

Original papers

Secretion of hydrolytic enzymes by fungal strains, isolated from patients with malignant tumors of head and neck, before, during and after radiotherapy[1]

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ABSTRACT. One method of treatment used in cancer therapy is radiotherapy which can injure the oral, pharynx or larynx mucosa and predisposes tissue to the development of fungal infections. The aim of the study paper was the mycological examinations of swabs from the oral cavity and pharynx of patients obtained prior to, in week 3, on the last day of and 3 weeks after radiotherapy, as well as isolation of fungi and identification of the selected parameter of strains pathogenecity, i.e. hydrolytic enzyme release. Forty-three patients with oral cavity, pharynx or larynx carcinoma were examined at four points during a course of radiotherapy: before treatment, in week 3 of treatment, on the last day of treatment and 3 weeks afterwards. The mycological examination was conducted based on a procedure introduced in the Department of Biology and Medical Parasitology, Medical University of Lodz. The activity of the hydrolytic enzymes was evaluated with a bioMerieux API ZYM test kit. More than 2/3 of the patients (68.2%) were found to have a fungal infection in the first examination, 4/5 (80%) in the second, about 3/5 (57.1%) in the third and all (100%) in the last examination. The release of enzymes varied, and on different stages show different inactive enzymes: at the start, α-chymotrypsin and α-mannosidase; at 3 weeks, β-glucuronidase and α-mannosidase; at the end, α-chymotrypsin; at 3 weeks after the end, trypsin, α-chymotrypsin, α-galaktosidase and α-fucosidase. The most frequent species isolated from the patients treated by radiotherapy is *Candida albicans* and *C. glabrata*. The latter is characterized by resistance to the majority of antimycotic medications. The isolated strains are characterized by the highest activity of leucine arylamidase, acid phosphatase and naphthol – AS-BI-phosphohydrolase. Considering the enzymes produced, most of the strains can be included to biotypes D_3, C_6 and A.

Key words: secrection, hydrolytic enzymes, fungal strains, radiotherapy

Introduction

One method of treatment used in cancer therapy is radiotherapy which, in cases of head and neck tumors, allows similar effects to be achieved as by surgery. In advanced cases, it is also an element of therapy associated with other methods; it is used in almost 60% of patients with malignant tumors and it gives 75–90% of cure [1]. However, radiotherapy can injure a large area of the oral, pharynx or larynx mucosa – resulting in xerostomia, as well as epitheliolisis with the mucosa demonstrating an acute post-radiation reaction, as well as a deteriorated quality of life and disturbances of chewing and swallowing. It also predisposes tissue to mechanical injures and the development of fungal infections [1–8].

The aim of the study paper was the mycological examinations of swabs from the oral cavity and pharynx of patients obtained prior to, in week 3, on the last day of and 3 weeks after radiotherapy, as well as isolation of fungi and identification of the selected parameter of strains pathogenecity, i.e. hydrolytic enzyme release.

Materials and methods

Forty-three patients (11 women and 32 men) aged between 45 and 85 (mean 63.09; SD=9.46) with oral cavity, pharynx or larynx carcinoma were

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Table 1. Hydrolitic enzymes estimated by API ZYM (bioMérieux) test

