scedosporium aurantiacum* to be the causes of fatal central nervous system infections and pneumonia in immunocompetent victims who have aspirated polluted water (4, 11, 12, 21, 22, 25, 30, 33, 57). Its significance as a potential pathogen of disaster evacuees has led to its recent inclusion in the Centers for Disease Control and Prevention list of infectious etiologies in persons with altered mental statuses, central nervous system syndromes, or respiratory illness.

P. boydii is thought to be an underdiagnosed fungus (60), and misidentification is one of the reasons that the mortality rate due to invasive pseudallescheriasis is high. Detection of invasive *P. boydii* infections, based on cytopathology and histopathology, is problematic since it can occur in tissue and bronchoalveolar and bronchial washing specimens with other hyaline septated fungi, such as *Aspergillus* and *Fusarium* spp. (7, 23, 53, 60), which exhibit similar morphological characteristics upon microscopic examination (2, 23, 24, 28, 37, 44, 53, 60). Early diagnosis of infection by *P. boydii* and differentiation from other agents of hyalohyphomycosis is imperative, since it is refractory to antifungal compounds, such as amphotericin B, that are commonly administered for the control of fungal infections (10, 39, 58).

The immunological diagnosis of *Pseudallescheria* infections has focused on the detection of antigens by counterimmunoelectrophoresis, and by immunohistological techniques using polyclonal fluorescent antibodies, but cross-reactions with antigens from other fungi, such as *Aspergillus* species, occurs (7, 19, 23). Pinto and coworkers (41, 42) isolated a peptidorhamnomannan from hyphae of *P. boydii* and proposed the antigen as a diagnostic marker for the pathogen. Cross-reactivity with *Sporothrix schenckii* and with *Aspergillus* have, however, been noted (23, 41). Furthermore, it is uncertain whether a similar antigen is present in the related pathogenic species *S. prolificans*, an important consideration in patient groups susceptible to mixed *Scedosporium* infections (6, 18).

Hybridoma technology allows the production of highly specific MAbs that are able to differentiate between closely related species of fungi (54, 55, 56). The purpose of this paper is to report the development of MAbs specific to *P. boydii* and certain closely related species and their use to accurately discriminate among *P. boydii*, *A. fumigatus*, and other human pathogenic fungi by using immunofluorescence and double-antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISAs).

Currently, the natural environmental habitat of *P. boydii* is

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TABLE 1. Details of fungi and results of ELISA specificity tests using MAbs GA3 and HG12

Organism	Isolate no.	Source[a]	Absorbance (450 nm)[b]	
			GA3	HG12
Absidia corymbifera	101040	CBS	0.021	0.021
Absidia glauca	2	CRT	0.027	0.053
Absidia spinosa	3	CRT	0.017	0.063
Acremonium alternatum	545.89	CBS	0.002	0.056
Acremonium atrogriseum	306.85	CBS	0.028	0.057
Acremonium blochii	424.93	CBS	0.010	0.041
Alternaria alternata	42	CRT	0.007	0.029
Apophysomyces elegans	658.93	CBS	0.032	0.044
Aspergillus clavatus	514.65	CBS	0.028	0.034
A. fumigatus	AF293	SK	0.016	0.041
A. fumigatus	181	CRT	0.012	0.029
Aspergillus flavus	91856iii	IMI	0.022	0.072
Aspergillus niger	102.40	CBS	0.026	0.063
A. niger	121.47	CBS	0.029	0.065
Aspergillus terreus var. *terreus*	601.65	CBS	0.037	0.053
Aspergillus versicolor	599.65	CBS	0.029	0.044
Aureobasidium pullulans var. *pullulans*	771.97	CBS	0.002	0.005
Candida albicans	SC5314	SB	0.016	0.023
Candida dubliniensis	8500	CBS	0.022	0.028
Candida glabrata	4692	CBS	0.018	0.022
Cladosporium herbarum	159.59	CBS	0.024	0.049
Cryptococcus neoformans	5728	CBS	0.027	0.047
C. neoformans	7779	CBS	0.033	0.037
Cunninghamella bertholletiae	182.84	CBS	0.019	0.062
Emericella nidulans var. *nidulans*	A4	FGSC	0.036	0.060
Exophiala dermatitidis	153.94	CBS	0.027	0.058
Exophiala jeanselmei var. *heteromorpha*	657.76	CBS	0.013	0.038
Fusarium solani	224.34	CBS	0.018	0.060
F. solani	80	CRT	0.028	0.066
F. solani var. *petrophilum*	102256	CBS	0.072	0.052
Fusarium verticillioides	539.79	CBS	0.006	0.044
Geotrichum capitatum	327.86	CBS	0.019	0.033
Graphium eumorphum	987.73	CBS	1.043	1.008
Graphium penicilliodes	320.72	CBS	1.507	1.518
L. fimeti	129.78	CBS	1.281	1.200
Madurella mycetomatis	868.95	CBS	0.013	0.027
Mucor plumbeus	96	CRT	0.027	0.066
Mucor racemosus	93	CRT	0.029	0.052
Paecilomyces variotii	339.51	CBS	0.000	0.002
Parascedosporium tectonae	127.84	CBS	1.517	1.462
Penicillium chrysogenum	105	CRT	0.029	0.040
Penicillium marneffei	101038	CBS	0.001	0.012
Penicillium marneffei	669.95	CBS	0.004	0.017
Penicillium purpurogenum	364.48	CBS	0.033	0.069
Petriella gutullata	362.61	CBS	1.456	1.509
Petriella setifera	109039	CBS	1.192	1.122
Petriellopsis africana	311.72	CBS	1.114	1.095
Phialophora richardsiae	483.80	CBS	0.028	0.022
Phialophora verrucosa	225.97	CBS	0.015	0.046
P. angusta	254.72	CBS	1.041	0.972
P. boydii	835.96	CBS	1.439	1.421
P. boydii	100393	CBS	1.301	1.184
P. boydii	100395	CBS	1.188	1.085
P. boydii	100870	CBS	1.200	1.071
Pseudallescheria desertorum	489.72	CBS	1.023	1.013
Pseudallescheria ellipsoidea	438.72	CBS	1.116	1.049
P. fusoidea	106.53	CBS	1.160	1.055
R. miehei	360.92	CBS	0.033	0.077
Rhizopus microsporus var. *rhizopodiformis*	102277	CBS	0.048	0.060
Rhizopus oryzae	395.54	CBS	0.086	0.019
Rhizopus sexualis var. *sexualis*	209090	IMI	0.010	0.038
Rhizopus stolonifer	G1	CRT	0.004	0.017
Saksenaea vasiformis	100390	CBS	0.038	0.074
S. aurantiacum	121926	CBS	1.431	1.243
S. aurantiacum	118934	CBS	1.302	1.271
S. apiospermum	117407	CBS	1.077	0.985
S. dehoogii	117406	CBS	0.026	0.049
S. prolificans	742.96	CBS	0.019	0.057
S. prolificans	100390	CBS	0.021	0.047
S. prolificans	100391	CBS	0.023	0.067
S. prolificans	102176	CBS	0.045	0.075
Sporothrix schenckii	345.53	CBS	0.034	0.043
Stachybotrys chartarum	485.48	CBS	0.016	0.040
Syncephalastrum racemosum	155	CRT	0.024	0.059
Trichoderma longibrachiatum	446.95	CBS	0.061	0.052
Wallemia sebi	196.56	CBS	0.022	0.046

