Occurrence of Serotypes Among *Histoplasma capsulatum* Strains

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ABSTRACT

KAUFMAN, LEO (Communicable Disease Center, Atlanta, Ga.), AND SHARON BLUMER. Occurrence of serotypes among *Histoplasma capsulatum* strains. J. Bacteriol. 91:1434-1439. 1966.—Extensive studies with a fluorescent antibody specific for the yeast form of *Histoplasma capsulatum* indicated that, although most strains of *H. capsulatum* stained intensely, others stained weakly and some did not stain. These observations suggested that antigenic variants of the fungus existed. To test this hypothesis, reciprocal cross-staining and adsorption procedures were performed with fluorescein-labeled antiglobulins to four strains of *H. capsulatum*. Fifty-six strains of *H. capsulatum* and 11 strains of *H. duboisii* were studied. The data indicate that at least five serotypes of *H. capsulatum* occur. One is closely related antigenically to *H. duboisii* and *Blastomyces dermatitidis*.

In a previous study, Kaufman and Kaplan (3) reported the development of a fluorescent antibody (FA) specific for the identification of the yeast form of *Histoplasma capsulatum*. They reported that, although all of the strains of *H. capsulatum* studied were stained with this reagent, the staining intensity varied among the strains. Later on, in a more extensive evaluation of this FA, they encountered a strain of *H. capsulatum* (strain B-703) that did not stain (*unpublished data*). More recently, Pine, Kaufman, and Boone (6) encountered two additional strains of *H. capsulatum* that failed to react with the specific FA. The variable staining reactions and the failure of some strains to react with the *H. capsulatum* FA reagent suggested that antigenic variants of this organism might exist. Apparent support for this hypothesis was recently provided by Markowitz (5), who studied by immunodiffusion techniques the antigenic properties of chemical fractions from various strains of *H. capsulatum*. His results suggested "the existence of components of differing serologic reactivity in the different strains" studied. However, he did not consider his work definitive, since his observations might have been based upon the chemical alterations that had occurred during the extraction or contamination of the material under study. The present study, with the use of reciprocal cross-staining and adsorption procedures, was undertaken to determine conclusively whether

serotypes exist among yeast-form strains of *H. capsulatum*.

MATERIALS AND METHODS

Antisera and antiglobulins. The antigens used for the production of antisera were prepared from four yeast-form cultures of *H. capsulatum* (strains 28, A-811, B-580, B-703) that had been grown in Brain Heart Infusion (Difco) broth. The cultures were shaken for 3 weeks while incubating at 37°C. All cultures were treated with formalin (0.5%) and, after appropriate sterility tests, were washed three times in formalin-treated (0.5%) physiological saline. The washed cells were stored at 4°C in formalin-treated saline. Vaccines were prepared by adjusting portions of these suspensions to a turbidity that yielded 42% light transmission at 550 mμ in a Coleman spectrophotometer (model 6A). Dosages of 1, 2, and 2 ml were injected intravenously into rabbits on 3 consecutive days for 4 consecutive weeks. Each rabbit was bled 1 week after the final injection. The antiglobulin fractions were obtained from each antiserum by three precipitations with half-saturated ammonium sulfate.

Conjugation of antiglobulin fractions. Antiglobulin fractions, adjusted to 2.0 g of protein per 100 ml, were labeled with fluorescein isothiocyanate according to the method of Riggs et al. (8). The labeled antiglobulins were purified by gel filtration with medium G-50 Sephadex (1) and stored at -20°C.

The titer of each conjugate was determined by staining smears of homologous and heterologous strains of *H. capsulatum* and strains of *H. duboisii* with serial twofold dilutions of each of the reagents.

Smears treated with the labeled antiglobulins were

examined with a Reichert microscope and a Reichert Fluorex unit that contained an HBO 200 maximal-pressure mercury vapor arc. A Corning 5113 exciter filter was used in combination with a Wratten 2A barrier filter.

Average cell wall staining ranging from 2+ to 4+ intensity was considered positive. Average cell wall staining less than 2+ was considered negative.

Adsorption procedure. Antigens for adsorptions were grown and harvested in the same manner as those used for vaccine production. However, they were washed and suspended in phosphate-buffered saline (pH 7.2) that contained Merthiolate (1:10,000). Antiglobulins were adsorbed with half volumes of packed cells at 37 C in a water bath for a period of 2 hr. Adsorptions were repeated until staining of the adsorbing strain had been eliminated.

