9 Preventive, Diagnostic and Therapeutic Applications of Baculovirus Expression Vector System

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Abstract

Different strategies are being worked out for engineering the original baculovirus expression vector (BEV) system to produce cost-effective clinical biologics at commercial scale. To date, thousands of highly variable molecules in the form of heterologous proteins, virus-like particles, surface display proteins/antigen carriers, heterologous viral vectors and gene delivery vehicles have been produced using this system. These products are being used in vaccine production, tissue engineering, stem cell transduction, viral vector production, gene therapy, cancer treatment and development of biosensors. Recombinant proteins that are expressed and post-translationally modified using this system are also suitable for functional, crystallographic studies, microarray and drug discovery-based applications. Till now, four BEV-based commercial products (Cervarix®, Provenge®, Glybera® and Flublok®) have been approved for humans, and myriad of others are in different stages of preclinical or clinical trials. Five products (Porcilis® Pesti, BAYOVAC CSF E2®, Circumvent® PCV, Ingelvac CircoFLEX® and Porcilis® PCV) got approval for veterinary use, and many more are in the pipeline. In the present chapter, we have emphasized on both approved and other baculovirus-based products produced in insect cells or larvae that are important from clinical perspective and are being developed as preventive, diagnostic or therapeutic agents. Further, the potential of recombinant adeno-associated virus (rAAV) as gene delivery vector has been described. This system, due to its relatively extended gene expression, lack of pathogenicity and the ability to transduce

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a wide variety of cells, gained extensive popularity just after the approval of first AAV-based gene therapy drug alipogene tiparvovec (Glybera®). Numerous products based on AAV which are presently in different clinical trials have also been highlighted.

9.1 Introduction

Baculovirus (family: *Baculoviridae*) derived its name from the Latin word "baculum" meaning "stick". They are rod-shaped (30–60 × 250–300 nm) large enveloped viruses with circular, supercoiled double-stranded DNA genomes, approximately 80–180 kb in size. While most of the baculoviruses infect their natural host, i.e., butterflies and moths (*Lepidoptera*), few are also known to infect sawflies (*Hymenoptera*) and mosquitoes (*Diptera*) (King et al. 2011). They have not been linked with any disease in any organism outside the phylum *Arthropoda* (Kost and Condreay 2002). Baculoviruses are well known for their role as biopesticides and are efficient tools for heterogeneous protein production in insect cells (Summers 2006). Morphologically, these enveloped viruses have been classified into two phenotypes: occlusion-derived viruses (ODVs) that are embedded in paracrystalline matrix forming polyhedral occlusion bodies (OBs) which are responsible for horizontal transmission between insects and the budded viruses (BuVs) present in the haemolymph which spreads infection from cell to cell (Luckow and Summers 1988). Occlusion body morphology was initially used to define two major groups of baculoviruses: nucleopolyhedroviruses (NPVs) and the granuloviruses (GVs). NPVs obtain their envelop from host nuclear membrane and are occluded within main occlusion protein polyhedrin forming large (1–15 μm) polyhedral inclusion bodies, while GVs obtain their envelop from cell membrane to make oval-shaped single virion structure called granule or capsule with diameter in the range of 0.2–0.4 μm (King et al. 2011). NPVs are further distinguished as single nucleopolyhedrovirus or multiple nucleopolyhedrovirus based on the number of nucleocapsids in a polyhedral inclusion body (O'Reilly et al. 1994). OBs allow virions to remain infectious for long period due to their highly resistant and stable structure.

Baculovirus-infected insect cell expression system has been used for the routine production of recombinant proteins, including several proteins of therapeutic nature over the last three decades. The establishment of this system begins from the production of human beta interferon (INF-β), the protein normally not produced in the cultured human cells. It was produced with a recombinant *Autographa californica* multiple nucleopolyhedrovirus (AcMNV) by exploiting its polyhedrin promoter (Smith et al. 1983). In this system, the protein coding sequence of human interferon gene was linked to the AcNPV polyhedrin gene promoter. The interferon gene was inserted at different positions relative to the AcNPV polyhedrin transcriptional and translational signals. The interferon-polyhedrin hybrid plasmid was then transferred to infectious AcNPV expression vectors by recombination within *S. frugiperda* insect cells, where more than 95% of biologically active glycosylated interferon was produced in the secreted form.

At the same time, another group successfully expressed *Escherichia coli* β-galactosidase gene in insect cells by using this system. A 9.2 kb plasmid construct was made of β-galactosidase gene (1 kb) after fusion with the N-terminal region of the polyhedrin gene (1.2 kb) of AcNPV. Co-transfection of this fused plasmid construct with wild-type AcNPV genomic DNA (134 kb) was performed in order to insert the foreign gene into the polyhedrin gene of AcNPV genome by the process of homologous recombination. Finally, the recombinant viruses were selected as blue plaques in the presence of β-galactosidase indicator X-gal medium. These discoveries mark the beginning of baculovirus expression system, facilitating the engineering and improvement of baculovirus vectors, modification of the sugar moieties of glycoproteins expressed in insect cells and scale up of the cell culture processes.

A baculovirus expression vector (BEV) platform has been tailored by taking advantage of baculoviruses' natural tendency to infect insect cells. There are almost 500 different types of baculoviruses, all of which specifically infect invertebrates. For laboratory research and manufacturing purposes, the most commonly studied baculovirus is *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV) which is often considered as a prototype of baculoviruses. It has a double-stranded circular DNA genome of 134 kb inside a rod-shaped nucleocapsid of size 25 × 260 nm (Fauquet et al. 2005). Its large size genome gives sufficient ability to accommodate a large foreign DNA or multiple genes together.

Typically, recombinant BEVs are constructed by co-transfecting a mixture of transfer plasmid and modified non-infectious and linearized *Ac*MNPV that lacks parental polyhedrin gene and a portion of ORF1629. Transfer plasmid contains the gene of interest (GOI), flanked upstream by strong polyhedrin or p10 promoter and downstream by an essential portion of ORF1629 of *Ac*MNPV for high-level protein expression in insect cells. The transfer plasmid and modified linearized *Ac*MNPV DNA undergo homologous recombination to generate de novo recombinant baculoviruses. After plating of these baculoviruses, single pure plaques of recombinant baculovirus are selected. Subsequently, this plaque is passaged through multiple rounds of insect cell infection to generate a high-titre stock. It creates a working virus bank (WVB) for utilization during downstream processes.

This system was further enhanced for manufacturing and commercialization purposes by multiple ways and technologies. Bacmid technology (Bac to Bac®, Life Technologies) was employed for the generation of recombinant *Ac*MNPV genomes in bacterial host system *E. coli*. FlashbackTM (Oxford Expression Technologies Ltd.) and BacMagicTM (Merck) BaculoOneTM (PAA) technologies are used to avoid the bacterial sequences in the final vector or rapid production of multiple recombinant viruses in a one-step procedure. MultiBac system is being used for the synthesis of multisubunit protein complex and OmniBac as multigene transfer vector for universal generation of recombinant baculoviruses. Sleeping beauty or PiggyBac transposon system are being exploited in highly efficient seamless excision of transposons from the genomic DNA and for its potential to target integration events to desired DNA sequences. For the production of AcMNPV vectors and

recombinant proteins, the *Spodoptera frugiperda Sf21* and its subclone *Sf9* and High Five cell lines are being used. These insect cells exhibit several properties like rapid growth, stress resistance and robust expression of recombinant proteins that make them suitable for the production of clinical biologics and commercial products.

