Poxvirus Vectors

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Abstract

The utility of poxviruses as expression vectors was first described in the early 1980s. Since then, poxviruses have been widely used as vaccine delivery platforms in human and veterinary medicine. The main features that make poxviruses excellent antigen delivery platforms and vaccine vectors are their large genome size with the presence of multiple immunomodulatory genes, the tolerance for large heterologous gene insertions, and their ability to induce cellular and humoral immunity. Initial attempts were focused on engineering vaccinia virus to express heterologous genes. Later, the potential of other poxviruses including avipoxvirus, parapoxvirus, and swinepox viruses as vectors was also explored with promising results. To address the safety concerns related to wild-type poxviruses, several highly attenuated, replication-defective strains have been developed mostly by serial passages in cell culture. Most of the recombinant poxviruses developed to date have targeted insertional inactivation of the thymidine kinase (TK) gene, in which the heterologous gene is inserted in the TK locus in the poxvirus genome. In recent years, other immunomodulatory genes have also been used to generate safer and multivalent poxvirus-vectored vaccine candidates. Poxvirus vectors have been shown to be very effective in heterologous prime-boost immunization regimes, where poxvirus vectors are used in combination with other killed or DNA vaccine formulations. To date multiple poxvirus-vectored vaccines have been licensed for use against a variety of animal pathogens including rabies virus (RabV), avian influenza virus (AIV), canine distemper virus (CDV), and West Nile virus (WNV).

Keywords

Poxvirus · Vector · Vaccine · Delivery platform · Immunity

Learning Objectives

After reading this chapter, you should be able to:

- Describe how poxviruses can be used in vaccine delivery platforms
- Explain how poxvirus vectors are constructed, selected, and utilized for vaccine delivery in domestic animal species
- Describe how poxvirus vectors are applied in veterinary medicine

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1 Introduction

Poxviruses are large complex viruses that belong to the family *Poxviridae*. The *Poxviridae* is divided into two subfamilies: the *Chordopoxvirinae* and *Entomopoxvirinae*. The subfamily *Chordopoxvirinae* comprises viruses that infect vertebrate animal species, whereas the *Entomopoxvirinae* contains viruses that mainly infect insects. Currently, there are 11 genera classified under the *Chordopoxvirinae* subfamily and 3 genera under the *Entomopoxvirinae* [1]. Poxviruses are classified in these genera on the basis of virus morphology, phylogeny, serological cross-reactivity, and host range [2]. The poxvirus species name usually refers to the host from which the virus was first isolated. While some poxviruses have restricted host range (i.e., variola virus known to infect only humans), there are many others, including cowpox virus (CPXV), buffalopox virus (BPXV), and monkeypox virus (MPXV), which have broad animal host range infecting multiple mammalian species including humans. Poxviral infections occur through different routes, including the skin (Orf virus), respiratory tract (variola virus), or oral route (ectromelia virus) [3] and are characterized by formation of skin lesions, usually evolving through the stages of papules, pustules, vesicles, nodules, and scabs [4].

Poxviruses are among the largest known viruses. Most poxviruses contain brick-shaped virions with a particle size ranging from 220 to 450 nm long × 140 to 260 nm wide × 140 to 260 nm thick. Parapoxviruses are oval-shaped with a particle size of 260 nm × 160 nm [4]. Virions are enveloped with the presence of surface tubules or surface filaments (Fig. 1a). Internally, the virions contain two lateral bodies and a dumbbell-shaped nucleoprotein core. The nucleoprotein core contains enzymes essential for virus replication and the nucleocapsid protein bound to the viral genome. The viral genome is a linear double-stranded DNA with the size ranging from 130 kbp to 375 kbp and encode between ~130 and 350 open reading frames (ORFs). The two strands of DNA are cross-linked at the termini due to presence of A+T-rich inverted terminal repeats (ITR) at the two ends of the genome. The central region of the genome is highly conserved across different poxviruses and encodes genes essential for viral transcription, replication, and virion assembly. Non-conserved genes that are involved in virus host range, immunomodulation, and virulence and pathogenesis are present at either end of the genome, flanking the conserved central genome core (Fig. 1b).

A unique feature of poxviruses is their replication site, which takes place in the cytoplasm of infected cells, making poxviruses an exception among DNA viruses. The replication mechanism of vaccinia virus (VACV) have been widely studied, and most of our understanding of poxvirus replication comes from VACV. Notably, transcription and expression of poxviral genes are temporally regulated, and the genes are classified as early, intermediate, or late genes based on the time of expression in relation to virus genome replication. In general, early genes are transcribed before replication, whereas intermediate and late genes are transcribed after the virus genome has been replicated. For VACV, for example, early, intermediate, and late genes are expressed in 20, 100, and 140 min after infection, respectively [5]. The poxvirus virion contains essential enzymes to initiate viral transcription upon infection. Therefore, early genes are transcribed within the virion core soon after the virus enters the cell, and mRNAs are extruded into the cytoplasm for translation. These early genes encode for transcription factors required for expression of intermediate genes. Some of the early proteins also play important roles in host immune evasion and modulation. Once early genes are expressed, uncoating of the virion core takes place, and DNA is released into the cytoplasm followed by viral DNA replication which occurs in discrete replication sites within the cytoplasm designated viral factories. The intermediate genes are expressed after DNA replication and encode for transcription factors required for the expression of late genes. The late genes encode proteins essential for virion assembly and early gene

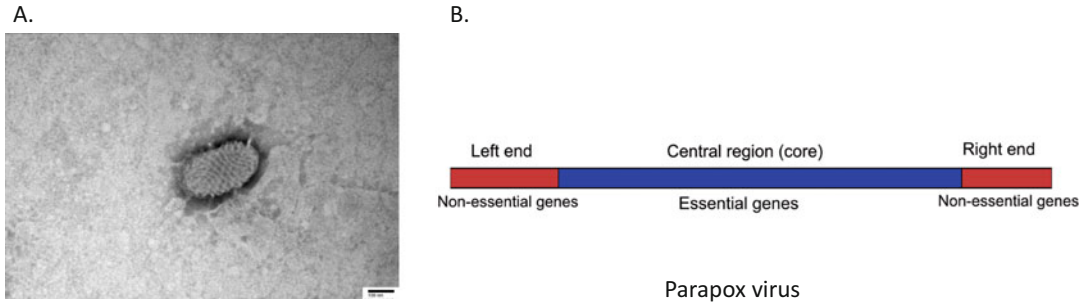

Fig. 1 Structure and genome of poxvirus. (**a**) Negative stain preparation of Orf virus (parapoxvirus). (**b**) Schematic representation of poxvirus genome. The essential genes are present in the central part of the genome. The non-essential genes which play role in immunomodulation are present in either end of the genome

transcription factors which will be packaged within the virion core. After virus assembly, enveloped virions are released by budding, whereas non-enveloped virions are released by cell lysis. The understading of these basic biological properties of poxviruses is important in designing poxvirus vectors.

2 Construction of Poxvirus Vectors

Poxviruses hold a unique place in the history of immunization. In 1796, Edward Jenner demonstrated that smallpox could be prevented by using CPXV as a vaccine [6]. Later, VACV was widely used to immunize people against smallpox, which culminated with the eradication of the disease in 1980. To date, smallpox remains the only human disease that has been eradicated. Although smallpox was eradicated, and vaccination was discontinued, the biological and immunomodulatory properties of VACV, the virus used as vaccine against smallpox, generated significant interest in poxviruses among scientists worldwide. Soon after the eradication of smallpox, a few studies describing genetically engineered VACV and the use of vaccinia as a eukaryotic expression system were published [7, 8]. In addition, recombinant VACV expressing single or multiple heterologous viral antigens were developed establishing the foundation for the use of poxvirus as vaccine delivery vectors [9–11].

