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Progress in Developing Virus-like Particle Influenza Vaccines

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Summary

Recombinant vaccines based on virus-like particles (VLPs) or nanoparticles have been successful in their safety and efficacy in preclinical and clinical studies. The technology of expressing enveloped VLP vaccines has combined with molecular engineering of proteins in membrane-anchor and immunogenic forms mimicking the native conformation of surface proteins on the enveloped viruses. This review summarizes recent developments in influenza VLP vaccines against seasonal, pandemic, and avian influenza viruses from the perspective of use in humans. The immunogenicity and efficacies of influenza VLP vaccine in the homologous and cross-protection were reviewed. Discussions include limitations of current influenza vaccination strategies and future directions to confer broadly cross protective new influenza vaccines as well as vaccination.

Keywords

Influenza virus; Virus-like particles; Vaccines; strain-specific protection; cross protection

1. Introduction

Influenza epidemics and the threat from novel pandemic outbreaks remain major health concerns since influenza can cause high morbidity and mortality in humans and animals. Influenza-associated disease affects all age groups, with the highest hospitalization rates occurring in children and the elderly, and claims an average of 36,000 deaths annually in the US alone [1, 2]. There are three types of influenza viruses: A, B and C. Human influenza A and B viruses cause seasonal epidemics of disease, outbreaks can occur all year round. The emergence of a new and very different influenza virus to infect people with effective

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transmissibility can cause an influenza pandemic. Influenza type C infections cause a mild respiratory illness and are not thought to cause epidemics. Influenza A viruses are divided into subtypes based on two proteins on the surface of the virus: the hemagglutinin and the neuraminidase. There are 18 different hemagglutinin subtypes and 11 different neuraminidase subtypes (H1 through H18 and N1 through N11 respectively) [3]. Each influenza A virus subtype can include many different strains with unique serologic antigenicity. Current subtypes of influenza A viruses commonly circulating in humans are A H1N1 and H3N2 subtype viruses. In the spring of 2009, a new influenza A (H1N1) virus emerged to cause pandemic illness in humans. This new virus was antigenically quite different from the human influenza A (H1N1) viruses circulating at that time and (often called “2009 H1N1”) has now replaced the H1N1 virus that was previously circulating in humans. Influenza B viruses do not have different subtypes but can be further divided into two different lineages and many strains. Currently circulating influenza B viruses belong to one of two lineages: B/Yamagata and B/Victoria. Homologous immunity refers to the immunity that protects host from exactly the same virus infection. The term heterologous immunity refers to the immunity against different strains within the same subtype virus.

In 2009, a new influenza A strain emerged and spread globally at alarming speeds, which resulted in declaring the first 21st century pandemic [4]. Effective vaccination is considered the most cost effective measure to prevent infectious diseases. Different platforms of influenza vaccines have been available on the market, which include inactivated whole virus, inactivated split vaccine (currently the most common type), live attenuated influenza vaccines (LAIV), and recombinant purified protein subunit vaccines. Trivalent vaccines contain current H1N1, H3N2, and a lineage of influenza B. A quadrivalent formulation includes two subtypes (H1N1, H3N2) of influenza A and both lineages of influenza B virus. LAIV is recommended for vaccination in the ages over 2 years old and inactivated or subunit vaccines are for a broader coverage with ages over 6 months old. Cell-based vaccines and recombinant HA-protein based vaccines are licensed although their current market-share is currently limited.

Recently, human infections by H5N1 and H7N9 avian influenza virus have brought real concerns and alert, emphasizing the priority of developing a broadly cross protective vaccine and an effective vaccination method [5]. Avian influenza virus outbreaks could threaten the supply of egg substrates for manufacturing influenza vaccines in the case of concurring pandemics. The experience with the 2009 H1N1 virus demonstrated that conventional influenza vaccine manufacturing process using the egg substrate significantly delayed the timely preparation and supply of vaccines to control the spreading a new pandemic. Critical shortages and delays in supply of the 2009 pandemic vaccine occurred, due in part to time-consuming laborious process and inferior growth in egg substrates compared to seasonal vaccines. Thus, approaches to produce egg substrate-independent influenza vaccines are highly desirable for supplying effective vaccines for future pandemics. Alternative immunogenic vaccines that can be prepared within a short time window and that do not rely on egg substrates would be highly desirable.

Influenza virus like particle (VLP) vaccines were able to be prepared within a short time frame of approximately 4 weeks and could be more immunogenic in naïve animals [6–8].

Influenza virus-like particles (VLPs) have been developed by several different laboratories. Previous and recent studies show that influenza VLP vaccination induces protective immune responses against seasonal, pandemic, and avian influenza viruses in animal models (Tables 1, 2, 3, 4). Herein, we discuss the progress of influenza VLP vaccines in which influenza VLPs present different influenza antigenic proteins and/or adjuvants in a membrane-anchored form. Vaccine immunogenicity and efficacies of different influenza VLPs in animal models and the current status of influenza VLP in clinical studies are reviewed.

2. Substrates for production of virus-like particles as effective vaccine platforms

Virus-like particles (VLPs) are formed by self-assembly competent proteins in cells expressing viral structural proteins. VLPs refer to a number of biological particles with morphology and structures similar to virus but they do not contain infectious genomic materials, and thus represent safe vaccine platforms. In addition, VLPs are highly immunogenic even in the absence of adjuvants compared to soluble subunit protein vaccines. Vaccination of single dose of H5N1 influenza VLPs provided protection against lethal challenge in mice. In contrast, vaccination of mice even with a 5 fold higher dose of soluble recombinant H5 HA vaccine was not effective inducing protection [9].

Commercial recombinant vaccines against hepatitis B virus (HBV) and human papilloma virus (HPV) include adjuvants and are based on recombinant VLP platforms, escalating the interests in new VLP vaccine technologies in recent years. Non-enveloped VLPs can be produced in insect, mammalian, yeast, plant or bacterial cells expressing self-assembly viral proteins such as recombinant HBV and HPV vaccines [10, 11]. Production and purification of enveloped VLPs are more complicated than those of non-enveloped VLPs. Enveloped influenza VLP vaccines have been produced mostly in mammalian cells and plants expressing the self-assembly core protein and protective surface target proteins. In particular, the recombinant baculovirus/insect cell-expression system was commonly utilized to produce influenza VLP vaccines that present influenza virus surface proteins, hemagglutinin (HA), neuraminidase (NA), and/or matrix protein 2 (M2) proteins in addition to the self-assembly competent matrix core protein 1 (M1) [12–15]. Electron micrographs of these influenza VLPs displayed 80–120-nm diameter particles with apparent spikes which resemble influenza virus [15, 16]. VLPs of influenza subtypes H1, H3, H5 and H9 have been generated in insect cells by co-expression of HA, NA, M1 and M2 or HA, NA and M1 or by co-expression of HA and M1, respectively. Plants can be an alternative system for VLP vaccine production owing to their capacity to produce large quantities of recombinant proteins and the post-translational modification. The recent development of plant virus-derived transient expression systems significantly improved the VLP production speed and yield [17]. Also, mammalian cells stably expressing structural proteins of influenza virus expressed influenza VLPs presenting high levels of HA and NA on the surfaces [18]. Besides these single-virus-derived VLPs, it is possible to produce chimeric VLPs by pseudotyping in which a viral antigen from one virus is displayed on the VLP core of another virus [19]. In terms of potential manufacturing scale up, the insect cell expression system would be more feasible compared to other substrates. Among the different sources of

cell lines, insect cells were most commonly used to manufacture VLPs, which is a focus in this review.

