DOI: https://doi.org/10.36233/0507-4088-30

© AUTHORS, 2021

Real-time PCR assay development for the control of vaccine against hemorrhagic fever with renal syndrome

Maria S. Egorova[1], Svetlana S. Kurashova[1], Aydar A. Ishmukhametov[1,2], Maria V. Balovneva[1], Andrei A. Deviatkin[3], Maria V. Safonova[4], Sergei V. Ozherelkov[1], Yusuf Kh. Khapchaev[1], Alexandra S. Balkina[1], Alla V. Belyakova[1], Tamara K. Dzagurova[1], Evgeniy A. Tkachenko[1]

[1]FSBSI «M.I. Chumakov Federal Scientific Center for Research and Development of Immune-and-Biological Products of Russian Academy of Sciences», 108819, Moscow, Russia;
[2]Institute for Translational Medicine and Biotechnology, FSAEI HE I.M. Sechenov First Moscow State Medical University of the Ministry of the Health of Russia, 119991, Moscow, Russia;
[3]Institute of Molecular Medicine, FSAEI HE I.M. Sechenov First Moscow State Medical University of the Ministry of the Health of Russia, 119991, Moscow, Russia;
[4]FSHI «Plague Control Center» of the Federal Service for Surveillance of Consumer Rights Protection and Human Wellbeing (Rospotrebnadzor), 119121, Moscow, Russia

Introduction. Hemorrhagic fever with renal syndrome (HFRS) holds a leading place among natural focal human diseases in Russian Federation. There is no etiotropic therapy for the disease now. The vaccine prophylaxis is the most effective method to control this infection. The main criteria for inactivated vaccines evaluation are its immunogenicity and specific activity.
The study **purposes** were to develop a sensitive and specific real-time PCR method for viral RNA quantification in the inactivated vaccine and to study the correlation between the viral RNA amount and vaccine immunogenicity.
Material and methods. L-segment fragments of the Puumala, Hantaan, and Sochi vaccine strains were selected as diagnostic targets for oligonucleotides and fluorescent probes. The immunogenicity of experimental vaccines was determined by the induction of neutralizing antibodies in BALB/c mice.
Results. A highly specific, sensitive and reproducible real-time PCR method has been developed. The analytical sensitivity was 1.24 ± 1.5 × 10^2 copies/ml for Puumala virus; 1.16 ± 1.4 × 10^2 copies/ml for Hantaan; 1.32 ± 1.8 × 10^2 copies/ml for Sochi, with a virus content of 1.5 ± 0.5 lg FFU/ml; 1.8 ± 0.5 lg FFU/ml and 2.2 ± 0.5 lg FFU/ml, respectively. The viral RNA amount in experimental vaccine preparations inactivated with β-propiolactone was proportional to the neutralizing antibodies titer observed in mice following the immunization.
Discussion. It was found that different virus inactivators differently affects the detected viral RNA amount, but not the vaccine immunogenicity, which indicates the same degree of the immunogenic proteins damage. The direct relationship between the viral RNA copy number and vaccine immunogenicity makes it possible to use this criterion for vaccine dosage preparation.
Conclusion. The developed method for viral RNA quantification is a promising tool for the specific activity control of the HFRS vaccine.

Key words: *hemorrhagic fever with renal syndrome (HFRS); inactivated vaccine; real-time quantitative polymerase chain reaction (qRT-PCR); formalin; β-propiolactone (β-PL); ultraviolet*

For citation: Egorova M.S., Kurashova S.S., Ishmukhametov A.A., Balovneva M.V., Deviatkin A.A., Safonova M.V., Ozherelkov S.V., Khapchaev Yu.Kh., Balkina A.S., Belyakova A.V., Dzagurova T.K., Tkachenko E.A. Real-time PCR assay development for the control of vaccine against hemorrhagic fever with renal syndrome. *Problems of Virology* (*Voprosy Virusologii*). 2021; 66(1): 65-73 (In Russ.). DOI: https://doi.org/10.36233/0507-4088-30

For correspondence: Maria S. Egorova, Ph.D. (Biol.), Senior Researcher of the Laboratory of Hemorrhagic Fevers, FSBSI «M.I. Chumakov Federal Scientific Center for Research and Development of Immune-and-Biological Products of Russian Academy of Sciences», 108819, Moscow, Russia. E-mail: centrglps@yandex.ru

Information about the authors:
Egorova M.S., https://orcid.org/0000-0002-6416-2573
Kurashova S.S., https://orcid.org/0000-0001-9934-699X
Ishmukhametov A.A., https://orcid.org/0000-0001-6130-4145
Balovneva M.V., https://orcid.org/0000-0003-2198-7521
Deviatkin A.A., https://orcid.org/0000-0003-0789-4601
Safonova M.V., https://orcid.org/0000-0002-5579-5694
Ozherelkov S.V., https://orcid.org/0000-0001-9778-0485
Khapchaev Yu.Kh., https://orcid.org/0000-0003-1613-5228
Balkina A.S., https://orcid.org/0000-0002-9704-7774
Belyakova A.V., https://orcid.org/0000-0003-4363-6399
Dzagurova T.K., https://orcid.org/0000-0002-6656-1682
Tkachenko E.A., https://orcid.org/0000-0002-6829-1241

Contribution: Egorova M.S. – concept and design of the study, data analysis, experiments with animals, statistical analysis, writing of the text; Kurashova S.S. – concept and design of the study, data analysis, experiments with animals, statistical analysis, writing of the text; Ishmukhametov A.A. – concept and approval of the final version of the article; Balovneva M.V. – purified viral material obtaining, analysis of the immune status; Deviatkin A.A. – full genome sequencing of the vaccine

strains, selection of the primers and probes; Safonova M.V. – selection of the primers and probes; Ozherelkov S.V. – purified viral material obtaining, analysis of the immune status; Khapchaev Yu.Kh. – purified viral material obtaining, analysis of the immune status; Balkina A.S. – technical support and editing of the text; Belyakova A.V. – technical support and editing of the text; Dzagurova T.K. – concept and approval of the final version of the article; Tkachenko E.A. – concept and approval of the final version of the article.

