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Live Turkey Herpesvirus vaccine against Marek's Disease: development, stabilization, and immunobiological evaluation

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Abstract

Background and Aim. Marek's disease (MD) is a highly contagious and economically significant viral infection of poultry, caused by Marek's disease virus (MDV), an alphaherpesvirus that induces lymphomas, paralysis, and immunosuppression in chickens. Kazakhstan currently lacks a domestically produced MD vaccine, resulting in dependence on imports and logistical challenges. This study aimed to develop and evaluate a national live vaccine against MD using a cloned strain of turkey herpesvirus (HVT, strain AV-0007), with optimized production and preservation technologies.

Materials and Methods. The AV-0007 strain was propagated in chicken embryo fibroblast (CEF) cultures using stationary, roller, and suspension cultivation methods. Virus yields were optimized by adjusting MOI, nutrient media, and harvest times. Stabilizing media for cryopreservation and freeze-drying were formulated. Experimental vaccine preparations in both forms were assessed for sterility, safety, biological activity, and immunogenicity in one-day-old chickens. Virus titers were measured using the focus-forming unit (FFU50) method, and immunogenicity was evaluated by virus neutralization tests.

Results. Virus titers exceeded 10^6 FFU50/cm^3 across optimized cultivation methods. Cryopreserved and freeze-dried vaccines preserved high viral activity after stabilization and storage. Both forms met international standards for sterility and were non-pathogenic in chicks even at 10× immunizing doses. Immunized birds developed virus-neutralizing antibodies with titers ranging from 1.67 to 2.33 log FFU50, indicating strong immunogenicity and protective potential.

Conclusion. The AV-0007-based vaccine formulations demonstrated safety, stability, and high immunogenicity. The study confirms the feasibility of producing a domestic Marek's disease vaccine in Kazakhstan and provides a foundation for the local development of poultry vaccines aligned with international quality standards.

Keywords: chicken embryo fibroblasts; immunogenicity; live vaccine; Marek's disease; turkey herpesvirus.

Introduction

Kazakhstan possesses significant potential for the advancement of its poultry industry, a sector anticipated to be pivotal in guaranteeing national food security and enhancing agro-industrial exports [1]. This potential is fundamentally reliant on the capacity to manage viral illnesses that jeopardize poultry health and productivity. Marek's disease (MD) is a notable lymphoproliferative condition induced by the extremely contagious alphaherpesvirus known as Marek's disease virus (MDV), which affects hens.

The ailment is marked by advancing paralysis, immunosuppression, and lethal lymphomas, frequently leading to significant economic detriment in commercial flocks [2, 3, 4, 5].

Currently, MD is solely controlled via immunization. The current vaccinations, however, do not provide sterile immunity [6]. While they reduce symptoms and the risk of death, they do not stop the virus from multiplying or spreading, which allows for the development of stronger MDV types. This constraint has initiated a worldwide quest for more efficacious vaccinations that can provide enhanced, preferably sterilizing, immunity [7, 8, 9].

Live vaccines made from turkey herpesvirus (HVT) [10] are the standard in commercial use because they don't cause disease in poultry and are similar enough to harmful MDV to trigger an immune response. These vaccines are usually made using fibroblast cultures from specially selected chicken embryos that are free from specific diseases, followed by steps to grow the virus, stabilize it, and store it using freezing or drying methods. The process is complicated and requires a lot of resources, involving large-scale cell growth in special containers, and is mainly used by major companies like Merial (France), Intervet (Netherlands), and ARRIAH (Russia) [11].

Despite the introduction of attenuated MDV strains [12, 13] and recombinant vector vaccines [14, 15, 16], they either pose production difficulties or generate diminished immune responses, particularly in flocks with previous MD exposure. Moreover, the specific production technology employed internationally remains undisclosed, creating a technological disparity for domestic manufacturers.

The lack of a domestically developed vaccine for MDV in Kazakhstan is both an epizootic and economic risk. This study fills the gap by creating a national technology for the production of a live vaccination against Marek's disease with a cloned strain of HVT. The objective was to develop a vaccine that complies with worldwide criteria for safety, immunogenicity, and viability while being tailored to local production conditions.

Materials and Methods

Materials: The research used hatching eggs from commercial sources and a specific type of turkey herpesvirus (strain AV-0007) that had a remaining biological activity of $10^{3.75}$ FFU/cm³, stored at -50 °C, along with Eagle's nutrient medium (with and without calcium), fetal bovine serum, and various tools and materials for growing cells. This included incubators, thermostats, roller devices, suspension culture fermenters, and lab glassware like 1.5 dm³ mattresses and 0.5 dm³ roller flasks. This encompassed incubators, thermostats, roller devices, suspension culture fermenters, and laboratory glassware, including 1.5 dm³ mattresses and 0.5 dm³ roller flasks.