3. Influenza VLP vaccines conferring protection against homologous virus

Studies have shown that influenza VLP vaccines provide protection against homologous influenza virus in animal models as summarized in Table 1 using mouse or ferret animal models. Hemagglutination inhibition (HAI) titers are a standard assay used for assessing the influenza vaccination efficacy and titers over 40 are considered protective serum antibodies. A range (104 – 1174) of protective HAI titers were induced in mice and ferrets intramuscularly immunized with influenza H3N2 VLPs containing M1, HA, and NA derived from A/Fujian/411/2002, and HAI titers were dependent on the VLP vaccine dose (0.024 – 3 µg HA) [7]. HAI titers induced by influenza virus H3N2 VLP vaccination were higher by 2 to 3 folds compared to those by recombinant HA proteins or whole inactivated influenza virus vaccine at equivalent doses [7]. IgG2a and IgG2b isotype dominant IgG antibodies were reported to be induced by influenza VLP vaccination indicating T helper type 1 (Th1) immune responses [7, 9, 12]. Follow up studies also demonstrated that VLP vaccines derived from H1N1 (A/PR/8/34, A/California/04/09, A/New Caledonia/20/1999, A/South Carolina/1/1918), H3N2 (A/Aichi/2/1968) were able to provide high efficacies of protection as evidenced by no or low levels of lung viral titers as well as no or minimum body weight loss in the absence of adjuvant [12]. The vaccine doses of influenza VLP vaccines used for immunization of mice were 3 to 10 µg total VLP proteins representing 0.3 to 1 µg in terms of HA protein antigen (Table 1). Current seasonal vaccines are trivalent or quadrivalent composed of predicted circulating influenza A virus strains H1N1, H3N2, and one or two influenza B virus strains. To mimic seasonal vaccines, the efficacy of a trivalent VLP vaccine was investigated in mice and ferrets, which was composed of HA, NA, and M1 derived from H1N1 (A/New Caledonia/20/1999), H3N2 (A/New York/55/2004), and influenza B virus (B/Shanghai/361/2002) [20]. The authors showed comparison data among the groups with 3 µg of trivalent VLP vaccines, monovalent VLP, or trivalent inactivated virus vaccine. HAI titers by the trivalent VLP vaccine (3 µg HA) were comparable to those by each monovalent VLP vaccine. Despite high HAI titers against seasonal H1N1, serum HAI titers and protective efficacy against mouse adapted H1N1 were low, suggesting a possibility that the challenge virus might have changed, behaving like a heterotypic virus [20]. Mice that were immunized with trivalent VLP vaccines elicited HA-specific CD8 T cell responses at higher levels than a commercial trivalent vaccine [20]. Ferrets that were immunized with 15 µg HA of trivalent VLP vaccine showed the lowest viral titers when challenged with the homologous H3N2 virus [20]. Thus, VLP-based seasonal influenza vaccines can be comparable to or better than commercial vaccines in animal model studies.

The routes of delivering influenza VLP vaccines include intramuscular (IM), intranasal route (IN), or microneedle (MN) immunization on the skin. The efficacy of influenza VLP vaccines via MN skin route was higher than that with IM immunization by enabling dose-sparing effects and rapid recall immune responses [21–23]. In addition, microneedle-based immunization using influenza VLP vaccines could have logistic advantages associated with avoidance of hypodermic needles and syringes, potential for rapid distribution and administration of microneedle patches, indicating a great impact on improving vaccination

efficacy, coverage, and public health. The mechanisms by which microneedle vaccination induces superior immunity to that with IM immunization are unknown. It has been suggested that vaccine antigens delivered to the skin are more likely to be captured by antigen-presenting cells, such as Langerhans cells and dermal DCs, and transported to the draining lymph nodes for B cells to respond [24–27]. Importantly, human skin experiments demonstrated that H1 and H5 VLP vaccines, when delivered via MN, stimulated Langerhans cells resulting in changes in cell morphology and a reduction in cell number in epidermal sheets [28]. Thus, this study using a viable human skin model implicates that MN skin delivery of influenza VLPs can promote trafficking skin dendritic cells to the draining lymph nodes for effective induction of protective immunity. The findings in this study are consistent with results from previous studies, explaining this phenomenon as passenger leukocyte concept in transplant models [29, 30].

It is likely that vaccine antigens delivered to the skin are effectively captured by cells which present antigens and stimulate B cells. In contrast, antigens delivered into the muscular tissues where antigen presenting cells are relatively sparse are passively drained to the regional lymph nodes via the lymphatic conduits or bloodstream [31, 32]. There are some limitations in the skin delivery of vaccines via solid MN patches. Dry process for coating of the vaccines onto solid steel MN patches involves a phase change from liquid to solid formulation and is associated with causing the loss of vaccine stability and addition of stabilizers may be required, which needs to be investigated for each type of vaccines. It remains to be determined whether coating vaccines would show efficacy during the mass manufacturing process of MN vaccine patches.

Adjuvants have a profound effect on the induction of protective immunity induced by VLP. Adjuvants flagelin, enterotoxin, cholera toxin, alum, CpG and MPL have been tested in VLP vaccine studies as seen in Table 1. Significantly decreased lung titers and body weight loss post-viral challenge were found in animals that received VLPs with adjuvants [33]. Also, the addition of adjuvant showed VLP vaccine dose-sparing effects with improved efficacy. The protective immune responses, efficacies of protection, and antigen-sparing effects were significantly improved by a second immunization as determined by the levels of neutralizing antibodies, morbidity post-challenge, lung viral titers, and inflammatory cytokines. The protective immunity induced by a single dose or two doses of influenza VLPs, dependent on antigen dosage and the presence of adjuvant.

Influenza M1 VLPs containing NA only without HA were generated to assist the protective role of NA, the second major glycoprotein [34]. Mice immunized with NA VLPs were 100% protected against lethal infection by the homologous virus A/PR/8/34 without weight loss as seen in Table 1.

Taken together, influenza virus-like particle vaccines containing HA or NA increased both the immunogenicity and efficacy against homologous virus challenges in mice. Influenza VLPs were demonstrated to provide protective immunity via either the intranasal or intramuscular route in the absence of adjuvants. Microneedle route immunization provides complete protection with dose-sparing effect. The presence of adjuvant significantly improved vaccine efficacy with showing antigen-sparing effects. Certainly, vaccine

efficacies were significantly improved by a second immunization compared to a single immunization.

