Functional Evaluation of <Mycotube> Roche, A New Kit for Taxonomical Identification of Pathogenic Yeasts

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A functional evaluation was made on <Mycotube> Roche for identifications of pathogenic and naturally occurring yeasts. When 10 species (40 strains), whose ID codes are described in CCIS index and very frequently isolated from patients with mycoses, were examined, 30 strains were simply and correctly identified by Mycotube alone or with supplementary tests including Mycoslide, while new ID codes were proposed to set for 4 species. Furthermore, examination of 19 clinically isolated strains showed that the identification of 18 strains by each hospital coincided with our simple Mycotube identification, whereas 1 strain was corrected to *Cryptococcus neoformans* by our tests. Mycotube tests of 28 species (66 strains) of yeasts not described in CCIS led to the setting of new ID codes for 24 yeast species frequently occurring in nature. The items for supplementary tests were set forth in order to differentiate from strains having the same ID code to reach the final identification.

Recent advances in chemotherapy have no doubt played important roles for decreasing serious infectious diseases caused by strong pathogens. On the other hand, opportunistic infections caused by weak pathogens like *Candida albicans*, *Torulopsis* sp. and *Cryptococcus neoformans* in cases of mycoses have rather increased, which had been little noted before^{1,2)}. Since the identification for these pathogenic yeasts are very complicated, time-consuming and requires empirical knowledges³⁾, it is still not easy in an ordinary clinical bacteriology laboratory to handle these yeasts aiming at a full identification. Saruta *et al.*⁴⁾, Yamazaki *et al.*⁵⁾ and others reported serological methods for identification of *Candida* species isolated from patients with mycoses. For these methods, however, it is a troublesome prerequisite to prepare antibodies covering every species that may be encountered in clinical scenes. In these cir-

cumstances, development of a new simple identification method has been desired for these yeasts.

We previously reported the functions and applicability of Enterotube^{6,7)} and OXI/FERM Tube⁸⁾, whose clinical use are now in full swing in Japan as a simple identification kit for Enterobacteriaceae and non-fermentative Gram-negative rods. Recently, a simple identification kit for yeasts, <Mycotube> Roche, having the same principle as the above kits, has been developed by Hoffmann-La Roche AG. This paper concerns to evaluate how simple and accurate the identification of pathogenic yeasts by this kit is and also describes its application to identification of a variety of yeasts widely occurring in nature.

MATERIALS AND METHODS

Strains used. Standard type culture strains of yeasts employed in the present study were either purchased newly from IFO and CBS or those stocked in our laboratory. Clinical isolates were obtained from University of Tokyo, Hok-

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kaido University, Niigata University, Keio University, Chukyo Hospital, Nobeoka Prefectural Hospital and Komagome Metropolitan Hospital. All strains have been maintained by oil overlaying method followed by a periodical culture transfer with malt agar at 37°C or 30°C.

The content and handling methods of kit, <Mycotube> Roche. The appearance and operation method of <Mycotube> Roche is similar to Enterotube^{6,7)} and OXI/FERM Tube⁸⁾. As shown in Fig. 1, 8 different media are included in 8 compartments and an inoculation needle is penetrated in the center of this tube through the compartments.

After grown on malt agar slant for 24 hours

at 30°C, the cells of each strain were inoculated into all compartments of Mycotube by picking them up with the right tip of the inoculation needle and passing it through all the compartments. The inoculation needle was immediately reinserted back into the first 4 compartments and then broken at a notch by bending, and the screw cap was replaced. After incubation for 2-4 days at 30°C, the positive or negative judgement for the production of acidic or alkaline substances after sugar assimilation were made by examination for color changes due to bromothymol blue or phenol red contained each compartment (Fig. 1) and fermentations of glucose and sucrose were determined by gas formation between wax overlay

Pick up this ID value from CCIS system

7310 *Saccharomyces cerevisiae*

Fig. 1. Appearance of Mycotube and coding of the reaction followed by CCIS reading.

and the basal agar medium embedded. The results obtained were codified as shown in Fig. 1.

Identification methods of yeasts with CCIS (Computer Coding of Identification System).

