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Original Article

Germ Tube Induction Test Comparing Total of Six Liquid and Three Solid Media in *Candida albicans*

Rivaldi Ruby[1], Erlangga Saputra Arifin[1], Sandy Vitria Kurniawan[2], Sem Samuel Surja[3*]

[1]School of Medicine and Health Sciences, Universitas Katolik Indonesia Atma Jaya, Jakarta, Indonesia

[2]Department of Pharmacology and Pharmacy, School of Medicine and Health Sciences, Universitas Katolik Indonesia Atma Jaya, Jakarta, Indonesia

[3]Department of Parasitology, School of Medicine and Health Sciences, Universitas Katolik Indonesia Atma Jaya, Jakarta, Indonesia

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ABSTRACT

Invasive candidiasis (IC) has a high mortality rate of 70%, thus diagnosis should be established without delay. Given its fast result, serological test such as β-d-glucan (BDG) test is one alternative diagnosis modalities. However, it lacks specificity. *Candida albicans* germ tube antibody (CAGTA) test is an alternative serological test which has a high sensitivity of 76.2% and specificity of 80.3%. Manufacturing CAGTA serological test requires provision of specific germ tube antigen. In this study, various culture media were tested to find the best media for germ tube induction. This study was an experimental in vitro study. The number and length of the germ tube were recorded in two- and three-hour incubation periods. A total of six samples containing one C. albicans ATCC 90028, four *C. albicans* wild type strains, and one *C. krusei* wild type strain were used. Nine media were tested to induce germ tube formation: human and sheep serum, fetal bovine serum, mueller hinton agar and broth, tryptic soy agar and broth, brain heart infusion agar and broth. At both incubation periods, the medium with the highest number of germ tube was human serum (p=0.001 and p=0). The longest germ tube was found in sheep serum at two-hour incubation period (p=0.005). Mueller hinton broth (MHB) showed comparable results with human and sheep serum (p>0.05). Human serum is a superior inducer of morphogenesis. However, the use of MHB is recommended in this study, since provision of fresh human and sheep serum on a regular basis is impractical.

Keywords: *Candida albicans*; germ tube; human serum; mueller hinton broth; sheep serum

Highlights: Several media could induce not only numbers of germ tube, but also its length. Therefore, they could benefit for easier diagnosis and also higher amounts of germ tube protein.

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* Corresponding Author:
sem.samuel@atmajaya.ac.id

INTRODUCTION

Candidiasis is a disease with a high prevalence rate globally. This disease generally affects the skin and mucosal tissue, causing mild conditions such as oral and vulvovaginal candidiasis.[1] At the systemic level, it has high mortality and morbidity, referred to as invasive candidiasis (IC). It is associated with prolonged intensive care unit (ICU) admission and immunocompromised conditions such as acquired immune deficiency syndrome (AIDS).[2] Globally, candidiasis occupies the top three incidences of diseases caused by fungi in the year 2017—the first is oral candidiasis with an incidence rate of 2,000,000, followed by esophageal candidiasis with 1,300,000, and then IC with 750,000 incidences.[1] Invasive candidiasis yields a high mortality rate of 70%.[3]

Invasive candidiasis is caused by *Candida spp*. with the most common etiology being *Candida albicans*.[4] In humans, this fungus is a normal flora of the skin, oropharynx, digestive, and urogenital tract.[5] Infection occurs when there is hyphal growth and biofilm formation in the tissue. These mechanisms also allow resistance of *C. albicans* to traditional antifungal agents.[6]