4. Influenza VLPs inducing cross-protection against heterologous virus

The influenza VLP vaccine capable of conferring heterologous cross-protection was first reported by Galarza et al, (2005) demonstrating that influenza VLPs containing M1 and HA derived from A/Udorn 72 (H3N2) provided protection against heterologous A/Hong Kong/68 (H3N2) virus [35]. Mice that were intramuscularly or intranasally immunized twice with influenza VLPs containing HA (1 µg) were protected against lethal challenge with 5 times 50% lethal dose (LD₅₀) of heterologous mouse-adapted A/Hong Kong/68 (H3N2) virus. Influenza M1-HA VLPs derived from the A/PR/8/34 H1N1 virus provided 100% protection against closely related heterologous strain H1N1 (A/WSN/33) with only moderate body weight loss of 4% [12]. As expected, the protective efficacy against homologous virus was significantly higher as assessed by lung viral titers with over a hundred fold lower in the homologous challenge group than those in the heterologous challenge group. Clearance of lung viral titers appeared to be consistent with higher neutralizing activity, HAI titers, and binding antibodies against homologous virus A/PR/8/34 (Tables 1 and 2). Influenza HA VLPs could provide significant protection against heterologous strain despite antigenic changes with approximately 91% amino acid homology in HA between A/PR/8/34 and A/WSN/33 viruses [12]. In contrast, A/Aichi/2/1968 HA VLP vaccine was not able to confer heterologous cross-protection against A/Philippines/1982 virus, indicating the significant antigenic drift between the two virus [36]. The amino acid identity between A/PR/8 and A/WSN/33 is similar to that between A/Philippines/82 and A/Aichi/2/68. However the amino acid differences between latter viruses is confined to well-recognized antigenic regions suggesting a larger antigenic difference. Thus A/WSN/33 and A/PR/8/34 are probably antigenically much more closely related to one another than A/Aichi/2/68 and A/Philippines/2/82, despite similar levels of sequence identity.

Single immunization with 2009 pandemic influenza HA VLPs was able to induce protective immunity against homologous virus in mice or ferrets, but a single dose of HA VLPs was not sufficient to confer protection against antigenically distant A/PR/8/34 virus [8, 22]. Nonetheless, prime-boost vaccination with 2009 pandemic HA VLPs induced significant cross-protection against the antigenically distinct viruses A/New Caledonia/20/99 and A/PR/8/34, indicating the requirement of multiple immunizations for heterologous cross-protection. Most subunit or killed vaccines such as killed polio vaccine, *Haemophilus* influenza type b polysaccharide conjugate vaccine, and hepatitis B recombinant protein vaccine require prime-boost or multiple immunizations [37]. Mice given 2 doses of vaccine with replication-deficient viral replicon particles expressing influenza HA showed high levels of IgG2a isotype antibodies contributing to lowering lung viral titers without strong virus neutralizing activities in vitro [38]. Consistent with this study, mice that received 2 or more doses of vaccine elicited high levels of cross-reactive IgG2a antibodies, likely contributing to increased clearance of virus against heterologous virus A/PR/8/34 or A/New Caledonia/20/99. Th1 type (IgG2a) immunity and/or opsonization of virus particles via Fc

receptors, complement-mediated lysis of infected cells or through ADCC might be possible mechanisms for heterologous cross-protection.

Influenza VLPs containing both HA and NA derived from pandemic virus A/California/04/09 were not able to provide sufficient protection against heterologous influenza virus A/Brisbane/59/07 in a ferret model, indicating significant antigenic changes between seasonal and pandemic virus [8]. The 1918 pandemic virus with high virulence and transmissibility affecting young healthy adults was recorded to cause the worst disease claiming 50 million deaths worldwide. 1918 H1N1 pandemic VLPs were highly immunogenic in intranasally immunized mice with or without CpG adjuvant, protecting against A/swine/Iowa/15/30 (H1N1) antigenically related to the 1918 virus [39]. Interestingly, VLPs containing both HA and NA derived from 1918 pandemic A/South Carolina/1/1918 fully protected aged mice from 2009 pandemic H1N1 virus challenge 16 months after vaccination of mice at young age [40]. These studies provide convincing evidence that VLP vaccines using a safe cell-based technology can protect against 1918 pandemic virus.

5. Novel VLPs to improve the cross protective efficacy

Developing a vaccine conferring heterosubtypic protection has been a challenge. Intranasal immunization of mice with a cocktail of VLPs containing H1, H3, H5, or H7 induced heterologous and heterosubtypic protection against influenza viruses including 1918 H1, 1957 H2, and avian H5, H6, H10, and H11 subtypes[41]. The Toll-like receptor 5 ligand flagellin, the major proinflammatory determinant of enteropathogenic *Salmonella*, was engineered to be expressed in a membrane-anchored form which was subsequently incorporated into influenza VLPs [42]. Thus, a chimeric influenza VLP (cVLP) vaccine containing HA and M1 from A/PR8/34 (H1N1), and flagellin as a molecular adjuvant was tested for their cross protective efficacy. Intranasal (IN) immunization of mice with H1 HA cVLPs induced higher levels of virus-specific antibodies mucosally and systemically compared to the normal H1 HA VLP immunization [43], enhancing the immunogenicity of cVLPs. The mice that were immunized with cVLPs containing flagellin molecular adjuvant also showed improved heterosubtypic cross -protection against H3N2 A/Philippines/82 virus [43]. IN immunization with cVLPs was more effective in lowering lung viral titers and conferring heterosubtypic cross-protection than IM immunization. However, the mice immunized with cVLPs showed severe weight loss over 15% and substantial levels of lung viral loads after heterosubtypic virus challenge, indicating the low efficacy of cross-protection by VLP vaccines based on HA immunity.

VLPs composed of influenza M1 and membrane-anchored HA contain lipid bilayers derived from host cells similar to enveloped viruses. A recent study described a novel protein transfer technology to enhance the potency of enveloped VLPs by decorating influenza VLPs with exogenously added glycosylphosphatidylinositol-anchored (GPI) granulocyte macrophage colony-stimulating factor (GM-CSF) [44]. Incorporation of immunostimulatory GM-CSF into influenza VLPs was dependent on the presence of GPI-anchor, and found to be stable and functional. Modified influenza VLPs with GPI anchor GM-CSF were effective in enhancing both heterologous and heterosubtypic protection .

NA is the second major glycoprotein on the surface of influenza viruses but its contribution to protection is relatively less well known than HA. A VLP platform presenting NA in a membrane-anchored form would mimic NA on influenza viruses without complication with HA immunity. Mice that were intranasally immunized with VLPs containing NA were well protected against homologous virus A/PR8 (H1N1) by lowering lung viral loads over 3 log₁₀ folds and preventing weight loss [34]. Notably, 100% survival protection against H3N2 heterosubtypic virus (A/Philippines/82) was observed in mice that were immunized with PR8 NA VLPs although these mice experienced severe weight loss of over 20% [34]. Since NA VLP vaccine was administered intranasally, mucosal responses such as IgA antibody might have contributed to heterosubtypic protection. Vaccination with adjuvanted recombinant NA proteins was demonstrated to induce broad heterologous but not heterosubtypic cross-protection against influenza virus in mice [45]. Presenting NA on VLPs is expected to be more effective in inducing cross protective immunity. In support of this, HA VLP vaccines were shown to be superior to recombinant soluble HA protein or split vaccines in inducing protection in mice and ferrets [8, 9, 46].

