



Solutions against emerging infectious and noninfectious human diseases through the application of baculovirus technologies

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Abstract

Baculoviruses are insect pathogens widely used as biotechnological tools in different fields of life sciences and technologies. The particular biology of these entities (biosafety viruses 1; large circular double-stranded DNA genomes, infective per se; generally of narrow host range on insect larvae; many of the latter being pests in agriculture) and the availability of molecular-biology procedures (*e.g.*, genetic engineering to edit their genomes) and cellular resources (availability of cell lines that grow under *in vitro* culture conditions) have enabled the application of baculoviruses as active ingredients in pest control, as systems for the expression of recombinant proteins (Baculovirus Expression Vector Systems—BEVS) and as viral vectors for gene delivery in mammals or to display antigenic proteins (Baculoviruses applied on mammals—BacMam). Accordingly, BEVS and BacMam technologies have been introduced in academia because of their availability as commercial systems and ease of use and have also reached the human pharmaceutical industry, as incomparable tools in the development of biological products such as diagnostic kits, vaccines, protein therapies, and—though still in the conceptual stage involving animal models—gene therapies. Among all the baculovirus species, the *Autographa californica multiple nucleopolyhedrovirus* has been the most highly exploited in the above utilities for the human-biotechnology field. This review highlights the main achievements (in their different stages of development) of the use of BEVS and BacMam technologies for the generation of products for infectious and noninfectious human diseases.

Key points

- *Baculoviruses can assist as biotechnological tools in human health problems.*
- *Vaccines and diagnosis reagents produced in the baculovirus platform are described.*
- *The use of recombinant baculovirus for gene therapy-based treatment is reviewed.*

Keywords Baculovirus · AcMNPV · BEVS · BacMam · Human diseases

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Introduction

Technologies in the life sciences have grown exponentially since the birth of genetic engineering in the 1970s, a central discipline within biotechnology. The possibility of intervening and modifying the double-stranded DNA molecules of organisms and their associated mobilomes (viruses, plasmids, transposons) has made possible an expansion of the general knowledge about living matter but has also facilitated the emergence of numerous goods and services that have improved the quality of human life. For example, baculoviruses, despite being insect parasites, have become highly useful tools for the development of beneficial products in human health. This review will describe the main technologies related

to baculoviruses and human health, the progress in this field, and the opportunities and prospects arising from that work. The recurring challenges of emerging human pathogens that rapidly spread locally or internationally, such as influenza viruses, arboviruses, and coronaviruses, plus the old problems that have plagued humanity for centuries (*e.g.*, cancer and coronary and genetic diseases), constitute a fertile field where baculoviruses and their associated technologies can provide benefits.

Survey methodology

Extensive literature research was conducted using the following electronic databases: PubMed; Science Direct; Google Scholar; and Scopus. The keyword combinations such as baculovirus and molecular biology, baculovirus and viral cycle, baculovirus and expression vector, baculovirus and diagnosis, baculovirus and vaccine, baculovirus and virus like particles, baculovirus and display, emerging and viral diseases, baculovirus and influenza virus, baculovirus and arbovirus, baculovirus and *Alphavirus*, baculovirus and *Flavivirus*, baculovirus and dengue virus, baculovirus and zika virus, baculovirus and yellow fever virus, baculovirus and West Nile virus, baculovirus and japanese encephalitis virus, baculovirus and chikungunya virus, baculovirus and coronavirus, baculovirus and SARS-CoV, baculovirus and MERS-CoV, baculovirus and SARS-CoV-2, baculovirus and gene therapy, baculovirus and adeno-associated viruses, baculovirus and BacMam, BacMam and gene therapy, BacMam and cancer, BacMam and vascular diseases, BacMam and tissue engineering, or BacMam and regenerative medicine were utilized to build literature review. All articles were exhaustively studied to be employed as references in the present work.

Original papers, mainly from the last decade, were selected where the BEVS platform was used for the diagnosis, treatment, and prevention of diseases that affected humanity in recent years. A similar criterion was applied for the conceptual use of baculoviruses as gene therapy vectors, selecting those studies that carried out tests on animals. Also, some older papers are considered when their inclusion is important for the topic development. All the articles were selected based on their scientific importance and publication year.

Baculoviruses

The planet's virome is a space rich in a diversity of parasitic entities of organisms, which abundance and variety offer promising opportunities for biotechnological applications (Paez-Espino et al. 2016). Many viruses that do

not infect humans or other mammals thus become tempting instruments for the development of associated technologies—for example, the production of recombinant proteins or the generation of viral vectors adapted to the delivery of specific sequences to target cells and tissues, among other possible applications (Mateu 2011). In this regard, baculoviruses stand out as a prominent group of viral entities with a major role in biotechnology.

Baculoviruses in nature

Baculoviruses are double-stranded DNA viruses that infect insects (larval stage) of the orders Lepidoptera, Hymenoptera, and Diptera (Fig. 1). The viral genomes are made up of a large circular molecule (about 80–180 kbp) that is packed in two different structures (Fig. 1C): budded viruses (BVs) or occlusion-derived viruses (ODVs). In both types of virions the viral DNA is associated with proteins conforming to a bacilliform structure known as a nucleocapsid (Nc) which in turn is enveloped by a lipid membrane that is different between BVs and ODVs (Rohrmann 2019). Essential proteins for supporting entry into susceptible cells are located in these membranes. Thus, the F and GP64 proteins are present in the BV envelopes (Westenberg et al. 2007), whereas the “*per-os*–infectivity factors” (the PIF complex), responsible for penetrating the midgut-epithelium cells of insects, occur in the ODV membranes (Wang et al. 2019b). We need to note that ODVs are, in turn, immersed in a protein crystal—mainly up made of polyhedrin (polh) or granulin depending on the species—denominated occlusion body (OB) that give them great stability in the environment (Fig. 1A). Hence, the name of *nucleopolyhedrosis* or *granulosis* that the diseases from these viruses also receive from the microscopically observable shape of their OBs in infected cells. Moreover, certain species of nucleopolyhedrosis carry several Ncs in their ODVs, while others carry only one unit, causing the former species to be referred to as “multiple nucleopolyhedroviruses” and the latter as “single nucleopolyhedroviruses.” Granuloviruses usually carry a single Nc per ODV (Fig. 2B) (Rohrmann 2019).

The viral cycle begins when susceptible larvae consume OBs. In the midgut, the crystals dissolve through the action of alkaline pH and proteases, and the ODVs are released. These viral forms, once in intestinal cells, will initiate the primary infection, which will then spread to different tissues of the insect through the production of BVs (secondary infection) generally ending in the death of the larva (Saxena et al. 2018).

The family *Baculoviridae* are composed of numerous species classified into 4 genera (Jehle et al. 2006): *Alphabaculovirus* (lepidopteran-specific nucleopolyhedroviruses); *Betabaculovirus* (lepidopteran-specific granuloviruses); *Deltabaculovirus* (dipteran-specific

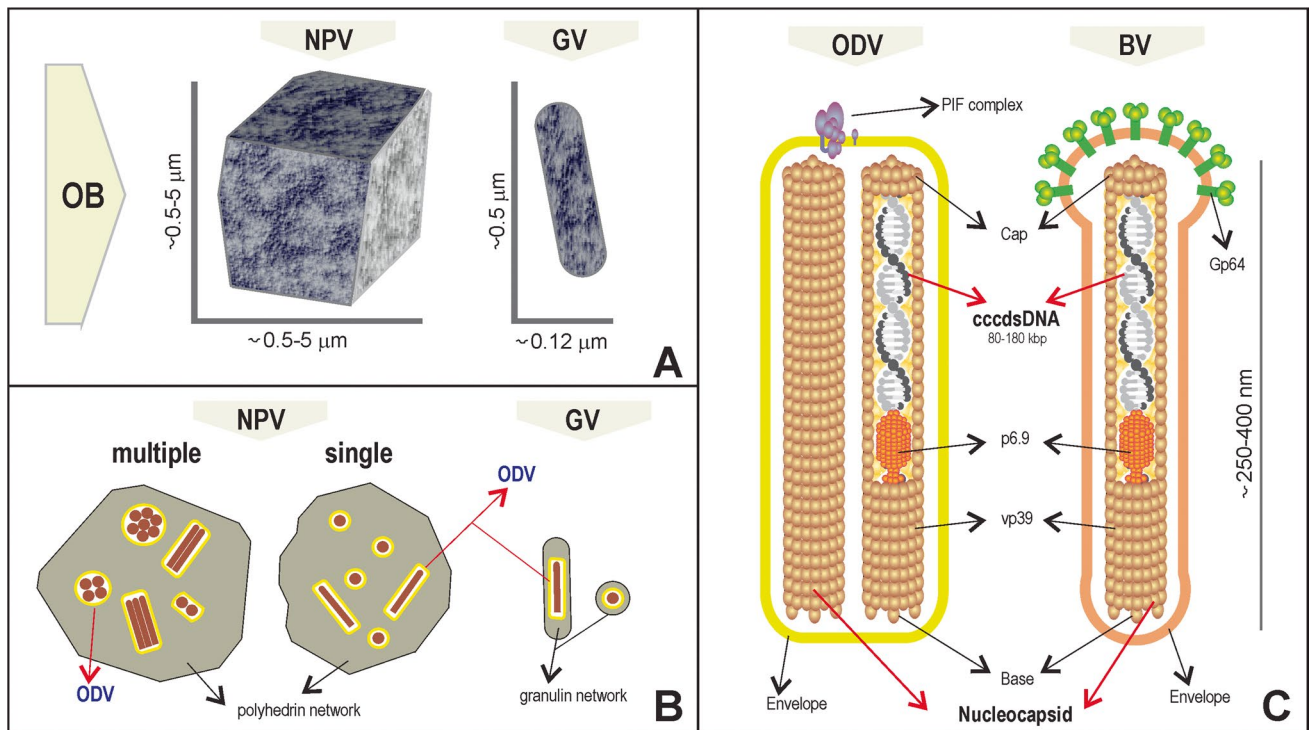


Fig. 1 Baculovirus virions. Illustrations describing the main characteristics of baculovirus morphologies. **A** Representation of typical shapes and dimensions of the occlusion bodies (OBs) of nucleopolyhedroviruses (NPV) and granuloviruses (GVs). **B** Representation of cross-sections on the OBs, revealing the occlusion-derived viruses (ODVs) present in multiple nucleopolyhedroviruses (MNPVs), single nucleopolyhedroviruses (SNPVs), and GVs. The latter illustrate two different cuts for each particle. **C** Comparative illustration between ODVs and budded viruses (BVs). The nucleocapsids are the same, but the envelopes are different (that of ODV is derived from the

nuclear membrane of the infected cell, whereas that of BV is derived from the plasma membrane). Ncs are polar structures manifesting a different composition and differing forms in the extreme ends and are composed of mainly the VP39 protein. The covalently closed circular double-stranded DNA (cccdsDNA) is associated with a basic protein called p6.9. The *per-os-infectivity* (PIF) complex is composed of various proteins and is present in only the ODV envelope. The fusogenic GP64 protein is present only in the BV envelope of Group I-*alphabaculoviruses* (e.g., in *AcMNPV*), while in the remaining clades this role is performed by the F protein

nucleopolyhedroviruses); and *Gammabaculovirus* (hymenopteran-specific nucleopolyhedroviruses). The genomes contain about 90–180 protein-encoding genes, of which 38 (known as core genes) are shared by all members (Miele et al. 2011; Garavaglia et al. 2012; Javed et al. 2017). Genes are expressed differentially throughout a viral cycle, either mediated by the host's transcription machinery (early stages) or by the virus-encoded RNA polymerase (late stages). The variable gene content generates a great intra- and interspecies—indeed, pangenomic—diversity (Garavaglia et al. 2012), with many auxiliary genes having been described that, for example, favor viral dissemination (Ishimwe et al. 2015) or produce notable changes in the physiologic state of the larvae (Gasque et al. 2019). The baculoviral prototype species is *Autographa californica multiple nucleopolyhedrovirus* (*AcMNPV*; of the genus *Alphabaculovirus*),

from whose properties most nonagricultural biotechnological applications are derived.

