DOI: 10.29326/2304-196X-2022-11-1-20-26

Study of resistance of pathogenic and opportunistic fungi to antimycotics

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SUMMARY

The widespread use of antimycotic agents for the treatment of mycosis in humans and animals highly concerns medical and veterinary specialists, due to the emergence of resistance in pathogenic and opportunistic fungi to antifungal agents. In recent years, information on the various molecular mechanisms underlying this phenomenon has been published, but in-depth studies are still needed to successfully predict the occurrence of resistance in various groups of fungi. To treat and prevent fungal infections, several groups of antimycotics are used, with azoles and allylamines being the most frequent ones. This, however, leads to the development of resistance in pathogenic and opportunistic fungi. The article presents the results of molecular identification methods of azole-resistant *Candida albicans* isolates and terbinafine-resistant *Trichophyton* isolates. The analysis of *ERG11* gene nucleotide sequences of 10 *Candida albicans* isolates, recovered from different animal species, enabled the division of phenotypically resistant and susceptible strains, but could not differentiate between strains that have dose-dependent resistance to azoles. The study of single nucleotide polymorphisms in the SQLE gene, that are associated with the development of resistance to terbinafine in 12 fungal isolates of the genus *Trichophyton*, did not allow to classify them by their resistance, which is likely associated with another resistance mechanism that can be observed in these strains. The obtained results can serve as a basis for using molecular methods for characterization of fungi belonging to *Candida* and *Trichophyton* genera. However, taking into the account biological features of pathogens from different groups, it would be reasonable to use several significant genome regions or the results of the whole genome sequencing, as well as the gene expression analysis, to predict the development of resistance.

Keywords: antimycotic resistance, *Candida*, *Trichophyton*, azoles, terbinafine, polymerase chain reaction, sequencing

Acknowledgements: The work was supported by the Rosselkhoznadzor as part of the research activities on the topic "Assessment of the animal mycosis spread and the risks of resistance to antimycotics". The authors express their gratitude to the staff members of the Department of Biotechnology L. E. Sakanyan and A. V. Putintseva for the technical support.

For citation: Kozlova A. D., Yatsentyuk S. P., Sokolov V. V., Manoyan M. G. Study of resistance of pathogenic and opportunistic fungi to antimycotics. *Veterinary Science Today*. 2022; 11 (1): 20–26. DOI: 10.29326/2304-196X-2022-11-1-20-26.

Conflict of interest: The authors declare no conflict of interest.

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УДК 619:616.992.28:615:616-076

Изучение резистентности патогенных и условно-патогенных грибов к противогрибковым препаратам

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РЕЗЮМЕ

Широкое применение антимикотических средств для терапии микозов у человека и животных вызывает беспокойство медицинских и ветеринарных специалистов в связи с возникновением резистентности патогенных и условно-патогенных грибов к противогрибковым препаратам. За последние годы накоплена информация о различных молекулярных механизмах, лежащих в основе данного явления, однако для успешного прогнозирования резистентности

в различных группах грибов необходимо провести углубленные исследования. Для терапии и профилактики грибковых заболеваний активно применяют несколько групп препаратов, среди которых наиболее часто используют азолы и аллиламины, что приводит к накоплению резистентности патогенных и условно-патогенных грибов к этим противогрибковым средствам. В работе представлены результаты использования молекулярно-генетических методов для выявления устойчивых к азолам изолятов *Candida albicans* и устойчивых к тербинафину изолятов грибов рода *Trichophyton.* Анализ нуклеотидных последовательностей гена *ERG11* 10 изолятов *Candida albicans*, выделенных от разных видов животных, позволил разделить фенотипически устойчивые и чувствительные штаммы, однако не дал возможности дифференцировать штаммы, обладающие дозозависимой устойчивостью к азолам. Изучение однонуклеотидных полиморфизмов в гене SQLE, ассоциированном с развитием устойчивости к тербинафину, у 12 изолятов грибов рода *Trichophyton* не позволило разделить их по резистентности, что, вероятно, связано с действием другого механизма устойчивости, который может наблюдаться у данных штаммов. Полученные результаты исследований служат основанием для использования молекулярно-генетических методов для характеристики грибов рода *Candida* и *Trichophyton*, однако, с учетом биологических особенностей патогенов разных групп, для прогнозирования резистентности целесообразно использовать несколько значимых участков генома или результаты полногеномного секвенирования, а также анализ экспрессии генов.

