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REVIEW

Live poultry vaccines against highly pathogenic avian influenza viruses

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ABSTRACT

The widespread circulation of highly pathogenic avian influenza viruses (HPAIVs) and their occasional transmission to humans creates a constant pandemic threat and leads to significant economic losses in the poultry industry. The development of an effective and safe vaccine for the broad protection of poultry from H5N1 HPAIVs remains an important goal. Prevention of the virus transmission between ducks and chickens is important for the efficient control of the spread of avian influenza. The oral administration of live vaccines corresponds to the natural route of infection that leads to virus replication in the intestinal epithelial cells that cause a well-balanced and broad immune response providing protection against the viruses of distant clades. The broad protection is the important advantage of live-attenuated influenza vaccines when compared to inactivated ones. Here, we give an overview of the latest approaches and results in the development of live poultry vaccine candidates against HPAIVs.

INTRODUCTION

The widespread circulation of HPAIVs causes significant economic losses in the poultry industry while the occasional transmission of these viruses to humans poses a continuous pandemic threat. In December 2014, the Eurasian HPAIVs of H5N8 and H5N2 subtypes reached United States (US) and caused the largest animal health emergency in US history resulting in death or the culling of more than 48 million birds [1].

Currently, the outbreaks of the HPAIVs are mainly controlled by the culling of all the birds in the affected regions. Vaccination can serve as a preventive measure to combat the virus infection in birds instead of eradication [2]. Thus, tens of billions of doses of inactivated vaccine in the form of oil-in-water emulsion containing whole virus with adjuvant were used in affected countries, particularly in China. This inactivated vaccine was based on reassortants engineered by reverse genetics, which contain the H5 hemagglutinin (HA) and N1 neuraminidase (NA) from H5N1 viruses and the remaining genes from A/Puerto Rico/8/1934 (PR8) virus [3, 4].

Inefficiency of inactivated vaccines in the prevention of virus spreading

The widespread campaigns of poultry vaccination with H5N1 vaccine unfortunately failed to prevent the long-distance transition of HPAIVs of H5 subtype. The immunization with inactivated virus did not prevent virus shedding in birds after being challenged by HPAIVs [5-8]. Therefore, the inactivated vaccines successfully protected layers and broiler chickens from disease and prevented the decrease in egg production but did not prevent the spread of the virus.

Influenza virus of the H9N2 subtype also continues to circulate in vaccinated chicken flocks in China because the protective efficacy of inactivated vaccines against antigenic drift variants is limited [9]. Since the effectiveness of inactivated vaccines strongly depends on the antigenic match of the HA in the vaccine and the HA of the field virus, the antigenic diversity of avian influenza viruses has been recognized as the main challenge for the eradication of HPAIVs [9-12].

The potential advantages of live attenuated influenza vaccines (LAIVs)

The use of live influenza vaccines offers substantial benefits compared to inactivated ones. The oral administration of live vaccines leads to the replication of the virus in epithelial cells and causes a well-balanced and broad immune response providing protection against the viruses of distant clades [13].

LAIVs have several advantages over traditional inactivated influenza vaccines. These vaccines can be produced rapidly, safely, and inexpensively [4, 14, 15]. It is generally recognized that live vaccines are superior to inactivated vaccines in preventing the circulation of the virus. The broad protection of live vaccines is ensured by development of both the humoral and cellular immunity to virus that replicates in relevant tissues. Moreover, mass vaccination with LAIV could be accomplished easily [4]. Today, several known approaches are used to develop live influenza vaccines.

Recombinant vector vaccines

A number of live recombinant H5N1 influenza vaccines have been developed using live virus vectors, such as duck enteritis virus (DEV), turkey herpes virus (HVT), Newcastle disease virus (NDV), fowlpox virus (FPV), and infectious laryngotracheitis virus (ILTV). These vaccines are cost effective and can provide protection against two viral diseases simultaneously [15, 16].

A polyvalent candidate vectored vaccine based on DEV carrying the *HA* gene from A/duck/Hubei/xn/2007 (H5N1) virus was developed by Zou *et al*. [17]. Ducks immunized with this live vaccine were shown to develop a long-lasting protection against homologous and heterologous H5N1 HPAIVs and DEV.

