Sensitivity of antifungal preparations of Candida isolates from sub-biotopes of the human oral cavity

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Introduction
Among the broad diversity of microorganisms, which as representatives of microbiota colonize sub-biotopes of the oral cavity in practically healthy people and patients with different pathological conditions, Archaea, bacteria and eukaryotes are distinguished (Kron et al., 2014; Lof et al., 2017). The oral cavity is an open system to the environment depending on the geographical living location, time of the day of sampling, diet, condition of the immune system (Costalonga et al., 2014). Over the recent decades, fungal diseases have rapidly increased in patients infected by the human immunodeficiency virus (HIV), and in patients receiving intensive chemo-and radiation therapy, or prolonged administration of broad-spectrum antibiotics (Aslani et al., 2018; Vipulanandan et al., 2018). The indicated factors have a cytotoxic effect on the immune protective mechanisms of the mucous membrane, change the composition of the microbiota of the oral cavity, causing xerostomia and hyposalivation. Impaired homeostasis of the oral cavity intensifies internal oral colonization, causing fungal infection. Potential pathogenicity for humans is posed by over 100 species of fungi, nosocomial infections are caused by no more than 20, of which the representatives of the Candida genus prevail (Cho et al., 2014). Candidiasis is an opportunistic infection often diagnosed in patients with oncopathology due to immune-suppression (Aslani et al., 2018; Vipulanandan et al., 2018). The literature sources indicate that the incidence of oral colonization by representatives of Candida ranges 43% to 90% among cancer patients (Jain et al., 2016; Aslani et al., 2018).

Candida albicans is the commonest fungus isolated during infections of the oral cavity of practically healthy persons and patients with pathology of different etiology (Montelongo-Jauregui et al., 2018). The studies indicate that in 78.0% of cases the cause of oral mucositis in cancer patients was C. albicans. This species remains the main source of diseases associated with immune suppression, despite the use of antifungal therapy, and can cause systemic infection related to the significant morbidity and mortality (de Sousa et al., 2016; Vaezi et al., 2017). The current tendency to the development of fungemia shows that in most cases this was caused by the species of Candida different from C. albicans, especially among patients of hematological, transplantation and intensive care departments (Taj-Aldeen et al., 2014).

Currently, there is recognition of the role played by species of Candida non-albicans (NACS) in the oral cavity as commensals and etiological agents of infection, including some interactions between different species of yeast-like fungi of the Candida genus (Rossoni et al., 2017).
Materials and methods

The studies were performed at the Department of Virology and Immunology of the O. O. Bohomolets National Medical University, O. O. Bohomolets Dental Medical Center, National Cancer Institute.

To achieve our goals, we examined a group of 50 oncology patients (experimental group) aged 18 to 78 (27 women, 23 men) and 73 practically healthy patients aged 18 to 50 (51 women, 22 men). Among pathologies, we diagnosed: lung cancer (27 patients), mammary gland cancer (8 patients), stomach cancer (9 patients), esophagus cancer (6 patients). Over the study, the examined patients received chemotherapy (26 patients), a complex of radiotherapy and chemotherapy (7 patients), 11 patients underwent surgical treatment, 6 patients were diagnosed for the first time. In total, 492 sub-biopoeses of the oral cavity were examined (200 – experimental group, 292 – control). We isolated 218 isolates (101 – experimental group, 117 – control).

The sensitivity of isolates towards the derivatives of alkyl (aryloxyethoxy) dialkylaminopropanol of the general formula:

\[
R_1(O(CH_2)O)_n\overset{+}{\text{N}}R_2(R_3\overset{-}{\text{Cl}})
\]

where \(R_1\) = 4-(1,1,3,3-tetramethylbutyl)phenoxy, \(R_2\), \(R_3\) = -(4-CH(CH_2)CH_3), \(R_4\) = -benzyl, \(n = 2, \text{Kc-2;}
\)

\(R_1\) = 4-(1,1,3,3-tetramethylbutyl)phenoxy, \(R_2\) – methyl, \(R_3\) – cyclohexyl, \(R_4\) = -benzyl, \(n = 2, \text{Kc-3;}
\)