Acknowledgment. The study was performed under the topic of Government Order No. 0837-2019-0006.
Conflict of interest. The authors declare no conflict of interest.
Received 12 November 2020
Accepted 18 February 2020

Разработка метода количественного определения вирусной РНК для контроля специфической активности вакцины против геморрагической лихорадки с почечным синдромом

Егорова М.С.[1], Курашова С.С.[1], Ишмухаметов А.А.[1,2], Баловнева М.В.[1], Девяткин А.А.[3], Сафонова М.В.[4], Ожерелков С.В.[1], Хапчаев Ю.Х.[1], Балкина А.С.[1], Белякова А.В.[1], Дзагурова Т.К.[1], Ткаченко Е.А.[1]

[1]ФГБНУ «Федеральный научный центр исследований и разработки иммунобиологических препаратов им. М.П. Чумакова РАН», 108819, Москва, Россия;
[2]Институт трансляционной медицины и биотехнологии, ФГАОУ ВО Первый Московский государственный университет им. И.М. Сеченова Минздрава России, 119991, Москва, Россия;
[3]Институт молекулярной медицины, ФГАОУ ВО Первый Московский государственный университет им. И.М. Сеченова Минздрава России, 119991, Москва, Россия;
[4]ФКУЗ «Противочумный центр» Федеральной службы по надзору в сфере защиты прав потребителей и благополучия человека (Роспотребнадзор), 119121, Москва, Россия

Введение. Геморрагическая лихорадка с почечным синдромом (ГЛПС) занимает ведущее место среди природно-очаговых болезней человека в Российской Федерации. Этиотропная терапия заболевания в настоящее время отсутствует. Наиболее действенным методом контроля этой инфекции может стать вакцинопрофилактика. Основными критериями оценки инактивированной вакцины являются её иммуногенность и специфическая активность.
Цель исследования – разработка чувствительной и специфичной методики проведения ПЦР в реальном времени с целью количественного определения вирусной РНК в инактивированной вакцине, а также установление корреляции между содержанием генетического материала вируса и выраженностью иммуногенности.
Материал и методы. В качестве диагностической мишени для олигонуклеотидов и флуоресцентных зондов выбраны фрагменты L-сегмента вакцинных штаммов вирусов ГЛПС Пуумала, Хантаан, Сочи. Иммуногенность экспериментальных вакцин контролировали по индукции нейтрализующих антител (нАТ) в ответ на иммунизацию мышей линии BALB/c.
Результаты. Разработана высокоспецифичная, чувствительная методика проведения ПЦР в реальном времени, имеющая высокий показатель воспроизводимости. Аналитическая чувствительность составила для вирусов Пуумала $1,24 \pm 1,5 \times 10^2$ копий/мл, Хантаан – $1,16 \pm 1,4 \times 10^2$ копий/мл, Сочи – $1,32 \pm 1,8 \times 10^2$ копий/мл при содержании вируса $1,5 \pm 0,5$, $1,8 \pm 0,5$ и $2,2 \pm 0,5$ lg ФОЕ/мл соответственно. Показано, что количество вирусной РНК в составе экспериментальных вакцинных препаратов, инактивированных β-пропиолактоном (β-ПЛ), пропорционально титру нАТ в ответ на иммунизацию.
Обсуждение. Установлено, что способ инактивирования вируса влияет на выявляемое количество копий РНК, не оказывая при этом воздействия на иммуногенность, что указывает на равнозначное повреждение белков-иммуногенов при разной степени повреждения вирусной РНК. Прямая зависимость между содержанием копий нуклеиновой кислоты вируса и иммуногенностью позволяет использовать этот критерий для дозирования вакцинного препарата.
Заключение. Разработанная система оценки количественного содержания вирусной РНК делает перспективным её применение для контроля специфической активности вакцины против ГЛПС.

Ключевые слова: *геморрагическая лихорадка с почечным синдромом; инактивированная вакцина; полимеразная цепная реакция в реальном времени (ПЦР-РВ); формалин; β-пропиолактон (β-ПЛ); ультрафиолет*

Для цитирования: Егорова М.С., Курашова С.С., Ишмухаметов А.А., Баловнева М.В., Девяткин А.А., Сафонова М.В., Ожерелков С.В., Хапчаев Ю.Х., Балкина А.С., Белякова А.В., Дзагурова Т.К., Ткаченко Е.А. Разработка метода количественного определения вирусной РНК для контроля специфической активности вакцины против геморрагической лихорадки с почечным синдромом. *Вопросы вирусологии.* 2021; 66(1): 65-73. DOI: https://doi.org/10.36233/0507-4088-30

Для корреспонденции: Егорова Мария Сергеевна, канд. биол. наук, старший научный сотрудник лаборатории геморрагических лихорадок, ФГБНУ «Федеральный научный центр исследований и разработки иммунобиологических препаратов им. М.П. Чумакова РАН», 108819, Москва, Россия. E-mail: centrglps@yandex.ru

Участие авторов: Егорова М.С. – разработка концепции и дизайна исследования, анализ данных, эксперименты с животными, статистический анализ, написание текста; Курашова С.С. – разработка концепции и дизайна исследования, анализ данных, эксперименты с животными, статистический анализ, написание текста; Ишмухаметов А.А. – разработка концепции и утверждение окончательного варианта статьи; Баловнева М.В. – получение очищенно-

го вирусного материала, анализ иммунного статуса; Девяткин А.А. – полногеномное секвенирование штаммов, подбор праймеров и зондов; Сафонова М.В. – подбор праймеров и зондов; Ожерелков С.В. – получение очищенного вирусного материала, анализ иммунного статуса; Хапчаев Ю.Х. – получение очищенного вирусного материала, анализ иммунного статуса; Балкина А.С. – техническая поддержка и редактирование текста; Белякова А.В. – техническая поддержка и редактирование текста; Дзагурова Т.К. – разработка концепции и дизайна исследования, анализ данных, написание текста; Ткаченко Е.А. – разработка концепции и утверждение окончательного варианта статьи.

Финансирование. Исследование выполнено по теме Госзадания № 0837-2019-0006.

