

# Immunodiffusion Test for Serodiagnosing Subcutaneous Zygomycosis

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**Culture filtrate antigens of *Basidiobolus ranarum* and *Conidiobolus coronatus* were analyzed by immunodiffusion (ID) with homologous rabbit antisera. *B. ranarum* and *C. coronatus* were each found to have five specific antigens. Results of tests with heterologous antisera indicated that all of the species shared at least one antigen. ID tests incorporating the specific precipitin bands as references were developed for detection of basidiobolomycosis and conidiobolomycosis. These tests were performed with sera from humans and horses with proven basidiobolomycosis and conidiobolomycosis as well as with control sera from humans and animals with and without heterologous mycotic and oomycotic infections. Only sera from cases of basidiobolomycosis and conidiobolomycosis produced lines of identity with the reference precipitates of *B. ranarum* and *C. coronatus*, respectively. The ID tests were found to be completely sensitive and specific for determining the etiology of zygomycosis caused by these two species. In addition they appeared useful for monitoring resolution of the infections.**

*Conidiobolus coronatus* and *Basidiobolus ranarum* are the prime etiologic agents of subcutaneous zygomycosis (syn. entomophthoromycosis) in humans and animals (7). *C. coronatus* frequently invades the nasal or adjacent tissue, producing tumoral masses, whereas *B. ranarum* most commonly infects the subcutaneous tissues of most other parts of the body, particularly the extremities, trunk, buttocks, and in some cases, the abdominal organs.

The clinical symptoms of these infections are similar and mimic those of other diseases, such as rhinocerebral zygomycosis, pythiosis, tuberculosis, neoplasia, and pyogenic abscess. The diagnosis of subcutaneous zygomycosis is based on isolation and identification of the etiologic agents, histopathologic results, or both. However, the etiologic agents are not always successfully cultured, and histologic studies do not permit a specific diagnosis (3). Antigenic studies by Yangco et al. (8) indicated that *B. ranarum* produces two antigens, one that is apparently distinct for *B. ranarum* and the other that is shared by the *Basidiobolus* and *Conidiobolus* species. The antigenic diversity demonstrated among these species supports the contention that serodiagnostic tests could be developed for subcutaneous zygomycosis. Except for a preliminary report on the applicability of immunodiffusion (ID) for diagnosing conidiobolomycosis (5), no routine serologic procedures have been described for diagnosing infections caused by *C. coronatus* and *B. ranarum* (7). This study was undertaken with the purpose of developing an ID test capable of diagnosing infections caused by either *B. ranarum* or *C. coronatus* in humans and animals.

## MATERIALS AND METHODS

**Cultures.** *B. ranarum* CDC B-4032, which was isolated from a human patient with basidiobolomycosis; *C. coronatus* C-6 (CDC B-4285), which was isolated from a horse with conidiobolomycosis; and *Pythium insidiosum* CDC B-4295, which was isolated from a horse with pythiosis, were used for preparing antigens and antisera. These cultures are

deposited in the Division of Mycotic Diseases, Centers for Disease Control, culture collection.

**Antigen production.** Cultures of *B. ranarum*, *C. coronatus*, and *P. insidiosum* were each transferred to brain heart infusion agar slants (Difco Laboratories, Detroit, Mich.) and incubated at 25°C for 3 days. Small portions of growth from the brain heart infusion slant cultures were transferred to each of three 1.0-liter flasks containing 500 ml of brain heart infusion broth. The cultures were incubated at 37°C on a shaker rotating at 150 rpm.

The flasks were removed after 5 days of incubation, and the cultures were killed with Merthiolate (0.02%) and filtered through Whatman no. 1 paper (Whatman, Inc., Clifton, N.J.) and then through a 0.45- $\mu$ m-pore-size membrane. The filtrate was concentrated by ultrafiltration under positive pressure in a stirred cell fitted with a membrane (nominal molecular weight limit, 10,000; PM-10 membrane; Amicon Corp., Lexington, Mass.). These concentrated culture filtrate antigen (CFA) preparations were used both for immunizing rabbits to obtain control antisera and as antigens in ID studies.