Biosecurity aspects for humans and the environment

Baculoviruses are abundant in nature and play fundamental ecological roles in the population dimension of the insects they infect (Cory and Hails 1997). Because most of these pathogens have a very narrow host range, they depend on particular species of invertebrates for their natural sustenance (Rohrmann 2019). Baculoviruses do not infect vertebrates and although BVs have been found to be able to transduce cells from animals that are not their natural hosts—including mammals (Airenne et al. 2011; Ono et al. 2018; Parsza et al. 2020)—viral DNA is unable to replicate and support progeny generation (Tija et al. 1983; Kost and Condreay 2002; Parsza et al. 2020). In fact, cell lines from mammals transduced with baculoviruses revealed

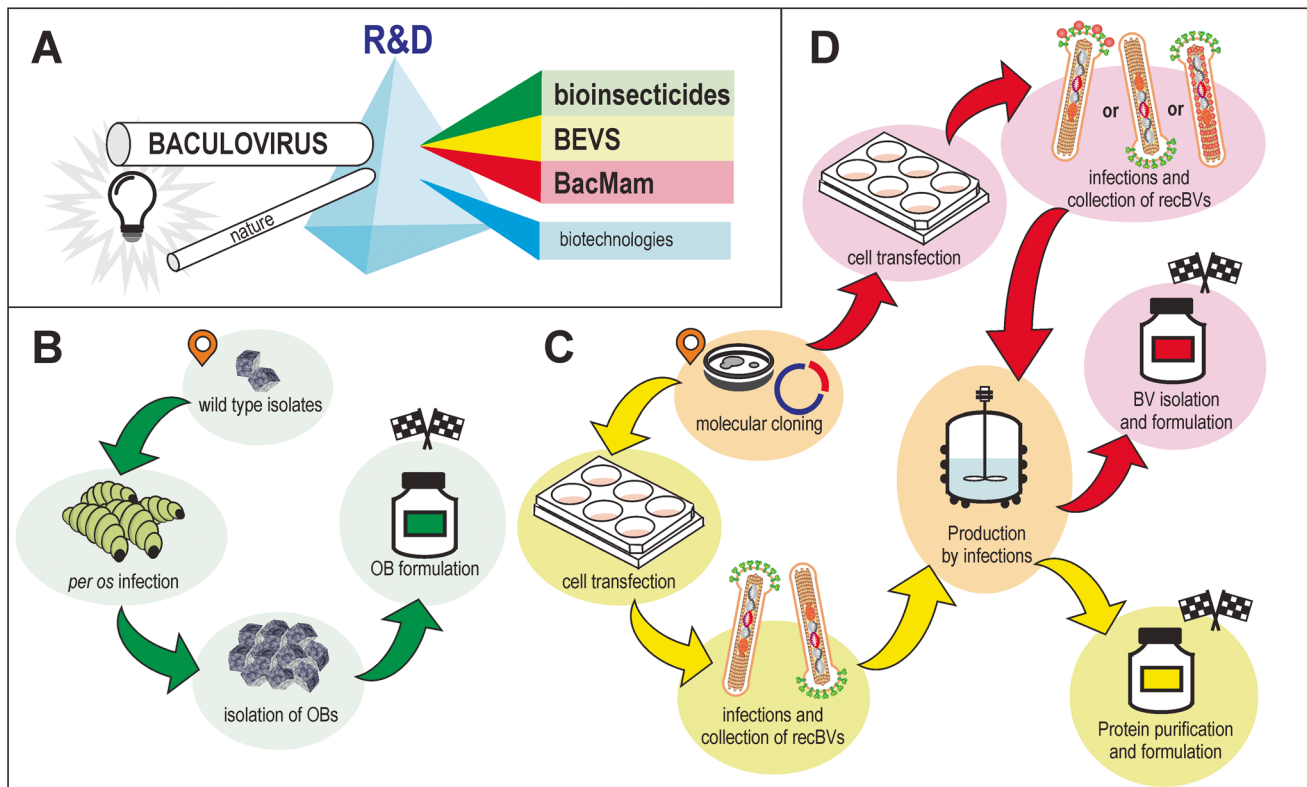


Fig. 2 Applications of baculoviruses. Illustrations indicating the main uses of baculoviruses. **A** Because of the research and development carried out on the biology of baculoviruses, 3 main applications have been generated: bioinsecticides, baculovirus expression vector systems (BEVS), and baculoviruses applied to mammals (BacMam). **B** Main procedures for the generation of bioinsecticide products based on occlusion bodies (OBs). **C** Main procedures for the generation

of protein products based on recombinant budded viruses (recBVs) infecting insect cells and subsequent downstream processes. **D**. Main procedures for the generation of virion products based on recBVs infecting insect cells and subsequent downstream processes. Only 3 alternatives of recBVs are illustrated: virions that display proteins in the envelope; virions that carry transgenes for the expression in mammal cells; virions that display proteins in the nucleocapsid

that some viral early genes (*e.g.*, PE38 and IE-01) evidenced a limited expression while the late viral genes (dependent on viral RNA polymerase) remained silent (Shin et al. 2020).

As to immunologic aspects, baculoviruses generate a low response in mammals, but do not evoke systemic antiviral reactions (Gronowski et al. 1999; Abe et al. 2003; Bocca et al. 2013), rather an induction of innate immunity that produces a type I interferon-mediated by Toll-like receptor-dependent and Toll-like receptor-independent pathways (Abe et al. 2005, 2009). Other transcriptomic analyses found similar evidence, including that baculoviruses slightly induce genes associated with Toll-like receptors, cytokine signaling, and complement (Shin et al. 2020).

The BV forms of baculoviruses handled under laboratory conditions are not infective to natural hosts—they would be infective only by injection into larvae—and are completely safe for human operators, as previously mentioned. In view of such considerations, these virions are generally regarded as entities that can be manipulated in facilities with biosafety level 1, as indicated in the guidelines for working with viral vectors of research-and-development laboratories from

prestigious universities and institutions worldwide. As has been demonstrated through their uses in agriculture, baculoviruses do not infect nontarget organisms and do not produce adverse effects on plants or humans and other animals (Kost and Condreay 2002).

Baculoviruses in biotechnology

The initial application of baculoviruses (Fig. 2) was the use as active ingredients for bioinsecticide products for the control of agricultural and forestry pests (Haase et al. 2015; Lacey et al. 2015). Currently recognized by the International Committee on Taxonomy of Viruses are 84 species—55 alphabaculoviruses, 26 betabaculoviruses, 1 deltabaculovirus, and 2 gammabaculoviruses (Harrison et al. 2018), most being pathogenic for specific arthropods that are harmful to crops. For this reason, numerous products have been developed and placed on the market, thus participating as one of the alternatives for integrated pest management since the second half of the twentieth century. This development has also enabled the regulatory agencies of many countries to

certify the safety of these viruses for humans and other non-target organisms. We need to emphasize that for this application in the agricultural sector wild-type baculoviral OBs are used and that their production is carried out mainly through infections in larvae reared in insectaries (Haase et al. 2015).

Subsequently, other uses emerged for members of *Baculoviridae* owing to (i) the facilities offered by genetic engineering through viral-DNA manipulation (Luckow et al. 1993), (ii) the natural condition that the viral genome is infective per se in host cells (Rohrman 2019), and (iii) the availability of more than 320 insect cell lines capable of multiplying baculoviruses under in vitro conditions (Lynn and Harrison 2016). Unlike the circumstance in the agricultural sector, most of these new applications derive from a single alphabaculovirus, AcMNPV. In particular, the identification of two main groups of applications for engineered baculoviruses is possible: (a) systems for the expression of recombinant proteins within eukaryotic contexts and (b) the use of recombinant virions in mammals for different goals. The former are generally called *Baculovirus Expression Vector Systems* or simply BEVS (O'Reilly et al. 1994; Possee et al. 2020) and comprise platforms composed of vectors for molecular cloning that enable modifications of the AcMNPV genome (many being based on bacmids) and susceptible insect cell lines plus their culture media or a whole insect as a biofactory (Martínez-Solís et al. 2019). The latter group contains engineered baculoviruses applied to mammals and is recognized as BacMam technologies (Airenne et al. 2013; Mansouri and Berger 2018; Ono et al. 2018). Unlike BEVS, which are a means for the generation of products (e.g., recombinant proteins, virus-like particles), in the BacMam technology, the virions are usually the final product for, among other applications, immunogenic or gene therapy uses (as protein-display systems or as gene vectors). These developments have led to different regulatory considerations because in some instances baculoviruses can be only a contaminant, while in others they are the main ingredient of the products. In contrast to the use of this technology in the production of bioinsecticides (where OBs of many viral species and production systems are used in larvae), genome-engineered BVs of AcMNPV multiplied on the Sf9 or Sf21 insect cell lines, among others, are generally the bases of the BEVS and BacMam technologies (Kwang et al. 2016; Possee et al. 2020).

Baculoviruses as tools for the control and diagnosis of emerging human infectious diseases

The BEVS is a simpler, safer, faster, and easier-to-scale-up method to produce recombinant proteins at a lower cost than traditional systems based on mammalian cell lines. In

recent years, this platform has been widely used in academia and industry to produce viral structural proteins (VSPs) for the development of vaccines and therapeutic and diagnostic assays to respond quickly in the event of epidemiologic emergencies (Kumar et al. 2018).

The AcMNPV is the most studied and implemented baculovirus for biotechnological purposes in the world (Premanand et al. 2018). Several commercially susceptible cell lines are available such as those mentioned above derived from the ovary of *Spodoptera frugiperda* larva (Sf21, Sf9, expresSF+) or *Trichoplusia ni* larva (BT1-Tn5B1-4, marketed as High Five™) (Martínez-Solís et al. 2019). As a low-cost alternative, the use of whole insect larvae or pupae as an ersatz bioreactor is possible. This technology enables a higher yield than insect cell lines in only a few weeks and at a lower cost. Among the lepidopteran species, the most exploited are *S. frugiperda*, *Rachiplusia nu*, and *T. ni*. In addition, species like *Bombyx mori* (silkworm) are widely exploited in Asian countries as small biofactories. This species and its derived cell line Bm5, however, are susceptible to another nucleopolyhedrovirus belonging to the family *Baculoviridae*, the *Bombyx mori nucleopolyhedrovirus* (Targovnik et al. 2016).

BEVS offers a eukaryotic environment providing adequate posttranslational modification, but the insects are not capable of producing human-type *N*-glycoproteins (Fabre et al. 2020). Since 1983, when the first protein was expressed by means of this system, different strategies have been implemented in order to improve the technology, upon considering both the host and the viral vector (Martínez-Solís et al. 2019). For instance, the *N*-glycosylation profile has been improved by the development of novel insect cell lines and viral vectors capable of producing human glycosyltransferase (PalMBERGER et al. 2013; Maghodia et al. 2021). In addition, the system provides strong promoters such as *polh* and *p10* (derived from the *polyhedrin* and *p10* genes, respectively) to produce recombinant proteins. Moreover, new chimeric promoters (*polh-pSeL*, *polhpB2*, *pB2p10*) and other elements have been incorporated into the viral vector to increase the yield and stability of the protein to be expressed (López-Vidal et al. 2013; Martínez-Solís et al. 2019).

In recombinant-protein production, the use of BEVS has two main methodological steps. First, the recombinant virus must be produced, and next the host has to be infected to achieve the final bioproduct. The whole process takes between 4 and 8 weeks (Cox 2012; Cox et al. 2015; Targovnik et al. 2016). Various approaches have been developed for the generation of recombinant baculovirus (re-baculovirus) including homologous recombination (i.e., BaculoGold™ system, BD Bioscience; BacPAK™ system, Clontech), site-specific transposition (i.e., Bac-to-Bac™ system, Thermo Fisher; Multibac™ system, Geneva Biotech) or combined technologies (i.e., flashBAC™ system,

Oxford Expression Technologies) (Martínez-Solís et al. 2019). Furthermore, the systems commercially available have enabled a simultaneous expression of two proteins, as in the example of the Bac-to-Bac™ system (Thermo Fisher), or a multiprotein complex with novel systems such as Multi-Bac™ (Geneva Biotech). Of course, this degree of expression can be expanded by using internal–ribosome–entry–site sequences or 2A peptides (or by customizing commercial vectors through the addition of new transcriptional units). Moreover, as mentioned before, the system is completely secure from biohazard and represents no form of risk to operators because the baculovirus cannot replicate in mammalian cells. For all of the above considerations, BEVS is a system with great biotechnological value for recombinant-protein production.