Antigens studied. Four strains of *H. capsulatum* (28, A-811, B-580, B-703) and 3 strains of *H. duboisii* (936, 1085, B-193) were routinely studied.

Complement-fixation (CF) test. CF titers of the rabbit anti-*H. capsulatum* sera to strains 28, A-811, B-580, and B-703 were determined against homologous and heterologous strain antigens according to the Communicable Disease Center Laboratory Branch Complement Fixation test (7). The antigenic titer for each strain was determined by a checker-board titration involving serial dilutions of proven human case histoplasmosis antiserum against serial dilutions of a 10% suspension of whole yeast-form cells.

RESULTS

Serological properties of unlabeled antisera and labeled antiglobulins. The CF test was used to characterize the various pools of *H. capsulatum* antiserum, since the quantitative data obtained provided preliminary information as to the level of antibodies present and the extent of cross-reactivity among the strains of *H. capsulatum* studied. Antisera were not used for fractionation and conjugation unless they demonstrated homologous CF antibody levels of 1:512 or higher. The CF tests revealed that the antisera prepared

against each of the four strains of *H. capsulatum* had homologous yeast-form titers that varied between 1:512 and 1:2,048, depending upon the strain. Moreover, the four antisera cross-titred to the same degree with the heterologous *H. capsulatum* yeast-form antigens. The effects of adsorptions on these antisera could not be determined by the CF technique, because in many instances these sera became anticomplementary after such treatment.

The staining properties of each of the non-adsorbed labeled antiglobulins were studied with the homologous and heterologous strains of *H. capsulatum* and also with three yeast-form strains of *H. duboisii*. The homologous staining titer of each of the conjugates varied from 1:16 to 1:64, depending upon the strain of *H. capsulatum* stained. Each of the labeled antiglobulins exhibited cross-staining with all of the *H. capsulatum* and *H. duboisii* strains examined. The cross-staining titers with the *H. capsulatum* strains did not vary more than fourfold from the homologous titers. All but one of the conjugates, however, had lower staining titers with the *H. duboisii* strains; those titers varied from 1:8 to 1:16. The one exception was the conjugate produced against *H. capsulatum* strain B-703. This conjugate stained *H. duboisii* at a dilution equivalent to the homologous titer.

The extensive cross-reactions with all of the nonadsorbed conjugates indicated that at least one antigen was common to the fungi studied. This common antigen was assigned the number 1. Homologous and heterologous adsorptions were employed to further elucidate the antigenic relationships among the four *H. capsulatum* strains.

Table 1 shows the effects of adsorptions on the staining properties of the *H. capsulatum* strain 28 conjugate. Adsorption of this labeled antiglobulin with cells of strain B-703 produced a

TABLE 1. Staining titers of unadsorbed and adsorbed fluorescein-labeled *Histoplasma capsulatum* strain 28 antiglobulins with homologous and heterologous antigens

<i>H. capsulatum</i> labeled antiglobulins	Adsorbing antigen	Staining titers ^a					Antigens homologous to antibodies
		<i>H. capsulatum</i> strain				<i>H. duboisii</i> (3 strains)	
		28	A-811	B-580	B-703		
Strain 28 ^b	None	64	32	32	16	8	1
Strain 28	B-703	8	4	4	0 ^c	0	2
Strain 28	A-811	0	0	0	0	0	None
Strain 28	B-580	2	U ^d	0	0	0	3

^a Reciprocal of dilution.

^b Showed no staining after adsorption with homologous antigen (strain).

^c No staining with undiluted conjugate.

^d Undiluted conjugate.

TABLE 2. Staining titers of unadsorbed and adsorbed fluorescein-labeled *Histoplasma capsulatum* strain A-811 antiglobulins with homologous and heterologous antigens

<i>H. capsulatum</i> labeled antiglobulins	Adsorbing antigen	Staining titers*					Antigens homologous to antibodies
		<i>H. capsulatum</i> strain				<i>H. duboisii</i> (3 strains)	
		28	A-811	B-580	B-703		
Strain A-811 †	None	32	32	32	16	8	1
Strain A-811	B-703	4	4	4	0 ‡	0	2
Strain A-811	A-811	0	0	0	0	0	None
Strain A-811	B-580	2	2	0	0	0	3

* Reciprocal of dilution.