Some of the features that made VACV a well-received and widely used vector are (i) the large genome size (190 kb), with the presence of many non-essential genes, which could be manipulated without severely impacting virus replication; (ii) the ability of VACV to tolerate insertion of up to 25,000 bp of foreign DNA [12]; (iii) the fact that the virus is a potent inducer of both humoral and cell-mediated immunities [13]; (iv) the ease of administration and its efficacy through different immunization routes [13]; and (v) the stability of the virus at room temperature when lyophilized, which obviates the need for cold chain [14]. Given that poxviruses share many common properties, the features described for VACV above also apply to other viruses in the family. The immunomodulatory properties of poxviruses and the efficacy of VACV as a vector platform led several groups to explore other poxvirus vector alternatives. Several studies showed the potential of other members of the family *Poxviridae*, including avipoxviruses (fowlpox [FWPV] and canarypox virus [CNPV]), swinepox virus (SWPV), and Orf virus (ORFV), as vectors for human and veterinary applications. These vectors are described below with the primary focus on their use to deliver veterinary vaccines.

3 Strategies Used in Developing Recombinant Poxvirus Vectors

Earlier studies in the 1960s showed that genetic recombination can occur between two different strains of related poxviruses when both viruses infect a single cell [15, 16]. This process known as homologous recombination involves the exchange of nucleotide sequences between two similar or identical DNA molecules [17]. Homologous recombination is now widely used for the generation of recombinant poxviruses [18–21]. This method requires the construction of a transfer plasmid (recombination plasmid) containing the foreign gene insert (heterologous gene) and the left and right homology DNA sequences flanking the insertion site from the parent poxvirus genome (Fig. 2). Homologous recombination and recombinant poxvirus generation are achieved by infecting permissive cells with the parent poxvirus and subsequently transfecting these cells with the recombination plasmid (infection/transfection). Within cells that were infected and transfected, homologous recombination between the parental virus and the recombination plasmid takes place, resulting in a new chimeric recombinant poxvirus. The recombinant poxvirus is purified by multiple rounds of limiting dilution and/or plaque assay (Fig. 2).

There are several factors that need to be considered to achieve homologous recombination, including the homology length and the DNA structure [22]. Higher recombination frequencies were obtained, for example, when homologous flanks with at least 100–350 bp and linear plasmid DNA were used in infection/transfection experiments with VACV [22]. In addition to the insertion site and homology length, the following factors need to be considered to design and generate poxvirus-based vectors:

1. *Promoters*. Given the temporal regulation of poxvirus gene transcription (early, intermediate, and late), selection of the promoter that will drive expression of the heterologous gene is a critical aspect of the design of poxvirus vectors. In general, promoters with both early and late activities are ideal for expression of foreign genes because they drive expression of the heterologous genes throughout the vector infection cycle, promoting sustained expression of the antigen and consequent stimulation of the immune system. Early promoters would also be preferable when the poxvirus vector is replication defective or when the vector is to be used in a non-permissive animal species, both of which preclude expression driven by late promoter, which takes place after virus replication. The most commonly used promoters to drive expression of heterologous genes by poxviruses include the native VACV early/late promoters ($P_{7.5}$ or $VV_{7.5}$), the modified early promoter (mH5), or synthetic promoters such as PrS, for which expression has been optimized by mutagenesis [23].
2. *Termination signal*. The presence of poxvirus early termination signal TTTTTTNT within the sequence of heterologous genes could potentially lead to premature transcription termination and consequently low expression levels or expression of a truncated protein [24]. Therefore, termination signals should be removed through site-directed mutagenesis or synthetic biology from the heterologous gene sequence before inserting the gene into the vector [20].
3. *Codon optimization*. Codon optimization of the heterologous gene may help to achieve higher expression levels especially when the recombinant is to be used in non-target animal species, in which replication and late gene expression are impaired. Codon optimization helps in the stability of the recombinant vector by removing non-desirable sequences [23]. Additionally, it may also be used when multivalent heterosubtypic viral vectors containing two or more viral genes from closely related virus strains are designed. Codon optimization and changes in the nucleotide sequence of one of the genes increase the stability of the vector by preventing or reducing the risk of intramolecular homologous recombination.

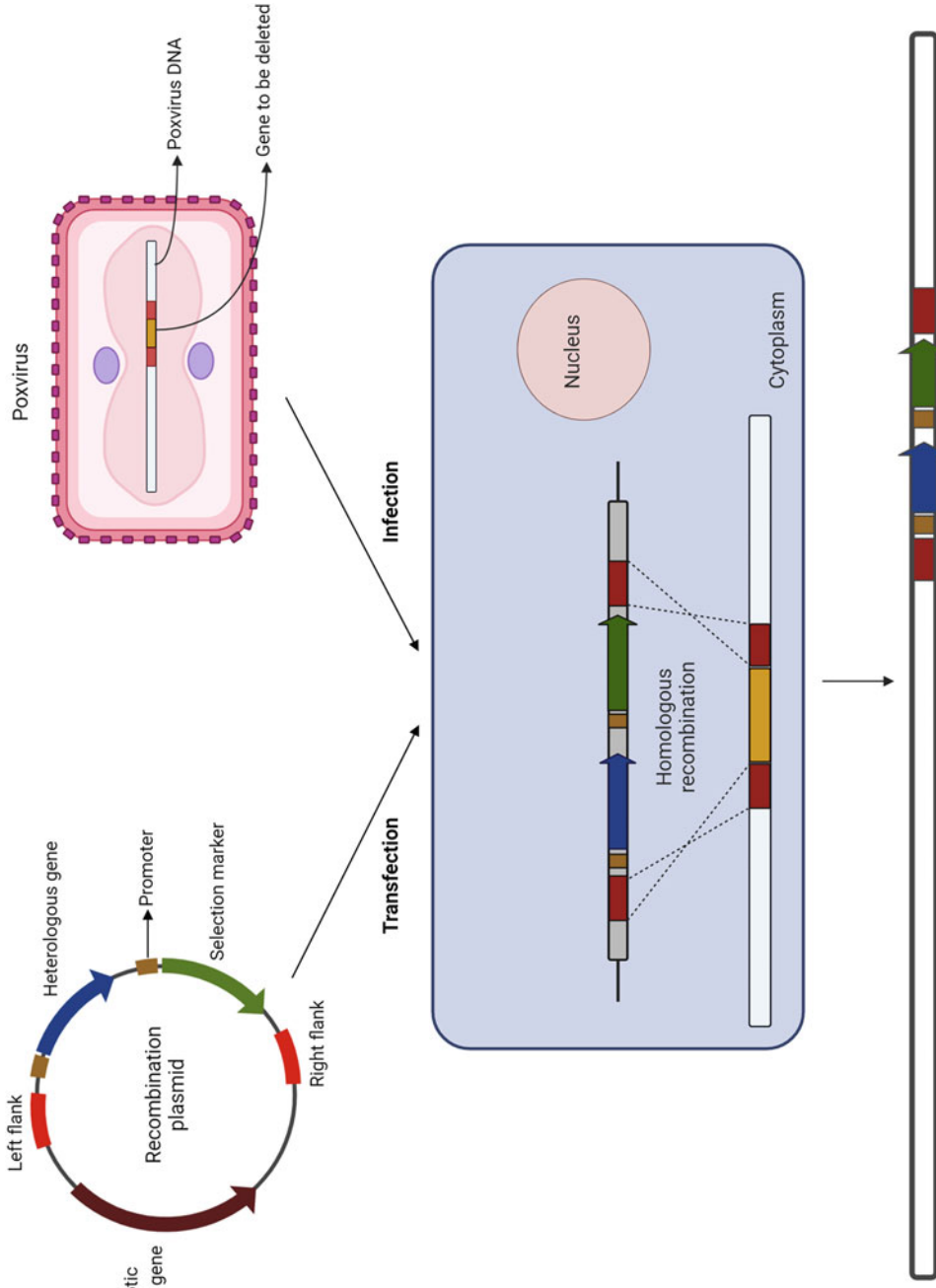

Fig. 2 Schematic representation of homologous recombination. Cells are infected with parental poxvirus and transfected with recombination plasmid. Homologous recombination between the plasmid and parental poxvirus genome occurs within the cytoplasm resulting in a chimeric recombinant poxvirus.