6. Influenza VLP vaccines against potential pandemic virus

An influenza pandemic can occur when a non-human novel influenza virus gains the ability for efficient and sustained human-to-human transmission and then spreads globally. The 2009 influenza pandemic clearly illustrated the limitations in the current influenza vaccination strategy and practice. There were significant shortages and delays in the global supply of the egg-based vaccines. VLP technology could be an attractive option because of safety, immunogenic characteristics, rapid and large-scale up production of vaccines. Influenza 2009 H1N1 VLPs were produced in insect cells expressing HA, NA, and M1 proteins of A/California/04/2009 using the recombinant baculovirus expression system [47]. Ferrets immunized with influenza 2009 H1N1 VLPs induced HAI activity at high titers, and inhibited replication of the challenge virus (A/Mexico/4482/09). A single dose (15 µg) of 2009 H1N1 VLPs resulted in complete virus clearance in the lungs of vaccinated ferrets [47]. Also, mice that received a single IM vaccination with VLPs containing 2009 H1 HA were 100% protected against A/California/04/2009 pandemic virus and partially protected against A/PR8 antigenically distinct virus [22]. Protective immune correlates include Th1 dominant IgG2a isotype antibodies, high HAI titers, and rapid recall IgG and IgA antibody responses. HAI responses against diverse geographic pandemic isolates were observed in immune sera from mice with 2009 H1 HA VLP vaccination. Thus, recombinant influenza VLP vaccines can be developed as an effective strategy against pandemic H1N1 virus and avoid the need to isolate high growth reassortants for egg-based influenza virus vaccine production.

The first outbreak of H5N1 highly pathogenic avian influenza virus infection in humans caused 6 deaths from the 18 confirmed cases in 1997 [48]. H5N1 avian influenza viruses are recognized as a continuous threat to humans and poultry farms as evidenced by the accumulated confirmed deaths of 608 people due to H5N1 virus infection. If avian origin high pathogenic pandemic occurs, conventional vaccine manufacturing facilities would be limited for producing H5N1 virus vaccines due to high pathogenicity and safety concerns on handling the live viruses. Inactivated H5N1 vaccines are not highly immunogenic and often

require higher doses of vaccines to elicit protective immune responses. In this respect, VLP technology is considered a highly attractive approach for producing vaccines against pathogenic avian influenza viruses. Many preclinical efficacy studies on avian origin influenza VLP vaccines were reported by different laboratories using mouse and ferret animal models. Homologous and cross-clade heterologous protective immune responses were induced in mice that were intramuscularly immunized with 0.6 – 3 µg HA of H5N1 VLPs produced in insect cells expressing either clade 1 (A/Viet Nam/1203/1204, A/VN) or clade 2 (A/Indonesia/05/2005, A/Indo) HA and NA in addition to M1 core matrix proteins [16]. The efficacy of homologous and heterologous protection by H5N1 VLPs was significantly higher compared to that by the recombinant HA protein vaccines. Interestingly, clade 2 H5N1 VLP vaccinated mice were protected against both clade 1 and 2 H5N1 reassortant viruses [16, 46]. The clade 1 H5N1 VLP vaccines were effective in eliciting protective immunity to the clade 1 virus but much less protective against the clade 2 H5N1 virus [16, 46]. Ferrets that were immunized with 0.6 to 15 µg of clade 2 H5N1 VLPs (A/Indonesia/05/2005) survived lethal challenge infection with clade 2 homologous (A/Indo) and clade 1 (A/VN) H5N1 virus [49]. Clade 0 H5N1 VLPs were produced in insect cells by baculoviruses co-expressing triple (HA, NA, M1) or quadruple (HA, NA, M1, and nucleoprotein NP) from A/goose/1996 (clade 0) [50]. Clade 0 H5N1 VLPs incorporating NP were more effective in generating immune responses protective against a heterologous lethal infection with H5N1 (clade 2.3.4) virus in vaccinated chickens [50]. Mice and ferrets that were intramuscularly immunized with influenza-pseudotyped VLPs with the murine leukemia virus Gag protein plus HA and NA from A/VN or A/Indo H5N1 virus also induced protective immunity without apparent morbidity [51]. Retrovirus Gag-derived VLPs presenting HA, NA, and M2 from H7N1 or H5N1 antigenic subtype viruses were effective in generating neutralizing antibodies at high titers in mice [52]. Influenza VLPs containing the HA, NA, and M1 proteins of H9N2 virus (A/Hong Kong/1073/99) were reported to induce antibodies that can inhibit replication of H9N2 virus in Balb/c mice [15] and to confer improved immunogenicity and protection with novasome adjuvant [53]. A/Multiple protections against H5N1, H7N1, and H9N2 viruses were demonstrated in ferrets that were intranasally immunized with triple-subtype influenza VLPs using a baculovirus expressing the H5, H7, and H9 genes from A/VN, A/New York/107/2003, and A/Hong Kong/33982/2009 [54]. These studies support development of VLP-based vaccines to prevent future potential pandemic due to highly pathogenic avian influenza H5, H7, and H9 subtype viruses. VLP vaccination can contribute to control of future pandemics but prevention of pandemics is difficult to envisage as it would require high vaccination coverage of regions where such viruses circulate in husbandry.

Cross protective efficacies of the 1918 H1N1 pandemic VLP vaccine were compared in preclinical studies of mice and ferrets after IN and IM immunizations [55]. This study demonstrated that IN immunization with 1918 H1N1 VLPs containing HA and NA was more effective in inducing heterosubtypic cross-protection against the H5N1 virus in both animal models. The longevity of cross protective immunity elicited by the 1918 pandemic H1N1 VLPs was investigated by vaccinating young mice (8 to 12 weeks) and then challenging the aged mice with 2009 pandemic H1N1 influenza virus [40]. The aged mice that were intramuscularly prime-boost vaccinated with 3 µg of 1918 H1N1 VLPs 16 months

earlier were protected against 2009 H1N1 pandemic virus and immune sera from the aged vaccinated mice conferred survival protection but not prevented morbidity. Thus, immunity acquired early in life by vaccinating influenza VLPs can provide survival protection but not prevention of infection in older ages in mice.

