



Hyphal Formation is Enhanced by 121°C Heat Inactivation of Serum Added to Culture Media

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Authors' contributions

This work was carried out in collaboration among all authors. Authors HH, YF, XW and JH drafted the manuscript. Author HH made critical revisions to include important intellectual content in the manuscript. Authors CL, XD and YF conducted the experiments, formatting and preparing the manuscript. All authors read and approved the final version.

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ABSTRACT

Background: A novel method for inducing hyphae formation, was described.

Methods: Mycelia of experimental and clinical strains from 100 patients with oral lichen planus were cultured in RPMI 1640 medium with 56°C or 121°C inactivated calf sera. After these strains were cultured for 2 h to 7 days, the average hyphae formation rate was observed under light microscopy.

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Results: The 121°C inactivated calf serum showed better outcomes than 56°C inactivated calf serum in terms of time and amounts of hyphae products.
Conclusions: For *Candida albicans* hyphae culture, the 121°C inactivated serum is superior to the 56°C inactivated serum.

Keywords: Serum inactivation; *Candida albicans*; hyphae; yeast; oral lichen planus.

ABBREVIATIONS

C. albicans : *Candida albicans*
OLP : Oral lichen planus
RPMI : Roswell Park Memorial Institute
A260/280 : Absorbance 260/280

1. BACK GROUND

The fungus *Candida albicans* can cause mucosal infections, such as oral mucosal lesions oral lichen planus, denture stomatitis and disseminated invasive infections including hospital-acquired bloodstream infection in susceptible individuals [1]. Hyphae formation is a research hot spot for attempting to inhibit clinical filament formation and pathogenesis of *C. albicans*. In our study, a novel method for inducing *C. albicans* hyphae formation is described.

Role of *C. albicans* in oral mucosal lesions [2-4], including premalignant disorders [5-8], leukoplakia and oral lichen planus (OLP) has been extensively studied in humans. Multiple studies have shown that *Candida* morphological switching and the specific genes regulate hyphae formation by blocking the transduction of signaling molecules [9-11]. In this context, we have identified and patented a novel method that was optimized for growing hyphae, and to the best of our knowledge, it might be the most effective method reported up to date.

Historically, *C. albicans* culture media includes solid media, such as corn medium, Lee's medium, milk Tween medium, and Spider medium *in vitro*. These procedures produce hyphae enriched with yeast-phase cells. Several studies have achieved very pure hyphae cells after 12 passages of seven days [12,13]. However, their mycelium collection process was all lengthy and tedious. Purer hyphae cells could be harvested with Roswell Park Memorial Institute (RPMI) 1640 medium, with 56°C inactivated fetal calf serum, at 37°C at pH 7.0 [12,13], but the time frame was restricted to ≤3 h, to avoid lateral bud growth affecting concentration due to over-growth. Other reports

indicated that pure mycelium could be induced more quickly at a constant temperature of 38°C [12,13]. In this study, to identify the best method for *C. albicans* hyphae culture, different brands of inactivated calf sera were utilized to induce hyphae cell growth in the same RPMI medium. Seven clinical and one standard laboratory strain were experimented. Hyphae formation rates were measured and the relationships between differently inactivated serum ingredients and mycelium induction were mentioned. This article wants to spread the conclusion: for *C. albicans* hyphae culture, to add 121°C inactivated serum is a superior choice.

This would facilitate the investigation of hyphae-specific genes and relevant signaling pathways, hopefully designing interventions against the morphological switching of *C. albicans*. Within the authors' fields, the ultimate goal would be to verify some possible etiology and progression of the precancerous disease oral lichen planus.

