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Immunization with live nonpathogenic H5N3 duck influenza virus protects chickens against highly pathogenic H5N1 virus

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Summary. – Development of an effective, broadly-active and safe vaccine for protection of poultry from H5N1 highly pathogenic avian influenza viruses (HPAIVs) remains an important practical goal. In this study we used a low pathogenic wild aquatic bird virus isolate A/duck/Moscow/4182/2010 (H5N3) (dk/4182) as a live candidate vaccine. We compared this virus with four live 1:7 reassortant anti-H5N1 candidate vaccine viruses with modified hemagglutinin from either A/Vietnam/1203/04 (H5N1) or A/Kurgan/3/05 (H5N1) and the rest of the genes from either H2N2 cold-adapted master strain A/Leningrad/134/17/57 (rVN-Len and rKu-Len) or H6N2 virus A/gull/Moscow/3100/2006 (rVN-gull and rKu-gull). The viruses were tested in parallel for pathogenicity, immunogenicity and protective effectiveness in chickens using aerosol, intranasal and oral routes of immunization. All five viruses showed zero pathogenicity indexes in chickens. Viruses rVN-gull and rKu-gull were immunogenic and protective, but they were insufficiently attenuated and caused significant mortality of 1-day-old chickens. The viruses with cold-adapted backbones (rVN-Len and rKu-Len) were completely nonpathogenic, but they were significantly less immunogenic and provided lower protection against lethal challenge with HPAIV A/Chicken/Kurgan/3/05 (H5N1) as compared with three other vaccine candidates. Unlike other four viruses, dk/4182 was both safe and highly immunogenic in chickens of any age regardless of inoculation route. Single administration of 10^6 $TCID_{50}$ of dk/4182 virus via drinking water provided complete protection of 30-days-old chickens from 100 LD_{50} of the challenge virus. Our results suggest that low pathogenic viruses of wild aquatic birds can be used as safe and effective live poultry vaccines against highly pathogenic avian viruses.

Keywords: influenza virus A; H5N1; poultry vaccine

Introduction

H5N1 highly pathogenic avian influenza virus (HPAIV) causes significant economic losses in the poultry industry. Control over H5N1 infection has mainly been achieved by culling of poultry in outbreak regions. Another effective way to combat avian influenza is vaccination of poultry. Most commonly used are inactivated oil-in-water emulsion-adjuvanted whole virus vaccines based on reverse genetically engineered (rg) reassortants with H5 hemagglutinin (HA) and N1 neuraminidase (NA) from H5N1 viruses, and the remaining genes from A/PuertoRico/8/1934 (PR8). Tens of billions of doses of such vaccines were used in affected countries, primarily in China (Chen, 2009; Spackman and Swayne, 2013).

Numerous studies have focused on determining the immune efficacy of inactivated whole virus vaccines against H5N1 virus infection in chickens and ducks. The fundamental problem for H5N1 vaccination is antigenic drift of field

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Abbreviations: AB = antibodies; dk/4182 = A/duck/Moscow/4182/2010 (H5N3); gull/3100 = A/Gull/Moscow/3100/2006 (H6N2); HA = hemagglutinin; HAI = hemagglutination-inhibiting assay; HPAIV(s) = highly pathogenic avian influenza virus(es); IAF = Infectious virus-containing allantoic fluids; IVPI = standard intravenous pathogenicity indices; LD_{50} = 50% lethal dose; MN = microneutralization; NA = neuraminidase; p.i. = post infection; rVN-PR8 = Vietnam/1203/04-PR8/CDC-RG (H5N1); $TCID_{50}$ = 50% tissue culture infection dose

viruses since immunological effectiveness of inactivated vaccines strongly depends on the antigenic match between the HA in the vaccine and the HA of the field viruses. Antigenic drift has been recognized as a plausible reason of failure of vaccination programs in poultry (Kim *et al.*, 2010; Tian *et al.*, 2010; El-Zoghby *et al.*, 2012).

Compared with inactivated vaccines, potential advantages of live vaccines are the possibility of mass inoculation and a potentially increased immune response to viral replication in relevant tissues involving cellular immunity to influenza (Spackman and Swayne, 2013). To date, a number of live recombinant H5N1 influenza vaccines have been developed using other virus vectors, such as fowl pox virus (FPV), Newcastle disease virus (NDV), turkey herpes virus (HVT), duck enteritis virus (DEV), and infectious laryngotracheitis virus (ILTV). These vaccines provide protection against two viral diseases, which is superior in terms of ease of administration and cost reduction. In total, over 11 billion doses of the bivalent recombinant NDV vector H5N1 AI vaccine were manufactured and used in chickens in China between 2006 and 2012 (Cornelissen *et al.*, 2012; Li *et al.*, 2014).

In recent years promising experimental reverse genetics-derived H5N1 live attenuated vaccines have been generated and characterized, including vaccines that are attenuated through temperature-sensitive mutation, replacement of the polybasic hemagglutinin cleavage site with an elastase motif, modulation of the interferon antagonist protein, or disruption of the M2 protein (Gabriel *et al.*, 2008; Steel, 2011). Nang *et al.* (2012) developed a vaccine comprising internal genes from a cold-adapted H9N2 influenza virus and hemagglutinin and neuraminidase derived from a highly pathogenic H5N1 influenza virus. Reassortants with modified HA genes from H5N1 clade 1 and clade 2 viruses and internal genes from a cold-adapted donor or a nonpathogenic H6N2 influenza virus were developed in our laboratory. These experimental live H5 vaccines were nonpathogenic for mice and provided protection against challenge with HPAIV infection 4 weeks after immunization. Preliminary testing demonstrated that these strains can protect chickens against highly pathogenic H5N1 virus (Boravleva *et al.*, 2011; Gambaryan *et al.*, 2012).

In the nineties, ideas of using waterfowl-origin isolates of influenza viruses for vaccine production had been discussed (Murphy *et al.*, 1982). Oral immunization with a live waterfowl-origin avian influenza virus (H5N9) effectively protected chickens from lethal H5AI virus challenge and blocked cloacal shedding of the challenge virus (Crawford *et al.*, 1998). Unfortunately, this research was not followed up.

In this study we have compared a nonpathogenic influenza virus isolated from a wild duck and four experimental reassortants as live poultry vaccines.

Materials and Methods

Viruses. The list of viruses used in this study is shown in Table 1. Virus Vietnam/1203/04-PR8/CDC-RG (H5N1) (rVN-PR8) was kindly provided by Dr. R. Donis, CDC, USA. Viruses A/Gull/Moscow/3100/2006 (gull/3100) and A/duck/Moscow/4182/2008 (dk/4182) were isolated from avian feces collected on the shore of a pond in Moscow, Russia (Lomakina *et al.*, 2009; Boravleva *et al.*, 2012). Attenuation of the highly pathogenic H5N1 virus isolate A/Chicken/Kurgan/3/05 was described previously (Lomakina *et al.*, 2011; Gambaryan *et al.*, 2012). The cold-adapted reassortants rVn-Len and rKu-Len and reassortants with HA from either rVN-PR8 or Ku-at and 7 other gene segments from the avian H6N2 virus A/gull/Moscow/3100/2006 were produced as described elsewhere (Boravleva *et al.*, 2011; Gambaryan *et al.*, 2012). Virus stocks were propagated in the allantoic cavity of 10-day-old embryonated SPF chicken eggs. Gull/3100, dk/4182, ch/Ku, Ku-at, rVN-gull and rKu-gull strains were incubated at 37°C and harvested 48 hr post infection (p.i.). Eggs inoculated with rVN-Len and rKu-Len were incubated at 32°C and harvested 90 hr p.i. Infectious virus-containing allantoic fluids (IAF) were pooled, divided into aliquots, and stored at -70°C. $TCID_{50}$ for each virus stock was determined by titration in MDCK cells. For detection or isolation of influenza viruses, samples of avian feces were suspended in two volumes of phosphate buffered saline (PBS) supplemented with 0.4 mg/ml gentamycin, 0.1mg/ml kanamycin, 0.01mg/ml amphotericin B and 2% MycoKill AB (PAA Laboratories GmbH) and centrifuged at 4000 rpm. The supernatant was used to inoculate 10-day-old embryonated chicken eggs (0.2 ml per egg). After incubation for 48 hr, IAFs were collected and tested by hemagglutination assay with chicken red blood cells. Three serial passages were performed for isolation of viruses.

