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Optimization of RHDV type 1 and 2 inactivation modes

E. D. Kunikova[1], N. V. Moroz[2], M. A. Dolgova[3], L. V. Malakhova[4], I. A. Komarov[5]

[1,2,3,5] FGBI "Federal Centre for Animal Health" (FGBI "ARRIAH"), Vladimir, Russia

[4] FSBEI HE "Kostroma State Agricultural Academy" (FSBEI HE Kostroma SAA), Karavaevo, Kostroma Oblast, Russia

[1] *ORCID 0000-0003-2384-8162, e-mail: kunikova@arriah.ru*

[2] *ORCID 0000-0002-9672-8594, e-mail: moroz@arriah.ru*

[3] *ORCID 0000-0001-8061-1116, e-mail: dolgova@arriah.ru*

[4] *ORCID 0000-0001-8011-5657, e-mail: van@ksaa.edu.ru*

[5] *ORCID 0000-0002-2084-4484, e-mail: komarov@arriah.ru*

SUMMARY

The purpose of these studies was to optimize RHDV type 1 and 2 (RHDV1 and RHDV2) inactivation modes to use the obtained antigens in inactivated vaccines and diagnosticums. The inactivating effect of aminoethylethylenimine and β-propiolactone was studied in different concentrations in correlation with the exposure time and temperature. The correlation between the inactivating effect of the compound used and the accepted test conditions (concentration, temperature, and exposure time) was studied on a group of rabbits, each of which was injected intramuscularly with 1 cm^3 of the inactivated material sample. At the end of the maximum exposure interval, a control sample of the viral material, kept under the same conditions without any inactivant added was similarly tested. Lethality was considered to evaluate the damaging action in the test and control groups: $L = m/n$, where m is the number of dead animals; n is the total number of rabbits in the group for testing of the inactivated material sample. The post-mortem diagnosis was confirmed by testing the rabbit liver tissue homogenate for relative antigens using ELISA. It was found that aminoethylethylenimine and β-propiolactone did not have the same effect on the studied variants of the virus. In order to preserve at maximum the antigenic structures of the virus, the following inactivation modes were considered to be optimal: for RHDV1 – aminoethylethylenimine at a concentration of 0.3% at 37 °C, exposure time – 72 hours, or β-propiolactone at a concentration of 0.1–0.3% at 25–37 °C, exposure time – 24–48 hours; for RHDV2 – aminoethylethylenimine at a concentration of 1% at 37 °C, exposure time – 72 hours, or β-propiolactone at a concentration 0.3% at 25 °C, exposure time – 24 hours.

Keywords: Viral hemorrhagic disease of rabbits, inactivated vaccine, aminoethylethylenimine, β-propiolactone, inactivation of rabbit hemorrhagic disease virus.

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For correspondence: Ekaterina D. Kunikova, Post-Graduate Student, Technologist, Laboratory for Avian Diseases Prevention, FGBI "ARRIAH", 600901, Russia, Vladimir, Yur'evets, *e-mail: kunikova@arriah.ru.*

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Отработка режимов инактивации вируса геморрагической болезни кроликов 1-го и 2-го типов

Е. Д. Куникова[1], Н. В. Мороз[2], М. А. Долгова[3], Л. В. Малахова[4], И. А. Комаров[5]

[1,2,3,5] ФГБУ «Федеральный центр охраны здоровья животных» (ФГБУ «ВНИИЗЖ»), г. Владимир, Россия

[4] ФГБОУ ВО «Костромская государственная сельскохозяйственная академия» (ФГБОУ ВО Костромская ГСХА), пос. Караваево, Костромская обл., Россия

