

REVIEW

REVIEW

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Modern approaches to the construction and use of recombinant viruses

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Abstract

The review describes certain viral vectors and considers various methods for constructing recombinant viruses with special attention paid to the homologous recombination and CRISPR/Cas9 system, and also describes the capabilities of using various cloning vectors (different plasmids, BAC etc.). The review also presents a comparative analysis of the effectiveness and safety of using various viral vectors, both for creating recombinant vaccines and for obtaining oncolytic viruses, as well as medicines for gene therapy.

Keywords: recombinant virus; CRISPR/Cas9; homologous recombination; cloning vector; plasmid; viral vector; recombinant vaccine; review

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НАУЧНЫЙ ОБЗОР

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Современные подходы к конструированию и применению рекомбинантных вирусов

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Резюме

В обзоре описан ряд вирусных векторов и рассмотрены различные методы конструирования рекомбинантных вирусов, особое внимание уделено системе гомологичной рекомбинации и CRISPR/Cas9, описана возможность использования разных клонирующих векторов (виды плазмид, BAC). Также в обзоре представлен сравнительный анализ эффективности и безопасности применения вирусных векторов как для создания рекомбинантных вакцин, так и для получения онколитических вирусов, препаратов для генной терапии.

Ключевые слова: рекомбинантный вирус; CRISPR/Cas9; гомологичная рекомбинация; клонирующий вектор; плазида; вирусный вектор; рекомбинантная вакцина; обзор

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Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Introduction

Vaccination is a cost-effective and efficient way to combat the spread of infectious diseases. Starting in 1796 (the first vaccination performed by E. Jenner), live attenuated vaccines were used, followed by inactivated and subsequently subunit vaccines [1–3]. In the initial stage of recombinant vaccine development, only poxvirus, herpesvirus and adenovirus vectors were primarily used. Currently, a significant number of recombinant vaccines based on DNA and RNA-containing recombinant viruses have been created [4], but in Russia, only the Gam-COVID-Vac vaccine is used in practice.

Currently, modern modifications of specific prophylaxis drugs include vaccines based on recombinant viruses, virus-like particles, as well as DNA and mRNA vaccines, which significantly increases the safety and effectiveness of vaccination and solves a multitude of problems that arise when using traditional live attenuated vaccines. Recombinant viruses are also used to create drugs for treating oncological and genetic diseases [1, 3, 5–8].

Typically, recombinant viral vector vaccines are developed using an attenuated heterologous viral vector as the target antigen producer (**Fig. 1**), as the use of a homologous virus as a vector predetermines the possibility of its virulence reverting upon recombination with the original virulent pathogen [3, 9]. Vaccine recombinant viruses contain a modified genome with a promoter upstream of the cloning sites for the insertion of one or more foreign genes encoding target protective antigens [10]. When vector vaccines are used, antigen expression is similar to that in natural infection, creating the possibility of delivering target antigens to specific cells and tissues [1, 11, 12]. Heterologous vector vaccines are developed based on two or more viral vectors encoding the same or different target antigens. A double-dose vaccination schedule elicits a more pronounced and long-lasting immune response compared to a single or double-dose vaccination with a single-vector vaccine [1, 13].

The highest level of safety is achieved by using single-cycle vaccines. These are vaccine recombinant viruses from whose genome a protein necessary for viral replication has been removed (typically, this is a protein involved in virion assembly). Simultaneously with the construction of a recombinant virus for the replication of this modified agent, a specialized cell line is being created in which the protein necessary for viral replication is synthesized. As a result, the defective recombinant virus replicates in a modified cell culture, and preparative quantities of it are produced. However, within the macroorganism, this virus loses its ability for productive infection and complete virion assembly during the synthesis of essential protective proteins. When such a recombinant virus is administered, both branches of the immune defense are activated: humoral and cellular, while virus itself lacks its contagiousness and ability to persist [14, 15].

For recombinant vaccines, the use of an adjuvant is necessary to enhance the immune response and increase its duration, although protection is possible without it [1, 3]. When administered orally or intranasally, the recombinant viral vaccine promotes the development of both a systemic immune response and a local reaction in the body, depending on the method of administration [1, 10].

