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Germ Tube Formation of *Candida albicans* in Corn Meal Broth Using the Non-Slip Slide Glass Incubation Method

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***Candida albicans* IFO 1385 yeast cells dominantly develop germ tubes in a dilute corn meal broth at 37˚C. For germ tube (GT) development the broth concentration is optimal at 75% of the original, and the inoculum is optimal at 10^5 cells/mL. Incubation procedures were compared and the non-slip slide glass incubation (NSSI) method was found to be more available than the tube incubation method or slid-covered slide glass incubation method. The GT formation was completed in 1 to 2 h in the NSSI method and clear-cut GTs were observed under optical microscopy. GTs were formed in all 111 test-strains of *C. albicans* but not at all in other species of *Candida*. Therefore, under optimal conditions, the rate of identifying *C. albicans* is 100%, demonstrating that GT detection with the NSSI method is a rapid and reliable identification method. The humid NSSI method is suited for continuous observation of a GT. The initially formed GT elongated to resemble a true hypha, attaching no chlamydoconidium. The hypha subsequently dissociated yeast-like cells.**

Key words: *Candida albicans*; corn meal broth; germ tube formation; non-slip slide glass incubation method

Since the germ tube (GT) is a characteristic morphology observed only in *C. albicans*, confirmation of GT is available as a rapid method for identifying *C. albicans*. GT formation was first reported by Reynolds and Braude (1956). They observed yeast cells transforming into filamentation (germ) tubes in human blood, sera, plasma and cerebrospinal fluids. Since then, many substances were reported as GT inducers, such as serum (Taschdjian et al., 1960; Mackenzie, 1962; Andleigh, 1964; Dolan and Ihrke, 1971), egg white (Buckley and Uden, 1963; Andleigh, 1964), *N*-acetylglucosamine (Simonetti et al., 1974), proline (Holmes and Shepherd, 1987), ethanol (Pollack and Hashimoto, 1985) and peptone (Joshi et al., 1973a, 1973b). Among them, serum is utilized as the most potent GT inducer in laboratory studies. However, all the present methods for GT induction have been, so far, time-consuming when the urgent identification of *C. albicans* is required. Thus, efforts to evaluate or improve the medium to achieve rapid GT induction has not yet ended.

Corn meal medium is usually used for the formation of pseudohyphae or chlamydoconidium of *Candida* with the slide culture method, in which fungi are inoculated onto a small piece of corn meal agar medium placed on a slide glass for culturing. Therefore, observing GT in this agar medium did not seem rational. However, the use of corn meal broth made it possible to induce high rates of GT formation, which led to the development of a rapid clinical mycological method for identifying *Candida*. In this study, using corn meal broth as the medium for GT formation, simple and rapid practical conditions were evaluated to obtain high rates of GT formation by establishing an optimal concentration of inoculating fungal solution or an optimal concentration of corn meal broth.

Abbreviations: GT, germ tube; NSSI, non-slip slide glass incubation; T_{50}, time for germ cells to reach at 50% of original; TI, tube incubation

Materials and Methods

Strains of Candida

C. albicans IFO 1385 was used as the standard strain. In addition, the following strains of *Candida* isolated and identified from clinical specimens were also used in this study: 111 strains of *C. albicans* and other species of *Candida* including 8 strains of *C. tropicalis*, 9 of *C. glabrata*, 4 of *C. parapsilosis*, 5 of *C. guilliermondii*, 2 of *C. lusitaniae*, 1 of *C. kekyr*, 1 of *C. krusei* and 1 of *C. lypolitica*. Fresh fungi cultured overnight at 30˚C with Sabouraud 4% dextrose agar were inoculated into the corn meal broth.

Medium

The corn meal broth was prepared as follows: corn meal agar medium (Nissui Seiyaku Co. Ltd., Tokyo, Japan) was modified to prepare the broth; it was mixed with cold distilled water, and continuously stirred overnight at 6˚C in a cold room. On the next day, insoluble components were removed by filtration, and the filtrate was autoclaved for use as an undiluted corn meal solution.

