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# OPTIMIZATION OF INFLUENZA A AND B VIRUSES CULTIVATION CONDITIONS FOR PREPARATION OF TRIVALENT SEASONAL INFLUENZA SPLIT VACCINE

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**Keywords:**

Influenza A and B viruses, cultivation conditions, chicken embryos, seasonal influenza vaccine

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**ABSTRACT:** This article presents the results of studies on determination of optimal parameters of recombinant strains A/NYMC X-217 (H3N2) and B/NYMC BX-49 of influenza virus cultivation in chicken embryos. It is found that a maximum virus accumulation is formed under the following cultivation conditions: age of chicken embryos is 9-11 days in case of inoculation of the allantoic cavity, inoculated virus dose — 1,000-10,000 $EID_{50}$, temperature and time of incubation — (34 ± 0.5) $^{o}$C and 48 h, respectively. If the cultivation parameters are satisfied, it is possible to obtain a highly active virus-containing material with infectious and hemagglutinating activity, as well as a content of hemagglutinin not less than 7.0lg $EID_{50}$/ml, 1:256 and 7.0μg/ml, respectively, that is enough to prepare a trivalent inactive seasonal influenza split vaccine.

**INTRODUCTION:** Influenza continues to be a serious problem for human health for quite a long time giving rise to annual epidemics when up to 10% of total population of the world fall ill. Moreover, during occasional pandemics of influenza, that number may grow up to 4-6 times. Furthermore, practically all influenza epidemics are attended by an increase of a mortality rate [1]. Influenza epidemics cause economic damage, estimated in billions of USA dollars.

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The main strategy of the fight against influenza is a vaccination. At present three types of inactive vaccines: whole virus vaccines, split vaccines and subunit vaccines are used in the world practice of preventive vaccination. Among these three types of influenza vaccines, whole virus vaccine contains the whole set of antigens of actual influenza virus strains, their immunogenicity is high, but due to the great number of antigens in these vaccines, they are the most reactogenic and a percentage of adverse reactions occurrence after their administration is a maximum. Due to that fact these vaccines have age restrictions for use.

Subunit vaccine contains only influenza virus surface antigens (HA and NA), which assures a good immunogenicity and minimal adverse events associated with administration (allergy to vaccine

components, such as chicken egg protein) [2]. Split vaccines are characterized by the significantly lower risk of adverse reactions, supposedly due to a destruction of viral dimensional structure. An advantage of split vaccines is that they contain both surface and core influenza virus antigens, so they are free from the foremost default of whole virus vaccines that is a presence of toxins. Split vaccines can be used in children at the age of 6 months and over.

They are recommended to prevent influenza in pregnant women. In fact, split vaccines present a "the golden mean" in the influenza prevention, as they are similar to subunit vaccines on level of adverse reactions, and are similar to whole virus vaccines on immunological efficiency. Prophylactic efficacy of vaccines of this class varies in the range of 75–96%. Split vaccines and subunit vaccines can be used for vaccination of people with immune defects, pregnant and nursing women; it is allowed to administer in the course of the immunosuppressive therapy. [3]

Vaccine formula is changed every year to assure a maximum protection against a wild-type virus. Depending on WHO prognosis regarding a type of virus which will circulate during the next season, different agents are included in the vaccine. Usually 3 types of virus antigens are in composition; these are two types A and one type B.

Up to date studies aimed to develop seasonal trivalent influenza vaccine for health service haven't been performed in Kazakhstan, and due to that fact there is a real threat to national safety in the case of an influenza pandemic. Works to prepare a trivalent seasonal influenza split vaccine are performed in the Scientific Research Institute for Biological Safety Problems to solve the above mentioned problem. A development of vaccine virus cultivation conditions with the aim of their accumulation is an important step on the way of viral vaccine development.

At present, chicken embryos (CE) are known to be the principal medium for influenza virus cultivation. A high efficiency of CE use has been proved as compared to various cell cultures (MDCK, Vero, HeLa, chicken embryo fibroblast) providing a possibility to obtain a highly active viral material applicable to produce inactive vaccines avoiding an expensive procedure of concentrating [4]. In the best way adapted to this system strains of AIV (avian influenza virus) result in rather high titers in CE (about $10^4$-$10^5$ hemagglutinating units per 1 embryo). In addition, it is not required to provide special aseptic conditions for an embryo isolated from an environmental effect that also increase their value in use [5, 6, 7].