Initially, insect-derived baculovirus expression vector (BEV) was recognized as a safe system for routine production of recombinant proteins both in insect and mammalian cells. During the last three decades, it has emerged as an effective tool for research as well as various applications in the field of biotechnology. It has shown tremendous potential as preventive, diagnostic and therapeutic agent against a myriad of diseases in the form of vaccination, tissue engineering, stem cell transduction, viral vector production and gene therapy (Airenne et al. 2013). It has been extensively used for functional studies, crystallography, biosensors, protein microarray and drug discovery. All these applications are based on different baculovirus-derived products such as heterologous proteins, protein/antigen displayed on baculovirus particle surface, heterologous viral vectors and gene delivery vehicles for mammalian cells (van Oers et al. 2015). In this chapter, we have presented the application of these products from a clinical point of view in three main categories, viz. preventive, diagnostic and therapeutic agents. Most of the approved biomolecules produced by using baculovirus expression system in insect cells have been discussed. As thousands of other products are being developed by BEVs, it seems ineffectual to include the entire list under the ambit of the present chapter; however few among them have been mentioned to have an understanding about the scope of this powerful expression system in the near future.

9.1.1 BEVs As Disease Preventive Agents

BEV exhibits many characteristics that make it suitable for the production of heterologous proteins in insect cells. It can be easily handled in the BSL1/2 laboratories due to its harmless nature to nontarget organisms. These viruses are environmentally safe due to their instability outside the laboratory. It is used to produce high level of proteins in insect cells or larvae where the eukaryotic environment provides the appropriate post-translational modifications. BEVs host insect cells are mostly free of human pathogens and do not require controlled oxygenic environment for their growth. Insect cells can be grown into serum-free medium, and the heterologous protein production can be enhanced to the level of pilot plant or larger bioreactors. Therefore, the proteins obtained by the BEVs can be used as vaccines either in the form of heterologous subunit proteins or virus-like particles (VLPs) formed by subunit proteins of virus.

Subunit vaccines are relatively safe as they are devoid of virus genetic material but exhibit poor immunogenicity that might be due to incorrect folding of the target protein. Structural proteins of viruses such as capsid and envelop proteins assemble into particulate structure similar to the naturally occurring virus or subviral particles. Therefore, virus-like particles (VLPs) that are non-infectious and

non-replicating due to the absence of viral genetic material can be produced in heterologous system (Yamaji 2014). VLPs are highly effective in eliciting both humoral and cellular immune response because of their densely repeated display of viral antigens in right conformation (Roy and Noad 2008). VLPs comparatively exhibit wide spectrum of clinical applications such as prevention of disease as vaccines, diagnostics as antigens for the detection of antibodies and therapeutics in the form of therapeutic vaccines and delivery agents. The use of heterologous proteins and VLPs as preventive agents in the form of vaccines against different diseases is being described (Table 9.1).

A decade back, only two veterinary products were manufactured using BEVs to prevent classical swine fever in pigs. Now, five more new vaccines have been approved, two of which are for humans, and many more products are in the development phase. Here, approved vaccines as well as development of other vaccines in preclinical stages have been highlighted.

9.1.1.1 Veterinary Vaccines

(a) *Subunit marker vaccine for classical swine fever*: Classical swine fever virus (CSFV) infection invariably develops antibodies against virus envelop proteins E^{RNS} and glycoprotein E2 and the non-structural protein NS3 in swine (Paton et al. 1991). However, injection of only glycoprotein E2 in pigs is reported to sufficiently provide protection to CSFV (Van Rijn et al. 1996). Therefore, a subunit vaccine has been produced on the basis of conserved glycoprotein E2 with a baculovirus vector in insect cells (Moormann et al. 2000). Glycoprotein E2 being expressed as envelop protein, its C-terminal transmembrane domain was removed to secrete it into the medium, and the residual baculovirus was inactivated with 2-bromoethyl-imminebromide. This vaccine was manufactured and commercialized as "Porcilis Pesti®" by MSD Animal Health. The same vaccine was also commercialized as "BAYOVAC CSF E2®/Advasurea" by Bayer AG/Pfizer Animal Health but was later discontinued.

(b) *Virus-like particle (VLP)-based vaccine for porcine circovirus type 2*: Porcine circovirus type 2 (PCV2) vaccine was developed based on VLPs. PCV2 is the primary causative agent of postweaning multisystemic wasting syndrome (PMWS) in swine. Two major open reading frames (ORF1 and ORF2) have been identified in PCV2. ORF2 encodes a major structural protein with type-specific epitopes and is found to be highly immunogenic. Therefore, ORF2 that encodes the capsid protein was used to develop the vaccine with a baculovirus in Tn5 insect cells (Liu et al. 2008). Insect Sf9 and Tn5 cells were infected with recombinant baculovirus AcPCV2-ORF2 that contains the complete PCV2 capsid protein. As compared to Sf9 insect cells, Tn5 expressed higher amount of PCV2 capsid protein as virus-like particles of size around 28-KDa. This vaccine was commercialized by two different names "Circumvent PCV and Porcilis PCV" in different geographical areas by MSD Animal Health (known as Merck Animal Health in the USA and Canada) (Felberbaum 2015). Vaccine for PCV2-based ORF2 was also commercialized as "Ingelvac CircoFLEX" by Boehringer Ingelheim Vetmedica Inc. (Desrosiers et al. 2009).

Table 9.1 BEVs produced biomolecules as disease preventive agents

Product name (company name, if any)	Targeted/used for	Expressed product	Used against	Product type	Development stage
Porcilis Pesti (MSD Animal Health)	Pigs	E2 glycoprotein	Classical swine fever	Protein subunit/ marker vaccine	Approved
Bayovac CSF E2 (Bayer Biologicals/Pfizer Animal Health)[a]	Pigs	E2 glycoprotein	Classical swine fever	Protein subunit/ marker vaccine	Approved
Circumvent PCV (MSD Animal Health)[b]	Pigs	Porcine circovirus ORF2	Porcine circovirus type 2	VLP vaccine	Approved
Porcilis PCV (MSD Animal Health)[b]	Pigs	Porcine circovirus ORF2	Porcine circovirus type 2	VLP vaccine	Approved
Ingelvac CircoFLEX (Ingelvac)#	Pigs	Porcine circovirus ORF2	Porcine circovirus type 2	VLP vaccine	Approved
AcAs3-PPV-VLP	Pigs	Viral capsid protein VP2	Porcine parvovirus (PPV)	VLP vaccine	Unapproved
BTV-1/BTV-4 VLP	Sheeps	BTV serotype 1 and 4	Bluetongue virus (BTV)	VLP vaccine	Unapproved
AI-H5N3 VLP	Ducks	Subunits HA, NA and M1	Avian influenza (AI)	VLP vaccine	Unapproved
IDBV-VLP	Chickens	Capsid proteins VP2, VP3 and VP4	Infectious bursal disease virus (IBDV)	VLP vaccine	Unapproved
RHDV-VLP	Rabbits	Capsid proteins VP60	Rabbit haemorrhagic disease virus (RHDV)	VLP vaccine	Unapproved
SIV-VLP	Primates	Precursor protein Pr56gag	Simian immunodeficiency virus (SIV)	VLP vaccine	Unapproved
Flublok (Protein Sciences)	Humans	Influenza HA	Trivalent flu vaccine	Protein subunit vaccine	Approved
Cervarix (GlaxoSmithKline)	Humans	Human papillomavirus L1 protein (serotypes 16 and 18)	Cervical cancer	VLP vaccine	Approved
Ebola-VLP	Humans	Ebola VP40 and GP protein	Ebola virus	VLP vaccine	Preclinical

