Review

Construction of recombinant capripoxviruses as vaccine vectors for delivering foreign antigens: Methodology and application

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ABSTRACT

Goatpox (GTP), sheeppox (SPP) and lumpy skin disease (LSD) are three severe diseases of goat, sheep and cattle. Their typical clinical symptoms are characterized by vesicles, papules, nodules, pustules and scabs on animal skins. The GTP, SPP and LSD are caused by goatpox virus (GTPV), sheeppox virus (SPPV) and lumpy skin disease virus (LSDV), respectively, all of which belong to the genus *Capripoxvirus* in the family *Poxviridae*. Several capripoxvirus (CaPV) isolates have been virulently attenuated through serial passaging *in vitro* for production of live vaccines. CaPV-based vector systems have been broadly used to construct recombinant vaccines for delivering foreign antigens, many of which have been demonstrated to induce effective immune protections. Homologous recombination is the most commonly used method for constructing recombinant CaPVs. Here, we described a methodology for generation of recombinant CaPVs by the homologous recombination, and further reviewed CaPV-vectored vaccines for delivering foreign antigens.

1. Introduction

Goatpox (GTP), sheeppox (SPP) and lumpy skin disease (LSD) are economically important diseases with severe impacts on the livelihoods of farmers in endemic regions. These three diseases cause tremendous losses of ruminants, and impose a major burden on economic activity and public health, especially in Africa [1]. The GTP and SPP are characterized by skin lesions of goats and sheep, which can occur over the whole body, or are restricted to the hairless areas of perineum, head, groin, axillae and mammary glands. LSD is an acute infectious disease of cattle, characterized by skin lumps, which may cover the whole body or are restricted to the head, neck, perineum, udder, genitalia or limbs. The GTP, SPP and LSD are caused by goatpox virus (GTPV), sheeppox virus (SPPV) and lumpy skin disease virus (LSDV), respectively, all of which belong to the genus *Capripoxvirus* in the subfamily *Chordopoxvirinae* of the family *Poxviridae*.

Many highly virulent capripoxviruses (CaPVs) have been successfully attenuated through serial passages *in vitro* for production of live vaccines, which generally confer effective immune protection for 1 year or longer following a single immunization [2–4]. Large packaging capacity of CaPVs for heterologous genes makes it possible to generate CaPV-vectored vaccines against other ruminant diseases, such as rinderpest [5], peste des petits ruminants [6–8], Rift Valley fever [9] and bluetongue [10,11]. Although the CRISPR-Cas9 system has been used to generate recombinant vaccinia virus (VV) [12,13], the most commonly used method to construct recombinant CaPVs (rCaPVs, including rGTPV, rSPPV and rLSDV) is still the homologous recombination. Here, we described a methodology for generation of rCaPVs by the homologous recombination, and further reviewed CaPV-vectored vaccines for delivering foreign antigens.

2. Characteristics of capripox

SPP, GTP and LSD are highly contagious, and can spread through aerosols [14] and/or close contact with infected animals. CaPVs can be introduced into small skin wounds by environmental contamination from diseased animals. Besides the regular routes of transmission, the CaPVs, especially LSDV, can be transmitted mechanically among susceptible animals by biting insects [15–20]. Clinical signs caused by the capripox generally vary between individuals and outbreaks in different areas. The clinical signs of SPP and GTP include mild to severe clinical disease development of erythematous macules, vesicles, papules, pustules and scabs on animal skins. LSD is characterized by fever, nodules (2–5 cm in diameter) on animal skins and mucous membranes, lesions in the respiratory and gastrointestinal tracts, and enlarged superficial lymph nodes [21].

Historically, the global distribution of SPP and GTP was wider than that of LSD, including northern and central Africa, across the Middle

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East and the Indian subcontinent, Iran, Iraq, Russia, Kazakhstan, Kyrgyzstan, Afghanistan, Pakistan, Nepal, Mongolia, China, Bangladesh, Vietnam and Chinese Taipei [1]. The LSD is widespread throughout Africa, except for Libya, Algeria, Morocco and Tunisia, which are still considered to be unaffected [22]. The LSD is causing concern in Europe, due to its recent outbreaks in Israel (2012), Turkey (2013), Azerbaijan (July 2014), Russia (July 2015), Greece (August 2015) and further spread into the Balkans.

Diagnostics of viral nucleic acid is specific and sensitive for detecting the CaPVs throughout the course of disease, regardless of emergence of antibody responses. Nucleic acid recognition methods mainly include conventional PCR [23], real-time PCR [24] and loop-mediated isothermal amplification [25]. The virus neutralization test is the only validated serological test available for LSD [26], and also is the most specific serological test for GTP and SPP [27]. Other serum-based methods, like antibody-detecting ELISAs [28–30], have been developed but are insufficiently validated to be recommended by the World Organization for Animal Health (OIE) for use.

