

Antimicrobial activities of human biofluids and their antimicrobial peptide fractions against *Candida albicans*

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ABSTRACT

It is known that human biological fluids contain antimicrobial protective factors that include antimicrobial peptides (AMP). Concentrations of AMP vary in different biofluids and tissues. The aim of the present study is to estimate the total antimicrobial activity of different human biofluids in comparison with the activity of their AMP fractions as markers of local immunity. The antimicrobial activities of serum (SE), vaginal fluid (VF), saliva (SA), water-soluble part of skin secretion (SS) and urine (UR) of 6 healthy women, 22-25 years old, were measured by the spectrophotometric method based on the ability of the above-mentioned biofluids to disrupt the cytoplasmic membrane of *Candida albicans* [Patent RF № 2602298]. The results were evaluated as the percent of dye penetrated into dead cells of treated yeast cell suspensions compared to the control ones. The medians of total and AMP activities are SE – 86.6% and 33.7%; VF – 24.8% and 21.9%; SA – 18.3% and 10.9%; SS – 12.8% and 2.8%; UR – 10.0% and 0.0%, respectively. The highest level of immune defense occurs in SA whereas the lowest in UR. The antimicrobial activity of AMP in VF is maximal compared to the other biofluids. Levels of total and AMP activities of SA, SS and UR

have high positive correlation between each other (Pirson's correlation coefficients $r \geq 0,700$), while none of them correlated with the activities of VF ($r \leq 0,300$). Using the most appropriate method of antimicrobial activity estimation the differences between biofluids of healthy human tissues of different localization are demonstrated for the first time.

KEYWORDS: antimicrobial peptides, human biofluids, *Candida albicans*.

ABBREVIATIONS

A	:	activity of specimen, %
AMP	:	antimicrobial peptides
OD _{control}	:	optical density of control supernatant
OD _{exp}	:	optical density of experimental supernatant
SA	:	saliva
SE	:	serum
UR	:	urine
VF	:	vaginal fluid

INTRODUCTION

Human biofluids contain immune defense factors such as complement proteins (CP), immunoglobulins (Ig) and antimicrobial peptides (AMP), all of them having a direct antimicrobial effect. While CP occur only in serum, Ig and AMP are found in

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serum and in all secretions of human body, for example vaginal and skin secretions, saliva, urine etc. [1-5]. The most common AMP include defensins, cathelicidins, secretory inhibitor of leucoprotease, lactoferrin, lysozyme, psoriasin, dermcidins, ribonuclease 7, calprotectin, histatin, hepcidin and lipocalin. Most of the CP and all Ig have a molecular mass above 150 kDa, but AMP are the family comprising of more than 20 peptide nature substances with mass up to 80 kDa. Concentrations of AMP vary in different biofluids and tissues. Recently we developed a useful method for estimating the AMP fraction activity in different human secretions [6]. The aim of the present study is the evaluation of total antimicrobial activity in different human biofluids in comparison with the activity of their AMP fractions as markers of local immunity, and estimation of a possible concordance of these parameters in different individuals.

MATERIALS AND METHODS

Volunteers for all experiments include 6 healthy women, 20-26 years old. Biofluids such as serum (SE), saliva (SA), urine (UR), vaginal fluid (VF) and water soluble part of skin secretion (SS) were investigated. All specimens were harvested in the morning prior to breakfast and any washing procedures except for SA which was obtained after tooth brushing and oral rinsing by water. Collection of VF was carried out by the following method: a tampon (OB Pro comfort) was inserted into the vagina for 10 minutes, and then the tampon was transferred to a plastic column of 15 x 75 mm and eluted with 7 ml of distilled water using a vacuum pump. Specimens of SS were collected from upper back skin site of 6 cm² as follows: a glass ring of 1 cm height and 24 cm diameter was placed on the skin, and the ring was filled with 3 ml of sterile distilled water. The skin inside the ring was intensively rubbed with plastic stick and the SS was collected along with the water. Every specimen from the different sites was filtered through the membrane filter Millipore having a pore diameter of 0.22 µ, freezed at -25 °C and then thawed out before the experiment, centrifuged for 5 min at 16000 rpm and the supernatant prior to the filtration was used for the further procedures (fraction "Total") or filtered

through a membrane of 100 kDa («Amicon ultra», Germany) (fraction "AMP").

Yeast strains used for all experiments were selected from the laboratory collection - *Candida albicans* № 927. Cultivation of yeast was carried out in Petri dishes with solid Sabouraud medium supplemented with antibiotics at 25 °C for 19 hours.

