Molecular and conventional methods for detection of *Candida* species isolated from a sample of immunocompromised Iraqi patients with pulmonary symptoms

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Objectives *Candida* species has emerged as a potentially pathogenic fungus rather than benefit mucosal commensal in patients with pulmonary diseases. Therefore, our study was carried out to detect *Candida* species in sputum samples from patients with pulmonary diseases using conventional and molecular methods.

Methods A total of 100 sputum samples obtained from patients with pulmonary symptoms such as chronic productive cough, shortness of breath, wheezing and fever were included in this study. Sputum samples were dispensed into three specimen parts; the first one was applicated for cultured on Sabouraud dextrose agar at 37°C for 48 h and then the purified colony of Candida underwent biochemical tests including API, Candida strips, and germ tube. The second part was undergone direct gram stain, while the third part was applicated for DNA extraction and then molecular diagnosis with PCR technique using specific primers.

Results Culture result revealed 43 positive samples for *Candida* species out of 100 samples. Among these positive samples, 23 (53.5%) were positive for *C. albicans* in each of culture and germ tube. API 20 Candida found that (40) samples were positive for Candida species as, 23 (57.5%) represent *Candida albicans*, 8 (20.0%) *Candida glabrata*, 4 (10.0%) *Candida parapsolosis*, 4(10.0%) *Candida tropicalis* and only one (2.5%) as *Candida krusei*. Molecular test revealed that forty one samples out of forty three culture isolates of Candida species were positive as follow twenty three (53.48%), belong *Candida albicans*, Nine (20.93%) belong *Candida glabrata*, Six (13.95%) *Candida parapsolosis*, Four (9.30%) belong *Candida tropicalis*.

Conclusion *Candida albicans* is highly prevalent among patients suffering from bronchopulmonary symptoms. The molecular and conventional methods gave concomitant results as detection tools for the diagnosis of such microorganisms.

Keywords *Candida albicans*, phospholipase B gene, OMNIgene, API 20 AUX

Introduction

Candidiasis is a mycotic infection caused by members of the genus *Candida*. Chiefly, *Candida albicans* is responsible for about (70–80%) of all *Candida* infection. The *Candida* as an opportunistic yeast pathogen which increases predominantly in patients with predisposing condition, including immunodeficiency such as HIV infections, prolong used of broad-spectrum antibiotics, corticosteroids, diabetic patients and infections with other debilitating disease.[1]

In immunocompromised patients, the clinical appearance of the *C. albicans* infection is often very complex and identification of the organism is difficult. Therefore, speedy diagnosis and management of candidiasis are crucial for these patients.[2] *Candida* was frequently isolated from the mucosal surface of normal individuals, is capable of initiating a variety of recurring diseases especially in the vagina, oral and gastrointestinal mucosa. It also can affect different organs of the body, as systemic candidiasis involves major organs including, heart, kidneys, liver, spleen, lungs, brain, peritoneum, joint, and skeletal muscles, and was referred to generalized dissemination of the pathogen.[3,4]

Candida pneumonia is one of the most challenging of all the *Candida* infections. Pneumonia due to infection with *Candida* spp. is extremely rare, but because of contamination with oral flora, these organisms are frequently cultured from respiratory secretions.[5] *Candida* species are the fourth common cause of lung infections in hospitalized patients, and the most commonly isolated species include *C. albicans, C. glabrata, C. tropicalis,* and *C. parapsilosis*.[6]

Methods

Sputum samples have been collected from 100 patients of age group ranged from 10 to 90 years old, with a mean age 47.23 ± 19.51. Some of these patients were suffering from systematic diseases such as tuberculosis, diabetes mellitus, leukemia, while others were with immunocompromised status. Those patients were attending and admitting to Al-Yarmouk Teaching Hospital, Al-Emamain Al-Kadhemain Teaching Hospital and Chest and Respiratory Diseases Institute/Baghdad Medical City during the period from September 2015 to February 2016. Each sputum sample was dispensed into three specimen parts. The first one was applied for culture on Sabouraud dextrose agar at 37°C for 48 hrs. Purified colonies from this culture had undergone biochemical tests including API *Candida* strips and germ tube. The second part was used in direct Gram stain while the third one was applied for molecular method. Standard strains of *C. albicans* ATCC 10231, was obtained from the National Institute of Health in Baghdad which was used as a positive control.