Incubation of chicken embryos: Developing chicken embryos (DCE) were acquired by incubating hatching eggs at 37-38 °C with a humidity of at least 50%, continuous air exchange, and periodic axial rotation. The ideal durations for egg storage before incubation, together with factors like temperature, humidity, aeration, and frequency of egg rotation, were established to enhance embryonic development.

Preparation of chicken embryo fibroblasts (CEF): Fibroblast cultures (CEF) were obtained from embryos of differing ages. The improved settings included the optimal age of the embryos for trypsinization, the total number of cells from each embryo, removal of feather debris, trypsin concentration, pH level, how often and how long to use trypsin, and the conditions for different culture methods like stationary, roller, and suspension techniques. Media replacement times for both nutrition and maintenance phases were established.

Virus biomass production: The amount of turkey herpesvirus produced was improved by looking at factors like the age and number of cells in the CEF culture, along with the condition of the cell layer before infection. Multiplicities of infection (MOI) from 0.1 to 0.001 $FFU_{50/cell}$ were evaluated. Viral titers were assessed at multiple times following infection to ascertain the optimal harvest time. The effect of freeze-thaw cycles on viral infectivity was also examined.

Vaccine production and formulations: Experimental vaccine batches were made using the AV-0007 virus strain at a concentration of $10^{6.75}$ FFU_{50/cm^3}, grown in CEF using the suspension method. Two formulations were developed: freeze-dried (lyophilized) and cryopreserved. Three vials of each formulation were utilized for testing. Freeze-dried samples were reconstituted in 0.9% sodium chloride and amalgamated, while cryopreserved samples were thawed in a 40 °C water bath for 1 minute and thereafter mixed.

Sterility testing: Sterility was assessed in accordance with GOST 28085, using standard microbiological culture techniques [17].

Safety assessment: Safety was assessed in 10 one-day-old chicks, each administered an intramuscular injection of 10 immunizing doses ($10^{4.0}$ $FFU_{50/0.2\ cm^3}$) of the vaccine. Birds were monitored for a duration of 10 days. The lack of clinical symptoms and death suggested that the immunization was deemed safe.

Biological activity testing: The amount of virus in both vaccine types was measured by making ten times weaker solutions and testing them on layers of CEF cells. Each dilution was injected with ten flasks. Focus-forming units (FFU) were enumerated, and titers were determined by the Reed and Muench methodology.

Immunogenicity testing: Immunogenicity was assessed in chicks inoculated with $10^{3.0}$ $FFU_{50/0.2cm^3}$. Specific antibodies were found in blood samples taken after vaccination using a test that checks for the ability to neutralize the turkey herpesvirus strain used in the study.

Statistical analysis: All experimental data were analyzed using conventional descriptive and inferential statistical techniques. Quantitative results are expressed as mean ± standard deviation (SD). Group means comparison (e.g., virus titers, antibody levels) was conducted utilizing one-way analysis of variance (ANOVA) accompanied by Tukey's post-hoc test to identify statistically significant differences among groups. A p-value of less than 0.05 was deemed statistically significant.

Results and Discussion

Determination of optimal cell density, cultivation period, and MOI for efficient virus accumulation. Determining the best conditions for infecting the CEF culture is a crucial step in increasing virus yield in vaccine manufacturing. This work included creating a single layer of chicken embryo fibroblasts (CEF) by placing 4×10^5 cells in each cubic centimeter and letting them grow for 4 days. On the third day of cultivation, the cells were infected with turkey herpesvirus at three distinct multiplicities of infection (MOIs): 0.1 FFU/cell, 0.01 FFU/cell, and 0.001 FFU/cell.

Following a 96-hour incubation period at 37.0-37.5 °C, all experimental flasks underwent a single freeze-thaw cycle (-40 °C for 24 hours, then thawed at ambient temperature), after which the virus titer was assessed by titration on a monolayer of fresh CEF cells.

Table 1 illustrates that the viral titer escalated in direct correlation with the MOI. At the minimal MOI of 0.001 FFU/cell, the total viral titer attained $10^{4.75}$ FFU/cm³. Conversely, at MOIs of 0.01 and 0.1 FFU/cell, the titers rose markedly to 106.00 and $10^{6.50}$ FFU/cm³, respectively. This illustrates a distinct positive association between infection dose and the resultant viral concentration in the biomass.

