EXPERT REVIEWS

Using plant cells as influenza vaccine substrates

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The reappearance of highly pathogenic avian influenza H5N1 in poultry in 2003, and the subsequent high-fatality zoonoses in Asia, Europe and Africa, has heightened the awareness of a potential pandemic and the need for global vaccine supply. Most manufacturers still use embryonated hens' eggs to produce influenza vaccines, a system that has demonstrated its value throughout six decades. There are, however, some challenges with this approach, both for seasonal and particularly for pandemic vaccine production. This review highlights some of these challenges and describes emerging alternative production platforms with the potential to deliver safe and effective vaccines to the global market in a timely fashion. A particular emphasis of this review will be on the production of recombinant influenza vaccines using transient plant expression systems.

Keywords: highly pathogenic avian influenza virus • influenza vaccine • influenza virus • plant-produced antigen • recombinant protein • subunit vaccine

The first influenza vaccines were made in the 1940s using purified, formalin-inactivated egg-grown influenza viruses. These vaccines were initially used in military personnel, but the indications were broadened to include the civilian population and particularly those at risk of serious illness and death. Throughout the 1960s and 1970s, the use of influenza vaccines slowly, but steadily, increased. The early vaccines were frequently reactogenic, mostly due to the presence of endotoxins and significant quantities of contaminating egg proteins. This reactogenicity was greatly reduced when detergents, to generate split-virus formulations, and zonal centrifugation, to obtain vaccines with higher purity, were used. Over time, processes have been optimized to further improve vaccine formulations. As a result, the highly purified vaccines of today are well tolerated. The production output of influenza vaccines has significantly increased during the last decade, reaching an estimated 565 million doses in 2007, and is expected to rise to 1 billion doses in 2010 [101].

The recent focus on influenza vaccines was spurred by the incident of highly pathogenic avian influenza (HPAI) viruses of the H5N1 subtype in man in Hong Kong in 1997, killing six of the 18 confirmed cases, raising a worldwide awareness that an influenza pandemic could be imminent. The reappearance of HPAI H5N1 viruses in poultry in 2003, and the subsequent zoonotic cases in Asia, Europe and Africa, has further heightened the potential risk of a pandemic. The H5N1 viruses are genetically unstable and since 1997 have evolved into ten distinct genetic clades with multiple subclades and antigenic clusters, complicating the pandemic vaccine development [102]. With nearly 400 reported human cases of H5N1 avian influenza since late 2003, mostly occuring in South-East Asia, and with a case–fatality rate of more than 60%, the situation today remains unresolved and a pandemic threat is still evident [103]. This threat was further highlighted in a WHO publication recommending expansion of surveillance and prophylaxis into developing world countries [1].

Up until the last decade, the use of vaccines against seasonal influenza has been mostly limited to industrialized countries. However, the steadily growing and affluent middle classes of the low- and middle-income countries are among the most recent users. A new and well-informed global market for seasonal influenza vaccines is now emerging, expecting a timely and uninterrupted annual vaccine supply. In the case of a pandemic, the global need for vaccine is even greater, requiring a staggering 6.5 billion vaccination courses, realizing that two doses per individual will be needed [2].

Current technology

Most manufacturers still use embryonated hens' eggs to produce their vaccines, a system that has demonstrated its value throughout six decades.