As we have emphasized, BEVS is widely used to produce VSPs (Mazalovska and Kouokam 2020). When one or more of these VSPs are coexpressed, they can self-assemble forming nonreplicative and nonpathogenic particles known as virus-like particles (VLPs). VLPs are one of the most promising tools in vaccine development. Various VLP-based vaccines that are currently at a clinical status have been manufactured through the use of BEVS, as has been extensively reviewed by Kumar et al. (2018). In this regard, vaccines against infectious diseases caused by *Ebola virus*, *Enterovirus*, *Parvovirus*, *Norwalk virus*, *Polyomavirus*, *Papilloma virus*, *Simian virus 40*, *Rotavirus*, *Human immunodeficiency virus*, and *Respiratory syncytial virus* are now being studied (Kumar et al. 2018). These vaccines represent a safer choice than established technologies, like inactivated or attenuated viruses, because those immunogens successfully mimic the virion morphology without the presence of any nucleic acid and, at the same time, are more likely to induce stronger cellular and humoral responses than single-protein vaccines. Within the context of emerging viral infections such as influenza, arboviral, or coronavirus-related diseases, VLPs represent an even more relevant technology in view of their faster development times and greater flexibility than with traditional approaches for endemic diseases like the influenza vaccine manufactured in eggs (Maranga et al. 2002; López-Macías 2012). For the development of these strategies, baculovirus technology is a powerful ally because of the ability to assemble viral particles with high protein yields, with safer and simpler handling, and in the absence of adventitious agents or egg-related contaminants.

In addition, and likewise, for vaccine purposes, the baculovirus itself can be used as an antigen-presenting vehicle by displaying immunogenic peptides or recombinant proteins on its surface (Tsai et al. 2020). This technology (being able to be included in BacMam approaches for vaccine uses) is known as *baculovirus display* and has the ability to mount a strong immune response. The antigen presentation on the

BV surface is achieved mainly by the fusion of heterologous proteins with GP64, the major surface glycoprotein of group-1 alphabaculovirus BVs (*cf.* the BV, Fig. 1C) (Kost et al. 2005; Xu et al. 2011). Moreover, baculoviruses can be used as DNA vectors to express the antigen in the tissue where the vaccine is injected, as is the circumstance with the new generation of recombinant adenovirus-based vaccines.

In summary, baculovirus technology has been successfully implemented (i) to express VSPs for use as vaccine candidates, with those immunogens exhibiting strong humoral and cellular responses, (ii) to develop diagnostic tests, and (iii) to produce VLPs with excellent results and comparative advantages *versus* established systems—including administration routes and the potential use as a delivery vector. All of these advantages indicate the widespread use of this technology as a tool for studying and developing strategies for future emerging virus threats and pandemics. In the next section, we will describe the use of baculovirus in the fight against the main emerging viral diseases, where most of the research work has been carried out in recent years (*i.e.*, influenza viruses, arboviruses, and coronaviruses). The use of the platform for the diagnosis and prevention of other clinically significant viral diseases, however, are comprehensively reviewed by Kumar et al. (2018).

Influenza vaccines and diagnostic-reagent production

Influenza—caused by the virus of the same name—is a highly contagious respiratory disease spread worldwide that exhibits high rates of morbidity and mortality. Among the four types of influenza viruses, A and B are responsible for the greatest number of seasonal infectious, producing around 5 million cases of virus-mediated severe flu and around 650,000 fatal cases per year all over the world (Harding and Heaton 2018; Basak et al. 2020). Furthermore, influenza A has caused four pandemics since 1918 (Harding and Heaton 2018), one being produced by the H1N1 influenza A in 2009 (Shim et al. 2019).

Influenza is a segmented negative-strand RNA virus belonging to the *Orthomyxoviridae* family. The virus envelope is formed by a lipid bilayer and three transmembrane surface proteins encoded by the viral genome: hemagglutinin (HA), neuraminidase (NA), and the proton-channel protein M2 (Veit and Thaa 2011). These components together with the viral-matrix protein 1 (M1) contribute to the virus assembly (Hilsch et al. 2014). Among the VSPs, the viral glycoprotein, HA and NA, are the most antigenic surface species involved in the viral pathogenesis—*i.e.*, attachment and release, respectively (Kash et al. 2004). At least 18 different HA subtypes exist (H1–H18) along with 11 NA subtypes (N1–N11) (Kosik and Yewdell 2019).

Serious influenza infection is prevented through a vaccination that reduces the impact of the disease (Fauci 2006). Because HA is the only influenza component responsible for the induction of neutralizing and protective antibodies in infected individuals, hemagglutinin is usually the principal component in vaccine production (Ting-Hui-Lin et al. 2019). Notwithstanding, antibodies directed against NA reduce the viral replication and the severity of the disease by blocking the viral receptor; therefore, NA can also be an effective ingredient for influenza vaccines (Deng et al. 2012). As both the antigens HA and NA are subject to selected point mutation, the formulation of the influenza vaccines must be annually updated in the fight against the seasonal flu (Fauci 2006). Often, new influenza subtypes with new antigenic properties can arise because of the reassortment of viral genome components among different virus strains. This trait shuffling may be a cause of pandemic outbreaks (Harding and Heaton 2018).

The conventional production platforms for the inactivated influenza virus vaccines are based on embryonated eggs and, in consequence, do not offer a rapid response in the circumstance of a pandemic. The production requires around 6 months after the circulating strain be detected (Ting-Hui-Lin et al. 2019). This use of eggs works well, although the approach has many other drawbacks. For example, the whole-virus manipulation requires specialized containment laboratories, thus complicating the scaling-up in a pandemic outbreak. In addition, an adaptation of the viral strain to multiplication in eggs is also usually necessary, a time-consuming process that is not always successful. Furthermore, the production requires the use of antibiotics and noxious chemicals for the final inactivation of the virus, and this type of vaccine is even not recommended for sufferers of egg-protein allergy. To overcome these limitations, different alternative systems to produce the influenza vaccines in a more rapid and cost-effective way are being explored (Harding and Heaton 2018; Ting-Hui-Lin et al. 2019). Among these approaches, the recombinant subunit protein-based vaccines have been extensively explored, with the associated manufacturing process not requiring specialized facilities for virus manipulation. The production involves the generation of the viral-envelope antigen via recombinant-DNA technology and further utilization of the purified antigen as the active ingredient. Consequently, BEVS emerged as a suitable alternative flexible platform for the fast production of the viral antigen by the infection of insect cells with rec-baculoviruses containing the HA gene (He et al. 2009). Within this context, in 2013 the Food and Drug Administration (FDA) in the USA licensed the first recombinant HA-based trivalent influenza-virus vaccine (a cocktail of three strains) named FluBlok® developed by the Sanofi Pasteur-Protein Science Corporation and produced in infected expresSF + insect

cells. This cell line was established to grow in serum-free medium at high densities in suspension cultures that are scalable in simple stirred tank reactors (Buckland et al. 2014). The three HA variants are expressed by coinfection with three independent rec-baculoviruses encoding different human hemagglutinins (Meghrouh et al. 2009). Standardized upstream and downstream processes have been developed to rapidly produce new HA variants and the entire procedure requires only 8 weeks (Cox et al. 2015). This vaccine has been demonstrated to be safe, immunogenic, and effective after several clinical trials were conducted (King et al. 2009); and in 2017, the trivalent vaccine was replaced by a quadrivalent version.

Moreover, with an aim at reducing the production costs and improving the antigen-expression levels, living insect larvae were also implemented as biofactories. Thus, the expression of the HA ectodomain—fused with the retention signal KDEL, the (Lys-Asp-Glu-Leu) endoplasmic-reticulum protein—in *T. ni* larvae infected with rec-baculoviruses was four-fold higher than that obtained with Sf21 insect cells (Gomez-Casado et al. 2011). In addition, the HA ectodomain fused with the viral signal peptide of GP64 was more highly expressed in the hemolymph of *Spodoptera litura* larvae (Hsieh et al. 2018). This signal peptide facilitated the necessary posttranslational modifications along with the secretion of the recombinant protein (Targovnik et al. 2019). The HA concentration in the hemolymph was around 100-fold higher than in Sf21 cell-culture medium (Hsieh et al. 2018). In all these reports, the larva-derived HA induced protective immunity in vaccinated mice.

Recently, BEVS has also been used as an antigen-expression platform for the development of diagnostic tests for influenza. Shim et al. (2019) developed a process to produce in insect cells the HA ectodomain as a suitable antigen to develop an enzyme-linked immunosorbent assay (ELISA) for detecting anti-HA antibody in serum samples derived from infected and vaccinated individuals (Shim et al. 2019).

Furthermore, vaccines based on NA have also been proposed to prevent viral spread (Faletti et al. 2014) such as FluNhanche™ (Sanofi Pasteur-Protein Science Corporation), which is an influenza-subunit vaccine based on a recombinant expression of the NA antigen in insect cells. This product is currently undergoing clinical trial (phase II challenge) and would be used as a booster of the already licensed vaccine to thus induce a broader and more protective immunity. Individuals vaccinated with FluNhanche™ in combination with the conventional influenza virus vaccine have developed milder illnesses (Johansson et al. 2002). We must also mention that NA has likewise been successfully expressed in insect larvae. In fact, the production of the same amount of NA that is generated in 153 mL of cell suspensions was possible with only six larvae of *R. nu* (Faletti et al. 2014).

In addition, BEVS were used to develop an influenza-VLP vaccine by coexpressing HA, NA, and M1 proteins in insect cells (Bright et al. 2007), thus representing a significant improvement in immunogenicity with respect to the recombinant-subunit vaccine. These VLPs can simulate the virus structure and induce protective immunity against the infection owing to the production of high anti-HA- and anti-NA-antibody titers (Bright et al. 2007; He et al. 2009). In this regard, different eukaryotic cells have been explored for the production of VLP vaccines against influenza (Deng et al. 2012). Nevertheless, the BEVS resulted in the best approach for that purpose because they provided an efficient platform to express multiple recombinant proteins simultaneously (Lai et al. 2019). Several reports demonstrated the effectiveness of VLPs produced in BEVS to confer protection against the influenza virus when those prototypes were administered via either the intramuscular or intranasal immunization routes in mice challenged with a lethal dose of the virus (Bright et al. 2007; Hahn et al. 2013; Smith et al. 2013; Ren et al. 2018; Lai et al. 2019). Within this context, Novavax manufactures a VLP vaccine for different influenza A and B strains through the coexpression of only the three structural proteins (HA, NA, and M1) in Sf9 insect cells infected with three different rec-baculoviruses (Hahn et al. 2013). The Novavax influenza VLP vaccine has proved both safe and immunogenic in phase I and phase II clinical trials (Deng et al. 2012; Hahn et al. 2013). The production, formulation, and inspection require only 2 months, after which time the product-release test needs 3 weeks more (Hahn et al. 2013). Recently, Lai et al. (2019) developed an improved method of manufacturing H7N9 VLP vaccine by coexpressing the H7, N9, and M1 proteins in High Five™ insect cells and an adequate level of dissolved oxygen (150 mmHg) at a high multiplicity of infection (Lai et al. 2019).

An alternative strategy has been established to produce vaccines based on nanoparticles. Thus, the production of NanoFlu® (a quadrivalent nanoparticle vaccine) involving the expression of HA through the use of BEVS has been developed by Novavax. Once the four HA variants are simultaneously expressed in Sf9 cells, the proteins are assembled into a nanoparticle during the purification. The vaccine is finally formulated with the patented saponin-based adjuvant Matrix M™ (Novavax) that improves the activation of innate immune cells and antigen presentation (Khalaj-Hedayati et al. 2020). The NanoFlu® vaccine is currently in phase III clinical trials (Shinde et al. 2021).

Unlike the variable HA proteins, the conserved M2 ectodomain has been studied as a presumed universal antigenic target for developing vaccines that produce cross-protective immunity. Kim et al. (2013) developed a VLP-based vaccine candidate by Sf9-cell coinfection with a rec-baculovirus expressing five tandem repeats of

the heterologous M2 ectodomain (M2e5x) from different influenza A strains and a baculovirus expressing the M1 matrix protein. To improve the incorporation of M2 into the VLPs, M2e5X was fused to the HA transmembrane domain (Kim et al. 2013). This vaccine induces lower neutralizing antibodies than the VLP based on HA expression, but the M2e5X's VLPs improved the effectiveness of the traditional attenuated influenza-virus-based vaccine and stimulated cross protection when tested in mice (Kim et al. 2013; Lee et al. 2019).