[a] CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CRT, C. R. Thornton; IMI, International Mycological Institute, Egham, England; SB, S. Bates, School of Biosciences, University of Exeter; SV, S. Krappman, Institute of Microbiology and Genetics, Department of Molecular Microbiology and Genetics, Georg August University, Gottingen, Germany.

[b] Each value is the mean for replicated samples. The threshold absorbance value for detection of antigen in ELISA is ≥0.100.

TABLE 2. Absorbance values from ELISAs with periodate-treated antigens by using MAbs GA3 and HG12 of IgM and IgG1, respectively

MAb	Time (h)	Absorbance (450 nm)[a]	
		Periodate	Control
GA3	20	0.220 ± 0.008	1.063 ± 0.022
	4	0.275 ± 0.014	1.065 ± 0.030
	3	0.276 ± 0.010	1.111 ± 0.012
	2	0.559 ± 0.028	1.127 ± 0.019
	1	1.033 ± 0.015	1.080 ± 0.018
HG12	20	0.197 ± 0.011	0.929 ± 0.022
	4	0.196 ± 0.008	0.935 ± 0.017
	3	0.264 ± 0.009	0.942 ± 0.011
	2	0.673 ± 0.018	0.936 ± 0.021
	1	0.907 ± 0.017	0.990 ± 0.012

[a] Each value is the mean for replicated values ± standard errors.

unknown, but nutrient-rich, brackish waters, such as estuaries, have been suggested (9, 17). In combination with a semiselective isolation procedure, I show how the DAS-ELISA can be used to rapidly and accurately track the pathogen in naturally infested estuarine muds, and in doing so illustrate the potential of the DAS-ELISA as a diagnostic platform for detection of *P. boydii* and related species within the *Pseudallescheria* complex.

MATERIALS AND METHODS

Fungal culture. All fungi were routinely cultured on Sabouraud agar (SA) under a 16-h regimen of fluorescent light.

Development of MAbs, preparation of immunogen, and immunization regimen. Mice were immunized with soluble antigens prepared from actively growing cultures of *P. boydii* (CBS strain 835.96). Five-day-old SA slope cultures of the fungus grown at 26°C were washed with 2 ml of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4 [pH 7.2]), and the resultant cell suspension was centrifuged for 5 min at 14,000 rpm. The supernatant, containing solubilized antigens, was used as the immunogen and as a source of antigens for antibody specificity tests and epitope characterization studies. For immunization, 6-week-old BALB/c female white mice were given four intraperitoneal injections (300 μl per injection) of surface washings containing 1.5 mg protein per ml PBS at 2-week intervals and a single booster injection 5 days before fusion.

Production and screening of hybridomas and determination of antibody specificity. Hybridoma cells were produced by a method described elsewhere (54), and the supernatants were screened by ELISA against surface antigens immobilized to the wells of Maxisorp microtiter plates (50 μl per well). For antibody specificity tests, fungi were grown on SA and surface washings prepared from actively growing cultures as described above. Protein concentrations, determined spectrophotometrically at 280 nm (Nanodrop, Agilent Technologies Limited, Berkshire, United Kingdom), were adjusted to 60 μg/ml buffer, and 50-μl volumes used to coat the wells of microtiter plates. After coating overnight at 4°C, wells were washed four times with PBS containing 0.05% [vol/vol] Tween 20 (PBST) and once each with PBS and distilled H_2O (dH_2O) and air-dried at 23°C in a laminar flow hood. The plates were stored in sealed plastic bags at 4°C in preparation for screening of hybridoma supernatants by ELISA as described below.