Methods for constructing recombinant viruses

Several methods have been developed for constructing recombinant viruses: homologous recombination (HR) [16], transposon-mediated insertional mutagenesis [17], nuclease methods ZFN [18], TALEN [19], as well as reverse genetics approaches [20, 21] and others.

Reverse genetics allows for the creation of a modified biologically active virus by transfecting cell lines infected with a viral vector with plasmids containing genes encoding target proteins. By including mutations into various target genes, it is possible to reduce the virulence and alter the antigenic properties of both the vector itself and the cloned viral genes [22].

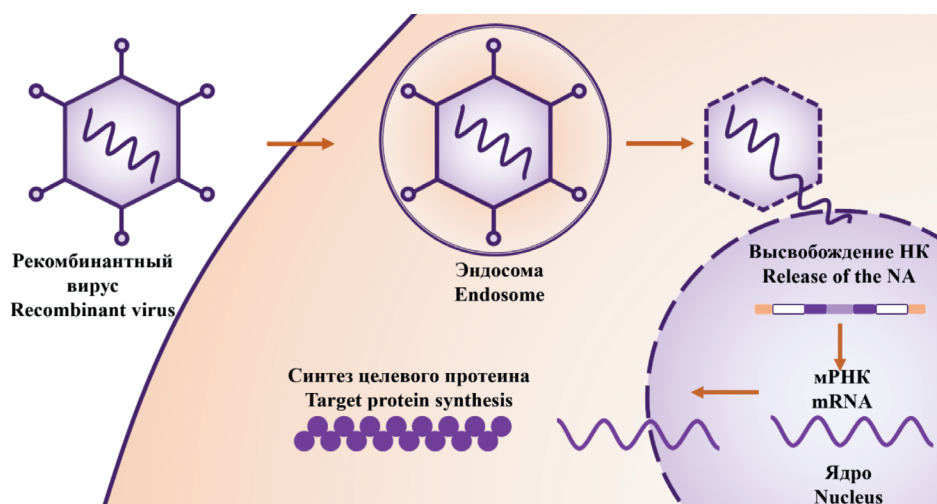


Fig. 1. Synthesis of the target protein by cellular ribosomes.

Рис. 1. Синтез целевого белка рибосомами клетки.

Typically, creating a recombinant virus requires a viral recipient vector and a donor plasmid (or synthetic) carrier of the target gene. For viral DNA vectors, the preparatory stage ends here. Genetic modification of RNA viruses requires a preliminary step of obtaining complementary DNA (cDNA) [16]. However, the synthesis of full-length cDNA can be hindered by a number of factors, such as toxicity to bacteria (which is addressed by the CPEC method (Circular polymerase extension reaction), where the vector is assembled sequentially from amplicons during reverse transcription polymerase chain reaction), hairpin formation, and others [21]. Further processes for obtaining recombinant RNA-containing viruses also have a number of differences that depend on the type of viral RNA: plus-strand genome, minus-strand genome, integrated genome, segmented genome, or double-stranded genome. For example, for recombinant RNA viruses with a «+» genome, efficient delivery (most often using liposomes) of the newly synthesized RNA into the cell cytoplasm is necessary, while for recombinant RNA viruses with a «-» genome, a helper virus or even the insertion of a modified full-length cDNA into a large DNA-containing viral vector is required, similar to how the marked recombinant vaccine against rinderpest (RPV) was obtained [23].

Regarding retroviral recombinants, it is known that the final product of the polymerase reaction is a double-stranded DNA provirus containing all viral genes and flanked by 3' and 5' LTRs (long terminal repeats). Pro-viral DNA, integrase (IN), and certain viral and cellular proteins form a viral pre-integration complex, which is imported into the nucleus, where IN catalyzes the integration of viral DNA into the cell's genome [24]. However, at the current stage, integrase-defective lentiviral vectors (IDLV) have been created, which, even after a single immunization, stimulate a long-lasting immune response and have a high level of safety [25, 26].

