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"QUALITY BY DESIGN"
IN LIPOSOMAL DRUGS CREATION

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Nanobiotechnological preparations creation is one of the promising areas of modern pharmacy, since it allows creating products of a qualitatively new level. The procedure development, based on an understanding of the product characteristics and the technological process, confirmed by reliable scientific data.

The article is devoted to the pharmaceutical development of liposomal drugs. On the basis of our own experience in the development of liposomal medicinal forms, as well as on the basis of literature data, the main components in their composition were detected and these components impact on the quality indicators of liposomes were studied. Individual lipids function in nanoparticle membrane and their interaction, which determines the stability both in the technological process and upon storage of the product, were considered. The advantages and disadvantages of cholesterol incorporation into liposomes with hydrophilic and hydrophobic active pharmaceutical ingredients were described. Cryoprotectors and buffer systems role in ensuring nanopreparation stability is discussed.

Key words: liposomes, phospholipids, cholesterol, cryoprotector, buffer system, Quality by Design.

The wide interest shown today to nanobiotechnological products, in particular to liposomal (Ls) drugs, in pharmacy is quite understandable — these drugs, possessing a wide spectrum of action, are intensively used for diseases of various etiologies diagnostics, prevention and treatment [1–6]. The Ls drugs creation is one of the promising areas of modern nanopharmacology [7–9].

The number of Ls medicinal forms on the world pharmaceutical market is more than 50 drugs, 5 of which are developed and licensed in Ukraine. Research begun in Ukraine in the early 90s led to the creation of Ls drugs of various directions: Lipin (pulmonology, cardiology, nephrology, obstetrics and gynecology); Lipodox (oncology); Lioliv (hepatoprotector) and two forms of Lipoflavone, namely eye drops (ophthalmology) and an injection form (cardiology) [10–14]. The indicated Ls preparations have found wide application in clinics of Ukraine: Lipin [3, 4, 15–21], Lipodox [4, 22–25], Lioliv [4, 26, 27], Lipoflavone [28–31]. In subsequent years, a team of authors proposed technologies for obtaining Ls preparations containing various active pharmaceutical ingredients (API), studied their physicochemical and pharmacological properties: irinotecan [32–34], cytochrome *c* [35–37], coenzyme Q10 [38, 39], curcumin [40, 41], cisplatin [42, 43], docetaxel [44–46] and a number of other products, including complex Ls preparations containing several APIs [40].

The advantage of Ls drugs in comparison with the free API form in an equivalent concentration is a decrease in toxicity (anticancer drugs irinotecan hydrochloride, doxorubicin hydrochloride, platinum drugs, etc.), prolonged action of API when administered; increased bioavailability for lipophilic substances (quercetin, curcumin, antral, etc.).

The aim of the work was to analyze the pharmaceutical development of Ls drugs. This
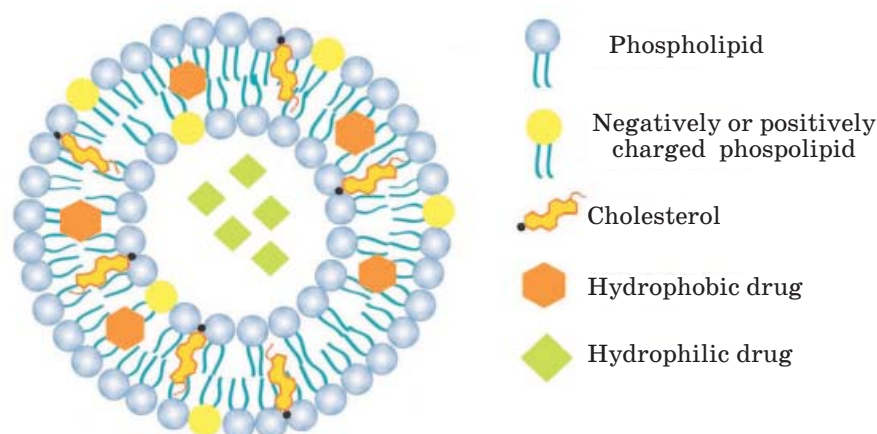
Fig. 1. **The structure of Ls preparations [47]**
In the developed preparations, hydrophobic substances are represented by quercetin, curcumin, phosphatidylcholine, etc., hydrophilic ones — by doxorubicin, irinotecan, oxaliplatin, etc.

report considered the issues of "Quality by design" in the creation of Ls drugs, namely the individual lipids role of nanoparticle membranes, their interaction, which determined the stability both in the process of technology and upon storage of the finished drug.

After analyzing the composition of the drugs proposed over the years, it should be noted that the Ls drugs include: API, phospholipids (PhL), cholesterol (Chol), polyethylene glycol derivatives (PEG), stabilizers (cryoprotectors); buffer systems providing the Ls structure and their physicochemical and pharmacological properties. The most important components of the Ls membrane are PhLs.

PhLs. The main membrane-forming components of Ls preparations are PhLs of various structure (natural and synthetic), differing in charge, saturation of fatty acids, properties of polar groups. To a certain extent, the effectiveness of Ls containing PhLs is based on the properties of the PhLs themselves [48−50].

The structure of PhL is characterized by the presence of hydrophobic and hydrophilic fragments in the composition of one molecule, as well as by the diversity of each of these fragments structure, which largely determines the role of PhL in a number of cellular processes [48]: structural function — the PhLs mixture should be able to form a stable bilayer for the functioning of membrane proteins (such PhLs include phosphatidylcholine (PC), phosphatidylglycerol (PG), sphingomyelin (SM), phosphatidylinositol (PI) and a number of others, and at the same time, there are some PhLs in the membrane that are capable of forming non-bilayer structures (phosphatidylethanolamine (PEA), diphosphatidylglycerol (DPG),

phosphatidic acid (PA), etc.) The formation of such highly curved membrane sections is necessary during contact between membranes (cell fusion process) or when certain proteins are bound in the membrane, which ensures the existence of the membrane in a functionally active state. During hydration in the aqueous phase, PhLs spontaneously form Ls due to their thermodynamic phase properties [48−50]. When choosing a PhL component, it is necessary to take into account the phase transition temperature, i.e. the temperature at which PhLs pass from the gel to the liquid crystal phase. The phase transition temperature depends on the structure of the PhL molecule, the saturation of fatty acids and the structure of the polar groups. It should be borne in mind that natural PhLs containing different fatty acids in two positions have a non-standard phase transition temperature, especially since natural, for example, egg PC (EPC) is represented by the PC family containing a number of fatty acids [48] and does not have a clear phase transition temperature. PhLs with long chain fatty acids have a higher phase transition temperature. When using PhLs with a temperature below the phase transition temperature, they are in the gel phase, which in turn gives Ls low fluidity and permeability. When Ls is obtained at a temperature higher than the phase transition temperature, the PhLs are in the liquid crystalline phase, which provides greater fluidity and permeability. It should be noted that such a structure can simultaneously impede the penetration of hydrophilic APIs through the Ls membrane.

An independent question is PhL selection for the basis of Ls. When choosing a PhL, we

were guided by a number of requirements: the maximal API inclusion in the bilayer or in the aqueous phase of Ls, Ls stability both upon obtaining the Ls emulsion and upon hydration after lyophilization, storage stability, absence of undesirable reactions upon introduction into the body, etc. Both natural (EPC, hydrogenated soy PC (HSPC), sunflower PC (SFPC)) and synthetic (dipalmitoylphosphatidylcholine (DPPC), dimyristoylphosphatidylcholine (DMPC), distearoylphosphatidylcholine (DSPC) and other have been studied previously. According to a number of researchers, the use of unsaturated PhLs should be avoided due to the fact that these lipids are subject to peroxidation processes. In our opinion, it is possible to use unsaturated PhLs, in particular EPC, and the use of this lipid has a number of significant advantages, including economic ones [4]. To prevent peroxidation of EPC, a number of protective measures are used: a) nitrogen or argon was used at the stage of lipid film obtaining and its hydration, and the process of obtaining Ls was carried out in a protective atmosphere of these gases; b) limiting illumination; c) in cases where it is possible, at the technological stages the storage mode of the liquid preparation at a temperature of 2−6 °C was used; d) Ls storage after lyophilization throughout the entire period at a temperature of about minus 10 °C; e) sealing of lyophilized preparations was carried out in a protective gas atmosphere [4]. The API used can also determine the choice of one or another PhL. At the same time, in our opinion, the influence of the API structure does not significantly affect the choice of PhL. Thus, when obtaining Ls forms of anthracyclines, several medicinal Ls forms have been created that do not differ in pharmacological action, the composition of which is different: Doxil (HSPC:Chol:PEG−2000−DSPE 56:38:5); Myocet (EPC:Chol 1:1); Daune−Home (DSPC:Chol 2:1); Lipodox (EPC:Chol 0.85:0.15). As can be seen from the data presented, various PhLs have been introduced into the Ls forms of anthracyclines: EPC, HSPC, DSPC. When determining PhL for Ls formation, it is imperative to study the effect of the molecule structure and its charge on the properties of nanoparticles, unsaturation degree of fatty acid residues and products of PhL peroxidation, and other factors [4, 33, 34].

PhL is used in the Ls forms: neutral (various PC), anionic (DPG, PG, PA, PI) and cationic (PEA, dioleylphosphatidyletanolamine (DOPE), 1,2-dioleyloxy-3- [trimethytlamine]-propane (DOTAP)). For example, in the composition of the drug Visudin (EPG); DepoCyt (DPPG); hepatitis A vaccine (DOPE). In previous studies, anionic PhLs (PI, DPPG, DPG) were used [4, 35, 36, 39, 45, 46]. The studies carried out on the anionic PhLs inclusion in the Ls membrane bilayer when obtaining the Ls forms of cytochrome c, docetaxel, ubiquinone, oxaliplatin, curcumin, etc. have shown the following advantages: an increase in the inclusion of an active substance in Ls; stabilization and standardization of Ls emulsion; increasing the manufacturability of the process due to increasing the filtration rate of the Ls emulsion; the stability of the Ls emulsion upon rehydration of the lyophilized preparation [4, 46, 51]. In addition, the introduction of anionic PhLs made it possible to prolong the effect of the drug. For example, DPPG inclusion from EPC into the membrane allows increasing the percentage of cytochrome c incorporation into Ls due to the interaction of negatively charged DPPG with positively charged cytochrome c, which is based on the specific cytochrome c interaction with anionic PhLs [35−37, 52]. The effectiveness of cytochrome c Ls form has been shown in a number of pharmacological models [36, 37, 53]. It should be noted that there is no unequivocal opinion on the use of negative and positive PhLs. Using cytochrome c as an example, some authors confirm the effectiveness of anionic PhLs, others — of cationic PhLs [54−59]. Complex Ls preparations were proposed containing two phenolic antioxidants: curcumin and quercetin. When creating the drug, several PhLs were studied: EPC, HSPC and SFPC. The optimal relationships between bioflavonoids and PC have been determined. Moreover, EPC was the most effective. Chol inclusion in the Ls composition did not lead to an improvement in curcumin and quercetin incorporation into nanoparticles and Ls stabilization. At the same time, DPPG introduction into Ls made it possible to increase the percentage of API inclusion in Ls, maintain uniformity of dimensions and improve the manufacturability of the production process. A synergistic effect of antioxidants complex has been shown [40, 60]. Ls compositions of antioxidants were obtained, in which the complex of quercetin and curcumin/ quercetin and ubiquinone was introduced. The incorporation of hydrophobic antioxidants into the Ls bilayer was no less than 85−95 %. The preparations were lyophilized, the size of nanoparticles in which during hydration was no more than 300 nm. A high antioxidant activity has been demonstrated, and each of the AFIs used in model experiments on animals acted on different markers of oxidative stress [61].

It should be noted that it was proposed to introduce Chol into a number of Ls drugs.

Chol. When analyzing the Chol content in Ls preparations, attention is drawn to the fact that Chol is mainly contained in products containing hydrophilic APIs (Doxil, DepoCyt, Lipodox, Ls form of irinotecan, etc.). Chol is a critical component [62] both in the formation of Ls and in the release of hydrophilic molecules from Ls. Chol influences the fluidity and permeability of the Ls membrane from EPC.

Chol was used to develop Ls medicinal forms containing hydrophilic APIs: doxorubicin and irinotecan. Both medicinal forms were obtained using the lipid film method, high pressure homogenization and the pH gradient method. When studying the dependence of irinotecan encapsulation degree on the Ls composition, the inclusion of API was found: EPC (100 wt %) — 33%; EPC:Chol (95:5 wt %) — 44%; EPC:Chol (85:15 wt %) — 57%; EPC:Chol (70:30 wt %) — 71%. As the Chol content increases, the degree of irinotecan encapsulation increases [33]. However, at an EPC:Chol ratio of 70:30 wt %, the membrane rigidity increases, which requires an increase in pressure when sterilizing filtration is used. Also, during the analysis of the particle size by laser diffraction, the presence of particles with a diameter of more than 5 µm was detected, which indicates the inhomogeneity of the emulsion, and also affects the possibility of sterilizing filtration. Based on the data obtained, the EPC:Chol ratio of 85:15 wt % was selected for further experiments. The experiment has showed that membranes made of EPC (100%), EPC: Chol (95: 5) do not have a high degree of encapsulation, and are also unstable upon lyophilization using various cryoprotectors. It has been also shown that Ls above 220 nm are absent. The Ls membrane based on EPC:Chol (85:15) shows a high degree of encapsulation and stability upon lyophilization. Trehalose at a concentration of 6.0% was used as a cryoprotector. Similar data were obtained during the development of doxorubicin Ls form [33]. Chol decreased the release of doxorubicin from Ls and, at the same time, the antitumor activity increased. It has been found that Chol inclusion in Ls can lead to an increase in the size of particles obtained by lyophilization, as well as a decrease in the amount of API included in Ls. Apparently, Chol presence decreases the rate of hydrophilic APIs passage into Ls and hydrophilic APIs escape from Ls. The composition of the preparation also determines the technological parameters of LS preparations lyophilization. It has been established that slow Ls freezing leads to an increase in the percentage of API inclusion after lyophilization and rehydration, in comparison with fast freezing [63]. Thus, "rigid" Ls containing Chol retained their structure to a greater extent during slow freezing than during fast freezing. API and PhL composition of Ls determines the need in Chol for nanoparticles. The Ls preparations developed by us with lipophilic APIs contain EPC as the main PhL, in which unsaturated fatty acids are mainly represented. Probably, lipophilic components forming lipid bilayer lead to membrane "rigidity" and do not require a steroid component in Ls. Also, it should be noted that Chol introduction into Ls with lipophilic components significantly complicates the sterilizing filtration process of the emulsion through membranes with a pore size of no more than 0.22 µm, which may be associated with an increase in the "rigidity" of the Ls membranes. Due to the fact that Chol presence in Ls did not lead to an increase in API inclusion in nanoparticles and worsened the technological parameters of Ls obtaining, it was decided to reject of sterols use for lipophilic substances: quercetin (Lipoflavon), antral (Lioliv), curcumin, coenzyme Q10 and others [11, 13, 14, 38, 39, 60]. It should also be noted that the presence of Chol high content in the Ls membrane led to a decrease in the penetration rate of the hydrophilic medicinal substance (anthracycline antibiotics — doxorubicin, epirubicin, idarubicin; fluorouracil, platinum preparations, irinotecan) into nanoparticles [4, 33, 42].

Cryoprotectors. The proposed Ls preparations are presented as lyophilized forms, which makes it possible to stabilize nanosize and provide a longer shelf life. To preserve the nanosize during lyophilization, it is necessary to introduce cryoprotectors into the Ls composition. When choosing a cryoprotector and determining its concentration, we were guided by the following requirements: preservation of Ls nanosize during the storage period; the product should be readily soluble when administered to humans; have no impact on the physicochemical and pharmacological properties of Ls; have no effect on the technological parameters of Ls obtaining. Disaccharides lactose monohydrate and trehalose dihydrate in various concentrations were introduced as cryoprotectors into the Ls preparations being developed. The amount of disaccharides in the proposed preparations is different [4, 10–14, 32, 35, 38, 45, 60, 63]. The content of cryoprotectors in the Ls emulsion is from 2.5% to 6.0%, depending on the composition of the preparation, which ensured the safety of Ls nanosize during lyophilization and storage.

Buffer systems — pH regulators. A phosphate saline buffer was used as the buffer solution. This buffer system is non-toxic and is widely used in the production of pharmaceuticals. For the technology of the pH gradient with API inclusion, in addition to phosphate saline buffer, citrate was used, which is the part of Ls form and is contained in the human body (tricarboxylic acid cycle). These buffer systems are widely used in pharmaceutical technology as pH stabilizers [64]. The use of buffer mixtures makes it possible to stabilize the pH of both the nanoparticles themselves and the APIs included in their composition. The pH of Ls preparations ranged from 5.0 to 7.4 [4, 12, 14, 32, 33, 35, 38, 46].

The analysis of the data obtained during the Ls drugs creation suggests that the development of "Quality by design" of the specified form of drugs requires a variety of experimental works: determination of the PhL components optimal ratio in the lipid membrane and their concentration, PhL charge and their fatty acid

composition; study of cryoprotector type and its content in the preparation; pH value and ionic strength of the buffer system, etc. An independent question is the determination of the need for Chol inclusion in the Ls membrane, taking into account its role in increasing the lipid structure rigidity, which can lead to the appearance of particle size heterogeneity both during Ls production and during lyophilization. In addition, the presence of Chol can reduce the effectiveness of sterilizing filtration. The composition of Ls lipid membrane is also determined by API structure and its content in the preparation. Ls drug design requires experimental determination of drug sublimation modes, nanoparticle formation method and technological procedures of API loading into Ls.

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"QUALITY BY DESIGN" ПРИ СТВОРЕННІ ЛІПОСОМАЛЬНИХ ЛІКАРСЬКИХ ПРЕПАРАТІВ

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Створення нанобіотехнологічних препаратів є одним з перспективних напрямів сучасної фамації, оскільки дає змогу створювати продукти якісно нового рівня. Стратегія «Quality by Design» передбачає системний підхід до фармацевтичної розробки, що ґрунтується на розумінні особливостей продукту і процесу його отримання, підтверджених надійними науковими даними.

Статтю присвячено фармацевтичній розробці ліпосомних лікарських препаратів. На основі власного досвіду, а також даних літератури з розробки ліпосомних лікарських форм визначено основні компоненти, що входять до їхнього складу, і встановлено вплив кожного з них на показники якості ліпосом. Розглянуто роль окремих ліпідів мембрани наночастинок, їх взаємодію, що визначає стабільність як у процесі технології, так і під час зберігання готового препарату. Описано переваги і недоліки введення холестеролу до складу ліпосом з гідрофільними та гідрофобними активними фармацевтичними інгредієнтами. Обговорюється роль кріопротекторів і буферних систем у забезпеченні стабільності нанопрепарату.

Ключові слова: ліпосоми, фосфоліпіди, холестерол, кріопротектор, буферна система, Quality by Design.

"QUALITY BY DESIGN" ПРИ СОЗДАНИИ ЛИПОСОМАЛЬНЫХ ЛЕКАРСТВЕННЫХ ПРЕПАРАТОВ

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Создание нанобиотехнологических препаратов является одним из перспективных направлений современной фармации, поскольку позволяет создавать продукты качественно нового уровня. Стратегия «Quality by Design» предполагает системный подход к фармацевтической разработке, основанный на понимании особенностей продукта и процесса его получения, подтвержденных надежными научными данными.

Статья посвящена фармацевтической разработке липосомальных лекарственных препаратов. На основе собственного опыта, а также данных литературы по разработке липосомальных лекарственных форм определены основные компоненты, входящие в их состав, и установлено влияние каждого из них на показатели качества липосом. Рассмотрена роль отдельных липидов мембраны наночастиц, их взаимодействие, определяющее стабильность как в процессе технологии, так и при хранении готового препарата. Описаны преимущества и недостатки введения холестерола в состав липосом с гидрофильными и гидрофобными активными фармацевтическими ингредиентами. Обсуждается роль криопротекторов и буферных систем в обеспечении стабильности нанопрепарата.

Ключевые слова: липосомы, фосфолипиды, холестерол, криопротектор, буферная система, Quality by Design.

REALITIES AND PROSPECTS OF FUTURE COMPLEX PROCESSING OF PLANT RAW MATERIALS INTO BIOTHETANOL AND BY-PRODUCTS

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The use of plant biomass as a primary source of energy is currently unacceptable both from an economic and environmental point of view. The experience of a number of industries, in particular hydrolysis production, enables to solve the problem of profitability of organic biomass treatment by its deep complex processing with the resulting components whose cost exceeds the cost of organic raw materials as fuel. Currently, the main results of complex processing of organic raw materials are still energy-intensive products — bioethanol and hydrolyzed lignin, which energy characteristics are commensurate with fossil fuels. Bioethanol production from starch-containing, sugar-containing or lignocellulosic raw materials requires the use of different technological stages and, accordingly, the cost of bioethanol for each type of raw material is different. Compared to bioethanol produced from sugar and starch raw materials, bioethanol from manufactured lignocellulosic raw materials is more expensive. Bioethanol from lignocellulosic raw materials is more expensive compared to bioethanol obtained from sugar and starch raw materials. The most energy-intensive in the technology of bioethanol obtaining from lignocellulosic raw materials is the stage of pretreatment of raw materials for hydrolysis, because the process of preliminary preparation and hydrolysis with dilute acids occurs at high temperatures and pressures. During enzymatic hydrolysis, the process temperature is maintained for a long time (up to several days). To ensure deep integrated processing of plant raw materials, as well as to reduce overall costs, it was proposed to improve the technology and equipment, which allow increasing the degree of conversion of raw materials into basic and by-products.

Key words: bioethanol, biomass, preliminary preparation, hydrolysis, lignin, rotary pulsation apparatus.

1. *Biomass as a renewable resource*

The demand of the world economy for energy resources is growing every year. Thus, global oil reserves (as at 2015) amounted to 1657.4 billion barrels. Given that world oil consumption is estimated at 99 million barrels per day, there is a need for widespread use of renewable energy sources. Biomass is the largest renewable resource used in the world economy (more than 500 million tons per year) [1]. The most important sources of biomass are wood and crops.

The annual growth of biomass in the world is estimated at 146 billion tons in terms of dry matter [2], which is energy equivalent to 80 billion tons of oil. The biomass potential in Ukraine is up to 20,2 million tons of oil equivalent [3]. These are straw, agricultural waste, waste from woodworking enterprises, etc.

Biomass is considered one of the key renewable resources of the future. Its amount reaches $2.423\,10^{12}$ t, or $\approx 550\,Gt$ of carbon (C), of which $\approx 80\%$ is accounted for by plants, among which terrestrial plants are predominate [4].