ELISA. Wells containing immobilized antigens were incubated successively with hybridoma supernatant for 1 h, followed with goat anti-mouse polyvalent (Ig classes IgG, IgA, and IgM) peroxidase conjugate (Sigma Chemical Company, Poole, United Kingdom) diluted 1 in 1,000 in PBST for a further hour. Bound antibody was visualized by incubating wells with tetramethyl benzidine substrate solution (54) for 30 min, and reactions were stopped by the addition of 3 M H_2SO_4. Absorbance values were determined at 450 nm with an MRX automated microplate reader (Dynex Technologies, Billingshurst, United Kingdom). Wells were given four 5-min rinses with PBST between incubations. Working volumes were 50 μl per well, and control wells were incubated with tissue culture medium (TCM) containing 10% (vol/vol) fetal bovine serum. All incubation steps were performed at 23°C in sealed plastic bags. The threshold for detection of antigen in ELISA was determined from control means (2 × TCM absorbance values) (51). These values were consistently in the range of 0.050 to 0.100. Consequently, absorbance values of >0.100 were considered positive for the detection of antigen.

TABLE 3. Absorbance values from ELISAs with protease-treated antigens by using MAbs GA3 and HG12 of IgM and IgG1, respectively

MAb	Temp (°C)	Absorbance (450 nm)[a]			
		Pronase	Pronase control	Trypsin	Trypsin control
GA3	4	1.046 ± 0.018	1.084 ± 0.024	1.084 ± 0.014	1.099 ± 0.017
	37	1.135 ± 0.011	1.101 ± 0.016	1.223 ± 0.015	1.188 ± 0.018
HG12	4	0.918 ± 0.011	0.932 ± 0.024	0.972 ± 0.010	0.933 ± 0.009
	37	0.939 ± 0.013	1.032 ± 0.013	1.034 ± 0.012	1.071 ± 0.013

[a] Each value is the mean for replicated values ± standard errors.

Determination of Ig subclass and cloning procedure. The Ig classes of MAbs were determined by using antigen-mediated ELISAs. Wells of microtiter plates coated with *P. boydii* surface washings were incubated successively with hybridoma supernatant for 1 h, followed with goat anti-mouse IgG_1-, IgG_{2a}-, IgG_{2b}-, IgG_3-, IgM-, or IgA-specific antiserum (Sigma) diluted 1 in 3,000 in PBST for 30 min and rabbit anti-goat peroxidase conjugate diluted 1 in 1,000 (Sigma) for a further 30 min. Bound antibody was visualized with TMB substrate as described above. Light-chain characterization was carried out by ELISA, by using goat anti-mouse κ-light chain-specific antibody (Bethyl Laboratories, Inc.) followed by rabbit anti-goat peroxidase conjugate and TMB substrate. Hybridoma cells lines were cloned three times by limiting dilutions, and cell lines were grown in bulk in a nonselective medium, preserved by a slow freezing in fetal bovine serum/dimethyl sulfoxide (92:8 [vol/vol]), and stored in liquid nitrogen.

Epitope characterization by periodate oxidation. Microtiter wells containing immobilized antigens were treated with sodium metaperiodate (20 mM $NaIO_4$ in 50 mM sodium acetate buffer [pH 4.5]), whereas control wells received only buffer. After incubation for the appropriate time period in darkness at 4°C, wells were washed three times with PBS and assayed by ELISA with MAbs GA3 and HG12 as described below. There were four replicates for each treatment.

Epitope characterization by protease digestion. Immobilized antigens were incubated with pronase (Protease XIV) (Sigma) (0.25 U per well) or trypsin (Sigma) solution (1 mg/ml in PBS) at 37°C or 4°C for 5 h and washed three times with PBS. Wells incubated with trypsin were treated for 10 min with a 0.1-mg/ml solution of trypsin inhibitor (Sigma) and given three more washes with PBS. Controls received PBS without pronase or trypsin and inhibitor but were otherwise treated similarly. The wells were assayed by ELISA with MAbs GA3 and HG12 as described below. There were six replicates for each treatment.

PAGE and Western blotting. Polyacrylamide gel electrophoresis (PAGE) was carried under denaturing conditions, with 4 to 20% (wt/vol) gradient polyacrylamide gels (Bio-Rad Laboratories Limited, Hemel Hempstead, United Kingdom). Washed conidia of *P. boydii* were used to inoculate flasks of TCM containing 10% (vol/vol) fetal bovine serum. The flasks containing 10^4 conidia/ml of medium were incubated at 37°C for 48 h with shaking, and the culture filtrates were centrifuged at 14,000 rpm for 5 min. The supernatants containing secreted antigens were mixed with Laemmli buffer and denatured by heating at 95°C for 10 min in the presence of β-mercaptoethanol prior to gel loading. Proteins were separated for 1.5 h at 23°C (165 V). Prestained broad-range markers (Bio-Rad) were used for molecular mass determinations. For Western blotting, separated proteins were transferred electrophoretically to a polyvinylidene difluoride membrane (Bio-Rad). Membranes were incubated for 4 h at 4°C with sodium metaperiodate solution or sodium acetate buffer only (control), prepared as described above. The membranes were blocked for 16 h at 4°C with PBS containing 1% (wt/vol) bovine serum albumin (BSA) and incubated with MAb supernatants diluted 1 in 2 with PBS containing 0.5% (wt/vol) BSA for 2 h at 23°C. After being washed three times with PBS, membranes were incubated for 1 h with goat anti-mouse IgM (μ-chain-specific) alkaline phosphatase conjugate (Sigma) or goat anti-mouse IgG (whole-molecule) alkaline phosphatase conjugate (Sigma), each diluted 1 in 15,000 in PBS containing 0.5% (wt/vol) BSA. Membranes were washed twice with PBS and once with PBST, and bound antibody was visualized by incubation in substrate solution. Reactions were stopped by immersion in dH_2O and air-dried between sheets of Whatman filter paper.

