Comparison between Methods for Serotyping of *Candida albicans* Produces Discrepancies in Results

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Serotyping of 101 clinical isolates of *Candida albicans* was done with two sets of Hasenclever original anti-*Candida* typing sera (HSN 1 and 2) and Iatron *Candida* Check factor 6 typing serum (IF6). The results of these two methods were compared with slide agglutination reactions of yeast with monoclonal antibody H9. Agglutination reactions with this antibody have been previously shown to correlate with serotype. Results indicate the following correlations: between HSN 1 and HSN 2 serotyping, 93% ($\kappa = 0.85$; 95% confidence interval [CI], 0.70 to 0.99); between IF6 and HSN 1, 60% ($\kappa = 0.39$; 95% CI, 0.19 to 0.58); and between IF6 and HSN 2, 74% ($\kappa = 0.77$; 95% CI, 0.64 to 0.90). Results with HSN 1 and 2 antisera correlated with H9 reactivity at 85 and 89% ($\kappa = 0.88$; 95% CI, 0.75 to 1.00; and $\kappa = 0.85$; CI, 0.70 to 0.99, respectively), while agreement between IF6 and H9 reactivities was $\leq 64\%$ ($\kappa \leq 0.43$; 95% CI, 0.14 to 0.60). Autoagglutination of yeast during IF6 serotyping occurred with 21 of the 101 (20.8%) yeast strains. In every case, these yeast strains were serotyped by the HSN methods without autoagglutination and were uniformly type B. This study implies that it may not be possible to make valid comparisons between studies which compare serotype prevalence unless the same methods are used to serotype the yeast. The practicality and utility of serotyping in epidemiological studies are discussed, as are some of the problems associated with the available methods.

Candida albicans, a dimorphic fungus and a member of the human endogenous flora, continues to gain importance as a serious pathogen in immunosuppressed patients (23), notably those with diseases such as hematologic malignancies and AIDS. Little is understood about the pathogenesis and epidemiology of candidiasis, although not for lack of studies. In the immunocompromised host, most *C. albicans* infection is thought to be of endogenous origin (27), and recent studies suggest that this may be a valid assumption (26, 39), but without adequate typing systems, this has been difficult to prove. Although various efforts have been made to establish a typing system (15, 20-22, 25, 26, 28-30, 39, 43, 46), none have been widely used or are completely satisfactory.

Nearly 3 decades ago, Hasenclever and Mitchell developed a method to serotype *C. albicans* (15). On the basis of differences in as-yet-undefined surface antigens, this method defines two major serogroups, A and B (13, 15, 41). According to the classical (in vitro) concept of serotyping, type A antigens are absent from the surface of type B yeast (33). Serotype C, thought to represent a third, rare antigenic type ungroupable by Hasenclever methods, was proposed but never widely accepted (25). In 1969, Fukazawa (12a) and Kanno and Suzuki (19) developed a yeast identification scheme based on agglutination patterns with reference and monospecific antisera and sucrose fermentation and assimilation. Agglutination of *C. albicans* by 1 of the 10 antisera, antiserum 6, reportedly differentiates type A yeast strains from type B.

Independent studies in the United States and Europe completed between 1961 and 1981 showed that the prevalent (65 to 95%) serotype isolated from patients with candidiasis was type A (10, 14, 15, 27, 32). In 1982, a single U.S. survey of 398 *C. albicans* isolates from patients with unspecified illness showed that type A and B strains accounted for 50.7 and 49.3% of the isolates, respectively (40). More recently, we found that immunocompetent individuals were as likely to carry type A isolates as they were to carry B isolates as

oral flora but that immunocompromised individuals, including AIDS patients, were almost twice as likely to be infected with type B strains as type A strains (6). Other recent studies of serotype prevalence have been done in the United States, Europe, and Africa (9, 11, 22, 31, 39, 44; works of Saballs et al. and Torres-Rodriguez et al. cited in reference 31). Although it is clear from these studies that the prevalence of type B isolates is increasing, the results are not always in agreement and vary widely (from 0 to 75.9% of isolated strains) from laboratory to laboratory (31). Many of these studies have been confined to the determination of the predominant oral serotype in AIDS patients. The typing methods used among these and other studies are diverse and have included Hasenclever tube agglutination (6, 14), a slide agglutination technique modified from the method of Hasenclever (10, 40), serotyping with Iatron factor 6 rabbit antiserum (IF6) (27a, 33), an indirect immunofluorescence assay with anti-A antiserum (1), and monoclonal antibodies which have been shown to correlate with serotype (6, 36).

