DOI 10.26724 / 2079-8334-2018-2-64-204-208
UDC 579.22:582.28:636.09:616.992.28:616.5

S.M. Kulynych, A.P. [1]Kabluchka, M.O. Petrenko, S.O. Kravchenko, N.S. Kanivets
Poltava State Agrarian Academy, Poltava
[1]Regional State Laboratory of the State Service of Ukraine for Food Safety and Consumer Protection in the Poltava region

BIOCHEMICAL PROPERTIES OF MICROSCOPIC FUNGI CULTURES ISOLATED FROM INJURED KEROID FORMATIONS OF SKIN

e-mail:fvmpoltava@ukr.net

From 2007 to 2016, 3694 animals were studied in nine farms of the Poltava and Kirovograd regions, including 3307 cows, 300 sheep and 87 horses. The total of 112 samples of the injured hoof horn taken from 83 animals were subject to mycological examination: 63 cows aged 3-5 years, 10 horses aged 5-9, and 10 sheep 4-7 years old. The sick animals were divided into groups according to their species affiliation. According to research results, 20 fungi species of 14 genera were allocated. From the microscopic fungi isolated from the destroyed keratinized horny capsule structures the highest keratinase, hemolytic phospholipase and lecithinase activity was found in S. brevicaulis fungus species ($p < 0.001$). The above convincingly proves the need for further in-depth studies aimed at identifying the factors that cause keratinized structures destruction by microscopic fungi.

Key words: microscopic fungi; keratinolytic, lecithinase, phospholipase and hemolytic activity.

The work was carried out in accordance with the plan of research work Study of pathogenesis and development of pathogenetic methods of therapy of inflammatory processes in surgical diseases of animals" (state registration number 1117U004721).

Microscopic fungi are known to gradually stratify keratin of a coffin and leads to the sole destruction, to the formation of crateroidal ulcers that reach the solar matrix. Uneven loads on the sole, hemorrhages and inflammatory processes in the solar matrix cause the formation of osteophytes and exostoses in the coffin bone [2]. Fungi of the Scopulariopsis (S) brevicaulis, Alternaria (A) alternata, Geotrichum (G) candidum species, isolating keratinase, lead to crevices, pareceratosis [8]. Their mycelium grows into the stratum corneum of the epidermis.

In its life course, it destroys keratin and discharges the products of metabolism [9]. At affection with microscopic fungi, splitting and dissection of the hoof horn, changes in its structure are observed. The disease has a subclinical nature until the animal displays lameness as a result of pododermatitis [3].

The purpose of the research was to study the biochemical properties of microscopic fungi isolated from destroyed structures of the coffin.

Materials and methods of the research. Studies of the destroyed solar horn was carried out on the basis of the Regional State Laboratory of the State Service of Ukraine for Food Safety and Consumer Protection in the Poltava region.

From 2007 to 2016, 3694 animals were studied, including 3307 cows, 300 sheep and 87 horses. The total of 112 hoof horn samples taken from 83 animals including 63 cows and 10 horses and sheep were studied by mycological methods. The injured animals with signs of the hoof corneous capsule destruction were divided into three groups according to their species affiliation. The first group included 63 cows aged 3-5 years, the second - 10 horses aged 5-9 years, and the third - 10 sheep 4-7 years old.

Criteria for exclusion of animals from the examination were aseptic and purulent pododromatitises without signs of destruction of the cornea capsule. The study also did not include animals with traumatic injuries to the hallux area, as well as animals with tyloma, phlegmonous processes of the coronet area and ulcers of the interdigital arches tissues.

From the affected coffin areas of animals, which were not treated for 10 days, samples from the destroyed horn were taken with curettes into sterile bacteriological test tubes and plated on Czapek and Sabouraud agar. Fungi cultures were identified by species using an indicator [6]. If necessary, their structure and growth rate were determined. Microscopic methods were used to study the nature of hyphae septate and the type of conidiogenic cells [7]. The keratolytic properties of the microscopic fungi were determined by the *in vitro* hairs perforation test (according to Cano J.) [11].

