Discriminant Analysis of Cellular Fatty Acids of *Candida* Species, *Torulopsis glabrata*, and *Cryptococcus neoformans* Determined by Gas-Liquid Chromatography

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We used discriminant analysis of cellular fatty acid compositions determined by gas-liquid chromatography to differentiate yeastlike fungi (a total of 190 strains; including 37 *Candida albicans* strains, 21 *Candida krusei* strains, 13 *Candida guilliermondii* strains, 37 *Candida tropicalis* strains, 10 *Candida pseudotropicalis* strains, 24 *Candida parapsilosis* strains, 32 *Torulopsis glabrata* strains, and 16 *Cryptococcus neoformans* strains). Previous results with a standard strain of *C. albicans* indicated that reproducible fatty acid chromatograms can be obtained with cells grown in a medium of 2% Sabouraud glucose agar at 35°C for between 48 and 72 h. These conditions were also maintained in cultures of the other organisms that we studied. The cellular fatty acid compositions of the organisms were determined quantitatively by gas-liquid chromatography and analyzed by discriminant analysis. The total correct identification expressed as relative peak percent was 95.8% (89.2% for *C. albicans* to 100% for *C. krusei*, *C. guilliermondii*, *C. pseudotropicalis*, *T. glabrata*, and *C. neoformans*). The total correct identification expressed as the common peak (palmitic acid) ratio was 94.7% (87.5% for *C. parapsilosis* to 100% for *C. pseudotropicalis*, *T. glabrata*, and *C. neoformans*). Both results suggest that cellular fatty acid compositions can be differentiated by this method.

Members of the genus *Candida* are indigenous human yeasts which colonize the skin and mucous membranes of healthy humans; but they may produce bloodstream invasion, thrombophlebitis, or opportunistic infections of the skin, urinary and respiratory tracts, and other organs. Recently, increasing numbers of compromised patients have led to concomitant rises in invasive fungal infections because, in part, of nosocomial infections and chemotherapy (3). It is therefore clinically important to identify *Candida* species other than *C. albicans* accurately and rapidly in the clinical laboratory. Identification methods for the genus *Candida* mainly include morphological, biochemical, and serological tests (3, 5).

Determination of cellular fatty acids (CFAs) of other clinical microorganisms, primarily bacteria, by gas-liquid chromatography (GLC) has been studied by Abel et al. (1) and Yamakawa and Ueta (17) as a means of identification. The development of computer systems has made possible the use of multivariate analysis as an identification method for differentiating CFAs of the organisms (2, 8, 13). Furthermore, investigators have designed a GLC method combined with a personal computer system, in which multivariate analysis is used as an objective identification method, to discriminate the CFA compositions of clinical microorganisms that were difficult to identify, that required extended growth periods, or that were excluded from routine studies because of questionable pathogenicity (11).

We report here the results of a stepwise linear discriminant analysis of CFA compositions of several members of the genus *Candida* (*C. albicans*, *C. krusei*, *C. guilliermondii*, *C. tropicalis*, *C. pseudotropicalis*, *C. parapsilosis*), *Torulopsis glabrata*, and *Cryptococcus neoformans* in which we used GLC and a modification of previously described pretreatment methods (10, 16).

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MATERIALS AND METHODS

Strains. A total of 190 strains of yeastlike cells were analyzed, including 153 strains selected from clinical isolates and 37 reference strains (Table 1). All strains were stock cultures maintained in the Department of Clinical Pathology, Hatanodai and Fujigaoka hospitals of Showa University; Teikyo Institute of Medical Mycology, Teikyo University; and the Department of Microbiology, Yamanashi Medical College. All clinical strains were identified by morphological tests (5), API 20C (Analytab Products, Plainview, N.Y.), and *Candida* Check (Iatrone Ltd., Tokyo, Japan) (14).