[1] *ORCID 0000-0003-2384-8162, e-mail: kunikova@arriah.ru*

[2] *ORCID 0000-0002-9672-8594, e-mail: moroz@arriah.ru*

[3] *ORCID 0000-0001-8061-1116, e-mail: dolgova@arriah.ru*

[4] *ORCID 0000-0001-8011-5657, e-mail: van@ksaa.edu.ru*

[5] *ORCID 0000-0002-2084-4484, e-mail: komarov@arriah.ru*

РЕЗЮМЕ

Цель настоящих исследований состояла в подборе режимов инактивации вируса геморрагической болезни кроликов 1-го (RHDV1) и 2-го (RHDV2) типов для использования полученных антигенов в составе инактивированных вакцин и диагностикумов. Изучали инактивирующее действие аминоэтилэтиленимина и β-пропиолактона в различных концентрациях в зависимости от времени экспозиции и температуры. Оценку инактивирующего эффекта используемого соединения соответственно принятым условиям испытания (концентрация, температура и время экспозиции) проводили на группе

кроликов. Каждому животному делали внутримышечную инъекцию пробы инактивируемого материала в объеме 1 см^3. По истечении максимального интервала экспозиции аналогичным образом испытывали пробу контрольного образца вирусного материала, который содержали при тех же условиях без добавления инактиванта. В опытных и контрольных группах повреждающее действие оценивали с помощью показателя летальности: $L = m/n$, где m – число погибших животных; n – общее количество кроликов в группе для испытания данной пробы инактивируемого материала. Посмертный диагноз подтверждали исследованием гомогената ткани печени кроликов на наличие соответствующих антигенов при помощи иммуноферментного анализа. Установили, что аминоэтилэтиленимин и β-пропиолактон не одинаково воздействовали на исследуемые варианты вируса. В целях максимального сохранения антигенных структур вируса считали, что оптимальными условиями инактивации будут следующие: для RHDV1 – аминоэтилэтиленимином в концентрации 0,3% при 37 °С и экспозиции 72 ч или β-пропиолактоном в концентрации 0,1–0,3% при 25–37 °С и экспозиции 24–48 ч; для RHDV2 – аминоэтилэтиленимином в концентрации 1,0% при 37 °С и экспозиции 72 ч или β-пропиолактон в концентрации 0,3% при 25 °С и экспозиции 24 ч.

Ключевые слова: Вирусная геморрагическая болезнь кроликов, инактивированная вакцина, аминоэтилэтиленимин, β-пропиолактон, инактивация вируса геморрагической болезни кроликов.

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Для корреспонденции: Куникова Екатерина Дмитриевна, аспирант, технолог лаборатории профилактики болезней птиц ФГБУ «ВНИИЗЖ», 600901, Россия, г. Владимир, мкр. Юрьевец, *e-mail: kunikova@arriah.ru.*

INTRODUCTION

Rabbit hemorrhagic disease (RHD) is an acute, highly contagious disease characterized by hemorrhagic diathesis in all organs, and high mortality (80–100%) [1]. The disease is caused by an RNA virus, a member of the family *Caliciviridae*, genus *Lagovirus*, which is genetically related to the European brown hare syndrome virus [2]. The virions are spherical, 20–40 nm in diameter and have some hemagglutinating properties [3, 4].

RHDV was first recognized in commercially bred Angora rabbits imported from Germany to Jiangsu Province, China, in 1984. Then the disease spread in Europe and Asia [5]. As of 2020, according to the World Organization for Animal Health (OIE), the following countries are recognized as RHD infected: Denmark, Benin, Iceland, Canada, the USA, Mexico, and Finland.

It is believed that weakly virulent variants of the RHDV have been circulating in nature indefinitely. However, in recent decades, the pathogen has undergone significant evolutionary changes, which have resulted in its significantly increased virulence [3].

In 2010, the RHDV type 2 (RHDV2) was isolated in France. The average homology between the genome region encoding the main capsid protein VP60 of the new virus variant and the known strains of this pathogen (RHDV1) was 87%. However, it is important to note that RHDV1 vaccines proved to be ineffective [6].

By 2018, several disease cases associated with RHDV2 were reported in Russia [7]. A characteristic feature that distinguishes the new type virus from RHDV1 is its ability to infect young rabbits under 2 weeks of age [8].

To date, only vaccines containing RHDV1 strains are available in the Russian market of veterinary medicinal products [9]. This fact helped to define the objectives of the study, that is to optimize RHDV type 1 and 2 (RHDV1 and RHDV2) inactivation modes in order to further use the obtained antigens in inactivated vaccines and diagnosticums.

