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Determination of OD Value and CFU Dilution for Modeling the Infection of Vulvovaginal Candidiasis on Experimental Mice

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Abstract

Vulvovaginal candidiasis (VVC) is also sometimes called a yeast infection, and it occurs when there is overgrowth of the normal yeast in the vagina. This infection is relatively common nearly 75% of all adult women have had at least one "yeast infection" in their lifetime and harmful for their immune system.

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To measure the number of colonies of Candida albicans in the treated mice induced by Candida albicans derived from clinical strain BALB/c at various clinical dosages in CFU dilution and to determine the optic density (OD) value in blood serums of the treated mice. Three mice clinical strain BALB/c, 18-12 weeks, weight 25-30 gr, were randomly selected and Candida albicans were transvaginally induced to the treated mice with 10^1µl, 10^2 µg and 10^3µg cilincal dosages respectively; and further were observed for 7 days. The antibody testing method of anti Candida albicans was ELISA Kit using Abcam ab53891 strain stock. The number of colonies of Candida albicans cultured in CFU dilution were 10^{-1}, 10^{-2}, 10^{-3},10^{-4} consecutively. Induction of Candida albicans at 10^2 µ clinical dosages in transvaginal area of the treated mice with the 10^{-3} colonies of Candida albicans in CFU dilution showed the increase of OD value compared to other dosages. Determination of the precise Candida albicans clinical dosage was significantly considered important to test the efficacy of both synthetic and natural medicines for therapy of Vulvovaginal Candidiasis.

Keywords: Vulvovaginal Candidiasis; Optic density (OD); CFU (Colony forming Units).

1. Introduction

Vulvovaginal Candidiasis (VVC) is an infection of the vaginal mucous membranes by *Candida albicans*, infected women sometimes whimper the presence of white and creamy and curd in appearance on their vagina along with dermatitis of vulva, vaginal itching and putrid odor [1]. Vulvovaginal Candidiasis is common in reproductive women (15-44 years) and approximately 70-75% of women are affected by this infection at least once in their lives, and nearly 50% of adult women will experience a second case [2]. Moreover, Candida albicans are also found in diabetes milletius patients and pregnant women [3]. Until now, data of infection spread of Vulvovaginal Candidiasis among infected individuals are not precisely identified yet since its clinical symptoms are not specific and clinical incidences of this infection are continuously invasive for more three decades ago due to late diagnoses [4] and inadequate classification of anti fungal medicines [5]. *Candida albicans* shows normal flora in 80% of healthy people, but they may become pathogenic [6] when this pathogen fungus is predisposed by several triggering factors causing Vulvovaginal Candidiasis, particularly in immunocompromised individuals [7]. *Candida albicans* is capable of a yeast-to-hyphal-phase transition (dimorphic transition); it is an important virulence factor, the only pathogenic form is the hyphal form [8]. Besides that, the use of corticosteroid may support the infection of this fungi [9]. Predisposition factors associated with the infection of *Candida albicans* are the alteration of vaginal pH level, obesity, sweat and chronic diseases and immunologic impairments as well as heat condition and moist, skin neatness, daily behavior and contact with infected individuals [10]. The infection of *Candida albicans* are associated with the alteration of Candida cells, from yeast become mycelium that has structure like roots which called rhizoid that penetrate skin mucous in mouth and vaginal area and epithelial cells in digestive tract, blood vessels causing septicemia [11]. This study used parameters of serums of anti *Candida albicans* using ELISA Kit testing method to measure the OD value in blood serums of the treated mice and determination of fungal culture to measure the number of colonies (CFU) of *Candida albicans*. Specific understandings of etiology of Candidiasis Vulvovaginalis is considered important to develop various medicines to test the efficacy of both chemical and natural medicines for therapy of Vulvovaginal Candidiasis.

2. Materials and Method

The study was conducted at the Microbiology Laboratory of Biomolecular and Immunology Department of Medicine Faculty, Hasanuddin University, Makassar. The duration of experimental procedure was 21 days.

2.1. Drug

The yeast strain of *Candida albicans* used in this study was Abcam *ab53891* strain which was diluted in 100% HFIP (*1,1,1,3,3,3-hexafluoro-2-propanol*) with 1 mg/ml concentration. The resulted solution was then incubated in room temperature for 1 hour while stirring the solution adequately. Further, the solution was vibrated for 10 minutes in sonicator (water tube). Peptide solution/ HFIP were dried under nitrogen gas flow. 100 DMSO was used to mix the peptide solution. Subsequently the solution was incubated at room temperature for 12 hours while stirring intermittently. The finished solution was then separated into sub-volumes and was stored at -80°C temperature. 500-100 µl *D-PBS* as adding solution (depending upon the last concentration) was put into the peptide broth stock and was then incubated in room temperature to avoid the agglutination of the peptide stock.

