Development of multiplex-PCR assay for rapid detection of *Candida spp*.

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Abstrak

Tujuan *Uji biokimia untuk identifikasi Candida spp. memakan waktu dan menunjukkan hasil yang tidak dapat ditentukan. Metoda deteksi spesifik untuk antibodi, antigen dan metabolit Candida spp. memiliki sensitivitas dan spesifisitas yang rendah. Pada penelitian ini kami mengembangkan metoda diagnostik cepat, Uji Reaksi Rantai Polimerasa Multipleks (Multiplex-PCR) assay untuk identifikasi Candida spp.*

Metode *Lima isolate Candida spp. dibiak, diidentifikasi menggunakan uji germ tub, dan kit API® 20 C AUX (BioMerieux® SA). Selanjutnya, DNA dipurifikasi dengan kit QIAamp DNA mini (Qiagen®) untuk uji Multiplex-PCR.*

Hasil *Batas deteksi DNA dengan uji Multiplex-PCR assay dari C. albicans, C. tropicalis, C. parapsilosis, C. krusei dan C. glabrata berturut-turut adalah 4 pg, 0,98 pg, 0,98 pg, 0,5 pg and 16 pg Uji ini lebih sensitif daripada biakan karena Multiplex-PCR dapat mendeteksi 2.6-2.9 x 100 CFU/ml, sementara biakan hanya 2.6-2.9 x 102 CFU/ml.*

Kesimpulan *Multiplex PCR menunjukkan sensitivitas yang lebih tinggi dari biakan. Uji ini dapat direkomendasikan sebagai uji yang sensitive dan spesifik untuk identifikasi Candida spp.* ***(Med J Indones 2010; 19: 83-7)***

Abstract

Aim *Candida spp.* infection commonly occur in immunocompromised patients. Biochemical assay for identification of *Candida spp.* is time-consuming and shows many undetermined results. Specific detection for antibody, antigen and metabolites of *Candida spp.* had low sensitivity and specificity. In this study, we developed a rapid diagnostic method, Multiplex-PCR, to identify *Candida spp*.

Methods Five *Candida spp.* isolates were cultured, identified with germ tube and API® 20 C AUX (BioMerieux® SA) kit. Furthermore, DNA was purified by QIAamp DNA mini (Qiagen®) kit for Multiplex-PCR assay.

Results DNA detection limit by Multiplex-PCR assays for C. *albicans, C. tropicalis, C. parapsilosis, C. krusei and C. glabrata* were 4 pg, 0,98 pg, 0,98 pg, 0,5 pg and 16 pg respectively. This assay was also more sensitive than culture in that *Multiplex-PCR* could detect 2.6-2.9 x 100 CFU/ml, whereas culture 2.6-2.9 x 102 CFU/ml.

Conclusion Multiplex-PCR is much more sensitive than culture and thus, can be recommended as a sensitive and specific assay for identification of *Candida spp*. ***(Med J Indones 2010; 19:83-7)***

Key words: *Candida spp., multiplex-PCR*

Recently, many fungal infections were caused by a group of *Candida spp*. The fungal infection constitutes an opportunistic infection that commonly causes candidiasis in immunocompromise patients. Fungal infections may be superficially acute or chronic infections, relating to mouth, oropharyngeal and esophagus mucosal skin, and systemic infection.[1-3]

Identifications of *Candida spp.* by conventional culture technique followed by biochemical tests are time consuming. In addition, biochemical tests sometime fail to identify the microorganisms, while API 20C AUX assay takes long time to complete, around 2-3 days. Dealing with this assay, Gundes *et al*. reported that 12% of tested isolates need additional treatment and 0.8% are not well identified.[4] The assays for detection of specific antibody, antigen and metabolite of Candida have already been developed, however their sensitivity and specificity are very low. The reason why an antibody detection is not applicable for candidosis detection, is that colonization of *Candida spp.* in intestinal tract and other sites of the body induce antibody response of either immunocompetent or immunocompromise people.[5]

To overcome this limitation, at this time, many molecular assays have been developed for detecting *Candida* specifically. Molecular approach is much more rapid, sensitive and specific compared to conventional techniques. Rapid identification is important for *Candida* species which is resistant towards azole group, so that

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an early alternative therapy can be administered.[6,7] Polymerase Chain Reaction (PCR) is a nucleic acid-amplification technique. This technique is very sensitive and can amplify a small amount of DNA target, even for a single cell DNA.[8,9] Multiplex-PCR is a rapid, sensitive and specific method which combining many specific-species primers in one PCR tube. Therefore, it can be used to detect the causative agents simultaneously.[10,11] In this study we developed Multiplex-PCR, a rapid diagnostic assay for identification of *Candida spp*.

