SERUM ACETALDEHYDE AS A POTENTIAL BIOMARKER FOR THE DETECTION OF PATHOGENIC BIOFILM FORMATION BY Candida albicans

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SERUM ACETALDEHYDE AS A POTENTIAL BIOMARKER FOR THE DETECTION OF PATHOGENIC BIOFILM FORMATION BY *Candida albicans*

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ABSTRACT

Candida albicans is a fungus that causes opportunistic infections in humans. It secretes toxic metabolites and induces the formation of biofilms. Fully developed C. albicans biofilms consist of planktonic yeast cells, pseudohyphae, and hyphae. The hyphae can invade tissues and bring toxic metabolites deep inside the organs and into the bloodstream. Biofilms cannot be identified by culture methods from feces or blood samples, necessitating the development of biomarkers to detect them. Here we show that the acetaldehyde level in serum can be used as a biomarker of gastrointestinal Candida biofilm formation. An in vitro C. albicans biofilm model is set up by culturing C. albicans in an Erlenmeyer flask, while an in vivo model is created in the rat gastrointestinal mucosa. A lack of nutrients induces biofilm formation in vitro, while in vivo biofilm formation is induced by treatment with antibiotics and corticosteroids. Identification of C. albicans biofilms is carried out macroscopically and microscopically using scanning electron microscopy. Analysis of acetaldehyde levels is performed by gas chromatography. The in vitro acetaldehyde level in the supernatant of the C. albicans culture is 0.9 ± 0.3 % (v/v) after a period of 48 h incubation, at which time biofilms start to form. The serum mean acetaldehyde level is 0.011 ± 0.003 % and 0.005 ± 0.001 % in the experimental and the control groups, respectively. The t-test results show that this difference is significant at $p < 0.05$, indicating that serum acetaldehyde can be used as a biomarker of C. albicans biofilm formation in the intestine.

Keywords: *acetaldehyde, biomarkers, biofilm, C.albicans, gaschromatography.*

INTRODUCTION

Candida is the most common of the commensal fungi in humans, primarily occurring in the mouth, gut, and vagina. They attach themselves to mucosal membranes, where they may give rise to opportunistic infections [1 - 3]. *C. albicans* is the dominant species in healthy subjects, but *Candida parapsilosis* and *Candida tropicalis* have also been found [2, 4]. The pathogenicity of *C. albicans* includes production of toxic metabolites and formation of biofilms, not only on biotic surfaces in the human body [5, 6] but also on abiotic surfaces such as those of central venous catheters, dentures, or tooth implants [7, 8].

C. albicans is a dimorphic organism that has two life

forms. The first one refers to a budding yeast that grows as blastospores, whereas the second one is a germ tube that grows as pseudohyphae. The buds develop as elongated spheres, while the germ tubes or hyphae, periodically form long tubiform extensions fragments. The pseudohyphae are potentially harmful to the host because of their ability to penetrate the host cells. Indeed, biofilm-related chronic mucocutaneous candidiasis (CMC) has been associated with a significant risk of oral cancer [9-11]. *C. albicans* biofilm isolated from these patients produces high levels of carcinogenic acetaldehyde (ACH) *in vitro*, thus providing one possible explanation for carcinogenesis [12]. Therefore, it is important to combat biofilms at the earliest stage possible.

Biofilms consist of a mixture of yeast and host cells, pseudohyphae, hyphae, and extracellular polymeric substances (β-1,3 glucan, chitin, and proteins) secreted by the fungus [13]. This extracellular polymeric material serves to reduce the exposure to antimicrobial compounds, host immune factors, and antimicrobial peptides [14]. In addition, chronic biofilm infections may cause inflammation, which increases the risk of carcinogenesis [15].

Several studies have detected the presence of *C. albicans* using a culture method from feces or blood samples. In contrast, *in vivo* detection of growing biofilms requires endoscopy, which is not easy, especially in the mucosa membrane of the digestive tract, and is painful. Therefore, we need a biomarker to determine the presence of metabolites resulting *C. albicans* biofilms. Our hypothesis is that ACH is a potential biomarker to determine the formation of *C. albicans* biofilm. We therefore investigate the production of acetaldehyde by *C. albicans* both *in vitro* and *in vivo*. We show that ACH levels are indeed significantly increased upon *Candida* biofilm formation. Our results may facilitate the discovery of novel antifungal agents that can inhibit *C. albicans* cell growth and biofilm formation, and reduce the carcinogenic possibility of *C. albicans* biofilms, which may be important when treating fungal biofilm infections.