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There are, however, some serious drawbacks with this approach. First; it is difficult to scale up production in an emergency, as quality-assured eggs have to be ordered many months in advance. This is usually not critical under normal seasonal circumstances when the manufacturers are ordering their egg supply based on calculated market projections and their production targets for the coming seasons. Second, in the case of a pandemic, an avian virus will most probably be lethal for chick embryos, thus creating problems for manufacturers using embryonated eggs. The use of reverse-genetics technology for creating nonpathogenic seed viruses can partially address this obstacle [3]. Even so, one must expect a time-delay of several weeks before the seed virus is approved and made available to the manufacturers [104]. Should a pandemic become a reality, every week of missed vaccine output would be critical. Third, the use of egg-adapted variants may select a seed virus not necessarily fully representing the original clinical isolate the vaccine strain was based upon [4,5]. On some occasions, an approved egg isolate for the selected vaccine strain may not be available or is delayed. This was shown in 2003–2004, when the preferred H3N2 vaccine strain A/Fujian/411/02, was not available in time to be included in the WHO strain recommendation for the Northern hemisphere. The previous season's A/Moscow/10/99-like strains were therefore selected, thus compromising vaccine efficacy [106]. In addition, eggs are vulnerable to contamination by pathogens that may be hazardous to the end user. This was clearly demonstrated in 2004 when one supplier to the US market could not meet its contractual delivery obligations due to bacterial contamination [105]. These are serious drawbacks of the egg-based system particularly when facing a potential pandemic. Therefore, some manufacturers have already established facilities for influenza vaccine production using approved cell lines (Vero, MDCK and PerC6) [107]. For safety concerns, as well as for technical and financial reasons, manufacturers opting for cell culture production platforms for pandemic influenza vaccines will need a nonpathogenic seed virus. Nonetheless, one manufacturer (Baxter) has developed and established facilities and processes for manufacturing influenza vaccine using wild-type virus strains [6]. Whether using embryonated eggs or cell culture as a vaccine substrate, there are some scale-up challenges that remain to be overcome, although the latter system is a far more flexible approach. In summary, there is a need for seamless and robust production systems, independent of propagating live virus and being able to modify or completely change the coding sequence for the target proteins if a critical strain adjustment is called for. In addition, these systems need to be able to rapidly produce safe, effective and affordable vaccines on a large scale.

Alternative substrates

The advent of recombinant DNA technologies has opened up possibilities for rapid production of vaccine proteins in large-scale industrial bioreactors. However, it remains to be determined whether new production platforms will generate authentic antigens that will elicit protective immune responses. In the context of influenza, both DNA vaccines and recombinant protein preparations produced in insect and *Escherichia coli* cell cultures have been used [7–11]. Of the approximately 80 ongoing or completed clinical trials with vaccines against avian influenza, nine have used alternative production platforms, two with DNA vaccines, five with recombinant proteins made in *E. coli*, and two with recombinant proteins made by engineered baculovirus in insect cells [108]. This is an important step forward and may circumvent some of the drawbacks of the traditional embryonated egg-based approach, offering scalability and flexibility in strain selection.

In this review, we will focus on another promising substrate for influenza vaccine production, namely transient antigen expression in plants. Plants offer competitive advantages to other expression systems, including safety, time and cost efficiency, target solubility and post-translational modification. These topics have been extensively discussed elsewhere [25,26]. Recent data demonstrating the immunogenicity of the engineered plant-produced proteins and their ability to elicit protective immunity in animal models will be discussed below.

Vaccine antigens made in plant cells

During the last decade, plants have been established as a suitable system for producing proteins of different origins, including vaccine antigens and therapeutic antibodies [12–15]. The main advantages of plant expression systems are safety, low cost, time efficiency and a greater potential for scalability as compared with other systems [16]. The more widely known strategy for the production of recombinant proteins in plants is via nuclear transformation [17], wherein the gene of interest is permanently incorporated into the plant nuclear genome. This approach has some limitations, including, potential gene silencing and long lead times. An alternative approach to nuclear transformation is the integration of target genes into the plastid genome, termed chloroplast transformation [18,19]. Undoubtedly, this approach provides significantly higher yields of target protein and is not subject to gene silencing; yet, it is limited by long lead times and minimal post-translational modification. Transient protein expression in plants has recently moved to the forefront due to the development of plant RNA virus expression vectors [20,21]. Plant RNA viral vectors facilitate rapid engineering and target production at higher yields compared with transgenic systems. However, plant virus vector-based transient expression systems also have their limitations, including, nonuniform target production in infected plants, and potential genetic instability of the viral vectors. Recently, 'launch vectors' that combine elements of plant RNA viral vectors and *Agrobacterium* binary plasmids were developed to address some of the limitations of nuclear, chloroplast, and viral RNA vector-based expression approaches [22,23]. In this system, the plant viral vector is introduced into growing plants – for example, *Nicotiana benthamiana* via the bacterium *Agrobacterium tumefaciens*. These new approaches enable uniformly high levels of target protein expression and rapid scale-up.

