UDC 579.841.11:577.114.083:578.865.1 https://doi.org/10.15407/biotech10.02.034

ANTIVIRAL ACTIVITY OF LIPOPOLYSACCHARIDES OF *Pseudomonas chlororaphis* subsp. *aureofaciens*

L. D. Varbanets[1]
S. L. Rybalko[2]
D. B. Starosyla[2]

[1]Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine, Kyiv
[2]Gromashevskyi Institute of Epidemiology and Infectious Diseases of the National Academy of Medical Sciences, Kyiv, Ukraine

E-mail: varbanets_imv@ukr.net

Received 03.01.2017

The aim of the study was to investigate the ability of lipopolysaccharides of two strains of *Pseudomonas chlororaphis* subsp. *aureofaciens* to inhibit *in vitro* the reproduction of human viruses: influenza A/FM/1/47 (H1N1), herpes simplex type 2 and bovine diarrhea, which is used as a model of hepatitis C virus, as well as to suppress hepatitis C virus production in model system of cells transfected with cDNA of this virus. It has been established that for both lipopolysaccharides in three types of cultures (*MDCK*, *Vero* and *MDBK*) the toxicity is not manifested even in a concentration of 100.0 μg/ml, and decreasing in infectious virus titer more than by 2.0 lg TCD_{50} (ED_{99}) was already achieved at concentrations of 1.55 mg / ml. Selectivity indexes determination of lipopolysaccharides preparations against the influenza A/FM/1/47 (H1N1) virus, herpes simplex virus type 2 and bovine diarrhea virus shows that lipopolysaccharides of *P. chlororaphis* subsp. *aureofaciens* UCM B-306 and UCM B-111 are effective inhibitors of investigated viruses reproduction: selectivity index is at least 64. In the model of *Jurkat* cells transfected with human hepatitis C virus cDNA, viral RNA loading was determined in cells treated with lipopolysaccharides of *P. chlororaphis* subsp. *aureofaciens*. The results of the studies indicate that when lipopolysaccharides of both strains are administered, the production of the hepatitis C virus is completely inhibited.

Key words: *Pseudomonas chlororaphis* subsp. *aureofaciens* lipopolysaccharides, influenza, herpes and hepatitis C viruses.

The problem of viral infections control is one of the more relevant in modern medicine. Against acquired immunodeficiency syndrome (AIDS), cytomegalovirus infection, hemorrhagic fevers (one of which is deadly Ebola fever), there are still no effective prevention tools. Influenza and other acute respiratory viral diseases occupy a leading place in the structure of human infectious diseases, quickly and widely distributed and cause significant morbidity and mortality worldwide. As pointed out by WHO experts, there is the tendency to constant growth of diseases [1].

Causative agents of these diseases often acquire partial or complete resistance to the treatment. Modern chemotherapy has a wide arsenal of antiviral agents — abnormal nucleosides, adamantane and thiosemicarbazone derivatives, synthetic amino acids, virucidal preparations, numerous interferons and interferonogens [2]. However, traditional methods of treatment using these antiviral preparations are often ineffective. The problem of lack of highly effective substance for prevention and treatment of viral infections is relevant not only due to their morbidity and possible serious complications, but due to provoking many others, including cancer. Therefore, the search for new alternative means of influence on viruses and viral infections is an urgent task of modern medicine and biotechnology.

Reports of bacterial lipopolysaccharides (LPS) or endotoxins ability to prevent the development of some experimental viral infections are present in the literature for long. It has been suggested that the antiviral effect of bacterial LPS can be caused by stimulation of endogenous interferon biosynthesis [3]. This assumption is confirmed by many experiments. So, it is shown [4] that