Trypsinized antigen from *C. coronatus* C-6 was prepared as described by Mendoza and Alfaro (5) and Miller and Campbell (6). Basically, this involved the use of the fungal mats from 10-day-old cultures grown on Sabouraud dextrose agar slants and dried at 37°C. The fungal mass was ground to a powder, and 1.0 g was added to 0.5 g of crystalline trypsin (Calbiochem-Behring, San Diego, Calif.) in 8 ml of 0.05 M phosphate buffer (pH 8.0). This mixture was incubated at 37°C for 24 h on a mechanical shaker rotating at 150 rpm. Following centrifugation at 3,000  $\times$  g for 20 min, the supernatant antigens were collected.

**Antiserum production.** Each of the CFA preparations of *B. ranarum*, *C. coronatus*, and *P. insidiosum* was used to inoculate four albino rabbits. The rabbits were bled before immunization. None of the preimmunization sera contained precipitins to the CFA antigens used. Each rabbit was injected intramuscularly in a single site with a mixture consisting of 0.5 ml of CFA and 0.5 ml of Freund incomplete adjuvant (Difco) on days 1, 2, and 3. One-half milliliter of the

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TABLE 1. Demographic and clinical characteristics of patients with entomophthoromycosis

Patient no.	Age (yr), sex, country	Type of disease	Basis of diagnosis
1	NA, <sup>a</sup> male, India	Chronic subcutaneous lesion of arm	<i>B. ranarum</i> culture, biopsy
2	7, NA, Mexico	Subcutaneous nodular lesions of thigh	<i>B. ranarum</i> culture, biopsy
3	69, male, United States	Abdominal and colonic mass	<i>B. ranarum</i> culture, biopsy
4	NA, male, India	Subcutaneous lesion of thigh	<i>B. ranarum</i> culture, biopsy
5	39, male, Ivory Coast	Nasofacial lesions, facial tumor	<i>C. coronatus</i> culture, biopsy (1, 2)

<sup>a</sup> NA, Not available.

same antigen was injected intravenously on days 7 and 14. One day 21, the rabbits were bled, and their sera were tested for precipitins by the ID test against the homologous antigens. Rabbit antisera demonstrating four to six strong precipitins were used as positive reference sera.

**Sera.** Serum samples from 15 horses with culturally proven conidiobolomycosis were examined. In addition, sera from 19 horses with biopsies containing hyphae suggestive of conidiobolomycosis, 6 horses with proven pythiosis, 3 horses with proven cutaneous habronemiasis, 3 horses with streptococcal pneumonia, 5 horses with high *Sporothrix schenckii* agglutinin titers, and 5 horses without evidence of any disease were studied. All the horses, except those with suspected sporotrichosis, were from Costa Rica. A serum sample from a cat with culturally positive pythiosis was also studied.

Sera from four humans with proven basidiobolomycosis and one human with conidiobolomycosis were also tested (Table 1). Basidiobolomycosis and conidiobolomycosis are used here to refer to zygomycosis caused by *B. ranarum* and *C. coronatus*, respectively. In addition, sera from five humans with aspergillosis, 5 humans with candidiasis, 2 humans with zygomycosis caused by *Rhizopus* spp., and 5 apparently healthy individuals were studied.

**ID test.** Agar gel double diffusion was carried out in plastic petri dishes (100 by 15 mm) into which 7.5 ml of 0.25% phenolized-1% purified agar (Difco) (4) was added. A seven-well pattern with 4-mm-diameter wells 4 mm apart was used. The reactants were added to the wells and incubated for 24 h in a humid chamber at room temperature.

## RESULTS

The reactivities of *B. ranarum*, *C. coronatus*, and *P. insidiosum* rabbit antisera with their homologous and heterologous antigens are shown in Fig. 1. The antigens and antisera demonstrated optimal reactivities when used undiluted. Rabbit antisera to the reference strains of *B. ranarum* and *C. coronatus* contained five distinct precipitins against