Live recombinant vector vaccine based on the HVT expressing the HA of H5N1 HPAIV was developed by Rauw *et al.* [18]. This vaccine protected vaccinated birds from challenge with the homologous and heterologous HPAIVs of H5N1 and H5N2 subtypes [19]. Recombinant HVT provided higher protection than the inactivated vaccine in the form of oil-in-water emulsion produced from a similar strain [20].

Live recombinant vector vaccine based on the HVT expressing the HA of H5N1 HPAIV (rHVT-H5/2.2) was examined in Pekin ducks and showed only 30% mortality reduction for the birds challenged with H5N1 HPAIV. When used together with an inactivated vaccine in a prime-boost regiment it provided only a minor additive effect on the reduction of virus shedding [21].

Tang *et al.* suggested that CRISPR/Cas9-based genome editing could be used as a powerful tool for the generation of the HVT recombinants expressing viral antigens [22].

In another effort to create a live vector vaccine, the NDV recombinant virus expressing the HA of HPAIV (H5N1) was generated. This recombinant virus protected chickens against lethal infection with H5N1 virus after the first immunization [23]. Immunization of chickens with NDV-vectored H5N1 vaccine provided a high level of protection against the clinical disease and mortality after lethal challenge with HPAIV of H5N1 subtype [24]. The NDV-vectored H7 and H5 vaccines were able to induce high antibody titers and completely protect chickens from challenge with the novel H7N9 and highly pathogenic H5N1 viruses [25]. The chimeric NDV vectored vaccine expressing the HA of A/Vietnam/1203/2004 (H5N1) was safe for 1-day-old chickens and provided a partial protection against challenge with A/Vietnam/1203/2004 HPAIV indicating the possibility of the early protection of chickens [26]. The NDV-based H5 vaccine that expressed a codon-optimized ectodomain of the HA of A/chicken/Iowa/04-20/2015 (H5N2) virus was also shown to be effective in chickens, demonstrating a lack of clinical signs and virus shedding after the challenge with HPAIV A/turkey/Minnesota/9845-4/2015 (H5N2) [27]. Thus, this type of vaccine can protect chickens against intercontinental HPAIVs of H5Nx subtype and, therefore, can be used for the mass vaccination of poultry. However, the pre-existing immunity to NDV vector of commercial chickens should be taken into consideration because it can reduce the vaccine efficacy.

Bacterial vectors can also be used to construct recombinant vaccines. One of the examples is the attenuated *Salmonella* gallinarum vaccine candidate, expressing the globular head (HA1) domain of H5 HA of low pathogenic avian A/spot-billed duck/Korea/KNU SYG06/2006 (H5N3) influenza virus. The immunization of chickens with this vaccine candidate demonstrated the faster clearance of H5N3 challenge virus. This recombinant vaccine can be used as a bivalent preparation against fowl typhoid and influenza diseases [28].

An attenuated strain of *Salmonella* typhimurium designed for the expression and delivery of H7N9 HA, NA, or the conserved extracellular domain of the matrix protein 2 (M2e) was constructed by Kim *et al.* [29]. It was shown that these vaccine candidates are safe and immunogenic in chickens. The single oral immunization of chickens with one or several strains expressing HA, NA, or M2e induced protective immunity against the lethal challenge with H7N9 virus.

Vaccines with truncated NS1 gene

One of the approaches for the generation of the attenuated influenza vaccine strain is the truncation of a gene encoding the nonstructural (NS) protein NS1. It was shown that the vaccine candidates carrying the shortened *NS1* gene are attenuated and protective against homologous and heterologous HPAIVs in animal models [30]. The high efficacy of NS1-truncated LAIV correlates well with the upregulation of interferon (IFN)-stimulated genes (ISGs) that promotes the rapid induction of adaptive immune response against influenza in chickens and increases the protective effect of vaccine [31].

