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Research Report

Genetic variability of *Candida albicans* in HIV/AIDS patient with and without ARV therapy and non HIV/AIDS

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ABSTRACT

Background: *Oral candidiasis is the mostly found oral manifestation in HIV/AIDS infected patient caused by immunocompromised especially immunodeficiency. Clinical symptoms is severe pain in oral cavity and dry mouth because of xerostomia which cause the loss of appetite. Candida albicans (C. albicans) is normal flora in oral cavity which plays as opportunistic pathogen and also the cause of oral candidiasis. Almost 90% of HIV–infected patient have oral candidiasis. This condition is clinical problem which has not been well-managed yet. C. albicans colonized oral mucous cavity has different genetic variability for each strain. Phenotype of C. albicans has been determined by genetic factor and environtment. This condition stimulate differences of genotype among various strain of C. albicans in the world.* ***Purpose:*** *The purpose of this research is to analyze the genetic variability of C.albicans which colonized in the mucous oral cavity of HIV/AIDS patient in Surabaya in the treatment with and without ARV therapy and non HIV/AIDS.* ***Methods:*** *This research has been identify and characterize the prevalent strain of C. albicans isolat in Surabaya (East Java) in HIV/AIDS infected patient with oral candidiasis by method of Iatron candidal check. The highlight of this research including cytology examination by Papanicoloau staining, C. albicans culture, spheroplast making, DNA isolation and genetic variability checking by randomly amplyfied polymorphism DNA (RAPD).* ***Results:*** *C. albicans colonizing oral mucosa of non-HIV patients had a predisposition of farther genetic relationship (genetic distance of 0.452) with C. albicans colonizing oral mucosa of HIV ARV and HIV non-ARV patients. The genetic distance was ranging between 0 and 1, where 9 was long genetic distance and 1 was short genetic distance. In contrast, C. albicans colonizing oral mucosa of HIV ARV have predisposition of closer genetic relationship (genetic distance of 0.762) with C. albicans colonizing oral mucosa of HIV non-ARV patients.* ***Conclusion:*** *The conclusion of this research were C.albicans colonizing HIV/AIDS patiens with and without ARV showed no high genetic variability between C.albicans isolate in HIV patients. There fore, the character of C.albicans colonizing HIV ARV and HIV non-ARV patients had similar genotype predisposition of closer relationship value with C.albicans colonizing oral mucosa non HIV patients.*

Key words: *Candida albicans, HIV/AIDS, oral candidosis, RAPD*

ABSTRAK

Latar belakang: *Oral candidiasis merupakan manifestasi kelainan rongga mulut yang paling sering timbul pada penderita HIV/AIDS karena kondisi immunocompromised terutama defisiensi imun. Gejala klinisnya berupa nyeri hebat di rongga mulut dan mulut kering karena xerostomia yang menyebabkan hilangnya nafsu makan. Candida albicans (C. albicans) berperan sebagai patogen oprtunistik dan merupakan penyebab Kandidiasis rongga mulut. Hampir 90% penderita terinfeksi HIV mengalami kandidiasis rongga mulut. Kondisi ini merupakan masalah klinis yang belum teratasi dengan baik. Kolonisasi C. albicans di mukosa rongga mulut mempunyai variabilitas genetic yang berbeda untuk tiap strainnya. Fenotip C. albicans ditentukan oleh faktor genetic dan lingkungan. Kondisi ini menstimulir perbedaan genotip di antara berbagai strain C. albicans di dunia.* ***Tujuan:*** *Tujuan penelitian ini adalah meneliti korelasi antara hubungan genetik yang menunjukkan variasi genetik kolonisasi C. albicans pada rongga mulut dan insidens kandidiasis rongga mulut pada penderita HIV/AIDS dan non-HIV/AIDS.* ***Metode:*** *Penelitian ini mengidentifikasi dan mengkarakterisasi strain Candida albicans isolat Surabaya (Jawa Timur) pada penderita HIV/AIDS dengan kandidiasis rongga mulut dengan metode iatron candidal*