Immunofluorescence and immunogold electron microscopy of spores and hyphae. Immunogold labeling was performed with washed conidia of *P. boydii* prepared as described elsewhere (55) using MAbs GA3 and HG12 and goat

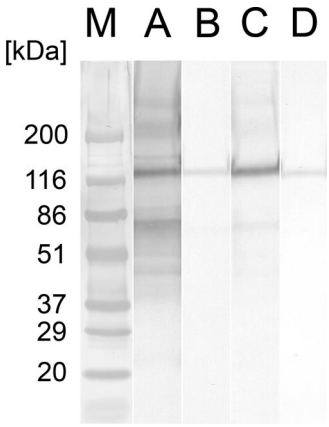

FIG. 1. Analysis of *P. boydii* 835.96 secreted antigens by using denaturing sodium dodecyl sulfate-PAGE and Western blotting. Lane M, molecular mass marker; lane A, Western immunoblot with MAb GA3 of culture filtrate, following acetate buffer (control) treatment; lane B, Western immunoblot with MAb GA3 of culture filtrate, following periodate treatment; lane C, Western immunoblot with MAb HG12 of culture filtrate, following acetate buffer treatment; lane D, Western immunoblot with MAb HG12 of culture filtrate, following periodate treatment. All wells were loaded with 1.6 μg of protein. Note the reduction in antibody binding to the major 120-kDa antigen following periodate treatment.

anti-mouse 15-nm-diameter gold conjugate (British Biocell International, Cardiff, Wales) as the secondary reporter molecule. Control grids were incubated with TCM instead of MAb supernatant but were otherwise treated the same. For immunofluorescence studies, washed conidia of *P. boydii* and *A. fumigatus* were suspended in sterile filtered (0.2 μM) 1% (wt/vol) glucose solution and transferred to the wells of multiwell slides. After incubation at 30°C for 16 h, slides were air dried and fixed as described elsewhere (55). Wells were incubated for 30 min with 50 μl of the *P. boydii*-specific MAb HG12 (IgG1), the *Aspergillus*-specific MAb JF5 (55), or TCM only. Slides were washed three times with PBS with gentle agitation and incubated for a further 30 min with goat anti-mouse polyvalent fluorescein isothiocyanate conjugate (Sigma) diluted 1 in 40 in PBS. Slides were given three 5-min rinses with PBS, and the wells were overlaid with coverslips mounted in PBS-glycerol mounting medium (Sigma). Slides were examined with a Zeiss Axiophot microscope fitted with an epifluorescence attachment, using a UV excitation filter of 365 nm and an absorption filter of 420 nm. All incubation steps were performed at 23°C in a moist environment, and slides were stored at 4°C in the dark in petri dishes containing moistened Whatman filter paper no. 1.

Detection of *P. boydii* in estuarine muds and identification of fungi by analysis of the ITS regions of the rRNA-encoding gene unit. Estuarine mud samples were taken from sites along the Exe estuary (Exmouth, Lympstone, and Topsham) in Devon, United Kingdom, and from sites along the Fal estuary system (Penryn, Restronguet, and Mylor), Cornwall, United Kingdom. A semiselective medium was used for recovery of fungi from mud samples and was a modification of a medium described elsewhere (46). The medium consisted of sterile autoclaved Dichloran-Rose-Bengal agar (Fluka) and contained, per liter, 6 mg benomyl and 0.1 g (each) of the antibiotics chloramphenicol, streptomycin sulfate, and ciprofloxacin. The medium supports growth of *Pseudallescheria* and *Scedosporium* species but is inhibitory to the growth of *Graphium*, *Parascedosporium*, and *Petriella* species. Ten-milliliter samples of estuarine mud contained in 50-ml sterile falcon tubes (Greiner) were mixed with 40 ml of sterile dH_2O for 1 h at 23°C and then allowed to stand for 15 min to allow sedimentation. Two hundred microliters of supernatant was removed and spread on the surface of selective medium using a glass spreader. After 7 days of incubation at 37°C in the dark, a 5-cm diameter cork borer was used to remove plugs of mycelium from the growing edge of hyphal colonies, or colonies of yeast cells. The plugs were placed in 1.5-ml Eppendorf tubes and macerated in 1 ml of PBST using a hand-held homogenizer. The tubes were centrifuged to remove cells and agar, and the supernatants were tested by DAS-ELISA. Solutions were adjusted with PBST to

