

Candida albicans Serotype Analysis by Flow Cytometry

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Candida albicans strains can be assigned to either of two major serogroups, A or B. Antigenic surface determinants present only in serotype A strains allow such a distinction, which has epidemiologic relevance. Reports have established that the relative distributions of the two serotypes can vary depending on the geographic origin of the isolates. A prevalence of susceptibility to an antifungal agent, flucytosine, was also observed with isolates of serotype A. More recently, it was suggested that the occurrence of serotype B isolates in various clinical forms of candidiasis is increasing. However, this latest finding remains controversial since serotyping results vary widely from one laboratory to another because of the lack of standardized methodologies. Difficulty in interpretation of results, which may lead to erroneous serotype identification, is the major setback associated with current methods. For this study, we thus devised a procedure that relies on flow cytometry and that may eliminate ambiguities in serotype determination. The validation of results was achieved with two types of serotype A-specific antisera, Iatron Factor 6 antiserum and an anti-*C. albicans* antiserum adsorbed on serotype B yeast cells. Agreement between results obtained with these two reagents was 100% with a wide array of *Candida* strains. These results confirmed the potential of the flow cytometric procedure as a reliable and reproducible method to establish the serotypes of *C. albicans* strains. Furthermore, some applications of this procedure to the epidemiological study of this human pathogen are presented.

The opportunistic yeast *Candida albicans* is at this time the most common fungal pathogen in humans. This yeast can cause mucosal as well as systemic infections in both immunocompetent and immunocompromised individuals. Several methods have been devised to classify *C. albicans* strains in subgroups below the species level (17). First described in the 1960s, the serotyping procedure separates strains of *C. albicans* into serotypes A and B (9). These two serogroups share the majority of their cell surface antigenic determinants; however, the presence of additional antigens in serotype A strains allows their distinction from serotype B strains (8, 9, 23, 28). Mainly used in association with other biotyping procedures, serotyping still contributes to our knowledge of the epidemiologies of *C. albicans* infections. For example, a correlation has been established between serotypes and susceptibility to the antifungal agent flucytosine (5FC) (1, 7, 19, 21, 22, 26). It has also been suggested that serotypes A and B differ in their geographic distributions, abilities to adhere to epithelial cells, and abilities to infect patients with different immunological statuses (3, 7, 10, 18, 19, 21, 26). However, these assumptions remain controversial since serotyping results can vary widely, reflecting discrepancies introduced by the use of numerous serotyping methodologies in different laboratories (21). Investigators have relied on serotyping techniques as diverse as slide agglutination, tube agglutination, and slide immunofluorescence assays using various antisera or purified antibodies that reportedly differentiate type A from type B yeast strains (1, 2, 9, 23, 24, 26). Recently, a comparative study suggested that it may not be possible to make valid comparisons between studies which evaluated serotype prevalence unless the same serotyping methodologies were used (2). This major setback was mainly

attributed to the difficulty involved in interpreting results visually, which may lead to erroneous serotype identification.

Analyses of *C. albicans* surface determinants previously suggested the potential of flow cytometry to determine yeast cell serotypes (4, 5). In the present study, we thus devised a method relying on flow cytometry which, by means of quantitative fluorescence monoparametric histogram outputs, was shown to eliminate the ambiguity of the interpretation of results. The labelling and washing conditions were optimized in order to distinguish clearly between serotype A and B strains, and the procedure was validated with different yeast cells from various *Candida* species and different serotype A-specific antisera. The usefulness of this procedure was further established by our performing a limited epidemiological study of *C. albicans* isolates from patients with either oropharyngeal or vulvovaginal candidiasis. Both groups of patients were shown to exhibit similar relative distributions of serotype A and B strains. Finally, we demonstrated that fluorescence patterns generated by flow cytometric analysis may be helpful in determining the origins of recurrent *C. albicans* infections, since even strains of the same serotype exhibit differences in their precise monoparametric histogram outputs.