check. Penekanan dalam penelitian ini termasuk pada pemeriksaan sitologi dengan pengecatan Papanicoloau, kultur C. albicans, pembuatan spheroplast, isolasi DNA dan pemeriksaan variabilitas genetik dengan randomly amplified polymorphism DNA (RAPD). ***Hasil:*** *C. albicans yang berkolonisasi di rongga mulut pada penderita non-HIV mempunyai predisposisi hubungan genetik (jarak genetik 0.452) dengan C. albicans yang berkolonisasi di rongga mulut pada penderita HIV yang mendapatkan terapi ARV dan non ARV. Jarak genetic bervariasi antara 0 dan 1, dimana 9 dalah jarak genetik terpanjang and 1 adalah jarak genetik terpendek. Sebaliknya, C. albicans yang berkolonisasi di rongga mulut pada penderita HIV yang menerima terapi ARV memiliki predisposisi hubungan genetic yang lebih dekat (jarak genetic 0.762) dibandingkan C. albicans yang berkolonisasi di rongga mulut pada penderita HIV non-ARV.* ***Kesimpulan:*** *Sebagai kesimpulan, penelitian ini menunjukkan bahwa C. albicans isolate Surabaya yang mengkolonisasi penderita HIV/AIDS dengan ARV dan non ARV memiliki hubungan kekerabatan genetik yang sama dibanding dengan pasien non HIV/AIDS.*

Kata kunci: *Candida albicans, HIV/AIDS, kandidiasis rongga mulut, RAPD*

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INTRODUCTION

HIV/AIDS cases is increasing and concern about the problem is growing. In Indonesia, the total number of HIV/AIDS cases up to the end of June 2006 was 10.859 cases (4.527 HIV cases and 6.332 AIDS cases), and the largest proportion, as much as 53%, belonged to age group of 20–29 years.[1] *Candida albicans* (*C. albicans)* causing oral candidiasis are frequently found along the course of HIV infection, resulting from immunocompromised condition that commonly accompany HIV/AIDS patients, and one of the complications with the highest incidence rate in oral cavity, reaching 90%.[2] Oral candidiasis occurs due to immunocompromised condition that commonly accompanying HIV/AIDS patients and may disturb host's immune response, one of which is the disturbed production of cytokines, such as IL-1α and TNF-α. In such condition, abnormalities in polymorphonuclear (PMN) and macrophage phagocytosis function are also found.[3,4]

Reduction of immunity system in the body of HIV/AIDS patients result in the absence of the host's defense against *C. albicans* attachment and colonization to oral mucosal epithelium. In HIV/AIDS patients, *C. albicans* infection in oral surface epithelium is recurrent and persistent. Such condition remains a clinical problem that cannot be solved satisfactorily up to the moment.[5] The increase of fungal infection is reported to raise morbidity and mortality rate of immunocompromised (HIV) patients.[1] *C. albicans* is a commensal organism in oral cavity. Within the oral cavity, there are various *C. albicans* strains with certain phenotype characteristics, determining them as commensal or pathogenic,[6] and it was suspected that *C. albicans* virulence in various strains is affected by genetic variability. The success of *C. albicans* attachment to oral mucosa epithelial surface is the beginning of *C. albicans* and oral candidiasis is one of the complications of HIV/AIDS with the highest incidence rate in oral cavity.[7,8] *C. albicans* infection on epithelial surface of oral mucosa is recurrent and persistent in DM patients. About 90% of HIV/AIDS population showed the presence of oral candidiasis. Such condition is a clinical problem that cannot be satisfactorily overcome,[6] since it is still endemic and the therapy has never been effective.

