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Correlation between Secreted Aspartyl Protease-5 (SAP5) on Increasing Vaginal Acidity in Vulvovaginal Candidiasis

Nur Sophia Matin[1], Regina Ayu Fristiyanti[1*], Safrina Dewi Ratnaningrum[2], Nurdiana Nurdiana[3], Sanarto Santoso[4], Bambang Rahardjo[5]
[1] Magister of Midwifery, Faculty of Medicine, Universitas Brawijaya, Malang, East Java, Indonesia
[2] Department of Anatomy Histology, Faculty of Medicine, Universitas Brawijaya, Malang, East Java, Indonesia
[3] Department of Pharmacology, Faculty of Medicine, Universitas Brawijaya, Malang, East Java, Indonesia
[4] Department of Clinical Microbiology, Faculty of Medicine, Universitas Brawijaya, Malang, East Java, Indonesia
[5] Department of Obstetrics and Gynaecology, Saiful Anwar General Hospital, Faculty of Medicine, Universitas Brawijaya, Malang, East Java, Indonesia

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CORRESPONDING AUTHOR*
Regina Ayu Fristiyanti
reginaafristy@gmail.com
Master Program of Midwifery, Faculty of Medicine, Universitas Brawijaya, Malang, East Java, Indonesia

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ABSTRACT

Introduction: Vulvovaginal candidiasis (VVC) is an infection of the vaginal mucosa that extends to the vulva due to excessive growth of *Candida albicans*, causing a progressive decrease of *Lactobacillus spp.*, as the main of normal flora. Decrease in the number of *Lactobacillus spp.* causes a reduction of lactic acid production that it was causing increasing vaginal pH. The increase in pH is due to the progressive growth of *C. albicans* colonies in the vaginal mucosa. In addition, the mechanism of candidiasis is also influenced by the secretion of virulence factors by *C. albicans*. *Secreted Aspartyl Protease*-5 (SAP5) is the dominant virulence factor that plays a role in changing the morphology of yeast cells into hyphae. This study aims to identify the correlation between SAP5 levels and vaginal pH.

Material and Methods: This research was conducted in vivo using a sample of *Rattus norvegicus* with a posttest randomized experimental group design approach. This study has two groups, i.e., Negative Control (NC) and Positive Control group (PC). SAP5 and pH samples were taken from vaginal fluids and then measured using ELISA and a digital pH meter. The statistical analysis used was Pearson Correlation to analyze the correlation between SAP5 and pH levels.

Results: There was a strong correlation (R=0.846) that means the increase of SAP5 is related to the increase of vaginal acidity in the PC group.

Conclusion: The increase in SAP5 levels is directly followed by an increase in the pH value of vaginal fluids.

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INTRODUCTION

Reproductive infection is one of the global health problems experienced by people in developing countries. WHO states that every year there are 357 million new cases of reproductive infections and sexually transmitted infections experienced by most women of childbearing age [1]. VVC is one of three types of reproductive tract infections in women of childbearing age with a high prevalence rate along with Bacterial Vaginosis (BV) and Trichomoniasis Trichomoniasis [2,3]. It is estimated that 70% of women of childbearing age experience VVC at least once in their lifetime, while the other 50% can experience VVC multiple times.

Based on the identification of demographic factors, 25-29 years is the age group that is most vulnerable to VVC [4]. Various predisposing factors increase the risk of VVC in women, including changes in fluctuations in the estrogen and progesterone hormones during pregnancy, menstruation, and while taking hormonal contraceptive drugs. Increased estrogen directly increases the virulence of the fungus by inducing the ability of *C. albicans* to avoid resistance from the immune system [5]. Meanwhile, progesterone contributes to increased blood glucose in the body, thereby triggering

high levels of nutrition for the growth of *C. albicans* [6]. Other predisposing factors can include having a degenerative disease such as diabetes, having a disease that suppresses immunity such as HIC/AIDs, and using broad-spectrum antibiotics and corticosteroid agents in the long term [4,7].

Based on epidemiological studies, it is stated that *C. albicans* cause 70 – 89% of VVC cases. In comparison, while another 10% of VVC cases can be caused by species other than *C. albicans*, such as *Candida glabrata*, *Candida tropicalis*, and *Candida krusei* [8]. VVC due to *C. albicans* growth generally can be asymptomatic and symptomatic. As commensal organisms that exist in the body with a percentage of 20-50%, *C. albicans* colonies can generally be found in the vaginal lumen without causing symptoms (asymptomatic). Symptomatic symptoms such as itching, burning sensation, presence of vaginal discharge, itching, irritation, and unpleasant abnormal secretions like a thick cottage cheese will occur when *C. albicans* colonies grow excessively, invade the epithelium, and secrete virulence factors that further support colony growth [9].

