Chapter 4

Preparing Live Influenza Vaccines against Potential Pandemic Influenza Using Nonpathogenic Avian Influenza Viruses and Cold-Adapted Master Donor Strain

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Additional information is available at the end of the chapter

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Abstract

As part of an influenza pandemic preparedness program, the WHO analyzes a range of potentially pandemic influenza viruses for appropriate vaccines development. Several vaccine candidates were prepared using classical genetic reassortment, with the cold-adapted A/Leningrad/134/17/57(H2N2) (Len/17) master donor strain (MDS) which is licensed in the Russia for the live influenza vaccine (LAIV) strains type A production for adults and children. The nonpathogenic avian viruses of different subtypes were used for reassortant vaccine strains preparation. All vaccine candidates demonstrated a high reproductive capacity and cold-adapted (*ca*-) phenotype in chick embryos. In mice, the LAIV of H5N2, H7N3, and H9N2 subtypes provided protection against infection with distant influenza viruses. The immunogenicity and protective efficacy of H7N3 LAIV was also demonstrated in ferrets. The H5N2 and H7N3 vaccine candidates demonstrated the inability to reproduce in chickens, which confirms the safety of their use in areas with highly developed agriculture. When tested in clinical trials, vaccine strains of H5N2 and H7N3 subtypes induce the conversions of antibodies homologous and antigenically distant variants. The use of LAIV can be effective against highly pathogenic influenza viruses even in the case of incomplete antigenic correspondence between the vaccine strain and the infectious virus.

Keywords: live influenza vaccine, avian influenza, attenuation, reassortment, cross-protection

1. Introduction

Influenza viruses belong to the family *Orthomyxoviridae*. These are RNA-containing viruses possessing a negative fragmented genome. To date, there are four types (serotype) of influenza viruses—influenza A, B, C, and D. Influenza A viruses affect humans and a wide range of mammals (horses, pigs, dogs, wild and domestic cats, seals, ferrets) and birds (chickens, wild waterfowl, gulls, etc.). Only influenza A viruses are known as causative agents of severe epidemics and pandemics. The antigenic properties of influenza A viruses are based on two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA).

Wild waterfowl are considered as a natural reservoir of influenza A viruses which is characterized by high divergence. The 16 HA subtypes and nine NA subtypes were detected in migratory waterfowl and poultry [1]. Sometimes, avian influenza viruses overcome the interspecies barrier and infect poultry and mammals. Avian influenza viruses of subtypes H5N1, H7N3, H7N7, H7N9, and H9N2 may become pathogenic for humans and occasionally cause very severe infections. As part of an influenza pandemic preparedness program, the World Health Organization (WHO) analyzes a range of zoonotic and potentially pandemic influenza viruses for the development of appropriate vaccines as seasonal influenza vaccination does not protect against pandemic avian influenza viruses [2].

After isolation of the first influenza viruses in 1933–1936, the development of influenza vaccines in England, the United States, Australia, and in the USSR began. The development of active immunization against influenza using live attenuated vaccines was conducted in Russia under the leadership of A.A. Smorodintsev since 1937, and in the USA since 1960, where the group of H.F. Maassab also obtained cold-adapted attenuated variants of influenza viruses A and B. At present, two types of LAIVs are commercially available. The first, based on cold-adapted master donor viruses (MDVs) A/Leningrad/134/17/57 (H2N2) and B/USSR/60/69 [3–5], was licensed in 1987 for the people 3 years and older as Ultravac (Microgen, Russia). The second, known as FluMist based on cold-adapted MDVs, A/Ann Arbor/6/60ca (H2N2), and B/Ann Arbor/1/66ca, was licensed in 2003 (MedImmune, Inc., USA). FluMist is used for the prevention of influenza in persons younger than 49 and older than 2 years of age [6]. According to World Health Organization (WHO), vaccination prevents influenza in 80–90% of vaccinated people, and the economic effect of influenza vaccinations is 10–20 times higher than the cost of vaccination. In the past 10 years, attention was paid due to the advantages of LAIV that cause the formation of systemic and strong local (secretory) immunity. By contrast, parenteral inactivated influenza vaccines (IIV) stimulate mainly the formation of serum strain-specific antibodies which offer only limited protection against newly emerging viruses [7]. Intranasal implementation of LAIV produces immune response similar to natural infection and therefore induces an earlier, broader, and more long-lasting protection than inactivated vaccines [8]. Besides, the cost of live vaccine is five times less than inactivated vaccine, and the productivity of the biotechnological production process is significantly higher which is also important in the event of pandemic.