C. albicans colonizing oral mucosa have various genetic variations in each strain. Profiles of a *C. albicans* character (profile) are determined by genetic factors and environmental factors.[9] Such condition results in different genotype among various *C. albicans* strains. Information on genetic correlation among individuals within and between species has several important benefits for improvement of an organism. In studying the outcome of genetic variations, assumption on the presence of genetic correlation will be useful for genotype identification. Knowledge on genetic data, such as the presence of genetic variance in *C. albicans* colonizing oral mucosa of HIV/AIDS patients based on the level of severity is necessary to improve therapy management of virulent *C. albicans* infection. So far, correlation between genetic relationship of *C. albicans* colonizing oral mucosa of HIV/AIDS patients and the severity of the disease remains unclear. Literature studies revealed that *C. albicans* virulence in various strains are supposedly affected by genetic variability. The objective of this study was analyze the genetic variability of C. albicans in HIV/AIDS patients in Surabaya with and without ARV therapy, and patients with no HIV/AIDS.

MATERIALS AND METHODS

Specimen for cytological examination was taken from the scrubbing of the tongue and buccal mucosa, and the result of scrubbing that had been swabbed onto object glass was fixed and stained with Papanicoloau procedure and mounted with DPX. Surabaya isolate of *C. albicans* were cultured for 2 × 24 hours in 37° C in Sabouraud Dextrose Agar (Difco). Then, gram staining and sugar fermentation test were performed. Sugar fermentation test with glucose, maltose, sucrose, and lactose were incubated in incubator 37° C for 3 × 24 hours. The existence of *C. albicans* was marked by color change. The obtained *C. albicans* were grown within Sabouraud Dextrose Broth (Difco) media, incubated for 18–20 hours in 37° C in orbital shaking,

centrifuged in 700 g for 5 minutes at 24° C, and washed with PBS 3 times.

Then the spheroplast production was done by inoculating *C. albicans* in dextrose-containing Yeast Pepton Dextrose Broth (YPD Broth, Difco laboratories). After aerobic incubation in 37º C for 18 hours in rotary incubator with 100 rev/min. *Candida* cells were harvested and washed 2× with PBS. Then, *Candida* cells were counted using hemocytometer and suspended to become 3.5×10^7 cell/ml in PBS. Pellet was resuspended with 600 ul sorbitol buffer containing 200 U Litycase. Incubation was performed in 30° C for 1 hour and the produced spheroplasts were harvested with centrifugation in 3000 rpm for 5 minutes. Pellet was suspended in 180 ul ATL (Qiagen) buffer, and added with 20 ul proteinase K into the supernatant and incubated in 55° C overnight.

After being incubated for 55° C overnight DNA isolation was done. Supernatant and 4 ul Rnase were added and treated with 200 ul buffer AL and incubated in 70° C for 10 minutes. Subsequently, 200 ul ethanol 96% was added and vortexed directly to become homogeneous. All supernatants were removed to Dneasy Spin column 2 ml collection tube and added with 500 ul buffer AW1. The tube was centrifuged in 800 rpm in 4° C for one minute, and Dneasy Spin Column in a new 2 ml collection tube and 500 ul buffer AW2 was added and centrifuged for 3 minutes in high speed. The Dneasy Spin Column was removed to 1.5 ml sterile microcentrifugation tube, and 200 ul AE buffer was pipetted directly to Dneasy membrane. It was incubated at room temperature for 1 minute and centrifugated in a speed of 8000 rpm. The AE buffer administration procedure was repeated one time. DNA purity and concentration were determined using UV/VIS, Jasco V-530.

PCR was done using EFB1 gene primer as internal control for *C. albicans* using EFB1 primer: 5'-ATTGAACGAATTCTTGGCTGAC-3' 5'-CATCTTCTTCAACAGCAGCTTG-3'. The final volume of PCR reaction mixture was 25 ul, comprising 10 x buffer Mg free, 25 mM MgCl2, 2.5 mM dNTP mix, 25 ng DNA, 20 uM primer and 100 U taq polymerase (Promega), and then mixed and eppendorf PCR was put into Master Cycler machine (Gene Amp PCR System 2499, Perkin Elmer). The PCR condition was as follows: denaturation at 94° C for 1 minute, annealing at 55° C for 1 minute and extension at 72° C for 1 minute, and the final step was extra extension at 72° C for 10 minutes. The number of PCR cycles was 45.