Biomass is the most important source of energy for 3/4 of Earth's population living in developing countries. In developing countries, biomass provides an average of 38% of primary

energy (in some up to 90%). Biomass is likely to remain an important global source of energy in the 21st century. The most common sources of biomass are plants (wood, peat, straw). In the process of their use and processing, a large amount of renewable waste is generated every year. It is estimated that only 25% of waste is used efficiently. Developing countries, at the cost of biomass, can theoretically cover 15% of energy needs, industrial — 4% [5].

2. The state of bioethanol production in Ukraine and the types of raw materials available in Ukraine

In 2018, there were about 13 bioethanol producers in Ukraine. Six of them are quite successful — Zarubinsky, Gaisinsky distilleries, private plants Ecoenergy and Fazor, as well as Uzinsky and Gnidavsky sugar factories. In 2017, they produced 80,000 tons of bioethanol, which went to the production of alternative fuels [6].

Currently, industrial plants in bioethanol production employ mainly two types of primary raw materials, namely starch from cereals and juice or molasses from sugar crops (first generation bioethanol) [7].

Energy crops for ethanol production in the European climate zone, which includes Ukraine, are plants with high sugar and starch content — cereals, potatoes, sugar beets, corn for grain [8].

Corn has the highest yield of bioethanol per product unit — an average of 40 liters per 1 centner of grain (with dry grinding). The result of corn complex processing (wet grinding) is starch, gluten, glucose-fructose syrups, lysine, food alcohol, or bioethanol, citric and lactic acids, protein-mineral vitamin concentrates [9].

Another type of raw material that can be used for bioethanol production in Ukraine is sugar beet. This culture provides the highest yield of ethanol from 1 ha, does not require additional costs for pre-treatment of raw materials, allows reducing greenhouse gas emissions [10]. By-products of sugar production are beet pulp, which is used as fodder for agricultural livestock, molasses, and filtration sludge [11].

Studies on the use of energy crops as raw materials for bioethanol production are energy willow, miscanthus, sugar sorghum [12].

The optimal raw material for bioethanol production in Ukraine is molasses — a by-
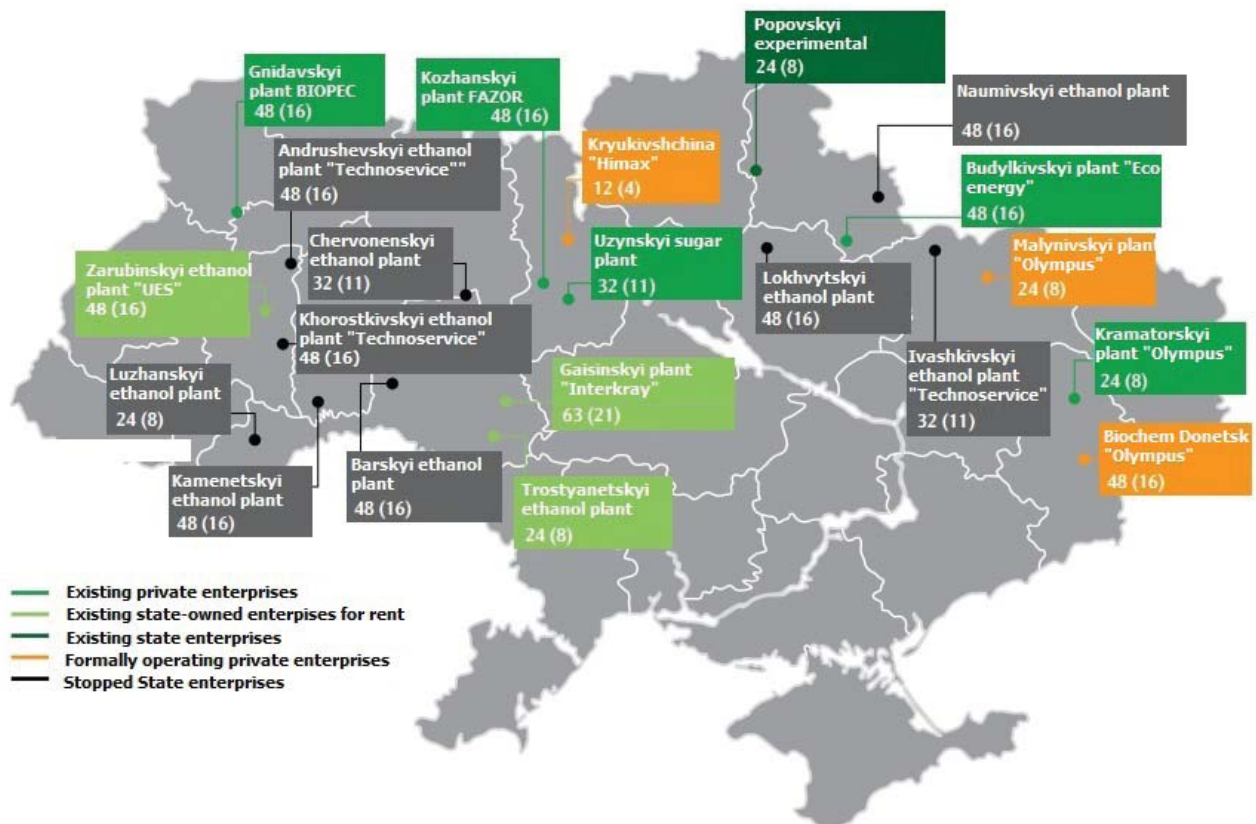
Fig. 1. **Ukrainian enterprises of various forms of ownership that produce bioethanol (for 2018) [6]**

product of sugar production. According to the authors of [13], the total energy consumption for one ton of bioethanol from molasses is 9 459 MJ, from corn — 56 565 MJ. The difference in production energy consumption is due to the presence of the stage of the operation of boiling raw materials and saccharification of starch by malt enzymes or enzyme preparations. The process of molasses preparation for fermentation consists only of homogenization of antiseptic molasses and dilution with water (spreading).

The main disadvantage of molasses as a raw material for bioethanol production is its limited amount. According to statistics, in 2019 the sugar factories of Ukraine received 832581.6 tons of sugar, 256067.6 tons of molasses, and 1297780 tons of pulp. That is, from 256067 tons it is possible to get 77896 tons of bioethanol, or (77896 tons · 0.64 = 49853.44 tons of oil equivalent) [14].

However, molasses are also used as a raw material for the production of food acids, baking, and feed yeast, as an additive to farm animal feed, as well as a binder in the lumping of fine coal [15].

The simplified block diagram of complex processing of grain raw materials for bioethanol obtaining with designation of by-products are shown in Fig. 2.

The comparative characteristics of different types of biomass as raw materials for bioethanol production are shown in Table 1.

3. Economic efficiency of different methods for bioethanol obtaining

It should be noted that the use of plant biomass as a primary energy source is currently unacceptable from both an economic and environmental point of view. The experience of a number of industries, in particular hydrolysis, allows solving the problem of profitability of organic biomass processing by its deep complex processing to obtain components whose cost exceeds the cost of organic raw materials as fuel.

The main results of the complex processing of organic raw materials are increasingly energy-containing products — bioethanol and hydrolyzed lignin, which energy characteristics are commensurate with fossil fuels.

Fig. 2. **Block chart of complex processing of grain raw materials and molasses for bioethanol obtaining with by-products designation**

Table 1. **Technological evaluation of raw materials for bioethanol production in Ukraine [16]**

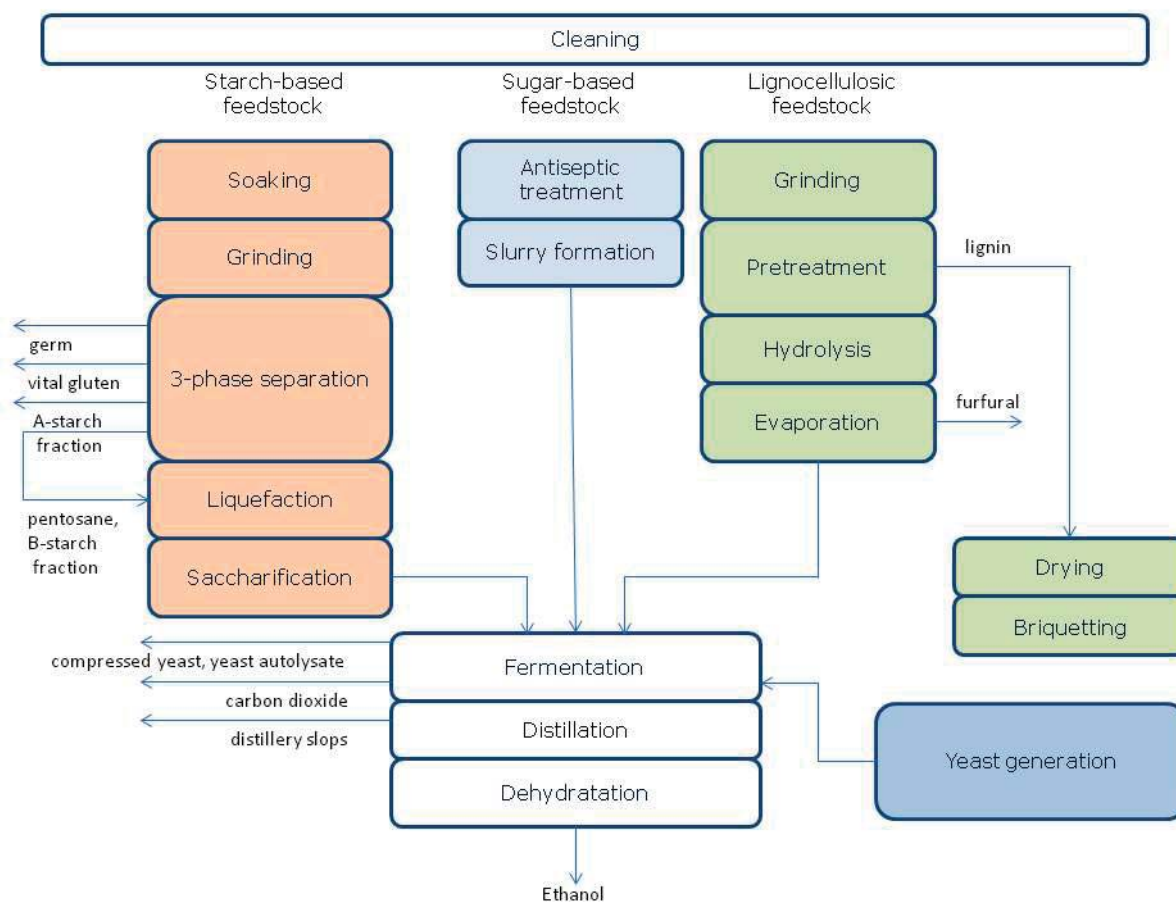| Type of raw material	Yield of bioethanol, th/tons of raw material	The required sowing area for the production of one ton of bioethanol, ha
Spiked cereals	0.3	1.24
Beet	0.08	0.42
Sorghum vulgare var. *saccharatum,* *Sorghum saccharatum*	0.09	0.14
Molasses	0.24	–
Wheat straw	up to 0.1*	–

Note: enzymatic hydrolysis with pretreatment by purified KOH [17].

The main direction of improving the process of complex processing of organic biomass, in particular plant raw materials, is the introduction of technologies and equipment that increase the degree of conversion of raw materials into basic and by-products without a significant increase in energy consumption.

The main factor that determines the efficiency of bioethanol production is energy consumption per unit of output.

Table 2 shows the average energy consumption of the plant for 1000 liters of bioethanol production. These indicators are typical but may vary depending on specific local conditions (according to VOGELBUSH Biocommodities) [18]. Steam consumption and supplied power vary depending on whether the drying of the post-alcoholic bard (wine concentration) takes place or not.

From the data given in Table 2 it can be concluded that the production of bioethanol from molasses is 44% less energy consuming compared to the production of bioethanol from wheat. The additional operation of drying the post-alcoholic bard, offered by the manufacturer, adds about 60% to energy

consumption (for molasses) and more than twice as much for wheat.

As an example of efficiency of deep grain processing, the material balance of typical plants offered by the VOGELBUSH group of companies is given (Table 3).

There are a number of studies on the cost-effectiveness of processing for various types of plant raw materials in order to obtain sugar with subsequent production of ethanol and by-products [20−22], in particular in [22] the fixed capital costs (buildings, equipment and plant construction costs, and production costs (including materials, labor, utilities, maintenance fees, and capital depreciation) (Fig. 3).

The figure shows that when investing in fixed capital, pulp processing has higher fixed costs than that of first-generation raw materials.

The acid-based pretreatment has the highest fixed cost because of the extra costs required to guard against equipment corrosion. The ionic liquid and organosolv pretreatments have the second highest fixed costs because of the additional equipment needed for recycling

Table 2. **Average energy consumption of the entire plant for 1000 liters bioethanol production (according to VOGELBUSH Biocommodities)**

Raw material	Part of starch (sugar*)	Quantity, kg	Steam, kg		Power, KW/h	
			Drying of post-alcoholic bard or vinassa concentration			
			no	yes	no	yes
Wheat	62	2420	1400	3150	115	260
Cereals	65	2285	1250	2750	110	220
Molasses	52*	3210	1700	3000	65	105
Cane juice *Sorghum vulgare* var. *saccharatum*, *Sorghum saccharatum*	18*	8640	1200	2550	60	90

Table 3. **Added value of typical plants (according to the VOGELBUSH group of companies) [19]**

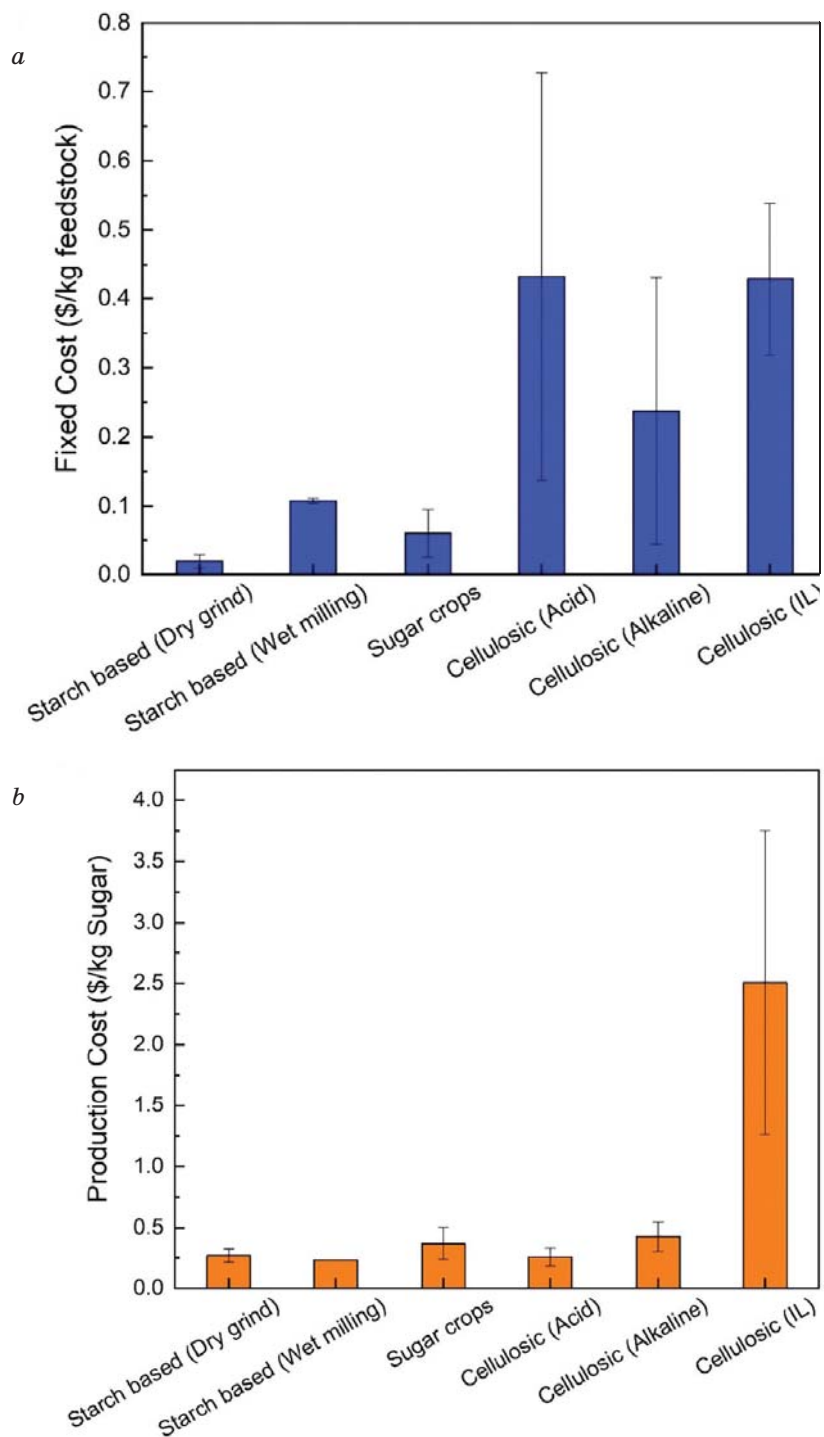| Type of enterprise	Wheat, thousand tons/year	Added value, million euros/year
A simple plant for grain processing into bioethanol	500	47.7
Deep processing plant grains	500	77.1
Plant for deep grain processing and bioethanol production	1000	123,3

Fig. 3. **Costs of sugar production from different types of feedstocks:**
a — fixed capital cost; *b* — production cost [22]

and facilities for storing the solvents. On the other hand, the least expensive sugar production operation used sugar crops because it required a relatively simple process for juice extraction.

Fixed costs for the wet grinding process are higher than for the dry grinding process due to operations related to the fractionation of corn grain.

Equipment used for raw material processing, pretreatment, and lignin separation is a major factor in increasing fixed capital investment for pulp raw materials.

As it is shown in Fig. 3, *b*, pretreatment of raw materials before hydrolysis using acid has the lowest cost of sugar production, because it has high efficiency and productivity of sugar. Lower sugar alkaline pretreatment productivity leads to higher sugar production costs.

The most promising lignocellulosic raw materials for complex processing are agricultural waste. Thus, in 2017, primary crop waste (straw and stems) amounted to 15039 thousand tons of oil equivalent, or 57.7% of the total biomass potential of agricultural enterprises of Ukraine, wood biomass — 16.3%, energy crops — 14.5% [23].

Fig. 2 shows that the common stages of the technological process of bioethanol obtaining, for these types of raw materials are the generation of yeast, fermentation, distillation, and dehydration of ethanol. The differences of the previous stages are determined by the type of raw material, the method for obtaining from it a sugar solution for fermentation and byproducts obtaining from it that are not used in ethanol production.

The most energy-intensive is the method of bioethanol obtaining from lignocellulosic raw materials, which consists of a stage of pretreatment for hydrolysis and direct hydrolysis. The main energy consumption is to maintain high temperatures and pressures during pretreatment and hydrolysis with dilute acids and to maintain a certain temperature for a significant time (up to several days) during enzymatic hydrolysis.

The main purpose of pretreatment of lignocellulosic raw materials for hydrolysis is the removal of lignin, which prevents the access of cellulolytic enzymes to cellulose fibers. Commercial pretreatment technologies are shown in Table 4 [24].

Cost distribution for the general lignocellulosic enterprise equipment is given in [24]. Pretreatment and steam production are the most cost-effective areas.

One of the ways to increase the efficiency of complex processing of lignocellulosic raw materials is the application of equipment that enables to increase the degree of raw materials conversion into basic and by-products. An example of the equipment that significantly intensifies heat and mass transfer in liquid multicomponent media are rotary pulsation devices, which are effective devices in technologies related to mixing, homogenization, dispersion, etc. [25]. In particular, there are a number of studies on the use of rotary pulsation devices in the technology of making wort from starch-containing raw materials [26, 27].

The research results on the use of rotary pulsation apparatus for delignification of wheat straw in the process of its preliminary preparation for hydrolysis are given in [28]. The hardware-technological scheme of the pretreatment stage for hydrolysis using a rotary pulsation apparatus and alkali as an agent for removing lignin is shown in Fig. 5. The receiving tank *1* and the rotary pulsation apparatus *2* are filled with an alkaline solution. After the engine start, the pre-crushed chips

Table 4. **Commercial technologies of of lignocellulosic raw materials prearrangement for hydrolysis**

| Type of process	Manufacturer	Characteristic
Steam explosion	Beta Renewables	Low xylose yield A large amount of enzyme preparation
One-stage treatment with dilute acid	Abengoa	High yield of xylose medium amount of enzyme preparation
Two-stage treatment with diluted acid	Poet−DSM	High yield of xylose Small amount of enzyme preparation
Ammonia and steam	Dupont	Requires a large amount of enzyme preparation
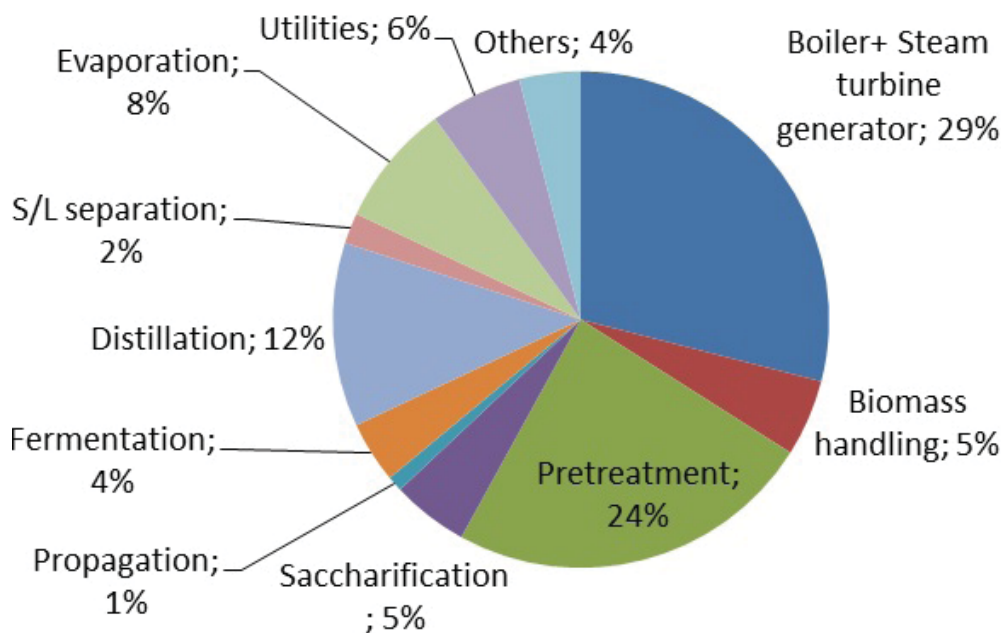
Fig. 4. **Cost distribution for equipment of the enterprise
for the bioethanol production from cellulose-containing raw materials [24]**

of annual plant raw materials are added into the solution moving in the reverse flow. The resulting dispersion passes through a rotary-pulsation apparatus. Processing is carried out in the recirculation mode at certain temperatures (up to 100 °C) and pressure (up to 0.1 MPa). The resulting dispersion is fed to filter *3* (optionally diluted with water), in which the filtrate containing lignin and the solid cellulose residue, which is fed to the stage of hydrolysis, is separated.