MATERIALS AND METHODS

Strains. The following virus strains were studied: RHDV1 – RHDV1/ARRIAH (infectious titer 3.00 ± 0.25 lg LD_{50}/cm^3); RHDV2 – RHDV2/ARRIAH (infectious titer 4.00 ± 0.25 lg LD_{50}/cm^3).

Virus-containing material. For the studies, a 10% tissue suspension (weight/volume) obtained from the liver tissue of rabbits infected with RHDV1 and RHDV2 was used. The homogenate was prepared in saline solution (0.9% NaCl solution). Dispersion was carried out using a laboratory tissue homogenizer at 10,000 rpm for 10 minutes. The tissue homogenate was stored in the refrigerator at –20 °C. Before use, chloroform (2% by volume) and high-molecular polyhexamethylene guanidine hydrochloride (0.1% by volume) were added to the thawed material, actively mixed and centrifuged at 500 g for 20 minutes. The supernatant was used for further work.

Hemagglutination test. Hemagglutinating activity of the virus was determined in accordance with the techniques set out in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals [1]. The hemagglutinating activity of the prepared virus-containing material was at least 1:1280–1: 2560 HAE.

Enzyme-linked immunosorbent assay (ELISA). RHDV antigen was detected using Ingezim RHDV DAS kit (Ingenasa, Spain) in accordance with the attached instructions [10].

Animals. For the purpose of the study, 360 Soviet chinchilla rabbits (aged 45 days, live weight 1.0–1.5 kg) not vaccinated against RHDV obtained from infectious disease-free farms were used. The animals were kept in groups in isolators equipped with automated drinking and feeding systems.

All tests in animals were carried out in strict compliance with the interstate standards for keeping and care of laboratory animals GOST 33216-2014 and GOST 33215-2014, adopted by the Interstate Council for Standardization, Metrology and Certification, as well as in accordance with the requirements of Directive 2010/63/EU of the European Parliament and of the Council of the European Union of 22.09.2010 on the protection of animals used for scientific purposes.

Rabbits were euthanized with trichloromethane vapours.

Inactivants. To inactivate virus-containing materials, aminoethylethylenimine was used in the form of a 15% aqueous solution (AEEA, LLC NPP Biochemservice, Russia) and *β*-propiolactone (Acros Organics, USA).

Inactivation. *β*-propiolactone was diluted with a phosphate buffer solution to a concentration of 10% before use. Previously, the pH of the AEEA working solution was adjusted to 8.2–8.3 using acetic acid. The prepared inactivants were added to the virus-containing material to the specified final concentrations. The inactivation procedure was performed at 25 and 37 °C. At specified time intervals, samples were taken to determine the completeness of virus inactivation. The control virus without inactivant was kept under the same conditions. Control virus samples were collected after the longest time exposure.

RESULTS

Inactivating effectiveness of the studied compounds in correlation with their concentration, temperature and exposure time was studied using 360 rabbits. Each experimental group consisted of three animals, each of which was inoculated intramuscularly with 1 cm^3 of the inactivated material sample. After the longest time exposure, the control sample was tested similarly.

The animals were clinically monitored for 10 days. Lethality was registered, and its specificity was confirmed by the following macroscopic findings: foamy/bloody exudate from the nostrils; enlarged liver with dystrophic changes; mottled lungs; kidney abnormalities (color from pale to dark red), kidney petechiation; intestinal hyperemia and hemorrhages, most pronounced at the top of the intestinal folds [11].

The damaging effect in both the experimental and control groups was assessed by the lethality index $L = m/n$, where m is the number of dead animals; n is the total number of rabbits in the group used for the inactivated material sample testing.

The post-mortem diagnosis was further confirmed by testing rabbit liver homogenate for RHDV antigen using ELISA. The completeness of inactivation of the studied materials was confirmed by the absence of RHDV antigen in the organs of the survived rabbits 10 days after their inoculation with the tested inactivated material samples.

Assessment of the inactivation effect of the compounds used are presented in the Table.