Method of GT formation

Fresh fungi were inoculated into various concentrations of the corn meal broth to establish a fungal suspension. That is, one loopful of fungi was mixed with a 0.2 mL phosphate buffered solution to create a dense fungal solution. Then, this was mixed with 1 mL broth to create a fungal suspension for incubation. The concentration thus prepared was approximately 10^6 cells/mL (Nakamoto, 1992). Moreover, various concentrations of inoculating fungal solutions were prepared based on this formula for preparing a fungal solution.

The 2 different incubation methods were comparatively evaluated, that is, the non-slip slide glass incubation (NSSI) method and the tube incubation (TI) method. The NSSI method was carried out as follows: 50 μL of the above fungal solution was placed on a sterile slide glass, and the slide glass was placed in a wet Petri dish without a cover glass. The Petri dish was further sealed with a vinyl bag, and the fungi were incubated at 37˚C. Meanwhile, the TI method was carried out as follows: 1 mL of the same fungi specimen was transferred to a test tube and dipped in a 37˚C-water bath for incubation.

Cultured specimens were collected at various time points, and fungal morphology was observed in an optical microscope to calculate the number of cells with extruding GT per 100 given yeast cells and the rate of GT formation. Moreover, the GT formation rate determined in 50% calf serum solution was used as the control.

Results

Conditions for GT formation

Figure 1 shows GT formation rates when 10^6 cells/mL of fungi were suspended in various concentrations of corn meal broth and incubated at 37˚C. The maximal GT formation rate was obtained by culturing in 75% corn meal broth, because of undiluted or overdiluted broth resulting in unsatisfactory GT formation. Figure 2 shows GT formation rates when 10^4–10^6

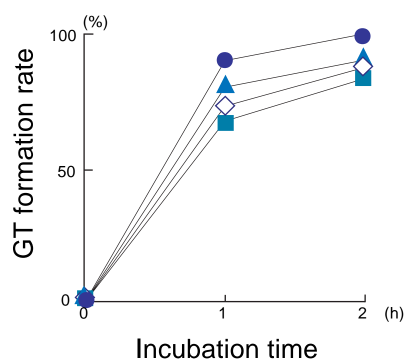

Fig. 1. The mean GT formation rate in various concentrations of corn meal broth. Seven strains of *C. albicans* including the standard strain (10^6 cells/mL each) were inoculated into various concentrations of corn meal broth, and incubated by the NSSI method at 37˚C. ◇, 25%; ▲, 50%; ●, 75%; ■, 100%.

cells/mL fungal solutions were incubated in 75% corn meal broth or 50% calf serum. When 10^5 or 10^6 cells/mL fungi were incubated for 1 h in 50% calf serum, GT formation rate was 100% in both concentrations of fungal solutions. When 10^5 cells/mL fungi were incubated in 75% corn meal broth, GT formation rates were 91% after a 1-h incubation and 100% after a 2-h incubation. Meanwhile, when 10^4 cells/mL fungi were incubated in 75% corn meal broth, the number of fungi in specimens was too small to observe, and the results could not be calculated.

Morphogenesis

Figures 3a and b show fungal morphology when 10^5 cells/mL fungi were incubated at 37°C for 2 h by the NSSI method in 75% corn meal broth or 50% calf serum. There were no morphological differences between fungi incubated with both media, and synchronized GT extrusions from yeast cells were observed. Figures 3c and d show fungal morphology when the same specimens were incubated by the NSSI method for 20 h. GTs formed in corn meal broth continuously elongated becoming similar to true hyphae measuring several times as long as

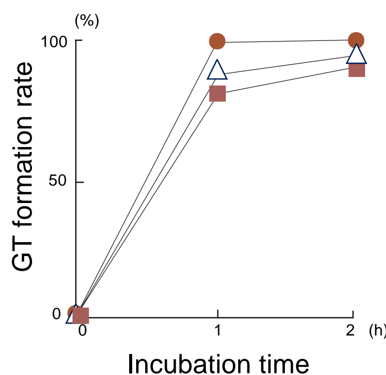

Fig. 2. Relationship between concentration of inoculating fungal solution and GT formation rate on culturing in 75% corn meal broth or 50% calf serum. *C. albicans* (10^5 cells/mL or 10^6 cells/mL) was inoculated into each medium, and incubated by the NSSI method at 37°C. 50% calf serum: ●, 10^5 cells/mL or 10^6 cells/mL fungal solutions. 75% corn meal broth: △, 10^5 cells/mL fungal solution; ■, 10^6 cells/mL fungal solution.