The occurrence of VVC is often associated with an imbalance between *C. albicans* colonies as an opportunistic pathogen and *Lactobacillus* spp colonies as the main normal flora in the vaginal mucosa. A healthy vaginal condition is characterized by an acidic vaginal pH <4.5. The acidity of vaginal pH occurs because the vagina epithelia are dominated by lactic acid bacteria, especially *Lactobacillus* species. The high number of *Lactobacillu*s is a natural defense in the vaginal environment that protects against bacteria and other pathogenic microorganisms. Decreasing the number of *Lactobacillus* will distort the conditions of microbiota homeostasis and facilitate the growth of pathogenic organisms [3,10].

In VVC, where the amount of *Lactobacillus* spp colonies has progressively decreased due to the dominance of *C. albicans*, it causes a decrease in lactic acid production, so VVC often experiences an increase in vaginal pH to become alkaline. Alkaline vaginal pH facilitates the progressive growth of pathogenic microorganisms. *C. albicans* was reported to neutralize the environment from acidic to alkaline pH. Under alkaline conditions, *C. albicans* can raise the pH from 4 to >7 in less than 12 hours, resulting in autoinduction of the transition from yeast to hyphae characterized by the secretion of certain virulence factors [11].

Besides pH, virulence factors secreted by *C. albicans* have an important role in the mechanism of candidiasis. There are several virulence factors for *C. albicans*, including phenotypic transitions, morphological dimorphism, thigmotropism, adhesion proteins, secretion of hydrolase enzymes, and biofilm formation [12]. The three main hydrolase enzymes secreted by *C. albicans* include Secreted Aspartyl Protease (SAP), phospholipase, and hemolysin [13,14]. Secreted Aspartyl Protease (SAP) produced by *C. albicans* is the main invasive protease enzyme and has an important role in the pathogenesis of *C. albicans* [15]. It was stated that SAP levels correlates with the development of vaginal candidiasis because they are involved in causing tissue damage, escaping from the immune system, both innate and adaptive [16]. In addition, the morphological changes from yeast cells to hyphae form cause damage to host tissue.

The transition from yeast cell form to hyphae is not only in the form of morphological changes, but there is a systematic process involving genetic processes in *C. albicans* cells. According to the coding gene, Secreted Aspartyl Protease (SAP) produced by *C. albicans* is classified into ten types. Each SAP has a specific role in the pathogenesis of the infection. SAP5 is the most frequently associated virulence factor with the onset of mucosal candidiasis immunopathology. The increased SAP5 levels reflect an important finding indicating tissue damage due to *C. albicans*' invasion. Based on this explanation, it is known that the mechanism for the occurrence of VVC is influenced by factors in the environment, host, and agent. Environmental factors were hormonal changes, factors present in the host's body, such as pH, and those in *C. albicans,* such as the virulence factor SAP5. This study aims to assess the correlation between SAP5 levels and pH in VVC female rats (*Rattus norvegicus*). There are limited studies that discuss the correlation between SAP5 level and vaginal pH. Even though SAP5 is one of the important virulence factors secreted by *C. albica*ns, SAP5 is a potential therapeutic target in candidiasis. In addition, vaginal pH is a strong predisposing factor that facilitates the growth of *C. albicans.*

MATERIAL AND METHODS

Experimental Protocol

This research is a true experiment with a posttest randomized experimental group design approach conducted in the laboratory in vivo. The sample in this study was female rats (*Rattus norvegicus*) of the Wistar strain. Before being used as research samples, rats were selected based on the inclusion and exclusion criteria, such as with ages 8-10 weeks and an average body weight of 90-110 grams. In addition, they must be in a healthy condition characterized by active movements, clear eyes, and thick white fur. The rats that had previously received chemical intake were disabled or died during the study and had changes in behavior, such as refusing to eat and being inactive, were excluded from this study. After being selected based on inclusion and exclusion criteria, thirty-two female rats were acclimatized for 7 days at the Experimental Animal Research Laboratory, Faculty of Medicine, Universitas

Brawijaya. Acclimatization aims to allow rats to adapt to the laboratory environment. After acclimatization, the rats were divided into two groups i.e., the negative control group and the positive control group. The negative control contains female rats that did not receive any intervention. Meanwhile, in the positive control group, female rats were made into a VVC model. In each group, there were 16 rats placed in 8 standard-size cages. Food and drink are provided ad libitum. Rats were set under room light with a light 12-hour cycle at 06.00 – 18.00 WIB and a dark 12-hour cycle at 18.00 – 06.00 WIB while the temperature and humidity of the room were set at a temperature of 27 – 28^{0}C.

