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Phenotyping Identification of *Candida albicans* for the Production of In House Helicase for Nucleic Acid-Based Detections for Fast Diagnosis

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ABSTRACT

Candida sp. was isolated from a local hospital in Kelantan, Malaysia. The isolated *Candida* sp. was identified presumptively by different culture media, microscopic examination, and germ tube test as well as physiological/biochemical test. Chromogenic agar, ChromID Candida was used to pre-identify the colonies based on colour code. The light and scanning electron microscopy were used to identify the morphology of the isolate. Germ tube test was carried out to find out the dimorphic and pathogenicity characteristic of *Candida* sp. A commercial Analytical Profile Index (API) Candida identification kit was utilised in this study as a phenotypic identification of *Candida* sp. *Candida albicans* was successfully isolated and identified phenotypically in this study.
Keywords: *Candida albicans*, scanning electron microscopy, germ tube test

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INTRODUCTION

In recent years, the incidences of fungal infections caused by the opportunistic fungal pathogens have increased tremendously, even though we are advance in health care and therapeutic methods. Moreover, most of the opportunistic fungal pathogens are the antifungal drug-resistant, resulting in high rate of fatality due to fungal infections. Of the causative agents, yeasts such as *Candida* species have been claimed as opportunistic fungal pathogens. Indeed, *Candida* species are the most common human commensal that able to cause a broad spectrum of disease in hosts [1-2]. In such case, *Candida albicans* has become one of the opportunistic fungal pathogen that colonizes mucosal surface of the oral and vaginal cavities as well as the gastrointestinal tract in host [3]. *C. albicans* may be isolated from persons with deficiency in immune system such as human immunodeficiency virus (HIV) infected patients [4]. In fact, *C. albicans* infections may exist in either superficial candidiasis or invasive candidiasis. The superficial candidiasis is the oral or vaginal thrush and the chronic mucocutaneous candidiasis whereas the invasive candidiasis is the candidemia and the disseminated candidiasis [5].

In general, *C. albicans* is a diploid and dimorphic fungus. Therefore, *C. albicans* has the ability to grow in two different ways; blastospore phase (reproduce by budding or yeast-like form) and hyphal phase [6]. This yeast exhibits different morphological forms under different environmental conditions. Therefore, the phenotypic transition of *C. albicans* can be induced by several external factors such as pH or temperature as well as different chemical compounds like *N*-acetyl-D-glucosamine, amino acids, and serum, etc. The ability of *C. albicans* to transit between yeast-like and hyphal form has been implicated in its pathogenicity [7]. As a result, *C. albicans* has been implied in molecular biology and pathogenicity studies.

Nowadays, various techniques are available for the isolation and identification of *Candida* species in patients. Besides that, *Candida* infections can be detected easily and accurately due to the rapid methods in clinical microbiology. Clinicians and scientists could be able to isolate and identify the *Candida* species from clinical samples in diagnostic laboratory based on the phenotypic and genotypic properties of *Candida* species [8]. Thus, the purpose of this study is to find out the phenotypic properties of local clinical isolate *Candida* species in hospital, Malaysia. The isolated *C. albicans* will be used for the production of in house Helicase for nucleic acid-based detections for fast diagnosis.

MATERIALS AND METHODS

Isolates

The clinical samples of *Candida* sp. were collected from School of Biological Sciences, University of Science, Malaysia (USM). The clinical strains were isolated from patients infected with *Candida* sp. The isolated strains of *Candida* sp. were stored in refrigerator at 4°C for further identification.

Culture media

The Sabouraud's dextrose agar (SDA) (Becton, Dickinson and Company, USA) mixture was prepared by dissolving 65.0 g of the powder in 1.0 L of sterile distilled water. Mix the mixture thoroughly. The mixture was autoclaved at 121°C for 15 minutes. SDA was prepared by pouring the mixture to the same thickness on sterilized petri plates. The isolated strains of *Candida* sp. were then grown at 37°C for 18 hours. The chromogenic culture media, ChromID Candida (bioMérieux, France) were used for rapid direct identification of *Candida* sp. The isolated strains of *Candida* sp. were subculture on the ChromID Candida plates and incubated at 37°C for 24 hours.