Bac-P1-3CD	Humans	EV71-P1 protein and 3CD protease	Enterovirus 71	VLP vaccine	Preclinical
VAI-VP705 (NIH/Meridian Life Science)	Humans	B19 VP1, VP2	Parvovirus B19	VLP vaccine	Phase I/II
NV-VLP (Baylor College of Medicine)	Humans	Capsid proteins NV CP	Norwalk virus (Nv)	VLP vaccine	Phase I
NV-VLP (Ligo Cyte Pharmaceuticals)	Humans	Capsid proteins NV CP	Norwalk virus (Nv)	VLP vaccine	Phase I
NV-VLP (Ligo Cyte Pharmaceuticals)	Humans	Capsid proteins NV CP	Norwalk virus (Nv)	VLP vaccine	Phase I/Phase I/II
PV-VP1-VLP	Humans	Major capsid protein VP1	Polyomavirus	VLP vaccine	Preclinical
SARS-CoV-VLP	Humans	SP, EP and MP	Severe acute respiratory syndrome-associated coronavirus (SARS-CoV)	VLP vaccine	Preclinical
SV40-VLP	Humans	VP1 or P1 and 3CD	Simian virus 40 (SV40)	VLP vaccine	Preclinical
RV-VLP	Humans	VP2, VP6 and VP7	Rotavirus	VLP vaccine	Preclinical
HIV-VLP	Humans	Gag protein	HIV	VLP vaccine	Preclinical
Influenza (Novavax)	Humans	A/California/04/09(H1N1) HA, NA	Influenza	VLP vaccine	Phase II
Influenza (Novavax)	Humans	A/Brisbane/59/07(H1N1), A/Brisbane/10/07, B/Florida/04/06 (H3N2)	Influenza	VLP vaccine	Phase IIa
RSV (Novavax)	Humans	RSV-F	RSV	VLP vaccine	Phase I

[a]Discontinued

[b]MSD Animal Health got the same products licensed by two names in different geographical areas

(c) *VLP-based vaccine for porcine parvovirus (PPV)*: PPV, a non-enveloped DNA virus, causes major reproductive failure in swine. Its viral capsid is made up of 50–60 molecules of VP2, the major structural protein that are being targeted for vaccine development. VP2 gene was expressed under the control of late p10 promoter of baculovirus and the *LacZ* gene under the control of *Drosophila hsp 70* promoter. The recombinant baculovirus AcAs3-PPV was used to infect Sp21 insect cell line to express VP2 that leads to self-assembled empty PPV VLPs in serum-free medium for safety point of view (Maranga et al. 2002). Earlier, it was also produced in *Sf9* cells in the presence of serum proteins. However, its commercialization at large scale still needs more developmental efforts.

(d) *VLP-based vaccine for sheep bluetongue virus (BTV)*: Bluetongue primarily causes disease in ruminants due to infection by BTV double-stranded RNA virus. Sheep generally shows more severe clinical signs than other cattle. Recombinant baculovirus expression system in *Sf9* insect cell lines shows great potential to develop VLP-based vaccine against BTV (de Diego et al. 2011). Monovalent and bivalent VLP vaccines are being developed for two serotypes 1 and 4 of BTV. BTV-1 exhibits more protection to virulent BTV live strain as compared to BTV-4. Earlier, VLP expressing capsid proteins VP2 and VP5 were developed by co-transfection of dual transfer vector DNA (pAcVC3/BTV-10-2/BTV-10-5) with wild-type AcNPV DNA in insect cells (French et al. 1990). Strong developmental efforts and further research are needed to commercialize robust and effective BTV vaccine.

Many more VLPs veterinary vaccines by baculovirus expression system in insect cells have been developed such as avian influenza (AI), (H5N3)-VLPs that consists of subunits haemagglutinin (HA), neuraminidase (NA) and matrix protein (M1) of AI virus for ducks (Prel et al. 2008); chimeric infectious bursal disease virus (IBDV)-VLPs consisting of structural protein VP2, VP3 and VP4 with varying degree of one of the capsid protein VP2 tagged with histidine of IBDV for chickens (Hu and Bentley 2001); rabbit haemorrhagic disease virus (RHDV)-VLPs made up of VP60 capsid protein for rabbits (Laurent et al. 1994); and simian immunodeficiency virus (SIV)-VLPs consisting of precursor protein $Pr56^{gag}$ for vaccine testing in non-human primates (Yamshchikov et al. 1995).

9.1.1.2 Human Vaccines

(a) *Subunit vaccine for influenza*: Influenza generally called as "the flu" is caused by RNA influenza viruses, designated from type A to C. Both type A and B influenza viruses possess haemagglutinin (HA) or neuraminidase (NA) glycoprotein spikes in their envelope which act as key antigens in the host immune response, therefore targeted for vaccine development. But HA and NA exhibit antigenic drift due to continuous mutations in the genetic material, and the vaccine based on these glycoproteins is required to be updated annually. However, type C influenza virus is not involved in annual influenza virus vaccine as they cause only mild respiratory disease in humans. The vaccine against influenza in *Spodoptera frugiperda Sf9* insect cells is developed by targeting dominant

surface glycoproteins HA of influenza virus. Recombinant viruses as vaccines are produced in Sf9 insect cells by co-transfecting linearized AcMNPV genomic DNA with the baculovirus transfer plasmids containing the HA gene. Recombinant plaques are selected on the basis of their morphology and virus stocks generated. These viral stocks are used for their amplification through passage in the fresh insect cell cultures. Commercial production of recombinant HA vaccine could begin within 45 days after identification of the new virus strain. It is commercialized by Protein Sciences Corporation by Flublok trademark (Cox and Hashimoto 2011).

(b) *VLP-based vaccine for human papillomavirus (HPV)*: HPV infection causes mostly all forms of cervical cancer in women. HPVs are icosahedral viruses with double-stranded circular DNA codes for two classes of genes; early (E) and late (L). Early genes regulate replication, transcription and other biological processes, whereas late genes (L1 and L2) are responsible for structural components of the viral capsid. L1 capsid proteins are known to form virus-like particles, therefore targeted for vaccine development against HPV. L1-based vaccine in insect cells that shows remarkable safety profile and clinical efficacy from the genotypes HPV16 and 18 was commercialized by GlaxoSmithKline by the trademark Cervarix (Monie et al. 2008). It has been produced in *Trichoplusia ni* insect cell lines Hi-5 Rix4446 by using baculovirus expression system.

With the advent of successful cases of approved VLP-based vaccines, researchers are indeed redirecting their efforts for the development of such products. Therefore, a number of vaccines have been produced against many viral diseases in humans; however many of them are either in preclinical or clinical trial stages. Prominent VLPs that are made up of multimeric proteins expressed in insect *Sf9* cells include Ebola by VP40 and glycoproteins (Sun et al. 2009); enterovirus by P1 and 3CD (Chung et al. 2010); human parvovirus B19 by B19 VP1, VP2 (Roldão et al. 2010); Norwalk virus (Nv) by capsid proteins (Jiang et al. 1992; Ball et al. 1999; Atmar et al. 2011; Frey 2011); polyomavirus by VP1 (Montross et al. 1991); severe acute respiratory syndrome-associated coronavirus (SARS-CoV) by SP, EP, MP and EN (Mortola and Roy 2004) and simian virus 40 (SV40) by VP1 or P1 and 3CD (Kanesashi et al. 2003). VLPs for rotavirus were prepared by using two (VP2 and VP6) to three (VP2, VP6 and VP7) capsid proteins expressed both in *Sf9* and High Five insect cells. It has also been expressed in *Sf larvae* with two capsid proteins VP2 and VP6 (Roldão et al. 2010). Combinations of capsid proteins from different strains of influenza were used in both *Sf9* and High Five insect cells such as HA (H1N1) with M1 (H3N2) and HA (H3N2) with M1 (H1N1) to produce higher amount of influenza A-VLPs. Other influenza A-VLPs formed by co-expression of M1 and ESAT6-HA were produced only in High Five cells. Strain-specific influenza HA and M1 capsid proteins were used to prepare influenza A H1N1-VLPs and influenza A H3N2-VLPs in both the insect cells (Krammer et al. 2010; López-Macías et al. 2011). Respiratory syncytial virus (RSV) vaccine was produced by using RSV-F protein (Mazur et al. 2015; Neuzil 2016). HIV VLPs were produced by targeting gag protein in rodents and rhesus macaques for preclinical trials (Pillay et al. 2009; Wagner et al. 1996).