A well-reproducible HR method is used to create the recombinant virus (Fig. 2 a). To obtain a recombinant vi-

rus, a plasmid vector is constructed with sequences flanking the target gene that are homologous to the insertion site in the vector virus genome [16]. The plasmid vector DNA is administered into virus-infected cells or co-transfected with the viral genomic DNA. In transfected cells, sequence exchange occurs between the plasmid and viral DNA containing homologous regions. Since a viral vector must have some kind of selective marker, recombinant viruses are selected by cloning based on the difference in replication between the original and the marker-containing recombinant virus [27].

Currently, the most effective and versatile method for editing the viral genome is CRISPR/Cas9 technology (Fig. 2 b) [19, 28]. It is characterized by high accuracy in editing DNA targets and low off-target cleavage activity, making it relatively simple to use, which is why the CRISPR/Cas9 system has replaced its predecessors, ZFN and TALEN [29, 30]. The CRISPR/Cas9 technology is based on the fact that instead of proteins (as in ZFN and TALEN), small guide RNA molecules (gRNA) are used to recognize the target sequence. The CRISPR/Cas9 genome editing system consists of a DNA-binding domain responsible for recognizing and binding to a specific DNA sequence, and an effector domain that mediates DNA cleavage [29]. Editing occurs in two stages: DNA cleavage and subsequent repair [28], which happens either thru non-homologous end joining (NHEJ) or homology-directed repair (HDR). To increase the efficiency and accuracy of the added changes, methods that suppress NHEJ and enhance HDR are used, based on chemical modulation and synchronized expression of overlapping homologous regions. The advantage of CRISPR/Cas9 is the ability to quickly create the guide RNA with ease, as well as the ability to modify multiple target genes. Nevertheless, CRISPR/Cas9 has limitations related to the difference in DNA cleavage rate compared to the virus replication rate, and the necessity to improve repair efficiency [28].

Widely sought-after genome editing technologies are rapidly and successfully developing. Yes, the recently

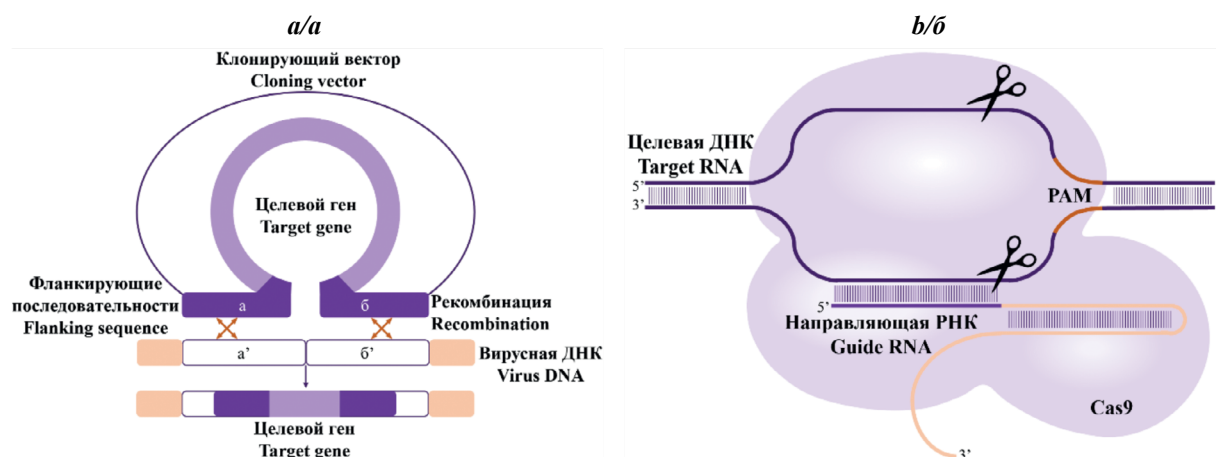


Fig. 2. The main methods for creating recombinant viruses.

a – homologous recombination; b – CRISPR/Cas9 system.

Рис. 2. Основные методы создания рекомбинантных вирусов.

a – гомологичная рекомбинация; б – система CRISPR/Cas9.

developed CRISPR/Cas9 system is being replaced by a new method called NICER, which is based on the use of Cas9 nickase, creating only single-strand breaks that are repaired without the risk of mutations, thus expanding the possibilities for correcting genetic disorders associated with certain diseases. [32].

