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OPTIMIZATION OF CULTIVATION CONDITION OF SUBTYPE H5 FLU VIRUS

Abstract. This study looks into optimal conditions for cultivating the recombinant strains of subtype H5 influenza virus. The study results in establishing optimal conditions (inoculation dose, incubation temperature, incubation time, and chicken embryos' age) for growing the influenza virus.

This study establishes optimum conditions for cultivating the A/Sichuan/26221/2014(H5N6)-PR8-IDCDC-RG42A and A/gyrfalcon/Washington/41088-6/2014(H5N8)-PR8-IDCDC-RG43A subtype H5 influenza virus recombinant strains in embryonated chicken eggs.

Data on culturing influenza virus recombinant strains presented herein indicates that they can be used in developing subtype H5 highly-pathogenic avian influenza vaccines. The results of this research will serve as a basis for developing a new inactivated emulgated vaccine following the process previously used by RIBSP to design its commercial vaccine.

These optimum conditions are an infective dose of 10000 EID_{50}/0.2 cm^3, an incubation temperature of 36±0.5^{0}C, an embryo age of 10 days for cultivating the recombinant strain A/Sichuan/26221/2014(H5N6)-PR8-IDCDC-RG42A. Using these culturing conditions allows a stable production of virus-containing materials with an infectivity level of not less than 8.45±0.24 log EID_{50}/cm^3, which is fully consistent with requirements for producing inactivated vaccines for avian influenza. And optimal conditions for growing recombinant strain A/gyrfalcon/Washington/41088-6/2014(H5N8)-PR8-IDCDC-RG43A in 10 days embryonated chicken eggs with the infective dose 100000 EID_{50} in the incubation temperature 35^{0}C. These optimum conditions are helping culturing a stable production of virus-containing materials with an infectivity level of not less than 8.74±0.06 log EID_{50}/cm^3, which is fully consistent with requirements for producing inactivated vaccines for recombinant strain A/gyrfalcon/Washington/41088-6/2014(H5N8)-PR8-IDCDC-RG43A.

An optimum incubation time of 48 hours, and relative air humidity of 55±5% for cultivating both recombinant strains.

Key words: influenza virus, recombinant strain, cultivation.

Introduction. The highly-pathogenic avian influenza (HPAI) is an extremely contagious pantropic disease affecting various species of poultry and wild birds that can take the form of epizootics and inflict major harm on poultry farming and trade [1]. To date, HPAI outbreaks caused by H5N1, H5N2, H5N6, H5N8, H9N2, H7N9 and other subtypes continue to occur in different regions of the world. A disease caused simultaneously by different viral subtypes is characteristic for recent avian influenza cases around the world [2-4]. Therefore, the strain composition of inactivated vaccines developed by various countries in early 2000s is considered lacking in relevance in terms of clade features compared with the epizootic strains of avian influenza virus of various pathogenicity levels circulating in the environment [5,6]. Thus,

it is important to update the strain composition of existing commercial avian influenza vaccines as part of measures to control this dangerous infectious disease. Major WHO service laboratories have started the production of recombinant strains to be used in manufacturing vaccines to prevent avian influenza.

In 2007, the Research Institute of Biological Safety Problems of the Science Committee under the Ministry of Education and Science of Kazakhstan (RIBSP) developed a technology for producing inactivated emulgated vaccine for clade 2.2, subtype H5 avian influenza. The Institute has an established production of inactivated emulgated vaccines for A/H5N1 avian influenza virus that is used for the specific prevention of subtype H5 avian influenza [7,8].

However, addressing only one clade and subtype of the influenza virus, as it is done at present, is not sufficient for the specific prevention and control of the highly-pathogenic avian influenza in Kazakhstan, given its vast territory. Therefore, like many other vaccine manufacturers throughout the world, we performed studies to establish optimal culturing properties of a range of recombinant viruses produced in service laboratories.

Materials and Methods. Our experiments used recombinant strains:

- A/Sichuan/26221/2014 (H5N6)-PR8-IDCDC-RG42A;
- A/gyrfalcon/Washington/41088-6/2014(H5N8)-PR8-IDCDC-RG43A.

Virus Cultivation

We used standard methodology to inoculate 10-, 11- and 12-day old chicken embryos [9]. We cultivated the recombinant strains in accordance with their data sheet information and RIBSP's existing technical guidance for inactivated emulgated AI vaccine. We incubated inoculated embryos for 48 and 72 hours at different temperatures (35±0.5°C, 36±0.5°C and 37±0.5°C).