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MATERIALS AND METHODS

***Candida albicans* strains and clinical isolates.** The clinical isolates of *C. albicans* were obtained from two groups of patients. Ninety-four strains were isolated from the vaginas of patients presenting with episodes of vulvovaginal candidiasis. Of that number, one isolate from each of two consecutive episodes of recurrent vulvovaginal candidiasis was obtained from 32 patients, recurrence being defined as the onset of three episodes of vaginitis in a period of 6 months (15). Isolates were also obtained from 29 human immunodeficiency virus (HIV)-infected individuals with oropharyngeal candidiasis (throat or esophagus). All strains were identified as *C. albicans* by their abilities to form germ tubes and produce chlamydospores (1). *C. albicans* collection strains of known serotype were obtained either from the American Type Culture Collection (Rockville, Md.) or

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from E. Drouhet (Institut Pasteur, Paris, France). Strains of other *Candida* species were obtained, unless otherwise mentioned, from the American Type Culture Collection. *C. stellatoidea* strains were a gift from J. Kwon Chung (National Institutes of Health, Bethesda, Md.) and S. Riggsby (University of Tennessee, Knoxville), while *C. dubliniensis* strains were provided by M. McCullough (University of Melbourne, Melbourne, Australia) and G. St-Germain (Laboratoire de Santé Publique du Québec, Montréal, Québec, Canada).

Phenotypic and genotypic typing procedures. The biotyping scores of *C. albicans* strains were determined according to the procedure of Odds and Abbott (20). Susceptibility testing to 5FC was performed as described by Auger et al. (1). Genotypic characterization was done as previously reported (15).

Serotype A-specific antisera. The serotype A-specific antiserum Ca\A-B was produced by a procedure based on the work of Hasenclever and Mitchell (9), who demonstrated that an antiserum raised against serotype A yeast cells of *C. albicans* can be rendered specific to serotype A yeast cells by a preadsorption procedure (1, 9). To perform this procedure, well-characterized strains of both serotypes were obtained from the American Type Culture Collection. The anti-*Candida* antiserum was raised in rabbits by injections of heat-killed (30 min, 100°C) yeast cells from the serotype A strain ATCC 32354. The titer of antibody raised against *C. albicans* was superior to 1:1,000; reaching such a high titer was necessary to retain sufficient reactivity of the antiserum after the successive adsorption steps. Adsorption was achieved by mixing equal volumes of antiserum with a suspension of heat-killed (3 h, 65°C) yeast cells of the serotype B strain ATCC 44373. The mixture was shaken at room temperature for 45 min to allow adsorption and then centrifuged for 10 min at $1,000 \times g$. This preadsorption procedure was repeated four more times, after which the resulting serotype A-specific antiserum (Ca\A-B) was diluted in phosphate-buffered saline (PBS) to obtain a final titer of 1:25. The titer of the antiserum was determined on serial dilution by slide immunofluorescence and defined as the highest dilution giving visible fluorescence. The other antiserum used for serotyping was a commercially available monospecific antiserum specific to *C. albicans* cells of serotype A (12, 13, 29); this antiserum, known as Iatron Factor 6 (IF6; also known as anti-IF6 or *Candida* Check no. 6), was purchased from Iatron Laboratories (Tokyo, Japan).

Slide immunofluorescence serotyping procedure. The serotypes of all strains were determined by an already described indirect immunofluorescence slide assay (6). The preadsorbed serotype A-specific antiserum Ca\A-B was used to detect the expression of antigens specific to serotype A yeast cells. Serotype A strain ATCC 32354 was included in each experiment as a positive control, and the serotype B strain ATCC 44373 was used as a negative control. Antibody binding was determined by visual observation by fluorescence microscopy, following incubation with an affinity-purified goat anti-rabbit immunoglobulin G (heavy and light) coupled to dichlorotriazinylamino fluorescein (DTAF), which was used as a secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, Pa.).