Конфликт интересов. Авторы заявляют об отсутствии конфликта интересов.

Поступила 12.11.2020
Принята в печать 18.02.2021

Introduction

Hemorrhagic fever with renal syndrome (HFRS) is caused by viruses belonging to the genus *Orthohantavirus* (the family *Hantaviridae*, order *Bunyavirales*) and representing a group of enveloped negative-sense RNA viruses. They are responsible for such human diseases as hemorrhagic fever with renal syndrome (HFRS), mainly in Europe and Asia, and hantavirus pulmonary syndrome (HPS) in North and South America [1–3].

The hantavirus genome is composed of three single-stranded negative-sense RNA segments: the large (L) segment encodes an RNA-dependent RNA polymerase; the medium (M) segment encodes 2 surface glycoproteins Gn/Gc, while the small (S) segment encodes a viral nucleocapsid protein (N) [4–6].

HFRS takes a leading place among natural focal diseases in the Russian Federation. Around 98% of the cases are associated with the Puumala virus (PUUV), while the others are caused by Hantaan (HTNV), Sochi (SOCHIV), Amur, Seoul and Kurkino viruses [7].

Currently, there is no etiotropic treatment for hantavirus infections to effectively eliminate the virus; non-specific preventive measures are frequently inefficient [8]. Therefore, preventive vaccination remains at the top of the agenda. At present, commercial, inactivated, whole-virion vaccines against HFRS are being manufactured in the People's Republic of China (PRC) and the Republic of Korea and are based on Hantaan and Seoul viruses [9]; however, they fail to provide cross-protection against Puumala virus, which is responsible for most of the infection cases in Europe and Russia. Considering that PUUV, HTNV and SOCHIV (the genotype of the Dobrava-Belgrade orthohantavirus) co-circulate in the Russian Federation [7], a multivalent inactivated, whole-virion vaccine candidate can be the best solution.

The specific activity and immunogenicity are the major criteria for assessment of an inactivated vaccine. In the manufacturing process, after the pathogen is inactivated, the specific activity is usually assessed either by the quantitative content of the target immunogenic protein or by the number of RNA copies in a unit of volume [10].

The study was aimed at development of a sensitive and specific method of quantification of RNA vaccine strains in inactivated vaccines against HFRS, as well as to find and analyze the correlation between the content of the viral genetic material and vaccine immunogenicity.

Materials and methods

Description of the vaccine materials. Cell culture and viruses. To obtain the virus-containing medium we used Vero continuous line of African green monkey (*Cercopithecus aethiops*) renal cells, which was received from the cell bank of the World Health Organization (WHO Vero cell bank ECACC, Accession number 991042) and which was certified and recommended by WHO and the Scientific Center for Expert Evaluation of Medicinal Products of the Ministry of Health of Russia (L.A. Tarasevich State Research Institute for Standardization and Control of Medical Biological Preparations (SRISCMBP)) as a permitted medium for manufacturing inactivated whole-virion vaccines.

The assessment of the viral RNA by a real-time polymerase chain reaction (real-time PCR, qRT-PCR) was performed on experimental monovalent vaccines (hereinafter vaccines) based on vaccine strains of Puumala (PUU-TKD/VERO) – VAC-PUU, Hantaan (HTN-P88/VERO) – VAC-HTN, and Sochi (DOB-SOCHI/VERO) – VAC-SOCHI, following the previously described procedure [11] and using different methods of inactivation: Formalin (F) at a final dilution of 1 : 4000 for 35 days; β-propiolactone (β-PL) at a final dilution of 1 : 6000 for 180 min; ultraviolet (UV) light at the layer thickness of 0.3 cm and at a distance of 24 cm from the shortwave UV light-emitting source at a wavelength of 253.7 nm for 3 min [12].

The whole-genome sequences of the above strains were registered in the GenBank database and assigned the following numbers: PUU-TKD/VERO: S – MH251331, M – MH251332, L – MH251333; HTN-P88/VERO: S – MH251328, M – MH251329, L – MH251330 and DOB-SOCHI/VERO: S – MH251334, M – MH251335, L – MH251336.

The quantitative content of the viral genetic material was estimated at the following stages: the 1st stage – the purified concentrate of monovalent candidates prior to the inactivation; the 2nd stage – the similar concentrate after the inactivation, and the 3rd stage – the semi-finished trivalent vaccine.

Real-time PCR assessment of the viral RNA content. Fragments of L-segments of vaccine strains of PUU, HTN and SOCHI viruses were chosen as a diagnostic target for oligonucleotides and fluorescent probes. The analysis of whole-genome sequences of vaccine strains as well as PUU, HTN and SOCHI viral genomes available at the NCBI GenBank (https://www.ncbi.nlm.nih.gov) was per-

formed to select primers and probes by using the MEGA-X (Molecular Evolutionary Genetics Analysis) program in compliance with the standard requirements. Parameters for primers were calculated with the online OligoCalc tool: Oligonucleotide Properties Calculator (http://biotools.nubic.northwestern.edu/OligoCalc.html); thermodynamic properties of fluorescent probes and their secondary structures were assessed by using The mfold Web Server (http://unafold.rna.albany.edu/?q=mfold/DNA-Folding-Form). Oligonucleotides were carboxyfluorescein labeled (FAM) at the 5' terminus, while the florescence quencher (BHQ2) was labelled at the 3' terminus.

The synthesis of primers and probes was performed at Evrogen CJSC (Russia). The designed probes and primers are shown in **Table 1**.