Ключевые слова: антимикотическая устойчивость, *Candida*, *Trichophyton*, азолы, тербинафин, полимеразная цепная реакция, секвенирование

Благодарности: Работа выполнена при поддержке Россельхознадзора в рамках научно-исследовательской работы по теме «Оценка распространенности микозов животных и рисков возникновения резистентности к антимикотическим средствам». Авторы выражают благодарность сотрудникам отделения биотехнологии Л. Э. Саканяну и А. В. Путинцевой за техническую помощь при проведении исследований.

Для цитирования: Козлова А. Д., Яцентюк С. П., Соколов В. В., Маноян М. Г. Изучение резистентности патогенных и условно-патогенных грибов к противогрибковым препаратам. *Ветеринария сегодня*. 2022; 11 (1): 20–26. DOI: 10.29326/2304-196X-2022-11-1-20-26.

Конфликт интересов: Авторы заявляют об отсутствии конфликта интересов.

Для корреспонденции: Яцентюк Светлана Петровна, кандидат биологических наук, заведующий отделом генодиагностики инфекционных болезней животных отделения биотехнологии ФГБУ «ВГНКИ», 123022, Россия, г. Москва, Звенигородское шоссе, 5, *e-mail: pcr-lab@vgnki.ru.*

INTRODUCTION

In recent years, there has been an unprecedented increase in the development of resistance to antimycotic agents in parasitic fungi, which cause severe diseases in humans. The risk group primarily includes people with weakened immune system. International organizations have called for increasing the intensity of research and control of the resistance to antifungals, also including the field of veterinary medicine. The spread of resistance in the population is a serious problem, since some socially significant fungal infections are transmitted from animals to humans, and vice versa.

The use of antifungals to treat mycosis in animals in the Russian Federation is spontaneous and is not regulated. This fact, certainly, contributes to the development of resistance to antimycotics in pathogenic and opportunistic fungi.

It is assumed that the widespread use of antifungal agents contributes to the development of drug resistance [1, 2]. With this being said, fungal resistance to antimycotic drugs is becoming a serious problem on a global scale.

Currently existing variants of antifungals are represented by several classes of substances that differ in both, chemical composition and the mode of action (Table 1).

The drugs usually affect plasma membrane, cell wall, nucleic acids, and fungal mitosis. Nowadays for public and animal health purposes, azoles (for example, fluconazole, voriconazole and posaconazole) and allylamines (for example, terbinafine) are mostly used for treatment and prevention of mycosis, caused by the fungi of genera *Candida*, *Microsporum,* and *Trichophyton*. As a result, in most cases resistance develops to these specific drugs [1, 3].

The resistance of *Candida* fungi to azoles is developed mainly due to the following mechanisms: changes in the drug targets, decrease in the intercellular concentration of the target enzyme, changes in the sterol biosynthetic pathway, overexpression of the antifungal target, or increased efflux of the drug across the cell surface. A specific target of azoles is the cytochromes P450-dependent enzyme lanosterol 1,4-*α*-demethylase encoded by the *ERG11* gene in yeast-like fungi. The protein product of this gene catalyzes the removal of the 1,4 *α*-methyl group from lanosterol. Binding of azole to a fragment of ferric iron at the heme-binding site blocks the enzyme's natural substrate, lanosterol, disrupting the biosynthetic pathway [4]. Amino acid substitutions in the drug target are a common mechanism of resistance to azoles in *Candida.* More than 140 substitutions have been reported in the resistant strains, many of which have an additive effect [5]. The most frequent alterations in *C. albicans* occur in two positions: R467K and G464S, which are in the close proximity to the heme-binding site [5, 6].