Assessing Viruses' Infectious Activity

We used common methodology in assessing the viruses' infectious activity. We calculated titration results based on L. Reed & H. Muench method and expressed them in decimal logarithms EID_{50}/cm^3 [10].

Statistic Processing

In calculating the average value of studied parameters, we considered $P<0.05$ as significant.

Study Results. At the initial phase of the study we inoculated 10-days' old embryos with 0.2 ml of 10^{-4} viral dilution and incubated them for 48 hours at 36±0.5°C and a relative air humidity of 55±5 %. We collected virus-containing allantoic fluid (VAF) once inoculated embryos had cooled and assessed their infective and hemagglutination activity and sterility. The study results are shown in table 1.

Table 1 – Control of the recombinant strains subtype H5 influenza virus strains of for consistency with its data sheet information

Strain Name	Hemagglutination activity	Infective Activity, ($\log_{10} EID_{50}$)	Sterility
A/Sichuan/26221	1:128	8.45±0.14	Sterile
A/gyrfalcon/Washington/41088-6	1:1024	8,74 ± 0,06	Sterile

As seen in table 1, the recombinant strains are consistent with its data sheet on all characteristics controlled.

The recombinant strains insert did not specify the age of chicken embryos for virus cultivation; and we had to assess the optimal age for virus cultivation at RIBSP. To do that, we tested chicken embryos that were 10, 11 and 12 days old. We incubated virus-inoculated embryos at various temperatures (35±0.5°C, 36±0.5°C and 37±0.5°C). We did this at a relative air humidity of 55 ± 5 % (figure, tables 2 and 3).

Table 2 – Quantity build-up of the A/Sichuan/26221/2014(H5N6)-PR8-IDCDC-RG42A strain depending on chicken embryo age and incubation temperature

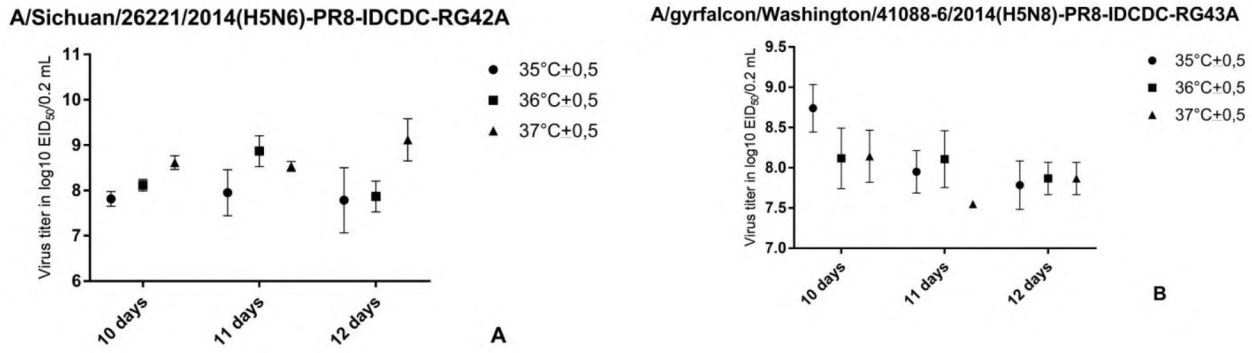
Embryo age	35±0.5°C		36±0.5°C		37±0.5°C	
	Titer in hemag-glutination test	Infective activity, log EID_{50}/cm^3	Titer in hemag-glutination test	Infective activity, log EID_{50}/cm^3	Titer in hemag-glutination test	Infective activity, log EID_{50}/cm^3
10	1:128	7.81 ± 0.06	1:512	8.12 ± 0.22	1:256	8.61 ± 0.07
11	1:256	7.95 ± 0.15	1:256	8.87 ± 0.15	1:256	8.53 ± 0.07
12	1:256	7.78 ± 0.07	1:256	7.87 ± 0.15	1:128	9.12 ± 0.15

As seen from table 2, the virus is well-adapted to the embryos, and the inoculated embryos do not die at incubation. The highest viral quantities are produced by incubating 10-day-old embryos at 36±0.5^{0}C.