The test results showed that:

– the RHDV1 strain turned out to be highly sensitive to *β*-propiolactone. Samples obtained after their exposure to all the tested inactivant concentrations under all the exposure conditions did not contain infective virus ($L = 0$);

– the RHDV1 strain showed a certain sensitivity to AEEA. Complete pathogen inactivation was observed at all temperatures only after the exposure to concentrations above 0.2%;

– the RHDV2 strain demonstrated a certain sensitivity to all the tested concentrations of *β*-propiolactone. However, for complete pathogen inactivation at 25 °C, the concentration of at least 0.3% and the exposure time of at least 24 hours were required. At 37 °C, regardless of the exposure time, all the tested concentrations provided complete inactivation of the pathogen;

– the RHDV2 strain demonstrated a relatively high resistance to inactivation with AEEA. At 25 °C and the inactivant concentrations of up to 1.0%, the pathogen completely retained its biological activity at almost all the time intervals. The exception was the interval of 72 hours, at which the mortality rate was $L = 2/3$ (inactivant concentration of 0.45%). Complete virus inactivation at 25 °C was observed after the exposure to 1.0% inactivant for 72 hours. At 37 °C and AEEA concentration of 1.0% the virus was completely inactivated regardless of the exposure time.

It should be noted that all the control samples of the materials containing viruses of both strains retained their biological activity under all the tested exposure conditions. This rules out the possibility of spontaneous virus inactivation during the experiment.

The results of testing using liver tissue homogenate obtained from dead rabbits of the experimental and control groups showed the presence of the RHDV antigen, which confirmed their specific death. The results of test conducted using homogenated liver tissue of the survived rabbits proved the completeness of virus inactivation.

DISCUSSION

The emergence of a new virus variant with significant antigenic differences is always an important event for this ecological niche. This phenomenon is clearly demonstrated by the epidemic situation in Australia, where RHDV2 was detected in May 2015. Retrospective studies have shown that viruses of this family have a rate of genetic evolution $(2.8–5.4) \times 10^{-3}$ substitutions per year, which occur uniformly in both non-structural and structural protein-coding regions of the genome. However, in RHDV2, changes in the region encoding VP60 (the external antigen of the virus) have been observed with a disproportionately high frequency. It has been suggested that this kind of evolutionary process is going on [12].

It is believed that at present, due to its immunological features, RHDV2 has a large epidemic potential, which is confirmed by data on the displacement of RHDV1 strains that previously circulated in the field in France, Spain and Portugal [13].

It should be noted that RHDV2 does not demonstrate clear advantages in terms of its replication in the macroorganism. The average (geometric mean) viral load in the livers of RHDV2-infected rabbits (3×10^8 capsid copies per mg of tissue) was comparable to that observed in RHDV1-infected rabbit livers (2×10^8 capsid copies per mg of tissue). This fact puts into question the association between the RHDV2 replication rate and its virulence.

As part of the study objectives, we determined the conditions under which *β*-propiolactone and AEEA completely inactivate RHDV types 1 and 2, which allows to obtain antigens that can be used to develop inactivated vaccines.

The immunological properties of the vaccine may fully depend on the choice of inactivation methodology. The main criteria for its effectiveness are complete and irreversibile virus inactivation, as well as the preservation of its original antigenic properties.

Table
Correlation between RHDV1 and RHDV2 inactivation completeness and the tested concentrations, temperature, and exposure time

Таблица
Оценка полноты инактивации RHDV1 и RHDV2 соответственно концентрациям химических соединений, температуре и времени экспозиции