4. *Selection method*. Selection of recombinant poxviruses is one of the most time-consuming steps in generating recombinant poxvirus-based vectors. Conventionally, selection of recombinant poxviruses has been based on expression of the β-galactosidase reporter gene. The 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) substrate is incorporated into the agarose overlay during plaque assay, and recombinants expressing β-galactosidase form blue plaques, which can be selectively picked and purified [18, 25]. Additionally, drug resistance genes like neomycin resistance gene can be used as a selectable marker for selection and isolation of poxvirus recombinants [26]. More recently, fluorescent proteins like the green fluorescent protein (GFP) have also been used successfully in recombinant poxvirus selection. The gene expressing GFP or other fluorescent proteins is inserted along with the gene of interest. The recombinant poxvirus expressing fluorescent protein can be selected by using plaque assay [20]. The presence of marker genes is not always recommended, as tandem expression of multiple genes can result in lower protein expression levels due to promoter interference. Therefore, strategies to develop markerless recombinant poxviruses have been recently developed. The most straightforward approaches involve selection of recombinant viruses by real-time PCR or immunofluorescence assays targeting the heterologous genes [27]. Whereas, more sophisticated approaches using excisable marker systems based on Cre/loxP recombination, which facilitate selection and subsequent removal of marker gene, were also developed and provide an efficient means to create markerless recombinants [28]. Recently, a marker-free system for construction of vaccinia virus vectors using CRISPR (clustered regularly interspaced short palindromic repeat)-Cas9 has also been reported [29].

4 Application of Poxvirus Vectors

Several poxvirus platforms have been developed and used as vaccine delivery vectors in veterinary species. Table 1 summarizes the poxvirus-vectored vaccines that are currently licensed and commercially available for use in veterinary medicine. Below we present a brief discussion of the main poxvirus vectors and their applications and/or uses in animals.

5 Orthopoxvirus-Based Veterinary Vaccines

Vaccinia virus (VACV), the type species of the *Orthopoxvirus* genus, has been widely used as a vector for vaccine delivery. Initially, parental moderately virulent VACV strains like Western Reserve (WR), Copenhagen, and Lister were used to develop recombinant vaccines. However, safety concerns with the use of these strains were raised especially in immunocompromised hosts which usually experienced moderate-to-severe adverse vaccine reactions [30]. These limitations led to the development of highly attenuated VACV strains. For example, the VACV strain LC16m8 was developed by sequential passage of the Lister strain in primary rabbit kidney (PRK) cells at 30 °C [31]. The modified vaccinia Ankara (MVA) has been developed by passage of VACV strain Ankara in chicken embryo fibroblasts (CEF) for 516 times [32]. The resulting virus lost ~15% of its genome during cell passaging [33], and it is replication deficient in most mammalian cells [34]. Another highly attenuated vaccinia virus strain NYVAC was derived from plaque-cloned isolate of the Copenhagen vaccine strain which contains select deletion of 15 non-essential genes [35]. The NYVAC strain is less pathogenic and has greatly reduced ability to replicate in a variety of mammalian cells (human, mice, and equine cells), but

Table 1 Licensed and commercially available poxvirus-vectored vaccines

Vaccine trade name[a]	Target pathogen	Target species	Insert gene	Poxvirus vector	References
RABORAL V-RG	Rabies	Wildlife canines	Glycoprotein	Vaccinia virus	[39, 166]
ProteqFlu	Equine influenza	Horses	HA	Canarypox	[167, 168]
RecombiTek Equine Influenza	Equine influenza	Horses	HA	Canarypox	[167, 168]
Recombitek West Nile Virus	West Nile virus	Horses	prM/E	Canarypox	[91, 169]
PUREVAX Feline Rabies	Rabies	Cats	Glycoprotein	Canarypox	[169]
PUREVAX Recombinant FeLV	Feline leukemia virus	Cats	Env, Gag/pol	Canarypox	[93, 169]
Recombitek Distemper	Canine distemper virus	Dogs	HA and F	Canarypox	[169, 170]
PUREVAX Ferret Distemper	Canine distemper virus	Ferrets	HA and F	Canarypox	[169]
Vectormune FP-MG	*Mycoplasma gallisepticum*	Poultry	40k and mgc	Fowlpox	[84]
Vectormune FP-LT	Laryngotracheitis	Poultry		Fowlpox	[171]
Vectormune FP-ND	Newcastle disease virus	Poultry	HN and F	Fowlpox	[73, 169]
TROVAC-AIV H5	Avian influenza	Poultry	HA	Fowlpox	[66]
TROVAC-NDV	Newcastle disease virus	Poultry	HN and F	Fowlpox	[76]

[a]Trade name might differ according to country and manufacturer

it retained the ability to induce immune response [36]. Although most of these highly attenuated vaccinia virus strains are known for their safety profile, their immunogenicity is often compromised due to high level of attenuation [37]. For example, higher doses or multiple doses of MVA-based vectored vaccines are required to achieve immune responses similar to wild-type VACV strains [38]. Nevertheless, both parental (e.g., Copenhagen, WR) and highly attenuated strains (e.g., LC16m8, MVA, NYVAC) have been used to develop recombinant vectored vaccines for veterinary use.

The first recombinant poxvirus licensed to be used as vaccine is a VACV-based vectored vaccine for rabies. This recombinant was constructed by inserting the rabies virus (RabV) glycoprotein (G) gene in the thymidine kinase (TK) locus of the Copenhagen strain of vaccinia virus [39, 40]. It has been used to control rabies in red foxes in several European countries, in coyotes and raccoons in the USA, and in raccoons in Canada [41, 42]. The vaccine is used as an oral bait which is dispersed in the wild habitat of the target species by hand or airplanes. This vaccine is safe and effective in foxes, raccoons, and coyotes [43–45]. It has been shown to be effective in vampire bats which are important reservoir for rabies virus [46]. However, it is less effective in skunks and in dogs when administered orally [41, 47, 48]. Also, as it is a live attenuated vaccine, safety concerns regarding exposure of live virus-based vaccine to non-target species have been raised. To develop safer alternatives to this vaccine, recombinant MVA, a highly attenuated VACV strain, expressing the RabV G was developed [38]. This recombinant vector was immunogenic in mice, dogs, and raccoons upon parenteral immunization. However, it was less immunogenic than the VACV Copenhagen-based recombinant vector and required a higher dose to induce immune response equivalent to the Copenhagen-based recombinant. Furthermore, the MVA recombinant failed to induce humoral immune

response when immunized orally making it unsuitable to use in wild animal populations [38]. These observations highlight the fact that there is a fine balance between protective efficacy and attenuation of poxvirus vectors.