Culture. For CFA determinations, each organism was streaked onto a plate of 2% Sabouraud glucose agar (peptone was from Daigo Chemicals, Ltd., Tokyo, Japan, and agar was from Difco Laboratories, Detroit, Mich.) and grown at 35°C for between 48 and 72 h. In preliminary experiments to determine the effect of medium in varying the CFA composition, however, *C. albicans* TIMM0134 (Teikyo Institute of Medical Mycology) was grown on 2 and 4% Sabouraud glucose agar (2SA and 4SA, respectively) and 2 and 4% Sabouraud glucose agar with 0.5% yeast extract (Difco)-containing medium (2YSA and 4YSA, respectively) at 35°C for 24, 48, and 72 h and 1 week. The 2SA medium was found to have the least effect on CFA composition and was used as the experimental medium. *C. albicans* TIMM0134 was also grown on 2SA at 25°C for the same time periods.

Preparation of samples. The CFAs of each strain were methyl esterified by a previously described method (10). Briefly, three loopfuls (3-mm loop) of yeastlike cells were transferred from the plate to a screw-cap tube (Pierce Chemical Co., Rockford, Ill.), suspended in 0.5 ml of 0.5 M NaOH in absolute methanol, and incubated at 70°C for 5 min. After the tubes were cooled at 4°C for 5 min, 1 ml of a 50% (wt/vol) boron trifluoride–methanol complex (Nakarai Chemical Industries, Kyoto, Japan) was added and the mixture was incubated at 70°C for 5 min. After the tubes

TABLE 1. Strains used in this study

Species	No. of clinical isolates	Reference strains[a]
C. albicans	36	TIMM0134
C. krusei	11	IFO0011, -0012, -0013, -0839, -0840, -0841, -1063, -1064, -1162, and -1395
C. guilliermondii	10	IFO0679, -0837, -0838
C. tropicalis	36	TIMM0313
C. pseudotropicalis	3	TIMM0300, -0301, -0302, and -1307 IFO0617, -0942, -1065
C. parapsilosis	18	TIMM0292 and -0289 and IFO0585, -0640, -1068, and -1022
T. glabrata	32	
C. neoformans	7	TIMM0304, -0360, and -0362; NIH A68, B112, C18, and D52; CDC B3187; and CBS132

[a] Abbreviations: TIMM, Teikyo Institute of Medical Mycology; IFO, Institute for Fermentation, Osaka, Japan; NIH, National Institutes of Health, Bethesda, Md.; CDC, Center for Disease Control, Atlanta, Ga.; CBS, Centaalbureau voor Schimmelcultures, Baarn, The Netherlands.

were cooled at 4°C for 5 min, the mixture was transferred to a test tube which contained 0.5 ml of a water-saturated NaCl solution and was stirred gently. Then, 2.5 ml of a chloroform-normal hexane mixture (1:4; vol/vol) was added, and the test tube was agitated for 5 min. The mixture was centrifuged at 3,000 × g at room temperature for 5 min, and the upper phase was transferred to another test tube and evaporated to dryness in an evaporator (Pierce Chemical Co.) at 50 to 60°C under a stream of nitrogen gas. The residue was redissolved in 0.2 ml of chloroform containing 0.03% (wt/vol) arachidic acid ($C_{20:0}$; subscript numbers are carbon number:number of double bonds), with the latter serving as an internal standard for each run. The chloroform-arachidic acid mixture contained no detectable fatty acids for any of the organisms tested. Dissolved extracts were kept at 4°C until analysis of a 2-µl sample.

GLC. A gas chromatograph (163; Hitachi Industries, Tokyo, Japan) equipped with a flame-ionization detector was used for all analyses. A glass column (length, 2 m; internal diameter, 3 mm) was packed with Unisole 3000 on 80- to 100-mesh Uniport C (Gaschro Kogyou Ltd., Tokyo, Japan). The injector port, detector, and column oven temperatures were kept at 250, 150, and 210°C, respectively. Pure nitrogen gas was used as a carrier gas at a flow rate of approximately 45 ml/min, which corresponded to a retention time of the arachidic acid methyl ester of approximately 25 min. A Chromatopac C-R2AX (Shimazu Industries, Kyoto, Japan) was used as an integrator. The reporting integrator was set at an attenuation of 32 and a chart speed of 1 cm/min. The integrator printed out retention times and calculated the peak area.