3. Characteristics of CaPVs

3.1. Viral structure

Both morphogenesis and structure of poxvirus virions are unique among viruses. They apparently lack any of the symmetry features common to other viruses such as helical or icosahedral capsids. CaPV-infected cells produce two types of progeny virions, namely mature virion (MV) (Fig. 1A) and enveloped virion (EV) (Fig. 1B). MVs are normally found exclusively inside cells and are liberated only by cell lysis. EVs consist of wrapped viruses which have been exocytosed via fusion of the outermost wrapped virus membrane with the plasma membrane, leaving an MV surrounded by one additional membrane that contains different proteins from those in the MV membrane. MVs are more stable than EVs, and are thought to mediate transmission among hosts, whereas the more fragile EVs are best suited to egressing from intact cells and to spreading within a host [31,32].

CaPV virions appear as large oval or brick-shaped particles with an irregular surface of projecting tubular or globular structure. Insights into the internal architecture of MV have been gained primarily through the use of ultrathin sections, revealing two distinct boundaries, the membrane, which surrounds the entire MV particle, and the core wall, which surrounds a dumbbell-shaped core. The core contains a viral genome and the full complement of virus-encoded enzymes that are required for synthesis and modification of early mRNA. Filling two concavities between the core wall and the membrane are two apparently amorphous masses, called "lateral bodies" [31].
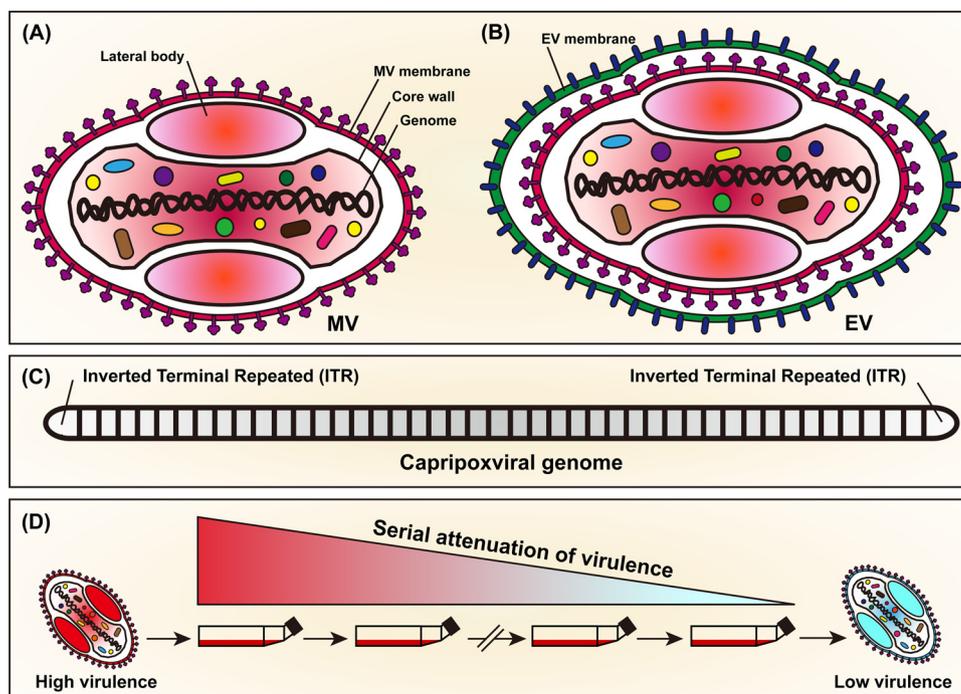
3.2. Viral genome

A CaPV genome consists of a linear double-stranded DNA, approximately 150 kb in length [33,34]. The genome contains a central coding region flanked by two inverted terminal repeat (ITR) sequences which are covalently-closed at their extremities (Fig. 1C). The ITR sequence is 2.1–2.3 kb in length. The CaPV genome contains approximately 150 putative genes, including conserved replicative and structural genes, as well as others likely involved in viral virulence and host range. Most genes within a central genomic core region are involved in basic replicative mechanisms and viral morphogenesis. Terminal genomic regions contain genes that possibly affect pathogenesis and host range functions [35,36].

SPPV and GTPV share an extremely high homology with each other at the nucleotide level. Both genomes are also similar to that of LSDV, and share about 97% nucleotide identity with it. All SPPV and GTPV genes are present in the genome of LSDV [37]. P32, one of the major immunogenic genes of CaPVs, is generally used for phylogenetic comparison among the SPPV, GTPV and LSDV. Phylogenetic analysis shows that SPPVs, GTPVs and LSDVs form distinct phylogenetic branches, and GTPVs have closer genetic relationship to SPPVs than to LSDVs [35].

3.3. Live-attenuated strains

Live-attenuated vaccines have been widely used for the control of capripox. The current attenuated strains include the Romanian and RM-65 strains (SPPV), the Mysore and Gorgan strains (GTPV), the South African Onderstepoort Neethling strain (LSDV), and the Kenyan O-240 strain (referred to as KS-1, LSDV) [38]. Due to sharing a closer homology of nucleotide with LSDV than with SPPV/GTPV, the Kenyan O-240 strain, albeit initially isolated from a sheep, is not SPPV but is

Fig. 1. Schematic representations of capripoxviral structures, genome and virulence attenuation. Capripoxvirus-infected cells produce two types of progeny virions, mature virion (MV) (A) and enveloped virion (EV) (B). A capripoxviral genome consists of a linear double-stranded DNA, and contains a central coding region flanked by two inverted terminal repeat (ITR) sequences which are covalently-closed at their extremities (C). A highly virulent capripoxvirus can be gradually attenuated by serial passages in cell culture (D).

actually LSDV [37]. This strain is clinically safe for vaccination in goats and sheep, but not in cattle [39]. Therefore, virus strain identity and attenuation properties must be ascertained when selecting vaccine strains for vaccination in cattle, sheep and goats.

