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 erythematosus, 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 peptidohammomannan 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
| Organism                          | Isolate no. | Source | GA3 | HG12 |
|----------------------------------|-------------|--------|-----|------|
| Abidia corymbifera               | 101040      | CBS    | 0.021 | 0.021 |
| *Abidia glauca*                  | 2           | CRT    | 0.027 | 0.053 |
| *Abidia spinosa*                 | 3           | CRT    | 0.017 | 0.063 |
| *Acrocnemium alternatum*        | 545.89      | CBS    | 0.002 | 0.056 |
| *Acrocnemium atrogriseum*       | 306.85      | CBS    | 0.028 | 0.057 |
| *Acrocnemium blochii*            | 424.93      | CBS    | 0.010 | 0.041 |
| *Alternaria alternata*           | 42          | CRT    | 0.007 | 0.029 |
| *Aphyllopsomyces elegans*        | 658.93      | CBS    | 0.032 | 0.044 |
| *Aspergillus clavatus*           | 514.65      | CBS    | 0.028 | 0.034 |
| *A. fumigatus*                   | AF293S      | SK     | 0.016 | 0.041 |
| *A. fumigatus*                   | 181         | CRT    | 0.012 | 0.029 |
| *Aspergillus flavus*             | 91858ii     | IMI    | 0.022 | 0.072 |
| *Aspergillus niger*              | 102.40      | CBS    | 0.026 | 0.063 |
| *Aspergillus terreus var. terreus* | 601.65   | CBS    | 0.026 | 0.065 |
| *Acremonium versicolor*          | 599.65      | CBS    | 0.002 | 0.005 |
| *Acremonium pullulans var. pullulans* | 771.97 | CBS    | 0.019 | 0.062 |
| *Candida albicans*               | SC5314      | SB     | 0.016 | 0.023 |
| *Candida dubliniensis*           | 8500        | CBS    | 0.002 | 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 | 0.060 |
| *Emericella nidulans*            | A4          | FGSC   | 0.027 | 0.058 |
| *Exophiala dermatitidis*         | 153.94      | CBS    | 0.013 | 0.038 |
| *Exophiala jeaneselmi var. heteromorpha* | 657.76 | CBS    | 1.281 | 1.200 |
| *Fusarium solani*                | 212.34      | CBS    | 0.018 | 0.060 |
| *F. solani var. petrophilum*     | 102256      | CBS    | 0.028 | 0.055 |
| *F. solani* var. petrophilum*    | 102277      | CBS    | 0.006 | 0.004 |
| *Geotrichum capitatum*           | 327.86      | CBS    | 1.043 | 1.008 |
| *Graphium eumorphum*             | 987.73      | CBS    | 1.507 | 1.518 |
| *Graphium penicilliodes*         | 320.72      | CBS    | 1.281 | 1.200 |
| *L. fimeti*                      | 129.78      | CBS    | 0.013 | 0.027 |
| *Madurella mycetomatis*          | 868.95      | CBS    | 0.028 | 0.052 |
| *Mucor plumbeus*                 | 96          | CRT    | 0.027 | 0.066 |
| *Mucor racemosus*                | 93          | CRT    | 0.029 | 0.052 |
| *Paracolomyces variotii*         | 339.51      | CBS    | 0.000 | 0.002 |
| *Paracolomyces tectonae*         | 127.84      | CBS    | 1.517 | 1.462 |
| *Pencillium 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 gutulata*             | 362.61      | CBS    | 1.456 | 1.509 |
| *Petriella setifera*             | 109039      | CBS    | 1.192 | 1.122 |
| *Pleurotus ostreatus*            | 311.72      | CBS    | 1.114 | 1.095 |
| *Phialophora richardiiae*        | 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*                      | 100870      | CBS    | 1.188 | 1.085 |
| *Pseudallescheria desertorum*    | 489.72      | CBS    | 1.200 | 1.071 |
| *Pseudallescheria ellipsoidea*    | 438.72      | CBS    | 1.071 | 1.049 |
| *P. fusoidea*                    | 106.53      | CBS    | 1.160 | 1.055 |
| *R. miehei*                      | 360.92      | CBS    | 0.033 | 0.077 |
| *Rhizopus oryzae*                | 395.54      | CBS    | 0.048 | 0.060 |
| *Rhizopus oryzae*                | 200900      | IMI    | 0.007 | 0.028 |
| *Rhizopus stolonifer*            | G1          | CRT    | 0.004 | 0.017 |
| *Saccharomyces vestivallis*      | 100390      | CBS    | 0.038 | 0.074 |
| *S. aureantiacum*                | 121926      | CBS    | 1.431 | 1.243 |
| *S. aureantiacum*                | 118934      | CBS    | 1.302 | 1.271 |
| *S. apiospermum*                 | 117407      | CBS    | 1.177 | 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 | 0.087 |
| *S. prolificans*                 | 102176      | CBS    | 0.045 | 0.075 |
| *Sporothrix schenckii*           | 345.53      | CBS    | 0.034 | 0.043 |
| *Sphaeroplastus chartarum*       | 485.48      | CBS    | 0.016 | 0.040 |
| *Syncaphalacia 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 |