Escherichia coli and *Salmonella typhimurium* LPS increased β-interferon secretion by cell culture of human skin fibroblasts, which in turn ensues protection against vesicular stomatitis virus. Data on the protective effect of bacterial endotoxins concern only some experimental viral infections, and most studied LPS were derived from the strains of pathogenic or opportunistic Gram-negative bacteria, which are characterized by significant toxicity. Unlike the works cited [5], it was previously shown by us that LPS of saprophytic bacteria of the genus *Pseudomonas*, which are widely inhabiting soil, water, plant rhizosphere, including *P. chlororaphis* subsp. *aureofaciens*, and used for biological crop protection from pests and pathogens are highly active antiviral agents [6]. During research in 2010–2012, they consistently demonstrated efficiency against the tobacco mosaic virus (TMV) in the plant of *Solanaceae* family. At LPS concentrations of 1–10 mg/ml the inhibition of virus infectivity was 98–100%, 0.1 mg/ml — 57–69%, 0.01 mg/ml — 43–44%. At a concentration of 1 mg/ml the reduction in infectivity of the virus at various plants-inductors — datura and tobacco — was from 10.2 to 46%. The activity of both LPS was similar. It is interestingly, that LPS derived from microorganisms belonging to other families and species (*Rahnella aquatilis, Ralstonia solanacearum*), were not active against the TMV, and sometimes even stimulated the formation of necrosis.

Therefore, the purpose of this study was to investigate the ability of lipopolysaccharides of *Pseudomonas chlororaphis* subsp. *aureofaciens* two strains to inhibit *in vitro* the reproduction of human viruses (influenza and herpes), bovine diarrhea virus, which is used as a model of hepatitis C virus, and also to inhibit the production of hepatitis C virus in the model system of cells transfected with the cDNA of this virus.

Materials and Methods

The objects of research were lipopolysaccharides (LPS 1 and LPS 2) of two strains of *Pseudomonas chlororaphis* subsp. *aureofaciens*: UCM B-306 and UCM B-111 respectively, kindly provided to us for research by prof. Kiprianova O. A. Cultures cultivation and LPS preparation were performed as described previously [5].

LPS ability to inhibit the development of viral infection was tested on three types of cell cultures: *MDCK, Vero* and *MDVK*. There are subinoculated cultures of dog kidney cells, African green monkey and bull respectively.
As a test objects there were used: 1) influenza virus — the strain A/FM/1/47 (H1N1) — infectious titer of allantois culture — 8,0 lg TCD_{50}, hemagglutinin titer — 1: 512 HAunits; 2) herpes simplex virus type 2 (HSV-2) strain VN, infectious titer by CPE in cell culture was 7.0 lg TCD_{50}. Prior to experimental studies virus was stored at −70 °C; 3) bovine viral diarrhea virus (VBVD), which is a surrogate test model of hepatitis C virus (HCV) [7], virus infectious titer after ten passages in *MDBK* cell culture was 6–7 lg TCD_{50}.

To determine the cytotoxic concentrations of LPS preparations, at least ten holes in plastic plots with cell culture for each preparation dilution (from 1.55 to 100.0 μg/ml) in culture medium were used. Plates with cell culture were incubated at 37 °C with supply of 5% CO_2 for 5 days. Test and control cultures were revised every day to detect the presence or absence of cytopathic effect (CPE) on cells. CPE degree was determined by the change in the morphology of cells (cell rounding and shrinkage, degenerated cells abruption from holes surface) by 4-plus system from “1+” to “4+”. The maximum non-toxic concentration (MNTC) of LPS preparation that does not cause degeneration of cells was determined.

For anti-flu LPS preparations activity determination *in vitro*, daily inoculated *MDCK* cell culture with continuous monolayer was used. Cells were grown in plates on the medium RPMI-1640 with 10% of fetal serum (Nunclon, Surface, Denmark) at 37 °C in thermostat with CO_2 supply. To increase the sensitivity of cells to infection with influenza virus the treatment with trypsin was carried out. Trypsin mother solution (Trypsin, TPCK) was prepared by adding of 10 ml of nutrient medium DMEM (Sigma) to 20 mg of enzyme. Cells were washed three times with this solution in a final concentration of 2 μl/ml by 50 μl per well. Growth medium was poured out, investigated LPS preparations in different concentrations were added to the cells and flu virus at a dose of 100 TCD_{50} was introduced. Cultures were incubated in thermostat with CO_2 supply for 3 days, daily monitoring using a microscope. After 48–72 hour of incubation, the culture fluid was collected and the influenza virus infectious titer was determined in cell culture by titration.

