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Molecular typing of *Candida albicans* isolated from oral cavity of cancer patients using randomly amplified polymorphic DNA

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Abstract

To facilitate studies on the epidemiology of Candida, *numerous phenotypic and genotypic typing systems have been developed. The polymerase chain reaction (PCR) can be adapted for pringerprinting microorganisms by using paired primers derived from previously characterized sequences for PCR amplification.*

The aim of the present study was to develop and use Randomly Amplified Polymorphic DNA (RAPD) for molecular genetic typing of Candida albicans. *RAPD which is based on PCR with arbitrary short primers, was used to evaluate a panel of 9* Candida albicans *that were isolated from oral cavity of cancer patients in Yogyakarta.*

Analysis of RAPDs with primer AT (5' GCGCACGG 3') gave 5 types of banding patterns. Primer NT (5' CCCTAGGA 3') yielded 8 types of banding patterns. These data indicate that many different substrains of Candida albicans *can infect patients with cancer disease causing either oral colonization or oral candidosis.*

This study indicates that because of its ease and reproducibility, RAPD analysis is useful in providing genotypic characters for confirming the identities of stock isolates, for typing such as Candida albicans, *as well as rapid identification of pathogenic fungi.*

Keywords: *Candida – RAPD – genotypic typing*

Introduction

The ubiquitous, dimorphic yeast *Candida albicans* is an important cause of vaginitis and nosocomial infections, including life-threatening infections in immuno-compromised patients. The most frequently encountered clinical problem is caused by *Candida albicans*. Many people are colonized by this yeast as a commensal organism. For this reason, immunosuppressed patients must be strictly monitored for the clinical presence of yeast or molds (Vasquez *et al.*, 1991; van Belkum *et al.*, 1994).

Oral complications of cancer therapy occur in most patients treated for head and neck malignancy and in approximately 40% of patients receiving chemotherapy for malignancies in other sites (National Institute of Health, 1990). Dreizen and his colleagues (1983) reported that approximately 70% of oral infections in patients with solid tumor are caused by *Candida albicans* and other fungi, while in patients with hematologic malignancies, 50% of oral complications are due to the same organism.

Several DNA typing assays for pathogenic fungi have been described (Kattak *et al.*, 1992; Odds *et al.*, 1992; Magee *et al.*, 1992; Caugant and Sandven, 1993). Recently, the polymerase chain reaction (PCR) has been adapted to detect interstrain variation in unicellular microorganisms (Lehman *et al.*, 1992; van Belkum *et al.*, 1992; Niester *et al.*, 1993).

Despite the advantages of sensitivity and reproducibility, routine identification of yeasts by these genotypic methods has the important disadvantages of being laborious and time consuming, as well as require specialized equipment and, sometimes, requiring prior knowledge of nucleotide sequences in target species (Lehmann *et al.*, 1992). However, the randomly amplified polymorphic DNA (RAPD) assay, described previously by Welsh and Mc Clelland (Weissing *et al.*, 1991 and Williams *et al.*, 1990), circumvents several of these problems.

RAPD assay uses a single or a pairwise combination of primers, typically 9 to 10 nucleotides in length, to amplify target genomic DNA by the polymerase chain reaction (PCR). Fragments of DNA are generated by PCR amplification if the target sites for the primer exist within approximately 5 kb of each other on opposite DNA strands. The DNA fragments are then analyzed by separation through an agarose gel and stained with ethidium bromide. The random nature of RAPD refers to specific primer used in the PCR; the primer is chosen at random from many permutations of nucleotides that are available for 9 or 10 mer-primers (Lechmann *et al.*, 1992).

The aims of the present study were to develop and use RAPD- PCR for molecular genetic typing of *Candida albicans*, and to compare results of 8- and 10 - mer primers in typing strains isolated from oral cavity of cancer patients. It is also expected that the result will contribute to the detection of the presence of concurrent *Candida albicans* by the application of this technique. This study will hopefully lead to an easy and simple means of identifying *Candida albicans* and in typing individual strains.

Materials and Methods

Sample preparation

Cancer patients from different hospitals were recruited for this study. *Candida albicans* samples were taken from mucosa of the oral cavity of the patients.

Collection of Samples

The oral cavity of cancer patients was swabbed asseptically using different cotton buds. Each sample was then immersed in Stuart Transport medium in a vial. The bottle was closed tightly. If sample was not processed within 4 h, it was stored in refrigerator at 4^0 C.