Highly virulent CaPVs can be attenuated by serial passages in cell culture [40–42], as schematically illustrated in Fig. 1D. For example, the O-180 isolate [43] was virulently attenuated by consecutive passages in bovine fetal muscle cell cultures. Its pathogenicity was lost between the 15th and 20th passages. The 18th passage was successfully and broadly used to control the disease under field conditions [41]. Likewise, a Romania pathogenic strain of SPPV was serially passaged in primary lamb kidney cell cultures. Consequently, a lyophilized vaccine prepared with the 30th passage did not confer any abnormalities in pregnant ewes, regardless of the stage of pregnancy at the time of vaccination. Primovaccination of lambs over 2 months of age produced a better immunity, especially when the lambs were born from vaccinated ewes. This lyophilized vaccine might be stored for over 1 year in a refrigerator [40].

In addition to the serial passaging, a method for deleting virulence-associated genes can also be used to lower viral virulence. In China, the most common vaccine strain is the GTPV AV41 that has been broadly used for vaccination against GTP and SPP. It was generated via consecutive passages of the high-virulence GPTV AV40 isolate in goat and sheep testis cells. Zhu et al. (2018) recently constructed a further attenuated GTPV AV41 (GTPV-TK-ORF) by deleting two virulence-associated genes, the thymidine kinase (TK) and the ORF8-18, from its genome. The GTPV-TK-ORF was proven to be safer than the wild-type GTPV AV41, possessed satisfactory immunogenicity, and could protect goats from the GTPV AV40 challenge. Thus, the GTPV-TK-ORF may be used as a novel vaccine vector with desirable safety performance [44].

Live-attenuated strains should not be able to perpetuate autonomously in a population. Generally, a monovalent vaccine is stable and safe to provide substantial protection from GTPV and SPPV for at least 1 year [3]. Immunity by vaccination with the Kenyan O-240 strain lasts over 1 year in sheep and goats, and the Romanian strain confers protection for at least 30 months [27]. However, the duration of protection provided by LSD vaccination is unknown [26]. A proper dose for inoculation depends on the vaccine strain used. CaPVs are highly susceptible to inactivation by sunlight, and therefore allowance should be made for loss of activity in the field. For instance, the minimum immunizing dose of the LSDV Neethling strain is $\log_{10}$ 3.5 $TCID_{50}$, although its minimum protective dose is $\log_{10}$ 2.0 $TCID_{50}$ [26].

It has been proven that CaPVs' infection is not host-specific, and that CaPV isolates from a heterologous host can confer immune protection from a homologous isolate [45]. In other words, CaPVs are cross-reactive within the genus *Capripoxvirus*. The Isiolo and Kedong GTPV strains, capable of infecting sheep, goats and cattle, are identified for potential use as broad-spectrum vaccine candidates against all capripox diseases [46]. In addition to the vaccine-based immunization, passive protection trials demonstrated that sheep immunized with immune serum against SPPV could also resist challenge with GTPV, and *vice versa* [47].

4. Methodology for generation of rCaPVs

Poxvirus-based vector systems have been widely used for developing recombinant vaccines to deliver heterologous antigens. These vaccines show considerable activities in clinical trials, and many of them have been registered and licensed [48]. Like other poxviruses, CaPVs have also been proven to be suitable as vaccine vectors to express foreign antigens in animals (Table 1). A conventional approach for generation of rCaPVs involves homologous recombination between a transfer plasmid and a replicating viral DNA *in vitro*. A general procedure includes: (i) construction of a recombinant transfer plasmid, (ii) infection with parental CaPV (pCaPV) and transfection with the recombinant transfer plasmid, (iii) plaque purification of recombinant

virus, (iv) PCR and sequencing analyses and (v) deletion of marker from recombinant if necessary. This section, according to our experience, described a methodology for generation of rCaPVs.

4.1. Key elements for generating rCaPVs

4.1.1. Nonessential genes for homologous recombination

Construction of recombinant virus involves the insertion of foreign gene into a nonessential gene of CaPV via homologous recombination. The most common gene used for homologous recombination is the TK gene (Table 1). The 177-aa TK is expressed early during infection and is switched off after 4 h by a translational repression mechanism [49]. An advantage in targeting the TK gene is that the TK-negative phenotype can be used to select for a recombinant without the need for additional selection markers [50]. Although the TK gene appears suitable as a site for the insertion of foreign genes, the virus is still dependent on some TK activity for normal growth [51]. In addition to the TK gene, other nonessential genes can also be used for generating recombinants (Table 1). For example, the GTPV 135 gene-encoded protein functions as an inhibitor of NF-κB and apoptosis. Using the 135 gene as an insertion site, the recombinant GPTV expressing peste des petits ruminants virus (PPRV) H gene can elicit stronger neutralizing antibody responses to PPRV and GTPV than using the TK locus insertion site [52].

