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Serotyping of *Cryptococcus neoformans* by Using a Monoclonal Antibody Specific for Capsular Polysaccharide

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The importance of epidemiological studies of cryptococcosis has increased since the beginning of the AIDS epidemic. *Cryptococcus neoformans* exists in two varieties defining four serotypes, *Cryptococcus neoformans* var. *neoformans* (serotypes A and D) and *Cryptococcus neoformans* var. *gattii* (serotypes B and C). The varieties are easy to distinguish by their differences of growth on diagnostic media. We propose here an easy serotyping method combining diagnostic media and a direct immunofluorescence assay with one monoclonal antibody (E1) specific for cryptococcal polysaccharide. The method was validated by the blinded testing of four to five reference strains of each serotype. Immunofluorescence patterns were characteristic of a given serotype provided that the variety of the strain had been defined before. For *C. neoformans* var. *neoformans*, a bright, homogeneous staining with several cell aggregates was characteristic of serotype A, whereas only a few serotype D cells were positive. For *C. neoformans* var. *gattii*, a completely negative isolate was serotype C, whereas the population of serotype B included a majority of negative cells but also included positive cells with a speckled pattern. The method was then used to serotype 156 clinical isolates from France and isolates from areas where *C. neoformans* var. *gattii* was endemic before the AIDS epidemic (13 strains from Rwanda and Zaire and 5 strains from Australia). The specificity of E1 was defined by its reactivity with various *Cryptococcus* spp. and analyzed according to the described cryptococcal antigenic factors. We conclude from this study that E1 provides a rapid and reliable means to serotype multiple isolates of *C. neoformans*.

Cryptococcus neoformans is an encapsulated yeast responsible for severe meningoencephalitis. The frequency of cryptococcosis has dramatically increased since the beginning of the AIDS epidemic because of the increased susceptibility of patients with cellular immunodeficiency to infections due to *C. neoformans* (5, 16). Cryptococcosis is the fourth most life-threatening infection during human immunodeficiency virus infection. *C. neoformans* exists in two varieties, *Cryptococcus neoformans* var. *neoformans* and *Cryptococcus neoformans* var. *gattii*, defining four serotypes. Serotypes A and D belong to *C. neoformans* var. *neoformans*, and serotypes B and C belong to *C. neoformans* var. *gattii* (1). The serotype of the infecting strain does not seem to influence its susceptibility to antifungal drugs (8), but *C. neoformans* var. *gattii* has been associated with more severe infections in humans (9). Infections due to *C. neoformans* var. *neoformans* are found worldwide, whereas those related to *C. neoformans* var. *gattii* have only been diagnosed in patients who live in or have travelled to tropical areas (1, 13). The AIDS epidemic seems to have changed the numbers (15). Only a few cases of cryptococcosis due to *C. neoformans* var. *gattii* have been reported in AIDS patients (4, 17, 19). Even in Central Africa where isolates were mainly *C. neoformans* var. *gattii* before 1980, AIDS patients are mainly infected with *C. neoformans* var. *neoformans* (15).

The two varieties are easily distinguished by their biochemical properties (2). Special media (7, 14) are reported to allow a rapid identification. However, up to now, the precise serotyping of clinical isolates required the preparation of various polyclonal immune sera, usually raised in rabbits against each serotype, and their reciprocal adsorptions against the other serotypes (12, 21). The antigenic structures allowing the distinction between serotypes are part of the glucuronoxylomannan, the main polysaccharidic component of the capsule (20). Differences in the amount of substitution (side chains and *O*-acetyl groups) of the α(1-3)-mannose backbone are found for the various serotypes (3). A few years ago, Ikeda et al. (10) described eight antigenic factors defined by the reactivity of appropriately adsorbed polyclonal antibodies. However, the precise structures corresponding to these antigenic factors remain to be determined.

Serotyping *C. neoformans* strains does not help diagnosis, which requires at least a positive detection of cryptococcal polysaccharide in body fluids and/or culture of *C. neoformans*. The main reason for serotyping *C. neoformans* strains is therefore epidemiological. We present here an easy and rapid method for the serotyping of *C. neoformans* strains by using only one monoclonal antibody (E1) specific for cryptococcal polysaccharide (6), in combination with two diagnostic media (7, 14). This method was validated by using reference strains of each serotype and then applied to serotype isolates from Africa, Australia, and France. E1 was tested on various *Cryptococcus* species in an attempt to define the antigenic factor recognized by the antibody.

