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Review

# Recombinant protein vaccines produced in insect cells

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ABSTRACT

The baculovirus-insect cell expression system is a well known tool for the production of complex proteins. The technology is also used for commercial manufacture of various veterinary and human vaccines. This review paper provides an overview of how this technology can be applied to produce a multitude of vaccine candidates.

The key advantage of this recombinant protein manufacturing platform is that a universal "plug and play" process may be used for producing a broad range of protein-based prophylactic and therapeutic vaccines for both human and veterinary use while offering the potential for low manufacturing costs. Large scale mammalian cell culture facilities previously established for the manufacturing of monoclonal antibodies that have now become obsolete due to yield improvement could be deployed for the manufacturing of these vaccines. Alternatively, manufacturing capacity could be established in geographic regions that do not have any vaccine production capability. Dependent on health care priorities, different vaccines could be manufactured while maintaining the ability to rapidly convert to producing pandemic influenza vaccine when the need arises.



**Contents**



## 1. Introduction

The majority of World Health Organization (WHO) recommended vaccines for routine immunization are derived from killed (inactivated) or live-attenuated infective agents. An overview of the twenty recommended vaccines, the etiological agent, the disease impact and the method of manufacturing is provided in Table 1. Nine are live-attenuated vaccines (LAV), which have historically been created by passaging a pathogenic organism in cultured cells. Such vaccines are almost or completely devoid of pathogenicity; however, this empirical attenuation may be unreliable and poses potential safety issues, i.e. reversion to pathogenic genotype. Advances in molecular virology are providing new ways of controlling viral replication and virulence and may lead to the new generation of safer, more widely applicable LAV vaccines [23]. The manufacturing of LAV requires growth of attenuated strains in large quantities. Thirteen (13) of these vaccines are inactivated or derivates of pathogens such as polysaccharides. These vaccines, while alleviating the potential safety concern posed by LAV, are obtained by cultivating often highly pathogenic organisms in large quantities that pose a potential exposure risk for the workers and the environment. Only two (2) out of twenty (20) recommended vaccines are recombinant protein vaccines. The first available recombinant sub-unit Hepatitis B vaccines, ENGERIX-B® (GSK) or RECOMBIVAX HB® (Merck), were licensed in 1986 and gradually replaced the plasma-derived hepatitis B vaccine [2]. This vaccine is a purified surface antigen (HBsAg) of the virus obtained by culturing genetically engineered *Saccharomyces cerevisiae* (*S.*

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**Table 1**
Vaccines recommended for routine immunization.

