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Differentiated sap (4–6) gene expression of *Candida albicans* isolates from HIV-positive patients with oral candidiasis and commensals in healthy individuals

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ABSTRACT

Gene expression of SAP 4–6 based on the detection of mRNA was observed in *Candida albicans* isolates from HIV-positive patients with oral candidiasis and commensal from healthy individuals. The species of *C. albicans* strains were selectively isolated from both sources using CHROMagar Chromogenic Media. The obtained isolates were then induced to express SAP 4–6 using SAP 4–6 gene inducer media. Analysis of gene expression was performed on a molecular basis using the RT-PCR method. Molecular analysis of gene expression showed that the isolates CH3 from HIV-positive patients with oral candidiasis could express SAP 4–6 gene, while commensal isolates from healthy people could not. Based on the results of this study, it could be concluded that, in terms of molecular detection, only isolates from HIV-positive patients (CH3) could express their SAP 4–6 gene.

1. Introduction

Oral candidiasis is mostly reported to attack people with immune system disorders, especially HIV-positive patients [1,2]. However, several cases also show that HIV-positive patients are not attacked by oral candidiasis [3]. Oral candidiasis is an infection caused by strains of the genus *Candida* [4], especially *Candida albicans* [5]. *C. albicans* is a normal microbiota in the human body and is not dangerous [6]. However, it possesses opportunistic nature so that it can cause infection in immunocompromised conditions [5,6]. This opportunistic nature cannot be separated from the support of its virulence factor, namely the Secreted Aspartyl Proteinase (SAP) enzyme [7] in addition to morphological changes (dimorphism) and adhesion [8]. This SAP enzyme encodes the multigene family sequentially from 1 to 10 (SAP 1–10) and has different levels of expression [8]. As a result, the infection process caused by *C. albicans* cannot be separated from the role of this enzyme, which encodes the SAP 1–10 gene.

SAP 4–6 gene is known to play an important role in oral candidiasis and hyphal formation during infection [7]. This is because the SAP 4–6 gene is expressed during hyphal formation in oral candidiasis [9]. In addition, the activation of the SAP 4–6 gene increases at the time of infection compared to SAP 1–3 gene based on the recombination-based in vivo expression technology (RIVET) technique [7]. Another study on gene expression using samples of oral candidiasis sufferers and Candida's carrier with the RT-PCR test showed that SAP 2 and SAP 4–6 genes were more predominantly expressed than SAP 1–3 [10]. For that reason, hyphae formation at the time of *C. albicans* infection is presumed to be closely related to SAP 4–6 gene expression.

Based on the explanation above, it is known that *C. albicans* is a normal microbiota of the human body. However, because of its opportunistic nature, it can turn into a pathogenic fungus. The pathogenicity can be seen in oral candidiasis infection in HIV-positive and non-HIV patients. *C. albicans* infection in oral candidiasis is influenced by the SAP enzyme encoded by the SAP 4–6 gene. Therefore, it is necessary to conduct further research on differences in SAP 4–6 gene expression and hyphal morphology during infection in HIV-positive patients with oral candidiasis and commensals in healthy individuals.

This study aimed to analyze SAP 4–6 gene expression of *C. albicans* isolates from HIV-positive patients with oral candidiasis and commensals in healthy individuals on the molecular and morphological basis by

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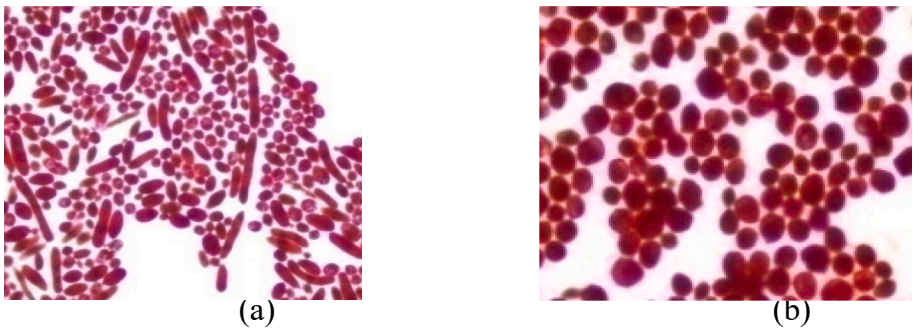

Fig. 1. Cell morphology of members of the genus *Candida* originating from healthy people; (a) *C. albicans* has round, oval, or oval-round cells, (b) *C. tropicalis* has round cells.

analyzing the length of hyphae and the speed of hyphal formation of *C. albicans* isolates formed in HIV-positive patients with oral candidiasis and commensals in healthy individuals.