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2. Avian influenza in humans

Most avian viruses are initially low virulent for birds, causing only transient asymptomatic intestinal infections in wild waterfowl [9]. Viruses of subtypes H5 and H7 can be widespread among poultry, while acquiring the increased pathogenicity. This was observed during outbreaks caused by H5N2 viruses in 1983 or 1994–1995 in North America [10, 11], subtype H7 (H7N7 or H7N2)—in Europe and in Australia [12]. For the first time, "bird plague," a disease caused (as is now known) by highly pathogenic influenza viruses, was described in 1878 during an outbreak among chickens in Italy. The outbreak causative agent was isolated in 1902 (virus A/Chicken/Brescia/1902 (H7N7)). During similar outbreaks, repeatedly observed in Europe and around the world, several other viruses of H7 subtype were isolated. In 1955, those viruses were identified as belonging to a group of influenza viruses [13]. The first of the highly pathogenic (HP) viruses of the H5N3 subtype—the A/Tern/South Africa/61—was isolated in 1961 [14]. HP avian influenza viruses can cause a mass death of chickens in a short time as a result of dissemination of infection in poultry with rapidly progressive neurologic symptoms, diarrhea, and fatal outcome. Until 1997, there was no obvious evidence of direct infection of humans with avian viruses. Nevertheless, serological studies revealed the presence of antibodies against avian viruses of various subtypes in human sera in southern China, Hong Kong, and East Asia, indicating exposure of some people to avian influenza viruses [15].

2.1. H5N1 influenza viruses

For the first time, attention to H5 avian influenza viruses as possible pandemic agents was brought in May 1997 in Hong Kong during a mass outbreak among chickens when the avian virus H5N1 was isolated from a child who died from viral pneumonia [16]. To the end of 1997, an infection with the virus H5N1 similar to poultry viruses identified in the region was confirmed in another 17 people, five of whom died [17].

It is possible that before the appearance of the virus H5N1 in humans, a series of reassortments during the circulation of a number of precursor viruses in birds have occurred. Thus, HA of H5N1 viruses isolated from humans were almost identical to those of the A/Goose/Guandong/1/96 (H5N1) [18], and NA may have been acquired from the virus H6N1 [24]. It is assumed that the internal genes were borrowed from the same H6N1 virus or H9N2 A/Qail/Hong Kong/G1/97 (H9N2) influenza viruses during transmission from waterfowl to quails and chickens [19].

The mechanisms of avian influenza viruses "step-by-step" adaptation to new hosts are well characterized [20]. The change in host cell specificity and the increase in the pathogenicity of influenza viruses can be influenced either by amino acid substitutions in the receptor binding site of HA or by substitutions affecting the conformation and steric availability of this center. In particular, this can be influenced by changes in the number of glycosylation sites or their localization. High pathogenicity of avian influenza viruses in mammals is polygenic in nature. The HA of H5 or H7 HP viruses with a polybasic cleavage site is known as a primary

virulence factor, although the unusual severity of clinical manifestations during human infection with influenza H5N1 viruses can also be associated with mutations in internal proteins (PB1-F2, PB2) and non-structural (NS) proteins.

From 1997 to 2001, the HA of H5N1 viruses remained antigenically conserved, although, since 2003, there has been an unusually high level of H5N1 viruses evolution. The HP H5N1 viruses isolated from poultry and humans separated into three branches that differ antigenically and genetically [21]. During the outbreak in 2005 on Lake Qinghai, a number of HP H5N1 viruses were isolated from wild waterfowl [22]. This may indicate a reverse drift of similar viruses from poultry to wild birds, which was not observed previously. Along with H5 HA evolution, the extensive reassortment of avian influenza viruses in birds in China resulted in new H5 viruses possessing different NA subtypes (H5N2, H5N5, H5N6, and H5N8) and internal protein genes. In 2014, HA gene segments of H5N1, H5N2, H5N5, H5N6, and H5N8 were designated as clade 2.3.4.4., which were detected in birds in 40 countries in Africa, Asia, and Europe [23].

WHO has consistently recorded cases of human infection caused by HP influenza H5N1 virus, many of which had fatal outcomes. The clinical features of human infection caused highly pathogenic H5N1 viruses are characterized not only by primary viral pneumonia but also by complications with acute distress syndrome and poly-organ lesions [24].

At present, cases of human infection with the avian influenza H5N1 virus were decreased compared to the early 2000s. From 2003 to 2009, 468 cases of this disease were registered in 16 countries, mainly in Vietnam, China, Indonesia, Thailand, and Egypt. In 2010–2014, the number of cases was two times decreased (233 people). In 2016, the virus continued to infect people in only one country—Egypt (10 cases, three of them with a fatal outcome). In 2017, again in Egypt three cases were recorded, one of which was fatal. Thus, even when the absolute number of cases was decreased, mortality remains extremely high. In total, according to WHO data, by mid-2017, 859 people were infected with influenza H5N1, 453 (53%) from which died [25].

During the outbreak in Hong Kong in 1997, there was no direct evidence of a sustained human-to-human transmission of H5N1 viruses, although antibodies against H5 viruses were detected in 3.7% of physicians who had contact with H5-infected patients [26]. In 2008, transmission of an infection with avian influenza H5N1 from a son to his father was registered in China [27]. Under conditions of the continuous appearance HP H5N1 viruses in the humans, there is a risk of such a transmission during close contacts.