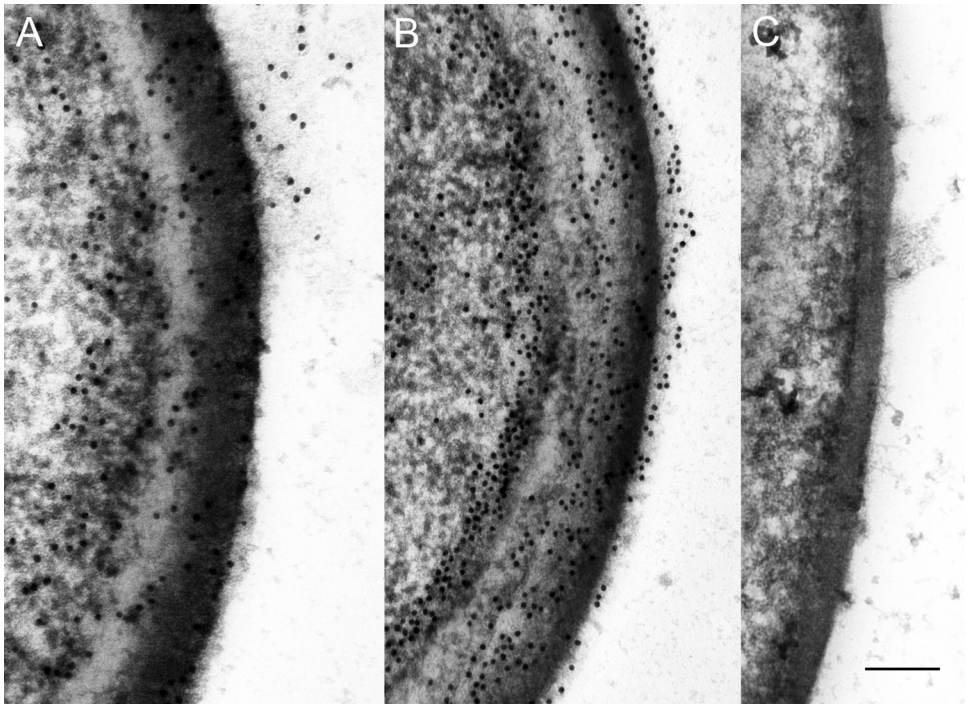

FIG. 2. Immunogold localization of the GA3 and HG12 antigens in spores of *P. boydii*. (A) Transverse section of conidial cell wall incubated with MAb GA3 and anti-mouse 15-nm gold particles, showing the distribution of the antigen in the cytoplasm, cell wall, and extracellular matrix of the spore. (B) Transverse section of the conidial cell wall incubated with MAb HG12 and anti-mouse 15-nm gold particles, showing the distribution of the antigen in the cytoplasm, plasma membrane, cell wall, and extracellular matrix of the spore. (C) Transverse section of the conidial cell wall incubated with TCM control and anti-mouse 15-nm gold particles, showing lack of staining. Bar, 0.2 μm.

contain 60 μg of protein/ml of buffer prior to the assay. Fungi were identified by internally transcribed spacer (ITS) region sequencing according to procedures described elsewhere (56).

DAS-ELISA of antigens from estuarine fungi. Wells of Maxisorp microtiter plates were coated overnight, at 4°C, with 50-μl volumes of MAb GA3 (IgM). Wells were washed three times (5 min each time) with PBST, washed once with PBS and once with dH_2O, and air dried at 23°C with a laminar flow hood. The plates were stored in sealed plastic bags at 4°C. Antigen extracts prepared in PBST as described above were incubated in the antibody-coated wells for 2 h. The wells were given four 5-min rinses with PBST and then incubated sequentially with HG12 MAb (IgG1) supernatant for 1 h, followed by goat anti-mouse IgG (γ-chain specific) peroxidase conjugate (Sigma), diluted 1 in 1,000 in PBST, for a further hour. Working volumes were 50 μl per well, and control wells were incubated with PBST antigen extract from *P. boydii* grown on semiselective medium under the same conditions. All incubation steps were performed at 23°C in sealed plastic bags. The wells were given an additional rinse with PBS prior to incubation with tetramethyl benzidine substrate solution for 30 min. Reactions were stopped, and the absorbance values of wells were determined as described above.

Nucleotide sequence accession numbers. Newly determined sequences were submitted to GenBank, and accession numbers FJ713051 to FJ713096 were obtained. For species designations, see Table 4.

RESULTS

Production of hybridoma cell lines, isotyping of MAbs, and specificities. A single fusion was performed, and 403 hybridomas were screened for MAb production. Nine cell lines produced MAbs with κ-light chains that reacted strongly in ELISAs with antigens from *P. boydii*. Three of the cell lines (HG12, PF5, and RA3) produced MAbs belonging to the Ig class G1 (IgG1), while the remaining six cell lines (GA3, LD9, RG5, SA5, SE8 and SH4) produced MAbs belonging to the Ig class M (IgM). MAbs were tested further for specificity against a wide range of related and unrelated fungi. With the exception of MAb RG5, which reacted strongly ($A_{450} > 1.0$) with antigens from all of the fungi tested (results not shown), the MAbs were highly specific, reacting with antigens from *Pseudallesheria* species, *Lophotrichus fimeti* and *Petriellopsis africana* (formerly *Pseudallescheria fimeti* and *Pseudallescheria africana*, respectively), *S. apiospermum*, and *S. aurantiacum* and from the related fungi *Graphium* and *Petriella*. The MAbs did not react with antigens from the closely related fungi *Scedosporium dehoogii* and *S. prolificans* or from a wide range of unrelated species. Results of ELISAs for specificity with two of the MAbs, GA3 and HG12, are shown in Table 1.

Characterization of antigen by using periodate and proteases. The epitopes bound by MAbs GA3 and HG12 were periodate sensitive (Table 2), showing that both antibodies recognize carbohydrate residues containing vicinal hydroxyl groups. There was no significant effect of pronase or trypsin on MAb binding, showing that the epitopes do not contain protein moieties (Table 3).