Flow cytometric serotyping procedure. *Candida* strains were grown to stationary phase in 10 ml of yeast nitrogen base (Difco, Detroit, Mich.) under agitation (250 rpm) at 30°C. Yeast cells from these cultures were pelleted by centrifugation at $1,000 \times g$ for 10 min; the cells were then washed with 10 ml of PBS, recentrifuged, and resuspended in PBS to approximately 5×10^6 cells/ml. One hundred microliters of the diluted *Candida* cells were transferred to flow cytometry tubes (Falcon 2052; Becton Dickinson Labware, Lincoln Park, N.J.) prior to the addition of the primary antiserum (5 μ l of the Ca\A-B antiserum or 1 μ l of the undiluted IF6 antiserum as provided by the manufacturer). This mixture was gently mixed and incubated on ice for 1 h. Cells were then washed by the addition of 3 ml of PBS to each sample. The tubes were centrifuged for 10 min at $1,000 \times g$, and the supernatants were carefully discarded, leaving the pellets in approximately 400 μ l of PBS. This washing procedure was repeated prior to the addition of 4 μ l of the affinity-purified goat anti-rabbit immunoglobulin G (heavy and light) antibodies coupled to DTAF to the yeast cells, now in 400 μ l of PBS. The mixture was vortexed gently and incubated on ice for 30 min. Finally, cells were washed twice in PBS as described previously and fixed by the addition of the same volume (400 μ l) of 2% paraformaldehyde for 30 min at 4°C. The flow cytometric analysis was performed with a FACStar flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) equipped with a 2-W argon ion water-cooled laser. The laser was set at 488 nm with a light output of 200 mW. The fluidic system was equipped with a 75- μ m-diameter nozzle tip. Green fluorescence (DTAF) was collected with a 530/30-nm bandpass filter. Multiparametric data for each sample were acquired in list mode for 10,000 events and analyzed with Consort 30 software. Data were analyzed in monoparametric histograms. All experiments included, as controls, collection yeast strains well established as either serotype A or B. A sample of yeast cells labelled solely with the DTAF conjugate was also included as a background control.

RESULTS

Validation of the flow cytometric serotyping procedure. The validation of our newly proposed flow cytometric procedure was achieved through the use of different groups of *Candida* strains labelled either by this procedure or by the classical indirect slide immunofluorescence assay (1, 6). The capacity of

the flow cytometric procedure to assign strains to a particular serotype was also evaluated with two antisera specific to serotype A yeast cells. The first, IF6, was a commercially available antiserum, while the second was prepared in our own laboratory by a standard preadsorption procedure, as described in Materials and Methods. The changing specificity of the latter, through the successive adsorption steps, was monitored by flow cytometry (Fig. 1). After five consecutive adsorption steps, the fluorescence intensity of the labelling for serotype B yeast cells was down to the background level observed when the complete labelling procedure was performed in the absence of the first anti-serotype A antiserum, while the fluorescence intensity observed with cells of serotype A remained several hundredfold higher. The antiserum (Ca\A-B) was thus considered a serotype A-specific antiserum.

Both primary antisera (IF6 and Ca\A-B) were then used to study the expression of serotype A-specific epitopes on *Candida* cells, with known antigenic characteristics. The capacity of the method to detect the labelling of yeast cells was evaluated for at least two representative strains of several *Candida* species. Analysis of flow cytometric recordings revealed that reactivity with both serotype A-specific antisera corroborated previous reports based mainly on slide agglutination assays. Yeast cells from *C. stellatoidea* type II, *C. tropicalis*, *C. glabrata*, and the newly described *C. dubliniensis* (27) were all positively labelled by either antiserum, while *C. stellatoidea* type I, *C. guilliermondii*, *C. kefyr*, and *C. krusei* all remained nonreactive (data not shown). Yeast cells from four collection strains of *C. albicans* were also submitted to the flow cytometric procedure. All strains were characterized by a single fluorescent peak with either antiserum (Fig. 2). The serotype A yeast cells displayed mean fluorescence intensities several hundredfold higher than those of controls and serotype B cells. Resulting serotypes were, in every case and with either antiserum, consistent with the known serogroups of these strains (Fig. 2).

We applied this analysis, which supported the potential of our procedure to facilitate serotype assignment of *C. albicans* strains, to a large number of *C. albicans* isolates from patients with oropharyngeal or vulvovaginal candidiasis. As already observed with collection strains, although the mean relative fluorescence intensities varied from strain to strain, it was always several hundredfold higher for cells from serotype A strains ($>10^2$). Levels of autofluorescence and nonspecific fluorescence of yeast cells were generally confined below fluorescence intensity values of 10^1 , and interference with the procedure resulting from autoagglutination of yeast cells, a major problem in some other serotyping procedures, was never observed. The monoparametric histogram outputs generated by flow cytometric analysis thus allowed us to easily assign a serotype to all strains tested. Identical results were consistently obtained with both serotype A-specific antisera.

