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Exoantigen Test for Differentiation of *Exophiala jeanselmei* and *Wangiella dermatitidis* Isolates from Other Dematiaceous Fungi

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Concentrated (25×) exoantigens of 105 isolates of pathogenic and saprophytic dematiaceous fungi and 3 isolates of *Sporothrix schenckii* were analyzed by the microimmunodiffusion method. The reagents used were nonadsorbed and adsorbed sera produced in New Zealand rabbits. One set of rabbits was immunized with soluble antigens of a 1-month-old culture of *Exophiala jeanselmei* (ATCC 34123), and the other set was immunized with soluble antigens from a culture of *Wangiella dematitidis* (ATCC 28869). The reference antigens were 25×-concentrated exoantigens of the above cultures. This exoantigen test permitted the differentiation of *E. jeanselmei* and *W. dermatitidis* from one another as well as from other *Exophiala* species, *Fonsecaea* species, *Phialophora* species, *Cladosporium* species, *Rhinocladiella* species, and *Sporothrix schenckii* by presence or absence of lines of identity or of partial identity, or lines of nonidentity. Using adsorbed serum eliminated the problems with cross-reactivity seen with nonadsorbed serum. Thus, with an adsorbed serum as the reagent, it was possible to presumptively differentiate *E. jeanselmei* and *W. dermatitidis* from one another and from other dematiaceous fungi.

Exophiala jeanselmei and *Wangiella dermatitidis* have been isolated from nature throughout the world. *E. jeanselmei* is an etiological agent of subcutaneous abscesses, mycetomas, and phaeohyphomycosis. *W. dermatitidis* has been associated with cutaneous and subcutaneous phaeohyphomycosis. These two dematiaceous pathogenic fungi are members of the group called the black yeasts which can be difficult and time consuming to identify. Both fungi may initiate growth as yeast-like organisms which develop mycelial elements with age. *E. jeanselmei* originally belonged to the genus *Phialophora* but was transferred to the genus *Exophiala*, owing to the predominant presence of annellides and annelloconidia (10). The genus *Wangiella* was established to contain the fungus *Phialophora dermatitidis* (*Hormiscium dermatitidis*), owing to the unique and predominant presence of phialides without collarettes in this species (9). Slide cultures for morphological studies as well as temperature tolerance and biochemical tests are used for the differentiation of these two fungi. These tests are time consuming and, in some instances, not reliable.

The exoantigen test has been used for a number of years for the serological differentiation or identification of several fungal pathogens (3, 5, 6, 11, 14–16). Antigenic differences between *E. jeanselmei* and *W. dermatitidis* have been demonstrated and factor sera for their differentiation have been produced (7). The study reported here was undertaken to develop specific adsorbed reference antisera for use in an exoantigen test which will differentiate these two dematiaceous fungi from each other, from other saprophytic and pathogenic dematiaceous fungi, and also from isolates of *Sporothrix schenckii*.

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MATERIALS AND METHODS

Cultures. A total of 105 isolates of saprophytic and pathogenic dematiaceous fungi and 3 isolates of *S. schenckii* 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) and proficiency cultures (Centers for Disease Control). Exoantigens from these isolates were tested against reference sera produced against type cultures of *E. jeanselmei* and *W. dermatitidis*. The genera and species represented by these cultures included 17 isolates of *E. jeanselmei*, 7 isolates of *Exophiala werneckii*, 5 isolates of *Exophiala spinifera*, 1 isolate of *Exophiala moniliae*, 13 isolates of *W. dermatitidis*, 11 isolates of *Fonsecaea pedrosoi*, 5 isolates of *Fonsecaea compacta*, 8 isolates of *Phialophora verrucosa*, 4 isolates of *Phialophora repens*, 6 isolates of *Phialophora richardsiae*, 4 isolates of *Phialophora hoffmannii* [*Lecythophora hoffmannii* (van Beyma) Gams and McGinnis, comb. nov.] (2), 8 isolates of *Cladosporium bantianum*, 7 isolates of *Cladosporium carrionii*, 2 isolates of *Cladosporium elatum*, 2 nonspeciated *Cladosporium* isolates, 5 isolates of *Rhinocladiella* species, and 3 isolates of *S. schenckii* (Table 1). Two type cultures used for the production of reference antigens and antisera were obtained directly from the American Type Culture Collection. These included *E. jeanselmei* ATCC 34123 and *W. dermatitidis* ATCC 28869.