Table 1 – Virus accumulation in the CEF depending on the dose of infection

Seeding concentration of CEF cells, cells/m³	Age of cell culture, days	MOI (FFU/cell)	Virus Titer (FFU/cm³, Mean ± SD)
4×10^5	4	0.1	$10^{6.50\pm0.10}$
		0.01	$10^{6.00\pm0.08}$
		0.001	$10^{4.75\pm0.12}$

The findings indicate that an MOI of no less than 0.01 FFU/cell is necessary to attain optimal viral yields for subsequent downstream processing and vaccine formulation. Although 0.1 FFU/cell produced the best results, using a slightly lower amount of 0.01 FFU/cell might be more cost-effective for large-scale production, while still generating a good amount of virus.

Determination of the optimal virus harvesting period and evaluation of freezing as a virus collection method. To ascertain the ideal timing for harvesting the virus from infected CEF cultures and to assess the efficacy of the freezing-thawing procedure for virus collection, virus-infected cell cultures were incubated under diverse conditions and durations. CEF monolayer cultures (stationary and roller) and suspension cultures were cultured at 37 °C and sampled at various time intervals: 72, 96, and 120 hours post-infection. In each instance, fifty percent of the cultures saw a singular freeze-thaw cycle (frozen at -40 °C for 24 hours, then thawed at ambient temperature), whilst the other samples were processed immediately without freezing.

The virus titer was assessed in all instances using monolayer titration. The results shown in Table 2 indicate that the highest amount of virus was found on the fifth day (96 hours) after infection for both stationary and roller monolayer cultures. The virus titer in stationary monolayers after freeze-thaw attained $10^{6.25}$ FFU/cm³, but in roller monolayers, the titer peaked at $10^{6.75}$ FFU/cm³. The figures exceeded those acquired without the freezing technique, where the titer was around 0.5-1.25 log units lower.

Conversely, in suspension cultures, the peak titers were recorded earlier, at 72 hours post-infection, attaining 105.50 FFU/cm³ without freeze-thaw and $10^{5.25}$ FFU/cm³ with freeze-thaw. Viral titers in suspension cultures decreased markedly at subsequent time points, signifying a reduction in cell viability and viral reproduction.

Table 2 – Virus titers established during production under different conditions

Virus-infected cell culture	Incubation period before collection of viral mass, days					
	3		5		7	
	Freezing. Defrosting.	No freezing	Freezing. Defrosting.	No freezing	Freezing. Defrosting	No freezing
CEF monolayer stationary	$10^{4.50\pm 0.10}$	not tested	$10^{6.25\pm 0.09}$	$10^{5.50\pm 0.11}$	$10^{4.25\pm 0.13}$	not tested
CEF monolayer roller	$10^{5.00\pm 0.10}$	not tested	$10^{6.75\pm 0.07}$	$10^{6.25\pm 0.08}$	$10^{5.50\pm 0.07}$	not tested
CEF suspension	$10^{5.25\pm 0.09}$	$10^{5.50\pm 0.08}$	$10^{2.75\pm 0.14}$	not tested	not tested	not tested

The results indicate that for monolayer cultures, especially those cultivated via the roller method, the virus should be harvested 96 hours post-infection after a freeze-thaw process, which facilitates virus release. In contrast, for suspension cultures, the ideal harvest time is 72 hours post-infection, and prolonged incubation results in diminished viral yields, irrespective of freezing.

Optimization of suspension culture parameters for enhanced virus titer. Due to the destitute virus yield in suspension cultures under conventional conditions, adjustments were implemented to improve virus productivity. The changes included using Eagle's nutritional medium with double the usual amount of amino acids and increasing the fetal bovine serum (FBS) level to 15%. The MOI was raised to 0.05 FFU/cell to evaluate if elevated initial infection doses would enhance viral yield.

Suspension cultures were inoculated with CEF at doses between 2.1×10^6 and 2.4×10^6 cells/cm³ and incubated for 72 and 96 hours. The virus titer was assessed in all instances using monolayer titration. Table 3 shows the best conditions for growing the virus in suspension culture: a cell concentration of 2.4×10^6 cells/cm³, a multiplicity of infection (MOI) of 0.05 FFU/cell, and 15% fetal bovine serum (FBS). Under these circumstances, the viral titer reached a maximum of $10^{6.75}$ FFU/cell at 72 hours. Prolonged incubation to 96 hours led to diminished titers, corroborating prior findings.