Timely diagnosis is required in order to reduce mortality. Currently, IC is diagnosed through the findings of hyphae on microscopic examination or through a time- consuming culture.[1] Serological tests provide relatively faster and easier way to diagnose IC. β-d-glucan (BDG) test is one widely used *Candida* serological test. However, it lacks specificity due to cross reaction with other fungi.[7] A serological test detecting antibody against germ tube could be used as an alternative to diagnose IC, namely *C. albicans* germ tube antibody (CAGTA) test.[8] Germ tubes are formed by *C. albicans* in a number of conditions such as starvation, presence of serum or N-acetylglucosamine, physiological temperature, and CO_2.[9] It has high sensitivity of 76.2% and specificity of 80.3% since morphological transition from yeast to germ tube and hyphae is important for pathogenicity of *C. albicans.*[8,10] Combination of BDG and CAGTA serological tests is recommended for IC early diagnosis.[11]

The first step in manufacturing CAGTA serological test is isolation of the germ tube antigen. It is important to seek the best medium for *C. albicans* since this antigen is obtained by inducing its growth in a suitable environment. Various media can be used for induction of germ tube, each with unique compositions and function. Human serum is the most used medium for germ tube test.[12] Its main limitation is the requirement of fresh human serum on a regular basis. For this reason, this study aims to find the best media in inducing germ tube formation of *C. albicans.* While previous studies mainly assessed the sensitivity of each medium for germ tube test, this study also measured the number and length of germ tube formed after certain incubation period.

MATERIALS AND METHODS

Study Design

This experimental in vitro study was conducted in the Parasitology Laboratory, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia from August 2020 to October 2020. Ethical clearance was obtained from the Atma Jaya ethical committee with the number 01/06/KEP-FKUAJ/2020.

Fungi Strains

Candida albicans wild type, *C. albicans* ATCC 90028, and *C. krusei* wild type were used in this study. All *C. albicans* wild type were obtained from patient's sputum in Microbiology Laboratory, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia, while *C. albicans* ATCC 90028 and *C. krusei* wild type were obtained from the collection of Department Parasitology, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia. Each strain

was identified and confirmed through macroscopic and microscopic examination. Macroscopic identification was made using CHROMAgar (Oxoid, United Kingdom) to analyze the color and characteristics of the fungal colonies. *Candida albicans* was characterized by the formation of green colonies, in contrast with *C. krusei* which appeared as pink colonies.[13] Microscopic identification was made through lactophenol cotton blue (LPCB) staining and germ tube test. Light microscope (Olympus CX21) is used to identify the morphologies. Ovoid and spherical yeast cell shapes are a characterization of *C. albicans*, distinguished from *C. krusei* that commonly appear as a more elongated (long grain rice) shape. A positive germ tube test is also only found in *C. albicans.*[14,15] A total of six isolates containing one *C. albicans* ATCC 90028, four *C. albicans* wild type, and one *C.krusei* wild type were used. *Candida albicans*ATCC 90028 and *C. krusei* were used as the positive and negative control, respectively.

Medium

The media used for induction of germ tube were serum, broth, and agar. Sera used were human serum, sheep serum, and fetal bovine serum (FBS, Biowest, France). The FBS used was not diluted with 100% concentration. Human serum was prepared by centrifugating blood from a healthy donor.[16] The broth and agar media used were mueller hinton agar (MHA, Oxoid, United Kingdom), mueller hinton broth (MHB, Conda, Spain), tryptic soy agar (TSA, Oxoid, United Kingdom), tryptic soy broth (TSB, Merck, Germany), brain heart infusion agar (BHIA, Oxoid, United Kingdom), and brain heart infusion broth (BHIB, Oxoid, United Kingdom). All media were obtained from the Department of Microbiology, Parasitology, and Pharmacology, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia.

Broth and agar media were prepared by combining 1 L of aquadest with 38, 21, 45, 30, 53, and 37 g of MHA, MHB, TSA, TSB, BHIA, and BHIB, respectively. These suspensions were heated until completely dissolved, followed by sterilization using an autoclave at 121˚C with a pressure of 15 Psi for 15 minutes. Broth medium was poured into the 1.5 ml test tube, while agar medium was poured into a petri dish. For all media, pH was adjusted at 7.4 which is confirmed by pH meter.