2. Materials and methods

2.1. Subjects and specimen collection

The isolates of *C. albicans* were collected using a cotton swab which was rubbed on the oral cavity of healthy people (having no signs of inflammation or mouth diseases, such as stomatitis. The ethical approval for conducting this study had been obtained from the Medical and Health Ethics Research Committee, Faculty of Medicine, Universitas Gadjah Mada/RSUP Dr. Sardjito on December 11, 2012. The samples were inoculated on *Saburoud Dextrose Agar* (SDA) media, then sub-cultured on CHROMagar (Oxoid) media.). The others source is archive candida from HIV patients who have been given a unique code and cannot be traced to their patients.

2.2. Candida albicans identification

Isolates that had been taken through a swab from the oral cavity of healthy people were then smeared on the SDA media. The results were then incubated at 35 - 37 °C for 48 h. The growing isolates were then identified based on their cell morphology using the Gram's stain method. The identification results that were suspected to be members of the genus *Candida* were then sub-cultured on CHROMagar media, in which the isolates were rubbed using the ose stick that had previously been glowing on a Bunsen burner. After that, they were incubated at 37 °C.

Detection and identification of *C. albicans* on CHROMagar media were carried out by observing the color of the colonies formed on CHROMagar media. This is because the color of the colonies formed by each species of the genus *Candida* members is different from one another. In other words, each species causes different colors, such as *C. albicans* produces green color, *C. krusei* has a reddish color, and *C. tropicalis* generates purple or dark blue color.

2.3. Hyphae formation and elongation

Hyphae morphology of *C. albicans* isolates can be observed by taking 100 μl of fetal bovine serum with pH 5.5 and added with 20 μl of cultured *Rowell Park Memorial Institute* 1640 (RPMI 1640), then incubating them at 37 °C for 1 h [11]. After that, the produced isolates were dropped on a slide microscope and closed using a cover glass. Subsequently, the morphology of the hyphae formed under a microscope was observed using a 400× magnification so that it was clear before being given a Gram's stain treatment.

Measurement of hypha elongation in each isolate was conducted using the method proposed by Gruber et al. [11] with slight modifications. A total of 2×10^5 cells/ml on RPMI 1640 media was inoculated into a microwell plate and then incubated at 37 °C. The morphology of *C. albicans* was determined microscopically after 6-h-incubation using a micrometer to measure its length. Each sample was measured by 3 replications per sample.

To test the ability of the yeasts to produce hypha, researchers applied Kusumawardani's method [12] with a slight modification. At first, researchers prepared fetal bovine serum with pH 5.5 added with 20 μl of cultured RPMI 1640 and then incubated at 37 °C for 1 h. The observation and the counting process for the number of hyphae formed and the total number of cells formed were carried out using a hemocytometer with a 400× magnification microscope. Counting the number of hyphae was carried out serially, which was conducted at incubation times of 1, 3, and 6 h.

2.4. SAP 4–6 genes expression

The initial step in the analysis of SAP 4–6 gene expression and the 18S rRNA housekeeping gene of *C. albicans* was to calculate the cell density, which was more than 10^5 cells/ml. After that, it was continued with the mRNA isolation stage. The mRNA isolation was carried out using TRIzol (Invitrogen) reagent. The results of mRNA isolation on the SAP 4–6 and 18S rRNA gene were then amplified using the RT-PCR method. The amplification results were then electrophoresed using agarose gel at a volume of 100 V. The DNA bands formed on the agarose gel were viewed over UV light using a UV transilluminator. After that, the results were analyzed further. The statistical analyses used in this study were the Mann-Whitney and Kruskal-Wallis tests (by SPSS application).

