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Exoantigen Test for Identification of *Petriellidium boydii* Cultures

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Cultures of *Petriellidium boydii* were serologically identified by detection of their exoantigens with an immunodiffusion procedure. The technique, which is specific and sensitive, allowed the rapid identification and differentiation of 12 isolates of *P. boydii* from numerous other morphologically similar *Hyphomycetes*. The antigen-antiserum reference system and the production, by two different techniques, of exoantigens used in the identification of *P. boydii* are described.

Petriellidium boydii (anamorph, *Scedosporium apiospermum*) has frequently been isolated from soil in the temperate areas of the world (1, 7, 9, 19a, 24). This fungus has been recognized as the most common etiological agent of eumycotic mycetoma in many countries, including Italy (17). *P. boydii*, moreover, can also cause other types of severe disease, such as meningomycosis (2, 4), pulmonary mycosis (13, 20, 22, 27), keratomycosis (3), prostatitis (16), and disseminated mycosis (21).

The identification of the perfect and imperfect forms (*S. apiospermum*) of this mold is basically founded on morphological features. Although not all of the isolates produce ascocarps, nutritional factors have been shown to play a significant role in the sporulation of this anamorph and of the teleomorph of this fungus (6, 10). Distinguishing *P. boydii* (anamorph, *S. apiospermum*) from other morphologically similar pathogenic or saprobic fungi at times is difficult. The fundamental importance of rapidity in the diagnosis and treatment of infections caused by *P. boydii* compelled us to develop a specific and rapid serological technique for the identification of cultures suspected of being *P. boydii*. Kaufman and Standard (11, 12, 25, 26) perfected an immunodiffusion technique which permitted the detection of cell-free antigens (exoantigens) that were specific for the identification of the mycelial forms of the most important dimorphic fungi. We chose to apply the exoantigen technique to the serological identification of *P. boydii* cultures. The results of our studies are presented in this report.

MATERIALS AND METHODS

Fungal isolates. A total of 12 isolates of *P. boydii* and 1 isolate each of the following fungi were used in this study: *Scedosporium apiospermum*, *Acremonium* sp., *Acremonium strictum*, *Aphanocladium* sp., *Beauveria* sp., *Beauveria bassiana*, *Ceratocystis minor*, *Chrysosporium parvum*, *Chrysosporium tropicum*, *Exophiala spinifera*, *Fonsecaea pedrosoi*, *Graphium penicillioides*, *Paecilomyces varioti*, *Petriella musispora*, *Petriella setifera*, and *Scopulariopsis brevicaulis*. We isolated some of the fungi from soil (19a) and also used clinical specimens from our institute. Other isolates were graciously furnished by Libero Ajello, Mycology Division, Centers for Disease Control, Atlanta, Ga., Robert Samson, Centraalbureau voor Schimmelcultures, Baarn, Holland, and David Lupan (14), Department of Microbiology, University of Reno, Reno, Nev. All of the cultures were maintained in sterile water at room temperature in our culture collection.

Control antisera. The control antisera were prepared by immunizing albino rabbits by the following procedure. One isolate of *P. boydii* (Università Cattolica del Sacro Cuore no. 0) was grown on Czapek dextrose agar (200 ml, Difco Laboratories) in 1-liter tissue culture bottles for 14 days at 25°C. Spores were successively suspended in cold 0.15 M NaCl, filtered through sterile gauze to remove mycelial fragments, and then centrifuged (3,000 rpm for 20 min at 4°C), and the spore pellets were washed three times. The washed spores were finally suspended in cold 0.15 M NaCl and adjusted to a final concentration of 10^7 spores per ml. The viability and purity of the spore suspension were determined by plating on potato dextrose agar and incubating at 25 and 37°C, respectively. The spore suspension was used to inoculate albino rabbits (2.5 to 3.0 kg) by the following immunization procedure. One milliliter of the suspension was injected subcutaneously once a week for 1 month. An additional 1.0 ml of antigen was administered after a rest period of 3 weeks. Seven days after the last injection, the animals were bled by intracardiac puncture (15). The serum was lyophilized and preserved at room temperature.