The code of four digits obtained from the example in Fig. 1 was compared to code number listed in CCIS index. This strain was identified as *Saccharomyces cerevisiae* by comparison of ID value. The CCIS index describes 30 codes for 13 species (7 genera) of common pathogenic yeasts¹⁰⁾. If plural species of yeast are described under the same ID code, supplementary tests based on morphological criteria such as the formation of pseudomycelium, true mycelium, chlamydospores, arthrospores and blastospores as well as on the biochemical reactions are provided for the final identification. The biochemical performance to be auxiliarily tested includes the fermentation and assimilation of various sugars and nitrogen sources and the cycloheximide sensitivity. Cycloheximide sensitivity was examined by <Myco-slide> Roche which consists of *side 1*; malt agar for incubation of yeasts, *side 2*; malt agar containing cycloheximide, and *side 3*; CLED agar for detection of bacteria, in a similar fashion as for <Urotube> Roche¹¹⁾. For the identification of some strains, especially those frequently occurring in nature but rarely found in clinical specimens, other supplementary tests including morphological examination were sometimes carried out.

RESULTS

Reproducibility of Mycotube reaction of each compartment.

In order to examine the reactivity of each compartment of Mycotube, 6 standard yeast strains were selected so as to give at least a positive reaction in each compartment and examined using 5 tubes per strain from 1 to 7 days. Those include *Candida albicans*, *Candida tropicalis*, *Candida pseudotropicalis*, *Rhodotorula* sp., and *Saccharomyces uvarum*. The macroscopic observation of Mycotube made either downwards or laterally gave the identical result for color change in each compartment, suggesting no problem for judgement of Mycotube reaction.

Results summarized in Table 1 indicate a good reproducibility with only minimal differences

being noted among individual tubes.

In the compartment of glucose, 5 strains showed a positive reaction at an early stage of culture. *Rhodotorula* (Strain 4) showed correctly a negative reaction for 2–4 days, but changed to a weakly positive reaction at 7 days. For gas production from glucose, all strains except *Rhodotorula* gave a positive reaction even at day 1 or 2, while *Rhodotorula* showed no reaction for 1–7 days. In the compartment of xylose, all strains showed a positive reaction: 5 strains reacted rapidly, but *Rhodotorula* was only weakly positive at 4–7 days. In No. 2 tubes for Strain 6, xylose reaction was changed from positive to negative after 2 days culture due to pH changes by further metabolism, which was judged as positive. In the compartment of sucrose, 5 showed a positive reaction from an early culture stage as expected and *Rhodotorula* was judged as negative, although 2 gave a weakly positive color at day 4. In the test of gas production from sucrose, 2 (Strain 2 and 6) showed a positive reaction from an early culture and 2 (Strain 3 and 4) indicated no reactivity. The other 2 strains (Strain 1 and 5) showed a somewhat delayed reaction where 1 out of 5 tubes was judged as negative. In the raffinose compartment, 2 strains showed a positive reaction and 4 strains gave correctly no reaction at all. Although Strain 2 changed the color to bluish due to a pH change to alkali, no problem was encountered in the final judgement for the reaction. In the compartment of lactose, 3 strains gave no reaction at all and a strain (Strain 6) gave a positive reaction as expected. Another lactose positive Strain 5 showed a delay in the reaction: 4 days observation was needed to be positive. A negative Strain 3 turned to positive in 2 out of 5 tubes tested. In the compartment of trehalose, a positive strain (Strain 2) and 3 negative strains reacted correctly. The other 2 strains showed a delayed reaction in 4 out of 5 tubes. Two strains changed the color of this compartment into bluish, though no trouble was recognized in the proper judgement. No problem was seen for citrate and urea compartments in which *Rhodotorula* gave a positive reaction and the other 5 strains showed properly a negative reaction.

As mentioned above, the range of deviation

Table 2. Effect of incubation temperature on Mycouteb reaction

Strain	Temp.	Tube	Glucose		Glucose gas production		Xylose		Sucrose		Sucrose gas production		Raffinose		Lactose		Trehalose		Cirtate		Urea	
			Days		Days		Days		Days		Days		Days		Days		Days		Days		Days	
			1	2	3	4	7	1	2	3	4	7	1	2	3	4	7	1	2	3	4	7
Strain 1	30	A*	●	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	○	○	○	○
	37	1 2	●	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	○	○	○	○
	26.5	1 2	●	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	○	○	○	○
Strain 2	30	A	●	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	○	○	○	○
	37	1 2	●	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	○	○	○	○
	26.5	1 2	●	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	○	○	○	○
Strain 3	30	A	●	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	○	○	○	○
	37	1 2	●	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	○	○	○	○
	26.5	1 2	●	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	○	○	○	○
Strain 4	30	A	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
	37	1 2	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
	26.5	1 2	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Strain 5	30	A	●	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	○	○	○	○
	37	1 2	●	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	○	○	○	○
	26.5	1 2	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Strain 6	30	A	●	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	○	○	○	○
	37	1 2	●	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	○	○	○	○
	26.5	1 2	●	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	○	○	○	○