2.2. Experimental mice and induction of Candida albicans

Three mice Balb/c strain were raised for 1 week in a cage to adapt with laboratory condition for 1 week at room temperature (25°C ± 2°), both dark and light cycles (12/12 hour), and those treated mice were given pellets and drinking water *ad libitum* according to the method designed by [12]. Selection of the treated mice was conducted randomly, and after the adaptive duration was ended, modeling the Candidiasis Vulvovaginalis infection to the experimental mice was made by placed the treated mice into the stereotaxic apparatus. Afterward, *Candida albicans* were transvaginally induced to the treated mice with 10^1µl, 10^2 µg and 10^3µg clinical dosages respectively, and then was observed for 14 days. Weighing and blood sampling of the treated mice were carried out at 0,1,2,5 and 7 days consecutively. Weighing the treated mice used Triple Beam Balance, HOUS and blood sampling was done at the tails of the treated mice Balb/c strain at 0.2 ml volume. Collecting the treated mice blood was done five times, the blood samples were centrifuged to obtain blood serum. The blood serums were put into the sterile tubes and were placed in freezer at 20°C until the measurement of OD (optic density) was done. Vaginal swabs were done at 0,1,2,5 and 7 respectively and then gram staining was conducted to observe the accumulative assessment of CFU (colony forming units) dilution.

2.3. Testing procedure of OD (optic density) value

Candida albicans serums of the samples were reacted with antibodies of anti-serum of *Candida albicans* which being absorbed on the surface of microtiters polystyrene on wells of microplates. After washing proteins, antibodies of anti-serum of *Candida albicans* were conjugated by adding horseradish peroxidase (HRP). Antibodies that labeled enzymes formed complex bindings of *Candida albicans* serums, and the washing was continued, the number of enzymes the complex bindings were measured by adding chromogenic 3,3',5,5'-tetramethylbenzidine (TMB*)* as the substrate. Quantity of enzyme bindings was proportionally varied with the concentrations of serums of *Candida albicans* of the tested samples. Atomic absorption at a 450 nm wavelength was determined in measuring the concentration of serums of *Candida albicans* of the testes samples.

Interpolarization of the quantity of serums of *Candida albicans* of the tested samples was made on the standard curves which being constructed and corrected for dilution of the tested samples [13].

In sum, the following procedure in measuring the serums of anti *Candida albicans* using ELISA Kit-ab533891 was as follows:

1) Removed precisely antibodies lining the wells of microplates, all reagents were equilibrated at room temperature. Provided all reagents, the tested samples or standard samples.
2) Added the tested samples or standard samples at each well of the microplates, and then incubated the tested samples in room temperature.
3) Aspired and washed each well of the microplate, and then added HRP previously prepared and was labeled using the second antibody detector. Incubated each sample in room temperature.
4) Aspired and washed each well of the microplate, and then added chromogen substrate into each plate. Further observed and noted the alteration of color of the tested samples.

2.4. Preparation of the samples

In general, the measurement for quantification of serums of *Candida albicans* of each sample was diluted prior to be used. For each phase of determination of 1/1,000 dilution was considered exact for part of serum/plasma samples. For absolute quantification of the measured samples was determined based on their distance out of the standard curve, which mean more or less of the desired measurement value. When levels of samples were doubted, dilution series with one or two prior representative samples were scuttled with the recommended samples. For preparation of 1/1000 dilution of the tested samples, took 5 µL of the sample to 496 µL once of each diluents. Here, 1/100 dilution was determined. Further, dilution of 1/100 of the sample from the removal of 30 µL until reached 270 µL of each dilution. 1/1,000 dilution should be gained from the samples, and the mixed precisely of each procedure.

2.5. Measurement procedure

All materials were equaled and provided reagents at room temperature prior to use. It is recommended to measure the whole standard, control and samples of the duplication

1) Pipetting 100 µL of each standard solution, including zero control in duplication into the prepared wells.
2) Pipetting 100 µL samples (in duplication) into the prepared wells.
3) Incubating the plates of microtitter at room temperature for 60 (60 ± 2) minutes. Closing the plates during incubation time.
4) Continuing the incubation, aspiring the materials from the wells.
5) Content of the well was diluted once with wash buffer precisely and the sample was aspirated. Repeating dilution three times and wash buffer was done four times. If manual procedure of the wash buffer was used, completing the filling up of each well using wash buffer, and then plates were reversed side and then poured the content into a washing container. Continued this process on the wells

carefully on the absorbing papers to remove residual buffer. Repeating the process three times for four washes.