We have been intrigued by the marked variability in results from studies comparing the prevalence of type B strains among AIDS patients and have sought an explanation for the discrepancies. No thorough studies have been done to demonstrate that serotype results between methods are comparable. Therefore, we have done extensive comparisons among two sets of Hasenclever antisera, the H9 monoclonal antibody, and IF6 typing sera.

MATERIALS AND METHODS

Organisms and culture conditions. Of the 101 yeast strains, all were clinical isolates, 61 were recent oral or blood isolates from immunosuppressed bone marrow transplant patients at Fred Hutchinson Cancer Research Center (Seattle, Wash.), and 38 were oral isolates from AIDS patients and immunosuppressed patients which had been used in a previous study (6) and had been maintained in and retrieved from freezer stocks. Two isolates (Ca9938 and Ca105),

although originally clinical isolates, have undergone numerous subcultures as laboratory strains. The identification of all *C. albicans* isolates was confirmed by germ tube and chlamydospore production, as well as by yeast identification strips (API 20C; Analytab Products, Plainview, N.Y.). Primary cultures were plated on Sabouraud dextrose agar (SDA), and each *C. albicans* strain was subcultured to the appropriate medium for serotype testing as specified by each method (see below).

Hasenclever serotyping methods. Serotyping of all isolates was determined in duplicate by using Hasenclever and Mitchell's original 526 and 792 antisera (both rabbit anti-B serotype *Candida* antisera) and 207 and B311 antisera (both rabbit anti-A typing sera) in tube agglutination assays, as previously described (15). Sera had been stored at -20°C , and cross-absorptions in preparation for serotyping were done as previously described (6). Antiserum was serially diluted in 1% fetal bovine serum (final concentration). Fifty-five yeast strains were serotyped by using two sets of Hasenclever sera, which have been designated HSN 1 and HSN 2 for this study. HSN 1 typing was done by using 207 (anti-A) and 526 (anti-B) cross-absorbed antisera. The original agglutinin titers were 480 to 960 and 240, respectively. HSN 2 serotyping was done by using cross-absorbed B311 (anti-A) and 792 (anti-B) antisera (original titers, 480 to 960 and 480, respectively) (15). The absorbed anti-A antiserum retained the ability to agglutinate a known type A control strain, Ca9938, at 1:320.

Agglutination tests with monoclonal antibody H9. Agglutination of 89 of 101 yeast isolates by H9 antibody, a mouse monoclonal antibody, was done as previously described (6) after growth to stationary phase in glucose (2%)-yeast extract (0.3%)-peptone (1%) broth (GYEP) for 24 h at 37°C with rotation (180 rpm). Yeast cell suspensions were washed twice and resuspended in saline to 5×10^8 cells per ml for slide agglutination, and 25 μl of yeast cell suspension was mixed with 25 μl of 1:40 diluted H9 monoclonal antibody in mouse ascites (original titer, 1:8,000). Standardization of qualitative and semiquantitative slide agglutination tests was achieved with ring slides (Scientific Products, Inc., Detroit, Mich.), and reactions were scored from no evidence of agglutination compared with the saline control (–) to maximum agglutination (4+), as previously described (3).