Microscopy examination

Isolated *Candida* sp. culture from the SDA plate was used for Gram's stain and lacto phenol cotton blue stain. 100.0 µl of culture from the Sabouraud's dextrose broth was inoculated and spread onto a SDA plate. The inoculated plate then incubated at 37°C for 18 hours prior to view under scanning electron microscope (SEM).

Germ tube test

Isolated *Candida* sp. culture from the SDA was inoculated into 1.0 mL of fetal bovine serum (FBS). The inoculated FBS was incubated at 37°C for 3 hours. After 3 hours incubation, 10.0 µl of culture was viewed under light microscope to identify the germ tube formation from the isolated *Candida* sp.

Biochemical test

A commercial Analytical Profile Index (API) yeast identification kit, namely API Candida (bioMérieux, France) was used to conduct the biochemical test on the isolated *Candida* sp. The API Candida strip consists of 12 biochemical tests. Yeast suspension was prepared as inoculum for the test. The prepared yeast suspension was distributed into each tubes of the test. One of the strips was used as negative control where sterile distilled water was used as inoculum. The biochemical tests of API Candida strip were shown in Table 1.

RESULTS AND DISCUSSION

At present, infections caused by *Candida* spp. are growing gradually; hence early diagnosis and prompt antifungal therapy are essential. In such reason, various methods of presumptive identification of *Candida* sp. are developed and available in clinical microbiology. In this study, clinical samples of isolated *Candida* sp. were subculture onto SDA. Based on the appearance of yeast colonies on SDA, the young colonies were cream-white with a soft consistency; and also the surfaces and margins of the colonies were round with convex and smooth (Figure 1A). The young colonies from SDA will then subculture onto ChromID Candida for further identification. After 24 hours of incubation at 37°C, yeast colonies had grown well,

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forming young colonies. The young colonies were appeared in smooth with blue colour (Figure 1B); hence, *C. albicans* was identified as stated in the manufacturer's instructions. The ChromID Candida allows the rapid and specific identification of *C. albicans* due to specific hydrolysis of a hexosaminidase chromogenic substrate, which resulting in formation of blue colour colonies [9, 10]. Williams and Lewis, 2000 reported that ChromID Candida has proven excellent for presumptive identification of *C. albicans*. As a result, chromogenic agar such as ChromID Candida was suitable to use as primary isolation with the combination of SDA [8, 11, 12].

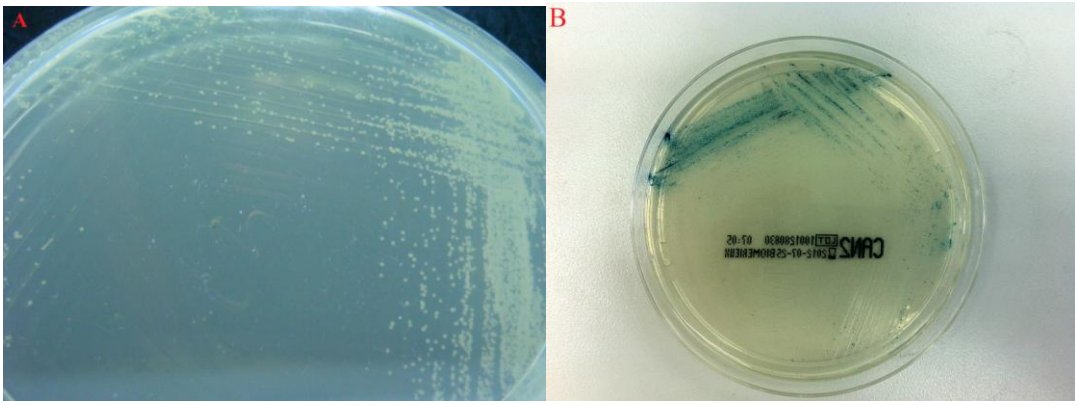

Figure 1A: Colonies appearance of isolated *Candida* sp. on SDA at 37°C for 18 hour.
Figure 1B: Colonies appearance of isolated *Candida* sp. on ChromID Candida.

In Figure 2A, gram's stains of smears showed gram-positive (dark blue/purple colour) budding yeasts without pseudohyphae. Gram-positive yeast cells are able to retain crystal violet stain due to thick peptidoglycan layer in the cell wall. Pseudohyphae are elongated cells formed from blastospores (budding cells). After that, the daughter cell elongated without break off from the mother cell [13]. On lacto phenol cotton blue wet mount examination, the isolate yeasts were stained in light blue as shown in Figure 2B. Lacto phenol cotton blue staining is widely used to stain the chitin in the fungal cell walls due to the simplicity in preparation [14, 15].