To the study of LPS preparations anti-herpetic activity, the herpes simplex virus type 2 (HSV-2) strain VN, infectious titer of

7.0 lg TCD_{50}, was used. CPE of HSV in cells morphologically manifested in symplasts or rounded cells formation in combination with the proliferation and giant multi-cells appearance. The study was performed on daily cell cultures *Vero* with their continuous monolayer. Cells were grown in plates on the medium RPMI-1640 with 10% of fetal serum (Nunclon, Surface, Denmark) at 37 °C in thermostat with CO_2 supply. Growth medium was poured out; investigated LPS preparation in different concentrations was added on the monolayer. After 1 hour of contact, herpes virus was added at a dose of 100 TCD_{50}. Cultures were incubated in thermostat with CO_2 supply for 5 days, daily monitoring with a microscope and noting the virus reproduction by HSV cytopathic action on *Vero* cells compared with control cultures where the monolayer is not subjected to any influence.

As a surrogate hepatitis C virus (HCV) the bovine viral diarrhea virus (VBVD) was used. Antiviral activity was studied in culture MDBK, in which various concentrations of LPS and VDVB at a dose of 100 TCD_{50} were added. Cultures were incubated in thermostat to specific CPE in control of the virus, and then the infectious titer in the culture medium was determined.

In all experiments, the virus infectious titer reduction under the action of preparation at 1.0 lg TCD_{50} (ED_{90}) or 2.0 lg TCD_{50} (ED_{99}) compared with the virus control was considered the indicator of inhibitory action.

Transfection was performed using a standard protocol with transfection reagent Turbofect (Thermo Scientific). The density of cells on the day of transformation was 5×10^4 (for the inoculated cells) and 5×10^5 (for suspension cells) in 1 ml of nutrient media. To obtain HCV-producing cell cultures, HCV RNA was isolated from patients with hepatitis C. Further cDNA was obtained on the matrix of HCV RNA via reverse transcription. 1 µg of cDNA was diluted in 100 µl of serum-free medium RPMI-1640, shaking the solution, and 2 ml of transfection reagent (Turbofect) were added. After pipetting or shaking in Vortex, specimens were incubated for 15–20 min at room temperature. Then 100 ml of transfection reagent containing cDNA were added dropwise to each well with cell culture. Plates were incubated at 37 °C in thermostat with CO_2. Cultures of transfected HCV cDNA were incubated at 36.6 °C in thermostat with 5% of CO_2 supply. Virus testing was performed by PCR at the second passage. All transfected cultures produced hepatitis C virus as on the 7^{th} and on the 9^{th} day of culture *Jurkat*-cDNA cultivation.

All experiments were performed at least in 3–5 replicates. Statistical analysis of experimental series results was performed by standard methods using Student t-test for the 5% significance level.

Results and Discussion

Lipopolysaccharides of investigated strains of *Pseudomonas chlororaphis* subsp. *aureofaciens* UCM B-306 and UCM B-111, which are components of gaupsin biopreparation, were isolated and described earlier [8]. Based on fatty acids and monosaccharides composition, including the presence of heptose, 2-keto-3-desoxyoctanoic acid and 3-hydroxy fatty acid, it is demonstrated that LPS of *P. chlororaphis* for the first time isolated by us contain all characteristic for these biopolymers components.

The influence of *P. chlororaphis* subsp. *aureofaciens* UCM B-306 and UCM B-111 LPS was studied on three types of viruses: influenza, herpes type 2 and bovine diarrhea viruses, which was used as hepatitis C virus model. They belong to different families: *Orthomyhoviridae, Herpesviridae* and *Flaviviridae,* respectively. These viruses were selected for studies concerning LPS influence on them, as they cause a series of life-threatening human diseases: influenza H1N1, which, since 1947, occasionally causes a flu epidemics; herpes simplex type 2, which infects the body of almost 90% of our planet population and capable of long-term persistence in the body, causing chronic and latent forms of infection with periodic exacerbations. Some herpes viruses are able to spread transplacentally and cause intrauterine and neonatal pathology; and hepatitis C virus, which causes dangerous liver disease capable in some cases to be complicated by cirrhosis and carcinoma. Therefore, finding of preparations able to retard the development of diseases caused by viruses described above represents the theoretical and practical interest.