MATERIALS AND METHODS

Strains examined for serotyping. Reference strains of the four serotypes were either kindly donated by K. J. Kwon-Chung and J. E. Bennett (National Institutes of Health, Bethesda, Md.) or purchased from the Centraalbureau voor Schimmelcultures (Delft, The Netherlands). Reference strains of the other *Cryptococcus* species were obtained from the Centraalbureau voor Schimmelcultures. All strains are listed in Tables 1 and 2.

Clinical isolates from patients with AIDS living in Rwanda or Zaire were sent by D. Swinne (Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium). These strains were

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TABLE 1. Results obtained with the diagnostic media and immunofluorescence patterns with E1 of authentic strains of *C. neoformans*

Strain	Known serotype	CGB[a]	Proline[b]	*C. neoformans* variety	IF pattern[c]	Resulting serotype
NIH 271	A	Green	−	*neoformans*	Bright, homogeneous	A
CDC B236	A	Green	−	*neoformans*	Bright, homogeneous	A
CDC B551	A	Green[d]	−	*neoformans*	Bright, homogeneous	A
CBS 5756	A	Green	−	*neoformans*	Bright, homogeneous	A
CBS 879	A	Green	−	*neoformans*	Bright, homogeneous	A
NIH 112	B	Blue	+	*gattii*	Speckled, variable	B
NIH 444	B	Blue	+	*gattii*	Speckled, variable	B
CDC B237	B	Blue	+	*gattii*	Speckled, variable	B
CBS 6289	B	Blue	+	*gattii*	Speckled, variable	B
CBS 6998	B	Blue	+	*gattii*	Speckled, variable	B
NIH 18	C	Blue	+	*gattii*	Negative	C
NIH 191	C	Blue	+	*gattii*	Negative	C
CDC B238	C	Blue	+	*gattii*	Negative	C
CBS 6994	C	Blue	+	*gattii*	Negative	C
NIH 52	D	Green[d]	−	*neoformans*	Homogeneous or speckled, variable	D
NIH 3501	D	Green[d]	−	*neoformans*	Homogeneous or speckled, variable	D
NIH 3502	D	Green[d]	−	*neoformans*	Homogeneous or speckled, variable	D
CBS 6885	D	Green[d]	−	*neoformans*	Homogeneous or speckled, variable	D
CBS 6886	D	Green	−	*neoformans*	Homogeneous or speckled, variable	D
CBS 6901	D	Green	−	*neoformans*	Homogeneous or speckled, variable	D
NIH 68	A-D	Green	−	*neoformans*	Bright, homogeneous	A
CBS 132	A-D	Green	−	*neoformans*	Homogeneous or speckled, variable	D

[a] CGB medium color.
[b] − and +, no growth and growth, respectively, in presence of proline.
[c] IF, immunofluorescence.
[d] Medium turned a little blue after 48 h of incubation.

isolated by J. Bogaerts (Kingali, Rwanda) and T. Muyembe (Kinshasa, Zaire). Other strains isolated from eight patients with AIDS were provided by J. J. Muyembe-Tamfum (Service de Microbiologie, Université de Kinshasa, Kinshasa, Zaire). Three strains isolated from *Eucalyptus camaldulensis* and two clinical isolates from one patient with AIDS and one seronegative patient (M4725) were generously given by D. H. Ellis and T. J. Pfeiffer (Adelaide Medical Center, North Adelaide, Australia). Finally, 156 clinical isolates were collected in France during 1991 and 1992 at the Reference Center for Mycoses (Institut Pasteur, Paris, France).

Separation of the two varieties of *C. neoformans*. The canavanine-glycine-bromothymol blue (CGB) agar and the disks of D-proline were prepared as described by Kwon-Chung et al. (14) and Dufait et al. (7), respectively. *C. neoformans* organisms to be tested were subcultured on Sabouraud-chloramphenicol agar for 48 h before being checked on the diagnostic media. The color of the CGB medium or growth in the presence of D-proline was assessed after 48 h of incubation at 27°C.