Recombinant VACV vectors expressing the hemagglutinin (H) and fusion (F) proteins of rinderpest virus have been developed. Two vaccinia recombinants were generated by inserting the H or F gene into the TK locus of VACV Wyeth strain. Immunization of cattle with either recombinant or with the mixture of the two recombinants provided 100% protection even when the immunized animals were challenged with 1000 times the lethal dose of rinderpest virus [49]. There was no transmission of the VACV vector from vaccinated animals to contact animals. Moreover, cattle vaccinated with the mixture of recombinant vectors presented solid immunity as indicated by absence of amnestic response after challenge infection with rinderpest virus [49]. The immunized animals, however, developed pock lesions at the site of immunization indicating that the vector was not completely attenuated. Additionally, the use and production of the mixed vector formulation, containing equivalent doses of two different recombinants, was cumbersome [50].

To address these drawbacks, a recombinant of VACV strain Wyeth expressing both H and F genes (vRVFH) was developed. The H gene was inserted into TK locus, and F gene was inserted into HA locus in the VACV genome. The insertional inactivation of TK and HA genes led to further attenuation of the vector. Consequently, no pock lesions were observed after intradermal immunization in cattle. The protective efficacy of the vaccine was not affected, and sterilizing immunity was observed in cattle against rinderpest virus challenge [50]. Later, another VACV-based recombinant expressing H and F genes (v2RVFH) was constructed using TK locus of the VACV Copenhagen strain. A strong synthetic VACV promoter was used instead of the natural $P_{7.5}$ VACV promoter which was used in previous constructs. This resulted in a threefold increase in the expression level of H and F genes as compared to vRVFH. Intramuscular vaccination of v2RVFH with a dose of 10^8 PFU provided sterilizing immunity in cattle for at least 16 months [51].

Interestingly, VACV strain Wyeth expressing H and F genes of rinderpest virus (vRVFH) provides protection to goats against peste des petits ruminants virus (PPRV) challenge [52]. Despite the inability of vRVFH to induce anti-PPRV neutralizing antibodies, complete protection was observed in goats against PPR [52]. Cell-mediated immunity or non-neutralizing antibodies might be responsible for the protection elicited by this recombinant vector in goats. Similarly, cross-protection has also been demonstrated for canine distemper virus (CDV) vectored by VACV vectors. Vaccinia virus recombinants expressing either the measles virus fusion (F) or hemagglutinin (H) glycoprotein have been shown to protect dogs against CDV [53]. The recombinants were generated by inserting measles virus F or H gene in the TK locus of VACV Copenhagen strain and using the H6 synthetic promoter. These recombinants fail to induce CDV neutralizing antibodies in dogs. However, inoculation of dogs with the recombinant VACV expressing H gene or co-immunization with the recombinant VACV expressing H and the recombinant expressing F protein was shown to protect dogs from lethal CDV challenge [53].

Vaccinia virus recombinant expressing glycoprotein (G) gene of vesicular stomatitis virus (VSV) induces protective neutralizing antibody responses in cattle. Neutralizing antibody levels increased by several fold after boosting. This recombinant provides partial protection to VSV challenge in cattle, and protection is correlated with neutralizing antibody levels [54]. The utility of VACV-based vectors in animals has also been demonstrated in chickens. A VACV expressing the Newcastle disease virus (NDV) F glycoprotein has been shown to protect chickens against live virulent NDV challenge [55]

Recombinant VACV vectors have also been used to develop vaccine candidates against protozoan parasites. A VACV expressing the LACK protein of *Leishmania* using the Western Reserve (rVV-LACK) or MVA (MVA-LACK) strains has

been developed [56]. Prime-boost immunization of dogs with a plasmid DNA expressing the LACK protein (DNA vaccination) followed by booster with rVV-LACK or MVA-LACK incudes both humoral and cellular immunities in dogs. Priming with DNA and boosting with rVV-LACK provided 50% protection in dogs, whereas boosting with MVA-LACK provided 75% protection in dogs. This study showed that boosting with non-replicative MVA vector elicited higher immune response than replication-competent Western Reserve (WR) vector [56]. Later, another MVA construct expressing TRYP protein of *Leishmania* was developed [57, 58]. Dogs receiving a DNA-TRYP/MVA-TRYP prime-boost vaccination strategy produced higher levels of TRYP-specific type 1 cytokine IFN-gamma and TRYP-specific IgG antibody in comparison to MVA-LACK construct [57]. These studies provided evidence for applicability of poxvirus vectors in prime-boost vaccination regimens. In fact, VACV can be used both for priming and boosting. This has been demonstrated for a VACV recombinant expressing Gn and Gc glycoproteins of Rift Valley fever virus (RVFV) [37]. The recombinant VACV vector was generated using the Copenhagen strain, and two virulence genes (B8R and TK) of the virus were inactivated. The RVFV Gn and Gc proteins were inserted in TK locus. A single vaccination with this recombinant provided 50% protection in mice, whereas animals immunized twice with this vaccine showed 90% survival rate after challenge. This recombinant was also tested in baboons (nonhuman primate model). All animals immunized with this recombinant mounted a strong anamnestic response to booster immunization [37].

6 Avipoxvirus-Based Vectors

Avipoxviruses naturally infect chickens, turkeys, and many other species of pet and wild birds. Currently there are ten species of avipoxviruses recognized by ICTV (International Committee on Taxonomy of Viruses) [59]. Given that avipoxviruses have been isolated from a wide range of hosts including crows, peacock, and ostrich, among others, there are many avipoxviruses which have been tentatively proposed as new species but remain officially unclassified [60]. Avipoxviruses share many characteristics with other poxviruses. Our understanding of molecular and biological characteristics of avipoxviruses comes mainly from fowlpox virus (FWPV) and canarypox virus (CNPV), which infect domestic poultry and canaries, respectively [61].

As described in the previous section, VACV has been widely used as a vaccine vector for human and veterinary applications. However, VACV-based recombinant vaccines developed for animals pose a risk of infection to humans because of the virus broad host range. Thus, it was desirable to construct host-restricted vectors, for instance, using recombinant avipoxviruses for use in mammalian species as these viruses only cause productive infection and disease in avian species [61]. The use of host-restricted or replication-incompetent poxvirus vectors would avoid the risk of genetic recombination and disease transmission between vaccinated animal species or humans. Avipoxviruses were initially proposed as vectors for vaccine delivery in poultry [62]. Later, the findings that recombinant FWPV initiate an abortive infection in non-avian tissue culture cells and express foreign antigens capable of inducing immune response in mammals sparked interest in using avipoxviruses as vectors for humans and other animal species [63]. Additionally, pre-existing immunity to orthopoxviruses does not affect immunogenicity of FWPV and canarypox virus (CNPV), which means they could be used as vectors in humans exposed to vaccinia virus or vaccinated against smallpox [60]. As a result, a large number of avipoxvirus recombinants based on FWPV and CNPV have been developed for use in humans and animals.