the mean GT initially observed. Along with the extension of GTs, yeast cells gradually appeared after a 2-h incubation and were recognized in specimens after a 20-h incubation period (Fig. 3c). In contrast, GTs formed during incubation in 50% calf serum grew to true hyphae with the development of a markedly small number of yeast cells, perhaps blastoconidia; moreover chlamydoconidium was not observed (Fig. 3d).

Figure 4 shows fungal specimens cultured by the TI method. Fungal clusters were formed by aggregating mutual GT-forming cells among scattering yeast cells in culturing by the TI method.

Table 1 shows the relationship between concentrations of fungal solutions and GT formation rates during GT formation in culturing by the NSSI and TI methods in 50% calf serum. Using my own experimental conditions, more satisfactory GT formation was generally achieved by the NSSI method than by the TI method even when the optimal concentration of fungal solution was roughly established by increasing it 5 times.

Quality of GT formation test using corn meal broth

GT formation in a total of 142 strains of *Candida* and related yeast-like fungi was tested by the NSSI method using 75% corn meal broth. GT formation was observed in all 111 strains of *C. albicans* only. The mean GT formation rate was 82.4% after a 1-h incubation, and GT formation with sufficient quality was confirmed under a microscope. Moreover, there was no GT formation observed in strains of other *Candida* species, although there is some concern regarding the small number of strains tested (Table 2).

Discussion

GT formation is a morphological characteristic of *C. albicans*, and it can be detected in a relatively short time when GT formation is accelerated by incubation at 37°C in a solution similar to serum containing abundant nutrients