Random amplified polymorphism DNA (RAPD) method was done using NT and AT Primers.11 NT Primer: 5' CCCGTCAGCA 3' and AT primer : 5' GCGCACGG 3'. To perform amplification in RAPD-PCR method, the following materials are needed: sterile distilled water (ddH_2O), 10× buffer (QIAGEN), dNTP (QIAGEN), Q-Solution (QIAGEN), TE pH 8, Taq polymerase, and DNA sample with PCR. PCR-RAPS conditions were as follows: Hot start 94° C for 5 minutes, denaturation in 94° C for 1 minute, annealing in 35° C for 1 minute and extension in 72° C for 2 minutes. Extra extension in 72° C for 5 minutes and the number of cycles were 45 cycles. To identify the success of PCR amplification, electrophoresis 2% was performed, Marker (DNA 174-Hae III digest and 1 Kb DNA ladder), and transluminator-UV polaroid gel camera.

RESULTS

EFB1 gene examination as internal control using primers 5'-ATTGAACGAATTCTTGGCTGAC-3' and 5'-CATCTTCTTCAACAGCAGCTTG-3' with following PCR conditions: Denaturation in 94° C for 1 minute, annealing in 55° C for 1 minute and extension in 72° C for 1 minute and the final step was extra extension in 72° C for 10 minutes. The number of PCR cycles was 45.

EFB1 gene is a housekeeping gene that acts as an internal control in *C. albicans*. The result of PCR of EFB1 gene in control group, HIV/AIDS patients with and without ARV were apparent in all samples (Figure 1). This indicates that candidap sp. samples was *C. albicans*.

The examination of *C. albicans* genotype infecting oral mucosa of HIV/AIDS patients with or withour ARV and control group was performed using Random Amplified Polymorphic DNA (RAPD) method. In this method NT primer 5' CCCGTCAGCA 3' and AT primer 5' GCGCACGG 3' with PCR-RAPD condition was used as follows: Hot start in 94° C for 5 minutes, denaturation 94° C for 1 minute, annealing in 35° C for 2 minutes and extension in 72° C for 2 minutes, extra extension in 72° C for 5 minutes and the number of cycles was 45. The result of RAPD was electrophoresized in 2% agarose gel separating *C. albicans* DNA fragment in a range of 250–3000 bp (Figure 2).

Based on unweight pair group method with arithmatic averages (UPGMA) clustering analysis using MVSP ver 3.1 (Kovach Computing service) program, by determining similarity value through simple matching coefficient method, indicated that RAPD result in genotype examination of *C. albicans* infecting oral mucosa of HIV/AIDS patients with and without ARV and control group (non-HIV/AIDS) using NT primer revealed two groups, that the similarity value of group I and II was 0.705 (70.5%).

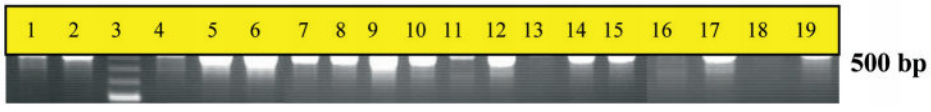

Figure 1. PCR result of EFB1 gene.
Note: Lane 3: 1 Kb DNA marker (Ladder Intron) Lane 24: 1 Kb DNA marker (Ladder Intron)