Isolation and Identification of Candida Species

Gram stain method was applied to each fresh sputum specimen and examined microscopically for detecting *Candida* species.

Sputum samples were streaked on Sabouraud's Dextrose Agar (SDA) and incubated at 37°C for 24–48 hrs. The isolates were re-identified by using API 20 C AUX and germ tube production. API 20 C AUX was performed according to the manufacturer's instructions. (Biomuriex, France) for the confirmatory identification of the *C. albicans* and other species. Germ tube production is a diagnostic characteristic method for *C. albicans*. A small part of yeast colony to be tested was emulsified with 0.5 ml of mammalian serum in a small test tube. The tube was incubated aerobically at 37°C in an incubator for 2 hrs. A drop of the serum was removed to a slide and examined microscopically using the ×10 and ×40 objective lenses. A cylindrical filament originating from the blastoconidium without any constriction at the point of origin and without obvious swelling along the length of the filaments indicates a germ tube positive yeast.[7]

Molecular Method for Diagnosis of Candida Species

The extraction of DNA was applied from each sample using sporeLYSE, DNA Genotek, purification kit (Canada) with modification by mixing 200 µl of the sputum sediment with the 40 µl of lysis buffer the suspension underwent a freezing–thawing technique by subjecting the samples to liquid nitrogen for 5 min; followed by boiling for 3 minute for five cycles.[8] (Freezing–thawing technique was added to the protocol as an efficient step and enhance the cell lysis)*. The primer sequences were used for the amplification for PLB genes of *Candida* species were selected according to Nabil S. Harmal et al.,[9] (Table 1). An internal control has been used to measure the efficiency of the DNA extraction process fluids, as well as the impact of external and internal factors on gene amplification process. Since DNA extracted from fluids can be variably degraded and may contain PCR inhibitors. The human beta-globin primers was taken from Saiki et al.,[10] and synthesized in Alpha DNA® (Canada). The thermocycling conditions with a cleaver scientific thermal cyclers (TC 32/80-UK) were as follows: After initial denaturation at 94°C for 5 min, the 30-cycle amplification profile consisted of 95°C for 30 s, 63°C for 35 s and 72°C for 1 min. Final elongation was occurred at 72°C for 10 min. PCR products were processed into a 2% (wt/vol) agarose gel (Merck-Germany) at 7 V/cm for 1.5 hr. A molecular marker (1-kb DNA ladder; Bioneer) was run concurrently. DNA bands were visualized and photographed under UV light after the gel was stained with ethidium bromide.

Table 1. **Sequence and product size of PLB genes primers**

Candida species	Primer name	Primer sequence (5′ → 3′)	Annealing temperature	Product size (bp)
C. albicans	CA F	TTGTGTTGCTACATCACCAAC	63°C	538 bp
	CA R	TTTGCTGGCAACTTGATTACC		
C. glabrata	Cg F	TCTCACACTCCATTGTCTCA	50°C	404 bp
	Cg R	AGCAGGTTTACCATCAGA		
C. parapsilosis	CPF	TCCATCGACGAATTGATTG	60°C	252 bp
	CPR	ACCGTTTTGAGACCTCAAG		
C. tropicalis	CTF	CCCATACGATTTATGGAAT	53°C	501 bp
	CTR	CCATTGACACAAGCATTTAC		

*Modifications Step

Statistical Analysis

Statistical Analysis System (SAS) software was used for all statistical analysis continuous variables were expressed in mean ± standard deviation (SD). The Pearson's Chi-square test or Fisher exact test was used for comparing the categorical variable. A two-sided significant level of 0.05 was considered to indicate a statistically significant difference.

Results

A total of 100 patients suffering from pulmonary diseases were enrolled in this study. 64 (64%) were males and 36 (36%) were females with a ratio of 1.8:1, Fig. 1. The principal findings were the ages ranged between 10 and 90 years with mean (47.23 ± 19.51) years.