* A: Average from Fig. 1. Abbreviations of Strain 1-6 and marks were shown in Fig. 1.

among Mycotube reactions was found remarkably narrow when the judgement was made during 2–4 days of culture as designated in the guideline for determination of Mycotube reaction¹⁰⁾. In certain compartments, some differences among tubes were recognized especially in the case of compartments exhibiting a weakly positive reaction. However, these deviations hardly affected the final identification by CCIS index (see the next chapter).

Effect of temperature on the reactivity of each compartment of Mycotube.

Table 2 compares Mycotube reaction observed at 26.5° and 37°C compared with that at 30°C for 6 strains. Reactions at 26.5° and 37°C in the glucose assimilation and gas production thereof, and sucrose assimilation were the same as that at 30°C. In the gas production from sucrose, 3 strains (Strain 3, 4 and 6) showed the same reaction at all temperatures, while with Strain 1, 2 and 5, the reaction turned negative at either 26.5° or 37°C, suggesting an optimal reactivity at 30°C. In the compartments of raffinose, citrate and urea, all strains showed the same reaction at all three temperatures. In the compartment of lactose, although 4 strains gave the same reaction at all temperatures, Strain 5 showed a positive reaction at 37°C and negative at 26.5°C, and Strain 3 showed no reaction at both 26.5° and 37°C. In the compartment of trehalose, 5 strains gave the same reaction at all temperatures, while Strain 1 became negative at 37°C. Thus, Mycotube was shown an optimal reactivity at 30°C.

Accuracy for identification of yeasts by Mycotube reaction through CCIS coding.

The reactions thus obtained can lead to identification of yeasts by coding into ID value by the method described in Materials and Methods, and then by comparing the value with CCIS index for known ID values¹⁰⁾. In some cases supplementary tests were needed for the final identification. The accuracy of this identification using the Mycotube system was examined with 40 strains from type culture collection, which had been previously identified correctly by conventional methods, and with 19 strains of new clinical isolates identified by university and

hospital laboratories.

Identification of type culture strains.

Identification of 40 strains of type culture collection by ID code from Mycotube reactions and supplementary tests is summarized in Table 3. Five strains of *Candida albicans* were correctly identified by Mycotube-CCIS system in which 3 strains needed supplementary testing. Four strains of *Candida guilliermondii* showed ID value 5111, which was directly indicated as *C. guilliermondii* in CCIS. ID value for 6 strains of *Candida parapsilosis* was 7101 that indicated *C. parapsilosis* in CCIS without supplementary test. Six out of 7 strains of *Candida tropicalis* showed ID value 7340 which was indicated as either *C. tropicalis* or *C. albicans* in CCIS. These strains were identified as *C. tropicalis* by the positive trehalose reaction and cycloheximide sensitivity by Mycoslide test. One strain (IFO 0589) of *C. tropicalis* showed ID value 7341 which was not described in CCIS. Furthermore, this strain showed properties different from other 6 strains of *C. tropicalis* because of the delay in trehalose reaction (positive only at 4 days) and cycloheximide resistance.

One of 2 strains of *Candida krusei* gave ID value 3100 correctly in 2–3 days culture, which, however, turned later into ID 7100 due to a positive xylose reaction at day 4. Another strain showed ID value 7100. The former can be identified as *C. krusei* by ID code 3100 and pseudomycelium formation. However, the latter as well as the later code for the former strain did not coincide with ID code listed in the index for *C. krusei*. Therefore, we propose that a new code 7100 should be set forth in CCIS for the *C. krusei* code. The fact that *C. albicans* also gave ID value 7100 in CCIS requires a supplementary test for the final identification: *C. albicans* and *C. krusei* could be easily differentiated by Mycoslide test because *C. krusei* was sensitive to cycloheximide.