Since the majority of these strains had been previously analyzed by a slide immunofluorescence assay, we compared the two sets of serotyping results; in the majority of cases, the serotype assignments of both methods were consistent. However, in some cases, the serotype of an infecting strain could be conclusively determined only by the flow cytometric procedure. In one sample originally assigned to serotype A by slide immunofluorescence, the flow cytometric procedure revealed the presence of two distinct fluorescent peaks; one population of cells fluoresced strongly, while the fluorescence intensity of a second population was comparable to that of the negative control in the absence of primary anti-serotype A antiserum (data not shown). Further analysis of this sample by colony

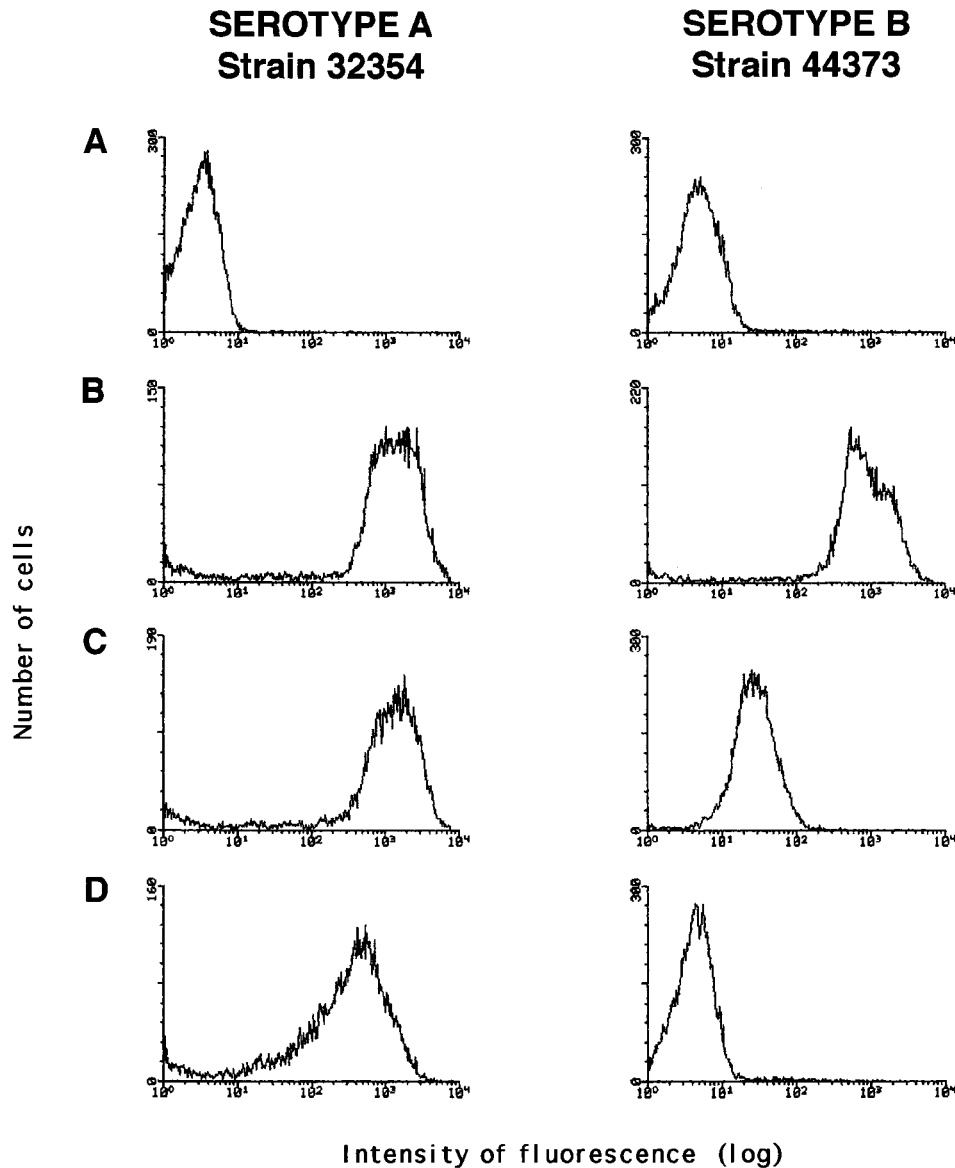


FIG. 1. Preparation of the preadsorbed serotype A-specific antiserum (CaA-B). The antiserum raised in rabbits following injection of heat-killed yeast cells from strain ATCC 32354 was rendered serotype A specific by successive adsorption with heat-killed serotype B yeast cells from strain ATCC 44373. The efficiency of adsorption was monitored by flow cytometry using a goat anti-rabbit immunoglobulin antibody coupled to DTAF. *C. albicans* cells of serotypes A and B were incubated in the absence of anti-*C. albicans* antiserum (A), with the anti-*Candida* antiserum prior to adsorption (B), with the anti-*Candida* antiserum adsorbed once (C), and with the anti-*Candida* antiserum after five successive adsorption steps (D).