Processing and identification of isolates

The sample was spread onto different plates of Sabouraud chloramphenicol agar. The yeast isolates were then identified as *Candida albicans* based on production of germ tubes after 3 h incubation in horse serum plasma and by the production of chlamydospores on cornmeal agar. Before genotyping, cells were grown for 48 h at 35°C on Sabouraud agar.

Confirmation of *Candida albicans*

Germ-Tube Test was done to confirm identification of *C. albicans*. A very small inoculum of yeast cells obtained from an isolated colony was suspended in 0.5 ml of horse serum plasma. The tubes were then incubated at 35°C to 37°C for no longer than 3.5 h. After incubation, a drop of the suspension was removed and placed on a

microscope slide. Examination was done under low power magnification for the presence of germ tubes. A germ tube is defined as an appendage that is one half the width and three to four times the length of the yeast cell from which it arises. In most instances, there is no point of constriction at the origin of the germ tube from the cell.

Another confirmatory test was chlamydospore formation. Colony from the primary culture medium was obtained and incubated on a plate of Cornmeal agar containing Tween 86 and trypan blue by making three parallel cuts about half inch apart at a 45° angle to the culture medium. A sterile coverslip was added to one area. The cornmeal agar plate was then incubated at 30°C for 48 h. After incubation, the areas where the cuts into the agar were made, were then removed and the presence of chlamydospora was examined.

Spheroplast formation and DNA extraction

After 2 days growth on Sabouraud-chloramphenicol agar, one colony of the yeast was grown in 1 ml of YPD broth (1% yeast extract, 2% peptone, 2% dextrose; Difco Laboratories). The mixture was then incubated for 18 to 22 h at 35°C with agitation. For spheroplast formation, cultures were washed with 1 M-sorbitol and the pellets were suspended at 30°C for 60 min in 1 ml SE buffer (1.2 M sorbitol, 0.1 EDTA pH 7.5) containing 3 mg of yeast lytic enzyme (Zymolase 100T, ICN Biochemicals)and 10 l of 2- µl mercapthoethanol. The resulting spheroplasts were harvested by centrifugation at 2500 rpm for 5 min, washed in SE buffer and resuspended in 0.5 ml of TE buffer (10mM Tris HCl [pH8]), 1mM EDTA (pH 8) and 100 µl of 10% SDS for 30 min at 65°C. Proteins were precipitated with 100 µl of 5 M potassium acetate and kept on -80°C for 30 min. The tube was then centrifuged at 12,000 rpm for 10 min, and the supernatant was treated with 50 µl of 10mg per ml proteinase K (Maniatis *et al.*, 1982) and RNAse (20 µl of 20 mg per ml stock solution) was added prior to overnight incubation at 55°C. The tube was centrifuged at 12.000 rpm for 10 min at 4 ^{o}C, and the supernatant was extracted twice with phenol-chloroform-isoamyl alcohol. The supernatant was precipitated in - 80°C for 15 min with 5M NaCl and followed by centrifugation at 12,500 rpm for 10 min. The resulting purified DNA was precipitated again with 1 ml of 0.1 M absolute ethanol. After mixing gently and storing at - 80°C for 30 min, DNA were recovered by spinning at 12,000 rpm for 30 min at 4°C. The supernatant was decanted and pellets were rinsed with 70% ethanol for 5 min. After decanting the supernatant again the pellets were either air dried or dried in Savant Automatic Environmental Speed Vac AES 2000 for 30 min. The DNA pellets were then resuspended in 100 µl of sterile distilled water and stored at 4°C.

Determination of DNA concentration

DNA concentration was determined by the intensity of ethidium bromide staining following electrophoresis of the samples on 0.9% (wt/vol) agarose gel in TBE buffer. DNA concentration was also determined spectrophotometrically using Beckman DU-650 spectrometer at a wavelength of 260 nm (Maniatis *et al.*, 1982).

Random Amplification of Polymorphic DNA

For RAPD profiles, the two primers used in this study were AT. [5'-GCGCACGG 3'] (Bostock *et al.*, 1993) and NT. [5'CCCGTC-AGCA 3'] (Pharmacia Biotech, 1997). The