Discrepant data regarding parameters of the influenza virus (IV) cultivation depending on a strain group and a degree of their adaptability to the biosystem are given in literature. For the most IV are cultivated in CE at the age of 10-13 days, are inoculated in allantoic cavity in doses of 100-10,000 $EID_{50}$ and incubated under the temperature of 34-38ºC. As seen regulations of IV strain cultivation in CE greatly vary, that forces many manufacturers of influenza virus vaccine to identify cultivation parameters of new vaccine strains of influenza virus for the purpose of production of reliable and highly efficient vaccines [6].

In this connection the aim of this study is to specify optimal cultivation parameters of A/NYMC X-217 (H3N2) and B/NYMC BX-49 recombinant strains to obtain a highly active virus-containing material (VCM) required developing a manufacturing method for seasonal trivalent inactive split vaccine against seasonal influenza.

## MATERIALS AND METHODS:

### Influenza viruses and chicken embryos:

For optimization of culture-based properties of influenza viruses A and B the following influenza virus strains have been used (according to WHO recommendations); NYMC X-217 (NIBSC code: 12/100) recombinant strain obtained in NIBSC (Great Britain) from A/Victoria/361/2011 (H3N2) and A/PR/8/34 (H1N1) strains by a reverse genetic method, gene ratio 6:2; NYMC BX-49 (NIBSC code: 11/244) recombinant strain obtained in NIBSC (Great Britain) from B/Texas/06/2011 (Yamagata line) strains by a reverse genetic method and BX-38-1P-1 (B/Lee/40-like high

growth reassortant/Panama/45/90 PA and NS genes), gene ratio 1:1:6, passage level 7, hemagglutinating activity 1:512; Chicken embryos (CE) from the Joint-Stock Company "Alel Agro" (Almaty Province, Kazakhstan).

**Optimization of viral cultivation parameters:**
For the purpose to specify an infective dose of A/NYMC X-217 (H3N2) and B/NYMC BX (IV) recombinant strains an inoculation was made into CE allantoic cavity in doses of 1 till 1,000,000 and from 1 till 100,000 $EID_{50}$, respectively. To identify and optimal age, temperature and time periods of infected EC incubation 9, 10, 11, 12, 13 — days CEs were infected with a dose of 1,000 EID50 and incubated during 24, 48, 72, 96 h under the temperature of (32 ± 0.5) ºC, (34 ± 0.5) ºC, (36 ± 0.5)ºC, (38 ± 0.5) ºC. A level of virus accumulation was estimated by titration in CEs, hemagglutination reaction (HR) and single radial immunodiffusion (SRID).

**Identification of an infective activity of viruses:**
An infective activity was identified by virus titration in CE using the uniform method 5. For this purpose ten-folds dilution of viral suspension in physiological buffer (PBS) from $10^{-1}$ till $10^{-10}$. 0.2 ml of each viral material dilution was inoculated in 4 CEs into allantoic cavity. CE cultivation was performed during 2 days under the temperature of 34 ± 0.5 ºC and 55 ± 5% relative humidity. The presence of virus in CE was identified after cooling by a drop-by-drop method in HR. Viral titer was calculated by [8] method and expressed in $log_{10}$ $EID_{50}$/ml.

**Identification of hemagglutinating viral activity:**
Hemagglutinating viral activity was identified by the uniform method in HR using 1 % suspension of chicken red blood cells. [9]

**Identification of hemagglutinin concentration in SRID:**
Identification of hemagglutinin concentration in HR was performed by single radial immunodiffusion (SRID) method according to (Palmer, 1975) [10].

**Statistical processing:** Arithmetic mean values of experimental variables as well as their standard error were determined. Significance of difference among measures was identified using statistical GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) program. *P*-value < *0.05* was considered to be significant. Correlation analysis between influenza viral titers obtained by HR and SRID method was performed using a random linear paired K. Pearson coefficient of correlation.

**RESULTS AND DISCUSSION:**
**Dose of CE infection:**
To specify optimal cultivation parameters of A/NYMC X-217 (H3N2) and B/NYMC BX-49 recombinant strains of influenza virus in CE, studies on investigating a level of virus accumulation in a case of various infecting doses have been performed. At that, from 1 till 1,000,000 EID50 were used for A/NYMC X-217 (H3N2) strain and from 1 till 100,000 EID50 were used for B/NYMC BX-49 strain. Results of the performed studies are shown in **Fig.1**.