The hair 5-7 cm long, used in the experiments, was pre-sterilized by physical methods and was taken from the tail of a clinically healthy cow. The hair was placed into a Petri dish with distilled water, to which a few drops of 10% yeast extract were added. Freshly grown cultures of each fungi type were introduced into each dish and incubated in a thermostat at 25° C for three weeks. For each culture, five Petri dishes were used. Petri dishes filled with medium and hair served as the control ones, where the fungi cultures were not sown. The keratolytic activity of the fungi was assessed by the existence and

intensity of the hair destruction (surface destruction of the cuticle, cortical layer, deep perforation with medulla destruction) on a four-point scale. The activity (++++) was considered to be sufficiently high if it caused destruction of the cuticle, cortical and medulla layers in each of the studied hairs; activity was considered high (+++) in case of the above destructions in most of the studied hairs; activity was assessed as moderate (++) when these signs were detected in half of the hairs; activity was low (+) if destructions of the cuticle only were detected in less than half of the hairs; it was negative (-) in the absence of destructions. To detect hemolytic properties in microscopic fungi cultures, Czapek agar was used, with 5% of the defibrinated ram blood. The 7-10- days culture of the microscopic fungus was sown on it and kept in a thermostat at 26 - 27ºC [1]. In determining the results, the presence and width of the hemolysis zone around the fungus colony were taken into account.

The proteolytic ability of microorganisms was determined on "hungry gelatin". To that effect, 20.0 g of gelatin was dissolved in 100 ml of demineralized water, heated to full dissolution, poured into 5 ml test tubes and sterilized with a flowing steam. Fungi cultures were sown directly into the test tube and incubated at 26° C. Starting from the third day of incubation, the test tubes were examined for the presence of rarefied medium. At the same time, the activity of the proteolytic enzymes complex was considered according to the height of the rarefied gelatin column [4]. Additionally, according to the assignments set before us, we determined lecithinase and phospholipase activity [5, 10]. The digital material is presented in tables, diagrams and processed by the variation statistics methods on a personal computer using the MS Excel software and R.B. Strelkov tables (1966). The probability of differences between the indices was estimated according to Student's criterion. The difference between the two values was considered probable at $p < 0.05$; $p < 0.01$; $p < 0.001$.

Results of the research and their discussion. According to the results of microscopic studies, it was found that the most common fungi isolated from samples of the hoof corn were S. brevicaulis (Fig. 1), A. alternata, A. flavus, A. fumigatus and Penicillium: their total part was nearly 73%. At this, the most numerous were fungi of the S. brevicaulis and other fungi species widely spread in the studied samples: A. alternata, A. flavus, A. fumigatus and Penicillium.

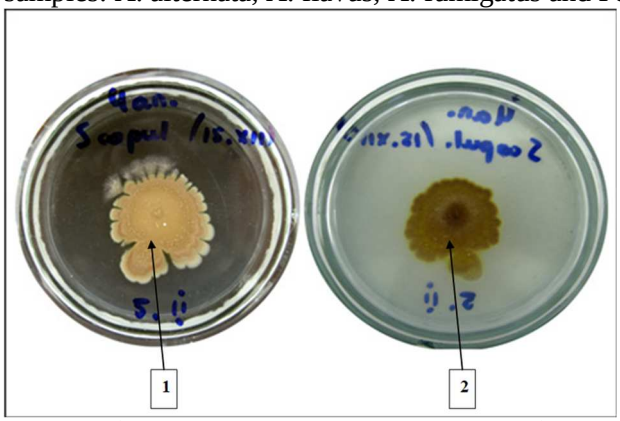

Fig.1 Colonies of S. brevicaulis culture on Czapek agar: 1. upper; 2. back side of the dish.

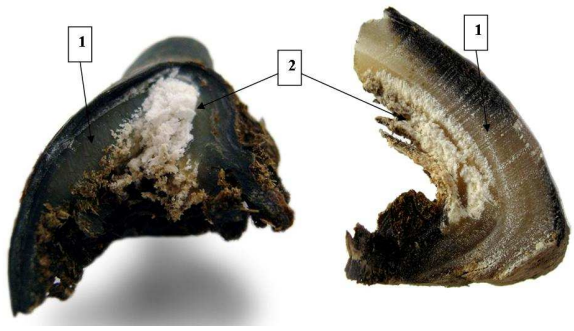

Fig.2 The view of the sheep's hoof horn sampled for mycological study: 1. hoof horn unchanged; 2. damaged areas