6.1 Fowlpox Virus-Based Vectors

Several fowlpox virus recombinant constructs targeting avian influenza (AI) have been developed. A fowlpox virus recombinant expressing

the influenza virus HA protein at the TK locus was able to induce hemagglutinin-inhibiting (HI) antibodies in chickens. These antibodies were detected as early as 9 days post immunization, and a boost effect was seen when chickens were re-immunized [62]. Interestingly, protection against AI has been observed in birds in presence of very low levels or even the absence of HI or neutralizing antibodies [64]. Cell-mediated immunity induced by immunization with the FWPV vector has been suggested as the effector mechanism of protection against AI in birds without significant levels of HI or neutralizing antibodies [64]. To enhance cell-mediated immunity induced by FWPV vectors, attempts were made to insert nucleoprotein gene (NP) of influenza virus along with HA gene. The co-expression of HA and NP by the FWPV vector, however, did not improve the efficacy of vaccine [65]. The FWPV-vectored avian influenza vaccine known as TROVAC-H5 has been licensed for emergency use in the USA and has full registration in Mexico, Guatemala, El Salvador, and Vietnam [66]. The vaccine consists of FWPV recombinant expressing the H5 gene from highly pathogenic AI isolate A/turkey/Ireland/1378/83 H5N8. This vaccine provides 90–100% protection against highly pathogenic Mexican avian influenza H5N8-type isolates. After a single immunization on day 1 of life, it confers protection for at least 20 weeks [67]. Good levels of protection have also been observed against some of the recent H5N1 Asian AI isolates A/chicken/South Korea/03 and A/chicken/Vietnam/04 [66].

Another recombinant FWPV vector co-expressing HA (H5 subtype) and neuraminidase (N1 subtype) can provide complete protection to chickens against AI H5N1 challenge. Protection was accompanied by high levels of HA- and N1-specific antibodies [68]. Notably, this recombinant is able to provide cross-protection against H5N1 and H7N1 highly pathogenic avian influenza (HPAI) virus challenge, presumably due to cross-reactive immunity conferred by the common N1 protein between these two HPAI types. This vaccine was licensed in China, and over 600 million doses of this vaccines were sold by 2009 [69]. Attempts have been made to co-express cytokines along with HA protein to enhance immunogenicity of FWPV-influenza recombinants. Improvement in protective efficacy has been reported by co-expression of cytokines like chicken interleukin-2 and chicken interleukin-18 along with the HA protein [70, 71].

A fowlpox vaccine recombinant expressing the H5 hemagglutinin gene provides protection against clinical signs and mortality in chicken following challenge by nine diverse highly pathogenic avian influenza viruses [67]. This vaccine overcomes limitations of many FWPV-influenza recombinants which fail to provide cross-protection to different influenza subtypes. The protection was correlated with the amino acid sequence similarity of H5 gene of challenge virus and the H5 gene inserted in the recombinant FWPV vector [67].

A recombinant FWPV expressing either Newcastle disease virus (NDV) hemagglutinin-neuraminidase (HN) or fusion (F) proteins or recombinants expressing both proteins have been developed [72–75]. Most of the recombinants use the VV7.5 or H6 early-late promoters to drive expression of HN or F gene in FWPV. Fowlpox virus recombinant vectors expressing HN and F proteins provide 100% protection to chickens against lethal velogenic NDV challenge [72, 73, 75]. One of these FWPV-based recombinant vectors, designated as TROVAC-NDV, has been licensed for commercial use in the poultry industry. A single dose of TROVAC-NDV can induce high levels of hemagglutination-inhibiting antibodies in chickens for up to 8 weeks post immunization [73]. Recombinant FWPV expressing the NDV fusion (F) gene is also capable of inducing anti-F protein antibody responses which can provide protection to the chickens from lethal NDV challenge [72, 76]. The expression levels of NDV fusion (F) gene can be increased by inserting F protein into non-essential genes in the inverted terminal repeats (ITR) of FWPV. This occurs because a foreign gene inserted into ITR region of a poxvirus vector is usually duplicated by homologous recombination, with one copy of the gene being

inserted in the 5′ and 3′ ITR, thus increasing the expression levels of the foreign protein [72]. This strategy, however, can also lead to loss of the gene insert due to increased instability of the resultant vector. Fowlpox-NDV recombinants administered through the intramuscular or wing-web method induce stronger immune response than that of oral or ocular inoculation [75, 76]. Several-fold increase in NDV HI titers is seen when chickens are primed with live or inactivated NDV vaccine and boosted with recombinant fowlpox virus expressing HN proteins [77].

Fowlpox vectors expressing the envelope glycoprotein of reticuloendotheliosis virus (REV) induce neutralizing antibodies and protection in chicken from viremia and runting-stunting syndrome following REV challenge [78]. Synthetic promoter P_s induces higher level of expression of envelope glycoprotein than vaccinia $P_{7.5}$ promoter. Similarly, recombinant FWPV expressing multiple genes of Marek's disease virus (MDV) have been constructed. Of these FWPV-MDV constructs, vectors containing one gene of MDV provide less than 50% protection in chickens. Whereas, a synergistic effect was observed when multiple genes of MDV are expressed resulting in increased protection up to 72%. Additionally, enhanced protection (94%) was seen when FWPV-MDV recombinants were given along with the MDV closely related turkey herpesvirus (HVT) [79].

Fowlpox vector expressing the VP2 protein of infectious bursal disease virus (IBDV) can protect chickens from mortality [80, 81]. However, these recombinants cannot protect chickens against damage to the bursa of Fabricius [80], and protection levels are lower than the oil-adjuvanted inactivated whole virus vaccine [82]. Recombinant FWPV (rFWPV) expressing VP2-VP4-VP3 polyprotein of IBDV inserted within TK gene under vaccinia P.L11 late promoter fails to develop protective antibodies against IBDV, whereas rFWPV expressing only VP2 under fowlpox early/late promoter inserted immediately downstream of TK gene can express five times more VP2 protein than the former construct and also induces antibodies to IBDV [82]. Therefore, the choice of promoter and insertion site can significantly affect the immunogenicity of poxvirus vectors. In addition to viral diseases, recombinant FWPV-vectored vaccines have been developed for non-viral diseases of poultry, including coccidiosis and mycoplasma [83, 84]. The Vectormune FP-MG consists of a FWPV recombinant vector expressing the 40k and *mgc* genes of *M. gallisepticum*, which is licensed in the USA for use in chickens and turkeys [84].

6.2 Canarypox Virus-Based Vectors

Another avipoxvirus that has been widely used as a vaccine vector is canarypox virus (CNPV). A plaque purified clone of CNPV designated as ALVAC is widely used as vector. This clone was obtained after serial passage of wild-type CNPV for 200 passages in CEF [85]. The safety and immunogenicity profile of CNPV ALVAC vector led to its use in human clinical trials as an HIV/AIDS vaccine candidate [86]. Additionally, there are several ALVAC-based vectored vaccines licensed for veterinary use, for example, ALVAC-AI-H5 (influenza virus), ALVAC-RV (rabies virus), and ALVAC-CDV-H/F (canine distemper virus) [87].