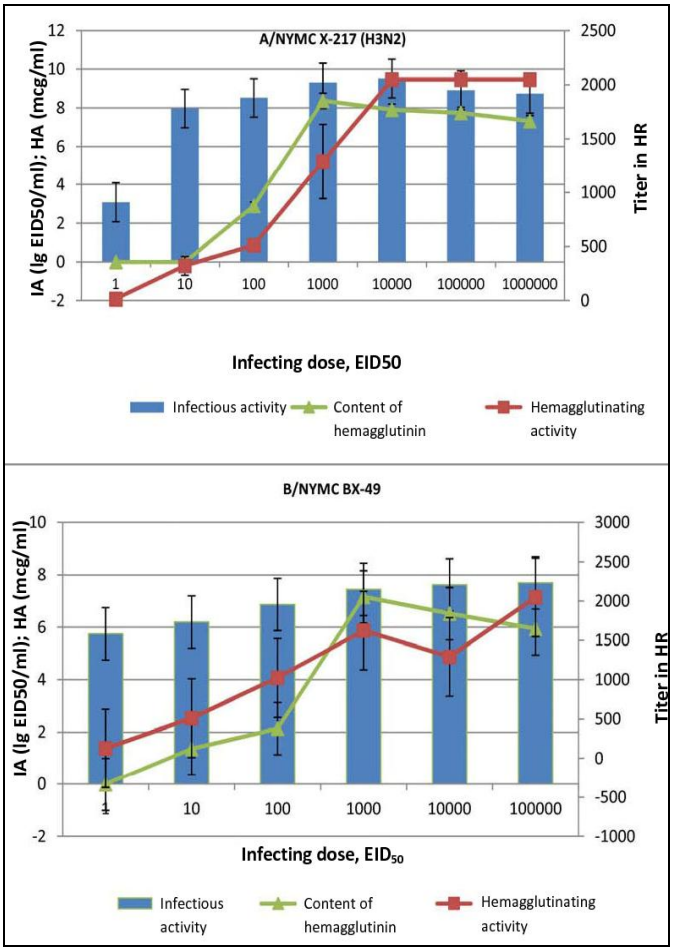


**FIG. 1: LEVEL OF A/NYMC X-217 (H3N2) STRAIN AND B/NYMC BX-49 VIRUS IN CE ACCORDING TO AN INFECTING DOSE.**

It can be seen in **Fig. 1**, that A/NYMC X-217 (H3N2) and B/NYMC BX-49 strains with all tested doses are capable of accumulation in CE with rather high titers. Maximum values of infectious and hemagglutinin viral activity were obtained by using doses of A/NYMC X-217 (H3N2) strain in the range of 1,000 — 10,000 $EID_{50}$ and B/NYMC BX-49 in the range of 1,000 — 100,000 $EID_{50,}$ at that it was found a dependence of viral accumulation level on infecting dose.

When the viral dose is increased from 1 till 10,000 $EID_{50}$ ($P < 0.05$) in CE an increase of such titers as an infectious and hemagglutinating activity is registered, as well as a quantitative content of hemagglutinin. Subsequently, with an infecting dose escalation no growth of infectious hemagglutinating activity was observed as well as of hemagglutinin content. A very high degree of correlation between values of A/NYMC X-217 (H3N2) and B/NYMC BX-49 influenza viral strains on HR and SRID was also determined, coefficient of correlation is r=0.9 and r=0.8, respectively.

Based on the above-mentioned and taking into account the highest value of an infectious, hemagglutinating activity and a hemagglutinin content as an optimal CE infecting dose for A/NYMC X-217 (H3N2) and B/NYMC BX-49 strains, a dose at the range of 1,000-10,000 $EID_{50}$ was accepted, since the further increase of an infecting dose didn't result in a statistically significant ($P > 0.05$) increase of the viral activity. The results we obtained are consistent with the data of some authors [11, 12, 13.]

**Temperature of viral incubation:**

In the following set of experiments a level of accumulation of A/NYMC X-217 (H3N2) and B/NYMC BX-49 influenza virus strains depending on temperature of the infected CE incubation. 10 days CE inoculated with 0.2 ml virus–containing material from each strain A/NYMC X-217 (H3N2) and influenza virus B/NYMC BX-49 in the dose of 1,000 $EID_{50}$ were used in the experiments. Subsequent virus cultivation was performed using the method mentioned in the section "Materials and methods". Infected CEs were incubated at different temperatures of 32, 34, 36, 38 ºC and relative humidity (60 ± 5) % during 48 h. After incubation a collection of allantoic fluid (AF) to identify an infectious, hemagglutinating activity and HA content was performed.