Table 3 – Quantity build-up of the A/gyrfalcon/Washington/41088-6/2014(H5N8)-PR8-IDCDC-RG43A strain depending on chicken embryo age and incubation temperature

Embryo age	35±0.5^{0}C		36±0.5^{0}C		37±0.5^{0}C	
	Titer in hemag-glutination test	Infective activity, log EID_{50} /cm^3	Titer in hemag-glutination test	Infective activity, log EID_{50} /cm^3	Titer in hemag-glutination test	Infective activity, log EID_{50} /cm^3
10	1:1024	8.74 ± 0,06	1:128	8.12 ± 0,22	1:128	8.14 ± 0,07
11	1:128	7.95 ± 0,15	1:256	8.11 ± 0,11	1:64	7.55 ± 0,07
12	1:256	7.78 ± 0,07	1:128	7.87 ± 0,15	1:32	7.87 ± 0,15

As seen from table 3, the highest viral quantities are produced by incubating 10-day-old embryos at 34±0.5^{0}C.



Growth characteristics of recombinant strains in eggs (A, B).
Infectious titers were measured as EID_{50}/ml (50% egg infectious dose per milliliter)

Data from figure shows that the various incubation temperatures selected did not have any notable effect on the viruses' accumulation rate or the recombinant strains' infective activity. We observed virtually no death of infected embryos during incubation. The higher virus accumulation rates occurred at the incubation temperature of 36^{0}C±0.5 in 10-day old embryos, as a result, we chose the incubation temperature of 35^{0}C±0.5 because A/Sichuan/26221/2014(H5N6)-PR8-IDCDC-RG42A had a high hemagglutination activity of 1:512 and an infectious activity of 8.12 ± 0.22 log10 EID_{50}/0.2 ml at this incubation temperature. The hemagglutination activity of A/gyrfalcon/Washington/41088-6/2014(H5N8)-PR8-IDCDC-RG43A at 35^{0}C±0.5 totaled 1:1024 while its infectious activity was at 8.74 ± 0,06 log10 EID_{50}/0.2 ml.

Then we performed an experiment to assess the optimal incubation time for inoculated embryos. We incubated inoculated embryos for 48 and 72 hours. During the incubation, we used a relative air humidity of 55 ± 5%.

Further on, we assessed the quantities of viral build-up depending on the infective dose, the aim being to increase the virus's infectivity. We performed our experiments at standard culturing conditions: a temperature of 36±0.5^{0}C for recombinant strain A/Sichuan/26221/2014(H5N6)-PR8-IDCDC-RG42A and 35±0.5^{0}C for recombinant strain A/gyrfalcon/Washington/41088-6/2014(H5N8)-PR8-IDCDC-RG43A, relative air humidity of 55±5%, with chicken embryos 10 days old. We introduced the virus into embryos' allantoic cavity in doses of 10 to 1000000 EID_{50}. As we incubated the inoculated embryos, we performed their ovoscopy every 3 hours. We recorded times of embryos' death. We assessed the quantities of viral build-up based on the infective and hemagglutinative activity. The results are shown in tables 4 and 5.

Table 4 – Build-up quantities of the A/Sichuan/26221/2014 (H5N6)-PR8-IDCDC-RG42A recombinant strain depending on the infective dose (X ± m), n=3)

Infective dose, EID_{50}	Number of dead / total chicken embryos in experiment	Activity	
		Hemagglutinative	Infective, $log_{10}EID_{50}$/ml
~10	0/30	1:64	8.45 ± 0.14
~100	0/30	1:128	8.70 ± 0.08
~1000	2/30	1:128	8.70 ± 0.17
~10000	0/30	1:128	8.45 ± 0.24
~100000	2/30	1:128	8.20 ± 0.2
~1000000	1/30	1:256	7.46 ± 0.12

As shown in table 4, almost no embryos died during incubation. The highest viral build-up is seen in embryos inoculated in doses from 1000 to 10000 EID_{50}. Viral hemagglutinative activity was stable, although much higher in the VAF taken from embryos inoculated with a dose of 1000000 EID_{50}.

