Study of *Candida albicans* Strains Isolated from Women with Various Forms of Vaginal Candidiasis

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Adhesive activity of *Candida albicans* towards vaginal epitheliocytes is hormone-dependent. Two types of *C. albicans* adhesins sensitive to polyphenyloxidase and asparaginase were detected. Laser irradiation nonspecifically modulated both adhesin types. Population relationships between fungi and lactobacilli in patients with vaginal candidal infection and in *C. albicans* carriers were studied. Genodiagnostic method for identification of *C. albicans* and morphogenesis-associated genes of these yeast-like fungi was approved.

Key Words: *Candida albicans; vaginal candidiasis; lactobacilli; genodiagnostic method; adhesion*

Vaginal candidiasis (VC) is the most prevalent gynecological disease. More than 75% women aged over 25 years have a history of at least one episode of VC and more than 5% women suffer from relapsing forms of this condition [5,13]. *Candida albicans* is responsible for 80-95% cases of VC; other yeast-like fungi (YLF) are identified rarely, but also can cause manifest infection [7]. *C. albicans* is characterized by dimorphism: the budding form is apathogenic, while the appearance of germ tubes and mycelium formation are considered as a manifestation of invasion [5,7]. Some authors regard YLF species often (25-50%) detected in healthy women as representatives of normal flora, while VC is considered as a manifestation of dysbiosis [4]. It is however known that *C. albicans* strains isolated from patients with manifest infection are characterized by higher virulence than strains isolated from Candida carriers [1], and therefore VC is suggested to be classified as a sexually transmitted infection (STI). However, there are still no persuasive epidemiological proofs of this viewpoint.

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Adhesion is an important factor for *C. albicans* interactions with vaginal epitheliocytes (VE). It can present as symbiotic relationships with the formation of colonization resistance of the econiche or as an infectious process. In the later case it becomes a factor of pathogenicity. Changes in adhesive activity of *C. albicans* under the effects of chemical and physical factors were described previously [3], and hence inclusion of drugs suppressing *C. albicans* adhesion to VE in standard therapy for VC is discussed [2,6,13].

Thus, there is no universal opinion about the pathogenesis of VC and approaches to its rational therapy. The type of population relationships between YLF and lactobacilli maintaining the colonization resistance of the vagina is to be determined. Several problems have to be solved with this aim in view. First of all, a specific method not only ensuring species diagnosis of *C. albicans*, but also detecting the corresponding genes associated with their pathogenicity has to be developed. One more task is to analyze species composition and quantitative characteristics of YLF and lactobacilli population in patients with VC and Candida carriers in order to elucidate the possibility of inhibiting *C. albicans* adhesion to VE by physical and chemical factors.

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MATERIALS AND METHODS

The study was carried out in 114 women aged 18-43 years. Group 1 consisted of 90 pregnant women, 44 of whom (48.9%) were healthy and 46 (51.1%) with threatened abortions (n=11), gestosis of the first (n=4) and second half of pregnancy (n=29), and anemia (n=2). Group 2 consisted of 24 patients tested for STI. YLF cultures were isolated from 55 women (48.2% examinees), 40 of these at different terms of gestation (6-40 weeks) from group 1 and 15 women from group 2.

Colonization of the vagina with YLF and lactobacilli was evaluated by Gould inoculation [8] into appropriate media; the results were expressed as decimal logarithm of the number of colony-forming units/ml vaginal secretion (lgCFU/ml). The capacity of *Candida* genus YLF culture to form germ tubes was evaluated using Kwon-Chung method [11].

Adhesive activity was studied in detail in 4 clinical strains of *C. albicans* isolated from three Candida carriers and one patient with VC. The strains were characterized as *C. albicans* by the PCR method and by their capacity to form germ tubes. Adhesion experiments were carried out on 24-h cultures grown in Saburo agar at 37°C and washed twice with 0.1 M buffered saline (pH 7.4) or 0.1 M phosphate buffer (pH 7.4).

Vaginal epitheliocytes (brush samples) were collected from 3 healthy parous women aged 29-30 years with regular menstrual cycle of 29-30 days, Rh(+), differing by AB0 blood groups. Suspension of VE was placed into buffered saline, washed 4 times, and adjusted to optical density 0.4 corresponding to a concentration of 10^6 cells/ml.

The effects of chemical and physical factors on YLF adhesion to VE were studied using asparaginase (Sigma) and polyphenoloxidase (Sigma) in concentrations 20 and 140 U/ml, respectively. All experiments with asparaginase were carried out using buffered saline; experiments with polyphenyloxidase were carried out in phosphate buffer, because enzyme activity increases in the presence of chlorine ions.