According to published data, overexpression of the *ERG11* is common among azole-resistant clinical isolates of *C. albicans* [7–9]. This directly contributes to the development of resistance, since an increase in the number of targets requires higher concentrations of the drug for inhibition [8], reducing its overall sensitivity. Overexpression of the *ERG11* has also been reported for azole-resistant isolates of other *Candida* species – *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei* [10–14]. The mechanism of overexpression in these species and its contribution to azole resistance remains largely unknown.

The most common mechanism of fungal resistance is the activation of membrane efflux pumps, which recognize

Table 1
Classification of major antifungals (according to A. K. Sahoo et al.) [3]

Group	Examples	Mechanism of action
Polyenes	nystatin, levorine, natamycin, amphotericin B	Fungistatic and fungicidal action by binding of the drug to ergosterol on the fungal cell membrane and thus destabilizing it
Azoles: *Imidazoles* *Triazoles*	ketoconazole, clotrimazole, miconazole, bifonazole, econazole, isoconazole, oxiconazole itraconazole, fluconazole, voriconazole, posaconazole, ravuconazole, pramiconazole, albaconazole	Fungistatic (less often – fungicidal) action by inhibiting 1,4*α*-demethylase that catalyzes the conversion of lanosterol into ergosterol
Allylamines	terbinafine, butenafine, naftifine	Fungicidal action by inhibition of the ergosterol synthesis. Unlike azoles, allylamines block earlier stages of biosynthesis by inhibiting the enzyme squalene epoxidase
Echinocandins	caspofungin, anidulafungin, micafungin, aminocandin	Fungistatic and fungicidal action by inhibiting synthesis of 1,3-*β*-D-glucan in the cell wall
Pyrimidines	flucytosine	Fungistatic and fungicidal effects associated with inhibited nucleic acid/protein synthesis
Sordarin derivatives	GR135402, GM237354	
Drugs of different groups		
	griseofulvin	Fungistatic effect by inhibition of cellular mitotic activity in metaphase and disruption of DNA synthesis
	amorolfine	Fungistatic and fungicidal action caused by damage of the fungal cell membrane structure
	nikkomycin	Fungistatic and fungicidal action by blocking chitin formation

various chemicals and contribute to multidrug resistance. Fungi have several different drug efflux systems that are encoded by at least ten different genes. Mutations in each of these genes can also affect the degree of pathogen resistance to the drug. In addition, multiple genomic alterations are associated with azole resistance, such as loss of heterozygosity in certain genomic regions, increase in the number of copies of chromosomes, as well as segmental or chromosomal aneuploidies [15]. The loss of heterozygosity is common for the regions containing genes that determine azole resistance, including *ERG11*. Analysis of *C. albicans* isolates with developed resistance revealed that mutations in these genes often arise in the heterozygous state and, then, become homozygous [16]. The prevalence of aneuploidies in azole-resistant isolates raised the question of whether azole exposure simply selects for the more resistant aneuploid variants or whether azole exposure contributes to the development of aneuploidies. It was found that the exposure to azole initiates aberrant cell cycle regulation with a tetraploid intermediate, which, ultimately, precedes the formation of aneuploidy in *C. albicans* [17].

It must be said that nowadays, the gene expression analysis is most widely used to study azole-resistance mechanisms. The technique is based on the comparison of the expression levels of certain genes in azole-susceptible and azole-resistant strains. However, to carry out this analysis, it is necessary to have fresh *C. albicans* cultures grown on a nutrient medium under the same conditions.

Thus, the development of resistance to azoles is a complex process that involves different biochemical processes and gene combinations.

