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REFINING OF STATIONARY CULTIVATION MODES OF THE EHV-1/K STRAIN OF EQUINE RHINOPNEUMONITIS VIRUS

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The purpose of these studies is to refine stationary cultivation modes to determine the most effective ways of viral antigen accumulation while monitoring the accumulation kinetics. The research novelty is the use of modern methods for analyzing the accumulation kinetics of the equine rhinopneumonitis virus in various modes. Modern production of immunobiological preparations and diagnostic test systems relies primarily on cost-effectiveness. Traditionally, for the accumulation of virus-containing material, a stationary cultivation method is used, which has a number of advantages, primarily associated with the absence of the need for expensive equipment, consumables, and with the great opportunities for controlling fungal and bacterial contamination. Additionally, even semiskilled staff may handle this method. Virological, microbiological, technological and analytical methods of management and control of stationary cultivation of the EHV-1 strain of equine rhinopneumonitis virus were used during the study.

The research findings served as a basis for the development of a mathematical model of stationary cultivation of the EHV-1 strain, factoring in the influence of external and internal factors on the kinetics of virus accumulation. Further, the findings were transferred to the production process. For the standard production process, the biological activity of the virus was 5.75 lg TCD_{50}/cm^3, after recalculation resulting in 500 thousand viral particles per 1 cm^3, and when the production process was modified using a mathematical model, the virus activity increased to 6.5 lg TCD_{50}/cm^3, after recalculation resulting in 3 million viral particles per 1 cm^3.

Key words: *equine rhinopneumonitis; virology; accumulation kinetics; cultivation; vaccine; EHV-1; equine herpesvirus.*

ЖЫЛҚЫ РИНОПНЕВМОНИЯСЫ ВИРУСЫНЫҢ EHV-1/К ШТАММЫНЫҢ СТАЦИОНАРЛЫҚ ӨСІРУ РЕЖИМДЕРІН ПЫСЫҚТАУ

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Бұл зерттеулердің мақсаты жинақтау кинетикасын бақылау кезінде вирустық антигеннің жинақталуының ең тиімді жолдарын анықтау үшін стационарлық өсіру режимдерін пысықтау болып табылады. Жүргізілген зерттеулердің жаңалығы әртүрлі режимдерде жылқы риноневмониясы вирусының жинақталу кинетикасын талдаудың заманауи әдістерін қолдану болып табылады. Иммунобиологиялық препараттар мен диагностикалық тест жүйелерінің заманауи өндірісі ең алдымен экономикалық тиімділікке сүйенеді. Дәстүр бойынша, құрамында вирусы бар материалды жинақтау үшін, ең алдымен, қымбат жабдықтардың, шығын материалдарының қажеттілігінің болмауына және персоналдың біліктілігі төмен болған кезде саңырауқұлақтар мен бактериялардың ластануын бақылаудың үлкен мүмкіндіктеріне байланысты бірқатар артықшылықтары бар стационарлық өсіру әдісі қолданылады. Жұмыста жылқы риноневмониясы вирусының EHV-1 штаммын стационарлық өсіруді басқару мен бақылаудың вирусологиялық, микробиологиялық, технологиялық және аналитикалық әдістері қолданылды.

Жүргізілген зерттеулер нәтижесінде вирустардың жинақталу кинетикасына сыртқы және ішкі факторлардың әсеріне негізделген EHV-1 штаммын стационарлық өсірудің математикалық моделі әзірленді, содан кейін нәтижелер өндіріс процесіне енгізілді. Стандартты өндіріс процесінде вирустың биологиялық белсенділігі 5,75 lg $ТСД_{50}$/см³ құрады, бұл қайта есептеу кезінде 500 мыңды құрайды. 1 см³ вирустық бөлшектер, ал математикалық модельді қолдана отырып, өндіріс процесін өзгерту кезінде вирустың белсенділігі 6,5 lg $ТСД_{50}$/см³ дейін көтерілді, бұл қайта есептеу кезінде 1 см³-те 3 миллион вирустық бөлшектер.