RNA isolation and real-time PCR. RNA was isolated from the chromatographically purified virus concentrate prior to the inactivation (control) and after the inactivation (F, β-PL, UV) by using phenol-chloroform extraction [13] and the commercial TRI Reagent® (Sigma-Aldrich, USA). The complementary DNA (cDNA) was synthesized via reverse transcription in 20 µl reaction mix containing 5 × Reverse Transcription (RT) Buffer (Thermo Fisher Scientific, USA), 2.5 mM mix of 4 unmodified highly-purified 2'-deoxynucleoside-5'-triphosphates – dNTPs (SibEnzym, Russia), 20 µM of N6 Random primer (SibEnzym), 1 U of reverse transcriptase Maxima Reverse Transcriptase 200 U/µl (Thermo Fisher Scientific) and 10 µl of RNA solution. The conditions for the reaction were the following: 10 min at 25 °C, 30 min at 42 °C and 6 min at 96 °C. The reaction mix volume for the qRT-PCR was 25 µl and included 10 × Hot Start Buffer (Thermo Fisher Scientific), 2.5 mM dNTPs, 5 µM of each primer and probe (Evrogen, Russia) (**Table 1**), 2 U of HS Taq DNA polymerase (Thermo Fisher Scientific), 2 µl cDNA and distilled water. The conditions met the following requirements: 2 min at 95 °C, then followed by 40 15-second cycles at 95 °C, 40 sec at 55 °C and 30 sec at 72 °C. The real-time amplification and detection were performed with the AriaMx 96 Bioanalyzer (Agilent Technologies, USA).

Standard curve. The qRT-PCR was used for quantification of the target RNA in the sample. The method involves building a standard curve, which shows the linear relationship between the threshold cycle value (C_q) and the initial concentration of RNA (copies/ml) (**Fig. 1**). These curves were obtained through the amplification of the 10-fold serial dilution series of the template having mean C_q values, with consideration for negative and positive control results. The same approach was used for serial dilution of RNA from the input material to identify the correlation between C_q and the virus concentration expressed as focus-forming units (FFU) per 1 ml. The templates were prepared from limiting 10-fold dilutions of the genetic material containing PUU, HTN and SOCHI vaccine strains at the specified concentration of the virus (FFU/ml). The study also included measurement of viral nucleic acid content in 1 ml of the diluted preparation and the obtained results were matched against the FFU/ml values.

The template concentration (copies/ml) was measured by the QX200 Droplet-Digital PCR (ddPCR) System (Bio-Rad Laboratory, USA); the obtained results were further adjusted to the number of copies/ml received by using the real-time PCR. Then, the inactivated samples were analyzed with the help of standard curves to quantify the target viral RNA.

Specificity of the system. The specificity of the selected probes and primers was evaluated through testing the RNA isolated from the monovaccine stock solution with other human pathogenic hantaviruses: Puumala (CG-Kazan-79, Halnaas 83-L20 and Sotkamo strains); Hantaan (strains Lee 83-61 and 76-118); Dobrava (Belgrade-1); Seoul (SR-11 and A9); Sin Nombre (strain CC/107) as well as with representatives of other families: viruses of Crimean-Congo hemorrhagic fever (strain IbAr 10200); dengue (strains 8356/10, 4397/11, 3140/09 and 3274/09); Japanese encephalitis (Nakayama); tick-borne encephalitis (Hochosterwitz); West Nile (MgAn 786/6/1995); Zika (MR766); Usutu (G39); yellow fever (Asibi); chikungunya (23161) and Lassa (Josiah strain).

Assessment of the specific activity by the enzyme-linked immunosorbent assay (ELISA). HANTAGNOST test systems (M.P. Chumakov Federal Scientific Center for Research and Development of Immunobiological

Table 1. Primers and probes

Strain	Name of the primer/probe	5'→3' sequence
PUU-TKD/VERO	Ufa_Z 3780	BHQ1-TGC TCC TGG GAT GGT AAA TAA CCC AAC T-FAM
	Ufa_F 3760	GTA TTA TGT ACG AGT AAA GTT GAG AGA
	Ufa_R 3800	CCT AAA GGT ATA GGG ATT AAA CTC CT
DOB-SOCHI/VERO	Sochi_Z 4500	BHQ1-ATC CCA GCT GTG ATT GGG TAC AAG T-FAM
	Sochi_F 4480	TCA CTG TGA GAG AGA AGG ATC GA
	Sochi_R 4530	TAG AAC ATC TGA CAT TTC ATC AAC TGT
HTN-P88/VERO	Hantaan_Z 3770	BHQ1-TAC AGC ACC AGG TAT GGT GAA CCA-FAM
	Hantaan_F 3750	GTG TAC TAG TAA AGT TGA ACG GTT GT
	Hantaan_R 3790	GTG TAT CAG CAT GCT TGA CTT GCA

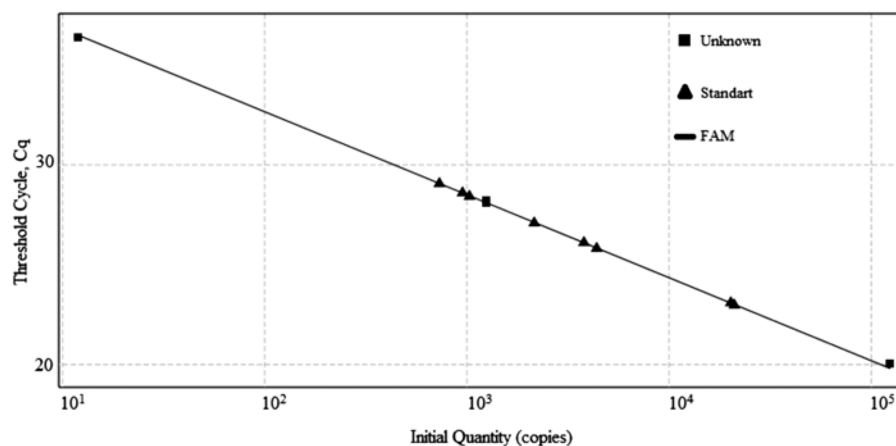

Fig. 1. Standard curve of the threshold cycle dependence on the initial concentration of Puumala virus RNA. The slope y = −3.44x + 39.59, correlation coefficient (R^2) = 0.998, and efficiency (E) = 110% of the linear regression curve. FAM is carboxyfluorescein (fluorescent tag).

Products (FSCRDIBP)) and the in-house HANTA-N test system were used for detection of hantavirus antigens in accordance with the manufacturer's manual [14].

Titration of focus-forming units of the virus. The previously described method was used for detecting FFUs on the monolayer of Vero E6 cells [15]. The number of infected colonies was estimated visually; the virus titer was expressed as lg FFU/ml.