\(R_1\) = 4-(1,1,3,3-tetramethylbutyl)phenoxy, \(R_2\), \(R_3\) = -(CH_2)6, \(R_4\) – benzyl, \(n = 2, \text{Kc-14;}
\)

\(R_1\) = 4-(1,1,3,3-tetramethylbutyl)phenoxy, \(R_2\), \(R_3\) = -(CH_2)6, \(R_4\) – benzyl, \(n = 2, \text{Kc-14;}
\)

\(R_1\) = 4-(1,1,3,3-tetramethylbutyl)phenoxy, \(R_2\), \(R_3\) = -(methyl), \(R_4\) = -(4-F-benzyl), \(n = 2, \text{Kp-18;}
\)

\(R_1\) = 4-(1,1,3,3-tetramethylbutyl)phenoxy, \(R_2\), \(R_3\) = -(methyl), \(R_4\) = -(4-methylbenzyl), \(n = 2, \text{Kp-19;}
\)

\(R_1\) = 4-(1,1,3,3-tetramethylbutyl)phenoxy, \(R_2\), \(R_3\) = -(methyl), \(R_4\) = -(4-F-benzyl), \(n = 0, \text{Kp-4;}
\)

\(R_1\) = 2-dibutylphenoxy, \(R_2\), \(R_3\) = -(CH_2), \(R_4\) = -(CH_2) in, \(n = 0, \text{Kp-8;}
\)

\(R_1\) = 2-dibutylphenoxy, \(R_2\), \(R_3\) = -(CH_2), \(R_4\) = -(CH_2) in, \(n = 2, \text{Kc-15;}
\)

\(R_1\) = 2-dibutylphenoxy, \(R_2\), \(R_3\) = -(CH_2), \(R_4\) = -(CH_2) in, \(n = 2, \text{Kc-16;}
\)

\(R_1\) = 2-dibutylphenoxy, \(R_2\), \(R_3\) = -(CH_2), \(R_4\) = -(CH_2) in, \(n = 2, \text{Kc-22;}
\)

The surveyed cultures were synthesized at the Institute of Chemistry of NAS of Ukraine by Candidate of Chemical Sciences Korotkyi Y. V. For obtaining compounds, target-oriented synthesis was made. We obtained 1-[4-(1,1,3,3-tetramethylbutyl)phenoxy-1-ethyloxy]-2-epoxypropane in the conditions of intra-phase catalysis (50.0% NaOH, tetrahydroammonium chloride, epichlorohydrin). Then, in alcohol solution, this substance interacted with secondary amines, producing 1-[4-(1,1,3,3-tetramethylbutyl)phenoxy-1-ethyloxy]-3-dialkylamino)-2-propanol. While heating in acetone, this substance was treated (isopropanol, acetonitrile) with halogenos alkyis, producing final compounds (Korotkyi & Smertenko, 2013). As commercial preparations, we compared amphotericin B and derivatives of azoles (fluconazole, itracozazole).

The material was collected from four sub-biopoeses of the oral cavity: the mucous membrane of the cheek (retromolar area), dorsal surface of the tongue, angle of the mouth (border of the mucous membrane and skin), area of the palate. The material was collected using sterile cotton swabs on wooden sticks. The studied biological material was inoculated onto the Sabouraud agar with addition of antibiotic (levomycetin in the concentration of 0.05 g/L) and cultivated at 30°C over 5 days. Pure culture of fungi was inoculated to Petri dishes with potato carrot agar and rice agar and incubated for 3 days at the temperature of 37°C. During the growth, we observed formation of chlamydospores which are a distinctive feature of C. albicans. Pure culture was inoculated onto the chromogenic agar for selective isolation of yeasts and direct identification (HiCrome Candida Agar). After 48 h, at the temperature of 37°C the colonies of C. albicans were light-green, C. tropicalis – light-blue, C. krusei – white, C. glabrata – pink. Parallel identification was conducted using test-systems ID 32 test strips manufactured by bioMerieux company, France.