Characterization of antigen by PAGE and Western blotting. Western blotting analysis of culture filtrate from *P. boydii* under denaturing conditions showed that MAbs HG12 and GA3 bind to a major secreted antigen with a molecular mass of approximately 120 kDa. Periodate treatment of the antigen resulted in a reduction in MAb binding, confirming ELISA results showing that the MAbs bind to carbohydrate epitopes containing vicinal hydroxyl groups (Fig. 1).

Localization of antigen by immunogold electron microscopy and immunofluorescence. Immunogold electron microscopy showed that the antigens bound by the MAbs are present in the plasma membrane, in the cell wall, and on the surface of conidia (Fig. 2). Immunofluorescence studies with MAb HG12 showed that the antigen is present on the surfaces of ungerminated conidia and on the hyphal surfaces of *P. boydii* germlings (Fig. 3). In mixed-species cultures, MAb HG12 reacted strongly with conidia and hyphae of *P. boydii* but did not react with cells of *A. fumigatus* (Fig. 4).

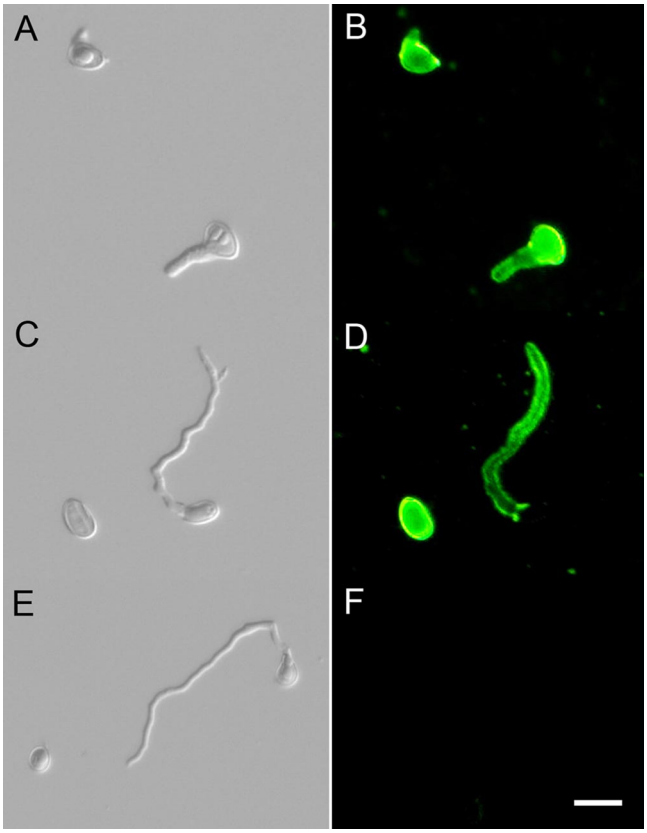

FIG. 3. Photomicrographs of *P. boydii* cells immunostained with MAb HG12 or TCM control and anti-mouse polyvalent Ig fluorescein isothiocyanate. (A and B) Germinating spores of *P. boydii* examined under a bright-field microscope (A) and under epifluorescence with MAb HG12 (B). (C and D) Ungerminated conidium of *P. boydii* and older germling with extended hypha examined under a bright-field microscope (C) and under epifluorescence with MAb HG12 (D). Note intense staining of the cell wall of the ungerminated spore and the cell wall of the hypha but the lack of staining of the germinated spore cell wall. (E) TCM control slide showing ungerminated conidium and germling of *P. boydii*. (F) Same slide shown in panel E but examined under epifluorescence. Bar, 6 μm.

Immunoassay of antigens from estuarine fungi. A diagnostic immunoassay was developed that comprised the MAbs HG12 and GA3. In the absence of clinical samples, the specificity of the assay was determined using fungi isolated from environmental samples. The assay was highly specific for detection of *P. boydii* only, with absorbance values of >0.600 in all cases (Table 4). The assay did not detect the related human pathogen *S. prolificans* and other related fungi in the Microascales (*Microascus* and *Scopulariopsis* species) or unrelated species, including the human pathogens *Mycocladus corymbiferus* (formerly *Absidia corymbifera*), *Pichia anomala*, *Rhizomucor miehei*, *Rhizomucor pusillus*, *Rhizomucor tauricus*, and *Issatchenkia*