Results of the performed studies are shown in **Fig. 2.**

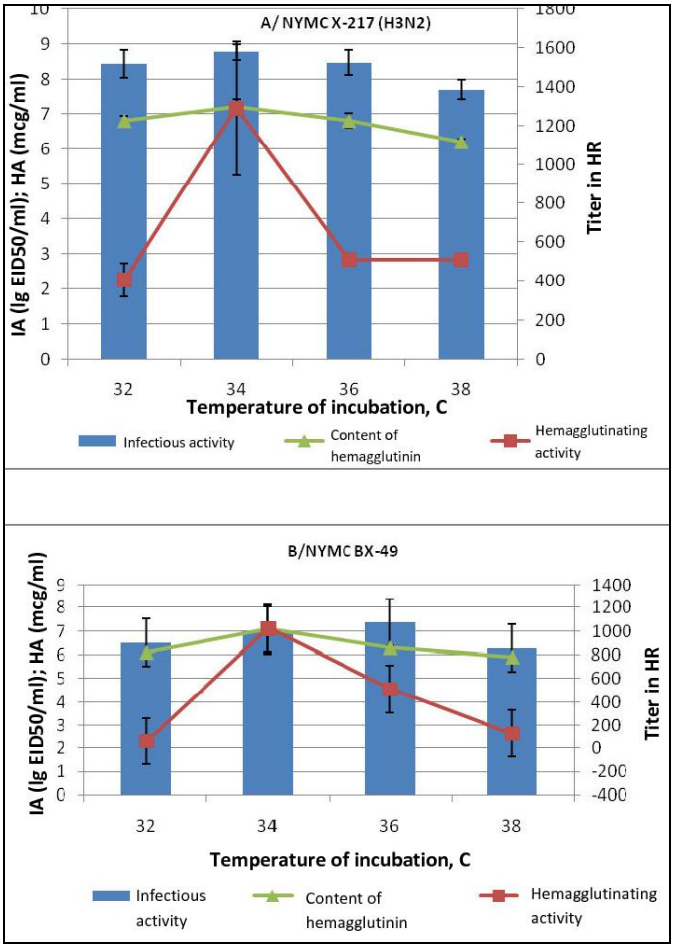


**FIG. 2: THE LEVEL OF ACCUMULATION OF A/NYMC X-217 (H3N2) AND B/NYMC BX-49 VIRUS STRAINS IN CE ACCORDING TO THE TEMPERATURE OF INCUBATION.**

Data of the **Fig. 2** point to a high level of reproduction of influenza virus strains regardless of the incubation temperature. Statistical analysis showed reliability of the results ($P < 0.05$), a maximum viral accumulation takes place at the temperature of 34 C and having in mind an economic efficiency and technological effectiveness of the manufacturing a temperature of (34 ± 0.5) °C has been chosen as an optimal incubation temperature for A/NYMC X-217 (H3N2) and B/NYMC BX-49 influenza virus strains in CE. Correlation analysis showed that mean coefficient of correlation (r=0.6) was registered between values of HR and SRID titers of

A/NYMC X-217 (H3N2) influenza virus strain and a very high coefficient of correlation (r=0.9) of B/NYMC BX-49 strain. Investigated strains IV had high reproductive performance in CE at cultivation temperature (34 ± 0.5) ºC; that is consistent with the literature data (Egorov, 1984; Tabynov, 2009) [14, 15].

**CE age for introduction of infection:**

According to the results of in-house studies on influenza virus (IV) reproduction on some extent the age of CE makes an impact. For the purpose of determination of the viral accumulation level according to CE age we performed the following series of studies. For this purpose CE of 9, 10, 11, 12, 13 days age were infected with the dose 1,000 $EID_{50}$. During 48 h after incubation at temperature of (34 ± 0.5) °C AF collection was performed and infectious viral activity was identified by the method of titration in CE, hemagglutinating viral activity was identified by hemagglutination test and a content of hemagglutinin was determined by SRID method. Results of the performed studies are shown in **Fig. 3**.