To determine the adhesive properties of the cultures isolated from the sub-biopoes of the oral cavity, we used generally the method introduced by Brilis et al. (1986). The method involves formalinized erythrocytes of humans with 0 (I) group of blood with positive Rh which is maximally approximated to the model of live structures of the human organism. The advantages of this method is complete in vivo correlation of the results with adhesion, because the survey on adhesion is conducted on cells of human origin; simplicity of obtaining erythrocytes in required amounts; a possibility of surveying a large number of the cultures, and performing express-analysis.

The erythrocytes were formalinized as follows: to fresh defibrinated human blood with Rh positive 0 (I) group diluted with normal saline (pH 7.2) in the proportion (1:1) we added a mixture comprising 20 mL of 40.0% formalin and 20 mL of double phosphate buffer (pH 7.2). These components were accurately mixed and incubated at the temperature of 37 °C over 2 h, carefully shaken every 15 min. After the incubation the erythrocytes were four times rinsed with normal saline using centrifugation for 10 min at 1,000 rpm. After rinsing, the erythrocytes were suspended in 400 mL of buffer and kept at 4 °C for 48 h. The supernatant was decanted, and the sediment was re-suspended in 400 mL buffer and then again kept at 4 °C for 48 h. After the repeated sedimentation, 50% suspension on buffer solution with 1% formalin was prepared from the sediment of erythrocytes. For preparation of 1% solution of formalin, we used 2.7 mL of 37% of industrial formalin, added 97.3 mL of phosphate buffer, obtaining 100 mL of 1% formalin. Before using erythrocytes, they were rinsed twice with 0.1 M solution of sodium phosphate and centrifuged at 1,000 rpm. On the buffer solution, we prepared the suspension of erythrocytes with concentration of 10^6 cells/mL. In order to perform the experiment, the surveyed culture was cultivated for 24 h on Sabouraud agar, and then, using 0.85% solution of NaCl, suspension of microorganisms was prepared in the concentration of 10^3 cells/mL. To the test tubes we added 0.5 mL of suspension of formalinized erythrocytes and 0.5 mL of the prepared suspension of microorganisms. The mixture was incubated for 30 min in 37 °C, being periodically shaken. Then, on a microscope slide we prepared a smear, dried it at room temperature, fixed and stained it using the method of Pappenheim. The adhesion was evaluated under the light microscope.

Interpretation of the results was performed based on the index of adhesiveness of microorganisms (IAM). IAM – average number of microbial cells which underwent adhesion on one erythrocyte that took part in the adhesion process.

This indicator was calculated using the formula: IAM = (AAI × 100 / PRRBC, where AAI – average adhesive indicator (i.e. average number of microorganisms which attached to one erythrocyte), PRBBRC – participation rate of red blood cells in adhesion (percentage of erythrocytes which had microorganisms that underwent adhesion on their surface). The microorganism consider as non-adhesive when IAM 1.75; low-adhesive 1.76–2.50; average-adhesive 2.51–4.00; and high-adhesive when IAM more than 4.00.

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The research on susceptibility of clinical isolates to antibiotics and newly-synthesized compounds was performed using the microdilution method of serial dilutions with the purpose of determining minimum inhibiting concentration (MIC) in liquid growth medium RPMI 1640 (Arendrup et al., 2015). For the experiment, we used daily culture of *Candida* grown in liquid glucose-containing medium. For the statistical comparison we used non-parametric Mann-Whitney U test. The results were statistically analyzed using program pack Statistica 8.0 (Statsoft Inc., USA). The data were presented in the form x ± SD (x ± standard deviation). For the comparison of independent selections, we used non-parametric Mann-Whitney U test. The results were considered reliable at values P < 0.05.

**Results**

Frequency of detecting representatives of *Candida* genus in the sub-biotopes of the oral cavity of patients with oncopathologies equaled 72.0%. The level of candidal carriage was 25.0%, and 47.0% were diagnosed as having candidiasis. The level of candidal carriage in the oral cavity among practically healthy patients without clinical signs was 56.4%. Among the representatives of the *Candida* genus, in the contents of the biotopes of the oral cavity the following were found: *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, both among the strains isolated from patients with oncopathology, and people of the control group. Among the strains of the control group, 76.0% were *C. albicans*, 12.8% – *C. glabrata*, 10.3% – *C. krusei*, 0.9% – *C. tropicalis*. Among the isolated clinical isolates, 70.3% were *C. albicans*, 9.9% – *C. glabrata*, 12.9% – *C. krusei*, 6.9% – *C. tropicalis* (Table 1).

