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Construction and Characterization of the Intestinal Biofilm Model of Candida spp

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ABSTRACT

Candida biofilm is difficult to be penetrated by antifungal agent, so that it shown *multi drug* resistent and caused incomplete treatment of candidiasis. Using a clinically relevant *Candida albicans,* the intestinal biofilm model was successfully established *in vivo* on intestine mucous membran of *Rattus norvegicus*, after treatment with a corticosteroid, gentamycin, streptomycin, tetracylin and *Candida albicans* inoculum. The biofilm began to appear on day 21, characterized by the presence of blastospore, budding yeast and the extracellular matrix. Hyphae began to appear on day 28. On day 35 began to appear a fibrous matter combined with the biofilm, which further thickened on days 42 and 49. The pathogenic *Candida* biofilm model in vivo in *Rattus norvegicus* mucous membranes is usefull for testing the natural ingredients omitting biofilm in the next research.

Keywords: intestinal biofilm, *Candida albicans*, *Rattus norvegicus.*

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INTRODUCTION

Biofilms are a protected form of parasitic microorganisms, where they are safe from host immune system and antibiotic treatment, creating a source of toxic metabolites and persistent infection. Biofilm not only provide a physical barrier to antimicrobial agents and host antibodies, but facilitate the exchange of antibiotic/antifungal-resistant genetic material. bacteria. *Candida* biofilm is difficult to be penetrated by antifungal agent causing incomplete treatment of candidiasis. It was reported *Candida. albicans* biofilm resistance to many antifungal, such flukonazole, ketoconazole, amphotericin B, klotrimazole, mikonazole, zaragozic acid B, terbinafine, cerulenin, chlorhexidine and nystatin in anaerobic growth conditions (Chandra *et al.*, 2001). Experiments of *Candida albicans* biofilm formation on polyvinyl chloride strip, evidenced that the biofilm state was 30-2000 times more resistant to flukonazole, amphotericin B, flucytosine, itraconazole and ketoconazole compared in cell-free state. Kill microorganisms in the biofilm structure would require 1000 times the dose of antibiotic required to achieve the same result in suspension without biofilm (Kojic and Darouiche, 2004).

Overgrowth of *Candida* spp and the biofilm formation in the human intestinal tract is a crucial but negligible chain increasing incidence of many diseases today, especially degenerative diseases. *Candida* spp can produce a variety of toxic metabolites, some of which interfere with the immune system. Therefore, when the infection is not immediately cured, can lower the human immunity in the site of collonization and causes complications.

Primary and secondary metabolites produced by *Candida* spp . are harmful to the body when there is in high amount, which is currently happening in the *Candida* overgrowth and biofilm. Toxic metabolites of *Candida* spp . includes ethanol, formaldehyde, acetaldehyde, D-arabinitol, and organic acid such as tartaric acid. Metabolites of *Candida* spp . some are denaturants, causing denaturation of host protein molecules. There is also a metabolite of *Candida* spp. that cause DNA mutations, resulting misfold and conformational changes in the protein molecule. Proteins are part of the molecular machinery of cells which is very important compilers. While protein is very sensitive to the denaturant compounds. Structural or conformational abnormality due to the presence of protein denaturants compounds resulting protein dissfunction, named conformational diseases or degenerative diseases. Around ten last year was understood that a large number of diseases with very different pathology, at the cellular level can be studied in the frame of protein missfolding, which include Alzheimer's disease, Parkinson's, prion, type 2 diabetes, amyloidosis, cystic fibrosis, sickle cell anemia , cataracts, and other (Mravec and Epp., 2006; Winter and Juckel , 2006; Tsang *et al*., 2007; Kuleta *et al*., 2009; Moir *et al*., 2010; David, 2012).

The formation of *Candida albicans* biofilm has been demonstrated in the previous in vitro studies (Baktir *et al*., 2012). In the present article we notify the formation of pathogenic *Candida albicans* biofilm in vivo on mucous membran of *Rattus novergicus* intestinal tract.

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MATERIALS AND METHODS

Materials

Material used in this experiment were *C. albicans* and *Rattus novergicus* strain Wistar. *C. albicans* strain was obtained from the culture collection of the microbiology research division, Airlangga University, Indonesia. The preparation of planctonic *C. albicans* were described in the previous article (Baktir *et al*., 2012). *Rattus novergicus* used as testing animal. Total testing animals were 20, devided into 2 groups each consist of 10 mice. They were randomly taken to be the control group and the treatment group using mercury. Adaptation period for each group is 2 weeks. After the adaptation period is completed the animals test entered the treatment period.