9.1.2 BEVs as Diagnostic Agents

Supposedly, both heterologous subunit proteins and VLP-based subunit vaccines can be used as vaccines as well as antigens for the detection of antibodies, given the condition that it satisfies the various diagnostic parameters like sensitivity, specificity, predictive values and likelihood ratios. These parameters have been well evaluated and found to be acceptable for diagnostic purposes for numerous BEV-derived products. However, commercialization of these vaccines/proteins demands further standardization and evaluation. Here, we have summarized some of the human as well as veterinary usage diagnostic molecules produced by BEV system in insect cells (Table 9.2).

Table 9.2 BEVs produced biomolecules as diagnostic agents

Targeted/used for	Expressed product	Used to detect	Test type
Rodents	Recombinant nucleocapsid protein	Sendai virus	ELISA
Swine	G-protein	Nipah virus (NiV)	ELISA
Swine	SVDV-VLP	Swine vesicular diseases virus (SVDV)	ELISA
Horse	EIAV-core gag and p26 protein	Equine infectious anaemia virus (EIAV)	ELISA and agar gel immunodiffusion (AGID)
Horse	HA	Equine influenza strain LP/93	ELISA
Cattles	VP7	Bluetongue (BTV) and epizootic haemorrhagic disease (EHDV)	Antigen capture competitive ELISA (Ag Cap c-ELISA)
Pigs and goats	VP1 capsid protein and 3C protease	Foot-and-mouth disease virus (FMDV)-type A	Blocking ELISA
Bovine	Recombinant-F protein	Bovine respiratory syncytial virus (BRSV)	Immunofluorescence
Ducks	E protein	Tembusu virus (TMUV)	E-ELISA
Birds	APMV2-HN	Avian paramyxovirus type 2	Haemagglutination inhibition (HI) test
Geese	VP1	Goose haemorrhagic polyomavirus (GHPV)	ELISA and haemagglutinin inhibition test
Humans	Nucleocapsid protein (N)	Lassa virus	ELISA
Humans	E1, E2 and polyprotein precursor	Rubella virus (RV)	Enzyme immunoassay (EIA) and immunoblot
Humans	Flagellar repetitive antigen (FRA)	*Trypanosoma cruzi*	ELISA

Table 9.2 (continued)

Targeted/used for	Expressed product	Used to detect	Test type
Humans	Glutamic acid decarboxylase (GAD65 and GAD67)	Insulin-dependent diabetes mellitus	Immunoassay
Humans	Nucleocapsid protein of strain SR-11	Hantavirus	Indirect immunofluorescence antibody (IFA)
Humans	E2 protein	Human papillomavirus (HPV)	ELISA
Humans	Hou/90 capsid	Human calicivirus (HuCV)	Immunoprecipitation and EIA
Humans	Fragment of gG comprising residues 321–580 of HSV-2	Herpes simplex virus (HSV)	Indirect ELISA
Humans	Capsid proteins	Human caliciviruses (HuCVs)	EIA
Humans	C-terminus truncated form of protein (Etr)	TBE complex virus	ELISA and immunoblot assay
Humans	Recombinant Fel dl (rFel dl Ch1 + Ch2)	Cat allergen	Radioimmunoassay (RIA) and ELISA
Humans	Recombinant human tissue TG (hu-tTG)	Autoantigen transglutaminase (TG)	ELISA
Humans	Envelop glycoproteins gB, gD, gC, gE and gG	Herpes B virus (HBV)	ELISA

9.1.2.1 Veterinary Applications

(a) *Rodent*: Recombinant nucleocapsid protein produced in baculovirus expression system-based enzyme-linked immunosorbent assay (ELISA) is reported to be more specific as compared to whole virion conventional ELISA for the detection of Sendai virus infection in rodents (Wan et al. 1995).

(b) *Swine*: Specific indirect ELISA method was developed for the detection of Nipah virus (NiV) infection in swine serum samples by cloning G-protein of NiV into pFASTBac HT vector (Eshaghi et al. 2004). Its further use as diagnostic reagent for humans needs to be explored. P1 and 3CD protein genes of swine vesicular disease virus (SVDV)-derived VLPs as antigens for detection of antibodies against SVDV in pigs by ELISA were also developed (Ko et al. 2005).

(c) *Horse*: Recombinant baculovirus expressing equine infectious anaemia virus (EIAV) core proteins Gag and p26 as antigens was found to possess high specificity and sensitivity in ELISA and agar gel immunodiffusion (AGID) to detect antibodies from infected horse sera (Kong et al. 1997). Haemagglutinin (HA)

protein of equine influenza strain, A/equine/La Plate/I/93 (LP/93), was produced in silkworm larvae with recombinant baculovirus for the detection of antibodies in horse sera by ELISA (Sugiura et al. 2001). Its efficiency was further tested by HA1 subunit of HA (Sguazza et al. 2013).

(d) *Cattle*: Baculovirus-derived antigen capture competitive ELISA (Ag Cap c-ELISA) for the diagnosis of bluetongue and epizootic haemorrhagic disease virus infection in cattle exhibits advantages in terms of easy production, standardization, less requirement of downstream processing and its non-infectious nature as compared to commercially available c-ELISA (Mecham and Wilson 2004). Blocking ELISA was developed by BEVs for the detection of antibodies against foot-and-mouth disease of cattle, pigs and goats by virus type A with a specificity of 99% (Ko et al. 2010). Bovine respiratory syncytial virus (BRSV) infection that causes lower respiratory tract disease in calves 1–3 months old can be detected by immunofluorescence analysis with recombinant F-protein as antigen (Pastey and Samal 1998).

(e) *Bird*: A variant of ELISA known as E-ELISA using eukaryotically expressed E protein as the antigen for the detection of Tembusu virus (TMUV) in ducks was developed with 93.2% specificity and 97.8% sensitivity (Yin et al. 2013). Recombinant avian paramyxovirus type 2 haemagglutinin (APMV2-HN) is found to be a useful alternative to APMV-2 antigens in haemagglutination inhibition (HI) test for the detection of APMV-2 infection in avians (Choi et al. 2014). Whole Sendai virus virion VLPs are being used as antigens for the detection of antibodies against virus for diagnostic purposes such as major capsid protein VP1 of goose haemorrhagic polyomavirus-VLPs for the detection of GHPV-specific antibodies in sera from flocks with haemorrhagic nephritis and enteritis of geese (HNEG) disease (Zielonka et al. 2006).

9.1.2.2 Application in Humans

Most of the recombinant proteins that are used as antigens have been expressed by baculovirus expression system in *Sf9* insect cells unless otherwise stated. Some of them are mentioned here.