3. Results and discussion

3.1. Selective isolation of C. albicans yeast

In total, 98 people were willing to be involved as samples in this study for screening the *C. albicans* in the oral cavity. Isolates from healthy individuals were then selected morphologically using selective isolation techniques, resulting in 23 isolates that were members of the genus *Candida* with cell morphology as shown in Fig. 1.

Fig. 1 shows that there are differences in cell shape between *C. albicans* and other members of the genus *Candida*, including *C. tropicalis*. *C. albicans* has special characteristics, among others. Its shape can be round, oval, or oval-round with a size of 2–5 μm x 3–6 μm to 2–5.5 μm x 5–28 μm (Tyasrini, 2006). This is because *C. albicans* is a dimorphic fungus that is influenced by external factors so that its cell shape can change, in which it may produce yeast cells, pseudo-hyphae, or hyphae [1]. However, sometimes members of the genus *Candida* are morphologically indistinguishable. Therefore, further detection and identification of *C. albicans* strains is highly needed.

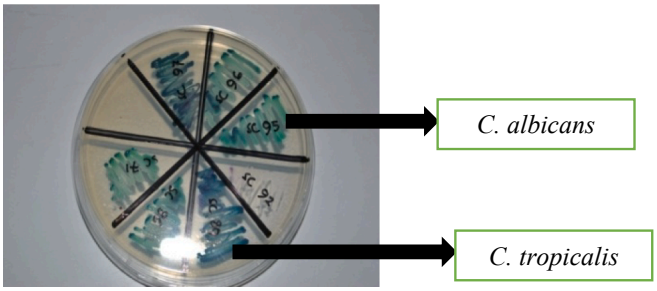

Fig. 2. Detection and Identification of *C. albicans* on CHROMagar media.

Table 1
The isolates of commensal *C. albicans* found in healthy individuals.

No.	Sources of Isolates	Isolate Code
1.	Female Sample	CK 29
2.	Female Sample	CK 32
3.	Female Sample	CK 34
4.	Female Sample	CK 37
5.	Male Sample	CK 49
6.	Male Sample	CK 55
7.	Female Sample	CK 68
8.	Female Sample	CK 69
9.	Male Sample	CK 71
10.	Male Sample	CK 74
11.	Male Sample	CK 77
12.	Female Sample	CK 89
13.	Female Sample	CK 95
14.	Female Sample	CK 96
15.	Female Sample	CK 97
Total		15 Isolates

Table 2
Distribution of isolates of members of the genus *Candida* in the oral cavity of healthy people.

No.	Names of Spesies	Total (n)	Percentage
1.	*C. albicans*	15 people	64.29%
2.	*C. tropicalis*	4 people	17.86%
3.	*C. glabrata*	2 people	10.71%
4.	*C. parapsilosis*	1 people	3.57%
5.	*C. krusei*	1 people	3.57%
Total		23 people	100%

3.2. Detection and identification of C. albicans

C. albicans isolates can be identified clearly, namely having a smooth surface and green color surrounded by a greenish halo, as illustrated in Fig. 2. Isolates identified as *C. albicans* from healthy individuals can be seen in Table 1.

Fig. 2 is the result of detection and identification of *C. albicans* on CHROMagar media, which shows that the colony of *C. albicans* isolates appears greenish on CHROMagar media, while the colony of *C. tropicalis* appears dark blue. This identification traces the study conducted by Odds & Bernaerts [13].

Table 1 shows that, out of 23 isolates from healthy people and identified as members of the genus *Candida*, 15 of them were *C. albicans*. These results indicate that *C. albicans* can be found in ±60% of a sample population that supports its existence as a normal microbiota [6,14].

3.3. Frequency of C. albicans in the sample population

The percentage of identification results of members of the genus *Candida* in healthy people shows that more than 60% of them (23 samples) carried *C. albicans* and the rest carried *C. tropicalis, C. glabrata, C. parapsilosis,* and *C. krusei* (see Table 2).

Table 3
Percentage of samples carrying commensal *C. albicans* on healthy individuals.