Viral RNA sequencing. Viral RNA was extracted from allantoic fluid using QIAamp Viral Mini Kit (Qiagen, Germany). cDNA fragments generated by reverse transcription followed by PCR were sequenced by dideoxy method using 3130x/Genetic Analyzer (Applied Biosystems, USA) with BigDye Terminator V3.1 Sequencing Kit. GenBank Acc. Nos. for the sequences are EU152234-EU152241, DQ323672-DQ323679, HQ724520-HQ724527, and KF885672-KF885679.

Animals. Chickens of Shaver Brown and Leghorn breeds and embryonated chicken eggs were purchased from State poultry farm "Ptichnoe" (Moscow, Russia). All studies with HP A/Chicken/Kurgan/3/05 virus were conducted in a BSL-3 containment facility of the Federal Center for Animal Health, Vladimir, Russia.

Ethics statement. Studies involving animals were performed in accordance with the European Convention for the Protection of Vertebrate Animals (1986). All operations were performed to ameliorate animal suffering. Highly pathogenic H5N1 influenza viruses cause rapid development of the symptoms of systemic infection and death of infected animals. The study design included assessment of the pathogenicity indexes of the viruses, i.e. determination of exact counts of sick and severely sick birds as well as birds dying

as a result of systemic infection. These circumstances were taken into account by the institutional Ethics Committee in the process of the study protocol approval. It was recommended to use alternative humane endpoints in all possible cases.

Assessment of pathogenicity index. The standard intravenous pathogenicity indices (IVPI) were assessed by inoculating 6-week-old chickens with 10^5 $TCID_{50}$ of the viruses. Birds were examined twice daily for up to 10 days. At each observation, each bird was scored 0 if normal, 1 if sick, 2 if severely sick, 3 if dead, and average IVPI for each group was determined (Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2012). If birds were too sick to eat or drink, they were humanely sacrificed and scored as dead at the next observation.

Oral, intranasal and aerosol routes of inoculation in chickens. For oral infection, chickens were left overnight without water. One drinking bowl per 3 chickens was placed in each cage in the morning. Each bowl contained 20 ml of diluted or undiluted IAF. Chickens were infected intranasally with 0.4 ml of undiluted stock viruses. Aerosol infection was performed as described below. Doses of the viruses and numbers of chickens per group are specified in the text.

Assessment of antibody levels. The levels of antibodies (AB) present in sera were assessed by ELISA with anti-chicken IgY (IgG) horseradish peroxidase-labeled antibodies (Sigma, USA), by hemagglutination-inhibiting assay (HAI), or by microneutralization (MN) assay using A/Chicken/Kurgan/3/05 virus (Suguitan *et al.*, 2006).

Aerosol challenge with HP A/Chicken/Kurgan/3/2005 virus. Aerosol challenge was performed as described previously (Gambaryan *et al.*, 2002). Briefly, the chickens were placed into a 200 liter transparent plastic chamber, which was connected to the Micropump Nebulizer (Aerogen, Ireland) generating virus aerosol. The aerosol entered the chamber through the inlet in its upper lid and was exhausted trough the outlet in the bottom part of the chamber connected via HEPA filter. Calculation of the virus deposition per animal was performed as described by Ovcharenko and Zhirnov (1994). The virus dose was $\approx 10^3$ $TCID_{50}$/chicken, which is equivalent to 100 LD_{50}. To unify the dose of the virus all chickens were marked and infected in the same chamber. After treatment chickens were divided into groups again and handled separately. Survival and body weight were monitored daily. Feces were sampled once daily on days 3–10 post challenge.

Results

Genome analysis of A/duck/Moscow/4182/2010 virus

Avian influenza virus (H5N3) A/duck/Moscow/4182/2010 was isolated from mallard feces collected in October 2010 on the shore of a pond in Troparevo Park, Moscow. Complete genome sequencing (KF885672-KF885679, GenBank) allowed to determine its genotype (G-G-D-5G-F-3B-F-1E) according to FluGenome nomenclature (Lu *et al.*, 2007). Analysis of viral proteins based on data obtained from the NIAID IRD (http://www.fludb.org) did not reveal any markers of pathogenicity or increased transmissivity. In particular, dk/4182 had the sequence PQRETRGLF at the HA cleavage site, 36A in PA, 678S in PB1, 199A, 701N, 627E, 661A and 702K in PB2, 16E and 55L in M2 (Suppl. S1).

Phylogenetic analysis of the full-length genomic sequences showed that all genes of A/duck/Moscow/4182/2010 were located in evolutionary clades containing exclusively low pathogenic viruses of wild aquatic birds (Suppl. S2).

Pathogenicity indices and virulence of viruses inoculated by different routes

Previously we reported that wild-type A/duck/Moscow/4182/2010 virus and reassortants rVN-gull, rKu-gull rVN-

Table 1.The influenza A viruses used in this study

Virus	Subtype	Abbreviation	Notes
A/chicken/Kurgan/3/05, wt	H5N1 clade 2	ch/Ku	HPAIV
A/chicken/Kurgan/3/05, attenuated	H5N1 clade 2	Ku-at	Substitutions in HA: Asp54Asn and Lys222Thr in HA1; Val48Ile and Lys131Thr in HA2
A/Vietnam/1203/04-A/Leningrad/134/17/57	H5N2 clade 1	rVN-Len	Cold-adapted reassortant 1/7*
A/Vietnam/1203/04-A/gull/Moscow/3100/2006	H5N2 clade 1	rVN-gull	Reassortant 1/7
Ku-at -A/Leningrad/134/17/57	H5N2 clade 2	rKu-Len	Cold-adapted reassortant 1/7
Ku-at -A/gull/Moscow/3100/2006	H5N2 clade 2	rKu-gull	Reassortant 1/7
A/duck/Moscow/4182/2010	H5N3	dk/4182	LPAIV
A/gull/Moscow/3100/2006	H6N2	gull/3100	LPAIV

*The HA from H5N1 virus and the remaining seven gene segments from a donor strain.

Table 2. Survival of chickens depending on age, dose and route of virus inoculation

Route of infection	Intravenous	Oral	Oral	Intranasal	Aerosol
Age (days)	**42**	**7**	**30**	**7**	**1**
Dose ($TCID_{50}$)	**10^5**	**10^7**	**10^7**	**10^6**	**10^5**
Strains					
Ku/05	0/10*	–	–	0/5	0/5
Ku/at	10/10	–	–	8/11	–
rKu-Len	10/10	5/5	–	20/20	20/20
rKu-gull	10/10	4/5	–	14/17	6/20
rVN-Len	10/10	5/5	–	19/19	20/20
rVN-gull	10/10	5/5	–	9/10	11/20
dk/4182	10/10	64/64	62/62	8/8	26/26
Gull/3100	10/10	–	–	8/8	5/5

*Number survived/number of infected birds. (–) = not tested.

