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CHEMICAL CHARACTERIZATION AND BIOLOGICAL ACTIVITY OF *ESCHERICHIA COLI* LIPOPOLYSACCHARIDES

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Lipopolysaccharides (LPS) are specific components of the cell envelope of gram-negative bacteria, located at the external surface of their outer membrane and performing a number of important physico-chemical and biological functions. The widespread in nature are representatives of Enterobacteriaceae family. Among them there are saprotrophic, useful human symbionts, as well as causative agents of acute intestinal infections. The role of saprophytic intestinal microbiota is not limited only to its participation in the digestion process. The endotoxin released as a result of self-renewal of the cell pool of Escherichia coli partially enters the portal blood and performs antigenic stimulation of the macroorganism. In addition, a small amount of endotoxin can also be released by live gram-negative bacteria, which, given the large population of E. coli in the intestine, can create a sufficiently high concentration of endotoxin. ***Aim.*** *The study of composition and biological activity of lipopolysaccharides of new E. coli strains, found in the human body.* ***Methods.*** *The objects of investigation were strains of Escherichia coli, isolated from healthy patients at the epidemiological center in Kharkiv. Lipopolysaccharides were extracted from dried cells by 45 % phenol water solution at 65–68° C by Westphal and Jann method. The amount of carbohydrates was determined by phenol-sulfuric method. Carbohydrate content was determined in accordance to the calibration curve, which was built using glucose as a standard. The content of nucleic acids was determined by Spirin method, protein − by Lowry method. Serological activity of LPS was investigated by double immunodiffusion in agar using the method of Ouchterlony.* ***Results****. In all studied E. coli LPS (2884, 2890, 2892), glucose was dominant monosaccharide (40.5, 41.1, 67.3 %, respectively). LPS also contained rhamnose (1.8, 22.9, 1.6 %, respectively), ribose (3.5, 6.1, 3.6 %, respectively) and galactose (4.1, 20.2, 18.3 %, respectively). E. coli 2884 LPS also contained arabinose (1.0 %) and mannose (44.8 %), while E. coli strains 2890 and 2892 LPS contained heptose (9.7 and 7.8 %, respectively). Lipid A composition was presented by fatty acids with a carbon chain length from C_{12} to C_{18}. As the predominant components were 3-hydroxytetradecanoic (39.2–51.3 %) as well as tetradecanoic (23.1–28.5 %), dodecanoic (8.9–10.9 %), hexadecanoic (4.3–7.2 %) and octadecanoic (1.8–2.4 %) acids. Unsaturated fatty acids: hexadecenoic (2.0–17.9 %) and octadecenoic (3.4–4.2 %) have been also identified. It was found that octadecanoic and octadecenoic acids were absent in the LPS of 2884 and 2892 strains, respectively. In SDS-PAAG electrophoresis, a bimodal distribution typical for S-forms of LPS was observed. The studied LPS were toxic and pyrogenic. Double immunodiffusion in agar by Ouchterlony revealed that the tested LPS exhibited an antigenic activity in the homologous system. In heterologous system E. coli 2892 LPS had cross reactivity with LPS of E. coli 2890 and M-17. Since the structure of the O-specific polysaccharide (OPS) of E. coli M-17 was established by us earlier, the results of serological reactions make it possible to suggest an analogy of the E. coli 2892 and 2890 OPS structures with that of E. coli M-17 and their belonging to the same serogroup.* ***Conclusions.*** *The study of the composition and biological activity of LPS of new strains of Escherichia coli 2884, 2890 and 2892, isolated from the body of almost healthy patients, expands our knowledge about the biological characteristics of the species.*

Keywords: Escherichia coli, lipopolysaccharide, monosaccharide and fatty acid composition, toxicity, pyrogenicity, serological activity.