The simultaneous coexpression of multiple proteins to achieve influenza vaccines involves the coordinate synchronous cell infection with several rec-baculoviruses. This strategy may be inefficient on large scale, or else would require baculovirus shuttle vectors that accept more than one heterologous gene so as to result in larger DNAs that are unstable (Sequeira et al. 2018; Martínez-Solís et al. 2019). To circumvent this obstacle, several reports indicated that recombinant insect cells would provide a promising platform to manufacture functional influenza VLPs (Matsuda et al. 2020). For instance, High Five™ cells stably transformed with HA and M1 efficiently produce VLPs at a yield comparable to those obtained with the baculovirus–insect-cell system (Matsuda et al. 2020). Another report developed a combined approach to express five HAs at the same time to produce a pentavalent HA VLP. The strategy involved High Five™ cells stably transformed with two different HA genes and the subsequent infection with a rec-baculovirus that encoded the other three HAs plus M1. This study demonstrated an efficient and scalable platform to produce multivalent VLPs (Sequeira et al. 2018).

The use of nonreplicative baculoviruses as gene-delivery DNA vectors for the assembly of VLPs in mammalian cells represents another promising strategy for influenza-vaccine development. Regarding this BacMam application, a rec-baculovirus was constructed, carrying influenza HA, NA, and M1 genes under the control of the human cytomegalovirus immediate-early enhancer and promoter. To improve targeted gene delivery, Gwon et al. (2016) incorporated into the baculovirus genome the envelope-glycoprotein-encoding sequence from the human endogenous retrovirus. As a result, mice immunized with the recombinant BVs produced a strong humoral response and neutralizing antibodies after the challenge with a lethal dose of influenza virus (Gwon et al. 2016).

Finally, the antigen-displaying alternative baculoviruses are also being explored as another approach to developing vaccines for influenza. The HA incorporation into the baculovirus surface demonstrated an ability to elicit strong humoral and cell-mediated immunity that protected animals from the lethal challenge of influenza viruses (Prabakaran et al. 2008; Prabakaran and Kwang 2014; Yu et al. 2020).

This vaccine can be administrated by the subcutaneous or intranasal route, thus enabling a new alternative in the development of vaccines against mucosal pathogens (Prabakaran and Kwang 2014; Sim et al. 2016). The rec-baculoviruses displaying HA antigens against influenza disease are extensively reviewed by Premanand et al. (2018).

Table 1 provides a summary of the principal influenza vaccines developed through the use of BEVS and BacMam platforms.

Table 1 Summary of vaccine produced in BEVS and BacMam platform in the campaign against influenza-virus infection

Bacu- lovirus tool	Host	Protein and Strain	Type of vector	Development stage	References
Protein or subunit expression					
	Sf9 cells	HA from A/H6N1	BaculoGold™ (BD Biosciences)	Exploratory	Faletti et al. (2014)
	Sf9 cells	HA from A/H1N1, H3N2, and two strain B. Contain saponin-based Matrix M adjuvant	ND	Phase-III clinical trial (Nanoflu™, Novavax)	Shinde et al. (2021)
	expresSF+ cells	NA (strain ND)	ND	Clinical trial II (FluNhanche™, Sanofi Pasteur-Protein Science Corporation)	Deng et al. (2012)
	expresSF+ cells	HA from A/H1N1, A/H3N2, and two strain B	ND	Approved (FlubloK, Sanofi Pasteur-Protein Science Corporation)	Buckland et al. (2014)
	<i>T. ni</i> larvae	HA ectodomain fused with KDEL from A/H1N1	ND	Preclinical	Gomez-Casado et al. (2011)
	<i>S. litura</i> larvae	HA from A/H6N1	Bac to Bac™ (Thermo Fisher)	Preclinical	Hsieh et al. (2014)
	<i>R. nu</i> larvae	NA from A/H1N1	BaculoGold™ (BD Biosciences)	Exploratory	Faletti et al. (2014)
VLP					
	Sf9 cells	HA, NA, M1 from H3N2 and H7N9	Bac to Bac™ (Thermo Fisher)	Preclinical	Bright et al. (2007); Smith et al. (2013); Ren et al. (2018)
	Sf9 cells	HA, NA, M1 from H5N1	Bac to Bac™ (Thermo Fisher)	Phase-I/II clinical trial	Khurana et al. (2011)
	Sf9 cells	M2e5x (five tandem repeat) from A/H3N2, two A/H1N1, and two A/H5N1	Bac to Bac™ (Thermo Fisher)	Preclinical	Kim et al. (2013)
	Sf9 cells	HA, NA, M1 from H7N9	ND	Phase-I/II clinical trial	Hahn et al. (2013)
	Sf9 cells	HA, NA, M1 from A/H1N1, A/H3N2 and two strain B	ND	Phase-IIa clinical trial (Novavax)	Kumar et al. (2018)
	High Five™ cells	HA, NA, M1 from A/H7N9	Bac to Bac™ (Thermo Fisher)	Exploratory	Lai et al. (2019)
Display					
	Sf9 cells	HA from A/H7N9	ND	Preclinical	Prabakaran et al. (2008)
	Sf9 cells	HA from A/H1N1	Bac to Bac™ (Thermo Fisher)	Preclinical	Sim et al. (2016)
Gene delivery					
	Sf9 cells	HA, NA, M1 from A/H1N1	Bac to Bac™ (Thermo Fisher)	Preclinical	Gwon et al. (2016)

M1 viral-matrix protein 1; HA hemagglutinin; NA neuraminidase; VLP virus-like particles. All preclinical tests were performed in mice; ND no data

Arboviruses vaccines and diagnostic-reagent production

Arboviruses (arthropod-borne viruses) are responsible for a large number of viral diseases that are transmitted to humans through the bites of arthropods (such as mosquitoes and ticks). While certain arboviral infections can be asymptomatic or cause mild febrile illness, others can be more severe causing encephalitis, hemorrhagic fever, joint pain, or even lead to death (Marchi et al. 2018). In fact, arboviral diseases represent 17% of communicable diseases worldwide, affecting millions of people and causing an estimated more than 700,000 deaths annually (Kading et al. 2020; World Health Organization 2020). In the last 50 years, these diseases have spread rapidly, causing epidemics around the world. Vaccination is the most effective method of preventing such infectious diseases, though few commercial vaccines have been approved for humans against certain arboviruses such as the *Yellow fever virus* (YFV), the *Dengue virus* (DENV), and the *Japanese encephalitis virus* (JEV), for which entities the vaccine development involves whole-virus manipulation (Krol et al. 2019). The recombinant-subunit and VLP vaccine approaches offer a quicker and lower-risk alternative to vaccine manufacturing, thus avoiding exposure to biohazardous agents (Cho et al. 2008). Accordingly, several eukaryotic expression platforms such as plant, yeast, mammalian, and insect cells have been exploited to produce recombinant antigens for controlling and preventing an imminent arbovirus outbreak (Martínez et al. 2012; Wilder-Smith et al. 2017; Girard et al. 2020). Since arboviruses replicate efficiently in arthropod cells, BEVS has emerged as a suitable and biosafe technology to produce authentic arbovirus proteins for the manufacture of new vaccines and diagnostic methods in the campaign against arboviruses transmitted by mosquitos, the former proteins mainly from the genera *Flavivirus* (Family *Flaviviridae*) and *Alphavirus* (Family *Togaviridae*), as summarized in Table 2.

Flaviviruses are enveloped, positive-sense, single-stranded RNA viruses whose genomes encode six non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B) essential for viral-RNA replication along with another three structural proteins present in the virion: the capsid (C), the membrane (M), and the envelope (E). In particular, the ectodomain of the E glycoprotein is divided into three structurally distinct domains (DomI, DomII, and DomIII), where DomIII is implemented in viral binding to the host-cell receptor and is the main antigenic component to induce protective immunity against flavivirus infection (Modis et al. 2003). Therefore, the E protein, and especially its DomIII, is a prime candidate antigen for vaccine and specific diagnostic-kit development. The *Flaviviridae* family contains five species with great impact on public health in recent years: the DENV, the *Zika virus* (ZIKV), and the YFV, which are

transmitted by mosquitoes of *Aedes* spp.; plus the JEV and the *West Nile virus* (WNV), which are transmitted by mosquitoes of *Culex* spp. (Gould et al. 2017).

Nowadays, DENV, the causative agent of dengue fever and dengue hemorrhagic fever, is one of the contagious viral diseases most widely distributed throughout the world. An increasing number of dengue outbreaks have occurred in recent times, resulting in around 400 million reported dengue-infection cases yearly (Pierson and Diamond 2020). Unlike other flaviviruses, the DENV is subdivided into four serotypes (DENV1 to DENV4). Although great efforts have been made to develop vaccines that simultaneously protect against all four variants (VanBlargan et al. 2013), only one vaccine for DENV infection is currently approved and with partial effectiveness depending on the serotype and the individual's basal immunologic status and age (Hernández-Ávila and Santos-Preciado 2016). Dengvaxia (Sanofi Pasteur) is a live-attenuated tetravalent vaccine based on the yellow fever viral backbone that expresses the precursor membrane (prM) and the E proteins from the four DENV serotypes (VanBlargan et al. 2013). In order to achieve a more effective vaccine, BEVS has currently been used to safely generate dengue antigens (Metz and Pijlman 2011). In this regard, the E ectodomain, comprising the three E domains, was successfully expressed in insect cells through the use of rec-baculoviruses (Delenda et al. 1994; Staropoli et al. 1997). The protein retained its antigenicity and was capable of eliciting neutralizing antibodies that protected mice from lethal virus infection (Eckels et al. 1994). In the second approach, the DENV-2 E was expressed, but fused to the prM translocation signal. As a result, an aggregated E was obtained with a native folding exposing functional epitopes that induced neutralizing antibodies in mice (Kelly et al. 2000). As a low-cost strategy, the production of dengue antigens in whole-insect larvae is possible. Within this context, Cerezo et al. (2020) demonstrated that the DomIII from DENV-1 and DENV-2 fused to hydrophobin (DomIIIHFB) and produced in insect larvae could elicit serotype-specific neutralizing antibodies in mice without cross-reacting against heterologous serotypes and other flaviviruses. Therefore, the DomIIHFB expression belonging to the four serotypes would be useful for providing reagents for the formulation of a low-cost tetravalent subunit vaccine (Cerezo et al. 2020).