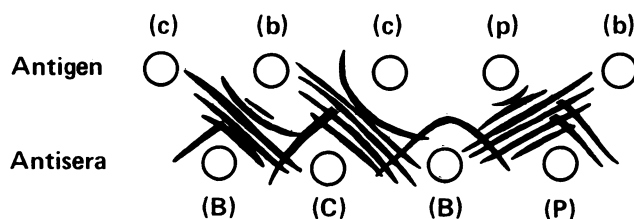


FIG. 1. Homologous and heterologous reactions of *B. ranarum*, *C. coronatus*, and *P. insidiosum* rabbit antisera and antigens. Wells b contained *B. ranarum* antigen, wells c contained *C. coronatus* antigen, and well p contained *P. insidiosum* antigen. Wells B contained *B. ranarum* antiserum, well C contained *C. coronatus* antiserum, and well P contained *P. insidiosum* antiserum.

their homologous antigens. In contrast, the rabbit anti-*P. insidiosum* serum demonstrated six distinct precipitins to its homologous antigens.

Cross-reactions were evident with all of the antigens. Both the *C. coronatus* and *P. insidiosum* antigens reacted with the *B. ranarum* antiserum to produce a single line indicative of a common antigen present in these species; this antigen was not detected in the homologous (*B. ranarum*) CFA. The *B. ranarum* antigen cross-reacted with both the *C. coronatus* and *P. insidiosum* rabbit antisera to produce one and two bands, respectively. Again, none of these bands showed any identity to those formed by using homologous antisera and antigens.

When the *B. ranarum*, *C. coronatus*, and *P. insidiosum* CFA preparations were tested against sera from horses with conidiobolomycosis and pythiosis, reactions similar to those obtained with the rabbit sera were noted. One to five precipitins were produced by the interaction of the conidiobolomycosis antigens and antibodies, and three to six precipitins were produced by interaction between the pythiosis antigens and antibodies (Fig. 2). The *B. ranarum* antigen produced two precipitin bands of nonidentity with the *P. insidiosum* reference system after reaction with the pythiosis horse serum. One band of nonidentity was noted when the *B. ranarum* antigen was tested with the conidiobolomycosis reference system. This band was identical to one of the two nonidentity bands that occurred with the *P. insidiosum* antigen.

Preliminary studies comparing the reactivities of the trypsinized and culture filtrate antigens of *C. coronatus* with rabbit and horse *C. coronatus* antisera revealed that the trypsinized preparation contained only one antigen while the CFA contained five to six antigens. The CFA was stable and

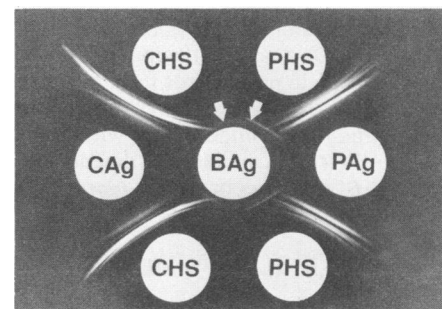


FIG. 2. Cross-reactivities of *B. ranarum* culture filtrate antigens with sera from horses with conidiobolomycosis and pythiosis. Well BAg contained *B. ranarum* antigen. Wells CHS contained conidiobolomycosis horse serum. Well CAg contained *C. coronatus* antigen. Wells PHS contained pythiosis horse serum. Well PAg contained *P. insidiosum* antigen. The *B. ranarum* antigen produced one (left arrow) and two (right arrow) bands, respectively, with sera from horses with conidiobolomycosis and pythiosis.

TABLE 2. ID reactions of sera from humans and horses with conidiobolomycosis, heterologous disease cases, and apparently healthy subjects with *C. coronatus* antigens

Serum source	Disease state	No. of cases:		No. of precipitins identical to those in <i>C. coronatus</i> C-6 reference antiserum
		Tested	Positive	
Horse	Proven conidiobolomycosis	15	15	1-5
Horse	Suspected conidiobolomycosis	19	13	1-5
Horse	Cutaneous habronemiasis	3	0	0
Cat	Pythiosis	1	0	0
Horse	Pythiosis	6	0	0
Horse	Streptococcal pneumonia	3	0	0
Horse	Suspected sporotrichosis	5	0	0
Horse	Normal	5	0	0
Human	Aspergillosis	5	1 <sup>a</sup>	0
Human	Basidiobolomycosis	4	0	0
Human	Conidiobolomycosis	1	1	3
Human	Candidiasis	5	1 <sup>a</sup>	0
Human	Zygomycosis	2	0	0
Human	Normal	5	0	0