Cloning vectors

Plasmid vectors have a mandatory minimal structure that includes an origin of replication (ori), a selective marker for antibiotic resistance, and a multiple cloning site (MCS) [33]. Other types of plasmids are also used as transfer vectors: fosmids based on the bacterial F-plasmid; cosmids containing lambda phage DNA with a cos sequence; yeast plasmids from *Saccharomyces* strains [34], linear plasmids from *Streptomyces*, etc. [35]. However, the accumulation and isolation of plasmids from yeast is a more complex, labor-intensive and expensive process compared to *Escherichia coli* [36].

BAC (Bacterial artificial chromosome) – low-copy vectors with high capacity (up to 300 kb) compared to plasmid vectors (up to 10 kb), used for cloning large viral genomes [27, 33, 37, 38]. The advantages of BACs are high replication accuracy, the absence of selective pressure on the viral genome in *E. coli*, and the lack of a toxic effect on bacteria [27].

The most popular method for obtaining cloning vectors is the restriction enzyme-ligation method, but there are also a number of alternative technologies: MCS and *in vivo* bacterial assembly [39], FastCloning [40], GATEWAY recombination cloning [41], SLiCE [42], Golden Gate [43], ligation-independent ELIC [44], SLIC [45], HAC [46], One-step SLIC [47] and In-fusion [48].

Virus vectors

Adenoviruses are often used as vectors because they have broad tropism, high transduction efficiency, and do not integrate into the host genome [1, 49]. Both replication-competent and replication-defective variants are chosen as adenovirus-based vectors: depending on whether they contain the entire early gene region responsible for modifying host gene expression and viral protein synthesis, or only a part of it [1, 50, 51]. For an effective immune response, the choice of adenovirus vector is based on the use of less common serotypes to avoid antibody-dependent inhibition of its replication [49]. Adenovirus type 5 (Ad5) was previously the standard choice as a vector, but its use is currently hindered by high seroprevalence among humans, reaching 90%. Therefore, to address this issue, other, rarer human adenovirus types (Ad26, Ad35, Ad11) and animal adenoviruses have been used [1, 52]. High seroprevalence leads to a reduced immune response, as demonstrated during the STEP candidate HIV vaccine trial: vaccinated individuals showed an increased susceptibility to HIV [53]. To protect against SARS-CoV-2, vaccines have been developed, including those approved for mass use, based on Ad5 [54–57], Ad26 [1, 54, 56, 58, 59], as well as their combination [54]. Vaccines based on Ad5 [60, 61] and Ad26 [62] were also developed against Ebola fever.

Various serotypes of chimpanzee adenovirus (ChAd), and particularly ChAdOx1 (a replication-deficient vector), have been used to create vector vaccine candidates against rabies virus (RABV), MERS, SARS-CoV-2 and others [1]. Although the candidate vaccine ChAdOx1 nCoV-19 was deemed safe, a high level of anti-platelet factor 4 (PF4) autoantibodies was found in those vaccinated, which is explained by the formation of a complex between PF4 and adenoviruses (including Ad5 and Ad26) [1, 63–65]. Chimpanzee adenovirus was used as a viral vector in the development of candidate vaccines ChAd3-EBO-Z against the Ebola virus [66], ChAdOx1-GnGc against Rift Valley fever [67].