Since the new outbreak of avian H7N9 virus in 2013, human cases of influenza A H7N9 infections became the highest number of approximately 275 deaths attributed to the avian H7 subtype virus until now. Soon after an outbreak of avian H7N9 virus, an H7N9 VLP vaccine was produced by infecting insect cells with baculovirus expressing HA, NA from A/Anhui/1/2013 (H7N9) and M1 from A/Indonesia/05/2005 (H5N1) within a month [56]. The immunogenicity and efficacy of H7N9 VLPs were investigated in BALB/c mice in comparison with H7N3 VLPs (A/Chicken/Jalisco/CPA1/2012) and H5N1 VLPs [56]. H7N9 VLP vaccines were immunogenic and elicited HAI titers over 64 against the homologous virus and cross-reactive HAI against H7N3 virus in addition to anti-NA antibodies. All mice that were immunized with H7N9 VLPs or H7N3 VLPs showed 100% survival protection against a lethal wild-type A/Anhui/1/2013 virus challenge [56]. Another study also reported the production of H7 HA VLPs containing HA derived from A/Anhui/1/2013 or A/Shanghai/1/2013 and M1 from A/Udorn/307/72 (H3N2) [57]. Single vaccination of mice induced protective immune responses including cross-reactive HAI titers and conferred survival protection against lethal challenge with H7N9 virus even with a low dose (0.03 µg HA) [57]. In addition, the ferrets that were vaccinated with H7N9 VLPs were protected against the homologous virus as evidenced by reductions in fever, weight loss, and virus shedding [58]. These studies support the concept that H7N9 VLP vaccines can be effective in generating protective immunity against avian influenza H7N9 virus with pandemic potential.

7. Clinical studies of influenza VLP vaccines

Baculovirus is a non-pathogenic insect virus, and proven to be harmless to vertebrates, and cannot replicate in mammalian cells [59, 60]. Insect cell culture derived trivalent seasonal influenza VLP vaccines were shown to be more immunogenic compared to the conventional egg-substrate split vaccines (a Phase II human clinical trial of the seasonal influenza VLP vaccine candidate, Novavax, Inc.). During the 2009 H1N1 pandemic, a phase II study was performed with the 2009 pandemic influenza VLP vaccine (5, 15, or 45 µg HA) in 4563 healthy adults, 18–64 years of age in Mexico [61]. Mild adverse events were observed in the higher dose groups (15 µg and 45 µg) compared to the low dose (5 µg) and placebo groups [61, 62]. The seroconversion rate (= or > 40 HAI) was high up to 82–92% even after a single vaccination [61]. In an FDA-approved phase I/II human clinical study, two doses of H5N1 VLPs (A/Indonesia/05/2005) at 15, 45, or 90 µg HA resulted in seroconversion and production of functional antibodies preferentially binding to the oligomeric form of hemagglutinin [62]. The two high dose groups showed cross reactivity against other clade 2 subtypes of H5N1 virus, HA antibodies reactive to diverse epitopes, and NA inhibiting antibodies [60]. A recombinant H7N9 VLP vaccine containing HA and NA from A/Anhui/1/13 with M1 (A/Indonesia/5/05) was evaluated in healthy adults (≥ 18 years of age) in a randomized, blinded, placebo-controlled clinical trial [6, 63]. Groups with 5 or 15 µg of two doses in the saponin-based ISCOMATRIX adjuvant formulation showed local and systemic

reactions although none of these adverse events was severe. The seroconversion rate as measured by HAI (≥ 40) was 5.7% and 15.6% in the subjects vaccinated with 15 ug or 45 ug of HA, respectively, without adjuvant. The adjuvanted groups with 5 ug or 15 ug HA H7N9 VLPs showed high seroconversion rates of 60% to 80.6% [6, 63]. Interestingly, N9 NA-inhibiting antibodies were observed in the 71.9% and 92% vaccinated individuals without and with adjuvant, respectively [6, 63]. These clinical studies demonstrated the safety and efficacy of influenza VLP vaccines produced in insect cells using the rBV expression system, further supporting the rationale for developing VLP vaccine technology.

VLP vaccines produced in plants as an alternative system have been tested in clinical studies. In a phase I human trial with 48 adult volunteers (18–60 years of age), safety and immunogenicity of plant-produced enveloped H5 HA (A/Indonesia/5/05) VLPs were evaluated with prime boost intramuscular doses of 5, 10, or 20 μ g HA of alum adjuvanted H5 VLP vaccines [64]. HAI antibodies over 40 were observed in the 16.7, 25, and 50% volunteers with 5, 10, or 20 μ g HA dose respectively [64]. Two doses of 20 μ g H5 HA VLPs were at least required to meet the protective criteria of seroprotection (single radial hemolysis $>70\%$), seroconversion (HAI $>40\%$), and geometric mean increase (>2.5) in microneutralization [65]. Plant-made HA VLPs were well tolerated and the adverse events were mild to moderate, demonstrating the safety in humans. In a phase II human clinical trial with 135 volunteers, the dose of 20 μ g H5 HA of plant-derived VLPs was shown to be optimal in older and younger individuals [66]. A study of phase I clinical trial has evaluated the plant-derived seasonal flu HA VLP vaccines of 2009 H1N1 pandemic virus [66]. A single dose of 5, 13, or 28 μ g HA of plant H1 VLPs was given to adult volunteers 18–49 years of age [66]. A single dose of 5 μ g HA of plant H1 VLPs was reported to induce detectable seroconversion immune responses [66]. Encouraging phase I clinical results support the rationale of planning a phase II human trial of quadrivalent plant HA vaccines composed of H1N1, H3N2, and two strains of influenza B viruses in adults. Finally, safety and immunogenicity of bacteriophage gH1-Qbeta-derived recombinant VLP vaccines were tested in a double-blinded, randomized phase I clinical trial in healthy volunteers [67].

8. Expert Commentary

VLPs can present glycoproteins in a membrane-anchored form mimicking the natural conformation of enveloped viruses, and are far more immunogenic than protein-based subunit vaccines. The particulate nature of VLPs is effective in stimulating antigen presenting cells and thus in eliciting CD4 and CD8 T cell immune responses. Antigens presented on VLPs appear to be processed by antigen presenting cells through cross-presentation for inducing CD8 T cells via MHC class I-loaded peptides as well as class MHC class II pathway for CD4 T cell responses. More importantly, a unique display of epitopes in a repetitive and high density on the VLP surfaces is another promising feature of VLP vaccines for cross-linking B cell receptors for generating protective antibody responses. Consistent with these cellular immune responses, influenza VLP vaccines are also known to effectively induce Th1 type IgG2a class and broadly reactive antibody immune responses. Together with non-infectious nature of VLPs, new vaccines based on VLP technology are worth continuing studies to develop effective vaccines against viruses with high antigenic variation. In the aspect of scale-up vaccine production, influenza VLPs

can be produced in suspension cultures of insect cells in a large fermenter, which is an advantage over the individual egg inoculation of current vaccine strains. The purification process of influenza VLPs can follow similar steps as current influenza vaccine manufacturing process. Influenza VLP vaccine is expected to take much less time for production since growth adaptation of vaccine strains is not needed.

The outbreaks of highly pathogenic avian influenza viruses will significantly affect the poultry farms economically as well as the supply of pathogen-free fertile eggs. In particular, during a pandemic, insufficient supply and limited manufacturing capacity would not meet the surge in high vaccine demand. Development of alternative influenza vaccine approaches independent of egg substrates has been a high priority during a last decade. Purified protein vaccines and mammalian cell culture-derived inactivated split vaccines are approved for human vaccination although their market shares are not significant. Purified protein subunit vaccines are relatively low immunogenic compared to VLP-based vaccines and the production cost may be high due to purification procedures, but protein vaccines are safe and would be effective in primed individuals. Cell culture derived split vaccines have similar efficacies to egg-produced split vaccines, and represent an alternative choice of vaccine production during a pandemic without the selection of high egg-growth reassortants.