Table 3 – Titers of turkey herpes virus in suspension cultivation in CEF culture, $FFU_{50/cell}$

Fibroblast concentration, cells/cm³	Dose of seed virus (MOI), $FFU5_{0/cell}$	Concentration of blood serum in the nutrient medium, %	Timeframe for determining virus titer, h	
			72	96
2.3×10^6	0.01	10	$10^{5.50\pm 0.08}$	$10^{4.25\pm 0.09}$
2.1×10^6	0.01	15	$10^{6.00\pm 0.06}$	$10^{5.50\pm 0.07}$
2.4×10^6	0.05	15	$10^{6.75\pm 0.08}$	not tested

The data show that increasing nutrient and serum levels, as well as a higher multiplicity of infection (MOI), significantly boosts virus production in suspension cultures. The time-dependent aspect of virus growth is a crucial element, as extended incubation negatively impacts titer.

Stability of intra- and extracellular Turkey Herpesvirus and development of preservation strategies. The stability of turkey herpesvirus inside and outside of cells was studied to see if there would be a loss of strength during normal storage and handling. This investigation was a vital measure for formulating efficient preservation strategies for forthcoming vaccine manufacture.

A suspension of live CEF cells containing the virus (intracellular virus) was segregated into two groups for this purpose. One portion was maintained at 4-6 °C, whereas the second underwent freezing at -20 °C for 3 hours, then thawed at ambient temperature, and then stored at 4-6 °C. Viral levels were measured right after treatment and again after 24 hours of storage using standard testing methods on CEF cell layers.

Table 4 illustrates that the internal virus exhibited greater stability than its extracellular counterpart. Following 24 hours of storage at 4-6 °C, the intracellular virus titer diminished marginally from $10^{6.00}$ to $10^{5.75}$ $FFU_{50/cell}$ (-0.25 log). The virus outside the cells, which came from broken cells after freezing, dropped from $10^{5.50}$ to $10^{4.75}$ $FFU_{50/cell}$ in the same period, showing a decrease of 0.75 log while thawing and another 0.5 log in the next 24 hours.

Table 4 – Titers of intra- and extracellular turkey herpesvirus under storage conditions

Material under study	Initial titer of virus in suspension	Terms and Conditions		Status of the virus	Research time, hours	
		processing	storage, °C		0	24
Chicken embryo fibroblast suspension containing turkey herpes virus	$10^{6.00}$	-	4-6	Intracellular	$10^{6.00}$	$10^{5.75}$
		Freezing at minus 20 °C and defrosting	4-6	Extracellular	$10^{5.50}$	$10^{4.75}$

The data demonstrate that the internal virus maintains stability during refrigerated storage, whereas the extracellular virus is considerably more susceptible to freeze-thaw cycles and extended exposure at 4-6 °C. Consequently, it is advisable to store virus-containing solutions intracellularly and safeguard them with stabilizing chemicals during freezing or drying procedures.

Preservation of virus biomass via cryopreservation and freeze-drying. Two preservation procedures, cryopreservation and freeze-drying, were developed to guarantee the long-term storage and biological stability of the vaccine virus. For each approach, virus-laden CEF suspensions were categorized into two groups: those with protective additives and those without.

The frozen samples were kept safe using a mixture made of 70% Eagle's nutritional medium, 20% fetal bovine serum, and 10% dimethyl sulfoxide. Following stabilization, samples were frozen at -70 °C and subsequently transferred to liquid nitrogen (-196 °C) for storage. Samples were made stable using a mix of 4% peptone, 8% sucrose, and 1% gelatin, and then dried out using a careful freezing method.

Biological activity was evaluated prior to stabilization, subsequent to stabilization, and following three months of storage. The findings are encapsulated in Table 5.

Table 5 – Virus titer before and after stabilization and storage (3 months)

Biomass form of turkey herpes virus	Virus titer in biomass before stabilization	Virus titer in biomass after stabilization	Virus titer in biomass after stabilization and storage for 3 months	Change in virus titer after storage, lg FFU_{50}
Cryopreserved with protective additive	$10^{6.75}$	$10^{6.25}$	$10^{6.25}$	0.0
Cryopreserved without protective additives	$10^{6.75}$	$10^{6.25}$	$10^{5.75}$	0.5
Sublimated with protective additive	$10^{6.00}$	$10^{5.75}$	$10^{5.50}$	0.25
Sublimated without protective additives	$10^{6.00}$	$10^{5.75}$	$10^{2.25}$	3.50

The data indicates that samples with protective additives preserved their biological activity nearly intact, exhibiting minimal loss after three months of storage. Conversely, virus biomass devoid of additives exhibited a notable decline in infectivity, especially in the freeze-dried state, when the titer fell by 3.5 log. This finding underscores the essential importance of adequate stability in maintaining vaccination viability.