An alternative approach involving the expression of synthetic consensus proteins based on the amino acid sequence belonging to all serotypes has emerged as an excellent alternative to achieve tetravalent vaccine antigens. For instance, a consensus protein of the dengue E ectodomain, has been expressed in Sf9 cells infected with rec-baculoviruses. The antigen was capable of eliciting high titers of specific protective antibodies against all the serotypes. Moreover, the vaccine could activate an antigen-specific T-cell response (Sun et al. 2017). In addition, a fusion of the E ectodomain

Table 2 Summary of products manufactured in BEVS and BacMam platform in the campaign against arbovirus infections

Arbovirus	Product	Baculovirus tool	Structural protein	Type of vector	Host	References
<i>Dengue virus</i>						
	Vaccine (preclinical stage)	Protein or subunit expression	E ectodomain from DENV-2	AcRP23-lacZ	Sf9 cells	Staropoli et al. (1997)
	Vaccine (preclinical stage)	Protein or subunit expression	Fusion of E ectodomain from DENV-1, DENV-3, and DENV-4	pVL (stratagene) and AcMNPV genome	Sf9 cells	Rantam et al. (2015)
	Vaccine (preclinical stage)	Protein or subunit expression	Consensus E protein	Bac to Bac™ (Thermo Fisher)	Sf9 cells	Sun et al. (2017)
	Vaccine (preclinical stage)	Protein or subunit expression	E full-length from DENV-2	pBlueBacIII + linearized AcMNPV (Invitrogen)	High Five™ cells	Kelly et al. (2000)
	Vaccine (preclinical stage)	Protein or subunit expression	DomIII from DENV-1 and DENV-2 fused to HFB	Bac to Bac™ (Thermo Fisher)	<i>R. nu</i> larvae	Cerezo et al. (2020)
	Vaccine (exploratory stage)	VLP	C, prM, E from DENV-2	Bac to Bac™ (Thermo Fisher)	<i>B. mori</i> larvae	Utomo et al. (2019)
	Two-step MAC-ELISA	Protein or subunit expression	Tandem repeat of DomIII from four serotype	pAcGP67/pSecG2T (BD Bioscience) AcPak6 DNA (Invitrogen)	Sf9 cells	Niu et al. (2015)
<i>Zika virus</i>						
	Vaccine (preclinical stage)	VLP	prM-E	Bac to Bac™ (Thermo Fisher)	Sf9 cells	Dai et al. (2018)
	Vaccine (preclinical stage)	Display	E ectodomain	Bac to Bac™ (Thermo Fisher)	Sf9 cells	Luo et al. (2020)
	Point-of-care IgG and IgM diagnosis	Protein- or subunit-expression platform	E, NS1	BaculoGold™ (BD Biosciences)	Sf9 cells	Kim et al. (2018)
<i>Yellow fever virus</i>						
	Vaccine (preclinical stage)	Protein or subunit expression	E, NS1	ND	Sf9 cells	Despres et al. (1991)
	IgM capture ELISA	Protein or subunit expression	E	pSynXIV/ vSynV1gal	High Five™ cells	Barros et al. (2011)
<i>West Nile virus</i>						
	Vaccine (preclinical stage)	Protein or subunit expression	E	pPSC12 (Protein Science Corporation)/ Bsu linearized AcMNPV	expresSF + cells	Bonafé et al. (2009)
	Vaccine (preclinical stage)	VLP	NS1	Bac to Bac™ (Thermo Fisher)	Sf9 cells	Qiao et al. (2004)
	Competitive ELISA	Protein or subunit expression	NS1	Bac to Bac™ (Thermo Fisher)	Sf9 cells	Yeh et al. (2012)
	IgG indirect ELISA	Protein or subunit expression	E	Bac to Bac™ (Thermo Fisher)	<i>T. ni</i> larvae	Alonso-Padilla et al. (2010)
<i>Chikungunya virus</i>						
	Vaccine (exploratory stage)	Protein or subunit expression	E1, E2	Bac to Bac™ (Thermo Fisher)	Sf21 cells	Metz et al. (2011)
	Vaccine (exploratory stage)	VLP	E1, E2	Bac to Bac™ (Thermo Fisher)	Sf21 cells	Metz et al. (2013)
	Vaccine (exploratory stage)	VLP	E1, E2	Bac to Bac™ (Thermo Fisher)	Sf9 Basic cells	Wagner et al. (2014)
	IgM indirect ELISA and immunochromatographic assay	Protein or subunit expression	C	Bac to Bac™ (Thermo Fisher)	Sf9 cells	Cho et al. (2008)
	IgG capture ELISA	Protein or subunit expression	E1	Bac to Bac™ (Thermo Fisher)	Sf9 cells	Kumar et al. (2014)

E envelope; *prM* premembrane; *C* capsid; *NS1* nonstructural protein 1; *DomIII* E protein domain III; *HFB* hydrophobin; *VLP* virus-like particles. All preclinical tests were performed in mice; *ND* no data

belonging to three distinct DENV serotypes (1, 3, and 4) was expressed with the ability to induce humoral and cellular responses (Rantam et al. 2015). A recombinant fusion E protein can also be useful as a reagent for efficient immunodiagnostic-kit development with the potential to detect all four serotypes at once. In this scenario, a tandem repeat of DomIII belonging to four serotypes was expressed in Sf9 cells infected with rec-baculoviruses. The protein was successfully used as an antigen for developing an ELISA capture to detect anti-DENV IgM antibodies in sera from patients at early stages of infection (Niu et al. 2015). In addition, the use of infected *R. nu* larvae to express DomIII from DENV-2 resulted in a low-cost platform to produce immunodiagnostic reagents. The VLP approach has also been employed to produce a dengue vaccine. There, the coexpression of the structural proteins C, prM, and E belonging to DENV-2 was found to lead to VLP formation when expressed in baculovirus-infected silkworm larvae (Utomo et al. 2019). Furthermore, DENV-2 VLPs were also produced by a transient-expression system through transfection of prM and E genes into Sf9 cells (Kuwahara and Konishi 2010).

ZIKV, for its part, is a flavivirus globally disseminated and associated with possible neurologic complications, the coexpression of whose structural proteins (prM and E) in Sf9 insect cells infected with rec-baculovirus also led to an assembly of VLPs. After administration to mice, the ZIKV VLPs induced a protective immunity that elicited neutralizing antibodies, virus-specific IgG titers, and a T-cell immune response (Dai et al. 2018). Recently, a report described for the first time the display of the E protein on the BV surface and the resulting immunogenic and protective effect against ZIKV. To the display, E was fused with the signal peptide and the transmembrane domain from AcMNPV GP64 (Luo et al. 2020). For the purpose of ZIKV diagnosis, the nonstructural proteins NS1 and E from the ZIKV were efficiently produced in insect cells infected with rec-baculoviruses. The purified antigen and monoclonal antibodies were used to develop a point-of-care diagnosis to rapidly detect IgG and IgM antibodies against ZIKV in sera from patients (Kim et al. 2018). The test appears as a novel technology and an alternative compared to traditional methods used in ZIKV diagnosis.

The E and NS1 proteins, or a fusion of both (E-NS1), have also been expressed in insect cells infected with rec-baculoviruses in order to produce a vaccine candidate against yellow fever (YF), a reemerging viral zoonosis caused by the YFV. Of all constructions tested, E-NS1 protein has resulted in the most immunogenic construction, whose administration proved to protect mice from a lethal viral challenge (Despres et al. 1991). YF infection is prevented by a highly effective vaccine (YFV-17D), but major outbreaks still occur due to the failure of vaccination

campaigns in certain regions, mainly belonging to Africa and Central and South America (Pijlman 2015). Despite the proven efficacy of the vaccine, it is based on attenuated viruses, vaccination is not recommended for persons older than 60 years or younger than 6 years of age, or for allergic and immunocompromised patients. Therefore, BEVS offers an alternative platform to develop immunogens that could be administered regardless of people's age or health status (Araujo et al. 2020). Furthermore, a YF diagnosis can be performed with the E antigen expressed in High Five™ cells as a reagent to develop an immunoassay to detect specific antibodies in the serum of infected patients (Barros et al. 2011).

Among the flaviviruses transmitted by *Culex* spp., one of the most widely distributed throughout the world is the WNV, causing a generally asymptomatic infection, but certain cases of infection can lead to severe clinical manifestations and even death (Krol et al. 2019). Several vaccine candidates have been developed against the WNV through the use of BEVS. For example, the soluble ectodomain E protein was expressed in expresSF+ cells, while WNV-antigenic VLPs were also produced in Sf9 cells by the coexpression of prM and E VSPs (Qiao et al. 2004; Bonafé et al. 2009). Both vaccine candidates resulted in stable, nontoxic, immunogenic preparations that were protective in model animals inoculated with WNV lethal doses. The diagnosis of WNV is currently carried out by an ELISA test involving the whole virus as a reagent. Nevertheless, the development of a cheaper and safer serological test through the use of the recombinant protein E expressed in *T. ni* larvae infected with rec-baculoviruses was possible. The antigenic component resulted correctly folded like the native counterpart and was recognized by the sera from infected animals (Alonso-Padilla et al. 2010). In another study, recombinant NS1 produced in rec-baculovirus-infected Sf9 insect cells was used as a reagent to develop a competitive ELISA assay (Yeh et al. 2012).

The JEV is another serious disease-producing flavivirus transmitted by *Culex* spp. mosquitoes. The infection is spread mainly in Asian countries causing around 50,000 cases with some 15,000 deaths per year (Nerome et al. 2018). Even though a vaccine against JEV has been approved for human use (IXIARO, Valneva), BEVS has been explored as an alternative platform to achieve more cost-effective prototypes. In this regard, the E protein has been successfully expressed in insect cells infected with rec-baculoviruses, where the recombinant protein induced neutralizing antibodies in mice. Moreover, through the expression of the E protein alone or the coexpression of the E with prM in rec-baculovirus-infected Sf9 insect cells, the production of JEV VLPs was possible that were 10 times more abundant than those produced in Chinese-hamster-ovary (CHO) cells (Yamaji and Konishi 2013; Du et al. 2015). In addition, stably transformed insect cells were developed for the

constitutive production of JEV VLPs (Yamaji and Konishi 2013). As a low-cost alternative, large amounts of VLPs were furthermore produced in silkworm pupae infected with rec-baculoviruses that subsequently induced protection in mice (Nerome et al. 2018).

Finally, the *Chikungunya virus* (CHIKV) belonging to *Alphavirus* genus is the causative agent of chikungunya fever, an infectious disease that has recently captured the scientific community's attention as an emerging threat to public health (Kading et al. 2020). No vaccine or specific treatment for CHIKV infection is currently available (Kuo et al. 2016). Like the *Flavivirus*, the *Alphavirus* contains enveloped positive-sense single-stranded RNA molecule as genome. The mature virion consists of three structural proteins: the Capsid (C); and the two major envelope surface glycoproteins, E1 and E2, that form the spike complex on the virion surface. E1 and E2 are highly immunogenic and are involved in mediating the fusion and interaction with the host receptor during the infection. BEVS were also extensively used in the production of alphavirus antigens to manufacture vaccine candidates and diagnostic tests. Several studies indicated that the structural proteins E1 and E2 expressed through the BEVS platform are correctly processed (Metz et al. 2011, 2013). At this point, an immunoassay to detect specific IgM and IgG antibodies from infected patients has been developed with the structural CHIKV proteins expressed in BEVS as antigens (Cho et al. 2008; Kumar et al. 2014). In addition, a stable Sf9 cell line was developed that expressed the CHIKV E2 as an antigen for the serodiagnosis of CHIK (Chua et al. 2016). A study has found that CHIKV structural proteins expressed in baculovirus-infected Sf9 cells self-assemble into VLPs (Metz et al. 2013; Wagner et al. 2014). To enhance the yield of CHIKV VLPs, a new Sf9 cell line, designated Sf9Basic, was developed with the ability to grow at high pH (Wagner et al. 2014). Moreover, CHIKV VLPs with Matrix MTM as an adjuvant were more immunogenic than a subunit vaccine in animal models and protected against lethal viral doses. Thus, these VLPs constitute promising candidates for vaccines to prevent CHIKV infection (Metz et al. 2013; Wagner et al. 2014).

Coronaviruses vaccines and diagnostic-reagent production

Coronaviruses—enveloped, positive-sense, single-stranded RNA viruses, with large genomes ranging from 26 to 32 kb—are phylogenetically divided into four genera: *Alpha*- and *Beta*- (those further subdivided into four lineages A, B, C, and D) plus *Gamma*- and *Deltacoronavirus* (Pallesen et al. 2017). To date, the seven known coronaviruses are able to infect humans, of which four (HCoV-OC43, HCoV-229E, HCoV-HKU1, and HCoV-NL63) circulate endemically in humans and are generally considered harmless causing

only mild respiratory diseases. In contrast, the Middle East respiratory syndrome coronavirus (MERS-CoV) isolated in 2012 in Saudi Arabia, the severe acute respiratory syndrome coronavirus (SARS-CoV) isolated in 2003 in south China, and the newly identified SARS-CoV-2 isolated in 2019 in Wuhan, China, produce much more severe clinical outcomes, with pulmonary, cardiovascular, and renal involvement. In 2002, the SARS epidemic produced a total of 916 deaths in 8,098 patients diagnosed in several countries around the globe, while the MERS epidemic caused 858 deaths in 2,254 cases (Song et al. 2019) and the current SARS-CoV-2 pandemic over 4 million deaths in more than 190 million cases worldwide (World Health Organization 2021).

Coronaviruses encode four structural proteins (Zeng et al. 2004): the membrane protein (M), essential for assembly and budding; the envelope protein (E), also involved in assembly; the spike protein (S), responsible for viral entry and membrane fusion; and the nucleocapsid protein (N). During infection, the spike behaves as the main antigenic determinant and target of neutralizing antibodies, with its receptor-binding domain being the region where most sites are concentrated for neutralizing-antibody recognition.

