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Progress and hurdles in the development of influenza virus-like particle vaccines for veterinary use

Virus-like particles (VLPs), which resemble infectious virus particles in structure and morphology, have been proposed to provide a new generation of vaccine candidates against various viral infections. As effective immunogens, characterized by high immunogenicity and safety, VLPs have been employed in the development of human influenza vaccines. Recently, several influenza VLP vaccines have been developed for veterinary use and successfully evaluated in swine, canine, duck, and chicken models. These VLP vaccine candidates induced protective immune responses and enabled serological differentiation between vaccinated and infected animals in conjunction with a diagnostic test. Here, we review the current progress of influenza VLP development as a next-generation vaccine technology in the veterinary field and discuss the challenges and future direction of this technology.

Keywords: Influenza, Vaccines, Virus-like particle, Veterinary

Introduction

Influenza A viruses (IAVs) are highly contagious viral pathogens that have been isolated from a wide variety of animals such as birds, humans, pigs, horses, dogs, cats, and marine mammals [1]. Because of their high impact on both human and animal health, IAVs have become a matter of great concern to public health concern garnering increased scientific interest over the past decade. The increased availability of genomic information on IAVs and greatly advanced molecular techniques have provided major advances in genetically engineered influenza vaccine development, such as recombinant subunit vaccines, virus-like particles (VLPs), DNA vaccines, and vector-vehicle vaccines. The use of such vaccines against animal influenza viruses is being actively explored in laboratory studies. These vaccines provide numerous advantages over the use of conventional inactivated whole-virus vaccines, including ease of production, immunogenicity, safety, and multivalency [2]. However, successfully translating these recent advances into the development of veterinary vaccines continues to be impeded by gaps in our understanding of differences between the goals of veterinary and human vaccines. In the past few years, several influenza VLP vaccines have been developed for veterinary use [3-8]. This review aims to give an overview of the most important results of these research efforts, and provides information about the progress and hurdles in the development of influenza VLP vaccines for veterinary use.

Influenza Virus in Animals

The IAV genome consists of 8 segments of single-stranded RNA, each of which encodes a different polypeptide including 2 surface glycoproteins (hemagglutinin [HA] and neuraminidase [NA]), nucleoprotein (NP), 3 polymerase proteins (polymerase basic 2, polymerase basic 1 and polymerase acidic), 2 matrix proteins (M1 and M2), and 2 nonstructural proteins (NS1 and NS2) [9]. IAVs are classified into subtypes based on the antigenic differences between their 2 surface glycoproteins, HA and NA. Sixteen HA subtypes (H1 to H16) and 9 NA subtypes (N1 to N9) have been identified among IAVs. All subtypes identified to date have been isolated from birds. The matrix protein surrounding the genome complex is enveloped in a lipid membrane covered by HA and NA. These surface antigens play an important role in protective immunity [10].

The pathogenicity of viruses in poultry species determines whether the virus is classified as low pathogenic avian influenza (LPAI) or highly pathogenic avian influenza (HPAI). HPAI has traditionally comprised either H5 or H7 subtypes [11]. The diseases caused by different avian influenza viruses (AIVs) vary in severity from causing high mortality to producing very mild forms or even inapparent infections. Sign of HPAI infection in Galliformes birds is the sudden onset of high flock mortality, which may approach 100% within a few days. LPAI viruses may cause mild-to-moderate clinical signs such as respiratory disease, depression, a reduction in egg production, and slightly elevated mortality. To prevent or eradicate AIV outbreaks, several strategies have been applied, including a high level of biosafety, movement control, stamping out, and vaccination [12]. Current vaccines against AIVs, when selected properly and administered correctly, can provide protection against clinical signs and mortality, reduce the levels and duration of virus shedding, and increase serum antibody levels, the elevation of which provides resistance against infection by raising the minimum infectious dose. Although vaccination against AIV in poultry is still a controversial topic and has been discouraged in the past, it has also been recommended as an alternative AIV control strategy [13]. From 2002 to 2010, more than 113 billion doses of AIV vaccine have been used in poultry: 95.5% as oil-emulsified, inactivated whole-AIV vaccines and 4.5% as live-vectored vaccines. The majority of vaccines have been used in the 4 H5N1 HPAI enzootic countries (China, 91%; Egypt, 4.7%; Indonesia, 2.3%; and Vietnam, 1.4%) where vaccination pro-

grams have been directed to all poultry [14]. In addition, due to the prevalence and zoonotic potential of the H9N2 viruses, an oil-based inactivated H9N2 LPAI vaccine was employed in the poultry industry in several countries [15-17].