generation of RAPD was carried out essentially as described by William *et al.* (1990) and William *et al.* (1991) with minor modifications. Every RAPD reaction mixture contained 100 ng genomic DNA, 100 pmol of oligonucleotide, 1.25 U *Taq* DNA polymerase (Pharmacia Biotech), 20mM (each) dATP, dCTP, dGTP and dTTP and 10x PCR buffer containing 10mM Tris. HCl pH 8.8, 50 mM-KCl, 15mM-MgCl2. The final volume of the reaction mix was 50 µl. After mixing, the tubes were placed in Mastercycler Eppendorf) Thermal Cycler for 45 cycles. For the AT primer, PCR was done as follows: 45 cycles of 1 min at 94 ^{o}C for denaturation, 1 min at 36 ^{o}C for annealing, and 2 min at 72 ^{o}C for elongation. The final extension step was prolonged to 10 min at 72 ^{o}C. For the NT primer, the PCR was programmed by using the conditions as follows; 30 sec at 94 ^{o}C for denaturation, 20 sec at 36 ^{o}C for annealing, and 2 min at 72 ^{o}C for elongation step. A final extenstion was performed at 7 min at 72 ^{o}C. Both conditions used 5 min at 95 ^{o}C for initial denaturating time.

Analysis of PCR products

Reaction products were analyzed by electrophoresis through 2% (wt/vol) agarose slabs with 1X TBE buffer using 100V for AT primer and 1.5% agarose gel was used for the NT primer. Gels were stained with ethidium bromide for 35-45 min, placed on translluminator UV and then photographed. The molecular size standards used were 100 bp ladder.

Results and Discussion

Nine patients were those who had undergone surgery recruited for this study (data of the patients's profile is available with the author). All patients were females with age ranged from 19-75 years. Each isolate was given a code that identifies the patient number. RAPD analysis was performed on 9 samples that are positive for *Candida albicans* for the 2 primer used in this study.

The 9 *Candida albicans* isolates from the 9 samples were genotypically characterizes using RAPD analysis with 2 primers. Both primers AT and NT successfully typed all isolates examined. Reproducibility was assessed by repeating the preparation of DNA from the same isolate and amplifying by PCR on three to four different occasions. Sometimes the bands produced were less intense, but their overall position and whether they were present or absent were consistent (Table 1 A and 1 B).

Table2 shows the RAPD patterns for primers AT and NT of the 9 *Candida albicans* isolates. Primer AT generated 6 types (Fig. 1), while primer NT gave 8 types. All 9 isolates were distinguishable from each other by RAPD. Some isolates showed that they are not polymorphic using AT primer (No. 2 , 4 and 5). Using both primers isolate No. 2 showed only one band. On the other hand, some isolates showed similar patterns as shown in Table 2. Among the RAPD patterns (Figs.1 and 2) obtained from both primers, the number of band ranged from a minimum of 1 to maximum of 7. The band sizes ranged from above 100 bp to higher than 1500 bp.

Table 1a. Reproducibility of RAPD profiles of 9 C *Albicans* isolates from oral cavity cancer patients using AT primer

Isolate	Replicate	Size in hundred bp																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	I						+			+		+				+					
	II						+			+		+				+					
	III						+			+		+				+					
2	I					+															
	II					+															
	III					+															
3	I					+															
	II					+															
	III					+															
4	I					+															
	II					+															
	III					+															
5	I					±															
	II					±															
	III					±															
6	I		+	±		+	+		±		+		+								
	II		+	±		+	+		±		+		+								
	III		+	±		+	+		±		+		+								
	IV		+	±		+	+		±		+		+								
7	I		+	±		+	+		±		+		+								
	II		+	±		+	+		±		+		+								
	III		+	±		+	+		±		+		+								
	IV		+	±		+	+		±		+		+								
8	I					+	+		±		+		+								
	II					+	+		±		+		+								
	III					+	+		±		+		+								
9	I		+			+	+		±		+					+					
	II		+			+	+		±		+					+					
	III		+			+	+		±		+					+					

Note: + band had good intensity, ± band had less intensity, faint or produced smear

Table 1b. Reproducibility of RAPD profiles of 9 C *Albicans* isolates from oral cavity cancer patients using NT primer

Isolate	Replicate	Size in hundred bp																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	I					+	+						+								±
	II					+	+						+								±
	III					+	+						+								±
2	I															+					
	II															+					
	III															+					
3	I		±				+	+			+					+			+		
	II		±				+	+			+					+			+		
	III		±				+	+			+					+			+		
	IV		±				+	+			+					+			+		
4	I				±		+	±						±		+					
	II				±		+	±						±		+					
	III				±		+	±						±		+					
5	I						+	±		+											
	II						+	±		+											
	III						+	±		+											
6	I								±		+										
	II								±		+										
	III								±		+										
7	I								±		+										
	II								±		+										
	III								±		+										
8	I		+	+					+		+					+			+		
	II		+	+					+		+					+			+		
	III		+	+					+		+					+			+		
9	I										+										
	II										+										
	III										+										