Identification of chromatographic peaks. Each peak in CFA compositions was identified by comparison of its respective retention time with a reference to an external standard containing methyl esters of capric acid ($C_{10:0}$), lauric acid ($C_{12:0}$), myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), palmitoleic acid ($C_{16:1}$), hepatadecanoic acid ($C_{17:0}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:3}$), nonadecanoic acid ($C_{19:0}$), and arachidic acid ($C_{20:0}$). Individual CFAs in yeast-like cells were also identified by gas chromatography-mass spectrometry (JEOL, Tokyo, Japan) under previously described chromatographic conditions, and the resulting mass spectrometry patterns were interpreted by standard methods (4).

Data analysis. The peak areas of CFA compositions were expressed in two ways: (i) by relative peak percentage (RPP), i.e., the area of each of the CFA methyl esters divided by that of the total methyl esters, and (ii) by common peak ratio (CPR), i.e., the area of each of the CFA methyl esters divided by that of a methyl ester of a $C_{16:0}$ acid, which has a common peak in all organisms used in this experiment and in a variety of bacteria (2). The mean, standard error, and Student's t test values of CFA compositions (RPPs) of C. albicans TIMM0134 under various culture conditions were computed (Canon Ltd., Tokyo, Japan). To identify the fungi, CFA compositions expressed as both RPP and CPR were analyzed by stepwise linear discriminant analysis to select peaks and test their ability to discriminate each fungal species from the others. The discriminant function was calculated from the following equation:

$$D_k = \sum_{i=1}^{n} (x_{ik})b_i + b_{n+1}$$

where D_k is the discriminant function, x_{ik} is the point in the datum space (explanatory variables), b_i is the coefficient of the classification equation, and b_{n+1} is a constant. The basic program list of the discriminant analysis (15) was programmed on a computer (PC-98091VX; NEC, Tokyo, Japan) and used in this experiment. The details of the discriminant analysis have been described by Marriott (9).

RESULTS

Initially, because it is conceivable that the CFA composition depends on the growth parameters, we tested (five times) the effects of various culture conditions on C. albicans TIMM0134 growth parameters (Table 2). Statistically significant differences were observed in almost all compositions when growth at 35°C was compared with that at 25°C by using 2SA and in $C_{16:0}$ and $C_{17:1}$ acids when growth for 48 h was compared with that for 24 h by using 2SA, 4SA, 2YSA, and 4YSA ($P < 0.05$). However, almost no significant differences were observed when growth for 48 h was compared with that for more than 72 h with any medium. Statistically significant differences in CFA composition were partially observed when growth on 2SA was compared with that on 2YSA and 4YSA, but only slight differences were observed when growth on 2SA was compared with that on 4SA. Therefore, in order to obtain an invariable CFA composition of C. albicans TIMM0134, the culture conditions required a regular medium, a growing temperature of 35°C, and a growing time of between 48 h and 1 week. These conditions were also maintained in cultures of the other organisms that we studied. We used 2SA in the experiment, but T. glabrata and C. neoformans grew weakly when compared with the growth of the other organisms studied here. To resolve this problem, we will carry out future experiments with 2YSA, which supports a higher rate of growth than does 2SA alone.

Gas chromatographs of CFA compositions of Candida species, T. glabrata, and C. neoformans are shown in Fig. 1. The peaks of $C_{16:0}$ acid (black peak) shown in Fig. 1 were commonly included in the gas chromatographs of all organisms. There were marked differences in CFA compositions among the three genera Candida, Torulopsis, and Cryptococcus. The gas chromatograms obtained for each of the Candida species showed similarities; but there were a number of obvious quantitative differences in CFA compositions

TABLE 2. Effects of various culture conditions on CFA composition of *C. albicans* TIMM0134

Medium	Growth temp (°C)	Growth time	Significant difference in the following fatty acid compositions[a]:							
			$C_{16:0}$[b]	$C_{16:1}$	Unknown	$C_{17:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
2SA	25	24 h	●	●○	○	●○	○	●	●	●○
		48 h	○	○		○	○	○		○
		72 h	●○	●○	●○	●○	○	○		○
		1 wk	●○	●○	●○	●		●○	●○	○
2SA	35	24 h	●		●	●				
		48 h								
		72 h	●						●	
		1 wk	●							
2YSA	35	24 h	●○	○		●		○		○
		48 h			○	○		○		○
		72 h				○	○	○	○	○
		1 wk						○		○
4SA	35	24 h	●○			●				
		48 h								
		72 h	○	●						
		1 wk	●							
4YSA	35	24 h	○	○		●		○		○
		48 h	○		○	○		○		
		72 h			○	●		○	○	
		1 wk			○			○	○	