The procedure for creating the VVC model in this study refers to the "Animal Models for Candidiasis" procedure [17]. On the 8th day, the positive control and six treatment groups were injected with 0.5 mg estradiol valerate dissolved in 100 µl sesame oil to induce pseudo estrus. On the 13th day, a vaginal smear was performed using a sterile cotton swab. After three rounds, the samples were placed on glass slides and allowed to dry. Then the sample was fixed using a bunsen burner, followed by Methylene Blue staining, and observed under a microscope. Isolate of *C. albicans* obtained from clinical specimens of a patient with vaginal candidiasis were sent to the Department of Microbiology, Faculty of Medicine, Universitas Brawijaya.

The isolate was identified with some microbiological procedures such as culture in *Saboraud Dextrose Agar* (SDA), Gram staining, potassium hydroxide test, and germinating tube test to analyze the suitability of *C. albicans* characteristics. For use in the experiment, *C. albicans* cells were cultured in SDA medium overnight before use and then diluted NaCl. Density was measured using a spectrophotometer with a wavelength of 520 λm. Pure *C. albicans* colony was isolated using a sterile inoculating loop and combined with 5 ml of 0.9% NaCl. The absorbance of the suspension was determined using spectrophotometry with a wavelength of 520 nm. On the 12th day, the positive control group was inoculated with *C. albicans* 2x10^7 CFU suspension dissolved in 0.02 ml PBS via the intravaginal route. Meanwhile, the negative control group was only inoculated with 0.02 ml of PBS. Before inoculation, the rats were anesthetized with 100 mg/kg BW of ketamine hydrochloride. On the 14th day, the establishment of *C. albicans* inoculation was confirmed by performing a vaginal lavage technique to collect 50 µl of vaginal fluid from the mice, then cultured in SDA medium and Giemsa stained. After the culture results in SDA media and Giemsa staining showed the characteristics of the *C. albicans* isolate, vaginal fluid was collected by vaginal lavage on the 15th day.

On the 15th day, the rats were anesthetized with 100 mg/kg BW of ketamine hydrochloride. Anesthesia was carried out to minimize the movement of the rats so vaginal lavage could be carried out optimally. 2 ml of vaginal fluid from each rat was placed in an Eppendorf tube to be used as a sample for measuring SAP5 and pH levels using ELISA and a digital pH meter.

Statistical Data Analysis

SAP5 and pH levels were analyzed using SPSS 21 software. Before being tested with the Pearson Correlation test to analyze the correlation between SAP5 levels and pH, the data was confirmed to fulfill the parameter requirements test using the Shapiro-Wilk test.

Ethics

All procedures in this study received ethical approval from the Ethical Commission of the Faculty of Medicine, Universitas Brawijaya, with an Ethical Approval Letter number 34/ EC/KEPK–S2/02/2023.

RESULTS

Table 1 is primary data from the measurements of SAP5 and pH levels in each subject in the negative control group (n=16) and the positive control group (n=16). The results of measurements of SAP5 levels showed that the group of mice that were inoculated with *C. albicans* intravaginally had a higher increase in SAP5 levels (mean=13.63 ng/ml) compared to the group that was not inoculated with *C. albicans* (mean=0.261 ng/ml). From the results of pH measurements, it can be seen that the pH in the group of mice that were inoculated with *C. albicans* intravaginally had an average pH of 6.5. In contrast, the pH in the group of mice that were not inoculated with *C. albicans* intravaginally had an average pH of 8.3. Based on the result of SAP5 and pH measurement, the subject in the negative control group showed that vaginal acidity did not exceed the neutral range of vaginal acidity. In the positive control group, vaginal acidity exceeded the neutral range of vaginal acidity.