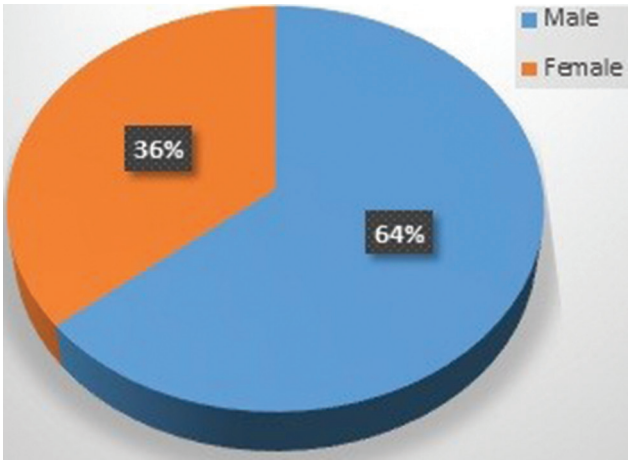

Fig. 1 **Gender distribution of patients with broncho pulmonary diseases.**

Table 2. **Correlation between *Candida* species and gender**

			PCR					Total
			Candida albicans	*Candida glabrata*	*Candida parapslosis*	*Candida tropicalis*	negative	
Sex	Female	Count	8	4	3	1	20	36
		%	34.8%	44.4%	50.0%	25.0%	34.5%	36.0%
	Male	Count	15	5	3	3	38	64
		%	65.2%	55.6%	50.0%	75.0%	65.5%	64.0%
Total		Count	23	9	6	4	58	100
		%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
P-value			0.899					

The correlations between *Candida* species infections and gender are shown in Table 2.

Out of 100 patients suffering from pulmonary diseases enrolled in this study, they were categorized according to their underlying diseases as follows; 34 patients with tuberculosis, 26 patients with diabetes mellitus, 14 patients with acute lymphoblastic leukemia, 12 patients with lung cancer, 10 patients with lymphoma, 8 patients with acute myelogenic leukemia, 5 patients with asthma, 3 patients with heart failure, 3 patients with renal failure, 2 patients with liver cancer, 2 patients with ovarian cancer, 2 patients with prostate cancer, 1 patient with chronic bronchitis, 1 patient with chronic myelogenic leukemia and only 1 patient with Rheumatoid arthritis, Fig. 2.

Percentages of Candida Species from Patients with Pulmonary Manifestations and Other Underlying Diseases

This relationship between *Candida* species isolated from patients with pulmonary manifestations and other underlying diseases are summarized in Table 3, which showed a statistical significant difference ($P < 0.01$).

Cultivation and Gram Stain

A total of 100 sputum samples were cultivated on Sabouraud's Dextrose agar and incubated for 2 days at 37°C. Forty three samples (43%) were positive for *Candida* species the colonies were mucoid and have a creamy color. Gram stain confirmed this result in that the 43 samples were gram positive.

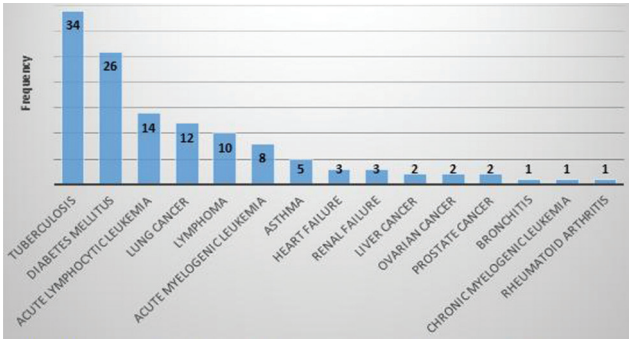

Fig. 2 **Distribution of patients with pulmonary manifestations and other underlying diseases.**

Comparison between Molecular and Culture as Detection Methods for Candida Species

Both methods gave positive results for 41 samples. Separately, one sample was negative by culture and positive by PCR method, and two out of 43 samples were negative by PCR and positive by culture method. The sensitivity of culture test was 97.6% with a specificity of 96.6%, while the positive predictive value was 95.4% and negative predictive value was 98.3% with a *P* value of < 0.001, Table 4.

Germ Tube Formation

A total of 43 culture samples were examined for germ tube. The result revealed that 23 (53%) were positive for *Candida albicans*, as shown in Fig. 3 and Table 5.

API 20 Candida Kit

A total of 43 culture samples were examined by API 20 AUX *Candida* strips, it was found that (40) samples were positive for *Candida* species, 23 (57.5%) represent *Candida albicans*, 8 (20.0%) *Candida glabrata*, 4 (10.0%) positive cases of *Candida parapslosis*, 4 (10.0%) *Candida tropicalis* and only 1 (2.5%) as *Candida krusei* (Table 6).