Encouraging results from the preclinical and clinical studies of VLP-based vaccines indicate that influenza VLP vaccines can be viable human vaccine candidates against seasonal and pandemic influenza vaccines. Both insect cell-derived influenza VLPs and plant-produced influenza VLPs appear to have similar efficacies in clinical trials. Given the comparable safety and efficacy of influenza VLP vaccines in clinical trials, it is expected that current egg-based seasonal vaccines would continue to dominate the influenza vaccine market. In the case of pandemic outbreaks, insect cell-produced pandemic VLP vaccines are expected to be manufactured faster than egg-growth vaccines. An advantage is that the manufacturing process of recombinant influenza pandemic VLP vaccines does not involve handling pathogenic avian flu live viruses that require high biosafety level facilities. Probably due to the intrinsic limitation of the plant-expression system, most plant-derived influenza VLP vaccines are based on the immunity to HA alone. Compared to the plant-derived VLPs, the recombinant baculovirus expression system in insect cells has high flexibility to engineer for co-expressing multiple genes to produce VLPs containing several influenza virus proteins (HA, NA, M1, M2). Vaccination with insect cell-derived VLPs containing HA and NA (and M2) influenza proteins was demonstrated to induce HAI, virus neutralizing antibodies and NA inhibiting antibodies in preclinical and clinical studies. Inducing immunity to both HA and NA is expected to be advantageous for broadening the breadth of cross-protection.

9. Five-year view

Most current influenza vaccines are manufactured using the fertilized egg substrates with selected high-growth reassortants containing HA and NA genes from the predicted circulating HA strains. Due to high mutability of influenza viruses in nature, it is often difficult to exactly predict the circulating influenza strains in the coming winter season. A promising approach for a future direction will be to develop influenza VLP vaccines

containing multiple HA and NA subtype proteins. An example is to develop multivalent HA subtype (H5+H7+H9) VLP vaccines as described [54].

It is a high demand to develop cross-protective influenza vaccines, which requires novel vaccines and new vaccination strategies. Heterosubtypic cross-protection can be mediated in animal models by serotype cross-reactive cytotoxic T lymphocytes that recognize conserved epitopes shared by different influenza viruses. In humans, cytotoxic T cells with cross-reactivity were reported to recognize the different subtype of influenza A virus [68]. In the absence of cross-reactive neutralizing antibodies, levels of influenza virus-specific CD4⁺ T cells and CD8⁺ T cells were found to have correlations with protection in humans [69, 70].

Development of cross protective universal influenza vaccines should be continued as a high priority. Recent studies suggest that cross protective neutralizing and non-neutralizing antibodies can significantly contribute to inducing cross-protection [13, 71] [72, 73]. Universal influenza vaccines are designed to present the highly conserved protective epitopes in an immunogenic form and to utilize effective adjuvants. The candidates of conserved epitopes include the extracellular domain of ion channel protein M2 (M2e), the stalk domain of HA, and nucleoprotein (NP). NA is less variable than HA and thus considered a target for inducing cross-protection within the same subtype [73]. Molecularly engineered tandem repeats of M2e epitopes were presented on the surfaces of VLPs at high density, highly immunogenic, and able to raise M2e antibodies cross protective against different subtypes of influenza viruses [72]. Headless HA stalk domains were engineered to be stably expressed on the cell surfaces, but their incorporation into VLPs was relatively low [74]. Headless HA stalk VLPs produced in transfected mammalian cells were able to confer cross-protection against selective strains of influenza viruses in vaccinated mice. Co-expression of HA, NP, and M1 in insect cells derived the incorporation of NP into HA VLPs that were more cross protective than HA VLPs alone [50]. Thus, VLP technology has high versatility in incorporating molecularly engineered influenza conserved epitopes into particulate forms. Development of VLPs containing multiple conserved epitopes would be a promising approach in future.

Antibodies to these conserved influenza epitopes (M2e, HA stalk, NP, NA) are not able to neutralize viruses and thus confer relatively weak cross-protection compared to the neutralizing HA antibodies. To overcome the limitation of strain-specific protection via HA neutralizing antibodies, a promising approach will be to apply influenza vaccination supplemented with cross protective conserved target epitopes presented on VLPs. It is expected that novel influenza VLP vaccines presenting conserved epitopes in an immunogenic form significantly improves the efficacy of cross-protection.

10. Key Issues

- Eggs independent influenza vaccine production: Influenza VLPs can be produced in various expression systems (insect cells, mammalian cells, plant cells).
- Influenza VLPs containing HA are more immunogenic and have higher protection efficacy compared to protein vaccines or inactivated whole

virus or split vaccines. Influenza VLP vaccines are able to induce cross-protection among the strains antigenically related.

- Seasonal influenza VLP vaccines provide an alternative manufacturing option in the case of shortage of vaccine supplies.
- Recombinant VLP technology provides versatility to incorporate immunostimulatory molecules into influenza VLP vaccines.
- Avian influenza VLP vaccines may offer a prophylactic measure to prevent future pandemic.
- Clinical trials of influenza VLP vaccines (seasonal, 2009 H1N1 pandemic, H5N1, H7N9) are promising in terms of acceptable safety and immunogenicity.

References

Reference annotations

* Of interest

** Of considerable interest

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Table 1

VLP vaccine against homologous influenza viruses

Subtype	Components / Adjuvant	Dose/Route	Prime/Boost	Virus Challenge	Protection Lung titer (PFU/ml)/% of decreased Body weight	Reference
H1N1 (A/PR/8/34)	HA, M1	VLP 10 μ g / IN	Three	H1N1	5 $\times$ 10 ¹ / 4% (D5)	[12]
H3N2 (A/Fujian/411/2002)	HA, NA, M1	VLP (HA 3 μ g-24ng)	Twice	-	-	[7]
H1+H3 (A/PR8/34 + A/Aichi/2/1968-x31)	HA(H1 + H3), M1	VLP 10 μ g / IM	Twice	H1+H3	0 / 0%	[36]
H1N1 (A/PR/8/34)	HA, M1 / Flagellin	VLP 10 μ g / IM	Twice	H1N1	0 / 0%	[42]
H1(NC/99), H3(NY/04), B(Shang/02)	HA, NA, M1	VLP (HA 3, 0.6, 0.12 μ g)	Twice	H1, H3	H1:6.3 $\times$ 10 ⁴ / 12%, H3:4.3 $\times$ 10 ³ / -	[20]
H1N1 (A/PR/8/34)	HA, M1 / mLT or CTB	VLP (HA 0.02 – 0.5 μ g) / IN	Twice	H1N1	0.02 μ g: 0.3 $\times$ 10 ⁴ , 0.5 μ g: 0/0%	[33]
H1N1 (A/California/04/2009)	HA, M1	VLP 0.1 – 10 μ g / IM	Prime Twice	H1N1	- / 12 % (D6) 0 / 0 %	[22]
H1N1 (A/PR8/34)	HA, M1	VLP 0.35–1.0 μ g / MN	Prime	H1N1	10 ⁴ ~ 10 ^{5.8} / 6–7 % (D7)	[21]
H1N1 (A/PR/8/34)	HA, M1 / Flagellin	VLP 10 μ g / IN	Twice	H1N1	- / 0 %	[43]
H1N1 (A/PR/8/34)	NA, M1	VLP 5 μ g / IM	Twice	H1N1	10 ² / 0%	[34]
H1N1 (A/South Carolina/1/1918)	HA, NA (A/Brevig Mission/1/1918), Gag / CpG oligonucleotides	VLP 3 μ g / IM	Twice	H1N1	- / 0%	[40]
H3N2 (A/canine/Korea/LBM412/2008)	HA, M1	VLP 3.75 – 15 μ g / IM	Prime	H3N2	- / -	[75]
H1N1 (A/PR/8), H3N2 (A/HK)	HA, M1	VLP 10 μ g / IN, IM	Twice	H1N1, H3N2	- / IN: 4 % (D4), IM: 7% (D3) - / IN: 5 % (D4), IM: 4% (D4)	[76]
H1N1 (A/PR/8/1934)	HA, M1 / CT, Alum, CpG, MPL	VLP 5 μ g / IN	Twice	H1N1	0.3 $\times$ 10 ⁵ / 0% (D8) 0.5 $\times$ 10 ⁵ / 2.5% (D8) 0.8 $\times$ 10 ⁵ / 3.5% (D8) 0.7 $\times$ 10 ⁵ / 3% (D8)	[77]