Control antigens. The same culture of *P. boydii* that was used for the production of the antisera was inoculated in tubes of Sabouraud dextrose agar and incubated for 48 h at 25°C. For producing control antigen, small squares of the culture were inoculated

into 1-liter flasks containing 400 ml of Sabouraud dextrose broth and incubated for 35 days at 25°C under stationary conditions. After this period, the broth cultures were killed with 0.5% Formalin and incubated for 24 h at 25°C to check the Formalin-killed cultures for viability and purity. After 48 h, the broth cultures were filtered through Whatman paper and collected in a glass flask. A double volume of cold acetone was slowly added to the filtrate that was maintained in constant agitation with a magnetic stirrer. When all of the cold acetone had been added, the mixture was agitated for 15 min at 4°C and then centrifuged (5,500 rpm for 30 min at 4°C), and the supernatant was discarded. The sediment was washed in cold acetone and removed after further centrifugation. The sediment was dried overnight in a desiccator jar containing $CaCl_2$, suspended in sterile distilled water to a volume 0.1 that of the filtrate, and centrifuged once more (5,500 rpm for 30 min at 4°C). The carbohydrate content of the supernatant was determined by the method of Dubois et al. (8).

Exoantigens. The production of cell-free filtrates (exoantigens) of *P. boydii* and the other test fungi was done with two different techniques. In the first technique, 100-ml flasks containing 30 ml of brain heart infusion broth were inoculated with a small square of mycelium from 48-h cultures that had been grown on Sabouraud dextrose agar and incubated, with shaking, for 3 to 6 days at 25°C. After this period, the broth cultures were killed with Merthiolate (1:5,000) and filtered, and the filtrate was centrifuged for 20 min at 3,000 rpm. The supernatant was concentrated 25 times with Amicon Minicon Macrosolute B-15 concentrators (25, 26). In the second technique, cultures showing luxuriant growth on Sabouraud dextrose agar after 10, 21, 30, and 85 days were covered with 8 ml of an aqueous solution of Merthiolate to a concentration of 1:5,000. Incubation was continued for an additional 24 h at 25°C. Five milliliters of the extract was finally concentrated 25 times with Amicon Minicon macrosolute B-15 concentrators (11, 12).

Serological technique. A double diffusion in agar was the serological technique used in this study. The medium preparation and execution of the test were carried out according to the recommendations of the Mycology Division, Centers for Disease Control, for the serological diagnosis of histoplasmosis by a microimmunodiffusion serological test (19).

RESULTS

Each fungal isolate used in this study had been previously identified according to its cultural and morphological characteristics. *P. boydii* induced the production of specific precipitins in the injected animals (5, 18, 23). An antigen with a carbohydrate concentration of 2,500 μg/ml was selected as the reference antigen. It gave three distinct precipitin bands when tested with the reference antiserum (Fig. 1). Differences in the serological reactions were not observed between the exoantigens produced from the broth culture filtrates and those extracted directly from Merthiolate-treated cultures grown in Sabouraud dextrose agar tubes. All of the 12 isolates of *P. boydii* studied produced homologous precipitin bands when tested, independently of their sources and regardless of the age of the cultures, with the reference antisera (Fig. 2). None of the other species studied, whether morphologically similar or taxonomically correlated or both, elicited homologous precipitinogens. *S. apiospermum* (Lupan 813), an anamorph of *P. boydii*, did elicit homologous antigen. Heterologous bands were produced by *Exophiala spinifera* (Fig. 3), *Fonsecaea pedrosoi* (Fig. 4), *Paecilomyces varioti* (Fig. 5), and *Scopulariopsis*

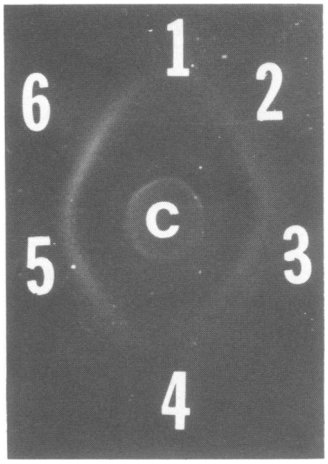

FIG. 1. *Reference antigen chosen for identification of P. boydii by immunodiffusion. Well contents: c, reference antiserum; 1 and 4, polysaccharide antigen (500 μg of carbohydrate per ml); 2 and 3, polysaccharide antigen (2,500 μg of carbohydrates per ml); 5 and 6, polysaccharide antigen (5,000 μg of carbohydrate per ml).*

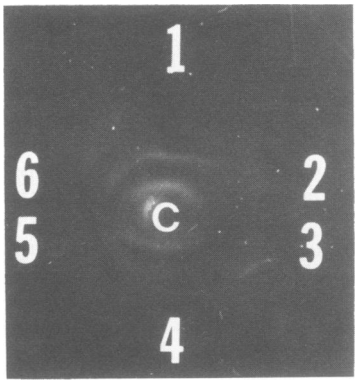

FIG. 2. *Specificity of the immunodiffusion exoantigen technique for P. boydii. Well contents: c, reference antiserum; 1 and 4, reference antigen; 2 and 3, Petriella musispora exoantigens; 5 and 6, P. boydii exoantigens.*