† Showed no staining after adsorption with homologous antigen (strain).

‡ No staining with undiluted conjugate.

TABLE 3. Staining titers of unadsorbed and adsorbed fluorescein-labeled *Histoplasma capsulatum* strain B-703 antiglobulins with homologous and heterologous antigens

<i>H. capsulatum</i> labeled antiglobulins	Adsorbing antigen	Staining titers*					Antigens homo- logous to antibodies
		<i>H. capsulatum</i> strain				<i>H. duboisii</i> (3 strains)	
		28	A-811	B-580	B-703		
Strain B-703 †	None	4	8	4	16	16	1
Strain B-703	28	0 ‡	0	0	8	8	4
Strain B-703	A-811	0	0	0	8	8	4
Strain B-703	B-580	0	0	0	8	4	4

* Reciprocal of dilution.

† Showed no staining after adsorption with homologous antigen (strain).

‡ No staining with undiluted conjugate.

fluorescent antibody that stained all of the *H. capsulatum* strains except B-703. This antigenic pattern was assigned the number 2. Adsorption of the strain 28 conjugate with cells of strain B-580 produced a reagent that stained only *H. capsulatum* strains 28 and A-811. The antigenic pattern that resulted from this adsorption was assigned the number 3. When the conjugate against strain 28 was adsorbed with cells of strain A-811, all staining activity was eliminated. This indicated that the antigens complementary to strain 28 antibodies were all present in *H. capsulatum* strain A-811.

The effects of cross-adsorption on the *H. capsulatum* A-811 conjugate revealed no new antigenic patterns. Conjugate A-811 was adsorbed in the same manner as was conjugate 28. It was found to have an antigenic pattern identical to strain 28, i.e., antibodies against antigens 1, 2, and 3 were present (Table 2). These results indicated that strains 28 and A-811 were closely related, if not identical, antigenically.

The effects of cross-adsorptions on the labeled

antiglobulin produced against *H. capsulatum* strain B-703 are shown in Table 3. When this reagent was adsorbed with cells of strains 28, A-811, or B-580, it failed to stain any of the *H. capsulatum* strains except B-703. This conjugate also stained the three strains of *H. duboisii*. This antigenic pattern was designated number 4.

Table 4 shows the results of the heterologous adsorptions on conjugate B-580. Adsorption of this conjugate with cells of strain B-703 showed that it possessed antigenic pattern 2; i.e., *H. capsulatum* strains 28, A-811, and B-580 were stained. The adsorption of conjugate B-580 with strains 28 and A-811 completely eliminated staining of all the fungi studied. This indicated that, in addition to their unique antigens, strains 28 and A-811 contained all the antigens homologous to strain B-580.

Table 5 summarizes the results of our antigenic studies with the four *H. capsulatum* strains. The four strains fall into three serotypes. The first serotype, typified by strains 28 and A-811, has the antigenic structures 1, 2, and 3. A second

TABLE 4. Staining titers of unadsorbed and adsorbed fluorescein-labeled *Histoplasma capsulatum* strain B-580 antiglobulins with homologous and heterologous antigens

<i>H. capsulatum</i> labeled antiglobulins	Adsorbing antigen	Staining titers*					Antigens homo- logous to antibodies
		<i>H. capsulatum</i> strains				<i>H. duboisii</i> (3 strains)	
		28	A-811	B-580	B-703		
Strain B-580†	None	32	32	32	32	16	1
Strain B-580	B-703	8	4	4	0‡	0	2
Strain B-580	28	0	0	0	0	0	None
Strain B-580	A-811	0	0	0	0	0	None

* Reciprocal of dilution.

† Showed no staining after adsorption with homologous antigen (strain).

‡ No staining with undiluted conjugate.

TABLE 5. Antigenic formulas for *Histoplasma capsulatum* strains

<i>H. capsulatum</i>	Antigenic formula
Strain 28.....	1, 2, 3
Strain A-811.....	1, 2, 3
Strain B-580.....	1, 2
Strain B-703.....	1, 4

serotype, typified by strain B-580, has antigens 1 and 2. The third serotype, represented by strain B-703, has antigens 1 and 4.