Two strains of *Candida stellatoidea* showed ID value 7100 which did not coincide with ID value for *C. stellatoidea* listed as 5100 in CCIS. ID value 7100 which adds the positive gas production from glucose to 5100 may well be listed as a new ID code for *C. stellatoidea*. *C. stellatoidea* can be distinguished from most *C. albicans* and *C. krusei*, both possibly having the same ID value

Table 3. Identification of type culture strains by Mycotube-CCIS system

Test organisms* (A)	No. of strains tested	ID value obtained	Supplementary tests needed	Species finally identified by Mycotube system (B)	Coinci- dence ratio (B/A)	ID value for (A) listed in CCIS
<i>Candida albicans</i>	2	7140	pseudomycelium	<i>C. albicans</i>	100%	5140, 7300
	1	7100	—	<i>C. albicans</i>		7100, 7340
	1	7300	—	<i>C. albicans</i>		7140, 7350
	1	7340	cycloheximide +	<i>C. albicans</i>		7150
<i>Candida guilliermondii</i>	4	5111	—	<i>C. guilliermondii</i>	100	5111, 5151
<i>Candida parapsilosis</i>	6	7101	—	<i>C. parapsilosis</i>	100	1101, 7101 5101
<i>Candida tropicalis</i>	6	7340	cycloheximide — Trehalose, early	<i>C. tropicalis</i>	85.7	7340, 7350
	1	7341		None		
<i>Candida krusei</i>	1	3100	pseudomycelium	<i>C. krusei</i>	50	3100
	1	7100**		<i>C. albicans</i>		
<i>Candida stellatoidea</i>	2	7100**		<i>C. albicans</i>	0	5100
<i>Candida pseudotropicalis</i>	2	7330	cycloheximide +	<i>C. pseudotropicalis</i>	100	7330
<i>Saccharomyces cerevisiae</i>	5	7310	—	<i>S. cerevisiae</i>	100	3310, 7700 7310
<i>Torulopsis glabrata</i>	1	3160	—	<i>T. glabrata</i>	50	1100, 1140
	1	7160**		None		3100, 3140 3160, 5140 7140
<i>Trichosporon cutaneum</i>	1	0003		<i>Cryptococcus</i>	0	1000
	3	4023**		None		
	1	5023		None		

* Species identified by conventional methods.
** These ID values were proposed for a new ID code.

7100, by positive coagulation test of litmus milk. On the other hand, the fact that some strains of *C. albicans* can be positive in this test requires further tests for clear identification. *C. stellatoidea* can be differentiated from these strains by negative sucrose assimilation and galactose fermentation test. Two strains of *Candida pseudotropicalis* showed ID value 7330 which was also attributable to *C. pseudotropicalis* and *Torulopsis sphaerica* in CCIS. *C. pseudotropicalis* was finally identified by differentiating from *T. sphaerica* with cycloheximide resistance.

All strains of *Saccharomyces cerevisiae* showed a single ID value 7310, which fits exclusively to the

value for *S. cerevisiae* in CCIS and hence no supplementary test was needed. *Torulopsis glabrata* showed ID values 3160 and 7160. The former can be identified as *T. glabrata* by ID code alone. ID value of the latter is obtained by adding a positive lactose reaction to ID code 7140 listed for *T. glabrata* in CCIS. Therefore, ID value 7160 should be set as a new ID code for lactose-positive *T. glabrata*.

Trichosporon cutaneum gave ID values 0003, 4023, and 5023 which were remarkably different from the listed ID code 1000 for *Trichosporon* in the index. Since 3 out of 5 strains showed ID value 4023, this ID value should be set as a new ID code for

Table 4. Identification of pathogenic isolates by Mycotube-CCIS system

Group	Species*	No. of strains	ID value obtained	Cycloheximide resistance by Mycoslide	Species finally identified
A	<i>Candida albicans</i>	8	7100	+	<i>Candida albicans</i>
B	<i>Candida albicans</i>	1	1103	—	<i>Cryptococcus neoformans</i>
C	<i>Crypt. neoformans</i>	4	1003	—	<i>Cryptococcus neoformans</i>
D	<i>Crypt. neoformans</i>	2	5113	—	<i>Cryptococcus neoformans</i>
E	<i>Crypt. neoformans</i>	1	1103	—	<i>Cryptococcus neoformans</i>
F	<i>Crypt. neoformans</i>	3	0003	—	<i>Cryptococcus neoformans</i>

* Species identified by original hospitals.
Mycotube tests were examined at 37°C.