Gem Tube Induction

Candida albicans and *C. krusei* were inoculated in sabouraud dextrose agar (SDA, Oxoid, United Kingdom) for 48 hours at room temperature (25°C). Two hundred µL of 3 McFarland fungi suspension was added into 800 µL of each serum and broth medium. The mixture was incubated for 24 hours at 37°C.[12] The number and length of the germ tube were recorded in two-, three-, and 24-hour incubation periods. Ten µL of the mixture was dripped into an improved Neubauer counting chamber and the germ tube was observed under the microscope (Figure 1a).[17] All processes were performed in duplicate.

Germ tube induction in agar media was conducted by dripping 10 µL of 0.5 McFarland fungi suspension into 1 x 1 cm^2 agar.[18] Cover slip was placed to facilitate easier examination. The agar was then incubated for three hours at 37°C. The number and length of germ tube induction were recorded in two- and three-hour incubation periods using the microscope (Figure 1b). All processes were performed in duplicate.

Germ Tube Calculation

This study measured the number and length of germ tubes formed. The number of germ tube was calculated in five small squares of the counting chamber using standardized formula (Figure 2).[19] Germ tube length measurement was conducted by comparing the length of the germ tube and the counting chamber small squares. The longest germ tube in the five small squares was

recorded. Germ tube length measurement was done only in serum and broth media. There are no difficulties in performing the measurement methods.

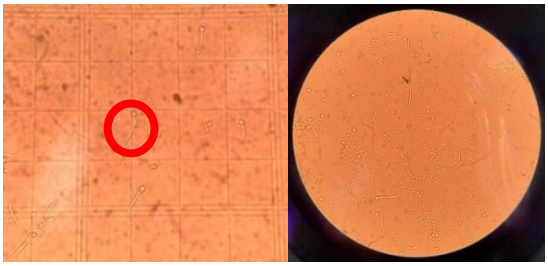
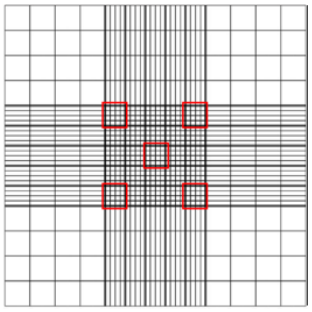


Figure 1. Germ tube appearance under the microscope in different preparations. (a) Improved Neubauer Counting Chamber – Germ tube (red circle); (b) Slide culture



$$Number\ of\ germ\ tube\ /\ \mu L = \frac{n}{V}$$

$$V = improved\ Neubauer\ counting\ chamber\ volume = \frac{1}{50} mm^3$$

$$n = total\ of\ germ\ tube\ in\ 5\ small\ squares$$

Figure 2. Improved Neubauer Counting Chamber

Calculation of the number of germ tube on agar media was carried out by taking three representative images using the high-power field (HPF) microscope. Then the number of germ tubes was grouped into several categories (Table 1).

Table 1. Germ Tubes Counts Categories Using Agar Media Inductions

Categories	Germ Tube Count/HPF
1+	1-10
2+	11-20
3+	21-30
4+	31-40
5+	41-50
6+	>50

HPF – high-power field

Data Analysis

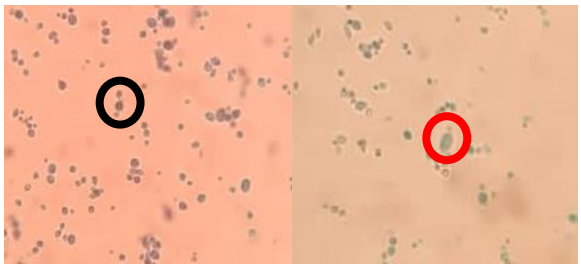
Data analysis was done using Statistical Product and Service Solution (SPSS) version 22. Data on serum and broth medium was analyzed using a One-Way ANOVA statistical test or Kruskal-Wallis test, depending on data normality. It was then followed by a Bonferroni post hoc test if significant results were found. In agar medium, Fisher-Exact or Chi-Square test were used depending on the terms and criteria of the statistical test. A significant value was yielded if $p<0.05$.