| Vaccine | Etiological agent | Disease impact | Production method |
|---|---|---|---|
| **Recommendations for all** | | | |
| BTG [1] | *Mycobacterium tuberculosis* (Mtb) | Tuberculosis (TB) – leading cause of human disease and death, particularly in developing countries. 16–20 million cases of TB worldwide, more than 8 million new cases and over 1.8 million deaths each year | Inactivated vaccine derived from *M. bovis* |
| Hepatitis B [2] | Hepatitis B virus (HBV) (*Hepadnaviridae*) | >2 billion people infected; ~360 million chronically infected and at risk of serious illness and death, mainly from liver cirrhosis and hepatocellular carcinoma | Recombinant vaccine produced in *S. cerevisiae* |
| Polio [3] | Polio virus serotypes (types 1, 2 or 3) (*Picornaviridae*) | Poliomyelitis is an acute communicable disease of humans; vaccination has led to polio control (and, since 1988, polio eradication) | Inactivated or live-attenuated oral vaccine derived from three serotypes |
| DTP (Diphtheria [4], Tetanus [5] and Pertussis [6]) | *Corynebacterium diphtheriae*, *Clostridium tetani* and *Bordetella pertussis* | Diphtheria is an acute disease caused by exotoxins from *C. diphtheria*; vaccination has resulted in case reduction of >90% (1980–2000); tetanus causes approximately 213,000 death annually; Pertussis (whooping cough) is an important cause of death in infants worldwide, est. 195,000 in 2008 | Inactivated vaccine based on growth of *C. diphtheria*; toxigenic strains of *C. tetani*; selected *B. pertussis* strains |
| Haemophilus influenzae [7] | *Haemophilus influenzae* type b (Hib) | Hib is estimated to be responsible for ~3 million cases of serious disease every year and ~386,000 deaths | Inactivated vaccine based on polyribosylribitol phosphate (PRP) (the capsular polysaccharide of Hib) conjugated to protein carrier |
| Pneumococcal (conjugate) [8] | *Streptococcus pneumoniae* | Most common cause of community-acquired bacterial pneumoni. WHO estimated in 2005 that 1.6 million people die of pneumococcal disease every year | Inactivated vaccine based polysaccharides derived from various serotypes, each conjugated to the non-toxic diphtheria CRM 197 protein |
| Rotavirus [9] | Rotavirus (*Reoviridae*) | Causes severe diarrhoeal disease in young children; 2004 estimates by WHO, 527,000 children aged <5 years | Live-attenuated vaccine |
| Measles [10] | Measles virus (*Paramyxoviridae*) | In 2007, worldwide coverage of the first dose of measles vaccine reached 82%; between 2000 and 2007, the estimated number of deaths from measles dropped from 750,000 to 197,000 | Live-attenuated vaccine originate from the Edmonston strain of measles virus, isolated by Enders and Peebles in 1954 |
| Rubella [11] | Rubella virus (*Togaviridae*) | Rubella is an acute, usually mild viral disease traditionally affecting susceptible children and young adults worldwide; large epidemics can lead to high levels of morbidity | Live-attenuated vaccine mostly based on RA 27/3 strain which is propagated in human diploid cells |
| HPV [12] | Human papilloma virus (>100 subtypes) (*Papillomaviridae*) | Viruses associated with cancers of the cervix, vagina, vulva, penis and anus; a subset of head and neck cancers; anogenital warts; and recurrent respiratory Papillomatosis. In 2005, there were about 500,000 cases of cervical cancer and 260,000 related deaths worldwide | Recombinant vaccine; purified L1 structural proteins produced in *S. cerevisiae* (GARDASIL®) or BEVS (CERVARIX®) |
| **Recommendations for certain regions** | | | |
| Japanese encephalitis [13] | Japanese encephalitis (JE) virus (*Flaviviridae*) | Japanese encephalitis (JE) is the most important form of viral encephalitis in Asia. The JE virus causes at least 50,000 cases of clinical disease each year, mostly among children aged <10 years, resulting in about 10,000 deaths and 15 000 cases of long-term, neuro-psychiatric sequelae | Live-attenuated vaccine produced in cell culture or inactivated vaccine grown in mice brain. |
| Yellow Fever [14] | Yellow fever virus (*Flaviviridae*) | Yellow fever (YF) is a mosquito-borne, viral hemorrhagic fever that is endemic in tropical regions of Africa and South America. WHO estimates that a total of 200,000 cases of YF occur each year, with about 30,000 deaths | Live-attenuated vaccine based on a wildtype YF virus (the Asibi strain) |
| Tick-borne encephalitis [15] | Tick-borne encephalitis virus (*Flaviviridae*) | Important cause of viral infections of the central nervous system in various geographic regions. Approximately 10,000–12,000 clinical cases of tick-borne encephalitis are reported each year | Inactivated vaccine produced in chicken embryo cells |
| **Recommendations for some high-risk populations** | | | |
| Typhoid [16] | *Salmonella enterica* serovar typhi | Typhoid fever is a serious systemic infection caused by the enteric pathogen *S. typhi*. WHO estimates the annual global incidence of typhoid fever at 21 million cases, of whom 1–4% end fatally | Live-attenuated vaccine or subunit vaccine consisting of purified Vi capsular polysaccharide from the Ty2 S. strain |