Methods

In vivo experiments using animal test *Rattus norvegicus* strain Wistar as many as 30, which are grouped into 10 groups, each group consisting of 3 members. Ten groups of mice were analyzed in succession on day 0, weeks 2, 3, 4 and so on until biofilms formed on the mucous membrane of the intestinal tract. In each group, the first member was control (no treatment), the second members was treated, the third member was prepared for backing up the second member if there was a death. All members were analysed, ie. feces and intestinal tissue colonization after sacrificed/dissected and SEM analysis.

Experimental

Cultivation of *Candida albicans*

A single colony of *Candida albicans* was inoculated into 10 mL YPD broth and shaked over night (24 hours) on rotary shaker at 155 rpm, room temperature. The culture suspension was harvested and centrifuged at 10,000 rpm, 4^{o} C for 15 minute. The cell pellets then washed twice with 0,1 M sterile phosphate buffer salin (PBS). The cell pellets then was suspended in Spider Media untill obtained optical density of 0,5 at 620 nm by spectrophotometer UV/Vis (Baktir *et al.,* 2012).

Construction of intestinal biofilm

Experimental animals used were *Rattus novergicus* strain Wistar, 3 months old, was orally took gentamycin, streptomycin and tetracylin for 4 days. On the fourth day, the mice were injected with 1.62 ml of cortisone subcutaneously. On the fifth day, mice was took suspension of C. albicans in spiders media orally (Baktir *et al*., 2012). Dissection of animal was done to observe biofilm in the gastrointestinal tract. Furthermore mucosal intestinal digestion was observed with SEM and counting cell colonization. Furthermore , disonde Wistar rats with enzyme extracts from

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Characterization of intestinal biofilm

The biofilm was firstly observed by macroscopis appearance of intact intestine and membrane mucouse intestine after dissection. The quantity of biofilm was estimated by measuring the total plate count of *C. albicans* in feces and membrane mucous samples. Biofilm characterization was done by SEM (Scanning Electron Microscope).

RESULTS

Total plate count of *C. albicans* in Rattus novergicus feces (Table 1) is a useful parameter to estimate the time of animal dissection for observation of the biofilm formation on the intestinal mucous membrane. The bar graph of Table 1 is in the Figure 1.

Table 1. Total plate count data of *C. albicans* in feces sample

Animal's number	TPC before treatment (CFU/mL/gram)	TPC after treatment* (CFU/mL/ gram)				
		Day-14	Day-14	Day-14	Day-14	Day-14
1	$3,7 \times 10^6$	$1,4 \times 10^4$	$1,2 \times 10^4$	$1,1 \times 10^4$	$2,0 \times 10^3$	$1,3 \times 10^4$
2	$1,0 \times 10^6$	$2,9 \times 10^4$	$1,8 \times 10^4$	$1,0 \times 10^3$	$9,0 \times 10^3$	$1,3 \times 10^4$
3	$6,9x\ 10^5$	$3,0 \times 10^4$	$3,0 \times 10^4$	$2,0 \times 10^4$	$2,9 \times 10^4$	$1,2 \times 10^4$
4	$1,0 \times 10^4$	$2,4 \times 10^4$	$2,4 \times 10^4$	$5,0 \times 10^3$	$3,0 \times 10^3$	$5,0 \times 10^1$
5	$9,3 \times 10^5$	$1,0 \times 10^4$	$1,5 \times 10^4$	$1,0 \times 10^3$	$2,0 \times 10^3$	$2,0 \times 10^4$
6**	$9,0 \times 10^4$	$1,4 \times 10^4$	$4,0 \times 10^4$	$1,5 \times 10^4$	$4,0 \times 10^3$	$4,0 \times 10^2$
7	$9,8 \times 10^5$	$1,1 \times 10^4$	$6,0 \times 10^4$	$5,1 \times 10^4$	$1,1 \times 10^4$	$8,0 \times 10^3$

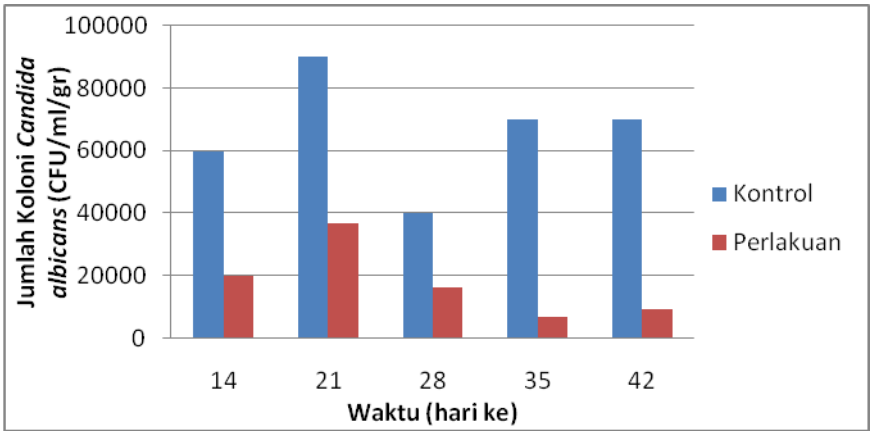


Figure 1 Total plate count bar graph of *C. albicans* in feces sample on days 14-42 after treatments.