Adeno-associated virus (AAV)-based vectors are popular for gene therapy and therapeutic antibody delivery [11]. Despite widespread immunity to AAV, some vaccines based on them generate a higher and more durable immune response compared to other types of vaccines [1]. The first recombinant AAV was created with herpes simplex virus type 2 followed by the development of rAAV-B11-Fc, which expresses antibodies with neutralizing activity against botulinum toxin type A [69], as well as the rAAV-COVID-19 candidate vaccine [70]. Several drugs have been developed and approved for gene therapy: Onasemnogene Apeparvovec based on AAV9 for treating spinal muscular atrophy in children [5, 6], Valoctocogene Roxaparvovec and Etranacogene Dezaparvovec based on AAV5 for treating hemophilia A and B [7, 71], and Voretigene neparvovec based on AAV2 for treating Leber's congenital amaurosis [8]. However, the use of rAAV, especially at high doses, can be limited due to immune system reactions that lead to toxic effects. Immunosuppression used to inhibit immune reactions to the AAV vector is often ineffective. The cause of toxic effects is the adaptive immune response to AAV capsid antigens, as well as excessive activation of the complement system. Side effects of AAV vector use include: thrombotic microangiopathy, liver circulatory disorder (fulminant hepatotoxicity), toxicity to the dorsal roots of spinal nerves, myocarditis, and allergic reaction. The development of toxic effects after AAV vector administration is influenced by: dosage, AAV serotype, route of administration, and individual patient characteristics (age, presence of certain diseases) [72].

Poxvirus vectors are characterized by high immunogenicity and the ability to induce a sustained and rapidly developing immune response, which can be enhanced when combined with two vectors [1, 73]. The advantage of poxvirus vectors is their largest capacity (~ 25 kb), which allows for the construction of multi-antigen vaccines [1, 74]. Attenuated orthopoxviruses are used most often, mainly MVA and NYVAC [75]. However, serious side effects are possible when a large dose (over 10⁸ PFU) is administered. Another disadvantage of poxvirus-based vaccines is the possible decrease in immunogenicity in individuals previously vaccinated against smallpox [75] {Citation}. Based on MVA, recombinant vaccine candidates SARS-CoV ADS-MVA [76], MVA-MERS-S against MERS-CoV [9], recombinant vaccine against cytomegalovirus infection (ALVAC-gB) was created based

on canarypox virus [77], and cowpox virus was used to construct LIPV-hIFN α and LIPV-mIFN α ; these recombinant viruses were developed in Russia and are characterized by high onco-selectivity and oncolytic activity [78]. In the Russian Federation, at the Institute of Chemical Biology and Fundamental Medicine of the Siberian Branch of the Russian Academy of Sciences, V.A. Richter and et al. created an oncolytic virus based on the vaccinia virus strain VV-GMCSF-Lact to combat breast cancer; phase I clinical trials have been conducted to date [79, 80]. To obtain VV-GMCSF-Lact, the parent strain LIPV of the vaccinia virus was used. The thymidine kinase gene fragment and growth factor were removed, and the genes for the antitumor immune response inducer cytokine GM-CSF (granulocyte-macrophage colony-stimulating factor) as well as the single-chain toxin lactaptin were inserted [79]. Comparative studies of the oncolytic activity of recombinant vaccinia virus strains LIPV-RFP and MVA-RFP with an inactivated thymidine kinase gene against solid tumors were also conducted in Russia [81].

Herpes simplex virus type 1 (HSV-1) is used as a viral vector for creating gene therapy drugs and treating oncological diseases. HSV-1 has a large capacity and also exhibits high activity in tumor destruction [82, 83]. Nevertheless, there are challenges in ensuring the safe and highly productive manufacturing of such drugs. The preferential tropism of HSV-1 proteins for surface receptors on nerve tissue cells and the possibility of deleting the Us3 gene, which causes the activation and synthesis of phosphatidylinositol-3-kinase, a signaling molecule in the processes of tumor cell proliferation and apoptosis, make HSV-1 an effective tool for treating central nervous system tumors. The main disadvantage of using HSV-1 as a vector virus for gene therapy is the toxicity associated with the vector's characteristics, as well as the induction of inflammation. Therefore, the goal of HSV-1 modification is to reduce its neurotoxicity and increase its ability to infect glioma cells, as well as to enable the expression of various transgenes that enhance the body's own antitumor immunity [84]. The antitumor properties of the described recombinant oncolytic herpesviruses have been tested both *in vitro* and *in vivo* models. For example, the HSV1716 strain has undergone three Phase I clinical trials [85].

Currently, there are already FDA-approved drugs: Imlygic for the treatment of malignant melanoma [86], and Vyjuvek for the therapy of dystrophic epidermolysis bullosa [87].