The filtrate from the filter is fed to the intermediate tank *4* and subjected to chemical treatment with sulfuric acid up to pH = 2, after which it is fed to filter *5*. The precipitated lignin remains on the filter and is fed to the drying stage. After filter *5* the filtrate is fed to the stage of hydrolysis.

If bioethanol is considered as an item, it has a stable high demand and turnover, which has a profitability of 15−30%. The production building is quite compact 30×21×24 meters. Of course, by building a complex for bioethanol production, the company has the opportunity to modernize processing and launch the production of baker's yeast, furfural or liquefied carbon dioxide, which are sold as separate products. Among the options for the future, the construction projects are considered for biogas production facilities through the methanation of bard and pulp with the production of green electricity, which

is implemented with a factor of 2.2−2.5. Every day, the plant, which processes 200 tons of molasses, produces 50 tons of bioethanol, 40 tons of carbon dioxide [29].

Lignin and bioethanol are energy-intensive products of plant biomass processing. The calorific value of dry lignin is 5500−6500 kcal/kg and is comparable to the calorific value of equivalent fuel (7000 kcal/kg). The caloric content of the product with a moisture content of 18−25% is 4400−4800 kcal/kg and the value of the product with a moisture content of more than 65% 1500−1650 kcal/kg, the caloric content of ethanol is respectively 6405 kcal/kg.

27 thousand tons of fuel equivalent per year could be produced from 43 thousand tons of absolutely dry lignin with a moisture content of 60%.

According to the world leader in lignin production Borregaard, 400 kg of cellulose, 50 kg of ethanol, 3 kg of vanillin, and 400 kg of hydrolyzed lignin are obtained from 1000 kg of wood after autoclaving, decolorization and drying.

According to various sources, the amount of lignin in Ukraine (waste from the yeast, feed, pulp, and paper industry) is from 5 to 15 million tons.

It is known that deep complex processing of plant raw materials can significantly increase the profitability of the relevant industries by obtaining additional products with high added
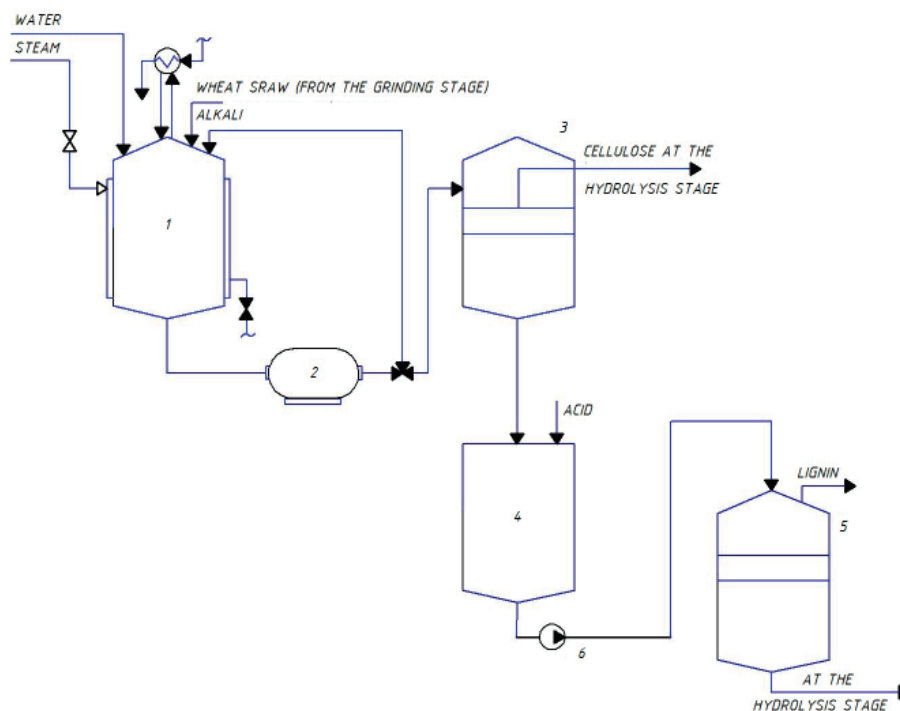
Fig. 5. **Hardware-technological configuration of the pretreatment stage
for hydrolysis using a rotary-pulsation apparatus:**
1 — receiving capacity; *2* — rotary pulsation aparatus; *3, 5* — filter; *4* — intermediate capacity; *6* — pump

value, thus reducing the cost of basic products and increasing the profitability of production as a whole. So, the substances obtained in the process of chemical destruction of organic raw materials in hydrolysis or yeast production are in great demand in the pharmaceutical, chemical, cosmetic industry, construction, and so on [5]. Much attention is paid to the integrated processing of organic raw materials in industrialized countries.

The centers for integrated processing of organic raw materials operate in Austria, France, Denmark, Germany, Canada, the Netherlands.

Thus, the Austrian company "Lenzing AG" produces furfural, acetic acid, sodium sulfite, potassium, lignin. At the same time, thermal and electrical energy is produced on the basis of lignin as an energy carrier.

In Canada, "Ensyn" recycles crop and wood waste, produces biodiesel, charcoal, binders, "green gasoline" (bioethanol), diesel, and aviation fuel (rapid pyrolysis is used). Biodiesel is used to produce heat and electricity, motor fuels, and a number of chemicals.

GEA Wiegand GMBH from Germany, producing bioethanol, additionally produces gluten flour, starches, dextrose, fructose syrup, and other high-value products.

Lignins, as products of complex processing of plant raw materials, have been used as dispersants, emulsifiers, binders and adhesives, sorbents for medical and technological purposes, sorbents for wastewater treatment [30, 31].

In the pharmaceutical field, lignin is considered as a promising material for hydrogels production, because lignin has antioxidant and antibacterial properties [32], it can be used as a material for 3D printers for the manufacture of dressings for wounds. The components of lignin decomposition are considered as antiviral drugs and antioxidants [33].

However, commercially lignins continue to be used as biofuels (fuel briquettes, pellets, including in a mixture with sawdust, coal and peat dust, fuel gas production, including the production of electricity in gas-piston gas generators, boiler fuel, etc.).

The most common type of use of hydrolyzed lignin with a humidity of $60–65\%$ is its combustion to produce heat.

Modern operating enterprises for lignocellulosic raw materials processing use lignin obtained during processing as fuel for steam production at the enterprise.

The use of lignin and fuel quality can cover up to 73% of the thermal energy demand for bioethanol production in an alkaline way [34].

Bioethanol obtaining from starch-containing, sugar-containing or lignocellulosic raw materials requires the use of different technological stages so the cost of bioethanol for each type of raw material is different accordingly. Compared to bioethanol with sugar-containing and starch-containing raw materials, bioethanol from lignocellulosic-containing raw materials is more expensive. The most energy-consuming is the stage of preliminary preparation of raw materials for hydrolysis in the technology of bioethanol obtaining from lignocellulosic raw materials. Increasing the profitability of bioethanol production from lignocellulosic raw materials is possible in two main ways, namely by its deep complex processing to obtain components whose cost exceeds the last one of organic raw materials as fuel and technology and equipment improvement enabling to increase the conversion of raw materials into basic and by-product materials.

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РЕАЛІЇ СЬОГОДЕННЯ ТА ПЕРСПЕКТИВИ КОМПЛЕКСНОЇ ПЕРЕРОБКИ РОСЛИННОЇ СИРОВИНИ НА БІОЕТАНОЛ ТА ПОБІЧНІ ПРОДУКТИ

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Використання рослинної біомаси як первинного джерела енергії на сьогодні є неприйнятним ні з економічного, ні з екологічного погляду. Досвід низки виробництв, зокрема гідролізного, дає змогу вирішити проблему рентабельності оброблення органічної біомаси шляхом глибокої її комплексної переробки з отриманням складових, вартість яких перевищує вартість вихідної органічної сировини як палива. Наразі головними результатами комплексної переробки органічної сировини залишаються енерговмісні продукти — біоетанол та гідролізний лігнін, які мають енергетичні характеристики, співставні з викопними паливами. Отримання біоетанолу з крохмалевмісної, цукровмісної або лігноцелюлозовмісної сировини потребує застосування різних технологічних стадій і, відповідно, собівартість біоетанолу для кожного виду сировини є різною. Порівняно з біоетанолом і цукровмісною та крохмалевмісною сировиною біоетанол з лігноцелюлозовмісної сировини є дорожчим. Найенерговитратнішою в технології отримання біоетанолу з лігноцелюлозовмісної сировини є стадія попередньої підготовки сировини до гідролізу, оскільки цей процес і гідроліз розведеними кислотами відбувається за високих температури та тиску. Під час ензиматичного гідролізу температура процесу підтримується протягом тривалого часу (до кількох діб). Для забезпечення глибокої комплексної переробки рослинної сировини, а також з метою зменшення загальних витрат запропоновано вдосконалення технології та обладнання, що уможливить збільшення ступеня конверсії вихідної сировини в основні та побічні продукти.

Ключові слова: біоетанол, біомаса, попередня підготовка, гідроліз, лігнін, роторно-пульсаційний апарат.

GASOMEDIATOR H$_2$S IN THROMBOSIS AND HEMOSTASIS

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This review was aimed to briefly summarize current knowledge of the biological roles of gasomediator H$_2$S in hemostasis and cardiovascular diseases. Since the discovery that mammalian cells are enzymatically producing H$_2$S, this molecule underwent a dramatic metamorphosis from dangerous pollutant to a biologically relevant mediator. As a gasomediator, hydrogen sulfide plays a role of signaling molecule, which is involved in a number of processes in health and disease, including pathogenesis of cardiovascular abnormalities, mainly through modulating different patterns of vasculature functions and thrombotic events. Recently, several studies have provided unequivocal evidence that H$_2$S reduces blood platelet reactivity by inhibiting different stages of platelet activation (platelet adhesion, secretion and aggregation) and thrombus formation. Moreover, H$_2$S changes the structure and function of fibrinogen and proteins associated with fibrinolysis. Hydrogen sulfide regulates proliferation and apoptosis of vascular smooth muscle cells, thus modulating angiogenesis and vessel function. Undoubtedly, H$_2$S is also involved in a multitude of other physiological functions. For example, it exhibits anti-inflammatory effects by inhibiting ROS production and increasing expression of antioxidant enzymes. Some studies have demonstrated the role of hydrogen sulfide as therapeutic agent in various diseases, including cardiovascular pathologies. Further studies are required to evaluate its importance as a regulator of cell physiology and associated cardiovascular pathological conditions such as myocardial infarction and stroke.

H$_2$S: from toxin to biological mediator

For many decades, hydrogen sulfide (H$_2$S), a simple gaseous molecule with the smell of rotten eggs, was considered to be a toxic gas that penetrates cells by simple diffusion [1]. Generations of researchers have investigated the toxicological effects of H$_2$S in various species, including human. Among the more recent studies: Attene-Ramos demonstrated the genotoxic effect of high doses of H$_2$S [2], Nicholson [3], Khan [4] and later Dorman [5] have directly showed the inhibition of cytochrome c oxidase activity *ex vivo* in tissues after H$_2$S exposure of experimental animals, and implicating these effect s in the disruption of respiratory and mitochondrial functions in the mammalian brain (and

other tissue). It is currently accepted that H$_2$S exerts its toxicological actions on the molecular level primary through the inhibition of mitochondrial Complex IV. Via this action, the consumption of O$_2$ is limited and mitochondrial electron transport and ATP generation is blocked. However, the toxicological mode of H$_2$S action is more complex, as it is capable of interacting with multiple intra- and extracellular proteins (for instance, sulfhydration etc.).

Following the discovery that mammalian cells are capable of producing H$_2$S, this molecule underwent a dramatic metamorphosis of recognition from dangerous pollutant to a biologically relevant molecule (as NO). Three enzymes have been shown to enzymatically

generate H$_2$S, cystathionine β-synthase (CBS), cystathionine γ-lyase (CTH or CSE) and 3-mercaptopyruvate sulfurtransferase (3MST) [6−8]. CBS and CSE participate in the interconversion of homocysteine to cysteine, known as the transsulfuration pathway; both enzymes are pyridoxal-5 phosphate dependent [9, 10]. It should, however, be kept in mind that CBS and CSE catalyze number of additional reactions that do not yield H$_2$S [9]. The gene expression of CBS and CSE has been detected in various tissues, including the liver, kidney, lymphatic system, vascular wall, cardiomyocytes and fibroblasts. While these enzymes contribute equally to the local production of H$_2$S in liver and kidney [11], one of the enzymes could be dominant in other contexts. There is prevalence of CSE in cardiovascular system [12]. Relatively high concentration of CSE is observed in arteries, and H$_2$S is produced by both endothelial cells [13] and smooth muscle cells of the vessel wall [14]. The key enzyme for H$_2$S synthesis in the central and peripheral nervous system is CBS [15].

H$_2$S dissolved in water is a weak acid and dissociates into H$^+$, HS$^-$, and S$_2^-$. At physiological pH (7.4), such as in blood and other physiological solutions, approximately 14% of the free sulfides are present as gaseous H$_2$S, more than 80% is present as HS$^-$, and the rest is S$_2^-$. It is still undetermined, which form is biologically active. H$_2$S itself, HS$^-$, polysulfides, as well as S/N hybrid species have been shown to affect a variety of signaling pathways leading to biological responses [16, 6, 17]. Hydrogen sulfide is also soluble in lipid membranes so that it has access to both intracellular and extracellular sites of target proteins [18]. A primary mechanism through which H$_2$S affects the activity of signaling proteins is a modification of reactive cysteine SH groups to persulfide groups (-SSH) [19]. This posttranslational modification is similar to S-nitrosylation, which is induced by NO, and could be an important signaling mechanism. Depending on the nature of the targeted protein, the effect of H$_2$S might take from seconds to days to manifest.

The field of H$_2$S biology has dramatically expanded over the last decade. Now endogenous hydrogen sulfide is recognized as a gasomediator of various physiological and pathological processes [1]. H$_2$S has been proven to be involved in vascular relaxation, hypertension, cellular proliferation, gene expression, cardioprotection, neuroprotection, intestinal secretion, diabetes, apoptosis, atherosclerosis and inflammation.

H$_2$S in vascular biology and thrombosis

Endogenous concentrations of hydrogen sulfide in human plasma are ranged from 30 μM to 65 μM [20]. Its physiological level in brain is threefold higher than in serum [21]. However, H$_2$S concentration in human tissues depends on the method used for measurement and the donor's age [20]. The primary action of H$_2$S in the vasculature is vasodilatory [6, 10, 1]. Although, biphasic responses to H$_2$S have been reported [22]. The first reports on vasoactive responses to endogenous H$_2$S were from Kimura's group, where they demonstrated the presence of H$_2$S-producing enzymes in vascular tissue, and showed the smooth muscle relaxant effect of H$_2$S, alone and in synergy with nitric oxide [23]. Latter studies, from Wang's laboratory demonstrated the importance of KATP for H$_2$S-triggered vasorelaxation [14]. Based on its ability to hyperpolarize endothelial and smooth cell membrane, its biological activity on small and/or intermediate conductance KCa channels, and its greater potency as a vasodilator in resistance versus conduit arteries, H$_2$S has been proposed as a candidate for endothelium-derived hyperpolarizing factor [24, 25]. Various groups have shown the protective effect of H$_2$S in organ injury and postischemic reperfusion disorders [26]. H$_2$S contributes to the maintenance of mean arterial blood pressure at physiological levels; pharmacological inhibition of H$_2$S production was shown to increase blood pressure [27]. Several laboratories have confirmed that H$_2$S drives angiogenesis by stimulating EC growth, motility, and organization into vessel-like structures [28−30]. Enhanced oxidative stress is a key event for diseases affecting the vessel wall including hypertension, atherosclerosis, and vascular diabetic complications. Hydrogen sulfide exhibits anti-inflammatory effects by inhibiting ROS production, but also eliminates ROS by direct scavenging, upregulation of GSH, and increased expression of antioxidant enzymes [31−33]. It was observed that H$_2$S causes apoptosis of human aortic smooth muscle cells and reduces the growth of atherosclerotic lesions [34].

Recent studies showed that H$_2$S exerts antithrombotic properties by inhibiting different steps of platelet activation (platelet adhesion, secretion and aggregation) and thrombus formation [35−39]. First it was demonstrated that NaHS (H$_2$S donor) prevented in a concentration-dependent manner human platelet aggregation induced
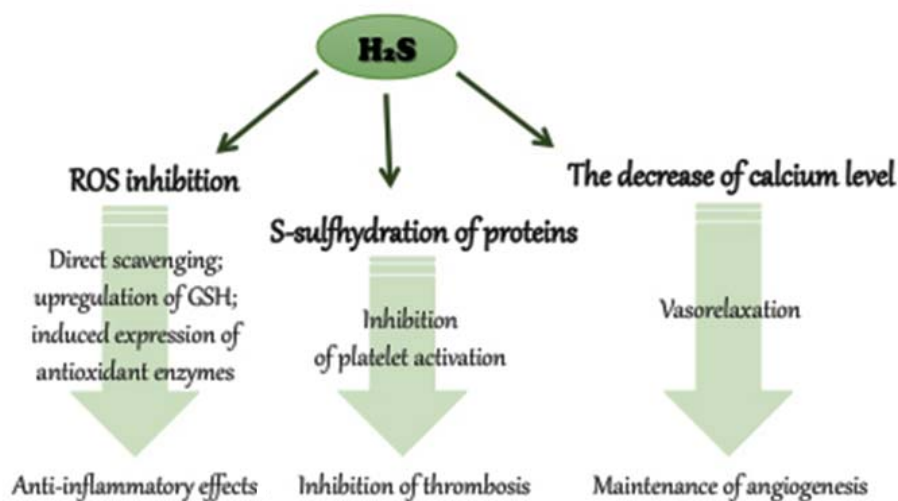
Gasomediator H$_2$S in vascular biology and thrombosis

by different agonists: ADP, U46619, collagen, epinephrine, thrombin and arachidonic acid [36]. Results of Nishikawa et al. showed that H$_2$S suppresses rabbit platelet aggregation (induced by collagen and ADP) by interfering with both upstream and downstream signals of cytosolic Ca^{2+} mobilization in cAMP-dependent manner [37]. Experiments of Grambow et. al. suggested that the anti-aggregatory effect of hydrogen sulfide might be due to S-sulfhydration of blood platelet proteins [39]. Next study demonstrated the inhibitory action of H$_2$S on blood platelet adhesion [38]. Moreover, hydrogen sulfide modifies the adhesive properties of collagen and fibrinogen [39]. The authors assume that the interaction of modified adhesive proteins may cause impaired adhesion [39]. Other research group observed that H$_2$S-releasing aspirin derivative ACS14 exerts strong antiaggregatory effects *in vitro* and *in vivo* via impairing the activation of fibrinogen receptor by mechanism involving increased intracellular cyclic nucleotides [40]. The study of Kram et al. has shown that H$_2$S has antithrombotic action, i.e. prolonging the time until both initial occlusion of blood flow. It was concluded that the anti-thrombotic efficacy of H$_2$S involves the NOS pathway [41].

The effects of hydrogen sulfide on the complex coagulation system and fibrinolysis are manifold due to its pleiotropic character. Olas and Kontek reported that activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT) of plasma treated with NaHS (H$_2$S donor) are prolonged *in vitro* [42]. The reduced fibrin polymerization of plasma in the presence of NaHS was also observed [42]. These results indicate the anticoagulant activities of H$_2$S. Modifications of various proteins of hemostatic system (including fibrinogen, plasminogen, and thrombin) induced by H$_2$S may be associated with changes of coagulation process and fibrinolysis. Other researchers demonstrated that compound with thiol group(s) enhances plasma factor XIII-mediated fibrinogen cross linking [43, 44]. It is possible that H$_2$S is involved in this process.

Some studies have demonstrated the role of hydrogen sulfide as therapeutic agent in various diseases, including cardiovascular diseases. An injectable Na$_2$S (IK-1001), which is H$_2$S donor, has been developed for clinical use [45]. S-allylcystein, which may be derived from garlic, reduced blood platelet aggregation, and this action may be mediated through H$_2$S [46]. Some proposal mechanisms of H$_2$S actions in vascular biology and thrombosis are summarized in Figure.

Hydrogen sulfide is a ubiquitous signaling molecule with important functions in many mammalian organs and systems. Although some beneficial properties of H$_2$S in hemostasis and thrombosis are well established, mechanistic insights into the molecular pathways implicated in disease prevention and treatment remain largely unexplored. In addition, acute regulation of H$_2$S production is still poorly understood and new researches delineating the pathways regulating the enzymes that produce H$_2$S will allow pharmacological manipulation of these pathways.

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ГАЗОМЕДІАТОР H$_2$S У ТРОМБОЗІ ТА ГЕМОСТАЗІ

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Метою огляду було стисло підсумувати наявні дані стосовно біологічної ролі газомедіатору сульфіду гідрогену в гемостазі та за розвитку серцево-судинних захворювань. Після відкриття здатності клітин ссавців ензиматично продукувати H$_2$S погляди на значення цієї молекули кардинально змінювалися: від віднесення його до небезпечних токсинів до визнання біологічно важливим регулятором. Як газомедіатор сульфід гідрогену відіграє роль сигнальної молекули, що залучається до низки процесів за норми та патології, включно з патогенезом серцево-судинних порушень, головним чином, модулюючи переважно різні аспекти функціонування судин та тромботичні явища. Нещодавно було отримано беззаперечні докази пригнічення сульфідом гідрогену активності тромбоцитів, що спостерігається на різних стадіях їх активації (тромбоцитарна адгезія, секреція та агрегація), а також власно формування тромбу. Більш того, H$_2$S модифікує структуру і функцію фібриногену та протеїнів системи фібринолізу. Сульфід гідрогену регулює проліферацію та апоптоз клітин гладеньких м'язів, модулюючи у такий спосіб ангіогенез і функціонування судин. Не викликає сумнівів, що H$_2$S також залучається до реалізації низки інших фізіологічних функцій. Наприклад, він виявляє протизапальні ефекти через інгібування утворення активних форм оксигену та підсилення експресії антиоксидантних ензимів. У деяких дослідженнях висвітлено роль сульфіду гідрогену як терапевтичного агента за різних захворювань, зокрема патологій серцево-судинної системи. Подальшого з'ясування потребує значення цього газомедіатору як регулятора клітинної фізіології за розвитку серцево-судинних хвороб, зокрема, інфаркту міокарда та інсульту.