Canarypox virus recombinants expressing the RabV G are known to elicit high levels of neutralizing antibodies in mice, cats, and dogs. The level of protection observed after challenge infection was comparable to that induced by replication-competent VACV vector [88]. A CNPV vector expressing hemagglutinin (HA) gene of equine influenza virus (rCNPV-EIV) induces both humoral and cellular immune responses against EIV. Cellular immune response is characterized by increased levels of IFN-γ in vaccinated ponies. Clinical signs and virus shedding were significantly reduced in rCNPV-EIV-vaccinated group after challenge infection [89]. A CNPV recombinant expressing the prM/E proteins of West Nile virus (WNV) has been licensed for use in horses [90]. A single dose of this vaccine protects horses against viremia caused by challenge with WNV-infected mosquitoes [91]. Two doses of this vaccine can

provide protection for at least 1 year post vaccination [90, 92]. Similarly, rCNPV expressing *env* and *gag* genes of feline leukemia virus (FeLV) provides protection to cats against oronasal challenge with FeLV. The cats are protected from contact challenge for at least 1 year [93, 94]. The rCNPV-FeLV vaccine has been licensed for commercial use under trade name E URIFEL FeLV [93]. Both rCNPV-WNV and rCNPV-FeLV can provide protection despite absence of measurable antibody responses [91, 94]. The protection observed might be related to the activation of cell-mediated immunity which requires relatively lower antigen load/dose. This phenomenon has been observed in rCNPV expressing glycoprotein (G) and fusion gene (F) of Hendra virus (HeV). The higher tested dose of rCNPV-HeV recombinant induced strong neutralizing antibodies in horses and hamsters, whereas at lower doses, partial protection was observed in hamsters despite the absence of detectable HeV-specific antibodies [95]. Canarypox virus vaccine vectors expressing glycoprotein (G) and fusion (F) gene of Nipah virus (NiV), when given in combination, can induce high levels of neutralizing antibodies in pigs and can provide solid protection from NiV challenge. In addition, vaccinated pigs show balanced Th1 and Th2 response with the induction of TNF-α, IL-10, and IFN-γ cytokines [96]. These rCNPV-HeV and rCNPV-NiV recombinants induce cross-neutralizing antibody against closely related Nipah virus (NiV) and Hendra virus (HeV), respectively.

The impact of maternal antibodies on the efficacy of avipoxvirus-vectored vaccines has been a subject of constant debate. This is important because layers are routinely immunized against FWPV which can impact FWPV vector-based vaccination in day-old chicks. It has been demonstrated that recombinant FWPV expressing the HN gene of NDV failed to induce immune response in chickens previously vaccinated with FWPV [77]. In contrast, there are reports that demonstrate that the FWPV or CNPV vectors remain effective in the presence of pre-existing immunity and can be used repeatedly without an adverse effect on the vaccine potency [60, 97, 98]. Additionally, pre-existing immunity against the heterologous gene inserted into avipox vector may interfere with the efficacy of the vaccine. For example, in the presence of maternally derived NDV antibodies, the humoral response provided by FWPV-NDV recombinant expressing HN protein of NDV was dampened [73]; nevertheless significant level of protection against NDV was still achieved.

7 Parapoxvirus-Based Vectors

The genus *Parapoxvirus* includes four species – *Bovine popular stomatitis virus* (BPSV), *Orf virus* (ORFV), *Parapoxvirus of red deer in New Zealand* (PVNZ), and *Pseudocowpox virus* (PCPV). The type species parapoxvirus ORFV has been widely used as vector. Some of the features that make ORFV an attractive candidate vector are (1) its restricted host range (sheep and goat), (2) its ability to induce humoral and cellular immune response even in non-permissive hosts [20, 27, 99], (3) its tropism that is restricted to the skin and the absence of systemic infection, (4) the fact that ORFV induces short-lived ORFV-specific immunity and does not induce neutralizing antibodies which allows repeated immunizations [20, 100, 101], and (5) the immunomodulatory properties of the virus [102]. In fact, inactivated ORFV is used as an immunomodulator in horses and has been shown to be effective in reducing clinical signs and shedding related to equine herpes virus type 1 (EHV-1) and *Streptococcus equi* (*S. equi*) infections [103]. There are several well-characterized immunomodulatory proteins (IMPs) present in Orf virus. These IMPs include an interleukin-10 homologue (vIL-10) [104], a chemokine-binding protein (CBP) [105], an inhibitor of granulocyte-monocyte colony-stimulating factor (GMC-CSF) [106], an interferon resistance gene (VIR) [107], a homologue of vascular endothelial growth factor (VEGF) [108], and at least four inhibitors of nuclear factor-kappa (NF-κB) signaling pathway [109–112]. The presence of these well-characterized IMPs provides unique opportunities for rational engineering of ORFV-based vectored vaccines.

Two strains of ORFV, D1701 and OV-IA82, have been explored as vectors for veterinary application.

The highly attenuated ORFV strain D1701 was obtained after serial cell culture passage of an ORFV isolate from sheep in African green monkey kidney cells (Vero cells). This virus is apathogenic in sheep and is well adapted to grow in cell culture [113]. Adaptation of ORFV strain D1701 in Vero cells led to further attenuation of the virus because of additional genomic deletions. This virus, designated D1701-V, is non-pathogenic even in immunosuppressed natural host sheep [114]. The utility of D1701-V strain as a vector has been explored in permissive and non-permissive animal species. In most constructs, the VEGF-E locus of D1701-V has been used to insert heterologous genes utilizing the early promoter of the VEGF-E gene. The D1701-V recombinant expressing the rabies G can stimulate high levels of rabies virus-specific neutralizing antibodies in mice, cats, and dogs [115]. Another recombinant expressing p40 protein of Borna virus provides protection to mice against Borna virus challenge and leads to the virus clearance from the infected brain eliminating persistent virus infection [116]. Similarly, ORFV recombinant (D1701-V) expressing the HA protein of influenza virus (H5N1) provides solid protection to mice against influenza A virus H5N1 and heterologous influenza A H1N1 challenge in a dose-dependent manner [117]. Orf virus recombinant (D1701-V) expressing the major capsid protein VP1 of rabbit hemorrhagic disease virus (RHDV) protected rabbits against lethal RHDV infection with a single immunization with a dose as low as 10^5 PFU [118]. Higher or multiple doses were required to induce significant humoral response; nevertheless single dose of 10^5 PFU was enough to provide protection. This dose is significantly lower than that of VACV or canarypox recombinants expressing RHDV VP1 which require 10^7–10^9 PFU for protection when given subcutaneously, orally, or intradermally [118–120].

The D1701-V has also been used as a vector in pigs. The D1701-V recombinant expressing glycoproteins gC and gD of pseudorabies virus (PRV) induces strong cellular and humoral immune response when used to boost the pigs primed with *Sindbis virus*-derived plasmid expressing gC and gD [121]. This type of heterologous prime-boost strategy, using DNA vaccine or baculovirus-expressed protein for priming followed by boosting with ORFV-based vaccine, has been shown to be more effective than homologous prime-boost strategy [122, 123]. Orf virus recombinant expressing the E2 glycoprotein of classical swine fever virus (CSFV) confers protection against CSFV challenge in pigs [123]. A single intramuscular immunization of this recombinant induces high levels of CSFV-specific neutralizing antibodies and IFN-γ production. Interestingly, multiple-site application was shown to be superior to single-site injection with the same dose. This might be due to effective antigen processing and presentation occurring at different lymph nodes at the same time [123].

Another ORFV strain that has been used as a vector is OV-IA82. ORFV strain IA82 (OV-IA82) was obtained from the nasal secretion of a lamb at the Iowa Ram Test Station during an Orf outbreak in 1982 and was isolated in ovine fetal turbinate cells (OFTu) [124]. Four genes of ORFV (ORFV002, ORFV024, ORFV121, ORFV073) that are involved in inhibition of host nuclear factor-kappa (NF-κB) pathway have been well-characterized [109–112]. Deletion of ORFV121 from viral genome attenuates the virus as evidenced by decreased pathogenesis in sheep which makes ORFV121 deletion mutant virus an attractive vector candidate for use in livestock [109]. An ORFV (OV-IA82) recombinant expressing the full-length spike (S) glycoprotein of porcine epidemic diarrhea virus (PEDV), containing a deletion of ORFV121 gene, induces PEDV-specific neutralizing antibodies in pigs and protects pigs from clinical signs of PED [20]. This ORFV-PEDV-S recombinant was also shown to induce passive immunity and transfer of PEDV-specific IgG, IgA, and neutralizing antibody to piglets via milk and colostrum. Upon challenge with virulent PEDV, decreased clinical signs and reduced mortality was observed in piglets born from ORFV-PEDV-S-vaccinated sows. Additionally, increased protection with 100% survival was obtained when sows were primed with live

PEDV and then boosted with the ORFV-PEDV-S recombinant [101].