Lassa virus infection causes Lassa fever mainly endemic in West Africa. Recombinant nucleocapsid protein acts as antigen for the detection of antibodies in Lassa virus-infected patient sera by ELISA (Barber et al. 1990; Saijo et al. 2007). Rubella virus (RV) normally causes a self-limiting disease, but its infection during the first trimester of pregnancy may cause foetal damage. Therefore, serological diagnostic test was developed by expressing E1, E2 and polyprotein precursor of rubella virus as antigen for enzyme immunoassay (EIA) and immunoblot analysis of patient sera (Seppänen et al. 1991). *Trypanosoma cruzi* causes Chagas' disease in Latin America. Flagellar repetitive antigen (FRA), part of *T. cruzi*-based improved diagnostic assay, was developed for Chagas' disease (dos Santos et al. 1992). Full-length human glutamic acid decarboxylases (GAD65 and GAD67) with histidine tag were produced in their natural conformations for the development of an immunoassay for the diagnosis of insulin-dependent diabetes mellitus (Mauch et al. 1993). Hantavirus which causes haemorrhagic fever with renal syndrome (HFRS)

nucleocapsid protein of strain SR-11 (rNP-SR-Sf9) was used as antigen for the indirect immunofluorescence antibody (IFA) diagnostic test that detects three serotypes (hantan 76-118, SR-11 and Puumala) of hantavirus (Yoshimatsu et al. 1993). Purified human papillomavirus (HPV) E2 protein was used to develop ELISA to detect IgG and IgA responses in cervical neoplasia patients (Rocha-Zavaleta et al. 1997). Houston/90 (Hou/90) is a human calicivirus (HuCV) strain in one of the three clades of Sapporo-like HuCVs that cause acute gastroenteritis in children. The viral capsid gene of Hou/90 capsid was used as antigen for immunoprecipitation and EIA (Jiang et al. 1998). Herpes simplex virus (HSV) infection is caused by two viruses HSV-1 and HSV-2. Diagnostic test that can distinguish between two strains has been developed that utilizes both type-specific and type-common HSV antigens in a single-step assay format to perform accurate diagnosis (Burke 1999; Wald and Ashley-Morrow 2002; Liu et al. 2015). Eight different strains of human caliciviruses (HuCVs) capsid proteins have been used to develop antigen-antibody detection assay by EIAs that are highly specific (Jiang et al. 2000). Causative agent of tick-borne encephalitis (TBE), C-terminus truncated form of protein E (Etr) of TBE complex virus tagged with histidine was used to develop sensitive and specific ELISA as well as immunoblot assay to detect the TBE virus-specific antibodies in infected individuals (Marx et al. 2001). Fel dl, the major allergen from cats, consists of two polypeptide chains, chain 1 (ch1) and chain 2 (ch2), which are usually linked with a disulphide bond. Recombinant Fel dl (rFel dl Ch1 + Ch2) protein construct in which two chains are linked together with glycine/serine linker was used as more potent antigen than bacterial-derived proteins for the detection of IgE and IgG antibodies by radioimmunoassay (RIA) and ELISA (Guyre et al. 2002). Coeliac disease (CD) is characterized by the presence of autoantigen transglutaminase (TG). Recombinant human tissue TG (hu-tTG) expressed with baculovirus system was used as antigen for ELISA that showed a sensitivity of 100% and a specificity of 98.6% (Osman et al. 2002). The envelope glycoproteins: gB, gD, gC, gE and gG are thought to be the primary targets of IgG antibody response in patients with Herpes B virus (HBV) infection. Therefore, ELISA test was developed by using the cocktail of these recombinant glycoproteins along with other capsid proteins with high sensitivity and specificity (Perelygina et al. 2005). Similarly, the recombinant proteins in single or multiple subunits for the diagnosis of different types of viral infections in humans have been developed with baculovirus expression system in insect cells.

9.1.3 BEVs as Therapeutic Agents

BEVs express products like growth factors, cytokines, chemokines, enzymes, hormones and monoclonal antibodies that can be used for human therapeutic purposes. More recently, BEV has also been exploited as effective tool for gene therapy. For simplicity, the applications of these products have been divided into two major groups: biological drug therapy and gene therapy. Over thousands of such biomolecules have been developed till now in this system, few among them are discussed here (Table 9.3).

Table 9.3 BEVs produced biomolecules as disease therapeutic agents

Therapy type	Targeted for	Product type	Product name	Expressed product	Development stage	Company name, if any
Immunotherapy	Prostate cancer	Recombinant fusion protein	Provenge or sipuleucel-T	PAP-GM-CSF^	Approved	Dendreon
Immunotherapy	Colorectal carcinoma	Monoclonal antibodies	Anti-GA733-2E	CO17-1A Mab (IgG2a)	Unapproved	
Immunotherapy	Haematolymphoid cells	Recombinant protein	Anti-Bcl-2-Mab	B-cell lymphoma leukaemia-2 (Bcl-2) protein	Unapproved	
Immunotherapy	Rotavirus	Single-domain antibodies (sdAbs)	3B2 and 2KD1 antibodies	Anti-VP6	Unapproved	
Immunotherapy	Breast cancer	Monoclonal antibodies	mAb-BR55/mAb-BR55K	HC and LC	Unapproved	
Immunotherapy	Antigen-presenting cells (APCs)	Adjuvant antibody	APCH1 antibody	Anti-MHC class II DR	Unapproved	
Immunotherapy	Immune cells	Cytokine	IL-2	Human interleukin 2	Unapproved	
Immunotherapy	Stem cells, macrophages	Cytokine	hGM-CSF	Human granulocyte-macrophages colony-stimulating factor	Unapproved	
Enzyme therapy	Purine salvage pathway	Enzyme	ADA	Human adenosine deaminase	Unapproved	

Hormone therapy	Hypoparathyroidism	Hormone	hPTH	Human parathyroid hormone	Unapproved	
Growth factor therapy	Wound healing	Growth factor	huEGF1	Human epidermal growth factor	Unapproved	
Growth factor therapy		Growth factor	huFGF2	Human fibroblast growth factor 2	Unapproved	
Growth factor therapy		Growth factor	huKGF1	Human keratinocyte growth factor 1	Unapproved	
Growth factor therapy	Alzheimer's disease	Growth factor	rhNGF	Human prepro (beta) nerve growth factor	Unapproved	
Enzyme-gene therapy	Familial lipoprotein lipase deficiency	Transgene	Glybera or LPL^{S447} × transgene	Lipoprotein lipase transgene	Approved	UniQure
Protein gene therapy	Haemophilia A	Transgene	AAV-FVIII	Factor VIII	Unapproved	
Protein gene therapy	Haemophilia B	Transgene	AAV8-hFIX19	Factor IX	Phase I	Spark Therapeutics
			AskBio009 (AAV8)		Phase I/II	Baxalta US Inc.
			scAAV 2/8-LP1-hFIXco		Phase I	St. Jude Children's Research Hospital
			AAV2-hFIX16		Phase I	Spark Therapeutics
Enzyme-gene therapy	Leber congenital amaurosis	Transgene	AAV2-hRPE65v2	Retinoid isomerohydrolase	Phase III	Spark Therapeutics
			rAAV2-CB-hRPE65		Phase I/II	Applied Genetic Technologies Corp

(continued)

Table 9.3 (continued)