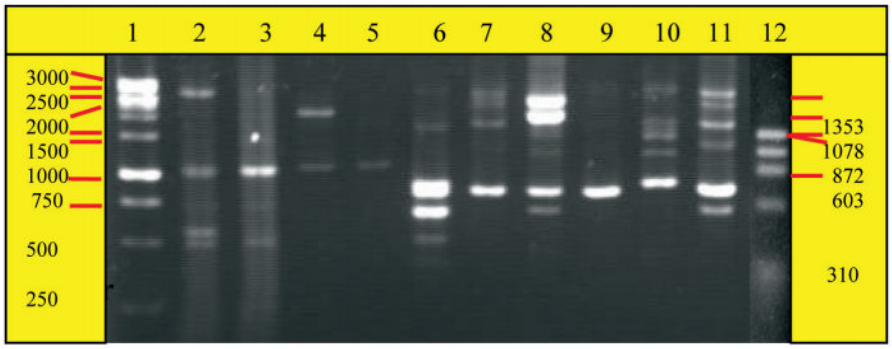

Figure 2. Result of RAPD with *C. albicans* NT primer in HIV/AIDS patients.
Note: Lane 1:1 Kb DNA marker (Ladder Intron) Lane 12: DNA marker 174-Hae III digest

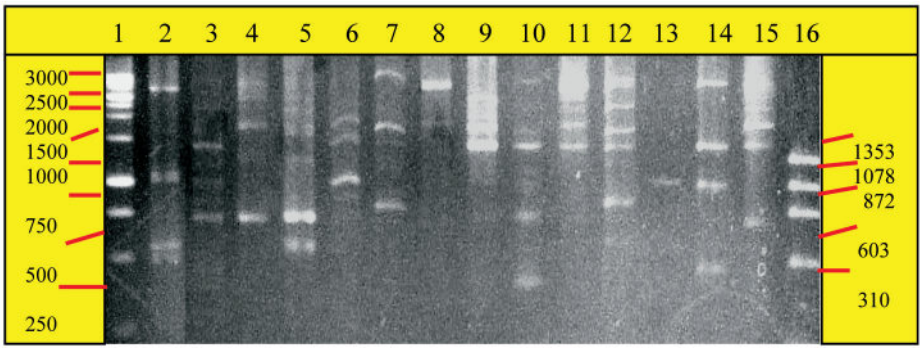

Figure 3. Result of RAPD with *C. albicans* AT primer in HIV/AIDS patients.
Note: Lane 1:1 Kb DNA marker (Ladder intron) Lane 16: DNA marker 174-Hae III diges