Note: + band had good intensity, ± band had less intensity, faint or produced smear

Table 2. RAPD profiles and overall PCR types of 9 Candida albicans isolates from oral cavity of cancer patients using AT and NT primers

Isolate code	AT-Fragment size (bp)	Overall PCR type[1]
1	600, 900, 1100, 1500	1
2	600*	2
3	600*	2
4	600*	2
5	600*	2
6	200, 300, 500, 600, 800, 1000, 1200	3
7	200, 300, 500, 600, 800, 1000, 1200	3
8	500, 600, 800, 1000, 1200	4
9	200, 500, 600, 800, 1000, 1500	5
Isolate code	**NT-Fragment size (bp)**	**Overall PCR type[1]**
1	500, 600, 1200	1a
2	1500*	2a
3	200, 600, 700, 1000, 1500, 1800	3a
4	400, 600, 700, 1300, 1500	4a
5	600, 700, 900	5a
6	800, 1000	6a
7	800, 1000	6a
8	200, 300, 800, 1000, 1500, 1800	7a
9	1000*	8a

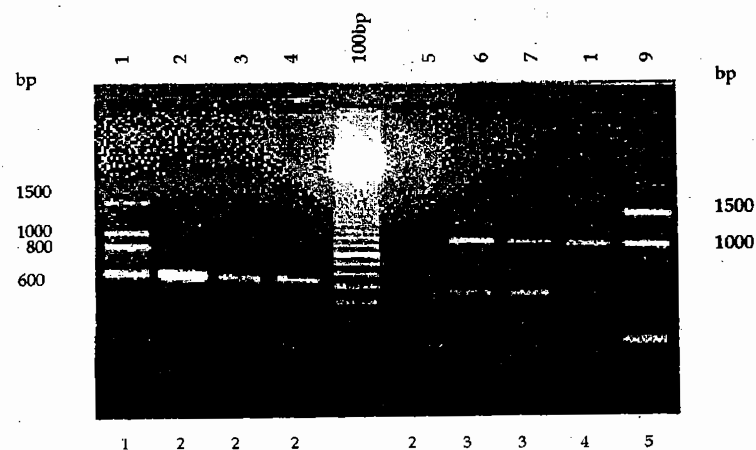

Figure 1. RAPD profiles of *C. albicans* using AT primer. Number above the lanes give the isolate code while those below the lanes represent the PCR overall type as indicated in Table 2.

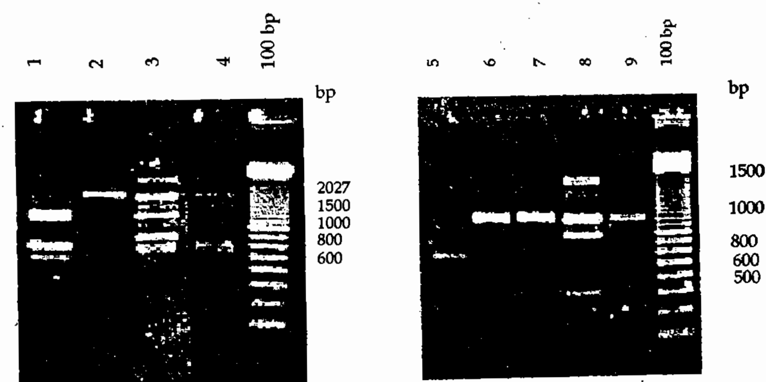

Figure 2. RAPD profiles of *C. albicans* using NT primer. Numbers above the lanes give the isolate code while those below the lanes represent the PCR overall type as indicated in Table 2.

Candida albicans is generally considered to have a world-wide distribution (Shepherd *et al.*, 1985). It is a harmless commensal of the oral cavity, commonly found in individuals undergoing no medical treatment. However the incidence of *Candida albicans* in various groups undergoing medical treatment is, in general, higher (Odds, 1985), with a mean figure of 42.9% for the oral cavity. This study shows that all cancer patients subjects had positive culture of *Candida albicans*.

In this study, 2 primers were used to obtain RAPD profiles from 9 isolates. The results showed that *Candida albicans* isolates can be characterized by using RAPD. The effect of the changing the primer on DNA fragment profiles was profound, as both the number and the size of DNA fragments were observed to change with each primer. For both primers, the profiles of DNA fragments found for some strains of *Candida albicans* were very similar and several common DNA fragments were observed. DNA length polymorphism was not always observed. For example, when using AT primer, 4 strains (2, 3, 4 and 5) were not polymorphic. These strains were polymorphic when NT primer was used except isolate No. 2. On the other hand NT primer revealed that 2 strains (2 and 9) were not polymorphic, but were polymorphic using the AT primer except isolate No. 2. Therefore, only one strains, No. 2 was not polymorphic using both primers.