Lipopolysaccharides (LPS) are specific components of the cell envelope of gram-negative bacteria, located at the external surface of their outer membrane and performing a number of important physico-chemical and biological functions. They play a significant role in the maintenance of membrane integrity, regulate its permeability for various substances, and participate in the contacts of the microorganism with other micro- and macroorganisms. Due to the surface location and structural properties, LPS are the main antigens of the gram-negative bacteria [1]. Since LPS are the endotoxins of a microbial cell, their presence in the organisms of higher animals and humans causes a wide range of endotoxic activities, which may result in septic shock. Due to these properties, LPS contribute to the pathogenic potential of gram-negative bacteria and are therefore among the factors of development of severe infectious diseases.

The widespread in nature are representatives of *Enterobacteriaceae* family. Among them there are saprotrophic, useful human symbionts, as well as causative agents of acute intestinal infections. The role of saprophytic intestinal microbiota is not limited only to its participation in the digestion process. The endotoxin released as a result of self-renewal of the cell pool of *Escherichia coli* partially enters the portal blood and performs antigenic stimulation of the macroorganism. In addition, a small amount of endotoxin can also be released by live gram-negative bacteria, which, given the large population of *E. coli* in the intestine, can create a sufficiently high concentration of endotoxin.

In this regard, the study of composition and biological activity of LPS of new *E. coli* strains, found in the human body, was **the aim** of this paper.

Materials and methods. The objects of investigation were *E. coli* strains, isolated from healthy patients at the epidemiological center in Kharkiv. Bacteria were grown on the solid meat-peptone agar (MPA) medium during 20–24 hours at 28° C. Cells were collected by centrifugation (20 min, 5000g), after that they were dried with acetone and ether.

LPS were extracted from dried cells by 45 % phenol water solution at 65–68° C by Westphal and Jann method [2]. The obtained aqueous fractions were dialyzed against tap water, and then distilled water to remove phenol [3].

The amount of carbohydrates was determined by phenol-sulfuric method [4]. The results were evaluated by color change during the phenol reaction with sulfuric acid in a spectrophotometer SF-26 at 490 nm. Carbohydrate content was determined in accordance to the calibration curve, which was built using glucose as a standard. The content of nucleic acids was determined by Spirin method [5], protein − by Lowry method [6].

LPS were purified from nucleic acids by ultracentrifugation (4 h at 100000 g, thrice) [3]. The presence of 2-keto-3-deoxyoctonic acid (KDO) in LPS was identified by reaction with thiobarbituric acid [3].

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAAG electrophoresis) was performed according to Laemmli [7] (4 % concentrating and 12 % separating gel, current 30 mA). The load on the gel lane was 15 μg. To identify polysaccharides, gels were stained with silver salts according to the Tsai method [8].

Identification of neutral monosaccharides was carried out after hydrolysis of preparations in 2 M HCl (5 h, 100° C). Monosaccharides were analyzed as acetates of polyols [9] on chromato-mass-spectrometer system Agilent 6890/5973N inert, column DB-225mS (30m×0.25mm×0.25μm), gas-carrier – helium, flow rate – 1 ml/min. The temperature of the evaporator was 250° C, interface – 250° C, thermostat – 220° C (isothermal regime). Monosaccharides were identified comparing the time of retention of acetates of polyols with the standards and using computer data base ChemStation. Quantitative ratios of individual monosaccharides were expressed as % from total sum of peak squares.

Fatty acid composition was identified after hydrolysis of the sample in 1.5 % acetyl chloride in methanol (100° C, 4 h), methyl ethers of fatty acids were analyzed on chromato-mass-spectrometer system Agilent 6890/5973 inert, column HP-5MS, length was 30 m, inner diameter – 0.25 mm, phase thickness – 0.25 μm, temperature regime – 150–250° C, temperature gradient – 4° C, gas-carrier – helium, flow rate – 1.2 ml/min, the temperature of evaporator was 250° C, flow distribution 1:100. Fatty acids were identified using computer database and standard mixture of methyl ethers of fatty acids [3].