Түйінді сөздер: *жылқы ринопневмониясы; вирусология; жинақтау кинетикасы; өсіру; вакцина; EHV-1; жылқы герпесвирусы.*

ОТРАБОТКА РЕЖИМОВ СТАЦИОНАРНОГО КУЛЬТИВИРОВАНИЯ ШТАММА EHV-1/К ВИРУСА РИНОПНЕВМОНИИ ЛОШАДЕЙ

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Целью данных исследований является отработка режимов стационарного культивирования, для определения наиболее эффективных путей накопления вирусного антигена при контроле кинетики накопления. Новизной проведенных исследований является применение современных методов анализа кинетики накопления вируса ринопневмонии лошадей в различных режимах. Современное производство иммунобиологических препаратов и диагностических тест-систем в первую очередь полагается на экономическую эффективность. Традиционно для накопления вируссодержащего материала применяется стационарный метод культивирования, имеющий ряд преимуществ, связанных в первую очередь с отсутствием необходимости дорогостоящего оборудования, расходных материалов, и большие возможности контроля грибковой и бактериальной контаминации, при низкой квалификации персонала. В работе использовались вирусологические, микробиологические, технологические и аналитические методы управления и контроля стационарного культивирования штамма EHV-1 вируса ринопневмонии лошадей.

В результате проведенных исследований была разработана математическая модель стационарного культивирования штамма EHV-1, основанная на влиянии внешних и внутренних факторов на кинетику накопления вирусов, с последующим внедрением результатов в производственный процесс. При стандартном производственном процессе биологическая активность вируса составляла 5,75 lg $ТЦД_{50}$/см³, что при перерасчете составляет 500 тыс. вирусных частиц в 1 см³, а при модификации производственного процесса с применением математической модели активность вируса была поднята до 6,5 lg $ТЦД_{50}$/см³, что при перерасчете составляет 3 млн вирусных частиц в 1 см³.

Ключевые слова: *ринопневмония лошадей; вирусология; кинетика накопления; культивирование; вакцина; EHV-1; герпесвирус лошадей.*

Introduction

Equine herpesviruses 1 & 4 (*EHV-1* and *EHV-4*) are two closely related pathogens of members of the *Perissodactyla* family, such as horses, zebras and donkeys. Based on the close genetic and antigenic affinity, *EHV-1* and *EHV-4* are classified as the *Varicellovirus* genus of *the Alphaherpesvirinae* family, *Herpesviridae* subfamily [1].

Equine herpesvirus 1 (*EHV-1*) is an alphaherpesvirus and a pathogen that can cause respiratory diseases, miscarriages and neurological diseases in horses [2.p.1]. The neurological form of the disease, equine herpesvirus myeloencephalopathy (EHM), is reported as a complication in nearly half of horses naturally infected with EHV-1 [3.p.1].

Several vaccines exist for the respiratory and abortive forms of equine rhinopneumonitis; however, they are limited by their short-lived immunity or insufficient protection. Outbreaks of *EHV-1* continue to occur in horses despite active immunization efforts [4.p.2].

According to OIE recommendations, the prevention of equine rhinopneumonitis depends on adapting vaccination protocols and monitoring their efficacy, which primarily relies on the strain used and its concentration in the final product [5.p.2].

Static cultivation offers the ability to produce a specific virus under minimal equipment requirements while maintaining strict control over cytopathic processes. Optimizing static cultivation processes can enhance the yield and productivity of the virus-cell system, facilitating the development of highly effective vaccines. Static cultivation should be refined to prevent undesired induction of apoptosis or autophagy at the early stages of viral infection. To fully realize the potential of static cultivation, each production process must include a thorough analysis of the cultivation conditions for the host-cell system, alongside the optimal conditions for EHV-1 strain infection. Additionally, factors influencing these processes must be considered [6.p.2].

Considering static cultivation as a system, the following subsystems can be distinguished:

1. Liquid medium that serves as a nutrient solution to sustain cell viability and prevent their drying;
2. Monolayer of cell cultures infected with the equine rhinopneumonitis virus.

The correlation between these subsystems, combined with the parameters under study, will enable the identification of the most effective static cultivation regimes.

In this context, the aim was to investigate the effects of static and dynamic pH and temperature regimes during the static cultivation of the EHV-1/K strain of equine rhinopneumonitis virus. This research is expected to support the development of effective mathematical models, where virus accumulation kinetics depend on various factors, such as viral replication rate and the impact of endogenous and exogenous factors on virus growth and accumulation, enabling to provide simplified formulas applicable across different parameter ranges [7.p.3].

To achieve this aim, the following objectives were outlined:

1. Analysis of virus cultivation based on growth phases to determine the quantity of replicated virus at different time intervals.
2. Optimization of pH influence regimes for the cultivation of the EHV-1 strain, involving three static and four dynamic regimes, to assess the effect of pH levels across growth phases and identify growth-limiting and stimulating factors.
3. Optimization of temperature influence regimes for the EHV-1 strain, involving three static and four dynamic regimes, to evaluate temperature effects across growth phases and determine limiting and stimulating growth factors.