Ключові слова: сульфід гідрогену, газомедіатор, гемостаз, тромбоз, фібриноліз, тромбоцити, серцево-судинні хвороби.

ГАЗОМЕДИАТОР H$_2$S В ТРОМБОЗЕ И ГЕМОСТАЗЕ

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Целью обзора было вкратце подытожить существующие сведения о биологической роли газомедиатора сульфида водорода в гемостазе и при развитии сердечно-сосудистых заболеваний. После открытия способности клеток млекопитающих энзиматически производить H$_2$S взгляды на значение этой молекулы кардинально изменялись: от отнесения его к опасным токсинам до признания биологически важным регулятором. Как газомедиатор сульфид водорода играет роль сигнальной молекулы, вовлекаемой в ряд процессов при норме и патологии, включая патогенез сердечно-сосудистых нарушений, модулируя главным образом различные аспекты функционирования сосудов и тромботические явления. Недавно были получены неопровержимые доказательства подавления сульфидом водорода активности тромбоцитов, наблюдаемые на разных стадиях их активации (тромбоцитарная адгезия, секреция и агрегация), а также собственно формирование тромба. Более того, H$_2$S модифицирует структуру и функцию фибриногена и протеинов системы фибринолиза. Сульфид водорода регулирует пролиферацию и апоптоз клеток гладких мышц, модулируя таким образом ангиогенез и функционирование сосудов. Не вызывает сомнений, что H$_2$S также участвует в реализации ряда других физиологических функций. Например, он проявляет противовоспалительные эффекты, ингибируя образование активных форм кислорода и усиливая экспрессию антиоксидантных энзимов. В некоторых исследованиях освещены роль сульфида водорода в качестве терапевтического агента при различных заболеваниях, в частности патологий сердечно-сосудистой системы. Дальнейшего выяснения требует значение этого газомедиатора как регулятора клеточной физиологии при развитии сердечно-сосудистых заболеваний, в частности инфаркта миокарда и инсульта.

Ключевые слова: сульфид водорода, газомедиатор, гемостаз, тромбоз, фибринолиз, тромбоциты, сердечно-сосудистые заболевания.

EXPERIMENTAL ARTICLES

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EXPRESSION OF NUCLEOCAPSID VIRAL PROTEINS IN THE BACTERIAL SYSTEM OF *Escherichia coli*: THE INFLUENCE OF THE CODON COMPOSITION AND THE UNIFORMITY OF ITS DISTRIBUTION WITHIN GENE

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A heterologous host has got a unique expression ability of each gene. Differences between the synonymous sequences play an important role in regulation of protein expression in organisms from *Escherichia coli* to human, and many details of this process remain unclear. The work was aimed to study the composition of codons, its distribution over the sequence and the effect of rare codons on the expression of viral nucleocapsid proteins and their fragments in the heterologous system of *E. coli*. The plasmid vector pJC 40 and the BL 21 (DE 3) *E. coli* strain were used for protein expression. The codon composition analysis was performed using the online resource (*www.biologicscorp.com*). Ten recombinant polypeptides were obtained, two of them encoding the complete nucleotide sequence of nucleocapsid proteins (West Nile and hepatitis C viruses) and the fragments including antigenic determinants (Lassa, Marburg, Ebola, Crimean-Congo hemorrhagic fever (CCHF), Puumala, Dobrava-Belgrade, Hantaan, and lymphocytic choriomeningitis viruses). Hybrid plasmid DNAs provided efficient production of these proteins in the prokaryotic system. The recombinant protein yield varied from 5 to 40 mg per one liter of bacterial culture. No correlation was found between the level of protein expression and the frequency of rare codon occurrence in the cloned sequence: the maximum frequency of rare codon occurrence was observed for the West Nile virus (14.6%), the minimum one was for the CCHF virus (6.6%), whereas the expression level for these proteins was 30 and 5 mg/l of culture, respectively. The codon adaptation index (CAI) values, calculated on the basis of the codon composition in *E. coli*, were in the range from 0.50 to 0.58, which corresponded to the average expressed proteins. The analysis of the CAI distribution profiles indicated the absence of rare codons clusters that could create difficulties in translation. Difference between the frequencies of the amino acids distribution and their content in E. coli was statistically significant for the nucleocapsid proteins of the Marburg, Ebola, West Nile, and hepatitis C viruses.

Key words: recombinant nucleocapsid proteins, expression, rare codons, codon adaptation index.

The development of recombinant DNA technology laid the foundation for the expression of proteins in a wide range of various cellular systems (from bacterial to eukaryotic and cell-free ones). Thus, the process of recombinant proteins production has become faster and easier in comparison with their natural counterparts. Until now, *Escherichia coli* remains the main host for protein production. The main advantages of this system are potentially very high expression levels, rapid growth of culture, low

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cost of media and simple cultivation conditions are the main advantages of this system. The scale of production of therapeutic, diagnostic, and industrially important proteins and/ or enzymes ultimately depends on protein expression the in a heterologous system. The expression ability of each gene is unique in a heterologous host, and not all proteins are successfully synthesized in *E. coli* cells. Many factors, such as a vector and a host, the promoter strength, the inducers concentration, the composition of the medium, and a number of others, affect the efficiency of protein production [1]. It has been known for a long time that gene expression in a heterologous host is disrupted due to differences in the use of codons by an organism [2]. Degenerate coding of 20 amino acids by 61 nucleotide triplets makes it possible to synthesize the same protein sequence using a huge number of synonymous mRNAs. Differences between synonymous sequences play an important role in the regulation of protein expression in the organisms from *E. coli* [3−5] to human, and many details of this process remain unclear [6]. Most literature on codon use are focused on a study of rare codons such as AUA codon for Ile and AGA, AGG and CGG for Arg, insufficient amount of the corresponding specialized tRNA [7]. However, this assumption was refuted by the studies on ribosome profiling, which showed that the net rate of translation-elongation, as a rule, was constant and did not depend on the use of codons [8]. The level of protein expression could be influenced not only by the presence of so-called rare codons, but also by their location and distribution within the gene. It has been reported that the presence of the AAA codon at position of +2 gene increases expression [9], while the NGG codon has the opposite effect at position +2 [10]. In this context, the codon composition of a heterologous gene can be optimized for expression in a particular host.

Studying the effect of mRNA sequence on protein expression is complicated by the fact that the changes in synonymous sequences (optimization) simultaneously affect many parameters, including identity, codon homogeneity and mRNA folding, as well as other features of local and whole sequences, ranging from the effects of codon pairs to theirs general content of A/U/C/G [11].

The aim of this research was to study the composition of codons, its distribution over the gene, and the effect of rare codons on the expression of viral nucleocapsid proteins and their fragments in the heterologous system of *Escherichia coli.*

Material and Methods

Lassa virus (LASV) (Josiach strain), Marburg virus (MARV) (Voege strain), Ebola virus (EBOV) (Zair strain) were obtained from Dr.G. van der Groen (Institute of Tropical Medicine, Belgium). Lymphocytic choriomeningitis virus (LCMV) (Armstrong strain) was obtained from the Center for Disease Control and Prevention (Atlanta, USA). Crimean-Congo hemorrhagic fever (CCHF) virus (strains 22263, Astrakhan and Uzbekistan); Hantaan virus (HTNV) (strain 4950); Dobrava-Belgrade virus (strain Aa 118) were obtained from the Ivanovsky Institute of Virology of RAMS (Moscow, Russian Federation). Puumala virus (PUUV) (strains CG-1820 and K-27) was obtained from the Center of the Ministry of Health of the Russian Federation for Combating Hemorrhagic Fever with Renal Syndrome (headed by Professor E.A. Tkachenko, Doctor of Medical Sciences) (Moscow, Russian Federation). Virus-containing fluid from a patient with laboratory-confirmed diagnosis of hepatitis C virus was used to obtain a nucleotide sequence encoding the hepatitis C virus nucleocapsid protein.

E. coli strain DH5 α (*supE44 lacU169 (f80 lacZ∆M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) was used for genetic engineering. *E. coli* strain BL21 (DE3) (*F− ompT gal dcm lon hsdSB* (rB —mB −) λ (DE3 [*lacI lacUV5-T7p07 ind1 sam7 nin5*]) [*malB+*]K-12 (λS)) was used for the expression of recombinant proteins. Plasmid pJC 40 [12] was used as an expression vector providing transcription of cloned genes under the control of the T7 polymerase promoter. The plasmid contains an additional fragment encoding 10 histidine residues (His), which is localized at the N-terminal part of the polypeptide during translation, allowing protein purification by metal chelate chromatography.

Topological analysis of nucleocapsid proteins amino acid sequences to search for antigensignificant sites was carried out using the computer program wxGeneBee.

Oligonucleotide sequences (primers) for the amplification of DNA fragments encoding regions of nucleocapsid proteins were synthesized by Primetech (Republic of Belarus).

Viral RNA obtained from virus-containing fluid was used as an initial template for reverse transcription. RNA was extracted using NucleoSpin RNA reagent kit (MACHERY-Nagel, Germany).

The reverse transcription reaction was performed using the Reverta-L reagents kit (Amplisense, Russian Federation) according

to the manufacturer's instructions. The complementary DNA (cDNA) was used for PCR. Reaction mixture contained 25 pmol of primers, 5 µl of 10x Taq buffer (Primetech, Republic of Belarus), 4 µl of 25mM MgCl2, 1 µl of dNTP (10 mM each), 10 µl of RT product (cDNA), 2.5 units of Taq DNA polymerase (Primetech, Republic of Belarus), deionized water to a final volume of 50 µl.

Analysis of DNA fragments synthesized in PCR was carried out by electrophoresis in 1.5–2% agarose gel. DNA was visualized in UV light by staining the gel with ethidium bromide.

DNA was hydrolyzed with restriction enzymes (1 U each) in accordance with the instructions (Thermo Scientific, USA) at 37 °C for 2 hours in a final volume of 20 µl. After incubation the enzymes were inactivated for 15 min at 65 °C.

DNA fragments were ligated in a volume of 20 µl at 22 °C for an hour. T4 DNA Ligase (Thermo Scientific, USA) was used as a ligating enzyme according to the manufacturer's instructions.

Isolation of recombinant plasmid DNA was performed using the QIAprep Spin Miniprep Kit (Qiagen).

Bacterial *E. coli* cells, BL 21 (DE3) strain, transformed with plasmid DNA, were cultured in a liquid LB medium containing ampicillin as a selective agent at a concentration of 50 µg/ml (Sigma, United States) with constant shaking at 37 °C until the cell culture reached the optical density OD600 = 0.3. Isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma, USA) was added to the medium and cells were further incubated for 3.5 hours under the same conditions. Inducer concentration, cultivation temperature, medium composition were optimized to provide the maximum yield of each recombinant polypeptide.

Analysis of recombinant polypeptides in polyacrylamide gel (PAGE) was performed according to the method proposed by Laemmli.

Analysis of the codon composition and calculation of the codon adaptation index (CAI) were performed using an online resource (www.biologicscorp.com). Each cloned sequence was divided into segments of 10 codons for assessing the distribution of CAI index values within a gene. The index value was determined for each of these segments.

Statistical data processing was carried out using the STATISTICA 6.0 package.

Results and Discussion

During development of immunodiagnostic kits for highly pathogenic viruses, the use of biotechnological methods is now of great importance, allowing the production of antigens and antibodies to them by artificial means. This eliminates the need to work with a highly contagious pathogen and is more cost-effective and technologically advanced. Recombinant technologies make it possible to express not only full-length proteins, but also their individual antigenic fragments, as well as mosaic molecules consisting of separate sections of one or several antigenic determinants.

The nucleocapsid protein (N-protein) packs the genome, acts as an RNA chaperone, provides intracellular protein transport, participates in DNA degradation and translation processes of the host cell. And it is commonly used as an antigen in the most serological studies, since it has a pronounced antigenic and immunogenic activity along with a high conservatism.

This article presents data on the expression of full-length nucleocapsid proteins and their fragments of ten highly dangerous viruses from various families of Arenaviridae (Lassa virus and lymphocytic choriomeningitis virus), Bunyaviridae (Hantaan, Puumala, Dobrava-Belgrade and Crimean-Congo hemorrhagic fever viruses), Filoviridae (Marburg and Ebola viruses), Flaviviridae (hepatitis C and West Nile viruses) in the prokaryotic system (*Escherichia coli* BL 21 (DE 3)) and expression vector pJC 40.

The potential antigenic determinants exposed on a molecule surface were found. The topological maps of the amino acid sequences of viral nucleocapsid proteins were compiled using the wxGeneBee computer program. The program is based on the scales of hydrophobicity and hydrophilicity proposed by Hopp and Woods (1981) and Kyte and Doolitle (1982). The evaluation criteria include such parameters as solubility, charge, distance from the NC backbone, the presence of helices, and the presence of sulfhydryl residues.

Analysis of antigenic determinants in nucleocapsid proteins showed their sequential arrangement throughout the entire sequence for the proteins of Lassa, LCM and hepatitis C viruses; C-terminal localization in Marburg, Ebola, West Nile viruses; placement at the N-terminus for Puumala, Hantaan, Dobrava-Belgrade viruses; central location in the CCHF virus. These studies served as a basis for selection and subsequent cloning into the expression vector of the following sequences: 432 amino acids (aa) of the Lassa virus (6/8 of amino acid sequence from position 137 aa to 569 aa); 320 amino acids of LCMV

(from 41 to 361 aa). For etiological agents of hemorrhagic fever with renal syndrome (HFRS), the most extended antigenic regions within the first 117 amino acids were found in the N-terminal part of the molecule (amino acids 1 to 117). For the CCHF virus the choice was made in favor of a sequence of 105 amino acid bases (from 202 to 306 aa), including one antigensignificant site with the highest antigenicity index located in the center of the protein. Five hydrophilic regions for the Marburg virus and six for the Ebola virus were identified as potential antibody binding sites in the C-terminal part of the protein. The most extended antigen-significant amino acid areas are localized in the region 442–695 aa of Marburg virus nucleocapsid, represented by four B-sites, and in region 434–739 aa of Ebola virus nucleocapsid, including all six antigenic determinants. The profile of antigenic sites in the nucleocapsid proteins of hepatitis C and West Nile viruses showed their uniform distribution in the sequence for the hepatitis C virus and the C-terminal location for the West Nile virus. Small size of the West Nile virus nucleocapsid protein and identification of only one antigenic determinant were the reasons to clone the full-length core proteins of these viruses. Specific fragments of 190 amino acids

and 103 amino acids encoding full-length nucleocapsid proteins of hepatitis C virus and West Nile virus, respectively, were cloned into the expression vector.

The results of the experiments showed that the level of recombinant polypeptides expression in optimal for each of them conditions varied to a large extent (by 8 times): from 40 mg per liter of culture for representatives of arenoviruses (Lassa virus, LCMV) to 5 mg per liter for the CCHF virus (Fig. 1). The highest yield of recombinant nucleocapsid proteins (30–40 mg/l of cell culture) was observed for the cloned sequences of three viruses: Lassa, LCM, and West Nile (Fig. 1, *A, B, C,* respectively). The average expression level was detected for the fragments of the Hantaan, Marburg, Ebola, Puumala, Dobrava-Belgrade, and hepatitis C viruses (from 25 to 15 mg/l of cell suspension) (Fig. 1, *C, E, F, H*). CCHF virus nucleocapsid was poorly expressed (5 mg/l) (Fig. 1, *D*).

One of the explanation for the different levels of protein biosynthesis may be the different number of codons that are rare for *E. coli* in the cloned viral sequence.

Amino acid codons rarely found in *E. coli* cells include AGG/AGA/CGA triplets encoding arginine (frequency of occurrence 1.1; 2.0;
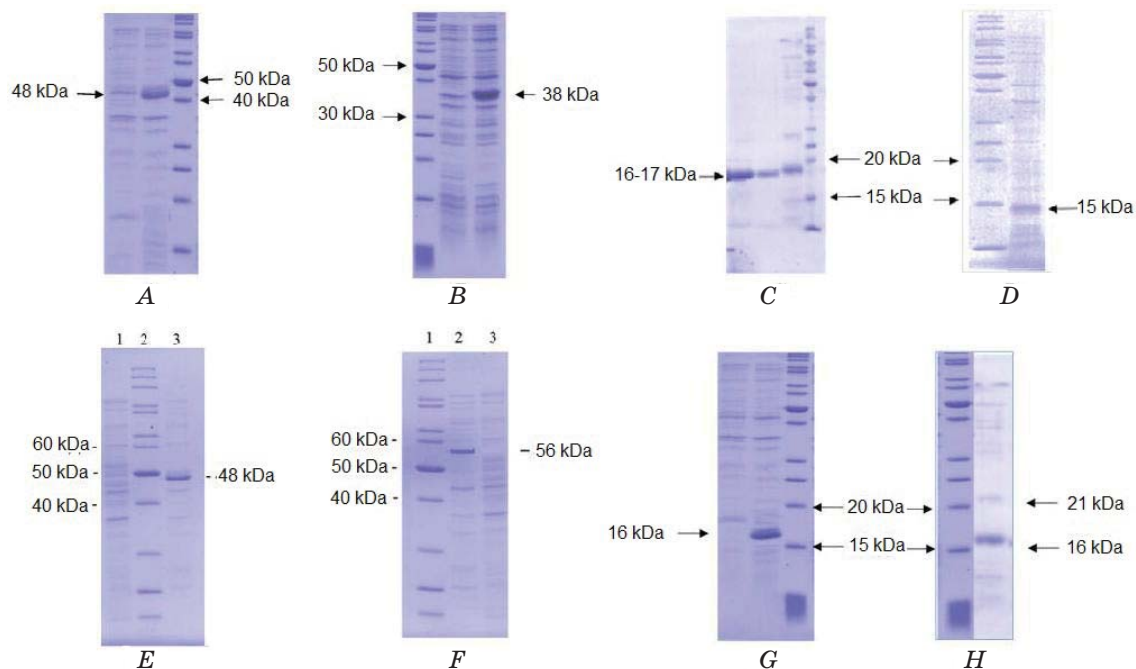

Fig. 1. **Electrophoretic analysis of recombinant viral nucleocapsid proteins expression**
Black arrows indicate the target viral proteins: LCM (*A*, lane 2); Lassa (*B*, lane 3); Hantaan, Dobrava-Belgrade, Puumala (*C*, lanes 1, 2, 3, respectively); CCHF (*D*, lane 2); Marburg (*E*, lane 3); Ebola (*F*, lane 2); West Nile (*G*, lane 2); hepatitis C (*H*, lane 2). The samples of bacterial lysates of the cell clones expressing recombinant viral nucleocapsid proteins after IPTG induction were used for the analysis

3.5 per 1000); CTA for leucine (3.8 per 1000); ATA for isoleucine (4.2 per 1000); CCC for proline (5.0 per 1000); GGA for glutamine (8.7 per 1000). If the distribution of codons was uniform, the frequency of occurrence for all codons would be about 16.4. The analysis of rare codon content in the cloned sequences is shown in the Table.

Analysis of the rare codons frequency (Fig. 2, *A*) showed that in the cloned sequences of most viral proteins (Lassa, LCM, Marburg, Ebola, Puumala, Dobrava-Belgrade, Hantaan) it ranged from 7.5 to 9.5%. The lowest of this value was for the CCHF virus polypeptide (6.6%), the highest one was for the triplets of the hepatitis C and West Nile viruses (12.1 and 14.6%, respectively). Based on this analysis, the maximum expression level is expected to be observed for the protein of the CCHF virus, and the minimum one should be for hepatitis C and West Nile viruses.

However, our data obtained by using pJC 40 expression plasmid, bacterial strain BL21 (DE3), and optimized for each polypeptide expression conditions indicated that the

maximum protein yield was observed for the amino acid sequences of the Lassa and LCM viruses (approximately 35−40 mg per liter of a culture), and minimum one was for the CCHF virus (5 mg/l) (Fig. 2, *B*). Thus, there is no clear correlation between the yield of recombinant proteins and the total frequency of rare codons in their sequences. It should be noted that a feature of the CCHF virus cloned sequence is the fact that the AGG codon, which is the rarest for *E. coli*, is located at position +2, the closest to the initiating codon. According to the literature, not only the presence of rare codons, but also their location can be critical for protein expression. The proximity to the initiating codon can explain the low synthesis efficiency (5 mg/l) of this protein in the heterologous system.

It is interesting to note that, despite the large number of "not typical" codons for the nucleocapsid protein of the West Nile virus (14.6%), its biosynthesis proceeded quite efficiently (30 mg/l). This can be partly explained by the fact that the main contribution to the percentage of rare codons in this case is not made by the most infrequent ones. The

Analysis of the rare codon number in cloned sequences

Virus (number of cloned amino acids)	Arg			Leu	Ile	Pro	Gln	General frequency of occurrence
	AGG (1.1%)	AGA (2.0%)	CGA (3.5%)	CTA (3.8%)	ATA (4.2%)	CCC (5.0%)	GGA (8.7%)	
Lassa virus (432 aa)	5/1.16*	12/2.78	–	3/0.69	6/1.39	4/0.93	11/2.55	41/9.5
LCM virus (320 aa)	8/2.5	9/2.81	–	4/1.25	2/0.63	2/0.63	2/0.63	27/8.4
Marburg virus (254 aa)	3/1.18	8/3.15	–	–	3/1.18	2/0.79	3/1.18	19/7.5
Ebola virus (306 aa)	3/0.98	8/2.61	1/0.33	3/0.98	1/0.33	6/1.96	3/0.98	25/8.2
Puumala virus (117 aa)	–	7/5.98	–	–	1/0.85	–	1/0.85	9/7.7
Dobrava-Belgrade virus (116 aa)	3/2.59	2/1.72	–	2/1.72	1/0.86	–	2/1.72	10/8.6
Hantaan virus (117 aa)	5/4.27	2/1.71	–	–	1/0.85	1/0.85	2/1.71	11/9.4
CCHF virus (106 v.)	1/0.94	–	1/0.94	–	3/2.83	–	2/1.89	7/6.6
West Nile virus (103 aa)	1/0.97	3/2.91	–	3/2.91	2/1.94	2/1.94	4/3.88	15/14.6
Hepatitis C virus C (190 aa)	7/3.68	1/0.53	1/0.53	1/0.53	–	10/5.26	3/1.58	23/12.1

* Absolute number of codons/frequency of occurrence, expressed as a percentage.

cloned sequence of this protein contains only one AGG triplet, located in the middle.

Codon adaptation index (CAI), which reflects the degree of non-uniformity of the codon composition of a gene, is an additional parameter that is widely used in various biological studies to determine the efficiency of translation, predict the level of cell protein synthesis and the expression of foreign genes in heterologous systems [13].