To determine the prevalence of the various serotypes of *H. capsulatum* in nature, adsorbed conjugates specific for antigens 2, 3, and 4, plus an unadsorbed conjugate, were used to survey 56 yeast-form strains of *H. capsulatum*. They had been isolated from humans, dogs, and soil, and from bat guano from various parts of the world. This survey revealed the existence of two new serotypes in addition to the three previously described. The two new types had the following antigenic pattern: 1, 2, 4; and 1, 2, 3, 4. The data in Table 6 show the percentage of strains that fall into each antigenic category and relate antigenic type to geographic source. Of particular interest is the observation that at present antigenic strains belonging to serotype 1, 2, 3 have been found only within the United States and that most of the isolates from Central and South America belong to serotype 1, 4.

The unadsorbed and adsorbed conjugates were also applied to 11 strains of *H. duboisii*. Only the unadsorbed conjugate and the labeled antibody complementary to antigen 4 stained the *H. duboisii* strains. An attempt was made to eliminate the cross-staining of *H. duboisii* and to retain the staining of *H. capsulatum* serotype 1, 4 by adsorption of *H. capsulatum* strain B-703 conjugate with yeast-form cells of *H. duboisii*.

TABLE 6. Correlation of serotype and geographical source of 56 *Histoplasma capsulatum* strains

Serotype	Per cent of total number	No. of isolates grouped according to geographical source*			
		U.S.	C-S.A.	Europe	Asia
1, 2	23	11	1		1
1, 4	46	6	16	1	3
1, 2, 3	20	11			
1, 2, 4	7	3	1		
1, 2, 3, 4	4	1	1		

* U.S. = United States; C-S.A. = Central and South America.

These adsorptions resulted in the loss of all staining activity. This indicated that the *H. duboisii* strains studied contained all the antigens present in *H. capsulatum* serotype 1, 4.

In further studies, we attempted to relate the *H. capsulatum* antibodies to reactivity with the yeast-form antigens of *Blastomyces dermatitidis*. Five strains of *B. dermatitidis* were examined as to reactivity with the unadsorbed and adsorbed *H. capsulatum* conjugates. Surprisingly, the observations were similar to those noted with the *H. duboisii* strains; i.e., the *B. dermatitidis* strains stained only with the unadsorbed and factor 4 conjugates. Attempts to eliminate this cross-reactivity and to render the factor sera specific for *H. capsulatum* serotype 1, 4 and *H. duboisii* by dilution or by adsorption of the *H. capsulatum* B-703 conjugate with *B. dermatitidis* yeast cells were not successful. It was thus evident that the yeast-form of *B. dermatitidis* and *H. duboisii* contained the antigens demonstrated by the *H. capsulatum* serotype 1, 4.

DISCUSSION

Although biochemical and serological studies had demonstrated differences among yeast-form

strains of *H. capsulatum* (3, 4), distinct serological entities had not been characterized by reciprocal adsorptions and cross-staining. We were able to demonstrate four distinct antigenic factors: 1, 2, 3, and 4. Factor 4 has been shown by immunodiffusion studies to be a single entity identical to a specific precipitin produced by adsorption of *H. capsulatum* B-703 antisera with yeast-form antigens of strain 28, A-811, or B-580. This precipitin reacts only with antigens from serotype 1, 4. The relationship of identity between the conjugated and nonconjugated antibodies was established against acetone-precipitated supernatant antigens derived from 3-week-old yeast-form Brain Heart Infusion broth cultures of *H. capsulatum*.

One of the most important contributions of this study was the realization that the specific FA reagent of Kaufman and Kaplan (3) failed to react with those *H. capsulatum* strains characterized as the 1, 4 serotype. Immunodiffusion studies demonstrated that this specific FA reagent was identical to the factor 2 conjugate.

In FA tests, maximal diagnostic coverage would require the use of factor 4 as well as factor 2 conjugates. Detection of antigen 4, however, would not necessarily establish a diagnosis of histoplasmosis, since this antigen is also found in the cell walls of *B. dermatitidis* and *H. duboisii*. The strong relationship of the latter two fungi with *H. capsulatum* serotype 1, 4 is supported by the fact that factor 2 sera can be produced by adsorption of *H. capsulatum* serotype 1, 2, 3 or 1, 2 antisera with the yeast-form cells of *B. dermatitidis* (hitherto used for specific FA production), *H. duboisii*, or *H. capsulatum* serotype 1, 4.