[a] The values (means ± standard errors) obtained from RPPs of CFAs were omitted from this table so that the data could be more easily understood. ●, Significant difference ($P < 0.05$) when growth for 48 h was compared with that for 24 h, 72 h, and 1 week on the same medium; ○, significant difference ($P < 0.05$) when growth on 2SA at 35°C was compared with that on 2SA at 25°C and on 2YSA, 4SA, and 4YSA at 35°C over the same growth period.

[b] Numbers to the left of the colon are the number of carbon atoms; numbers to the right of the colon are the number of double bonds.

among them, such as some with $C_{16:0}$, $C_{16:1}$, unknown, $C_{17:1}$, $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$ acids.

We analyzed both RPPs and CPRs of the CFA compositions of *Candida* species, *T. glabrata*, and *C. neoformans* by discriminant analysis. The results of discrimination expressed as RPP (190 strains) are given in Table 3. A set of eight explanatory variables was used for this analysis, consisting of peak areas of $C_{16:0}$, $C_{16:1}$, unknown, $C_{17:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$ acids. The total correct identification was 95.8% and ranged from 89.2% for *C. albicans* to 100% for *C. krusei*, *C. guilliermondii*, *C. pseudotropicalis*, *T. glabrata*, and *C. neoformans*. False-positive identifications ranged from 0% for *C. krusei*, *C. pseudotropicalis*, *C. parapsilosis*, and *T. glabrata* to 13.3% for *C. guilliermondii*.

The results of discrimination expressed as CPR (190 strains) are given in Table 4. A set of seven explanatory variables was used for this analysis, consisting of peak areas of $C_{16:1}$, unknown, $C_{17:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$ acids. The total correct identification was 94.7% and ranged from 87.5% for *C. parapsilosis* to 100% for *C. pseudotropicalis*, *T. glabrata*, and *C. neoformans*. False-positive identifications ranged from 0% for *C. krusei* and *T. glabrata* to 14.2% for *C. guilliermondii*.

RPP was incorrect for eight strains (*C. albicans* TIMM0134 and three clinical isolates, *C. tropicalis* TIMM0313 and one clinical isolate, and *C. parapsilosis* TIMM0829 and one clinical isolate). CPR was incorrect for 10 strains (*C. albicans*, two clinical isolates; *C. tropicalis*, three clinical isolates; *C. parapsilosis* TIMM0829 and two clinical isolates; *C. krusei*, one clinical isolate; and *C. guilliermondii*, one clinical isolate). Five incorrect strains (*C. albicans*, three clinical isolates; *C. tropicalis*, one clinical isolates; *C. parapsilosis* TIMM0829) were common to both measurements.

DISCUSSION

In our laboratory, we are studying the use of GLC to identify a variety of clinical organisms on the basis of extracted CFAs. Based on the observations of Gangopadhyay et al. (6), Lategan (7), and Moss et al. (12) that certain yeasts can be identified by GLC, we attempted to define the CFA compositions of *Candida* species, *T. glabrata*, and *C. neoformans* and to use these CFA profiles as a possible means of identifying yeasts through multivariate analysis of their RPPs and CPRs.

We chose a stepwise linear discriminant analysis method because it is suited to a smaller number of explanatory variables and requires approximately 20 strains to form a statistically valid group. With smaller groups of organisms, there is the risk of spurious discrimination (9). Therefore, there was difficulty in discriminating three species, *C. guilliermondii* (13 strains), *C. pseudotropicalis* (10 strains), and *C. neoformans* (16 strains), although the correct identifications of each strain were higher (Tables 3 and 4). The reliability of identification improves as more strains in each species are analyzed. Since the total correct identifications expressed as RPP and CPR were high, 95.8 and 94.7%, respectively, it not only appears that these values are sufficient to discriminate between species but it is also suggested that both CPR and RPP are suitable for discriminant analysis.