Assessment of immunogenicity. Immunogenicity of experimental vaccine candidates against HFRS, which were exposed to different methods of inactivation, was assessed during tests on clinically healthy adult female BALB/c mice whose weight ranged from 18 to 20 g and who were supplied by the Andreevka Branch of the Scientific Center for Biomedical Technologies (SCBT) of the Federal Medical and Biological Agency (FMBA) of Russia. The studies on animals were performed in compliance with the ethical principles adopted by the European Convention for the Protection of Vertebrate Animals and by the Ethics Committee of M.P. Chumakov FSCRDIBP.

The animals from the experimental groups were vaccinated with the studied vaccine candidate at a dose of 0.5 ml of the finished candidate product, non-diluted (n/d) and diluted, intramuscularly, three times at 2-week intervals. The animals from the control group were administered injections of 0.9% sodium chloride in the same amount (C-SC).

The BALB/c mice were divided into groups, depending on the injected experimental vaccine candidates:

1 – VAC-PUU-F, 2 – VAC-PUU-β-PL, 3 – VAC-PUU-UV;

4 – VAC-HTN-F, 5 – VAC-HTN-β-PL, 6 – VAC-HTN-UV;

7 – VAC-SOCHI-F, 8 – VAC-SOCHI-β-PL, 9 – VAC-SOCHI-UV.

Neutralization test (NT/FFU$_{50}$). Neutralizing antibodies (nAbs) were detected with the neutralization test by 50% inhibition of focus-forming units (NT/FFU$_{50}$) in the Vero E6 cell culture, following the previously described procedure [15].

Statistical analysis. The validity of the findings was assessed in 3 series of tests conducted in identical conditions. All the obtained data were analyzed by using the GraphPad Prism v. 8.2.0 software. The statistical significance was assessed by using the one-way analysis of variance (ANOVA) and Tukey's multiple comparison test to compare the means of each set of data; p <0.05 values were accepted as significant. The statistical significance was reported as not significant (NS); p <0.05; p <0.01; p <0.005 and p <0.0001.

Results

FAM-based fluorescence was recorded only in RNA tests of PUU, HTN and SOCI vaccine strains. The tests of the other pathogens (including other strains of PUU, HTN and SOCHI viruses) turned out negative, thus demonstrating the absence of false-positive results and proving the high specificity of the method.

To compare the sensitivity of real-time PCR and ELISA, we used samples of serial dilutions of vaccine strains of PUU, HTN and SOCHI viruses. The sensitivity of the real-time PCR was as follows: for PUUV – 1.2 ± 1.5 × 10^2 copies/ml or 1.5 ± 0.5 lg FFU/ml; for HTNV – 1.16 ± 1.4 × 10^2 copies/ml or 1.8 ± 0.5 lg FFU/ml, and for SOCHIV – 1.32 ± 1.8 × 10^2 copies/ml (2,2 ± 0,5 lg FFU/ml). The ELISA sensitivity was much lower: for PUUV – 1 : 256 or 3.5 lg FFU/ml, for HTNV – 1 : 128 or 2.8 lg FFU/ml, and for SOCHIV – 1 : 128 (3.2 lg FFU/ml) (**Table 2**). The C_q indices were linear and correlated with the PUUV, HTNV and SOCHIV RNA concentration; the correlation coefficient R^2 = 0.99. The comparison of the qRT-PCR, ELISA and focus-forming assay (FFA) results demonstrated that the method of molecular genetic detection of virus presence is significantly more sensitive than ELISA. The test was repeated three times with respective negative controls; all the repeated tests were included in calculation of the standard deviation (M ± m, where M – the arithmetic mean, m – the standard error of the mean). The above data were used for identifying the correlation between FFU/ml and RNA copies/ml.

Real-time PCR-based quantitative assessment of the viral RNA content in the inactivated vaccine. The quantification of PUUV, HTNV and SOCHIV RNA content showed that the virus concentration in the samples matched the standards containing the specific RNA concentration of the above agents (**Fig. 1**). The curve was used to estimate the level of RNA copies in the semi-finished vaccine after the inactivation (**Table 3**).

The exposure of PUUV, HTNV and SOCHIV RNA to inactivators revealed the statistically significant difference between the control samples (prior to the inactivation) and the samples inactivated by different methods (p <0.0001) (**Fig. 2**). The maximum loss in RNA was observed after the UV treatment; the minimum loss was observed after the β-PL treatment. It should be noted that such losses are unavoidable and are caused by inactivating agents.

Assessment of immunogenicity. One pool of the virus harvest having the specified titer and inactivated by different methods was used for assessment of immunogenicity of the vaccine candidates.

No side effects (both local and general) were observed after the BALB/c mice had been immunized with the formalin, β-PL and UV-inactivated vaccines. The nAbs titer did not exceed the 2.32 log2 level when the immunogenicity was assessed by using the NR/FFU_{50} method in the negative control groups, and the above titer value was

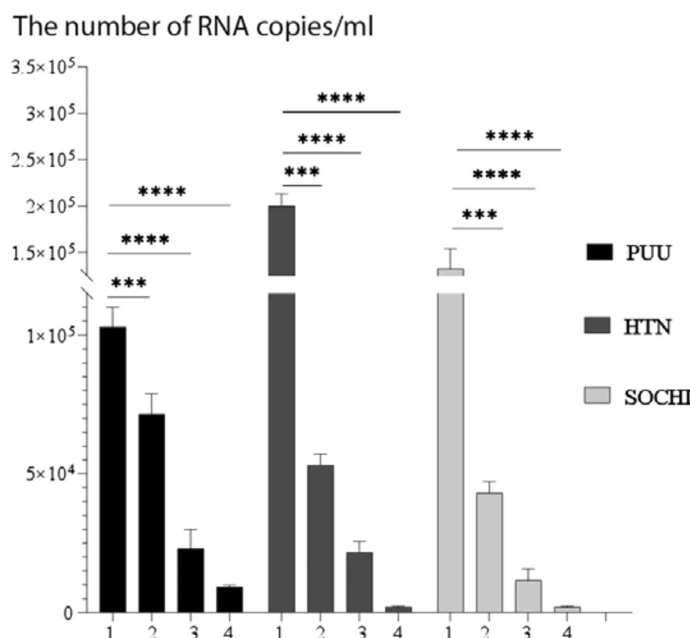

Fig. 2. Analysis of the dependence of the number of RNA copies on the inactivating agent/ml. The combined results of 3 independent experiments using a one-sided analysis of variance (ANOVA) with Tukey's multiple comparisons test are shown. *1* – control; *2* – β-propiolactone; *3* – formalin; *4* – UV radiation.