Out of 83 affected tissues samples (Fig. 2), 14 fungi genera were isolated, represented by 20 species, for 8 of them a definite geographical location was identified, which is explained by the microflora features of different farms. Thus, the cultures of C. albicans are isolated from biomaterials of animals in two farms, namely the State Enterprise DP DG "Stepne" and the State Enterprise DP NDG "Yubileynyi"; the culture of Cladosporium genus – in the Private farm PAF "Pershe Travnya" and in the Private Enterprise PP "Agroecologia"; the Microascus culture – in PE PP "Agroecologia" and in the private sector; the Trichoderma viridae culture – in te State Enterprise NDG "Dzherelo" and in PAF "Pershe Travnya"; the Verticillium culture – in DP NDG "Dzherelo" (Table 1). In addition, isolated cases of microscopic fungi growth of Acremoniella and Trichophyton genera were observed. Taking this fact into account, it is difficult to consider these genera playing an important role in the destruction foci formation.

The fungi species A. alternata, A. flavus, A. fumigatus, Penicillium spp. and others, considered to be commonly spread, were found in almost all the samples. It is quite probable, that the frequency of these fungi isolation directly depends on their role in the pathogenesis of the hoof corneous capsule destruction. When performing the test for hair perforation (Table 2), it was found that after 13 days of incubation in dishes with A. flavus culture, the destruction of the hair structure was taking place. A growth of mycelium was observed on the hair surface. The hair surface had a slight peeling and

lamination of surface keratinous cuticle scales and partial melting of the hair's cortex layer.

Table 1

Frequency of certain fungi species isolation from the damaged corneous capsule of animals at different farms (n = 83)

ВИДИ ГРИБІВ	ENTERPRISES										
	PAF «PODOLYAKA» n=10	DP NDG «YUVILEYNYI»	DPNDG «DZHERELO»	PAF «PERHE TRAVNYA TRATRAVNYA»	DP DG «STEPNE»	PP «AGROECOLOG»	VAT «POLTAVA ПЛЕМСЕРВІС»0	PRIVATE SECTOR	ОНУФРІЇВСЬКИ STUD FARM n=10	**TOTAL**	
		n=63								**ABSOLUTE NUMBER**	**%**
Acremoniella spp.						1				1	0.3
A. flavus	2	8	4	8	9	4				35	11.9
Alt. alternata	7	2	4	3	6	6	1		4	33	11.2
A. fumigatus		7	2	5	7	7		1		29	9.8
C. albicans		3			3					6	2
Cladosporium hordei				6		3				9	3
Fusarium spp.		3			3	2		1	7	16	5.4
Microascus spp.						2		2		4	1.4
Mucor spp.		4	2	3	3		1	3	3	19	6.4
Penicillium spp.	4	5	8	2	5	5	1		3	33	11.2
Rhizopus spp.		3	1		3	1				8	2.7
S. brevicaulis	11	12	10	13	12	11	4	6	6	85	28.8
Trichoderma viridae			6	2						8	2.7
Trichophyton spp.				1				1	1	3	1
Verticillium spp.			6							6	2
TOTAL	*24*	*47*	*43*	*43*	*51*	*42*	*7*	*14*	*24*	*295*	99.8

Table 2

Results of the hair perforation test (n = 5)

The studied culture	Destruction of the cuticle and the cortex layer	Destruction of the medulla and the hair perforation
A. flavus	+++	++
A. fumigatus	+	-
Cladosporium hordei	++	+++
C. albicans	+++	+++
Trihoderma viridae	++	++
A. alternata	++++	++++
S. brevicaulis	++++	+++
Penicillium spp	+	+

where: - (++++) - very high activity; (+++) - high activity; (++) - moderate; (+) - low; (-) negative.