Sex	Total Samples	Carrying *C. albicans*	Percentage (%) of*C. albicans*
Male	40 people	5	12.50
Female	58 people	10	17.24

Table 4
Distribution of commensal *C. albicans* on healthy individuals by sex.

Sex	Carrying *C. albicans* in the oral cavity	Percentage (%)
Male	5 people	33.00
Female	10 people	67.00

Table 5
Percentage of samples carrying *C. albicans* by occupation.

Occupations	Total	Percentage (%)	Carrying *C. albicans* in the oral cavity
Labors	23 people	23.47	2 people
Farmers	5 people	5.10	2 people
Housewives	21 people	21.43	4 people
Students	44 people	44.90	7 people
Civil servants/ teachers	5 people	5.10	–

Table 6
Percentage of samples carrying *C. albicans* by age.

Age	Total	Carrying *C. albicans* in the oral cavity	Percentage (%)
20–29	49 people	8 people	50.00
30–39	24 people	2 people	24.49
40–49	14 people	3 people	14.29
50–59	9 people	–	9.18
60–69	2 people	2 people	2.04

Table 2 shows that members of the genus *Candida* are normal microbiota of the human body, which are found mainly in the mucosal area [6], including the oral cavity [15]. According to the results of a study, members of the genus *Candida* that are found in the oral cavity are *C. albicans* (64.29%), *C. tropicalis* (17.86%), *C. glabrata* (10.71%), *C. parapsilosis*, and *C. krusei* (3.57%). This is in line with the results of a study conducted by Molero et al. [16] that *C. albicans* is a dimorphic fungus that is found in the oral cavity, vagina, and digestive tract.

The identified *C. albicans* strains come from the sample population which can be grouped by sex (see Table 3), occupation, and age. Grouping the samples according to sex shows a not too far comparison between men and women. However, the isolation results from all samples carrying *C. albicans* showed that 67% of the growing isolates were from the female samples and the remaining 33% were from the male samples (see Table 4).

Table 4 shows that commensal *C. albicans* is more common in women. However, there is no statistically significant difference of *C. albicans* found between the two samples (men and women). Another characteristic that can be identified is based on the type of occupation which can be seen in Table 5.

The grouping of isolates obtained in the samples according to the type of occupation shows that 46.67% of the isolates that grew are from samples who have a work background as a student. Table 5 indicates that there is no relationship between the level of education and the tendency for a culture of clean life so that people who have a better work background do not necessarily have a better level of cleanliness. This is in line with a study conducted by Sariningrum & Irdawati [17] that

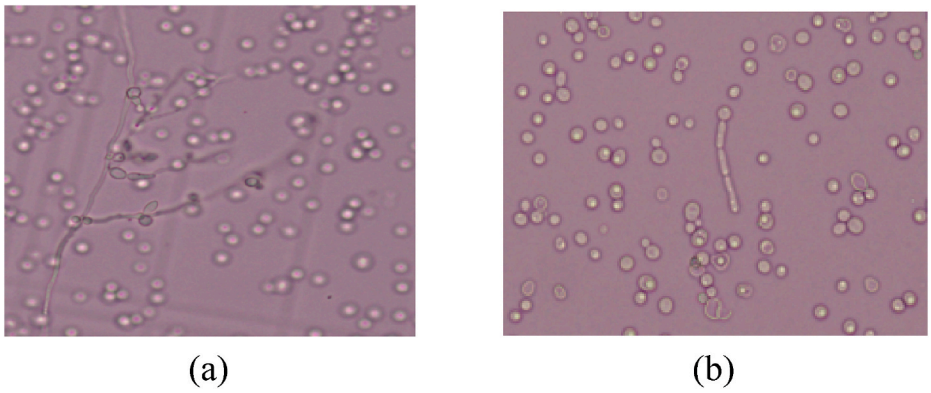

Fig. 3. Observation of *C. albicans* morphology from 1-h incubation; (a) isolates originating from oral candidiasis patients with HIV-positive (CH3), (b) isolates originating from commensals in healthy people (CK68).