Allylamines is another group of drugs, to which resistance is often evolved. Among its representatives, terbinafine is most actively used worldwide to treat many diseases caused by different species of dermatophyte fungi. The terbinafine's target is squalene epoxidase (SQLE), responsible for ergosterol biosynthesis that catalyzes the conversion of squalene into 2,3-oxidosqualene. Terbinafine inhibits squalene oxidase activity, resulting in ergosterol depletion and squalene accumulation [18]. Mutations in the SQLE gene that give rise to amino acid substitutions lead to structural changes and decreased binding ability of terbinafine to the protein without causing dysfunction in ergosterol biosynthesis [19].

The mechanism of resistance to this allylamine among *Trichophyton* species is attributed to single nucleotide polymorphism (SNP) mutations in the SQLE gene. Point mutations in the SQLE gene lead to single amino acid substitutions at one out of four positions (Leu^{393}, Phe^{397}, Phe^{415}, His^{440}), which corresponds to the increased values of minimum inhibitory concentrations (MICs) for terbinafine *in vitro* [20]. On the contrary, newly occurring missense mutations in the SQLE gene lead to H440Y/F484Y and I121M/V237I substitutions, which were detected in low-resistant isolates [21].

In the study by Yamada T. et al., the group tested 2,056 isolates of *T. rubrum* and *T. interdigitale* for their susceptibility to terbinafine. The authors found that only 17 of them (less than 1%) were resistant to this antifungal agent. By analyzing the genetic sequence of the SQLE gene, they found point mutations in four positions responsible for the exhibiting resistance phenotype. In addition

to L393F and F397L, seven new mutations were identified, including one in the L393 residue and two in the F397 residue. These mutations were studied through the expression of the corresponding amino acid substitutions using *T. mentagrophytes* as a recipient organism. The strains that harbored mutated genes were less susceptible to terbinafine. At the same time, proteomics revealed no other significant differences between the mutated strains and the ones in the control group, indicating that the increased terbinafine resistance was caused by mutations [20].

The aim of the work was to evaluate the possibility of identifying azole-resistant strains of *C. albicans* and terbinafine-resistant strains of *Trichophyton* spp. by using molecular-genetic methods without assessing the expression levels of certain genes.

MATERIALS AND METHODS

In the study, ten isolates of *C. albicans* were taken from cattle and dogs, isolates of *T. interdigitale* and *T. rubrum* were taken from nail plates of individuals with onychomycosis signs, isolates of *T. verrucosum* were taken from the cattle wool, and isolates of *T. mentagrophytes* were taken from the coat of dogs with the signs of dermatomycosis.

The susceptibility (MICs) of *C. albicans* to fluconazole (FLU), itraconazole (ITR), and voriconazole (VRC) was determined in accordance with EUCAST E.Def 7.3.2 [22]. The MICs were interpreted in accordance with Breakpoint tables for interpretation of MICs for antifungal agents, version 10.0, 2020 (FLU: MIC more than 4.0 mg/L – resistant, from 2.0 to 4.0 mg/L – susceptible at increased exposure, less than 2.0 mg/L – susceptible; ITR: MIC more than 0.06 mg/L – resistant, less than 0.06 mg/L – susceptible; VRC: MIC more than 0.25 mg/L – resistant, from 0.06 to 0.25 mg/L – susceptible at increased exposures, less than 0.06 mg/L – susceptible) [23].

The isolate was identified as "resistant" or "sensitive" to azoles using the following model: an isolate resistant to three drugs was considered as resistant; an isolate resistant or showing intermediate resistance to one or two drugs was considered was considered to exhibit dose-dependent sensitivity; an isolate susceptible to all drugs was considered as sensitive to azoles.

Data on *C. albicans* isolates, their susceptibility to azole drugs, and characterization of their susceptibility are given in Table 2.