It was shown that the mutant influenza virus lacking NS1 protein (named delNS1) is highly attenuated in IFN-competent subjects [32-35]. Poor virus replication and lack of disease symptoms following the delNS1 virus immunization were accompanied by an enhanced IFN induction. Therefore, the viruses with *NS1* deletion or truncated *NS1* gene are attenuated for animal hosts and could be used as live attenuated vaccine candidates. Using this approach, a number of vaccine viruses were produced and their effectiveness was demonstrated in mice and chickens.

The properties of H5N1 avian influenza virus reassortants with NS1 protein terminated at amino acids (aa) 48, 70, 73, or 99, along with a modified HA protein were analyzed by Shi *et al.* [36]. The recombinant virus with NS1, truncated at aa 73, demonstrated the protection of chickens from the broad range of H5N1 influenza viruses. A dual LAIV carrying viruses with *HA* and *NA* genes from an avian H5N2 and H9N2 viruses, constructed on the PR8 backbone with truncated *NS1* genes was attenuated in mice [37]. This vaccine induced a powerful IFNβ response and completely protected mice from a lethal challenge with heterologous highly pathogenic H5N1 virus and highly virulent virus of H9N2 subtype.

The mutant virus of H9N2 subtype with NS1-128 truncation was more immunogenic than the corresponding inactivated vaccine and protected chickens from challenge by homologous and heterologous H9N2 avian influenza viruses [38].

Vaccines based on temperature-sensitive mutants

The attenuation of influenza virus through the acquisition of temperature-sensitive mutations is another approach for the generation of live influenza vaccines [39]. Live cold-adapted influenza vaccine candidates were developed by serial passages of H9N2 viruses in chicken embryos at low temperature. It was shown that the obtained mutant viruses protect chickens from homologous and heterologous strains of H9N2 subtype [9, 40].

Hickmann *et al*. constructed the live attenuated avian influenza vaccine candidate on the base of genetically modified temperature-sensitive A/guinea fowl/Hong Kong/WF10/1999 (H9N2) strain carrying the mutations in *PB1* and *PB2* genes [41]. Genes encoding the HA and NA antigens of vaccine candidate were originated from the Asian H5N1 virus. This virus was administered *in ovo* to 18-day-old chicken embryos. Challenge of the hatched chickens with HPAIV of H5N1 subtype led to 60% protection for the 4-week-old chickens and to 100% protection for the 9-12-week-old birds.

Another vaccine candidate comprising the genes that encode the internal proteins from a cold-adapted influenza virus A/chicken/Korea/S1/2003 (H9N2) (obtained by serial passages in chicken embryos at 25°C) and *HA* and *NA* genes from a highly pathogenic H5N1 influenza virus was generated by Lee *et al*. [42]. The immunized chickens developed substantial humoral and cellular immunity and were protected from lethal challenge with the homologous and heterologous influenza viruses of H5N1 or H9N2 subtypes.

Pena *et al*. reported that a vaccine strain with the rearranged genome of an avian H9N2 influenza virus that expressed the H5 HA open reading frame (ORF) from the segment 8 viral RNA protected mice and ferrets against lethal H5N1 challenge as well as against a potentially pandemic H9 virus [43].

Vaccine lacking neuraminidase protein

A novel live experimental H5 vaccine candidate EscEgg50A lacking the NA protein was developed by passaging of HPAIV A/Cygnus cygnus/Germany/R65/2006 (H5N1) in embryonated chicken eggs in the presence of a neutralizing serum. The resulting mutant strain lost the large section of the gene encoding NA but preserved the polybasic cleavage site in the HA protein. A single immunization of chickens, mice, and ferrets with this virus seven and three days before a lethal challenge with A/Cygnus cygnus/Germany/R65/2006 protected all the animals from the signs of disease and virus shedding [44].

The examples presented here confirm that live influenza vaccine could be more effective in the protection of birds against circulating drift variants and in the prevention of virus spreading when compared to inactivated vaccine.

Live vaccines made from viruses with genes of apathogenic avian viruses

Considering the low virulence of naturally selected low pathogenic avian influenza viruses (LPAIVs) isolated from waterfowls, many researchers tried to use the genes of these viruses for the development of live influenza vaccines. For the first time, this approach was used by Murphy *et al*. for the generation of live attenuated reassortants between low pathogenic avian and human influenza A viruses [45, 46]. Oral immunization of chickens with a live waterfowl-originated avian H5N9 influenza virus effectively protected birds from lethal challenge with H5 virus and prevented cloacal shedding of the virus [47].

