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Exoantigen Test for *Cladosporium bantianum*, *Fonsecaea pedrosoi*, and *Phialophora verrucosa*

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Exoantigens from 10-day-old cultures of 100 isolates of pathogenic and saprophytic dematiaceous fungi were analyzed by the exoantigen test. Antisera to *Cladosporium bantianum* ATCC 10958, *Fonsecaea pedrosoi* CDC AMO-B06, and *Phialophora verrucosa* CDC AMO-C12 were prepared in New Zealand rabbits immunized with soluble antigens from 1-month-old cultures. Absorbed and nonabsorbed antisera and exoantigens from the same organisms were used as reference reagents. Serologic reactions were analyzed in terms of the presence or absence of lines of identity or nonidentity. These reactions allowed presumptive differentiation of *C. bantianum*, *F. pedrosoi*, and *Phialophora verrucosa* from other dematiaceous fungi, including *Cladosporium* spp. (28 isolates), *Exophiala* spp. (18 isolates), *Fonsecaea* spp. (17 isolates), *Lecythophora hoffmannii* (4 isolates), *Phaeoannellomyces werneckii* (3 isolates), *Phialophora* spp. (17 isolates), *Wangiella dermatitidis* (9 isolates), and *Rhinocladiella* spp. (4 isolates).

Like other dematiaceous fungi, *Cladosporium bantianum* (synonym, *Cladosporium trichoides*), *Fonsecaea pedrosoi*, and *Phialophora verrucosa* are found in nature throughout the world. McGinnis and Borelli concluded that *C. bantianum* and *C. trichoides* are conspecific and that, based upon priority, *C. bantianum* is the correct name (17). *C. bantianum* grows at temperatures up to 42 to 43°C, produces long, sparsely branched chains of blastoconidia, and is neurotropic in humans and experimental animals, in which it produces phaeohyphomycosis. Human brain infections caused by this fungus have been reported from all over the world in hosts without apparent immunodeficiencies. *C. bantianum* is similar in basic morphology to *Cladosporium carrionii*, a causative agent of chromoblastomycosis. Physiological reactions like urea hydrolysis, gelatin liquefication, and decomposition of casein, xanthine, hypoxanthine, and tyrosine are not of diagnostic value in separating these two species because of variable results produced by isolates of *C. carrionii* (6).

F. pedrosoi is a polymorphic dematiaceous fungus which is characterized by the presence of the following four types of conidial development: phialides with distinct collarettes (observed sporadically), a *Cladosporium*-like form of blastoconidia in chains (observed regularly), a *Rhinocladiella*-like form of sympodial conidiogenous cells, and a series of conidia forming complex conidial heads, which is the most distinctive stable and unusual conidial arrangement observed in this species (9, 15, 16).

Phialophora verrucosa is characterized by dematiaceous phialides with distinct collarettes which produce balls of one-celled conidia borne directly on the hyphae and at the apices of short lateral branches that arise from aerial hyphae (2, 15). Both *Phialophora verrucosa* and *F. pedrosoi* have been reported as etiologic agents of chromoblastomycosis. This is a chronic mycotic infection which is generally limited to skin and superficial subcutaneous tissues. The infecting fungi appear as thick-walled, dark, planate dividing structures called sclerotic bodies or, more recently, muriform cells (9, 16). Sclerotic bodies apparently represent an intermediate vegetative form which is phenotypically arrested between yeast morphology and hyphal morphology. The formation of sclerotic cells in vivo may be induced by cellular events and other factors (16).

The taxonomy of the etiologic agents of chromoblastomycosis is relatively stable, but there is disagreement regarding the proper genus for *Fonsecaea compacta* and *F. pedrosoi*. A thorough evaluation of the different arrangements of conidia produced by these two fungi has confirmed that *Fonsecaea* is the proper genus (16). *C. bantianum*, *F. pedrosoi*, and *Phialophora verrucosa* are identified mainly by morphological characteristics and the tests mentioned above. Honbo et al. (7) were able to further differentiate serologically four species of *Cladosporium* (*C. carrionii*, *C. bantianum*, *Cladosporium herbarum*, and *Cladosporium cladosporioides*) by using monospecific factor sera obtained through adsorption procedures.