The pyrogenic activity of LPS was studied on rabbits weighing 2.0–3.5 kg by intravenous administration of a minimum pyrogenic dose of LPS with further thermometry of the animals for 3 hours [3].

The toxic properties of LPS were tested on outbred mice (20 g), which were injected with different amounts of LPS (from 0.02 to 600 mkg in 0.2 ml of saline). The control group was injected with saline (0.2 ml). The survival rate of mice in groups was determined within 48 hours. Lethal dose LD_{50} was calculated using the Spearman-Karber method [10]:

$\log LD_{50} = \log X100 - \log Fd\ (\sum t - n/2)$,

where:

LD_{50} = 50 % lethal dose

log X100 = logarithmic dose giving 100 % mortality

log Fd = constant logarithm recording interval

n = number of mice per dose

$\sum t$ = total number of dead animals

O-antiserum toward heated *E. coli* cells (boiling water bath, 2.5 h) was obtained. Rabbits were immunized intravenously five times with an interval of four days, cell concentration was 2×10^9/ml (from 0.1 to 1 ml). On the 5th day after the last injection, blood (20–30 ml) was taken from the ear vein to obtain O-antisera. The titer of sera was determined by the ring precipitation reaction. Antigen activity of LPS was investigated by double immunodiffusion in agar using Ouchterlony method [11].

Results of investigations were statistically tested according to the method proposed for pharmaceutical preparations [3]. Each experiment was performed in 3–5 replications. In all tables and graphics the mean values and their standard deviations are presented. The data were statistically tested by Student's t test, and also with Excel 2000 computer programs. $P<0.05$ were considered to be statistically significant.

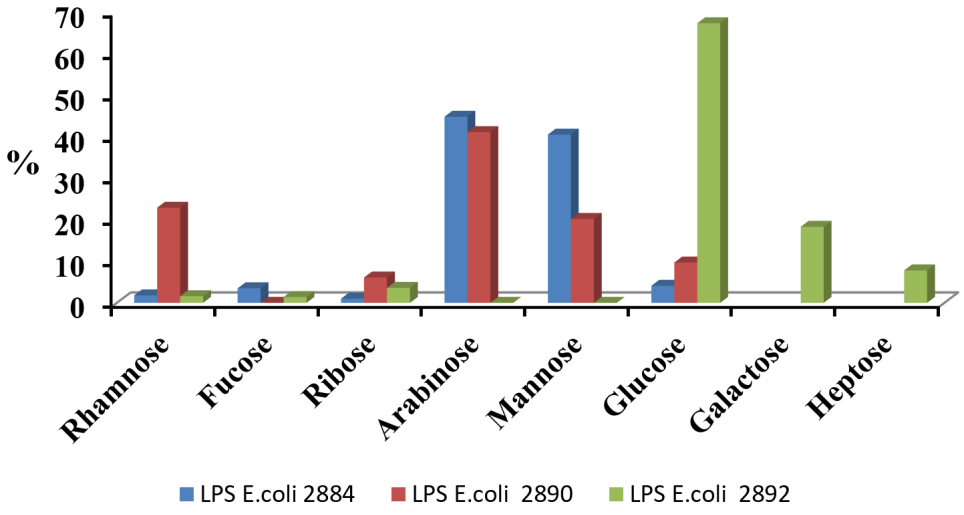
Results. *E. coli* strains 2884, 2890 and 2892 were isolated from the organisms of healthy patients. The LPS yield from dry bacterial cells was 3.2–4.7 %, depending on the strain (Table 1). When studying the biopolymer composition by specific reactions to each component, it was found that the content of carbohydrates in LPS of *E. coli* 2884 is 41.6 %, while in LPS of *E. coli* strains 2890 and 2892 it is 2 times higher (71.1 and 88.3 %, respectively). Protein in all LPS is present in trace amounts of 0.6–0.7 %, and nucleic acids from 5.0 to 6.6 % (depending on the strain). The content of KDO in the LPS of *E. coli* 2884 is 0.54 %, and in the LPS of *E. coli* strains 2890 and 2892 – 0.07 %.