Based on the data normality test using the Shapiro Wilk test (n < 50) as a parametric test requirement, the primary data for the negative control group and the positive control group in Table 2 show p-values (0.546 and 0.875> 0.05), which means that the data distribution is normal. Findings of normal data distribution fulfill the requirements for the Pearson Correlation test. The results of the Pearson Correlation test for SAP5 levels at the pH of the negative control group and the positive control group showed a value of R = 0.546 and 0.875 > 0.5, which means that the correlation between SAP5 levels and pH in the negative control group showed a relatively low correlation. Whereas in the positive control group, SAP5 levels showed a high correlation with pH.

Table 1. SAP5 Levels and pH Measurement in Each Subject of Negative and Positive Control Groups

Group	Subjects	SAP5 (ng/ml)	pH
Negative Control (NC)	1	0,145	5,8
	2	0,269	5,9
	3	0,331	7
	4	0,179	6,8
	5	0,315	7,2
	6	0,178	6,9
	7	0,193	6,2
	8	0,211	5,9
	9	0,245	6,5
	10	0,298	7,1
	11	0,367	7,1
	12	0,389	7,2
	13	0,294	6,3
	14	0,344	6,4
	15	0,225	6,8
	16	0,197	6,4
Mean		0,261	6,5
Positive Control (PC)	1	14,289	8,5
	2	13,668	8,1
	3	17,639	8,7
	4	16,252	8,6
	5	12,965	8,4
	6	12,582	8,2
	7	12,965	8,2
	8	11,895	8,2
	9	12,667	8,3
	10	11,906	7,8
	11	11,625	7,9
	12	16,407	8,6
	13	12,582	8,1
	14	15,895	8,5
	15	12,889	8,2
	16	11,885	7,9
Mean		13,63	8,3

DISCUSSION

Secreted Aspartyl Protease (SAP) is a protein that has an important role in maintaining the fungus's life in the host's body. In the case of mycosis in humans, SAP production is often associated with the presence of *C. albicans, C. parapsilosis*, and *C. tropicalis*. SAP secretion is related to genetic activity in fungal cells. Among SAP4-6, only SAP5 plays a role in tissue colonization, penetration, and invasion [18,19]. Secreted Aspartyl Protease 5 (SAP5) has an important role in the pathophysiology of infection in both mucosal candidiasis and candidemia because it is identified as the most dominant virulence factor expressed by hyphae. Hyphae become an important morphology in dimorphic fungal species that cause damage to mucosal tissue as an initial stage of the emergence of an immunopathological response.

The presence of SAP5 plays an important role in supporting dimorphic properties in *C. albicans* because SAP5 is involved in the mechanism of adhesion, colonization, invasion, and tissue damage by *C. albicans* [20]. One of the dominant virulence factors expressed when there is a morphological change from yeast to hyphae is SAP5. Then. SAP5 specifically facilitates the adhesion of *C. albicans* to the mucosa along with the ALS3 protein, predominantly involved in the transition mechanism from yeast cells to hyphae and invasion of tissues [21,22]. Based on the results of measurements of SAP5 levels (Table 1), the group of mice that were

Table 2. Pearson Correlation

		pH
SAP5 Negative Control Group	Pearson Correlation	0.546*
	Sig. (2-tailed)	0.029
	N	16
SAP5 Positive Control Group	Pearson Correlation	0.875**
	Sig. (2-tailed)	0.000
	N	16

*Correlation is significant at the 0.05 level (2-tailed)
**Correlation is significant at the 0.01 level (2-tailed)

inoculated with *C. albicans* (PC) showed higher levels of SAP5 than the group of mice that were not inoculated with *C. albicans* (NC). The PC and NC groups experienced various increases in SAP5 levels, but the increase in the PC group showed a relatively higher increase compared to NC. The aspartate protease enzyme is one type of the endopeptidase enzyme group typically found in the tissues of prokaryotic, eukaryotic, plant, and animal organisms.

The aspartate protease enzyme plays a role in regulating the activity of various proteins, modulating protein interactions, and contributing to cellular information processes [23]. So, an increase in SAP5 levels in the NC group is physiological. Meanwhile, significantly increased levels of the aspartate protease enzyme in cases of candidiasis indicate a process of degradation of various host proteins, both cell membrane proteins [24,25]. In candidemia patients, vulvovaginal candidiasis and dental caries levels and expression of SAP5 can be identified. SAP5 is one of the important virulence factors in the pathogenesis of *C. albicans*. Levels of SAP5 are called an essential target in modeling potential antifungal agents [9].