| Species | Sub-biotopes of the oral cavity | angle of the mouth | sub-biotope of the tongue | mucus membrane of the cheek | area of the palate | *E* | *C* | E | C | E | C | E | C |
|---------|--------------------------------|------------------|--------------------------|------------------------|-----------------|-----|----|---|---|----|---|----|---|
| *C. albicans* | 60.00 | 73.3 | 77.3 | 80.6 | 69.6 | 71.4 | 81.0 | 73.3 |
| *C. glabrata* | 14.3 | 15.6 | 4.6 | 8.3 | 8.7 | 21.4 | 9.5 | 9.1 |
| *C. tropicalis* | 8.6 | 0.0 | 9.1 | 0.0 | 8.7 | 7.2 | 0.0 | 0.0 |
| *C. krusei* | 17.1 | 11.1 | 9.1 | 11.1 | 13.0 | 0.0 | 9.5 | 13.6 |
| Total | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |

**Table 1** Species range of yeast-like fungi of *Candida* genus isolated from the examined patients (%)

Highest level of *Candida* colonization among the sub-biotopes in patients with oncopathology was observed on the mucus membrane of the wall of the tongue (Fig. 1). During research on the posterior wall of the tongue in cancer patients, we isolated 33 strains. Out of them, 21 (60.0%) were *C. albicans*, 6 (14.3%) – *C. glabrata*, 6 (14.3%) – *C. krusei*, 3 (8.6%) – *C. tropicalis*. The parameter of the semination of this sub-biotope equaled 34.7%. In this sub-biotope we observed a singular case of association of *C. glabrata* and *C. krusei*. In the comparison group, no significant differences were observed. However, in the control group, from the wall of the tongue we isolated *C. tropicalis*. The lowest level of semination was seen in the zone of the palate, equaling 22.8%. From that sub-biotope we isolated 23 strains from the patients with oncopathology, including *C. albicans* accounting for 16 (69.6%), *C. glabrata* – 2 (8.7%), *C. krusei* – 3 (13.0%), *C. tropicalis* – 2 (8.7%). Associations of fungi of this species were not observed. The control group was observed to have a much lower level of colonization of the palate. No *C. krusei* was isolated from practically healthy patients. The level of colonization with fungi of *Candida* genus was noted on the mucus membrane of the cheek accounting for 21.8%. In that sub-biotope, 22 strains were isolated, including *C. albicans* equaling 17 (77.3%), *C. glabrata* – 1 (4.5%), *C. tropicalis* – 2 (9.1%), *C. krusei* – 2 (9.1%). Association of fungi of this genus was observed in two cases. Fungi *C. albicans* and *C. tropicalis* were also seen co-existing. In that sub-biotope, among practically healthy people, no *C. tropicalis* were isolated. In the sub-biotope of the angle of the mouth, the incidence of representatives of *Candida* genus was 20.8%. A total of 21 strains was isolated from the patients suffering from oncopathologies, including *C. albicans* accounting for 17 (81.0%), *C. glabrata* – 2 (9.5%), *C. krusei* – 2 (9.5%). We observed a single case of association of *C. albicans* and *C. glabrata*. In the control and experimental groups, no strains of *C. tropicalis* were isolated. Thus, among all the isolated strains, the prevailing one in all the 4 sub-biotopes was *C. albicans*. In 4 sub-biotopes, associations of two species of *Candida* were found.

![Fig. 1](Image 313x394 to 532x531)

**Fig. 1.** Incidence of fungi of *Candida* genus in the sub-biотopes of the oral cavity of the patients with oncopathologies and practically healthy people (AM – angle of the mouth, ZP – zone of the palate, ST – surface of the tongue, MMC – the mucus membrane of the cheek) grey – experimental group (patients with oncopathologies), black – control group (practically healthy people)