The exoantigen test has been used for a number of years for serological identification of many fungal pathogens (4, 8, 11–13, 19, 21–23). In this paper we describe an exoantigen test which can be used for serological identification or differentiation of *C. bantianum*, *F. pedrosoi*, and *Phialophora verrucosa* from one another and from several other saprophytic and pathogenic dematiaceous genera and species.

(Some of the results were presented previously [Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, F77, p. 377].)

MATERIALS AND METHODS

Cultures. A total of 100 isolates of saprophytic and pathogenic dematiaceous fungi obtained from the Medical College of Virginia/Virginia Commonwealth University culture collection and from the collection of M. R. McGinnis (University of North Carolina, Chapel Hill) were used in this study. These included clinical and environmental isolates, as well as known type cultures (American Type Culture Collection, Rockville, Md.) and proficiency cultures (Centers for Disease Control, Atlanta, Ga.). Exoantigens of these

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isolates were tested against reference sera produced against authentic cultures of *C. bantianum*, *F. pedrosoi*, and *Phialophora verrucosa*. The cultures studied included 8 isolates of nonpathogenic *Cladosporium* species, 9 isolates of *C. bantianum*, 7 isolates of *C. carrionii*, 2 isolates of *Cladosporium elatum*, 2 isolates of *Cladosporium sphaerospermum*, 13 isolates of *Exophiala jeanselmei*, 5 isolates of *Exophiala spinifera*, 6 isolates of *F. compacta*, 11 isolates of *F. pedrosoi*, 4 isolates of *Lecythophora hoffmannii* (synonym, *Phialophora hoffmannii*) (5), 3 isolates of *Phaeoannellomyces werneckii* (synonym, *Exophiala wereneckii*) (18), 3 isolates of *Phialophora repens*, 6 isolates of *Phialophora richardsiae*, 8 isolates of *Phialophora verrucosa*, 2 isolates of *Rhinocladiella aquaspersa*, 2 isolates of *Rhinocladiella atrovirens*, and 9 isolates of *Wangiella dermatitidis* (Tables 1 through 4). For the production of reference sera the following three cultures were used: *C. bantianum* ATCC 10958, *F. pedrosoi* CDC AMO-B06, and *Phialophora verrucosa* CDC AMO-C12.

Antigens for animal immunization. Reference antigens were obtained as previously described (4). One-month-old cultures of *C. bantianum*, *F. pedrosoi*, or *Phialophora verrucosa* grown on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.) were harvested with sterile saline, and 500-ml volumes of Sabouraud dextrose broth (Difco) were inoculated with 1-ml portions of the resulting fungal suspensions (ca. 10^7 CFU). The broth cultures were grown in an incubator shaker (160 rpm) for 4 weeks at 25 to 28°C. The cultures then were treated with 0.5% Formalin for 48 h at 4°C; samples were cultured for sterility control. Conidia and hyphal elements were separated from the supernatants by centrifugation and were washed three times with sterile physiological saline. The cells were broken with a Braun cell homogenizer for 3 min. Microscopic observations confirmed that there was a large percentage (ca. 90%) of cell breakage. Soluble antigens were separated by centrifugation at 1,700 × *g* for 30 min and then at 10,000 × *g* for 30 min. The protein contents of the soluble antigens were determined by the method of Lowry et al. (14). These soluble antigens were used for the production of reference antisera.

Reference antigens. After harvesting, the supernatants of the cultures described above were collected and filtered through 0.20 μm membrane filters (Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.). The filtrates were then concentrated (25×) with a stirred ultrafiltration cell and a type PM10 filter membrane (Amicon Corp., Danvers,

TABLE 1. Immunodiffusion reactions between nonadsorbed and adsorbed *C. bantianum* antisera and exoantigens from 100 pathogenic and saprophytic fungi

Exoantigen source[a]	No. of isolates	Immunodiffusion reaction with:[b]	
		Nonadsorbed serum	Adsorbed serum
Cladosporium spp.	8	Id or A	A
C. bantianum	9	Id (1 or 3)	Id or A
C. carrionii	7	Id or P	P or A
C. elatum	2	Id (2)	Id
C. sphaerospermum	2	Id or A	A
E. jeanselmei	13	Id or A	A
E. spinifera	5	Id	A
F. compacta	6	Id (1 or 2)	A
F. pedrosoi	11	Id (1 or 2) or A or P	A
L. hoffmannii	4	A	A
Phaeoannellomyces werneckii	3	Id or A	A
Phialophora repens	3	A	A
Phialophora richardsiae	6	A	A
Phialophora verrucosa	8	Id	A
R. atrovirens	2	Id	A
R. aquaspersa	2	Id	A
W. dermatitidis	9	Id or P or A	A

[a] Species identification as received and deposited in our culture collection.
[b] Abbreviations: Id, identity line(s); P, partial identify; A, absence of lines. The numbers in parentheses are the numbers of identity lines.