2.2. H7N9 influenza viruses

On March 31, 2013, the first three cases of human infection with the avian influenza H7N9 virus were registered in China. In all three cases, an infection of the respiratory tract was complicated with severe pneumonia. Two patients died, the third was in a critical condition for a long time, but recovered. Since then, the number of laboratory-confirmed cases in China has increased every day. In addition to severe and lethal cases, the sero-diagnostics methods have proved the asymptomatic course of the disease in workers of poultry farms. From March

2013, there were 1566 cases of avian influenza H7N9 in the world, of which 613 (39%) were fatal [28]. At the same time, 88% of the infected developed severe pneumonia, 68% was hospitalized in the intensive care unit. Mortality in different years ranged from 31 to 39%.

Experts believe that the virus H7N9 is not likely transmitted from person to person, but can spread with prolonged contact, especially when people care for sick family members. Moreover, the reassortment of several viruses is also not excluded. Genome analysis of human-isolated H7N9 viruses has shown adaptive evolution and convergent changes in eight viral genes, including sites in the PB2 gene (Q591K, E627K, and D701N), in HA (R156K, V202A, and L244Q), and in NA (R289K). These substitutions are known as playing a role in crossing species barriers from avian to human [29].

2.3. H9N2 influenza viruses

The H9N2 influenza viruses readily transmit from birds to animals and humans due to the easy appearance of variants that have an affinity for sialic receptors in mammals [30]. Sero-epidemiological studies revealed antibodies to viruses H9N2 among 15% of poultry workers in China [31]. Viruses H9N2 were isolated from people with symptoms of respiratory infection in Hong Kong and China from 1997 to 2009 [32] belonging mainly to the antigenic G1 line, unlike other H9N2 viruses isolated from swine and poultry belonging to the antigenic variety G9 [33]. Phylogenetic analysis showed that after 1994, Eurasian H9N2 after complex genetic reassortment of G1 and G9 viruses circulating among wild and domestic birds formed several antigenic lines [34]. The H9N2 viruses, which caused human cases, were not HP as they did not possess highly cleavable HA and were not highly virulent for poultry, although molecular analysis demonstrated similarity of genes for internal proteins with HP H5N1 viruses, which caused an outbreak among people in 1997 [35]. Due to the fact that the avian influenza viruses of the H9 subtype are transmitted to humans, have genetic similarity to the H5N1 viruses, and are widespread in Asia, Europe, and the Middle East, the WHO has included H9N2 vaccine development in the overall plan for pre-pandemic training [36].

2.4. H6N1 influenza viruses

Serological studies in Southern China revealed that 13% of people from different provinces have antibodies to the influenza virus of H6 subtype [37]. Phylogenic analysis of influenza A viruses indicates that the closely related genes coding the internal proteins could be found in influenza A viruses of different subtypes and that the reassortment between the avian and human influenza viruses is possible [38]. It was also shown that some of the fragments of the NP and NA genes of highly pathogenic H5N1 viruses originated from the H6 virus of wild ducks [18, 39]. Therefore, the avian influenza viruses of H6N1 subtype may represent a potential danger for humans.

Thus, various avian influenza viruses can pose a threat to humans that necessitates the development of a corresponding vaccine strain for the protection of humans from possible infection. As part of an influenza pandemic preparedness program, the WHO monitors the

number of zoonotic and potentially pandemic influenza viruses to schedule candidates for the development of appropriate vaccines [40].

3. Development of live influenza vaccines against potentially pandemic avian influenza

The LAIVs preparation against potentially pandemic avian influenza viruses is conducted in two directions: the preparation of vaccine strains using classical genetic reassortment in chick embryos or through reverse genetics (RG) technique. The first attenuated A/Ann Arbor/6/60 (H2N2)-based vaccine strains were obtained by reverse genetics shortly after H5 influenza outbreaks in Hong Kong in 1997 [41].

The vaccine candidates containing internal genes from the attenuation donor, and the surface antigens from viruses A/Hong Kong/156/97 (H5N1) or A/Hong Kong/483/97 (H5N1) with RG-modified HA demonstrated an attenuated phenotype for ferrets and chickens. Both reassortants caused seroconversions in chickens, which confirm the sensitivity of chickens to these vaccine strains despite the *att*-phenotype [42]. By RG methods, three reassortant strains based on the A/Ann Arbor/6/60 (H2N2) were prepared in the Vero cell line [43]. As a source of surface antigens, viruses H5N1 of 1997, 2003, and 2004 years of isolation with RG-modified HA were used. It was shown that a double immunization with LAIV from a strain isolated in 1997 completely protected mice from infection with later "wild" isolates, including the isolate obtained in 2005—A/Indonesia/05/2005 (H5N1). The use of HP viruses requires increased biosecurity level laboratories, certified cell lines, and RG techniques. The HP avian viruses found in nature cannot be used directly to prepare influenza vaccines because they would not grow in eggs and might be dangerous to people. The RG-modified viruses do not cause severe illness in birds and that also will grow well in chicken eggs (so that vaccine manufacturers can use it to produce vaccine). An alternative approach is to use low pathogenic surrogate viruses that show antigenic similarity to HP viruses. In this regard, the identification of non-pathogenic variants, which are antigenically close to potentially pandemic strains, may be very important.