Antigens for animal immunization. Sabouraud dextrose broths (Difco Laboratories) in 500-ml volumes were inoculated with 1-ml suspensions in sterile saline (ca. 10^7 CFU/ml) of a 1-month-old culture (Sabouraud dextrose agar slants) of either *E. jeanselmei* or *W. dermatitidis*. Cultures were incubated in an incubator shaker (160 rpm) for a period of 4 weeks at 25 to 28°C. At the end of this period, the cultures

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TABLE 1. Culture list for 108 isolates of pathogenic and saprophytic fungi[a]

Species (no.)	Collection accession no. of isolates from indicated source:					
	MCV/VCU			NCMH/UNC		
	e	c	unk (or other)	e	c	unk (or other)
E. jeanselmei (17)	29.10, 29.11, 29.28	29.17, 29.18, 29.21, 29.26	29.7 (M3-006), 29.9 (M6-009), 29.24 (AM0-C13), 29.30 (ATCC 34123), 29.35			135 (ATCC 10224), 262, 694, 702
E. werneckii (7)			29.36 (Philpott 1288) 29.37 (Philpott 2943) 29.38 (Philpott 2676)		18, 75, 137, 765	
E. spinifera (5)	29.29			819, 820	1361	152 (ATCC 18218)
E. moniliae (1)					721	
W. dermatitidis (13)	29.27, 29.41, 29.42, 29.43, 29.44	29.32	29.31 (ATCC 28869), 29.45	1070, 1188	147	461, 1222
F. pedrosoi (11)		19.7	19.1, 19.5 (M3-014), 19.9 (M5-016), 19.12 (AM9-B06)		11, 707, 899, 925, 1032, 1302	
F. compacta (5)					684, 904, 1000, 1271	10 (ATCC 10222)
P. verrucosa (8)	29.14, 29.46		29.5, 29.23 (AM0-C12)	489, 649	102	1060 (ATCC 10223)
P. richardsiae (6)		29.25		144	490, 728	491, 1359
P. hoffmannii (4)			29.48	19, 639	1039	
P. repens (4)	29.16		29.47	227	184	
C. bantianum (8)		19.10, 53.12	53.30 (ATCC 10958)		112, 115, 121, 122	113 (ATCC 10958)
C. carrionii (7)			53.18 (M5-017), 53.27 (AM0-C16)		779, 781, 784, 791, 1415	
C. elatum (2)				634, 636		
Cladosporium spp. (2)		53.16	53.13			
Rhinocladiella atrovirens (2)					1444	1590
R. musae (1)						1285
R. aquaspersa (2)					76, 1260	
S. schenckii (3)		38.96, 38.102	38.61 (M6-008)			

[a] Culture collections: MCV/VCU, Medical College of Virginia, Virginia Commonwealth University; NCMH/UNC, North Carolina Memorial Hospital, University of North Carolina. Isolates were from the following sources: e, environmental, c, clinical; unk, unknown; "M" and "AM" numbers, Centers for Disease Control Proficiency Testing Program: Mycology; Philpott numbers, C. Philpott, Bedfordshire, England.

were treated with 0.5% Formalin for 48 h at 4°C. Samples then were cultured for sterility control. Conidia and hyphal elements were separated by centrifugation and then washed three times with sterile physiological saline. The cells were then broken in a Braun cell homogenizer for 3 min. Microscopic observations were performed to confirm a large percentage (ca. 90%) of cell breakage. The soluble antigens were separated first by centrifugation at 1,700 × *g* for 30 min and a second time at 10,000 × *g* for another 30 min. Protein contents of the soluble antigens were determind by the method of Lowry et al. (8).

Reference antigens. After being harvested, the resulting supernatants were collected and filtered through 0.20-μm membrane filters (Nalgene Labware Division). The filtrates were then concentrated (25×) with a stirred ultrafiltration cell and a PM 10 filter membrane (Amicon Corp.). These filtrates were stored at −20°C in small volumes and used as the reference antigens.

Exoantigens. Fungal isolates to be tested were grown on Sabouraud dextrose agar (Difco) 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 1,700 × *g*, filtered through a 0.45-μm membrane or treated with 0.5% Formalin (17), and concentrated (25×) with Minicon B15 concentrators (Amicon). Randomly selected samples of the exoantigens were cultured to confirm the sterility of the preparations.