The feasibility of plants as a system for producing vaccine antigens that elicit protective immune responses has been validated in numerous reports and reviews [24–26]. More specifically, plants have been used to produce vaccine antigens targeting seasonal,

as well as potentially pandemic, strains of influenza. Strategies for plant-produced vaccines have focused on a subunit-based approach. As is the case with most subunit influenza vaccines, plant-based approaches are using HA as the main target for generating protective immune responses.

Plant-produced seasonal influenza vaccines

Two approaches have been used to engineer HA as vaccine candidates produced in plants. Mett *et al.* described the engineering and production in plants of the globular domain (GD) and stem domain (SD) of HA from A/Wyoming/3/03 (H3N2) as in-frame fusions with the thermostable enzyme, lichenase (LicKM) [27]. When tested in ferrets, the combination of GD and SD was immunogenic, eliciting high serum hemagglutination-inhibition (HI) and virus-neutralizing (VN) antibody titers, and was protective against a homologous virus challenge in the presence of alum adjuvant. In addition, the vaccine candidate elicited high levels of cross-reactive antibodies to variant strains of H3N2, A/Sydney/5/97 and A/California/07/04. This was the first demonstration of the use of the GD as a potential target for influenza vaccine development and the first report to demonstrate the feasibility of plant cells as a substrate for seasonal influenza vaccine development.

In a subsequent publication, Shoji *et al.* produced full-length HA from A/Wyoming/3/03 (ppH3HAwy) in *N. benthamiana* plants [28]. The plant-produced HA demonstrated appropriate antigenicity as determined by ELISA and single-radial immunodiffusion (SRID) assays. ppH3HAwy was immunogenic in mice when combined with the adjuvant Quil-A, eliciting significant serum HI and VN antibody titers against homologous H3N2 virus. Unsurprisingly, this adjuvanted ppH3HAwy also generated a mixed Th1/Th2 response with production of both IFN-γ and IL-5 cytokines and IgG_1 and IgG_{2a} antibodies. In influenza virus infections, IgG_1 antibody subclass plays a pivotal role in virus neutralization and protection, while IgG_{2a} antibody subclass has been associated with viral clearance [29]. Therefore, stimulation of both IgG_1 and IgG_{2a} could be important for effective influenza vaccine development. The quality of the immune responses generated by ppH3HAwy further supports plants as a potential system for producing effective seasonal influenza vaccines. However, additional studies are required to determine the protective efficacy of ppH3HAwy vaccine against a live virus challenge.

Plant-produced pandemic influenza vaccines

As the development of vaccines against potentially pandemic avain influenza strains of H5N1 subtype has become an issue of paramount importance, plants, among others, have been assessed as candidate novel substrates with the capacity to provide large quantities of vaccine antigens at an affordable cost. A recent study demonstrated the feasibility of producing HA from A/Indonesia/05/05 (H5N1) (ppH5HA-I) in plants [30]. The antigenicity of ppH5HA-I was assessed by ELISA, and mice immunized with ppH5HA-I in the presence of Quil-A, mounted H5-specific immune responses, including high serum HI and VN antibody levels. The HI antibodies were found to cross-react with variant H5N1 strains including, A/Anhui/1/05 (clade 2.3), A/Bar-headed Goose/Qinghai/1A/05 (clade 2.2) and A/Vietnam/1194/04 (clade 1). In addition, ferrets immunized with ppH5HA-I were fully protected against a challenge with 10 ferret 50% lethal dose (10 FLD_{50}) of homologous virus. Although, relatively high vaccine doses (90 and 4 µg) were used in this particular study, subsequent studies in mice have shown that high HI and VN titers can be elicited by an antigen dose as low as 5 µg (data not shown).