Vaginal acidity (pH) is an important indicator for understanding vaginal physiology and the development of genital tract diseases. During the reproductive period, an increase in the hormone estrogen in the body triggers various anatomically, physiological, and biochemically changes in the body, including in the genital tract. The high estrogen hormone causes changes in the structure of the vaginal epithelium to become multi-layered, with the middle layer containing lots of glycogen [26]. The presence of *Lactobacillus spp*. with a relatively large number and dominating the composition of other normal flora gives an advantage to women. *Lactobacillus spp*. colonies, which are gram-positive bacteria capable of producing lactic acid through glycogen fermentation in the middle layer of the vaginal epithelium. So that the glycogen in this layer is a nutrient-rich compartment to support the natural life of *Lactobacillus spp*. and other diverse and dynamic normal flora.

Glycogen fermentation to produce lactic acid is related to the acidic pH's stability, which provides a protective effect on the urinary tract and genital tract against pathogens that cause infection. The vaginal pH of non-pregnant women under normal conditions is 3.8 – 4.5 [27]. Until now, vaginal adaptation has become a concern and makes pH regulation more complex than that of mammals. The difference in the composition of normal flora colonies in humans and mammals makes the pH of the human vagina more acidic, which under normal circumstances is 4.5, while the normal pH of mammals is, on average, 5.7 – 7.3 [28]. This significant difference is related to the dominance of Lactobacillus and high levels of glycogen in the human vagina. Conditions of the lower acidity in women's vaginal pH because generally, women are more at risk of experiencing genital infections throughout their life cycle due to longer periods of sexual intercourse, experiencing more complex menstrual periods, pregnancy, and postpartum to post menopause compared to mammals [29].

Refers to the research results, the level of SAP5 in the negative control group has a low correlation with pH. The aspartate protease enzyme is a type of endopeptidase enzyme group normally found in the tissues of prokaryotic, eukaryotic, plant, and animal organisms. aspartate protease plays a role in regulating the activity of various proteins, modulating protein interactions, and contributing to cellular information processes [23]. So an increase in SAP5 levels in the NC group is a physiological thing. In the negative control group, pH <7.3 indicates that the pH is neutral in that group. This is to research that the neutral pH value of rats is in the range of 5.8 – 7.3. Although there were differences in the vaginal pH value of the female and the rat, the rat tended to maintain a neutral pH value while the female pH value showed high acidity. This is due to differences in the microbiota composition in women and mice. In women, the normal flora in the vagina tends to be dominated by *Lactobacillus*, while in mice, it is dominated by *Streptococcus*. Although both are species of lactic acid bacteria, *Lactobacillus* has a higher potential to ferment glycogen in the vaginal compartment, so it is normal for there to be differences in lactic acid levels in the female and rat vaginal compartments. However, this does not make the VVC model studies using mice do not reflect the condition of VVC in humans. In mice and

humans, high estrogen levels are homologous conditions that trigger acute or chronic infections. In addition, the acidity of the pH in the female vagina is considered atypical, which reflects a condition of the pathogenicity of the infection because only women are mammal species that have an acidic pH value [30].

The positive control group that was inoculated with *C. albicans* showed an increase in SAP5 levels followed by an increase in pH> 7.3. This indicates that *C. albicans* inoculation in the positive control group increased levels of the aspartate protease enzyme in cases of candidiasis, indicating process of degradation of various host proteins, both cell membrane proteins [24,25]. High levels of SAP5 correlate with the level of membrane protein damage and the progressive growth of *C. albicans* colonies. This is consistent with the results of a study which stated that the dominance of *C. albicans* over lactic acid-producing normal flora such as *Lactobacillus spp*. and *Streptococcus* could increase the vaginal pH to be alkaline due to decreased lactic acid production. Increasing this pH value will further facilitate the growth of *C. albicans* to become more progressive, marked by increased levels of the proteolytic carboxylase enzyme, which triggers the sloughing of squamous cells, inflammation in the vaginal mucosa and the production of abnormal secretions in the vagina [31].

CONCLUSION

Increased levels of SAP5 in mice inoculated with *C. albicans* intravaginally had a high correlation with an increase in pH values above normal. The results of this study indicated that the high levels of SAP5 in the positive control group reflected the high damage to the rat vaginal epithelial tissue due to *C. albicans* hyphae invasion. In addition, high levels of SAP5 in vaginal fluid indicate an increase in the number of *C. albicans* colonies, thereby suppressing the number of lactic acid bacteria, reflected in an increase in pH into the alkaline range.

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CONFLICT OF INTEREST

The authors declare there is no conflict of interest regarding the publication of this article.

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