Number	Enzyme	Substrate	pH	Classification*
e_1	Alkaline phosphatase	2-naphthyl phosphate	8.5	3.1.3.1
e_2	Esterase (C4)	2-naphthyl butyrate	6.5	3.1.1.6
e_3	Esterase lipase (C8)	2-naphthy caprylate	7.5	3.1.1.3
e_4	Lipase (C14)	2-naphthyl myristate	7.5	3.1.1.3
e_5	Leucine arylamidase	L-leucyl-2-naphthylamide	7.5	3.4.11.14
e_6	Valine arylamidase	L-valyl-2-naphthylamide	7.5	3.1.4.1.11.14
e_7	Cystine arylamidase	L-cystyl-2-naphthylamide	7.5	3.4.11.14
e_8	Trypsin	N-benzoyl-DL-arginine-2-naphthylamide	8.5	3.4.4.4
e_9	α-chymotrypsin	N-glutaryl-phenyl-alanine-2-naphthylamide	7.5	3.4.4.5
e_{10}	Acid phosphatase	2-naphthylphosphate	5.4	3.1.3.2
e_{11}	Naphthol-AS-BI-phosphohydrolase	Naphthol-AS-BI-phosphate	5.4	3.1.3.31
e_{12}	α-galactosidase	6-Br-2-naphthyl-α-D-galactopyranoside	5.4	3.2.1.22
e_{13}	β-galactosidase	2-naphthyl-βD-galactopyranoside	5.4	3.2.1.23
e_{14}	β-glucuronidase	Naphthol-AS-BI-βD-glucuronide	5.4	3.2.1.31
e_{15}	α-glucosidase	2-naphthyl-αD-glucopyranoside	5.4	3.2.1.20
e_{16}	β-glucosidase	6-Br-2-naphthyl-β-glucopyranoside	5.4	3.2.1.21
e_{17}	N-acetylo-b-glucosyloaminidase	1-naphthyl-N-acetyl-βD-glucosaminidase	5.4	3.2.1.50
e_{18}	α-mannosidase	6-Br-2-naphthyl-αD-mannopyranoside	5.4	3.2.1.24
e_{19}	α-fucosidasae	2-naphthyl-αL-fucopyranoside	5.4	3.2.1.51

*according to Nomenclature Committee of International Union of Biochemistry and Molecular Biology (1992)

examined at four points during a course of radiotherapy: before treatment, in week 3 of treatment, on the last day of treatment and 3 weeks afterwards.

The mycological examination was conducted based on a procedure introduced in the Department of Biology and Medical Parasitology, Medical University of Lodz [9]. The activity of the hydrolytic enzymes was evaluated with a bioMerieux API ZYM test kit, which is a semiquantitative method of determining the levels of 19 hydrolytic enzymes (Table 1). The results were read according to the instructions provided by the producer. Enzyme activity was expressed in nanomoles of hydrolysed substrate according to the intensity of a colour reaction on a 5-point scale: 0 – no reaction; 1 – 5 nanomoles; 2 – 10 nanomoles; 3 – 20 nanomoles; 4 – 30 nanomoles; 5 – 40 nanomoles and more. The biotyping of the strains was performed according to Kurnatowska [10], the results if which are presented in Table 2.

Regarding statistical analyses, prevalence and correlation were performed with the Rho-Spearman method. All calculations were performed using STATISTICA v.7.1.

Results

More than 2/3 of the patients (68.2%) were found to have a fungal infection in the first examination, 4/5 (80%) in the second, about 3/5 (57.1%) in the third and all (100%) in the last examination.

The release of enzymes varied, and on different stages show different inactive enzymes: at the start, α-chymotrypsin and α-mannosidase; at 3 weeks, β-glucuronidase and α-mannosidase; at the end, α-chymotrypsin; at 3 weeks after the end, trypsin, α-chymotrypsin, α-galaktosidase and α-fucosidase. The percentage of strains showing enzyme activity measured in nanomoles of hydrolysed substrate are presented in Tables 3-6.

Table 2. Biotypes of fungi strains based on enzymatic activity [10]

Grupe	Biotype	Valine arylamidase	Naphthol-AS-BI-phosphohydrolase	α-glucosidase	N-acetylo-β-glucosyloaminidase
A	A	+	+	+	+
B	B_1	–	+	+	+
	B_2	+	–	+	+
	B_3	+	+	–	+
	B_4	+	+	+	–
C	C_1	–	–	+	+
	C_2	–	+	–	+
	C3	–	+	+	–
	C_4	+	–	–	+
	C_5	+	–	+	–
	C_6	+	+	–	–
D	D_1	–	–	–	+
	D_2	–	–	+	–
	D_3	–	+	–	–
	D_4	+	–	–	–
E	E	–	–	–	–

In the first examination, leucine arylamidas, esterase, valine arylamidase, acid phosphatase and naphthol-AS-Bi-phosphohydrolase were the most active enzymes, much more so than alkaline phosphatase and N-acetyl-β-glucosaminidase ($p<0.05$). Detailed data is presented in Table 3.