Len, and rKu-Len generated in our laboratory were safe for mice and provided protection against HPAI H5N1 virus (Boravleva *et al.*, 2012; Gambaryan *et al.*, 2012). Here we compared the pathogenicity of these viruses for chickens (Table 2).

To determine the IVPIs, 10^5 $TCID_{50}$ of each virus (0.2 ml of stock viruses diluted at 1:10) were administered intravenously to groups of ten 6-week-old chickens. The IVPIs of wild type avian viruses dk/4182 and gull/3100, attenuated virus Ku/at and the four reassortants were 0. The chickens

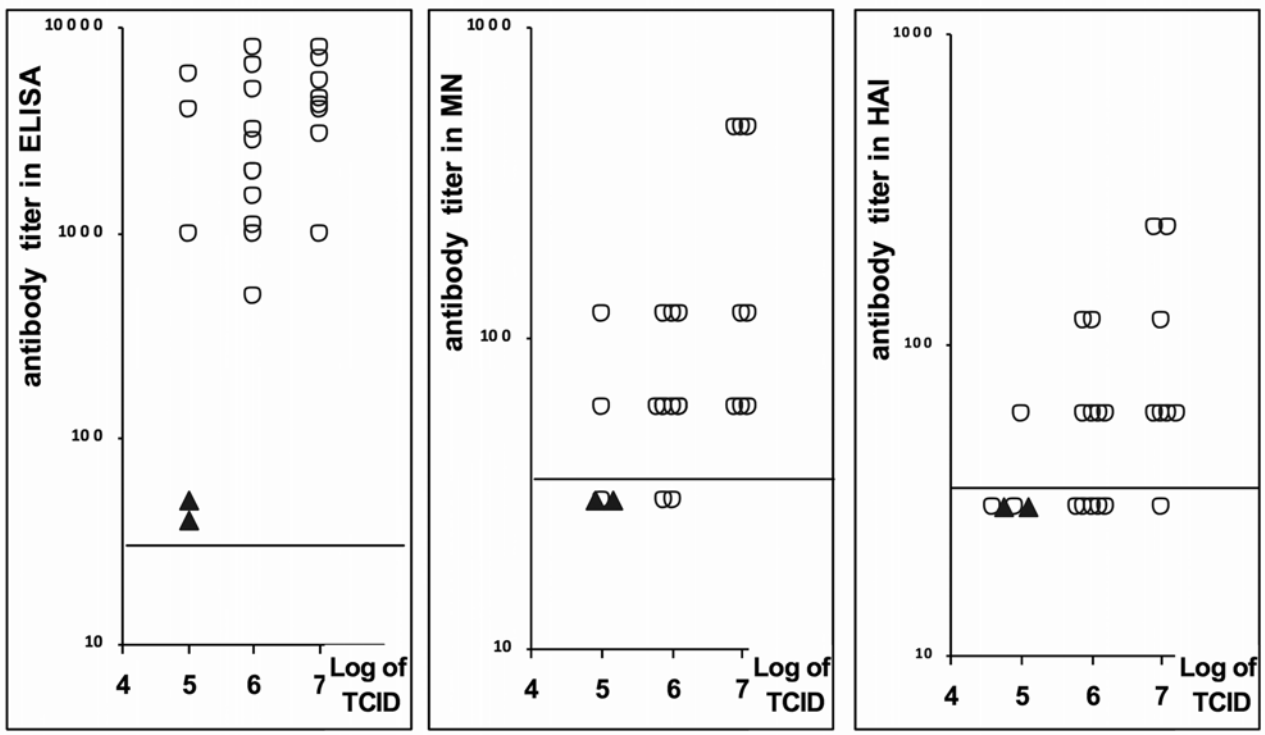

Fig. 1

Parallel testing of antibody titers in sera using ELISA, microneutralization and hemagglutination inhibition assays

Trial 1. Five, ten and eight 30 days-old chickens were vaccinated with 10^5, 10^6 and 10^7 $TCID_{50}$ of dk/4182 virus added to drinking water, respectively. Sera were collected at 14 day post immunization, e.g. one week before challenge with 100 LD_{50} of HPAIV A/chicken/Kurgan/3/2005. Black triangles represent sera samples taken from the chickens that died after challenge. Horizontal lines show lower limits of detection of the assays. Data points representing results below detection limit of the assay are shown under the lines.

in these groups showed no signs of the disease. The wild type HPAIV A/chicken/Kurgan/3/2005 used as a control showed an IVPI of 2.9, as all chickens died as a result of infection.

Oral and intranasal infection of 7-day-old chickens with Ku/at, rKu-gull and the rVN-gull led to death of some chickens. The highest mortality was observed following aerosol infection of 1-day-old chickens. In this group, rKu-gull killed most of the chickens and rVN-gull killed about half of them. rKu-Len, rVN-Len and native virus isolates dk/4182 and gull/3100 did not kill chickens infected by any of the routes tested.

Immunogenicity assessment

Immunogenicity of the viruses was assessed by measuring antibody titers in chicken sera collected on day 14 p.i. using hemagglutination-inhibition (HAI) assay, microneutralization (MN) assay and ELISA. An example of parallel testing of serum samples from chickens infected with dk/4182 virus using these methods is shown in Fig. 1. Challenge with HPAIV A/chicken/Kurgan/3/2005 was performed 21 days after immunization. ELISA reliably detected AB levels in almost all sera, while HAI and MN tests detected AB only in sera with titers above 1000 in ELISA. The antibody titers were, therefore, further determined by ELISA.

Pathogenicity, immunogenicity and protective activity of the experimental vaccines after aerosol infection of chickens

Comparison of oral, intranasal and aerosol infection led to the conclusion that aerosol administration of viruses to 1-day-old chickens is the most powerful method of infection (Table 2). In addition, younger chickens are more sensitive to challenge (data not shown). Therefore, this experimental design is very suitable to analyze the efficacy of experimental vaccines. For these reasons, we compared different strains using aerosol infection and challenge.

rKu-gull and rVN-gull viruses caused weight loss and significant mortality in chickens. All chickens that survived the infection by these viruses developed similar high levels of antibodies (Fig. 2 and Table 3) and were protected against subsequent challenge with 100 LD_{50} of H5N1 HPAIV A/chicken/Kurgan/3/2005. Reassortant viruses with cold-adapted backbones (rKu-Len and rVN-Len) caused no disease symptoms or weight loss, but they were significantly less immunogenic and offered lower protection against lethal challenge as compared with 3 other experimental vaccines. The best results were obtained with the A/duck/Moscow/4182/2010 virus, which caused no mortality or visible signs of the disease but stimulated the development of high levels of antibodies and offered 100% protection against lethal challenge.