Table 5 – Build-up quantities of the A/gyrfalcon/Washington/41088-6/2014(H5N8)-PR8-IDCDC-RG43A recombinant strain depending on the infective dose (X ± m), n=3)

Infective dose, EID50	Number of dead / total chicken embryos in experiment	Activity	
		Hemagglutinative	Infective, $log_{10}EID_{50}$/ml
~10	0/30	1:32	6.45 ± 0.14
~100	0/30	1:64	7.70 ± 0.08
~1000	2/30	1:128	7.70 ± 0.17
~10000	0/30	1:256	8.17 ± 0.11
~100000	2/30	1:512	8.74 ± 0.06
~1000000	1/30	1:512	7.46 ± 0.12

As shown in table 5, almost no embryos died during incubation. The highest viral build-up is seen in embryos inoculated in doses 100000 EID_{50}. Viral hemagglutinative activity was stable 1:512.

Thus, we determined that the optimal infective doses of the A/Sichuan/26221/2014 (H5N6)-PR8-IDCDC-RG42A influenza recombinant strain for chicken embryos range from 1000 to 10000 EID_{50}; these dosages enable the development of a virus-containing material with a high infective and hemagglutinative activity. And optimal infective doses of the A/gyrfalcon/Washington/41088-6/2014(H5N8)-PR8-IDCDC-RG43A influenza recombinant strain for chicken embryos range 100000 EID_{50}; these dosages enable the development of a virus-containing material with a high infective and hemagglutinative activity.

Discussion and Conclusion. Our study establishes optimum conditions for cultivating the A/Sichuan/26221/2014(H5N6)-PR8-IDCDC-RG42A and A/gyrfalcon/Washington/41088-6/2014(H5N8)-PR8-IDCDC-RG43A subtype H5 influenza virus recombinant strains in embryonated chicken eggs. Data on culturing influenza virus recombinant strains presented herein indicates that they can be used in developing subtype H5 highly-pathogenic avian influenza vaccines. The results of this research will serve as a basis for developing a new inactivated emulgated vaccine following the process previously used by RIBSP to design its commercial vaccine.

These optimum conditions are: an infective dose of 10000 EID_{50}/0.2 cm^3, an incubation temperature of 36±0.5^{0}C, an embryo age of 10 days, an incubation time of 48 hours and a relative air humidity of 55±5% for cultivating the recombinant strain A/Sichuan/26221/2014(H5N6)-PR8-IDCDC-RG42A. Using these culturing conditions allows a stable production of virus-containing materials with an infectivity level of not less than 8.45±0.24 log EID_{50}/cm^3, which is fully consistent with requirements for producing inactivated vaccines for avian influenza.

We identified the following optimal conditions for growing A/gyrfalcon/Washington/41088-6/2014(H5N8)-PR8-IDCDC-RG43A recombinant strain in embryonated chicken eggs: the infective dose

100000 EID_{50}; the embryo age 10 days; the incubation temperature 35^{0}C, and incubation time 48 hours. These optimum conditions are helping culturing a stable production of virus-containing materials with an infectivity level of not less than 8.74±0.06 log EID50/cm3, which is fully consistent with requirements for producing inactivated vaccines for recombinant strain A/gyrfalcon/Washington/41088-6/2014(H5N8)-PR8-IDCDC-RG43A.

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ҚҰС ТҰМАУЫНЫҢ H5 СУБТИПІН ӨСІРУ ЖАҒДАЙЫН ОҢТАЙЛАНДЫРУ

Аннотация. Мақалада H5 субтипті тұмау вирусының A/Sichuan/26221/2014 (H5N6)-PR8-IDCDC-RG42A және A/gyrfalcon/Washington/41088-6/2014(H5N8)-PR8-IDCDC-RG43A рекомбинантты штамм өсірудің оңтайлы параметрлері ұсынылған. Нәтижесінде тұмау вирусын өсірудің оңтайлы параметрлері (жұқтыру мөлшері, инкубация температурасы, инкубация мерзімі және тауық эмбриондарының жасы) анықталған.

Зерттеудің бастапқы кезеңінде, штамм паспортына сәйкес 10 тәуліктік эмбриондарға 0,2 мл көлемде 10^{-4} есе сұйылтылған вирус жұқтырылып, 48 сағат бойы түрлі температурада және ауаның салыстырмалы ылғалдылығы 55±5% инкубацияланды. Вирусқұрамды аллантоис сұйықтығын (ВАС) жинау инфекция жұқтырылған эмбриондар салқындағаннан кейін жүргізілді және олардың инфекциялық және гемагглютиндеуші белсенділігі, сондай-ақ ВАС зарарсыздығы анықталды.