Establishment of methods for standardization of freeze-dried and cryopreserved Turkey Herpesvirus. The evaluation of vaccine quality is grounded in factors that positively influence the vaccinated organism while ensuring no harm to the animals or the environment. Therefore, the methods used to ensure both types of vaccines are safe include checking for cleanliness from unwanted germs, measuring the strength of the vaccine virus, and testing how well it triggers an immune response, as well as assessing the physical and technical quality of the vaccine. A biological model vulnerable to Marek's disease was selected from this list of approaches as the target for evaluating the preparation's safety. The biological model consisted of one-day-old chicks. The procedures outlined in GOST 28085 "Biological preparations. Methods for determining sterility" were selected to evaluate sterility. The biological activity was evaluated using a titration approach to quantify the turkey reproductive herpes virus through plaque- or focus-forming units in a monolayer culture of chicken embryo fibroblasts contained in penicillin vials. A biological model susceptible to the Marek's disease virus, specifically one-day-old hens, was selected to evaluate the vaccine's immunogenicity and safety. Standard methods were used to check the physical and technical features of the vaccine, such as how it looks, how well it dissolves (only for the dry form), the condition of the packaging, how well it is sealed, the internal vacuum (only for the dry form), nitrogen levels in the Dewar flask (only for the liquid form), and the correctness of the labels. Table 6 presents the compilation of selected and established methodologies for the standardization of the cryopreserved and freeze-dried turkey herpes virus vaccine.

Table 6 – List of methods for standardization of turkey herpes virus vaccine in cryopreserved and dry forms

Vaccine parameter being assessed	Method of evaluation by vaccine forms		Indicators for forms	
	Cryopreserved	Dry	Cryopreserved	Dry
1	2	3	4	5
Appearance, color, purity from impurities	Frozen liquid of yellow color, without impurities	Dry tablet-shaped porous mass without impurities of cream color	Corresponds	Corresponds
Presence of vacuum in vaccine vials	-	Vacuum according to GOST 28083	-	Vacuum according to GOST 28083
The presence of liquid nitrogen in the Dewar vessel in which the vaccine is stored	Liquid nitrogen with signs of boiling and evaporation	-	Corresponds	-
Solubility	-	For up to 2 minutes in physiological sodium chloride solution	-	Corresponds
Sterility	GOST 28085		Corresponds	Corresponds
Harmlessness	Inoculation with the vaccine intramuscularly at a dose of at least 10^4 $PFU_{50/0.2}$ cm^3 in one-day-old chickens in an amount of at least 10 heads		Harmless	Harmless

Continuation of table 6

Biological activity by virus titer, FFU50/cm3	Titration on a monolayer culture of chicken embryo fibroblasts by infecting the cell culture with tenfold dilutions of the vaccine in at least 4 flasks	Not less than $10^{6.0}$	Not less than $10^{6.0}$
Immunogenicity by antibody titer in vaccinated chickens, lg	Immunization of at least 20 one-day-old chickens by intramuscular administration of the vaccine at a dose of 10^3 $FFU_{50/0.2}$ cm^3.	Not less than 1.5	Not less than 1.5

The information in Table 6 shows that the main qualities of the vaccine, like being free from germs, being safe, working effectively through virus titer, and triggering an immune response, are all standardized using the same methods. The dry vaccine formulation has supplementary methods for assessing the look and color of the preparation, as well as verifying the presence of a vacuum within the vials containing the vaccine. The evaluation of the cryopreserved preparation includes not just the main procedures but also checking the vaccine's appearance and color, as well as confirming the presence of liquid nitrogen in the Dewar vessel used for its transport.

Preparation of experimental samples of freeze-dried and cryopreserved forms of the vaccine against Marek's disease, and their standardization according to immunobiological properties. Samples of freeze-dried and cryopreserved Marek's disease vaccine were made from turkey herpes virus with a strength of $10^{6.75}$ FFU_{50/cm^3}, using special chemicals meant for freeze-drying and cryopreservation. One hundred vials of freeze-dried and cryopreserved vaccine formulations were manufactured.

The vaccine's qualities were standardized by checking for cleanliness, safety, how well it works based on virus levels, and its ability to trigger an immune response.

Three vials of the vaccine were utilized for assessing each parameter, both freeze-dried (dry) and cryopreserved (liquid). The freeze-dried vaccine from the three vials was mixed with a saltwater solution, combined into one vial, and the average of that sample was used for testing. The cryopreserved vaccine was thawed in a water bath at 40 °C for 1 minute, pooled, and an average sample was collected for testing.