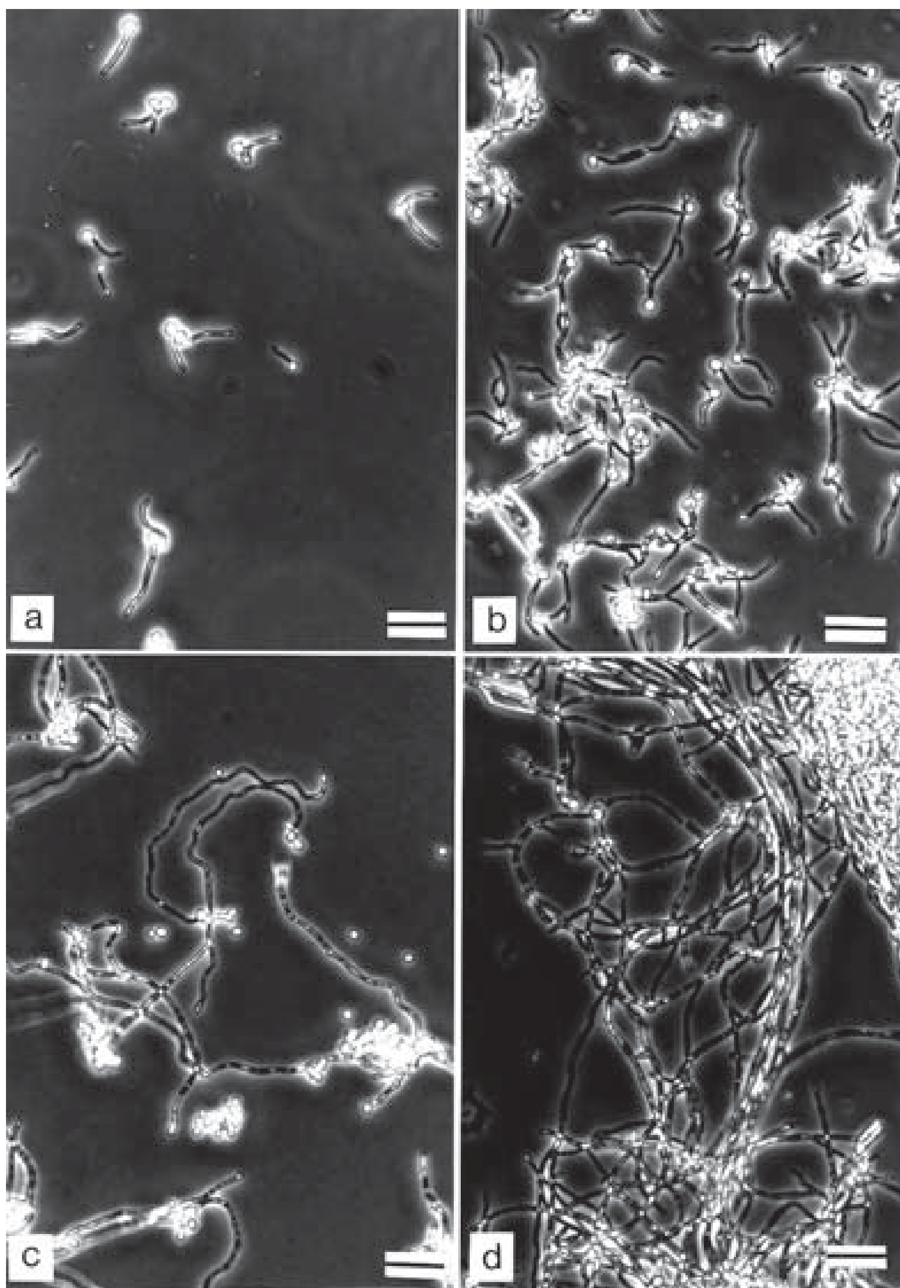

Fig. 3. Morphology of *C. albicans* IFO 1385 inoculated into 2 kinds of inducer solutions, 75% corn meal broth and 50% calf serum, and incubated by the NSSI method at 37˚C for 2 h and 20 h. **a**: *C. albicans* inoculated into 75% corn meal broth at a concentration of 10^5 cells/mL, and incubated for 2 h. **b**: *C. albicans* inoculated into 50% calf serum at a concentration of 10^6 cells/mL, and incubated for 2 h. **c**: *C. albicans* was inoculated into 75% corn meal broth at a concentration of 10^5 cells/mL, and incubated for 20 h. GT extended like relatively short true hyphae, and the small number of yeast cells were observed in the medium. **d**: *C. albicans* was inoculated into 50% calf serum at a concentration of 10^6 cells/mL, and incubated for 20 h. In contrast to culturing in corn meal broth, well developed true hyphae-like fungal morphology was observed together with diffused blastoconidium-like yeast cells, while chlamydoconidia were not observed in the medium. Bar indicates 10 μm.

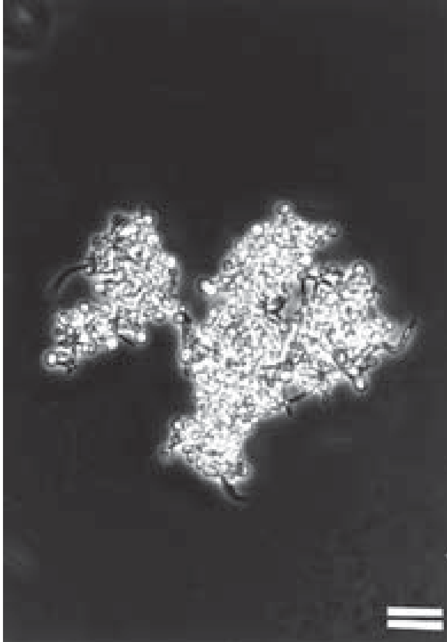

Fig. 4. Fungal morphology of *C. albicans* IFO 1385 inoculated into 50% calf serum, and incubated by the TI method at 37˚C for 2 h. Bar indicates 10 μm.