In all studied *E. coli* LPS (strains 2884, 2890, 2892) (Fig. 1), glucose was predominant monosaccharide (40.5, 41.1, 67.3 %, respectively). LPS also contained rhamnose (1.8, 22.9, 1.6 %, respectively), ribose (3.5, 6.1, 3.6 %, respectively) and galactose (4.1, 20.2, 18.3 %, respectively).

Table 1

Chemical characterization of *E. coli* LPS

Components		LPS of *E. coli* strains:		
		2884	2890	2892
LPS yield	mg	190	900	600
	%	3.2	4.7	4.2
% to the dry mass of the preparation				
Carbohydrates, %		41.6	71.1	88.3
Protein, %		0.6	0.6	0.7
Nucleic acids, %		5.0	6.6	5.1
KDO, %		0.54	0.07	0.07
Phosphorus, %		0.65	0.59	0.34
Heptose, %		–	0.6	0.2

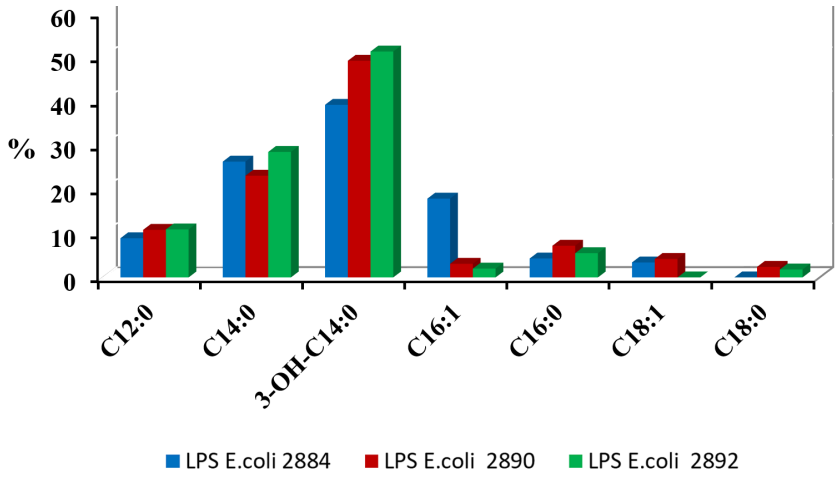


F i g. 1. Monosaccharide composition of *E. coli* LPS

E. coli 2884 LPS also contained arabinose (1.0 %) and mannose (44.8 %), while *E. coli* strains 2890 and 2892 LPS contained heptose (9.7 and 7.80 %, respectively) (Fig. 1).

Analysis of LPS fatty acids of the studied *E. coli* strains showed that it was close to that of other representatives of the *Enterobacteriaceae* family and presented by seven fatty acids with a carbon chain length from C_{12} to C_{18} (Fig. 2).