Animal immunization. Male New Zealand rabbits were immunized in the foot pads with 0.6-ml volumes (0.3 mg of protein in 0.3 ml) of soluble antigens and Freund complete adjuvant on weeks 1, 2, and 3. Subcutaneous boosters of 0.6 ml of the same antigens were given on week 6. Final injections of 0.5 ml of the 25×-concentrated filtrates to be used as reference antigens were given intravenously on week 10. The rabbits were bled on weeks 2, 4, 6, and 10, and their sera were tested against homologous reference and cytoplasmic-soluble antigens to determine the presence of distinctive

TABLE 2. Immunodiffusion reactions between nonadsorbed and adsorbed *E. jeanselmei* antisera and exoantigens from 108 pathogenic and saprophytic fungi

Exoantigen from following species as received (no. of isolates)	Immunodiffusion reaction with[a]:	
	Nonadsorbed serum	Adsorbed serum
E. jeanselmei (17)	Id (2 or 3)	Id (1) or P or A
E. werneckii (7)	P or Id or P + Id	P or A
E. spinifera (5)	P	P or A
E. moniliae (1)	P + Id (2)	P
W. dermatitidis (13)	P or Id or P + Id (1)	P or A
F. pedrosoi (11)	P or Id or P + Id (1)	P or A
F. compacta (5)	Id (1)	A
P. verrucosa (8)	P or Id or P + Id (1)	P or A
P. repens (4)	P or Id or P + Id (1)	P or A
P. richardsiae (6)	Id (1)	A
P. hoffmannii (4)	Id (1)	A
Cladosporium spp. (2)	A or Id	A
C. elatum (2)	A or Id	A
C. bantianum (8)	P or Id or P + Id (1)	P or A
C. carrionii (7)	P or Id or P + Id (1)	A
Rhinocladiella spp. (5)	Id (1)	A
S. schenckii (3)	A	A

[a] Abbreviations: Id, identity line(s); P, partial identity; A, absence of lines. Numbers in parentheses are the numbers of identity lines.

TABLE 3. Immunodiffusion reactions between nonadsorbed and adsorbed *W. dermatitidis* antisera and exoantigens from 108 pathogenic and saprophytic fungi

Exoantigen from following species as received (no. of isolates)	Immunodiffusion reaction with[a]:	
	Nonadsorbed serum	Adsorbed serum
W. dermatitidis (13)	Id (1–3)	Id or A
E. jeanselmei (17)	A or Id	A
E. werneckii (7)	A or Id	A
E. spinifera (5)	A or Id	A
E. moniliae (1)	Id	A
F. pedrosoi (11)	Id	A
F. compacta (5)	A or Id	A
P. verrucosa (8)	A or Id	A
P. repens (4)	A	A
P. richardsiae (6)	A	A
P. hoffmannii (4)	A or Id	A
Cladosporium spp. (2)	A or Id	A
C. elatum (2)	A or Id	A
C. bantianum (8)	A or Id	A
C. carrionii (7)	A or Id (2)	A
Rhinocladiella spp. (5)	A or Id	A
S. schenckii (3)	A	A

[a] Abbreviations: Id, identity line; A, absence of lines. Numbers in parentheses are the numbers of identity lines.

and multiple-precipitating bands of antibody. The rabbits were exsanguinated at week 11, and sera were collected and stored at −20°C for further testing.

Adsorption of sera. Immunized rabbit sera were adsorbed according to the method of Kaufman and Lopez (4). Concentrated (25×) lyophilized exoantigen culture filtrates of *F. pedrosoi* (Centers for Disease Control reference number AM9-B06), and *E. spinifera* (ATCC 18218), prepared in the manner described above, were mixed 1:1 (vol/vol) with the *E. jeanselmei* antiserum. Only the exoantigen filtrate culture of *F. pedrosoi* was mixed (2:1) with the *W. dermatitidis* antiserum. The mixtures were incubated for 2 h at 37°C and 48 h at 4°C. They then were centrifuged at 800 × *g* for 30 min.

Exoantigen tests. The exoantigen or microimmunodiffusion tests were performed as previously described (15, 16), using commercially available immunodiffusion plates (Meridian Diagnostics, Inc.). The 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. Plates were incubated in a moist chamber at 25°C and examined at 24 and 48 h.