<sup>a</sup> Precipitin band of nonidentity.

lost no activity after 2 years of storage, whereas the trypsinized antigen showed relatively weak reactivity following 1 month of storage. The trypsinized preparation contained an antigen common to the CFA preparation and demonstrated a single weak precipitin band with sera from horses with proven conidiobolomycosis. Because the *C. coronatus* CFA preparations exhibited greater antigenic complexity and stability than those of the trypsinized antigens, we used CFA preparations in all the subsequent studies.

The ID precipitin reactivities of sera from control and diseased humans and animals with *C. coronatus* C-6 CFA were ascertained and compared against the reactivity of the homologous reference rabbit antiserum. Sera from all 15 horses with proven conidiobolomycosis contained between one and five precipitins that were identical to those of the reference serum (Table 2). Sera from 13 of 19 horses with suspected conidiobolomycosis were positive and had similar precipitin reactions. Sera from neither the cat with pythiosis nor any of the normal horses or horses with heterologous diseases were precipitin positive. Of the 22 human serum samples tested, only one contained precipitins identical to those in the reference precipitate. The latter serum sample, with three specific precipitins, was from a patient infected with *C. coronatus*. All sera from humans with heterologous disease were negative, except for one serum sample from a patient with aspergillosis and one serum sample from a patient with candidiasis, which produced bands of nonidentity that were considered nondiagnostic.

Sera from humans with proven basidiobolomycosis, from humans and animals with heterologous diseases, and from controls were tested against the *B. ranarum* CFA. The rabbit reference strain *B. ranarum* B-4032 antiserum containing five precipitins was included in each test (Table 3). In the sera from four human patients with basidiobolomycosis, between one and five precipitins were observed in each sample. In each case, one to two of those precipitins produced bands of identity. None of the sera from humans

TABLE 3. ID reactions of sera from humans with basidiobolomycosis, humans and horses with conidiobolomycosis or heterologous disease, and apparently healthy subjects with *B. ranarum* antigens

Serum source	Disease state	No. of cases:		No. of precipitins identical to those in <i>B. ranarum</i> B-4032 antiserum
		Tested	Positive	
Horse	Conidiobolomycosis	5	3 <sup>a</sup>	0
Horse	Cutaneous habronemiasis	3	0	0
Cat	Pythiosis	1	0	0
Horse	Pythiosis	6	1 <sup>a</sup>	0
Horse	Suspected sporotrichosis	5	0	0
Horse	Normal	5	0	0
Human	Aspergillosis	5	1 <sup>a</sup>	0
Human	Basidiobolomycosis	4	4	1-2
Human	Candidiasis	5	0	0
Human	Conidiobolomycosis	1	0	0
Human	Zygomycosis	2	1 <sup>a</sup>	0
Human	Normal	5	0	0

<sup>a</sup> Precipitin band(s) of nonidentity.

with conidiobolomycosis, pythiosis, or other heterologous disease or from the controls produced bands of identity. However, one of the serum samples from a human with aspergillosis and zygomycosis, one serum sample from a horse with pythiosis, and serum samples from three horses with conidiobolomycosis reacted with the *B. ranarum* antigens to produce a single band of nonidentity.

We obtained three serial serum specimens from patient 1 (Table 1). One, which was taken prior to treatment with potassium iodide, demonstrated five precipitins. Two of these precipitins were identical to two of five precipitins in the reference *B. ranarum* antiserum. The second specimen, which was taken after 3 months of therapy, when the patient showed significant disease regression, demonstrated only one precipitin. A third serum specimen, which was taken 6 months later, was serologically negative.

Ten of the horses with conidiobolomycosis and demonstrating multiple serum precipitins were treated successfully with iodides, surgery, or both. Eight showed a decline in the number of precipitins 3 months after treatment. No precipitins were detected 7 months after treatment.