Based on our observations with *Moraxella catarrhalis* (10), it is conceivable that the CFA composition of yeasts might depend on their growth parameters. Consequently, we determined the effects of various culture conditions on the CFA composition of replicate cultures of *C. albicans* TIMM0134 (Table 2). Statistically significant differences in CFA composition were observed when growth on 2SA was
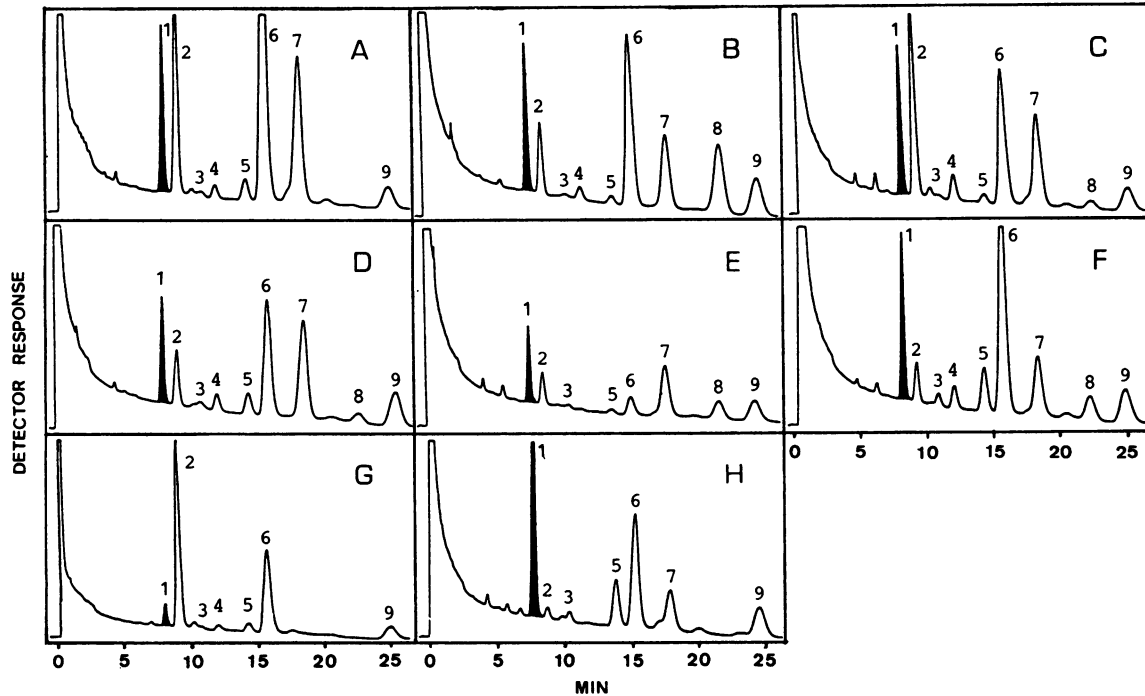
FIG. 1. Gas chromatograms of CFA compositions of *C. albicans* TIMM0134 (A), *C. krusei* IFO0011 (B), *C. guilliermondii* IFO0679 (C), *C. tropicalis* TIMM0313 (D), *C. pseudotropicalis* TIMM0302 (E), *C. parapsilosis* TIMM0292 (F), a *T. glabrata* clinical isolate in the Fujigaoka Hospital (G), and *C. neoformans* TIMM0304 (H). Peak numbers were as follows: 1, $C_{16:0}$ acid (close peak); 2, $C_{16:1}$ acid; 3, unknown; 4, $C_{17:1}$ acid; 5, $C_{18:0}$ acid; 6, $C_{18:1}$ acid; 7, $C_{18:2}$ acid; 8, $C_{18:3}$ acid; and 9, $C_{20:0}$ acid as an internal standard.

compared with that on 0.5% yeast extract-containing medium, and when growth at 37°C was compared with that at 25°C. In contrast, statistically significant differences were rarely observed when growth for 48 h was compared with that for 72 h and 1 week. These data indicate that a consistent incubation temperature and medium as well as growth for 48 h to 1 week are necessary for obtaining highly reproducible CFA chromatograms for *C. albicans* TIMM0134. This observation extended to other *Candida* species, *T. glabrata*, and *C. neoformans* as well.