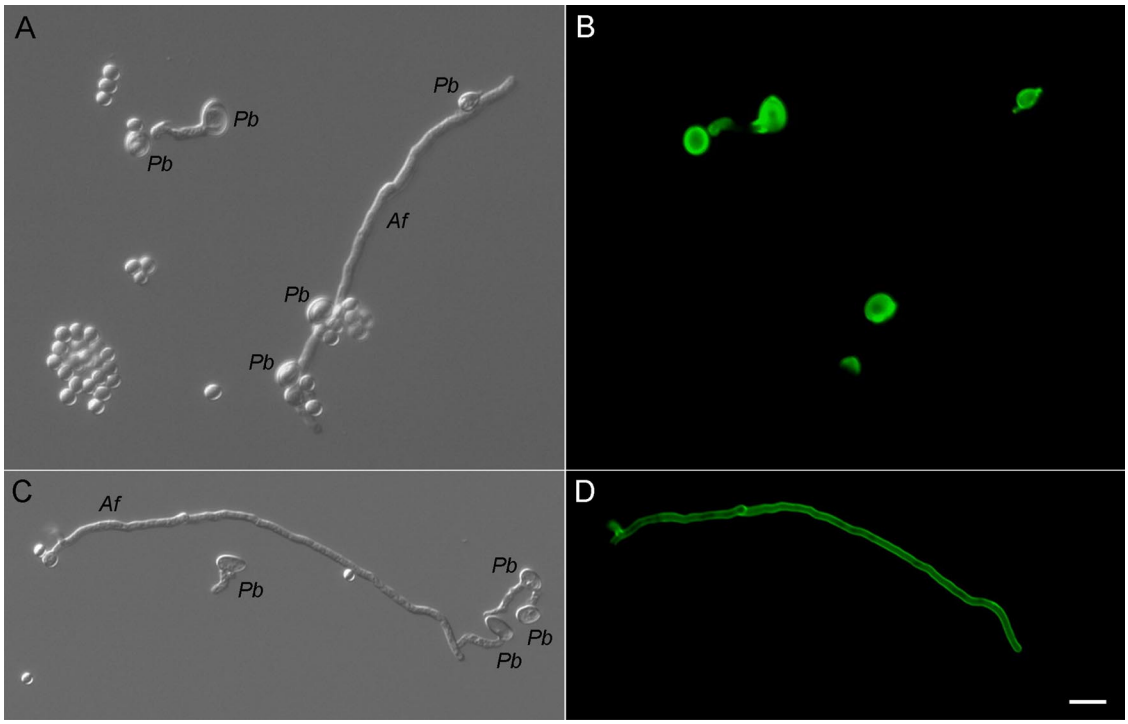

FIG. 4. Differentiation of *P. boydii* and *A. fumigatus* cells using the *P. boydii*-specific MAb HG12 or the *Aspergillus*-specific MAb JF5 and anti-mouse polyvalent Ig fluorescein isothiocyanate. (A and B) Ungerminated spores and germlings of *P. boydii* (*Pb*) and *A. fumigatus* (*Af*) examined under a bright-field microscope (A) and under epifluorescence with MAb HG12 (B). Note the lack of staining of *A. fumigatus* hypha and ungerminated *A. fumigatus* spores but the intense staining of *P. boydii* cells. (C and D) Germinating spores of *P. boydii* and *A. fumigatus* examined under a bright-field microscope (C) and under epifluorescence with MAb JF5 (D). Note the lack of staining of *P. boydii* cells but the intense staining of *A. fumigatus* hypha. Bar, 6 μm.

orientalis (anamorph *Candida krusei*). In all cases, these fungi gave absorbance values in the DAS-ELISA that were lower than the threshold value for detection, 0.100.

DISCUSSION

This paper describes the generation of MAbs against extracellular antigens from the emerging human pathogen *P. boydii*. The MAbs display a high degree of specificity, reacting with *P. boydii* and related fungi within the *P. boydii* complex and with *Graphium* and *Petriella* species but not with a wide range of related and unrelated fungi of clinical significance, including *S. prolificans*.

P. boydii was previously considered synonymous with *Pseudallescheria angusta*, *Pseudallescheria ellipsoidea*, and *Pseudallescheria fusoidea* (44), but a recent taxonomic study suggests that *P. boydii* is a complex comprising six species (*P. boydii*, *P. angusta*, *P. ellipsoidea*, *P. fusoidea*, *Pseudallescheria minutispora*, and *S. aurantiacum*) (13). The MAbs reacted with antigens from these fungi and with antigens from *Pseudallescheria africana* and *Pseudallescheria fimeti*, fungi recently reclassified as *Petriellopsis africana* and *L. fimeti*, respectively (15). Reactivity was also displayed with *S. apiospermum* and with species in the related genera *Graphium* and *Petriella* but not with *S. prolificans* or the proposed new species *S. dehoogii* (14). Cross-reactivity with *S. apiospermum* and *Graphium* was expected, since these are considered to be synanomorphs of *P. boydii* (45), although a recent study considered *S. apiospermum* to be a species separate from *P. boydii* (14), contradicting numerous previous reports that refer to this species as the anamorph (asexual state) of *P. boydii*.

Cross-reaction of the MAbs with *Graphium* and *Petriella* species might be regarded as problematic with respect to the use of the MAbs in diagnosis of *P. boydii* infections. However, *Petriella* infections of humans have never been recorded, and only a single infection by a *Graphium* species has been reported to date (26). Similarly, infections by *Pseudallescheria* species other than *P. boydii* are infrequent, although *P. fusoidea* was recently reported as the cause of osteomyelitis (29). A major benefit of the MAbs is their ability to differentiate between *P. boydii*/*S. apiospermum* and *S. prolificans*. The need to discriminate these fungi is imperative for immunocompromised patient groups known to be susceptible to mixed *Scedosporium* infections (6, 18), since the species exhibit differing sensitivities to antifungal drugs, such as voriconazole and the echinocandins (7, 16, 18, 59).