2. MATERIALS AND METHODS

2.1 Subjects

Strains were collected from patients diagnosed with OLP who visited the Stomatology Hospital and the Second Hospital, affiliated to the School of Medicine, Zhejiang University between August 2011 and June 2014. The patients were divided into two groups. The non-erosive group included 59 patients with an average age of 53.2 years. There were 23 males with an average age of 53.8 years and 36 females with an average age of 52.6 years. The erosive group included 41 patients with an average age of 53.0 years. There were 14 males with an average age of 48.7 years and 27 females with an average age of 56.0 years. All participants signed informed consent forms.

Samples were collected by rubbing the lichen planus lesions with sterile cotton swabs, which were transferred within 30 min to clinical microbiology laboratories of the Stomatology Department and the Second Hospital, affiliated to School of Medicine, Zhejiang University.

2.2 Strains

Twist cotton (Cixi Ningbo China) was coated on Sabouraud Lloyd agar (SDA, Nanjing SenBeiJia Biological Technology Co., Ltd. PRC), cultured for 24~48 h at 37°C (Beijing Fu Yilian Medical Equipment Co., Ltd. PRC) until colonies appeared, before transferring to French CHROMagar chromogenic medium for another 24 h, in which colonies appearing emerald green were initially identified as *C. albicans*. Colonies that appeared dark green were suspected as *C. parapsilosis*, and were rechecked after another 24~48 h culture on SDA. Emerald green colonies were confirmed as *C. albicans*, while dark green colonies were confirmed as *C. parapsilosis*.

Typical colonies were picked and inoculated on SDA plates three times to achieve separation and purification. After culturing for 48 h on SDA, the colonies were milky white, cheese-like and circular in shape. They formed germ tubes after 4~6 h at 37°C on rice Tween agar (Hangzhou microbiology reagents factory, China), and continued to grow well after 48 h incubation on SDA. Light microscopy (Phoenix Optical Instrument Group, China) showed Gram-positive structures with large cell volume, circular or oval layers by modified Gram staining. API 20C AUX *Candida* identification system confirmed that the results were in accordance with databases provided by BioMerieux. Finally, cinnamon peptone (Shanghai Qianchen Biotechnology Co., Ltd. PRC) strains were stored at -20°C and regularly sub-cultivated.

Strains were designated as a~h, in which a, c, g isolates were from female non-erosive OLP patients, while b, e, f isolates were from female erosive OLP patients, d isolate was from male erosive OLP patients, and h was the standard experimental strain (ATCC 16220) used as a random control afforded by the clinical microbiology laboratory of Affiliated Second Hospital, School of Medicine, Zhejiang University.

The cinnamon peptone was rapidly thawed at 37°C in a water bath; 10µL of solution was plated on SDA medium on a super clean bench, and cultured for 48 h in a 37°C constant temperature incubator.

2.3 Dimorphic Incubation and Quantity

For yeast phase culture of *C. albicans*, yeast colonies of 2 mm diameter were added to a cell culture flask with 5mL yeast extract peptone

dextrose YPD (Shanghai Xinfan Biological Technology Co., Ltd. PRC) (121°C 30 min, autoclaved, Japan machine) at an adjusted concentration of 1×10^6 /mL, and shaken in a 37°C, 200 rpm thermo-shaker (Thermo Fisher Scientific, America) for 24 h. The yeast phase was cryopreserved at -20°C.

For hyphal phase culture of *C. albicans*, hyphal colonies of 2 mm diameter were added to 4.5 mL RPMI 1640 (Shanghai Beinuo Biological Technology Co., Ltd) + 0.5 mL calf serum (Nanjing SenBeiJia Biological Technology Co., Ltd.) of 56°C 30 min inactivation complement and cultured in a water-jacketed thermostatic constant incubator. The concentration was adjusted to 1×10^6 /mL for 7-day culture, and passaged 12 times (the first 2 days, passaged every 8 h, the next 2 days, passaged every 12 h, and the last 3 days, passaged every 24 h).