The results of evaluation of adhesion revealed that regardless of the sub-biotope the highest parameter of IAM among the clinical strains belonged to *C. albicans* accounting for 3.16 ± 0.02. The representatives of non-*albicans* group exhibited less active ability to adhesion: *C. tropicalis* – 2.45 ± 0.76, *C. glabrata* – 2.51 ± 0.03, *C. krusei* – 2.37 ± 0.08. The survey on the minimum inhibiting concentration of commercial antifungicotics and derivatives of alkyl (aryl oxy ethoxy) diakil-amipropionat towards clinical isolates of the *Candida* allowed us to determine the level of activity of this group of preparations against these yeast-like fungi, and thus to compare these indicators with museum strains. The effectiveness of the examined compounds varied depending on the strain and species. The synthesized compounds exerted higher efficiency compared with the commercial preparations. Sensitivity of *Candida* to the substances and preparations with antifungicotic effect was tested towards the representatives of *albicans* and non-*albicans*. According to the results of the study, non-*albicans* displayed higher resistance to this group of preparations (Table 2).

The data demonstrated in Figures 2 and 3 show that the studied substances significantly differed by the level of antifungicotic effect against *C. albicans* and non-*albicans*. Among the studied newly-synthesized compounds, Kp19 exhibited the highest activity towards the representatives of non-*albicans*, the concentration of 0.97 ± 0.71 µg/mL inhibited 83.0% of the studied strains. Average value of the inhibiting activity of the compound against the museum strain equaled 3.25 ± 0.65 µg/mL. Signifi-
The latter exhibited antimycotic effect only in the initial concentration of 1.0 ± 0.74 µg/mL.

Clinical isolates of *C. albicans* were observed to have higher sensitivity to antifungal agents. Among the alkyl acyl amino alcohols, the highest activity was demonstrated by compound Kc15. MIC of Kc15 for 42.0% of strains equaled 0.24 ± 0.07 µg/mL, for 50% – 0.48 ± 0.14 µg/mL. Compound Kp4 had lower antifungal effect, killing 33.0% of the strains with the concentration of 0.24 ± 0.07 µg/mL. The concentration of 0.48 ± 0.14 µg/mL exhibited antifungal effect against 58.0% of *Candida*. Compounds Kk2, Kk3, Kk5 and Kk6 in the concentrations of 0.48 ± 0.14 µg/mL displayed antifungal action against 50.0% of the strains. Against *C. albicans* ATCC 10231, the MIC of the compounds equaled 3.25 ± 0.65 µg/mL for Kk3 and 1.30 ± 0.33 µg/mL for Kk15. These indicators were most efficient regarding the reference strain of *C. albicans*. The inhibiting effect of Kk22 against the reference strain was 2.60 ± 0.86 µg/mL. Among all commercial antifungal preparations the antifungal effect was exerted by amphotericin B. Activity of this preparation was seen in the concentration of 0.12 ± 0.3 µg/mL. A total of 50.0% of the strains were inhibited by the dose of 0.48 ± 0.14 µg/mL. Fluconazole in the concentration of 0.97 ± 0.34 µg/mL killed 25.0% of strains. To inhibit 50.0% of clinical isolates, a concentration of over 31.25 ± 6.11 µg/mL is needed. Antimycotic action of itraconazole took no significant effect.

### Table 2

Minimum inhibiting concentration of the derivatives of amino alcohols and antifungal agents (µg/mL, x ± SD, n = 3, P < 0.05)