Physical modulation of adhesion was carried out using laser exposure at λ=890 nm and 1000 Hz frequency (magnetic infrared laser Milta for quantum therapy, State Industrial and Designing Firm for Humanitarian Information Technologies).

In the first and second experimental series the enzymes in the studied concentrations were added to YLF suspension in buffered saline (in studies of asparaginase and laser effects) or phosphate buffer (in studies of polyphenyloxidase effect) with $OD_{\lambda=540\ nm}$=1.0 (corresponding to 10^9 CFU/ml) and the mixture was incubated at 37°C for 1 h with regular shaking. In the third experimental series *C. albicans* were exposed to laser for 1 min, and in the fourth series *C. albicans* were exposed to laser and enzymes. After treatment the suspension was washed twice, suspension of VE was added (1:3), and the mixture was incubated at 37°C for 1 h with regular shaking. After incubation VE were washed in homologous solution 5 times. Smears were fixed in the fire and stained with Gentian violet by the routine method. At least 30 cells were counted. The mean adhesive capacity of VE was estimated as the ratio of the *C. albicans* number per one VE in the control and experiment. Thus, affinity of *C. albicans* adhesins to VE was in fact evaluated.

DNA studies (PCR) were carried out at Laboratory of Gene Engineering Systems LAGIS. DNA specimens from cell cultures were isolated by modified Boom's method [9].

The reaction mixture for PCR (final volume 25 µl) contained 10 mM Tris HCl (pH 8.7), 50 mM KCl, 2 mM $MgCl_2$, 0.25 mM each dNTP, 10 pmol each primer, 1 U Taq polymerase, and 5 µl DNA preparation. Amplification was carried out in a Tertsik programmed thermostat (DNK-Tekhnologiya Firm) according to the traditional three-step program. *C. albicans* DNA and sterile water were used as positive and negative controls, respectively. An aliquot of the resultant reaction mixture was analyzed in 1.5% agarose gel stained with ethidium bromide.

The results were statistically processed using software developed at Bioavtomatika Center (Nizhny Novgorod) and Statistica 5.0 software. The significance of differences was evaluated using Student's t test. The relationships of lactobacilli and YLF populations and hormone-dependent affinity of *C. albicans* adhesins to VE receptors were evaluated using regression analysis by the least squares method.

RESULTS

VC was diagnosed clinically and confirmed by laboratory findings in 13.2% cases (11.1% pregnant and 20.8% nonpregnant women). On the other hand, *C. albicans* were isolated from 66.7% women (70% pregnant and 60% nonpregnant). Other YLF strains (not *C. albicans*) were isolated from 12.7 women (10% pregnant and 20% nonpregnant), in whom VC was associated with less severe inflammation than in patients infected with *C. albicans* (the mean number of leukocytes in smears from patients infected with Candida genus YLF was 10.60±0.08 *vs.* 15.80±0.13 in patients infected with *C. albicans* YLF).

Analysis of case histories revealed at least one episode of the disease in 60% patients with VC and in only 26% carriers.

These data allow different interpretation. Presumably, the colonization resistance of the vagina deter-

TABLE 1. Characteristics of PCR Test System Primer Pairs and Results of PCR Analysis of *Candida* YLF Cultures (%)

Primer pair	Presumable specificity	Incidence of positive signal in PCR			
		C. albicans		other carriers	
		patients (*n*=11)	carriers (*n*=37)	patients (*n*=4)	carriers (*n*=3)
Can (ribosomal gene fragment)	*Candida spp.* genus	100	100	100	100
Caa (ribosomal gene fragment)	*C. albicans* species	100	100	0	0
Car (retrotransposon)	*C. albicans* species	91	92	0	0
Cash(chitin synthetase gene)	*C. albicans* morphogenesis marker	100	100	0	0
CaNrg (hypha formation negative regulation gene)	*C. albicans* morphogenesis marker	100	100	0	0

Note. Selected target for primer pair is shown in parentheses.

mined by lactoflora is impaired in VC patients. It cannot also be excluded that YLF strains isolated from VC patients were more virulent than the strains isolated from healthy carriers. Transformation of YLF from the budding into micellar form is a manifestation of aggressiveness of *C. albicans* population [5,7,11]. We hypothesized that *C. albicans* strains isolated from carriers were defective by morphogenesis-associated genes. Therefore, at the initial stage of the study we carried out a genetic analysis of cultures isolated from patients with various forms of candidal infection. All cultures tested at this stage of the study were positive in the Can-test system (LAGIS). The choice of target genes for primer pairs was based on published *Candida* sequences [10,12,14]. The use of several test systems allowed typing of the resultant cultures not only by the genus- and species-specific genetic markers, but also by genes associated with pathogenicity factors (Table 1).