For each primer, the array of DNA fragments consisted of bands of high or low intensities of ethidium bromide staining. The higher-intensity bands may be due to the amplification of repetitive DNA sequences, the influence of neighboring sequences on hybridization to the target sequence, or fewer mismatches to the target sequence. In contrast, the low intensity bands may have been generated inefficiently because of higher degree of mismatch between the primer and the target sequence (Goodwin and Annis, 1991).

A program of PCR used for AT primer requires 5.5 h to complete, and 4 hr needed for the of NT primer condition. This result indicates that at the beginning of the study, it is essential to test a variaty of reaction conditions to determine those that are best suited for the particular *Candida albicans* strains under study. RAPD analysis is simpler to perform and interpret than most other genotypic methods used for finger-printing of fungi. Another advantage of RAPD is that gel-to-gel variation is reduced in RAPD analysis since standard-sized fragments of DNA can be used to define the sizes of the limited number of fragments observed.

In spite of its name, RAPD is not a random collection of DNA products (William *et al.*, 1990). The sequence of each primer is chosen at random from many possible permutations of bases composing the oligomers, but then the primer is tested for its ability to prime the generation of useful selection of DNA fragments in PCR. For a given DNA preparation, each primer generates a characteristic and repeatable pattern of DNA fragments. Therefore, once a primer has been selected for RAPD analysis, it is employed to generate DNA fragments in a specific manner, and these fragments can then be used as genetically defined characters (William *et al.*, 1990). However the best primers and the number of fragments needed for a particular purpose have to be determined empirically.

Some isolates showed the same patterns and close to one another and may have corresponded to genetically related strains. However, these isolates were from different patients from different hospitals. The data in the present study do not support the suggestion that there may be restricted number of strains causing oral candidosis or

local colonization of *Candida albicans*. The data idicate that many different substrains of *Candida albicans* can infect patients with cancer disease and cause oral candidosis or oral colonization of this yeast. Indeed, the results suggest that each individual patient is colonized with a unique strain of *Candida albicans*. This is consistent with the report from other laboratories using more elaborate molecular techniques. Miyasaki *et al* ., 1989 (*cited* by Powderly *et al*., 1992) using DNA fingerprinting to analyze isolates from 28 patients with oral candidosis showed that different strains infected different patients

The utility of RAPD profiles for the primary identification and typing of *Candida albicans* isolates from oral cavity of cancer patients will require further evaluation. Even if different laboratories can adopt the method with ease, the cost of the assay will increase substantially if several different RAPD primer per isolate are found to be needed for strain identification. Whatever the cost beneficial should RAPD profiles provide valid identification of strains (Lechman *et al*., 1992).

Both oral infection or oral colonization by *Candida albicans* is an increasing problem especially in cancer patients (Saral, 1991). Since there are at present no reliable, reproducible, and fast assays, this study investigated the applicability of the RAPD in the development of a test system useful for clinical applications. Because of the increasing incidence of nosocomial infections caused by *Candida* species (Goodwin and Annis, 1991), there is an urgent need for a rapid and simple procedure for typing yeast in clinical specimens. Such a procedure would allow analysis of epidemiology, outbreaks and the incidence of person-to-person transmission associated with these organisms.

Conclusion

Strains of *Candida albicans* were isolated from oral cavity of cancer patient for molecular typing analysis using Randomly Amplified Polymorphic DNA (RAPD).

The following findings were obtained:

1. There is increased susceptibility of *Candida albicans* infection of the oral cavity for patients cancer.
2. RAPD is a suitable technique to differentiate *Candida albicans* strains. RAPD with AT primer [5'd(GCGCACGG)-3'] was less discriminatory than primer NT, yielding only 5 types. Using NT primer [5' d(CCCGTCAGCA)-3' yielded 8 types out of 9.
3. The shorter arbitrary sequence used, the longer time needed for denaturation and annealing steps, as in the case of the AT primer a 8 mer sequence. Using the NT primer (10 mer) sequence, shorter time for amplification was required.
4. The data indicate that many different substrains of *Candida albicans* can infect patients with cancer disease and cause oral candidosis or oral colonization. Indeed, the results suggest that each individual patients is colonized with unique strains of *Candida albicans*.

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