Note. *** – p <0.005, **** – p <0.0001.

Table 2. Comparison of sensitivity of real-time qPCR and ELISA

Puumala			Hantaan			Sochi		
Virus titer, lg FFU/ml	ELISA	qPCR	Virus titer, lg FFU/ml	ELISA	qPCR	Virus titer, lg FFU/ml	ELISA	qPCR
	Antigen titer	copies/ml		Antigen titer	copies/ml		Antigen titer	copies/ml
5.5	1 : 2048	1.24×10^5	5.8	1 : 4096	3.06×10^5	5.2	1 : 2048	1.01×10^5
4.5	1 : 1024	3.55×10^4	4.8	1 : 1024	2.16×10^4	4.2	1 : 1024	2.25×10^4
3.5	1 : 256	6.91×10^3	3.8	1 : 1024	9.53×10^3	3.2	1 : 128	5.01×10^3
2.5	n.d.	8.58×10^2	2.8	1 : 128	3.39×10^2	2.2	n.d.	1.32×10^2
1.5	n.d.	1.24×10^2	1.8	n.d.	1.16×10^2	1.2	n.d.	n.s.
0.5	n.d.	n.s.	0.8	n.d.	n.s.	0,2	n.d.	n.s.

Note. * – ELISA test system «HANTAGNOST» was used; ** – ELISA test system «HANTA-N» was used; n.d. – not defined; n.s. – no fluorescence signal.

adopted as the cut-off value. The value of the geometric mean titer (GMT) above 4.32 log2 was adopted as the acceptable level of nAbs induction.

No statistically significant difference was observed in the nAbs titers after the animals were immunized with formalin, β-PL and UV-inactivated vaccines (**Table 3**). Although the number of RNA copies detected in the dose of the vaccine inactivated by different methods was different, the immune response did not demonstrate any critical differences (nAbs titers were not statistically different), thus proving that immunogenic epitopes were not affected by the applied methods of inactivation.

To study the correlation between the number of RNA copies and the immunogenicity, the BALB/c mice were immunized with β-PL-inactivated VAC-PUU (VAC-PUU-β-PL) in specified dilutions. The number of genetic material copies per 1 ml was estimated in each of them and correlated with the titer of nAbs detected after the immunization. The test results showed a direct relationship between the number of RNA copies and the nAbs titer (**Table 4**).

Discussion

Previously, the specific activity of vaccines was assessed by detecting viral antigens with ELISA using monoclonal antibodies against N- and/or G-proteins of hantaviruses [14]. This study offers a more sensitive and advanced method of assessment – real-time PCR based on the whole-genome sequence of vaccine strains; the method demonstrated high specificity, sensitivity and reproducibility in quantification of the specified strains in samples of β-PL-inactivated inactivated vaccine products. Compared to the traditional methods (ELISA), qRT-PCR provides high reproducibility of quantitative measurement.

The significant difference was observed in the number of RNA copies before and after the inactivation of the semi-finished vaccine by using different methods, including application of formalin, β-PL and UV light. At the same time, the immunogenicity of the vaccine products did not show any critical differences in the number of induced nAbs, thus indicating the similar damage to immunogenic proteins, while the viral RNA demonstrated different levels of destruction caused by the tested inactivators. During the extension studies aimed at assessment of specific activity of vaccines by using real-time PCR, the decision was made to select β-PL-inactivated vaccine candidates. As compared to formalin and UV-inactivated vaccines, the β-PL-inactivated vaccines reduce the content of ballast proteins by reducing their aggregation. This results in more efficient purification of the virus during clarification filtration and gel-filtration as well as in decreased losses of the target component of vaccines, which are caused by sterilizing filtration. The obtained data on correlation of the number of RNA copies with the nAbs titer in the blood serum of BALB/c mice after their immunization make it possible to calculate the immunization dose of the vaccine by the quantitative content of viral

Table 3. Control specific activity of the vaccine prefabricated Puumala. Hantaan and Sochi viruses products

Vaccine material	Method of inactivation	Virus titer, lg FFU/ml	qPCR ($M \pm m$), copies/ml	ELISA. antigen titer***	GMT nAbs ($M \pm m$), log2**
VAC-PUU	Control*	5.50	$1.2 \pm 0.3 \times 10^5$	1 : 2048	–
	Formalin	–	$2.3 \pm 0.4 \times 10^4$	1 : 1024	8.8 ± 0.1
	β-propiolactone	–	$7.0 \pm 0.3 \times 10^4$	1 : 2048	8.8 ± 0.1
	UV radiation	–	$9.2 \pm 0.4 \times 10^3$	1 : 2048	8.8 ± 0.1
VAC-HTN	Control*	5.80	$3.0 \pm 0.3 \times 10^5$	1 : 4096	–
	Formalin	–	$2.2 \pm 0.4 \times 10^4$	1 : 2048	9.4 ± 0.1
	β-propiolactone	–	$5.3 \pm 0.4 \times 10^4$	1 : 4096	9.6 ± 0.1
	UV radiation	–	$2.0 \pm 0.3 \times 10^3$	1 : 4096	9.3 ± 0.1
VAC-SOCHI	Control*	5.20	$1.0 \pm 0.3 \times 10^5$	1 : 2048	–
	Formalin	–	$1.2 \pm 0.4 \times 10^4$	1 : 1024	6.7 ± 0.1
	β-propiolactone	–	$4.3 \pm 0.4 \times 10^4$	1 : 2048	6.9 ± 0.1
	UV radiation	–	$2.0 \pm 0.3 \times 10^3$	1 : 2048	6.8 ± 0.1

Note. *The control is the initial vaccine prefabricated product before inactivation; **GMT is geometric mean titer of neutralizing antibodies. log2; *** – ELISA test systems «HANTAGNOST» and «HANTA-N» were used.