Table 4. **Comparison between molecular and culture methods for detection of *Candida* species**

			PCR		Total
			positive	negative	
culture	positive	Count	41	2	43
		%	95.3%	3.4%	43.0%
	negative	Count	1	56	57
		%	1.8%	96.6%	57.0%
Total		Count	42	58	100
		%	42.0%	58.0%	100.0%
P-value			< 0.001		
Sensitivity			97.6%		
Specificity			96.6%		
Positive predictive value			95.4%		
Negative predictive value			98.3%		

Table 3. **Percentages of isolated *Candida* species from patients with pulmonary manifestations with different underlying diseases**

	PCR					Total	*P*-value
	Candida albicans	*Candida glabrata*	*Candida parapslosis*	*Candida tropicalis*	Negative		
Hematological malignancies	5	2	2	2	11	22	---
%	21.7%	22.2%	33.3%	50.0%	19.0%	22.0%	0.0014**
Solid tumor	9	5	1	1	12	28	---
%	39.1%	55.6%	16.7%	25.0%	20.7%	28.0%	0.0031**
Ashmatic patients	1	0	1	0	3	5	---
%	4.3%	0.0%	16.7%	0.0%	5.2%	5.0%	0.0149**
DM	7	4	1	1	13	26	---
%	30.4%	44.4%	16.7%	25.0%	22.4%	26.0%	0.0035**
TB	4	1	2	1	25	33	---
%	17.4%	11.1%	33.3%	25.0%	43.1%	33.0%	0.0049**

Molecular Detection

Conventional PCR was done for the amplification of PLB gene by using a specific set of primer sequences. The results showed that, this gene (PLB gene) was present in 23 out of 100 sputum samples, PCR product of this gene was 538 bp which represent *Candida albicans*. Nine *Candida glabrata*, PCR product of this gene was 404 bp. Six *Candida parapslosis* PCR product of this gene was 252 bp. Four *Candida tropicalis* PCR product of this gene was 501 bp (Figs. 4 and 5).

Discussion

Candida infections are still an important problem, especially for immunosuppressed individuals.[11] Inability or delay in diagnosing fungal infection defers the administration of appropriate therapy. This has grave implications for the prognosis of the patient: reliable and rapid diagnostic tests for systemic mycoses are imperative to improve rates of patient survival.[12]

In the present study, results indicated that there is no relationship between the infection rate with *Candida* species and gender. The percentage of infected males were 26 out of 43 (60.4%) and females were 17 out of 43 (39.5%), hence there is no significant difference between male and female infection rate upon existing both in the same environment.

This result was disagreed with other study done by Saba Sabeeh,[13] who found that *Candida* infection was more frequent among females than males, and disagreed with that done by Ibrahim,[14] who found that infection was more frequent in males than in females. The possible explanation for such discrepancy may be due to nature of the societies and duration of time for sample collection.

In the present study, a relatively high percentage of *Candida* species infection was found among patients with hematological malignancies, solid tumor, asthma, diabetes mellitus and from patients with tuberculosis. These results are in accordance with those obtained by Lindau et al.[15] and Ansari et al [16] who proved that a fungal infection represents a growing problem in patients with hematologic malignancies particularly during chemotherapy induced neutropenia and other chronic debilitating diseases.

Microscopic examination of sputum using staining methods, remain popular in the diagnosis of pulmonary infection especially in low-income countries, due to its rapidity, low cost, relatively easy to perform and high positive predictive value.[15] On the other hand, culture is considered to be the "gold standard" method for the diagnosis of pulmonary infections but require 20 to 100 viable organisms per sample, and this is a cumbersome in partially treated patients. Culture also labor intensive and time consuming.[16]

In this study, positive cultures were tested by germ tube and biochemical API 20. Results of germ tube revealed that 53.4% of positive culture were *C. albicans*. All germ tube samples were positive for *C. albicans* by API 20 AUX. Regarding other *candida* species tested by biochemical API 20, results revealed that 8 (20.0%) *Candida glabrata*, 4 (10.0%) positive cases of *Candida parapslosis*, 4 (10.0%) *Candida tropicalis* and only 1 (2.5%) as *Candida krusei*. That's mean API 20 *Candida* and germ tube technique provides a convenient and reliable method for identification of *Candida* species.