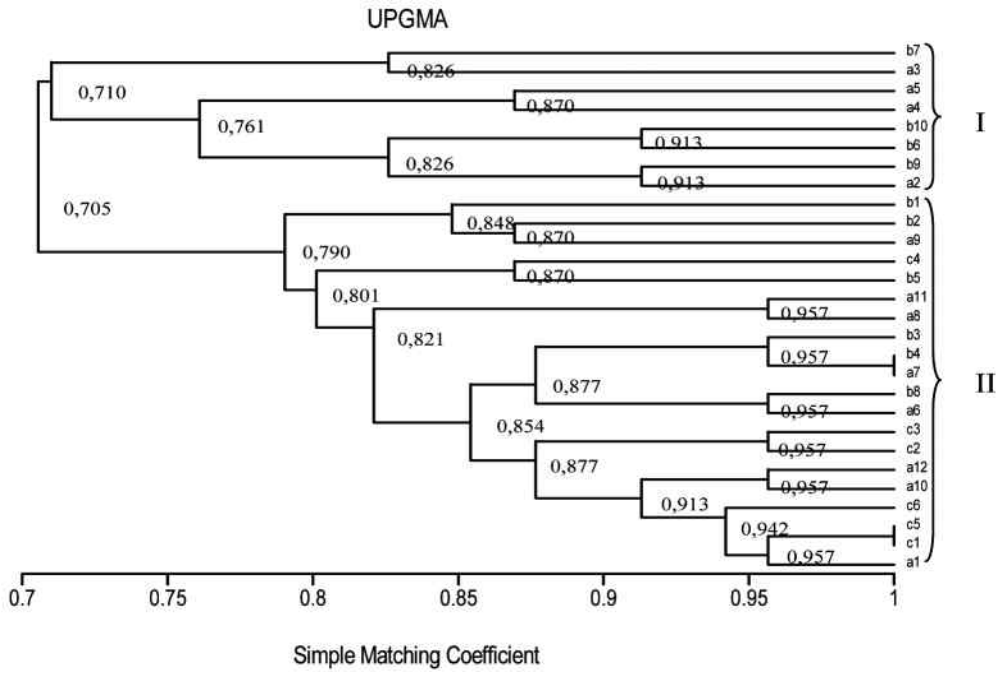

Figure 4. Dendogram from RAPD with *C. albicans* NT primer in HIV/AIDS patients.
Notes: a = *C. albican* in HIV/AIDS patients with ARV, b = *C. albican* in HIV/AIDS patients without ARV, c = Control (*C. albicans* in Non-HIV/AIDS)

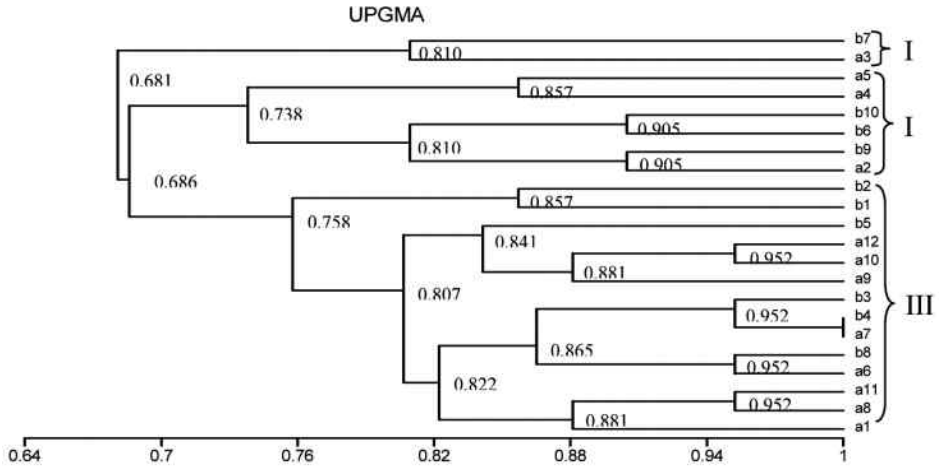

Figure 5. Dendogram from RAPD with *C. albicans* AT primer in HIV/AIDS patients.
Notes: a = *C. albicans* in HIV/AIDS patients with ARV, b = *C. albicans* in HIV/AIDS patients without ARV

Based on UPGMA clustering analysis using MVSP ver 3.1 (Kovach Computing service) program by determining similarity value through simple matching coefficient method indicated that RAPD result in genotype examination of *C. albicans* infecting oral mucosa of HIV/AIDS patients with and without ARV using AT primer showed that there were three groups. Above data indicated that similarity value in group II and III was 0.686 (68.6%), while the similarity value in group II and III to group I was 0.681 (68.1%).

Table 1. Similarity matrix between sample groups

Sample groups	HIV ARV	HIV non ARV	non - HIV
	B	C	A
B	-	0.762	0.476
C	-	-	0.428
A	-	-	-

Genetic relationship and variability of *C. albicans* colonizing HIV ARV, HIV non-ARV and Non-HIV, based on the genotype was measured through similarity matrix of DNA fragments based on genetic distance. Between HIV ARV and HIV non-ARV the genetic distance was 0.762. Between HIV ARV and non-HIV the genetic distance was 0.476, and between non-HIV and HIV non-ARV the genetic distance was 0.428.