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

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, Göttingen, Germany.

b Each value is the mean for replicated samples. The threshold absorbance value for detection of antigen in ELISA is \( \geq 0.100 \).
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 regimens. 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 Na2HPO4, and 1.5 mM KH2PO4 [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 immunobilized to the wells of microtiter plates (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.

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 successively washed with hybridoma supernatant for 1 h, followed with goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA, 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 cell 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 NaIO4 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% 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 230 V (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 metabolite 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 4°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 diH2O and air-dried between sheets of Whatman filter paper.

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

| TABLE 2. Absorbance values from ELISAs with periodate-treated antigens by using MAbs GA3 and HG12 of IgM and IgG1, respectively. |
|-----------------------------------------------|
| | MAb | Time (h) | 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 |
| | | 1 | 0.137 ± 0.026 | 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 | 0.956 ± 0.021 |
| | | 1 | 0.907 ± 0.017 | 0.990 ± 0.012 |

* Each value is the mean for replicated values ± standard errors.

| TABLE 3. Absorbance values from ELISAs with protease-treated antigens by using MAbs GA3 and HG12 of IgM and IgG1, respectively. |
|-----------------------------------------------|
| | MAb | Temp (°C) | Pronase | Pronase control | Trypsin | Trypsin control |
|-----------------------------------------------|
| | GA3 | 4 | 0.1066 ± 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 |

* Each value is the mean for replicated values ± standard errors.

(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.

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

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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.

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 dH2O 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. 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.](http://cvi.asm.org/article-pdf/16/5/759/1642477/1642477.pdf)

![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.](http://cvi.asm.org/article-pdf/16/5/759/1642477/1642477.pdf)
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 PBS and once with dH2O 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 (A450 > 1.0) with antigens from all of the fungi tested (results not shown), the MAbs were highly specific, reacting with antigens from *Pseudallescheria* 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* germings (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).

**Immunobob assay of antigens from estuarine fungi.** A diagnostic immunobob assay 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 pathogens *S. prolificans* and other related fungi in the Microscalles (*Microascus* and *Scopulariopsis* species) or unrelated species, including the human pathogens *Mycocladium corymbiferis* (formerly *Absidia corymbifera*), *Pichia anomala*, *Rhizomucor miehei*, *Rhizomucor pusillus*, *Rhizomucor tauricus*, and *Issatchenka
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 fusoides* (44), but a recent taxonomic study suggests that *P. boydii* is a complex comprising six species (*P. boydii*, *P. angusta*, *P. ellipsoidea*, *P. fusoides*, *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. fusoides* 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...
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

| Site          | Isolate no. | Absorbance (450 nm) | 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        | PB2(7)      | 0.753                | *P. boydii*           | FJ713059              |
|               | PG5(3)      | 0.018                | *Mucor circinelloides*| FJ713068              |
| Restrounguet  | R6(6)       | 0.019                | *Coprins corporea*    | 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                | *Coprins corporea*    | 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. corymbfirus*      | 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              |
| Lymstone      | L1B         | 0.000                | *Pichia fermentans*   | FJ713081              |
|               | L1C         | 0.000                | *I. orientalis*       | FJ713082              |
|               | L1D         | 0.000                | *Candida catenulate*  | FJ713083              |
|               | L4A         | 0.002                | *M. corymbfirus*      | FJ713084              |
|               | L6A         | 0.004                | *S. prolificans*      | FJ713085              |
|               | L6B         | 0.000                | *Kazachstania telluri*| FJ713086              |
|               | L7C         | 0.000                | *Arasa adeninivorans* | FJ713087              |
|               | L10A        | 0.005                | *Klebsiellaceae 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* | FJ713086              |

* 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.
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 lyophilophymocysis, 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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