Another vaccine candidate based on A/Ann Arbor/6/60, containing HA and NA from virus A/duck/Hokkaido/69/2000 (H5N3), A/chicken/Hong Kong/G9/97 (H9N2), or A/Chicken/British Columbia/CN-6/04 (H7N3) was prepared by classical genetic reassortment methods in the chick embryos (CE) [44–46]. The vaccine strains exhibited *ts*-, *ca*-, and *att*-phenotype and provided protection against infection with the wild-type virus in mice and ferrets.

3.1. Development of reassortant vaccine strains based on a/Leningrad/134/17/57 (H2N2) MDV

To prepare vaccines based on A/Leningrad/134/17/57 (H2N2) MDV, several non-pathogenic avian viruses of different subtypes (A/duck/Potsdam/1402–6/1986 (H5N2), A/mallard /The Netherlands/12/2000 (H7N3), A/Hong Kong/1073/99 (H9N2), A/quail/Hong Kong/G1/1997 (H9N2), and A/herring gull/Sarma/51 s/2006 (H6N1)) were used. The HP avian influenza viruses of subtypes H5 and H7 contain a HA insertion from several positively charged amino

acid residues (lysine and arginine) in the proteolytic cleavage site [47], which causes effective cleavage of HA by intracellular proteases expressed in most organs and tissues of birds and mammals. Unlike HP avian influenza viruses, non-pathogenic viruses contain a single arginine residue (R) in the cleavage site [44]. For non-pathogenic viruses proteolytic activation, the presence of trypsin-like enzymes is required, which is expressed by a limited range of cells and is found in the airways.

The reassortant vaccine strains were prepared in the Virology Department, Institute of Experimental Medicine, using classical genetic reassortment in CE as previously described [48]. The H5N2 reassortant virus inherited only the HA gene from the H5N2 parent virus, and the remaining seven genes from the Len/17 MDV (7,1 genome composition) [49]. The reassortants of subtypes H7N3, H9N2, and H6N1 inherited the HA and NA from parental avian influenza viruses (6,2 genome composition). All the reassortant strains were studied for temperature-sensitive (*ts*-) and cold-adapted (*ca*-) phenotype [49–52]. For those purposes, the reassortant viruses were propagated in CE for 2 days at 25, 34, and 40°C. The yield of "wild-type" avian influenza viruses at 40°C was the same or greater than at 34°C. Only when the temperature was increased to 41°C, the reproduction of these strains was partially limited. Thus, the high degree of temperature resistance of all the above viruses was demonstrated. In contrast to parental avian viruses, all vaccine candidates poorly reproduced at 40°C in titers not exceeded 1.5–1.8 $\log_{10}$ EID_{50}/ml. At the same time, these reassortant strains grew well at low temperatures. Thus, all obtained reassortants acquired the genes of internal and nonstructural proteins from the A/Leningrad/134/17/57 (H2N2) MDV inherited the *ts*- and *ca*-phenotype. The pronounced difference in optimal reproductive conditions between the temperature-resistant viruses of avian influenza and the cold-adapted attenuation donor is due to the properties of viral polymerases [53]. This difference in the temperature optimum of the parental viruses may facilitate the isolation of the reassortant viruses possessing the desired gene composition after selective passages at a lower temperature.

3.2. Immunogenicity and cross-protection in mice

The ability of LAIV to induce antibodies not only to the homologous variant subtype but also to cross-reacting antibodies to antigenically different variants including HP variants was shown in several mouse studies [50–52, 54–56].

Among all vaccine candidates based on non-pathogenic avian influenza viruses, the H6N1 LAIV was characterized by the highest HI titers in mice after a single administration (GMT = 17.4). The LAIV of H7N3 subtype raised serum antibodies not only against the homologous virus but also against H7N9, which possessed the difference of 3% in the HA amino acid sequence. In the sera from mice double-vaccinated with H7N3 LAIV, serum HI titers against H7N9 were 20–40 times higher than against H7N3 ($P < 0.05$) [56]. At the same time, local IgA levels were higher against homologous H7N3 compared with H7N9 after vaccination with LAIV. The H5N2 LAIV induced detectable HI and neutralizing antibody titers only against the homologous H5N2 virus, perhaps due to the genetic differences between H5N2 vaccine strain and infectious viruses H5N1 isolated in 1997, 2003, and 2005 (10–12% differences of the HA1 amino acid sequence).

Nevertheless, immunization using virus H5N2 of 1986 resulted in a significant level of protection in experimental infection of mice (**Figure 1**).