RESULTS AND DISCUSSION

Fungi Identification

Macroscopic identification of all samples was done using CHROMagar. It is depicted in Figure 3. Moreover, the morphologies found in microscopic identification using LPCB confirmed the samples' species (Figure 4).

Figure 3. Macroscopic characterizations on CHROMagar. The green colonies are *C. albicans* and the pink colony is *C. krusei*



(a) **(b)**

Figure 4. Microscopic characterizations of *Candida* species grown in SDA. (a) Ovoid appearance of *C. albicans* (black circle) (b) Elongated appearance of *C. krusei* (red circle)

Germ Tube Induction in Serum and Broth

All Candida samples were used in germ tube induction. *Candida albicans* ATCC 90028 and *C. krusei* function serve as a positive and negative control, respectively. In all media, *C. albicans* ATCC 90028 showed positive germ tube results, while *C. krusei* had negative results. *Candida albicans* wild type was used for the evaluation of each media's ability to induce germ tube formation.

Several media could facilitate germ tube formation. Results were variable between media at two- and three-hour incubation period. At two-hour incubation period, it was found that the medium with the highest number of germ tube was human serum. Sheep serum also showed comparable results in germ tube induction. Roughly, the order of media that could facilitate germ tube induction was as follows: human serum, sheep serum, FBS, MHB, TSB, and BHIB; while at three-hour incubation period, the order was as follows: human serum, MHB, sheep serum, FBS, TSB, and BHIB as shown in Table 2.

Table 2. Number of Germ Tubes Formed on Serum and Broth Media (number/µL)

Time	Fungi	Media						p-value
		Human Serum	Sheep Serum	FBS	MHB	TSB	BHIB	
2 hours	CA 1	38125	40625	26875	11250	5625	5000	0.001
	CA 2	23125	20625	10000	22500	11250	3750	
	CA 3	25000	23125	8750	15625	13750	0	
	CA 4	21250	15000	18125	11250	0	625	
3 hours	CA 1	42500	16250	13750	15625	3750	0	0
	CA 2	26250	15000	5000	37500	15625	6875	
	CA 3	32500	20625	7500	25000	16250	0	
	CA 4	31875	13125	18750	15000	3750	0	

*Post hoc (2 hours) Human Serum vs. BHIB p=0.003; Human Serum vs. TSB p=0.024
**Post hoc (2 hours) Sheep Serum vs. BHIB p=0.006
***Post hoc (3 hours) MHB vs. BHIB p=0.004
****Post hoc (3 hours) Human Serum vs. BHIB p=0; Human Serum vs. Sheep Serum p=0.03; Human Serum vs. TSB p=0.001; Human Serum vs. FBS p=0.003

No difference was shown between MHB and human serum in post hoc analysis (p>0.05). The number of germ tubes between broth media were highest in MHB, compared to TSB and BHIB (p=0.015 and p=0.009 at two- and three-hour incubation periods, respectively) as shown in Table 3. At the 24-hour incubation period, the fungi experienced rapid growth into hyphae. Therefore, further analysis was not performed in this incubation period.

Table 3. Number of Germ Tubes Formed on Broth Media (number/µL)

Time	Fungi	Media			p-value
		MHB	TSB	BHIB	
2 hours	CA 1	11250	5625	5000	0.115
	CA 2	22500	11250	3750	
	CA 3	15625	13750	0	
	CA 4	11250	0	625	
3 hours	CA 1	15625	3750	0	0.009
	CA 2	37500	15625	6875	
	CA 3	25000	16250	0	
	CA 4	15000	3750	0	

*Post hoc (2 hours) MHB vs. BHIB p=0.015
**Post hoc (3 hours) MHB vs. BHIB p=0.009

Table 2. Number of Germ Tubes Formed on Serum and Broth Media (number/μL)