TABLE 2. Immunodiffusion reactions between nonadsorbed and adsorbed *F. pedrosoi* antisera and exoantigens from 100 pathogenic and saprophytic fungi

Exoantigen source[a]	No. of isolates	Immunodiffusion reaction with:[b]	
		Nonadsorbed serum	Adsorbed serum
Cladosporium spp.	8	Id or A	A
C. bantianum	9	Id or A	A
C. carrionii	7	Id (1 or 2)	A
C. elatum	2	Id or A	A
C. sphaerospermum	2	Id or A	A
E. jeanselmei	13	Id or P or A	A
E. spinifera	5	Id	A
F. compacta	6	Id (1 or 2)	A
F. pedrosoi	11	Id (2 or 3)	Id
L. hoffmannii	4	A	A
Phaeoannellomyces werneckii	3	A	A
Phialophora repens	3	A	A
Phialophora richardsiae	6	A	A
Phialophora verrucosa	8	Id (1 or 2) or P or A	A
R. atrovirens	2	Id	A
R. aquaspersa	2	Id	A
W. dermatitidis	9	Id or P or A	A or P

[a] Species identification as received and deposited in our culture collection.
[b] Abbreviations: Id, identity line(s); P, partial identify; A, absence of lines. The numbers in parentheses are the numbers of identity lines.

TABLE 3. Immunodiffusion reactions between nonadsorbed and adsorbed *Phialophora verrucosa* antisera and exoantigens from 100 pathogenic and saprophytic fungi

Exoantigen source[a]	No. of isolates	Immunodiffusion reaction with:[b]	
		Nonadsorbed serum	Adsorbed serum
Cladosporium spp.	8	Id or A	A
C. bantianum	9	Id or P or A	A
C. carrionii	7	Id (1 or 2)	P or A
C. elatum	2	A	A
C. sphaerospermum	2	Id or A	A
E. jeanselmei	13	Id or P or A	A
E. spinifera	5	Id	A
F. compacta	6	Id (1 or 2) or P	A
F. pedrosoi	11	Id or P or A	A
L. hoffmannii	4	Id or A	A
Phaeoannellomyces werneckii	3	Id or A	A
Phialophora repens	3	Id or A	A
Phialophora richardsiae	6	Id or A	A
Phialophora verrucosa	8	Id (2)	Id
R. atrovirens	2	Id	A
R. aquaspersa	2	Id	A
W. dermatitidis	9	Id (1 or 2) or P	A

[a] Species identification as received and deposited in our culture collection.
[b] Abbreviations: Id, identity line(s); P, partial identity; A, absence of lines. The numbers in parentheses are the numbers of identity lines.

TABLE 4. List of isolates of pathogenic and saprophytic fungi[a]

Taxon	No. of isolates	Collection accession no. of isolates					
		MCV/VCU collection			NCMH collection		
		Environmental isolates	Clinical isolates	Source unknown (or other)	Environmental isolates	Clinical isolates	Source unknown (or other)
Cladosporium spp.	8	53.39	53.16	53.10, 53.13, 53.19, 53.21, 53.22, 53.23			
C. bantianum	9		19.10, 53.12	53.30 (= ATCC 10958)	29.54	112, 115, 121, 122	113 (= ATCC 10958)
C. carrionii	7			53.18 (= M5-017) 53.27 (= AMO-C16)		779, 781, 784, 791, 1451	
C. elatum	2				634, 636		
C. sphaerospermum	2						1199, 1402
E. jeanselmei	13	29.28	29.18, 29.21, 29.26	29.7 (= M3-006), 29.9 (= M6-009) 29.24 (= AMO-C13), 29.30 (= ATCC 34123)			135 (= ATCC 10224) 262, 694, 1410
E. spinifera	5	29.39			839, 820	1361	152 (= ATCC 18218)
F. compacta	6					185, 684, 904, 1000, 1271	10 (= ATCC 10222)
F. pedrosoi	11		19.7	19.1, 19.5 (= M3-104) 19.9 (= M5-016) 19.12 (= AMO-B06)		11,707, 899, 925, 1032, 1302	
L. hoffmannii	4		29.28		19,639	1039	
Phaeoannellomyces werneckii	3				75,137,765		
Phialophora repens	3	29.16		29.47		184	
Phialophora richardsiae	6		29.25		144	490, 728	491, 1359
Phialophora verrucosa	8	29.14		29.5, 29.24 (= AMO-C12)	489, 659	102	1060 (= ATCC 10223)
R. atrovirens	2					1444	1590
R. aquaspersa	2					76, 1260	
W. dermatitidis	9	29.32, 29.44		29.31 (= ATCC 28869), 29.45	1070	147	461, 1222, 702