Another strategy for influenza vaccine development that has demonstrated broad cross-clade protection in animal models is virus-like particles (VLPs) [31,32]. A recent report by D'Aoust *et al.* demonstrated the production of VLPs with HA from A/Indonesia/5/05 (H5N1) in plants via transient expression [33]. These plant-produced VLPs elicited strong virus-specific immune responses in mice and provided protection against challenge with 1 LD_{50} of heterologous virus A/Vietnam/1994/04 at a dose as low as 0.5 µg when administered in the presence of alum adjuvant. It should be noted, however, that the challenge dose used in this study was significantly lower compared with that reported in the literature for a VLP-based vaccine against A/Indonesia/05/05 using an insect cell system [34]. Nevertheless, these are promising results and demonstrate that plants can be utilized to generate both recombinant HA- as well as VLP-based influenza vaccines.

Expert commentary

Since the pandemic threat was recognized, there have been continued efforts to address some of the shortcomings of the current vaccine technology, such as dependence upon timely availability of licensed seed viruses, as well as lack of time efficiency, capacity and strain flexibility. As a result, new technologies that have higher flexibility and the potential to provide larger quantities of vaccine in a relatively shorter period of time are being evaluated. These efforts are particularly emphasized in the initiatives by the US government in supporting development and establishment of the infrastructure for cell culture-based production of influenza vaccines. Additionally, there are several other production systems that have been evaluated for influenza vaccine development. For example, vaccine antigens produced in insect cell cultures and *E. coli* have moved into clinical evaluation. One particular vaccine based on HA and produced in insect cells has been through Phase II/III clinical trials demonstrating 54% reduction of US CDC-defined influenza-like illness [35]. A subunit HA vaccine against H5N1 strain A/Vietnam/1203/04 produced in the same system was shown to elicit protective levels of antibody in 52% of subjects after two intramuscular doses of 90 µg [36]. Furthermore, influenza vaccines based on VLPs have been successfully tested in preclinical studies [37] and are currently being evaluated in human trials. Similarly, plants have been used to make influenza vaccines based both on subunit HA antigens as well as VLPs. Vaccine antigens produced in plants using both approaches have shown protection in preclinical trials [27,28,30,33]. This particular methodology has certain advantages that makes it an attractive system for influenza vaccine manufacturing, including safety, scalability and time efficiency. Plants are extremely versatile and have been shown to produce proteins of viral, bacterial, fungal and parasitic agents [25].

In the context of influenza, we have successfully expressed HA molecules from a range of influenza virus A subtypes, including H3N2, H1N1, H5N1 and H7N2 as well as several B viruses. Moreover, since there is no need for seed viruses, the vaccine development can be initiated using synthesized or cloned cDNA sequences. The use of synthetic DNA, in turn, allows for codon optimization to enhance target expression in plants. Recombinant technologies, plants in particular, have the potential for rapid scale-up of production, delivery of affordable and safe vaccines, and to satisfy the global need for influenza vaccines. Continued investment into these alternative vaccine production technologies is important to ensure security of future supply for all vaccines, including seasonal and pandemic influenza.

Five-year view

There will be a steadily growing demand for and use of seasonal vaccines throughout the world. The emerging markets, particularly in South America, India and China, will claim an increasing part of the global output. It is recognized that the current egg-based production system may not be sufficient to meet this growing demand in the event of a pandemic. This has facilitated the development and implementation of alternative approaches, particularly recombinant subunit vaccine technologies. The first vaccines based on recombinant HA antigens produced in heterologous systems are already in human clinical trials, and it is possible that the first HA-based subunit vaccines will be approved in the next 5 years. Plants are one of the new promising production systems, and HA molecules made in plants have been show to be effective in preclinical studies. In the next 5 years, antigens made in plants will be tested in human trials leading up to the validation of this approach as a viable alternative for production of vaccines against influenza as well as other diseases. As mentioned previously, since the first human case of H5N1 was detected in 1997, these HPAI viruses have been shown to be genetically unstable [109]. As it is not possible to predict which strain of influenza may result in a pandemic outbreak, it is most critical to have a technology with rapid response capabilities to provide a vaccine against emerging strains in the shortest period of time possible. Transient expression in plants using 'launch vectors' is a particularly promising technology in this respect. As these technologies mature into products, new regulatory guidelines will be developed and a sector of industry using alternative production platforms will be established, leading to an overall increase of global vaccine manufacturing capacity. In addition, it is likely that during this time the transfer of some of these production technologies to developing countries will have been initiated.