METHODS

A. Isolates of *Candida spp*

Candida albicans ATCC 10231 and *Candida glabrata* isolates were obtained from Clinical Microbiology Laboratory, Faculty of Medicine, University of Indonesia (FMUI). *Candida tropicalis*, *Candida parapsilosis*, and *Candida krusei* were obtained from Parasitology Laboratory, FMUI. All isolates were identified by using the germ tube technique and API® 20 C AUX (BioMerieux® SA) according to the manufacturer's instruction.

B. DNA extraction

DNA genome of *Candida spp* was extracted and purified by QIAamp DNA mini (Qiagen®) Kit. Briefly, single colony of fungi was taken by stick applicator sterile and inoculated into 5 ml YPD medium. The medium was incubated on orbital incubator at 30°C and 250 rpm for 16-24 h. Spectrophotometer was used to measure the cell density. Three milliliter medium containing cell density of 1.2 x 10^8 cells/ml (OD_{600} = 5–10) was centrifuged at 7.500 rpm for 5 min. Pellet was suspended with 600 µl Sorbitol Buffer and 200 U lyticase (Sigma®). The mixture was then incubated at 30°C for 30 min, and centrifuged at 7.500 rpm for 5 min. For obtaining DNA genome, pellet was extracted by QIAamp DNA mini (Qiagen®) following manufacturer's instruction. Fifty micro liters of final elute containing DNA was stored at– 20°C until used. [12]

C. Multiplex-PCR

Multiplex-PCR was performed with a total volume of 30 µl, consists of: 1x hotstar buffer solution (Qiagen®), 1 mM $MgCl_2$ solution (Qiagen®), 0,2 mM dNTP Mix (Biogen), 0,4 µM each of ITS1 and ITS2 primers, 1.2 µM each of CA3 and CA4 primers, 0,025 U of HotStar taq DNA polymerase (Qiagen®), and 2 µl (2-6 ng/reaction) of DNA template. The primer sequences used in this reaction were universal primers (ITS1 [5'-TCCGTAGGTGAACCTGCGG-3'] and ITS2 [5'-GCTGCGTTCTTCATCGATGC-3'], and specific primers (CA3 [5'-GGTTTGCTTGAAAGACGGTAG-3']) and CA4 [5'–AGTTTGAAGATATACG TGGTAG-3']).[10] Products were amplified using the following conditions (PCR thermal cycler MJ Mini PCR System BioRad): 95°C for 15 min, then 40 cycles of 94°C for 30 sec, 60°C for 60 sec, and 72°C for 45 sec followed by one cycle of 72°C for 5 min. For annealing temperature, primer was reacted with all five DNA templates (*C. albicans, C. tropicalis, C. parapsilosis, C. krusei* and *C. glabrata*) at 60.0, 60.6, 61.5, 62.8, 64.4, 65.6, 66.6 and 67.0°C. This reaction was aimed to know the optimal annealing temperature of all five DNA templates. For detection of amplification results, the PCR products were run on 9% polyacrylamide gel at 100 volt for 1.5 h. Gel was stained with 0.04% ethidium bromide for 5 minutes. The DNA bands were detected by Gel Doc XR with ultra violet transiluminator. A positive test result was claimed if a DNA band corresponding to each *Candida* species was detected on polyacrylamide gel, except for *C. albicans* in that two DNA bands had to be detected.

D. Sensitivity

Minimal DNA detection of Multiplex-PCR was tested towards all five DNA templates of *Candida spp*. A series of the two-fold DNA dilution (0.5 - 0.00025 ng/µl) in 1x PBS was conducted. Sensitivity of Multiplex-PCR assay was performed by comparing it with conventional culture. Ten-folded dilutions (stock: 2 x 10^5 cells) of *C. albicans* in 1x PBS were carried out. For each dilution, 200 µl suspension was applied for culture in SDA medium and another 200 µl was tested by Multiplex-PCR. After cells isolated from the medium, it were incubated at 35°C for 48 h, the number of fungal colonies were then calculated (CFU/ml).

E. Specificity

The specificity of Multiplex-PCR assay was evaluated for *Aspergillus niger, Aspergillus flavus* and *Aspergillus fumigatus*, *Streptococcus pneumoniae, Streptococcus alpha haemolyticus, Streptococcus beta haemolyticus*, *Streptococcus gamma haemolyticus, Staphylococcus aureus, Staphylococcus epidermidis, Morraxella, Diptheroid, Haemophilus influenzae, and Neisseria sica*.