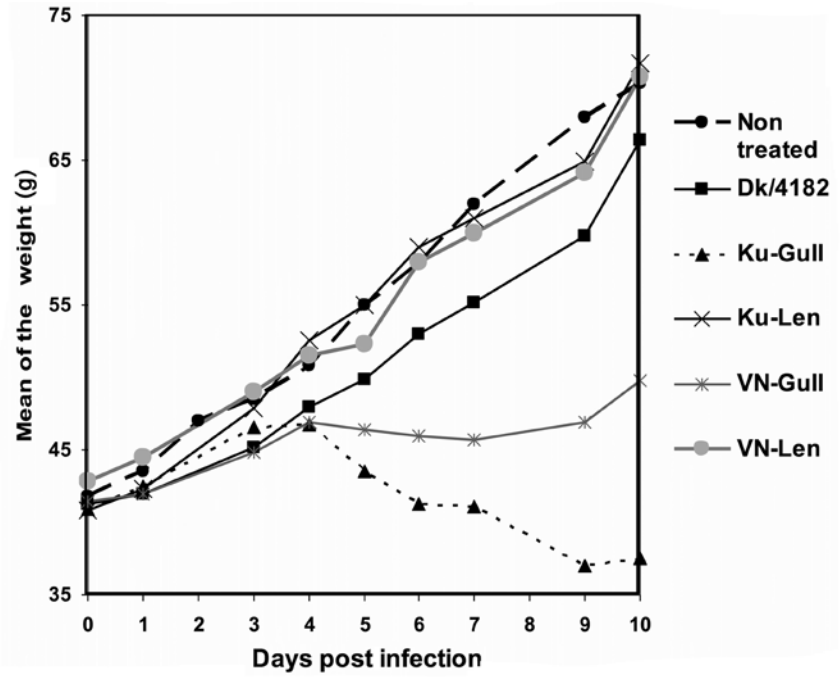

Fig. 2

Weight dynamics of 1-day-old chickens after aerosol infection with viruses dk/4182, rVN-Len, rVN-gull, rKu-Len and rKu-gull
Doses of viruses, numbers of birds and survival rates are indicated in legend to Table 3.

Table 3. Safety, immunogenicity, virus shedding and protective efficacy of dk/4182, rVN-Len, rVN-gull, rKu-Len and rKu-gull after aerosol infection of 1-day-old chickens

	Dose	Post vaccination			Post challenge	
Virus	$TCID_{50}$	Survival*	Ab**	Shedding	Survival	Shedding
rKu-Len	10^6	20/20	155	–	5/20	+
rKu-gull	10^5	6/20	2696	+	6/6	–
rVN-Len	10^6	20/20	167	–	4/20	+
rVN-gull	10^5	11/20	2063	+	10/10	–
dk/4182	10^5	26/26	1423	+	20/20	–
Control		10/10	–		0/10	+

*Survived/total number after vaccination or after challenge. **Geometric mean titer of antibodies. ***Shedding of the virus with feces. (–) = no shedding detected in any of the birds tested. (+) = shedding detected at least once.

Thus, immunogenicity of reassortants rKu-Len and rVN-Len, on the one hand, and rKu-gull and rVN-gull, on the other hand, directly correlated with their pathogenicity. By contrast, immunization with wild type avian dk/4182 virus resulted in potent and uniform immune response and protection without causing any signs of disease.

Unlike chickens immunized with rKu-Len and rVN-Len, chickens infected with rKu-gull, rVN-gull and dk/4182, shed the vaccine virus with feces on days 3–10 p.i. However, these chickens did not shed HPAIV A/chicken/Kurgan/3/2005 after the challenge.

Protective efficacy of A/duck/Moscow/4182/2010 virus administered via drinking water

Effectiveness and ease of application of AIV vaccines is a major factor in poultry vaccination, as the H5N1 HPAIV is primarily a problem of under-developed countries and small-scale family poultry farms. Vaccine administration via drinking water is, thus, an attractive way of immunization. To explore the capabilities of this method of vaccination, we evaluated dk/4182 virus as an oral live vaccine candidate for preventing disease and cloacal shedding of the virus following HP H5N1 challenge. Five trials with chickens from Shaver Brown and Leghorn breeds were conducted as described below (Figs. 1, 3–5). The antibody responses of 3 to 30-day-old chickens after infection with different doses of viruses with different vaccination schemes were measured, and protective efficacy against lethal H5N1 challenge was assessed.

Dose-dependence of vaccination efficacy

30-day-old chickens were vaccinated with 10^5, 10^6 and 10^7 $TCID_{50}$ of dk/4182 virus via drinking water. Sera were collected 14 days after vaccination; challenge with 100 LD_{50} of HPAIV A/Chicken/Kurgan/3/2005 was performed 21 days after vaccination. All chickens receiving 10^6 and 10^7 $TCID_{50}$ of the virus had protective levels of AB and survived the challenge. Antibody responses to immunization with the lowest dose (10^5 $TCID_{50}$) varied. Two of the 5 chickens had very low AB levels and died after challenge, whereas the three other chickens showed strong response and survived the challenge (Fig. 1).

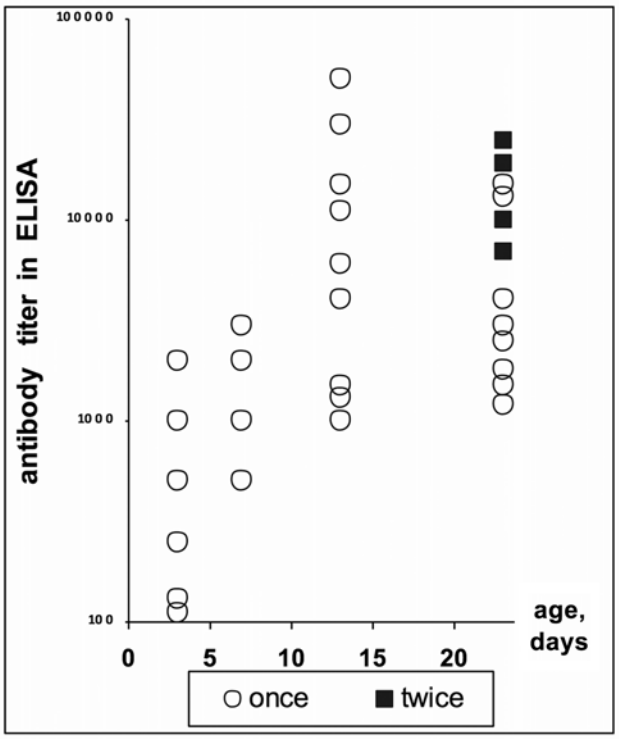

Fig. 3

Optimization of age for oral vaccination of chickens

Trial 2. Six, four, nine and eight chickens were vaccinated at the age of 3, 7, 13 or 23 days, respectively, via drinking water with 10^7 $TCID_{50}$ of the dk/4182 virus. 4 chickens vaccinated at the age of 3 days were repeatedly vaccinated at the age of 23 days. Sera were collected at 14 day post immunization. Circles and black squares represent sera samples obtained from chickens vaccinated once or twice, respectively.

The optimal age of vaccination

In trial 2, chickens were vaccinated with 10^7 $TCID_{50}$ of dk/4182 virus via drinking water at the age of 3, 7, 13 and 23 days. Four chickens were vaccinated twice: 3 days after hatching and 23 days after hatching. Sera were collected 14 days post vaccination. AB responses in 3-day-old chickens were weak and uneven. 7-day-old chickens developed higher AB responses, and 13-day-old and 23-day-old chickens showed the highest AB titers. All chickens that were vaccinated twice had stable and high AB titers (Fig. 3).

Optimization of the vaccination scheme

In trials 3, 4 and 5, we evaluated the antibody response after vaccination of Shaver Brown or Leghorn chickens via drinking water with 10^6 $TCID_{50}$ of dk/4182 with a single dose at either 7 or 30 day of age, or with two doses at 7 and 30 day of age. Vaccination of 30 day-old chickens was more effective than vaccination of 7 day-old chickens. Vaccination with two doses was more effective (Fig. 4). We found no significant differences between antibody responses in Shaver Brown and Leghorn chickens.

