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Drug Sensitivity of *Candida* Yeast Isolated from Patients with Allergic Diseases

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Viability of 40 *Candida spp.* cultures was studied after long-term exposure to antifungal drugs in minimum inhibitory concentrations. The fungicidal effect decreased in the series: pimafucin—nitrofungin—diflucan—orungal—levorine—clotrimazole—exoderil. Nizoral in a concentration of 4 μg/ml was ineffective; in the rest cultures the effect was either fungistatic (of different degree) or null. Pimafucin, diflucan, nitrofungin, orungal, levorine, and exoderil possessed individual fungicidal effects.

Key Words: *Candida; antifungal drugs; sensitivity; viability*

Intensive use of antifungal drugs leads to incessant increase in the number of resistant fungal strains retaining viability due to their resistance mechanisms. This necessitates investigation of the sensitivity/resistance of clinically significant fungi. *Candida albicans* is the best studied yeast for today, and the best studied drugs are azole preparations ketoconazole, intraconazole, and clotrimazole. Great attention to *C. albicans* is not accidental. Unlike other yeast, it belongs to obligate commensals; they are extremely rare in nature but highly incident in humans: more than 40% adults are carriers [3]. The virulence (a matter of virulence is, *e. g.* adhesion capacity) of clinical strains of *C. albicans* varies, but, as in any yeast infection, the balance between transitory carriership, commensal colonization, or parasitism depends on the physiological status of the host [3].

We have found no reports about specific features of *Candida* fungi isolated from patients with various diseases.

Our collection of yeast-like fungi isolated from patients with allergic diseases is constantly increasing. Previously we described the species variability of isolated strains and their sensitivity to 8 antifungal drugs [1].

Now we evaluated the viability of *Candida spp.* cultures after long-term incubation with antifungal drugs *in vitro.*

MATERIALS AND METHODS

Cultures of yeast isolated from patients with skin and bronchopulmonary allergic diseases were used [1]. The isolates were identified using software "Yeast identification program version".

Two-day yeast cultures were grown on oblique modified Saburo agar at 27°C. The initial content of viable cells in liquid synthetic test medium was 10^3-10^4 CFU/ml. The medium contained pH indicator; color changes was indicative of culture growth.

Concentrations of some antifungal drugs were selected depending on the minimum inhibitory concentrations (MIC) reported by NCCLC. The final concentrations of diflucan was 64 μg/ml, orungal 4 μg/ml, and nizoral 4 μg/ml. For clotrimazole, the most characteristic MIC for azoles was selected: 4 μg/ml. As exoderil showed no activity in concentrations 2-16 μg/ml, it was used in a concentration of 64 μg/ml. Levorine, pimafucin, and nitrofungin, previously used in concentra-

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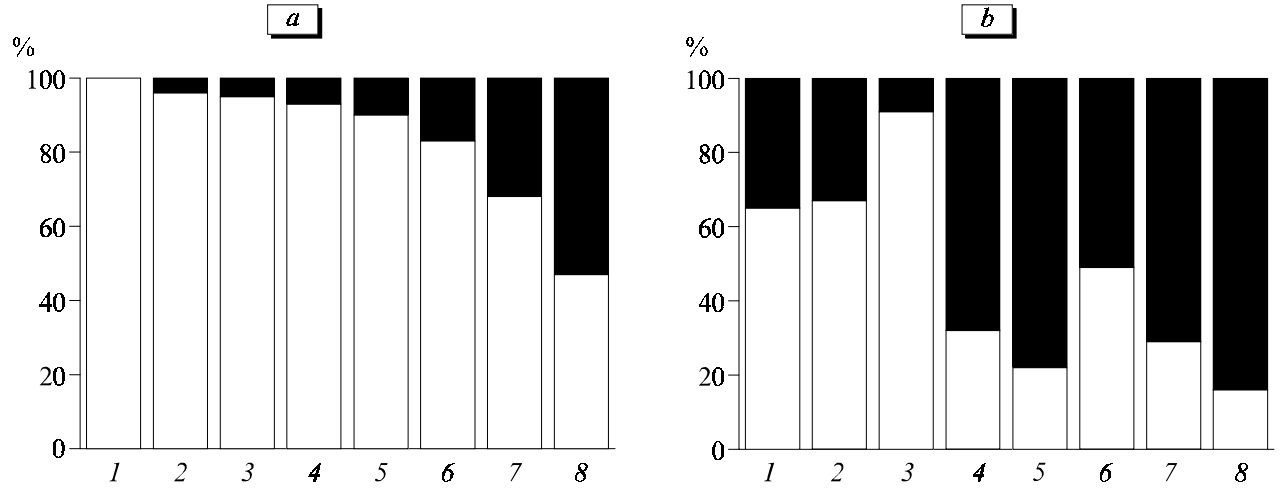