The activity of the hydrolytic enzymes expressed by the strains isolated during the second examination revealed that leucine arylamidase, acid phosphatase, naphthol-AS-Bi-phosphohydrolase and β-glucosidase demonstrated the highest activity; that their activity was significantly higher than that of alkaline phosphatase, cystine arylamidase, β-glucuronidase and α-mannosidase ($p<0.05$).

Table 3. The percentage of strains showing activity of particular enzymes in batch 1 (n=29)

Enzyme	Nanomoles					
	0	5	10	20	30	40 and >
Alkaline phosphatase	33.3	56.7	3.3	6.7	0	0
Esterase (C4)	6.7	33.3	30.0	20.0	6.7	3.3
Esterase lipase (C8)	26.7	36.7	30.0	6.7	0	0
Lipase (C14)	90.0	10.0	0	0	0	0
Leucine arylamidase	3.3	13.3	30.0	26.7	13.3	13.3
Valine arylamidase	43.3	23.3	10.0	3.3	16.7	3.3
Cystine arylamidase	63.3	36.7	0	0	0	0
Trypsin	96.7	3.3	0	0	0	0
α-chymotrypsin	100	0	0	0	0	0
Acid phosphatase	3.3	50.0	13.3	30.0	0	3.3
Naphthol-AS-BI-phosphohydrolase	0	40.0	33.3	23.3	3.3	0
α-galactosidase	93.3	6.7	0	0	0	0
β-galactosidase	93.3	6.7	0	0	0	0
β-glucuronidase	90.0	10.0	0	0	0	0
α-glucosidase	66.7	30.0	0	0	0	3.3
β-glucosidase	96.7	3.3	0	0	0	0
N-acetylo-β-glucosyloaminidase	60.0	36.7	0	3.3	0	0
α-mannosidase	100	0	0	0	0	0
α-fucosidasae	96.7	3.3	0	0	0	0

Table 4. The percentage of strains showing activity of particular enzymes in batch 2 (n=24)

Enzyme	Nanomoles					
	0	5	10	20	30	40 and >
Alkaline phosphatase	29.2	58.3	4.2	8.3	0	0
Esterase (C4)	0	33.3	50.0	12.5	4.2	0
Esterase lipase (C8)	8.3	87.5	0	4.2	0	0
Lipase (C14)	95.8	4.2	0	0	0	0
Leucine arylamidase	0	12.5	37.5	29.2	12.5	8.3
Valine arylamidase	50.0	25.0	25.0	0	0	0
Cystine arylamidase	66.7	33.3	0	0	0	0
Trypsin	95.8	4.2	0	0	0	0
α-chymotrypsin	91.7	8.3	0	0	0	0
Acid phosphatase	12.5	29.2	29.2	16.7	4.2	8.3
Naphthol-AS-BI-phosphohydrolase	0	25.0	37.5	25.0	8.3	4.2
α-galactosidase	95.8	4.2	0	0	0	0
β-galactosidase	75.0	20.8	0	0	0	4.2
β-glucuronidase	100	0	0	0	0	0
α-glucosidase	37.5	58.3	0	0	4.2	0
β-glucosidase	83.3	8.3	8.3	0	0	0
N-acetylo-β-glucosyloaminidase	66.7	33.3	0	0	0	0
α-mannosidase	100	0	0	0	0	0
α-fucosidasae	91.7	8.3	0	0	0	0

Detailed data is presented in Table 4.

In the third examination, acid phosphatase, leucine arylamidase and naphthol-AS-Bi-phosphohydrolase were expressed the most, more so than lipase, cystine arylamidase, tripsine, α-chymotrypsine, α-galactosidase, β-glucuronidase or α-mannosidase ($p<0.05$). More detailed data is given in Table 5.

In the fourth examination, only leucine arylaminidase had a high level of activity, much higher than that of cysteine arylamidase and α-mannosidase. The details are in Table 6.