Phenogram shows that *C. albicans* colonizing oral mucosa of non-HIV patients had a predisposition of farther genetic relationship (genetic distance of 0.452) with *C. albicans* colonizing oral mucosa of HIV ARV and HIV non-ARV patients. The genetic distance was ranging between 0 and 1, where 9 was long genetic distance and 1 was short genetic distance. In contrast, *C. albicans* colonizing oral mucosa of HIV ARV have predisposition of closer genetic relationship (genetic distance of 0.762) with *C. albicans* colonizing oral mucosa of HIV non-ARV patients.

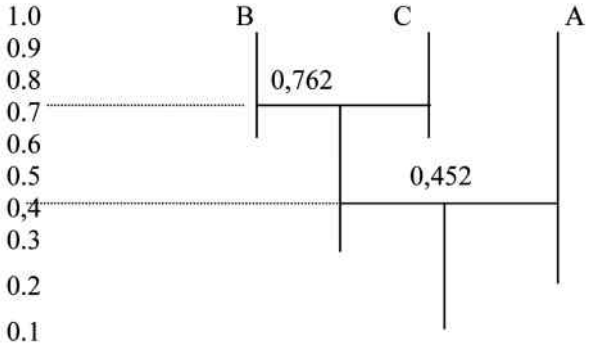

Figure 6. Phenogram based on similarity matrix of RAPD-resulted *C. albicans* DNA fragment.

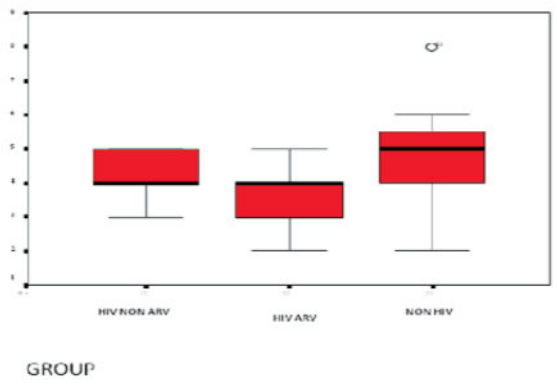

Figure 7. Distribution of the number of bands resulting from *C. albicans* RAPD.

Result of statistical analysis revealed that the mean of polymorphism (number of bands) in non-HIV (4.91 ± 2.02) was higher than the mean polymorphism in HIV-ARV (3.55 ± 0.82) and HIV non-ARV (4.27 ± 0.65).

DISCUSSION

Oral candidiasis is commonly found anytime during of HIV infection. This is due to immunocompromized condition generally accompanying HIV/AIDS patients and one of complications with highest rate of incidence in oral cavity. Various literatures reported that factors affecting the incidence of *C. albicans* in oral cavity of HIV/AIDS patients are the immunodeficiency resulting from immunocompromized condition, so the patients are susceptible to oral infection. In HIV/AIDS patients, the factor is a predisposition of superficial and systemic infection, including *C. albicans* infection. This is because immunocompromized condition in host cells results in immunodeficiency and disordered cytokines production, leading to disturbed phagocytic function of PMN and macrophage. In oral cavity live various strains of *C. albicans* with certain phenotype characteristics that determine its nature as commensal or pathogenic. Up to the moment, it remains unclear what cell types and receptor as the primary target of HIV virus in oral mucosa. However, it is reported that the HIV-resulted change of oral epithelial cells causes changes in CD4 T cells in the mucosa and the reduction of Th1 cytokine within the saliva of chronic HIV patients. This triggers the occurrence of opportunistic infection.

Knowledge on genetic data, such as the presence of genetic variations in *C. albicans* colonizing oral mucosa of HIV/AIDS patients, should be improved to determine the virulence, both qualitatively and quantitatively. Knowledge on genetic variation can be used as a basis for improving the management for solving various infection cases resulting from *C. albicans* in HIV/AIDS patients.