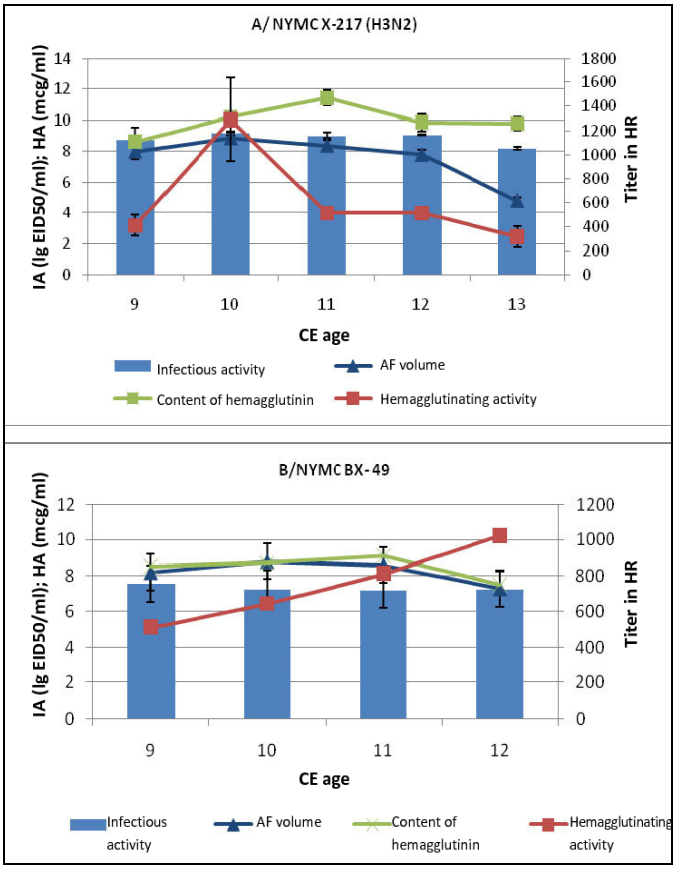


**FIG.3: THE LEVEL OF ACCUMULATION OF A/NYMC X-217 (H3N2) AND B/NYMC BX- 49 INFLUENZA VIRAL STRAINS IN CE DEPENDING ON THEIR AGE**

From the **Fig. 3** it follows that the level of reproduction of two A/NYMC X-217 (H3N2) and B/NYMC BX-49 influenza virus strains is high irrespective of CE age: an infectious activity is from (7/20 ± 0.14) till (9.16 ± 0.12) $log_{10}$ $EID_{50}/cm^3$; hemagglutinating activity is from 322 ± 86.42 till 1290 ± 345.45; HA content is from (7.53 ± 0.9) till (11.43 ± 0.49)μg/ml; reliability of the results ($P < 0.05$). At that viral reproduction in 12-13 days CE results in a decrease of egg-derived virus fluid volume and a degradation of its quality.

A very weak correlation (r=0.2) between the values of IV titers on HR and SRID of A/NYMC X-217 (H3N2) strain and a weak negative correlation (r = -0.5) between a hemagglutinating activity and a content of hemagglutinin were shown. Thus, according to the results of the performed studies CE of 9-11 days age are recommended to grow A/NYMC X-217 (H3N2) and B/NYMC BX-49 strains, that is consistent with the literary data (Ershebulov, 2010; Ershebulov, 2012; Ewasyshyn, 1986; Wang, 1986).[11, 16, 17]

**Time period of virus cultivation in CE:**

The final stage in the study on optimization of cultivation conditions for A/NYMC X-217 (H3N2) and B/NYMC BX-49 influenza virus strains was to determine an optimal time period for viral cultivation in CE. For this purpose CE of 10 days age were infected by influenza virus in 1,000 $EID_{50}$ dose and incubated at the temperature of (34 ± 0.5) °C. In 24, 48, 72 and 96 h infected embryos were cooled during 12-14 h at the temperature 4 ± 1 °C and AF was collected to specify an infectious, hemagglutinating activity and a content of HA of the accumulated virus. The results of the study are shown in **Fig.4**.