The comparison of enzyme activities estimated during all examinations revealed that the activities of only 3 enzymes varied during the course of the study: alkaline phosphates, esterase and α-gluco-

Table 5. The percentage of strains showing activity of particular enzymes in batch 3 (n=16)

Enzyme	Nanomoles					
	0	5	10	20	30	40 and >
Alkaline phosphatase	18.8	75.0	6.3	0	0	0
Esterase (C4)	0	25.5	56.3	12.5	0	6.3
Esterase lipase (C8)	12.5	50.0	31.3	0	6.3	0
Lipase (C14)	81.3	18.8	0	0	0	0
Leucine arylamidase	0	18.8	50.0	25.0	0	6.3
Valine arylamidase	43.8	31.3	18.8	6.3	0	0
Cystine arylamidase	68.8	31.3	0	0	0	0
Trypsin	87.5	12.5	0	0	0	0
α-chymotrypsin	100	0	0	0	0	0
Acid phosphatase	0	31.3	37.5	18.8	0	12.5
Naphthol-AS-BI-phosphohydrolase	0	25.0	25.0	50.0	0	0
α-galactosidase	87.5	12.5	0	0	0	0
β-galactosidase	68.8	31.3	0	0	0	0
β-glucuronidase	81.3	18.8	0	0	0	0
α-glucosidase	62.5	25.0	0	6.3	6.3	0
β-glucosidase	75.0	18.8	6.3	0	0	0
N-acetylo-β-glucosyloaminidase	43.8	43.8	6.3	6.3	0	0
α-mannosidase	93.8	6.3	0	0	0	0
α-fucosidasae	81.3	18.8	0	0	0	0

Table 6. The percentage of strains showing activity of particular enzymes in batch 4 (n=10)

Enzyme	Nanomoles					
	0	5	10	20	30	40 and >
Alkaline phosphatase	60.0	30.0	10.0	0	0	0
Esterase (C4)	0	50.0	50.0	0	0	0
Esterase lipase (C8)	10.0	80.0	10.0	0	0	0
Lipase (C14)	90.0	10.0	0	0	0	0
Leucine arylamidase	0	40.0	30.0	30.0	0	0
Valine arylamidase	20.0	60.0	20.0	0	0	0
Cystine arylamidase	80.0	20.0	0	0	0	0
Trypsin	100	0	0	0	0	0
α-chymotrypsin	100	0	0	0	0	0
Acid phosphatase	0	50.0	20.0	30.0	0	0
Naphthol-AS-BI-phosphohydrolase	0	40.0	30.0	30.0	0	0
α-galactosidase	100	0	0	0	0	0
β-galactosidase	70.0	30.0	0	0	0	0
β-glucuronidase	90.0	10.0	0	0	0	0
α-glucosidase	80.0	0	10.0	0	10.0	0
β-glucosidase	80.0	20.0	0	0	0	0
N-acetylo-β-glucosyloaminidase	60.0	20.0	10.0	0	10.0	0
α-mannosidase	80.0	20.0	0	0	0	0
α-fucosidasae	100	0	0	0	0	0

sidase. The activity of alkaline phosphatase was significantly lower in the fourth examination than the third, while the activities of esterase and α-glucosidase were significantly lower in the fourth than in the first two examinations.

Using the Rho-Spearman method, the activities of particular hydrolytic enzymes and *Candida albicans* (the most common species) were correlated and the results are presented in Table 7 (n – analysis is not possible due to the small dispersal of enzyme activity). Analyzing the relationship during the first examination, it was found that an increase of cysteine arylamidase and a decrease of alkaline phosphatase and β-galactosidase activity were characteristic of *C. albicans,* while in the second examination, esterase lipase and N-acetyl-β-