Data on the protective efficacy of reassortant vaccine strains against intranasal challenge with avian influenza viruses are summarized in **Table 1**. When the mice were challenged with HP H5N1 viruses following immunization with H5N2 LAIV, the infectious viruses were not isolated from nasal passages or from the brain [54, 55]. Limited reproduction of HP viruses in the respiratory tract of mice and preventing a systemic infection, including neuro-infections, are important advantages of LAIV, especially in respect with data on the neurogenic pathway of generalization of infection caused by HP H5N1 viruses [58]. The absence of nasal infection correlated with high titers of secretory virus-specific IgA viruses in nasal swabs. The local immune response of the mucous membranes of the body serves as the first and most significant barrier for many viral infections, including influenza [59]. Due to their polymeric structure, IgAs have several times higher anti-hemagglutinating and neutralizing activity compared to IgG [60] and are also more stable and more cross-reactive. In addition, IgA can interact with the surface proteins of the influenza virus intracellularly, during trans-cytosis [61]. With respect to LAIV, it is still unclear how antibody-mediated immune response is related to protective efficacy. Mechanisms of cross-immunity in influenza are mediated by several factors, among which the cellular immune response is very important. Cellular immunity is involved in virus clearance and in activating the humoral immune response. In this regard, the production of Th1 and Th2 marker cytokines *in vitro* by splenocytes from mice immunized with H5N2 LAIV and whole-virion H5N2 IIV was compared [55].

Both LAIV and IIV caused the cytokines production by splenocytes of immunized mice in response to stimulation with both whole H5N1 virus and recombinant H5 HA. While immunization with LAIV caused higher levels of IFN-γ production by splenocytes of mice stimulated with H5N1 viruses, immunization with IIV induced IL-4 and IL-10 production. Interestingly,

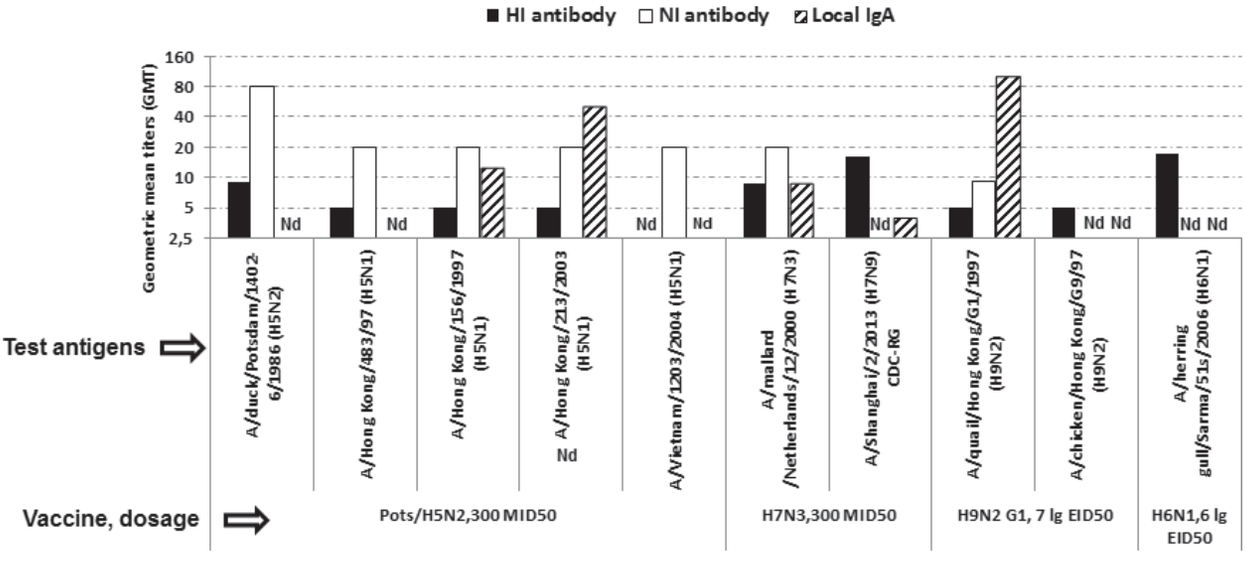

Figure 1. Influenza virus-specific serum antibodies and local IgA in mice after intranasal (i.n.) immunization with LAIV [50–52, 54–56]. *Nd, not done.

Challenge virus	Dosage	Vaccine groups	Protection				Refs.
			Virus titers ($log_{10}EID_{50}$/ml)			Lethality	
			Lung	Noses	Brain		
A/Hong Kong/483/97 (H5N1)	50 LD_{50}	H5N2 LAIV	1.9	≤0.8	≤0.8	0%	[54]
		PBS	5.9	4.0	4.3	100%	
A/Hong Kong/213/2003 (H5N1)	100 MID_{50}	H5N2 LAIV	1.8	Nd*	Nd	Na**	[54]
		PBS	5.3	Nd	Nd	Na	
A/Vietnam/1203/2004 (H5N1)	200 LD_{50}	H5N2 LAIV	1.6	0.8	0.8	0%	[55]
		PBS	6.1	4.7	4.5	100%	
A/chicken/Kurgan/02/2005 (H5N1)	27 LD_{50}	H5N2 LAIV	Nd	Nd	Nd	13%	[57]
		PBS	Nd	Nd	Nd	100%	
A/mallard/The Netherlands/12/2000 (H7N3)	7 lg EID_{50}	H7N3 LAIV	≤1.5	≤1.5	≤1.5	Na	[50]
		PBS	5.7	4.2	≤1.5	Na	[50]
A/chicken/Hong Kong/G9/97 (H9N2)	7 lg EID_{50}	H9N2 LAIV	3.4	1.1	Nd	Nd	[51]
		PBS	6.9	2.0	Nd	Nd	[51]

*Nd, not done.