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Key issues

- A major challenge is to meet the steadily increasing global demand for seasonal influenza vaccines.
- The highly pathogenic avian influenza virus H5N1 has the potential to cause a pandemic.
- Current manufacturing technology is unlikely to meet the global demand for pandemic vaccine in time.
- We need to achieve the availability of rapid and scalable technologies with emergency response capabilities.
- Progress will involve facilitating the transfer of technologies into developing countries for creating local vaccine manufacturing capacity.
- Another aim is to gain industry acceptance of plants as an alternative means for vaccine production.
- Regulatory frameworks for plant production platforms are crucial.

References

Papers of special note have been highlighted as:
• of interest
•• of considerable interest

1 WHO. Influenza Vaccines. *Wkly Epidem. Rec.* 75(35), 281–288 (2000).

• **The WHO publishes for the first time the need not only for influenza surveillance but also for prophylactic interventions in the developing world. Important political statement.**

2 Options for the use of human H5N1 influenza vaccines and the WHO H5N1 vaccine stockpile. Presented at: *WHO Scientific Consultation*. Geneva, Switzerland, 1–3 October 2008.

3 Webby RJ, Perez DR, Coleman JS *et al.* Responsiveness to a pandemic alert: use of reverse genetics for rapid development of influenza vaccines. *Lancet* 363(9415), 1099–1103 (2004).

4 Schild GC, Oxford JS, de Jong JC, Webster RG. Evidence for host-cell selection of influenza virus antigenic variants. *Nature* 303(5919), 706–709 (1983).

• **Seminal paper describing how growing influenza virus in embryonated eggs or in cell culture selects different antigenic variants.**

5 Kodihalli S, Justewicz DM, Gubareva LV, Webster RG. Selection of a single amino acid substitution in the hemagglutinin molecule by chicken eggs can render influenza A virus (H3) candidate vaccine ineffective. *J. Virol.* 69(8), 4888–4897 (1995).

6 Ehrlich HJ, Muller M, Oh HM *et al.* A clinical trial of a whole-virus H5N1 vaccine derived from cell culture. *N. Engl. J. Med.* 358(24), 2573–2584 (2008).

7 Chen MW, Cheng TJ, Huang Y *et al.* A consensus-hemagglutinin-based DNA vaccine that protects mice against divergent H5N1 influenza viruses. *Proc. Natl Acad. Sci. USA* 105(36), 13538–13543 (2008).

8 Goji NA, Nolan C, Hill H *et al.* Immune responses of healthy subjects to a single dose of intramuscular inactivated influenza A/Vietnam/1203/2004 (H5N1) vaccine after priming with an antigenic variant. *J. Infect. Dis.* 198(5), 635–641 (2008).

• **Demonstrates that vaccination 8 years earlier with a clade 0 H5N1 vaccine will boost the subsequent response to a clade 1 vaccine, even without demonstrable prevaccination serum hemagglutinin-inhibition antibodies. Holds promise for prime–boost protocols.**

9 Treanor JJ, Schiff GM, Couch RB *et al.* Dose-related safety and immunogenicity of a trivalent baculovirus-expressed influenza-virus hemagglutinin vaccine in elderly adults. *J. Infect. Dis.* 193(9), 1223–1228 (2006).

10 Huleatt JW, Nakaar V, Desai P *et al.* Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin. *Vaccine* 26(2), 201–214 (2008).