Table 1 (*Continued*)

| Vaccine | Etiological agent | Disease impact | Production method |
|---|---|---|---|
| Cholera [17] | *Vibrio cholerae* | Cholera is a rapidly dehydrating diarrhoeal disease caused by ingestion of toxigenic serogroups (O1 and less commonly O139) of *Vibrio cholerae*. WHO estimates 178,000–237,000 cases of cholera and 4000–6300 deaths from cholera | Widely used vaccine = monovalent inactivated vaccine based on formalin and heat-killed whole cells (WC) of *V. cholerae* O1 (classical and El Tor, Inaba and Ogawa) plus recombinant cholera toxin B subunit |
| Meningococcal [18] | *Neisseria meningitides* (various serotypes) | Meningococcal disease is associated with high case-fatality rates (5%, 15%); Globally, about 500,000 cases and 50,000 deaths are caused by this pathogen each year | Purified, heat-stable, lyophilized capsular polysaccharides from meningococci of the respective serogroups |
| Hepatitis A [19] | Hepatitis A virus (HAV) (*Picornaviridae*) | Hepatitis A is an acute, usually self-limiting disease of the liver with an estimated 1.5 million clinical cases occurring annually | Inactivated vaccine produced in cell culture |
| Rabies [20] | Rabies virus (RABV) (*Rhabdoviridae*) | The vast majority of the estimated 55,000 deaths caused by rabies each year occur in rural areas of Africa and Asia | Inactivated vaccine produced in cell culture or embryonated eggs |
| **Recommendations for immunization programs with certain characteristics** | | | |
| Mumps [21] | Mumps virus (*Paramyxoviridae*) | Viral infection mostly occurring in children, primarily affecting the salivary glands | Live-attenuated vaccine |
| Influenza [22] | Influenza virus (*Orthomyxoviridae*) | Influenza virus types A and B are both common causes of acute respiratory illnesses | Trivalent inactivated vaccine or live-attenuated vaccine produced in chicken embryo cells or cell culture |

*cerevisiae*) cells. The antigen is purified by several physicochemical steps and formulated as a suspension of the antigen adsorbed on aluminum hydroxide. It took nearly twenty years before the next recombinant vaccines, GARDASIL® (Merck) and CERVARIX®, (GSK) were licensed [12]. This human papilloma virus vaccine consists of purified L1 structural protein (major capsid) produced either in *S. cerevisiae* cells (GARDASIL) or in the baculovirus expression vector system (CERVARIX). The advantage of recombinant vaccines is that they do not contain the pathogen or its genetic material and therefore cannot cause disease. In addition, recombinant vaccines do not depend on the cultivation of (pathogenic) organisms and offer the potential to utilize flexible multipurpose manufacturing facilities. However, both inactivated and recombinant vaccines have in general been less efficacious than their LAV counterparts and, therefore, often require the use of adjuvants.

The baculovirus-insect cell expression system, often referred to as BEVS, is well known as a tool for producing complex proteins, and providing rapid access to biologically active proteins. This protein production platform has been extensively explored for the production of viral and parasitic antigens [24] and, more recently, vaccines have been commercialized demonstrating its potential as a commercial manufacturing technology [25]. Baculoviruses are insect pathogens that can cause fatal disease in lepidopteran, dipteran and hymenopteran larvae, resulting in their use as biocontrol agents of insect pests in agriculture and forestry. They are characterized by their narrow host range [26] and their inability to replicate in vertebrates, including man. Baculoviruses are commonly found on green vegetables and, therefore, are part of the daily diet of healthy individuals. For example, a typical serving of coleslaw contains 112 million polyhedra, each containing multiple baculovirus virions [27]. The baculovirus particles or virions contain a large double-stranded DNA genome that on average, depending on the virus species, is 130 kb pairs in size. It can be easily characterized, genetically manipulated and propagated in cell lines derived from a.o. the fall armyworm *Spodoptera frugiperda* (SF) or the cabbage looper *Trichoplusia ni* (*T. ni*) [28], both of which grow well in suspension cultures [29].

Summers and Smith demonstrated in the 1980s that polyhedrin, the major capsule protein, was not essential for the propagation of the virus in a cell cultures and that its open reading frame could be exchanged for sequences encoding proteins of medical importance such as β-interferon [30]. This marked the beginning of the BEVS expression era and since then thousands of proteins have been produced using the polyhedrin promoter or later the p10 promoter to drive expression. Insect cells have the capability of performing many of the post-translational modifications such as glycosylation, disulfide bond formation and phosphorylation required for the biological activity of many complex proteins [31]. The protein of interest is usually produced under the control of the polyhedrin promoter, one of the strongest promoters known in nature.