Two of the MAbs, an IgG1 (HG12) and GA3 (IgM), were used to develop a DAS-ELISA for the detection of *P. boydii* antigens. In the absence of clinical specimens, specificity of the assay was determined using environmental samples. While *P. boydii* has been isolated from soils, stagnant water, and sewage (9, 17, 50), its natural ecological niche has yet to be established; however, estuary waters have been suggested (17). The assay was therefore used, in combination with a semiselective plate-enrichment technique, to detect the pathogen in estuarine

TABLE 4. Absorbance values from DAS-ELISAs of fungi isolated from estuarine muds and species designations of recovered isolates as determined by ITS sequence analysis

Site	Isolate no.	Absorbance (450 nm)[a]	Species	GenBank accession no.
Mylor	MG2(8)	1.181	*P. boydii*	FJ713055
	MG4(2)	0.026	*R. miehei*	FJ713069
	M7A(1)	0.808	*P. boydii*	FJ713063
	M7B(5)	1.052	*P. boydii*	FJ713057
Penryn	P4B(7)	0.753	*P. boydii*	FJ713059
	PG5(3)	0.018	*Mucor circinelloides*	FJ713068
Restronguet	R6(6)	0.019	*Coprinopsis cinerea*	FJ713066
	R9(9)	0.053	*Microascus trigonosporus* var. *trigonosporus*	FJ713076
Topsham	T3B	0.044	*R. pusillus*	FJ713079
	T3D	0.000	*Geotrichum cucujoidarum*	FJ713071
	T6A	0.000	*Coprinopsis cinerea*	FJ713078
	T6C	0.000	*I. orientalis*	FJ713073
	T7B	0.000	*Saccharomyces paradoxus*	FJ713072
	T9A	0.000	*Pichia fermentans*	FJ713080
	T11A	0.618	*P. boydii*	FJ713064
	T11B	1.051	*P. boydii*	FJ713060
	T12A	1.127	*P. boydii*	FJ713061
	T13A	0.054	*Mucor circinelloides*	FJ713074
	T14A	0.001	*M. corymbiferus*	FJ713070
	T22A	1.117	*P. boydii*	FJ713054
Exmouth	E2A	0.013	*Mucor circinelloides*	FJ713065
	E5A	0.017	*R. miehei*	FJ713075
	E7A	0.000	*Pichia anomala*	FJ713067
	E12A	0.011	*R. pusillus*	FJ713077
	E13A	0.953	*P. boydii*	FJ713051
Lympstone	L1B	0.000	*Pichia fermentans*	FJ713081
	L1C	0.000	*I. orientalis*	FJ713082
	L1D	0.000	*Candida catenulate*	FJ713083
	L4A	0.002	*M. corymbiferus*	FJ713084
	L6A	0.004	*S. prolificans*	FJ713085
	L6B	0.000	*Kazachstania telluris*	FJ713086
	L7C	0.000	*Arxula adeninivorans*	FJ713087
	L10A	0.005	*Kluyveromyces nonfermentans*	FJ713088
	L10B	1.023	*P. boydii*	FJ713062
	L11B	0.006	*S. prolificans*	FJ713089
	L12A	0.007	*Pichia spartinae*	FJ713090
	L12B	0.006	*S. prolificans*	FJ713091
	L12C	0.077	*Simplicillium lamellicola*	FJ713056
	L12D	0.012	*Mucor circinelloides*	FJ713092
	L12E	0.769	*P. boydii*	FJ713053
	L14A	0.008	*Emericellopsis minima*	FJ713093
	L15A	0.699	*P. boydii*	FJ713052
	L18A	1.093	*P. boydii*	FJ713058
	LB1A	0.045	*R. tauricus*	FJ713094
	LB3A	0.000	*Scopulariopsis chartarum*	FJ713095
	LB3B(ii)	0.000	*Fennellomyces linderi*	FJ713096

[a] The threshold absorbance value for detection of antigen in DAS-ELISA is ≥0.100. The absorbance value of the control (*P. boydii* 835.96) is 1.005.

muds. The accuracy of the assay in detecting *P. boydii* was confirmed using antigens extracted from species of thermotolerant fungi recovered from estuarine muds and subsequent identification of isolates using ITS sequencing. The assay detected *P. boydii* only. No reaction was found with other members of the *Microascaceae* isolated, including *S. prolificans*, *Microascus trigonosporus*, and *Scopulariopsis chartarum*, known invasive pathogens of humans and animals (6, 7, 18, 20, 31, 35, 45, 59, 62). Of the other nonyeast human pathogenic fungi isolated from the estuarine mud samples, the zygomycetes predominated. *Mycocladus* (*Absidia*), *Mucor*, *and Rhizomucor* species have all been described as human pathogens (40, 48), and cutaneous zygomycosis has been described in a case of near drowning with pulmonary *P. boydii* infection (4). The assay did not detect these fungi, and no cross-reaction was found with the yeast species isolated, including *I. orientalis* (*Candida krusei*), *Candida catenulata*, and *Pichia anomala*, which have been reported as emerging nosocomial pathogens of humans (40, 58, 59).

PCR has been used to develop diagnostic assays for the

discrimination of *Scedosporium* species (61, 63), but these have not yet been tested with clinical samples. Similarly, the MAbs described here have not yet been used to detect *P. boydii* in clinical samples. However, both the immunofluorescence assay and the DAS-ELISA show their potential in differentiating *P. boydii* from other agents of hyalohyphomycosis, such as *A. fumigatus* and *S. prolificans*, which exhibit similar morphological characteristics upon microscopic examination. For *A. fumigatus*, a galactomannan-based diagnostic test is available for the detection of invasive aspergillosis (43), but there is no such test for *P. boydii*. While a peptidorhamnomannan from hyphae of *P. boydii* has been proposed as a diagnostic marker for the fungus, like galactomannan (43), it is evident that other invasive pathogens may also possess the glycoprotein, making differential diagnosis problematic (23, 41). MAbs HG12 and GA3 are currently being used to generate a lateral-flow device for detection of circulating *P. boydii* antigen in serum, bronchoalveolar lavage, and urine samples, similar to the lateral-flow device recently developed for the rapid serodiagnosis of invasive aspergillosis (55).

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