On day 42 after *Candida*, antibiotic and steroid treatments, *Rattus novergicus* were sacrificed to observe the intestinal biofilm. Intestine was separated, dissected and cleaned from any matter that are still attached to the mucous membrane, then washed with PBS

repeatedly until clean of blood. Early biofilm identification was done through macrocopic observation of intact intestine. The appearance intact intestine before dissection are in Figure 3. The outside of the intestine containing biofilm (Figure 3A) was looked darker compared to the control groups (Figure 3B).

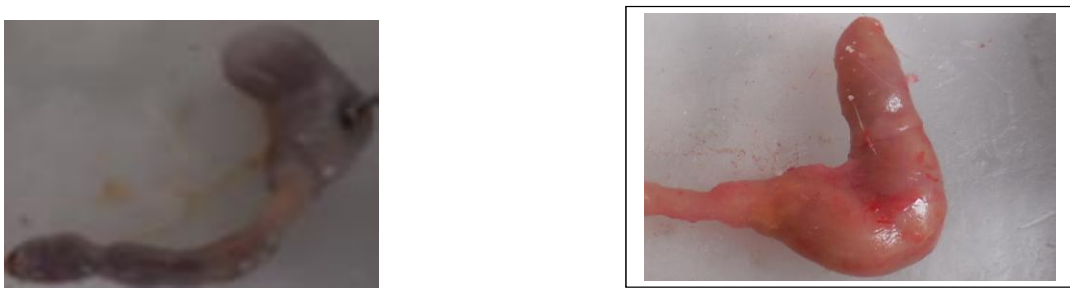
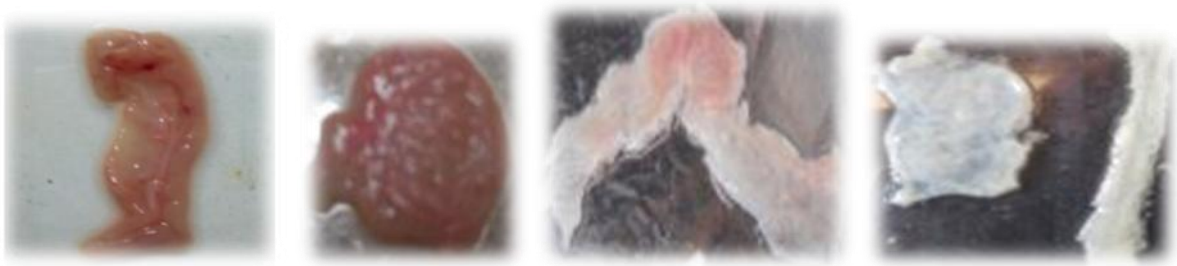


Figure 3. Surface appearance of *Rattus novergicus* intestine of test- (A) and control-groups (B).

Table 2. Total plate count data of *C. albicans* on intestinal mucous membrane

Number	Day	Total plate count data of *C. albicans* on intestinal mucous membrane (CFU/cm^2)	
		Control group	Treatment group
1	14	6,0 x 10^2	2,9 X 10^2
2	21	7,0 X 10^2	1,2 X 10^3
3	28	7,3 X 10^2	6,0 X 10^4
4	35	1,9 X 10^2	2,1 X 10^5



A **B** **C** **D**

Figure 4 Macroscopic appearance of *C albicans* biofilm on intestinal mucous membrane of *Rattus novergicus*. A, B, C and D: non-treated, after treatment on day 7, 14 and 21 respectively.

The macroscopic appearance of biofilms showed as a white layer mass overlaid the surface of mucous membrane of the intestine (Figure 4). The biofilm attach to the mucous membrane and was not easily removed by PBS solution.