The potential of the BEVS platform is enormous as its transient nature makes it an attractive "plug and play" protein production system – a single well characterized cell line is used for the production of all proteins, thereby eliminating the time-consuming process of preparing, qualifying and securing regulatory approval of a new cell line for each new protein. By developing a universal protein purification process, one can begin to imagine that a single multi-product production facility could be established to produce a multitude of vaccines to combat a broad range of diseases.

This potential is illustrated here by commercially available vaccines, those that are in advanced clinical development, and the applicability to produce a variety of WHO recommended vaccines and vaccines for unmet medical needs. Manufacturing of recombinant vaccines will offer the opportunity to produce a broad range of vaccines in multi-purpose production facilities at lower costs.

## 2. Protein based vaccines produced in insect cells

### 2.1. Commercially available vaccines

The BEVS technology has been established as a versatile and robust vaccine manufacturing platform [25]. Five commercially available vaccines for four different indications produced in insect cells are summarized in Table 2.

The first commercially available veterinary vaccine produced in insect cells was a classical swine fever virus (CSFV) vaccine. This vaccine was based on the E2 antigen and received European Market Authorization in 2000. CSF is on the WHO for Animal Health list of notifiable diseases and is one of the most important contagious

**Table 2**
Approved vaccines for human or veterinary use.

| Disease | Brand name(s) | Originator | Protective antigen | Reference(s) |
|---|---|---|---|---|
| **Vaccines for human use** | | | | |
| Cervical cancer | CERVARIX® | GSK | L1 protein | [32] |
| Prostate cancer | PROVENGE® | Dendreon | PSA | [33] |
| **Vaccines for veterinary use** | | | | |
| PCV2 | Porcilis® PCV | Merck | PCV2 ORF2 protein | [34] |
| PCV2 | CircoFLEX® | B. Ingelheim | PCV2 ORF2 protein | [35] |
| Classical swine fever | Porcilis Pesti® | Merck | E2 protein | [36] |

diseases of pigs. In its classical clinical form, it is an acute hemorrhagic disease accompanied by high fever, depression, anorexia, and conjunctivitis. Morbidity and mortality are both very high and may reach 100%. It took nearly seven years for the second veterinary vaccine, PCV2, to receive market authorization. PCV2 is the major pathogen in the etiology of post-weaning multisystemic wasting syndrome. The PCV2 vaccine is based on the protective open reading frame 2 or ORF2 protein of the virus and is manufactured by both Merck and B. Ingelheim.

The first human vaccine produced in insect cells, CERVARIX, was licensed by the European Medicines Agency (EMA) in 2007 and by U.S. Food and Drug Administration (FDA) in 2009. CERVARIX is a bivalent human papilloma virus vaccine indicated for the prevention of cervical cancers (see Table 1). It contains 20 μg of HPV-16 L1 protein and 20 μg of HPV-18 L1 protein that self-assembles to form virus like particles (VLPs) resembling HPV types 16 and 18. These proteins are produced in *T. ni* cells, purified and adsorbed onto a proprietary $ASO_4$ adjuvant system containing 500 μg of aluminum hydroxide and 50 μg of 3-O-desacyl-4′-monophosphoryl lipid A [12]. The second product for human use licensed by the FDA was PROVENGE®, an autologous prostate-cancer therapy product for which the antigen prostate surface antigen (PSA) is produced in *S. frugiperda* cells.

### 2.2. Other vaccine candidates in human clinical development

Other vaccines in human clinical development are summarized in Table 3. These products were the subject of a recent review by Mena and Kamen [25]. The recombinant influenza vaccine FluBlok® based on the hemagglutinin (HA) surface antigen will likely be the next BEVS derived vaccine to receive market authorization.

### 2.3. Applicability of technology for WHO recommended vaccines

The status of recombinant vaccine development using different protective antigen targets for the thirteen viral vaccines recommended by WHO is summarized in Table 4. Other than for influenza all vaccine candidates are in preclinical development. The high development costs for a new medicine product often prohibit the development of a product that ultimately could be produced at much lower cost than the current vaccines. For example, the development of FluBlok has taken nearly twenty years and the estimated development costs approach $100 million even though the scientific challenges in this program were limited because hemagglutinin (HA) is the established protective antigen for influenza and the disease is quite well understood, making the clinical development rather straightforward. Therefore, it is not surprising that most progress in recombinant vaccine development has been made for those vaccines where high prices can be charged such as the HPV vaccine or where public support enables the development of recombinant vaccines. For example, the Center for Diseases and Control (CDC) price for one dose of CERVARIX vaccine is $96 versus the price of a combination MMR (measles, mumps, rubella) vaccine dose of only $19 [57].