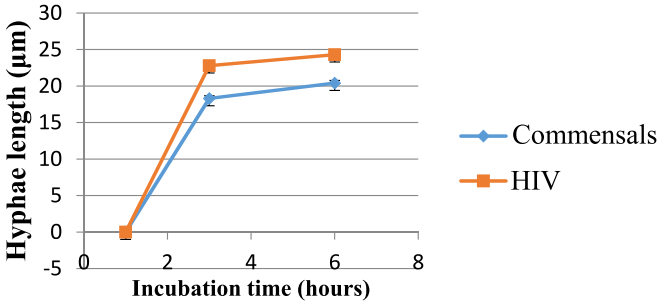

Fig. 4. Mean length of hyphae isolates of *C. albicans* originating from oral candidiasis patients with HIV-positive and commensals in healthy people with an incubation time of 1, 3, and 6 h.

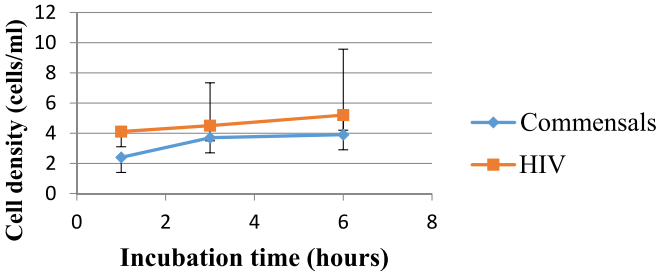

Fig. 5. The mean density of *C. albicans* cells from oral candidiasis patients with HIV-positive and commensals in healthy people with an incubation period of 1, 3, and 6 h.

there is no significant relationship between the level of parental education and the incidence of caries in children under five in Jatipurno Kindergarten. Meanwhile, if being viewed based on the age of the samples, the results can be seen in Table 6.

Table 5 shows that the grown *C. albicans* isolates are found 53% from samples aged 20–29, 13.33% from samples aged 30–39, 20% from samples aged 40–49, and 13.33% from samples aged 60–69. This shows that oral hygiene is not related to a person's age. Therefore, it can be assumed that the higher the age of a person is, the less maintained the condition of the oral cavity of the person will be. This is supported by the opinion of Anonymous [18] that, at an advanced age, a person no longer pays attention to and takes good care of their teeth because their physical health is disturbed. For that reason, it can be stated that the older a person is, the more susceptible the person to all diseases will be. It is including diseases in the oral cavity.

3.4. Analysis of secreted aspartyl proteinase 4–6 gene expression and 18S rRNA housekeeping gene

The detection of SAP 4–6 gene expression morphologically showed that isolates from both sources (HIV-positive patients and healthy individuals) could be expressed. However, isolates from the two sources had slightly different morphological forms (see Fig. 3).

Fig. 3 shows that the hyphae formed in isolates from oral candidiasis patients with HIV-positive had a fairly complex shape because they had not only one hypha and there was already a pseudo-hyphae form found. Meanwhile, isolates from healthy people had a fairly simple shape because, during incubation time, only one short hypha was formed. Therefore, it was presumed to be closely related to the speed of hypha morphogenesis in the two sources of isolates.

The size of *C. albicans* hyphae can be observed by growing them on RPMI 1640 media. In this study, the results of observation showed that the size of *C. albicans* hyphae originating from healthy people was shorter than those from oral candidiasis patients with HIV-positive (see Fig. 4).

Fig. 4 is supported by the results of statistical analysis tests which show that there is a significant difference between the length of hyphae in *C. albicans* isolates from oral candidiasis patients with HIV-positive and commensals in healthy people. These results are also supported by a study conducted by Gruber et al. [11] that the hyphae size of *C. albicans* isolates originating from HIV-1 patients is larger than commensals in normal people. In other words, the length of hyphae originating from HIV patients differed significantly from those of healthy individuals.

Hyphae is a change in the morphological form of *C. albicans* as a marker of invasion [19]. This change in shape is strongly influenced by the environment of host cells detected by *C. albicans* during the invasion process [20]. However, these morphological changes can be observed in vitro using the addition of serum to the media [21]. The results of observations carried out on two different isolate sources (oral candidiasis patients with HIV-positive and commensals in healthy people) with different incubation periods (1, 3, and 6 h) showed differences in cell numbers (see Fig. 5), shape, and morphological changes (see Fig. 6, Fig. 7, and Fig. 8).