In mammalian species, swine influenza virus (SIV) is not only an important respiratory pathogen in pigs but also a potent threat to human health. Swine influenza is an acute and highly contagious respiratory disease caused by IAVs subtype H1N1, H1N2, or H3N2 [18]. Since porcine respiratory epithelial cells possess receptors for both avian and human influenza viruses, pigs are thought to be the “mixing vessels” that generate new recombinant strains [19]. The outbreak of the 2009 influenza pandemic underscored the important role of pigs in the evolution and emergence of pandemic influenza viruses. Thus, the control and prevention of SIV in pigs is required for both human and animal health. To control swine influenza, vaccination is the most common practice in swine industry. Current swine influenza vaccines are adjuvanted, inactivated, whole-virus vaccines prepared typically from viruses propagated in embryonated chicken eggs [20].

Dogs are susceptible to natural influenza virus infections via transmission from avian (H3N2 and H5N1), equine (H3N8), or human (pandemic H1N1/2009 and H3N2) virus reservoirs [21-24]. Historically, dogs were not considered natural hosts for the influenza virus. However, beginning in 2004, an equine-origin H3N8 influenza virus was first isolated from racing dogs affected with acute respiratory disease in the United States [25]. Subsequent outbreaks were reported, and the canine influenza virus (CIV) H3N8 spread rapidly across the United States. Avian-origin CIV H3N2 was reported from clinically ill dogs in southern China in 2006 and 2007 [26]. In addition, an outbreak of avian-origin CIV H3N2 in pet dogs occurred in South Korea in 2007 [27]. In beagles, experimental avian-origin CIV H3N2 infection causes mortality and clinical signs such as high fever, depression, and respiratory signs [28]. In the United States and South Korea, inactivated vaccines against equine-origin CIV H3N8 (Intervet/Schering-Plough Animal Health, Elkhorn, NE, USA) and avian-origin CIV H3N2 (Green Cross Veterinary Products Co., Yongin, Korea) have been developed and were licensed in 2009 [29,30].

Influenza VLP Vaccines

Current human influenza vaccines contain soluble forms of the viral surface split antigens produced by treating chemically inactivated virus with detergent. In addition, a live-at-

tenuated influenza vaccine, FluMist (MedImmune), is licensed for intranasal delivery in humans [31]. VLPs, which resemble infectious virus particles in structure and morphology and have multiple antigenic epitopes, have been proposed as a new generation of vaccine candidates against various viral infections (Fig. 1). Influenza VLPs have been developed as non-egg-based, cell culture-derived vaccine candidates against influenza infection [32]. Influenza VLP vaccines containing influenza HA and NA antigens are produced easily in insect or mammalian cells via the simultaneous expression of HA and NA along with a viral core protein such as influenza M1 [33]. VLPs do not have viral genomes and therefore have a solid safety profile. The highly organized form of antigens on VLPs can induce strong B-cell responses [34]. The protective mechanisms of influenza VLP vaccines, that is, induction of neutralizing antibodies and HA inhibition, are similar to those of commercial influenza vaccines [32]. In addition, VLPs can stimulate antigen presentation cells and induce CD4 T-cell proliferation and cytotoxic T-cell immune responses and thus induce both B- and T-cell responses [35,36]. However, VLPs are less effective than live-attenuated vaccines at induc-

ing cytotoxic CD8 T-cell and helper CD4 T-cell responses, as VLPs do not replicate [34]. To enhance the immunogenicity and protective efficacy of VLPs, Wang et al. [37] incorporated a membrane-anchored form of the Toll-like receptor (TLR) 5 ligand flagellin into VLPs. The results demonstrated that flagellin-containing VLPs enhanced humoral and specific cellular responses.

Several animal viruses have been investigated as candidates for the development of VLP-based vaccines for veterinary use. For mammalian species, the bluetongue virus, canine parvovirus, equine rhinitis A virus, foot and mouth disease virus, feline calicivirus, influenza virus, porcine circovirus type-2, papillomavirus, porcine encephalomyocarditis virus, porcine parvovirus, Rift valley fever virus, rotavirus, rabbit hemorrhagic disease virus, and mink enteritis virus have been investigated. For poultry species, the chicken anemia virus, goose parvovirus, infectious bursal disease virus, influenza virus, Muscovy duck parvovirus, and Newcastle disease virus (NDV) have been examined, and for fish species, the nervous necrosis virus has been studied [38,39]. In particular, several influenza VLPs have been developed and suggested as vaccine candidates for animal use, including VLPs for AIV subtype H5N3 and H9N2, HPAI H5N1, SIV H1N1, and CIV H3N2 (Table 1). All of the influenza VLPs for veterinary use have been produced using baculovirus/insect cell technology (mainly because of the high expression levels of insect cell expression systems in comparison to mammalian cell expression systems) and emulsified with an oil adjuvant. Prel et al. [6,40] developed an AIV H5N3 VLP vaccine and evaluated in Muscovy ducks. Lee et al. [3,4] developed CIV H3 VLP and LPAI H9 vaccines and evaluated in beagles and SPF chickens, respectively. The LPAI H9 and CIV H3 VLP vaccines contain HA and M1 but not NA. A single dose of vaccination with these VLP vaccines induced high antibody titers and lessened viral shedding. Park et al. [5] developed an HPAI H5N1 VLP vaccine without purification step and evaluated in SPF