The sum of microbiological and genetic findings indicates that all isolated strains belong to YLF *Candida*. The same strains were positive in the Can-test system, which confirmed genus specificity of this system. PCR detected chitin-synthetase gene and gene of negative regulation of hypha formation in all cultures microbiologically identified as *C. albicans* and the absence of these genes in cultures identified as non-*C. albicans* YLF.

C. albicans strains isolated from females with manifest and inapparent infection had no appreciable structural changes in gene regulating hypha formation and gene encoding chitin-synthetase. None studied strains lacked at least one of these genes, but all these strains were capable of hypha formation. Presumably, PCR detection of genes associated with invasive activity of *C. albicans* YLF, in contrast to prokaryotes, cannot be considered as sufficient proof of pathogenicity. It is more likely that functional activity of these genes is regulated by differential expression. Detection of non-*C. albicans* YLF in VC patients suggests that dimorphism is not the only condition determining invasion.

False-negative results in the Car-test system (absence of positive PCR signals in 8-9% of *C. albicans* strains) indicates that the system is unfit for recognition of this species. This peculiarity of the Car-test system can be explained by the absence of target DNA in some isolated *C. albicans* strains.

At the second stage of the study we analyzed the dynamics of populations of YLF and lactobacilli in different VC variants (Table 2). The number of *C. albicans* and non-*C. albicans* YLF were similar. The

TABLE 2. Mean Density of *Candida* YLF and *Lactobacillus spp.* (lgCFU/ml)

Group		*C. albicans*/ *Lactobacillus spp.*	YLF other than *C. albicans*/ *Lactobacillus spp.*
VC patients:	pregnant	5.22±0.31*/4.00±0.42	4.53±0.60*/4.17±0.88
	nonpregnant	5.07±0.58/3.90±0.10	—
Candida carriers:	pregnant	3.45±0.11*/6.63±0.25	3.16±0.17/5.57±0.30
	nonpregnant	3.40±0.10*/6.40±0.40	—

Note. *$p<0.05$ compared to patients with vaginal candidiasis or Candida carriership.

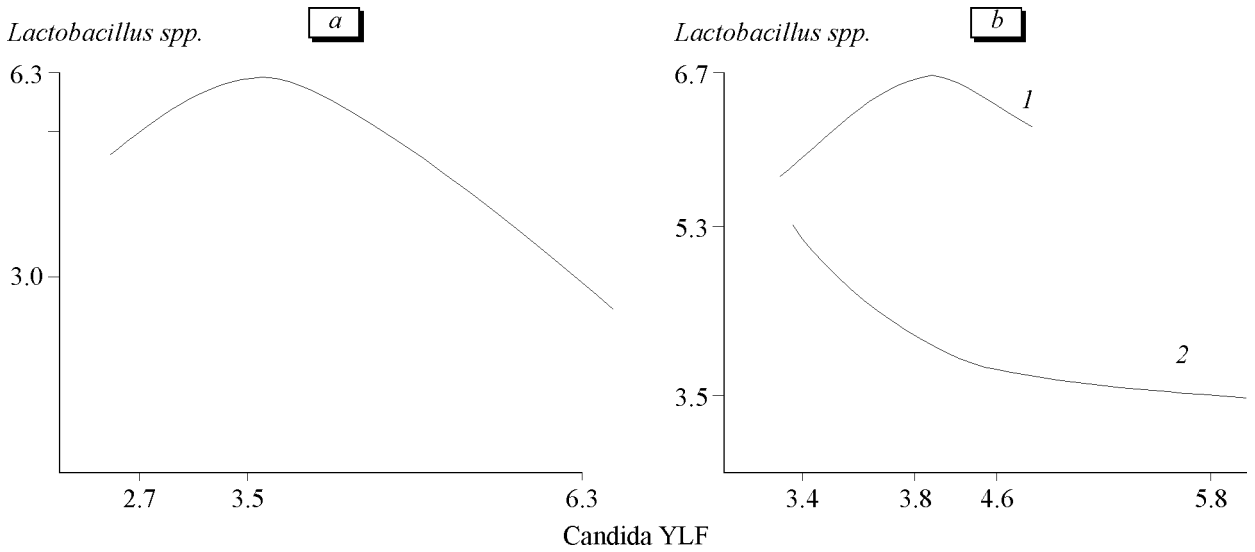

Fig. 1. Relationship between the number of lactobacilli and fungi population density (lg CFU/ml). *a*) total sample; *b*) ratio of Candida carriers (*1*) to patients with vaginal candidiasis (VC) (*2*).