Protective levels of antibodies

Vaccinated chickens from trials 1, 3, 4 and 5 (Fig. 1 and 4) were challenged with 100 LD_{50} of H5N1 virus A/chicken/Kurgan/3/2005 at 21–87 days post last vaccination. To determine the levels of protective antibodies, antibody titers in sera collected the day before challenge were measured. Fig. 1 and 5 show the titers of antibodies and the outcomes of the challenge for individual chickens. Chickens with AB titers in ELISA below 100 generally died simultaneously with the control chickens on day 3 after infection. All chickens with AB titers above 1000 survived the challenge. Chickens with AB titers between 100 to 1000 either survived or died 4–11 days after infection. (Fig. 1 trial 1 and Fig. 5 trial 3).

The viruses in the organs and feces of chickens

Dk/4182 and ch/Ku viruses were detected in the lungs, kidneys, brains, cloacae and feces on days 3–9 post vaccination or challenge in trials 1, 4, 5. More than 200 samples of feces and about 50 samples of internal organs were tested. The dk/4182 virus was regularly detected in the cloacae and feces and was not detected in the lungs, kidneys or brains of chickens after vaccination via drinking water. Ch/Ku challenge virus was detected in internal organs and in feces of control chickens as well as in lungs and kidneys of vaccinated chickens, which died on day 3 after challenge. However, ch/Ku was had never been detected in feces of vaccinated chickens.

Transmission of dk/4182 virus in chickens

Despite the fact that dk/4182 virus is excreted in feces, we did not find any evidence of infection in chickens kept in cages together with infected ones. The chickens did not

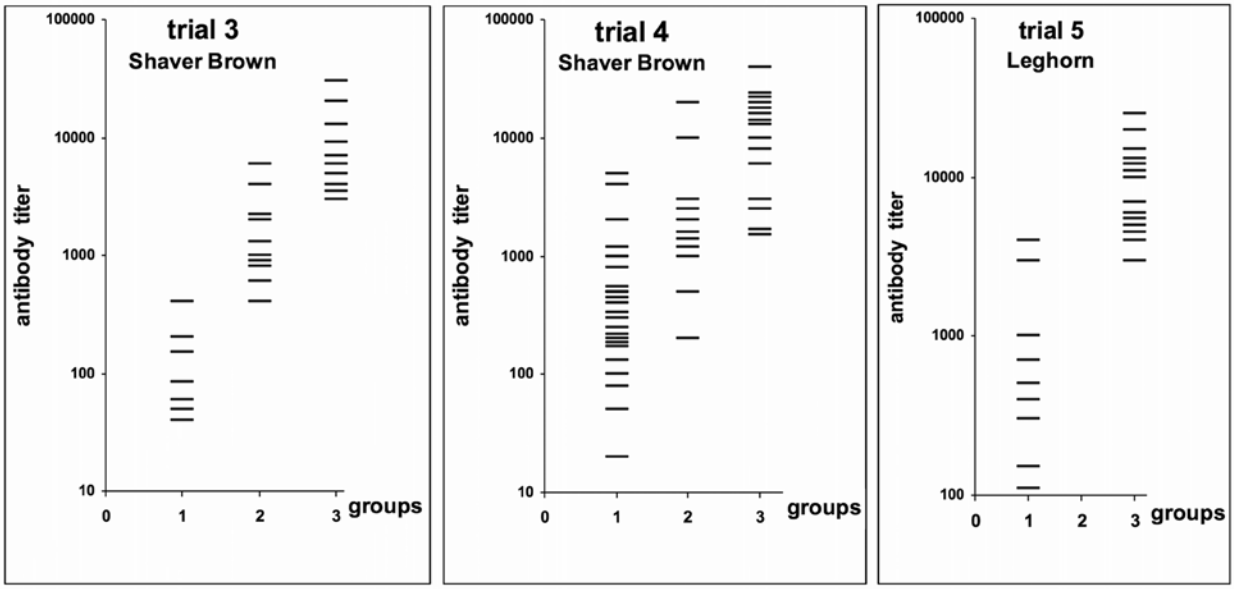

Fig. 4

Antibody titers in sera of chickens infected via drinking water after single and double vaccination

Trials 3, 4 and 5. Chickens were infected with 10^6 $TCID_{50}$ of dk/4182 virus with a single dose at either 7 or 30 days of age (groups 1 and 2, respectively), or with two doses at 7 and 30 days of age (group 3). Sera were collected at 14 day post immunization.

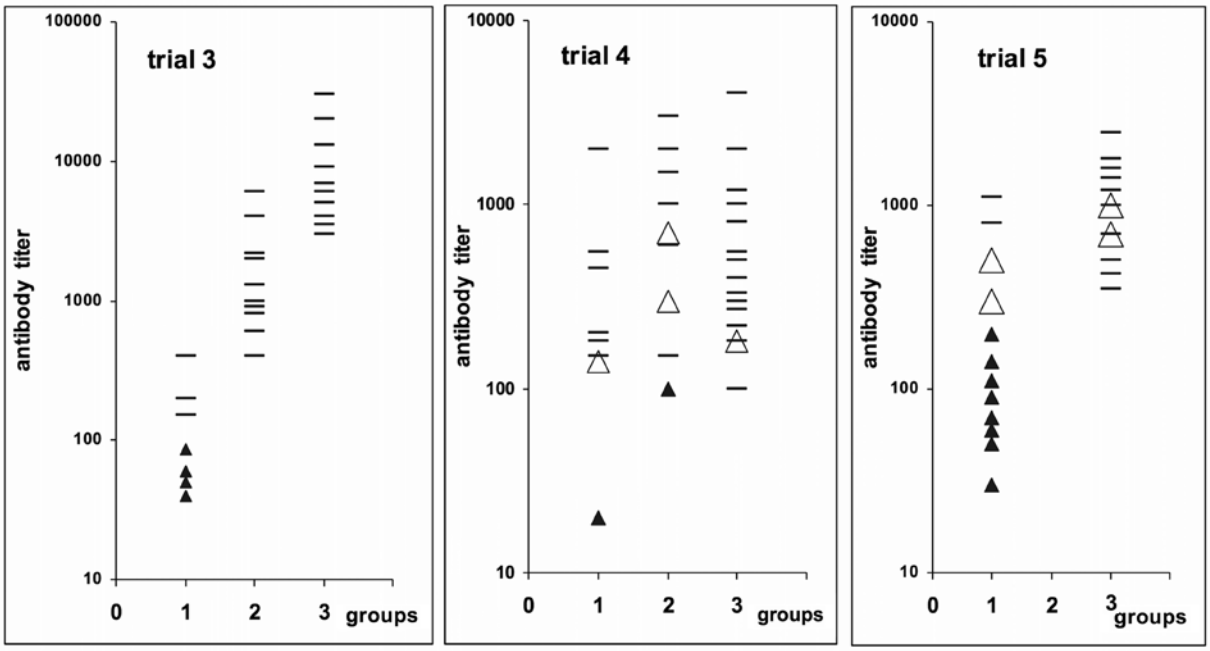

Fig. 5

Titers of anti-H5 antibodies in sera of individual chickens before challenge and outcomes of the challenge

Groups 1, 2 and 3 were vaccinated with a single dose at the age of 7 days, a single dose at the age of 30 days, or with two doses at the age of 7 days and 30 days, respectively. The challenge was performed at the age of 58 days in trial 3; at the age of 93 days in trial 4 and at the age of 74 days in trial 5. Lines, filled triangles and empty triangles represent, respectively, chickens that survived the challenge, died 3–5 days after challenge and died 6–11 days after challenge.

excrete the virus and had no antibodies to dk/4182 virus. Furthermore, our attempts to passage dk/4182 virus by fecal – oral route were unsuccessful as the virus was lost after the first passage. These results can be explained by the fact that a very high dose of dk/4182 virus is required for successful infection of chickens (Fig. 1). Our observations match with previous reports of inefficient replication and transmission of aquatic bird viruses in chickens (Ito *et al.*, 2001 and Cilloni *et al.*, 2010).