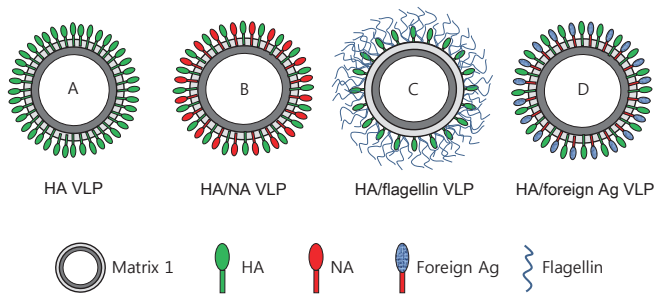


Fig. 1. Schematic diagrams of influenza virus-like particles (VLP). (A) Influenza VLP containing matrix protein M1 and hemagglutinin (HA). (B) Influenza VLP containing HA, neuraminidase (NA), and matrix protein M1. (C) Chimeric influenza VLP containing matrix protein M1, HA, and flagellin as molecular adjuvant. (D) Chimeric influenza VLP containing matrix protein M1, HA, and foreign antigens.

Table 1. Influenza virus-like particles as candidate vaccines for veterinary use

Virus	Composition	Animal	Adjuvant	Expression system	Purification	References
Avian influenza H5N3	HA, NA, M1	SPF muscovy duck	Oil-emulsion	Baculovirus (Sf9)	Sucrose density gradient	Prel et al. (2008) [6]
Avian influenza H9N2	HA, M1	SPF chicken	Oil-emulsion	Baculovirus (Sf9)	Sucrose density gradient	Lee et al. (2011) [4]
Pandemic H1N1	HA, NA, M1	Influenza free pigs	Oil emulsion	Baculovirus (Sf9)	Sucrose density gradient	Pyo et al. (2012) [7]
Avian influenza H5N1	HA, NA, M1	SPF chicken	Oil emulsion	Baculovirus (Sf9)	-	Park et al. (2013) [5]
Canine influenza H3N2	HA, M1	SPF beagle dog	Oil-emulsion Aluminum hydroxide	Baculovirus (Sf9)	Sucrose density gradient	Lee et al. (2013) [3]

chickens, as a vaccine that can be produced without a purification process costs less than the highly purified VLP vaccines reported in other studies. Although this H5N1 VLP was prepared without purification, the vaccine provided high immunogenicity and complete protection from lethal HPAI H5N1 infection. Using insect cells, Pyo et al. [7] generated VLP antigens consisting of HA, NA, and M1 proteins derived from pandemic H1N1/2009 virus, A/California/04/2009(H1N1). This H1N1 VLP elicited robust levels of humoral and mucosal immune responses and protected pigs against experimental infection. Pigs inoculated with this vaccine and subsequently infected with the wild-type virus displayed reduced lung lesions and inhibition of virus replication in the lungs [7].

Even though vaccination against AIV is an effective control measure for the eradication of LPAI or HPAI, it may result in issues related to the international trade of poultry products and AIV surveillance programs [41]. Conventional live-attenuated and inactivated veterinary vaccines have a limitation, namely, it is impossible to differentiate vaccinated from infected animals (DIVA), and this fact hampers serological surveillance. Therefore, several different DIVA strategies have been suggested that use appropriate vaccines accompanied by serological tests for discriminating between infected and vaccinated animals. These strategies include the use of sentinels, subunit vaccines, the heterologous NA strategy, and the NS1 strategies [41]. VLP vaccines, accompanied by corresponding diagnostics, have been suggested as a useful tool both for DIVA strategies and for the prophylactic protection of animals [39]. Previous studies have reported that LPAI H9 and HPAI H5N1 VLP vaccines allowed the differentiation of AIV-infected chickens from vaccinated chickens using an enzyme-linked immunosorbent assay for NP. Thus, these VLP vaccines offer a promising strategy for DIVA [4,5].