PFU: plaque forming units, HA: Hemagglutinin, NA: Neuraminidase, M1: Matrix protein, IM: intramuscular, IN: intranasal, -: Not detected. Twice: prime boost, Three: prime and 2 boosts, D#: The time of day representing indicated peak weight loss post challenge.

Table 2

VLP vaccine against heterologous influenza viruses

Subtype	Components / Adjuvant	Dose / Route	Prime/Boost	Virus Challenge	Protection Lung titer (PFU/ml)/% of decreased Body weight	Reference
H3N2 (A/udorn/72)	HA, M1/IL-12	VLP (HA 1 μ g) / IN, IM	Twice	H3N2 (A/Hong Kong/68)	- / 10% (D5)	[35]
H1N1 (A/PR8/34)	HA, M1	VLP 10 μ g / IN	Three	H1N1 (A/WSN/33)	2 $\times$ 10 ⁴ /4% (D5)	[12]
H1+H3 (A/PR8/34 + A/Aichi/2/1968-x31)	HA(H1 + H3), M1	VLP 10 μ g / IM	Twice	H3N2 (A/Philippines/2/1982) H1N1 (A/WSN/33)	10 ⁷ /36% (D10) 0 / 0%	[36]
H1N1 (A/California/04/2009)	HA, M1	VLP 10 μ g / IM	Prime	H1N1 (A/PR8/34)	*10 ⁵ /20% (D8)	[22]
H1N1 (A/California/04/2009)	HA, NA, M1	VLP 3.75–15 μ g / IM	Twice	H1N1 (A/Mexico/4482/09)	15 μ g: 10 ¹ /5.6% (D3), 3.75 μ g: 13.1% (D8)	[47]
H1N1 (A/California/04/2009)	HA, M1	VLP (HA 3 μ g) / IM, VLP (HA 15 μ g) / IN	Twice	H1N1 (A/New York/18/2009)	- / IM: 6% (D5), IN: 5% (D5)	[8]
H1N1 (A/California/04/2009)	HA, M1	VLP (HA 15 μ g) / IN	Twice	H1N1 (A/Brisbane/59/2007)	- **7% (D12)	[8]
H1N1 (A/California/04/2009)	HA, NA, M1	VLP 15 μ g / IM	Three	H1N1 (A/SW/Manitoba/MAFR132/2009)	- / -	[78]
H1N1 (A/South Carolina/1/1918)	HA, NA (A/Brevig Mission/1/1918), Gag / CpG oligonucleotides	VLP 3 μ g / IM	Twice	H1N1 (A/Mexico/4108/2009)	10 ⁵ /4% (D4)	[40]
H1N1 (A/California/04/2009)	HA, M1	VLP 10 μ g / IM	Three	H1N1 (A/PR/8/1934) H1N1 (A/New Caledonia/20/99)	0.2 $\times$ 10 ⁵ /5% (D7) 1.8 $\times$ 10 ⁵ /0%	[79]
H1N1 (A/South Carolina/1/1918), H3N8 (A/pintail/Ohto/339/1987), H5N1 (A/mallard/Maryland/802/2007) H7N3 (A/Environment/Maryland/261/2006)	HA, M1 (A/New York/312/2001 (H1N1))	VLP 1.5 for H1, H3, H5: 6 μ g for H7/IN	Twice	H5N1 (A/Vietnam/1203/1204) H7N9 μ g (A/Anhui/1/2013)	H5: 7% (D6) H7: 0/0%	[41]

* Partial protection was found with 75% of survival rate.

** Virus titers in nasal washes were determined and the protection was not found.

PFU: plaque forming units, HA: Hemagglutinin, NA: Neuraminidase, M1: Matrix protein, IM: intramuscular, IN: intranasal, -: Not detected. Twice: prime boost, Three: prime and 2 boosts, D#: The time of day representing indicated peak weight loss post challenge.

Table 3

VLP vaccine against heterosubtypic influenza viruses

Subtype	Components/Adjuvant	Dose/Route	Prime/Boost	Virus Challenge	Protection Lung titer (PFU/ml) / % of decreased Body weight	Reference
H1N1 (A/PR8/34)	HA, M1/Flagellin	VLP 10 µg /IM	Twice	H3N2 (A/Philippines/2/82/x-79)	1.6 × 10 ⁴ /16% (D8)	[42]
H1N1 (A/PR/8/34)	HA, M1/Flagellin	VLP 10 µg /IN	Twice	H3N2 (A/Philippine/2/82)	- /12% (D8)	[43]
H1N1 (A/PR/8/34)	NA, M1	VLP 5 µg /IN	Twice	H3N2 (A/Philippine/82)	10 ⁴ /16% (D9)	[34]
H1N1 (A/South Carolina/1/1918), H3N8 (A/pintail/Ohio/339/1987), H5N1 (A/mallard/Maryland/802/2007)	HA, M1 (A/New York/312/2001 (H1N1))	VLP 1.5 for H, H3, H5, 6 µg for H7/IN	Twice	H2N1(A/Green Wing Teal/Ohio/175/1986) H6N1 H10N1	H2: 0.4x10 ³ /16%/ (D3) H6: 10 ⁴ /5% (D7) H10: 10 ⁵ /10% (D6) H11: 2% (D4)	[41]
H7N3 (A/Environment/Maryland/261/2006)				H11N9(Green Wing Teal/Ohio/340/1987)		

PFU: plaque forming units, HA: Hemagglutinin, NA: Neuraminidase, M1: Matrix protein, IM: intramuscular, IN: intranasal, -: Not detected. Twice: prime boost, Three: prime and 2 boosts, D#: The time of day representing indicated peak weight loss post challenge.