decrease in lactobacillus titer did not depend on the agent species (the differences are insignificant). Colonization with YLF in manifest VC was higher that in other cases and was associated with a decrease in the number of lactobacilli to 3.9:4.0. Regression analysis of the relationship between the density of YLF and lactobacillus populations showed that the increase in the number of YLF to a certain critical level (3.5-3.8 lgCFU/ml) stimulated multiplication of lactobacilli, while further increase led to decrease in the number of lactobacilli (Fig. 1, *a*). An opposite relationship was observed for YLF: their number decreased with increasing the number of lactobacilli.

In patients with VC the effect of stimulation of lactobacillus growth was absent, because YLF contamination was initially above the critical level (Fig. 1, *b*).

At the same time, in carriers the increase in the number of lactobacilli in parallel with increase of YLF population to a critical level was clearly seen (Fig. 1, *b*). It is noteworthy that lactoflora in Candida carriers actively suppressed multiplication of YLF. This attests to a protective effect of lactobacteria and autoregulation of vaginal normocenosis in the absence of extreme exo- and endogenous factors.

Hence, vaginal bacterial biocenosis is an intricate autoregulated balanced system in which the agent of VC is maintained at a low population level. It cannot be excluded that imbalance in this system promotes selection of highly virulent variants of *C. albicans* [1].

Analysis of the mean adhesive capacity of VE showed no appreciable differences in this parameter depending on the blood group of VE donors and on the studied strains. The mean adhesive capacity of VE increased significantly depending on the day of the menstrual cycle. On day 7 it was 4.36±0.06, on day 11 4.76±0.05, on day 14 5.48±0.10, on day 17 6.14±0.08, on day 21 6.39±0.10, and on day 26 9.34±0.17 (differences between days 17 and 21 were significant at $p<0.05$, for the rest pairs at $p<0.01$).

Affinity of *C. albicans* for VE receptors decreased under the effect of enzymes and laser exposure. After polyphenyloxidase treatment the number of adhesive YLF per one VE decreased to 19.4±4.1%, after asparaginase treatment it was 37.0±4.7%, and after laser exposure 53.4±4.8%. The decrease in adhesin affinity after combined treatment (enzymes+laser) was similar to that after laser exposure alone (50.0±3.9%). This suggests that the effect of laser exposure on the surface structures of YLF responsible for adhesion is

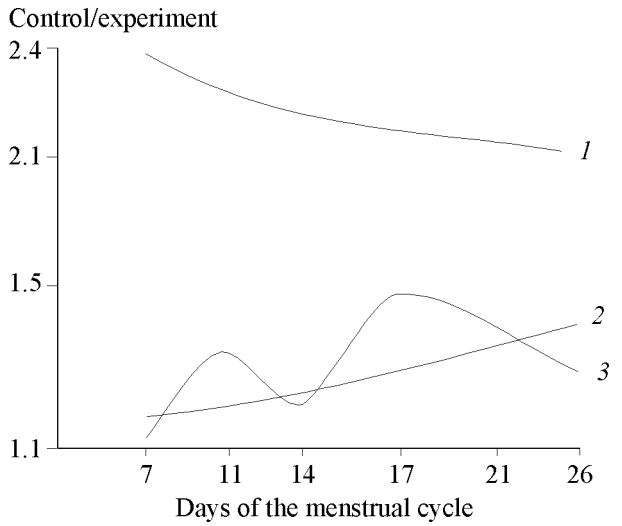

Fig. 2. Changes in the affinity of *C. albicans* adhesins to vaginal epithelium receptors under the effects of chemical and physical factors on different days of the menstrual cycle. *1*) laser exposure (LE); *2*) polyphenyloxidase (PPO); *3*) asparaginase (ASG).