IF6 typing system. Strains of *C. albicans* were prepared for slide agglutination serotyping with IF6 (Iatron Laboratories, Inc., Higashi-Kanda, Chiyoda, Tokyo, Japan) as suggested in the package insert. Yeast cultures were grown on peptone (1%)-yeast extract (0.5%)-glucose (2%)-agar (PYEG agar) for 48 h at 25°C , and a "small amount" (no further specifications on package insert) of the yeast cells was mixed directly with 1 drop (ca. 50 μl) of IF6 antiserum on a ring slide (as described above) and rotated for 1 to 2 min. Yeast strains which autoagglutinated when placed directly into antiserum (as recommended in the kit instructions) were washed three times in saline or distilled water and retested. As stipulated in the package insert, agglutination reactions were interpreted as "strong positive, moderate positive, weak positive, or negative" when compared with a saline control. Any degree of agglutination indicates presence of IF6 antigen (type A). The absence of agglutination indicates that the isolate is type B. To test whether the density of the yeast suspension affected interpretation of results, we made heavy suspensions (ca. 5×10^8 cells per ml) and light suspensions (ca. 1×10^8 cells per ml) prior to the addition of IF6 sera. To allow direct comparison of H9 and Iatron slide agglutination tests, we arbitrarily also chose to grade Iatron

positive agglutination from – to 4+ (described above for H9) as well as weak, moderate, or strong positive.

Expression of serotype antigen as a function of the growth medium and temperature of incubation. To examine whether changes in growth environment or nutrition affected agglutination and autoagglutination, 48 of the 101 yeast isolates were grown for 48 h at 25°C on SDA and PYEG agar, and agglutination tests with IF6 and H9 antibody were compared. Comparisons of IF6, H9, and HSN serotype results were also made among 16 strains grown to stationary phase (24 h) in PYEG broth at 37°C and 180 rpm. Except for changes in the culture medium and incubation temperature, other aspects of the slide and tube agglutination techniques for determination of serotype remained unchanged.

Statistical analyses of results. κ (11) and a 95% CI were calculated to compare the degree of agreement between two tests. κ is corrected for chance and ranges from -1 to 1 , where $\kappa = 1$ (-1) indicates perfect agreement (disagreement) and $\kappa = 0$ indicates agreement expected by chance alone. Two κ values are significantly different at the 0.05 significance level if their 95% CIs do not overlap.

RESULTS

Concordance between HSN 1 and HSN 2 serotype. The four high-titered Hasenclever anti-rabbit antisera used in this study had been produced in four different animals, had been previously tested by Hasenclever, and had been stored at -20°C . The sets of anti-A and anti-B antisera were paired at random for testing. The agreement between serotype results with antisera HSN 1 and 2 (92.7%) was comparable to the agreement between HSN 1 and H9 serotyping results (94.5%) but slightly better than the concordance between HSN 2 and H9 (86.5%) (Fig. 1B, D, and E and Table 1). However, because the CIs overlap, these percentages are not statistically different from one another, and the degree of agreement between typing tests with H9 and Hasenclever antisera was comparable and relatively high (Table 1).

Comparison of Hasenclever serotype and H9 agglutination. Comparisons between H9 agglutination and HSN serotype (Fig. 1B and D and Table 1) confirmed the strong ($\kappa = 0.77$ to 0.88) but not absolute correlation we have previously reported (8). In short, when cells were grown to stationary phase in GYEP broth, weak (1 to 2+) or, rarely, negative H9 reactivity correlates with type B, while strong H9 reactivity (3 to 4+) correlates with type A. However, neither HSN 1 nor HSN 2 serotype results correlated as strongly with H9 agglutination (94.5 and 86.5%, respectively) (Table 1) as had results with isolates from a previous study (98% [6]).

Comparisons between Hasenclever and Iatron serotyping results. Correlation between Hasenclever serotype results and Iatron results varied depending on the set of HSN antisera used, but because the CIs overlapped, the differences between HSN 1 and IF6 results and HSN 2 and IF6 results were not statistically significant (Fig. 1A and C and Table 1). κ indicates considerably more discrepancy in typing results between HSN 1 and IF6 typing methods (0.39) than between HSN 1 and H9 methods (0.88). When the 20 to 24% of the isolates that could not be tested by the IF6 methods (because they autoagglutinated in saline after growth on PYEG agar) are considered, agreement between these two serotyping methods was, at best, 74.2% ($\kappa = 0.39$ to 0.57) (Table 1). Washing yeast cells in saline or distilled water before testing did not alleviate autoagglutination. However, all of the yeast strains which autoagglutinated in slide tests after growth on PYEG agar serotyped as B strains