E. coli refers to the organisms in which the preferential use of certain codons is observed depending on the gene expression level, while the translation efficiency correlates with the uneven use of codons [14]. Highly expressed genes of *E. coli* (genes of ribosomal proteins, transcription and translation factors, outer membrane proteins) are characterized by high CAI values (over 0.75).

The CAI values for the cloned viral sequences, calculated on the basis of the codon composition in *E. coli*, are in the range from 0.50 to 0.58, which corresponds to the average expressed proteins. Therefore, the codon composition of viral nucleocapsid proteins is relatively optimal for *E. coli*. For example, green fluorescent protein (GFP), which is also foreign to *E. coli* and according to the literature [15] is synthesized in bacterial cells with a high yield, has a coefficient of 0.58. However, despite the proximity of the CAI values to each other, the results of the experiments indicate that viral polypeptides with a similar content of rare codons in the nucleotide sequence are expressed with different efficiencies.

Different levels of the recombinant proteins expression can also be associated with the peculiarities of the rare codons location. Additional information for assessing the spatial distribution of rare codons can be obtained by studying the segmental distribution of CAI values within a gene. The obtained data are represented in graphical form in Fig. 3. As a comparison the coding sequences of highly expressed *E. coli* proteins (*rspB 30S* — ribosomal protein S2, *rec A* — recombinase A), as well as heterologous green fluorescent protein GFP (*gfp*) were analyzed.

The CAI distribution profiles for cloned sequences are different. For example, more flattened curve corresponds to the genes of the Dobrava-Belgrade and CCHF viruses (Fig. 3, *A*) while an alternation of pronounced peaks are evidenced for the nucleotide sequences of the Ebola, LCM and Marburg viruses (Fig. 3, *B*). In general, distribution of CAI values for genes of viral nucleocapsid proteins is similar to that for proteins with an average expression level. The curves for *E. coli* proteins are differed by higher values of the index, which are typical for highly expressed proteins. Analysis of the CAI distribution
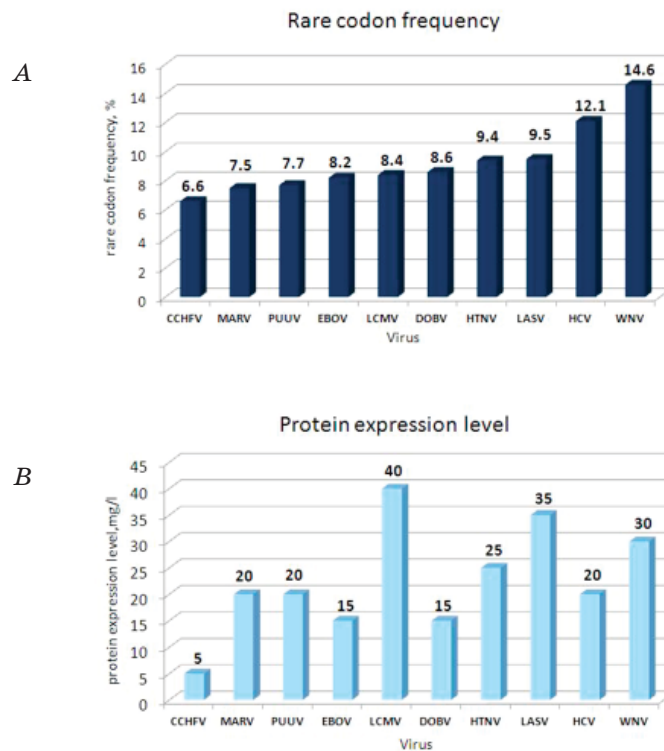

Fig. 2. **Analysis of rare codon occurrence in cloned sequences
and recombinant proteins expression level**

profiles in the cloned sequences did not reveal areas of sharp decreases with extremely low (less than 0.2) index values. This may indicate the absence of rare codon clusters capable of embarrassing the translation.

As far as is known, the use of synonymous codons in the coding genetic sequences of various organisms is not accidental. Analysis of the codon adaptation index values, as well as the distribution of its values over the cloned sequence for the causative agents of LCM and HFRS, which are the most relevant for the Republic of Belarus zoonotic viral diseases, made it possible to identify some patterns in the use of codons. Higher CAI values for all studied viral sequences indicate a potentially more efficient translation of viral proteins in humans compared to *E. coli* (Fig. 4).

Almost similar distribution of CAI along the analyzed sequences of LCM, Puumala, and Dobrava-Belgrade viruses in the case of their expression in humans and rodents (Fig. 5) indicates that the coding strategy of nucleocapsid proteins of these viruses is adapted for optimal replication of pathogens in natural hosts.

The data obtained confirm the existing assumption that one of the manifestations of viruses adaptation to the host during their evolution is the use of certain synonymous codons in the viral genome, which corresponds to codon preferences that are specific for the host [16].

The frequency of the amino acids occurrence is another factor that can affect the level of heterologous protein expression in *E. coli*.

If the amino acid composition of the recombinant protein is skewed compared to the typical *E. coli* proteins, heterologous expression may result in translation disruptions. Premature termination, reading frame shift, or amino acids misincorporation can lead to a decrease in the amount or quality of the expressed protein [17−20].

Analysis of the cloned sequences amino acid composition in comparison with that typical to *E. coli* proteins [18] showed the uneven content of amino acids in the recombinant proteins. The polypeptides of the Lassa, LCM, and CCHF viruses were the closest to bacterial proteins in the percentage of amino acids in their composition (Fig. 6, *A*). For nucleocapsid
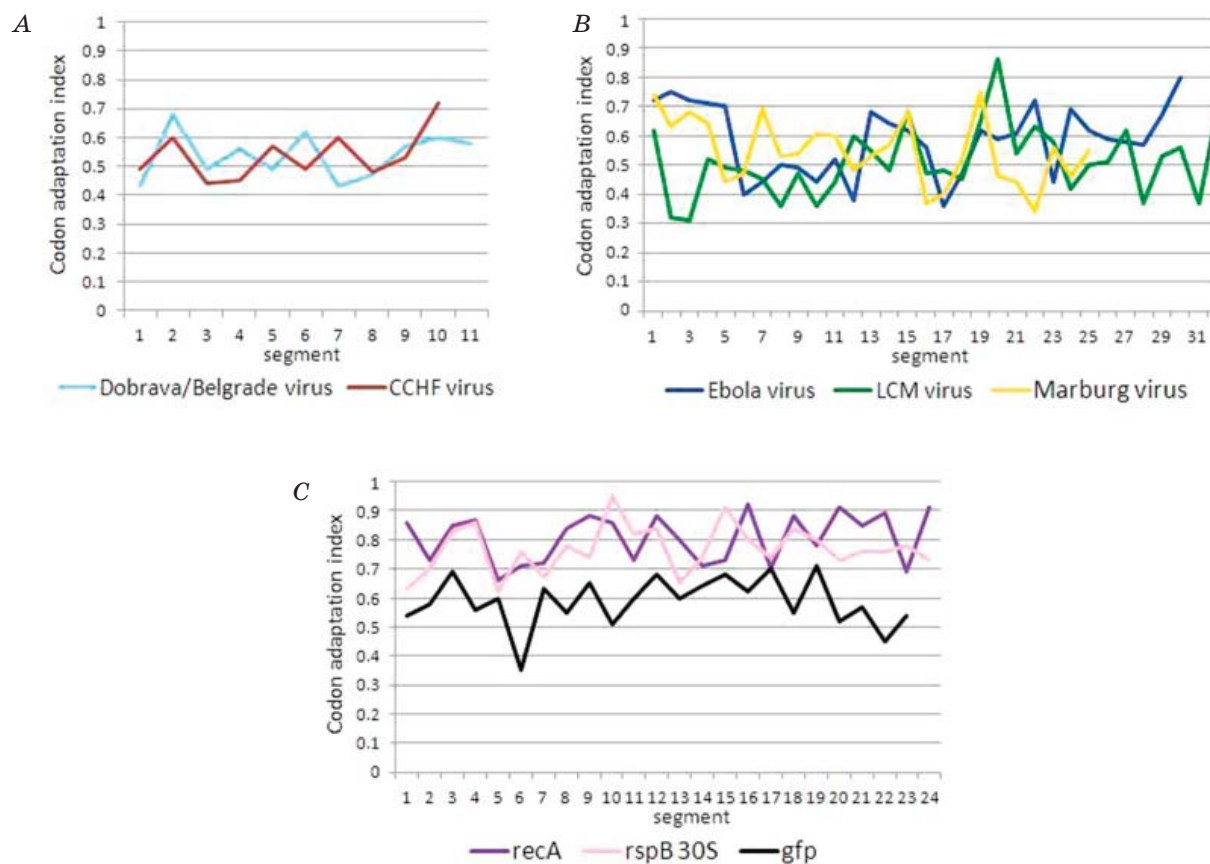

Fig. 3. **Segmental distribution of the CAI values within genes encoding the nucleocapsid viral proteins and highly expressed *E. coli* proteins**
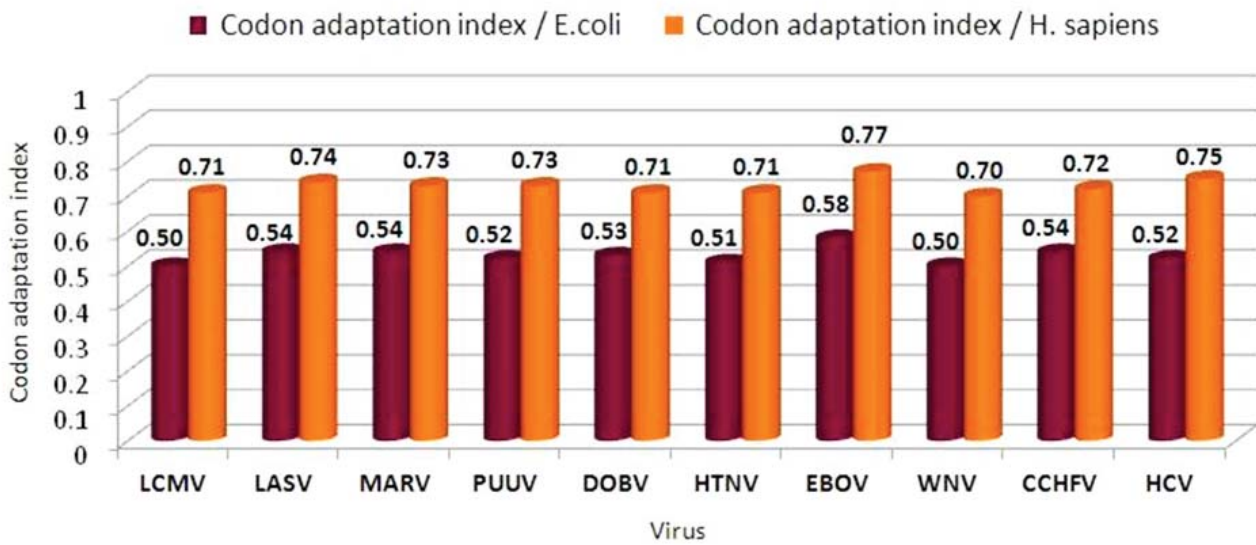
Fig. 4. **CAI values for viral sequences, calculated based on the codon composition of *E. coli* and humans**
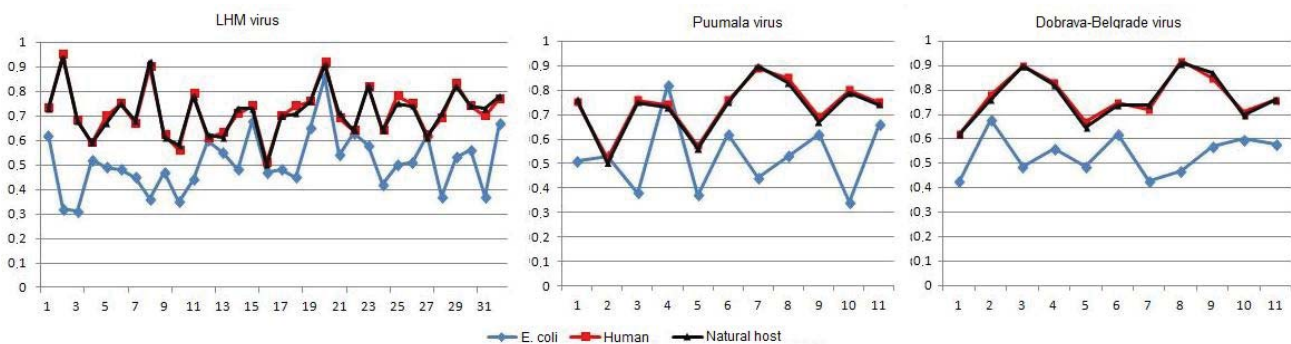

Fig. 5. **Distribution of CAI along the analyzed sequences of LCM, Puumala, and Dobrava-Belgrade viruses when expressed in humans and rodents**

proteins of Marburg, Ebola, West Nile and hepatitis C viruses, a statistically significant difference was observed between the frequencies of distribution of amino acids in the cloned sequences and their content in *E. coli* ($\chi^2 = 36.79$, $P = 0.008$; $\chi^2 = 37.60$, $P = 0.007$; $\chi^2 = 32.29$, $P = 0.029$; $\chi^2 = 33.96$, $P = 0.019$, respectively) (Fig. 6, *C*).

Analysis of the data obtained demonstrated the multidirectional nature of existing deviations. The frequency of various amino acids occurrence in viral proteins differed from that in the bacterial proteome both upward and downward. For example, the amino acids Cys, Trp, and Phe, which were rare for *E. coli* proteins, were not contained in the sequences of the Puumala, Dobrava-Belgrade, and Hantaan nucleocapsid proteins. For a number of cloned sequences, there was a significant excess in the frequency of occurrence of both the most rare amino acids for *E. coli* proteins (Cys in the Lassa virus sequence, Trp in the hepatitis C virus sequence), and relatively uniformly presented in the bacterial proteome (Asp in the sequences of Marburg, Ebola, Puumala, Dobrava-Belgrade viruses; Pro in the sequences of Marburg, Ebola, hepatitis C viruses, Lys in the sequences of viruses Dobrava-Belgrade, West Nile, CCHF viruses).

Our studies have shown that the nucleotide sequences of ten nucleocapsid proteins of belonging to various families dangerous and especially dangerous human viruses are expressed in a prokaryotic heterologous system quite efficiently: from 5 to 40 mg per liter of bacterial culture. Bioinformatic analysis
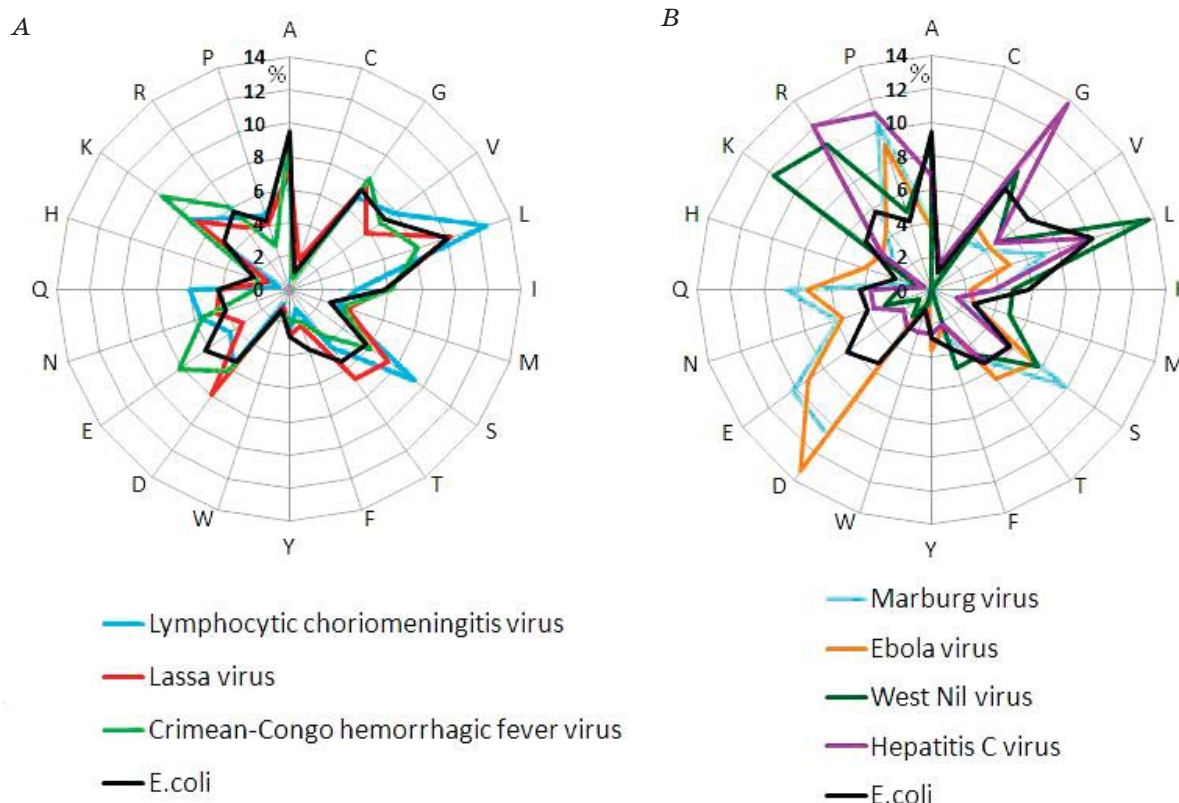
Fig. 6. **Histogram of the amino acid composition of the cloned viral sequences
and the amino acid composition typical for *E. coli* proteins**

of the codon adaptation index classifies recombinant polypeptides as proteins with an average level of xpression in the *E. coli* system. At the same time, the efficiency of viral proteins translation in the human as a typical host is potentially higher than in *E. coli*, as evidenced by the higher CAI values for all viral sequences.

Nevertheless, significant differences in the frequency of rare codon occurrence in sequences and the absence of its correlation with the level of protein expression were revealed, which confirms the complexity of forecasting the of the protein biosynthesis process in a heterologous system and its unpredictable nature.

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ЕКСПРЕСІЯ НУКЛЕОКАПСИДНИХ ВІРУСНИХ ПРОТЕЇНІВ У БАКТЕРІАЛЬНІЙ СИСТЕМІ *Escherichia coli*: ВПЛИВ КОДОНОВОГО СКЛАДУ ТА РІВНОМІРНОСТІ ЙОГО РОЗПОДІЛУ ВСЕРЕДИНІ ГЕНА

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Експресійна здатність кожного гена є унікальною у гетерологічного хазяїна. Відмінності між синонімічними послідовностями відіграють важливу роль у регуляції експресії протеїну в організмах від *Escherichia coli* до людини, і багато деталей цього процесу ще не з'ясовано. Метою дослідження було вивчити склад кодонів, його розподіл у послідовності та вплив рідкісних кодонів на експресію вірусних нуклеокапсидних протеїнів і їхніх фрагментів у гетерологічній системі *E. coli*. Для експресії протеїнів використовували плазмідний вектор рJC 40 і штам BL 21 (DE 3) *E. coli*. Аналіз складу кодонів виконано з використанням on-line ресурсу (*www.biologicscorp.com*). Отримано десять рекомбінантних поліпептидів, з них два, що кодують повну нуклеотидну послідовність нуклеокапсидних протеїнів (віруси Західного Нілу і гепатиту С) та їхні фрагменти, що містять антигенні детермінанти (вірус Ласса, Марбург, Ебола, Кримської-Конго геморагічної лихоманки (ККГЛ), Пуумала, Хантаан, Добрава-Белград і лімфоцитарного хоріоменінгіту (ЛХМ)). Гібридні плазмідні ДНК забезпечують ефективне продукування цих протеїнів у прокаріотичній системі з виходом рекомбінатного протеїну, що варіює у 8 разів: від 5 до 40 мг на 1 літр бактеріальної культури. Не виявлена кореляції рівня експресії протеїну з частотою народження рідкісних кодонів у клонованій послідовності: максимальна частота народження рідкісних кодонів у клонованій послідовності спостерігалася для вірусу Західного Нілу (14,6%), мінімальна – для вірусу ККГЛ (6,6%), тимчасом як рівень експресії для цих протеїнів становив 30 і 5 мг/л культури відповідно. Значення індексу адаптації кодонів (CAI), розраховані на основі кодонового складу у *E. coli*, для клонованих вірусних послідовностей знаходяться в діапазоні від 0,50 до 0,58, що відповідає середньоекспресованим протеїнам. Проведений аналіз профілів розподілу CAI у клонованих послідовностях свідчить про відсутність кластерів рідкісних кодонів, здатних створювати труднощі за трансляції. Статистично значущу відмінність між частотами розподілу амінокислот у клонованих послідовностях та їхнім змістом в *E. coli* спостерігали для нуклеокапсидних протеїнів вірусів Марбург, Ебола, Західного Нілу і гепатиту С.

Ключові слова: рекомбінантні нуклеокапсидні протеїни, експресія, рідкісні кодони, індекс адаптації кодонів.

PROSPECTS FOR APPLICATION OF BOVINE PERICARDIAL SCAFFOLD FOR CARDIAL SURGERY

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The aim of the study was to estimate the properties of the scaffold obtained by decellularization with of bovine pericardium a 0.1% solution of sodium dodecyl sulfate. The experiment included standard histological, microscopic, molecular genetic, and biomechanical methods. Scaffold was tested *in vitro* for cytotoxicity and *in vivo* for biocompatibility. A high degree of removal of cells and their components from bovine pericardium-derived matrix was shown. Biomechanical characteristics were of artificial scaffold were the same same as those of the native pericardium. With prolonged contact, no cytotoxic effect on human cells was observed. The biointegration of the scaffold in laboratory animals tissues was noted, thus confirming the potential possibility of the implant application in cardiac surgery.

Key words: pericardium, scaffold, decellularization, cardioimplant, tissue engineering.

Today, cardiovascular diseases are the leading cause of death in the world. According to WHO statistics, heart diseases kill more than 17 million people each year, which is 31% of all deaths [1]. Congenital heart defects occur with a frequency of about 9 % [2]. To date, cardiac surgery is performed in almost all congenital heart defects, where in most cases a complete anatomical correction is performed using artificial implants. The total postoperative mortality from these surgeries in the world's leading clinics is less than 3%. However, the use of artificial prostheses has a number of disadvantages that significantly impair the quality of patients life in the postoperative period. About a third of patients those get operated require repeated surgery at various times in the long term. Patients usually need lifelong anticoagulation therapy. A promising direction in overcoming the above problems may be the use of biological implants. However, they also have a number of unresolved issues, such as complete or partial biodegradation and calcification after implantation [3].