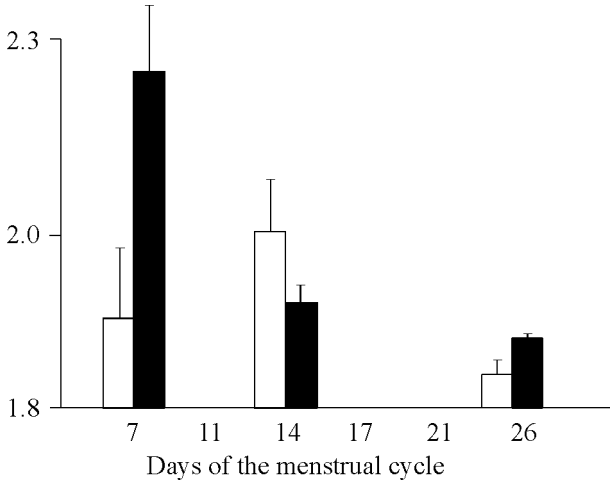

Fig. 3. Affinity of *C. albicans* adhesins to vaginal epitheliocyte receptors after laser irradiation and enzyme treatment. Light bars: laser+ASG; dark bars: laser+PPO.

nonspecific. On the other hand, the enzymes selectively decreased affinity of certain adhesins, which was confirmed in study of the relationship between their inhibitory activity and the phase of menstrual cycle when VE were collected. The inhibitory effect of polyphenyloxidase increased throughout the cycle (Fig. 2), while asparaginase treatment decreased adhesion of YLF cultures to VE collected during the proliferative phase of the cycle; the inhibitory effect of the enzyme decreased during ovulation and again increased during the secretory phase. Laser exposure inhibited adhesion by the end of the cycle. These findings suggest that VE has receptors to different types of *C. albicans* adhesins (polyphenyloxidase- and asparaginase-dependent). The expression of these receptors is a dynamic process and reaches the maximum during the secretory phase of the cycle. The decrease in affinity of *C. albicans* adhesin for VE receptors after polyphenyloxidase treatment was less pronounced after laser exposure, *i. e.* laser irradiation destroyed polyphenyloxidase-dependent adhesins (Fig. 3). Inactivation of these adhesins by laser radiation increased by the end of the menstrual cycle, when the expression of homologous receptors on VE was most pronounced. Combined treatment with asparaginase and laser (differences between the parameters on days 7 and 14 of the cycle were insignificant) suggested lower sensitivity of asparaginase-dependent adhesins to laser, because the inhibitory effect of this enzyme was still observed during the period of the maximum expression of the corresponding receptors (day 14 of the cycle).

REFERENCES

1. T. S. Bogomolova, *Morphobiological Characteristics of Candida*, Abstract of Cand. Med. Sci. Dissertation, Leningrad (1990).
2. K. Z. Kartvelishvili, *Vestn. Ros. Ass. Akush. Gin.*, No. 1, 100-103 (2000).
3. T. V. Kolganova, A. V. Ermolaev, and R. J. Doyle, *Byull. Eksp. Biol. Med.*, **133**, No. 1, 71-74 (2002).
4. S. J. F. Priestley, V. M. Jones, J. Dhar, and L. Goodwin, *Zabolevaniya Peredavaemye Polovym Putem*, No. 4, 12-18 (1997).
5. V. N. Prilepskaya, A. S. Ankirskaya, G. R. Bairamova, and V. V. Murav'eva, *Vaginal Candidiasis* [in Russian], Moscow (1997).
6. V. E. Radzinskii, E. M. Mikhailenko, and K. A. Zakharov, *Drugs and Bioactive Additives in Obstetrics and Gynecology* [in Russian], Elista (1998).
7. A. Yu. Sergeev and Yu. V. Sergeev, *Candidiasis* [in Russian], Moscow (2001).
8. Yu. M. Fel'dman, L. G. Makhaneva, A. V. Shapiro, and V. D. Kuz'menko, *Lab. Delo*, No. 10, 616-618 (1984).
9. R. Boom, C. J. A. Sol, M. M. M. Salimans, *et al.*, *J. Clinical Microb.*, **28**, No. 3, 495-500 (1990).
10. J. L. Chen-Wu, J. Zwicker, A. R. Bowen, and P. W. Robbins, *Mol. Microbiol.*, **6**, 497-502 (1992).
11. K. J. Kwon-Chung and J. E. Bennett, *Medical Mycology*, Philadelphia (1992), pp. 61-62.
12. A. M. A. Murad, P. Leng, M. Straffon, *et al.*, *EMBO J.*, **20**, No. 17, 4742-4752 (2001).
13. J. D. Sobel, S. Faro, R. W. Force, *et al.*, *Am. J. Obstet. Gynecol.*, **178**, 203-211 (1998).
14. Z. Weissman, I. Berdicevsky, and B. Cavari, *J. Med. Vet. Mycol.*, **33**, No. 3, 205-207 (1995).