Осылайша A/Sichuan/26221/2014 (H5N6)-PR8-IDCDC-RG42A және A/gyrfalcon/Washington/41088-6/2014(H5N8)-PR8-IDCDC-RG43A рекомбинантты штамм көрсеткіштері бойынша паспортта көрсетілген деректермен сәйкес келеді.

Рекомбинантты штамм алуда ілеспе құжатта вирус өсіру үшін тауық эмбриондарының жасы көрсетілмеген және БҚПҒЗИ жағдайында эмбриондардың рекомбинантты штамм өсірудің оңтайлы жасын анықтау қажеттігі шықты. Осы мақсатта 10, 11 және 12 тәуліктік ТЭ сынақтан өткізілді. Инфекцияланған эмбриондар түрлі температура режимінде (35, 36 және 37±0,50 С) инкубацияланды. Ауаның салыстырмалы ылғалдылығы 55 ± 5 % құрады.

Зерттеу нәтижелері бойынша вирус эмбриондарға бейімделіп, инфекция жұқтырылған эмбриондар алдыңғы тәжірибедегідей инкубациялау кезінде эмбриондар өлімі тіркелмеді. Вирустың жиналу деңгейінің жоғары көрсеткіші жұқтырылған эмбриондарды 35±0,5^{0}С және 36±0,5^{0}С температурада инкубациялау кезінде 10 тәуліктік тауық эмбрионында байқалды және зиянсыз.

Мұнан кейін инфекция жұқтырылған эмбриондарды инкубациялаудың оңтайлы мерзімін анықтау бойынша зерттеулер жүргізілді. Зарарланған эмбриондар 48 және 72 сағат бойы инкубациялауға салынды. Ауаның салыстырмалы оңтайлы ылғалдылығы 55±5% құрады. Инкубациялау нәтижесінде гемагглютиндеуші белсенділігі 1:128 A/Sichuan/26221/2014 (H5N6)-PR8-IDCDC-RG42A рекомбинантты штамынан вирусқұрамды материалдар алынды. Осы себептен одан әрі зерттеу үшін тұмау вирусын 36^{0}С+0,5 кезінде 48 сағат бойы инкубациялау мерзімі пайдаланылды. Ал A/gyrfalcon/Washington/41088-6/2014(H5N8)-PR8-IDCDC-RG43A рекомбинантты штамм үшін оптималды инкубациялау мерзімі 48 сағат оңтайлы 35^{0}С+0,5 температура режимінде гемагглютиндеуші белсенділігі 1:1024 вирусқұрамды материал алынды.

Вирустың инфекциялық белсенділігін арттыру мақсатында кейінгі тәжірибеде вирустың жұқтырушы дозаға байланысты жиналу деңгейі анықталды. Зерттеулер жалпы қабылданған өсіру параметрлерін пайдалану арқылы жүргізілді, яғни температура 36±0,50^{0}С, ауаның салыстырмалы ылғалдылығы 55±5%, тауық эмбрионының (ТЭ) жасы 10 тәулік екені анықталды. Эмбриондар аллантоис қуысына 10-нан 1000000 $ЭИД_{50}$-ге дейінгі мөлшерде жұқтырылды. Инфекция жұқтырылған эмбриондарды инкубациялау барысында әрбір 3 сағат сайын овоскоптау жүргізілді, сонымен қатар эмбриондардың өлу уақыты тіркелді. Жоғары инфекциялық және гемагглютиндеуші белсенділігі бар жоғары белсенді вирусқұрамды материалды көбейтуге мүмкіндік беретін 1000-нан 10000 $ЭИД_{50}$-ге дейінгі дозасы тұмау вирусының A/Sichuan/26221/2014 (H5N6)-PR8-IDCDC-RG42A ТЭ үшін тиімді жұқтырғыш дозасы болып саналады. A/gyrfalcon/Washington/41088-6/2014(H5N8)-PR8-IDCDC-RG43A рекомбинантты штамм үшін ТЭ жұқтыру дозасы 100000 EID_{50} болып анықталды, сонымен қатар аталмыш доза жоғары инфекциялық және геммаглютиндігі белсенді вирусқұрамды материал алуға болатындығы дәлелденді.

Түйін сөздер: тұмау вирусы, рекомбинанттық штамм, өсіру.