Two examples of suitable candidates for further development using the insect cell production platform – rabies and Japanese encephalitis – are discussed in greater detail below.

Rabies, a form of encephalitis, that causes more than 55,000 deaths each year would be an excellent disease candidate for vaccine development. Current vaccine costs are high and typically exceed $1000 for a course of rabies immune globulin and five doses of vaccine given over a four (4)-week period [58]. Thus, there is an urgent need for a cheaper rabies vaccine. The rabies virus (RABV) belongs to the genus *Lyssavirus* in the family *Rhabdoviridae*. The RNA of RABV encodes five proteins, including the G glycoprotein that carries the main antigenic sites. Human infection usually occurs following a transdermal bite or scratch by an infected animal. Already in 1993, Fu et al. [56] showed that protein G produced in insect cells was effective in vaccinating racoons against rabies. Unfortunately, and surprisingly, not much additional progress has been made since.

Japanese encephalitis (JE) is also an excellent candidate for subunit vaccine development. JE, a mosquito-borne disease, is the most important form of viral encephalitis in Asia. The JE virus causes at least 50,000 cases of clinical disease each year, mostly among children aged <10 years, resulting in about 10,000 deaths and 15,000 cases of long-term, neuro-psychiatric sequelae. The JE virus is a member of the genus *Flavivirus* of the Flaviviridae family, which comprises about 70 viruses including dengue, yellow fever, and West Nile viruses. The virion consists of a single-stranded RNA molecule enclosed by the core membrane and the envelope (E) protein. The E protein contains the antigenic determinants responsible for hemagglutination and neutralization and induces protective immunity in the host. Therefore, the E antigen is a promising target for vaccine development. The antigen produced in insect cells forms particulates that are biochemically and biophysically equivalent to the authentic antigens obtained from infected C6/36 mosquito and is able to induce neutralizing antibody titers in mice [52].

### 2.4. Applicability for vaccines that address unmet medical needs

Vaccines are desperately needed for broad range of diseases including malaria, HIV, emerging highly pathogenic arboviruses, and, of course, the neglected diseases caused by various protozoa.

Most recent estimates of malaria suggest several hundred million clinical cases and 800,000 deaths annually [59]. Many malaria vaccine candidates are being produced using the insect cell production system [24,25]. Unfortunately, insufficient progress has been made, and most vaccine candidates remain “stuck” in preclinical development. This lack of progress is caused in part because the disease mechanism is not well enough understood, the complexity of conducting clinical studies in endemic regions and the absence of economic incentives.

HIV is another disease where even though human clinical trials with GP160 variants produced in insect cells by MicroGeneSys [60,61] were already conducted in the early nineties not much progress has been made since. Initially this was caused by a lack in understanding how to combat the virus once it enters the body and, later, the availability of relative effective anti-viral drugs.

**Table 3**
Vaccines candidates for human use in clinical development.

| Disease | Protective antigen | Originator | Development stage | Reference(s) |
|---|---|---|---|---|
| Influenza | HA | Protein Sciences | Under FDA review | [37] |
| Diabetes | GAD | Diamyd | Phase III | [38] |
| Hepatitis E | ORF 2 | GSK | Phase II | [39] |
| Influenza | NA | Protein Sciences | Phase II | [40] |
| Influenza | HA/NA/M1 | Novavax | Phase II | [41] |
| ParvovirusB-19 | Parvovirus VLP | Meridian Life Sciences | Phase II | [42] |
| Influenza H5 | HA | Protein Sciences | Phase I | [43] |
| Norwalk | Norwalk capsid VLP | Ligocyte | Phase I | [44,45] |

The opportunities to use BEVS to develop arthropod-borne arbovirus vaccines such as Chikungunya, Dengue, West Nile, Rif Valley Fever, and Blue Tongue Viruses were recently reviewed elsewhere [62]. This excellent review discusses the threat of emerging vector-borne viral diseases as a result of increased global interaction combined with climate changes and increased population density and further describes vaccines already commercially available and those in development.