**Na, not applicable.

The virus was not lethal for mice.

Table 1. Protection against infection with avian influenza viruses.

after immunization with the H2N2 MDV, the IFN-γ production by splenocyte of mice occurred only in response to stimulation with whole virus H5N1, but not purified HA. This may indicate the directivity of hetero-subtypic immunity to conserved epitopes of viral proteins [55].

3.3. Pathogenicity for chickens

Several experiments with vaccine candidates H5N2 and H7N3 were performed at Southeast Poultry Research Laboratory, GA, USA. Those studies demonstrated that the *ca*- reassortants of avian viruses adapted to a lower temperature of reproduction were unable to either infect a bird or be released into the environment. This was confirmed by the absence of virus isolation from the gastrointestinal tract of birds, as well as the impossibility in the determination of specific antibodies (**Table 2**).

A high degree of attenuation of H5N2 and H7N3 reassortants in chickens (up to a total inability) to reproduce confirms the safety for poultry farms during the production and use of such strains.

3.4. Study in primates

If the genetically homogeneous population using linear mice is the most appropriate model for assessing the molecular mechanisms of pathogenicity, the use of genetically heterogeneous

Virus	I.v. pathogenicity test*		I.n. pathogenicity and infectivity data**					Refs.
			Virus isolation on day 3 p.i.		Seroconversions (AGID).	Morbidity	Mortality	
	Morbidity	Mortality	Oropharyngeal swabs	Cloacal swabs				
Len/17	0/8	0/8	0/5	0/5	0/5	0/5	0/5	[53]
Len17/H5N2	0/8	0/8	0/5	0/5	0/5	0/5	0/5	
H5N2-wt	0/8	0/8	0/5	0/5	3/5	0/5	0/5	
Len17/H7N3	0/8	0/8	0/5	0/5	0/5	0/5	0/5	[49]
H7N3-wt	5/8	5/8	2/5($10^{1.1}$)***	1/5($10^{0.91}$)***	5/5	0/5	0/5	

*Groups of eight 5-week-old specific pathogen-free (SPF) chickens were infected intravenously (i.v.) with and observed daily for 10 days for clinical signs and death.

**Groups of five chickens were infected intranasally (i.n.) with 6 $\log_{10}$ EID_{50}/0.1 ml. The oropharyngeal and cloacal swabs were collected 3 days post infection (p.i.) and titrated in eggs for assessing viral replication. The chickens were observed for clinical signs of disease and death for 14 days. To determine infectivity, sera were collected 21 days p.i. and tested for the presence of antibodies by agar gel immunodiffusion (AGID) test.

***Mean virus titers (EID_{50}/0.1 ml).

Table 2. Pathogenicity and infectivity data for chickens.

animals (ferrets, primates) better allows one to assess the effect of natural host defense factors in mammalian infection by avian influenza viruses. The use of primates is one of the most promising areas in the study of human infectious pathology. The evolutionary relationship and biological similarity between humans and monkeys make them unique objects in the modeling of infectious diseases. However, the lower primates, while remaining closest to humans than other mammals, differ significantly in physiological characteristics from them. In experiments on the hybridization of nuclear DNA, it has been established that the similarity of man to chimpanzee reaches 90–98%, with lower monkeys—50–75% whereas in rodents, this index is not more than 20% (unpublished data).

The use of lower primates as models makes it possible to establish the duration and sequence of biochemical, metabolic, and physiological responses in the course of the development of the disease, which are then used to evaluate various preventive and therapeutic measures [62]. The use of primates for the modeling of the pathogenesis of influenza H5N1 in people of preclinical evaluation of vaccine preparations by a group of scientists from The Netherlands is described [63].

Before the clinical trials, the safety, immunogenicity, and protective properties of the LAIV based on strain A/17/duck/Potsdam/86/92 (H5N2) were studied by intranasal immunization of Java macaques [64]. None of the four monkeys immunized with H5N2 LAIV at a dose of 6.9 $\log_{10}$ EID_{50}/ml showed no adverse reactions with either temperature or behavioral changes or weight loss. The vaccine virus multiplied in the upper respiratory tract and was isolated in two of four monkeys, on days 3–5 after the first vaccination with the maximum titer of 4.2 lg EID50/ml. The absence of viremia and a temperature reaction in the same period indicates the local immunization process. In three of four monkeys, double immunization caused neutralizing antibodies to H5 viruses in titers 1:40–1:160. Twenty-one days after the end of the immunization cycle, the

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animals were infected in a combined method using intratracheal and intranasal administration of 7.5 lg EID_{50}/ml primate-adapted influenza virus A/Chicken/Kurgan/2/05 (H5N1). According to the summary data on clinical reactions and virus isolation from the respiratory tract, the vaccine protected at least 50% of immunized animals against the H5N1 infection.