Determination of vaccine sterility. The results of the sterility tests, done according to GOST 28085 standards, showed that both vaccine types are free from unwanted germs. In the vaccine samples grown on specific nutrient media, no germs were found during the observation period. In the vaccine samples grown on special nutrient media MPA, MPB, MPPB, and Sabouraud, no germs were found during the observation time.

Determining the safety of a vaccine. To assess safety, ten one-day-old chickens were vaccinated intramuscularly with an average sample of each preparation form containing 10^4 $FFU_{50/0.2cm^3}$ of turkey herpes virus, administered at a dose of 0.2 cm^3. The chickens underwent clinical observation for a duration of 10 days. The safety was evaluated according to the clinical status of the birds. The outcomes of evaluating this parameter from the experimental vaccine series are presented in Table 7.

Table 7 – Data on the safety assessment of the experimental series of the vaccine against Marek's disease

Test drug	Number of vaccinated chickens, heads	Vaccine dose, $FFU_{50/head}$	Results of observation over 10 days	Vaccine evaluation
Marek's disease vaccine dry	10	10^4	0/10	Harmless
Marek's disease vaccine dry	10	10^4	0/10	Harmless
Note: The denominator is the number of vaccinated chickens, the numerator is the number of chickens with general and local pathologies detected				

The information in Table 7 shows that all 10 chickens given the freeze-dried Marek's disease vaccine and all 10 chickens given the cryopreserved Marek's disease vaccine, at a dose ten times higher than the usual amount, stayed healthy and alive for 10 days after vaccination, proving that the vaccine is safe for chickens.

Determination of biological activity of vaccine preparations. The biological efficacy of the freeze-dried and cryopreserved experimental vaccine formulations was assessed by measuring the viral titer in monolayer cultures of CEF cells utilizing the focus-forming unit (FFU) method. To achieve this, 10-fold serial dilutions were created using pooled average samples of each vaccine formulation (reconstituted or thawed), and 10 flasks of monolayer CEF cultures were inoculated with each dilution.

Focus-forming units were detected and enumerated 96 hours post-infection. The virus titers were subsequently determined by the Reed and Muench method. Table 8 reveals that the freeze-dried vaccine exhibited a titer of $10^{6.00}$ $FFU5_{0/cm^3}$, whereas the cryopreserved variant demonstrated a marginally elevated titer of $10^{6.50}$ FFU_{50/cm^3}.

Table 8 – Results of titration of experimental series of freeze-dried and cryopreserved Marek's disease vaccine

Material under study	Virus dilutions						Virus titer
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	
Marek's disease vaccine dry	10/10	10/10	9/10	6/10	0/10	0/10	$10^{6.0}$
Marek's disease vaccine liquid	10/10	10/10	10/10	10/10	0/10	0/10	$10^{6.5}$
Note: in the denominator is the number of flasks with infected cell culture, in the numerator is the cell culture in flasks with focus-forming units							

These findings show that both vaccination formulations maintain robust biological efficacy post-processing and stabilization. The cryopreserved version showed slightly better ability to infect, possibly because the viral material was handled more gently compared to the lyophilization process. Both formulations, however, achieved the requisite potency level of $\geq 10^6$ FFU_{50/cm^3}, thereby qualifying for immunogenicity assessment.

Evaluation of vaccine immunogenicity. To evaluate the immunogenic potential of the vaccine, one-day-old hens were inoculated with $10^{3.00}$ $FFU_{50/0.2\ cm^3}$ of either the freeze-dried or cryopreserved vaccine formulation. Blood samples were taken from the vaccinated hens (15 in each group) 21 days after vaccination and tested with a virus neutralization test using a turkey herpesvirus strain at a strength of $10^{6.50}$ FFU_{50/cm^3}.

The difference in virus neutralization levels before and after vaccination was used to evaluate the production of specific antibodies. Table 9 shows that the average level of neutralizing antibodies was 1.67 log FFU_{50} in hens given the freeze-dried vaccine and 2.33 log FFU_{50} in those given the cryopreserved version. This result signifies that both vaccinations elicited the generation of virus-specific antibodies at levels adequate to provide protective protection.