A. Hasenclever serotype (HSN 1) vs. Iatron (IF6) serotype ($\kappa = .39$)

	A ^{HSN1}	B ^{HSN1}	auto ^{HSN1}
A ^{IF6}	20	9	
B ^{IF6}		13	
auto ^{IF6}		13	

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B. Hasenclever (HSN 1) serotype vs. H9 agglutination ($\kappa = .88$)

	A ^{HSN1}	B ^{HSN1}
S ^{H9}	18	1
w ^{H9}	2	34

55

C. Hasenclever (HSN 2) serotype vs. Iatron serotype ($\kappa = .77$)

	A ^{HSN2}	B ^{HSN2}	auto ^{HSN2}
A ^{IF6}	48	5	
B ^{IF6}		27	
auto ^{IF6}		21	

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D. Hasenclever (HSN 2) serotype vs. H9 agglutination ($\kappa = .85$)

	A ^{HSN2}	B ^{HSN2}	auto ^{HSN2}
S ^{H9}	38	1	
w ^{H9}	9	41	
auto ^{H9}		2	

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E. Hasenclever (HSN 1) serotype vs. Hasenclever serotype (HSN 2) ($\kappa = .85$)

	A ^{HSN1}	B ^{HSN1}
A ^{HSN2}	19	4
B ^{HSN2}		32

55

F. Iatron serotype vs. H9 agglutination ($\kappa = .43$)

	A ^{IF6}	B ^{IF6}	auto ^{IF6}
S ^{H9}	38		
w ^{H9}	13	19	19
auto ^{H9}			

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G. Degree of agglutination: Iatron vs. H9 ($\kappa = .31$)

	S ^{IF6}	w/- ^{IF6}	auto ^{IF6}
S ^{H9}	30	10	
w/- ^{H9}	9	21	16
auto ^{H9}		2	1

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FIG. 1. Contingency tables of results from comparisons of Hasenclever, H9, and Iatron serotyping tests. The correlation between Hasenclever serotype results and H9 agglutination results is strong ($\kappa = 0.77$ to 0.88). Correlation between Hasenclever serotyping or H9 agglutination results and Iatron serotyping results is weaker ($\kappa = 0.39$ to 0.58). A maximum of 101 strains were compared, and the total number of strains tested in each comparison is given at the right lower corner of each contingency table. Abbreviations: A, serotype A; B, serotype B; auto, number of strains which autoagglutinated; S, strong agglutination reaction of yeast by H9 antibody; w, weak agglutination reaction with H9 antibody; w/-, weak or negative agglutination by H9 antibody; HSN 1, serotype results with Hasenclever antisera 207 (anti-A) and 526 (anti-B); HSN 2, serotype results with Hasenclever antisera B311 (anti-A) and 792 (anti-B); IF6, serotype results with IF6 typing serum.

in concomitant tests with PYEG broth-grown yeast and HSN antisera. When autoagglutinating strains were not considered, agreement between the two methods improved (78.6 and 93.8%) but discrepant results increased (21.4 and 6.2%) (Table 1).

Comparison of slide agglutination methods for serotyping: IF6 versus H9. Typing results determined by these two methods were in agreement for only 64% ($\kappa = 0.43$; 95% CI, 0.28 to 0.58) of the yeast isolates tested when the recommended Iatron interpretations were followed and the autoagglutinating strains were included in the calculations (Fig. 1F; Table 1). Because direct comparison of IF6 agglutination and H9 agglutination was difficult when criteria for interpretation of the Iatron kit were followed, we also interpreted IF6 agglutination by H9 standards, i.e., IF6 results were graded as negative or 1+ to 4+. Correlations did not improve when comparisons were made in this way ($\kappa = 0.31$;

95% CI, 0.14 to 0.48) (Fig. 1G; Table 1). Moreover, 27% (19 of 70) of the strains were strongly agglutinated by one antibody but weakly agglutinated by the other (data not shown), suggesting that the antigens which serve as the basis for serotyping with IF6 and H9 antibodies are not the same.

The results of IF6 serotyping were easier to interpret if a moderate (1×10^8 cells per ml) rather than a heavy (5×10^8 cells per ml) yeast suspension was tested. Weak positives were difficult to differentiate from negatives, and the degree of reactivity was less obvious when the inoculum was heavy (data not shown).