In addition, 5 mL RPMI 1640, 4.5 mL RPMI 1640 + 0.5 mL calf serum (no inactivation) and 4.5 mL RPMI 1640 + 0.5 mL calf serum (121°C, 30 min inactivation) were used as controls, all the strains were cultured at pH 7.0 and mycelium cell formation was observed under light microscopy. The number of cells forming hyphae among 100 cells was counted three times at 2 h, 3 h, 6 h, 8 h, 12 h, 24 h, and 7 days under high power microscope, and the average hyphae formation rate was calculated.

2.4 Statistical Analysis

The Chi-square test was used for statistical analyses. Statistical significance was determined at $p < 0.05$. All calculations were performed using SPSS 16.0 software.

3. RESULTS

The media used in this study were 5 mL RPMI 1640, 4.5 mL RPMI 1640 plus 0.5 mL calf serum (no inactivation), 4.5 mL RPMI 1640 + 0.5 mL calf serum (inactivated at 56°C for 30 min), and 4.5 mL RPMI 1640 + 0.5 mL calf serum (inactivated at 121°C for 30 min). The strains were cultured at pH 7.0 for 2 h, 3 h, 6 h, 8 h, 12 h, 24 h and 7 days for 12 passages and the consequent mycelium formation are shown in Table 1, Table 2 and Figs. 1-3. Hyphae of *C. albicans* showed aggregation that was proportional to the hyphae formation rates.

The hyphae formation rates of experimental strains and clinical isolates cultured in RPMI 1640 with calf serum (no inactivation) were

significantly low, only 6% at 3 h and 49% at 6 h (blue curves in Fig. 1-3 and Table 1). The 3 h hyphae formation rates with 56°C and 121°C inactivated sera could reach 58% and 75%, while the 6 h hyphae formation rates with those sera were 96% and 98%, respectively. The 7-day hyphae formation rates after 12 generations could reach 99%, and these pure hyphae cells were collected in both 56°C and 121°C

inactivated sera (Tables 1 and 2, Figs. 1 and 2). The 6 h hyphae (Figs. 1-3) formation rate at pH 7.0 with RPMI 1640 + heat-inactivated serum could reach above 95% and the most apparent aggregation of hyphae can be observed in 121°C inactivated medium. These mycelium phase cells were pure enough to be utilized to study hyphae phase of *C. albicans*.

Table 1. Average hyphae formation rates of experimental standard strain

	2 h	3 h	6 h	8 h	12 h	24 h	7 d
1640 control	0	0	15%	7%	0	0	2%
1640 + no inactivation	0	2%	49%	13%	3%	0	5%
1640 + 56°C inactivation	21%	47%	96%	41%	13%	0	99%
1640 + 121°C inactivation	35%	75%	98%	48%	18%	2%	99%