Antibodies for serotyping. The eight factor sera were obtained a few years ago from T. Shinoda (Department of

TABLE 2. Reactivity of the monoclonal antibody E1 on *Cryptococcus* species

Strain	Species	Serotype and slide agglutination with the following factor[a]:									Diagnostic medium:		Binding of E1
		S[b]	1	2	3	4	5	6	7	8	CGB[c]	Proline[d]	
CDC B551	*C. neoformans* var. *neoformans*	A	+++	+++	+++	−	−	−	+	−	Green	−	Bright, homogeneous
NIH 52	*C. neoformans* var. *neoformans*	D	+++	+++	+++	−	−	−	−	++	Green[e]	−	Variable[f]
NIH 112	*C. neoformans* var. *gattii*	B	+++	+++	−	++	+	−	−	−	Blue	+	Variable
NIH 18	*C. neoformans* var. *gattii*	C	+++	−	−	++	−	+	−	−	Blue	+	Negative
CBS 142	*C. albidus* var. *albidus*	A	+++	+++	+++	−	−	−	+	−	Green	−	Variable
CBS 571	*C. humicolus*	D	++	+++	++	−	−	−	−	+	Blue	−	Variable
CBS 570	*C. curvatus*	A-D	+++	++	+++	−	−	−	+	+	Blue	+	Variable
CBS 1926	*C. kuetzingii*	Unt[g]	++	++	+	−	−	−	−	−	Green	−	Variable
CBS 943	*C. luteolus*	Unt	++	++	+	−	−	−	−	−	Green[e]	−	Variable
CBS 1895	*C. terreus*	Unt	+	+	+	−	−	−	−	−	Green	−	Variable
CBS 155	*C. albidus* var. *aerius*	C	++	−	−	++	−	+	−	−	Blue	−	Negative
CBS 1927	*C. gastricus*	Unt	−	−	−	−	−	−	−	−	Green	−	Negative
CBS 1730	*C. uniguttulatus*	Unt	−	−	−	−	−	−	−	−	Green	−	Negative
CBS 139	*C. laurentii*	Unt	−	−	−	−	−	−	−	−	Blue	−	Negative

[a] Reproduced after Ikeda et al. (10); + to +++, positive agglutination; −, negative agglutination.
[b] S, serotype.
[c] CGB medium color.
[d] − and +, no growth and growth, respectively, in presence of proline.
[e] Medium turned a little blue after 48 h of incubation.
[f] Positivity varied from cell to cell.
[g] Unt, untypeable.

Microbiology, Meiji College of Pharmacy, Tokyo, Japan). They were used as described previously (10) to serotype the isolates according to their patterns of agglutination.

The purified monoclonal antibody E1 (6) was conjugated with fluorescein isothiocyanate (FITC, isomer I; Sigma Chemical Co., St. Louis, Mo.). Briefly, a mixture of E1 (5 mg/ml in 50 mM carbonate buffer, pH 9.5) and FITC (30 μg/mg of antibody) was rotated for 2 h at room temperature. Removal of free dye was obtained by filtration on a small Sephadex G-25 column (Pharmacia, Uppsala, Sweden). The reactivity of the FITC-labeled E1 (E1-FITC) was checked on a *C. neoformans* serotype A strain (NIH 271). The optimal concentration was close to 5 μg/ml.

Immunofluorescence assay. *Cryptococcus* strains to be tested were grown in Sabouraud broth for 18 h at 27°C in an orbital shaker (150 rpm). Ten microliters of the suspension (approximately 2×10^6 to 3×10^6 cells) was then added to wells of a Multiscreen-HV filtration plate (hydrophilic Durapore membrane with a pore size of 0.45 μm; Millipore Corp., Bedford, Mass.). The screen allows an easy washing by filtration without loss of the cells during the procedure and the screening of multiple samples at the same time. After four washings with phosphate-buffered saline (PBS; 10 mM, pH 7.4), 100 μl of appropriately diluted E1-FITC was added to each well and incubated for 30 min at room temperature in the dark. All the strains were incubated with the same concentration of E1-FITC. After four washings with PBS, the cells were resuspended in 100 μl of PBS. Ten microliters of the suspension was then placed on a glass slide to allow examination under an epifluorescence microscope. Before being tested, all the reference strains were coded. Immunofluorescence patterns were noted by two different examiners (O.R. and F.D.) who knew only whether the strain was from *C. neoformans* var. *neoformans* or *C. neoformans* var. *gattii*. Experiments were repeated at least three times. The influence of the culture (stationary or exponential phase and growth on Sabouraud agar or broth) and reproducibility of the patterns after cloning of the strains were checked.