From the data in **Fig. 4** it can be seen that in case of infecting 10 days CE by A/NYMC X-217 (H3N2) and B/NYMC BX- 49 strains the highest viral accumulation is registered in 48-96 h of incubation. The mean degree of correlation (r=0.7) for A/NYMC X-217 (H3N2) strain and mild degree of correlation (r=0.3) for B/NYMC BX — 49 strain between the values of IV titers on HR and SRID were specified. For the purposes to reduce technological process duration, a regimen of 48 h of incubation has been chosen as an optimal time

period. Based on the performed comparative analysis of the obtained results with literature data it may be concluded that the period of 48h incubation is an optimal period for influenza virus (Ershebulov, 2010; Ewasyshyn, 1986). [11, 16]

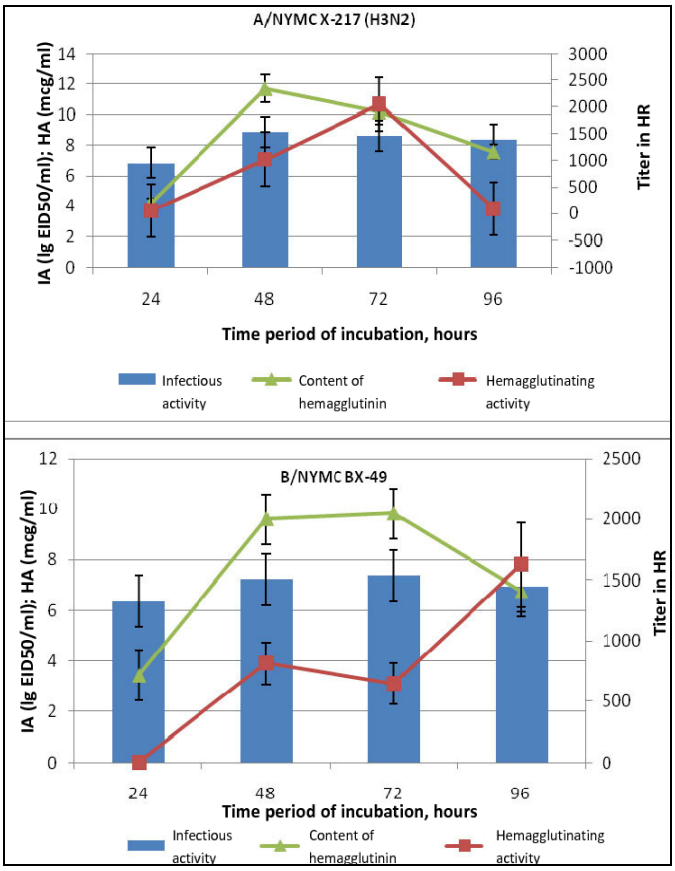


**FIG.4: THE LEVEL OF ACCUMULATION OF A/NYMC X-217 (H3N2) AND B/NYMC BX-49 VIRUS STRAINS IN CE ACCORDING TO A TIME PERIOD OF INCUBATION**

The presented results show that in the process of optimization of cultivation parameters for recombinant influenza virus strains A/NYMC X-217 (H3N2) and B/NYMC BX-49 in CE high values of activity have been achieved; those had no significant difference among themselves. The major advantage of our work is a determination of hemagglutinin accumulation in SRID as additional criteria of evaluation. SRID allows quality of inactive influenza vaccine (IIV) characterizing by HA concentration (in µg/ml) in vaccine product.

At the same time SRID has a pinpoint accuracy and reproducibility, less result dependency on conditions of experimental set up, that allows to characterize strictly quantitatively all components of multivalent influenza vaccines. The latest factor is critically important for the manufacturing practice and IIV control and their further development (Kastrikina, 1990) [18]. Thus, it was shown, that a content of hemagglutinin determined by SRID is in the range of 7.0µg/ml which is within the acceptable WHO limits.

Thus, as a result of performed study necessary data on optimal cultivation parameters of A/NYMC X-217 (H3N2) and B/NYMC BX- 49 recombinant strains were obtained. It should be noted that parameters of another NIBRG — 121xp (H1N1) recombinant strain of trivalent seasonal influenza vaccine were determined in our early studies ([b]Tabynov, 2012).[19]

**CONCLUSION:** Based on the performed studies the optimal cultivation conditions for A/NYMC X-217 (H3N2) and B/NYMC BX-49 recombinant strains in CE, including CE age, infective viral dose, temperature and incubation duration and their observance gives a possibility to obtain virus-containing suspension with infectious and hemagglutinating activity not less than 7.0 $\log_{10}$ $EID_{50}$/ml and 1:256, respectively, and a content of HA not less than 7.0 µg/ml. Achieved viral activity is sufficient to prepare a trivalent inactive seasonal influenza split vaccine meeting the WHO requirements.

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