[a] MCV/VCU, Medical college of Virginia Commonwealth University; NCMH, North Carolina Memorial Hospital, University of North Carolina. The designations beginning with M and AM are designations of the Centers for Disease Control Proficiency Testing Program (Mycology).

Mass.) These filtrates were stored at −20°C in small volumes and used as the reference antigens to perform the exoantigen test.

Exoantigens. Exoantigens were extracted as previously described (4). Fungal isolates to be tested were grown on Sabouraud dextrose agar slants. After 10 days of incubation at 25°C, the slants were covered with 8 ml of sterile distilled water and left at 25°C for another 24 h. The culture extracts were centrifuged for 20 min at 1700 × *g*, filtered through a 0.45-μm membrane filter or treated with 0.5% Formalin (24), and concentrated (25×) with Minicon B15 concentrators (Amicon). Randomly selected samples of the exoantigens were cultured to confirm the sterility of the preparations. These were the antigens which were tested against the reference antisera and antigens described above in the exoantigen test.

Animal immunization. Immune sera were produced as previously described (4). Male New Zealand rabbits were immunized in their footpads with 0.6-ml volumes (0.3 mg of protein in 0.3 ml) of solutions containing soluble antigens and Freund complete adjuvant (1:1) during weeks, 1, 2, and 3. Subcutaneous 0.6-ml boosters of the same antigens were given during week 6. Final 0.5-ml injections of the 25×-concentrated filtrate used as reference antigen were given intravenously during week 7. The rabbits were bled during weeks 2, 4, 6, and 7, and their sera were tested by immunodiffusion against homologous reference antigens to determine the presence of distinctive and multiple precipitating bands of antibody. The rabbits were exsanguinated during week 8, and sera were collected and stored at −20°C for further testing.

Adsorption of sera. Immunized rabbit sera were adsorbed by the method of Kaufman and Lopez (10). Concentrated (25×), lyophilized exoantigen culture filtrates of *F. pedrosoi* CDC AMO-B06, *C. bantianum* ATCC 10958, *F. compacta* ATCC 10222, and *C. carrionii* CDC AMO-C16 were prepared in the manner described above for reference antigens. *F. pedrosoi* antiserum was mixed 2:1 (vol/vol) with *C. bantianum* and *F. compacta* antigens. *Phialophora verrucosa* antiserum was mixed with *C. carrionii* and *F. pedrosoi* antigens. The exoantigen filtrate culture of *F. pedrosoi* was mixed 2:1 with *C. bantianum* antiserum. The

mixtures were incubated for 2 h at 37°C and for 48 h at 4°C, and then they were centrifuged at 800 × *g* for 30 min.

Exoantigen tests. The exoantigen or microimmunodiffusion tests were performed as previously described (22, 23) by using commercially available immunodiffusion plates (Meridian Diagnostics, Inc., Cincinnati, Ohio). Immune serum was placed in the center well, reference antigens were placed in wells 1 and 4, and unknown antigens were placed, in duplicate, in lateral wells. A 10-min period was allowed between addition of antiserum and subsequent addition of antigens. The plates were incubated in a moist chamber at 25°C and were examined after 24 and 48 h.