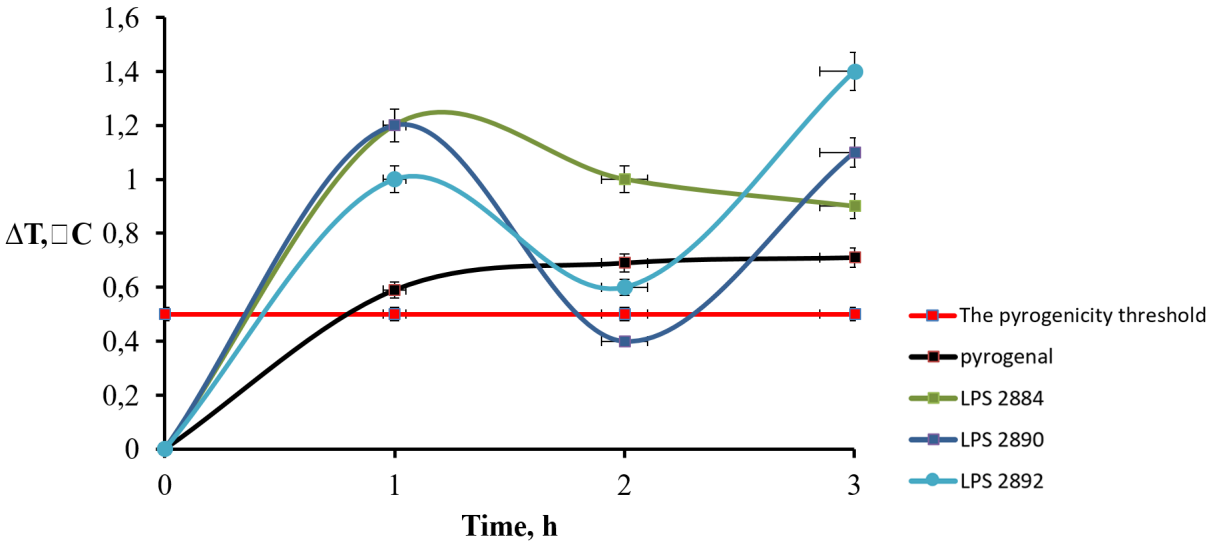
They contained 3-hydroxytetradecanoic acid as the predominant component (39.2–51.3 %) as well as tetradecanoic (23.1–28.5 %), dodecanoic (8.9–10.9 %), hexadecanoic (4.3–7.2 %) and octadecanoic (1.8, 2.4 %) acids. Unsaturated fatty acids: hexadecenoic (2.0–17.9 %) and octadecenoic (3.4–4.2 %) have been also identified. It was found that octadecanoic and octadecenoic acids were absent in the LPS of 2884 and 2892 strains, respectively.



F i g. 2. Fatty acid composition of *E. coli* LPS

The results of thermometry (Fig. 3) showed that the LPS of *E. coli* 2884 exhibited the greatest pyrogenic effect, an hour after the introduction of which the rabbit temperature increased by +1.2° C, and then gradually decreased. LPS of *E. coli* strains 2890 and 2892 were also pyrogenic, but the dynamics of temperature change was somewhat different: two hours after their introduction the temperature in experimental animals decreased and then increased again.

The study of toxicity showed that the LPS of all three strains differ in this indicator. Thus, the most toxic was *E. coli* 2890 LPS, and the least toxic – *E. coli* 2884 LPS (Table 2).



F i g. 3. Pyrogenic activity of *E. coli* LPS

Table 2

Determination of acute toxicity of *E. coli* LPS

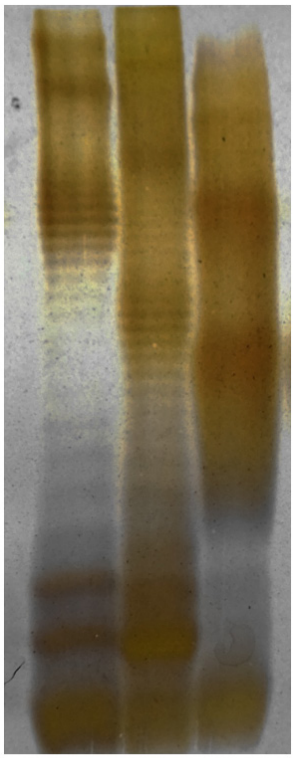
LPS of *E.coli*	d, mkg LPS per mouse					LD_{50}	
	d=37.5	d=75	d=150	d=300	d=600	mkg LPS per mouse	mkg LPS per kg of mouse body weight
2884	0/4	1/4	2/4	3/4	4/4	150	75000
LPS of *E.coli*	d, mkg LPS per mouse					LD_{50}	
	d=0.02	d=0.2	d=2	d=20	d=200	mkg LPS per mouse	mkg LPS per kg of mouse body weight
2890	0/4	0/4	1/4	2/4	3/4	158.87	7943.28
2892	0/4	0/4	0/4	0/4	1/4	355.66	17782.79

Legend: 1/4 – the number of dead (numerator) and the total number (denominator) of animals after the introduction of various doses (d) of LPS.