Discussion

In this study we have tested five live experimental vaccines against highly pathogenic H5N1 viruses. Our experiments with viruses rVN-Len and rKu-Len agree with previous observations, in which viruses with internal gene segments from cold adapted donors were overly attenuated for chickens and did not provide reliable protection from lethal challenge with HPAIV (Suguitan *et al.*, 2006, 2009). In contrast, all three viruses with internal genes from viruses of wild birds provided complete protection against HPAIV A/Chicken/Kurgan/3/2005, despite the fact that the hemagglutinin of rVN-gull belongs to another clade of H5N1 viruses, and that A/duck/Moscow/4182/2010 belongs to a distant evolutionary clade of subtype H5 viruses. However, among three viruses offering protection, rVN-gull and rKu-gull were not attenuated enough and caused partial death of chickens. Both viruses have 7 gene segments from the gull/3100 parent strain, which is completely safe for chickens. Hemagglutinin of rKu-gull contains mutations, which reduce the fusion pH from 5.6 to 5.1, and is attenuated for mice (Gambaryan *et al.*, 2012). Nevertheless, rKu-gull caused death of 1-day-old chickens due to the presence of the multibasic cleavage site, which is considered to be the main pathogenicity marker of AIV. However, in addition to multibasic cleavage site, some other specific properties of the HA are required for the high virulence of HPAIV in chickens (Stech *et al*, 2009). Thus, although the HA of rVN-gull lacked the multibasic cleavage site, this virus was able to kill 1-day-old chickens. We speculate that the high pH optimum of HA-mediated fusion can be responsible for the pathogenicity of this virus. Dependence of replication efficiency and pathogenicity of influenza viruses on fusion pH was well documented previously (Brown *et al.*, 2001; Hsu *et al.*, 2006; Reed *et al.*, 2010; Shelton *et al.*, 2013).

The risk of pathogenicity restoration is the main disadvantage of live-attenuated vaccines based on HPAI viruses.

Restoration of virulent infectious laryngotracheitis virus resulting from recombination between distinct attenuated vaccine strains has been shown (Lee *et al*. 2012). It has been shown that attenuated vaccines, particularly those produced by passaging in chicken embryos, could be transmitted to non-vaccinated birds, shed by the host, and revert to virulence after *in vivo* passage (Coppo *et al*., 2013). Reassortment with field strains or reverse mutation could repair the virulence of a virus. Because of the presumable risks of such reversions to pathogenicity, live vaccines against H5N1 virus are not approved for use anywhere except China (Spackman & Swayne, 2013).

To address some of these issues, new-generation live vaccines have been developed (Steel, 2011). Nevertheless, "regulatory barriers are probably insurmountable for avian influenza viruses based live vaccine in poultry" (Spackman and Swayne, 2013).

It is generally believed that HPAI viruses emerge from low-pathogenicity avian predecessors (Alexander, 2003). Indeed, it has been shown that outbreaks of HPAI in Pennsylvania (1983–1984), Mexico (1994–1995), and Italy (1999–2000) were caused by originally non-virulent viruses that had evolved to high pathogenicity during circulation in poultry (Van Der Goot *et al*., 2003). However, phylogenetic analyses showed that all mentioned episodes of rapid acquisition of pathogenicity happened in the cases when the low-pathogenicity avian predecessors were closely related to poultry pathogenic viruses and had only recently been re-introduced into natural wild bird reservoir. All viruses of the Pennsylvania/1983 lineage had RKKRG in the cleavage site, and the difference between the HAs of the virulent and non-virulent strains was the lack of an asparagine-linked carbohydrate on the virulent HA1 polypeptide at residue 11 (Deshpande *et al*., 1987). Both HPAI and LPAI were found among viruses of the Mexican/1994 lineage, but all of them were relatively far from the LPAI viruses that circulated in wild ducks (data not shown).

LP viruses of wild aquatic birds differ significantly in this respect from LP poultry-adapted viruses. The wild duck viruses do not replicate efficiently in poultry. For example, all attempts to propagate the LPAI H5N3 virus A/whistling swan/Shimane/499/83 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 (Ito *et al*., 2001). Introduction of four basic amino acid residues at the HA cleavage site and ten subsequent consecutive passages in air sacs of chickens were needed for acquisition of intravenous pathogenicity of a non-pathogenic H9N2 virus in chickens (Soda *et al*., 2011). In our study we show that A/duck/Moscow/4182/2010 virus also poorly replicated in the internal organs of chickens and was not transmitted to contact birds.

Inefficient replication of aquatic birds viruses in chickens depends, at least partially, on the differences in receptors on target cells in these species (Gambaryan *et al*., 2002). Adaptation of duck viruses to gallinaceous poultry is accompanied by changes in the viral receptor specificity and neuraminidase activity, owing to acquisition by poultry-adapted viruses of additional glycosylation sites near the receptor-binding site of the HA and to deletions in the stem region of their NA (Matrosovich *et al*., 1999, 2001; Banks *et al*., 2001; Gambaryan *et al*., 2008). These adaptive changes in the HA and NA of poultry-adapted viruses occur before the acquisition of multibasic cleavage site and high pathogenicity, which is the final stage in the evolution of AIV in gallinaceous poultry.

High virulence is not typical for viruses of wild hosts. HPAIVs are only occasionally transmitted from poultry to aquatic birds and, as a rule, are not maintained in wild birds. In the wild, quick death of the host is the barrier to spread of the virus. By contrast to influenza viruses in natural reservoirs, increase in virulence is not prevented by natural selection under specific conditions of poultry farms with constant crowding of the birds with efficient transmission by both fecal-oral and airborne routes. During years of circulation of H5N1 viruses in domestic chickens they accumulated multiple mutations in many genes that increase pathogenicity (Hatta *et al*, 2001, Seo *et al*., 2002; Gabriel *et al*, 2005; Jiao *et al*., 2007; Stech *et al*, 2009; Imai *et al*., 2010). On the other hand, influenza viruses of wild birds had been subjected to natural selection toward non-virulence for centuries.

Considering naturally selected lack of virulence of waterfowl-origin LPAIV, many researchers tried to use the genes of these viruses for the development of live influenza vaccines (Murphy *et al*., 1982, Crawford *et al*., 1998, Van Der Coot *et al*., 2003; Shi *et al*., 2007; Wu *et al*., 2010; Zhang *et al*., 2012; Pena *et al*., 2013). Our data confirm that the H5N3 wild duck virus represents a promising live candidate vaccine for protection of poultry from H5N1 HPAI viruses. Parallel testing of experimental strains rVN-Len, rKu-Len, rVN-gull, rKu-gull and duck virus dk/4182 showed that only the wild duck virus A/duck/Moscow/4182/2010 meets requirements for the "perfect AIV vaccine for poultry", such as high safety and high potency with protection after a single vaccination, low cost and the possibility of vaccine administration without handling each bird individually (Peyre *et al*, 2009; Spackman and Swayne, 2013). Indeed, our experiments show that dk/4182 is safe for chickens of any age, regardless of the method of application. The virus is also apathogenic for mice and likely other mammals. It offers protection from the lethal challenge and prevents shedding of the challenge virus after a single immunization, can be administered via drinking water (mass application) and meets the formal criteria of «DIVA» owing to the presence of N3 neuraminidase.

Since dk/4182 virus is antigenically equidistant from all the H5N1 viruses, including the ch/Ku, which was used for the challenge, it could be expected that it will be effective against a broad range of H5N1 strains.