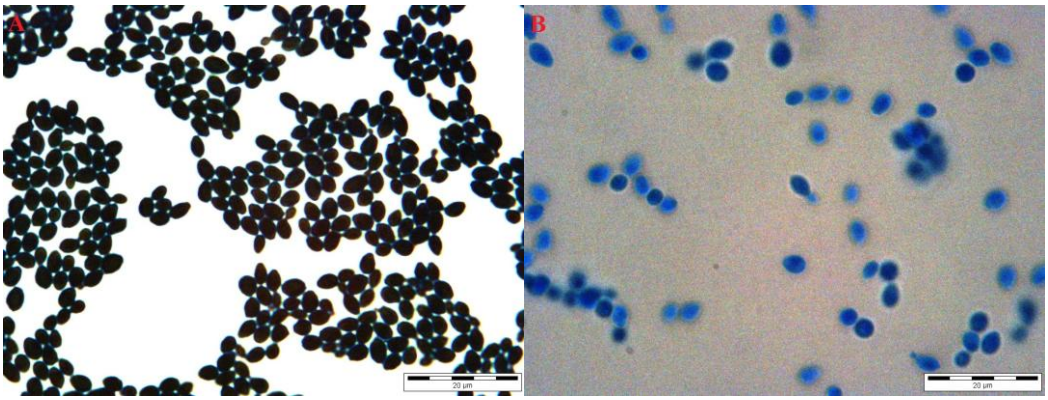

Figure 2A: Gram's stain of isolated yeast Cells. 2B: Lacto phenol cotton blue stain of isolated yeast cells.

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The morphology of isolated yeast cells was further identified via observation under scanning electron microscope. The isolated yeast cells were cultured onto SDA at 37°C for 18 hours. After 18 hours incubation, yeast culture on SDA was processed and viewed under scanning electron microscope. The morphology of isolated yeast cells were in ovoid or spherical with budding as shown in Figure 3. Microscopic examination via scanning electron microscope (SEM) is an essential step to identify the morphology of isolated yeast cells precisely compare to staining method.

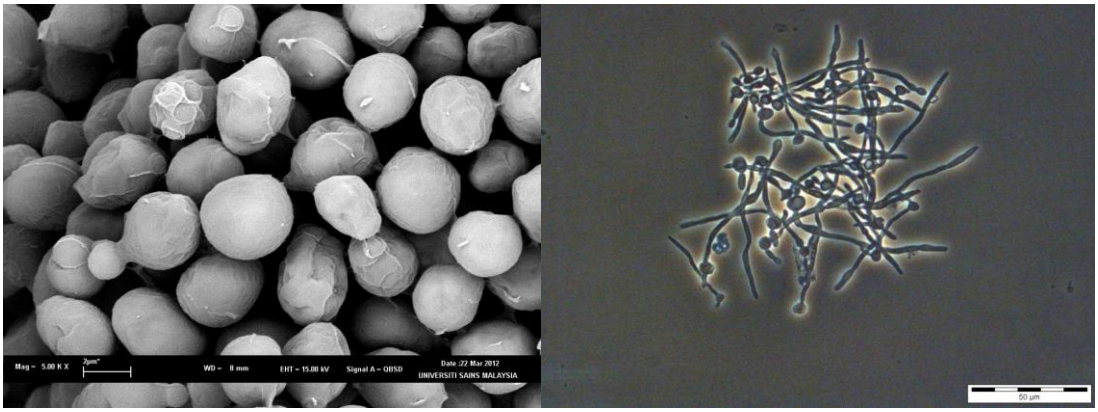

Figure 3: SEM micrograph of the isolated *Candida* sp. cultured on SDA at 37°C for 18 hours.
Figure 4: Formation of germ tubes from isolated *Candida* sp. in FBS at 37°C for 3 hours.