The susceptibility of *Trichophyton* to terbinafine (TBF) was determined by the method developed in the Department of Mycology in FSBI "VGNKI", based on EUCAST E.Def 9.3.1 [24]. Susceptibility was interpreted on the basis of MIC ranges for terbinafine (mg/L): more than 32 mg/L – resistant, from 16 to 32 mg/L – exhibit dose-dependent sensitivity, less than 16 mg/L – sensitive.

Data on *T. verrucosum*, *T. mentagrophytes*, *T. interdigitale* and *T. rubrum* isolates, their susceptibility to antifungal drugs and characterization of their susceptibility to the drug are given in Table 3.

DNA was extracted using a commercial kit "DNA-sorb-C-M", and amplification was performed using reagents manufactured by AmpliSens® (FBIS CRIE, Russia) by "Tertsik" machine (DNA Technology LLC, Russia).

To amplify DNA of *C. albicans,* primers described earlier by M.-K. Lee et al. [25] were used. To identify *Trichophyton* resistance to terbinafine, the following selected primers for amplification of the informative site of the SQLE gene were used: Tr-terb-F 5′-CTTAGTCCAGAGGCCGTACC-3′ and Tr-terb-R 5′-AGGATGACCCTGCAGGCAGT-3′. Cycles of polymerase chain reaction for *Trichophyton* isolates were as following: one cycle at 95 °C for 5 min, then 42 cycles at 95 °C for 10 sec., 60 °C for 10 sec., 72 °C for 10 sec., then one cycle at 72 °C for 1 min.

The Sanger sequencing was performed for the amplification of fragments using ABI PRISM Big Dye v. 1.1 cycle

Table 2
Data on *Candida albicans* isolates used in the study

Isolate	Source	Minimum inhibitory concentration, mg/L			Characterization
		FLU	ITR	VRC	
C. albicans No. 1	cattle, udder	4.0	0.125	0.5	resistant
C. albicans No. 2	cattle, udder	4.0	0.125	0.5	resistant
C. albicans No. 3	cattle, udder	8.0	0.25	0.5	resistant
C. albicans No. 4	cattle, milk	8.0	0.125	0.5	resistant
C. albicans No. 5	dog, stomatitis	4.0	0.125	0.5	resistant
C. albicans No. 6	dog, stomatitis	1.0	0.03	0.03	sensitive
C. albicans No. 7	cattle, milk	2.0	0.06	0.25	dose-dependent
C. albicans No. 8	cattle, udder	2.0	0.06	0.25	dose-dependent
C. albicans No. 9	cattle, udder	1.0	0.03	0.03	sensitive
C. albicans No. 10	cattle, milk	2.0	0.06	0.125	dose-dependent

Table 3
Data on dermathophyte isolates used in the study

No.	Isolate	Source	Minimum inhibitory concentration TBF, mg/L	Characterization
1	*T. verrucosum* No. 1	cattle wool	16	dose-dependent
2	*T. verrucosum* No. 2	cattle wool	16	dose-dependent
3	*T. verrucosum* No. 3	cattle wool·	16	dose-dependent
4	*T. verrucosum* No. 4	cattle wool·	16	dose-dependent
5	*T. mentagrophytes* No. 1	dog's coat	64	resistant
6	*T. mentagrophytes* No. 2	dog's coat	32	dose-dependent
7	*T. mentagrophytes* No. 3	dog's coat	32	dose-dependent
8	*T. interdigitale* No. 1	human nails	64	resistant
9	*T. interdigitale* No. 2	human nails	64	resistant
10	*T. interdigitale* No. 3	human nails	64	resistant
11	*T. interdigitale* No. 4	human nails	8	sensitive
12	*T. interdigitale* No. 5	human nails	16	dose-dependent
13	*T. rubrum* No. 1	human nails	64	resistant
14	*T. rubrum* No. 2	human nails	64	resistant
15	*T. rubrum* No. 3	human nails	64	resistant

sequencing kit (Applied Biosystems, USA), according to the manufacturer's instructions with the use of automated capillary sequencer ABI-3100 PRISM Genetic Analyzer (Applied Biosystems, USA). The obtained chromatograms were analyzed using the Chromas software. The nucleotide sequences were analyzed using the Vector NTI Advance 11.5.0 program.