Table 5. Assimilation and fermentation tests of clinically isolated yeasts on sugars

Pathogenic yeasts	Assimilation test				Fermentation test	
	Inositol	Galactitol	Rhamnose	Glucose	Glucose	Maltose
B	+	+	(+)	+	—	—
C	+	+	+	+	—	—
D	(+)	+	+	+	—	—
<i>Candida albicans</i>	—	—	—	+	+	+

1. B, C, and D correspond to those of pathogenic yeasts in Table 4.
2. After inoculation of yeast in 10 ml of medium which consisted of 0.67% Yeast Nitrogen Base and 0.5% sugar, each tube was incubated for 5–21 days at 25°C. Results of assimilation were obtained by measurement of OD 600 nm Spectronic 20.
Fermentation of sugars were tested in 10 ml of medium, containing Durham tube, which consisted of 0.18% yeast extract, 0.3% polypeptone, sufficient amount of Bromothymol blue and 2% sugar. After incubation of 5–25 days at 25°C, production of air bubble in Durham tube gave a positive reaction in fermentation test.

T. cutaneum.

Identification of clinically isolated *C. albicans* and *Cryptococcus neoformans*.

Table 4 shows the test results on 9 clinically isolated strains of pathogenic *C. albicans* and 10 strains of *Cryptococcus neoformans* identified by each university and hospital. Eight strains of *C. albicans* showed ID value 7100 to give their final identification by the code in CCIS (A in Table 4). Cycloheximide resistance gave supportive evidences for the identification.
The strain described in B in Table 4 showed ID value 1103, which was considered as *Cryptococcus neoformans* by ID code as well as by cycloheximide sensitivity. This identification was further confirmed by examination with morphological criteria as well as assimilation and fermentation

tests on sugar as shown in Table 5.
One strain (E in Table 4) and 3 strains (F in Table 4) of *Cryptococcus neoformans* showed ID values 1103 and 0003, respectively, which were identified as *Cryptococcus neoformans* by ID code, together with cycloheximide sensitivity. Four strains (C in Table 4) and 2 strains (D in Table 4) showed ID values 1003 and 5113, respectively. Although these ID values are not described in CCIS, they should be included newly as ID codes for *Cryptococcus neoformans*, because their assimilation and fermentation tests on sugars (Table 5) together with other characteristics have proven that these belong to *Cryptococcus neoformans*.
Examination of Mycotube identification for non-pathogenic yeasts not listed in CCIS index.

Table 6. New ID values proposed for various naturally occurring yeasts

Organisms	No. of strains tested	ID values obtained	Cycloheximide resistance by Mycoslide	New ID value proposed
<i>Candida brumptii</i>	2	0000	—	
<i>Candida valida</i>	2	0000	—	
<i>Hansenula holstii</i>	1	0000	+	
<i>Pichia membranaefaciens</i>	3	0000	—	
	1	0002		
<i>Candida lipolytica</i>	1	0003	+	0003
<i>Rhodotorula glutinis</i>	2	0002		0002
<i>Hanseniaspora uvarum</i>	1	0000	+	0100
	1	0100		
<i>Candida zeylanoides</i>	1	0000	—	1000
	1	1000	+	1001
	1	1001	+	
<i>Saccharomyces rouxii</i>	4	3000	—	3000
	1	0000	—	
<i>Leucosporidium scottii</i>	2	4003	—	4003
<i>Rhodotorula</i> sp.	1	4003	—	4003
<i>Rhodotorula rubra</i>	1	4003		0003
	1	0003		
<i>Rhodotorula minuta</i>	2	4003	—	4003
<i>Cryptococcus laurentii</i>	1	4002	—	4002
	1	4032	—	4032
<i>Metschnikowia pulcherrima</i>	1	5100	—	5100
<i>Torulopsis candida</i>	1	5100	—	5100
	1	5110	—	5110
	2	5140	—	5140
	2	5150	—	5150
<i>Debaryomyces hansenii</i>	1	5101	—	5101
	1	5121	—	5121
<i>Cryptococcus albidus</i>	1	5043	—	5043
	1	0000	—	
<i>Pichia farinosa</i>	2	7100	—	7100
<i>Hansenula californica</i>	2	7100	—	7100
<i>Candida utilis</i>	2	7300	—	7300
<i>Candida intermedia</i>	1	7301	—	7301
	1	7311	—	7311