3.5. Study of the H5N2 and H7N3 reassortants in phase I clinical trials

The randomized, double-blinded, placebo-controlled phase I trials were conducted in healthy adults at the St. Petersburg Institute of Influenza [65, 66]. Both H5N2 and H7N3 LAIV were safe for volunteers. In the genome of the isolated vaccine virus, all the mutations known for the MDV were conserved [65, 66]. Data on the LAIV when used in humans confirm the concept of attenuation of influenza viruses by reassortment with MDV and association of the ca- phenotype with an attenuation for people. For the vaccine virus isolation from the nasal washes, two to three passages were required on MDCK cell culture, indicating a very low content of the virus. These data confirmed LAIV implementation safety for contact persons.

The post-vaccination antibody response was assessed using the HI test, which is still posing as the "gold standard" for the evaluation of influenza vaccine immunogenicity, the microneutralization (MN) test which is supposed to be more sensitive compared to the HI in the detection of serum antibodies after immunization against potential pandemic subtypes. Local IgA response in nasal washes was estimated using ELISA (**Figure 2**).

According to the results of three tests, more than 80% of the vaccinated subjects responded to immunization with a significant increase in serum or local antibodies [65, 66]. Moreover, after double vaccination with H5N2 LAIV, 30.8% of vaccinated volunteers responded to the HA antigen of the A/Indonesia/05/2005xPR8 IBCDC-RG (H5N1). When serum samples of volunteers vaccinated with H7N3 LAIV were tested for the anti-H7N9 HI antibodies, the

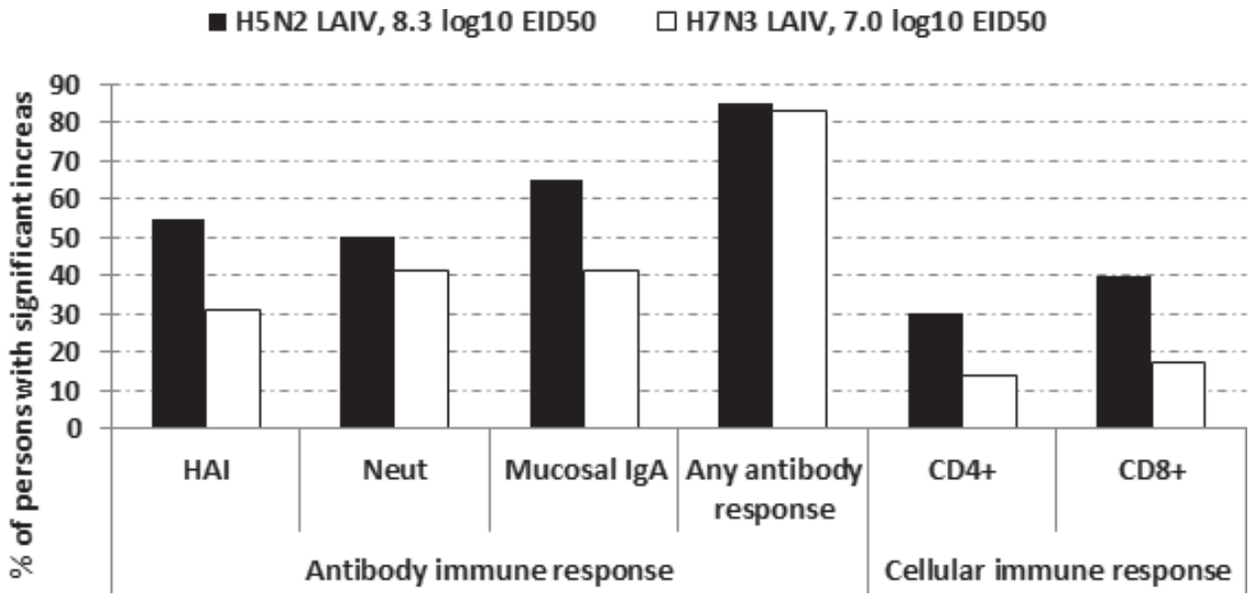

Figure 2. Immunogenicity of H5N2 and H7N3 LAIV in volunteers after boost immunization [65–67].

Vaccine	Groups	NI data				Ref.
		Number of ≥2-fold antibody rises	Geometric mean titers (GMT)		GMT fold-rise	
			Before vaccination	After revaccination		
LAIV H5N2 (6.9 $\log_{10}$ EID_{50}/0.5 ml)	LAIV	6 (33.3)	5.0	9.9	2.0*	[71]
	Placebo	0 (0)	9.9	7.5	0	

*P < 0.05.

Table 3. Serum NA-inhibiting antibodies against H5N2 LAIV 21 days after second vaccination.

seroconversions were found among 44.8% of vaccinated persons [67]. These data indicate the substantial level of cross-reactive antibodies induced by LAIV against distant avian influenza viruses. The two doses of LAIV raised both CD4 and CD8 T-memory-cell responses in peripheral blood of healthy volunteers on day 21 after boost immunization [67].