Table 9 – Titer of virus-neutralizing antibodies in blood serum samples of chickens vaccinated with freeze-dried and cryopreserved Marek's disease vaccine

The vaccine used for the vaccination	Number of serum samples in the pool	The titer of the virus in a mixture with blood serum collected		Difference in virus titers
		before vaccination	21 days after vaccination with the vaccine	
Marek's disease vaccine dry	15	$10^{6.00}$	$10^{4.33}$	$10^{1.67}$
Marek's disease vaccine liquid	15	$10^{6.50}$	$10^{4.17}$	$10^{2.33}$

The information in Table 9 shows that the amount of turkey herpes virus mixed with blood serum samples from chickens before they were vaccinated with freeze-dried Marek's disease vaccine was about 106.00 FFU50, while it was about $10^{6.50}$ FFU_{50} when mixed with serum samples from chickens before they received the cryopreserved Marek's disease vaccine. Conversely, the titer of the same virus in a mixture with serum samples from the same chickens 21 days post-vaccination was $10^{4.33}$ FFU_{50} and $10^{4.17}$ FFU_{50}, respectively. The disparity in viral titers was 101.67 and $10^{2.33}$ FFU_{50}, respectively, within the mixture of blood serum samples obtained before and after inoculation with freeze-dried and cryopreserved vaccines.

The observed disparity in viral titers is large and ensures the presence of robust protection in the bodies of vaccinated animals.

The standardization results show that the experimental vaccine series, whether cryopreserved or freeze-dried, do not contain any harmful microbes and are safe for one-day-old chickens when given a much higher dose ($10^{4.0}$ FFU_{50} per chicken) through an injection into the muscle. The Marek's disease vaccine, made from the "AV-0007" strain of the turkey herpesvirus, shows it can effectively trigger an immune response in chickens when given as an injection at a dose of $10^{3.0}$ FFU_{50} per bird. In immunized hens, antibodies are produced at a titer of 1.67-2.33 lg FFU_{50} after 21 days.

To mitigate the significant danger of Marek's disease in industrial poultry, farmers implement preventive measures by vaccinating all chicks on their first day of life. The lack of such procedures results in the affliction of juvenile birds and their widespread mortality [18]. In our nation's chicken business, a vaccine manufactured abroad is utilized specifically for the prevention of Marek's illness. The vaccines coming from faraway countries, even with regular air shipping, often don't meet the needs of chicken farms because of problems like late deliveries, breaking storage and transport rules, and not having enough for all the baby birds since they weren't ordered early enough. The utilization of imported vaccinations creates a degree of dependency on pharmaceutical partners and suppliers. The aforementioned reasons underscore the importance of developing a homegrown vaccination for Marek's disease as well as other perilous infectious diseases. Investigations involving the turkey herpes virus, which underpins the vaccination for Marek's disease, are among the most intricate [19, 20, 21, 22].

This paper discusses the successful creation, standardization, and first tests in live hens of a vaccine made from the AV-0007 strain of turkey herpesvirus (HVT), showing strong safety, effectiveness, and ability to provoke an immune response in one-day-old hens.

This work's principal achievement was the finding of optimal viral generation parameters in CEF culture. Our results indicate that the virus production is directly affected by the multiplicity of infection (MOI), with titers rising proportionately from 0.001 to 0.1 FFU/cell. An MOI of 0.01 FFU/cell was found to be both affordable and effective, generating viral levels over 10^6 FFU/cm³, similar to those seen in standard vaccine production using traditional HVT strains.

Our study of growing methods showed that using the roller monolayer method, particularly with a 96-hour incubation followed by freeze-thaw treatment after infection, resulted in the highest virus levels (up to $10^{6.75}$ FFU/cm³). This finding supports previous research that shows gentle rolling and better air flow during incubation improve the health of fibroblasts and how well the virus replicates. Even though suspension cultures initially had lower yields, changes to the nutrient mix and the number of infections significantly improved results, proving that suspension systems can work well for large-scale production.

An essential component of this study was the examination of viral stability under diverse storage and processing conditions. We noted that intracellular virus (inside living CEF) exhibited substantially greater stability than extracellular virus in solution, which experienced substantial destruction during freeze-thaw cycles. This conclusion corresponds with previous research indicating that cell-associated HVT had more infectivity than free virus, owing to its protection against physical and enzymatic destruction.

So, all later versions, like frozen and dried vaccine preparations, used the virus inside fibroblast suspensions. This method was crucial for maintaining the virus's biological activity during extended storage periods.

The research further illustrated the significance of integrating stabilizing chemicals in vaccination preservation. Cryopreserved and freeze-dried virus samples kept higher levels of the virus when they

were mixed with the right protectants (FBS and DMSO for cryopreservation; peptone, sucrose, and gelatin for freeze-drying). In contrast, the virus levels dropped by up to 3.5 log in freeze-dried samples that didn't have stabilizers. Conversely, viral titers decreased by as much as 3.5 log in freeze-dried samples without stabilizers. This finding highlights how important the formulation is for keeping vaccines effective and matches earlier studies on freeze-dried viral vaccines.