Among viral RNA constructs, vectors based on vesicular stomatitis virus (VSV) are of particular interest, which have a high level of reproduction and are characterized by low antibody prevalence in humans [1, 88, 89], broad tropism [89], high immunogenicity after a single administration, and a prolonged immune response. Their immunogenicity is much higher compared to replication-deficient RABV-based vaccines [10]. The disadvantages of VSV vectors include: low efficacy upon re-administration, and weak tropism for cancer cells compared to other oncolytic viruses [89]. The VSV vector was used for the already approved Ebola virus vaccine (rVSV-ZEBOV) [1, 88]; as

well as for candidate vaccines to prevent Marburg hemorrhagic fever [90], Lassa fever [91] and others.

Integrase defective lentiviral vectors (IDLVs) stimulate an intense and long-lasting immune response after a single immunization and have a high safety profile [1, 25, 26]. IDLVs are derived from HIV or simian immunodeficiency virus by removing the region responsible for viral replication from the genome and mutating the long terminal repeats of the packaging signal and the integrase gene [1, 10]. However, IDLV is characterized by a significantly lower level of expression compared to integrating lentiviruses [92, 93], as well as a lower risk of insertional mutagenesis [94]. Based on IDLV, recombinant viruses have been created to enhance the transduction efficiency of dendritic cells [95], and IDLV has also been used to deliver influenza H1N1 virus antigens [96, 97].

Due to its high genomic stability, parainfluenza virus (PIV) serotypes 1, 2, 3, and 5, as well as B/HPIV3 – a chimeric bovine and human parainfluenza virus – are used as vectors. The existence of a possible expansion of tropism and increased pathogenicity has been established when using PIV5 [98]. Based on PIV, candidate vaccines have been developed for the prevention of human parainfluenza type 2, COVID-19 [99], respiratory syncytial virus infection [100] and Ebola fever [101].

It is generally accepted that the measles vaccine virus MeV strains Schwarz and Moraten are safe viral vectors [10]. MeV allows for the creation of polyvalent recombinant vaccines. Prior measles vaccination usually does not reduce the immunogenicity of MeV-based vaccines, and MeV is also highly stable [102]. However, it was found that pre-existing immunity to measles can affect the effectiveness of immunization depending on the antigen chosen [103]. Candidate vaccines based on MeV have been developed for the prevention of West Nile fever [104], Chikungunya fever [105] and Zika fever [106].

Sometimes a viral vector based on a low-virulence Newcastle disease virus (NDV LaSota, B1) is also used, as NDV administration enhances interferon induction, and its proteins have adjuvant properties [10, 107]. It is important to note that humans can be susceptible to the Newcastle disease virus, but the course of the illness is usually mild and does not cause complications [108]. However, two fatal cases have recently been recorded following infection in immunosuppressed individuals [109]. The effectiveness of revaccination using NDV as a vector can be reduced due to the presence of antibodies against it, which limits its potential for continuous use [4]. NDV has been used to develop candidate vaccines against COVID-19 [110], as well as Ebola fever [111].

The characteristics of viral vectors are presented in the Table. (Table выделить пж)

Conclusion

In modern approaches to human and animal immunization, recombinant viral vaccines are increasingly being used. The use of recombinant vaccines based on a homologous replicating vector carries a high risk of restoring its pathogenic properties [1, 3, 9]. To avoid reversion of virulence in the pathogen, an alternative option with a higher level of safety

Table. Comparative characteristics of viral vectors [52, 55, 83, 84, 88, 98, 102, 108, 112–116]**Таблица.** Сравнительная характеристика вирусных векторов [52, 55, 83, 84, 88, 98, 102, 108, 112–116]