Previously, when studying the immunogenicity of inactivated vaccines based on potentially pandemic avian influenza viruses, both the experiment and the clinical trials showed a low immunogenicity of such preparations, according to generally accepted criteria for seroconversion of HI antibodies. The European Committee for the Control of Medicines has established the following criteria for the immunogenicity of vaccine preparations based on both epidemic and potentially pandemic influenza viruses: the multiplicity of antibody growths of at least 2.5 for individuals 18–60 years old and the development of reliable seroconversion in 40% of the vaccinated [68]. Obviously, the detection of only strain-specific HI antibodies is not sufficient to fully characterize the immunogenicity of the LAIV [69]. Moreover, it remains unclear what antibody titer can be considered protective against potentially pandemic viruses—1:20 or 1:40. Recently, it was shown that neuraminidase-inhibiting (NI) antibody titers better correlate with protection and can be an independent predictor of reduction of influenza disease severity [70]. Therefore, neuraminidase immunity should be considered when studying susceptibility after vaccination as a critical target in future influenza vaccine platforms. In this connection, the NI antibodies in the sera of volunteers after H5N2 immunization were estimated (**Table 3**). The two doses of the monovalent LAIV H5N2 raised a statistically significant increase in the NI antibodies against vaccine strain. More than twofold increase in antibodies was obtained among 19.5–33.3% of those vaccinated. The MN test and NI assay titers in the same sera of the vaccinated volunteers were 73.2% corresponded and suggested a statistically significant correlation between the values in antibody titers revealed in both tests ($p = 0.04$).

4. Conclusions

- The use of non-pathogenic avian viruses as a source of surface antigens combined with the use of cold-adapted "donors" of attenuation can be a significant advantage in the development of vaccine strains for LAIV against potentially pandemic influenza using classical genetic reassortment in CE. Low pathogenic avian influenza viruses do not contain a

polybasic amino acid insertion in the cleavage site and therefore do not require modification by reverse genetics methods prior to reassortment.

- A high degree of attenuation of the reassortants of subtypes H5N2 and H7N3 in chickens, up to a total inability to reproduce, confirms the safety for poultry farms during the production and use of such strains. The high yield of the obtained reassortants in the CE makes it possible to produce a large amount of viral material, which allows their use for the production of both LAIV and IIV.
- In preclinical and clinical studies, LAIV based on non-pathogenic avian influenza viruses causes the formation of systemic and secretory antibodies including those against antigenically distant viruses. In animal models, LAIV based on non-pathogenic avian influenza viruses provided protection against HP variants that appeared much later. Protection from lethal infection with HP viruses was observed even in the absence of HI antibodies. This suggests that the use of LAIV may be effective against HP influenza viruses even in the case of incomplete antigenic correspondence between the vaccine strain and the infectious virus.
- In general, studies in mice represent an adequate preclinical model for studying the properties of reassortants of non-pathogenic avian influenza viruses, since data on the safety, immunogenicity, and cross-reactivity of post-vaccinal antibodies obtained in mice were confirmed in clinical trials.
- In the clinical trials of LAIV of potentially pandemic subtypes, the detection of only strain-specific HI antibodies is not sufficient to fully characterize the positive effect of immunization on the stimulation of antiviral immunity, which in this case is mediated by a variety of other factors, both humoral and cellular.

5. Future perspectives

In the face of a pandemic threat, only live vaccines can eliminate the risk of losses from increased morbidity and mortality, as it was demonstrated in the cases with smallpox eradication and polio control. The conducted studies clearly showed that the classical genetic reassortment method allows obtaining high-yield, harmless and immunogenic LAIVs on the basis of an attenuated donor virus. In the post-pandemic period, when the direct threat of infection recedes, the main task is the search for optimal regimens for the use of new pandemic vaccines, including (1) the possibility of including such vaccine strains in the composition of polyvalent live vaccines; (2) prime-boost schemes using both LAIV and IIV; (3) the development of recommendations for vaccination of people with an increased risk of influenza infection complications; (4) a comprehensive study of the immune mechanisms of vaccination with influenza vaccines against emerging variant viruses; (5) the development of the most reliable and standardized assays to measure post-vaccination immune response.

Currently, the FluMist LAIV, which was withdrawn from use in the USA and Europe in 2015 due to reduced LAIV effectiveness against A/H1N1pdm09, was returned to the practice by

the CDC's Advisory Committee on Immunization Practices (ACIP) (http://www.cidrap.umn.edu/news-perspective/2018/02/cdc-vaccine-panel-brings-back-flumist-2018-19-season). It was noted that after the replacement of A/H1N1pdm09 vaccine strain in the quadrivalent LAIV, an immune response was achieved similar to that of highly immunogenic seasonal A/H1N1 viruses circulating before 2009. Therefore, at present, much attention is paid to influenza vaccine strategies that target more broadly reactive antibodies which also apply to potentially pandemic vaccine strains.

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Conflict of interest

The author confirms that this manuscript content has no conflict of interest.

Abbreviations

AGID	agar gel immunodiffusion
CE	chick embryos
ca-	cold-adapted
EID_{50}	fifty percent egg infectious dose
HI	hemagglutination inhibition
HP	highly pathogenic
i.n.	intranasal
LAIV	live attenuated influenza vaccine
MDCK	Madin-Darby canine kidney
MDV	master donor virus
MN	microneutralization
p.i.	post infection
WHO	World Health Organization
NI	neuraminidase-inhibiting

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