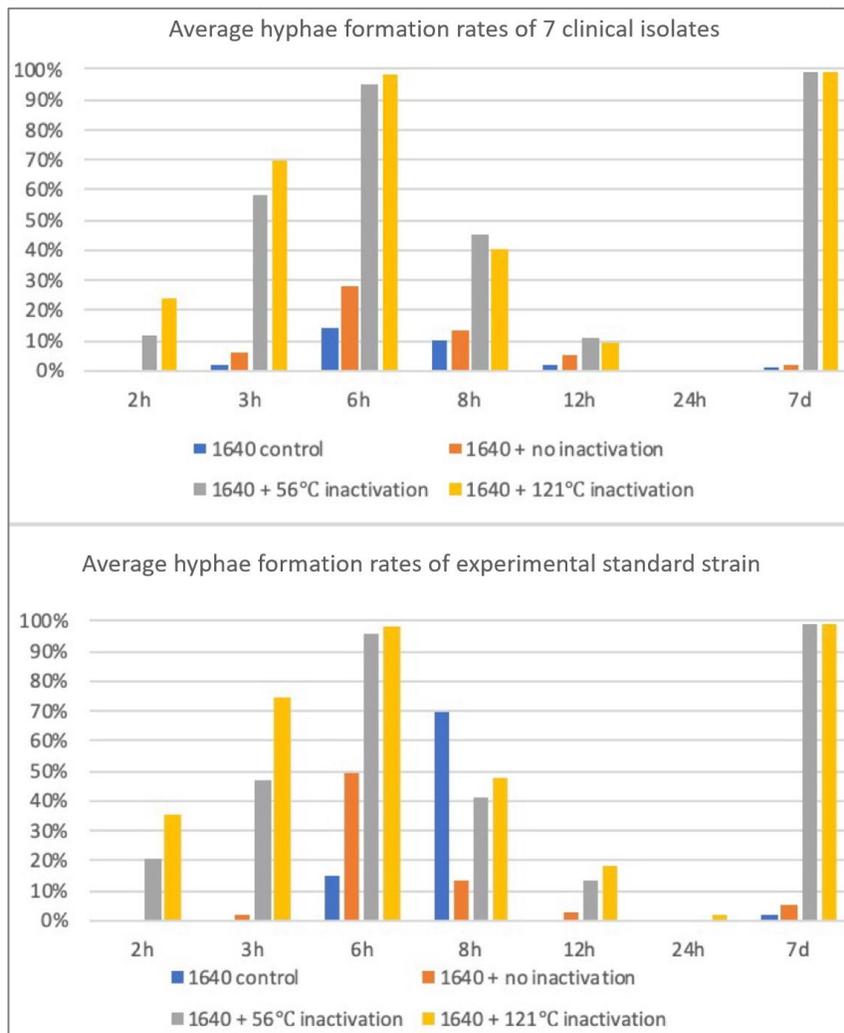


Fig. 1. Hyphae culture rates of clinical and standard strains. Y-axis: Rate of Hyphal formation (%). X-axis: Time after inoculation

Table 2. Average hyphae formation rates of 7 clinical isolates

	2 h	3 h	6 h	8 h	12 h	24 h	7 d
1640 control	0	2%	14%	10%	2%	0	1%
1640 + no inactivation	0	6%	28%	13%	5%	0	2%
1640 + 56°C inactivation	12%	58%	95%	45%	11%	0	99%
1640 + 121°C inactivation	24%	70%	98%	40%	9%	0	99%

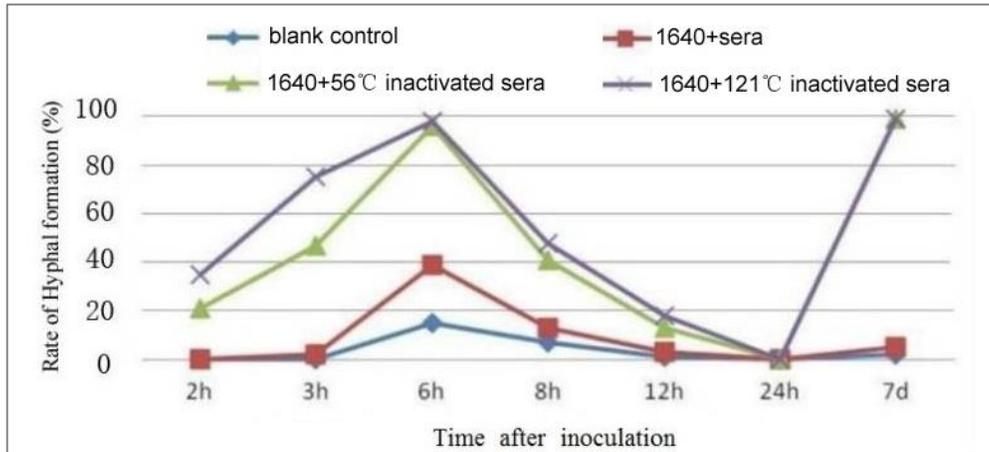


Fig. 2a. Hyphae formation rates of the experimental strain in four different media. 1640 medium without serum (blue), supplemented with the calf serum (red), with 56°C inactivated calf serum (green) and with 121°C inactivated calf serum (purple). Y-axis: Rate of Hyphal formation (%). X-axis: Time after inoculation