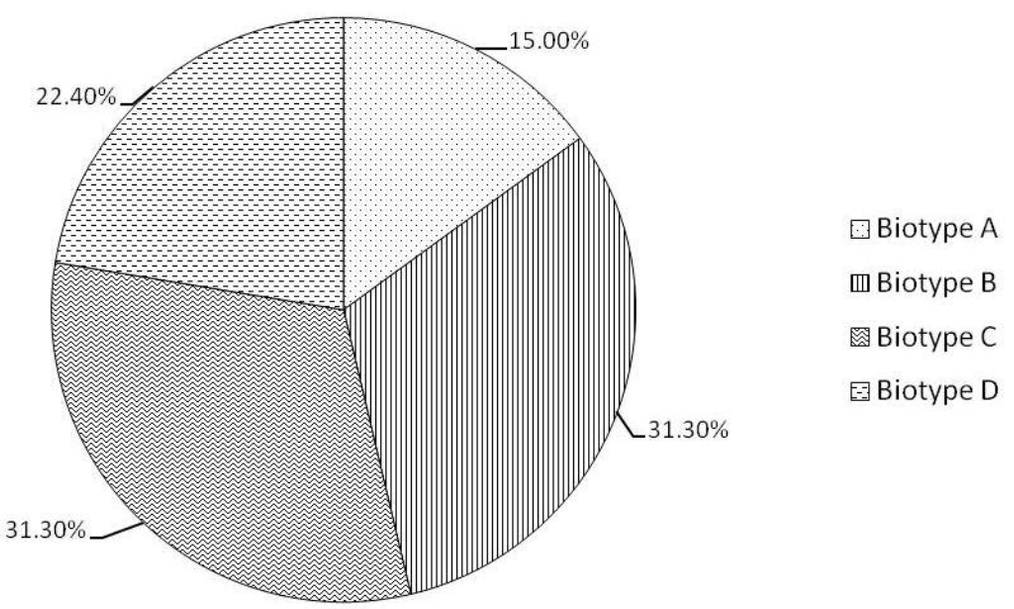

Fig. 1. Biotypes of strains

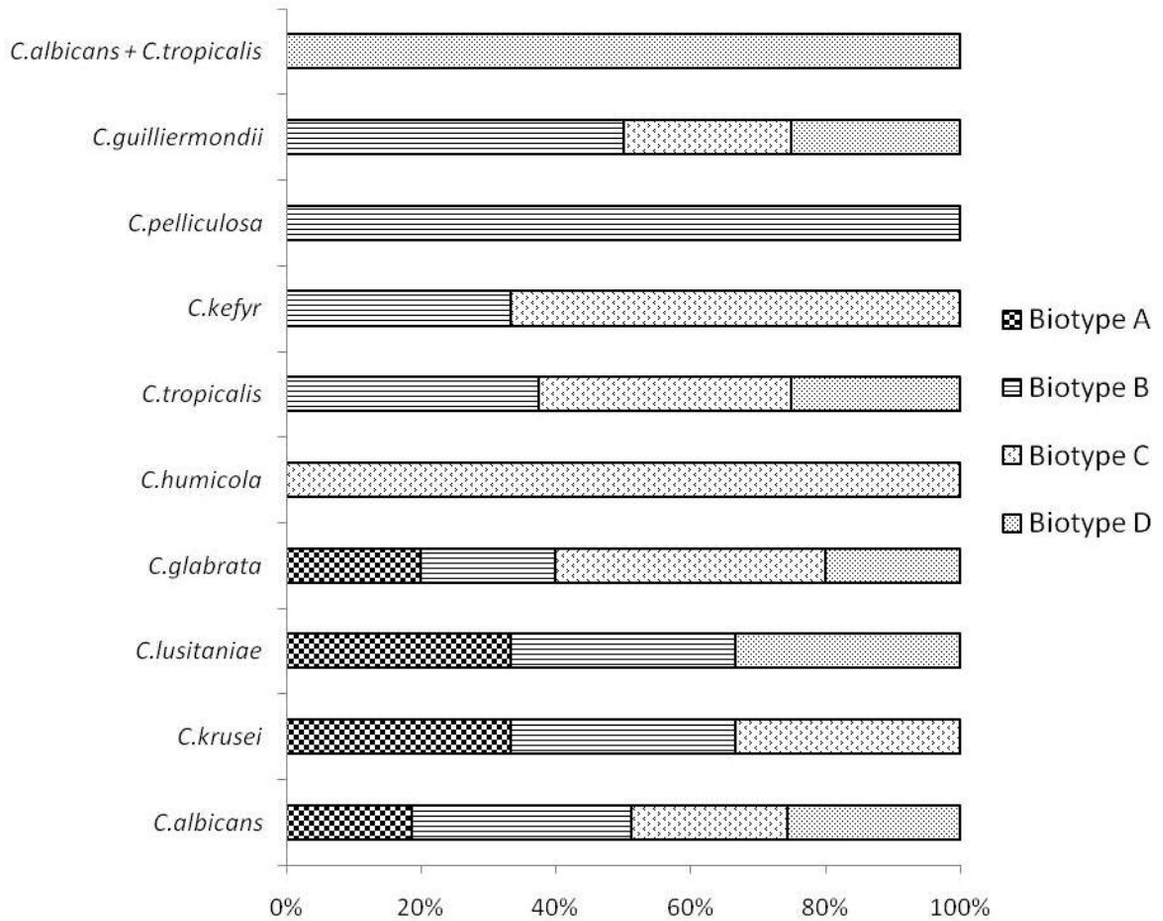

Fig. 2. Biotypes of particular species

glucosamidase activity was increased, and acid and alkaline phosphatase activity was decreased. In addition, in the third examination, the activity of β-glucosidase was found to be lower. In the final examination, esterase, cystine arylamidase and α-mannosidase were seen to have the highest activity.

Taking into account the ability of fungal strains to release selected hydrolytic enzymes, 4 biotypes characteristic of particular species of *Candida* were defined. Most strains could be categorised as biotypes B and C (at 31%), slightly less for D (23%), and the least for biotype A (15%). This data is illustrated in Fig. 1.

Regarding the different biotypes of the various species of *Candida,* it was found that the greatest diversity (classification in all four biotypes) was demonstrated in the case of *C. albicans* and *C. glabrata*, the first of which were classified mainly as biotype B, while the second as biotype C. Interestingly, *C. krusei* and *C. lusitaniae* demonstrated a similar distribution into three biotypes: in the first case to biotypes A, B and C, while in the second – the A, B and D. In addition, examples of the three strains were found which were classified as only one biotype. Detailed information is graphically presented in Fig. 2.

After taking into account the subcategories of the analyzed biotypes, it was found that the most common fungal biotypes were D_3 (22.5%), C_6 (17.5%) and A (15%), while the least common were C_3 (7.5%), B_1 (7.5%) and B_2 (1.3%). Precise information regarding classification of the different strains of the biotypes is summarized in Table 8.