RESULTS

Exoantigens of 100 saprophytic and pathogenic dematiaceous fungi were tested against the nonadsorbed sera of *C. bantianum, F. pedrosoi*, and *Phialophora verrucosa* (Tables 1 through 3). When each nonadsorbed antiserum was tested with the corresponding reference antigens, at least two lines of precipitation were observed (Fig. 1). When the nine exoantigens of *C. bantianum* were reacted with their nonabsorbed reference antiserum, seven of the nine (78%) produced two or three distinctive precipitation bands. Heterologous exoantigens of the isolates studied also shared antigenic characteristics or cross-reacted when they were tested with nonadsorbed *C. bantianum* antiserum, as shown by the presence of identity lines close to the antibody wells (Fig. 1A). Similar cross-reactions were observed when heterologous exoantigens of the isolates were tested with the nonadsorbed *F. pedrosoi* and *Phialophora verrucosa* antisera (Fig. 1B and C). When the exoantigens extracted from 8 isolates of *Phialophora verrucosa* and 11 isolates of *F. pedrosoi* were tested, one or two identity bands with nonadsorbed antiserum were evident with the corresponding antisera (Fig. 1B and C). Also, a variety of lines of nonidentity and partial identity were observed; these did not affect evaluation of the test.

Exoantigens of two of the nine isolates which were received as *C. bantianum* failed to give more than one precipitin band with the respective nonadsorbed reference serum. These isolates also failed to grow at 41°C and were not neurotropic in mice. Two isolates which were received as *C. elatum* reacted in the same manner as homologous isolates with both *C. bantianum* nonadsorbed serum and *C. bantianum* adsorbed antiserum. Further investigation revealed that the identification of these isolates was preliminary and was made prior to temperature and animal inoculation studies; these isolates subsequently were deposited in another collection as *C. bantianum* when growth at 41 to 42°C and neurotropism in mice were demonstrated.

After adsorption of the antisera, only single distinctive precipitin lines of identity were observed with the reference and homologous antigens (Fig. 2). Such single lines of identity were observed when we tested adsorbed antisera to *C. bantianum*, *F. pedrosoi*, and *Phialophora verrucosa* with their homologous antigens. The exceptions were the four isolates mentioned above. When we tested the two of nine isolates received as *C. bantianum* which gave only one precipition band in tests with nonadsorbed antisera against adsorbed reference antisera, no precipitin lines were observed.

DISCUSSION

Like identification of any other fungus, identification of a dematiaceous fungus is based primarily on morphological characteristics. Many of the dematiaceous pathogens are