Fig. 1. Sensitivity of *Candida spp.* to antifungal drugs evaluated by changes in color of pH indicator 3 (*a*) and 14 (*b*) days after inoculation. Light part of each bar represents the percentage of isolates sensitive to the drug and the dark part is the percentage of cultures resistant to the drug. Here and in Fig. 2: *1*) diflucan; *2*) pimafucin; *3*) nitrofungin; *4*) clotrimazole; *5*) nizoral; *6*) orungal; *7*) levorine; *8*) exoderil.

tions 2 and 16 μg/ml, were active only in the latter concentration for the majority of studied cultures.

The viability of *Candida spp.* cultures was evaluated on day 14 of culturing at 27°C. To this end, aliquots from cultures in which pH indicator did not change its color, *i. e.* fungal growth was inhibited, were inoculated in agar-treated medium.Viability control in cultures with changed indicator color was also carried out by inoculation into agar-treated medium. All tested cultures were viable and retained high population. The activity of drugs was tested 3 times during the entire period of culturing by inoculating 2-day cultures in media containing the drugs and pre-incubated at 27°C. All drugs retained their activity towards control cultures during the entire period of observation.

RESULTS

By the moment of our experiment, the collection of *Candida* yeast identified by species consisted of 40 strains: 17 *C. albicans,* 8 *C. haemulonii,* 5 *C. bombicola,* 4 *C. vesratilis,* 2 *C. zeylanoides* strains, and *C. azyma, C. blankii, C. glabrata,* and *C. terebra* 1 strain each. On day 3 of culturing, the growth of all strains was inhibited by diflucan (64 μg/ml) and only 47% cultures were sensitive to exoderil (Fig. 1, *a*). However the situation changed during subsequent days, and on day 14 the sensitivity to all drugs except nitrofungin increased 1.5-4.5 times (Fig. 1, *b*).

The variants with sharp growth inhibition (pH indicator not changing color) were tested for cell viability.

The effect was considered fungicidal if after 14 days of incubation the culture contained no live cells, highly fungistatic, if the content of live cells was no more than 10^4 CFU/ml (0-10-fold growth in comparison with the initial population), and slightly fungistatic, if the culture contained 10^4-50×10^4 CFU/ml (10-50 times increase in the population number).

In the cultures with changed indicator color the number of viable cells was much higher than 5×10^4 CFU/ml.

On day 14 all drugs except pimafucin had only a fungistatic effect in the majority of cultures (Fig. 2). Nizoral in a concentration 4 μg/ml exerted no fungicidal effect, but only a fungistatic. Pimafucin (16 μg/ml) possessed the highest fungicidal effect: 56% pimafucin-sensitive cultures contained no live cells. Interestingly that of all isolated strains sensitive to nitrofungin after long-term incubation only 29% were completely nonviable. After 14-day incubation, 38% cultures incubated with pimafucin contained no live cells, for nitrofungin this value was 26%, for diflucan 23%, for orungal 10%, for levorine and clotrimazole

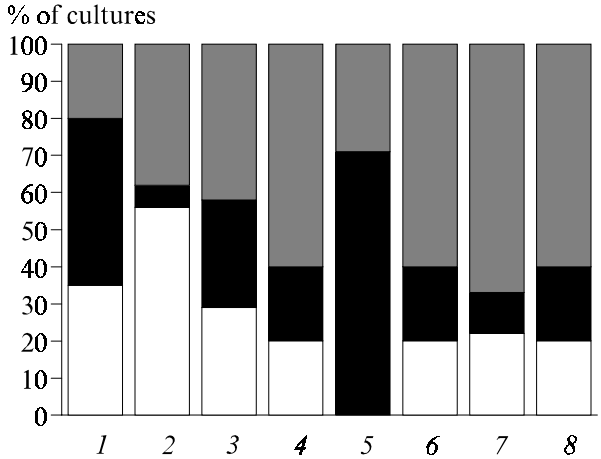

Fig. 2. Evaluation of viability of *Candida spp.* cultures sensitive to antifungal drugs after 14-day incubation. Light part of each bar shows fungicidal, dark part strong fungistatic, and cross-hatched weak fungistatic effect of the drug.