Table 1. The mean GT formation rate in 10 strains of *C. albicans* inoculated in 50% calf serum at 37˚C: relationship between concentration of inoculating fungal solution and incubation method

Incubation time (h)	Concentration of fungal suspension (cells/mL)	Mean GT formation rate (%)	
		TI method	NSSI method
1	1.4×10^6	90	95
	2.7×10^6	84	97
	4.1×10^6	68	96
	5.5×10^6	54	95
	6.8×10^6	44	97
2	1.4×10^6	97	100
	2.7×10^6	91	100
	4.1×10^6	79	100
	5.5×10^6	66	100
	6.8×10^6	53	100

GT, germ tube; NSSI, non-slip slide glass incubation; TI, tube incubation.

(Taschdjian et al., 1960; Mackenzie, 1962; Andleigh, 1964; Dolan and Ihrke, 1971; Nakamoto, 1996). Meanwhile, chlamydoconidia, another morphological characteristic of *C. albicans*, are formed when *C. albicans* is incubated in scanty nutrients, corn meal medium, at a low temperature between 25 and 30˚C. In this case, the corn meal medium used was applied as agar but not a liquid for studying chlamydoconidial formation, whereas few reports have been published describing chlamydoconidial morphogenesis even in corn meal liquid medium (Takagi and Nagata, 1962; Vidotto et al., 1986; Nakamoto, in press). Moreover, no report has been published regarding GT formation in corn meal broth. Since the composition of corn meal broth was the same as that of corn meal agar medium, commonly used

Table 2. GT formation test in 75% corn meal broth at 37˚C by the NSSI method

Strains	Number of strains	Number of GT-formed strains	Mean GT formation rate (%)	
		1-h Incubation	1-h Incubation	2-h Incubation
C. albicans	111	111	82.4 [44–100]	94.1 [74–100]
C. tropicalis	8	0	0	0
C. glabrata	9	0	0	0
C. parapsilosis	4	0	0	0
C. guilliermondii	5	0	0	0
C. lusitaniae	2	0	0	0
C. kekyr	1	0	0	0
C. krusei	1	0	0	0
C. lypolitica	1	0	0	0

GT, germ tube; NSSI, non-slip slide glass incubation.

for chlamydoconidial detection, except for agar, GT formation has been overlooked. In this study, it was confirmed that high rates of GT formation were achieved when yeast cells were incubated at 37˚C in the corn meal broth though diluted to some degree. Concerning the concentration of inoculating fungal solution, which influenced the efficiency of GT formation, it was already reported that the extension of GT was more accelerated at a lower concentration of fungal solution (Mackenzie, 1962; Nakamoto, 1996). Gunasekaran and Hughes (1977) reported that satisfactory GT formation was induced in 10^5 cells/mL fungal solution after incubation in a chitosan trypticase Tween-80 agar medium, while Strippoli and Simonetti (1975) also reported it to be between 10^5 and 10^6 cells/mL after incubation in a medium containing *N*-acetyl-D-glucosamine. It was also reported that GT induction was achieved by culturing yeast cells with sera at a similar range of fungal concentration, and further increases in the concentration of fungal solution resulted in decreased GT formation rates and inhibited GT extension (Mackenzie, 1962; Joshi et al., 1973a; Nakamoto, 1996). In this study, the optimal concentration of fungal solution in the corn meal broth was established, which was similar to those reported in previous studies. However, compared to GT induction by serum, it was confirmed that increased fungal concentration (10^6 cells/mL) resulted in slightly delayed GT formation and decreased GT formation rate. However, regarding GT formation during induction by serum, it was reported (Nakamoto, 1996) that the initial velocity of GT formation after a 20-min lag time was 4.25% per min, and the time for germ cells to reach 50% of the original (T_{50}) was 33 min. Among many reports regarding T_{50} (Walkeret et al., 1984; Pollack and Hashimoto, 1985; Sevilla and Odds, 1986; Holmes and Shephered, 1988), Sevilla and Odds (1986) recorded the shortest T_{50} at 40 min. Therefore, a further reduction of T_{50} was recorded under optimal conditions. In this study, it was considered that the rate of GT formation in 75% corn meal broth was as good as that in serum.