EXPERIMENTAL

Materials and methods

C. albicans stock was acquired from the Laboratory of Microbiology, Faculty of Dentistry, Airlangga University. The serum was obtained from two groups of 16 male white Wistar rats (Rattus norvegicus) aged approximately 2-3 months. They were in a good condition weighing approximately 200 g. The solution of phospate salin buffer (PBS) contained NaH_2PO_4, $Na_2HPO_4.7H_2O$, NaCl and KCl

Growth and formation of *in vitro C. albicans* biofilm

In vitro C. albicans biofilm formation was studied using Erlenmeyer flasks. 5 % of *C. albicans* inoculum was added to 30 mL Yeast Peptone Dextrose (YPD) growth medium. The culture was incubated at 37°C using a rotary shaker at 140 rpm. *C. albicans* growth in culture was monitored by measuring the optical density at 600 nm (OD_{600}) with a UV/VIS spectrophotometer after 0 h, 8 h, 16 h, 24 h, 32 h, 40 h, 48 h, 56 h, 64 h, and 72 h of incubation. *C. albicans* biofilm appeared on the wall of the Erlenmeyer flask during incubation and was examined.

Formation of *in vivo C. albicans* biofilm

The experimental animals were adapted for one week and randomly divided into two groups (a control and an experimental group, each containing 16 rats). The groups were orally given the antibiotics streptomycin (20 mg/kg), tetracycline (25 mg/kg), and gentamicin (7.5 mg/kg) each day for 4 days. On the 4th day, the animals were injected with cortisone acetate (225 mg/kg) as an immunosuppressant. On the 5th day, they were orally inoculated with *C. albicans*. During the treatment, the animals were fed with AIN-93 [16] and spider medium (peptone, yeast, beef extract, NaCl, mannitol, K_2HPO_4).

Identification of *C. albicans* biofilm

The biofilm identification was conducted macroscopically (small intestine and cecum were split and examined through a magnifying glass) and microscopically by scanning electron microscopy (SEM).

Scanning electron microscopy of *in vitro*-produced biofilms

Biofilms were obtained in Erlenmeyer flasks after

growing *C. albicans* for 72 h as described above. 5 ml of *C. albicans* suspension was centrifuged at 10,000 rpm. The supernatant was removed and the residue was washed once with PBS. The mixture was centrifuged again, and the supernatant was removed. The *C. albicans* biofilm residue was dried overnight at 37°C, coated with gold/palladium (40/60 % ratio) and observed under a scanning electron microscope in a high vacuum mode at 10 kV.

Scanning electron microscopy of *in vivo*-produced biofilms

Biofilms were created in the rat gastrointestinal mucosa as described above. The intestine specimens were rinsed with PBS and subjected to consecutive overnight incubations in the fixative reagents glutaraldehyde and osmic acid. Samples were subsequently washed with distilled water, dehydrated through a series of ethanol washes (70 % for 10 min, 95 % for 10 min, and 100 % for 20 min), dried using critical point drying (CPD), stuck on a stub (holder) and coated with pure gold using a vacuum evaporator. The surface topographic features of the biofilm were visualized by scanning electron microscopy.

Acetaldehyde measurement

For the determination of ACH concentrations, a Hewlett Packard 6890 Series Agilent 19095P-Q04 gas chromatography instrument was used that was equipped with an HP-Plot Q column and a flame ionization detector. Helium gas was used as a mobile phase. The GC temperature on injector A was set at 220°C. The initial temperature was fixed at 150°C, the initial time was 15 min, and the temperature was raised automatically by 5.0°C/min. The final temperature was 200°C, the final time was 30 min, and the flow rate of the carrier gas passing through the large column was 68 mL/min. Acetaldehyde was detected by flame ionization.

Three milliliter samples of *C. albicans* culture from *in vitro* biofilms were centrifuged at 10,000 rpm for 10 min. Each supernatant was injected and its acetaldehyde content was analyzed by gas chromatography.

Samples (0.5 ml) of rat serum were placed in the centrifuge tube and 0.5 ml 5 % TCA (trichloroacetate) was added. Then they were centrifuged at 3000 rpm for 5 min. The supernatant was injected and analyzed for acetaldehyde content by gas chromatography.