In the world medical practice, bioimplants made of xenogenic tissues, for example from the pericardium of pigs, horses, cattle, which have the elasticity of the material close to human tissues, are increasingly used [4]. To obtain such an implant, the native material is subjected to decellularization, which is the complete elimination of donor cells and purification from antigenic molecules while maintaining the structure of the extracellular matrix. Today, scaffolds are successfully used in the clinic for tissue engineering and regenerative medicine [5–8]. Decellularized extracellular matrix made of bovine pericardium is a promising biomaterial for cardiovascular tissue repair, as the structure of collagen-elastin components of the framework is satisfactorily preserved, and antigenic molecules are properly eliminated and thus reduces the antigenicity of such material [9–11].

Sodium dodecyl sulfate (SDS) is one of the most commonly used anionic detergents to create extracellular matrices, as it can more

efficiently washout cytoplasmic proteins and remnants of nuclear components from tissues than other detergents [12, 13]. For example, it was the main detergent used to decellularize cardiac perfusion in all rats [12]. Thus, in a number of studies, colleagues described protocols for material SDS processing in accordance with the standard requirements for complete removal of cells and elimination of at least 90% of host DNA from tissues and organs of different species [14–17].

Another detergent often used in conjunction with SDS is Triton X-100, a non-ionic detergent that can remove cellular contents and help wash residual SDS from the ECM [12, 18]. Although the use of Triton X-100 eliminates cells from the heart valve, it is less effective in clearing the myocardium and aortic wall of cellular residues [19].

Unfortunately, today there is no ideal biomaterial that would meet all the requirements of cardiac surgery and has athrombogenicity, elasticity, durability, minimal antigenicity, lack of immunogenicity and cytotoxicity, and strength, i.e. was close to the characteristics of natural tissues. Therefore, the search for methods of biotechnological transformation of xenotissue, their development and improvement, which could provide high quality material obtaining, that will significantly improve the life quality of children with congenital heart disease, reduce the number of repeated cardiac surgeries and the cost of treatment.

Materials and Methods

The procedure for tissue obtaining

The material for the study was the cattle pericardium. The pericardial sac was extracted from outbred 12–18-month-old bulls after slaughter at the TOV "Antonovsky Meat Processing Plant". All animals underwent veterinary inspection. In the process of organ removal, the rules of asepsis with the maximal available atraumaticity and taking into account the anatomical features of animals, as well as in accordance with the basic principles of bioethics and bioethical expertise, consistent with the provisions of the "European Convention for the Protection of Vertebrate Animals which are used for experimental and other purposes" (Strasbourg, France, 1985) and in accordance with the Law of Ukraine № 3447-IV "On Protection of Animals from Cruel Treatment" (2006, latest edition 2009). The biomaterial was transported to the laboratory for one hour in a sterile Hanks solution in a container on ice. Then the pericardial sac was carefully prepared, separating the serous layer from the fibrous one. Fatty appendages and excess connective tissue were removed from the latter. To wash the fragments of the isolated pericardium from the remnants of blood components, it was placed in flasks with distilled water with a volume of 1 000 ml and stirred continuously (70 rpm) for 3 hours at 4 °C.

Protocol for decellularization of the bovine pericardium

Cattle pericardial samples were decellularized as follows: a 40×40 mm pericardial sample was placed in a 200 ml bottle containing 100 ml of 0.1% solution of SDS (Sigma-Aldrich, USA) with constant shaking (200 rpm) for 40 days at 4 °C [11]. The next step was to stabilize and fix the obtained samples. We provided stabilization and fixation of all obtained samples in a solution of 70 % ethanol for 24 h at t 4 °C with constant stirring at 200 rpm. Then all fragments of the decellularized pericardium were washed with sterile NaCl solution for 24 h at 4 °C with constant stirring at 200 rpm. An additional stage of chemical decellularization was achieved using the crosslinking method: EDC/NHS solution — MES (10 mM of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 10 mM of N-Hydroxy-succinimide) and MES solution (pH 5.6) 6 (0.05 M of 2 — morpholinoethane sulfonic acid). Native pericardium was used as a control.

Histological staining (hematoxylin-eosin and DAPI)

Hematoxylin — eosin. Histopathological examination of tissue samples includes fixation in 10 % solution of neutral buffered formalin (pH 7.4) for at least 3 days at a temperature of 4 °C. Tissues were fixed after rapid freezing and sectioning with a cryostat (6 μm thick). Validation of cell nucleus removal was performed according to the standard hematoxylin and eosin (H&E) staining protocol. Stained samples were examined using an Olympus BX 51 light microscope.

DAPI. DAPI staining (4′,6-diamidino-2-phenylindole, dihydrochloride) was performed directly by incubating a tissue sample (luminal side up) with 25 μg/ml DAPI diluted in PBS for 2 min (in the dark). Achieving maximal tissue thickness, adventitia and middle layer were removed using the approach proposed by Jelev et al. [20]. The ECM was fixed on the plate with

forceps under a microscope. The intima layer was carefully removed by stretching it with forceps, simultaneously with cutting the edges with a scalpel, followed by restoration in PBS. DAPI stains were also applied to paraffin-embedded cross-sections (5 µm thick) following standard fixation, dehydration, embedding, cutting, dewaxing, rehydration, and staining protocols. Images were taken using an Olympus BX 40 fluorescent microscope (Tokyo, Japan).

DNA quantitative evaluation

DNA extraction was performed using Easy Blood and Tissue DNA kit (Qiagen, Germany). 10−25 mg of tissue from external materials before decellularization, or from decellularized samples, were briefly treated with proteinase K to quantify the total amount of nucleic acid, ng/mg of dry tissue, and calculate the percentage of DNA removal after decellularization. Fluorescence measurements (photons per second) were performed at room temperature in a room of 23-24 °C, using a spectrofluorometer Qubit 3.0 to count photons. The limit of DNA detection was 0.2 ng/µg of nucleic acid.

Biomechanical testing. The biomechanical properties of bovine pericardium for flaps with a size of 20×40 mm were analyzed. To determine the maximal tensile strength at break (F_{max}), the pericardial samples were loaded separately between 2 steel rods and clamped vertically in a test machine (IMADA, MX2 — 110, Japan).

In vitro cytotoxicity assessment

To determine cytotoxicity, the matrix samples were cultured in a suspension of human fibroblasts. From the obtained material by 3 fragments from each sample were cut with an area of about 8−10 mm^2. Cells were inoculated by applying 150 µl of concentrated cell suspension on decellularized pericardial samples moistened with standard growth medium (DMEM + 10% of serum). Impregnation lasted 30 minutes. The number of inoculated on fragments cells was 300 thousand. They were then transferred to a standard 6-well tissue culture plate and immersed in growth medium DMEM + 10% of serum, cultivation was performed under standard conditions of 37 °C and 5% of CO_2 [21]. For histological examination, DEM samples were fixed in 10% buffered formalin embedded in paraffin. Sections were made (5 µm). Hematoxylin / eosin samples were obtained (light microscopy) [22]. Images were taken using an Olympus BX 51 light microscope (Tokyo, Japan).

In vivo biocompatibility test

For the study, 10 Wistar rats were kept in a pathogen-free environment. Decellularized and pre-sterilized bovine pericardium was implanted subcutaneously in the interscapular space and explanted after 8 weeks. The animals were divided into 2 groups. The groups were as follows: Control group: native bovine pericardium ($n = 5$), group 1: bovine pericardium decellularized with 0.1% solution of SDS ($n = 5$). Wool was removed from the skin surface of rats in the area of the operating field and treated with a 70% solution of ethyl alcohol. The operation was performed under sterile conditions. Xylazine (Alfasan, Netherlands) at a dose of 1 mg/kg of body weight in combination with ketamine (Biolik, Ukraine) at a dose of 10 mg/kg was used for intramuscular anesthesia. An incision of 2 cm was made in the back of the animal, and the subcutaneous pockets were formed with a pointed spatula, separating the subcutaneous tissue from the muscle layer. The prepared 1×1 cm implants were placed in the lumen of the pocket, which were fixed at the corners to the muscle tissue with noose sutures "Polypropylene" ("Golnit", Ukraine). The skin was closed with a continuous suture, the thread, which did not delaminate, was treated with an antiseptic — a solution "Betadine" ("EGIS Pharmaceuticals, PLC", Hungry). The implants were removed for histological analysis 3 months after surgery.

Statistical analysis. Statistical significance was analyzed using variation analysis and t-test, if necessary. $P < 0.05$ value was regarded as statistically significant. Variation and statistical processing of the obtained results was performed using StataIC software.

Results and Discussion

It is known that cellular components, including nucleic acids of xenogeneic tissues, are strong antigens that trigger immunological reactions in the recipient, which provokes graft rejection. A decellularization protocol is considered to be effective, using which all components of the cell and nucleic residues are completely removed [23].

To assess the decellularization process, histological examination of samples stained with hemotoxylin-eosin and by the DAPI method was performed. Basophilically stained bovine pericardial cells and the bright glow of fluorescent dye were detected in the control samples, which confirms the presence of nucleic acids (Fig. 1). At the same time, the

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absence of cells in hematoxylin-eosin staining and the absence of glow in DAPI were recorded for the bovine decellularized pyricard of the experimental group. The absence of nuclear elements was seen by staining in both ways after 21 days of decellularization. There was also no obvious difference in the structure and distribution between the collagen and elastin fibers of the decellularized extracellular matrix and the native bovine pericardium.

In the samples of the experimental group, the DNA concentration was recorded at the level of 5 ng / mg on the 35th day of decellularization (Fig. 2). Thus, the degree of matrix purification of decellularized bovine pericardium tissue from nucleic acid residues was 99.8 % compared with native samples (Table 1). A statistically significant difference was found between the DNA concentrations of the experimental and control groups ($P < 0.05$).

The study of biomechanical properties showed that the decellularized matrix not only did not change its natural properties, but also gained greater strength after the process of purification and cross-linking. This is evidenced by an increase almost 2 times of the maximal tensile strength value in the samples of the experimental group (Table 1).

The study of the cytotoxic effect of the decellularized extracellular matrix showed that after two months of cultivation the culture of human fibroblast cells were placed in a uniform layer on the surface of the sample. Some groups of fibroblasts penetrated to a depth of 350 µm, but only in places where the fiber bundles were less dense (Fig. 3). Collagen

and elastin components of the matrix are well expressed, fiber bundles are strong, ordered.

The ultimate goal of implantation is bioimplant integration into the host tissue, with its subsequent regeneration [24]. Fig. 5 presents a histological examination of the explanted pericardium in control rats implanted with untreated/native bovine pericardium. As expected, the pericardium is completely degraded and eliminated, there is only connective and muscle tissue of the operated animal. The tissue is infiltrated with leukocytes, which indicates inflammatory processes in this area.

At the same time, in comparison with the control, the histology data indicate the successful biointegration of the implant in the rats of the experimental group (Fig. 6). In the tissues, implant replacement with growing immature connective tissue was noted. In the area of implant, the increased vascularization of connective tissue is also observed, capillaries are formed, which are filled with erythrocytes. There was a lower level of macrophages and monocytes filtration in decellularized tissues of the pericardium compared to non-decellularized tissues of the control group.

This study made it possible to obtain a scaffold made of xenotissue, which in its biomechanical and biological characteristics is similar to the native bovine pericardium. Ionic detergent SDS was used in the decellularization process. The scientific literature confirms the effective use of this detergent, the action of which is aimed at cleaning the matrix by solubilizing the cytoplasmic and nuclear
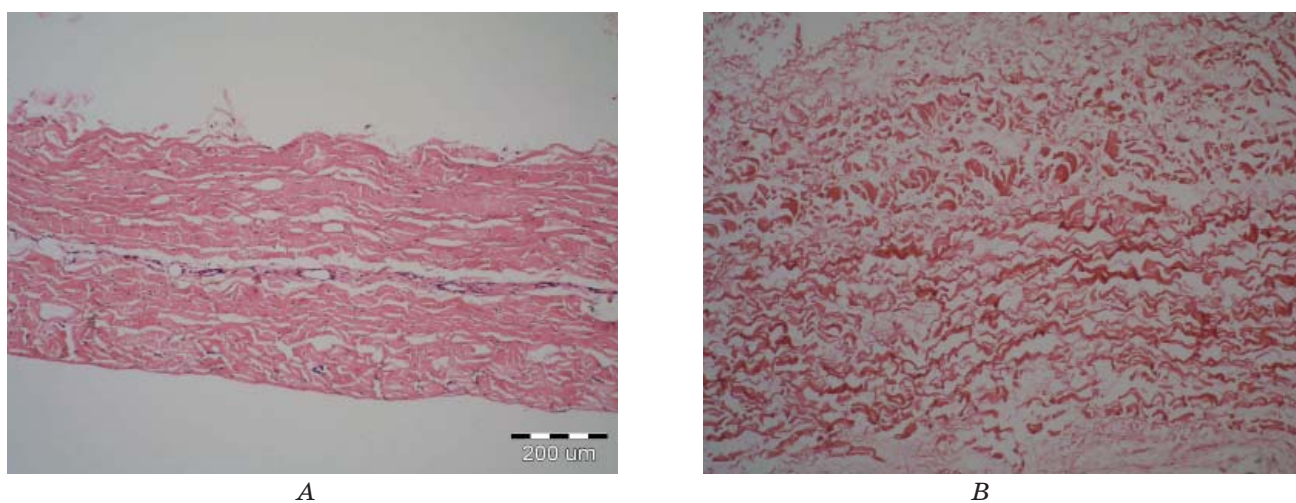

Fig. 1. **Imaging of histological sections of bovine cellular and decellulorized pericardium. Hematoxylin and eosin staining (light microscopy, ×200) and DAPI (fluorescence microscopy, ×200):**
A — control group (native pericardium); *B* — experimental group (0.1% solution of SDS)

Table 1. **Comparative evaluation of native and decellularized bovine pericardium**

Research / indicators	Control group	Experimental group
Histology	Presence of basophilically stained cells	Absence of cells
DAPI	The presence of the glow of cells nuclear material	No glow of cells nuclear material
Average DNA content (ng/mg), $n = 5$	1436 ± 116.8	$0.5 \pm 0.448*$
Fmax, maximal tensile strength (kgf)	6.84 ± 0.69	$9.548 \pm 0.65*$
Cytotoxicity	-	None after 2 months
Biocompatibility, inflammatory reaction**	$++++$	$+$

* statistically significant compared with the control group ($P < 0.05$).

** To obtain more objective data, a semi-quantitative assessment of inflammatory reaction severity was performed: 0 — no signs; + — single cells (neutrophils, lymphocytes, macrophages, eosinophils); ++ — small foci; +++ — separate and massive foci; ++++ — large infiltrates.
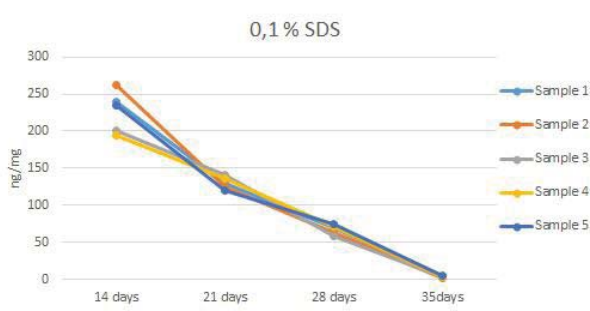

Fig. 2. **DNA concentration (ng/mg) in pericardium samples decellularized with 0.1% solution of SDS**

membranes, denaturation of proteins and removal of nuclear residues [25–27].

At the same time, it is known that SDS can bind to and deform collagen fibers. Due to hydrogen bonds of collagen fibers rupture, tissue edema may develop [23, 28]. SDS is reported to interact very strongly with extracellular matrix proteins, making it difficult to remove SDS completely [29]. Ning Lia and colleagues also noted that residual SDS in decellularized tissues can lead to insufficient settling of host cells after implantation, causing less implant durability and inhibiting growth. This was clearly correlated in rat implantation studies, where peri-implant necrosis was found around about 1% of SDS-treated implants [22]. Based on these facts, it was hypothesized that the use of a low concentration of 0.1% solution of SDS will allow for effective decellularization, while maintaining the architectonics of the

matrix and not affect the biocompatible properties of scaffold. This study confirms the hypothesis and demonstrates the effective use of low-concentration SDS detergent for decellularization of the bovine pericardium. Tran Ha Le Bao et al. also showed that 0.1% solution of SDS is better fit for porcine pericardial decellularization than 0.3% or 0.5% solution of SDS, because the biological properties of scaffold were better preserved [31].

In our study, the absence of cells and their components was confirmed by histological and molecular genetic studies. Examination of hematoxylin-eosin-stained samples showed the absence of nuclear elements and the preservation of scaffold matrix structure. Complete removal of all cellular components is virtually impossible with any method of decellularization [32]. Quantitative evaluation of residual DNA can be used as an additional marker in determining the effectiveness of the decellularization process. DNA analysis showed that decellularization was able to remove 99.8% of nucleic acids from the extracellular matrix.

The absence of scaffold toxic effects on donor cells is an important part of selection for further transplantation. In this study, the cytotoxicity of scaffold was determined by comparing different microscopy samples to identify the number and location of human fibroblasts cultured on the obtained tissue sample. Histological analysis made it possible to establish the levels of structural changes in the fibers together with the position of fibroblast cells after prolonged cultivation. In our study, we did not observe any *in vitro* cytotoxic effect for a decellularized matrix
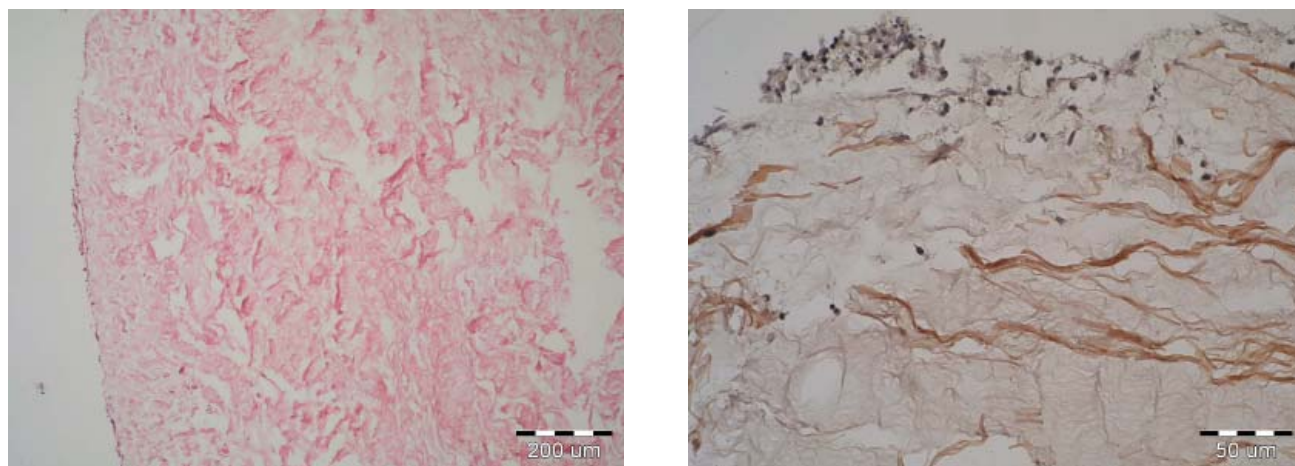
Fig. 3. **Histological examination for cytotoxicity in samples of the experimental group (pericardium decellularized with 0.1% solution of SDS) after 2 months of cultivation**
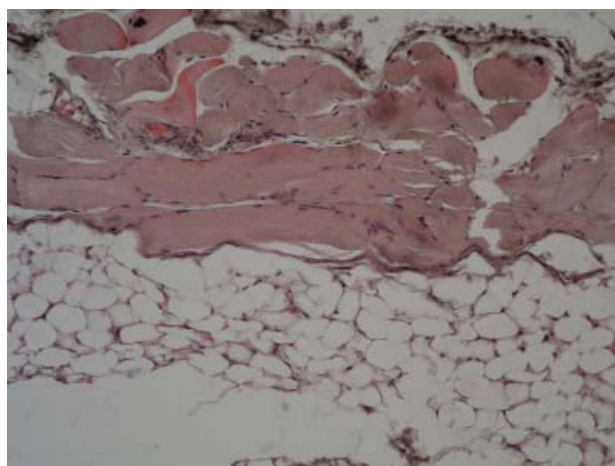(Congo staining and H/E, light microscopy, × 200, ×50)

Fig. 4. **Histological examination of the explanted pericardium of control rats**
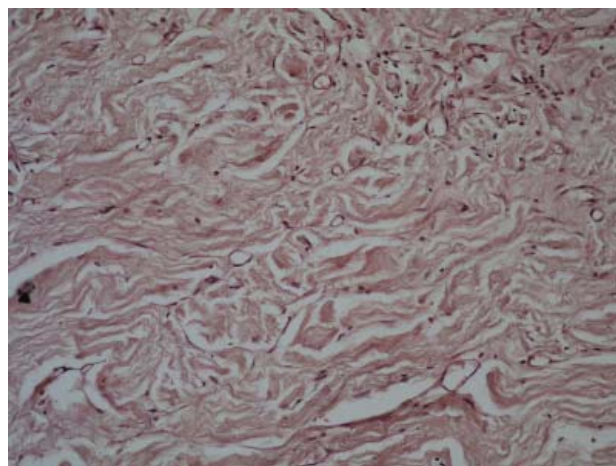(tricolor Masson staining method, light microscopy, ×200)

Fig. 5. **Histological analysis of decellularized pericardial implants and evaluation of rat tissue response after subcutaneous implantation** (tricolor Masson staining method, light microscopy, ×200)

with a law concentration of 0.1% solution of SDS for 2 months of cultivation. Although in most experiments the cytotoxic effect for a short period of time (24 to 48 hours) was studied, long-term studies are more effective for the transplant stage [25–27, 31].

The ideal scaffold should not only be free of any cells, have sufficient stability, be able to withstand mechanical loads for a long time, while maintaining the structure of the extracellular matrix, but also when implanted to integrate into the donor tissue [23]. That is, the created matrix is the basis for its settling by donor cells. One of the most important requirements for the safety of the scaffold is the biocompatible properties of the biomaterial determination [33, 34]. Our study has demonstrated a high level of

scaffold biocompatibility with tissues of Wistar rats. The bioimplant was not only destroyed, but also became a full-fledged part of the tissues of experimental animals, as evidenced by its replacement by immature connective tissue.

Thus, the scaffold from bovine pericardium was obtained by decellularization using a low concentration of 0.1% solution of SDS which can then be used in cardiac surgery, subject to a series of preclinical and clinical tests.