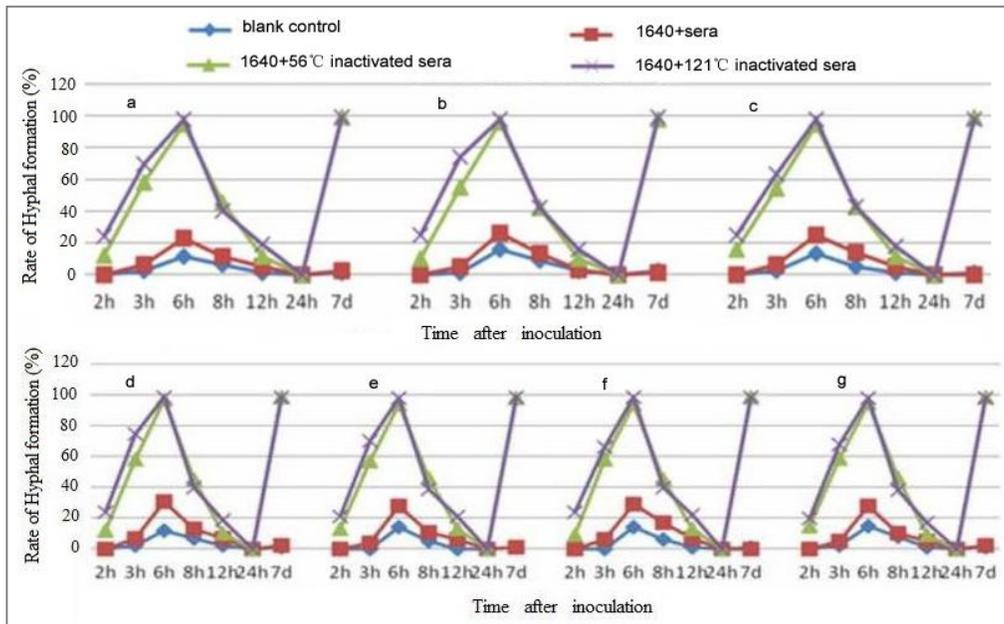


Fig. 2b. Hyphae formation rates of 7 clinic isolates in four media a to c (left to right in upper panel) and d to g (Left to right in the lower panel). 1640 medium without serum (blue), supplemented with the calf serum (red), with 56°C inactivated calf serum (green) and with 121°C inactivated calf serum (purple). Y-axis: Rate of Hyphal formation (%). X-axis: Time after inoculation