To determine the cytotoxicity of investigated preparations and their effects on viruses reproduction, LPS were tested at concentrations ranging from 1.55 to 100.0 µg/ml.

The results of experiments to determine the MNTC show that both LPS on three types of cultures: *MDCK, Vero* and *MDVK* were non-toxic and did not cause degeneration of cells even at a concentration of 100.0 µg/ml.

LPS minimal concentration that can inhibit the reproduction of the virus by 1.0–2.0 lg TCD_{50}, was equal to influenza, herpes and bovine diarrhea viruses (Fig. 1–3). The reduction of viruses' infectious titer at least by 2.0 lg TCD_{50} influenced by LPS was observed even at a concentration of 1.55 μg/ml for both LPS. In fact, LPS preparations inhibited influenza virus reproduction by 5.0–6.0 lg TCD_{50}, VBVD (surrogate HCV) — by 4.0–5.0 lg TCD_{50}, herpes virus type 2 — by 3 lg TCD_{50}.

According to the $MNTC/ED_{99}$ ratio, indices of selectivity of *P. chlororaphis* subsp. *aureofaciens* UCM B-306 and UCM B-111 LPS preparations against the influenza virus A/FM/1/47 (H1N1), herpes simplex type 2 and bovine diarrhea accounted for at least 64. Therefore, investigated LPS are effective inhibitors of investigated viruses reproduction.

The purpose of the following studies was to perform anti-virus LPS screening on the model of cell culture *Jurkat* producing human hepatitis C virus. As a result of suspension cultures *Jurkat* transfection with cDNA of HCV from a patient using *Turbofect* we received producing cell cultures transfected with cDNA of HCV that provide stable production of HCV with viral loading of $2.9 \cdot 10^3$, $1.6 \cdot 10^5$, $1.1 \cdot 10^5$, $7.6 \cdot 10^4$, $1.0 \cdot 10^5$ genome/equivalents.

In the model of transfected human hepatitis C virus with viral loading of $2.9 \cdot 10^3$ genome/equivalents the effect of LPS was determined. Into producing culture *Jurkat* HCV the LPS preparations in dilutions of 1:1000 were added. After 5 days in each sample the HCV RNA loading was determined. The study shows that the introduction of LPS 1 and 2 fully inhibits the HCV production, i.e. in producing cultures *Jurkat* HCV the viral loading was not found, while in the control cultures *Jurkat* HCV the viral loading was $3.83 \cdot 10^2$ genome/equivalents.

Thus, it is revealed that LPS of *P. chlororaphis* subsp. *aureofaciens* UCM B-306 (LPS 1) and UCM B-111 (LPS 2) are highly active antiviral agents. They inhibit the reproduction of influenza viruses A/FM/1/47 (H1N1), herpes simplex type 2, and bovine viral diarrhea that is surrogate hepatitis C virus. Although the mechanism of LPS antiviral activity until today is not deciphered, we can assume that there are two such mechanisms. Since the majority of LPS biological activity types is due to lipid A, the most conservative part of the molecule, as evidenced by almost the same fatty acid composition [8], we can assume that lipid A is responsible for the antiviral effect that in *P. chlororaphis* subsp. *aureofaciens* UCM B-306 and UCM B-111 was similar. We cannot exclude the impact of the O-specific polysaccharide (OPS), the most variable part of LPS molecule. Although earlier [9] we found that there are significant differences in the OPS structure of two studied strains of *P. chlororaphis* subsp. *aureofaciens*: one OPS represented by linear trisaccharide while another by branched tetrasaccharide, they have one common characteristic: contain the residues of 2-amino-2-deoxy-galacturonic acid (GalNAcA). This fact is very important because it is known that receptors of some viruses are galactose-specific.