Time	Fungi	Media						p-value
		Human Serum	Sheep Serum	FBS	MHB	TSB	BHIB	
2 hours	CA 1	14	28.89	22.22	17.775	24.445	2.22	0.005
	CA 2	26	38	37.78	22.22	26	17.78	
	CA 3	20	33.335	26.665	23.33	31.115	0	
	CA 4	30	55.555	38	33.335	0	10	
3 hours	CA 1	26.665	53.33	26	31.115	14	0	0.155
	CA 2	57.78	50	42	77.775	95.555	48.89	
	CA 3	28.89	36	34	37.78	57.78	0	
	CA 4	77.78	44.665	22	73.335	26.665	0	

*Post hoc (2 hours) BHIB vs. Sheep Serum p=0.003
*Post hoc (2 hours) BHIB vs. FBS p=0.039

In the measurement of length, the longest germ tube was found in sheep serum at two-hour incubation period, followed by FBS, MHB, TSB, human serum, and BHIB. No difference was found at the three-hour incubation period as shown in Table 4. No broth media was found superior to the other in terms of the germ tubes length as shown in Table 5.

Table 5. Length of the Germ Tube Formed on Broth Media (μm)

Time	Fungi	Media			p-value
		MHB	TSB	BHIB	
2 hours	CA 1	17.775	24.445	2.22	0.998
	CA 2	22.22	26	17.78	
	CA 3	23.33	31.115	0	
	CA 4	33.335	0	10	
3 hours	CA 1	31.115	14	0	0.132
	CA 2	77.775	95.555	48.89	
	CA 3	37.78	57.78	0	
	CA 4	73.335	26.665	0	

Germ Tube Induction in Agar

The number of germ tubes formed was highest in MHA. However, results were not significant both in two- and three-hour incubation periods as shown in Table 6.

Table 6. Length of the Germ Tube Formed on Agar Media (μm)

Time	Fungi	Media			p-value
		MHA	TSA	BHIA	
2 hours	CA 1	+3	+1	0	0.408
	CA 2	+6	+1	+2	
	CA 3	+4	+1	+1	
	CA 4	+1	0	0	
3 hours	CA 1	+1	0	0	1
	CA 2	+6	+1	+1	
	CA 3	+2	+1	+1	
	CA 4	0	0	0	

Hyphal morphogenesis is one of the most investigated virulence attributes of *C. albicans*. The ability of *C. albicans* to undergo reversible morphological transition could be triggered by variety of environmental condition.[20–22] Serum, especially human serum, contains several important components that promote germ tube formation; therefore, it accounts as strong inducer for yeast-to-hyphae formation. Burch et al. (2018) stated that human serum fraction revealed signs of bacterial peptidoglycan (PGN)-like molecules which highly active for hyphae induction.[23] Glucose could also act as morphogen, which in the certain amount could stimulate morphogenesis of *C. albicans*.[24,25]

Human serum, sheep serum, and FBS have approximately 1 mM to 10 mM of glucose.[26–28] This amount of glucose is optimal for germ tube formation according to previous study.[24] Moreover, combined with exposure of 37°C and neutral pH environment, serum could inhibit NRG1 transcription, a potent inhibitor for hyphal formation (Su et al. 2018).[29] This exposures to 37°C and neutral to alkaline pH, could induce hyphal growth through the Cek1 mitogen-activated protein kinase pathway (MAPK pathway) and the Rim101-pH sensing pathway, respectively.[21] Studies by Hilmioglu et al. (2007) found that human serum was superior with the highest number of positive germ tube.[30] In concordance to previous data, present study found that human and sheep serum were capable of inducing the highest number and longest germ tube, respectively, compared to other

media. This study also found that extended incubation period on serum led to an increasing number of hyphae. Long incubation period causes deprivation of nutrient and energy which leads to more effective hyphal growth.[31,32]