RESULTS

Exoantigens of 105 saprophytic and pathogenic dematiaceous fungi and 3 isolates of *S. schenckii* first were tested against the nonadsorbed sera of *E. jeanselmei* and *W. dermatitidis* antisera (Tables 2 and 3). When each nonadsorbed serum was tested with the corresponding reference antigens, at least two lines of precipitation were observed (Fig. 1). When the 17 exoantigens of *E. jeanselmei* were reacted with the nonadsorbed reference antiserum, 15 of 17 (88%) produced two to three distinctive precipitation bands. Heterologous exoantigens of the isolates studied also shared antigenic characteristics or cross-reacted when tested with nonadsorbed *E. jeanselmei* antiserum, as shown by the presence of identity lines closest to antibody wells (Fig. 1A). Similar cross-reactions were observed when heterologous exoantigens of the isolates were tested with the nonadsorbed *W. dermatitidis* antiserum (Fig. 1B). When the 13 exoantigens extracted from *W. dermatitidis* isolates were tested, 1 to 3 identity bands with nonadsorbed antiserum were evident with 11 of 13 isolates (85%). Also, a variety of lines of nonidentity, partial identity lines, and absence of lines was observed; the presence of lines of partial identity and lines of nonidentity did not affect the evaluation of the test. None of the heterologous antigens reacted with the outer precipitin line of the nonadsorbed reference antisera.

Exoantigens from 2 of 17 isolates listed in our culture collection as *E. jeanselmei* produced only lines of partial identity or failed to give any precipitin bands with their respective nonadsorbed reference antiserum. Three isolates which were previously identified as *W. dermatitidis*, two from an environmental study (same site) and one from a clinical source, also failed to demonstrate more than one precipitin line close to the antibody well when each was

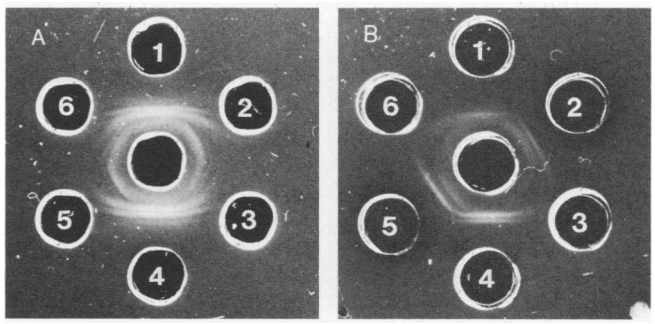

FIG. 1. Nonadsorbed antisera. (A) Immunodiffusion reactions of nonadsorbed *E. jeanselmei* antisera (center well) with corresponding reference antigens (wells 1 and 4), exoantigens of *F. pedrosoi* (wells 2 and 3), and exoantigens of *C. bantianum* (wells 5 and 6). (B) Immunodiffusion reactions of nonadsorbed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

reacted with its respective nonadsorbed reference serum. We will refer to these five isolates as "problem" isolates.

After adsorption of the sera, only one distinctive precipitin line of identity was seen with the reference antigen and homologous test antigens (Fig. 2A and B). Such single lines of identity were observed when testing adsorbed antisera to both *E. jeanselmei* and *W. dermatitidis* with their respective homologous antigens. The exceptions were the problem isolates mentioned above. When the two problem isolates of *E. jeanselmei* were tested against adsorbed reference antisera, lines of partial identity or absence of precipitin lines were observed. When reacting the three problem isolates previously identified as *W. dermatitidis*, the lines of identity observed with nonadsorbed serum were not seen with adsorbed serum. This was the type of response seen with the heterologous exoantigens.

These problem isolates were later reidentified in three different laboratories (our own, that of M. McGinnis, and that of D. Dixon, Department of Biology, Loyola College, Baltimore, Md.), primarily on the basis of morphology. Temperature tolerance tests also were used in our laboratory. The three problem isolates of *W. dermatitidis* failed to grow at 40°C. Two were identified as *Phaeococcomyces exophialiae*, and the third was identified as *E. jeanselmei*. Two problem isolates of *E. jeanselmei* were grown in slide cultures and tested for temperature tolerance. One isolate grew between 40 to 42°C on three different occasions and on slide culture showed the morphology of *W. dermatitidis*, i.e., primarily a yeast-like morphology with conidia apparently produced from characteristic phialides without collarettes. When the exoantigen from this isolate was tested against the *W. dermatitidis* reference serum, it behaved as the other *W. dermatitidis* antigens with nonadsorbed and adsorbed sera. The second isolate did not grow at 40°C; however, morphologically, it showed predominant apical phialides without collarettes. Serologically, it behaved as did the first isolate.