BEVS can be a useful technology for studying coronaviruses and developing diagnostic tests and vaccines, as this platform has already been successfully implemented in the production of commercially available vaccines such as those for influenza (Cox and Hashimoto 2011). Examples of BEVS-mediated immunogens and reagents are summarized in Table 3. Thus, BEVS were used to produce the recombinant S protein derived from the Urbani Strain of SARS-CoV that evoked high titers of neutralizing antibodies in mice thus immunized that strikingly cross-neutralized other strains including Tor2, GD03T13, SZ3, and Palm Civet's SARS-CoV (He et al. 2006). Dai et al. (2020) also described a universal design for use against all beta coronaviruses (SARS, SARS-CoV-2, and MERS-CoV), a vaccine based on a dimeric form of the S-protein–receptor-binding domain, which immunogen induced a high level of neutralizing antibodies in mice and conferred protection against intranasal challenge with MERS-CoV (Dai et al. 2020). A SARS-CoV-2–subunit vaccine was also developed by Novavax, produced from the full-length spike glycoprotein expressed in Sf9 insect cells, which antigen was demonstrated to have increased thermal stability compared to the wild-type spike protein, an essential variable to consider in view of the known stringent temperature requirements for novel RNA vaccines. When used to immunize mice and baboons in combination with the Matrix-MTM adjuvant, the recombinant antigen elicited high titers of anti-S antibodies and strong multifunctional T- and B-cell responses (Tian et al. 2021). The Novavax vaccine is currently in phase III human trials, and the safety and efficacy of the vaccine have

Table 3 Summary of products manufactured in BEVS and BacMam platform in the campaign against coronavirus infections

Coronaviruses	Product	Baculovirus tool	Structural protein	Vector type	Host	References
<i>MERS-CoV</i>						
	Vaccine (preclinical stage)	Protein or subunit expression	RBD-dimer	Bac to Bac™ (Thermo Fisher)	Sf9 cells	Dai et al. (2020)
	Vaccine (preclinical stage)	VLP	M, E, S	Bac to Bac™ (Thermo Fisher)	Sf9 cells	Wang et al. (2017b)
	Vaccine (preclinical stage)	VLP	RBD	Bac to Bac™ (Thermo Fisher)	Sf9 cells	Wang et al. (2017a)
	Indirect and IgG-ELISA sandwich	Protein or subunit expression	S	Bac to Bac™ (Thermo Fisher)	Sf9 cells	Lee et al. (2018)
<i>SARS-CoV</i>						
	Vaccine (preclinical stage)	Protein or subunit expression	S	Bac to Bac™ (Thermo Fisher)	expressSF+	He et al. (2006)
	Vaccine (exploratory stage)	VLP	M, E, S	BaculoGold™ (BD Biosciences)	Sf21 cells	Ho et al. (2004)
	Vaccine (preclinical stage)	VLP	M, E, S	Bac to Bac™ (Thermo Fisher)	Sf21 cells	Lu et al. (2007)
	Vaccine (preclinical stage)	VLP	E, M from Sars-CoV and S from bat-CoV	Bac to Bac™ (Thermo Fisher)	Sf21 cells	Bai et al. (2008a)
	Vaccine (exploratory stage)	VLP	M, N, E, S	pAcP102X/pAcVC3/linearized AcM-NPV	Sf9 cells	Mortola and Roy (2004)
	Vaccine (preclinical stage)	VLP	S with influenza M1	Bac to Bac™ (Thermo Fisher)	Sf9 cells	Liu et al. (2011)
	Vaccine (preclinical stage)	Display	S	Bac to Bac™ (Thermo Fisher)	Sf9 cells	Feng et al. (2006)
	Vaccine (preclinical stage)	Gene delivery	N, S	Bac to Bac™ (Thermo Fisher)	Sf9 cells	Bai et al. (2008b)
	Immunofluorescence assay	Protein or subunit expression	N195 (N) and Sc (S) fusion protein	Bac to Bac™ (Thermo Fisher)	Sf9 cells	He et al. (2005)
	IgG indirect ELISA	Protein or subunit expression	N	pAc-cHis/linearized AcMNPV	Tn5 cells	Saijo et al. (2005)
<i>SARS-CoV-2</i>						
	Vaccine (exploratory stage)	Protein or subunit expression	S	BmNPV bacmid (Qd04)/pFastBac1	<i>B. mori</i> larvae	Fujita et al. (2020)
	Vaccine (phase III clinical trial, Novavax)	VLP technology	S with Matrix M adjuvant	BacVector™ (Millipore)	Sf9 cells	Tian et al. (2021)
	Vaccine (exploratory stage)	VLP	M, E, S	Bac to Bac™ (Thermo Fisher)	ExpiSf9™	Mi et al. (2021)
	Indirect IgG ELISA	Protein or subunit expression	S complete and RBD	Bac to Bac™ (Thermo Fisher)	High Five™ cells	Amanat et al. (2020)
	Indirect IgG ELISA	Protein or subunit expression	S	Bac to Bac™ (Thermo Fisher)	<i>R. nu</i> larvae	Smith et al. (2021)

RBD receptor-binding domain; *VLP* virus-like particles; *M* membrane; *E* envelope; *S* spike; *N* nucleoprotein. All preclinical tests were developed in mice, except MERS-CoV VLP from Wang et al. (2017b) that was performed in macaques and Sars-CoV-2 VLP from Tian et al. (2021) was performed in baboons and mice; *ND* no data

been verified when coadministered with seasonal influenza vaccines (Jhaveri et al. 2020).

As an alternative to cultured insect cells, whole living insects have also been studied as biofactories for the

production of coronavirus recombinant proteins. For instance, the silkworm larvae successfully expressed the SARS-CoV-2 spike protein (Fujita et al. 2020), thus indicating that the system could be a viable vehicle for generating

diagnostic kits and therapeutic proteins for coronaviruses. One such kit for the serological testing of COVID-19 based on the expression of the S protein from SARS-CoV-2 in rec-baculovirus-infected *R nu* larvae has already been produced with excellent results (Smith et al. 2021). Likewise, Escribano et al. (2020) have proposed the use of pupae from *T. ni* as a living bioreactor for the large-scale production of recombinant proteins, a technology they named CrisBio®. This approach was successfully used for the production of veterinary-virus vaccines (Escribano et al. 2020) and is now shifting into human zoonotic diseases with a successful proof-of-concept test for avian flu (Sisteré-Oró et al. 2020) and a subsequent grant to develop a COVID-19 vaccine as well.

Baculoviruses were also tested as vectors to mediate the gene expression for bat-coronavirus proteins in mammalian cells under the control of mammalian promoters (a BacMam approach). The immunization of mice with such viruses induced the expression of high titers of antibodies as measured by ELISA assays along with Th1-cell responses as visualized by ELISPOT (Bai et al. 2008b). Another alternative approach is the use of a baculovirus-facilitated surface display of the spike protein, which approach was tested by Feng et al. (2006), who accordingly demonstrated the capability of inducing a serum-neutralizing activity against SARS-CoV (Feng et al. 2006). A baculovirus display of the S protein can also be used to study the physiopathology of SARS-CoV along with the contribution of specific amino acids in triggering immune responses, as proven by Chang et al. (2004), the knowledge of which specificity can aid in targeted-drug design (Chang et al. 2004). The BacMam approach for coronavirus vaccine development is summarized in Table 3.

The development of serological tests can also be achieved with BEVS, because of the technology's ease of use and substantial protein yields. BEVS has accordingly been successfully implemented to produce ELISA and/or immunofluorescence assays for SARS-CoV (He et al. 2005; Saijo et al. 2005), MERS (Lee et al. 2018), and SARS-CoV-2 (Amanat et al. 2020).

Many attempts have been made to achieve a VLP vaccine via the baculovirus system since the first SARS outbreak. First Ho et al. (2004) and then Mortola and Roy (2004) and Lu et al. (2007) provided evidence that VLPs composed of the S, M, and E proteins from SARS-CoV form correctly when simultaneously expressed in infected-Sf9 cells (Ho et al. 2004; Mortola and Roy 2004; Lu et al. 2007). What is still not clear, though, is if the incorporation of the N protein is necessary for VLP formation or the latter depends on viral species and the expression system used (Naskalska et al. 2018). VLPs for SARS-CoV-2 have also been successfully produced in ExpiSf9 cells—a nonengineered derivative of the Sf9 insect cell line adapted for the first time to grow at high-density and with a faster duplication rate and adequate morphology (Mi et al. 2021). In addition, hybrid

SARS-like VLPs—formed by the S protein from Bat-CoV and the M and E proteins from SARS-CoV—were found to up-regulate the level of costimulatory molecules for optimal activation of T cells along with the secretion of cytokines by immature dendritic cells at much higher levels than obtained with monoligated SARS-CoV VLPs (Bai et al. 2008a), a finding of great utility in view of the need for strong cellular immunity on top of humoral neutralizing responses. Chimeric SARS-CoV VLPs were also successfully produced in insect cells by coexpression of the SARS-CoV S protein combined with the influenza M1 protein (Liu et al. 2011), which immunogens protected mice against infection without the need for adjuvants as opposed to the full-length S protein alone, which did require adjuvants. Chimeric VLPs like these could prove a powerful option since influenza antigens can stimulate the memory of CD4 cells from past infections and accelerate the activation of the antigen-specific B-cells against coronaviruses. Furthermore, VLPs produced in insect cells were also effective against MERS-CoV infection in nonhuman primates and mice, through eliciting high specific antibody titers and Th1 cellular responses (Wang et al. 2017a, b).

The administration route is also an essential aspect to consider against respiratory infections such as coronaviruses because of the need for a mucosal-antibody response to combat viral upper respiratory tract replication. The nasal administration in mice of VLPs produced in insect cells induced production of detectable IgA against the S protein in saliva, pulmonary mucosa, intestines, and the urinary tract; and although serum IgG levels were higher with intraperitoneal administration, the neutralizing activity was higher with intranasal administration (Lu et al. 2010), thus proving the versatility and effectiveness of the baculovirus system for these kinds of strategies.

The study of VLPs usually concentrates on two main topics, the particle assembly and the ability to elicit an immune response, but as indicated by Naskalska et al. (2018), the SARS-Cov-2 VLPs can also be useful as delivery vectors for cells expressing the angiotensin-converting enzyme 2, the specific receptor for SARS-CoV-2 (Naskalska et al. 2018). These kinds of vectors would exhibit a narrow tissue tropism, similar to what was observed with antibody–drug conjugates, thus highlighting one more tool that the baculovirus provides in the fight against coronaviruses and other infections.

Baculoviruses as tools for the treatment of noninfectious human diseases via gene therapy

Baculoviruses represent a flexible tool for producing biologics for therapeutic purposes. Certain recombinant proteins produced in BEVS are being analyzed for the treatment of

noninfectious diseases and are in different stages of medical evaluation. Already on the market is a recombinant protein (Provenge®) produced in BEVS for the treatment of prostate cancer developed by Dendreon (Kumar et al. 2018), but in recent years, most of the studies have been directed mainly toward evaluating the use of baculoviruses for gene therapy-based treatment, either by producing adeno-associated viruses (AAVs) or through the BacMam technology.

BEVS associated with gene therapy: baculovirus and AAV

Gene therapy is a medical procedure that bases the treatment of diseases on the use of genetic sequences as active ingredients. Accordingly, different virus species have been engineered to generate viral vectors that enable the possibility of directing the active ingredients to the targeted cells—via approaches involving an *in vivo* administration to the patient's body or an *ex vivo* modification of patient cells or tissues similar to a cell therapy followed by reintroduction into the patient—but without the recombinant's replication or progeny generation in either approach. Among the most widely studied options that have reached the pharmaceutical market are gene therapies mediated by AAVs (Ginn et al. 2018).