4.1.2. Markers for purification of rCaPVs

Recombinant viruses must be purified from the progeny virus stock, where only small percentages (usually 10^{-4} to 10^{-3}) of the total viruses are recombinants. Thus, the isolation of recombinants implies the selection of low-frequency viruses, a task that would be facilitated by the incorporation of one or more genetic markers into a transfer plasmid. Genetic markers generally include genes that can be: (i) detected by staining (e.g., β-galactosidase and β-glucuronidase) or fluorescence (e.g., enhanced green fluorescent protein, eGFP), (ii) selected by specific drugs (e.g., mycophenolic acid), (iii) required for virus spread (e.g., viral genes, A27 L or F13 L) and (iv) required for virus replication in particular cell lines (e.g., host range genes) [53].

4.1.3. Deletion of markers from rCaPVs

Although the selectable markers work well and greatly facilitate the purification of recombinant viruses, their use inevitably results in the creation of an unwanted product, which may affect the overall fitness of viruses [54]. Therefore, the genetic markers should be selectively deleted from rCaPVs if necessary. The Cre/loxP site-specific recombination system is widely used to excise reporter genes from recombinant large DNA viruses [55–59]. This system mainly consists of a Cre recombinase and two Loxp sequences (ATAACTTCGTATANNNTANNNT ATACGAAGTTAT) [60]. An unwanted sequence between two Loxp sites, both of which are oriented in the same direction, can be accurately excised off under the action of Cre recombinase. The Cre/loxP system can be implemented without inserting any extra supporting proteins or sequences. An rCaPV would be considered as a safer vector, if its selectable markers are removed from its genome using the Cre/loxP system.

4.1.4. Promoter and terminator for mRNA transcription

A recombinant transfer plasmid generally contains an efficient promoter sequence, such as the VV p7.5 K promoter. This promoter is a constitutive early/late promoter, and therefore foreign proteins can be expressed both early and late during viral infection under its regulation. Other promoters (e.g., p11 K promoter) may be used to achieve selective expression during only part of the life cycle if desired [61]. The VV early genes terminate transcription approximately 50 bp downstream of a "TTTTTNT" sequence [62], which must be taken into consideration when designing a sequence of foreign gene to be inserted. The termination signal sequence (TTTTTNT) is used early in infection, but not

Table 1
Construction of recombinant capripoxviruses for evaluating their immune effects.

Capripoxvirus vectors	Insert sites	Target pathogens	Antigens	Animal experiments			Ref.
				Animals	Challenge 1 (Pathogen 1)	Challenge 2 (Pathogen 2)	
LSDV KS-1	TK gene	RPV	RPV F protein	Cattle	Protection (RPV)	Protection (LSDV)	[65]
LSDV KS-1	TK gene	RPV	RPV H protein	Cattle	Protection (RPV)	NA	[5]
LSDV KS-1	TK gene	PPRV	RPV F or (and) H protein	Goats	Protection (PPRV)	NA	[6]
LSDV KS-1	TK gene	BTV	BTV VP7	Sheep	Partial protection (BTV)	NA	[11]
LSDV KS-1	TK gene	PPRV	PPRV H protein	Goats	Protection (PPRV)	NA	[69]
LSDV KS-1	TK gene	PPRV	PPRV F protein	Goats	Protection (PPRV)	NA	[68]
LSDV KS-1	TK gene	BTV	BTV VP2, VP7, NS1 and NS3	Sheep	Partial protection (BTV)	NA	[10]
LSDV KS-1	TK gene	RVFV	RVFV Gn and Gc proteins	Sheep	Protection (RVFV)	Protection (SPPV)	[9]
LSDV KS-1	TK gene	RVFV	RVFV NSm and Gn proteins	Mice	Partial protection (RVFV)	NA	[74]
LSDV KS-1	TK gene	RVFV	RVFV Gn	Goats	NA	NA	[75]
LSDV KS-1	TK gene	PPRV	PPRV H and F proteins	Goats	Protection (GTPV)	Partial protection (PPRV)	[85]
LSDV KS-1	GPCR gene	PPRV	PPRV H protein	Goats	Partial protection (GTPV)	Protection (PPRV)	[86]
LSDV Neethling	RR gene	RV	RV G protein	Cattle	NA	NA	[83]
LSDV Neethling	TK gene	RVFV	RVFV Gn and Gc proteins	Mice	Protection (RVFV)	NA	[73]
LSDV Neethling	TK gene	BEFV	BEFV glycoprotein	Cattle	No full protection (BEFV)	NA	[73]
LSDV Neethling	flank I/II	HIV	Grttn	Mice	NA	NA	[80]
LSDV Neethling	flank I/II	HIV	Grttn	Monkey	NA	NA	[81]
GTPV AV41	TK gene	PPRV	PPRV H protein	Goats	Protection (GTPV)	NA	[8]
GTPV AV41	TK gene	PPRV	PPRV H, F and H/F proteins	Sheep/goats	Protection (PPRV)	Protection (SPPV)	[7]
GTPV AV41	gp_24/gp_24.5	FMDV	FMDV mP1-2A	Sheep	NA	NA	[82]
GTPV AV41	135 gene	PPRV	PPRV H protein	Sheep	NA	NA	[52]
GTPV AV41	TK gene	*EG*	*EG* EG95 antigen	NA	NA	NA	[79]
GTPV AV41	TK gene	*Brucella*	*Brucella* OMP25	Mice	NA	NA	[78]