The findings from this research can be applied to the algorithmic and software design of calculations for biotechnological processes, such as substrate and biomass recirculation. This will enable practical implementation of biotechnological processes through optimized ratios [8.p.1].

Modern veterinary immunobiological production, in compliance with international GMP (Good Manufacturing Practice) standards, should focus on quality and safety while maintaining economic efficiency. This approach will make a significant contribution to combating equine rhinopneumonitis in conjunction with current anti-epizootological strategies [9.p.5].

Materials and methods of research

The research took place during the period from July to November 2022 at the Kazakh National Agrarian Research University at the Department of biological safety, as well as in the Virology laboratory of the Scientific and Production Enterprise "Antigen" LLP.

Virus

Equine herpesvirus 1 (*EHV-1)* isolated from abortive material with typical properties of equine rhinopneumonitis virus. The virus was adapted to static cultivation. The cytopathogenic effect included cell rounding and reduction in size, followed by cell detachment from the glass surface.

Cell culture

The passaged *E. Derm* cell culture was provided by the "Cell Culture" laboratory of the Scientific and Production Enterprise "Antigen" LLP (Kazakhstan, Almaty) and was originally obtained from *ATCC*® (*NBL – 6*).

Nutrient medium

Cultivation was conducted using Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Life Technologies, USA) spiked with 5% fetal bovine serum (FBS) (Gibco, Life Technologies, USA), without antibiotics. The medium contained phenol red (phenolsulfonphthalein), a colorimetric pH indicator providing integrated pH control.

Static cultivation

Static cultivation was carried out in BINDER BD 115 incubators, with daily monitoring of cytopathogenic effects via daily microscopy using AE31 Trinocular inverted microscope at 60X/0.80 magnification in bright field.

Static cultivation

Static cultivation of the *EHV-1* strain was performed in ventilated flasks on monolayers of 2- to 3-day-old *E. Derm* cell cultures, using varying infection doses. Samples were maintained for up to 96 hours in the incubator under temperature regimes ranging from +36°C to +38°C. Daily microscopic examination was conducted to assess the level of cytopathogenic effect using inverted microscope.

pH level control

The pH level was adjusted using 1% sodium bicarbonate solution and 0.1% sulfuric acid solution. These solutions were added to the nutrient medium as needed to correct fluctuations in pH levels. The pH was monitored using phenol red and the SevenCompact S220 pH/ion meter (Mettler Toledo).

Virus activity monitoring

The biological activity of the *EHV-1* strain was assessed through serial titration of *EHV-1*cell cultures following the Reed-Muench method. [10.p.3]

Research results

EHV-1 strain growth analysis

The growth analysis of the EHV-1/K strain was based on the manufacturing technology of the rhinopneumonitis vaccine and the list of standard operating procedures of SPE Antigen LLP. The analysis results are shown in Table 1.

Table 1. – Standard static cultivation

No.	Name	Growth phase/hour				Concentration
		I	II	III	IV	
1	*EHV-1/K*	24-30	24-72	72-96	96-120	5.5 lg TCD_{50}/cm^3

According to the above table, the stationary growth phase is notably prolonged, lasting from 24 to 30 hours before the first signs of cytopathogenic effect become evident. This is attributed to the replication cycle of the equine rhinopneumonitis virus within the host cells, followed by subsequent infection of neighboring monolayer cells. The exponential growth phase spans from 24 to 72 hours, during which the virus extensively infects the monolayer. The duration of the stationary growth phase is between 72 and 96 hours, characterized by a slowdown in the accumulation of virus-containing material due to the infection of monolayer cells at this stage, limiting further viral propagation. The growth deceleration phase occurs between 96 and 120 hours and is marked by a decline in the concentration of viable viruses, as those produced during the initial replication phase (Phase I) begin to degrade. Figures 1 and 2 illustrate the distinct differences between infected and uninfected *E.Derm* cell cultures.