DISCUSSION

Identification of a dematiaceous fungus, like that of any other fungus, is based primarily on morphological characteristics, including especially the type of conidial development. Most of the dematiaceous pathogens grow slowly. *E. jeanselmei* and *W. dermatitidis* may start growing as yeasts and sometimes will require several passages onto fresh media before hyphal elements develop. Temperature tolerance and biochemical tests (13) can aid in their identification, but these are time consuming and not reliable or applicable to the other different species or genera in this group. The study of morphological characteristics requires specialized expertise and, in many instances, the use of phase-contrast microscopy.

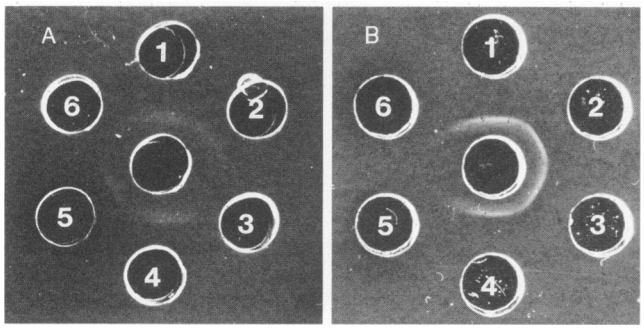

FIG. 2. Adsorbed antisera. (A) Immunodiffusion reactions of adsorbed *E. jeanselmei* antisera (center well) with corresponding reference antigens (wells 1 and 4), exoantigens of *E. jeanselmei* (wells 2 and 5), and exoantigens of *W. dermatitidis* (wells 3 and 6). (B) Immunodiffusion reactions of adsorbed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

In this study, serological differentiation was possible in less than 2 weeks by using adsorbed antisera as reagents. We were able to differentiate isolates of either *E. jeanselmei* or *W. dermatitidis* from each other and from other dematiaceous fungi and from cultures of *S. schenckii* (Tables 2 and 3). Five problem isolates represented an exception. The latter was later shown by morphology and temperature tolerance tests to belong to different genera and species than as originally identified. Two problem isolates previously identified by conventional methods as *W. dermatitidis* were identified as *Phaeococcomyces exophialiae*, and the third was identified as *E. jeanselmei*. Both of the two problem isolates previously identified as *E. jeanselmei* were identified morphologically and by temperature tolerance studies as *W. dermatitidis* which correlated with results of our exoantigen tests.

Kaufman et al. (7) have previously reported on the use of a serum factor as a potential tool to serologically diagnose isolates of *E. jeanselmei* from *W. dermatitidis*. They have used sera produced in rabbits immunized with culture filtrates as reagents. We used antibodies 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 *E. jeanselmei*, it was necessary to further adsorb the serum with *E. spinifera* antigens, owing to cross-reactions observed with two of six isolates of *E. spinifera* after adsorption of serum with *F. pedrosoi* antigens. The presence of common antigens has been reported previously among the members of dematiaceous pathogens. Neilsen and Conant (12) have found that *W. dermatitidis* (*H. dermatitidis*) and *E. jeanselmei* (*P. jeanselmei*) contained antigenic components common to each other and to *E. werneckii* (*Cladosporium werneckii*). Cooper and Scheidau (1), using nonadsorbed sera, have found that *P. verrucosa* and *C. carrionii* also have common antigens which indicated that those two fungi were closely related to one another. We found that when nonadsorbed antisera of either *E. jeanselmei* or *W. dermatitidis* were tested against the members of dematiaceous pathogens included in this study, common antigenic characteristics and cross-reactions were demonstrated. However, after adsorption, it was possible to obtain reference sera which could be used as a potential tool to serologically differentiate *E. jeanselmei* and *W. dermatitidis* from one another and also from other dematiaceous fungi. No cross-reactions were observed with the isolated of *S. schenckii*.

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