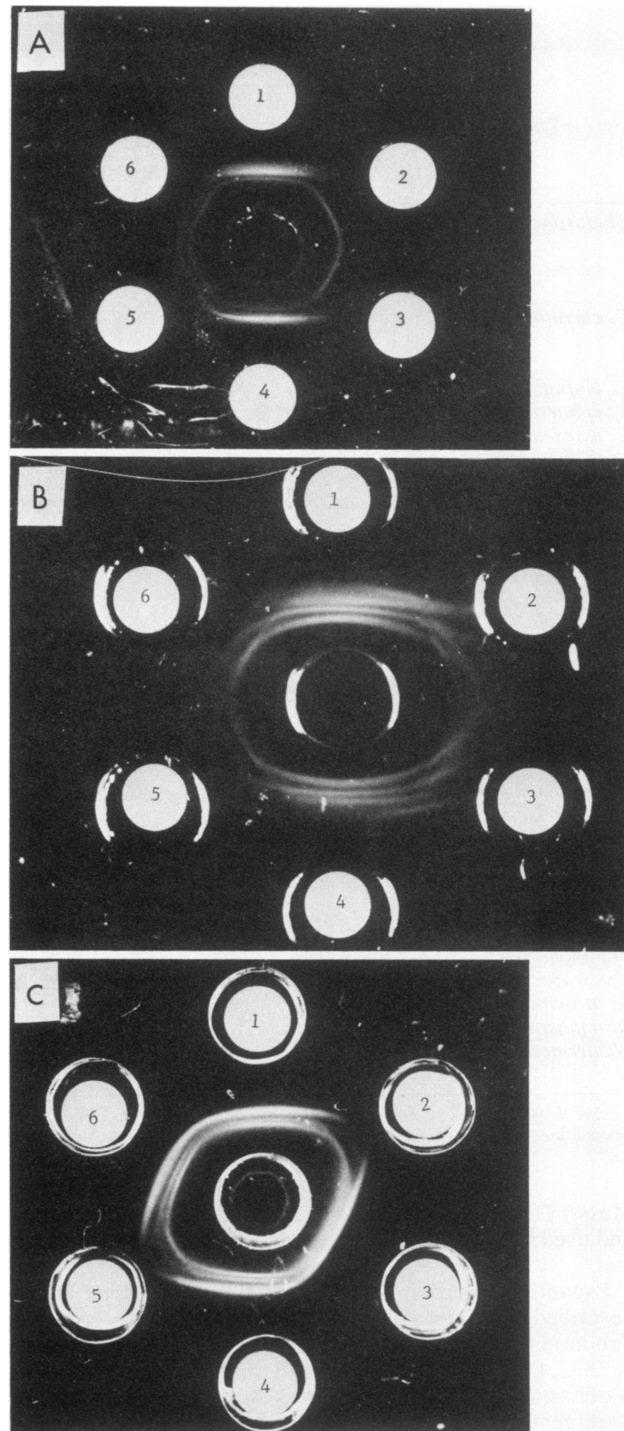

FIG. 1. Nonadsorbed antisera. (A) Immunodiffusion reactions of nonabsorbed *C. bantianum* antiserum (center well) with corresponding reference antigens (wells 1 and 4), *F. pedrosoi* exoantigen (wells 2 and 3), and *C. bantianum* exoantigen (wells 5 and 6). (B) Immunodiffusion reactions of nonabsorbed *F. pedrosoi* antiserum (center well) with corresponding reference antigens (wells 1 and 4), *F. compacta* exoantigens (wells 2 and 5), and *W. dermatitidis* exoantigens (wells 3 and 6). (C) Immunodiffusion reactions of nonadsorbed *Phialophora verrucosa* antiserum (center well) with corresponding reference antigens (wells 1 and 4), *F. pedrosoi* exoantigens (wells 2 and 5), and *Phialophora verrucosa* exoantigens (wells 3 and 6).