In addition to ORFV121, another NF-κB inhibitor, ORFV024, has also been used as a site for foreign gene insertion. The immunogenicity of two ORFV-IA82 recombinants expressing the RabV G either in the ORFV121 or ORFV024 gene loci was evaluated in pigs and cattle [27]. Both recombinants induced robust neutralizing antibody response against RabV in pigs and cattle. Additionally, both viruses induced long-lasting memory B cell responses to RabV, as evidenced by anamnestic responses following a single-dose booster on day 390 post primary immunization [27]. Notably, the neutralizing antibody titers induced by ORFV121 deletion mutant were higher than that of ORFV024 deletion mutant [27]. This type of differential regulation of innate and adaptive immune response has also been reported for NF-κB inhibitor proteins encoded by vaccinia virus (VACV), where deletion of one NF-κB inhibitor (A52R) leads to higher immune response against heterologous antigen when compared to other NF-κB inhibitors (B15, K7) [125].

Despite restricted host range, infections of humans have been reported for parapoxviruses. The virus can cause self-limiting infections in immunocompromised people or farmers who work in close contact with infected animals and present abrasions or cuts in the skin. These infections are restricted to the areas surrounding the sites of virus entry and usually involve the hands [126–128]. Since both recombinant ORFV vectors that have been used as platforms (D1701 and OV-IA82Δ121) are known to be non-pathogenic in the natural sheep and goat hosts and the strain IA82 presents marked growth impairment in human cells, it is safe to assume that the risk of human infections with these recombinants should be very low.

8 Swinepox Virus-Based Vector

Swinepox virus (SPV) is the only member of genus *Suipoxvirus*. SPV causes mild self-limiting infection in pigs. Because of its narrow host range restricted to pigs, SPV-based recombinant vectored vaccine candidates have been developed mostly targeting pigs. One of the first attempts to use SPV as a vector involved a recombinant SPV targeting Aujeszky's disease (pseudorabies virus (PRV)) [129]. The gp40 and gp63 genes of PRV were inserted into TK locus of SPV under the early/late $VV_{7.5}$ promoter. At 21 days post immunization, 90% of the pigs vaccinated by scarification developed serum neutralizing antibody against pseudorabies virus, whereas 100% of the animals vaccinated by the intramuscular route developed neutralizing antibodies. Significant level of protection was observed in pigs upon challenge with virulent PRV [129]. A recombinant SPV expressing the E2 glycoprotein of classical swine fever virus (CSFV) in the TK locus expresses the E2 protein in a dimeric form in the cytoplasm of the infected cells [130]. However, this recombinant was not tested in animal model. SPV recombinant expressing HA1 gene of swine influenza virus (SIV; H1N1) elicits humoral and cellular immune responses and provides complete protection against SIV in swine and mice [131]. Although neutralizing antibody titers were low (1:8 to 1:32), potent Th1 and Th2 responses were observed as evidenced by increased levels of IL-4 and IFN-γ, which may have contributed to protection against SIV challenge. Co-expression of the HA gene of H1N1 and H3N2 subtype provides complete protection against H1N1 and H3N2 challenge in pigs [132].

Swinepox virus recombinants expressing immunodominant epitopes of certain viral pathogens have also been shown effective. A recombinant SPV expressing the A epitope of transmissible gastroenteritis virus (TGEV) spike protein induced neutralizing antibodies and strong Th1 and Th2 cytokine responses against TGEV. Notably, when neutralizing antibodies purified from vaccinated animals were fed to piglets, they were protected against severe disease and mortality after challenge with TGEV [133]. Similarly, a recombinant SPV expressing three repeats of a conserved six-amino acid epitope present in the N-terminal ectodomain of the GP3 protein of porcine reproductive and respiratory syndrome virus (PRRSV) induced cellular

and humoral response in pigs [134]. Swinepox virus has also been used to develop recombinant vaccine candidates against bacterial diseases. A recombinant swinepox virus expressing M-like protein (SzP) of *Streptococcus equi* spp. *zooepidemicus* (SEZ) provided significant protection against SEZ infection [135].

Swine are the major reservoir of SPV, and the virus does not infect other mammalian or avian species. Initially, SPV was known to infect only cells of porcine origin; however, recent findings have shown that SPV exhibit a relatively broad cell culture host range in vitro [136, 137]. Recombinant SPV can infect, replicate, and express foreign genes in cells of non-porcine origin including human, monkey, hamster, and rabbit cell lines [137]. Moreover, when inoculated intradermally, SPV causes productive infection in rabbits [138]. These findings have opened opportunities for the use of SPV vector in different animal species. A SPV recombinant expressing the *gag* and *env* proteins of feline leukemia virus (FeLV) fails to replicate in feline cells, but FeLV gag virus-like particles were produced in feline cells and incorporated into SPV intracellular mature virion (IMV) [139]. The immunogenicity of this recombinant virus, however, was not tested in cats.

Deletion of the TK gene has been widely used in recombinant poxvirus vector construction. However, deletion of the TK gene may not be the best strategy to generate SPV-based recombinant vectors as it results in severe attenuation of the vector and consequently in lower vaccine efficiency. It has been shown that deletion of the TK gene results in decreased neutralizing antibody levels and in Th1- and Th2-mediated immune responses [140]. The ability of the virus to replicate in non-permissive cells also decreases significantly in TK deletion mutants [140]. Insertion of foreign antigens in intergenic or non-coding regions of the SPV genome could be used as an alternative approach. A SPV recombinant expressing the porcine IL-18, the capsid protein of porcine circovirus 2, and the SzP protein of SEZ in the intergenic region between SPV020 and SPV021 open reading frames induces immune responses against both PCV2 and SEZ, which was comparable to the immune responses elicited by commercial vaccines [141]. These findings suggest that SPV can be used as a vector platform for multivalent vaccines against diseases of swine.

9 Capripoxvirus-Based Vector

The genus *Capripoxvirus* includes *Goatpox virus* (GTPV), *Sheeppox virus* (SPPV), and *Lumpy skin disease virus* (LSDV). SPPV and GTPV infect sheep and goats, respectively, with some isolates being able to infect both species. LSDV causes disease in cattle and buffalo [142]. These three species of CPV share 96–97% nucleotide identity [143, 144]. Because of the high degree of sequence conservation, cross-immunity is observed among the three viruses. An attenuated LSDV deficient of an IL-10 gene homologue (ORF005) has been shown to provide protective immunity against virulent capripoxvirus (CPV) challenge in sheep and goats [145]. Thus, theoretically, an attenuated strain of any capripoxvirus should be able to protect against SPPV, GTPV, and LSDV [146]. Using these viruses as vectors, it is possible to generate multivalent vaccines against CPV and other target pathogens of ruminants. Moreover, the replication of capripoxviruses is restricted to ruminants with no evidence of human infections. These traits make CPVs good candidates for developing recombinant vectored vaccines.