During the microscopic studies of the hair changes, in part of them, we found ingrowth of the fungus hyphae into the hair's thickness, followed by its fragmentation and the medulla destruction. The A. fumigatus culture formed a poorly developed mycelium around the hairs, keratinous cuticle scales practically did not change, remained clearly expressed; a large number of spores was detected around the hair, somewhere forming a kind of casing. The Cladosporium hordei culture, on the 19th day of being kept in the thermostat on the surface of the hair, destroyed a number of keratinized scales in its outer layer. At the ends of the hair there was a loosening and porosity of structural tissues due to the perforation of the cortical and medulla layers. In the mycological study of hairs with the C. albicans fungus culture, on the 14th day, we found signs of hairs destruction, in particular, the cuticle was absent on the greater part of the hair, erosions of the hair surface occurred and the cavitation began, occasional hair fouling with the fungus mycelium and the formation of its ingrowth into the medulla was observed. After 15 days of incubation for the hair perforation test with the culture of Trihoderma viridae fungi species, the hair fouling with mycelium and the lamination of surface keratinized scales on its cuticle were detected. The culture of A. alternata had a developed mycelium, which in some places was fastened to the hairs, but did not grow around them, significant clumps of macroconidia were detected. The places of hairs perforation with hyphae were visualized. Hairs lost their strength and could be torn with the slightest effort. The ends of the hair were acerated, the clump of mycelium was noticed around them.

The S. brevicaulis fungi species formed a thin, pale weakly developed mycelium on the surface of a hair. In some places, it tightly ingrew into a hair, on the periphery of the hair a clump of spores was observed. The microscopic studies detected a homogeneous mass of mycelium and remains of destroyed

tissues along the hair. In addition, the hyphae ingrowing through the hair cuticle, cracks in it and wedge-shaped foveae into the thickness of the cortex layer (Fig. 3) were observed. The Penicillium spp. fungus formed a poorly developed mycelium, thin, short, sometimes attached to the surface of the hair. At the microscopic examination of the hair, it was found that the scales were weakly noticeable, with fuzzy contours. Any damage to the hair's integrity was not detected. Thus, in assessing the isolated fungi cultures, it was found that in relation to the hair, the greatest keratinolytic activity was demonstrated by the A. alternate, S. brevicaulis, C. albicans fungi species. It was manifested as the cuticle destructions in the form of holes, erosions, longitudinal cracks and sphenoidal foveae with the mycelium penetration into the cortex layer, perforation in most of the studied hair and with the ingrowth of hyphae through a hair.

In other cultures, in most of the hairs samples, the surface lysis of the hair cuticle and the cortex layer were detected. One of the important features that characterizes the pathogenicity of microorganisms is their ability to hemolysis. To determine it, we used the Czapek medium with 5% of the defibrinated ram's blood. On the seventh day of incubation (Fig. 4), there was clearly seen a wide area of hemolysis caused by the A. fumigatus culture. The clear rounded, transparent area of β-hemolysis was also noticeable in S. brevicaulis fungi, but in Penicillium spp., around the colony there was a slightly noticeable area of clarification with an olive shade (α-hemolysis).

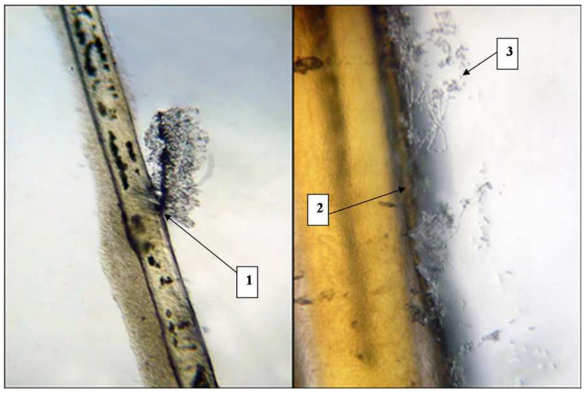

Fig. 3. Test for perforation of a hair with the S. brevicaulis culture: 1. ingrowth of mycelium into the hair; 2. destroyed cuticle; 3. Mycelium.

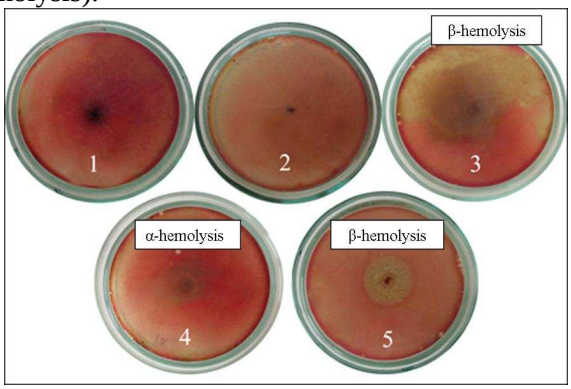

Fig. 4. Hemolytic activity test. 1. A. alternata; 2. A.flavus; 3. A. fumigatus; 4. Penicillium spp. 5. S. brevicaulis.