By examining their ability to produce hydrolytic enzymes, it was found in this study that the fungal strains demonstrate different activities. In the first examination, α-chymotrypsin and α-mannosidase were inactive, in the second – β-glucuronidase and α-mannosidase, in the third – α-chymotrypsin, while in the fourth – trypsin, α-chymotrypsin, α-galactosidase and α-fucosidase. However, hydrolase activity differed between examinations; leucine arylamidase was found to have the highest activity in all four, while acid phosphatase and naphthol-AS-BI-phosphohydrolase demonstrated the highest activity in studies I-III. Moreover, strains isolated

Table 7. The relationship between enzymes activity and species of fungi (the variable coded as: 1 – *C . albicans*, 0 – other species) during particular batches – results of Rho-Spearman correlation

Enzyme	Batch			
	1 (n=30)	2 (n=24)	3 (n=16)	4 (n=10)
Alkaline phosphatase	–.307t	–.389t	.289	□.285
Esterase (C4)	.273	.243	.122	816**
Esterase lipase (C8)	.008	.356t	327	.456
Lipase (C14)	n	n	n	n
Leucine arylamidase	.101	–.333	.355	.339
Valine arylamidase	.017	n	–.073	.323
Cystine arylamidase	.339t	.000	.051	.612t
Trypsin	n	n	n	n
α-chymotrypsin	n	n	n	n
Acid phosphatase	.051	.396t	–.014	.424
Naphthol-AS-BI-phosphohydrolase	.104	–.132	.238	–.151
α-galactosidase	n	n	n	
β-galactosidase	n	–.024	–.221	–.535
β-glucuronidase	n	n	n	n
α-glucosidase	–.167	–.292	.143	.152
β-glucosidase	n	–.241	–.650**	–.408
N-acetylo-β-glucosyloaminidase	.128	.530**	.314	.321
α-mannosidase	n	n	n	n
α-fucosidasae	n	n	n	n

during the first study were characterized by a high activity of esterase and valine arylamidase.

Pytko-Polończyk et al. [11] studied the enzymatic activity of 63 *C. albicans* strains isolated from patients undergoing radiotherapy for cancer of the oral cavity and oropharynx. Fourteen enzymes were found to be active; acid phosphatase, leucine arylamidase and naphthol-AS-BI-phosphohydrolase were found to be highly active, while no activity at all was seen for trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase or α-fucosidase.