AAVs are small (about 20–25 nm) icosahedral nonenveloped single-strand (ss-) DNA viruses (genus: *Dependovirus*; family: *Parvoviridae*) whose infections in humans manifest no significant clinical consequences despite being very widespread. In particular, AAVs require helper viruses (*e.g.*, adenoviruses, herpes-simplex viruses) to sustain their generation of progeny. The ssDNA (about 4.7 kb in length) is flanked by inverted terminal repeats (ITRs; of about 145 b, generating hairpin structures) and encode two sets of proteins: those associated with replication (Rep proteins); and the virion structural elements (Cap proteins). The ITRs govern the main processes of the multiplication cycle—*i.e.*, double-stranded (ds-) DNA generation, replication, packaging—and, together with the Rep proteins, are responsible for the possible genomic integration (at a *locus* located in the long arm of chromosome 19) when helper viruses are not present (Mezzina and Merten 2011). AAV-based viral vectors have been extensively evaluated for gene therapy because of their simplicity, satisfactory immune profile, the occurrence of serotypes with different tissue tropisms, and the ability to transduce nondividing cells, thus ensuring a long-term expression of the transgene—*n. b.*: the nucleic acid remains episomal in the absence of Rep proteins (Keeler and Flotte 2019). In essence, the recombinant AAVs (rAAV) carry an ssDNA that contains the gene of interest (GOI, which is replacing certain viral genes) flanked by the ITRs. Products such as Glybera® (alipogene tiparvovec), Luxturna® (voretigene neparvovec), or Zolgensma®

(onasemnogene abeparvovec) are AAV-based gene therapies to treat lipoprotein-lipase deficiency, inherited retinal diseases, and spinal muscular atrophy, respectively (Ginn et al. 2018; Keeler and Flotte 2019).

The initial procedures for producing rAAVs involved transfecting mammalian-cell lines (mainly human-embryonic-kidney HEK 293 cells) with plasmids that carried the therapeutic sequence and the ITRs, plus other helpers with the AAV genes and those needed from other viruses (generally, adenovirus genes). Although undergoing improvements over time with the purpose of achieving scalable systems (including, for example, the development of mammalian packaging cells), the approach did not enable productions in the yield necessary for therapy in human beings (Galibert and Merten 2011). Those limitations led to studies that evaluated the rAAV production by complementation systems that involved adenoviruses, herpes-simplex viruses, or baculoviruses (Aponte-Ubillus et al. 2018). In the first report where BEVS were employed for this purpose, three rec-baculoviruses were used (rep and cap genes in two independent BVs and the ITR-GOI-ITR construct in the third: the so-called *ThreeBac* system) along with Sf9 cells (Urabe et al. 2002). Subsequently, different optimizations were performed including the use of only two rec-baculoviruses—*i.e.*, the TwoBac system; one combining both the rep and the cap genes and the other carrying the ITR-GOI-ITR (Wu et al. 2019) plus the later generation of a OneBac system consisting of packaging-Sf9 cells that expressed rep and cap genes when infected with a rec-baculovirus that carried the therapeutic nucleic acid (Mietzsch et al. 2015). Subsequent optimizations of the OneBac platform enabled quite good yields of whole particles with little contaminating DNA (Mietzsch et al. 2015; Joshi et al. 2019). In another study, an alternative OneBac system was developed that involved a rec-baculovirus expressing both rep and cap genes and carrying the ITR-GOI-ITR construct (Wu et al. 2018). What is interesting to us about this work is that these studies enabled the insect larval platform to produce rAAV, thus significantly lowering the overall costs. In another report, the genomic stability of rec-baculoviruses was evaluated in accordance with the genetic-engineering platforms to produce those recombinants, either through transposons or homologous recombination (Aurelién et al. 2021). All these current contributions are enabling the insect-cell-rAAV-production platform to remain a viable alternative for the gene-therapy industry, thus underscoring that approach as a principal milestone after Glybera® was produced by a ThreeBac system.

One of the main problems in rAAV production is the generation of empty capsids or capsids carrying contaminating DNA. For this reason, all the technologies developed for this purpose not only aim at high general yields but also must ensure a definitive production of complete particles free of helper DNA. These remaining aspects of the present state

of the art constitute a significant challenge that makes this bioprocess one that still requires critical improvement on any of the production platforms and, of course, leaves open the opportunity for innovations.

BacMam technology associated with gene therapy

In addition to the application of BEVS to produce protein-based drugs for numerous noninfectious human diseases, baculoviruses can also be used as viral gene-delivery vectors for therapeutic or immunogenic purposes. These BacMam applications emerged in the mid-1990s with the report that BVs constructed with the prototype species AcMNPV could transduce, but would not infect, mammalian cells (Hofmann et al. 1995); thus enabling the expression of a given GOI assembled with a typical mammalian-gene syntax (as opposed to the insect syntax used when applying BEVS or a baculovirus display platform). Later ODVs (Fig. 1C) were found to not possess this capability (Mäkelä et al. 2008), with the result that the range of the baculovirus species sharing this characteristic was then expanded by adding studies about the BVs of *Bombyx mori nucleopolyhedrovirus* (Kenoutis et al. 2006; Liu et al. 2017) and the *Anticarsia gemmatalis nucleopolyhedrovirus* AgMNPV (Parsza et al. 2020). In particular, these benefits in mammalian cells were reported to be due to the GP64 protein present in the BV envelopes of group 1-alphabaculoviruses (Kataoka et al. 2012; Luz-Madrigal et al. 2013); consequently narrowing down the feasibility of such applications to this specific subgroup of species, for which the viral entry mechanism would be based on clathrin-mediated endocytosis (Fujita et al. 2006; Liu et al. 2014; Hu et al. 2019b). Even variants of lentiviruses containing different GP64 proteins evidenced cell-expanded transduction efficiencies (Sinn et al. 2017).

The reasons that led so many researchers to explore this application of AcMNPV BVs (Ono et al. 2014; Mansouri and Berger 2018) resides mainly in biosafety issues: quite simply stated, the virus does not infect mammals (Kost and Condreay 2002) and furthermore exhibits a natural efficiency in transducing different mammalian cells including stem cells (Airenne et al. 2011). In addition to those essential properties, the virus is easy to manipulate for generating recombinant virions (Luckow et al. 1993; Airenne et al. 2011) and producing those constructs on an industrial scale (He et al. 2005; Kwang et al. 2016); possesses an extensive capacity for carrying exogenous DNA of up to at least 38 kbp, thus enabling the insertion of several GOIs at once (Cheshenko et al. 2001); and is characterized by an excellent immunogenic profile when administered in vivo as a result of the lack of preexisting immunity in mammals (Bocca et al. 2013). Another advantage is the compatibility of the baculovirus system carrying GOIs that will express suicide proteins in mammalian cells (useful in cancer treatments),

but not in insect cells (where the viral vector is produced) owing to the use of introns and other typical elements of mammalian genetics (Chen 2019).

Despite these definitive benefits, the usually short-expression time in vivo of the GOI in mammals (around 7–14 days postinoculation) should be considered, which timing limits readministrations (Luo et al. 2013), mainly because of certain generic immunologic responses that baculoviruses trigger—those being more pronounced upon systematic administration, though lesser upon local inoculation (Ono et al. 2014). Among such responses, the innate immune system is a clear example because the administration of BVs has been reported to upregulate the RIG-1-like receptor (Balasundaram et al. 2017) and the Toll-like-receptor-9 signaling pathways (Abe et al. 2009; Boulaire et al. 2009), thus activating type-I interferon (Hervas-Stubbs et al. 2007; Abe et al. 2009) and natural-killer cells (Moriyama et al. 2017). In addition, a few other genes are induced by baculoviruses, such as those encoding complement factors and adhesion molecules, or those involved in cytokine-cytokine receptor interaction (Shin et al. 2020). Other in vitro studies revealed that the cGAS-STING nucleic-acid-sensing pathway would act as a decisive agent in generating an antiviral state produced by interferon types I and III (Amalfi et al. 2020). In this regard, the unmethylated CpG DNA of baculoviruses would be one of the main causes of the low transgene expression in certain cells triggering the different responses mentioned (Ono et al. 2018). At around the same time, baculovirus-transduced cell transplantation in animal models (associated with *ex-vivo*-gene-therapy approaches) gave very good results and did not elicit a systemic induction of monocytes and CD8 + T cells as did other viruses (Chuang et al. 2009). Another relevant aspect to be considered is regarding the GP64 viral protein of BVs (Fig. 1C). Although this fusogenic factor is useful and sufficient for the baculoviral Ncs to enter the cells and then, in turn, penetrate the nucleus in an actin-dependent manner (Fujita et al. 2006; Long et al. 2006), that factor—though generally considered polytropic—can manifest differences according to the mammalian cells targeted (Airenne et al. 2011; Kataoka et al. 2012; Luz-Madrigal et al. 2013).

Nevertheless, a lot of effort is being made to overcome the limitations (Aulicino et al. 2020) that other viral vectors used in gene therapy do not escape, where those vectors can generate much greater immune responses as a result of being derived from mammalian pathogens and have other serious disadvantages including the limited duration of the transgenes they carry and the costs of scaled-up production. Thus, several investigations have been reported that have offered improvements in baculoviral vectors for BacMam-type applications. In this, list of developments can be mentioned: (i) the pseudo-typed BVs, which constructs express in insect cells the stomatitis-vesicular-virus glycoprotein

(Barsoum et al. 1997; Kolangath et al. 2014) or other factors from eukaryote parasites and viruses (Tamura et al. 2016; Hu et al. 2019a) to increase transduction capability in mammals; (ii) the development of baculoviral-hybrid vectors that achieve a longer duration of the GOI expression by, for example, the presence of the ORI P of the Epstein-Barr virus, either alone (Shan et al. 2006; Suzuki et al. 2009) or combined with the FLP/FRP, ϕ C31/attB-P or Cre/Loxp recombinase systems (Lo et al. 2009, 2017; Sung et al. 2013); (iii) the inclusion of the inverted terminal repeats and the Rep gene of the adeno-associated virus (Wang 2008) or the inverted terminal repeats and the transposase gene of the Sleeping-Beauty transposon (Turunen et al. 2014); and (iv) genome modifications to increase the BV yield in insect cells (Graves et al. 2018), among other optimizations. In addition, progress has been made in different formulations to achieve better results after the administration of recBVs in mammals. Thus, binary complexes between rec-baculoviruses and nanoparticles composed of the HIV trans-activator-of-transcription protein and DNA molecules encoding therapeutic genes have been studied (Paul et al. 2011) to combine the benefits of viral horizontal-gene-transfer systems with those based on nonviral complexes. Similar efforts have been made involving galactosylated-polyethyl-eneimine–DNA complexes (Kim et al. 2009), cationic poly-amidoamine-dendrimer synthetic nanoparticles (Paul et al. 2013), or nanomagnetic particles (Zhu et al. 2020) in combination with rec-baculoviruses. This last approach, combined with the use of magnetic fields, helps overcome serum inactivation and achieves local expression of the GOI after even administrations that are systemic. Moreover, recombinant BVs that express complement-regulatory proteins on the virion surfaces exhibited a higher complement resistance than nonmodified variants (Kawai et al. 2018), while other studies tested the use of different compounds in baculovirus formulations to inhibit the complement system (Kaikkonen et al. 2010) or to improve transduction by decreasing the endosomal pH (Hu et al. 2019b). Of interest to this field was that baculoviruses expressing the adenovirus receptor have been developed to perform combined gene therapies with both viral vectors (Hong et al. 2017). Furthermore, the downstream processing of vectors during *in vivo* expansion received attention in order to improve the quality and quantity of BVs recovered from infected insect cells (Kwang et al. 2016; Nasimuzzaman et al. 2018) including the development of different chromatographic methods (Nasimuzzaman et al. 2016; Lothert et al. 2020).

Although no BV-vector-based therapies have yet reached a clinical stage, several biodistribution and efficacy studies in different animal models utilizing recombinant AcMNPVs have been performed in the last 10 years. These approaches were designed mainly for the treatment of cancer, heart and/or vascular diseases, and tissue engineering and/or

regenerative medicine and include preclinical assays in mice, rats, rabbits, dogs, and pigs with various types of therapeutic sequences and different BV technologies (Table 4).

We need to mention that certain studies were carried out in nonhuman primates (Balasundaram et al. 2017) or using whole-human-blood samples (Georgopoulos et al. 2009) and cells from human donors (Bak et al. 2011; Paul et al. 2011; Graves et al. 2018; Wang et al. 2019a, 2020). In addition, owing to the excellent transport capability of baculoviruses, new approaches in synthetic biology are being explored *in vitro* that enable, for example, the incorporation of complex gene circuits that can express a therapeutic gene in target cells and not in others (Lin et al. 2018).