The tested vaccination series, checked according to veterinary standards and GOST rules, showed strong sterility, safety, and ability to trigger an immune response. Both freeze-dried and frozen versions produced strong neutralizing antibody responses (1.67–2.33 log FFU50), similar to or better than the levels usually associated with protection against Marek's disease. Both the freeze-dried and frozen versions of the vaccine produced strong antibody responses (1.67–2.33 log FFU50), which are similar to or better than the levels usually associated with protection against Marek's disease. No adverse reactions or fatalities were documented during safety assessments, further corroborating the appropriateness of these formulations for neonatal chicks.

This research establishes a basis for the domestic manufacturing of a Marek's disease vaccine in Kazakhstan, diminishing dependence on imported vaccines and enhancing food security and poultry health infrastructure. Using the AV-0007 strain as a base for the vaccine, along with improved growing and stabilizing methods, offers a practical and expandable way to produce vaccines locally.

While the AV-0007-based vaccine demonstrated strong immunogenicity and biological activity under laboratory conditions, it is important to contextualize these results against existing commercial Marek's disease vaccines. Widely used HVT-based vaccines, such as those produced by Merial (France), Intervet (Netherlands), and FGBI ARRIAH (Russia), typically achieve virus titers of 10^6–10^7 $FFU_{50/cm3}$ in production, with established immunogenicity inducing antibody responses in the range of 1.5–2.5 log FFU_{50} in SPF chickens.

In our study, both the cryopreserved and freeze-dried formulations reached titers $\geq 10^{6.0}$ $FFU_{50/cm3}$ after optimization, which is consistent with the international standard for live poultry vaccines. Furthermore, the virus-neutralizing antibody response induced in one-day-old chicks (1.67–2.33 log FFU50) aligns with or slightly exceeds those reported for commercial HVT vaccines under controlled conditions. These results indicate that the vaccine candidate is competitive in both potency and immunogenicity.

Notably, commercial production protocols often involve industrial-scale roller bottle systems or bioreactors, which are not fully accessible in Kazakhstan. However, the methods proposed in this study – including suspension culture in optimized media – offer a scalable and cost-effective alternative adapted to local infrastructure. This supports the feasibility of establishing national production capabilities without requiring immediate large-scale investment in industrial platforms.

Additionally, the inclusion of both cryopreserved and lyophilized forms provides flexibility in storage and distribution, comparable to international vaccine options, many of which offer only one preservation form. The stability results obtained here, especially for formulations with protective media, are on par with imported analogues, which typically guarantee viability for 3-6 months under cold chain conditions.

This study focused on small-scale production and initial testing in living animals; future research should focus on larger pilot batches, longer tests for immune response (more than 21 days), and trials in real chicken farming settings. Furthermore, investigating the genetic stability of the AV-0007 strain and its effectiveness in conjunction with other vaccines (e.g., bivalent or trivalent formulations) could improve its practical use in strategies for managing poultry diseases comprehensively.

Conclusion

A production matrix lot of the cloned turkey herpesvirus strain (AV-0007) with a baseline titer of $10^{5.5}$ FFU_{50/cm^3} was refreshed and used as a foundation for generating high-yield viral biomass. Standardized protocols for obtaining developing chicken embryos (RCE) and preparing chicken embryo fibroblast (CEF) cultures were established using both stationary and roller monolayer methods, as well as for maintaining fibroblast viability in suspension. Technological parameters for virus propagation in CEF cultures were developed and optimized across all three cultivation systems-stationary monolayer, roller monolayer, and suspension-resulting in consistent production of viral suspensions with titers of at least $10^{6.0}$ FFU_{50/cm^3}.

A scalable technological scheme for the production of HVT biomass in CEF culture was successfully developed. Formulations of stabilizing media were also optimized to preserve viral infectivity during cryopreservation and lyophilization. Using these systems, experimental and pilot series of the Marek's disease vaccine were prepared and evaluated. In accordance with international standards for veterinary viral vaccines, comprehensive methods for vaccine quality assessment were selected and implemented, including sterility testing, safety evaluation in one-day-old chickens, determination of biological activity by viral titer, and immunogenicity testing via virus-neutralizing antibody titers.

These results validate the feasibility of developing a domestically produced vaccine against Marek's disease in Kazakhstan, which meets international quality benchmarks for biological safety and efficacy.

Authors Contributions

LK and BM: Conceptualization, formal analysis, designed the study, writing - original draft. BM, LK, AT: Conducted an extensive literature review and analyzed the data. GZh, TT, AT, K.B: ШInvestigation. BM, LK, AT: Bata Curation, Writing - Review & Editing. BM: supervision. All authors have read, reviewed, and approved the final manuscript.

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