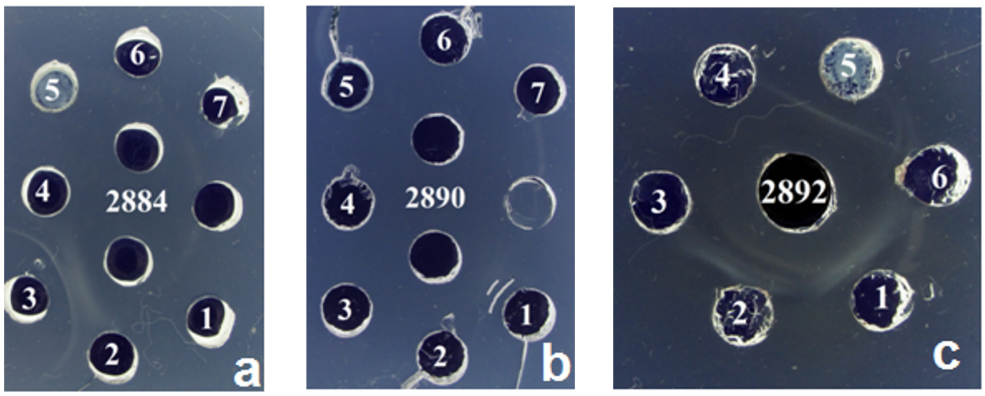
It's known [12] that most clinical isolates of *E. coli* are characterized by a smooth colony form due to the presence of the S form of LPS, which is heterogeneous and consists of three structural components: O-specific polysaccharide (OPS), oligosaccharide core (OG-core) and lipid A. Heterogeneity of the LPS molecule is very good manifests itself in SDS-PAAG electrophoresis (Fig. 4). The fast-moving zones correspond to the non-substituted OG-core, and the slow-moving ones correspond to the core oligosaccharide, which is replaced by a different number of O-specific chains, which differ in molecular weights. This feature determines the formation of a classic profile in the form of a staircase on electrophoregrams. In the upper part of the gel of *E. coli* strains 2884, 2890 LPS lines with a high molecular weight, corresponding to an O-specific polysaccharide, are well identified.

The well-known and best studied is the functional role of LPS as the main thermostable antigens of a microbial cell. Their introduction into the organism of animals and humans leads to the production of antibodies complementary to them, directed against certain structural sites in the LPS molecule, which is used for immunization [13]. When conducting serological studies, polyclonal antisera obtained against heated cultures of *E. coli* 2884, 2890 and 2892 were used. LPS isolated from the cultures of these strains served as antigens. In the reaction of ring precipitation, the working titer of O-antisera obtained for heated cultures was determined: 1:40000 (for *E. coli* 2884 and 2892) and 1:20000 (for *E. coli* 2890).

Double immunodiffusion in agar by Ouchterlony revealed that the LPS tested exhibited an antigenic activity in the homologous system. In heterologous system *E. coli* 2892 LPS give cross reactivity with LPS of *E. coli* 2890 and M-17 (Fig. 5). Since the structure of the O-specific polysaccharide M-17 was established by us earlier [14], the results of serological reactions make it possible to suggest an analogy of the *E. coli* 2892 and 2890 OPS structures with that of *E. coli* M-17 and their belonging to one of the same serogroup.