Furthermore, *in vivo* and *ex vivo* studies involving animals and human cells have demonstrated the feasibility and safety of a BV-mediated gene therapy, thus creating new possibilities for the future approval of clinical trials and giving us the ability to realistically imagine the clinical approval of the first BV-based gene therapy in the near future. Overall, BVs have been used to treat several noninfectious diseases but mostly are in use for cancer therapy and tissue engineering and/or regenerative medicine.

Concluding remarks and perspectives

Baculoviruses, and especially AcMNPV, have been exploited in diverse biotechnological uses for years, generating products and conceptual tests in different technological fields that include agriculture (the first and most obvious biotech use), but also animal and human health. All these new technological opportunities emerged because of the basic studies carried out on baculoviruses in general, and on AcMNPV in particular—it is chosen as the prototype of this family, which includes more than 80 species and hundreds of isolates (Fig. 3). Although baculoviruses (or at least their effects) have been known since the nineteenth century through the consequences they produced in colonies of *B. mori* larvae reared for silk production, only by the middle of the twentieth century did the baculoviruses begin to be identified as viral entomopathogens (Rohrmann 2019). In the early 1970s, a baculovirus isolated from *Autographa californica* larvae began to be extensively characterized (Vail et al. 1971). Thus, owing to the development of the insect-cell lines Sf21, Sf9, and High Five™ that were useful for multiplying viruses under laboratory conditions (Vaughn et al. 1977; Wickham et al. 1992), AcMNPV virions became subjected to multiomics studies that included the genome sequencing (Ayres et al. 1994), the determination of differential gene expression by means of transcriptomic approaches (Yamagishi et al. 2003; Chen et al. 2013), and the identification of the proteomic composition from both BVs (Wang et al. 2010) and ODVs (Braunagel et al. 2003).

Table 4 Some successful preclinical gene-therapy assays in mammals based on BacMam technology

Disease	AI (sequence)	Type of vector	Animal model	In vivo/ ex vivo	Dose (PFU)	Reference
<i>Cancer</i>						
Hepatoma	Gene encoding Apoptin from the chicken anemia virus	Bac to Bac™ (Thermo Fisher) Pseudotyped with VSV-G	Mice	In vivo (intra-tumor)	$1 \times 10^8 - 1 \times 10^{10}$	Pan et al. (2010)
Glioblastoma	Gene encoding thymidine kinase from herpes simplex virus	Bac to Bac™ (Thermo Fisher)	Mice	Ex vivo (human MSC-like cells transduced)	MOI 100	Bak et al. (2011)
Prostate and ovarian tumors	Gene encoding an antiangiogenic fusion protein	Bac to Bac™ (Thermo Fisher) Hybrid system with Sleeping Beauty transposon	Mice	In vivo (intra-tumor)	3×10^9	Luo et al. (2013)
Hepatocellular carcinoma	Gene expressing LncRNA PTENP1	Hybrid system with Sleeping Beauty transposon	Mice	In vivo (intra-tumor)	1×10^9 Bac-SB + 2×10^8 Bac-GOI	Chen et al. (2015)
Bladder tumor	Genes encoding CD40 ligand and IL-15	BacPAK™ (Clontech)	Mice	In vivo (intra-tumor)	1×10^8	Ang et al. (2016)
Pituitary tumor	Gene expressing shRNA	BacPAK™ (Clontech)	Mice	In vivo (intra-tumor)	1×10^8	Gottardo et al. (2018)
Hypopharyngeal carcinoma	Gene encoding sodium iodide symporter (NIS)	Bac to Bac™ (Thermo Fisher) Hybrid system with AAV	Mice	Ex vivo (human bone marrow mesenchymal stem cells -BMSCs- transduced) combined with I therapy	MOI 400	Wang et al. (2019a); Wang et al. (2020)
<i>Cardiovascular diseases</i>						
Myocardial infarction	Gene encoding angiopoietin-1	BaculoGold™ (BD Biosciences) and Tat/DNA nanoparticles	Rats	In vivo (peri-infarct regions)	1×10^{10}	Paul et al. (2011)
Atherosclerosis related cardiovascular diseases	Gene encoding vascular endothelial growth factor (VEGF)	Bac to Bac™ (Thermo Fisher) Evaluation variants Pseudo-typed with VSV-G	Rabbits	In vivo (skeletal muscle)	1×10^9	Heikura et al. (2012)
Myocardial infarction	Gene encoding angiopoietin-1	BaculoGold™ (BD Biosciences) and Tat/DNA nanoparticles	Rats	Ex vivo (modified human adipose tissue derived stem cells -hASCs- transduced)	MOI 100	Paul et al. (2012)
In-stent restenosis	Gene encoding vascular endothelial growth factor (VEGF)	BaculoGold™ (BD Biosciences) with cationic Polyamidoamine dendrimer synthetic nanoparticles (PAMAM)	Dogs	In vivo (baculovirus based gene eluting stent)	5×10^{12}	Paul et al. (2013)

Table 4 (continued)

Disease	AI (sequence)	Type of vector	Animal model	In vivo/ ex vivo	Dose (PFU)	Reference
Myocardial infarction	Gene encoding vascular endothelial growth factor (VEGF)	Bac to Bac™ (Thermo Fisher). Use of Bac-GOI/ORI P (EBV) + Bac-recombinase (FLP)	Rabbits	Ex vivo (rabbit adipose-derived stem cell -ASC-transduced)	MOI 150 Bac-GOI/ORI P + MOI 50 Bac-FLP	Yeh et al. (2014)
Limb ischemic disease	Gene encoding vascular endothelial growth factor (VEGF)	Bac to Bac™ (Thermo Fisher). Use of Bac-GOI/ORI P (EBV) + Bac-recombinase (FLP)	Mice	Ex vivo (mouse adipose-derived stromal cells -ADSC-transduced)	MOI 150 Bac-GOI/ORI P + MOI 15 Bac-FLP	Makarevich et al. (2015)
Ischaemia-reperfusion (I/R) injury associated with kidney transplantation	Gene encoding manganese superoxide dismutase (SOD-2)	flashBAC ULTRA™ DNA (Oxford Expression Technologies Ltd)	Porcine kidneys	Ex vivo (human kidney 2 -HK-2- cells transduced)	MOI 150	Hitchman et al. (2017)
Peripheral arterial disease	Gene encoding a mutant oxygen-resistant hypoxia-inducible factor 1-alpha (mHIF-1 α)	Bac to Bac™ (Thermo Fisher)	Rabbits	In vivo (skeletal muscle)	1 \times 10 ⁹	Giménez et al. (2020)
<i>Tissue engineering/regenerative medicine</i>						
Massive segmental bony defects following trauma or tumor resection	Genes encoding bone morphogenetic protein-2 (BMP2) and vascular endothelial growth factor (VEGF)	Bac to Bac™ (Thermo Fisher) Use of Bac-GOI/ORI P (EBV) + Bac-recombinase (FLP)	Rabbits	Ex vivo (rabbit adipose-derived stem cells -ASCs-transduced)	MOI 100 Bac-GOI/ORI P + MOI 100 Bac-FLP	Lin et al. (2011); Lin et al. (2014)
Massive segmental bony defects following trauma or tumor resection	Genes encoding bone morphogenetic protein-2 (BMP2) and vascular endothelial growth factor (VEGF)	Bac to Bac™ (Thermo Fisher) Use of Bac-GOI/ORI P (EBV) + Bac-recombinase (FLP optimized)	Minipigs	Ex vivo (rabbit adipose-derived stem cells -ASCs-transduced)	MOI 150 Bac-GOI/ORI P + MOI 100 Bac-FLP	Lin et al. (2015)
Osteoporosis	Gene expressing a miR-214 sponge	Bac to Bac™ (Thermo Fisher) Use of Bac-GOI/ORI P (EBV) + Bac-recombinase (Cre)	Rats	Ex vivo (rat adipose-derived stem cells -ASCs-transduced)	MOI 150 Bac-GOI/ORI P + MOI 100 Bac-Cre	Li et al. (2016); Li et al. (2017)
Degenerative disc disease	Gene encoding bone morphogenetic protein-7 (BMP-7)	Bac to Bac™ (Thermo Fisher)	Rats	Ex vivo (rat bone marrow-derived mesenchymal stem cells -BMDMSC-transduced)	MOI 25	Liao (2016a)
Unstable spine	Gene encoding bone morphogenetic protein-7 (BMP-7)	Bac to Bac™ (Thermo Fisher)	Rabbits	Ex vivo (rat bone marrow-derived mesenchymal stem cells -BMDMSC-transduced)	MOI 25	Liao (2016b)

Table 4 (continued)

Disease	AI (sequence)	Type of vector	Animal model	In vivo/ ex vivo	Dose (PFU)	Reference
Rheumatoid arthritis	Gene encoding Coxsackievirus receptor (CAR) in BV + human adenovirus type 5 (HAdV5)-PUMA (p53 upregulated modulator of apoptosis)	Bac-N-Blue™ (Thermo Fisher)	Rats	In vivo (intra-articular)	1×10^5 BVs + 1×10^9 HAdV5-PUMA	Hong et al. (2017)
Peripheral nerve injuries	Gene encoding CRISPRa system for multiplexed activation of brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF) and nerve growth factor (NGF) genes	Bac to Bac™ (Thermo Fisher) Use of Bac-GOI/ORI P (EBV) + Bac-recombinase (Cre)	Rats	Ex vivo (rat adipose-derived stem cells -ASC- transduced)	MOI 150 Bac-GOI/ORI P + MOI 100 Bac-Cre	Hsu et al. (2019)
<i>Other diseases</i>						
Hepatic encephalopathy	Gene encoding the glutamine synthetase	Bac to Bac™ (Thermo Fisher)	Rats	In vivo (intramuscular)	9×10^7	Torres-Vega et al. (2015)

AI active ingredient; PFU plaque-forming unit; MOI multiplicity of infection; AAV adeno-associated virus; GOI gene of interest; SB Sleeping Beauty transposase; EBV Epstein-Barr virus; HAdV human adenovirus

(Ignoffo 1999), most the other products registered for vertebrates (human and nonhuman) are derived from this species. In this regard, the statement is appropriate that the first 4 commercial products reaching the market derived from the use of BEVS platforms in human health were Cervarix® (a human VLP vaccine against human papillomavirus; GlaxoSmithKline, 2007); Provenge® (a recombinant protein for prostate-cancer immunotherapy; Dendreon, 2010); Flublok® (a human-subunit vaccine against influenza; Protein Sciences Corporation, 2013); and Glybera® (human gene-therapy product for the treatment of lipoprotein-lipase deficiency based on recAAV produced by BEVS) (Kumar and Gong 2018). In addition, 8 products for veterinary use produced in BEVS are already on the market. Among those that can be highlighted are Porcilis Pesti (MSD), the first veterinary subunit vaccine against swine flu, and Circumvent PCV (MSD), the first veterinary VLP vaccine against swine circovirus type 2, both produced in insect cells. In addition, Virbagen® Omega (Virbac) was the first veterinary protein therapy produced in insect larva for the treatment of feline viral diseases.

In the coming years, owing to the development of more extensive knowledge about baculoviruses, which new perspective will enable the development of the next generation of vectors for producing better-engineered virions that would be more useful for the aforementioned applications and for other innovations, we could expect that these technologies will be well valued by and appropriate for more human- and veterinary-health companies. Furthermore—and in view of the approvals received in recent years by various human-gene-therapy products based on viral vectors (Ginn et al. 2018) and the new generation of vaccines that use virions (Rawat et al. 2021)—we would expect that in this third decade of the twenty-first century, clinical trials in humans may begin to promote BacMam technologies and bring those procedures to the pharmaceutical market (Aulicino et al. 2020). The usefulness of the application of baculoviruses in human and animal health is already indisputable. The joint efforts between the baculovirus-genome engineering and improvement in the production and the downstream processes will enable baculovirus and/or insect-cell systems to be more productive and less expensive. Moreover, a greater number of registered products will favor a higher level of knowledge in regulatory agencies. All these propitious conditions in combination will enhance the use of baculoviral systems and derived technologies in the pharmaceutical industry.

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Declarations

Ethics statement This article does not contain any studies with human participants or animals performed by any of the authors.

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