Therapy type	Targeted for	Product type	Product name	Expressed product	Development stage	Company name, if any
			tgAAG76 (rAAV 2/2.hRPE65p.hRPE65)		Phase I/II	University College, London
			rAAV2/4.hRPE65		Phase I/II	Nantes University Hospital
			rAAV2-CBSB-hRPE65		Phase I	University of Pennsylvania
			rAAV2-hRPE65		Phase I	Hadassah Medical Organization
Enzyme-gene therapy	Leber's hereditary optic neuropathy	Transgene	AAV2-ND4	NADH-ubiquinone oxidoreductase chain 4	Phase I	John Guy, University of Miami
Enzyme-gene therapy	Age-related macular degeneration	Transgene	AAV2-soluble Flt1	Soluble fms-like tyrosine kinase	Phase I	Genzyme, a Sanofi Company
Enzyme-gene therapy	Canavan disease	Transgene	AAV-ASAP	Aspartoacylase	Unapproved	
Growth factor-gene therapy	Alzheimer's disease	Transgene	AAV-NGF or CERE-110	Beta-nerve growth factor	Phase I	Sangamo Therapeutics (Ceregene)
Enzyme-gene therapy	Parkinson's disease	Transgene	AAV2-GAD	Glutamic acid decarboxylase	Phase II	Neurologix, Inc.
Protein gene therapy			AAV2-NTN or CERE-120	Neurturin	Phase II	Ceregene
Enzyme-gene therapy			AAV-hAADC-2	Aromatic L-amino acid decarboxylase	Phase I	Genzyme, a Sanofi Company
			AAV2-hAADC		Phase I	Voyager Therapeutics

Protein gene therapy			AAV2-GDNF	Glial cell line-derived neurotrophic factor	Phase I	National Institutes of Health Clinical Center (CC)
Protein gene therapy	Duchenne muscular dystrophy	Transgene	rAAV2.5-CMV-minidystrophin	Mini-dystrophin	Phase I	Asklepios BioPharmaceutical, Inc.
Protein gene therapy	Becker muscular dystrophy	Transgene	rAAV1.CMV.huFollistatin344	Follistatin	Phase I	Nationwide Children's Hospital
Protein gene therapy	Limb girdle muscular dystrophy	Transgene	AAV1-gamma-sarcoglycan	Gamma-sarcoglycan	Phase I	Genethon
Protein gene therapy	Spinal muscular atrophy	Transgene	scAAV9.CB.SMN	Survival motor neuron	Phase I	AveXis, Inc.
Enzyme-gene therapy	Acute intermittent porphyria	Transgene	rAAV2/5-PBGD	Porphobilinogen deaminase	Phase I	Digna Biotech S.L.
Enzyme-gene therapy	Alpha 1-antitrypsin deficiency	Transgene	rAAV1-CBhAAT	Alpha 1-antitrypsin	Phase II	Applied Genetic Technologies Corp
Enzyme-gene therapy			rAAV2-CBhAAT		Phase I	University of Massachusetts, Worcester
Enzyme-gene therapy			AAVrh.10halpha1AT		Phase I	Adverum Biotechnologies, Inc.
Enzyme-gene therapy	Aromatic amino acid decarboxylase deficiency	Transgene	AAV2-hAADC	Aromatic L-amino acid decarboxylase	Phase I/II	National Taiwan University Hospital
Protein gene therapy	Choroideremia	Transgene	rAAV2.REP1	Rab-escort protein 1	Phase I	University of Oxford

(continued)

Table 9.3 (continued)

Therapy type	Targeted for	Product type	Product name	Expressed product	Development stage	Company name, if any
Enzyme-gene therapy	Chronic heart failure	Transgene	AAV1-CMV-SERCA2a	Sarcoplasmic reticulum calcium ATPase	Phase II	Imperial College London, Assistance Publique—Hôpitaux de Paris and Celladon Corporation
Protein gene therapy	Gastric cancer	Transgene	AAV-DC-CTL	Carcinoembryonic antigen	Phase I	Tianjin Medical University Cancer Institute and Hospital
Enzyme-gene therapy	HIV	Transgene	AAV-2 HIV vaccine (tgAAC09)	Gag, protease and reverse transcriptase parts	Phase I	International AIDS Vaccine Initiative
Antibody-gene therapy			rAAV1-PG9DP	PG9 antibody	Phase I	International AIDS Vaccine Initiative
Receptor-gene therapy	Inflammatory arthritis	Transgene	tgAAC94	TNFR:Fc fusion gene	Phase 1/II	Targeted Genetics Corporation
Protein gene therapy	Late infantile neuronal ceroid lipofuscinosis	Transgene	AAVrh.10CUCLN2	Neuronal ceroid-lipofuscinosis 2	Phase 1/II	Weill Medical College of Cornell University
Protein gene therapy			AAV2CUhCLN2	Neuronal ceroid-lipofuscinosis 2	Phase I	Weill Medical College of Cornell University
Trinucleotide-gene therapy	Pompe disease	Transgene	rAAV1-CMV-GAA	Normal GAA	Phase 1/II	University of Florida

9.1.3.1 Biological Drug Therapy

BEVs have been utilized as eukaryotic expression vectors in insect cells for the production of therapeutic or immunotherapeutic proteins such as monoclonal antibodies, cytokines and chemokines, growth factors, etc. that require post-translational modifications, more importantly glycosylation. The baculovirus expression system has been accepted as one of the most efficient and powerful technologies for the production of biological recombinants in terms of achievable quantity, purity and ease of the eukaryotic processing (Luckow and Summers 1988). Therapeutic recombinant protein production is considered as an essential section of the emerging biotechnology industries. This system has the potential for the development of high commercial value industry.

(a) *Immunotherapy*: Over the years, numerous tumour immunotherapies achieved early-stage successes but failed in clinical trials Phase-III (Goldman and DeFrancesco 2009). Baculovirus-derived Dendreon's Provenge (Seattle; sipuleucel-T) for prostate cancer is among the first therapeutic cancer vaccines to complete Phase-III trail successfully and to receive FDA approval. Provenge (Sipuleucel-T) is an autologous active cellular immunotherapy that has shown evidence of reducing the risk of death among men with metastatic castration-resistant prostate cancer (Kantoff et al. 2010). It consists of autologous peripheral-blood mononuclear cells (PBMCs), including antigen-presenting cells (APCs), which have been activated ex vivo with a recombinant fusion protein (PA2024). The fusion protein PA2024 contains prostate antigen, prostatic acid phosphatase which is fused to an immune-cell activator called granulocyte-macrophage colony-stimulating factor. PA2024 is produced by BEV in *Sf21* insect cells.

Monoclonal antibody CO17-1A was prepared against colorectal cancer cells by using pFastBac vectors (Park et al. 2011). The BEVs expressed proteins that are being utilized for the production of monoclonal antibodies against Bcl-2 (B-cell lymphoma leukaemia-2). It is an integral membrane oncoprotein that regulates programmed cell death (apoptosis) in haematolymphoid cells (Reed et al. 1992). Single-domain antibodies (sdAbs) that are prepared against rotavirus infection are also known as nanobodies or VHHs. They have characteristically high stability, solubility and very high affinity for their antigens. These antibodies were first produced in the insect larvae *Trichoplusia ni* which serve as living bio-factories for the production of these biomolecules (Gómez-Sebastián et al. 2012). Anti-breast cancer monoclonal antibodies (mAb) BR55, with or without fusion with KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum retention signal, were prepared. The heavy chain (HC) and light chain (LC) genes of mAb BR55 were cloned in pFastBac Dual vector under the control of polyhedrin (P_{PH}) and p_{10} promoters, respectively, in Sf9 insect cells (Lee et al. 2014). Antibody response was enhanced against two recombinant subunit vaccines by tagging the vaccines with adjuvant recombinant single-chain antibody APCH1. It recognizes the MHC Class II DR and produced in *Trichoplusia ni* insect cells (Gil et al. 2011).

Human interleukin 2 (IL-2) was prepared in insect larvae of *T. ni* by placing the IL-2 gene under p10 promoter of BEV (Pham et al. 1999). Human granulocyte-macrophages colony-stimulating factor (hGM-CSF) was prepared in *Bombyx mori* (silkworm) nuclear polyhedrosis virus (BmNPV) (Shi et al. 1996). Other cytokines and chemokines are being produced by using this expression system in a similar manner.