When the developmental process of GT-forming cells was observed, GTs observed at the initial stage of culturing were morphologically similar to those observed during GT induction by serum (Fig. 3b), demonstrating that at least morphologically similar GT could be induced in corn meal broth. These GTs extended like true hyphae without chlamydoconidia after a long-term incubation (Fig. 3c). Furthermore, dissociated proliferation of yeast cells was gradually observed after a 2-h incubation. Considering the unexpected consistency between the time that dissociated proliferation of yeast cells started and the time that the GT formation rate reached 100%, nutrients contained in a limited medium became rapidly insufficient. This phenomenon might be related to the findings that *C. albicans* grew to yeast phases when incubated at 37˚C in common media. From the results obtained in this study, it was speculated that GT formation and the development of true hyphae transiently occurred before the development of the yeast phase when *C. albicans* was incubated in 75% corn meal broth at 37˚C. However, further detailed experimental models were necessary to analyze this hypothesis. Meanwhile, it was recently reported that when *C. albicans* was incubated in corn meal broth at 23˚C, short pseudohyphae were aggregated and radially arranged in three dimensions to form clusters of specific hyphae with chlamydoconidia on their external tips (Nakamoto, in press). Therefore, despite the same media components, *C. albicans* showed a temperature-dependent induction of the morphological phase, which was considered different from the dimorphism observed on culture in common media. In general, corn meal broth is an excellent medium for the development of hyphae. However, it can be speculated that the morphogenesis of *C. albicans* using this corn meal broth has led to GT formation and the subsequent development of true hyphae without chlamydoconidia when the incubation temperature was initially established at 37˚C, while the morphogenesis of *C. albicans* has led to the development of pseudohyphae without GT formation and the subsequent formation of specific fungal clusters with chlamydoconidia

when the incubation temperature was initially established at 23˚C.

The TI method and the slide glass incubation method are commonly used for morphological observation of *C. albicans*. The slide glass incubation method is usually carried out by placing a drop on the medium with a cover glass. In this study, though GT formation could be observed by the TI method using corn meal broth, aggregation of GT-formed fungi occurred as shown in Fig. 4. Therefore, evaluation of the GT formation rate was markedly difficult. Concerning the slide glass incubation method, though satisfactory GT formation was observed in the fungal solution around the edges of the cover glass, poor GT formation was observed in the middle of the cover glass. Therefore, it was considered that contact with air was necessary for GT formation even in a limited specimen of fungal solution. Moreover, since the medium easily evaporates in the slide glass incubation method, observation of a long-term culturing is difficult (Nakamoto, 1994). To improve these culture conditions and to increase contact with air, I employed the NSSI method, in which a cover glass was not placed over the fungal solution on the slide glass, but the slide glass was sealed inside a vinyl bag to create an environment with high humidity. As a result, a long-term incubation experiment lasting for 1 or 2 days became possible. In the NSSI method, when a cover glass was first placed on the slide glass before microscopic examination, cells diffusing in the fungal solution were observed across all areas under the cover glass, but there were no fungal clusters due to aggregated GT-formed cells observed. Therefore, this culture condition provided an advantage, which facilitated precise evaluation of the GT formation rate. In addition, it was revealed that the NSSI method had an advantage in maintaining high rates of GT formation even though several times the optimal concentration of inoculating the fungal solution was used without strictly establishing the optimal fungal concentration in comparison to the TI method (Table 1). This finding may further facilitate clinical mycological techniques. On the basis of these findings, the NSSI method is markedly useful for rapid identification of *C. albicans* by confirmation of GT.

Under optimal conditions, GT formation was tested by the NSSI method in various strains. As a result, the quality of identifying *C. albicans* after a 1-h incubation was 100%. The GT formation rate in each strain ranged between 44 and 100%, which allowed no doubt in the evaluation.

These results concluded that corn meal broth could replace serum, which was commonly used for GT formation test, and that the use of corn meal broth was far more advantageous than serum with regard to cold storage, cost efficiency and biohazard safety.

Because the NSSI method is technically simple, this method facilitates the rapid diagnosis of *C. albicans* infection.

Finally, the reason why GT was formed by removing agar from corn meal medium remains to be clarified. Further information is necessary about culturing *C. albicans* by adding agar to the optimal 75% corn meal broth.

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