Capripoxviruses have been mainly used to develop recombinant vaccines for use in ruminants. Most of the recombinants have been generated using Kenya strain-1 (KS-1), which was isolated from sheep and passaged in lamb testis and baby hamster kidney (BHK) cells [147]. Recent molecular studies have shown a close relationship between KS-1 and LSDV, suggesting KS-1 may actually be LSDV [143]. The majority of KS-1-based constructs have been generated by inserting the foreign gene into the TK locus. Two individual KS-1 recombinants generated by insertion of the fusion (F) protein or hemagglutinin (H) protein of rinderpest virus (RPV) into TK gene locus under the

control of vaccinia virus late promoter P11 protect cattle against rinderpest virus after lethal challenge with a virulent RPV isolate. The recombinant with the H gene insert showed better protective efficacy than the vector expressing the F gene. Both recombinants protected cattle against LSDV challenge in addition to rinderpest [148, 149]. Interestingly, these recombinants protect goats from lethal challenge with peste des petits ruminants virus (PPRV) challenge [150]. Another recombinant capripoxvirus expressing the H and F gene of PPRV has been developed using AV41 strain of GTPV. A single dose of this recombinant elicits seroconversion in ~80% of immunized sheep and goats. Neutralizing antibodies are detected up to 6 months after vaccination. Two doses of this vaccine completely overcome the interference caused by pre-existing immunity to caprinepox virus [151]. Another recombinant CPV expressing both H and F genes of PPRV confers an earlier and stronger immune response against PPR and GTPV [152].

A KS-1 recombinant expressing the VP7 protein of bluetongue virus (BTV) provides partial protection in sheep [153]. Partial protection against BTV was achieved even when sheep were immunized with the combination of KS-1 recombinants individually expressing four proteins (NS1, NS3, VP2, VP7) of BTV [154]. Capripoxvirus has been used to develop recombinant vaccine candidates against Rift Valley fever virus (RVFV) [155, 156]. A recombinant KS-1 virus expressing Gn and Gc glycoproteins of RVFV induced neutralizing antibodies to RVFV in sheep, and two doses of the vaccine candidate provided significant protection against RVFV and SPPV challenge in sheep.

In addition to the KS-1 strain, an attenuated strain of LSDV, Neethling strain, has also been used as vector. This strain is used as vaccine against lumpy skin disease in Africa. The ribonucleotide reductase gene of Neethling strain has been identified as potential insertion site for recombinant vector generation [157]. The Neethling strain has been used to develop recombinant vaccines against RabV and bovine ephemeral fever and RVFV viruses [142]. Recombinant LSDV (Neethling strain) generated by inserting RabV G in the ribonucleotide reductase gene locus induced strong cellular and humoral response in cattle [157]. Neutralizing antibody titers as high as 1513 IU/mL were observed in cattle. This recombinant also induced robust humoral and cellular immunity in non-permissive hosts (mice and rabbits). Immunization of mice with this recombinant vector protected the animals from an aggressive intracranial RabV challenge [158].

Because of the cross-protection offered by CPVs, theoretically it should be possible to develop a universal recombinant CPV vector that would provide protection against all capripoxviruses in addition to target pathogen. However, the geographical distribution of the different CPV limits that possibility. Sheeppox and goatpox viruses are endemic to Asia, Middle East, and Africa south of the equator, whereas LSDV is mainly present in sub-Saharan Africa [142]. A country would refuse to use capripoxvirus-vectored vaccine if the vector is not endemic. Future research should aim at identifying immunomodulatory genes and virulence factors encoded by CPV, which would allow the development of safer recombinant CPV-based vectors that could potentially be used in both endemic and non-endemic countries.

10 Leporipoxvirus as Vector

Myxoma virus (MYXV), the type species of genus *Leporipoxvirus*, specifically infects rabbits and hare (leporides). MYXV was used as biological agent to control European rabbit population in Australia. This method, however, was not sustainable because of the coevolution of the virus and the rabbit which led to the adaptation of the virus to the novel host species [159].

MYXV has been used as vector for both leporide and non-leporide species. A recombinant myxoma virus expressing capsid protein (VP60) of rabbit hemorrhagic disease virus (RHDV) has been shown to protect rabbit from myxomatosis and RHDV [120]. MYXV expressing hemagglutinin (HA) of influenza virus can induce high

levels of anti-HA antibodies in rabbits as efficiently as VACV vector [160]. MYXV recombinant has been shown to be effective in protecting cats from feline calicivirus [161]. The possibility of using MYXV as a non-replicative vector in small ruminants (sheep) has been demonstrated [162]. Moreover, the use of MYXV-vectored immunocontraception as a means to control wildlife species has been assessed [163]. Myxoma virus expressing immunocontraceptive antigen (zona pellucida 3 [rZp3] glycoprotein of rabbit) was able to induce autoimmune infertility in 70% of rabbits at the first breeding [164].

MYXV encodes several proteins that are known to cause immunosuppression in rabbits [165]. Deletion of those immunosuppressive genes is essential to enhance the safety and immunogenicity of the MYXV vector.

11 Future Directions and Potential for Other Applications

Poxvirus-based vectors have long been used as vaccine delivery platforms in many animal species. The overall immunogenicity, safety, and broad disease and species applicability of these viral vectors make them especially attractive for vaccine delivery in veterinary medicine. After decades of research, there is a general consensus that replication-competent poxvirus vectors can induce better immune responses in target species, but they have potential for infecting non-target hosts (e.g., vaccinia virus). In contrast, replication-deficient poxviruses are safer but induce comparatively lower immune responses. Hence, future studies focusing on rationally designed poxvirus vector platforms could lead to more balanced vectors, with an improved safety profile and immunogenicity. Poxviruses are known for encoding several immunomodulatory proteins (IMPs) that target host immune responses to allow efficient virus infection and replication. Most importantly, several of these genes encode for virulence determinants that contribute to poxvirus disease pathogenesis. By targeting those genes, one could expect to attenuate a given poxvirus vector and, perhaps, simultaneously enhance its immunogenicity in target animal species. As additional poxviral IMPs are identified and characterized, we are likely to see the development and refinement of poxviral vectors.

In addition to modulation of poxvirus vector safety and immunogenicity, the field would greatly benefit from additional studies focused on identifying better promoters for expression of heterologous genes. Promoters that would allow sustained gene expression following immunization would likely result in more robust and long-lasting immune responses. There is also a need for better recombinant selection methods. The use of CRISPR/Cas9 in combination with fluorescent activated cell sorting is a promising approach that may facilitate and speed up the selection process of poxvirus vectors. Additional research assessing the effect of dose and route of immunization, the stability of the recombinants, the sustained heterologous gene expression, and perhaps even the use of host cytokines to enhance T-cell responses will be pivotal in developing safe and immunogenic poxvirus-based vectored vaccines in the future.

12 Summary

Since the first demonstration of recombinant vaccinia virus as an expression vector in 1982, we have seen a huge improvement in different aspects of poxvirus recombinant technology from generating safer poxvirus strains to improved methods of recombinant selection. We now have better understanding of poxvirus infection biology which has enabled us to generate safer and more immunogenic vectored vaccines. There are many poxvirus-based recombinant vaccines that have been licensed for commercial use, and clinical trials are in progress for many other infectious diseases of humans and animals. Finally, given their utility, broad species applicability, and immunogenicity, one can expect that poxviruses will continue to evolve and remain as one of the main vaccine delivery platforms both in human and veterinary medicine.

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