TABLE 1. Effects of Antifungal Drugs on *C. albicans*

Drug	Duration of incubation, days					
	3		14			
	—	+	—	fungistatic effect		fungicidal effect
				weak	strong	
Diflucan	0	100	36	7	43	14
Pimafucin	0	100	50	21	0	29
Nitrofungin	6	94	7	50	22	21
Clotrimazole	0	100	57	29	7	7
Nizoral	0	100	72	14	14	0
Orungal	12	88	51	21	21	7
Levorine	29	71	71	29	0	0
Exoderil	59	41	93	7	0	0

Note. "-" no growth inhibition; "+" growth inhibition.

6%, and after incubation with exoderil only 3% cultures contained no live cells.

For *Candida albicans* (17 isolates) on day 3 of incubation the regularities were in general the same as in all *Candida spp.* (Table 1), but after 14 days of incubation the fungicidal effects of nizoral, levorine, and exoderil were null. Even diflucan which is considered to be highly active towards *C. albicans* decreased its activity after long-term exposure: only 14% cultures contained no live cells, and 36% isolates remained absolutely viable. It seems that higher drug resistance can be regarded as indirect evidence of higher virulence of our *C. albicans* cultures in comparison with other *Candida*, which is in line with published reports [3].

The mechanisms of the effects of test drugs consist mainly in impairment of cell membrane structure and/or function. Ergosterol is the main component of cell membrane and fungal mitochondria and serves as the bioregulator of their fluidity, asymmetry, and hence, intactness. Azole drugs diflucan, orungal, nizoral, and clotrimazole inhibit cytochrome P-450-dependent hem-containing 14α-demethylase participating in biosynthesis of ergosterol. As a result, accumulation of ergosterol precursors in the cell impaired membrane structure and function. Polyenic drugs pimafucin and levorine react with membrane ergosterol forming pores, through which vitally important low-molecular components of the cytoplasm are released. Allylamines (in our case exoderil) inhibit early stages of ergosterol biosynthesis at the level of squalenepoxidase, and cells die because of squalene accumulation, which increases membrane permeability, thus leading to impairment of cell structure. There are no published reports about the mechanism of effect of nitrofungin (2-chloro-4-nitrophenol). We can only hypothesize that like 2,4-dinitrophenol, it can act by dissociating the oxidation and phosphorylation processes in the respiratory chain of yeast mitochondria [2].

TABLE 2. Fungicidal Effects of Antifungal Drugs on Yeast Cultures

Drug	Sensitive cultures, %	Sensitive to this preparation only, %
Azole derivatives		
diflucan	23	10
orungal	10	3
clotrimazole	6	0
nizoral	0	0
Polyenes		
pimafucin	38	21
levorine	6	3
Allylamines		
exoderil	3	3
Nitrofungin (2-chloro-4-nitrophenol)	26	10

A yeast cell developed numerous resistance mechanisms in response to antifungal drugs [5,6]. These mechanisms are hyperproduction of target enzyme due to which the drug cannot completely inhibit the relevant biochemical reaction; alteration of the target as a result of mutation or selection, leading to modification of its affinity to the drug; release of the drug outside by means of a special pump. These mechanisms can prevent the realization of fungicidal effects of azole derivatives, polyenes, and allylamines (Table 2). In addition, drug entry into the cell can be blocked at the level of cell membrane or cell wall; the loss of function because of inhibition by the drug can be compensated via a bypass pathway; and finally, fungal cell

enzymes can be inhibited, which transfer inactive drug into its active form. The presence of inducible enzymatic systems promoting drug degradation can provide pimafucin resistance [4].

However even functioning of several mechanisms of resistance cannot guarantee the preservation of population in the presence of antifungal agents. It is particularly interesting to observe individual fungicidal effect of a drug in a yeast population resistant to all drugs except one. This can indicate effective functioning of all resistance mechanisms except the one responsible for cell protection from this particular drug. The most active drugs (diflucan, pimafucin, and nitrofungin) demonstrated individual fungicidal effect in half of the cases (Table 2). Yeasts which were not resistant belonged to different species.

Interestingly that exoderil was characterized by low fungicidal activity, but in the only case when it manifested (towards *C. haemulonii*), other drugs showed no fungicidal effect (Table 2). Bearing in mind that the mechanisms of resistance to allylamines are almost unknown, it is very important to have such cultures of yeast-like fungi in the collection.

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