Utilization of human serum for germ tube induction has few drawbacks despite its superiority to other media. Serum has to be fresh otherwise stored serum could decrease germ tube production.[12] Some serum could have biological inhibitor present in it. Wich et al. (2021), found that human serum antibodies have the capability to inhibit adherence of *C. albicans* to epithelial cells.[33] Moreover, Ding et al. (2014) stated that germ tube formation in RPMI 1640 medium was delayed in the initial stage of the culture (within 90 min) in the presence of serum, although the number of hyphae was gradually increased to normal after extension of incubation period (from 2h to 3h).[31] Presence of these biological inhibitor could cause inconsistent result in different batches of serum. Lastly, serum preparation poses possible risk of biohazard.[12] In this study, several standardized media were studied to find comparable alternative of serum. Application of commercially available media also facilitates further culture and production of germ tube antigen for serological test.

Broth medium also have essential substances for the growth and morphogenesis of *C. albicans*. MHB, TSB, and BHIB were tested in their ability to promote germ tube formation. As stated above, elevated temperature and neutral pH were the one main inducer for morphogenesis.[29] Moreover, all media contains nutritive compounds that are necessary for fungal growth. Amino acids are one potent inducer present in the medium. It promotes yeast-to-hyphal transition through the cAMP-PKA pathway.[21] In this study, MHB, TSB, and BHIB contain amino acids which further facilitate hyphal formation.[21,22] Furthermore, in MHB, *C. albicans* showed the highest number of germ tube formation. One possible explanation is that it contained starch components with protective colloid roles against toxic compounds in the medium.[34] BHIB is the most nutritious medium with combination of brain and beef infusion (a total of 17.5 g/l), protease peptone (10 g/l), and dextrose (2 g/l) which provide carbon, nitrogen, amino acids, and other nutrients.[34] However, *C. albicans* showed lowest number of germ tube in BHIB. An exact explanation of this phenomenon is unknown. It seems that high nutrient provision is not an inducer of morphogenesis. According to Mba et al. (2020), *C. albicans* exhibits metabolic flexibility and filamentous growth in the condition of nutrient starvation.[34] It is difficult to identify which media facilitate better morphogenesis based on previous studies, since results were mostly conflicting. Hilmioglu et al. (2007) showed that BHIB surpassed TSB as morphogenesis inducer, while Yakasiri et al. (2020) concluded that TSB exceeds MHB and BHIB.[30,35] Different results could be attributed to different strain, media quality, and research conditions.

Interestingly, Atalay et al. (2017) found that MHA was the best media for germ tube induction compared to human serum.[18] Distinct factor affects the yeast-to-hyphal transition in agar media. Villa et al. (2020) stated that *C. albicans* hyphal growth in agar is influenced by the embedded surroundings or conditions. This is achievable through the upregulation of CZF1 transcription factor when the fungi are inoculated within the agar matrix.[9] In present study, higher number of germ tube was found in MHA compared to other agar media although the result was not significant.

STRENGTH AND LIMITATION

To our knowledge, this is the first study that compares several media by measuring the number and length of the germ tubes. However, this study has several limitations. Lack of fungal strain prevents generalization of the result to other strain of *C. albicans*.

Wider variety of culture medium could be used, since RPMI 1640 and YEPD broth also showed potential in previous studies.[12,31]

CONCLUSIONS

This study showed that certain media in a specific environmental condition could facilitate hyphal growth that initially appears as germ tube formation. Human serum is a strong inducer of morphogenesis. Incubation of *C. albicans* in standardized medium such as MHB and TSB, coupled with 37°C environmental temperature and neutral pH, is also adequate to facilitate such phenomenon. Those media are preferred to human serum because it is readily available, routinely used in daily microbiology laboratories, and it provides stable result between batches.

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ETCHICAL CLEARANCE

Ethical clearance was obtained from the Atma Jaya ethical committee with the number 01/06/KEP-FKUAJ/2020.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTION

Designed the study, collected and analyzed the data, and also prepared the manuscript: RR and EAS. A scientific adviser in the field of mycology: SS and SVK. All authors read and approved the final manuscript.

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