BEFV: bovine ephemeral fever virus; BTV: bluetongue virus; *EG*: *Echinococcus granulosus*; FMDV: foot-and-mouth disease virus; GPCR: G-protein-coupled chemokine receptor; Grttn: Gag, reverse transcriptase, Tat and Nef as a polyprotein; GTPV: goatpox virus; HIV: human immunodeficiency virus; LSDV: lumpy skin disease virus; NA: not available; OMP: outer membrane protein; PPRV: peste des petits ruminants virus; RPV: rinderpest virus; RR: ribonucleotide reductase; RV: rabies virus; RVFV: Rift Valley fever virus; SPPV: sheeppox virus; TK: thymidine kinase.

late [63].

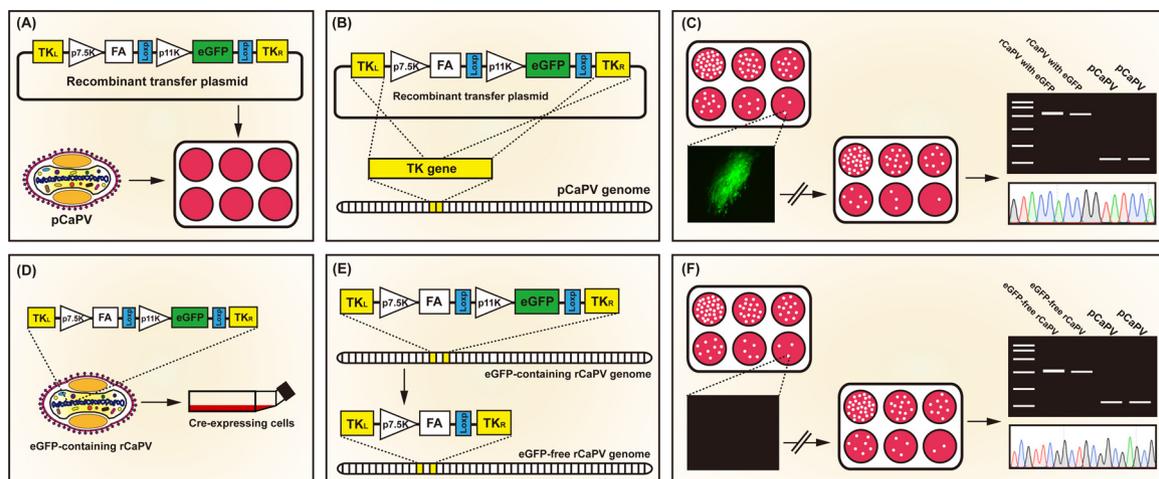### 4.2. General protocol for generating rCaPVs

4.2.1. Construction of recombinant transfer plasmid

A functional map of recombinant transfer plasmid is schematically shown in Fig. 2A. This plasmid is designed with the VV p7.5 K promoter embedded within the TK-encoding region. Downstream of the promoter is a foreign antigen with the termination signal sequence (TTTTTNT). The genetic marker is an eGFP sequence, which is regulated by the VV p11 K promoter. In order to facilitate the deletion of eGFP from

recombinant virus, the complete sequence of eGFP and p11 K promoter is flanked by two Loxp sites that are oriented in the same direction.

4.2.2. Co-infection/transfection

The procedure of co-infection/transfection is shown in Fig. 2A. Briefly, cells (e.g., OA3.T cells) are seeded into a 6-well plate in 2 mL of DMEM supplemented with 10% FBS and cultured at 37 °C with 5% CO_2 for 1 d. Cell monolayers at 70–90% confluency are infected with pCaPVs (MOI = 1) for 1–2 h, and then the supernatant is removed. The pCaPV-infected cells are transfected with the recombinant transfer plasmid (3–4 μg/well), and then cultured at 37 °C with 5% CO_2 for 3–4

Fig. 2. Flow chart for constructing marker-free recombinant capripoxvirus. A general procedure includes: co-infection/transfection (A) for generation of eGFP-containing rCaPV by homologous recombination (B), serial plaque purifications for isolating eGFP-containing rCaPV followed by PCR and sequencing analyses (C), infection of Cre-expressing cells with eGFP-containing rCaPV (D) for generation of eGFP-free rCaPV by Cre/loxP-mediated homologous recombination (E), and serial plaque purifications for isolating eGFP-free rCaPV followed by PCR and sequencing analyses (F). eGFP: enhanced green fluorescent protein; FA: foreign antigen; p7.5 K and p11K: p7.5 K and p11 K promoters; pCaPV: parental capripoxvirus; rCaPV: recombinant capripoxvirus; TK$_L$ and TK$_R$: left and right arms of thymidine kinase gene.

d, during which homologous recombination occurs between the pCaPV and the plasmid to generate rCaPVs. The recombination mechanism is illustrated in Fig. 2B. Cell cultures are harvested at 72–96 h post co-infection/transfection, and subjected to three freeze-and-thaw cycles for collecting progeny viruses, which are composed of parental and recombinant viruses.