Medium comparison studies. We have previously shown that growth of *C. albicans* in various broths and agar induces variability in H9 reactivity (3) but has no effect on Hasenclever serotype (6). In this study, 7 of the 48 strains tested agglutinated more strongly (4+ versus 2+) after growth on SDA than after growth on PYEG agar (data not shown).

TABLE 1. Correlation between serotyping methods

Methods compared	n	% Agreement ^a	% Disagreement ^b	No. of reactions involving autoagglutination ^c	κ ^d
HSN 1 and IF6	55	60 (78.6)	16.4 (21.4)	24	0.39 (0.19 to 0.59)
HSN 1 and H9	55	94.5	5.5		0.88 (0.75 to 1.00)
HSN 2 and IF6	101	74.2 (93.8)	5.0 (6.2)	20.8	0.58 (0.46 to 0.70)
HSN 2 and H9	89	86.5 (88.5)	11.2 (11.5)	2.3	0.77 (0.64 to 0.90)
HSN 1 and HSN 2	55	92.7	7.3		0.85 (0.70 to 0.99)
IF6 (A/B ^e) and H9	89	64 (81.4)	14.6	21.4	0.43 (0.28 to 0.58)
IF6 (w/S ^f) and H9	89	57.3 (72.9)	21.5 (27.1)	19	0.31 (0.14 to 0.48)

^a Percent agreement = no. in agreement/total no. tested. The number in parentheses = no. of results that agree/(total no. tested - no. of autoagglutinating strains).

^b Percent disagreement = no. of discrepant results/total no. tested. The number in parentheses = no. of discrepant results/(total no. tested - no. of autoagglutinating strains).

^c Autoagglutination of yeast with either method that prevented interpretation of results and serotype comparisons.

^d $\kappa = I_o - I_e/1 - I_e$, where I_e = agreement observed and I_o = chance agreement. The number in parentheses is the 95% CI.

^e A/B, Serotypes A and B.

^f w/S, Weak and strong agglutination by H9 antibody.

However, type B strains, regardless of growth conditions, did not change from IF6 nonreactive (type B) to IF6 positive (type A), nor did reactors convert to nonreactors. Growth on PYEG agar, in PYEG broth, and on SDA did not significantly decrease autoagglutination (data not shown). Three *Candida* strains which were type B by HSN methods autoagglutinated during H9 agglutination after growth in GYEP broth (Fig. 1G).

DISCUSSION

As reported in a number of recent studies, the incidence of oral candidiasis caused by type B strains appears to be increasing in AIDS patients. However, striking variability between some studies has been noted (31). These differences may be related to as-yet-undefined host or fungal factors, differences in geographic distribution, or antifungal drug selective pressures. For instance, type B strains are known to be more resistant to flucytosine (2, 24). More work will be needed to determine the role that these factors play. However, variability may, at least in part, reflect discrepancies introduced by differences in serotype methodology. Particularly in Europe, where it is well accepted, several of the studies have used the IF6 antiserum for serotyping (31, 32). Although the Iatron Serological *Candida* Check kit has been used by some for identification purposes, commercially available biochemical as well as automated yeast identification systems have become the U.S. standard. One study has favorably compared the Iatron system with the API 20C system (Analytab, Plainview, N.Y.) (37) and reported serotype correlations on only 7 type B and 10 type A isolates.

In this study, we have found statistically significant discrepancies between results obtained with the Hasenclever serotyping tube agglutination techniques and with the IF6 serotyping results. In addition, two different lots of Hasenclever antisera did not produce uniform serotype results among all strains, but agreement between the two was still very good ($\kappa = 0.85$; 95% CI, 0.70 to 0.99). No explanation for the inconsistency between lots of HSN antisera is available, but it may be associated with the fact that the original antisera were produced in different animals. Incomplete cross-absorption prior to testing is not the source of the discrepancies, since when we reabsorbed the antiserum and retested the discrepant organisms, the results were the same (data not shown). To evaluate whether this lack of correlation reflected differences in agglutinin titers, lower dilutions

of HSN 2 antisera were tested. However, correlation between HSN 1 and 2 did not improve, because several of the yeast strains subsequently agglutinated in the autologously absorbed HSN 2 antisera, precluding serotype interpretation. Nonetheless, these sera produced comparable results in 51 of 55 instances (92.7%).