Mortality after the inoculation of the virus suspension containing β-propiolactone							
type of virus	D, %	25 °C			37 °C		
		24 hours	48 hours	72 hours	24 hours	48 hours	72 hours
RHDV1	0.10	0/3*	0/3	0/3	0/3	0/3	0/3
	0.20	0/3	0/3	0/3	0/3	0/3	0/3
	0.30	0/3	0/3	0/3	0/3	0/3	0/3
	control**	3/3	3/3	3/3	3/3	3/3	3/3
RHDV2	0.10	1/3	1/3	1/3	0/3	0/3	0/3
	0.20	1/3	1/3	1/3	0/3	0/3	0/3
	0.30	0/3	0/3	0/3	0/3	0/3	0/3
	control	3/3	3/3	3/3	3/3	3/3	3/3
Mortality in rabbits after their inoculation with the virus suspension containing AEEA							
type of virus	D, %	25 °C			37 °C		
		24 hours	48 hours	72 hours	24 hours	48 hours	72 hours
RHDV1	0.10	3/3	2/3	1/3	3/3	2/3	1/3
	0.20	1/3	1/3	1/3	1/3	1/3	1/3
	0.30	0/3	0/3	0/3	0/3	0/3	0/3
	0.45	0/3	0/3	0/3	0/3	0/3	0/3
	1.00	0/3	0/3	0/3	0/3	0/3	0/3
	control	3/3	3/3	3/3	3/3	3/3	3/3
RHDV2	0.10	3/3	3/3	3/3	3/3	3/3	3/3
	0.20	3/3	3/3	3/3	3/3	3/3	3/3
	0.30	3/3	3/3	3/3	3/3	2/3	1/3
	0.45	3/3	3/3	2/3	3/3	2/3	1/3
	1.00	1/3	1/3	0/3	0/3	0/3	0/3
	control	3/3	3/3	3/3	3/3	3/3	3/3

* Ratio of dead animals to the total number of animals in the group;
** suspension without inactivant; D – inactivant concentration (%).
Conditions under which no lethal effect were observed are highlighted in color.

* Отношение погибших животных к общему количеству в группе;
** суспензия без добавления инактиванта; D – концентрация инактиванта (%).
Цветом выделены условия, при которых летальный эффект отсутствовал.

Of the chemical compounds, two types of inactivants are used most often: reticulating (loosening) and alkylating. The agents that reticulate proteins include aldehydes (formaldehyde, glutaraldehyde, and glycidaldehyde). The alkylating agents that affect the structure of nucleic acids include β-propiolactone, ethylenimine, and others [14].

Under optimal conditions, β-propiolactone inactivates, for example, ND virus without changing its

hemagglutinating, neuraminidase and hemolytic activity significantly. This also applies to the chicken infectious bronchitis virus, which, after the exposure to this compound, retains its antigenic properties at a satisfactory level. However, increased concentration of β-propiolactone can result in an undesirable reaction with viral proteins and, thus, to the decrease of antigenic activity [14].

Compounds that interact with the viral genome are called genotoxic. In aqueous solutions, such inactivants break down to form highly active derivatives having an excess positive charge – the so-called electrophilic group (for example, chloroethylamine). The electrophilic group interacts with the negatively charged (nucleophilic) groups of DNA or RNA molecules to form a stable covalent bond. During replication, such a nucleotide, bound to the inactivant molecules, may not be read or read incorrectly by the polymerase, which blocks its replication or results in lethal mutations [15]. An important fact is that nitrogen-containing heterocyclic compounds inactivate viruses in a first-order reaction, and the inactivation rate as well as the endpoint can be determined with sufficient accuracy. This allows to objectively assess the safety of the final product [16].

Ethylenimine and its N-acetyl derivative inactivate a wide range of viruses belonging to several different families under conditions that do not affect the enzymatic or serological properties of a number of proteins [17]. The results of special studies of the properties of FMDV of various types inactivated with ethylene derivatives showed that aziridine-type preparations minimally altered the protein structures of the virion responsible for antigenicity [18]. The study of the structure of the FMDV antigen after inactivation with ethylenimine showed a high percentage of 140S component corresponding to virions with conserved capsid architecture [19]. When the ILT virus was inactivated with formalin, β-propiolactone, methylenimine, and ethylenimine, it was found that the antigen obtained after the exposure to ethylenimine was the most immunogenic [20].

The effectiveness of the inactivant depends on at least three factors: the specified concentration, temperature, and exposure time. According to the PCR results, the concentration of binary ethylenimine 0.001 M did not affect AIV glycoprotein gene during 8 hours of exposure, while the concentration of 0.01 M changed the structure of this gene after 4 hours [21]. The example with FMDV demonstrates that with the increase of temperature from 25 to 37 °C acetylethylenimine at a concentration of 0.01% accelerated the inactivation process by more than an order of magnitude [22]. The pathogen still remained infective when FMDV suspension was treated with 0.05% acetylethylenimine at 37 °C for 8 hours, while with the increase in the exposure time to 12 hours, the virus was completely inactivated [23].