isolation confirmed the presence of yeast cells from two strains of different serogroups. In four other cases, the serotype could not be conclusively determined since the serotyping results were contradictory: one isolate assigned to serotype A by slide immunofluorescence was later identified as serotype B by flow cytometry, while three strains originally assigned to serotype B by slide immunofluorescence were instead identified as serotype A by flow cytometry. These isolates were further studied in order to determine which serotyping method is more likely to be accurate when such discrepancies arise. Thus, we looked more closely at correlations between serotype and other phenotypic or genotypic traits of *C. albicans* strains that might give us indications of the actual serotypes of these four strains. First, a well-documented correlation between serotype and susceptibility to 5FC of *C. albicans* had been previously estab-

lished (19, 21). Briefly stated, most serotype A yeast strains are susceptible to 5FC while serotype B strains are mainly resistant to 5FC. Another correlation between serotype and a major dimorphism observed at the DNA level among *C. albicans* strains was also recently discovered (16). This dimorphism is due to the presence of a newly described self-splicing intron in the sequence coding for the 25S rRNA in 40% of *C. albicans* strains (14). A previous analysis revealed that all strains presenting the intron and having serotypes that could be unambiguously established were of serotype A. In contrast, intronless strains were mostly but not exclusively of serotype B (16). Among the strains presenting ambiguous serotyping results, three of these were assigned to serotype B by flow cytometric analysis but were assigned to serotype A by slide immunofluorescence assay. These three strains were shown to be intron-