RESULTS

1. Annealing Temperature of Multiplex-PCR Assay

The optimal temperature was determined by visually examination of the DNA band intensity on polyacriyamide gel. The optimal annealing temperature was defined as the annealing temperature that yielded the highest specific PCR products corresponding to particular species of *Candida spp*. At annealing temperature of 61.5°C, a 110 bp-DNA band intensity corresponding to *C. albicans* showed a faded DNA band, and it disappeared at annealing temperature of 62.8°C (Figure 1). For *C. tropicalis, C. parapsilosis, C. krusei* and *C. Glabrata*, their specific DNA bands showed the considerable DNA intensities at annealing temperature of 60.0 and/or 60,6°C. Therefore, we decided that the optimal annealing temperature of primers used in this assay for all five DNA was 60.0°C (Figure 1)

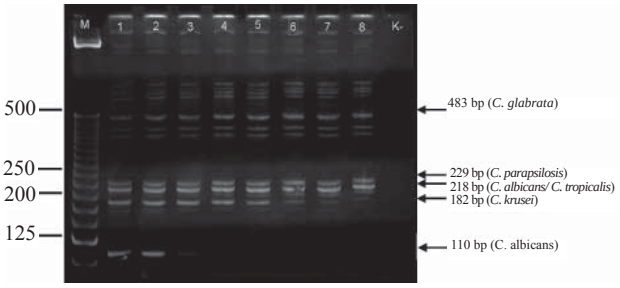

Figure 1. Annealing temperature of Multiplex-PCR assay. M: DNA ladder. Line 1-8: annealing temperatures at 60.0, 60.6, 61.5, 62.8, 64.4, 65.6, 66.6 and 67.0oC respectively. K-: PCR negative control

2. Sensitivity

In this study, DNA detection limits by Multiplex-PCR assay for *C. albicans, C. tropicalis, C. parapsilosis, C. krusei* and *C. glabrata* were 4.0, 0.98, 0.98, 0.5, and 16 picogram (pg) respectively (data not shown). To know the interferences among primers, we compared the results of PCR reactions using all primers (mixture of universal primer and *C. albicans* specific primers) and only a universal primer pair. These studies showed that DNA detection limit using a universal primer pair was 0.4 pg, while mixture of all primers was 4 pg (data not shown). It explained that the mixture of primers in a Multiplex-PCR system decreased by ten-fold DNA detection.

Moreover, sensitivity of Multiplex-PCR system was also analyzed by comparing it with conventional culture (Table 2). The Multiplex-PCR assay was able to detect *C. albicans* cells at dilution of 2 x 10^2 corresponding to 2.6 – 2.9 x 10^1 CFU/ml, while culture could only detect *C. albicans* cells at dilution of 2 x 10^3 corresponding to 2.6 – 2.9 x 10^2 CFU/ml. These results showed that the Multiplex-PCR assay developed in this study was ten times more sensitive than conventional culture for identification of *Candida spp*.

Table 1. Sensitivity of Multiplex-PCR assay compared with conventional culture

Fungi	Dilution in 1x PBS	Conventional culture* (duplo)		Number of colonies (CFU/ml) (duplo)		Multiplex-PCR*
		I	II	I	II	
C. albicans	2 x 10^5	+	+	TD	TD	+
	2 x 10^4	+	+	2.6 x 10^3	2.9 x 10^3	+
	2 x 10^3	+	+	-	-	+
	2 x 10^2	-	-	-	-	+
	2 x 10^1	-	-	-	-	-
	2 x 10^0	-	-	-	-	-

3. Specificity

Multiplex-PCR assay developed in this study generated negative results against the following fungus: *A. niger, A. flavus*, and *A. fumigatus*. Besides fungus, this assay also yielded negative results against a selection of bacteria, including S. *pneumoniae, S. alpha haemolyticus, S. beta haemolyticus, S. gamma haemolyticus, S. aureus, S. epidermidis, Morraxella, Diptheroid, H. influenzae,* and *N. sica*.