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ПЕРСПЕКТИВА ВИКОРИСТАННЯ СКАФФОЛДУ З ПЕРИКАРДА ВЕЛИКОЇ РОГАТОЇ ХУДОБИ ДЛЯ ПОТРЕБ КАРДІОХІРУРГІЇ

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Метою дослідження було оцінити властивості скафолду, отриманого після децелюляризації перикарду великої рогатої худоби 0.1% розчином додецилсульфату натрію. Експеримент включав стандартні гістологічні, мікроскопічні, молекулярно-генетичні, біомеханічні методи. Скаффолд протестований на цитотоксичність *in vitro* та біосумісність *in vivo*. Показано високу ступінь очистки позаклітинного децелюляризованого матриксу від клітин та їх компонентів. Біомеханічні характеристики були такими самими, як і нативного перикарда. При довготривалому контакті не спостерігали цитотоксичного впливу на клітини людини. Відмічено біоінтеграцію скаффолду в тканини лабораторних тварин, що підтверджує потенційну можливість використання імпланту в кардіохірургічний практиці.

Ключові слова: перикард великої рогатої худоби, скафолд, децелюляризація, кардіоімплант, тканинна інженерія.

POLYCLONAL ANTIBODIES AGAINST HUMAN PLASMINOGEN: PURIFICATION, CHARACTERIZATION AND APPLICATION

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The plasminogen/plasmin system plays a crucial role in both fibrinolysis and the of cell functions in a wide range of normal and pathological processes. Investigation of plasminogen/plasmin functions requires the availability of well-characterized and effective molecular tools, such as antibodies. In the present work, the isolation and characterization of rabbit polyclonal antibodies against human plasminogen are described, and approaches for the identification of plasminogen and its fragments using the purified antibodies are demonstrated. For the antibodies isolation, standard animal immunization and blood collection procedures, serum isolation, protein salting out and affinity chromatography were performed. For the antibodies characterization and application, the following methods were used: enzyme linked immunoassay (ELISA), Western blotting, FITC-protein conjugation, flow cytometry and spectrofluorometry. Obtained polyclonal rabbit anti-human plasminogen antibodies interact with human Glu- and Lys-plasminogen, kringles 1−3 and 1−4 of plasminogen, mini-plasminogen, the heavy and light chain of plasmin. We propose the application of anti-plasminogen antibodies for the direct ELISA, Western blot analysis, and for flow cytometry and spectrofluorometric analysis of plasminogen binding with cells. Produced anti-plasminogen antibodies may represent promising tools for the investigation of plasminogen/plasmin system functions, either fibrinolytic or signaling.

Key words: plasminogen, rabbit polyclonal antibodies, affinity chromatography, ELISA, Western blotting, FITC-coupling, flow cytometry, spectrofluorometry.

Plasminogen is a key component of the fibrinolytic system and an inactive precursor of the main fibrinolytic enzyme plasmin (3.4.21.7). Plasminogen conversion to plasmin is mediated by plasminogen activators, either tissue-type or urokinase-type ones, on cells and fibrin surface. Plasmin regulates fibrin clot lifetime, maintains normal circulation and blood vessels passability [1], mediates extracellular matrix proteolysis during cell migration, and influences cell signaling [2].

Human plasminogen is a one-chain glycoprotein with a molecular weight of 92 kDa and 791 amino acid residues. Intact zymogen, normal for the circulation, contains glutamic acid on N-terminus (Glu-plasminogen). Partially degraded plasminogen molecule contains lysine (Lys-plasminogen) and appears on primary stages of fibrinolysis or under pathological conditions as a result of proteolytic activity in blood [3].

Plasminogen molecule consists of N-terminal domain, 5 kringle domains (K1-5) and serine-protease domain. Glu-plasminogen molecule is folded due to the N-terminal peptide interaction with K4 and K5, whereas N-terminal peptide cleavage in Lys-form of the zymogen leads to deployment of the molecule. Kringle domains are responsible for the substrate and cell receptor binding and serine-protease domain after the zymogen activation is able to cleave substrates. Degradation of plasminogen molecule by proteases (elastase, trypsin, plasmin, etc.) leads to plasminogen kringle-containing fragments formation,

which have antiangiogenic properties and thus are called angiostatins [4].

Plasminogen/plasmin interacts with a wide range of cell types. Plasminogen receptors were found on platelets, neutrophiles, macrophages, endotheliocytes, smooth muscle cells, fibroblasts, neuronal cells and different types of cancer cells [5]. Plasminogen/plasmin mediates cell signaling during inflammation, inducing proinflammatory cytokines production and macrophages chemoattraction, as well as metastasis, cell migration and proliferation, angiogenesis, tissue remodeling, myogenesis, neuritogenesis [6–8].

Plasminogen fragments, known as angiostatins, have mostly opposite function than parent molecule, inhibiting cell proliferation, migration, wound healing, and sometimes exhibit their functions through distinctive cell receptors [9, 10].

Investigation of plasminogen/plasmin functions requires an availability of well-characterized and effective instruments, such as antibodies. Polyclonal antibodies against plasminogen can be used for measurement of plasminogen level and identification of the zymogen/enzyme and its degraded forms in different type of samples, studying of plasminogen/plasmin interaction with cells and tissues, blockage of plasminogen functional activity, etc.

The aim of the present work was to isolate and characterize rabbit polyclonal antibodies against human plasminogen and to develop approach for the identification of plasminogen interaction with cells.

Materials and Methods

Proteins isolation
Glu-plasminogen was purified from citrate donor blood plasma using the Lysine-Sepharose 4B affinity chromatography in the presence of 1000 IU/ml aprotinin (AWD pharma, Germany) with subsequent salting out [11]. Salting out was performed with $(NH_4)_2SO_4$ (0.4 g per 1 mL of eluted protein solution) overnight at 4 °C, precipitate was centrifuged at 2,000 g and 4 °C for 30 min and then dissolved in 50 mM tris/HCl buffer with 150 mM NaCl (TBS), pH 7.4, in the presence of 100 mcM *p*-nitrophenyl guanidine benzoate to avoid plasmin activity. Plasminogen solution was dialyzed against TBS, pH 7.4, using 12 kDa dialysis tubing (Merck-Millipore, Germany) and stored at −20 °C.

Lys-plasminogen was purified from the blood plasma fraction III$_{2,3}$ by Cohn using the abovementioned method.

Plasmin was obtained by activation of Glu-plasminogen with urokinase-Sepharose 4B [12] and stored at −20 °C in TBS, pH 7.4, with 50% glycerol.

Plasminogen fragment kringle 1-3 was isolated from plasminogen hydrolysate, obtained by elastase-mediated hydrolysis, using affinity chromatography as described elsewhere [13].

Plasminogen fragments kringles 1−3 + 1−4 and mini-plasminogen were kindly provided by Dr. Artem Tykhomyrov (Palladin Instiute of Biochemistry of the National Academy of Sciences of Ukraine).

Tissue-type plasminogen activator used in the study was from Boerhinger Ingelheim (Actilyse).

Plasminogen-Sepharose synthesis
Plasminogen conjugation to Sepharose was performed using CNBr-activated Sepharose 4B (Sigma Aldrich, USA) according the manufacturer's recommendation. CNBr-activated Sepharose was swelled in cold 1 mM HCl for 30 min and washed with 0.1 M NaHCO$_3$ buffer containing 0.5 M NaCl, pH 8.3−8.5 (coupling buffer). Plasminogen was dialyzed against coupling buffer and conjugated with CNBr-Sepharose during 8 h(5 mg protein per cm^3 of gel), then washed with 5 gel volumes of coupling buffer. Sepharose uncoupled CNBr-groups were blocked with 100 mM glycine in coupling buffer for 2 h at 4 °C and washed with TBS, pH 7.4. Plasminogen-Sepharose was stored in TBS, pH 7.4, containing 100 mcM *p*-nitrophenyl guanidine benzoate to avoid possible plasmin activation and conserved with 0.02% sodium azide.

Rabbit immunization and antiserum preparation
Rabbit immunization was performed in accordance with the recommendations [14]. Male rabbits weighing about 3 kg were kept on the standard diet in the animal house of Palladin Institute of Biochemistry of NASU. Each rabbit was immunized by an emulsion containing 0.1 mg of human plasminogen in 0.5 ml TBS, pH 7.4, and 0.5 ml of complete Freund`s adjuvant (Sigma Aldrich, USA). Six subcutaneous injections were given symmetrically in dorsal thoracic and lumbar regions. In 2 weeks, animals had boost immunization with incomplete Freund`s adjuvant (Sigma Aldrich, USA). In 10 days, the blood serum was tested for ELISA and the presence and titer of antibodies specific to the introduced antigen. In 12−15 days, blood was collected for 3 times.

Blood was collected from marginal ear vein by venipuncture into glass tubes and incubated at 4 °C for 16 hours for clot formation. After the clot removal serum was centrifuged to remove the debris at 200 *g* for 15 min at 4 °C. The supernatant was used for the antibody purification.

Anti-plasminogen antibodies isolation

The globulin fraction of blood proteins was isolated by salting out from blood serum using saturated solution of $(NH_4)_2SO_4$ in volume ratio 1:1 during 16 hours with subsequent centrifugation 15 min at 1,000 *g* and 4 °C. The precipitate was dissolved in TBS, pH 7.4, and dialyzed. Total immunoglobulin G fraction was isolated from globulin solution using affinity chromatography on Protein A-Sepharose (Sigma Aldrich, USA) as described elsewhere [14].

Immunoglobulin G solution, purified on Protein A-Sepharose, was applied on Plasminogen-Sepharose equilibrated with TBS pH 7.4 in volume ratio of protein solution : affinity gel 1.5:1, then the column was washed with TBS pH 7.4. Plasminogen-specific antibodies were eluted with 200 mM glycine, pH 2.8, and immediately mixed with 1 M tris-HCl, pH 8.5, in volume ratio 100:8. Protein concentration in eluate was controlled by light absorption at 280 nm. Fractions, containing the antibodies, were collected, mixed, dialyzed against TBS, pH 7.4, and concentrated using Amicon 100 centrifugal devices (Merck-Millipore, Germany). Purified antibodies were stored with glycerol (volume ratio 1:1) at −20 °C.

FITC-labelling of plasminogen-specific antibodies

Antibodies-FITC conjugation was carried out using FITC (fluorescein isothiocyanate, Sigma Aldrich, USA) [15]. FITC stock solution 1 mg/ml in DMSO was added to antibodies (2 mg/ml in 0.1 M sodium carbonate buffer, pH 9.0) in volume ratio 50: 1000 and the reaction mixture was incubated for 8 hours at 4°C in the dark, than the excess of the label was blocked by NH_4Cl in a final concentration of 50 mM during 2 hours 4 °C in the dark and dialyzed against 0.05 M sodium-phosphate buffer with 0.15 M NaCl (PBS), pH 7.4, overnight at 4 °C. F/P ratio was 3.2. FITC-labeled antibodies were stored with 25% glycerol at −20 °C.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) (Fluka, Germany) was performed using standard method [16]. For the protein purity evaluation, gels after the procedure were stained with 0.025% Coomassie Brilliant Blue R250 in 25% isopropanol with 10% acetic acid (w/v/v) and washed with 7% acetic acid overnight. For Western blot analysis gels were washed twice with distilled water to remove SDS without staining.

Western and far-blot analysis

Western blotting was performed using standard approach [17]. Electrophoresis was performed without reducing agents, except plasmin sample, which contained 1% β-mercaptoethanol. Proteins were transferred from polyacrylamide gel onto 0.45 mcm pore size nitrocellulose membrane (GVS, Italy). The membrane then was blocked with 5% fat-free skim milk solution during 2 h at 37 °C, incubated 2 h with 1 mcg/ml anti-plasminogen antibodies in PBS pH 7.4 and then 1 h with anti-rabbit secondary peroxidase-conjugated antibodies (Sigma Aldrich, USA) diluted according to the manufacturer recommendations. After each antibody, the membrane was washed 5 times × 5 min with PBS, pH 7.4, containing 0.1% Tween-20 and 5 times × 5 min with PBS, pH 7.4. Washed membrane was stained with 4-chloro-6-naphtol (Sigma Aldrich, USA) methanol solution 30 mg/mL and 3% hydrogen peroxide in PBS, pH 7.4 (volume ratio 10 : 90 : 2).

Enzyme linked immunoassay

Proteins (1 mcg in 100 mcL of PBS, pH 7.4) were immobilized in 96-wells high-sorption microplates (NUNC Maxi-Sorp, Denmark) for 2 h at 37 °C and thoroughly washed by PBS, pH 7.4, containing 0.1% Tween-20 and PBS, pH 7.4. Then wells were blocked by 200 mcL of 1% BSA solution in PBS, pH 7.4, for 1 h at 37°C. After blocking, plasminogen-specific antibodies (0-5 mcg/100 mcL of PBS, pH 7.4) were added into the wells and incubated for 1 h at 37 °C and washed. For the determination of the primary antibodies interaction with the immobilized proteins, goat secondary anti-rabbit antibodies conjugated with alkaline phosphatase (Sigma Aldrich, USA) were used as recommended by the manufacturer, and after washing, developed for 30 min using chromogenic phosphatase substrate — 500 mcg/mL *p*-nitrophenyl phosphate (Sigma Aldrich, USA) in 10% diethanolamine, pH 9.8, and measured by Multiscan microplate reader (Thermo Scientific, USA) at 405 nm.

Platelet preparation

Blood was collected from two healthy volunteers was collected by venipuncture and

immediately mixed with anticoagulant (3.2% sodium citrate) in 9:1 volume ratio. Platelets were isolated from blood using the approach described elsewhere [18]. Platelets count was performed using an aggregometer (Solar AT-02, Belarus).

Platelets (5×10^6 cells) were incubated with Glu-plasminogen (1 mcM final concentration) in 20 mM HEPES buffer, containing 137 mM NaCl, 4 mM KCl, 0.2 mM $MgCl_2$, pH 7.4, for 10 min at 37 °C, washed twice by centrifugation (1,000 g for 10 min at 25 °C), and then incubated with 5 mcg/mL of FITC-labeled anti-plasminogen antibodies in PBS with 1% BSA, pH 7.4, for 20 min at 37 °C and washed twice.

Spectrofluorometric analysis
Binding of FITC-labeled plasminogen-specific antibodies was analyzed using flow cytometry and steady-state spectrofluoro-metry.

Flow cytometry was performed using Coulter Epics XL flow cytometer (Beckman Coulter, USA) via FL1 channel (515−535 nm) for FITC label. For each sample 30,000 events were chosen as sample size. Population of untreated platelets was used as a control.

Steady-state spectrofluorometry was performed using QuantaMaster spectrofluo-rometer (Photon Technology International, Canada), excitation wavelength was 490 nm, emission was measured at 520 nm.

Data analysis
Data was analyzed and presented using standard tools of GraphPad Prism 7 software. The flow cytometry results are presented using "FCS Express V3" software.

Results and Discussion

Glu-plasminogen used for rabbit immunization and Plasminogen-Sepharose synthesis was electrophoretically pure (Fig. 1, *a*). Blood serum of immunized animals contained 150-kDa protein fraction, complying the immunoglobulin G molecular weight (Fig. 1, *b*, Ser). The antibodies purified with subsequent use of protein A- and Plasminogen-sepharose were electrophoretically pure (Fig. 1, *b*, AB). Specific antibodies yield was 7.1%.

Analysis of the purified antibodies binding to Glu- and Lys- forms of human plasminogen demonstrates the same affinity: saturation of 1 mcg of immobilized plasminogen is reached at the antibody concentration 0.5 mcg/ml, for Glu-plasminogen $K_d = 5.02 \pm 1.20$ nM, for Lys-plasminogen $K_d = 5.40 \pm 1.27$ nM

(Fig. 2). Recommended antibodies dilution for the direct ELISA is 0.5 mcg/mL. Tissue-type plasminogen activator was used in the assay as negative control to discern possible antibodies cross-reaction with kringle domain-containing non-plasminogen proteins and it is confirmed that the obtained antibodies have specificity to plasminogen.

To clarify which plasminogen domain contains antigenic determinant for the antibodies, Western-blot analysis was applied (Fig. 3, *a*). Optimal antibodies dilution (1 mcg/mL) was chosen using dot-blot analysis (data not shown). The ability of the antibodies to interact with plasminogen, heavy and light chains of plasmin, plasminogen fragments K 1-3, K 1-4 and mini-plasminogen was demonstrated. Moreover, the use of anti-plasminogen antibodies allows to distinct different forms of plasminogen fragments in the mixture (Fig.3, track K1-4+K1-3), which can represent different angiostatins [4, 19, 20]. Western blot analysis of platelet lysate (whole cell, 10^6 cells/track, in 5 x Laemmli buffer) confirms plasminogen and its fragments to be presenced in platelets (Fig. 3, *b*), among which is typical one with molecular weight near 50 kDa complying angiostatin that can be released by platelets [21]. Therefore, the obtained antibodies can be applied for the determination either plasminogen or plasminogen fragments, including angiostatins.

Plasmin sample contains β-mercapto-ethanol to divide heavy (63 kDa) and light (26 kDa) chains. Western blotting was applied for the analysis.
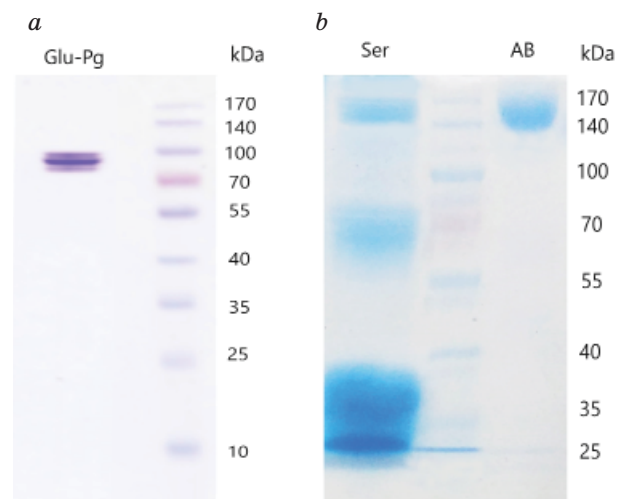

Fig. 1. **Electrophoregram of (*a*) purified human Glu-plasminogen, used for the animal immunization and (*b*) the rabbit serum (Ser) and affinity purified anti-plasminogen antibodies (AB)**

Investigation of plasminogen or plasminogen fragments interaction with cells can be carried out using fluorometric techniques. For this purpose, the anti-plasminogen antibodies are conjugated with different fluorescent labels and applied for the fluorometric analysis. To test the obtained antibodies suitability for these applications, we performed FITC-labeling of the antibodies and demonstrated Glu-plasminogen binding to platelets with flow cytometry and steady state spectrofluorometric analysis.
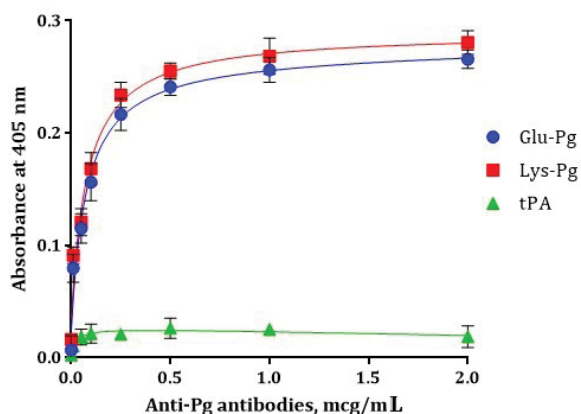

Fig. 2. **Binding of purified polyclonal antibodies against plasminogen with Glu-plasminogen (Glu-Pg), Lys-plasminogen (Lys-Pg), and tissue-type plasminogen activator (tPA) evaluated by ELISA**

Fig. 4 demonstrates typical fluorescence histograms of native washed human platelets, bound with anti-plasminogen FITC-labeled antibodies before and after incubation with exogenic plasminogen. Data obtained with flow cytometry and presented with FCS Express 3 software. As a control, platelet autofluorescence was monitored. Results indicate that washed platelets absorb plasminogen from plasma on their surface and, when isolated, can bind additional exogenous zymogen amount (median value is 4.7 for the platelets-FITC-antibodies interaction, and 8.3 for the platelets-plasminogen-FITC-antibodies interaction, comparing with 1.03 of platelets autofluorescence).

Steady-state fluorometry analysis indicates the same results (Fig. 5, *a*): washed platelets surface contains plasminogen or its fragments and can bind additional exogenous plasminogen. Washed platelets can bind additionally FITC-antibodies after the preincubation with exogenous plasminogen comparing with the labeled antibodies binding level without plasminogen addition (Fig. 5, *b*).

Platelets autofluorescence serves as a control. Mean values obtained for washed platelets of two healthy volunteers.