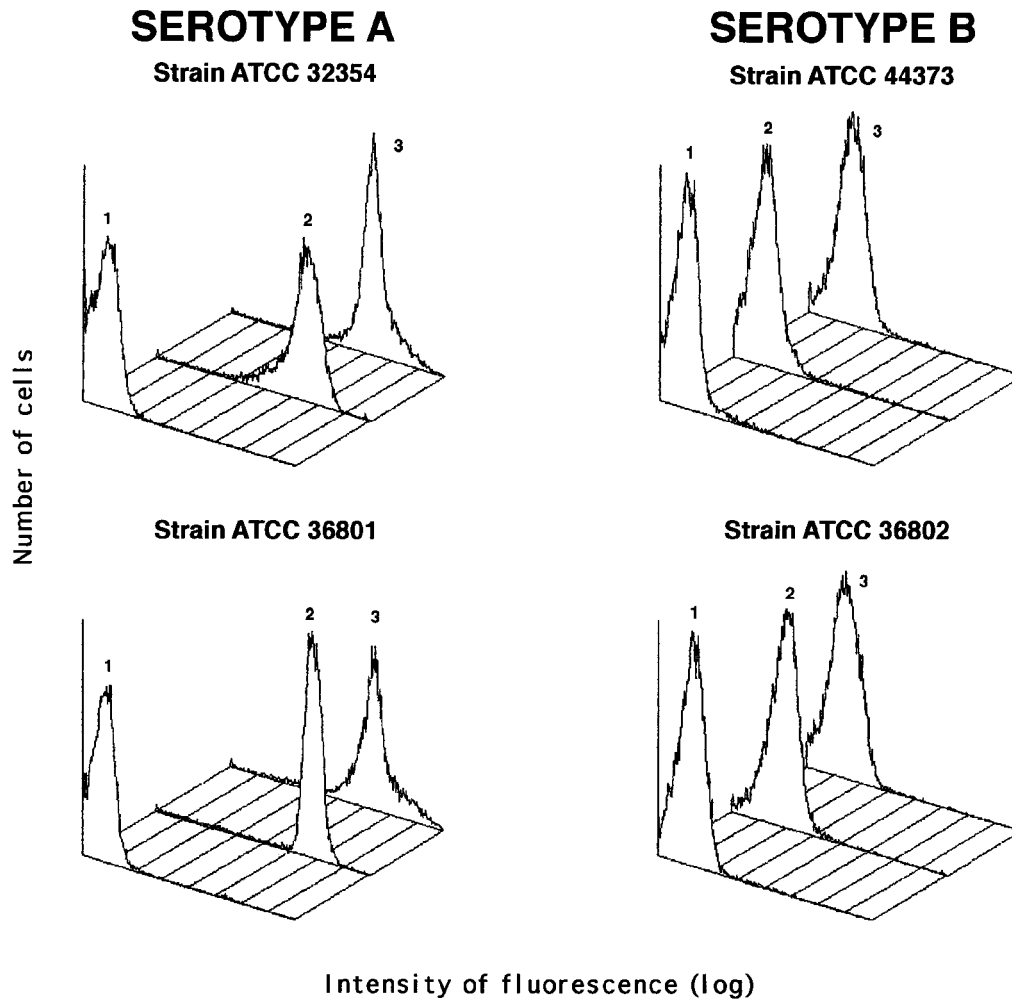


FIG. 2. Comparative analysis of serotyping results using IF6 and preadsorbed serotype A-specific antiserum (Ca\A-B). *C. albicans* cells from four collection strains of known serotype (serotype A, ATCC 32354 and ATCC 36801, and serotype B, ATCC 44373 and ATCC 36802) were analyzed by the flow cytometric serotyping procedure. The numbering of curves in each panel refers to different incubation conditions in the first step of the indirect procedure: 1, in the absence of antiserum (negative control); 2, in the presence of IF6 antiserum; 3, in the presence of the Ca\A-B antiserum.

less strains resistant to 5FC; since these characteristics are mostly associated with strains of serotype B, these results support the serotype B assignment obtained by flow cytometry rather than the result of slide immunofluorescence. Similarly, for the remaining strain, the assignment to serotype A obtained by flow cytometry seemed most likely since this strain was intron-bearing and susceptible to 5FC.

Application of the flow cytometric serotyping procedure to the epidemiology of candidiasis. Apart from validating the method, the analysis of *C. albicans* clinical isolates by the flow cytometric serotyping procedure was used to evaluate the usefulness of this method to study serotype distribution in *C. albicans* infections. In our study, a total of 123 clinical isolates from patients with either oropharyngeal or vulvovaginal candidiasis were analyzed by the flow cytometric serotyping procedure. Among both groups of strains, serotype A was predominant (Table 1). In the first group, *C. albicans* strains were isolated from the oropharynxes of 29 HIV-infected individuals and 69% of these isolates were assigned to serotype A. In the second group, 94 strains from patients with vulvovaginal candidiasis were analyzed by flow cytometry and 56.4% were of serotype A. This last panel of strains included 32 pairs of

isolates from consecutive episodes of recurrent vulvovaginal candidiasis. The relative distribution of serotype A and B strains in isolates from cases of recurrent vulvovaginal candidiasis was comparable to the relative distribution in the com-

TABLE 1. Flow cytometric analysis of serotype A and B prevalence in *C. albicans* strains isolated from patients with vulvovaginal or oropharyngeal infections^a

Type of strain ^b	No. of strains (% of total) of:	
	Serotype A	Serotype B
Vulvovaginal	53 (56.4)	41 (43.6)
Oropharyngeal	20 (69.0)	9 (31.0)
Total	73 (59.3)	50 (40.7)

^a Serotyping was performed with IF6 and Ca\A-B antisera with the same results.

^b Oropharyngeal isolates of *C. albicans* were obtained from HIV-infected individuals. Vulvovaginal isolates were obtained from otherwise healthy patients presenting no underlying risk factors for candidiasis.