Recently, the intravesical instillation of transgene devoid baculovirus is found to elicit local immune stimulation by upregulating a set of Th-1-type cytokines in orthotopic bladder tumours in mice (Ang et al. 2016). However, the application of such strategy for non-muscle invasive bladder cancer (NMIBC) in humans is awaited.

(b) *Enzyme and hormonal therapy*: Enzyme human adenosine deaminase, a key purine salvage enzyme required for immune competence, has been produced both in *Trichoplusia ni* and *Spodoptera frugiperda* insect cells as well as larvae. This enzyme possessed specific activity of 70 units/mg in crude homogenate that is 70–350 times higher than its two most abundant natural sources thymus and leukemic cells. Such biologically active, inexpensive, rapid and huge production of the enzymes by this baculovirus system opens up the avenues for other biologically active molecules. Human parathyroid hormone (hPTH) was produced both in *Bombyx mori* cells and larvae. Both of the host systems have been reported to be suitable for efficient synthesis and secretion of the correctly processed hPTH (Mathavan et al. 1995). Similarly, recombinant full length human growth hormone (hGH) was produced in *Bombyx mori* nuclear polyhedrosis virus (vBmhGH) (Sumathy et al. 1996).

(c) *Growth factors therapy*: Growth factors are naturally signalling molecules required for myriads of biological processes for which the requirement of consistent, cost-effective and clinically efficient technologies is indispensable. Wound healing is one of such complex biological processes that requires the collaborative efforts of different tissues, cells and molecules. The repair process of wounds after injury is initiated by the release of various growth factors (GFs). GFs act as functional messenger molecules between cells which control the cellular processes in the regulatory network and sometimes require recombinant protein therapies. Currently, wound healing is being focussed on GFs and/or human skin substitutes, required for decreasing healing time by modifying inflammation and accelerating the proliferative phase. The beneficial effects of GFs to attract different kinds of cells at the site of wound healing have been demonstrated by many studies. Wider clinical and commercial applications of such GFs depend on their scalable cost-efficient production. BEVs have been successful in unblocking the bottlenecks for such inevitabilities. Three fully functional human GFs, the human epidermal growth factor 1 (huEGF1), the human fibroblast growth factor 2 (huFGF2) and the human keratinocyte growth factor 1 (huKGF1), have been produced with BEVs in *Trichoplusia ni* insect larvae (Dudognon et al. 2014). The expression of huKGF1 was found to be enhanced further when it was expressed by tagging it with human antibody IgG fragment crystallisable region (Fc).

Human prepro (beta) nerve growth factor that has been suggested as a therapeutic agent for the treatment of Alzheimer's disease was produced in insect cells as recombinant virus, mature human beta nerve growth factor (rhNGF). It was found to be biologically active in cholinergic cell survival (Barnett et al. 1990). Similarly, different strategies are being worked out with BEVs in insect cells or larvae for biologically active, cost-effective, therapeutic and commercial scale production of numerous highly variable molecules.

9.1.3.2 Gene Therapy

Today, gene therapy potential has reached to the point whereby it can be exploited to treat many diseases that were earlier thought to be untreatable. The requisite modalities for such gene drugs such as safety, generation, immune response, duration of expression and the gene delivery capacity are being successfully realized by baculovirus-based vectors. Baculoviruses have been found to deliver genes into a wide range of vertebrate cells and species. However, the exact mechanism of entry of baculovirus in to the host cells is still not fully understood. Recently, phagocytic-like mechanism of entry into mammalian cells was found to be more convincing than pinocytosis (Long et al. 2006). Baculovirus progeny production occurs in two forms, budded virus (BuV) and occlusion-derived virus that only differ in their envelops. BuV derives its envelop from cell membrane and spreads the infection within host, whereas occlusion-derived virus envelop is derived from nuclear membrane and spreads infection between hosts. BuV is the most widely used form in biotechnology that enters the insect and other hosts through endocytosis mechanism, although the tenet of exact endocytic mechanism still needs to be build.

AcMNPV is the prototype of baculoviruses and widely used for different applications including gene therapy. It is able to transduce both dividing and non-dividing mammalian cells and activates the transgene in the target cells that it carries under the control of specified promoter. It indicates that the nucleocapsid of the baculovirus transports its genome across the intact host cell nuclear membrane through nuclear pore complex. However, the detailed molecular mechanism of baculovirus transduction in mammalian cells demands further investigation for efficient gene delivery. BEVs gene delivery capability have been exploited in understanding the mechanism of vertebrate cell transduction, preclinical studies, vaccination, cartilage and bone tissue engineering, cancer gene therapy, assay development, drug screening and generation of other gene therapy vectors (Airenne et al. 2013). We would like to emphasize on the use of recombinant adeno-associated vectors (rAAV) as gene therapy tools which are highly important from bioprocess and therapeutic perspective.

BEVs-Derived Recombinant Adeno-Associated Viruses (rAAVs) for Gene Therapy Recombinant AAVs that carry therapeutic DNA turn out to be the attractive gene delivery vectors because of their suitability for in vivo gene therapy potential, relatively long-term gene expression, lack of pathogenicity and ability to transduce wide variety of both dividing and non-dividing cells. Nine different serotypes of rAAVs are used for gene therapy whereby each serotype exhibits different

propensity for tissue-specific infection and infection kinetics (Zincarelli et al. 2008). The major limitation of low production quantity was addressed recently by optimizing the BEVs platforms and adjusting different parameters such as multiplicity of infection, cell density and fermentation mode that produced up to 10^4 vector genomes per litre (Mena et al. 2010).

The strategy for rAAV production requires the production of three AAV capsid proteins, VP1, VP2 and VP3. These capsid proteins assemble within BEV-transduced insect cells to produce icosahedral VLPs (Aucoin et al. 2007). More efficient rAAVs require co-infection of insect cells with three different kinds of baculoviral vectors. The first one is Bac-Rep, expressing the major AAV replication enzymes Rep 78 and Rep 52 essential for AAV genomic replication and packaging, respectively. Second is Bac-Cap, expressing the AAV virion capsid proteins (VP1, VP2 and VP3), and third is Bac-GOI, expressing the gene of interest flanked by AAV inverted terminal repeat elements required for the rescue, replication and packaging of the heterologous gene. Co-infection with these three vectors in insect cells produces efficiently replicated and encapsulated single-stranded AAV vector genome (Weyer and Possee 1991). Further enhancement of AAV in terms of stability, robustness, scalability and high-titre production involves both Rep and Cap protein expression from a single baculovirus (Bac-Rep Cap), i.e. expression of both Rep 78 and Rep 52 transcription from a single mRNA and genetic modifications of the original Bac-Rep and Bac-Cap constructs (Virag et al. 2009). The development of such robust gene delivery vehicles was based on the fact that AAV genome is efficiently replicated in *Sf9* and *Sf21* insect cell lines in a Rep-dependent fashion. Some of the diseases that are being targeted by gene therapy using rAAV are discussed below:

(a) *Gene therapy against lipoprotein lipase deficiency (LPLD)*: It is a rare autosomal recessive genetic and metabolic disorder in which inactivation of familial lipoprotein lipase enzyme occurs due to mutation in gene LPL. Functional lipase is required for plasma triglyceride hydrolysis under normal condition. Inactivated enzyme results into hypertriglyceridemia characterized by frequent abdominal pain and fatty deposits in the skin and retina that in severe cases can lead to fatal pancreatitis, diabetes and onset of cardiovascular diseases. Earlier therapies targeted to lower the plasma triglycerides have not been proved much effective. Alipogene tiparvovec (also known as AAV1-LPL^{S447X} in the early phases of clinical trial) is the first adeno-associated virus (AAV)-mediated gene therapy manufactured by UniQure that got market authorization and government approval in Europe. It is an AAV1 (serotype 1) vector expressing naturally occurring variants of LPL transgene, LPL^{S447X} linked with improved lipid profile and is commercialized by the name of Glybera (Gaudet et al. 2010). It is injected through intramuscular route in the patients that results in natural gain of function of LPL gene variants to muscle tissues. Glybera use significantly lowers plasma triglycerides by increasing the lipoprotein lipase enzyme activity.