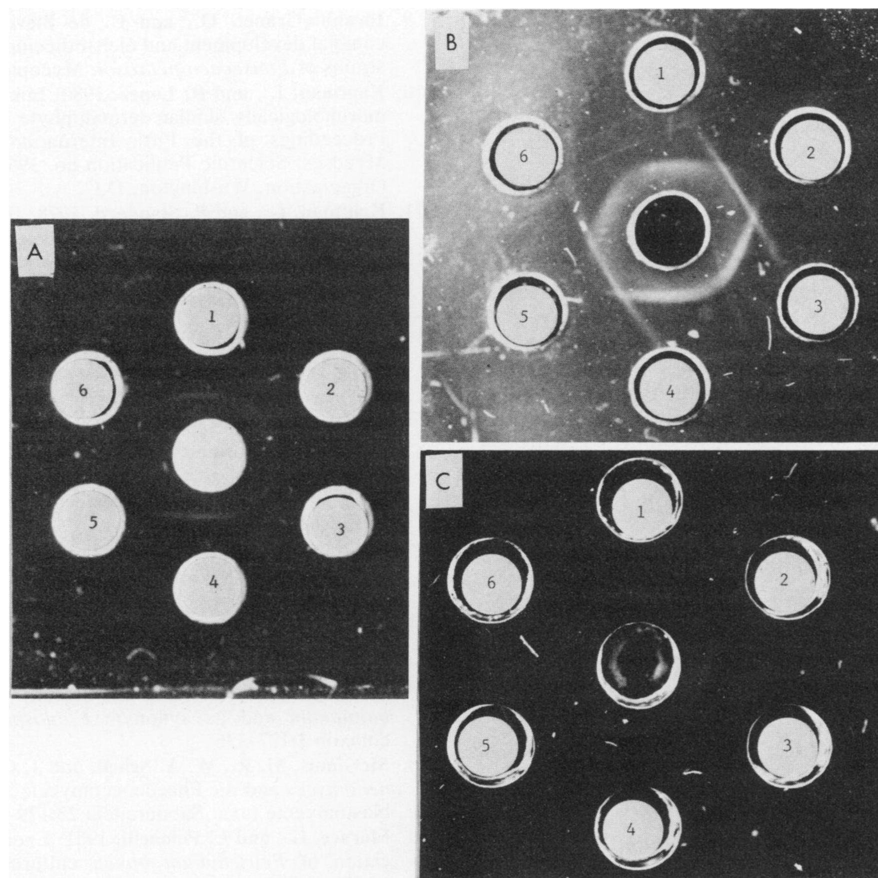

FIG. 2. Adsorbed antisera. (A) Immunodiffusion reactions of adsorbed *C. bantianum* antiserum (center well) with corresponding reference antigens (wells 1 and 4) *E. jeanselmei* exoantigens (wells 5 and 6), and *C. bantianum* exoantigens (wells 2 and 3). (B) Immunodiffusion reactions of adsorbed *F. pedrosoi* antiserum (center well) with corresponding reference antigens (well 1 and 4), *W. dermatitidis* exoantigens (wells 2 and 5), and *F. pedrosoi* exoantigens (wells 3 and 6). (C) Immunodiffusion reactions of adsorbed *Phialophora verrucosa* antiserum (center well) with corresponding reference antigens (wells 1 and 4), *Phialophora verrucosa* exoantigens (wells 3 and 6), and *F. pedrosoi* exoantigens (wells 2 and 5).

polymorphic, with the form(s) of growth dependent upon growth conditions and upon the individual isolate. Determination of the most characteristic mode of conidial ontogeny can be a tedious task which requires the expertise of a specialist. Temperature tolerance, biochemical tests, and animal pathogenicity tests can aid in the identification process, but these are time consuming and not reliable or applicable to all species in this complex group.

In this study, the use of adsorbed antisera in the exoantigen test permitted differentiation of isolates of *C. bantianum, F. pedrosoi,* or *Phialophora verrucosa* from each other and from other dematiaceous fungi in less than 2 weeks. Four isolates were exceptions. These were later shown by temperature tolerance tests and animal inoculation studies to belong to different species than originally thought. Two isolates previously identified as *C. bantianum* did not grow at 40°C and did not show neurotropism in mice. Serologically, they had the same reaction as the nonpathogenic *Cladosporium* isolates. The two isolates received as *C. elatum* were reidentified morphologically and by temperature tolerance and mouse inoculation tests as *C. bantianum*; this finding correlated with the results of our exoantigen tests.

Honbo et al. (7) previously described the use of monospecific factor sera as potential tools to serologically differentiate isolates of *C. bantianum, C. carrionii, C. herbarum,* and *C. cladosporioides* from each other. These authors used sera that were produced in rabbits immunized with culture filtrates as reagents. We used antibodies that were prepared by immunizing rabbits with soluble cell antigens. However, in both studies it was necessary to perform subsequent adsorptions of the sera because of cross-reactions. In the case of testing for *C. bantianum*, it was necessary to adsorb only with *F. pedrosoi* antigen. With *F. pedrosoi* it was necessary to adsorb the serum with both *C. bantianum* and *F. compacta* antigens. *Phialophora verrucosa* antiserum was adsorbed with *F. pedrosoi* and *C. carrionii* antigens.

Common antigens have been reported previously among the members of the dematiaceous pathogen group. It was reported that *W. dermatitidis* and *E. jeanselmei* contained antigenic components which were common to each other and to *Phaeoannellomyces werneckii* (13, 20). Cooper and Schneidau (3) found that *Phialophora verrucosa, C. carrionii,* and *F. pedrosoi* share common antigens, as well as other antigens that are genus or species specific. These authors also proved serologically that there were more common antigens between *Phialophora verrucosa* and *C. carrionii* than between either species and *F. pedrosoi*, which indicated that these two fungi are closely related to one another. Buckley and Murray (1) reported that *F. pedrosoi*

and *F. compacta* shared more antigens with each other than with *Phialophora verrucosa*. We found that when nonadsorbed antisera of either *E. jeanselmei* or *W. dermatitidis* were tested against other dematiaceous fungi, common antigenic characteristics and cross-reactions were observed (4).

In this study, *C. bantianum* had more antigens in common with *F. pedrosoi* and *F. compacta* than with any other species of *Cladosporium* studied. On the other hand, *F. pedrosoi* shared more common antigens with *Phialophora verrucosa*, *C. carrionii*, and *F. compacta* than with any of the other species tested. *Phialophora verrucosa* was serologically more closely related to *C. carrionii*, *F. pedrosoi*, and *W. dermatitidis* than to any other species of *Phialophora*. However, after adsorption it was possible to obtain a reference serum that could be used as a potential tool to serologically distinguish *C. bantanium*, *F. pedrosoi*, and *Phialophora verrucosa* from one another and also from other dematiaceous fungi (Tables 1 through 3 and Fig. 1 and 2).

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