| Preparation               | *C. albicans* ATCC 10231 | *C. albicans* (clinical strains) | *C. non-albicans* (reference strains) | *C. non-albicans* (clinical strains) |
|---------------------------|--------------------------|----------------------------------|---------------------------------------|-------------------------------------|
| Kc2                       | 1.62 ± 0.33              | 5.26 ± 2.71                      | 9.12 ± 3.91                           | 16.03 ± 5.49                       |
| Kk3                       | 3.25 ± 0.65              | 4.29 ± 1.73                      | 10.42 ± 6.51                          | 13.27 ± 4.67                       |
| Kc14                      | 2.92 ± 0.98              | 3.92 ± 1.39                      | 7.81 ± 3.91                           | 24.09 ± 9.36                       |
| Kk15                      | 1.30 ± 0.33              | 4.94 ± 2.57                      | 9.77 ± 4.56                           | 34.51 ± 18.87                      |
| Kc16                      | 2.27 ± 0.87              | 5.61 ± 2.71                      | 19.53 ± 11.72                         | 17.41 ± 10.21                      |
| Kk22                      | 2.60 ± 0.86              | 3.11 ± 1.80                      | 27.35 ± 6.32                          | 12.12 ± 4.15                       |
| Kp4                       | 2.92 ± 0.65              | 8.83 ± 5.17                      | 23.44 ± 13.02                         | 29.05 ± 11.51                      |
| Kp8                       | 2.92 ± 0.98              | 3.62 ± 1.36                      | 9.77 ± 5.86                           | 18.31 ± 9.94                       |
| Kp19                      | 3.25 ± 0.65              | 4.04 ± 0.88                      | 5.86 ± 3.26                           | 1.70 ± 1.23                        |
| Fluconazole               | 10.4 ± 2.26              | 9.28 ± 4.31                      | 145.8 ± 62.5                          | 140.8 ± 7.47                       |
| Amphotericin B            | 0.40 ± 0.08              | 25.21 ± 20.62                    | 1.54 ± 0.04                           | 49.82 ± 40.34                      |
| Itraconazole              | 145.8 ± 87.5             | 895.8 ± 71.9                     | 30.21 ± 5.21                          | 100.0                              |

![Fig. 2. Sensitivity of *C. non-albicans* to substances with antifungal effect: Kc2, Kc3, Kc14, Kc 15, Kc16, Kc22, Kp4, Kp8, Kp19 – derivatives of amino alcohols; Flc – fluconazole, Amph B – amphotericin B, Itr – itraconazole](image)

![Fig. 3. Sensitivity of *C. albicans* to substances with antifungal effect: Kc2, Kc3, Kc14, Kc15, Kc16, Kc22, Kp4, Kp8, Kp19 – derivatives of amino alcohols; Flc – fluconazole, Amph B – amphotericin B, Itr – itraconazole](image)

### Discussion

In the study we observed spread of candidal carriage among practically healthy people, and also a high parameter of candidiasis infection in patients with oncopathology. Our conclusions coincide with the studies by Jain et al. (2016), Jayachandran et al. (2016). In our population of patients, the level of oral colonization was 72.0%, which corresponds to other similar studies (Aslani et al., 2018). Among the clinical isolates, the prevailing species was *C. albicans*, as well as in the results of surveys by de Sousa et al. (2016), Aslani et al. (2018). In the studies by Jain et al. (2016) the most frequently isolated species was *C. tropicalis*, confirming the theory on spread of the pathogens depending on the geographical locations where patient lives. The main commercial fungus among practically healthy patients was *C. albicans*, correlating with the study by Aslani et al. (2018).

During our studies, we analyzed topographic peculiarities of the colonization of the oral cavity by representatives of *Candida*. In 4 patients with oncopathology we found associations of fungi. On the mucous membrane of the tongue, we isolated association of *C. glabrata* and *C. krusei*, on the mucous membrane of the cheek – *C. albicans* and *C. tropicalis*, on the mucous membrane of the angle of the mouth – *C. albicans* and *C. glabrata*. Studies by Tati et al. (2016) indicate that *C. glabrata* is often found in association with *C. albicans*, especially in patients with immune deficiencies. Detected associations of fungi are relevant and require detailed study.

In the study, we found resistance of clinical isolates to fluconazole, correlating with the studies by Budice et al. (2017), Deorukhkar et al. (2017). All the clinical isolates were resistant to itraconazole, by contrast to the surveys of Zomorodian et al. (2016). Both studies confirm the fact of high resistance of *C. albicans* to itraconazole. The clinical isolates were most susceptible to amphotericin B, as in the surveys by Bagirova & Dmitrieva (2016).

Currently, a tendency towards spread of the candidiasis is observed, therefore the problem of rational therapy requires great attention (Tkačenko & Šklyar, 2017). Observations on the dispensary group showed that patients (63.4%) with pathogen *C. albicans* were diagnosed with relapse of the disease after 2–3 years. Taking this to account, in cases of relapses of the disease, a combined therapy is used (Glazunov, 2015). This fact indicates necessity of determining the sensitivity of each clinical strain to modern antifungics. This would allow rational antifungal therapy and...
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