The review of Van Oers [24] also describes the potential of the BEVS to develop vaccines for diseases caused by protozoa. However, because the protective antigens for hookworm disease and schistosomiasis, also known as bilharziasis, are not yet well understood,

**Table 4**
Antigen targets for recommended viral vaccines.

| Vaccine | Etiological agent | Protective antigen | Status of development | Reference |
|---|---|---|---|---|
| Hepatitis B | Hepatitis B virus (HBV) | HbSAg | Subunit vaccine produced in yeast cells is approved. The immunogenicity of recombinant hepatitis B surface antigen (HBsAg) produced in the baculovirus/insect cell expression system was compared to a commercially available yeast-derived recombinant HBsAg vaccine preparation and shown to be equivalent | [46] |
| Polio | Polio virus serotypes (types 1, 2 or 3) | VP1 and VP4 | No work has been published for insect cells, but authors showed that regions from VP1 and VP4 can neutralize the virus suggesting that VP1 and VP4 may be suitable candidates for vaccine development | [47] |
| Rotavirus | Rotavirus | VP6, VP7 and major outer capsid protein | Co-expression of VP2, VP6, and VP7 produced triple-layered VP2/6/7, which were similar to native infectious rotavirus particles. No virus neutralization data was provided | [48] |
| Measles | Measles virus (genus *Morbillivirus*, family *Paramyxoviridae*) | H and F proteins H, F, and N viral proteins | No work has been published for insect cells; however, the potential of subunit H and F was already demonstrated in 1987 by Varsanyi et al. [49] | [50] |
| Rubella | Rubella virus (=togavirus of the genus Rubivirus) | E1 | While no work has been published for insect cells, the E1 glycoprotein proved to be best immunogen in an early study | [51] |
| HPV | Human papilloma virus (>100 subtypes) | L1 structural protein | Approved (CERVARIX) (produced in insect cells). The authors showed that L1 protein produced in insect cells had the intrinsic capacity to assemble into empty capsid-like structures whose immunogenicity is similar to infectious virions | [32] |
| Japanese encephalitis | Japanese encephalitis (JE) virus (*Flaviviridae*) | Glycoprotein E | Viral E antigen produced in insect cells forms biochemical and biophysical particulates equivalent to the authentic antigens obtained from infected C6/36 mosquito that is able to induce neutralizing antibody titers in mice | [52] |
| Yellow fever | Yellow fever virus (*Flaviviridae*) | E, and E/NS1 | Proof of concept in mice. Solid protection against lethal YFV encephalitis was achieved after immunization with cell lysates containing the E protein. The NS1 protein appeared to enhance the immune response | [53] |
| Tick-borne encephalitis | Tick-borne encephalitis virus (*Flaviviridae*) | E and C | Protein E and C produced in insect cells triggered CD4 T-cell immune responses. Significance needs to be further established | [54] |
| Hepatitis A | Hepatitis A virus (HAV) | Polyproteins | Recombinant baculoviruses were constructed that contained the hepatitis A virus (HAV) open reading frame (ORF). This HAV antigen had a buoyant density in cesium chloride gradients similar to HAV empty capsids, and elicited HAV neutralizing antibodies in mice. Early work by Hughes and Stanton suggests that VP3 may be candidate for a subunit vaccine | [55] |
| Rabies | Rabies virus (RABV) (*Rhabdoviridae*) | Protein G | Authors demonstrated that protein G produced in insect cells was effective in vaccinating racoons against rabies | [56] |
| Mumps | Mumps virus (*Paramyxoviridae*) | Protein H and N | No work has been published for insect cells | [50] |
| Influenza | Influenza virus (*Orthomyxoviridae*) | HA, NA, HA- NA- M1 VLP | HA is the protective antigen and antibodies against HA are associated with protection against the disease. Various vaccine candidates are in development | [37,40,41] |