Organisms	No. of strains tested	ID values obtained	Cycloheximide resistance by Mycoslide	New ID value proposed
<i>Hansenula saturnus</i>	6	7310	—	7310
<i>Hansenula anomala</i>	2	7310	—	7310
<i>Saccharomyces diastaticus</i>	2	7310	—	7310
<i>Saccharomyces pretoriensis</i>	1	7310	—	7310
<i>Kluyveromyces fragilis</i>	1	7310	+	7310
	1	7330	+	7330
<i>Kluyveromyces lactis</i>	1	7330		7330
<i>Saccharomyces uvarum</i>	2	7330	—	7330

Mycotube tests were examined at 30°C except *Leucosporidium scotii*, which was incubated at room temperature.

It is obvious that there must exist a large number of yeast species not described in the present CCIS and yet frequently occurring in nature or used in the fermentation industry. In order to know whether any of them possess the identical ID code with the above mentioned pathogenic yeast or, if any, how to differentiate those in the identification of clinical strains, the Mycotube reaction was examined for 66 strains of 28 species (Table 6).

Candida brumptii, *Hansenula holstii* and *Candida valida* showed ID value "0000". *Pichia membranaefaciens* showed either ID value "0000" (3 strains) or ID value 0002 (1 strain). ID value of *Rhodotorula glutinis* was 0002 which was differentiated from *Pichia membranaefaciens* by production of carotenoid pigments. *Candida lipolytica* showed ID value 0003 which was finally identifiable when the formation of true mycelium was observed. *Hanseniaspora uvarum* showed ID values of 0000 and 0100. *Candida zeylanoides* showed ID values of 0000, 1000 and 1001. In the case of ID 1000, *C. zeylanoides* was distinguished from *Trichosporon* by its failure of arthrospore formation.

Since 4 out of 5 strains of *Saccharomyces rouxii* showed ID value 3000 and one strain showed ID value of 0000, a new ID code "3000" would be

reasonably set up for *S. rouxii*. *Leucosporidium scotii*, *Rhodotorula* sp., *Rhodotorula rubra* and *Rhodotorula minuta* gave the identical ID value of 4003. *L. scotii* was differentiated from *Rhodotorula* genera by failure of carotenoid pigment production. Differentiation between *R. rubra* and *R. minuta* was possible by the fact that the former could assimilate maltose and raffinose, while the latter could not. One strain of *R. rubra* showed ID value 0003 but was distinguished from *Cryptococcus neoformans* of the same ID value by production of carotenoid pigments. *Cryptococcus laurentii* showed ID values 4002 and 4032.

Metschnikowia pulcherrima (ID 5100), *Torulopsis candida* (ID 5100, 5110, 5140, 5150), *Debaryomyces hansenii* (ID 5101, 5121) and *Cryptococcus albidus* (ID 5043, 0000) were detected as strains showing ID value of 5000 level. *M. pulcherrima* was distinguished from other strains having the same ID value of 5100, by production of pulcherrimin, while *Torulopsis candida* was distinguished from them by failure of pseudomycelium and arthrospore formation. *Debaryomyces hansenii* (ID 5101) was differentiated from *C. parapsilosis* by the formation of ascospores. *Torulopsis candida*, which showed ID value 5140, was differentiated from *Torulopsis glabrata* by positive growth at 40°C.