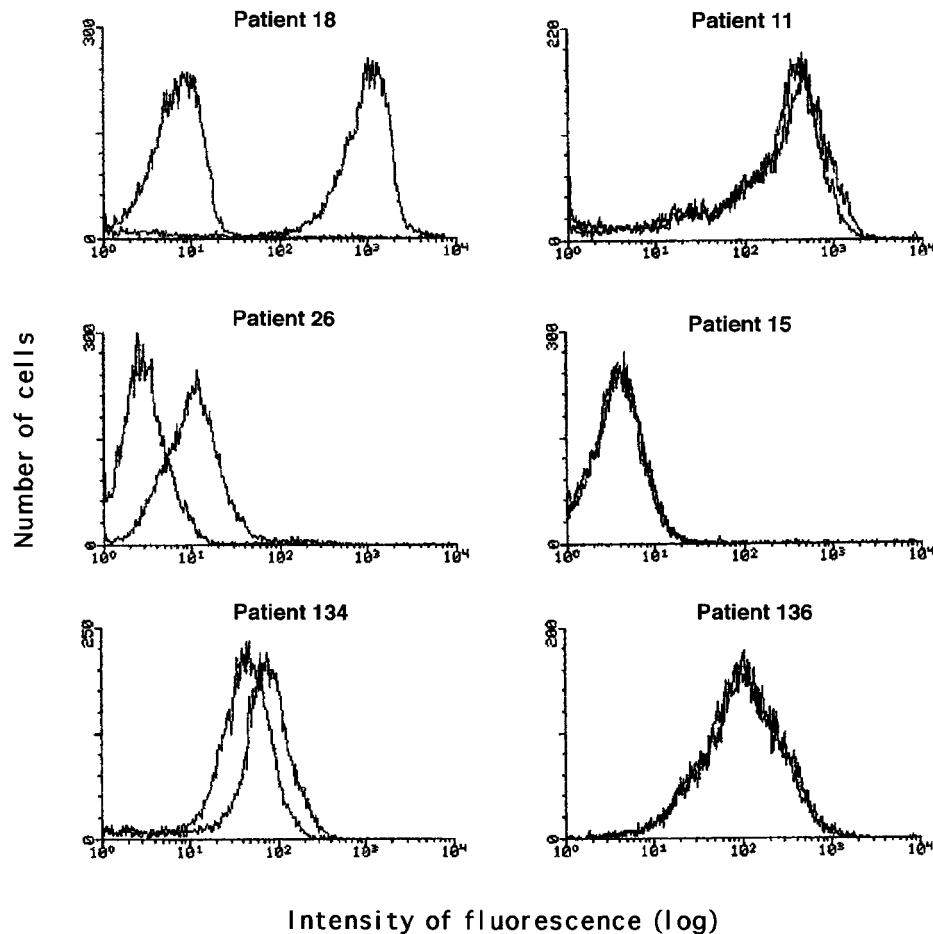


FIG. 3. Representative examples of serotyping results obtained with *C. albicans* isolates from patients with recurrent vaginitis. The relationship between isolates from consecutive episodes of vaginitis was evaluated by flow cytometry following labelling of yeast cells with IF6 antiserum. The curves in each panel represent the fluorescence patterns observed for both isolates from the same patient. The negative control was omitted in each panel in order to facilitate the comparison of curves.

plete panel of vaginal strains (data not shown). The same serotype was generally isolated from consecutive episodes of vaginitis, with the exception of three cases, in which we identified a change in the serotype of the infecting strain (for example, Fig. 3, patient 18). In addition, comparison of the monoparametric histogram outputs for isolates from each of two consecutive episodes of vulvovaginal candidiasis presenting the same serotype, revealed that their precise fluorescence patterns can sometimes be different (for example, Fig. 3, patients 26 and 134). When these results were compared with the previously reported results of biotyping by the triplet scoring procedure (20) and genotype analysis by hybridization pattern with a *C. albicans*-specific probe (15), it was observed that fluorescence patterns were a reflection of strain lineage. Strains with identical fluorescence patterns presented similar biotypes and genotypes, while isolates of the same serotype, exhibiting differences in their fluorescence patterns, also possessed different biotypes and genotypes.