## DISCUSSION

Clinically, *B. ranarum* and *C. coronatus* infections must be differentiated from such diseases as neoplasia, infections by other zygomycetes, and pythiosis. Cultural evidence, although unequivocal, is not always attainable. Histologically, infections with *B. ranarum* and *C. coronatus* are indistinguishable, and their diagnosis may be further complicated by similarities exemplified by fungi of the order *Mucorales* and the oomycete *P. insidiosum*. Early diagnosis of basidiobolomycosis and conidiobolomycosis is important, since these diseases cause extensive deformities and can possibly be fatal. ID tests present an alternative means for rapidly and inexpensively diagnosing these diseases. Our studies indicated that concentrated *B. ranarum* and *C. coronatus* brain heart infusion culture filtrate antigens not only elicited multiple precipitins in rabbits following 2 weeks of immunization but served as excellent ID precipitinogens. The culture filtrate antigens of *C. coronatus* were preferable to the trypsinized mycelial antigens used by Mendoza and

Alfaro (5) because they contained more specific antigens and were more stable. Five specific antigens were apparent in the CFA preparation, and only one was apparent in the trypsinized product. The additional antigens could contribute to making the CFA a more sensitive reagent and more valuable in prognostic studies.

Five antigens were consistently recognized in the ID tests involving the homologous antigens and rabbit antisera to *B. ranaarum* and *C. coronatus*, and six antigens were recognized with the *P. insidiosum* homologous reactants. Although minimal cross-reactivities were noted with all of the antigen preparations, the reference precipitates appeared to be totally specific. A single identical antigen was shared by *B. ranaarum*, *C. coronatus*, and *P. insidiosum*. In addition, *B. ranaarum* contained a second cross-reacting antigen, which was evident upon reaction with *P. insidiosum* antisera. These reference and cross-reactive antigens were evident in tests with both rabbit and horse antisera (Fig. 1 and 2). The specific antibodies formed lines of identity with one or more of the reference system precipitates. In contrast, the cross-reacting antibodies produced lines of nonidentity. Our results suggest that detection of antibodies identical to those in the reference systems for *B. ranaarum*, *C. coronatus*, and *P. insidiosum* would be tantamount to a specific diagnosis. This capability was confirmed when the *C. coronatus* and *B. ranaarum* antigens, using homologous antisera for reference purposes, were extensively tested against a variety of undiluted human and animal case and control sera (Tables 2 and 3).

The *C. coronatus* C-6 antigen-antibody reference system proved to be specific. Of the serum samples from 15 horses and one human with proven conidiobolomycosis, all were diagnostically positive in the ID test. No diagnostic precipitins were detected in any of the normal sera or in those obtained from animals or humans with heterologous infections (Table 2). Furthermore, studies with the control Costa Rican horse sera suggest that environmental exposure does not give rise to false-positive *C. coronatus* precipitins in the absence of disease.

The *B. ranaarum* ID reference precipitates proved to be 100% specific. Forty-seven serum samples representing proven and suspected cases of heterologous fungal infections, four serum samples from humans with basidiobolomycosis, and serum samples from normal controls were tested. Of these, only sera from humans with basidiobolomycosis produced diagnostic bands of identity (Table 3).

Our preliminary studies suggest that the ID tests may have prognostic value. With *C. coronatus* infections in horses, precipitins diminished in numbers or disappeared during successful treatment or several months thereafter. Similarly,

in serum samples from patient 1, who had basidiobolomycosis and from whom pre- and posttreatment serum samples were available, precipitins were noted to decline in number after successful therapy.

Histologically, these infections may be suspected when sparsely septate hyphae surrounded by an eosinophilic sleeve are observed. Recognition of this Splendore-Hoeppli phenomenon, however, does not permit distinction between *B. ranaarum* and *C. coronatus*. Although *B. ranaarum* and *C. coronatus* share antigens and exhibit cross-reactivity with sera from patients and horses with aspergillosis, candidiasis, pythiosis, and zygomycosis, the ID tests are reliable diagnostically as long as only bands of identity are considered positive and all other reactions are regarded as indeterminate. Our results indicate that patients with subcutaneous zygomycosis are immunoresponsive. By using these tests, *B. ranaarum* and *C. coronatus* infections can readily be etiologically serodiagnosed and distinguished from clinically similar heterologous fungal infections and pythiosis.

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