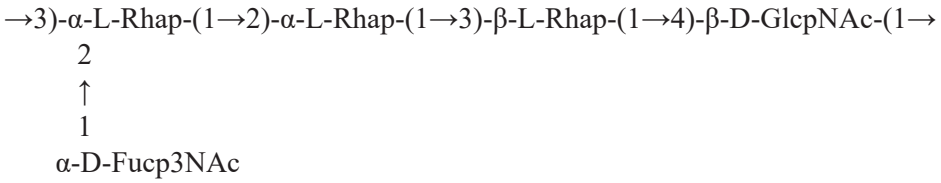
F i g. 4. SDS-PAAG electrophoresis of *E. coli* LPS (1 – strain 2884, 2 – strain 2890, 3 – strain 2892)



F i g. 5. The reaction of double immunodiffusion in agar by Ouchterlony between O-antiserum to *E. coli* strains: 2884 (a), 2890 (b), 2892 (c) and LPS isolated from *E. coli* strains 2890 (1), 2892 (2), 2884 (3), 126 (4), M-17 (5), 58 (6), L-19 (7)

Discussion. The representatives of *Enterobacteriaceae* family have been among the first studied microorganisms, since they are an indispensable part of the biosphere and are extremely abundant in various environments. The activity of these bacteria considerably affects diverse aspects of human life. Therefore, the study of the properties of new strains of *Enterobacteriaceae*, in particular representatives of such a widespread species as *E. coli*, is important, as it expands our knowledge of the biological characteristics of this species. Earlier we studied LPS of a number of *E. coli* strains, both widely known, such as *E. coli* M-17 [14], which is a part of colibacterin, and isolated from a healthy patient (*E. coli* L-19) [15] or a patient with diarrhea (*E. coli* 126) [16]. *E. coli* strains 2884, 2890, and 2892 studied in this work were isolated from the body of healthy patients. In the monosaccharide composition of the LPS of the studied *E. coli* strains, both qualitative and quantitative differences were revealed, while in the fatty acid composition they differed little from each other, although differences were observed in the presence or absence of some unsaturated fatty acids. Since lipid's A fatty acid portion of the LPS molecule is responsible for its endotoxic properties, the differences in toxicity and pyrogenicity observed between strains may be due to differences in the structure of the LPS molecule. In particular, the symmetric or asymmetric distribution of fatty acids in it, their length and quantity. In SDS-PAAG electrophoresis, a bimodal distribution of typical LPS S forms was observed. At the same time, LPS of *E. coli* 2992 differed in the quantitative content of O-specific polysaccharide chains from LPS of 2884 and 2890 strains.

The results of serological cross-reactions between antisera to the investigated strains of *E. coli* 2884, 2890, and 2892 and LPS of the strains we studied earlier turned out to be interesting. Thus, it was shown that antiserum to *E. coli* 2892 cross-reacts with LPS of *E. coli* 2890 and M-17. The structure of the OPS of *E. coli* M-17 was established by us earlier [14]. Unfortunately, the structure of the OPS of this strain was not unique, but similar to the structure established earlier by other researchers [17]. It is typical to OPS structures of *E. coli* representatives, belonging to serogroup O2.



The results obtained give a possibility to suppose that all 3 strains belong to the same O2 serological group. To confirm or refute this assumption, further research is needed to establish OPS structures.

The study of LPS in a significant number of *E. coli* strains indicates a large variety of their structures, in particular, O-specific polysaccharide chains, on the basis of the subtle variations of which structures the intraspecies serological classification schemes for gram-negative bacteria are created.

Conclusion. Thus, at present, more than 180 unique structures of O-specific polysaccharides are identified in the LPS of various *E. coli* strains, on the basis of which a serological classification scheme has been created. The number of serogroups in this scheme is constantly expanding. Therefore, the study of the properties and functions of new *E. coli* strains is of considerable fundamental interest.