The obtained nucleotide sequences of *C. albicans* were compared with X13296 sequence from the GenBank database, which was recognized as a reference sequence for fluconazole-susceptible isolate in the article by M.-K. Lee et al. [25]

The obtained nucleotide sequences of the *Trichophyton* genome fragment were checked for the presence of point mutations responsible for amino acid substitution Leu393Phe, Leu393Ser (nucleotide position 1177 in the gene SQLE), Phe397Leu, Phe397Ile, Phe397Val (nucleotide position 1189 in the gene SQLE), Phe415Ile, Phe415Ser, Phe415Val (nucleotide position 1305 in the gene SQLE), His440Tyr (nucleotide position 1380 in the gene SQLE) [20].

RESULTS AND DISCUSSION

Analysis of C. albicans resistance to azoles. Nucleotide sequences of the *ERG11* gene were obtained for all 10 isolates of *C. albicans*. The resistance of *C. albicans* to azole drugs was evaluated, based on the analysis of both nucleotide and amino acid sequences of the *ERG11* gene.

The obtained results of comparing the isolate nucleotide sequences with the reference sequence are presented in Table 4.

Substitutions in only 10 out of 30 analyzed nucleotide positions in the *ERG11* gene lead to changes in the amino

Table 4
Amino acid and nucleotide substitutions in the *ERG11* gene of *C. albicans* strains

Amino acid substitution	Reference nucleotide in X13296	Isolates of *C. albicans*									
		1	2	3	4	5	6	7	8	9	10
		R	R	R	R	R	S	M	M	S	M
F105F	T	C	C	C	C	C	.	C	C	.	C
D116E	T	.	**A**	**A**	**A**	**A**	.	**A**	.	.	.
K119K	A	.	G	G	G	G	.	G	.	.	.
K128T	A	.	.	C	.	C	.	C	.	.	.
S137S	C	.	.	.	.	.	.	.	.	.	.
K143R	A	.	.	.	.	.	.	.	.	.	.
H183H	T	.	.	.	.	.	C	C	C	C	C
L220L	C	T	T	T	T	T	T	T	T	T	T
E266D	A	**C**	**C**	.	**C**	.	.	.	**C**	.	**C**
L320L	G	.	.	.	.	.	.	.	.	.	.
V332V	T	N	.	.	.	.	C	.	C	C	C
L340L	A	.	G	.	.	G	.	G	.	.	.
K342K	A	G	.	.	.	.	.	.	G	.	G
S361S	A	.	.	.	.	.	G	.	.	G	.
L370L	C	T	T	T	T	T	T	T	T	T	T
F380F	T	.	.	.	.	.	.	.	.	.	.
Y401Y	T	.	.	.	.	.	.	.	.	.	.
V404I	G	.	.	.	.	.	.	.	.	.	.
P419P	T	.	.	.	.	.	.	.	.	.	.
D428D	T	.	.	.	.	.	.	.	.	.	.
V437I	G	.	.	.	.	.	.	.	.	.	.
G448G	G	.	.	.	.	.	.	.	.	.	.
F449V	T	.	.	.	.	.	.	.	.	.	.
V452V	T	.	.	.	.	.	.	.	.	.	.
V456V	T	.	.	.	.	.	.	.	.	.	.
R467K	**G**	.	.	.	.	.	.	.	.	.	.
Q474K	**C**	.	.	**A**	.	**A**	.	.	**A/C**	.	**A/C**
L480L	A	.	G	G	G	G	G/A	G	G/A	G	G/A
N490N	T	.	C	C	C	C	C/T	C	C/T	C	C/T
V509M	G	.	.	.	.	.	.	.	.	.	.