A new approach for the expression and/or delivery of foreign antigens was developed by the substitution of the extracellular domain of the low pathogenic avian A/chicken/Jiangsu/11/2002 (H9N2) virus M2 protein with the HA1 from PR8 virus [48]. The resulting hybrid virus named H9N2-PR8/HA1 had low pathogenicity and was genetically stable. Intranasal immunization of Balb/c mice with H9N2-PR8/HA1 induced both anti-H9N2 and anti-PR8 HA antibodies and provided protection against lethal challenge with either H1N1 or H9N2 viruses.

The avian influenza virus A/duck/Zhejiang/1028/2009 (H7N3) isolated from ducks was evaluated as a potential live influenza vaccine candidate. This virus turned out to be low pathogenic and immunogenic for mice and chickens after intranasal administration [14]. The presented data suggest that the duck influenza virus could be used as a candidate for the development of a live vaccine in order to mitigate the severity of the possible pandemic that could be caused by the newly emerging H7N9 virus.

Parallel evaluation of different live influenza vaccine candidates

Parallel evaluation of apathogenic wild duck influenza virus A/duck/Moscow/4182/2010 (H5N3, named dk/4182) and attenuated experimental reassortants made on the base of different donors was conducted in order to compare their safety, immunogenicity, and protective efficacy against H5N1 HPAIV [49, 50]. Two experimental reassortants were constructed on the base of cold-adapted master strain of LAIV for humans A/Leningrad/134/17/1957 (H2N2) (Len). These reassortants inherited all the genes from the master strain Len except the gene encoding the HA. The gene encoding the HA was originated from the A/Vietnam/1203/2004 (H5N1) (VN) strain lacking the polybasic HA cleavage site, or from the H5N1 virus A/chicken/Kurgan/3/2005 (Ku/wt) attenuated by means of the amino acid substitutions 54Asp→Asn and 222Lys→Thr in HA1 and 48Val→Ile and 131Lys→Thr in HA2 while maintaining the polybasic HA cleavage site (named Ku/att). These reassortants were named as VN-Len and Ku-Len, respectively.

Two more reassortants were generated on the base of apathogenic H6N2 virus A/gull/Moscow/3100/2006. These viruses inherited the same *HA* genes as two viruses described above – from VN and Ku/att viruses respectively, while all the other genes were originated from A/gull/Moscow/3100/2006 virus. The obtained reassortants were named as VN-Gull and Ku-Gull respectively [51-54].

All obtained viruses were tested in chickens using intravenous, intranasal, aerosol, and oral routes of infection and proved to be apathogenic for chickens. Viruses VN-Len and Ku-Len were over-attenuated for chickens

[39, 55] and did not provide reliable protection from lethal challenge with HPAIV [49, 50]. In contrast, the viruses VN-Gull and Ku-Gull ensured the complete protection of chickens. However, these viruses were not attenuated enough and caused the partial death of 1-day-old chicks.

On the contrary, the wild duck virus dk/4182 was safe, highly immunogenic and protective in chickens regardless of inoculation route.

The impact of the vaccine dose and age of chickens on the vaccination efficacy

Vaccine administration via drinking water is an attractive way for the immunization of poultry. To explore the capabilities of this method of vaccination, the dk/4182 virus was tested as a live oral vaccine candidate. The impact of vaccine dose and the age of chickens on the vaccination efficacy were studied in order to find the optimal immunization conditions.

Single immunization of 30-day-old chickens with 10^6 $TCID_{50}$ (tissue culture infective dose 50) of dk/4182 virus via drinking water completely protected chickens from the lethal dose (100 LD_{50}) of the HPAIV of H5N1 subtype [56]. The antibody response to immunization with lower doses varied between the birds and was considered unsatisfactory. Young chickens (10-day-old or younger) showed the weak humoral immune response to the vaccination. The vaccination of chickens older than 20 days resulted in reasonable antibody titers. All the chickens that were vaccinated twice on the 7th and 30th day after hatching showed a high and stable antibody response.