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ХІМІЧНА ХАРАКТЕРИСТИКА ТА БІОЛОГІЧНА АКТИВНІСТЬ ЛІПОПОЛІСАХАРИДІВ *ESCHERICHIA COLI*

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Резюме

Ліпополісахариди (ЛПС) − специфічні компоненти клітинної оболонки грамнегативних бактерій, розташовані на зовнішній поверхні їх зовнішньої мембрани і виконують ряд важливих фізико-хімічних та біологічних функцій. Поширеними в природі є представники родини *Enterobacteriaceae.* Серед них є сапротрофи, корисні для людей симбіонти, а також збудники гострих кишкових інфекцій. Роль сапротрофної кишкової мікробіоти не обмежується лише її участю в процесі травлення. Ендотоксин, що виділяється в результаті самовідновлення клітинного пулу кишкової палички, частково потрапляє в портальну кров і здійснює антигенну стимуляцію макроорганізму. Крім того, невелика кількість ендотоксину може також виділятися живими грамнегативними бактеріями, які, враховуючи велику популяцію кишкової палички в кишечнику, можуть створити досить високу концентрацію ендотоксину. **Мета**. Вивчення складу та біологічної активності ліпополісахаридів нових штамів кишкової палички, виділених з організму людини. **Методи**. Об›єктом досліджень були штами кишкової палички, виділені від здорових пацієнтів в епідеміологічному центрі м. Харкова. ЛПС екстрагували з висушених клітин 45 %-ним водним розчином фенолу при 65–68° С методом Вестфаля та Янна. Кількість вуглеводів визначали фенольно-сірчаним методом, їх вміст − відповідно до калібрувальної кривої, яку будували з використанням глюкози як стандарту. Вміст нуклеїнових кислот визначали методом Спірина, білка − Лоурі. Серологічну активність ЛПС досліджували подвійною імунодифузією в агарі методом Оухтерлоні. **Результати**. У складі всіх досліджених ЛПС *E. coli* (2884, 2890, 2892) глюкоза була домінуючим моносахаридом (40.5, 41.1, 67.3 % відповідно). ЛПС також містив рамнозу (1.8, 22.9, 1.6 % відповідно), рибозу (3.5, 6.1, 3.6 % відповідно) та галактозу (4.1, 20.2, 18.3 % відповідно). ЛПС *E. coli* 2884 також містив арабінозу (1.0 %) та манозу (44.8 %), тоді як ЛПС штамів *E. coli* 2890 та 2892 містили гептозу (9.7 та 7.8 % відповідно). Склад ліпіду А був представлений жирними кислотами з довжиною вуглецевого ланцюга від C_{12} до C_{18}. Переважаючими компонентами були 3-гідрокситетрадеканова (39.2–51.3 %), а також тетрадеканова (23.1–28.5 %), додеканова (8.9–10.9 %), гексадеканова (4.3–7.2 %) та октадеканова кислоти (1.8–2.4 %). Також були виявлені ненасичені жирні кислоти: гексадеценова (2.0–17.9 %) та октадеценова (3.4–4.2 %). Було встановлено, що октадеканова і октадеценова кислоти відсутні в ЛПС штамів 2884 та 2892. Результати SDS-PAAG електрофорезу свідчать про бімодальний розподіл, типовий для S-форм ЛПС. Досліджувані ЛПС були токсичними та пірогенними. Подвійною імунодифузією в агарі за Оухтерлоні показано, що досліджувані ЛПС проявляли антигенну активність в гомологічній системі. У гетерологічній системі ЛПС *E. coli* 2892 давав перехресну реакцію з ЛПС *E. coli* 2890 та М-17. Оскільки структура О-специфічного полісахариду М-17 була встановлена нами раніше, результати серологічних реакцій дають змогу припустити аналогію структур О-специфічних полісахаридів *E. coli* 2892 та 2890 із структурою *E. coli* М-17 та належність їх до тієї ж серогрупи. **Висновки**. Дослідження складу і біологічної активності ЛПС нових штамів *Escherichia coli* 2884, 2890 та 2892, виділених із організму практично здорових пацієнтів, розши-

рює наші знання щодо біологічної характеристики виду.

Ключові слова: *Escherichia coli*, ліпополісахарид, моносахаридний та жирнокислотний склад, токсичність, пірогенність, серологічна активність.

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