It is well established that platelets are able to bind plasminogen, and the cells washed from platelet reach plasma interact with the zymogen through different partner proteins, which can be removed from the cell surface by gel-filtration on Sepharose 2B, but not washing by centrifugation [22−25],
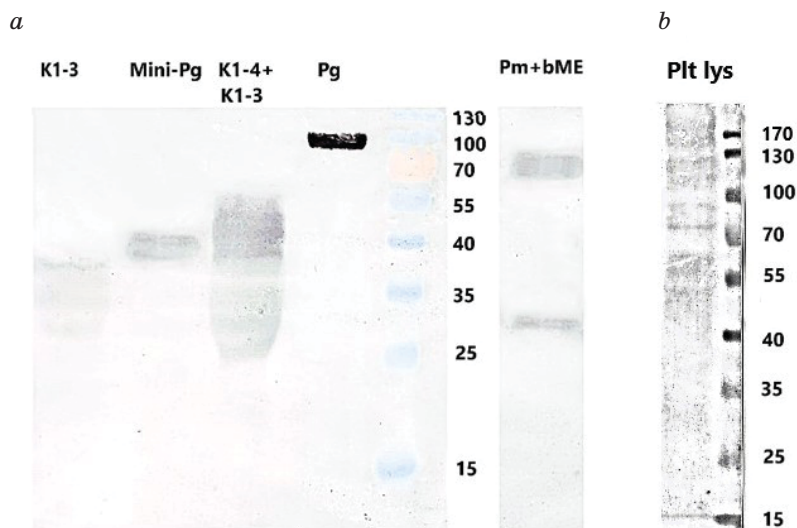

Fig. 3. **Interaction of purified polyclonal antibodies against plasminogen:**
a — Glu-plasminogen (Glu-Pg), mini-plasminogen (Mini-Pg), plasminogen kringles 1-4+1-3 (K14+K1-3), kringles 1-3 (K1-3) and plasmin (Pm+bME); *b* — platelets lysate (Plt lys).
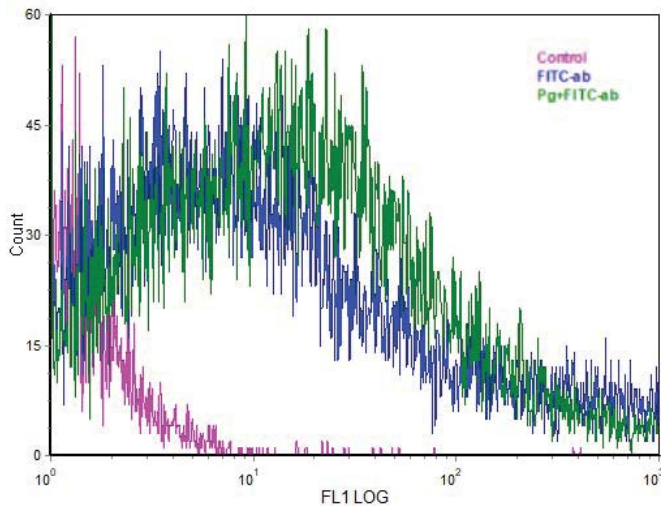
Fig. 4. **Typical fluorescence histograms of platelets distribution of untreated (Control), only FITC-antibodies treated (FITC-ab) and plasminogen and FITC-antibodies treated (Pg+FITC-ab) platelets**
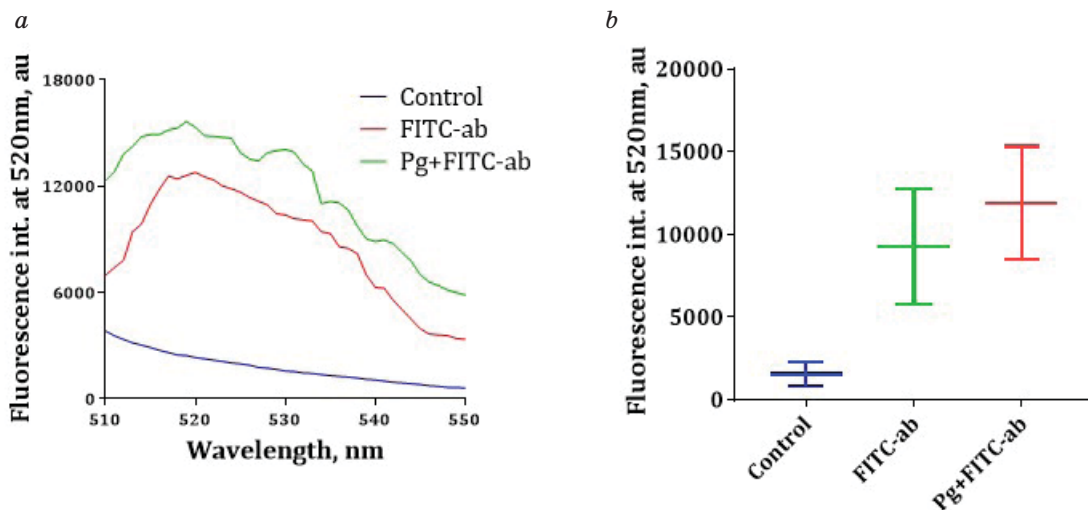

Fig. 5. **Spectrofluorometric analysis of Glu-plasminogen binding to intact platelets using FITC-labeled anti-plasminogen antibodies:**
a — Typical fluorescence spectra of untreated — autofluorescence (Control), only FITC-antibodies (FITC-ab) treated and plasminogen and FITC-antibodies treated (Pg+FITC-ab) platelets; *b* — Fluorescence intensity FITC-antibodies (FITC-ab) treated and plasminogen and FITC-antibodies treated (Pg+FITC-ab) platelets at maximum FITC emission wavelength (520 nm)

thus the results obtained using FITC-labeled anti-plasminogen antibodies comply with the previously published data.

The results of the present work demonstrate that rabbit polyclonal antibodies against human Glu-plasminogen can be used for the detection of full-length molecules of Glu- and Lys-forms of zymogen, as well as kringle- and protease-domain containing fragments. FITC-labeled anti-plasminogen antibodies are suitable for plasminogen detection on cells using fluorometric approaches and allow evaluating changes in bound plasminogen levels depending on the different functional states of cells. To perform the identification of intact or fragmented forms of the zymogen, the Western blot analysis can be applied.

The obtained antibodies can represent a promising tool for the investigation of plasminogen/plasmin system functions, either fibrinolytic or signaling, for example platelet physiology, cell migration, tumor growth, angiogenesis, inflammation, etc.

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ПОЛІКЛОНАЛЬНІ АНТИТІЛА ДО ПЛАЗМІНОГЕНУ ЛЮДИНИ: ОЧИЩЕННЯ, ХАРАКТЕРИСТИКА ТА ЗАСТОСУВАННЯ

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Плазміноген/плазмінова система відіграє вирішальну роль у фібринолізі та регулюванні функцій клітин у широкому діапазоні нормальних та патологічних процесів. Дослідження ролі плазміногену/плазміну потребує добре охарактеризованих та ефективних інструментів, зокрема антитіла. У роботі описано виділення поліклональних антитіл кроля проти плазміногену людини та надано їх характеристику, а також запропоновано підходи до ідентифікації плазміногену та його фрагментів з використанням одержаних антитіл. З метою виділення антитіл застосовували стандартну процедуру імунізації тварин і забору крові, виділення сироватки, висолення протеїнів та афінну хроматографію. Для характеристики та застосування антитіл використовували такі методи: імуноензимний аналіз, вестерн-блот, кон'югацію протеїнів, протокову цитометрію та спектрофлуорометрію. Показано, що антитіла взаємодіють з Glu- і Lys-плазміногеном людини, крінглами 1-3 та 1-4 плазміногену, міні-плазміноном, важким та легким ланцюгами плазміну. Пропонується застосування антитіл до плазміногену у прямому імуноензимному аналізі, вестерн-блот-аналізу та після мічення флуоресцентною міткою у протоковій цитометрії та спектрфторометричному аналізі зв'язування плазміногену з клітинами. Отримані антитіла до плазміногену є перспективними інструментами для дослідження як фібринолітичних, так і сигнальних функцій плазміноген/плазмінової системи.

Ключові слова: плазміноген, поліклональні антитіла кроля, афінна хроматографія, імуноензимний аналіз, вестерн-блот, FITC-мічення, протокова цитометрія, спектрофлуориметрія.

DEVELOPMENT OF RECOMBINANT POSITIVE CONTROL FOR AFRICAN SWINE FEVER VIRUS PCR DETECTION

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Recombinant plasmids containing target sequences are widely used as positive controls for PCR laboratory diagnostics. The aim of the study was development of recombinant positive control containing a fragment of *B646L* gene of African swine fever virus. The sequence of interest encodes targets of all the PCR assays for African swine fever laboratory diagnostics recommended by World Organisation for Animal Health. A plasmid containing 1763 bp insertion was cloned in *E. coli* DH5α strain. After purification, the plasmid ten-fold serial dulutions were used as a positive control while PRC testing. A minimal detectable copy number was 20 copies per reaction for both conventional and real-time PCR assays. The developed plasmid could be used as a safe and effective positive control while ASF laboratory diagnostics by PCR.

Key words: African swine fever virus, molecular cloning, PCR, positive recombinant control.

African swine fever (ASF) is a viral disease, affecting domestic pigs and wild boars. Acute ASF form is characterized by hemorrhagic fever and results in high mortality. The causative agent is the ASF virus (ASFV), a giant enveloped DNA virus, the only member of the *Asfarviridae* family. African wild boars and soft ticks of the *Ornithodoros* genus are the natural virus hosts. In the wild boar reservoir the infection is usually asymptomatic [1, 2]. Since a vaccine and treatment are unavailable, the disease causes significant economic losses and poses a threat to pig industry in all European countries. For these reasons, ASF is a notifiable disease according to the World Organization for Animal Health [3].

ASF is endemic in sub-Saharan countries and Sardinia. In 2007 ASFV was detected in Georgia for the first time. Probably, the virus of the genotype II from Africa was introduced to the new territory via sea. Since 2007 ASFV has rapidly spread to Azerbaijan, Armenia, Russia, Belarus, Ukraine, Poland, Estonia, Lithuania, Latvia, Moldova, the Czech Republic, Romania, Hungary, Bulgaria, Serbia, the Slovak Republic and Germany, where it caused numerous outbreaks among domestic pigs and wild boars. In September 2018 ASFV has been detected in wild boars in Belgium for the first time since eradication in 1985. Belgium is the first country of Western Europe, where the disease reoccurred after its introduction to Georgia [4, 5]. In August 2018 ASF for the first time was confirmed in China, which is the world biggest pork producer. Until now the disease has spread to Vietnam, Cambodia, Indonesia, Mongolia, the Republic of Korea, the Democratic People`s Republic of Korea, Myanmar, Philippines, Laos, Papua New Guinea, India and other Asian countries [5]. Thus, rapid virus detection and strict quarantine measures implementation is crucial for the disease control.

Different laboratory tests are used for ASF diagnostics. Assays, directed towards virus

isolation, antigen or antibodies detection are time-consuming and may generate false-negative results. Molecular methods, such as conventional and real-time PCR, are highly sensitive and rapid technique for virus detection. They are appropriate for routine diagnostics and surveillance of the ASF and could be used for a wide isolates variety of different virus genotypes testing. According to the OIE guidelines, conventional and real-time PRC assays are recommended for ASFV detection [6]. These assays are aimed at identifying the sequences in positions 115–371 [7], 648–714 [8] and 1627–1877 [9] of gene *B646L* that encodes viral protein p72.

PCR assays require using positive controls for valid results obtaining. It`s preferable not to use virus culture as a positive control for biosafety and biosecurity providing. Plasmid positive controls use is reliable for such purposes.

The aim of the study was to develop a universal positive recombinant control containing all the mentioned sequences, wich can be used for both conventional and real-time PCR assays recommended by OIE.

Materials and Methods

The plasmid map was designed using the Clone Manager Professional 9 (Scientific and Educational Software, USA). The insertion of 1763 bp *B646L* gene fragment in positions 115–1877 was synthesized using conventional PCR. The following primers were used for this assay:

Primer PPA-1: AGTTATGGGAAACCCGACCC [7];

King 2: GATACCACAAGATCAGGCCGT [9].

ASF virus strain Georgia 2007 was used as a positive DNA template. The reference DNA of the ASF virus was obtained from the European Union Reference Laboratory for African Swine Fever (Spain). Reaction mix was prepared according to the following protocol (per 1 sample): AmpliTaq Gold polymerase (Applied Biosystems, USA) 1.25 U/50 mcl — 0.13 mcl, PE-buffer 1X — 2.5 mcl, dNTP mix 0.2 mM — 0.5 mcl, $MgCl_2$ 1.5 mcM — 1.5 mcl, primer PPA-1 10 mcM — 0.5 mcl, Primer-2 10 μM — 0.5 mcl, DNA matrix — 5 mcl, adjusted with water up to the final volume of 20 mcl. Thermal cycling was performed using the following program: one cycle at 95 °C for 5 min, followed by 40 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, final elongation at 72 °C for 10 min. Amplification was verified by amplicons visualising in 1% agarose gel.

The size of the PCR product was 1763 bp. The amplicons were isolated from the gel using GeneJET Gel Extraction Kit (Thermo Scientific, USA). The DNA concentration was determined using a DS-11 spectrophotometer (DeNovix, USA).

PCR product was inserted into the vector using InsTAClone PCR Cloning Kit (Thermo Scientific, USA) with the vector to insertion ratio 1:5. Ligation was performed overnight at +4 °C. *E. coli* DH5α (Thermo Scientific, USA) Calcium Chloride competent cells were transformed via heat-shock with the ligated plasmid. Transformed cells were verified using blue-white screening and confirmed by the mentioned above PCR. After the transformed bacteria cultivation, the plasmid was isolated using GeneJET Plasmid Miniprep Kit (Thermo Scientific, USA).

Isolated plasmids were used as a positive template for PCR assays with PPA1/2 [7] and King 1/2 [9] sets. The reaction mixture for conventional PCR per 1 reaction contained 12.5 mcl of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific, USA), 1 mcl of 10 pM PPA-1 primer, 1 mcl of 10 pM PPA-2 primer, 5.5 mcl of water and 5 mcl of a template. The cycling was performed using the following protocol: one cycle at 95 °C for 5 min, 40 cycles at 95 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s, final elongation at 72 °C for 5 min and 257 bp amplicons were visualized by electrophoresis in 1.5% agarose gel. Real-time PCR reaction mixture was prepared using 10 mcl of TaqMan Fast Advanced Master Mix (2X) (Applied Biosystems, USA), 0,5 mcl of 10 pM probe, 0,4 mcl of each 20 pM primer, 6,7 mcl of water, 2 mcl of a template. Real-time PCR was carried out according to the following protocol: one cycle at 95 °C for 5 min, 40 cycles at 95 °C for 15 s, 58 °C for 30 s, 60 °C for 30 s, the fluorescent signal was recorded at FAM channel at every 60 °C step.

Results and Discussion

The plasmid with a 1763 bp *B646L* gene fragment ligated into the pTZ57R/T vector was constructed. The total length of the plasmid is 4649 bp (Fig. 1).

The vector encodes an ampicillin resistance and a *lacZ* genes, which were used as selective markers for transformed *E. coli* DH5α clones. Ten white single colonies were screened with conventional PCR and confirmed as containing a 1763 bp *B646L* gene fragment.

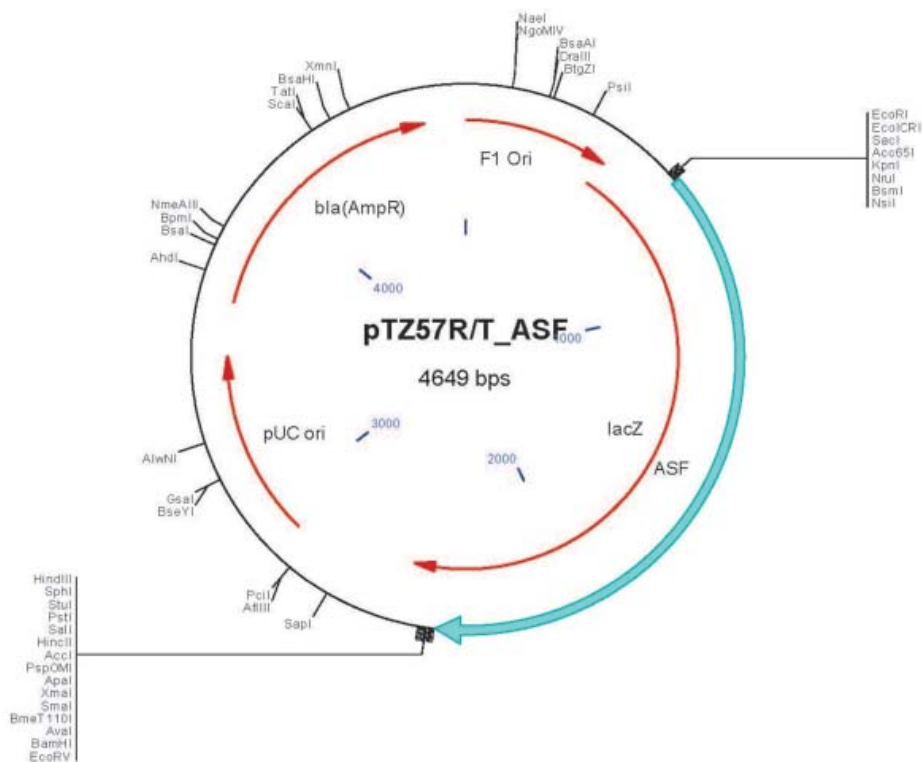The series of ten-fold dilutions, containing 10^5, 10^4, 10^3, 100 and 10 copies of

Fig. 1. **The plasmid pTZ57R/T_ASF map**

the plasmid DNA in 1 mcl, were prepared for a minimum copy number detection testing. The pTZ57R/T_ASF plasmid is detected by both real-time PCR (Fig. 2) and conventional PCR (Fig. 3) at dilution up to 10 copies per µl, corresponding to 20 copies per reaction. The successful result for both target regions proved that the whole sequence of interest was ligated correctly into the vector and can be used as a positive control sample.

PCR is considered to be a "gold standard" test for an early ASFV detection. However, its results can be regarded as true only in case of positive and negative controls use, which ensure performance of the reaction and help to avoid false-posive and false negative results obtaining [10].

Substantions of different types containing the gene of interest can be used as positive control. DNA extracted from reference culture of microorganism is an optimal kind of positive control sample. However, this approach has limitations while dangerous diseases diagnostics, as laboratory must meet special biosafety requirements to be permitted to cultivate that kind of agents. Even though a laboratory has the permittion, a cultivation of hazardous agents, biomass producing and concentration is associated with additional

risks. In case of inactivated microorganisms use, resiaduals of inactivating substances (formaldehyde, ethanol, etc.) may affect PCR acting as inhibitors [10]. DNA extracted from clinical material can also be used as positive control. Advantage of this type of material is a close simulation of tested samples, so the influence of sample matrix on result eliminates. However, variation of Ct values obtained during real-time PCR testing of samples from different patients/ animals as positive control should be taken into account [11].

Recombinant plasmids are widely used as both inner and outer positive controls while PCR testing. Many kits available commercially include recombinant plasmid containing gene of interest as a positive control sample. OIE suggests using recombinant plasmid DNA as positive control while diagnostics testing by PCR [12]. They can be easily obtained in big amount from transformed bacteria during short time. This process does not require special conditions and can be performed even in BSL-1 laboratory as bacteria strains used for cloning are safe for a researcher and environment. Plasmid DNA is also an excellent material for the absolute quantification and qPCR assay validation [13]. Plasmid purification kits ensure that the final product contains
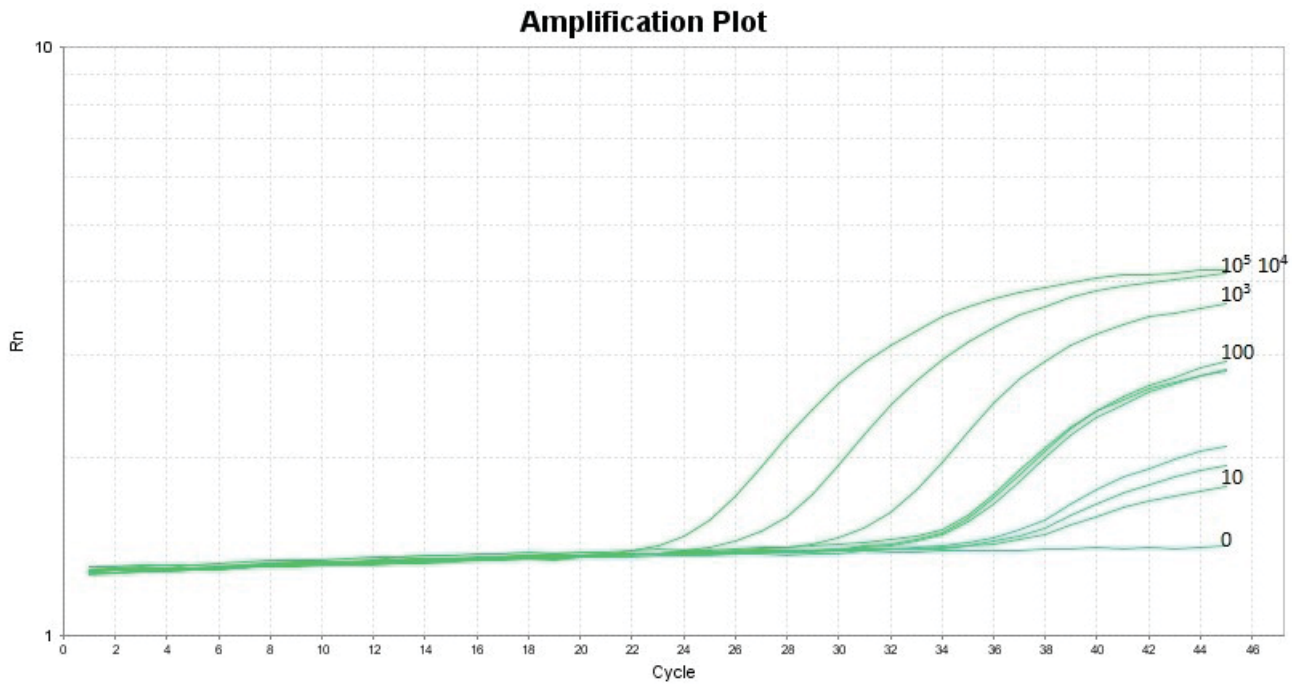
Fig. 2. **The results of pTZ57R/T_ASF minimal copy number detection using real-time PCR**

Fig. 3. **The results of pTZ57R/T_ASF minimal copy number detection using conventional PCR**

pure plasmid DNA. Thus, gene copy number calculation in plasmid DNA sample is more accurate than in one containing DNA of both host organism and pathogen.

Plasmid DNA containing different fragments of ASFV genome (p72 gene, topoisomerase II gene etc.) are commonly used as a positive control in plentiful diagnostic kits and during scientific studies [14−17]. We have developed the recombinant plasmid containing region of *B646L* gene, which includes target sequences for all the PCR assays recommended by OIE for ASF diagnosis. It makes the developed plasmid the universal positive control for ASFV

genome detection by PCR according to OIE guidance [6].

However, there are some limitations for work with plasmids. Since plamid is a circular molecule, this structure is more stable than liner DNA. In case of multicopy plasmid (including pTZ57R/T) use, a huge amount of plasmids are produced during cloning procedure. These issues should be considered while working with plasmids, as they can result in contamination of reagents, equipment or surfaces. Even though plasmid use in the laboratory diagnostics poses a threat of contamination, plasmid contamination of the sample can be easily distinguished from the cross-contamination by

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native viral DNA using vector-specific primers (e.g., M13 primers).

Thus, being quantifiable, renewable and stable, recombinant plasmid is a good choice for PCR positive control during laboratory diagnostic testings.

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РОЗРОБЛЕННЯ РЕКОМБІНАНТНОГО ПОЗИТИВНОГО КОНТРОЛЬНОГО ЗРАЗКА ДЛЯ ДЕТЕКЦІЇ ВІРУСУ АФРИКАНСЬКОЇ ЧУМИ СВИНЕЙ ЗА ДОПОМОГОЮ ПЛР

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Рекомбінантні плазміди, що містять цільові послідовності, часто використовують у лабораторній діагностиці як позитивний контроль для ПЛР. Метою дослідження було розроблення рекомбінантного позитивного контролю, що містить фрагмент гена вірусу Африканської чуми свиней. Ця послідовність кодує цільові ділянки усіх ПЛР-методик, що їх рекомендує для лабораторної діагностики АЧС Всесвітня організація охорони здоров'я тварин. Плазміду, що містить вставку розміром 1763 пн, клонували у *Escherichia coli* штаму DH5α. Після очищення десятикратні серійні розведення плазмідної ДНК було використано під час проведення ПЛР. Мінімальна кількість копій плазміди, що детектується класичною ПЛР та ПЛР у режимі реального часу, становила 20 копій на реакцію. Розроблену плазміду можна використовувати як безпечний і ефективний позитивний контрольний зразок у разі лабораторної діагностики АЧС за допомогою ПЛР.

Ключові слова: вірус африканської чуми свиней, молекулярне клонування, ПЛР, рекомбінантний позитивний контроль.