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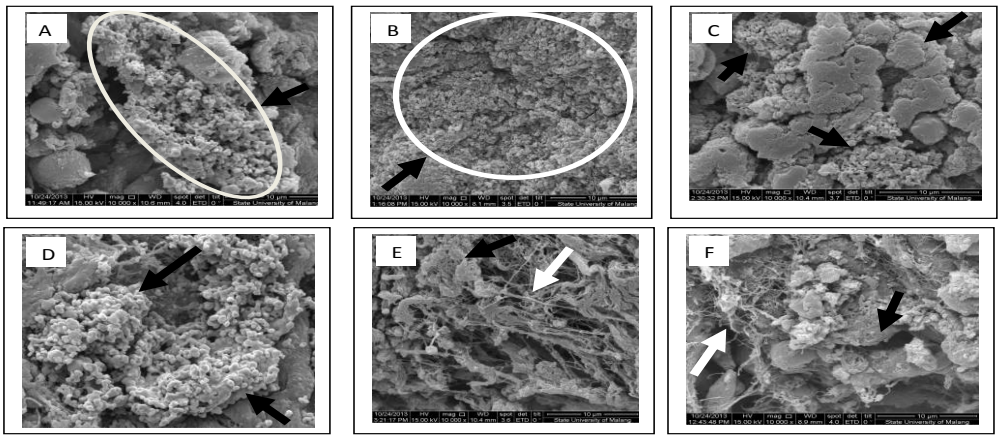

Figure 5 SEM images of *C. albicans* biofilm attach to intestinal mucous membran of Rattus norvigatus on day 7 (A), 14 (B), 21 (C), 28 (D), 35 (E) and 42 (F). Black arrow indicate biofilm, white arrow indicate fibrous mass growed arround biofilm.

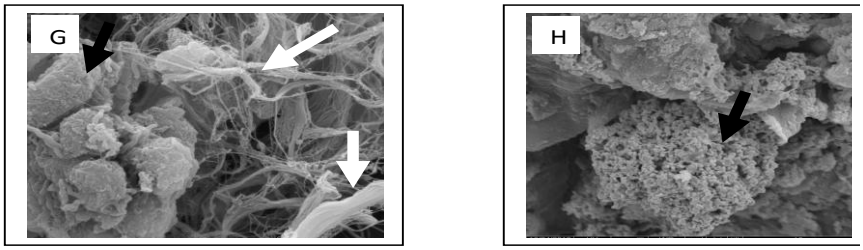

Figure 6. Fibrous mass growed arround biofilm from membran mucous
G: fiber mass observed; H: intestinal membran mucous from non-treated animal test, fibrous mass was not produced, appear microbial collonization (black arrow).

DISCUSSION

Candida albicans biofilm formation in vivo in rat model system has been frequently reported on a variety of frequently implanted biomaterials, including urinary and vascular catheters, joint and voice prostheses, and ocular lenses (Ricicova *et al*., 2010; Nett *et al*., 2010). All these devices can serve as an ideal niche for biofilm structures, which can then become a potential systemic infection. However, intestinal mucous membran is also good habitat for *Candida* biofilm growth, which become a potential systemic distribution of toxic metabolites of *Candida* in other organs, although in the immunocompetent individu. This is the first article report about in vivo rat model system of *Candida* biofilm on intestinal mucous membran.

Based on the Table 1, the stool colonization are very diverse. *C. albicans* colonization in feces between day 14 and 21 in the whole animal samples showed a decrease in the number of *C. albicans* . On day 35 and day 42 in the overall feces sample of experimental

animals showed a decrease in the number of *C. albicans*. Therefore surgery was done on day 42.

Decrease in faecal colonization data caused by moving of *C. albicans* cells from feces to the intestinal epithelial cells. This was confirmed by the data of the intestinal colonization, increasing sharply with increasing time , along with a decrease in stool colonization (Table 2) .

C. albicans biofilm formation begins with the attachment of yeast cells on a solid surface to form microcolonies, which further develops and produces 3 -dimensional structure composed of yeast cells, budding yeast , hyphae and extracellular matrix (Verstrepen *et al*., 2006) . In this study, the results of SEM biofilm formed on day 7 after taking mice with *C. albicans* inoculum (Figure 5). The image appears biofilm characteristics which include yeast cells, budding yeast, hyphae and extracellular matrix. Biofilm structure on next days become thicker which the same characteristics except the fibrous mass formed on days 35, 42 (Figure 5) and 49 (Figure 6).

According to SEM data in Figure 5 E and F, the fibrous structure appears around the biofilm. Comparing with SEM control data from non-treated animal mucous membran (Figure 6), the fibrous mass was not detected. The fibrous structure probably derived from the polymerization of globular protein contained in the mucous membrane, like happened in the other organ disorder as amyloid protein. The fibrous protein need to be isolated and molecular proteomic characterized in the next works.

CONCLUSIONS

Model of *C. albicans* biofilms in the intestinal mucous membranes was successfully established in the small intestine and cecum of Rattus novergicus, Wistar, after treatment with corticosteroids, antibiotics and *Candida* albicans inoculum, with a start time of the formation of day 7, characterized by the presence of budding yeast and the extracellular matrix. On day 28 appears hyphae, and on day 35 began to appear around the fibrous mass of the biofilm, which further thickens on days 42 and 49.

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