The major concern for using such vector-based gene therapy is to prevent both humoral and cell-mediated immune response elicited against viral capsid

proteins that may impact the efficacy and safety of these drugs. Intramuscular injections of Glybera has been proved clinically safe and efficient drug that does not elicit any additional systemic and local immune response harmful for humans. This approach was found to be relevant and promising for the treatment of thousands of single gene disorders. Similar strategies are being investigated in diverse range of therapeutic areas, and many products for the treatment of human diseases are in different stages of clinical development. These AAV gene therapy drugs at different clinical development phases are being discussed here.

(b) *Haemophilia*: It is a blood clotting disorder caused by the mutation in the clotting factor IX gene. Presently, four clinical trials are going on that involve rAAV serotype 2 or 8, designed to express factor IX.

Haemophilia A, the most common severe inherited bleeding disorder caused by mutation in factor VIII gene, is significantly more problematic for this treatment because of a larger size of cDNA that prevents in achieving the adequate level of transgene expression and elicits the anti-factor VIII immune response (High et al. 2014).

(c) *Retinal degeneration*: Recombinant AAV has been used to treat a number of animal models but is limited by carrying capacity, slow onset of expression and limited ability to transduce some of the retinal cell types from the vitreous. Next-generation AAVs have been produced to address these issues by creation of self-complementary AAV vectors for faster onset of expression and specific mutations of self-exposed residues to increase transduction. Such vectors were further improved for broader applicability and advantageous characteristics by directed evolution through an iterative process of selection (Day et al. 2014). Age-related macular degeneration (AMD) that leads to the central vision loss in elderly individuals due to choroidal neovascularization is marked by proliferation of blood vessels and retinal pigment epithelial (RPE) cells. It leads to photoreceptor death and fibrous disciform scar formation. Treatment of AMD patients requires neutralization of vascular endothelial growth factor (VEGF) for which expression of modified soluble Flt1 receptor was designed and expressed in AAV2-sFLT01 vector. Presently, this study is in Phase 1 trial (MacLachlan et al. 2011). Leber congenital amaurosis (LCA) is an autosomal recessive blinding disease that occurs due to mutations in RPE65 gene. Subretinal administration of AAV2-hRPE65v2 has been reported both safe and efficient for at least 1.5 years after injection. Currently six clinical trials, either in stage 1 or 2, are going on to treat this retinal disease (Simonelli et al. 2010).

(d) *Neurological diseases*: rAAV has been used as an effective gene delivery system for the treatment of central or peripheral nervous system with almost no adverse effects in many clinical trials. First time, its clinical use in the human brain has been used to treat Canavan disease, a childhood leukodystrophy also known as Van Bogaert-Bertrand disease caused by the deficiency of enzyme aspartoacylase (ASAP). It involves neurosurgical administration of approximately 10 billion infectious particles of recombinant adeno-associated virus (AAV) containing the aspartoacylase gene (ASPA) directly to the affected

regions of the brain (Janson et al. 2002). To treat Alzheimer's disease, transfer of gene encoding nerve growth factor (NGF), which is essential for healthier nerve cells, is transduced by an adeno-associated nerve growth factor (CERE-110) (Bakay et al. 2007). Transduction of glutamic acid decarboxylase (GAD) and trophic factor neurturin was assessed successfully in different Phase 1 and 2 clinical trials for the treatment of Parkinson's disease (Marks et al. 2010; Kaplitt et al. 2007).

(e) *Duchenne muscular dystrophy (DMD)*: DMD is a severe recessive X-linked muscle disorder caused by mutations in gene encoding dystrophin. Gene therapy to treat DMD is a challenge due to the large size of DMD gene. However, alternative gene delivery strategies like exon skipping, trans-splicing, micro- and mini-dystrophin in Phase II/III clinical trials have been found to be promising (Jarmin et al. 2014).

A number of Phase I/II/III clinical trials are underway for the treatment of numerous diseases such as acute intermittent porphyria, alpha 1-antitrypsin deficiency, aromatic amino acid decarboxylase deficiency, Becker muscular dystrophy, choroideremia, chronic heart failure, gastric cancer, HIV, inflammatory arthritis, late infantile neuronal ceroid lipofuscinosis, Leber's hereditary optic neuropathy, limb girdle muscular dystrophy, macular degeneration, Pompe disease, spinal muscular atrophy, etc. (Felberbaum 2015).

The future prospectives of baculovirus gene delivery applications in stem cell transduction, cancer gene therapy and cartilage and bone tissue engineering are also quite optimistic. Great interest in regenerative medicine begins with the advancement in identification, isolation and derivation of human stem cells, specifically the generation of human-induced pluripotent stem cells. Prolonged expression of transgenes has been demonstrated in multiple multipotent stem cells such as mesenchymal, neural, umbilical cord, bone marrow, adipose tissue, human embryonic stem cells (hESCs) and pluripotent stem cells. These baculoviruses have also been customized for stable gene expression in stem cells by genomic integration for downstream therapeutic applications, for example, deriving unlimited numbers of genetically corrected functional adult cells for cell replacement therapy (Kotin et al. 1991).

De-differentiated chondrocytes transduced with baculovirus vector (Bac-CB) expressing bone morphogenetic protein-2 (BMP-2) result into sustained expression of BMP-2 with passaged chondrocytes in vitro. It was further improved by co-expression of transforming growth factor beta with baculovirus vectors (Chen et al. 2008). These chondrocytes were further used to grow cartilage-like tissues in rotating shaft bioreactors that demonstrated the potential of baculovirus in cartilage tissue engineering, but their clinical utility in humans is yet to be proved.

Bac-CB-based BMP-2 transduction into human bone marrow-derived mesenchymal stem cells (BMSCs) is also demonstrated to directing ontogenies of naïve BMSCs. Implantation of these transduced cells induced ectopic bone formation in nude mice and promoted calvarial bone repair in immunocompetent rats (Chuang et al. 2009). For massive repairing of bone, sustained expression of genes promoting

osteogenesis (BMP-2) and angiogenesis (VEGF) in adipose-derived stem cells (ASCs) was performed by dual baculovirus vector system. Transplantation of these ASCs in NZW rabbit resulted in accelerated healing, improved bone quality and angiogenesis. Same technique was also tested in rabbits, and the results altogether support the viability of baculoviruses for stem cell engineering and bone formation (Luo et al. 2011).

The propensity of baculoviruses for effective high-level transgene expression has been exploited for cancer gene therapy. Baculovirus vectors have been tailored with suicide, tumour suppressor, pro-apoptotic, immune-potentiating and anti-angiogenesis genes and studied in animal tumour models under in vivo conditions in many anticancer strategies (Luo et al. 2012; Wang and Balasundaram 2010). Recently, stem cells transduced with suicide genes have proved beneficial for curbing primary, solid and metastatic tumours (Zhao et al. 2012).

Today, baculovirus technology has matured to the level that it can be used for plethora of applications. The studies conducted on model organisms in the context of therapeutic applications are encouraging and support further development of baculoviruses from preclinical applications to clinical trials and for human diseases treatment. A deeper and holistic understanding of antigenic and target cell transduction molecular mechanisms will be helpful in enhancing the clinical utility of this unique and powerful gene delivery system.

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