Table 4

VLP vaccine against pandemic influenza virus

Subtype	Components / Adjuvant	Dose / Route	Prime /Boost	Virus Challenge	Protection Lung titer (PFU/ml) / % of decreased Body weight	Reference
H9N2 (A/Hong Kong/1073/99)	HA, NA, M1	VLP 10 µg / SC	Twice	H9N2 (A/Hong Kong/1073/99)	10 ⁶ / 12% (D3)	[15]
H9N2 (A/Hong Kong/1073/99)	HA, NA, M1/Novasome B52CCPCE	VLP (HA 0.12 – 15 µg) / IM	Twice	H9N2 (A/Hong Kong/1073/99)	10 ⁴ / 10% (D4)	[53]
H1N1 (A/South Carolina/1/1918)	HA, NA, M1, M2/CpG	VLP (HA 1 µg) / IN	Twice	H1N1 (A Swine/Iowa/15/30)	8 × 10 ⁴ /0%	[39]
H5N1 clade1 (A/Viet Nam/1203/2004) H5N1 clade2 (A/Indonesia/05/2005)	HA, NA, M1	VLP (HA 0.6 – µg) / IM	Prime or Twice	H5N1 (A/Viet Nam/1203/2004) H5N1 (A/Indonesia/05/2005)	- / 5% (D6)	[16]
H5N1 clade2.1 (A/Indonesia/05/2005)	HA, NA, M1	VLP (HA 0.6 – 15 µg) / IM	Twice	H5N1 (A/Viet Nam/1203/2004) H5N1 (A/Indonesia/05/2005)	- / 15 µg: 5% (D6), 0.6 µg: 7% (D1) - / 15 µg: 2% (D4), 0.6 µg: 3% (D4)	[49]
H5N1 clades 1+2 (A/Viet Nam/1203/2004)	HA, NA, M1	VLP (HA 0.6 µg-) / IM	Twice	H5N1 (A/Viet Nam/1203/2004) H5N1 (A/Indonesia/05/2005)	- / 0% 5.25 × 10 ³ / 0%	[46]
H5N1 clade1 (A/Viet Nam/1203/2004)	HA, NA, M1	VLP (HA 0.1 – 0.3 µg-) / IN	Twice	H5N1 (A/Viet Nam/1203/2004)	10 ⁴ /0.3 µg: 0% (D7), 0.1 µg: 10% (D7)	[80]
H5N1 (A/Hubei/489)	HA, NA, M1	VLP 10 µg- / IM	Twice	H5N1 (A/Hubei/489)	10 ⁵ /6% (D6)	[81]
H5N1 clade1 (A/Viet Nam/1203/2004) H5N1 clade2 (A/Indonesia/05/2005)	HA, NA, Gag	VLP (HA 0.7 – 1 µg-) / IM, IP	Twice	H5N1 (A/Viet Nam/1203/2004)	- / 1% (D9)	[51]
H1N1 (A/South Carolina/1/1918)	HA, NA, M1	VLP (HA 5 µg-) / IN, IM	Twice	H1N1 (A/South Carolina/1/1918) H5N1 (A/Viet Nam/1203/2004)	0/0% IN: 10 ⁴ , IM: 10 ⁶ / IN: 17% (D5), IM: 25% (D6)	[55]
H5N1 (A/Hanoi/30408/2005)	HA, NA, M1, M2	VLP 0.3 – 10 µg- / IM	Twice	H5N1 (A/Hanoi/30408/2005)	- / 10 µg: 8% (D8), 0.3 µg: 25% (D8)	[18]
H5N1 (A/Viet Nam/1203/2004)	HA, M1	VLP (HA 0.4 – 2 µg) / IM	Prime	H5N1 (A/Viet Nam/1203/2004)	0 – 10 ⁴ /0%	[9]
H5N1 (A/Indonesia/5/2005)	HA, M1	VLP 30HAU / IM	Prime	H5N1	- / 8% (D4)	[71]
H9N2 (A/Chicken/Korea/01310/2001)	HA, M1/ISA70	VLP 2 – 20 µg- / IM	Prime	H9N2	- / -	[82]
H5N1 (A/chicken/Korea/ES/2003)	HA, NA, M1 / ISA70	VLP 2 ⁸ – 2 ¹⁰ HAU / IM	prime	H5N1	- / -	[83]
H5N1 (A/Viet Nam/1203/2004)	HA, NA, M1	VLP (HA 15 µg-) / IM	Twice	H5N1 (A/Indonesia/5/2005)	- / 8–15% (D5–D6)	[84]

Subtype	Components / Adjuvant	Dose / Route	Prime /Boost	Virus Challenge	Protection Lung titer (PFU/ml) / % of decreased Body weight	Reference
H5N1 (A/Indonesia/5/2005, A/Whooper Swan/Mongolia/244/2005, A/Anhui/1/2005)	HA, NA, M1	VLP (HA 3-15 μ g:) / IM	2-3 times	H5N1 (A/Whooper)	- / 0%	[85]
H5N1 (A/Viet Nam/1203/2004), H7N2 (A/New York/107/2003), H9N2 (A/Hong Kong/33982/2009)	HA, NA, M1	VLP (HA 15 μ g:) / IN	Twice	H5N1, H7N2, H9N2	- /H5N1: 4.7% (D6), H7N2: 4.7% (D6), H9N2: 1.8% (D6)	[54]
H5N1 (A/chicken/Korea/Gimje/2008)	HA, M1	VLP (HA 10 μ g:) / IN	Prime	H5N1	- / -	[86]
H7N9 (A/Anhui/1/2013)	HA, NA, M1/MF59, Iscomatrix	VLP 10 μ g: / IP	Twice	H7N9 (A/Anhui/1/2013)		[87]
H7N9 (A/Shanghai/1/2013)	HA, NA, M1/Iscomatrix	VLP (HA 5, 15 μ g:) / IM	Twice	H7N9 (A/Shanghai/1/2013)	- / -	[63]
H7N9 (A/Anhui/1/2013)	HA, NA, M1/Iscomatrix	VLP (HA 15 μ g:) / IM	Twice	H7N9 (A/Anhui/1/2013)	- / 3.5% (D7)	[58]
H5N1 (A/meerkat/Shanghai/SH-1/2012)	NA, HA, M1 / CFA	VLP 10 μ g: / IM	Twice	H5N1 (A/meerkat/Shanghai/SH-1/2012)	0 / -10 ² /0% (D3)	[88]
H5N1 (A/goose/GD/1996)	HA, NA, M1, NP	VLP (HA 1 μ g:) / IM	Twice	H5N1 μ g: 2.3.4 A/duck/Fujian/31/2007)	VLP3: 50% protected VLP4(with NP): 100% protected	[50]

PFU: plaque forming units, HA: Hemagglutinin, NA: Neuraminidase, M1: Matrix protein, IM: intramuscular, IN: intranasal, -: Not detected, Twice: prime boost, Three: prime and 2 boosts, D#: The time of day representing indicated peak weight loss post challenge.