11 Song L, Nakaar V, Kavita U *et al.* Efficacious recombinant influenza vaccines produced by high yield bacterial expression: a solution to global pandemic and seasonal needs. *PLoS ONE* 3(5), e2257 (2008).

12 Yusibov V, Mett V, Mett V *et al.* Peptide-based candidate vaccine against respiratory syncytial virus. *Vaccine* 23(17–18), 2261–2265 (2005).

13 Chichester JA, Musiychuk K, de la Rosa P *et al.* Immunogenicity of a subunit vaccine against *Bacillus anthracis*. *Vaccine* 25(16), 3111–3114 (2007).

14 Mett V, Lyons J, Musiychuk K *et al.* A plant-produced plague vaccine candidate confers protection to monkeys. *Vaccine* 25(16), 3014–3017 (2007).

15 Hull AK, Criscuolo CJ, Mett V *et al.* Human-derived, plant-produced monoclonal antibody for the treatment of anthrax. *Vaccine* 23(17–18), 2082–2086 (2005).

16 Karasev AV, Foulke S, Wellens C *et al.* Plant based HIV-1 vaccine candidate: Tat protein produced in spinach. *Vaccine* 23(15), 1875–1880 (2005).

17 Twyman RM, Schillberg S, Fischer R. Transgenic plants in the biopharmaceutical market. *Expert Opin Emerg. Drugs* 10(1), 185–218 (2005).

18 Kumar S, Daniell H. Engineering the chloroplast genome for hyperexpression of human therapeutic proteins and vaccine antigens. *Methods Mol. Biol.* 267, 365–383 (2004).

19 Streatfield SJ. Engineered chloroplasts as vaccine factories to combat bioterrorism. *Trends Biotechnol.* 24(8), 339–342 (2006).

20 Yusibov V, Rabindran S, Commandeur U, Twyman RM, Fischer R. The potential of plant virus vectors for vaccine production. *Drugs R & D* 7(4), 203–217 (2006).

•• **Discusses the advantages of plant virus vectors, their development and the immunological experiments that have demonstrated the principle of plant virus-derived vaccines.**

21 Fitzmaurice WP, Holzberg S, Lindbo JA *et al.* Epigenetic modification of plants with systemic RNA viruses. *Omics* 6(2), 137–151 (2002).

22 Musiychuk K, Stephenson N, Bi H *et al.* A launch vector for the production of vaccine antigens in plants. *Influenza Other Resp. Viruses* 1, 19–25 (2007).

•• **Describes in detail the launch vector system for engineering and expressing target antigens from pathogens, including influenza.**

23 Marillonnet S, Giritch A, Gils M *et al.* *In planta* engineering of viral RNA replicons: efficient assembly by recombination of DNA modules delivered by *Agrobacterium*. *Proc. Natl Acad. Sci. USA* 101(18), 6852–6857 (2004).

24 Chichester JA, Yusibov V. Plants as alternative systems for production of vaccines. *Hum. Vaccin.* 3(4) (2007).

25 Yusibov V, Rabindran S. Recent progress in the development of plant derived vaccines. *Expert Rev. Vaccines* 7(8), 1173–1183 (2008).

•• **Discusses the advantages of plant-based vaccines and the probable impact of plant systems on the vaccine industry.**

26 Mett V, Farrance CE, Green BJ, Yusibov V. Plants as biofactories. *Biologicals* 36(6), 354–358 (2008).

27 Mett V, Musiychuk K, Bi H *et al.* A plant-produced influenza subunit vaccine protects ferrets against virus challenge. *Influenza Other Resp. Viruses* 2(1), 33–40 (2008).

• **First demonstration of the use of the globular domain of hemagglutinin as a potential target for influenza vaccine development and the first report to demonstrate the feasibility of plant cells as a substrate for seasonal influenza vaccine development.**

28 Shoji Y, Chichester JA, Bi H *et al.* Plant-expressed HA as a seasonal influenza vaccine candidate. *Vaccine* 26(23), 2930–2934 (2008).