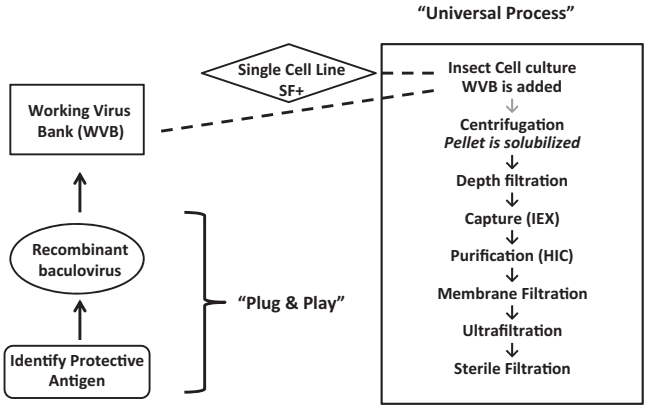


**Fig. 1.** Overview of universal "plug and play" recombinant protein production process. The protective antigen is inserted into the baculovirus to generate the recombinant virus ("Plug & Play") that is amplified in insect cells to generate the Working Virus Bank (WVB). The WVB is expanded and used to infect the universal insect cells. A series of protein purification steps are performed to purify the protein of interest.

it may be a while before vaccines for these diseases will become available.

## 3. Manufacturing of recombinant vaccines made in insect cells

Manufacturing costs are important for vaccines, especially those for emerging diseases that are primarily endemic in the developing world, which usually do not carry high-profit margins. The capital investment required to establish production capacity and the production yield are key drivers for the cost of goods. For example, doubling the capital investment cost from $70 to 140 million increases the cost per dose by 40%, and doubling the yield (or output per L) reduces the cost per dose by 100%. The BEVS may be an attractive choice as manufacturing capacity exists, thereby reducing the investment to an absolute minimum and recombinant protein yields are high and multiple opportunities for further improvement exist as described below. The process steps to produce a recombinant protein in insect cells are shown in Fig. 1. As described earlier the protective antigen is inserted into the baculovirus to generate the recombinant virus ("plug and play") that is amplified in insect cells to generate the Working Virus Bank (WVB). The insect cells are grown in a *bioreactor* and infected with the WVB that has been expanded in insect cells at a scale that is approximately 100-fold smaller than the protein production bioreactor. Cells are separated from the media using *centrifugation* and, dependent on the product that is being produced, either the cell paste or the supernatant is further processed. The protein of interest is solubilized (when applicable) and processed using *depth filtration*. It is then captured using column *chromatography* and further purified using additional chromatography. Potential further contaminants can be removed, if required, using *membrane filtration* technology and, finally, the product is brought into its final buffer composition using *ultrafiltration*. The process steps indicated in *italics* are routinely used in monoclonal antibody production. Many of such production facilities have become obsolete as a result of yield improvements achieved in mammalian cell culture manufacturing processes and these facilities could be used for insect cell based production processes.

This technology is also particularly suitable to address health care emergencies currently posed by pandemic influenza as the manufacturing technology can be readily and economically transferred to other countries. It is estimated that sufficient monovalent bulk protein capacity exists worldwide to produce approximately 9 million doses containing 15 μg of rHA in a 5-day cycle. As reported by Fedson and Dunnill [63] 425 million doses of vaccine containing 10 μg/dose could be produced within one month if 25% of the global bioreactor capacity (or 500,000-L) were to be allocated to rHA vaccine production. In order to address the potential threat of a pandemic, WHO has taken a major initiative to increase the global and equitable access to influenza vaccine through technology transfer [64]. Eleven vaccine manufacturers based in Brazil, Egypt, India, Indonesia, Iran, Mexico, Republic of Korea, Romania, Serbia, Thailand and Vietnam were selected to participate in this program. The majority of the effort was based on producing inactivated influenza vaccines in embryonated chicken eggs. Tremendous progress has been made; however, it has become apparent that it is difficult to maintain production capacity for a pandemic in the absence of a regular buyer for vaccine [65]. Hence, sustainability of vaccine supply cannot be guaranteed unless there is an economic motive to maintain that capacity. A BEVS production facility could rapidly be changed over to produce a pandemic influenza vaccine when needed from making other vaccines that are more needed in the absence of a pandemic threat.