DISCUSSION

Phenotyping procedures are still widely used to characterize clinical isolates of *C. albicans* on the basis of their distinctive assimilation, resistance, or antigenic properties. Although subdivision of *C. albicans* in two subgroups may seem of limited relevance, the serotyping procedure still contributes to our

ever-growing knowledge of the epidemiology of *C. albicans* infections. However, the value of serotyping as an epidemiologic tool was recently questioned following the publication of widely divergent results concerning serotype distributions among patients with candidiasis (21). This lack of interlaboratory reproducibility was attributed to the subjectivity involved in result interpretation and the reliance of investigators on widely divergent procedures and reagents (2). In an effort to circumvent these setbacks, we evaluated a novel serotyping procedure that relies on flow cytometric analysis.

The use of monoparametric histograms generated by flow cytometric analysis prevents subjective interpretations of results. Subjective interpretations may lead to incorrect serotype assignment when agglutination or slide immunofluorescence assays are used. Autoagglutination, known to generate high levels of false positives in agglutination assays with IF6 (2), had no adverse effect on the flow cytometric procedure, allowing serotyping of all strains examined.

In order to further evaluate the reliability of the flow cytometric procedure, 94 strains isolated from patients presenting vulvovaginal candidiasis were analyzed in parallel by a slide immunofluorescence serotyping assay with the preadsorbed serotype A-specific antiserum Ca\A-B. In this comparative analysis, the flow cytometric procedure appeared more precise than the slide immunofluorescence assay. In fact, the flow

cytometric method allowed us to detect the presence of two different strains in a supposedly pure culture and enabled the serotyping of four strains whose serotypes could not be determined by slide immunofluorescence. However, the serotypes of four other isolates remained questionable since results from both techniques were contradictory. Further analyses of these strains, using previously established correlations between serotype and other phenotypic or genotypic traits, suggest that flow cytometry is more reliable than slide immunofluorescence for serotype assignment.

The results of previous epidemiological studies of *C. albicans* have been questioned essentially because of reliability problems associated with serotyping procedures. We thus applied our new flow cytometric procedure as an alternative method to clarify the distribution of serotypes in two well-defined groups of *C. albicans* strains (15, 25). This study revealed that serotype distributions are similar (56.4% serotype A and 43.6% serotype B) in patients with single or recurrent episodes of vulvovaginal candidiasis. Accordingly, previous studies of vulvovaginal candidiasis reported that serotype B strains are more prevalent in North America than in Europe, where serotype A strains account for approximately 90% of vulvovaginal isolates (19). Our second group of strains consisted of clinical isolates from the oropharynxes of HIV-infected individuals. The distribution of serotypes in this group of patients remains controversial. Indeed, data were presented showing that serotype B isolates were more likely to be isolated from immunocompromised hosts while in other instances, strains of serotype A were shown to be predominant (4, 11, 21, 30). Our results tend to support these latter reports; however, they must be taken with caution since only a limited number of strains was studied.

Another application of the flow cytometric procedure was suggested by a closer look at multiple fluorescence patterns which revealed differences among strains of the same serotype. The observation that similar fluorescence patterns occur in numerous isolates from patients with consecutive episodes of vulvovaginal candidiasis suggests a close relationship among strains from the same patient. The value of the flow cytometric method was strongly supported when the serotyping results were compared with the previously established phenotypes and genotypes of these strains. Differences in fluorescence intensity patterns were observed only when isolates were evaluated as different by genotypic typing methods. The essentially identical fluorescence patterns observed in identical strains from the same patient were also further evidence supporting the reliability of the flow cytometric procedure. Differences in the exact patterns of fluorescence observed among different strains of the same serotype were likely to reflect differences in the expression levels of antigens at the surfaces of the cells. The capacity of the flow cytometric procedure to finely discriminate among strains would potentially be helpful, since the origin of the infecting strain is often questioned when clinicians encounter patients with repeated episodes of *Candida* infections. Moreover, in clinical settings, when a cytometer is available, the procedure is probably more cost efficient than a commercially available kit for serotype determination per se.

In conclusion, the flow cytometric procedure coupled to the widely used IF6 antiserum could certainly provide investigators with a reproducible, accurate and reliable tool to determine the serotype of *C. albicans* strains. The technique was used with success and with consistent results by three different investigators on our team. The technique was also performed in another center with a different cytometer (a FACScan from Becton Dickinson) with similar results. However, further evaluations of the interlaboratory reproducibility of this method

will be necessary before it can be routinely applied to answer questions about discrepancies observed between studies concerned with serotype distribution.

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