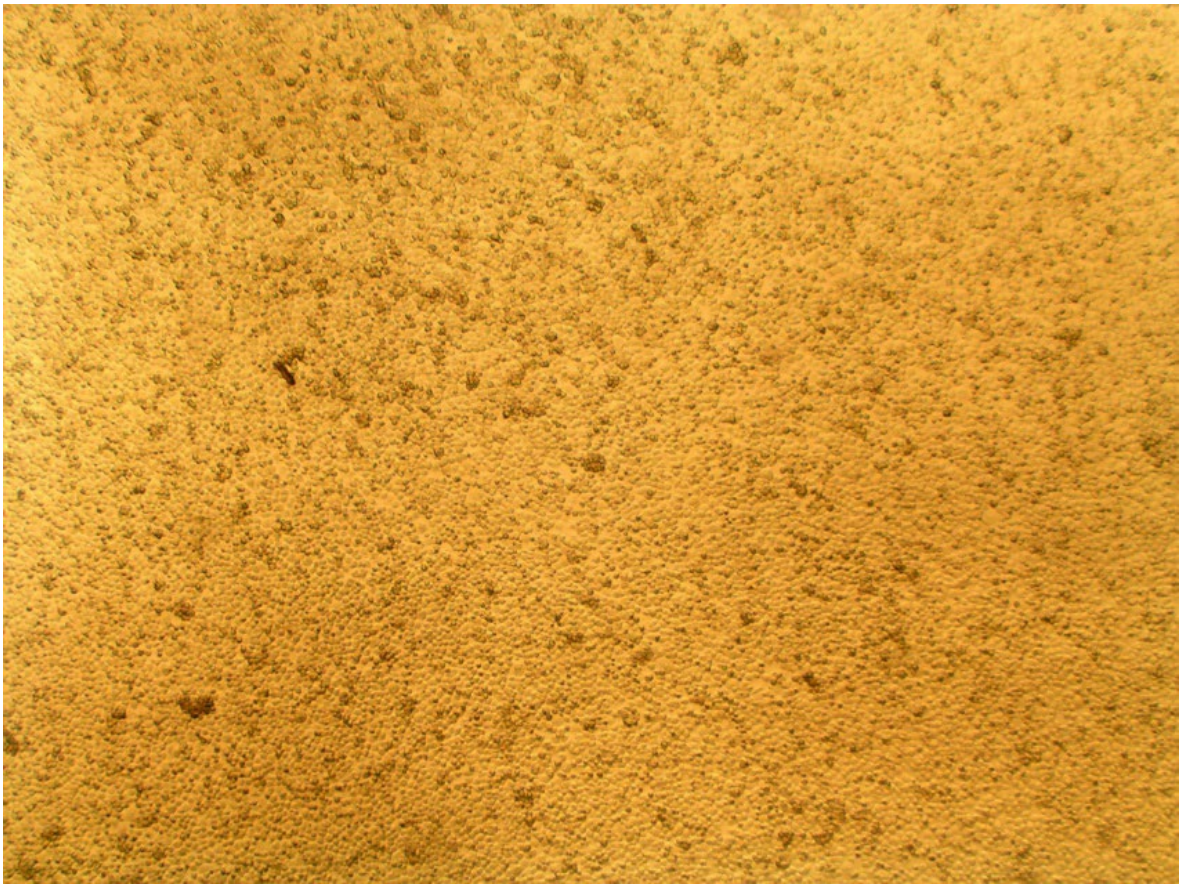

Figure 1 – *E. Derm* cell culture not infected with *EHV-1* without signs of CPE, at 60X/0.80 magnification in bright field