Determination of the fungi ability to the proteolytic activity due to dilution of the medium was determined by the height of the formed fluid column on the 14th day of incubation. The test was carried out on the "hungry gelatin" medium. It was found that the S. brevicaulis microscopic fungus manifested the highest activity (6,0 ± 0,1 mm), it was probably the highest ($p < 0,001$) in comparison with other cultures. The lower proteolytic activity was observed in A. Flavus: by 16.7% (5.0 ± 0.1 mm) lower than the above mentioned, by 50% lower proteolytic activity compared with S. brevicaulis was manifested by A. alternata fungus (3.0 ± 0.1 mm). The A. fumigatus fungus demonstrated six times lower proteolytic activity. The presence and relative activity of hydrolytic enzymes were determined by the width of the clarification area (lecithinase) or the zone of precipitation (phospholipase) around the fungus colony in millimeters (Table 3).

Table 3

Comparison of the results of lecithinase and phospholipase activity determination (n = 5)

Culture under study	Activity	
	lecithinase, mm	phospholipase, mm
A. alternata	9.4±0.86	–
A. flavus	–	5±0.43
A. fumigatus	–	9.2±0.43
Penicillium spp		4.6±0.21
S. brevicaulis	11.2±0.43	14.4±0.86*

Note: *– $p<0,001$ activity compared to other explored cultures

As we can see, lecithinase activity was displayed by microscopic fungi of the two species: A. alternate - 9,4 ± 01 mm and S. brevicaulis - 12 ± 01 mm, while in the latter it was significantly higher on the 19.1 %. The effect on phospholipids in another test was demonstrated by four out of the five tested microorganisms, except A. alternate. It should be noted that in the S. brevicaulis the zone of precipitation was 2-3 times higher than that of other fungi species ($p < 0,001$).

Phospholipase and lecithinase are enzymes of the same group, but there are four main families of these enzymes that differ in the way of effecting the substrate. The fact that in various tests we obtained different results suggests that S. brevicaulis synthesizes several varieties of the said hydrolase.

Conclusion

On the basis of the performed research data, it can be concluded that in the microscopic fungi, isolated from the destroyed keratinized structures of the hoof corneous capsule, the highest keratinase, hemolytic phospholipase and lecithinase activity was demonstrated by the S. brevicaulis species fungus. Among the other species, the high activity was expressed by the A. alternate species fungus with the exception of the hemolytic activity. Consequently, we believe that the determination of these fungi activity indices in vitro can indicate their pathogenicity and the ability to destroy keratinized structures.

Prospect of further research. The above data convincingly prove the need for further in-depth studies aimed at identifying the factors that cause keratinized structures destruction by microscopic fungi.

References

1. Donetskaya EG. Klinicheskaya mikrobiologiya: Rukovodstvo dlya spetsialistov klinicheskoy laboratornoy díagnostiki. M. GEOTAR. 2011; 480 s. [in Russian]
2. Izdepskiy VI, Kulinich SN. Rol gribov pri gnoyno-vospalitelnykh protsessakh konechnostey u korov. Veterinariya. M. 2008; 3: 27-30. [in Russian]
3. Izdepskyi VY, Kulinich SN, Kabluchka AP. Deyaki etiolohichni faktory urazhen kopyt u tvaryn. Visnyk Zhytomyrskoho natsionalnoho ahroekolohichnoho universytetu. 2012; 1(32) 2: 43-47. [in Ukrainian]
4. Lyuta VA, Kononov OV. Mikrobiolohiya z tekhnikoyu mikrobiolohichnykh doslidzhen, virusolohiya ta imunolohiya: Pidruchnyk. K. Medytsyna. 2017; 576 s. [in Ukrainian]
5. Muradov PZ, Kasumova SYu, Babayeva IKh. Issledovaniye letsitinaznoy aktivnosti nekotorykh mikromitsetov. Nauka i sovremennost. 2011; (9-1): 23–27. [in Russian]
6. Satton D, Fotergill A, Rinaldi M. Opredelitel patogennykh i uslovno patogennykh gribov. per. s angl. Tarasova KL, Kovaleva YuN. M. Mir. 2001; 486 s. [in Russian]
7. Chkhenkeli VA. Veterinarnaya mikologiya. Uchebnoye posobiye Irkutsk. IrGSKHA. 2010; 116 s. [in Russian]
8. Apprich V, Spergser J, Rosengarten R, Hinterhofer C, Stanek C Scanning electron microscopy and fungal culure of hoof horn from horses suffering from onychomycosis. Vet Dermatol. 2010 Aug; 21(4): 335-40.
9. Călin M, Drăcea O, Răut I, Vasilescu G, Badea Doni M, Arsene ML, et al. In vitro biodegradation of keratinized substrates by keratinophilic fungi. Scientific Bulletin. Series F. Biotechnologies 2016 Vol. XX: 248-53.
10. Sacristán B, Blanco MT, Galán-Ladero MA, Blanco J, Pérez-Giraldo C & Gómez-García AC Aspartyl proteinase, phospholipase, hemolytic activities and biofilm production of *Candida albicans* isolated from bronchial aspirates of ICU patients. Medical Mycology. 2011 Jan; 49: 94-7.
11. Sharma M, Sharma M, Mohan Rao V In vitro biodegradation of keratin by dermatophytes and some soil keratinophiles. African Journal of Biochemistry Research. 2011 Jan; 5(1): 1-6.