One of the important advantages of such inactivants as β-propiolactone and ethylenimine is that they are completely hydrolyzed within a few hours to form non-toxic products, and therefore there is no need to neutralize them [14].

It is known that formaldehyde and theotropin (A-24) can be used to inactivate rabbit hemorrhagic disease virus [24, 25]. In some instructions for the use of inactivated vaccines against this disease, formalin is indicated as an inactivant [26, 27].

There are several inactivated vaccines available on the Russian market of veterinary medicinal products for RHD prevention: Tissue inactivated hydroxyaluminium vaccine against "Rabbit Hemorrhagic Disease Virus" (FRCVM, Russia) [26]; "Lapimun Gem-2" (BioTestLab, Ukraine) [27]; "Pestorin" (Bioveta, Czech Republic) [28]; "Rabbivak-V" (LLC "TD "Biagro", Russia) [29]. In addition to that, a vector vaccine ("Nobivac® Myxo-RHD", Intervet International B.V., Netherlands) was registered in 2018. It contains a recombinant myxoma virus with the inserted capsid gene of RHDV [30].

However, only "Lapimun Gem-2" contains RHDV1 and RHDV2 antigens, which is officially declared.

Since the level of cross-protection after the immunization with monovaccines is insufficient, the only appropriate solution is to use a combined vaccine containing antigens of both types of the virus [1]. Thus, it was considered reasonable to develop methods for producing such antigens.

CONCLUSION

The results obtained during the assessment of the inactivating effect of the agents used in the study showed that AEEA and β-propiolactone did not have the same effect on RHDV of types 1 and 2. RHDV1 was significantly more sensitive to the used inactivants. In order to preserve at maximum the antigenic structures of the virus, the following inactivation modes were considered to be optimal:

– for RHDV1 – AEEA at a concentration of 0.3% at 37 °C, exposure time 72 hours or β-propiolactone at a concentration of 0.1–0.3% at 25–37 °C, exposure time 24–48 hours;

– for RHDV2 – AEEA at a concentration of 1% at 37 °C, exposure time 72 hours, or β-propiolactone at a concentration of 0.3% at 25 °C, exposure time 24 hours.

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INFORMATION ABOUT THE AUTHORS / ИНФОРМАЦИЯ ОБ АВТОРАХ

Ekaterina D. Kunikova, Post-Graduate Student, Technologist, Laboratory for Avian Diseases Prevention, FGBI "ARRIAH", Vladimir, Russia.

Natalia V. Moroz, Candidate of Science (Veterinary Medicine), Head of Laboratory for Avian Diseases Prevention, FGBI "ARRIAH", Vladimir, Russia.

Maria A. Dolgova, Candidate of Science (Biology), Junior Researcher, Laboratory for Avian Diseases Prevention, FGBI "ARRIAH", Vladimir, Russia.

Lyudmila V. Malakhova, Candidate of Science (Veterinary Medicine), Associate Professor, Department of Epizootology, Parasitology and Microbiology, FSBEI HE Kostroma SAA, Karavaevo, Kostroma Oblast, Russia.

Ilya A. Komarov, Junior Researcher, Laboratory for Avian Diseases Prevention, FGBI "ARRIAH", Vladimir, Russia.

Куникова Екатерина Дмитриевна, аспирант, технолог лаборатории профилактики болезней птиц ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Мороз Наталья Владимировна, кандидат ветеринарных наук, заведующий лабораторией профилактики болезней птиц ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Долгова Мария Алексеевна, кандидат биологических наук, младший научный сотрудник лаборатории профилактики болезней птиц ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Малахова Людмила Васильевна, кандидат ветеринарных наук, доцент кафедры эпизоотологии, паразитологии и микробиологии ФГБОУ ВО Костромская ГСХА, пос. Караваево, Костромская обл., Россия.

Комаров Илья Александрович, младший научный сотрудник лаборатории профилактики болезней птиц ФГБУ «ВНИИЗЖ», г. Владимир, Россия.