6) Pipeting once precisely 100 µL conjugate of enzyme-antibodies into each well. Incubating in room temperature for 30 minutes (30 ± 2). Protecting the plates using the cover in dark period during incubation.
7) Washing residual stains on the well.
8) Pipeting 100 µL TMB substrate solution into each well.
9) Incubating in dark period at room temperature for 10 minutes.
10) Adding 100 µL stop solution on each well
11) Determining the atomic absorption of the samples at a 450 nm wavelength.
12) Calibrating the plate reader for its specification.

2.6. Measurement

Reading the duplication standard was equal for each standard, sample and blank control. Blank control of the average results was reduced. The previous determination of the average resulted standard showed the contrary concentrations, and reversed curves was made cutting into plots to make a standard curve. Part of software of the plate reader or graph was determined in the form the exact values and bend curves. The equation consisting of four parameters of algorithm (4PL) is fair. Other equations can be used to determine the accuracy of the measurement (linear, semi-log, log/log, 4-parameter logistic). It is important to consider protein concentrations for unknown sample concentration. Sample control of the standard curve should be considered precisely. Samples revealed signals more than the standard value and the subsequent dilution was conducted in one incubation buffer and reanalysis was done for each sample. Further duplicated the resulted concentration using exact dilution factor.

2.7. Procedure of CFU (colony forming units) determination

Determination of the number of colonies used the former staining method of the culture [14]. The procedure was as follows. Vaginal swabs were diluted and cultured on the disc glasses for 24-48 hours making the growth of *Candida albicans* showed well reproduction and forming colonies using a dilution method with direct eyesight measurement. The number of microbial populations was determined by multiplying the number of colonies with dilution factor on disc glasses and used colony forming units abbreviated in cfu/mL.

3. Results

The effects of *Candida albicans* induction based on the measurement of OD (optic dnsity) values using ELISA Kit as the method to test serums of anti *Candida albicans* were shown in Table 1 which showing the data of prior incubation of *Candida albicans* at 0 day (abbreviated in S0) and later induction of *Candida albicans* was done at the next day, first day after injection/24 hour post induction (abbreviated in SI), at the second day (abbreviated in S2) and the last day of the seventh day (abbreviated in S3) as the last week. On the other hand, the first and the second code showed the treated mice at various clinical dosages, as stated the following:

A1 to A3 : A1 (yellow code showed head area = 10^1 µg), A2 (yellow code showed back area = 10^2 µg), A3 (yellow code showed tail area = 10^3 µg)

Tabel 1: Measurement results of OD values in serums of *Candida albicans*

Treated Mice	***Serum of Candida albicans***					
	1	*2*	*3*	*4*	*5*	*6*
SO (A)	*0,92*	*0,91*	*0,97*	*0,96*	*0,95*	*0,95*
S1 (B)	*1,20*	*1,20*	*1,31*	*1,26*	*1,25*	*1,25*
S2 (C)	*1,35*	*1,35*	*1,39*	*1,40*	*1,45*	*1,45*
S3 (D)	*1,38*	*1,36*	*1,48*	*1,48*	*1,55*	*1,55*
Blank (F)						
Blank (G)						
Blank (H)						

Table 1 also showed the results of duplication for each clinical dosage and each dosage was counted based on the mean value of each dosage as given in the Table 2.

Table 2: The mean value of measurement results of OD values of *Candida albicans* of the transvaginal areas of the treated mice

Treated Mice	***The mean value of OD level using Elisa Kit***		
	1	*2*	*3*
SO (A)	*0,920*	*0,970*	*0,954*
S1 (B)	*1,205*	*1,292*	*1,253*
S2 (C)	*1,355*	*1,403*	*1,455*
S3 (D)	*1,377*	*1,484*	*1,553*

Table 2 showed the significant increase of the induction values of *Candida albicans* at the transvaginal area of the treated mice at dosage = 10^2 µg. To verify this result precisely, this figure could be compared to the Figure

1.

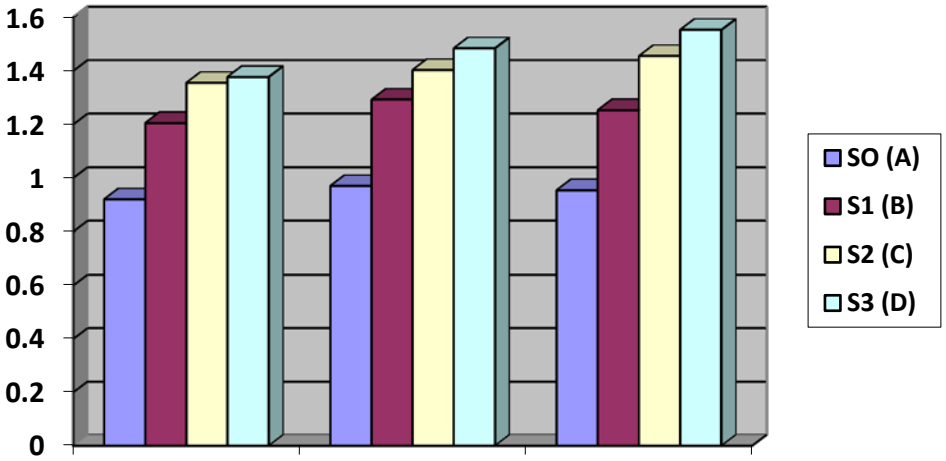

Figure 1: Diagram of OD values of *Candida Albicans*

Results of the analysis of CFU dilution in vaginal swabs, as seen in the following figures.