A germ tube test was carried out in this study to differentiate *C. albicans* from *Candida* spp. This germ tube test is widely known as presumptive test for identification of *C. albicans*. Figure 4 showed that the formation of germ tubes from isolated *Candida* sp. in fetal bovine serum (FBS) at 37°C for 3 hours. Generally, a hypha is formed by elongation of a germ tube produced by the round mother cell, *C. albicans*, in the presence of inducer such as serum [13, 16]. Based on the results, number of germ tube formed was more than five (n>5), thus no false-positive results in this test. Hereby, the isolated *Candida* sp. can be identified as *C. albicans*. However, further biochemical identification is needed for confirmation.

A biochemical identification of isolated *Candida* sp. was performed in order to confirm the species of isolated organism. There was a series of biochemical tests in API Candida test strip. This API Candida system is specific designed for *Candida* sp. identification. The API Candida system proven that able to produced good results without extra tests in the identification of majority of yeasts, especially *Candida* sp. isolated in clinical microbiology laboratories compared to ID 32C test system [17]. Thus, the physiological or biochemical identification of *Candida* sp. can be determined easily and rapidly via this API Candida system [18]. The reading of API Candida system was based on the changes in turbidity or colour changes in each well of a test strip. The numerical codes obtained from the test results were used to identify the test organism with database provided by the manufacturer. The numerical codes were confirmed using the api*web*™ identification software (bioMérieux, France). Figure 5A showed the negative control of API Candida test strip, in which no inoculum was added in each well. On the other hand, Figure 5B showed the positive results of *C. albicans* in API

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Candida test strip. Total of 12 biochemical tests were performed in API Candida system (see Table 1).The positive results showed that changes of colour in GLU, GAL, SAC, TRE and βNAG. The positive results indicated that the yeast cells have acidification reaction with glucose, galactose, saccharose and trehalose under anaerobic condition, except raffinose. Besides that, the yeasts cells also showed positive results in N-Acetyl-β-Glucosaminidase [19]. Hence, we can conclude that the isolated *Candida* sp. was *C. albicans* based on the numerical codes of positive results in API Candida system.

Figure 5: (A) Negative control without inocula. (B) Positive results showed changes colour in API Candida system after incubation at 37°C for 24 hours.

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CONCLUSIONS

In this study, several isolation and identification methods were carried out. These methods included the identification via conventional techniques and the used of commercial identification kits. Clinical sample of *Candida* sp. was isolated and identified as well as recognised according to the phenotypic characteristics of *Candida* sp. Despite of only a presumptive identification, we can deduce that the isolated *Candida* sp. is *Candida albicans*. However, deeper studies such as genome analysis or genotypic characterisation of the *Candida* sp. should be suggested in future work in order to find out the species and also the strain of *Candida*.

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Table 1: The list of biochemical tests in API Candida.

*Tests	Active Ingredients	Qty (mg/well)	Reactions	Results	
				Negative	Positive
1) GLU 2) GAL 3) SAC 4) TRE 5) RAF	D-glucose D-galactose D-saccharose D-trehalose D-raffinose	1.4 1.4 1.4 1.4 1.4	Acidification (GLUcose) Acidification (GALactose) Acidification (SACcharose) Acidification (TREhalose) Acidification (RAFfinose)	violet / grey-violet	yellow green / grey
6) βMAL	4-nitrophenyl-βD-maltopyranoside	0.08	β-MALtosidase	colourless	pale yellow / bright yellow
7) αAMY	2-chloro-4-nitrophenyl-αD-maltotrioside	0.168	α-AMYlase	colourless	pale yellow / bright yellow
8) βXYL	4-nitrophenyl-βD-xylopyranoside	0.095	β-XYLosidase	colourless / very pale yellow/ blue / green	pale yellow / bright yellow
9) βGUR	4-nitrophenyl-βD-glucuronide	0.063	β-GLUcuRonidase	colourless / blue/green	pale yellow / bright yellow
10) URE	urea	1.68	UREase	yellow-pale orange	red
11) βNAG	5-bromo-4-chloro-3-indoxyl-N-acetyl-βD-glucosaminide	0.09	N-Acetyl-β-Glucosaminidase	colourless / yellow	blue / green
12) βGAL	5-bromo-4-chloro-3-indolyl-βD-galactopyranoside	0.0815	B-GALactosidase	colourless / yellow	blue / green

*Wells from number 1-5 and 10 were covered with mineral oil for anaerobic incubation.

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