Table 7. Differentiation by proposed supplementary tests among species having the same ID values

ID code	Possible species	Supplementary tests proposed
0003	<i>Cryptococcus neoformans</i> <i>Candida lipolytica</i> <i>Rhodotorula rubra</i>	cycloheximide —, carotenoid pigments — pseudomycelium, true mycelium carotenoid pigments
1000	<i>Candida zeylanoides</i> <i>Trichosporon</i>	arthroconidia — arthroconidia +
4003	<i>Leucosporidium scottii</i> <i>Rhodotorula rubra</i> <i>Rhodotorula minuta</i>	carotenoid pigments — carotenoid pigments +, mal. & raff. assim. carotenoid pigments +, mal. & raff. not assim.
5100	<i>Candida stellatoidea</i> <i>Geotrichum candidum</i> <i>Metschnikowia pulcherrima</i> <i>Torulopsis candida</i>	cycloheximide +, pseudomycelium, chlamydospore cycloheximide —, arthroconidia + cycloheximide —, pulcherrimin + cycloheximide —, pseudomycelium —, chlamydospore —
5101	<i>Candida parapsilosis</i> <i>Debaryomyces hansenii</i>	pseudomycelium ascospores
5140	<i>Torulopsis candida</i> <i>Torulopsis glabrata</i>	no growth at 40 °C positive growth at 40 °C
7100	<i>Hansenula californica</i> <i>Pichia farinosa</i> <i>Candida krusei</i> <i>Candida albicans</i> <i>Candida stellatoidea</i>	nitrate & sucrose assim., ascospores nitrate & sucrose not assim., ascospores galactose not assim., ascospores — nitrate not assim., sucrose assim. litmus milk coagulation, sucrose not assim. galactose not ferm.
7300	<i>Candida albicans</i> <i>Candida utilis</i>	cycloheximide + cycloheximide —
7310	<i>Saccharomyces cerevisiae</i> <i>Saccharomyces diastaticus</i> <i>Saccharomyces pretoriensis</i> <i>Kluyveromyces fragilis</i> <i>Hansenula anomala</i> <i>Hansenula saturnus</i>	nitrate, lactose & sol. starch not assim. galactose ferm. nitrate & lactose not assim., sol. starch assim. galactose not ferm. closely similar to <i>S. cerevisiae</i> and <i>S. diastaticus</i> nitrate not assim., lactose assim. cycloheximide + nitrate assim., hat-shaped ascospores nitrate assim., saturn-shaped ascospores
7330	<i>Saccharomyces uvarum</i> <i>Kluyveromyces fragilis</i> <i>Kluyveromyces lactis</i> <i>Torulopsis sphaerica</i> <i>Candida pseudotropicalis</i>	cycloheximide — cycloheximide +, positive growth at 40 °C, ascospores cycloheximide +, no growth at 40 °C, ascospores cycloheximide +, no growth at 40 °C ascospores — cycloheximide +, positive growth at 40 °C ascospores —, pseudomycelium

Abbreviations: mal. maltose
raff. raffinose
assim. assimilation
ferm. fermentation

Table 8. Comparison of ID value between perfect and imperfect state of yeasts

Perfect form	Imperfect form
<i>Pichia membranaefaciens</i> ID: 0000 and 0002	<i>Candida valida</i> ID: 0000
<i>Debaryomyces hansenii</i> ID: 5101 and 5121	<i>Torulopsis candida</i> ID: 5100, 5110, 5140 and 5150
<i>Kluyveromyces lactis</i> ID: 7310 and 7330	<i>Torulopsis sphaerica</i> ID: 7330
<i>Kluyveromyces fragilis</i> ID: 7330	<i>Candida pseudotropicalis</i> ID: 7330

Both strains of *Hansenula californica* and *Pichia farinosa* gave ID value 7100. These two strains can be led to final identification by supplementary tests since the former can, and the latter can not, assimilate nitrate, although these strains can form ascospores. *Candida utilis* showed ID value 7300 which was differentiated from *C. albicans* by cycloheximide sensitivity. *C. intermedia* showed both ID values 7301 and 7311. *Saccharomyces diastaticus*, *S. pretoriensis*, *Kluyveromyces fragilis*, *Hansenula saturnus* and *H. anomala* showed ID value 7310. The final identification of all these strains by single supplementary test is too complicated to differentiate their characteristics resembling each other, but possible by combination of the following facts: *S. diastaticus* does not assimilate both nitrate and lactose but does soluble starch; *K. fragilis* is resistant to cycloheximide; *H. saturnus* can assimilate nitrate and form saturn-shaped ascospores; *H. anomala* can assimilate nitrate and form hat-shaped ascospores. As *S. pretoriensis* was closely similar to *S. cerevisiae* and *S. diastaticus*, a simple identification method among these strains could not be obtained.