There is a high potential for lowering the cost of goods further in recombinant protein production through yield improvements. Opportunities include exploration of alternative baculovirus promoters, such as the p10/p6.9 chimaeric promoter [66,67], modified baculoviruses such as the Δ cathepsin-/chitinase-negative AcMNPV bacmid [68] or development of fed-batch fermentation processes [69–71]. Yield improvement has also frequently been reported as a result of improved cell culture media. Additions of plant hydrolysates, other growth and production enhancing factors and control of proteolysis were reviewed by Ikonomou et al. [72] and offer promise for yield improvement. Specifically, adding the plant hydrolysate, Hypep 1510 to an insect cell culture resulted in a doubling of expression of a reporter gene [73] but, also, simple changes in pH may offer great benefit [74]. Finally, it has been shown that viral and host modifications can improve cell survival and production of heterologous proteins. Modifications to the host insect cell line, for example by including the anti-apoptotic gene Bcl-2, may limit the cytopathic effects of the baculovirus and may result in enhanced expression such as was recently reported for Sindbis virus in a mammalian cell line [75]. Co-expression of chaperones may also be a promising prospect for efficient production of recombinant secretory proteins in insect cells as was recently reported by, for instance, Kato et al. [76].

## 4. Conclusions

The approval of various vaccines including more recently CERVARIX – GSK's human papilloma virus vaccine produced using insect cells – has clearly demonstrated that the BEVS production technology has matured into a commercial manufacturing technology.

Now that various products made in insect cells have been approved for commercial use, the product development uncertainty is greatly reduced. A large number of products are being developed and, therefore, we can expect to see an acceleration of products manufactured in insect cells in the near future [24,25]. We may also see follow-on products, or generics, developed and enter the field within the next years.

In this review an overview was provided for the broad applicability of this technology for already available vaccines and many unmet medical needs. The "plug and play" nature of this technology provides the potential for sustainability of vaccine supply in developing world counties as production facilities can be used to produce vaccines that are most relevant to the needs of a particular country.

Production costs for vaccines have to be low, and while currently available recombinant vaccines are characterized by high costs, BEVS technology offers the intrinsic possibility for affordable vaccines. The HA production levels obtained using the BEVS technology are 4–7× greater than those obtained when growing influenza viruses in MDCK cells [77], which results in substantially lower cost of goods for this influenza vaccine. Furthermore, there are many opportunities for process improvements that will enable an even greater reduction in production cost, including molecular biology approaches, media development and alternative cell culture strategies. It was shown previously that 40-fold improvements in antibody production in mammalian cells could be obtained by implementation of a continuous fed-batch process [78]. The worldwide overcapacity for mammalian protein manufacturing reduces the capital investment required for BEVS production to an absolute minimum. This is in sharp contrast to the substantial capital investment for the biological containment facilities that are required when influenza viruses are cultivated in cell lines as exemplified by the Novartis investment estimate of $600 million [79].

The BEVS technology is also likely to offer a powerful first line defense in combating emerging new viruses due to the increased contact between human and wildlife [80]. Vaccines against zoonotic diseases caused, for example, by Human Immunodeficiency Virus (HIV), West Nile Virus, Chikungunya Virus, Marburg Virus and Ebola Virus are desperately needed and the BEVS technology provides a great opportunity to develop such vaccines. Surface antigens offer a promising fast approach as exemplified by the virus neutralizing antibodies induced by the spike protein antigen derived from SARS coronavirus [81]. However, it is important to acknowledge that it may not be easy or even feasible to identify the antigen that offers protection as demonstrated by the failure to develop an effective vaccine against HIV over the past two decades. While the insect cell production technology could be deployed to develop other inexpensive, safe and efficacious vaccines listed in Table 4, it should be noted that subunit vaccines do not always generate potent immune responses needed for the protection against certain pathogens. Especially for diseases like polio, rotavirus, measles, rubella, yellow fever, and mumps, where safe, effective and affordable LAV candidates are available, it may not be useful to pursue development of recombinant vaccines at this time. It would also be useful to explore safe and effective adjuvants to stimulate immune response.

Insect cell-derived recombinant proteins are also used as vaccines against cancer [82–84]. Thus, commercialization and further scale-up of this manufacturing technology beyond viral vaccines may have broad implications for disease control and treatment in general.

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