A disadvantage of the dk/4182 vaccine may be high dose of virus required for immunization via drinking water (at least 10^6 EID per animal), which increases vaccination costs. This disadvantage should be compensated by the ease and low costs of the vaccine production. The most primitive poultry farms can produce enough material for immunization from a small amount of the stock virus. Another disadvantage of dk/4182 vaccine is that the vaccine virus is excreted in feces of chickens and could theoretically get out of control. However, in the case of focused vaccinations in regions where HPAI H5N1 viruses already circulate and cause problems, the LPAI H5N3 duck virus is clearly a lesser evil. Furthermore, a possible spillover of the dk/4182 virus into the environment should not be overdramatized because the virus already widely circulates in wild aquatic birds.

Supplementary information is available in the online version of the paper.

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References

Alexander DJ (2003): Should we change the definition of avian influenza for eradication purposes? Avian Dis. 47 (Suppl. 3), 976–981. http://dx.doi.org/10.1637/0005-2086-47.s3.976

Banks J, Speidel ES, Moore E, Plowright L, Piccirillo A, Capua I, Cordioli P, Fioretti A, Alexander DJ (2001): Changes in the haemagglutinin and the neuraminidase genes prior to the emergence of highly pathogenic H7N1 avianinfluenza viruses in Italy. Arch Virol. 146, 963–973. http://dx.doi.org/10.1007/s007050170128

Boravleva EY, Lomakina NF, Gambaryan AS (2012): Isolation of influenza A viruses from birds on ponds of Moscow. Kazarka 15, 13–30 (in Russian).

Boravleva EY, Lomakina NF, Kropotkina EA, Rudneva IA, Yamnikova SS, Rudenko LG, Drygin VV, Gambaryan AS (2011): The generation and characteristics of reassortant influenza A virus with H5 hemagglutinin and other genes from nonpathogenic virus H6N2. Voprosi Virusologii 6, 9–14 (in Russian).

Brown EG, Liu H, Chang Kit L, Baird S, Nesrallah M (2001): Pattern of mutation in the genome of influenza A virus on adaptation to increased virulence in the mouse lung: Identification of functional themes. Proc. Natl. Acad. Sci. USA 98, 6883–6888. http://dx.doi.org/10.1073/pnas.111165798

Chen H (2009): Avian influenza vaccination: the experience in China. Rev Sci Tech. 28, 267–274. http://dx.doi.org/10.20506/rst.28.1.1860

Cilloni F, Toffan A, Giannecchini S, Clausi V, Azzi A, Capua I, Terregino C (2010): Increased pathogenicity and shedding in chickens of a wild bird-origin low pathogenicity avian influenza virus of the H7N3 subtype following multiple in vivo passages in quail and turkey. Avian Dis. 54 (1 Suppl.), 555-557. http://dx.doi.org/10.1637/8919-050809-Reg.1

Coppo MJ, Noormohammadi AH, Browning GF, Devlin JM (2013): Challenges and recent advancements in infectious laryngotracheitis virus vaccines. Avian Pathol. 42, 195–205. http://dx.doi.org/10.1080/03079457.2013.800634

Cornelissen LA, de Leeuw OS, Tacken MG, Klos HC, de Vries RP, et al. (2012): Protective efficacy of Newcastle disease virus expressing soluble trimeric hemagglutinin against highly pathogenic H5N1 influenza in chickens and mice. PLoS One 7, e44447. http://dx.doi.org/10.1371/journal.pone.0044447

Crawford JM, Garcia M, Stone H, Swayne D, Slemons R, Perdue M L (1998): Molecular characterization of the hemagglutinin gene and oral immunization with a waterfowl-origin avian influenza virus. Avian Dis. 42, 486–496. http://dx.doi.org/10.2307/1592675

Deshpande KL, Fried VA, Ando M, Webster RG (1987): Glycosylation affects cleavage of an H5N2 influenza virus hemagglutinin and regulates virulence. Proc. Natl. Acad. Sci. USA 84, 36–40. http://dx.doi.org/10.1073/pnas.84.1.36

El-Zoghby EF, Arafa AS, Kilany WH, Aly MM, Abdelwhab EM, Hafez HM (2012): Isolation of avian influenza H5N1 virus from vaccinated commercial layer flock in Egypt.Virol. J. 9, 294. http://dx.doi.org/10.1186/1743-422X-9-294

European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, Strasbourg, 18.III.1986. www.conventions.coe.int/Treaty/en/Treaties/Word/123.doc

Gabriel G, Dauber B, Wolff T, Planz O, Klenk HD, Stech J (2005): The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. Proc. Natl. Acad. Sci. USA 102, 18590–18595. http://dx.doi.org/10.1073/pnas.0507415102

Gabriel G, Garn H, Wegmann M, Renz H, Herwig A, Klenk HD, Stech J (2008): The potential of a protease activation mutant of a highly pathogenic avian influenza virus for a pandemic live vaccine. Vaccine 26, 956–965. http://dx.doi.org/10.1016/j.vaccine.2007.11.052

Gambaryan A, Webster R, Matrosovich M. (2002): Differences between influenza virus receptors on target cells of duck and chicken. Arch. Virol. 147, 1197–1208. http://dx.doi.org/10.1007/s00705-002-0796-4

Gambaryan AS, Lomakina NF, Boravleva EY, Kropotkina EA, Mashin VV, Krasilnikov IV. Klimov AI, Rudenko LG

(2012): Comparative safety, immunogenicity and efficacy of several anti-H5N1 influenza experimental vaccines in a mouse and chicken models. Influenza Other Respir. Viruses 6, 188–195. http://dx.doi.org/10.1111/j.1750-2659.2011.00291.x

Gambaryan AS, Tuzikov AB, Chinarev AA, Juneja LR, Bovin NV, Matrosovich MN (2002): Polymeric inhibitor of influenza virus attachment protects mice from experimental influenza infection. Antivir. Res. 55, 201–205. http://dx.doi.org/10.1016/S0166-3542(02)00020-7

Gambaryan AS, Tuzikov AB, Pazynina GV, Desheva JA, Bovin NV, Matrosovich MN, Klimov AI (2008): 6-sulfo sialyl Lewis X is the common receptor determinant recognized by H5, H6, H7 and H9 influenza viruses of terrestrial poultry. Virol. J. 5, 85. http://dx.doi.org/10.1186/1743-422X-5-85

Hatta M, Gao P, Halfmann P, Kawaoka Y (2001): Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science 293, 1840–1842. http://dx.doi.org/10.1126/science.1062882

Hsu CN, Wang CH (2006): Sequence comparison between two quasi strains of H6N1 with different pathogenicity from a single parental isolate. J. Microbiol. Immunol. Infect. 39, 292–296.