Positions that lead to amino acid substitutions are emphasized in bold.
R – azole-resistant; S – azole- sensitive; M – exhibit dose-dependent sensitivity

acid sequence. Among studied *C. albicans* isolates, no amino acid substitutions were observed in azole-susceptible isolates № 6 and № 9 in this gene region, and in four out of five isolates, substitutions in the amino acid sequence were observed in 2 out of 10 positions. The most informative positions were **D116E** and **E266D**.

As indicated in the Table 4, the obtained data generally corresponds to declared phenotypic resistance of the strains. Interestingly, even a single amino acid substitution in the *ERG11* gene, detected in isolate № 1, was associated with the development of resistance to azole drugs. However, the study failed to differentiate fully azole-resistant and dose-dependent strains. In dose-dependent isolates two substitutions were also identified: one in position **E266D** and the other in position **Q474K** in isolates № 8 and № 10 in the heterozygous state.

Marichal P. et al. in their work [26] identified three "hotspot regions" in the amino acid sequence of the *ERG11* gene, based on the compilation of the *ERG11* mutations, which are reported to be associated with azole-resistance. These "hot spots" include amino acid regions 105–165, 266–287, and 405–488. The detected nucleotide substitutions fall within these "hotspot regions", which can serve as the evidence of the reliability of the obtained results.

Analysis of resistance to terbinafine in *Trichophyton* spp. For this part, primers were selected for the amplification and subsequent sequencing of the 440 bp SQLE gene fragment, covering all positions of single-nucleotide substitutions leading to the development of resistance to terbinafine. 12 *Trichophyton* isolates, both resistant and sensitive to terbinafine, were analyzed, based on mycological test results.

The analysis of obtained chromatograms and multiple sequence alignment using the Vector NTI Advance 11.5.0 program, all isolates were identified as terbinafine-sensitive. In 2 out of 12 isolates, a point mutation was detected at the position 1,177 (substitution of TTA with CTA), but it is not significant and does not lead to amino acid substitution.

The discrepancy in the data on terbinafine resistance obtained by molecular-genetic and cultural methods can be explained by the appearance of additional resistance mechanisms, which can be observed in the strains. This hypothesis is confirmed by the numerous studies of the fungal resistance to antimycotic agents. For example, azole resistance in clinical isolates of *C. albicans* is associated with several mechanisms, including missense mutations in the *ERG11* and overexpression of genes encoding carrier proteins of several drugs. Combined effects of such mechanisms were observed in the same clinical isolate resistant to azoles [27]. It is possible that overexpression of genes encoding efflux pumps is also involved in terbinafine resistance in the studied isolates.

CONCLUSION

The widespread use of azole and allylamine compounds in the treatment and prevention of mycoses contributes to the emergence of strains and isolates resistant to these antifungal drugs. The resistance is conventionally tested using cultural methods, however, for certain types of fungi that are important in the field of veterinary medicine, there are no standards and control points used for classification of isolates into "susceptible" or "resistant", which makes it difficult to interpret the clinical effects of a minimum inhibitory concentration. The current goal is to find reliable methods for detecting resistance, based on the genetic analysis. This paper describes methods for detecting the resistance of *C. albicans* to azoles and of *Trichophyton* dermatophytes to terbinafine by analyzing certain genes. It should be noted that the results obtained so far indicate that the analysis of nucleotide sequences of *C. albicans ERG11* gene and SQLE gene of *Trichophyton* fungi has limited informative value for predicting resistance to antifungal drugs (azoles and terbinafine, respectively). The obtained information on the structure of these genes can be used for the characterization of strains. However, in order to increase the reliability of molecular-genetic tests for resistance, it is necessary in the future studies to use more fungal isolates characterized phenotypically, and include several genes associated with antimycotic resistance in the analysis.

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Received 14.09.2021
Revised 20.10.2021
Accepted 11.11.2021

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