29 Huber VC, McKeon RM, Brackin MN *et al.* Distinct contributions of vaccine-induced immunoglobulin G1 (IgG1) and IgG2a antibodies to protective immunity against influenza. *Clin. Vaccine Immunol.* 13(9), 981–990 (2006).

30 Shoji Y, Bi H, Musiychuk K *et al.* Plant-derived hemagglutinin protects ferrets against challenge infection with the A/Indonesia/05/05 strain of avian influenza. *Vaccine* (In press) (2009).

31 Mahmood K, Bright RA, Mytle N *et al.* H5N1 VLP vaccine induced protection in ferrets against lethal challenge with highly pathogenic H5N1 influenza viruses. *Vaccine* 26(42), 5393–5399 (2008).

32 Bright RA, Carter DM, Daniluk S *et al.* Influenza virus-like particles elicit broader immune responses than whole virion inactivated influenza virus or recombinant hemagglutinin. *Vaccine* 25(19), 3871–3878 (2007).

33 D'Aoust ML, PO. Couture, MMJ. et al. Influenza virus-like particles produced by transient expression in *Nicotiana benthamiana* induce a protective immune response against a lethal viral challenge in mice. *Plant Biotechnol. J.* 6, 930–940 (2008).

34 Bright RA, Carter DM, Crevar CJ *et al.* Cross-clade protective immune responses to influenza viruses with H5N1 HA and NA elicited by an influenza virus-like particle. *PLoS ONE* 3(1), e1501 (2008).

35 Treanor JJ, Schiff GM, Hayden FG *et al.* Safety and immunogenicity of a baculovirus-expressed hemagglutinin influenza vaccine: a randomized controlled trial. *JAMA* 297(14), 1577–1582 (2007).

36 Treanor JJ, Campbell JD, Zangwill KM, Rowe T, Wolff M. Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. *N. Engl. J. Med.* 354(13), 1343–1351 (2006).

37 Cox MM. Progress on baculovirus-derived influenza vaccines. *Curr. Opin Mol. Ther.* 10(1), 56–61 (2008).

Websites

101 WHO. Projected supply of pandemic influenza vaccine sharply increases www.who.int/mediacentre/news/releases/2007/pr60/en/index.html

102 WHO. Antigenic and genetic characteristics of H5N1 vaccine viruses developed for potential use as human vaccines www.who.int/csr/disease/avian_influenza/guidelines/h5n1virus/en/index.html

- **Official WHO summary of the antigenic and genetic diversity of H5N1 strains and the availability of seed virus for vaccine development. The site is regularly updated.**

103 WHO. Cumulative number of confirmed human cases of avain influenza A/(H5N1) reported to WHO www.who.int/csr/disease/avian_influenza/country/cases_table_2008_09_10/en/index.html

104 WHO. WHO guidance on development of influenza vaccine reference viruses by reverse genetics www.who.int/csr/resources/publications/influenza/WHO_CDS_CSR_GIP_2005_6.pdf

105 CIDRAP. Becker AL. Scrambling for vaccine: a sampling of responses www.cidrap.umn.edu/cidrap/content/influenza/general/news/oct2804fluseries.html

106 CDC. Influenza vaccine effectiveness studies www.cdc.gov/od/oc/media/pressrel/fs040115.htm

107 WHO. Patriarca PA. Use of cell lines for the production of influenza virus vaccines: an appraisal of technical, manufacturing, and regulatory considerations www.who.int/vaccine_research/diseases/influenza/WHO_Flu_Cell_Substrate_Version3.pdf

108 WHO. Tables on the clinical trials of pandemic influenza prototype vaccines www.who.int/vaccine_research/diseases/influenza/flu_trials_tables/en/index.html

109 WHO. Antigenic and genetic characteristics of H5N1 viruses and candidate H5N1 vaccine viruses developed for potential use in human vaccine www.who.int/csr/disease/avian_influenza/guidelines/200809_H5VaccineVirusUpdate.pdf

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