RESULTS

Immunofluorescence patterns of the reference *C. neoformans* strains (Table 1). The determination of the *C. neoformans* variety was easily performed by using the diagnostic media. Results agreed for all reference strains. After being classified as belonging to one of the two varieties, the reference *C. neoformans* strains were studied by immunofluorescence with E1.

As suspected during the characterization of E1 (6), the pattern of fluorescence varied from one serotype to the other when the same antibody concentrations were used. Serotype C strains were consistently negative and remained negative even after increase of E1 concentration (data not shown). Serotype A strains exhibited a bright, homogeneous pattern. Almost all cells were stained, the fluorescence being at the capsule level. Moreover, many cell aggregates were seen. For serotype B and D cells, the amount of fluorescence varied from cell to cell. The fluorescence intensity was lower than on A cells, the majority of the cells being negative. The positive cells could be as bright as the A cells or could have intermediate intensity. Positive B cells always appeared hazy and speckled, whereas the fluorescence on D cells was either speckled or homogeneous (with a rim reinforcement identical to that observed for A cells). This explained the need for the prescreening of the isolates with diagnostic media. With this preliminary distinction of the two varieties, all the strains were correctly serotyped. The heterogeneity of the fluorescence obtained with serotypes B and D could be related to a mixture of serotypes or to differences in capsule size. However, the fluorescence pattern of B and D cells was still found on eight different expanded clones (data not shown). To address the question of the influence of capsule size, cryptococcal cells obtained after experimental infection were tested. A direct immunofluorescence test was performed on the minced brains from infected mice. All cryptococcal cells had an enormous capsule, and the variability in fluorescence intensity was exactly similar to that seen for cells grown on agar that have a small capsule (data not shown).

TABLE 3. Serotypes of isolates from Australia, Africa, and France

Country	No. of isolates	No. of *C. neoformans* var. *neoformans*		No. of *C. neoformans* var. *gattii*	
		Serotype A	Serotype D	Serotype B	Serotype C
Australia	5	0	1	4	0
Zaire	9	8	0	1	0
Rwanda	4	0	0	3	1
France	156	124	32	0	0

When selecting the reference strains, we included two strains that were reported to be recognized by anti-A (factor 7) and anti-D (factor 8) polyclonal sera (10) and classified as A-D. They had a pattern typical of serotype A for NIH 68 and of serotype D for CBS 132 when E1 was used.

Serotyping of clinical isolates from Australia, Africa, and France (Table 3). With serotyping of clinical isolates from Australia, Africa, and France, the diagnostic media were used before precise serotyping with E1. Two clinical isolates from Australia were sent to us because the classical methods of serotyping did not permit their classification in a given serotype. One was *C. neoformans* var. *gattii* and the other one was *C. neoformans* var. *neoformans* as determined by the diagnostic media. The first one was agglutinated by both anti-B and anti-D polyclonal sera and was serotyped B by the binding of E1-FITC. It was isolated from a patient who tested negative for human immunodeficiency virus infection. The second one was untypeable by polyclonal immune sera and had a typical D pattern in our hands. The three strains isolated from environmental sources and already known as serotype B were confirmed by E1 binding pattern.

Among isolates from Africa, five were *C. neoformans* var. *gattii*, four were serotype B, and one was serotype C. Since no isolate of the C serotype has ever been found in Central Africa, we confirmed these results by using the factor sera (10). All these patients had AIDS. The other strains isolated in Zaire were *C. neoformans* var. *neoformans*, and all were serotype A.

Of the 156 clinical isolates collected in France, all were *C. neoformans* var. *neoformans*, 124 were serotype A, and 32 were serotype D. When randomly checking 10 of these isolates (7 of serotype A and 3 of serotype D) with the factor sera, we found the same results except for one A isolate that was untypeable by the conventional method.

Immunofluorescence pattern with E1-FITC on *Cryptococcus* species. To better define the specificity of E1, we tested reference strains of various *Cryptococcus* species that had been used by Ikeda et al. (10) in their first description of antigenic factors. The results are presented in Table 2. The agglutination patterns in the presence of serum factors are

those described and published by Ikeda et al. (10). It should be noted first that diagnostic media used with all the listed strains were valueless for the subsequent serotyping interpretation.