Estimation of antimicrobial activity was carried out by the following procedure: 50 µl suspension of test-culture *C. albicans* № 927 (final concentration of cells about 10⁸ CFU/ml) was combined with 400 µl of specimen in an Eppendorf tube. Control sample contained 400 µl of normal saline solution instead of the analyzed specimen. The mixture was incubated on a shaker at 32 °C for 2 hours, and after that centrifuged for 5 mins at 16000 rpm; the supernatant was removed and 300 µl of the dye 2 mM bromocresol purple in 1.25 M phosphate buffer (pH 4.6) was added and incubated again for 45 mins. After centrifugation 50 µl of the supernatant was dissolved in 2.5 ml of the same buffer and the optical density was measured at a wavelength of 440 nm using a spectrophotometer ("Genesys 10S UV-Vis", USA). These procedures were carried out three times.

The antimicrobial activity was calculated according to [7] using the following formula:

$$A = (OD_{\text{control}} - OD_{\text{exp}}) * 100 / OD_{\text{control}}$$

A - activity of specimen, %;

OD_{control} - optical density of control supernatant;

OD_{exp} - optical density of experimental supernatant.

Statistical evaluation of obtained results was carried out by parametric and nonparametric methods of 'MS Excel', and 'Statistica for Windows'.

RESULTS

The method of estimation of antimicrobial activity is based on the ability of CP, Ig and AMP to destroy the cytoplasmic membrane. As a result of such destruction the dye - bromocresol purple – inserts into the damaged *Candida* cells and its concentration in the incubation medium decreases. The control specimen of test-culture, in which majority of cells (70-80%) are alive, absorbs less

amount of dye than the experimental specimen, and hence the optical density in the control supernatant is always higher than that in the experimental one. The results of investigation of the activities of the different biofluids against *C. albicans* are presented in Table 1 and Figures 1 and 2.

Based on the obtained data one can see that SE demonstrated the maximal candidacidal effect compared to other biofluids in total fraction as well as in AMP fraction. However, unlike VF and SS, SE, SA and UR are native liquids because of the methods of their collection, whereas methods of VF and SS collection include the stage of elution (VF) or washing (SS) by distilled water. Therefore these biofluids become diluted compared to the natural secretions. Nevertheless SE is the most active among native liquids.

Comparison of the average values of the obtained parameters allows to estimate the percent of AMP activity in total activity against *C. albicans* (Table 1). Clearly, the maximal contribution of AMP is found in VF, and the minimal in UR. Medians of the parameters show even more impressive data (Figure 2): contribution of AMP in total activity of UR is equal to zero. Correlations between antimicrobial activities of different biofluids from 6 healthy volunteers are shown in Table 2. Levels of total activities of SS, UR and SA have high positive correlation between each other ($r \geq 0,700$), while none of them correlated with the activities of VF ($r \leq 0,300$). The same situation occurs with AMP activities.

DISCUSSION

Antimicrobial activities of five human biofluids, including activities of their AMP fractions, against *C. albicans* were investigated for the first time. Traditionally studied AMP are defensins, cathelicidins, secretory inhibitor of leucoprotease, lactoferrin, lysozyme, psoriasin, dermcidins, ribonuclease 7, calprotectin, histatin, hepcidin, and lipocalin. Some of the AMP occurred in all the mentioned biofluids: these are defensins, cathelicidins, secretory inhibitor of leucoprotease, lactoferrin, lysozyme, psoriasin, and lipocalin. Besides, the average concentrations of the prevailing AMP in the different biofluids are as follows:

- for SE – lysozyme - about 3 $\mu\text{g/ml}$ [8] and calprotectin - 3 $\mu\text{g/ml}$ [9];
- for VF – lysozyme - 13 $\mu\text{g/ml}$, lactoferrin - 8 $\mu\text{g/ml}$ and calprotectin - 34 $\mu\text{g/ml}$ [10];
- for SA – defensin h β D-1 - 10 $\mu\text{g/ml}$ [11], secretory inhibitor of leucoprotease - 18 $\mu\text{g/ml}$ [12], lactoferrin - 8 $\mu\text{g/ml}$ [13], lysozyme - 4 $\mu\text{g/ml}$ [14], psoriasin - 14 $\mu\text{g/ml}$ [15] and histatin - 33 $\mu\text{g/ml}$ [16];
- for SS – dermcidins - 6 $\mu\text{g/ml}$ [17] (information about concentrations of other SS AMP was not found);
- for UR – RNAse 7 - 2 $\mu\text{g/ml}$ [18].