4.2.3. Plaque purification of rCaPV

The procedure of plaque purification is shown in Fig. 2C. Briefly, cells are seeded into a 6-well plate, incubate until 90% confluent, and infected with the collected supernatant in 5-fold serial dilutions. After 1–2 h adsorption, the inocula are removed and the cell monolayers are overlaid with selection medium containing 1% low-melting-point agarose. After 7–10 days of incubation, a typical fluorescent plaque is picked from a well (with the highest dilution) into 0.5 ml of growth medium, followed by three freeze-and-thaw cycles to collect supernatant for further plaque purification up to 5–8 times. The putatively purified rCaPV is analyzed by PCR and then confirmed by Sanger sequencing (Fig. 2C).

4.2.4. Deletion of eGFP from rCaPV

If necessary, the genetic marker should be removed from the rCaPV genome by the Cre/loxP system. Firstly, Cre recombinase-expressing cells are seeded into a T-25 flask, incubate until 70% confluent, and infected with purified rCaPV (MOI = 1) for 1–2 h (Fig. 2D). Subsequently, the supernatant is removed and replaced with DMEM. Virus-infected cells are cultured at 37 °C with 5% CO_2 for 3–4 d, during which Cre/Loxp-mediated homologous recombination occurs, consequently removing the eGFP sequence from the rCaPV genome. The eGFP-deleting mechanism is schematically showed in Fig. 2E. The cell culture is harvested at 72–96 h post infection, and subjected to three freeze-and-thaw cycles for collecting progeny viruses, which are composed of eGFP-free and -containing viruses.

4.2.5. Purification of marker-free rCaPV

The marker-free rCaPV must be isolated from the progeny viruses by serial plaque purifications as described in Subheading 4.2.3. However, unlike picking a green plaque out from numerous white plaques (Fig. 2C), purification of marker-free recombinant would follow the principle of picking a white plaque out from numerous green plaques (Fig. 2F). Generally, the marker-free rCaPV can be thoroughly isolated by serial three plaque purifications. The putatively purified eGFP-free rCaPV is analyzed by PCR and then confirmed by Sanger sequencing (Fig. 2F).

5. CaPV-vectored vaccines

The strategy of homologous recombination for gene-modifying viruses has become a valuable tool in development of CaPV-vectored vaccines (Table 1). The rCaPVs have many attractive features (Fig. 3), including (I) ease of genetic manipulation, (II) genomic capacity for large payloads, (III) relatively high genetic stability, (IV) relatively high thermostability, (V) induction of cellular and/or humoral immunities, (VI) long duration of protective immunity, and (VII) potential in differentiation of infected from vaccinated animals (DIVA). The recombinant vaccines have been shown to protect animals from many diseases. This section described generation and use of CaPV-based multivalent vaccines in detail.

5.1. Anti-rinderpest vaccines

Rinderpest, also known as cattle plague, was one of the most deadly diseases, mainly affecting buffalo and cattle, with occasional overspill into wild animals. The disease was caused by infection with the rinderpest virus (RPV), a member of the genus *Morbillivirus* in the family *Paramyxoviridae*. In 2011, the OIE and the Food and Agriculture Organization of the United Nations (FAO) jointly declared global freedom from the rinderpest [64].

Anti-rinderpest CaPV vaccines were explored as early as the 1990's, when two rCaPVs (LSDV KS-1 strain) were constructed to express RPV H and F proteins, respectively. Both vaccines individually prepared from these two recombinant viruses protected cattle from lethal challenges with virulent RPV and LSDV. However, protection was achieved using H protein-expressing vaccine doses lower than those with using the F protein-expressing vaccine [5,65]. A follow-up study showed that if both vaccines were equally mixed for clinical vaccination, approximately 50% of the vaccinated cattle were solidly protected from a RPV challenge at 2 years after vaccination; at 3 years approximately 30% were fully protected. In the case of a LSDV challenge, all of 4 vaccinated cattle were completely protected at 2 years after vaccination; 2 of 5 vaccinated cattle were completely protected at 3 years. This H/F-expressing combined vaccine showed no loss of potency for 1 year when lyophilized for storage at 4 °C [66].

5.2. Anti-peste des petits ruminants vaccines

Peste des petits ruminants is a highly contagious disease, mainly affecting goats and sheep, occasionally wild small ruminants and even large ruminants. Its clinical signs are usually characterized by pyrexia, mucopurulent oculo-nasal discharges, diarrhea, stomatitis and pneumonia. The etiological agent of peste des petits ruminants is PPRV, which belongs to the genus *Morbillivirus* in the family *Paramyxoviridae*, and shares a high homology with the RPV at the genomic level [67].