Statistical analysis

A t-test was performed to determine the significance of the ACH data.

RESULTS AND DISCUSSION

Formation of *in vitro* biofilms

The growth profile of *C. albicans* in YPD medium showed a short logarithmic phase and a long stationary phase (Fig. 1). After 48 h, the optical density decreased

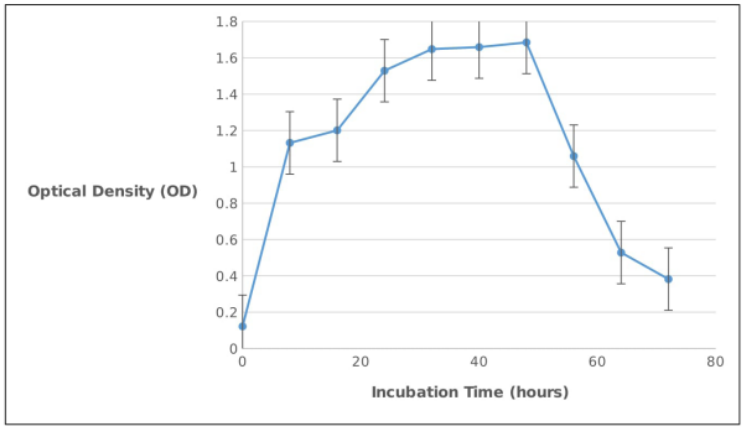


Fig. 1. Growth profile of *C. albicans* cultured in YPD medium in Erlenmeyer flasks.

Fig. 2. Macroscopic observation of *C. albicans* biofilm adhering to the wall of the Erlenmeyer flask after a period of 48 h incubation (see the black arrow).

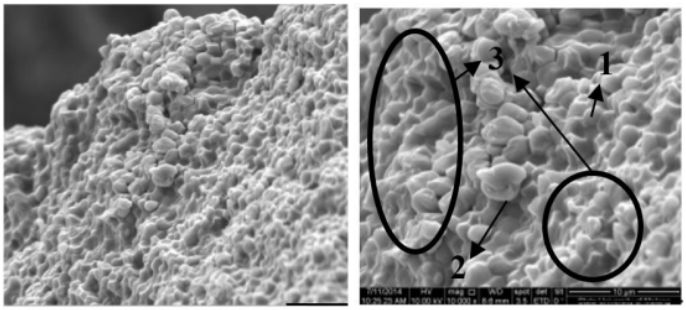
sharply. Biofilm began to appear at the end of the stationary phase just before the death phase as a white coat attached to the flask surface. This biofilm formation was induced by the lack of nutrients in the YPD medium. The biofilm production continued during the subsequent growth stages and appeared as a piece of cotton-like substance floating on the top of the liquid culture in the Erlenmeyer flask (Fig. 2).

The thick white plaque adhering to the wall of the Erlenmeyer flask (Fig.2) is examined. In correspondence with the defense mechanism of *C. albicans* the lack of nutrient or nutrient depletion triggers *C. albicans* adhering to the solid surface of the flask. Then they undergo morphogenesis to produce a dense layer of cells of mixed morphology embedded in a extracellular matrix rich in polymer 1,3 glucan. SEM analysis is carried out revealing that it contained a mass of *Candida* cells in a biofilm form. The *in vitro* biofilm consists of yeast cells, budding yeast and extracellular matrix (Fig. 3).

Formation of *In vivo Candida* biofilms

In vivo C. albicans biofilms were successfully formed in the white rat intestinal mucosa by four types of inductions: tetracycline, streptomycin, and gentamicin antibiotics administered daily for 4 days. On the 4th day the animals were injected with cortisone acetate as an immunosuppressor, *C. albicans* inoculum, and spider medium.

We monitored the growth of *C. albicans* in the course of an *in vivo* formation of a biofilm by analyzing the presence of the organism in the feces. The number of colonies of *C. albicans* (in Colony Forming Units, CFUs) in the rat's feces slightly increased from day 3 to 7 after the fifth inoculation and sharply increased on day 14. The antibiotics and immunosuppressant were responsible for this, as they disrupted the normal intestinal microflora balance and led to growth obstruction of some



(A) (B)

Fig. 3. SEM image of *C. albicans* biofilms obtained after 48 h growth on YPD. (A) 5000x magnification. (B) 10,000x magnification. (1) An yeast form of *C. albicans* cells, (2) *C. albicans* budding yeast cells, (3) *C. albicans* cells coated by an extracellular matrix (scale bar = 10 µm).