According to the type and concentrations of AMP the maximal activity should be expected in SA, although the most active undiluted biofluid (and its AMP fraction) is SE. However the minimal

Table 1. Candidacidal activities of different biofluids from 6 healthy volunteers.

Biofluids	Antimicrobial activity, %		Significance level of differences between total and 100 kDa activities	Percent of AMP activity compared to total activity, %
	Total	AMP		
Serum	86.9 \pm 1.3	38.3 \pm 6.8	$p \leq 0.01$	44.1
Vaginal fluid	24.8 \pm 8.3	20.8 \pm 3.5	$p > 0.05$	83.9
Saliva	21.3 \pm 8.1	10.9 \pm 5.8	$p > 0.05$	51.2
Water soluble part of skin secretion	14.0 \pm 4.9	5.7 \pm 5.4	$0.01 \leq p \leq 0.05$	40.7
Urine	10.2 \pm 6.4	1.5 \pm 2.2	$0.01 \leq p \leq 0.05$	14.7

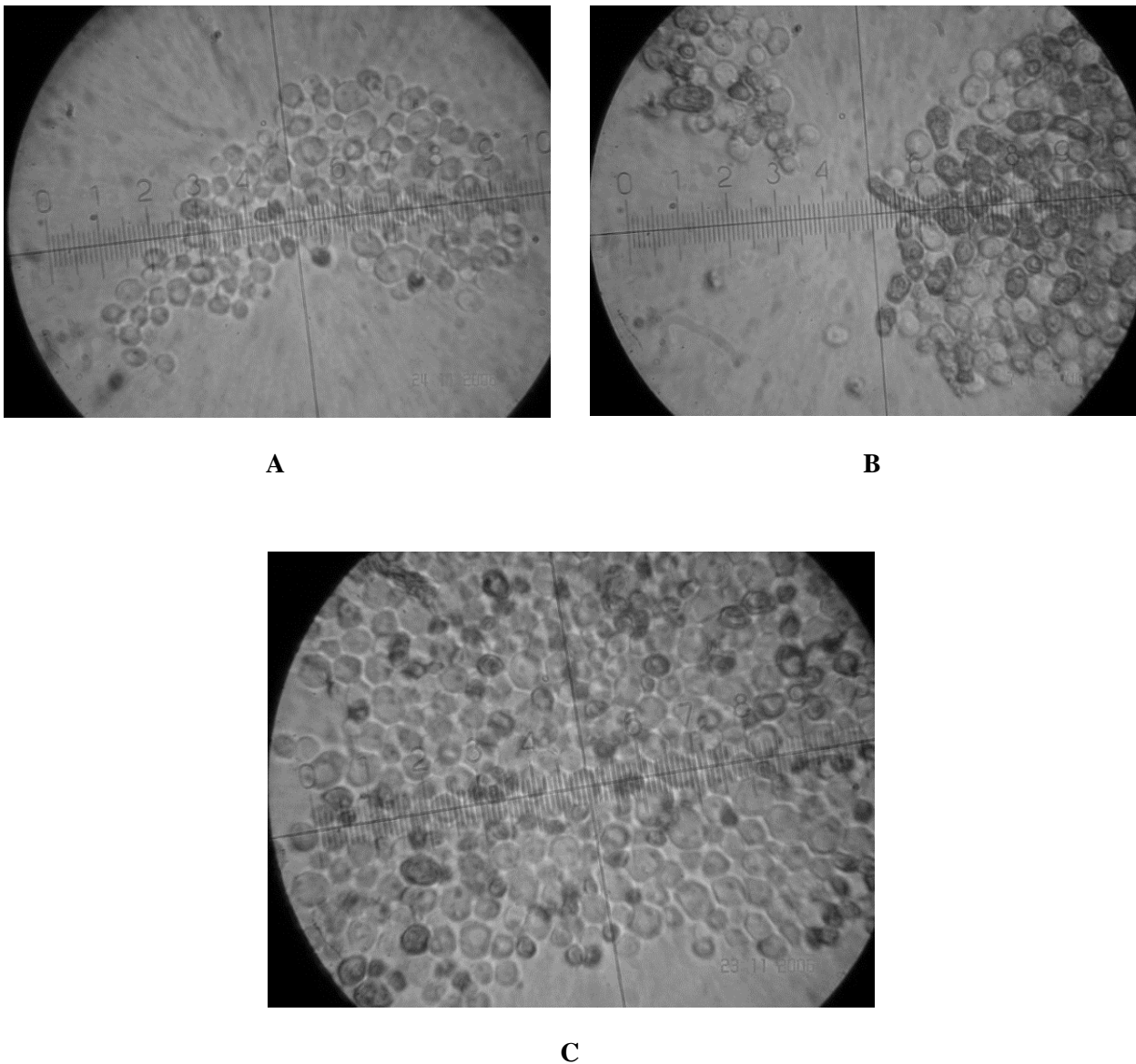


Figure 1. Effect of different saliva fractions on *Candida albicans* cells.
A – control specimen; **B** – total saliva; **C** – the AMP fraction (< 100 kDa) of saliva.

known concentrations of AMP as well as the minimal estimated AMP activities occurred in SS and UR. The overall AMP activity apparently is not the simple sum of their individual activities.