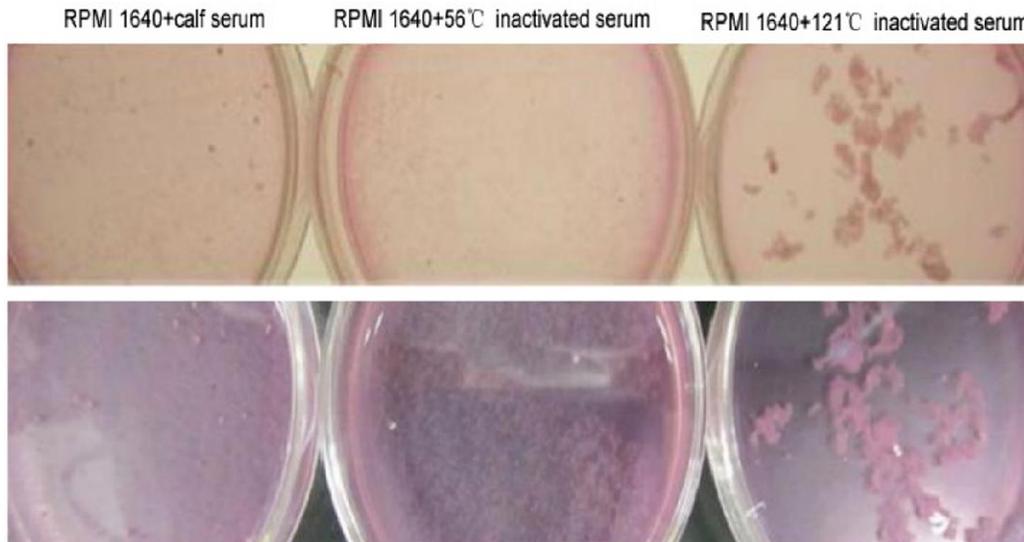


Fig. 3a(Upper):clinical isolates

Fig. 3b(Lower):experimental strain

Fig. 3a. Cultures of the clinical isolates at 6 h. 3a left: No inactivation. 3a middle: 56°C inactivated. 3a right: 121°C inactivated. Aggregation of hyphae is most apparent in 121°C inactivated medium

Fig. 3b. Cultures of the experimental strain at 6 h. 3b left: No inactivation. 3b middle: 56°C inactivated serum. 3b right: 121°C inactivated serum. Aggregation of hyphae is most apparent in 121°C inactivated medium

4. DISCUSSION

4.1 Current Research Attention on Hypha Growth

C. albicans is a unicellular yeast-like fungus, which has two morphological forms, yeast spore and hyphae [14]. Hypha, which can be easily identified at sites of infection, is the pathogenic form with mass propagation by which *C. albicans* invades the host, and is also known as bacteria phase switching. Hypha is considered to be responsible to virulence with specific transcription [15]. More mycelium indicates higher infection rates. Mutant strains that cannot form mycelia have low or no virulence.

Reports such as Vivek Gupta reported [16], *C. albicans* infection or in combination with other cofactors may induce epithelial dysplasia and even malignant disease, and they found the growth of *C. albicans* in severe form of leukoplakia and oral squamous cell carcinoma, while no *C. albicans* growth in mild and moderate form of leukoplakia. Endotoxin or metabolites of *C. albicans* could affect proliferation. For instance, there are inhibitory substances such as second messenger cAMP in oral mucosal

epithelial cells, which stop oral mucosal hyperkeratosis, abnormal cell proliferation and even cancer. Secreted by *C. albicans*, the fungal cytolytic peptide toxin candidalysin [17] damages epithelial membranes. In addition to the cAMP-PKA pathway, it is reported that N-acetylglucosamine (GlcNAc), serum or neutral pH can induce hyphal formation efficiently in log phase of cell growth by Brg1-mediated removal of Nrg1 inhibition [18,19]. Consequently, the growth condition of *C. albicans* hyphae cells have been extensively studied [18]. An improved method for more effective cultivation of hyphae is important for molecular research.

4.2 Different Conditions of Serum for Hyphae Formations

Hyphae formations of *C. albicans* are significantly different in various culture conditions, which vary in terms of pH, CO₂, temperature, serum, etc. Acidic media increases spore formation, which easily fall off during epithelial adhesion; while alkaline media shorten hyphae length, which also hinders epithelial adhesion. Hence, a 5% w/v sodium bicarbonate gargle is commonly used to prevent adhesion and growth of *C. albicans*.

Mycelial growth is inhibited when the temperature is $<35^{\circ}\text{C}$, while spores are easily damaged or die when the temperature is $>40^{\circ}\text{C}$. The body's normal temperature of $36.3\sim 37.2^{\circ}\text{C}$ is thus very suitable for *C. albicans* hyphae formation. Hence, clinicians advise patients with *C. albicans* infections to drink $40\sim 60^{\circ}\text{C}$ warm water, and keep the mouth moist.