Correlation between IF6 and HSN 1 and IF6 and HSN 2 indicated significant disagreement between the two methodologies ($\kappa = 0.39$ and 0.58). H9 serotype results correlate well with HSN 1 and 2 results ($\kappa = 0.77$ and 0.88, respectively) but poorly with IF6 results ($\kappa = 0.43$), suggesting that these antisera do not recognize the same surface antigens. Our previous studies indicate that H9 antigen and Hasenclever type A antigen are not identical (6).

Attempts to compare results of serotype prevalence studies among laboratories may be jeopardized if different methods have been used to serotype strains. As shown here, perhaps as many as 24% of *C. albicans* isolates cannot be tested with IF6 antiserum because they autoagglutinate in saline when grown on the recommended medium. The Iatron kit offers no alternatives for testing strains which autoagglutinate. Growing yeast cultures in other broth or agar media, washing cells prior to agglutination, and allowing cells to grow for longer periods at 25°C did not reduce autoagglutination or improve correlations. In every instance, the yeast strains which autoagglutinated were type B as determined by Hasenclever typing and H9 agglutination. Consequently, if IF6 is used in serotype prevalence studies, the results may be slanted in favor of type A prevalence if the autoagglutinating isolates are removed from consideration for lack of an alternative serotyping method. In addition, care must be taken to include saline controls to identify autoagglutinating type B strains which would be misinterpreted as type A because they (auto)agglutinate in the presence of IF6 antiserum.

Serotyping with Hasenclever antisera also has drawbacks. In addition to lack of absolute concordance from lot to lot, antiserum production and cross-absorption is labor-intensive and expensive. Each serotyping test requires a set of 4 serial twofold dilutions per organism. At our institution, injection of live pathogenic organisms for the production of antisera (as required in Hasenclever's methods), except in very limited circumstances, is discouraged. With intravenous injections of heat-killed yeast, we have had no success in producing high-titered antisera whose reactivity and high titer withstand the rigorous cross-absorption techniques

required by the method. Also, use of certain adjuvants is in disrepute; others are expensive and untested in this circumstance. Recent studies which have focused on candidiasis in immunocompromised patients (particularly those with AIDS) have addressed the question of whether yeast strains with increased virulence have emerged as pathogens (6, 6a, 12, 17, 18, 20, 35, 38, 42, 45). In one study of *C. albicans* isolates from AIDS patients, it was concluded that these strains were not unique or hypervirulent (45). However, this assessment was based on antifungal drug susceptibility, biochemical differentiation, and DNA fingerprints of the yeast. Serotype associations were not considered. *C. albicans* surface antigens, known to be variably expressed (3–5, 7, 16, 33), are undoubtedly involved in immune recognition and adherence. Therefore, to conclude that serotype-related surface antigens are not related to virulence and pathogenicity may be premature. To determine whether a relationship between the increase in type B superficial infection or colonization and the increase in invasive infections exists warrants examination.

Effective tools to biotype strains and determine virulence are of primary importance in understanding the epidemiology and pathogenesis of candidiasis. The ability to divide *C. albicans* into only two serogroups is an ineffective epidemiological tool. Whether the classical concept of serotyping can be universally applied to *C. albicans* has been questioned because we and others (3–5, 33, 34) have reported antigenic variability in this organism during yeast phase and morphogenesis. Although we previously showed that variability of HSN serotype antigens does not occur during growth, the closely related H9 antigen is present in large quantities on serotype B cells in logarithmic phase but dramatically decreases in stationary phase (3, 6). Others have reported the possible synthesis of serotype A antigens by serotype B cells and suggest that serotypes may be a manifestation of phenotype, not genotype (for a review, see reference 33). On the other hand, biotyping, isoenzyme analysis, and karyotyping produce unwieldy numbers of groups. Therefore, until a better system of typing *Candida* isolates is developed, precise use of the methods presently available for comparison studies must be made.

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