Fig. 5 shows that the average of the total of *C. albicans* cells derived from oral candidiasis patients with HIV-positive was higher than that from commensals in healthy people with an incubation period of 1, 3, and 6 h. These results are supported by statistical analysis tests which show that there are significant differences between the two sources. In addition, the results are also supported by a study conducted by Gruber et al. [11] on HIV-1 patients and normal people, which indicates that the number of cells in isolates from HIV-1 patients was higher than that from normal people.

By referring to the results shown in Figs. 6–8, in addition to different cell numbers, the two sources of isolates also showed differences in the

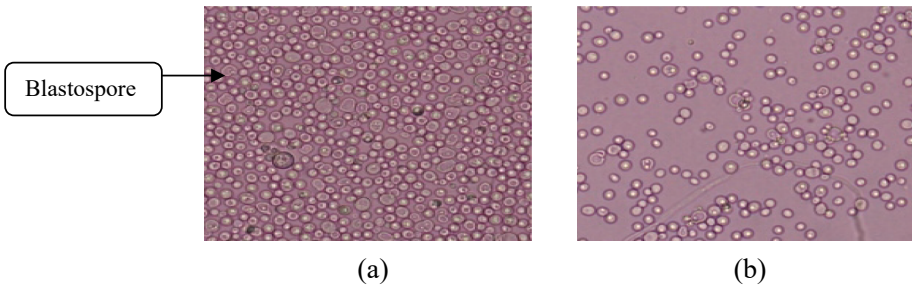

Fig. 6. The shape of the hyphae of *C. albicans* at 1-h incubation time. (a) Isolates originating from oral candidiasis patients with HIV-positive (CH3) (b) Isolates originating from commensals in healthy people (CK68).

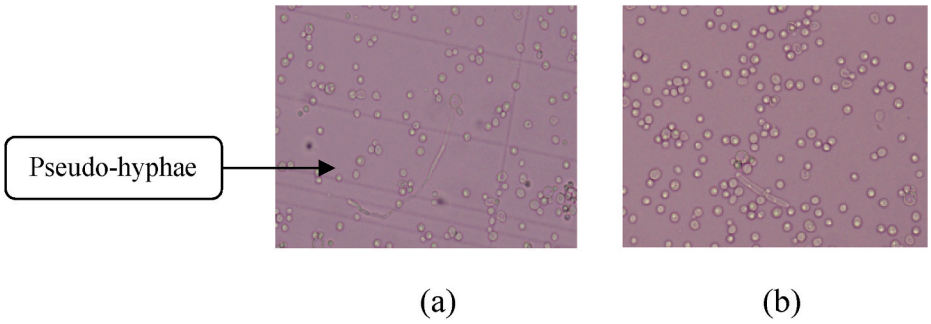

Fig. 7. The shape of the hyphae of *C. albicans* at 3-h incubation time. (a) Isolates originating from oral candidiasis patients with HIV-positive (CH3) (b) Isolates originating from commensals in healthy people (CK68).

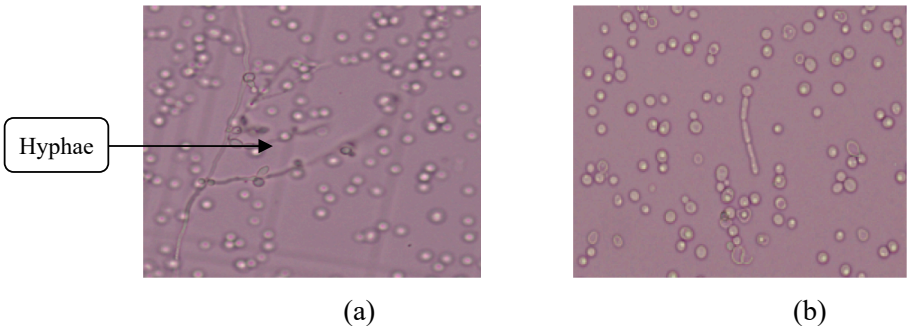

Fig. 8. The shape of the hyphae of *C. albicans* at 6-h incubation time. (a) Isolates originating from oral candidiasis patients with HIV-positive (CH3) (b) Isolates originating from commensals in healthy people (CK68).