Saccharomyces uvarum, *Kluyveromyces fragilis* and *K. lactis* gave ID value of 7330. Differentiation among these can be made by the supplementary tests: *S. uvarum* is sensitive to cycloheximide, whereas *K. fragilis* is resistant to cycloheximide, can grow at 40°C and forms ascospores. *K. lactis* is resistant to cycloheximide and can not grow at 40°C and forms ascospores.

New ID codes obtained from above results are summarized in right column of Table 6. If plural species give the same ID code, simple sup-

plementary tests for further differentiation among them are proposed as in Table 7. As shown in Table 8, it is noted that yeasts representing either perfect or imperfect forms of the identical species have a tendency to give the identical or very similar ID value.

DISCUSSION

Identification by Mycotube of 10 species (40 strains) of yeast type culture and 2 species (19 strains) of clinically isolated yeasts was examined in detail. *C. albicans*, *C. guilliermondii*, *C. parapsilosis*, *C. pseudotropicalis* and *S. cerevisiae* were clearly identified by ID code alone or ID code plus specified supplementary tests. One of 2 strains tested of *C. krusei* gave ID value of 7100 which indicated only *C. albicans* in CCIS index. This value may be registered as a new ID code for *C. krusei*.

Six out of 7 strains of *C. tropicalis* were correctly identified by ID value plus supplementary tests, but one strain showed a different reaction in the citrate compartment of Mycotube and the specified supplementary test. *C. stellatoidea* showed ID value 7100 which indicates the additional gas production from glucose to the ID code 5100 listed in CCIS. This value 7100 was proposed as a new code of *C. stellatoidea*. *T. glabrata* gave ID values 3160 and 7160 which indicated a positive lactose reaction in addition to ID codes 3140 and 7140 listed for *T. glabrata* in CCIS. Therefore, ID value 7160 is also to be set up as a new code for *T. glabrata*. *Trichosporon cutaneum* gave ID values 0003, 4023 and 5023 which were remarkably different from ID code 1000 for *Trichosporon*. ID value 4023 among these values is to be set up as a new code for *T. cutaneum*.

Eight strains of *C. albicans* and 10 strains of *Cryptococcus neoformans* isolated from clinical sources were also identified correctly as such by the Mycotube system. A clinical isolate turned out to be *Cryptococcus neoformans* by Mycotube with the aid of Mycoslide test. Furthermore, ID values 1003 and 5113 thus obtained are considered to be listed newly in CCIS as new codes for *Cryptococcus neoformans*. Examination for the effect of temperature on Mycotube reaction suggested that 30°C would probably be the optimal tem-

perature for growth of yeasts.

All of the above results indicate that Mycotube system would serve as a rapid, simple, enough accurate and useful identification tool for pathogenic yeasts, although further improvement is desirable for CCIS index. For the identification of some species, supplementary tests were necessary where the cycloheximide sensitivity was most useful. For this test, Mycoslide that was originally devised as a selective isolation plate of yeasts from clinical materials was found very useful in terms of convenient versus accuracy.

Number of ID codes set in the present CCIS (only for 16 species) is by no means sufficient. This is especially true when one intends to use Mycotube for the identification of a wide variety of yeasts occurring in nature. Furthermore, examination by Mycotube of yeasts that were not listed in CCIS, might lead to misidentification when the same ID value for different strains are listed in CCIS. In order to overcome this problem, 28 species of yeasts belonging mainly to genera *Saccharomyces* and *Candida* widely distributing in nature as reported by Lodder¹²⁾, Soneda¹³⁾ and Banno¹⁴⁾ were examined for Mycotube reactions aiming at the setting of new ID values. New ID codes thus obtained as well as supplementary tests necessary for the final identification (Table 6 and 7) from these results enable to identify a total of 40 species by adding 33 new ID codes in CCIS in a simplified fashion that need not any profound knowledges in yeast taxonomy. If the validity of these new codes is confirmed and the CCIS index will be thus improved, Mycotube test would also be applicable for identification of important industrial and naturally occurring yeasts, in addition to clinical isolates. In the identification of these species, certain supplementary tests were in most cases needed to differentiate among species having the identical ID values (Table 3 and 4). Nevertheless, we consider that the simplicity and time saving by the use of Mycotube still outstands the conventional methods.

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