DISCUSSION

Primers employed for determination of Multiplex-PCR were similar to primers used by Chang, et al. namely, universal fungi-primers (ITS1 and ITS2) and specific primers for *Candida albicans* (CA3 and CA4). ITS1 and ITS2 primers are targeted to the conserved parts of 18S rDNA and 5.8S rDNA. These universal primers produced varies DNA fragments of *Candida spp*. *Candida albicans* specific primers, CA3 and CA4, amplified ITS2 region of *Candida albicans*.[7] Based on Fujita, et al, ITS region was located between 18S gene and 28S rRNA. ITS was divided into ITS1 (located between 18S and 5.8S rRNA genes) and ITS2 (located between 5.8S and 28S rRNA genes). The sequence differences in ITS1-5.8S rDNA-ITS2, ITS1 and ITS2 regions among fungus lead the regions to be used as

targets for fungal detection and identification.[13] Lau, et al. reported that universal primers targeted to ITS1 region for identification of pathogenic fungi were an ideal approach, because there were multi copies (approximately ≥ 100 copies) of this region in single fungal genome; therefore increasing the sensitivity assay. Also, this region contained the high variable sequences that were useful for species identification.[14]

In this research, sample was inoculated on YPD medium and then incubated for overnight *(*16-18 hours) before DNA extraction. Lysis of fungal cell with special lysis enzyme to produce spheroplast was an important step for extraction of fungal DNA. For obtaining a maximum DNA concentration, suspension containing fungal cells should be incubated overnight until achieving 10^5 fungal cells before the addition of lysis enzyme**.** [15]

Concerning about the sensitivity, Multiplex-PCR assay was able to detect *C. albicans* cells at dilution of 2×10^2 corresponding to $2.6 – 2.9 \times 10^1$ CFU/ml. According to Einsele, et al., detection limit of PCR using primers with target sequence of 18S rDNA was 1 CFU/mL blood.[7] In this research, primers with the same sequence target of 18S rDNA were also applied, but there were result differences as reported by Einsele, et al. These differences may be caused by technique applied. Einsele and colleagues were applied a hybridization method that was more sensitive than conventional PCR, while in this study we only applied conventional PCR.

The result of this research was also not agreed with study reported by Chang, et al. They reported that the detection limit of Multiplex-PCR using same primers as performed in this study was is 20 CFU/ml.[10] This differences in specimen type may be influence the result obtained between Chang, et al study and ours. In this case, Chang used blood, whereas in our study we use throat swab. As reported by Fredricks, et al. cited by Pryce, et al., blood contains inhibition factors such as hemoglobin and lactoferin In addition, specimens containing BACTEC and BacT/ALERT compounds are also not applicable for PCR due to the present of inhibition factors such as sodium polyanetholesulphonate (SPS).[16]

Multiplex-PCR sensitivity towards DNA *Candida spp* were varied, sensitivity of Multiplex PCR towards DNA *Candida glabrata* was lower compare to others. Sensitivity of fungal molecular determination was 10 fg, however, 1 – 10 fungal cells per mL.[17] According to Khlif, et al., determination of *Candida spp* using PCR method can detect less than 1 genome cell per 1 ml sample, he applied Nested PCR Method and used ITS1-ITS4 primers. Amplification in Nested PCR method was applied twice, the first amplicon was amplified further to increase sensitivity.[18] The differences are in the primer used, between pair of ITS1-ITS4 and ITS1-ITS2 was not affecting the sensitivity, however size or length of the amplicon detected was affected. ITS1-ITS4 primers amplified regions 3' 18S which bind to ITS1 region, 5.8S rDNA that bind to ITS2 region and 28S rDNA region.[13,18] In accordance to Chang, et al., sensitivity towards DNA *Candida albicans* obtained was 4 pg and this result was the same with the sensitivity found in this research. Based on Jaeger, et al., detection limit of fungal cell DNA using molecular method obtained was 10 pg and predicted similar to 100 cells, in which 1 *Candida albicans* cell containing of 37 fg DNA.[10,19] Primer sensitivity towards *Candida spp* DNA in their research is varied. According to Jordan, et al., the different observed can be due to some factors, including primer design applied or sensitivity different of cell wall chitin shell against fungi lysis enzyme.[15]

Inconclusions, multiplex-PCR (2,6-2,9 x 100 CFU/ml) was more sensitive, accurate and rapid, compared to culture (2.6-2.9 x 102 CFU/ml) in identifying *Candida spp.* with sensitivity towards DNA of *C. albicans* ATCC 10231, *C. tropicalis* and *C. parapsilosis*, *C. krusei* and *C. glabrata* were 4 pg, 0.98 pg, 0.5 pg, and 16 pg, respectively. While Multiplex-PCR DNA control positive concentration for *C. albicans* ATCC 10231, *C. tropicalis* and *C. parapsilosis*, *C. krusei* and *C. glabrata* obtained were 0.6 ng/µl, 0.2 ng/µl, 0.4 ng/µl, and 1.4 ng/µl, respectively. In the future, this assay would be evaluated using sufficient clinical specimens to study the validity of this assay

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