Table 4. Correlation between RNA copy number and nAbs titer in the blood of BALB/c mice following VAC-PUU-β-PL immunization

Vaccine dilution	qPCR ($M \pm m$), copies/ml	nAbs titer, log2
u/d*	$7.0 \pm 0.3 \times 10^4$	8.8 ± 0.1
1/2	$4.3 \pm 0.4 \times 10^4$	6.3 ± 0.1
1/8	$7.6 \pm 0.3 \times 10^3$	5.4 ± 0.1

Note. *u/d – undiluted vaccine preparation.

nucleic acid copies in a unit of volume. Based on the findings of our study, the minimum immunization dose of the β-PL-inactivated vaccine product, which induces nAbs in the 4.32 log2 titer in 10 out of 10 immunized BALB/c mice, is equivalent to $7.5 \pm 0.2 \times 10^2$ RNA copies/ml. The method is highly specific and reliably reproducible, thus having promising future in assessment of the specific activity of HFRS vaccines.

Compliance with the ethical standards. The animals under study were kept in similar conditions. The tests on experimental models were performed in compliance with the international and national requirements for animals care and use. The above requirements are based on the ethical principles specified by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, adopted on March 18, 1986 and revised in Strasbourg on June 1, 2006 (ETS N 123), (https://www.coe.int/t/e/legal_affairs/legal_cooperation/biological_safety_and_use_of_animals/laboratory_animals/GT123(2002)63%20E%20PART%20B%20Ferrets%20rev2.pdf), the principles adopted by the Ethics Committee of M.P. Chumakov FSCRDIBP, and the rules incorporated in the Good Laboratory Practice (GLP) (http://docs.cntd.ru/document/1200101144, https://www.nc3rs.org.uk/the-3rs) (GOST R-53434-2009 The Principles of the Proper Laboratory Practice).

Conclusion

Preventive vaccination against any infectious disease requires proper control of the quality of vaccine products. The designed system of real-time PCR quantification of viral RNA, due to its characteristics, can be seen as promising for assessment of the specific activity of HFRS vaccines.

REFERENCES

1. Kruger D.H., Figueiredo L.T., Song J.W., Klempa B. Hantaviruses – globally emerging pathogens. *J. Clin. Virol.* 2014; 64: 128–36. https://doi.org/10.1016/j.jcv.2014.08.033.
2. Lähdevirta J. Nephropathia epidemica in Finland. A clinical, histological and epidemiological study. *Ann. Clin. Res.* 1971; 3(Suppl. 8): 1–154.
3. Schmaljohn C., Hjelle B. Hantaviruses: a global disease problem. *Emerg. Infect. Dis.* 1997; 3(2): 95–104. https://doi.org/10.3201/eid0302.970202.
4. Maes P., Li S., Verbeeck J., Keyaerts E., Clement J., Van Ranst M. Evaluation of the efficacy of disinfectants against Puumala hantavirus by real-time RT-PCR. *J. Virol. Methods.* 2007; 141(1): 111–5. https://doi.org/10.1016/j.jviromet.2006.11.037.
5. Plyusnin A., Vapalahti O., Vaheri A. Hantaviruses: genome structure, expression and evolution. *J. Gen. Virol.* 1996; 77(Pt. 11): 2677–87. https://doi.org/10.1099/0022-1317-77-11-2677.
6. Schmaljohn C.S., Dalrymple J.M. Analysis of Hantaan virus RNA: evidence for a new genus of Bunyaviridae. *Virology*. 1983; 131(2): 482–91. https://doi.org/10.1016/0042-6822(83)90514-7.
7. Tkachenko E.A., Ishmukhametov A.A., Dzagurova T.K., Bernshtein A.D., Morozov V.G., Sinyugina A.A., et al. Hemorrhagic fever with renal syndrome, Russia. *Emerg. Infect. Dis.* 2019; 25(12): 2325–8. https://doi.org/10.3201/eid2512.181649.
8. Liu R., Ma H., Shu J., Zhang Q., Han M., Liu Z., et al. Vaccines and therapeutics against hantaviruses. *Front. Microbiol.* 2019; 10: 2989. https://doi.org/10.3389/fmicb.2019.02989.
9. Barkhaleva O.A., Vorob'eva M.S., Ladyzhenskaya I.P., Tkachenko E.A., Dzagurova T.K. Vaccine against hemorrhagic fever with renal syndrome *[Vaktsina protiv gemorragicheskoi likhoradki s pochechnym sindromom]. BIOpreparaty. Profilaktika, diagnostika, lechenie.* 2011; (1): 27–30 (in Russian).
10. Jiang W., Ma N., Hui Q., Chen B., Qiu Y., Sun H., et al. Type inactivated bivalent hantavirus vaccines using dual fluorescence quantitative real time PCR: Establishment and evaluation. *J. Med. Virol.* 2017; 89(1): 10–6. https://doi.org/10.1002/jmv.24599.
11. Tkachenko E.A., Ishmukhametov A.A., Dzagurova T.K., Sinyugina A.A., Korotina N.A., Nabatnikov P.A., et al. Manufacturing techniques and methods of control of the inactivated vero cell-derived vaccine against HFRS has been developed in Russia *[Razrabotka eksperimental'no-promyshlennoi tekhnologii proizvodstva vaktsiny dlya profilaktiki gemorragicheskoi likhoradki s pochechnym sindromom]. Remedium. Zhurnal o rossiyskom rynke lekarstv i meditsinskoi tekhnike.* 2015; (6): 47–53 (in Russian).
12. Kurashova S.S., Ishmukhametov A.A., Egorova M.S., Balovneva M.V., Dzagurova T.K., Tkachenko E.A. Comparative characteristics of inactivation agents for HFRS vaccine development *[Sravnitel'naya kharakteristika inaktiviruyushchikh agentov dlya sozdaniya vaktsiny protiv gemorragicheskoi likhoradki s pochechnym sindromom]. Epidemiologiya i vaktsinoprofilaktika.* 2018; 17(4): 26–8. https://doi.org/10.31631/2073-3046-2018-17-4-26-29 (in Russian).
13. Eprintsev A.T., Popov V.N., Fedorin D.N. *Identification and Study of Gene Expression: A Teaching Aid for Universities [Identifikatsiya i issledovanie ekspressii genov: Uchebno-metodicheskoe posobie dlya vuzov].* Voronezh; 2008: 14–5 (in Russian).
14. Dzagurova T.K., Solopova O.N., Sveshnikov P.G., Korotina N.A., Balovneva M.V., Leonovich O.A., et al. Development of ELISA on the basis of monoclonal antibodies for detecting specific activity of the vaccine against hemorrhagic fever with renal syndrome *[Razrabotka immunofermentnoi test-sistemy na osnove monoklonal'nykh antitel dlya opredeleniya specificheskoi aktivnosti vaktsiny protiv gemorragicheskoi likhoradki s pochechnym sindromom]. Voprosy virusologii.* 2013; 58(1): 40–4 (in Russian).
15. Dzagurova T.K., Tkachenko E.A., Bashkirtsev V.N., Okulova N.M., Apekina N.S., Korotina N.A., et al. Isolation and identification of strains of hantavirus-causative agents of HFRS in the European part of Russia *[Vydelenie i identifikatsiya shtammov khantavirusov – vozbuditelei GLPS v evropeiskoi chasti Rossii]. Meditsinskaya virusologiya.* 2008; 25: 142–50 (in Russian).