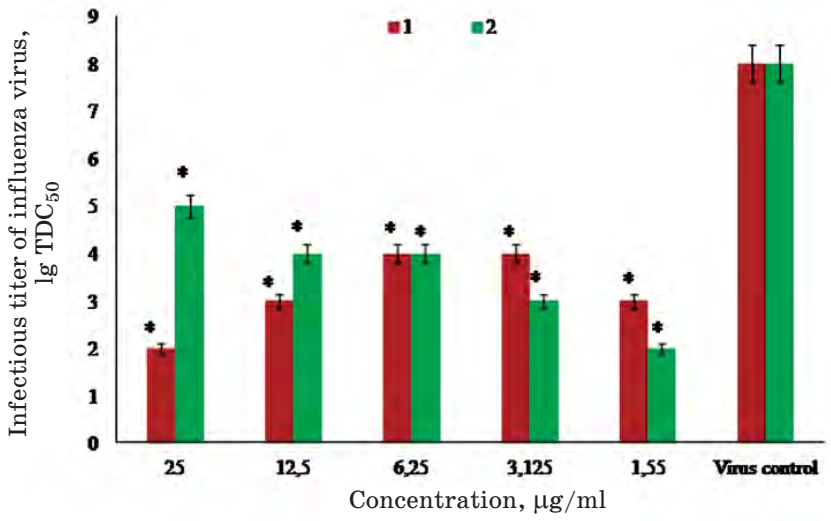

Fig. 1. **ED_{99} of LPS preparations (1 and 2) of *P. chlororaphis* subsp. *aureofaciens* against the influenza virus, $P < 0.05$ versus control**

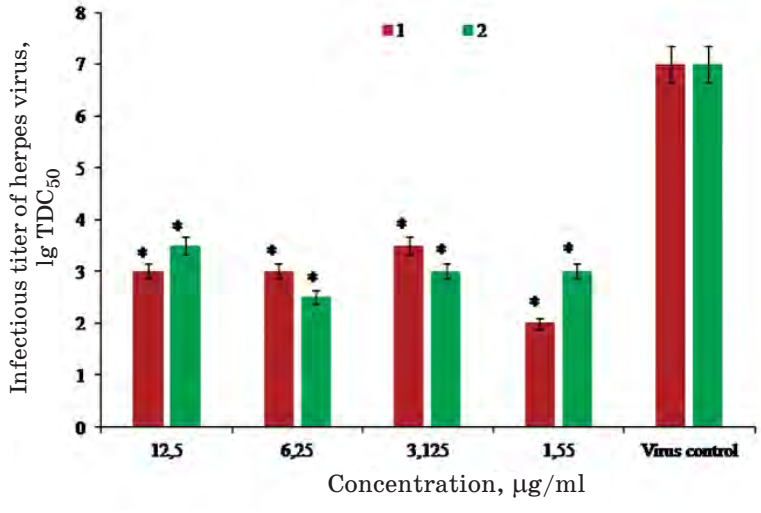

Fig. 2. **ED_{99} of LPS preparations (1 and 2) of** *P. chlororaphis* **subsp.** *aureofaciens* **against the herpes virus, $P < 0.05$ versus control**