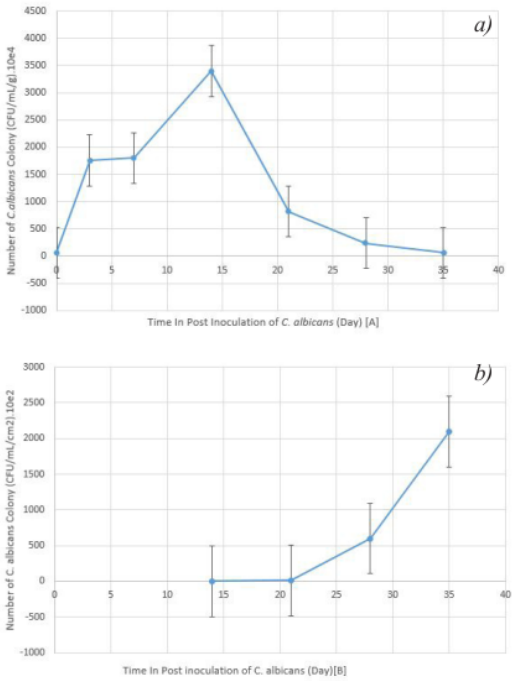


Fig. 4. Profile of *Candida* colonization on (A) feces and (B) rat intestinal mucosa that had been induced by antibiotics and immunosuppressants.

microflora. On the other hand, *C. albicans* survived and even developed an uncontrolled growth (overgrowth) as more nutrients became available. *C. albicans* formed colonies in the excess of food and flew out with the feces. Therefore, the increasing CFU number of *C. albicans* colonies in the feces is a *Candida* overgrowth parameter. The overgrowth of *C. albicans* occurred 14 days after inoculation. On day 21, the CFU number of *C. albicans* colonies in the feces decreased again, but it increased in the gastrointestinal mucosa, as shown in Fig. 4. This is consistent with the increased colonization of the rat intestinal mucosa by *C. albicans* cells during the early stage of biofilm formation. Indeed, on day 35, the *C. albicans* CFU in feces decreased even more accompanied by a concomitant drastic increase of the *C. albicans* CFU in the gastrointestinal mucosa.

Fig. 5 shows the presence of *C. albicans* biofilms developed in rat intestinal mucosa. They appear as white plaques on the surface of the rat intestinal mucosa. They are neither easy to peel off with surgical instruments or be washed off by PBS buffer solution.

The SEM image of *in vivo* biofilms (Fig. 6) shows clearly *C. albicans pseudohyphae* which cannot be seen in the *in vitro* biofilms. These pseudohyphae are potentially harmful to the host because of their ability to penetrate host cells. In immunocompromised individuals the hyphae and toxins produced by *C. albicans* can end up in the bloodstream. In immunocompetent individuals the *C. albicans* cells are blocked by the immune system prior reaching the bloodstream. However, toxins may

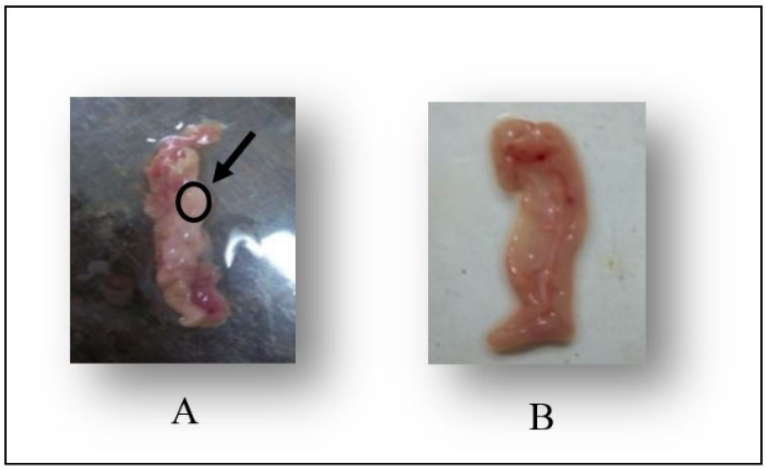


Fig. 5. Rat intestinal mucosa in presence (A) and absence (B) of *C. albicans* biofilm. The black arrow indicates the thickest plaque on the surface of rat intestinal mucosa.

reach the bloodstream because they are not recognized by the immune system.