Реферати

БІОХІМІЧНІ ВЛАСТИВОСТІ МІКРОСКОПІЧНИХ КУЛЬТУР ГРИБІВ, ІЗОЛЬОВАНИХ З УРАЖЕНИХ РОГОВИХ УТВОРЕНЬ ШКІРИ.

Кулинич С.М., Каблучка А.П., Петренко М.О., Кравченко С.О., Канівець Н.С.

З 2007 по 2016 рр. у 9 фермах Полтавської та Кіровоградської областей було вивчено 3694 тварин, у тому числі 3307 корів, 300 овець та 87 коней. Всього було проліковано 112 зразків ушкодженого рогу копита, взятих від 83 тварин: 6 корів у віці 3-5 років, 10 коней у віці 5-9 років та 10 овець 4-7 років. Хворі тварини були поділені на групи відповідно до їх видової приналежності. За результатами досліджень було виділено 20 видів грибів з 14 родів. З мікроскопічних грибів, виділених із зруйнованих кератинізованих рогових капсульних структур, найвища активність кератинази, гемолітичної фосфоліпази та лецитинази була виявлена у видах грибів S. brevicaulis (p <0,001). Вищезгадане переконливо доводить необхідність подальших глибинних досліджень, спрямованих на виявлення факторів, що спричиняють руйнування кератинізованих структур мікроскопічними грибами.

Ключові слова: мікроскопічні гриби; кератинолітична, лецитиназна, фосфоліпазна та гемолітична активність.

Стаття надійшла 27.03.18р.

БИОХИМИЧЕСКИЕ СВОЙСТВА МИКРОСКОПИЧЕСКИХ КУЛЬТУР ГРИБОВ, ИЗОЛИРОВАННЫХ ИЗ ПОРАЖЕННЫХ РОГОВЫХ ОБРАЗОВАНИЙ КОЖИ.

Кулинич С.М., Каблучка А.П., Петренко Н.А., Кравченко С.А., Канивец Н.С.

С 2007 по 2016 год в 9 хозяйствах Полтавской и Кировоградской областей изучалось 3694 животных, в том числе 3307 коров, 300 овец и 87 лошадей. Всего 112 образцов поврежденного рога копыта, взятых от 83 животных, были подвергнуты микологическому исследованию: 63 коровы в возрасте 3-5 лет, 10 лошадей в возрасте 5-9 лет и 10 овец 4-7 лет. Больных животных разделили на группы по их видовой принадлежности. Согласно результатам исследований было выделено 20 грибковых видов из 14 родов. Из микроскопических грибов, выделенных из разрушенных кератинизированных роговых капсульных структур, наибольшая активность кератиназы, гемолитической фосфолипазы и лецитиназы была обнаружена у видов гриба S. brevicaulis (p <0,001). Вышеизложенное убедительно доказывает необходимость дальнейших углубленных исследований, направленных на выявление факторов, вызывающих разрушение кератинизированных структур микроскопическими грибами.

Ключевые слова: микроскопические грибы; кератинолитической, лецитиназной, фосфолипазной и гемолитической активности.

Рецензент: Старченко І.І.