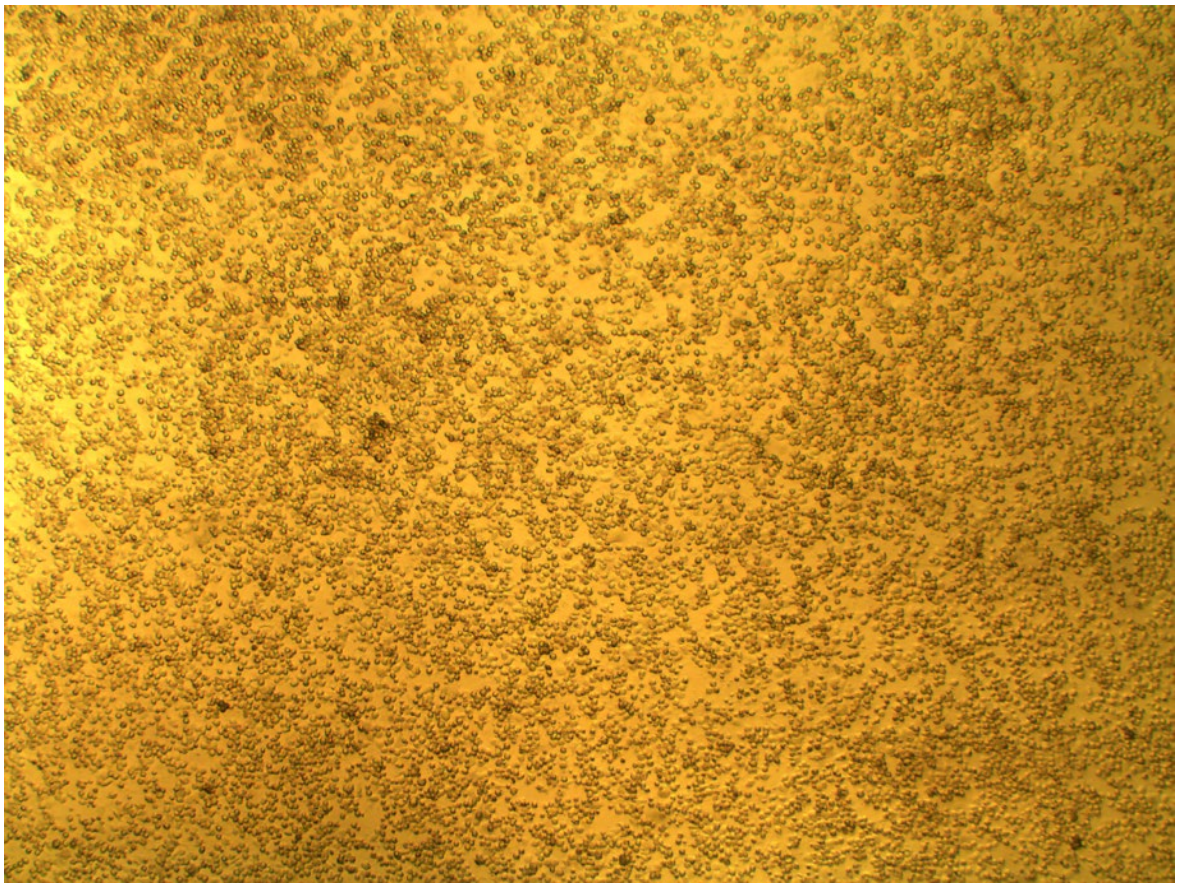

Figure 2 – *E. Derm* cell culture infected with *EHV-1* with signs of CPE,
at 60X/0.80 magnification in bright field

Figure 1 demonstrates that the cells exhibit a well-preserved, intact structure, uniformly distributed across the surface area of the flask wall. In contrast, Figure 2 shows clear signs of CPE characterized by cell rounding, peeling, and the appearance of cell-free gaps on the surface of the culture flask wall.

Optimization of pH Influence on EHV-1 strain cultivation

To develop a mathematical model with a parameter of the pH effect on the cultivation process of the EHV-1 strain, we investigated the following regimes:

(I) 6.0;

(II) 8.0;

(III) pH maintained at 6.0 during the first two growth phases and adjusted to 7.0 for the subsequent two growth phases;

(IV) pH maintained at 6.0 during the first two growth phases and adjusted to 8.0 for the subsequent growth phases;

(V) pH maintained at 8.0 during the first two growth phases and adjusted to 7.0 for the subsequent growth phases;

(VI) pH maintained at 8.0 during the first two growth phases and adjusted to 6.0 for the subsequent growth phases;

(VII) 7.0.

The presented pH regimes for studying the influence of pH on the cultivation of the EHV-1 strain allow for a comparison of static upper and lower pH limits, as well as dynamic transitions from upper to lower pH levels and vice versa. It is essential to consider the specific characteristics of the host-cell and virus interaction system during the cultivation process.

Table 2. – Effect of pH on *EHV-1* virus growth

pH regime	pH/hour				The final viral titer
	24-30	48-72	72-96	96-120	
1	6,0	6,0	6,0	6,0	5.75 lg TCD_{50}/cm^3
2	8,0	8,0	8,0	8,0	5.75 lg TCD_{50}/cm^3
3	6,0	6,0	7,0	7,0	6.00 lg TCD_{50}/cm^3
4	6,0	6,0	8,0	8,0	5.75 lg TCD_{50}/cm^3
5	8,0	8,0	7,0	7,0	6.5 lg TCD_{50}/cm^3
6	8,0	8,0	6,0	6,0	5.75 lg TCD_{50}/cm^3
7	7,0	7,0	7,0	7,0	6.00 lg TCD_{50}/cm^3

According to the above table, the significant impact of pH on the kinetics of virus accumulation and the progression rate through different growth phases is evident. For example, under the fifth regime, elevated pH levels during the first two phases enhance cell viability, while lower pH levels during phases III and IV are more favorable for virus viability. Conversely, lower pH levels during the initial two phases reduce cell viability and sensitivity to EHV-1.