"Pure" (90%) hyphae phase *C. albicans* could be obtained at 3h after incubation in RPMI 1640 medium at 37°C and pH 7.0, with serum inactivated at 56°C [13]. *C. albicans* may grow lateral buds when the induction time is too long, which affects the mycelium purity. In the present study, we found that hyphae formation abilities varied between *C. albicans* obtained from different sources. Within an oral cavity mucosal lesion of OLP, 6 h would be the peak time to yield the purest hyphae growth.

4.3 Possible Mechanism for Hyphal Formation in Different Conditions of Serum

Although the mechanism controlling hyphae formation remain unclear, serum properties might play a role. The serum is an important component of cell culture media, which facilitates cell growth. Growth factors (hormones, interleukins, etc.) within the serum can promote growth and attachment factors can promote cell adherence. Meanwhile, serum proteins such as serum albumin, globulin, either act as nutrients, or neutralize the pancreatic enzymes. The serum can play a role in detoxification of fatty acids, heavy metals and certain proteases. The serum can also protect cells from mechanical damage. The animal serum can even act as pH buffer to facilitate cell growth.

In this study, we found that hyphae formation rate in RPMI 1640 serum-free control was similar to that of RPMI 1640 with non-inactivated serum, suggesting that non-inactivated serum possessed no obvious advantages for hyphae formation. Furthermore, our results reveal that hyphae formation rate in non-inactivated serum was significantly lower than that in 56°C inactivated serum, which is a conventional method for serum inactivation to destroy complement components, mycoplasma as well as other microorganisms in serum. This indicates that complement components or/and other humoral immunity factors might partially suppress hyphae formation of *C. albicans in vitro*. In our experiments with seven different clinical

isolates and one standard laboratory strain, hyphae formation started earlier in 121°C inactivated serum medium than in 56°C inactivated serum. Although compliant peaks were produced at 6 h of hyphae cultivation with these two sera, higher quantities of the eight strains were obtained in 121°C inactivated serum medium than in 56°C inactivated serum medium. Consequently, 121°C inactivated serum was found to be superior to 56°C for hyphae culture. The underlying mechanism requires further study, which would contribute to research about molecular mechanism involving hyphal development and the etiology, progression and treatment of the oral precancerous disease OLP targeting the virulence of *C. albicans* filamentation

5. CONCLUSION

A 6 h incubation in RPMI 1640 medium with serum, at 37°C and at pH 7.0, is optimal for mycelium collection or of *C. albicans* strains.

The 121°C 30 min inactivated calf serum is probably and currently the best way for culturing pure hypha of *C. albicans*, meanwhile, the underlying mechanism remains unknown.

Minerals in sera might play a role in mycelium morphogenesis and pathogenesis of *C. albicans*. This is need to be supported by further experiment data.

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7. AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

CONSENT AND ETHICAL APPROVAL

The study was carried out in accordance with the Helsinki Declaration of 1975, as revised in 2000,