Molecular Method for the Detection of Candida Species

The PLB gene of *Candida* species is a novel target which shows a high variability of sequences among *Candida* species. The nucleotide sequence variability between the different species of *Candida* can reach 95%.[17] Thus, it is possible through designing a specific set of primers to target the unique sequence of PLB gene.

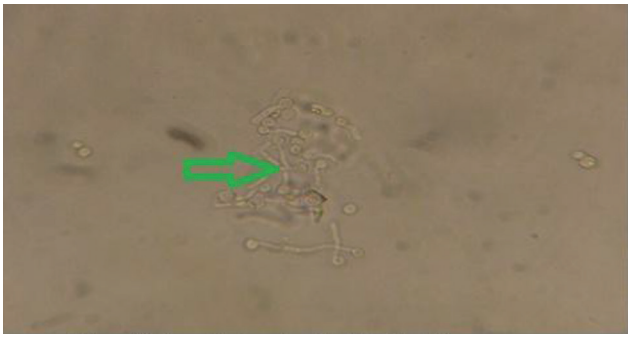

Fig. 3 **Germ tube formation test of *Candida albicans*.**

Table 5. **Percentages of germ tube formed by *Candida albicans***

Germ tube	Number	Percentage (%)
Negative	77	77.00
Positive	23	23.00
Total	100	100%
Chi-square (χ^2)	---	12.792**
P-value	---	0.0001

**($P < 0.01$).

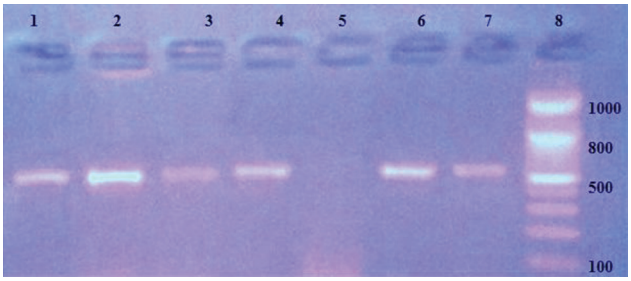

Fig. 4 **Agarose gel electrophoresis (2% agarose, 7v/cm2, and 1.5 hrs) of the PCR products of PLB gene 538 pb of *Candida albicans* lane 8: 100 bp DNA ladder, lane (1–4 and 6) positive sample for *Candida albicans*, lane 5 negative control, lane 7 positive control.**

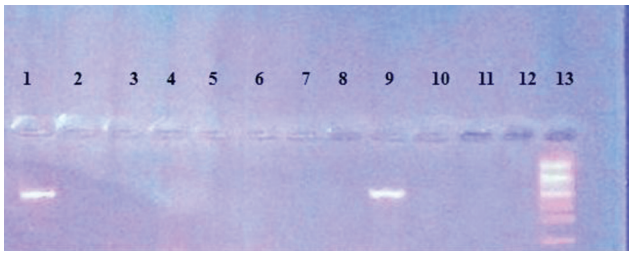

Fig. 5 **Agarose gel electrophoresis (2% agarose, 7v/cm2 and 1.5 hrs) of the PCR products of PLB gene of *Candida glabrata*. Lane 13: 100 bp DNA ladder, lane (1 and 9) positive sample for *Candida glabrata*, PCR product of this gene was 404 bp.**

Being used the same set of primers as in the current study Harmal et al[9] proved that species-specific PCR assay could identify and differentiate between the four most common *Candida* species isolated from clinical specimens namely, *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis*. Distinctive product size for each of these 4 species allow specific identification directly from the gel e ectrophoresis without the need for further genotyping. Based on the molecular weight of the amplicon product from that PCR product of this gene, it was 538 bp in *Candida albicans*, 404 bp in *Candida glabrata*, 252 bp in *Candida parapslosis* and 501 bp in *Candida tropicalis*.[18,19]

In this study, 23 samples were positive for PLB gene which is specific for *Candia albicans*, nine belong to *Candida glabrata*, six belong *Candida parapslosis*, and four belong *Candida tropicalis*. A study done by Cheang Pey Shyuan (19) strongly suggest that PLB is a significant virulence determinant of *albicans* species. However, the data generated here would provide the vital groundwork for elucidating the intrinsic functional role of PLBs in the virulence and pathogenesis of the *Candida albicans* and non-albicans *Candida* species.