Optimization of temperature regimes for the EHV-1 strain

To optimize temperature conditions for the cultivation process of the EHV-1 strain, the following temperature parameters were studied:

(I) 36.0;

(II) 38.0;

(III) temperature maintained at 36.0 during the first two growth phases and adjusted to 37.0 for the subsequent two growth phases;

(IV) temperature maintained at 36.0 during the first two growth phases and adjusted to 38.0 for the subsequent growth phases;

(V) temperature maintained at 38.0 during the first two growth phases and adjusted to 37.0 for the subsequent growth phases;

(VI) temperature maintained at 38.0 during the first two growth phases and adjusted to 36.0 for the subsequent growth phases;

(VII) 37.0.

The presented temperature regimes consider both static temperature parameters, which remain constant throughout the cultivation process, and dynamic parameters, which shift from higher to lower values or vice versa depending on the growth phases.

Table 3. – Effect of temperature on *EHV-1* virus growth

Temperature regime	Temperature/hour				Concentration
	24-30	48-72	72-96	96-120	
1	36,0	36,0	36,0	36,0	6.00 lg TCD_{50}/cm^3
2	38,0	38,0	38,0	38,0	5.75 lg TCD_{50}/cm^3
3	36,0	36,0	37,0	37,0	6.25 lg TCD_{50}/cm^3
4	36,0	36,0	38,0	38,0	5.75 lg TCD_{50}/cm^3
5	38,0	38,0	37,0	37,0	6.25 lg TCD_{50}/cm^3
6	38,0	38,0	36,0	36,0	6.5 lg TCD_{50}/cm^3
7	37,0	37,0	37,0	37,0	6.00 lg TCD_{50}/cm^3

According to the above table, the effect of temperature on the kinetics of equine rhinopneumonitis virus accumulation during static cultivation is evident. The most optimal temperature regime is regime 8, where the temperature is maintained at 38.0°C during the first two phases and at 36.0°C during the last two

phases. This regime resulted in a viral titer of 6.5 log TCD_{50}/cm^3, which, after converting via the antilogarithm, corresponds to 3 million viral particles per 1 cm^3.

Conclusion

Optimizing the pH and temperature regimes will enhance the stationary cultivation conditions, facilitating their integration into technological processes for the production of virological immunobiological products and diagnostic test systems. Specifically, the biological activity of the *EHV-1* strain initially measured at 5.75 log TCD_{50}/cm^3 (equivalent to 500,000 viral particles per 1 cm^3) was increased to 6.5 log TCD_{50}/cm^3 (equivalent to 3 million viral particles per 1 cm^3) through modifications in the production process.

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СЕРОЛОГИЧЕСКИЙ МОНИТОРИНГ НА НАЛИЧИЕ ВИРУСНЕЙТРАЛИЗУЮЩИХ АНТИТЕЛ У ВАКЦИНИРОВАННЫХ ПРОТИВ БЕШЕНСТВА ДОМАШНИХ ПЛОТОЯДНЫХ

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В статье представлены результаты серологического мониторинга вакцинированных против бешенства собак, обитающих в городских и сельских территориях Республики Казахстан. В результате экспедиционных выездов в города Петропавловск, Алмата, Шымкент, в сельские округа районов Северо-Казахстанской (Есильский, Кызылжарский), Жамбылской (Байзакский, Жамбылский) областей согласно календарному плану научного проекта, группой научных исследователей был произведен отбор проб сыворотки крови от привитых против бешенства собак вакцинами разных производителей, независимо от половой принадлежности, в возрасте от 1 года до 15 лет. Указанные места отбора проб крови были определены по результатам изучения эпизоотологической характеристики территории страны за последние десять лет по бешенству животных.

Сыворотку исследовали доступным методом, а именно «Иммуноферментная тест-система для определения уровня антител к вирусу бешенства в сыворотках крови животных, вакцинированных против бешенства методом непрямого иммуноферментного анализа» (производитель ФГБНУ «ФЦТРБ-ВНИВИ», Россия).

Результаты поствакцинальных исследований сыворотки крови, взятой через 1,5-5 мес. от собак разных возрастных групп и половой принадлежности, обитающих в северном и южном регионе республики, иммунизированных инактивированными вакцинами против бешенства, свидетельствуют о создании необходимого защитного барьера для восприимчивой популяции домашних плотоядных.