Cryptococcus albidus var. *albidus* appeared bright, with almost all the cells exhibiting a homogeneous peripheral staining. The fluorescence intensity and pattern looked almost the same on *Cryptococcus kuetzingii*. On both species, though more often on *C. kuetzingii*, some cells looked hairy with fluorescent prickles. *Cryptococcus albidus* var. *aerius*, on the contrary, was negative. *Cryptococcus humicolus*, *Cryptococcus curvatus*, and *Cryptococcus terreus* exhibited the same kind of fluorescence as *C. neoformans* serotype D, with a majority of the cells being negative. All the other species tested were negative. Here again, the results were checked three times and blind testing was used for reading the slides. All the negative strains were retested with higher concentrations of E1 and remained negative.

DISCUSSION

Since serotyping of *C. neoformans* strains is performed mainly for epidemiological studies, it seems necessary to have a rapid and reliable way of screening multiple isolates. The combination of diagnostic media and a rapid direct immunofluorescence assay using one monoclonal antibody at a unique concentration allows the four serotypes of *C. neoformans* to be distinguished. The biochemical tests that were included in a recently published method of serotyping (11) were deliberately neglected in favor of the common procedure of identification. This includes direct examination and assimilation tests completed here by using the diagnostic media. The advantage of our method is the use of a unique reagent that is a monoclonal antibody. This ensures reproducibility of the results and rapidity of the test. The preparation of factor sera or serotype-specific sera in fact requires long immunization procedures and adsorption experiments lowering the titers of the sera. The second advantage is in allowing screening of multiple isolates during the same experiment.

The evaluation of this serotyping method was performed blindly after separation of authentic *C. neoformans* strains into the two varieties, *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii*. Immunofluorescence patterns were then specific for a given serotype. When a strain was known to be *C. neoformans* var. *neoformans*, a bright homogeneous pattern with several cell aggregates was specific of serotype A whereas a lower intensity of fluorescence with a majority of negative cells was characteristic of serotype D. For strains of *C. neoformans* var. *gattii*, the distinction was even more striking since serotype C cells were always negative whereas serotype B cells exhibited a fluorescence variable in intensity and similar to that obtained with strains of serotype D. The patterns were not dependent on the medium used for the culture, the age of the cells, or the size of the capsule. They were reproducible after cloning of the strains. The combination of diagnostic media and immunofluorescence study seems therefore rapid and perfectly reliable since all the reference strains were correctly classified at the end of the blinded screening. The only discrepant result concerns strains that were previously serotyped A-D because both factor 7 and factor 8 were detected. With our method, we easily classified these strains as A or D. Nevertheless, the epidemiological significance of serotype A-D has not yet been established.

The method was tested on isolates from France or from areas where *C. neoformans* var. *gattii* is known to be endemic (Africa and Australia). The majority (79%) of the isolates from France were serotype A, and 21% were serotype D. The percentage of serotype D is a little higher than the one published by Kwon-Chung and Bennett (13). However, it could be explained by the fact that only 21 strains were then available. Using our method, and confirming the results with the polyclonal sera, we discovered the first *C. neoformans* serotype C in Africa. Finally, three isolates (serotypes A, D, and B, respectively) that were untypeable with polyclonal sera were easily serotyped by using E1.

The differences between the conventional serotyping methods and ours are mainly the disappearance of the ambiguous serotype A-D in our hands and, up to now, the absence of untypeable strains. Several studies using polyclonal antisera have reported 1 to 5% untypeable strains (13, 21) and a number of strains agglutinating both anti-A and anti-D sera (1, 10, 13, 21). Hypotheses can be made to explain these differences. First, an immunofluorescence assay is far more sensitive than an agglutination assay. Second, E1 may recognize an epitope different from those defined by polyclonal sera. The results of E1 binding on *C. neoformans* serotypes A, B, and D suggest a specificity for factor 2 (10) that was confirmed by the study of E1 reactivity on other *Cryptococcus* species. Our data showed also that the epitope has a variable degree of expression from one serotype to another (A versus B or D) and from cell to cell within a given serotype (D or B). Variation within serotypes of *C. neoformans* has been noted by immunological (18) and biochemical methods (20). All these results suggest that the variability in the amount of antigen detected during an infection may not only be related to the severity of the colonization but also to the variation in antigen expression in *C. neoformans* serotypes. More experiments are needed to find out whether antigenic variation at the capsule level can influence the pathogenicity of an isolate.

In conclusion, E1 in combination with simple diagnostic media is, in our point of view, a rapid and reliable method to serotype clinical isolates. Molecular typing methods could then be used on specific isolates to better discriminate among them within a given serotype (15).

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