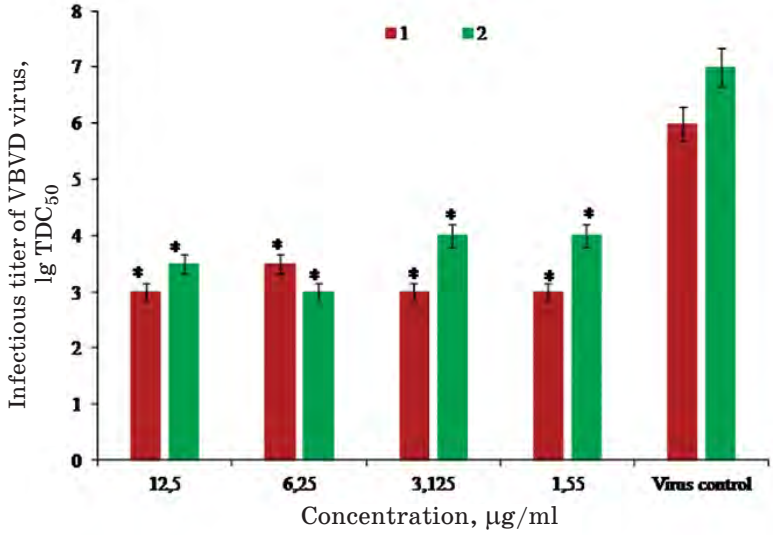

Fig. 3. **ED_{99} of LPS preparations (1 and 2) of** *P. chlororaphis* **subsp.** *aureofaciens* **against the VBVD virus, $P < 0.05$ versus control**

REFERENCES

1. *Babushkina A.V.* Upper respiratory diseases and bronchoobstructive syndrome. *Ukr. Medychny chasopys.* 2011, (81) (1), 1–11. (In Russian).
2. *D'yachkova S.YA., Nikolayevskiy V.A.* Antiviral means. *Voronezh*, 2008, 149 p. (In Russian).
3. *Solov'ev V.D., Bektemirov T.A.* Interferon. Theory and Applications. *N. Y.: Plenum Press.* 1973, 304 p.
4. *Helfgott D.C.* Bacterial lipopolysaccharides (exotoxin) enhances expression and secretion of B2 interferon by human fibroblastes. *J. Exp. Med.* 1987, 166 (5), 1300–1309.
5. *Kiprianova E.A., Varbanets L. D., Shepelevich V. V., Voichuk S. I.* Antiviral activity of lipopolysaccharides of *Pseudomonas chlororaphis* subsp. *aureofaciens*. *Biotechnologia Acta*. 2013, 6 (2), 68–73. (In Russian).
6. *Kiprianova E. A., Varbanets L. D., Rybalko S. S., Shepelevich V. V.* The strain *Pseudomonas chlororaphis* subsp. *aureofaciens* — producer of lipopolysaccharide with antiviral activity. *Patent Ukrainy № 76689* C12N 1/100 MPK 10.01.2013. (In Ukranian).
7. *Buckwold V. E., Beer B. E., Donis R. O.* Bovine *viraldiarrhea* virus as a surrogate model of hepatitis C virus for the evaluation of antiviral agents. *Antivir. Res.* 2003, V. 60, P. 1–15. doi. org/10.1016/SO166-3542(03)00174-8.
8. *Varbanets L. D., Zdorovenko E. L., Kiprianova E. A., Avdeeva L. V., Brovarskaya O. S., Rybalko S. L.* Characterization of the

lipopolysaccharide of *Pseudomonas chlororaphis*. *Microbiology (Moscow)*. 2015, 84 (6), 781–790.
9. *Zdorovenko E, Kadykova A., Varbanets L., Shashkov A. S., Kiprianova E., Brovarskaya O., Knirel Yu.* Structure of the O-specific polysaccharides of *Pseudomonas chlororaphis* subsp. *chlororaphis* UCM B-106. *Carboh. Res.* 2016, 433, 1–4.

АНТИВІРУСНА АКТИВНІСТЬ ЛІПОПОЛІСАХАРИДІВ *Pseudomonas chlororaphis* subsp. *aureofaciens*

Л. Д. Варбанець[1]
С. Л. Рибалко[2]
Д. Б. Старосила[2]

[1]Інститут мікробіології і вірусології ім. Д.К. Заболотного НАН України, Київ
[2]Інститут епідеміології та інфекційних хвороб ім. Л.В. Громашевського НАМН України, Київ