Very intriguing is the fact that the role of AMP is varied in each biofluid. AMP fraction estimated as the percentage of AMP activity compared to the total activity decreases in the following sequence: VF-SA-SE-SS-UR. Therefore the antimicrobial defense of vagina is provided mainly by AMP,

whilst urinary AMP do not play significant role in this locus.

The reason for high correlations between antimicrobial activities of UR-SS-SA (and low correlations with activities of VF) could be attributed to common embryogenesis of the tissues which secrete each of these biofluids. However it is well known that AMP:

- ◆ of SE are produced by neutrophils originating from mesoderm;

- ◆ of VF – by cells of vaginal epithelium - from ectoderm;
 - ◆ of SA – by salivary glands - from endoderm;
 - ◆ of SS – by epidermal sweat glands – from ectoderm;
 - ◆ of UR – by epithelium of urinary bladder (endoderm) and kidneys (mesoderm).
- From the above it is obvious that this hypothesis of correlation between UR-SS-SA does not have enough confirmation.

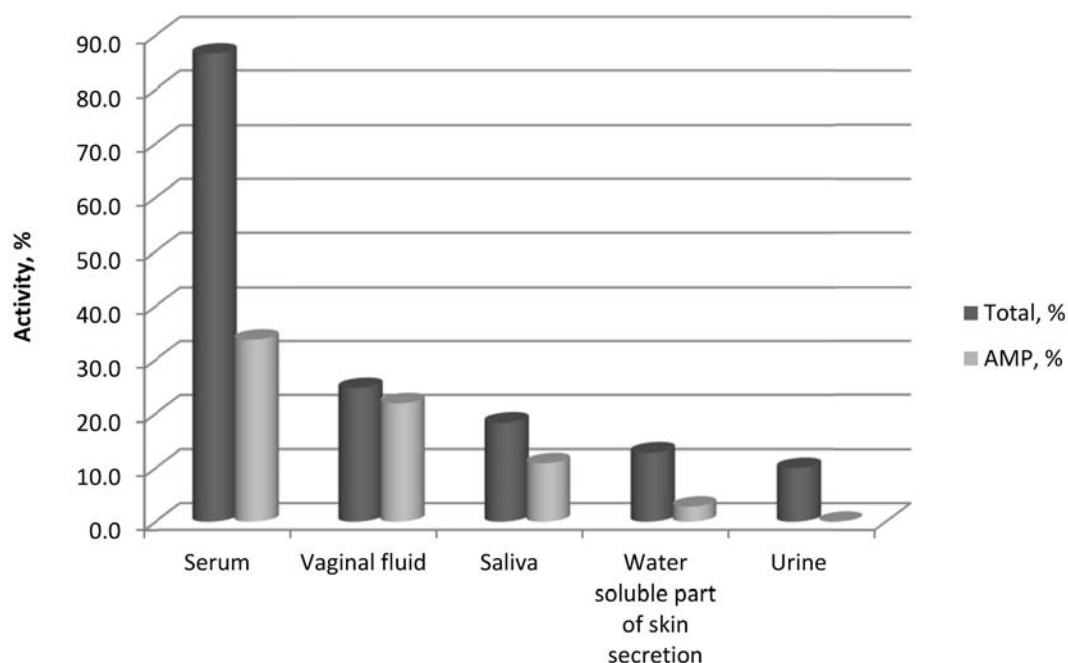


Figure 2. Medians of candidacidal activities of different biofluids from 6 healthy volunteers.