Plomer-Niezgoda et al. [12] compared the enzymatic activity of strains isolated from patients with primary lymphoma of the skin and bullous diseases treated with immunosuppressive drugs, and found that the fungi produced 11 of the 19 enzymes: alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, β-glucosidase and N-acetyl-β-glucosyloaminidase; the authors found statistically significant differences ($0.0004<p<0.0003$) between the production of valine arylamidase and N-acetyl-β-glucosylo-aminidase in the strains isolated from patients and in those derived from healthy individuals.

By evaluating the enzymatic activity of strains of *C. alhicans* from oral cancer patients between 1999 and 2003, Lukaszuk et al. [13] found no significant differences in the hydrolase activity of strains isolated during these periods. However, while the highest enzyme activity was demonstrated by leucine arylamidase, esterase and cystine arylamidase in 1999, these had been supplanted in 2003 by leucine arylamidse, esterase lipase and esterase.

Pytko-Polończyk et al. [11] classified half the samples of *C. albicans* strains isolated from patients treated with radiation therapy (49.2%) as biotype A, according to Kurnatowska [10], the other half mainly as B_4 (42.9%), and a single example as C_6 (3.17%) and B_1 (1.59%). The majority of the strains isolated by Plomer-Niezgoda et al. [12] from patients with primary lymphoma of the skin, and bullous diseases treated with immunosuppressive drugs were classified as biotypes B_3, B_1 and D_3.

The majority of *C. albicans* biotypes isolated by Krajewska-Kułak et al. [14] from patients with oral cavity cancer were classified as B_4 (30%), and much fewer to biotype E (4.8%), while strains of

Table 8. The percentage of particular species classified to different biotypes

Species	Biotype								
	A	B_1	B_2	B_3	B_4	C_2	C_3	C_6	D_3
C. albicans	18.6	11.6		14.0	7.0	9.3	2.3	11.6	25.6
C. krusei	33.3				33.3			33.3	
C. lusitaniae	33.3	33.3							33.3
C. glabrata	20.0				20.0			40.0	20.0
C. humicola							100.0		
C. tropicalis			12.5	12.5	12.5	12.5	12.5	12.5	25.0
C. kefyr				33.3				66.7	
C. pelliculosa					100				
C. guilliermondii				25.0	25.0		25.0		25.0
C. albicans+C. tropicalis									100.0
In general	15.0	7.5	1.3	11.3	11.3	6.3	7.5	17.5	22.5

C. albicans isolated by Brajer et al. [15] from the sputum of patients with lung cancer were most often classified as C_6 (36.4%), B_3 (24.2%) and C_1 (24.2%), much more rarely as A (3.03%), B_4 (3.03%) and D_4 (3.03%) according to Kurnatowska [10].

Pytko-Polończyk et al. [11] most often determined the biotypes of *C. albicans* isolated from the oral cavity of patients with nasopharyngeal cancer who underwent radiation therapy as A (49.2%) and B_4 (42.9%), while rarer biotypes were C_6 (3.17%) and B_1 (1.59%) according to Kurnatowska [10]. Nowak [16] found that the most frequently occurring biotype for *C. albicans*, *C. glabrata* and *C. parapsilosis* isolated from patients with implanted voice prosthesis was C_6, while for C. *tropicalis* strains it was B_4; among strains isolated from patients without voice prosthesis, biotype B_3 was most often observed, while D_3 was much rarer; *C. glabrata* and *C. parapsilosis* strains were classified as D_3.

From the above, the literature data suggests that *Candida* strains isolated from patients are characterized mainly as biotypes A, B_1, B_3, B_4, C_1, C_3, C_6, D_1, D_3, D_4 and E.

Conclusions

On the basis of the research performed the following conclusions can be drawn:

1. The most frequent species isolated from the patients treated by radiotherapy is *Candida albicans* and *C. glabrata*. The latter is characterized by resistance to the majority of antimycotic medication.

2. The isolated strains are characterized by the highest activity of leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Considering the enzymes produced, most of the strains can be included to biotypes D_3, C_6 and A.

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