Like H- and F-expressing CaPVs against RPV, both PPRV H- and F-expressing CaPVs (LSDV KS-1 strain) can also induce immune responses against challenges with virulent PPRVs. However, the lowest protection dose of the former is 100 times higher than that of the latter [68,69]. More recently, Fakri et al. (2018) compared 3 different rGTPV vaccines expressing PPRV H, F or HF proteins to determine which of those proteins was most efficient to protect animals against PPRV infection. Results showed that GTPV-HF vaccine conferred both stronger and earlier antibody responses than the others. A better immune response was obtained with recombinant H or HF than with F alone, confirming an important role of PPRV H protein in the immunity process. This result [7] was totally inconsistent with those of the above-mentioned two studies [68,69], both of which demonstrated that the F protein was more immunogenic than the H protein. Factors leading to the opposite conclusions remain unclear.

5.3. Anti-Rift Valley fever vaccines

Rift Valley fever, caused by the Rift Valley fever virus (RVFV; genus *Phlebovirus*, family *Phenuiviridae*), is a mosquito-borne disease that is highly pathogenic to both animals and humans. The disease is characterized by high rates of abortions and mortalities in newborn sheep, cattle, and goats, as well as a transient febrile illness in humans with occasional complications that can progress to hemorrhagic fever, neurological disorders, or blindness in approximately 1–2% of patients [70].

The Gn and Gc proteins are two RVFV envelope glycoproteins arranged on the surface of virions, and can induce neutralizing antibodies and protective immunity against RVFV in sheep [71,72]. A Gn/Gc-expressing LSDV (Neethling strain) was proven to be capable of protecting mice from a challenge with virulent RVFV [73]. Another Gn/Gc-expressing LSDV (KS-1 strain) could also confer protective immunity against RVFV in mice. Moreover, sheep vaccinated with this recombinant virus twice also developed neutralizing antibodies, and were significantly protected from RVFV and SPPV challenges [9].

The NSm protein is a nonstructural protein of RVFV. An NSm/Gn-expressing LSDV (KS-1 strain) could not simply induce neutralizing antibodies, but also stimulate lymphocyte proliferation, whereas only provided partial protection from a RVFV challenge in inbred MBT/Pas
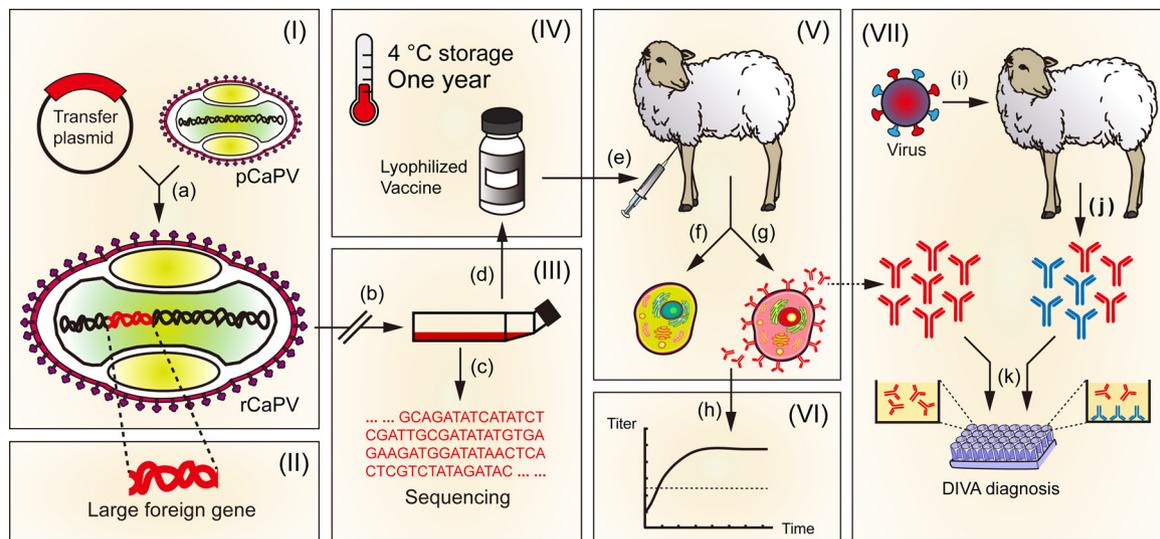
Fig. 3. Confirmed and potential advantages in construction and use of capripoxvirus-vectored vaccines. These advantages include: (I) ease of genetic manipulation, (II) genomic capacity for large payloads, (III) relatively high genetic stability during serial passages *in vitro*, (IV) relatively high thermostability, (V) induction of cellular and/or humoral immunities, (VI) long duration of protective immunity, and (VII) potential in DIVA. (a): co-infection/transfection; (b): serial passages of rCaPV *in vitro*; (c): Sanger sequencing of foreign gene; (d): production of rCaPV-vectored vaccine; (e): vaccination of animal with rCaPV vaccine; (f): induction of cellular immunity; (g): induction of humoral immunity; (h): surveillance on duration of antibody response; (i): natural infection of animal with target virus; (j): induction of target virus-specific antibodies; (k): DIVA diagnosis. DIVA: differentiation of infected from vaccinated animals; pCaPV: parental capripoxvirus; rCaPV: recombinant capripoxvirus.

mice [74]. More recently, a Gn-expressing LSDV (KS-1 strain) was generated for immunization of goats. The result suggested that Th2 cells mediated immunity mainly through the significant production of IL4, which, coupled with a decrease in IFN-γ, might be involved in the replication of the recombinant virus. Additionally, CD4$^+$ cells possibly played a role of helper cells in B cell responses for neutralize antibody production in anti-CaPV humoral immunity [75].