The results of the current study showed that the PLB gene provides a novel target that could be used for the identification and detection of medically important *Candida* species from the clinical samples.

From this study, we concluded that *Candida albicans* is the most dominated isolates from patients suffering from pulmonary manifestations. Culture method is still the gold standard one in comparison with the molecular method.

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Conflict of Interest

The authors declare that they have no competing interests.

References

1. Estrada-Mata E, Navarro-Arias MJ, Pérez-García LA, Mellado-Mojica E, López MG, Csonka K, et al. Members of the Candida parapsilosis Complex and *Candida albicans* are differentially recognized by human peripheral blood mononuclear cells. Front Microbiol J. 2016;6:1527.
2. Ryan KJ, Ray CG. An introduction to infectious diseases 4th ed. New York, Sherris medical microbiology. 2004;pp. 661–663.
3. Ruchel R. Cleavage of immunoglobulines by pathogenic yeast of genus Candida, Microbial Sci. 1986;3:316–319.
4. Greenwood D, Richard C, Slack S, et al. Medical Microbiol 5th ed. Churchill Livingstone, London. 1997.
5. D'Eça Júnior A, Silva AF, Rosa FC, Monteiro SG, de Maria Silva Figueiredo P, de Andrade Monteiro C. In vitro differential activity of phospholipases and acid proteinases of clinical isolates of Candida. Rev Soc Bras Med Trop. 2011;44:334–338.
6. Mohandas V. Ballal M. Distribution of Candida Species in different clinical samples and their virulence: Biofilm formation, proteinase and phospholipase production: A study on Hospitalized Patients in Southern India. J Global Infect Dis. 2011;3:4–8. doi: 10.4103/0974-777X.77288.
7. Donghwa K, Woon S, Kyoung H. Rapid differentiation of Candida albicans from other Candida species using its unique germ tube formation. Yeast J. 2002;19:957–962. doi.org/10.1002/yea.891.
8. Reischl U, Pulz M, Ehret W, Wolf H. PCR-based detection of Mycobacteria in sputum samples using a simple and reliable DNA extraction protocol. Biotechniques. 1994;5:844–5.
9. Nabil S, Harmal, Alireza KH, Mohammed A, Alshawsh FJ, Zamberi SK. Simplex and triplex polymerase chain reaction (PCR) for identification of three medically important Candida species. African J Biotechnol. 2012;11:12895–12902. doi: 10.5897/AJB12.1708.
10. Saiki RK, Gelfand DH, Stoffel S, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science. 1988;239:487–491.
11. Shokohi T, Hashemi Soteh MB, Saltanat Pouri Z, Hedayati MT, Mayahi S. Identification of Candida species using PCR-RFLP in cancer patients in Iran. Indian J Med Microbiol. 2010;28:147–151.
12. Pfaller MA, Diekema DJ. Epidemiology of invasive Candidiasis: a persistent public health problem. Clin Microb Rev. 2007;20:133–163.
13. Saba Sabeeh. Molecular detection of Candidemia in Iraqi acute leukemic patients. 2011 (Thesis).
14. Ibrahim AA. Biochemical and immunological studies on Candida albicans proteinase. 2002 (Thesis).
15. Lindau S, Nadermann M, Ackermann H, et al. Antifungal therapy in patients with pulmonary Candida spp. colonization may have no beneficial effects. J Inten Care. 2015;3:31.
16. Ansari SH, Shirzadi E, Elahi M. The prevalence of fungal infections in children with hematologic malignancy in Ali-Asghar Children Hospital between 2005 and 2010. Iran J. Ped Hematol Oncol. 2015;5:1–10.
17. Nabil S, Harmal, Alireza Khodavandi, Mohammed A, Alshawsh, et al. Identification and differentiation of Candida species using specific polymerase chain reaction (PCR) amplification of the phospholipase B gene. African J Microbiol Res. 2013;7:2159–2166.
18. Lau A, Halliday C, Chen SCA, Playford EG, Stanley K. Comparison of whole blood, serum, and plasma for early detection of candidemia by multiplex-tandem PCR. J Clin Microbiol. 2010;48:811–816.
19. Cheang Pey Shyuan. Molecular cloning and gene expression analysis of the phospholipase b genes of non-albicans Candida species. 2005 (Thesis).