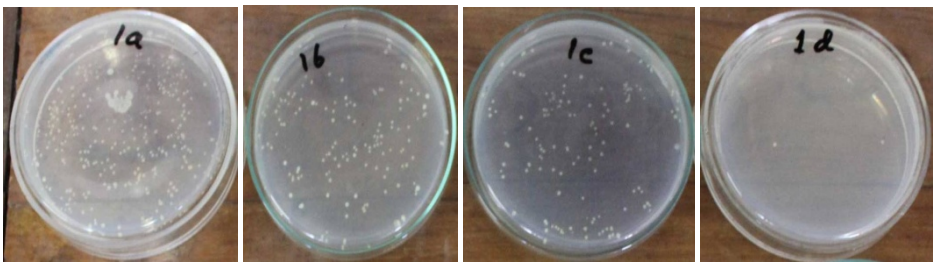

Figure 1 (10-1) **Figure 2** (10-2) **Figure 3** (10-3) **Figure 4** (10-4)

Figures 1, 2, 3 and 4 showed 10^{-1} µg, 10^{-2} µg, 10^{-3} µg and 10^{-4} µg clinical dosages respectively. Results of the test revealed the number of measured colonies for 10^{-3} µg dilution was given in figure 3, whereas, both 10^{-1}µg and 10^{-2} dilutions were given in Figure 1 and Figure 2 respectively where both of them were difficult to count, and Figure 4 showed no colonies of *Candida albicans.*

4. Discussion

Reduced acidity level in genitalial areas of women was the main factor that affect the immune system and the current fungal therapy is limited [3]. Infection of *Candida albicans* is categorized both acute and subacute, besides it may infect vaginal area, it infects also the mouth mucosa, sin, nail, bronchi or lung, causing septikemia, endocarditis or meningitis [15]. *Candida. albicans* has complex and dynamic cell wall, each cell wall is 100-400 nm in thickness. According to Segal & Bavin , a cell wall of *C. albicans* consists of five

different layers. Overgrowth of *Candida albicans* 'sometimes occur due to the use of toilet consisting many Candida spp after defecation, polluted nails or water while bathing in toilet [16]. In normal condition, normal pH could be maintained by vaginal bacteria, but the reduced acidity or pH is considered as the main predisposition of Vulvovaginitis Candida [7].

Our investigation revealed that induction of *Candida albicans* at transvaginal area in mice BALB/c strain could induced the symptoms of Vulvovaginitis Candida. Former studies showed the incidence of leucorrhea and itching.

Previous study conducted by Wahyuningsih [17] showed that anti *Candida albicans* was formed 48 hours after induction of *Candida albicans* at transvaginal area with the following procedure identification. To select several colonies in separated growth, those colonies were suspended into 1 mL aquadest with 10^5 sel/ml concentration, suspended culture was determined in chromogenic media, viz., Chrom-agar Candida (CAC) and the culture was covered with aluminum foil to avoid light effect, and then the culture was incubated at 35-37^{o}C temperature for 48 hours, species was analyzed based on the color of growth colonies. Molecules of *Candida albicans* colonies was then identified using *Direct Colony PCR* [18]. Several theories and references state that specific characteristics of *Candida albicans* effects, viz., lesion on vagina with the presence of white and creamy and curd in appearance near the vaginal area [19].

5. Recommendations

Initial symptoms of the infection as proved in our study showed that *Candida albicans* could detected at the second day of the vaginal area. Culture method in analyzing the number of colonies revealed that cognitive ability of the treated mice *BALB/c* strain showed damaged vagina and chronic inflammation occurred, even at the next phase due to the decreased immune system. On the whole, results of our investigation showed faster physiological alteration of the treated mice at the fifth day that pave a way to do advanced studies to test the efficacy of both synthetic and natural medicines for therapy of Vulvovaginal Candidiasis.

6. Conclusion

These data suggest that antibodies are not readily present in vaginal secretions of infected mice and thus have a limited natural protective role against infection. Determination of the precise Candida albicans clinical dosage was significantly considered important to test the efficacy of both synthetic and natural medicines for therapy of Vulvovaginal Candidiasis.

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