5.4. Anti-bluetongue vaccines

Bluetongue is an economically important, non-zoonotic arboviral disease of certain wild and domestic species of cloven-hooved ungulates. The disease is caused by infection with the bluetongue virus (BTV), a member of the genus *Orbivirus* in the family *Reoviridae*. Occurrence of BTV infection is distinctly seasonal in temperate regions of the world, and dependent on the presence of biting midge vectors [76].

Unlike the rCaPV vaccines against RPV and PPRV, anti-BTV vaccines only provided partial protection of sheep from virulent BTV challenges. Wade-Evans et al. (1996) constructed an rLSDV (KS-1 strain), which could effectively express the core protein VP7 of BTV serotype 1 (BTV-1) in lamb testis cells. Sheep vaccinated with this recombinant virus developed VP7 antibodies but no neutralizing antibodies, prior to a challenge with BTV-1 or BTV-3. Following the challenge with the virulent BTV-3, all of the animals developed clinical signs of disease. Although all of the control animals died, six of the eight animals recovered fully [11]. This partial protection was not dependent on antibody neutralization of BTV, suggesting that the VP7 played a crucial role in protection possibly via the cell-mediated immunity.

In another study, four rLSDVs (KS-1 strain) were constructed to express BTV-2 NS1, NS3, VP2 and VP7, respectively. The four recombinants were administered to goats and sheep. Immune responses were evaluated in both species, and sheep were challenged with a pathogenic BTV-2. Seroconversion was observed against NS3, VP7 and VP2 in both species, and a lymphoproliferation specific to BTV antigens was demonstrated in goats. Finally, partial protection was observed in sheep that were challenged with a virulent BTV-2 3 weeks after administration [10]. Besides the rCaPVs, a recombinant canarypox virus

coexpressing BTV-17 VP2 and VP5 was also revealed to confer effective protection in sheep against a homologous challenge [77].

5.5. Other CaPV-vectored vaccines

In addition to the above-mentioned vaccine candidates, CaPVs are also used to construct recombinants for delivering heterologous antigens from bacterium [78], parasite [79] and other viruses [73,80–83]. In a recent study, Sun et al. (2019) constructed an rGTPV expressing the *Brucella* outer membrane protein 25. This recombinant could elicit anti-*Brucella*-specific immunoglobulin G responses. Mice vaccinated with it did not exhibit pathological alterations in their kidneys and livers [78]. In another recent study, an rGTPV was generated to express *Echinococcus granulosus* EG95 antigen. An *E. coli*-expressed EG95 subunit vaccine has been commercially available in China, and can induce a robust immune response in goats and sheep. Thus, if proven to be efficient in resisting *Echinococcus granulosus* and CaPVs in future animal experiments, the EG95-expressing rGTPV would become a bivalent vaccine candidate [79].

The LSDV is a replication-deficient virus in non-ruminant hosts [84], therefore having a potential in development of vector vaccines for other animals. Shen et al. (2011) constructed an rLSDV, which expressed HIV-1 subtype C Gag, reverse transcriptase, Tat and Nef as a polyprotein (grttn). The rLSDV-grttn was demonstrated to be non-pathogenic in immunocompromised mice, and to be immunogenic in mice particularly in prime-boost regimens. The rLSDV-grttn might be useful for enhancing, in particular, HIV-specific CD4 IFN-γ and IL-2 responses induced by a priming vaccine [80]. A subsequent trail revealed that the rLSDV-grttn, in combination with a modified VV Ankara-grttn, induced high-magnitude, broad and balanced CD4$^+$ and CD8$^+$ T-cell responses, as well as transient activation of the immune response [81]. Both studies support further development of LSDV-vectored vaccines for non-ruminant hosts.

6. Conclusions

CaPVs genetically modified to express foreign antigens are an established tool for the development of novel vaccine candidates. A

system of homologous recombination can be used either to insert a foreign gene or to delete a viral gene for construction of rCaPVs. This system mainly includes a transfer plasmid and pCaPVs, between which recombinant viruses would be generated by the homologous recombination. The rCaPV can be isolated from parental viruses by plaque purification for further deletion of genetic markers if necessary.

During the past decades, CaPV-vectored vaccine candidates have been shown to elicit full and partial immune protections from rinderpest, peste des petits ruminants, Rift Valley fever and bluetongue. Their ability to express foreign antigens, which have a potential in inducing cellular and (or) humoral immune responses *in vivo*, make them appealing for use as vaccines in the developing world. Furthermore, their thermostability has been notably improved by freeze-drying, thus facilitating their storage, transport and distribution in tropical and subtropical regions. However, they may face regulatory issues in some CaPV-free countries, where CaPV-derived vaccines would not be permitted to use.

The past trend in the development of CaPV-vectored vaccines mainly focused on constructing recombinant viruses for evaluation of their immune effects. Major directions for future researches will aim to screen out foreign antigens with DIVA characteristics, and to optimize technology further for vaccine production.

Declaration of interest statement

The authors declare that there are no conflicts of interest.

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