E-mail: varbanets_imv@ukr.net

Метою роботи було дослідити здатність ліпополісахаридів двох штамів *Pseudomonas chlororaphis* subsp. *aureofaciens* пригнічувати *in vitro* репродукцію вірусів людини — грипу A/FM/1/47(H1N1) та простого герпесу 2 типу, бичачої діареї, що використовують як модель вірусу гепатиту С, а також інгібувати продукцію вірусу гепатиту С в модельній системі клітин, трансфікованих кДНК цього вірусу. Встановлено, що для обох ліпополісахаридів на трьох типах культур: *MDCK*, *Vero* та *MDBK* токсичність не виявлялась навіть в концентрації 100,0 мкг/мл, а зниження інфекційного титру вірусу більш ніж на 2,0 lg $ТЦД_{50}$ (ED_{99}) досягалось вже за концентрації 1,55 мкг/мл. Визначення індексів селективності препаратів ліпополісахаридів стосовно вірусу грипу A/FM/1/47(H1N1), простого герпесу 2 типу та бичачої діареї шляхом встановлення співвідношення максимальної нетоксичної концентрації до ED_{99} свідчить, що ліпополісахариди *P. chlororaphis* subsp. *aureofaciens* УКМ В-306 та УКМ В-111 є ефективними інгібіторами репродукції досліджуваних вірусів: індекс селективності становить щонайменш 64. На моделі клітин *Jurkat*, трансфікованих кДНК вірусу гепатиту С людини, визначали навантаження РНК цього вірусу в клітинах, оброблених ЛПС *P. chlororaphis* subsp. *aureofaciens*. Результати досліджень свідчать, що у разі введення ліпополісахаридів обох штамів репродукція вірусу гепатиту С повністю інгібується.

Ключові слова: ліпополісахариди *Pseudomonas chlororaphis* subsp. *aureofaciens*, віруси грипу, герпесу та гепатиту С.

АНТИВИРУСНАЯ АКТИВНОСТЬ ЛИПОПОЛИСАХАРИДОВ *Pseudomonas chlororaphis* subsp. *aureofaciens*

Л. Д. Варбанец[1]
С. Л. Рыбалко[2]
Д. Б. Старосила[2]

[1]Институт микробиологии и вирусологии им. Д.К. Заболотного НАН Украины, Киев
[2]Институт эпидемиологии и инфекционных заболеваний им. Л.В. Громашевского НАМН Украины, Киев

E-mail: varbanets_imv@ukr.net

Целью работы было исследовать способность липополисахаридов двух штаммов *Pseudomonas chlororaphis* subsp. *aureofaciens* ингибировать *in vitro* репродукцию вирусов человека: гриппа A/FM/1/47(H1N1), простого герпеса 2 типа и бычьей диареи, используемых как модель вируса гепатита С, а также ингибировать продукцию вируса гепатита С в модельной системе клеток, трансфицированных кДНК этого вируса. Установлено, что для обоих липополисахаридов на трех типах культур: *MDCK*, *Vero* и *MDBK* токсичность не проявлялась даже в концентрации 100,0 мкг/мл, а снижение инфекционного титра вируса более чем на 2,0 lg $ТЦД_{50}$ (ED_{99}) достигалось уже при концентрации 1,55 мкг/мл. Определение индексов селективности препаратов липополисахаридов по отношению к вирусу гриппа A/FM/1/47(H1N1), простого герпеса 2 типа и бычьей диареи путем установления соотношения максимальной нетоксической концентрации к ED_{99} свидетельствует, что липополисахариды *P. chlororaphis* subsp. *aureofaciens* УКМ В-306 и УКМ В-111 являются эффективными ингибиторами репродукции исследуемых вирусов: индекс селективности составляет не менее 64. На модели клеток *Jurkat*, трансфицированных кДНК вируса гепатита С человека, определяли нагрузку РНК этого вируса в клетках, обработанных ЛПС *P. chlororaphis* subsp. *aureofaciens*. Результаты исследований свидетельствуют о том, что при введении липополисахаридов обоих штаммов репродукция вируса гепатита С полностью ингибируется.

Ключевые слова: липополисахариды *Pseudomonas chlororaphis* subsp. *aureofaciens*, вирусы гриппа, герпеса и гепатита С.