Genotype examination of Surabaya isolate *C. albicans* colonizing oral mucosa in HIV/AIDS and non-HIV/AIDS patients was aimed to detect polymorphism at DNA level. Polymorphism is a different shape from the basic structure to find the variability of *C. albicans* using molecular epidemiology. DNA markers are widely used in studying genetic variations in *C. albicans* is RAPD.[9,11,12] RAPD technique analysis have several advantages, such as shorter processing time, requiring less DNA samples (0.5–50 nm), and need no radioisotope. RAPD also needs no early DNA sequence information, it has more simple procedure and larger number of samples which can be processed rapidly. Profile of *C. albicans* (phenotype) character depends on genetic factors and environmental factor. Such condition result in different genotype in various *C. albicans* strains worldwide.

Genotype-based genetic relationship and variability of *C. albicans* colonizing patients with HIV/AIDS with ARN, Non-ARV and non-HIV/AIDS was measured using similarity matrix of DNA fragments based on genetic distance. The result of dendogram (Figure 4) through UPGMA clustering analysis using MVSP ver 3.1 program describes that *C. albicans* colonizing oral mucosa of HIV/AIDS patients with ARV, non-ARV with NT primer had predisposition of closer relationship value with genetic distance of 0.705 (70.5%). However, the result of Dendogram analysis with AT primer (Figure 5) indicated that *C. albicans* colonizing oral mucosa of HIV/AIDS patients with ARV and non-ARV had predisposition of relatively closer relationship value as well (genetic distance 0.681). From both results it was apparent that *C. albicans* isolated from patients with HIV/AIDS with ARV and non-ARV, either using AT or NT primer, had predisposition of homogeneous genetic distance, ranging between 0.681 and 0.705. This means that *C. albicans* colonizing HIV/AIDS patients with and without ARV, both using NT and AT primers, showed no high genetic variability between *C. albicans* isolate in HIV patients. Therefore, the character of *C. albicans* colonizing HIV ARV and HIV non-ARV patients had similar genotype predisposition.

The result of phenogram analysis shows that *C. albicans* colonizing oral mucosa of non-HIV had a predisposition of farther relationship value (genetic distance 0.452) with *C. albicans* colonizing oral mucosa of HIV ARV and HIV non-ARV patients. In contrast, *C. albicans* colonizing oral mucosa of HIV ARV patients have a predisposition of closer relationship value (genetic distance 0.762) with *C. albicans* colonizing oral mucosa of HIV non ARV patients (Figure 6).

The result of statistical analysis shows that the mean polymorphism in non-HIV (4.91 ± 2.02) was higher than the mean of polymorphism in HIV ARV (3.55 ± 0.82) and HIV non ARV (4.27 ± 0.65) (Figure 7). This difference is interesting since ARV therapy in HIV patients may possibly influence *C. albicans* genotype. This was also supported by the effect of oral environmental conditions, such as saliva quality and quantity, diet pattern, nutritional status, and host immune response. Nevertheless, it requires further analysis to find the target gene in *C. albicans* that are subjected to mutation due to the use of ARV for HIV/AIDS patients.

Several references reported that oral environment condition also facilitates certain strains of *C. albicans* to colonize oral mucosa. From several genotypes that present as the existence of *C. albicans* genetic variance, serotype A is reported to have a predisposition of polymorphism higher than that of polymorphism in serotype B. This is possibly because serotype A has higher virulence and adherence capability compared to serotype B through glycomannoprotein receptor that present on *C. albicans* cell wall. However, patients with immunodeficiency due to immunocompromized condition are highly susceptible to infection, particularly by *C. albicans*, so that *C. albicans* may become virulent and have higher adherence capability, facilitating the occurrence of infection in the oral mucosa of HIV/AIDS patients. The conclusion of this research were *C. albicans* colonizing HIV/AIDS patients with and without ARV showed no high genetic variability between *C. albicans* isolate in HIV patients. Therefore, the character of *C. albicans* colonizing HIV ARV and HIV non-ARV patients had similar genotype predisposition of closer relationship value (genetic distance 0.762) with *C. albicans* colonizing oral mucosa non HIV patients.

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