Lategan (7) showed that it is possible to characterize *C. krusei*, *C. parapsilosis*, *C. tropicalis*, *C. albicans*, and *C. guilliermondii* by determining the volatile fatty acids produced in the culture medium; but more species and isolates must be studied for conclusive results. We showed that characterization is also possible in *T. glabrata* and *C. neoformans* by determining the CFAs that are nonvolatile

fatty acids. Moss and colleagues (12) were able to divide a variety of groups (GI, *Saccharomyces* species; GII, *T. glabrata*; GIII, most *Candida* species; GIV, *Cryptococcus* species, *Rodotorula* species, and *C. curva*) on the basis of large quantitative differences in CFA compositions. Data for our organisms corresponded to those for groups GII, GIII, and GIV as defined by Moss and colleagues (12).

Results of our analysis of yeast CFAs expressed as either CPR or RPP were similar, with the number of explanatory variables reduced from eight in the latter to seven in the former. RPP and CPR gave 8 and 10 incorrect results, respectively. These results indicate that these two ways of interpreting chromatograms correspond closely with each other. However, the results also indicate that complete discrimination is limited by discriminant analysis, as all strains for which incorrect results were obtained were correctly identified by usual methods (5).

TABLE 3. Discrimination results expressed as RPP of CFA compositions of yeastlike cells

Group[a]	No. of strains	No. of correct identifications	% Correct identifications	No. of strains classified as follows[a]:							
				G1	G2	G3	G4	G5	G6	G7	G8
G1	37	33	89.2	33	0	2	2	0	0	0	0
G2	21	21	100	0	21	0	0	0	0	0	0
G3	13	13	100	0	0	13	0	0	0	0	0
G4	37	35	94.6	2	10	0	35	0	0	0	0
G5	10	10	100	0	0	0	0	10	0	0	0
G6	24	22	91.7	0	0	0	1	0	22	0	1
G7	32	32	100	0	0	0	0	0	0	32	0
G8	16	16	100	0	0	0	0	0	0	0	16

[a] G1, *C. albicans*; G2, *C. krusei*; G3, *C. guilliermondii*; G4, *C. tropicalis*; G5, *C. pseudotropicalis*; G6, *C. parapsilosis*; G7, *T. glabrata*; G8, *C. neoformans*. The percentages of false-positive identifications, expressed as [number of incorrect identifications/(number of correct identifications + number of incorrect identifications)] × 100, were 5.7, 0, 13.3, 7.9, 0, 0, 0, and 5.9 for groups G1 through G8, respectively. The percent total correct identification, expressed as (number of total correct identifications/total number of strains) × 100, was 95.8.

TABLE 4. Result of discrimination expressed in CPR of CFA compositions of yeastlike cells

Group[a]	No. of strains	No. of correct identifications	% Correct identifications	No. of strains classified as follows[a]:							
				G1	G2	G3	G4	G5	G6	G7	G8
G1	37	35	94.6	35	0	0	2	0	0	0	0
G2	21	20	95.2	0	20	1	0	0	0	0	0
G3	13	12	92.3	0	0	12	0	0	0	0	0
G4	37	34	91.9	2	0	0	34	0	0	0	1
G5	10	10	100	0	0	0	0	10	0	0	0
G6	24	21	87.5	0	0	1	1	1	21	0	0
G7	32	32	100	0	0	0	0	0	0	32	0
G8	16	16	100	0	0	0	0	0	0	0	16

[a] See footnote a of Table 3 for group definitions. The percentages of false-positive identifications (calculated from the equation given in footnote a of Table 3) were 5.4, 0, 14.2, 8.1, 9.1, 4.5, 0, and 5.9 for groups G1 through G8, respectively. The percent total correct identification (calculated from the equation given in footnote a of Table 3) was 94.7.

Our results suggest that an identification method based on CFA compositions with computer analysis of the data by multivariate analysis could potentially differentiate almost all *Candida* species, *T. glabrata*, and *C. neoformans*, extending the number of organisms that can be identified by discriminant analysis. It appears that this is a powerful method for rapidly obtaining more accurate identifications when the results of usual methods (5) are doubtful. Therefore, this procedure may be of value for diagnostic centers handling a large number of cultures (12).

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