The antigenic relationship of *H. capsulatum* with other fungi as revealed by this and previous studies is worthy of consideration. The antigens shared in common by *H. capsulatum* and other fungi, such as *B. dermatitidis*, *Geotrichum candidum*, and species of *Candida* and *Cryptococcus*, are evidently synonymous to factor A shown previously to be shared by the yeast and mycelial forms of *H. capsulatum* and *B. dermatitidis* (4) and to factor 1 as described in this study. Elimination of factor 1 antibody from *H. capsulatum* antisera in the present study yielded either a specific *H. capsulatum* antibody (factor 2) or one that cross-reacted with *B. dermatitidis* and *H. duboisii* (factor 4). Accordingly, if one could successfully adsorb antisera against *H. capsulatum* serotype 1, 2, 3, 4 with an organism such as a *Candida* species that contained only factor 1 in common with the *Histoplasma* species, one might successfully produce a polyvalent reagent from a single antiserum. This hypothesis is currently under investigation in our laboratory.

A review of Kaplan and Kaufman's (2) report on the production of a specific *B. dermatitidis* conjugate by adsorption of *B. dermatitidis* antibodies with *H. capsulatum* yeast cells reveals that one of the adsorbing strains used was a 1, 4 serotype. The fact that such an adsorption resulted in the production of a specific *B. dermatitidis* antibody indicates that *B. dermatitidis* is more complex antigenically than *H. capsulatum* serotype 1, 4.

One of the more interesting observations of this study is that *H. capsulatum* strain B-703 (serotype 1, 4), isolated from a patient in Ohio, and other isolates also of non-African origin, appear to be very closely related, if not identical antigenically, to *H. duboisii*. *H. duboisii* is the etiological agent of African histoplasmosis. This species is not known to occur elsewhere. All efforts to distinguish strain B-703 from *H. duboisii* strains by immunological procedures have been unsuccessful to date. However, in vivo studies with hamsters have shown that strain B-703 is a typical *H. capsulatum*. It consistently produces only small yeast cells (3 to 5 μ in diameter) and not the large tissue-form cells (7 to 15 μ in diameter) that are characteristic of *H. duboisii*.

Labeled antibody solutions representing the four factor conjugates permitted the grouping of 56 strains of *H. capsulatum* into combinations representing 5 serotypes: 1, 2; 1, 4; 1, 2, 3; 1, 2, 4; and 1, 2, 3, 4. The correlation of *H. capsulatum* serotypes with geographical source indicates that, except for serotype 1, 2, 3 which has been found hitherto only in the United States, the serotypes have a world-wide distribution. Another interesting observation was the predominance of serotype 1, 4 in Central and South America. More strains need to be examined from various parts of the world to determine whether these geographical patterns will hold.

A problem which we encountered with certain of our (adsorbed) sera and which we have not as yet solved was the reagent's loss of staining with storage. Adsorbed reagents had greater stability when treated with Merthiolate and refrigerated than when frozen. Under the former conditions, reagent shelf life was approximately 3 months.

The value of the *H. capsulatum* serotypes as antigens in the CF test is presently being evaluated. Currently, the antigen of choice in the diagnostic CF test used in our laboratory is strain A-811 which is serotype 1, 2, 3. This yeast-form proved superior in antigenicity and sensitivity when compared with yeast-form cells representing the more complete antigenic serotype 1, 2, 3, 4 and the less complete 1, 4 (strain B-703) as evaluated in titrations with proven human histoplasmosis antiserum. The lack of antigenicity and

sensitivity noted with the yeast antigens containing factor 4 might be attributed to a relative paucity of factors 1 to 3, to the masking or blocking of these antigens by factor 4, or to the possibility that the antigenic factors demonstrated by the FA and agar-gel diffusion tests are not operative in the CF test. The antigens containing factor 4 might have potential value in those instances where the host response is predominantly or exclusively due to antigen 4. The value of these serotypes in diagnostic CF tests has yet to be determined.

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