ЛИТЕРАТУРА

1. Kruger D.H., Figueiredo L.T., Song J.W., Klempa B. Hantaviruses – globally emerging pathogens. *J. Clin. Virol.* 2014; 64: 128–36. https://doi.org/10.1016/j.jcv.2014.08.033.
2. Lähdevirta J. Nephropathia epidemica in Finland. A clinical, histological and epidemiological study. *Ann. Clin. Res.* 1971; 3(Suppl. 8): 1–154.
3. Schmaljohn C., Hjelle B. Hantaviruses: a global disease problem. *Emerg. Infect. Dis.* 1997; 3(2): 95–104. https://doi.org/10.3201/eid0302.970202.
4. Maes P., Li S., Verbeeck J., Keyaerts E., Clement J., Van Ranst M. Evaluation of the efficacy of disinfectants against Puumala hantavirus by real-time RT-PCR. *J. Virol. Methods.* 2007; 141(1): 111–5. https://doi.org/10.1016/j.jviromet.2006.11.037.
5. Plyusnin A., Vapalahti O., Vaheri A. Hantaviruses: genome structure, expression and evolution. *J. Gen. Virol.* 1996; 77(Pt. 11): 2677–87. https://doi.org/10.1099/0022-1317-77-11-2677.
6. Schmaljohn C.S., Dalrymple J.M. Analysis of Hantaan virus RNA: evidence for a new genus of Bunyaviridae. *Virology*. 1983; 131(2): 482–91. https://doi.org/10.1016/0042-6822(83)90514-7.
7. Tkachenko E.A., Ishmukhametov A.A., Dzagurova T.K., Bernshtein A.D., Morozov V.G., Sinyugina A.A., et al. Hemorrhagic fever with renal syndrome, Russia. *Emerg. Infect. Dis.* 2019; 25(12): 2325–8. https://doi.org/10.3201/eid2512.181649.

8. Liu R., Ma H., Shu J., Zhang Q., Han M., Liu Z., et al. Vaccines and therapeutics against hantaviruses. *Front. Microbiol.* 2019; 10: 2989. https://doi.org/10.3389/fmicb.2019.02989.
9. Бархалева О.А., Воробьева М.С., Ладыженская И.П., Ткаченко Е.А., Дзагурова Т.К. Вакцина против геморрагической лихорадки с почечным синдромом. *БИОпрепараты. Профилактика, диагностика, лечение.* 2011; (1): 27–30.
10. Jiang W., Ma N., Hui Q., Chen B., Qiu Y., Sun H., et al. Type inactivated bivalent hantavirus vaccines using dual fluorescence quantitative real time PCR: Establishment and evaluation. *J. Med. Virol.* 2017; 89(1): 10–6. https://doi.org/10.1002/jmv.24599.
11. Ткаченко Е.А., Ишмухаметов А.А., Дзагурова Т.К., Синюгина А.А., Коротина Н.А., Набатников П.А., и др. Разработка экспериментально-промышленной технологии производства вакцины для профилактики геморрагической лихорадки с почечным синдромом. *Ремедиум. Журнал о российском рынке лекарств и медицинской технике.* 2015; (6): 47–53.
12. Курашова С.С., Ишмухаметов А.А., Егорова М.С., Баловнева М.В., Дзагурова Т.К., Ткаченко Е.А. Сравнительная характеристика инактивирующих агентов для создания вакцины против геморрагической лихорадки с почечным синдромом. *Эпидемиология и вакцинопрофилактика.* 2018; 17(4): 26–8. https://doi.org/10.31631/2073-3046-2018-17-4-26-29.
13. Епринцев А.Т., Попов В.Н., Федорин Д.Н. *Идентификация и исследование экспрессии генов: Учебно-методическое пособие для вузов.* Воронеж; 2008: 14–5.
14. Дзагурова Т.К., Солопова О.Н., Свешников П.Г., Коротина Н.А., Баловнева М.В., Леонович О.А., и др. Разработка иммуноферментной тест-системы на основе моноклональных антител для определения специфической активности вакцины против геморрагической лихорадки с почечным синдромом. *Вопросы вирусологии.* 2013; 58(1): 40–4.
15. Дзагурова Т.К., Ткаченко Е.А., Башкирцев В.Н., Окулова Н.М., Апекина Н.С., Коротина Н.А., и др. Выделение и идентификация штаммов хантавирусов – возбудителей ГЛПС в европейской части России. *Медицинская вирусология.* 2008; 25: 142–50.