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ОПТИМИЗАЦИЯ УСЛОВИЙ КУЛЬТИВИРОВАНИЯ ВИРУСОВ ГРИППА СУБТИПА H5

Аннотация. В данной работе представлены оптимальные параметры культивирования рекомбинантных штаммов A/Sichuan/26221/2014 (H5N6)-PR8-IDCDC-RG42A и A/gyrfalcon/Washington/41088-6/2014(H5N8)-PR8-IDCDC-RG43A вируса гриппа субтипа H5. В результате чего установлены оптимальные параметры культивирования (доза заражения, температура инкубации, срок инкубации и возраст куриных эмбрионов) вируса гриппа. На начальном этапе исследований, согласно паспортных данных штаммов, куринные эмбрионы (КЭ) 10 сут возраста были заражены вирусом в разведении 10^{-4} в объеме 0,2 мл и инкубированы при различных температурах и относительной влажности воздуха 55±5% на протяжении 48 часов. Сбор вируссодержащей аллантоисной жидкости (ВАЖ) проводили после охлаждения инфицированных эмбрионов и определяли их инфекционную и гемагглютинирующую активность, а также стерильность ВАЖ.

Таким образом, рекомбинантные штаммы A/Sichuan/26221/2014 (H5N6)-PR8-IDCDC-RG42A и A/gyrfalcon/Washington/41088-6/2014(H5N8)-PR8-IDCDC-RG43A по всем показателям соответствует паспортным данным.

При получении рекомбинантных штаммов в сопроводительном документе не были указаны возраст КЭ для культивирования вируса и необходимо было определить оптимальный возраст эмбрионов в условиях НИИПББ. Для этого были апробированы КЭ 10, 11 и 12 сут. возрастов. Инфицированные эмбрионы инкубировали при различных температурных режимах (35, 36 и 37±0,5°С). Относительная влажность воздуха составляла 55 ± 5 %.

Согласно результатам исследования вирус адаптирован к КЭ, инфицированные эмбрионы не погибают при инкубировании как в предыдущем опыте. Более высокие показатели накопляемости вируса отмечаются при температуре инкубирования инфицированных эмбрионов 36±0,5^{0}С и 35±0,5°С на 10 сут. КЭ и являются безвредными.

Далее были проведены исследования по определению оптимальных сроков инкубирования инфицированных КЭ. Зараженные КЭ были заложены для инкубирования на протяжении 48 и 72 часов. Относительная влажность воздуха составляла 55±5 %. В результате инкубирования получены вируссодержащие материалы с одинаковой гемагглютинирующей активностью (1:128). Поэтому для дальнейшего исследования использованы сроки инкубирования вирусов гриппа в течение 48 часов при 36°С+0,5 для рекомбинантного штамма A/Sichuan/26221/2014 (H5N6)-PR8-IDCDC-RG42A. А для рекомбинантного штамма A/gyrfalcon/Washington/41088-6/2014(H5N8)-PR8-IDCDC-RG43A оптимальные сроки инкубирования 48 часов, температура инкубирования была 35°С+0,5, при этом гемагглютинирующяя активность составила 1:1024.

С целью повышения инфекционной активности вируса определяли уровень накопления вируса в зависимости от заражающей дозы. Исследования проводили с использованием общепринятых параметров культивирования – температура 36±0,5^{0}С, относительная влажность воздуха 55±5%, возраст КЭ 10 суток. КЭ инфицировали в аллантойсную полость в дозах от 10 до 1000000 ЭИД$_{50}$. В процессе инкубирования инфицированным КЭ через каждые 3 часа проводили овоскопирование. Регистрировали время гибели КЭ. Таким образом, оптимальной заражающей дозой рекомбинантного штамма A/Sichuan/26221/2014 (H5N6)-PR8-IDCDC-RG42A вируса гриппа для КЭ является доза от 1000 до 10000 ЭИД$_{50}$, а также оптимальная заражающая доза для КЭ является доза 100000 ЭИД$_{50}$ позволяющая наработать высокоактивный вируссодержащий материал с высокой инфекционной и гемагглютинирующей активностью. Для получения оптимальной инфекционной дозы рекомбинантного штамма гриппа A/gyrfalcon/Washington/41088-6/2014(H5N8)-PR8-IDCDC-RG43A для КЭ составляют доза заражения 100000 EID$_{50}$; Эти дозировки позволяют создать вируссодержащий материал с высокой инфекционной и гемагглютинирующей активностью.

Ключевые слова: вирус гриппа, рекомбинантный штамм, культивирование.

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