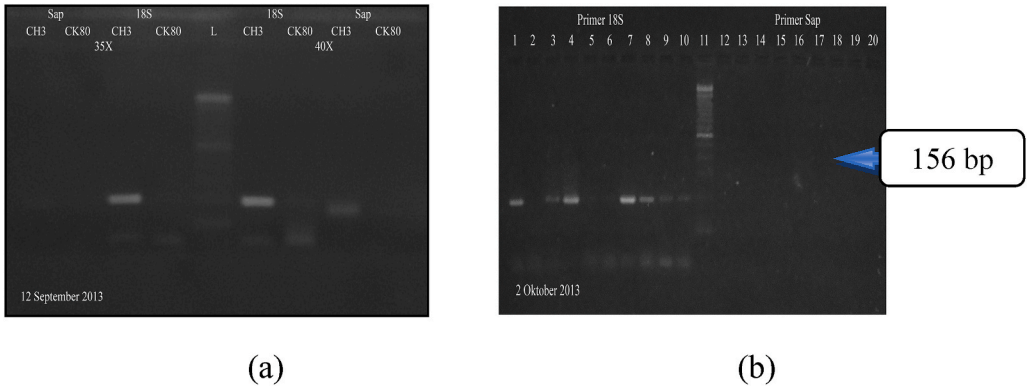

Fig. 9. Electropherogram visualization of SAP 4–6 gene isolates of *C. albicans* using the RT-PCR method (a) *C. albicans* isolates of CH3 and CK80 that express SAP 4–6 gene (b) *C. albicans* isolates of CH3 and CK80 that do not express SAP 4–6 gene.

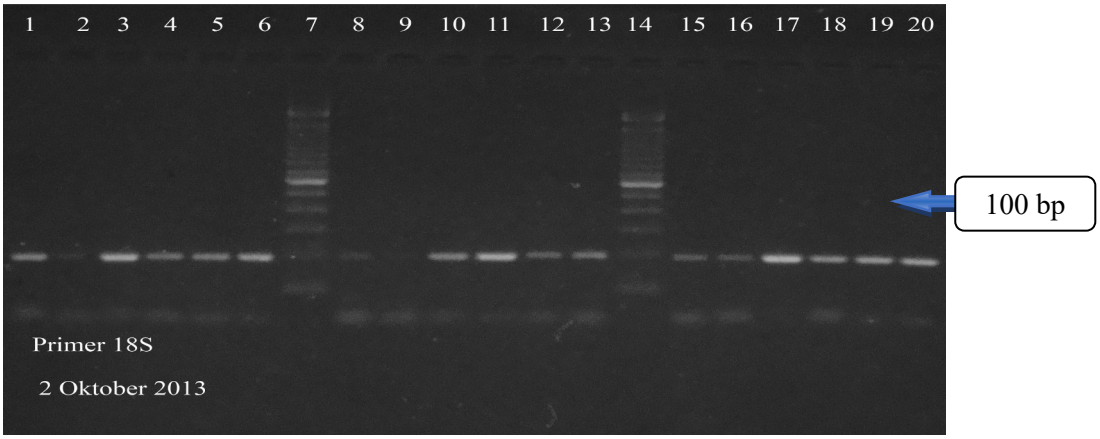

Fig. 10. Visualization of the 18S rRNA gene for *C. albicans* isolates using the PCR method.

shape and speed of changes based on the morphology of *C. albicans* cells from one cell to pseudo-hyphae and hyphae at different incubation times.

Observations on the two sources of isolates during the first 1-h incubation showed that the cells or germ tubes formed in isolates from oral candidiasis patients with HIV-positive were round in shape with very close cell spacing and having several oval cells. However, the characteristics of CH3 isolates in the middle of the cell looked like a dot or circle, indicating that the cell will immediately divide to form new cells. Meanwhile, the shape of isolates from commensals in healthy people (CK68) was round, having a not too close spacing between cells, and rarely having oval cells. In CK68 isolate, only a few cells looked like they were going to divide.