Future Directions and Conclusions

Vaccination in veterinary fields has greatly improved animal health, welfare, productivity of industry animals, and eventually contributed to food safety [2]. In particular, vaccination has been proven to be a cost-effective measure for the prevention and eradication of infectious diseases. Additionally, veterinary vaccines have already made enormous impacts not only on veterinary fields but also on public health. The major goals of vaccination for industry animals are significantly different from those for human vaccination. The ultimate goals of veterinary vaccines are to improve the health

and welfare of companion animals, increase production of livestock in a cost-effective manner, and prevent zoonotic infection. The final successful outcome of vaccine development in the veterinary field is the generation of a product that will be available in the marketplace at a reasonable cost or that will be used in the field to achieve desired outcomes [42].

With livestock animals, there is a considerable focus on the direct and indirect costs of vaccines. Vaccine production cost has been thought to be an important issue that influences the feasibility of recombinant subunit vaccines including VLP for veterinary use. In terms of the manufacturing advantages of VLPs, HPAI VLP vaccines have potential advantages over egg-based inactivated influenza vaccines because VLPs can be produced without the need for expensive biocontainment facilities, which are required for the propagation of HPAI strains in vaccine production. Sucrose density gradient purification using ultracentrifugation is a popular method for purifying cell-assembled VLPs in most laboratories (Table 1). However, this procedure is not economically scalable. To reduce the production cost of VLP vaccines, Park et al. [5] developed a H5N1 VLP vaccine that was prepared without using expensive and time-consuming purification procedures (aside from low centrifugation to remove large cell debris). The use of crude, unpurified VLPs could be a promising method for decreasing the production cost of VLP vaccines for veterinary use. However, these crude VLPs may contain high titers of baculovirus and insect cell derived proteins. Although baculovirus is known to be essentially nonpathogenic to mammals [43], baculovirus contamination may contribute to the overall immunogenicity of the VLP preparation and thus cause safety concerns and regulatory complications. However, previous studies have shown that residual baculovirus in baculovirus-derived VLPs can enhance immunogenicity by activating the innate immune response [44]. As long as the crude VLPs are only employed for veterinary use, these residual baculoviruses may exhibit significant adjuvant activity without causing safety concerns.

In addition, since a major challenge is to develop cost-effective production platforms that can express VLPs while significantly reducing production times and costs, robust and flexible production platforms are emerging to produce a range of VLPs targeting different viruses. For example, commercial VLP production has been achieved with *Escherichia coli*, yeast, baculovirus-insect cell expression systems, and CHO mammalian cell expression systems [45]. As shown in Table 1, only the baculovirus-insect cell expression system has been used

for the production of influenza VLPs for veterinary use, as insect cells can readily co-express multiple proteins and assemble them into structurally complex VLPs. Both *E. coli* and yeast offer high yields and ease of scale-up, with the yeast expression system providing for post-translational modification. Thus, for cost-effective VLP production, expression of VLPs using alternative systems such as yeasts and plants for large-scale production need to be explored.

Stimulating the innate immune system is important for inducing potent adaptive immunity. Viruses can interact with innate immune system through pattern recognition receptors such as TLR and thus are highly effective at inducing adaptive immune responses. Incorporation of immunostimulatory molecules including TLR agonists into VLP antigens would enhance the immunogenicity of influenza VLPs and thus produce dose-sparing effects and broader cross-protection [37]. Up until now, several immunostimulatory molecules have been evaluated as molecular adjuvants for VLPs for human viruses, but this approach has not yet been tried for the development of veterinary VLP vaccines. In addition, chimeric influenza VLP antigens, which are composed of HA protein hetero-subtypes or immunogenic proteins from different virus, have been developed [8,46]. In particular, a chimeric VLP vaccine that contained M1 and HA from AIV and a chimeric protein containing the cytoplasmic and transmembrane domains of NA from AIV and the ectodomain of the HA-NA protein from the NDV induced antibody responses against both AIV and NDV [8]. In terms of cost-effectiveness and broad cross-protectivity, the incorporation of molecular adjuvant and heterologous epitopes into VLP antigens could be promising strategies for the development of VLP vaccines for veterinary use.

In conclusion, VLPs can be considered to provide an innovative influenza vaccine platform in the veterinary field that both induces protective immune responses against animal influenza viruses and has a solid safety profile. In addition, VLP vaccines may be useful in DIVA strategies and so are more attractive than whole viruses for vaccine development in the veterinary field. However, there are technical and practical considerations that may limit the application of VLP vaccines. Production cost is an important issue that influences the feasibility of VLP vaccines in veterinary use. Thus, development of large-scale production platform using various systems is required for cost-effective VLP production. Further, the development of VLPs as a platform for the co-expression of molecular adjuvants or foreign antigens can broaden their

potential applicability. Overall, an amalgamation of bioengineering and chimeric VLP technologies will accelerate the development and application of new generation influenza VLP vaccines for veterinary use.

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