Family, genome size Семейство, размер генома	Viral vector Вирусный вектор	Vector capacity Емкость вектора	Advantages Достоинства	Disadvantages Недостатки
<i>DNA viruses</i> ДНК-содержащие вирусы				
<i>Adenoviridae</i> 30–45 kb/т.п.о.	Ad5, Ad26, ChAd, ChAdOx1	6–15 kb/т.п.о.	Broad tropism, efficient transduction, lack of integration into the host genome Широкий тропизм, эффективная трансдукция, отсутствие интеграции в геном хозяина	Risk of thrombosis. Ad5: high antibody prevalence in humans Риск тромбозов. Ad5: высокий уровень превалентности антител у людей
<i>Parvoviridae</i> 4,7 kb/т.п.о.	AAV	4,5 kb/т.п.о.	A strong and sustained immune response Высокий и устойчивый иммунный ответ	Риск тяжелых токсических эффектов Risk of severe toxic effects
<i>Poxviridae</i> 200–300 kb/т.п.о.	MVA, NYVAC, VOV	25 kb/т.п.о.	High immunogenicity, long-lasting immune response, high capacity Высокая иммуногенность, длительный иммунный ответ, большая емкость.	Высокие дозы повышают побочных эффектов, низкая иммуногенность, у привитых осповакциной High doses increase side effects, low immunogenicity in smallpox vaccinees
<i>Herpesviridae</i> 152 kb/т.п.о.	HSV-1	15–25 kb/т.п.о.	High capacity, high oncolytic activity Большая емкость, высокая онколитическая активность	Обеспечение безопасности на производстве, токсичность вектора Ensuring safety in production, vector toxicity
<i>RNA viruses</i> РНК-содержащие вирусы				
<i>Rhabdoviridae</i> 11 kb/т.п.о.	VSV	Up to 6 kb До 6 т.п.о.	Low antibody prevalence in humans, high immunogenicity Низкая превалентность антител у людей, высокая иммуногенность	Невозможность повторного введения, низкая селективная онколитическая активность Inability to re-administer, low selective oncolytic activity
<i>Retroviridae</i> 10,7 kb/т.п.о.	IDLV	Up to 10 kb До 10 т.п.о.	High level of safety, long-lasting immune response, reduced risk of insertional mutagenesis Высокий уровень безопасности, длительный иммунный ответ, сниженный риск инсерционного мутагенеза	Низкий уровень экспрессии генов Low gene expression level
	PIV1, PIV2, PIV3, PIV5, B/HPIV3	1,5–2,5 kb/т.п.о.	High genomic stability Высокая геномная стабильность	Возможное расширение тропизма и патогенности Possible expansion of tropism and pathogenicity
<i>Paramyxoviridae</i> 15 kb/т.п.о.	MeV	5 kb/т.п.о.	Stability, the possibility of creating polyvalent vaccines Стабильность, возможность создания поливалентных вакцин	Редко: негативное влияние иммунизации от кори на иммуногенность рекомбинантных вакцин Rarely: the negative impact of measles immunization on the immunogenicity of recombinant vaccines
	NDV	4,5 kb/т.п.о.	High immunogenicity, adjuvant properties Высокая иммуногенность, адъювантные свойства	Decreased effectiveness upon repeated administration Снижение эффективности при повторном введении

was developed – vaccines based on heterologous vectors and replication-deficient vectors – single-cycle [3].

The creation and application of recombinant viruses for vaccine prevention, cancer therapy, gene therapy, and the study of the viral genome significantly improve the arsenal of protective measures in modern medicine, veterinary medicine and biology in general. Currently, the methodology for obtaining recombinant vaccines based on CRISPR/Cas9, homologous recombination, etc., has

increased their safety and vaccination efficacy, made it possible to create new treatments for oncological and genetic diseases, and research in this area remains one of the key prospects in modern virology.

However, the use of recombinant viruses as vaccines remains a poorly studied area in both epidemiology and epizootology. A large number of recombinant vaccines have been developed and are successfully used in veterinary medicine, which is fully justified since their

use in animals provides statistically significant material for medical researchers when analyzing the efficacy and safety of recombinant vaccines. The spread of viruses within a target population always implies the possibility of a viral vector coming into contact with its virulent counterpart, creating the conditions for a partial or complete restoration of pathogenic properties. The rate of viral evolution should also be considered, which is approximately 10^{-2} – 10^{-4} nucleotide substitutions per site per year for RNA viruses and 10^{-5} – 10^{-6} nucleotide substitutions per site per year for DNA viruses [117, 118]. Therefore, the question of using recombinant virus vaccines should be decided based on joint research by epidemiologists, epizootologists, virologists and molecular biologists.

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