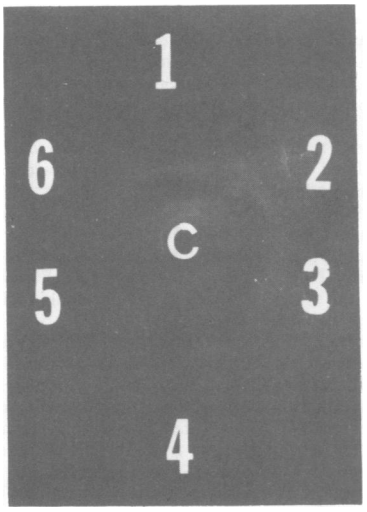

FIG. 3. *Specificity of the immunodiffusion exoantigen technique for P. boydii. Well contents: c, reference antiserum; 1 and 4, reference antigen; 2 and 3, E. spinifera exoantigens; 5 and 6, P. boydii exoantigens.*

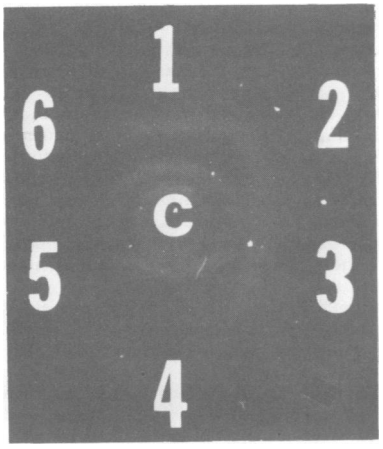

FIG. 4. *Specificity of the immunodiffusion exoantigen technique for P. boydii. Well contents: c, reference antiserum; 1 and 4, reference antigen; 2 and 3, F. pedrosoi exoantigens; 5 and 6, P. boydii exoantigens.*

brevicaulis (Fig. 6). These fungi, however, caused no diagnostic problems, as the bands that they produced were quite separate from the homologous bands produced by *P. boydii*.

DISCUSSION

The importance of *P. boydii* as an etiological agent of eumycotic mycetoma and the increasing incidence of pulmonary and other types of infection have made it imperative to accurately identify isolates suspected of being *P. boydii*. Conventional laboratory procedures based on culture studies are limited by the morphological analogies between *P. boydii* and other related genera and species and by the frequent difficulty in inducing the production of ascocarps. The detection of specific *P. boydii* exoantigens is not a difficult procedure because of the simplicity of directly extracting the exoantigens from cultures with an aqueous Merthiolate solution. Among the fungi studied, the exoantigen technique was shown to be specific. It permitted the accurate identification of *P. boydii* isolates within 3 days. The exoantigen technique has been used successfully at our institute as an aid in the diag-

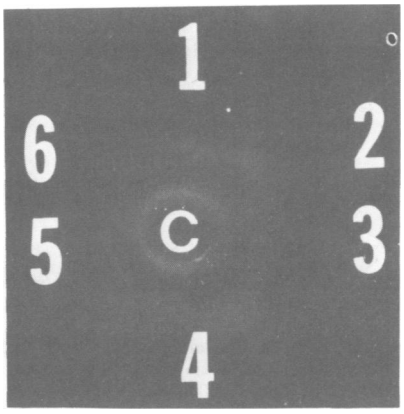

FIG. 5. *Specificity of the immunodiffusion exoantigen technique for P. boydii. Well contents: c, reference antiserum; 1 and 4, reference antigen; 2 and 3, P. varioti exoantigens; 5 and 6, P. boydii exoantigens.*

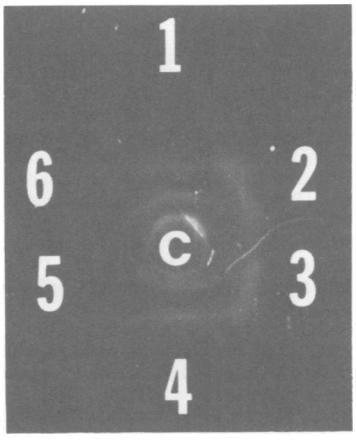

FIG. 6. *Specificity of the immunodiffusion exoantigen technique for P. boydii. Well contents: c, reference antiserum; 1 and 4, reference antigen; 2 and 3, S. brevicaulis exoantigens; 5 and 6, P. boydii exoantigens.*

nosis of a case of petriellidiosis encountered by Giovanni Fadda in the Sassari area of Sardinia, a location from which we had previously isolated *P. boydii* from soil (19a).

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