Table 2. Correlations between antimicrobial activities of different biofluids from 6 healthy volunteers.

Compared bioliqids	Pirson's correlation coefficients	
	Total antimicrobial activities	AMP activities
Vaginal fluid - Water soluble part of skin secretion	-0.517	-0.348
Vaginal fluid - Urine	-0.579	-0.068
Vaginal fluid - Serum	-0.490	-0.015
Vaginal fluid - Saliva	-0.692	0.055
Serum - Urine	0.530	0.081
Serum - Water soluble part of skin secretion	0.195	0.439
Serum - Saliva	0.343	0.563
Saliva - Urine	0.897	0.571
Saliva - Water soluble part of skin secretion	0.713	0.689
Urine - Water soluble part of skin secretion	0.846	0.814

CONCLUSION

On the basis of the obtained experimental data one can conclude that SE is the most active human liquid against the yeast *C. albicans*, but the maximal activity of AMP fraction in comparison with the total activity was noted in VF. Values of total and AMP activities of SA, SS and UR have high positive correlation between each other, while none of them correlated with the activities of VF. Using the most appropriate method of antimicrobial activity estimation the differences between biofluids of healthy human tissues of different localization were demonstrated for the first time. Anyway the investigated parameters may be significant in the diagnostics of human epithelial tissue immunity. Further investigation would need additional groups of volunteers/patients and test cultures of other opportunistic microorganisms.

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CONFLICT OF INTEREST STATEMENT

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REFERENCES

1. Levy, O. 2000, *Blood*, 96, 2664.
2. Dale, B. A. and Fredericks, L. P. 2005, *Curr. Issues Mol. Biol.*, 7, 119.
3. Arzumanian, V., Malbakhova, E. and Vartanova, N. 2012, *A Search for Antibacterial Agents*, V. Bobbarala (Ed.), InTech, Croatia, 125.
4. Arzumanian, V. and Kabaeva, T. 2008, *Experimental'naya i klinicheskaya dermatokosmetologia (Russ.)*, 3, 47.
5. Nienhouse, V. 2012, *Master's Theses, Univ. of Chicago*, 727.
6. Arzumanian, V., Malbakhova, E., Foshina, E., Artemeva, T., Butovchenko, L., Vartanova, N. and Shmeleva, O. 2015, *Patent RF № 2015113069/15*.
7. Arzumanian, V., Shmeleva, O. and Michailova, N. 2017, *Med. Mycol. Open Access*, 3, 23.
8. Johansson, B. and Malmquist, J. 1971, *Scandinavian Journal of Clinical and Laboratory Investigation*, 27, 255.
9. Meuwis, M., Vernier-Massouille, G., Grimaud, J., Bouhnik, Y., Laharie, D., Piver, E., Seidel, L., Colombel, J. and Louis, E. 2013, *J. Crohns Colitis*, 7, 678.
10. Valore, E., Park, C., Igteti, S. and Ganz, T. 2002, *J. Obstet. Gynecol.*, 187, 561.
11. Kucukkolbasi, H., Kucukkolbasi, S., Ayyildiz, H. F. and Dursun, R. 2013, *West Indian Med. J.*, 62, 58.
12. Wahl, S., McNeely, T., Janoff, E., Shugars, D., Worley, P., Tucker, C. and Orenstein, J. 1997, *Oral Dis.*, 1S, 64.
13. Berlutti, F., Pilloni, A., Pietropaoli, M. and Polimeni, A. 2011, *Curr. Issues Mol. Biol.*, 7, 119.
14. Jenzano, J., Hogan, S. and Lundblad, R. 1986, *J. Clin. Microbiol.*, 24, 963.
15. Giusti, L., Sernissi, F., Donadio, E., Ciregia, F., Giacomelli, C., Giannaccini, G., Mazzoni, M. R., Lucacchini, A. and Bazzichi, L. 2016, *J. Transl. Med.*, 14, 262.
16. Tsai, H. and Bobek, L. 1998, *Crit. Rev. Oral Biol. Med.*, 9, 480.
17. Rieg, S., Steffen, H., Seeber, S., Humeny, A., Kalbacher, H., Dietz, K., Garbe, C. and Schitteck, B. 2005, *J. Immunol.*, 174, 8003.
18. Spencer, J., Schwaderer, A., Dirosario, J. and McHugh, K. 2011, *Kidney International*, 80, 174.