and was reviewed and approved by the local ethical committee (No.20150012). All the patients gave their written informed consent for participation in the study.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Lohse MB, Gulati M, Johnson AD, Nobile CJ. Development and regulation of single- and multi-species *Candida albicans* biofilms. *Nat Rev Microbiol*. 2018;16(1): 19–31.
DOI: 10.1038/nrmicro.2017.107
2. Arora S, Verma M, Gupta SR, Urs AB, Dhakad MS, Kaur R. Phenotypic variability and therapeutic implications of *Candida* species in patients with oral lichen planus. *Biotech Histochem*. 2016;91(4):237-241.
3. Mehdipour M, Taghavi Zenouz A, Hekmatfar S, Adibpour M, Bahramian A, Khorshidi R. Prevalence of *Candida* species in erosive oral lichen planus. *J Dent Res Dent Clin Dent Prospects*. 2010; 4(1):14–16.
4. Li JY, Sun HY, Zhang QQ. Antifungal susceptibility test of genotypes of *Candida albicans* from patients with atrophic or erosive oral lichen planus. *Shanghai Kou Qiang Yi Xue*. 2011;20(3):300–303. [Chinese]
5. Eisen D. The clinical features, malignant potential and systemic associations of oral lichen planus: A study of 723 patients. *J Am Acad Dermatol*. 2002;46(2):207–214.
6. Gainza-Cirauqui ML, Nieminen MT, Novak Frazer L, Aguirre-Urizar JM, Moragues MD, Rautemaa R. Production of carcinogenic acetaldehyde by *Candida albicans* from patients with potentially malignant oral mucosal disorders. *J Oral Pathol Med*. 2013;42(3):243–249.
7. Kramer IR, Lucas RB, Pindborg JJ, Sobin LH. Definition of leukoplakia and related lesions: An aid to studies on oral precancer. *Oral Surg Oral Med Oral Pathol*. 1978;46(4):518–539.
8. Lodi G, Scully C, Carrozzo M, Griffiths M, Sugerman PB, Thongprasom K. Current controversies in oral lichen planus: Report of an international consensus meeting. Part 2. Clinical management and malignant transformation. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2005; 100(2): 164–178.
9. Fan Y, He H, Dong Y, Pan HB. Hyphae-specific genes HGC1, ALS3, HWP1, and ECE1 and relevant signaling pathways in *Candida albicans*. *Mycopathologia*. 2013; 176(5-6):329–335.
10. Naseem S, Min K, Spitzer D, Gardin J, Konopka JB. Regulation of hyphal growth and N-Acetylglucosamine catabolism by two transcription factors in *Candida albicans*. *Genetics*. 2017;206(1):299–314.
11. Manoharan RK, Lee JH, Kim YG, Lee J. Alizarin and chrysazin inhibit biofilm and hyphal formation by *Candida albicans*. *Front Cell Infect Microbiol*. 2017;7:447. [Published 2017 Oct 16]
DOI: 10.3389/fcimb.2017.00447
12. Sudbery PE. Growth of *Candida albicans* hyphae. *Nat Rev Microbiol*. 2011;9(10): 737-748.
13. Carbon source induced yeast-to-hypha transition in *Candida albicans* is dependent on the presence of amino acids and on the G-protein-coupled receptor Gpr1. Maidan MM, Thevelein JM, Van Dijck P. *Biochem Soc Trans*. 2005;33(Pt1): 291-293.
14. Gulati M, Nobile CJ. *Candida albicans* biofilms: Development, regulation and molecular mechanisms. *Microbes Infect*. 2016;18(5):310–321.
DOI: 10.1016/j.micinf.2016.01.002
15. Hebecker B, Naglik JR, Hube B, Jacobsen ID. Pathogenicity mechanisms and host response during oral *Candida albicans* infections. *Expert Rev Anti Infect Ther*. 2014;12(7):867-879.
16. Vivek Gupta, Khare Abhishek, Shreedhar Balasundari, Niranjana K Devendra, Kalim Shadab, Mahendra Anupama. Identification of *Candida albicans* using different culture media and its association in leukoplakia and oral squamous cell carcinoma. *J Oral Maxillofac Pathol*. 2019;23(1):28–35.

17. David L. Moyes, Duncan Wilson, et al. Candidalysin is a fungal peptide toxin critical for mucosal infection. *Nature*. 2016; 532(7597):64–68.
18. Su C, Yu J, Sun Q, Liu Q, Lu Y. Hyphal induction under the condition without inoculation in *Candida albicans* is triggered by Brg1-mediated removal of NRG1 inhibition. *Mol Microbiol*. 2018;108(4):410-423.
19. Su C, Yu J, Lu Y. Hyphal development in *Candida albicans* from different cell states. *Curr Genet*. 2018;64(6):1239-1243.
20. Yan Y, Tan F, Miao H, Wang H, Cao Y. Effect of shikonin against *Candida albicans* biofilms. *Front Microbiol*. 2019; 14;10:1085.
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