Further observations were conducted after the cells were incubated for 3 h. The results showed that many CH3 isolates had divided into two cells and even three cells. Meanwhile, in CK68 isolates, only a small proportion had split (see Fig. 7).

The results above showed that isolates from oral candidiasis patients with HIV-positive (CH3) divided faster (had formed pseudo-hyphae) than those from commensals in healthy people (CK68). This is because the isolates from oral candidiasis patients with HIV-positive (CH3) had experienced an increase in pathogenicity, which caused these isolates to be more aggressive in dividing [21].

The results of hyphae observations after incubation for 6 h showed that isolates from oral candidiasis patients with HIV-positive (CH3) had formed hyphae, while isolates from commensals in healthy people (CK68) were still in the form of pseudo-hyphae. It can be assumed that isolates originating from oral candidiasis patients with HIV-positive undergo morphogenesis faster than those from commensals in healthy people. In relation to this, Gruber et al. [11] stated that the morphogenesis that occurred in isolates from HIV-1 patients was faster than isolates from normal people so that the size of the hyphae formed was also longer than that from normal people.

The results of the RT-PCR method on 30 *C. albicans* isolates showed that only one isolate expressed SAP 4–6, namely CH3 isolates (see Fig. 8). These results indicated that molecularly only one isolate (CH3) from oral candidiasis patients with HIV-positive can express SAP 4–6 genes. This is in contrast to the results of the morphological analysis which showed that all genes were expressed (see Fig. 8). Therefore, these results (see Fig. 9) are not in line with a study conducted by Taylor et al. [22]; that the induction of host cells on SAP 4 and SAP 5 gene expression causes morphological changes of *C. albicans* from the yeast to the hyphae and SAP 4, SAP 5, and SAP 6 simultaneously are responsible for virulence [19]. In addition, this indicates that Secreted Aspartyl Proteinase (SAP) is not possessed by all strains of the species member, whereas according to Naglik et al. [7]; SAP is owned by all strains of members of *C. albicans* which act as a virulence factor [5]. Several factors are considered to influence the final RT-PCR results, including (i) the composition of the induction medium is not appropriate to induce the samples of the SAP 4–6 gene in *C. albicans* isolates [11,23,24], and (ii) it is possible that not all SAP 4–6 genes can be expressed [24,25].

To ensure that the RNA isolated had a good quantity and quality and the RT-PCR procedure was carried out correctly, the PCR of the 18S rRNA gene was performed as a loading control. The results can be seen in Fig. 10.

Based on the PCR results of the 18S rRNA gene, all isolates expressed the 18S rRNA gene. This means that all isolates have the same housekeeping gene (18S rRNA), indicating that all isolates belong to the Eucarya domain, which is the domain of *C. albicans* [26]. Therefore, it can be concluded that molecular detection of SAP 4–6 gene expression indicates that only one isolate from samples of oral candidiasis patients with HIV-positive is expressed, while none of the isolates from commensals in healthy people is expressed.

4. Conclusions

Based on the results of this study, it can be concluded as follows.

(i) The colonization frequency of *C. albicans* in healthy people is ±15% of the total sample (98 people).
(ii) The SAP 4–6 gene of *C. albicans* isolated from the oral cavity of oral candidiasis patients with HIV-positive was only expressed in one isolate with a visible thin band (CH3). Meanwhile, the SAP 4–6 gene of *C. albicans* isolated from the oral cavity of healthy people was not expressed in vitro.
(iii) There are differences in SAP 4–6 gene expression among *C. albicans* isolated from the oral cavity of oral candidiasis patients with HIV-positive and commensals in healthy people.
(iv) The size and speed of hyphal formation of *C. albicans* from oral candidiasis patients with HIV-positive have significant differences based on statistical analysis.

Author statement

All the authors have equal contribution in preparation of the manuscript. The first, second and last author have original idea, conceptualization, methodology, analysis and validation. The third author contributed in revision, editing, review and improvement of the first draft of the manuscript. First and third authors did organization of the manuscript including language corrections and formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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