Once the *in vivo* and *in vitro* biofilm models had been proven, the next step was *C. albicans* metabolite observation. Metabolite analysis was carried out by GC of the *in vivo* and *in vitro* biofilm samples aiming to detect ACH.

Analysis of ACH

Gas chromatography was used to detect the presence of ACH in the *in vitro* and *in vivo* biofilm samples. The amount of ACH in the *C. albicans* culture supernatant after 48 h incubation was 0.9 ± 0.3 % (v/v) or 154 ± 50 µM. These results indicate that the *C. albicans* used in this study is a strain that could potentially promote carcinogenesis by producing ACH. Previous studies indicated that some *Candida* species were able to produce ACH from ethanol [17 - 19]. *C. albicans, C. tropicalis*, and *C. parapsilosis* especially produce ACH in higher quantities than other species, well above the concentration that is considered to be carcinogenic (>100 µM) [17].

The results obtained with the *in vivo* production of ACH in biofilms are presented in Fig. 7. The ACH levels in the Wistar rat intestinal mucosa show a homogeneous normal distribution. The *t*-test carried out shows that the ACH levels in the serum of the control and treated rats are essentially different ($p < 0.05$). Forty-two days

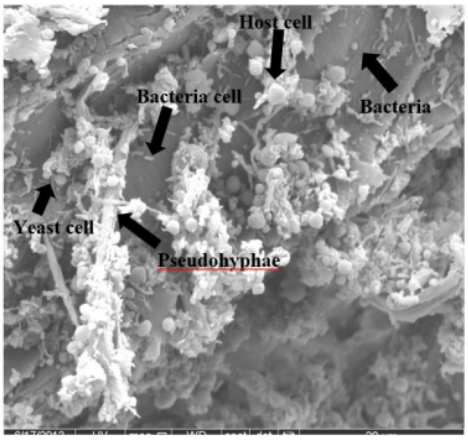


Fig. 6. A SEM image of *C. albicans* biofilm on mouse cecum membrane mucosa at 5000x magnification.

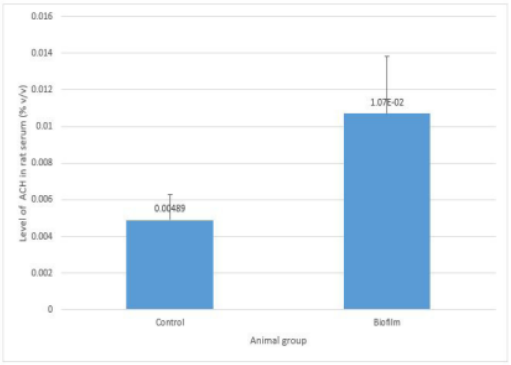


Fig. 7. Levels of ACH in the serum of control and biofilm rats.

after inoculation with *C. albicans*, the serum ACH level is increased by a factor of 2.3. Under conditions of biofilm formation, the amount of *C. albicans* attached to rat intestinal mucosa is significantly higher than in the normal state; therefore, it produces more ACH, likely because of glucose fermentation and alcohol production. Thus, elevated ACH levels in the serum can be used as a biomarker of biofilm formation.

ACH produced as a metabolite of alcohol catabolism is highly toxic, mutagenic, and carcinogenic. ACH can be produced in many organs such as the intestine, liver or blood, where *C. albicans* may be present. The higher level of ACH caused by biofilm formation is harmful to the organs. In this study, the rats from the biofilm group had serious liver damage (data not shown).

CONCLUSIONS

Acetaldehyde is a highly toxic, mutagenic and carcinogenic product of alcohol fermentation and metabolism in microbes, especially in yeast. In *C. albicans*, acetaldehyde is a by-product of the pyruvate bypass that converts pyruvate into acetyl-Coenzyme A (CoA). Our results show that *C. albicans*-infected (biofilm) rats are capable of producing carcinogenic amounts of ACH from ethanol which affects greatly the levels of control rats serum ACH. The latter of the biofilm rats is increased by a factor of 2.3. The difference is significant enough to be used as a biomarker for biofilm formation of *C. albicans* in the intestinal tract in vivo.

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PAGE 1

PAGE 2

PAGE 3

PAGE 4

PAGE 5

PAGE 6

PAGE 7