Shedding and transmission of dk/4182 and Ku/wt viruses in chickens and ducks

Ducks and chickens infected with Ku/wt or dk/4182 viruses shed the viruses in feces from day 3 to day 10 post infection. Despite that, contact of infected birds with uninfected chickens led to their infection with Ku/wt virus but did not result in infection with dk/4182 as they did not excrete this virus and did not develop antibodies to it. These results can be explained by the fact that a very high dose of dk/4182 virus is required for the productive infection in chickens. In contrast, the chickens being in contact with the ducks infected with Ku/wt virus became sick, shed the virus, and died [57]. When ducks were primarily infected with dk/4182 and challenged with H5N1 HPAIV from 14 to 90 days after infection, the challenge virus was not found in their feces. Naïve chickens that were placed in contact with these ducks did not show any signs of the disease, any excretion of the virus with feces, or present any antibody response.

These results show that the low pathogenic virus of wild aquatic birds dk/4182 can prevent the transmission of H5N1 viruses between ducks and chickens.

Perspectives of use of live poultry vaccines against H5N1 HPAIV

The potential risk of the restoration of pathogenicity of the attenuated vaccine strain is the reason for the existing strong regulatory barriers against live poultry vaccines created from avian influenza viruses [4]. It is believed that reverse mutations could restore the virulence of a vaccine virus. Indeed, it has been shown that outbreaks of HPAIVs in Pennsylvania (1983-84), Mexico (1994-95), and Italy (1999-2000) were caused by originally non-virulent viruses that became highly pathogenic in the course of circulation in poultry [58]. However, phylogenetic analyses of these viruses showed that all the mentioned episodes of rapid acquisition of pathogenicity happened when the low-pathogenic avian predecessors were closely related to pathogenic poultry viruses and had been recently reintroduced into natural wild bird reservoir [59].

The emergence of a new evolutionary branch of HPAIVs is a rare phenomenon. Wild bird influenza viruses do not cause disease in their natural host waterfowls. Wild ducks differ from chickens in terms of ecology and spreading of influenza viruses. The duck-adapted viruses derived from the natural reservoir in contrast to the poultry-adapted viruses, do not replicate effectively in poultry. All attempts to propagate the LPAIV A/whistling swan/Shimane/499/83 (H5N3) by intranasal, intratracheal, and intracerebral inoculation into 1-day-old chickens were unsuccessful. This virus acquired partial virulence in 2-day-old chickens only after 11 passages through air sacs [60]. The virus dk/4182 also poorly replicated in the internal organs of chickens and and was not transmitted to contact birds. One of the factors of the virus host range restriction is the pH at which the HA undergoes the conformational change necessary for the fusion of virus and cellular membranes in order to deliver the virus genome to the cytoplasm. This pH is known as the pH of fusion or pH of activation. Virus dk/4182 as a typical duck virus is characterized by a low pH of fusion (pH 5.2), which makes it resistant to the acidic pH of the intestinal tract – the main site of virus replication. Chicken viruses, on the contrary, have an elevated pH of fusion (pH 5.6-5.8) and, therefore, are unstable at acidic pH. Chicken viruses replicate predominantly in the oropharyngeal tract and viruses with the polybasic cleavage site disseminate to other internal organs [50].

Phylogenetic analysis of the full-length genomic sequences showed that all the genes of dk/4182 virus belong to evolutionary clades containing exclusively low pathogenic viruses of wild aquatic birds. Since dk/4182 virus is antigenically equidistant from all HPAI H5 viruses, it is likely that vaccine based on this virus should be effective against a broad range of H5 HPAIVs [49, 50].

Since the administration of dk/4182 H5N3 to chickens and ducks via drinking water ensured the complete protection of birds from lethal viral challenges and prevented the transmission of the challenge virus, one can conclude that the dk/4182 H5N3 virus represents a promising candidate for the development of a live vaccine for the protection of poultry from H5N1 HPAI viruses [56, 57].

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CONFLICT OF INTERESTS

The authors declare no commercial or financial conflict of interest.

CITATIONS

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