Imai H, Shinya K, Takano R, Kiso M, Muramoto Y, Sakabe S et al. (2010):The HA and NS genes of human H5N1 influenza A virus contribute to high virulence in ferrets. PLoS Pathog. 2010 Sept. 16; 6, e1001106. http://dx.doi.org/10.1371/journal.ppat.1001106

Ito T, Goto H, Yamamoto E, Tanaka H, Takeuchi M, Kuwayama M, Kawaoka Y, Otsuki K (2001): Generation of a highly pathogenic avian influenza A virus from an avirulent field isolate by passaging in chickens. J. Virol. 75, 4439–4443. http://dx.doi.org/10.1128/JVI.75.9.4439-4443.2001

Jiao P, Tian G, Li Y, Deng G, Jiang Y, Liu C, Liu W, Bu Z, Kawaoka Y, Chen H (2008): Amino acid substitution in the NS1 protein changes the pathogenicity of H5N1 avian influenza viruses in mice. J. Virol. 82, 1146–1154. http://dx.doi.org/10.1128/JVI.01698-07

Kim J, Kayali G, Walker D, Forrest HL, Ellebedy AH et al. (2010): Puzzling inefficiency of H5N1 influenza vaccines in Egyptian poultry. Proc. Natl. Acad. Sci. USA 107, 11044–11049. http://dx.doi.org/10.1073/pnas.1006419107

Lee SW, Markham PF, Coppo MJ, Legione AR, Markham JF, Noormohammadi AH, Browning GF, Ficorilli N, Hartley CA, Devlin JM (2012): Attenuated vaccines can recombine to form virulent field viruses. Science 337,188. http://dx.doi.org/10.1126/science.1217134

Li Ch., Bu Zh, Chen H (2014): Avian influenza vaccines against H5N1 'bird flu'. Trends Biotechnol. 1133, 1–10. http://dx.doi.org/10.1016/j.tibtech.2014.01.001

Lomakina NF, Boravleva EY, Kropotkina EA, Yamnikova SS, Drygin VV, Gambaryan AS (2011): Attenuation of A/Chicken/Kurgan/3/2005 (H5N1): Influenza Virus Using Selection in an Environment Simulating the Life Cycle of Wild Duck Viruses, Mol. Gen. Mikrobiol. Virusol. 26, 132–139. http://dx.doi.org/10.3103/S0891416811030025

Lomakina NF, Gambaryan AC, Boravleva EY, Kropotkina EA, Kirillov IM, Lavrientev MV, Yamnikova SS (2009): Character of Nonpathogenic Influenza A Viruses Found in Moscow. Mol. Gen. Mikrobiol. Virusol. 24, 37–45. http://dx.doi.org/10.3103/S0891416809010078

Lu G, Rowley T, Garten R, Donis RO. (2007): FluGenome: a web tool for genotyping influenza A virus. Nucleic Acids Research; http://flugenome.org/

Matrosovich M, Krauss S, Webster R (2001): H9N2 influenza A viruses from poultry in Asia have human-virus-like receptor specificity. Virology 281, 156–162. http://dx.doi.org/10.1006/viro.2000.0799

Matrosovich MN, Zhau N, Kawaoka Y, Webster R (1999): The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties. J. Virol. 73, 1146–1155.

Murphy BR, Sly DL, Tierney EL, Hosier NT, Massicot JG, London WT, Chanock RM, Webster RG, Hinshaw VS (1982): Reassortant virus derived from avian and human influenza A viruses is attenuated and immunogenic in monkeys. Science 218, 1330–1332. http://dx.doi.org/10.1126/science.6183749

Nang NT, Song BM, Kang YM, Kim HM, Kim HS, Seo SH (2013): Live attenuated H5N1 vaccine with H9N2 internal genes protects chickens from infections by both Highly Pathogenic H5N1 and H9N2 Influenza Viruses. Influenza Other Respir. Viruses 7, 120–131. http://dx.doi.org/10.1111/j.1750-2659.2012.00363.x

Ovcharenko AV, Zhirnov OP (1994). Aprotinin aerosol treatment of influenza and paramyxovirus bronchopneumonia of mice. Antiviral Res. 23, 107–118. http://dx.doi.org/10.1016/0166-3542(94)90038-8

Pena L, Sutton T, Chockalingam A, Kumar S, Angel M, Shao H, Chen H, Li W, Perez DR (2013): Influenza viruses with rearranged genomes as live-attenuated vaccines. J. Virol. 87, 5118–5127. http://dx.doi.org/10.1128/JVI.02490-12

Peyre, M., Fusheng, G., Desvaux, S., Roger, F., (2009a): Avian influenza vaccines: a practical review in relation to their application in the field with a focus on the Asian experience. Epidemiol. Infect. 137, 1–21. http://dx.doi.org/10.1017/S0950268808001039

Reed ML, Bridges OA, Seiler P, Kim J-K, Yen H-L, Salomon R, Govorkova EA, Webster RG, Russell CJ (2010): The pH of Activation of the Hemagglutinin Protein Regulates H5N1 Influenza Virus Pathogenicity and Transmissibility in Ducks. J. Virol. 84, 1527–1535. http://dx.doi.org/10.1128/JVI.02069-09

Seo SH, Hoffmann E, Webster RG (2002): Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. Nat. Med. 8, 950–954. http://dx.doi.org/10.1038/nm757

Shelton H, Roberts KL, Molesti E, Temperton N, Barclay WS (2013): Mutations in haemagglutinin that affect receptor binding and pH stability increase replication of a PR8 influenza virus with H5 HA in the upper respiratory tract of ferrets and may contribute to transmissibility. J. Gen. Virol. 94, 1220–1229. http://dx.doi.org/10.1099/vir.0.050526-0

Shi H, Liu XF, Zhang X, Chen S, Sun L, Lu J (2007): Generation of an attenuated H5N1 avian influenza virus vaccine with all eight genes from avian viruses. Vaccine 25, 7379–7584. http://dx.doi.org/10.1016/j.vaccine.2007.08.011

Soda K, Asakura S, Okamatsu M, Sakoda Y, Kida H (2011): H9N2 influenza virus acquires intravenous pathogenicity on the introduction of a pair of di-basic amino acid residues at the cleavage site of the hemagglutinin and consecutive passages in chickens. Virol. J. 10, 64. Spackman E, Swayne DE (2013): Vaccination of gallinaceous poultry for H5N1 highly pathogenic avian influenza: Current questions and new technology. Virus Res. 178, 121–132.

Stech O, Veits J, Weber S, Deckers D, Schröer D, Vahlenkamp TW, Breithaupt A, Teifke J, Mettenleiter TC, Stech J (2009): Acquisition of a polybasic hemagglutinin cleavage site by a low-pathogenic avian influenza virus is not sufficient for immediate transformation into a highly pathogenic strain. J. Virol. 83, 5864–5868. http://dx.doi.org/10.1128/JVI.02649-08

Steel J (2011): New strategies for the development of H5N1 subtype influenza vaccines: progress and challenges. BioDrugs 25, 285–298. http://dx.doi.org/10.1007/BF03256169

Suguitan AJ, Marino MP, Desai PD et al. (2009): The influence of the multi-basic cleavage site of the H5 hemagglutinin on the attenuation, immunogenicity and efficacy of a live attenuated influenza A H5N1 cold-adapted vaccine virus. Virology 395, 280–288. http://dx.doi.org/10.1016/j.virol.2009.09.017

Suguitan AJ, McAuliffe J, Mills KL, Jin H, Duke G, Lu B, Luke CJ, Murphy B, Swayne DE, Kemble G, Subbarao K (2006): Live, Attenuated Influenza A H5N1 Candidate Vaccines Provide Broad Cross-Protection in Mice and Ferrets. PLoS Med. 3, 1541–1554. http://dx.doi.org/10.1371/journal.pmed.0030360

Tian G, Zeng X, Li Y, Shi J, Chen H (2010): Protective efficacy of the H5 inactivated vaccine against different highly pathogenic H5N1 avian influenza viruses isolated in China and Vietnam. Avian Dis. 54, 287–289. http://dx.doi.org/10.1637/8707-031709-ResNote.1

Van Der Goot JA, De Jong MCM, Koch G, Boven M (2003): Comparison of the transmission characteristics of low and high pathogenicity avian influenza A virus (H5N2): Epidemiol. Infect. 13, 1003–1013. http://dx.doi.org/10.1017/S0950268803001067

WORLD ORGANISATION FOR ANIMAL HEALTH (OIE) (2013): Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Chapter 2.3.4. Avian Influenza, pp. 436–452.

Wu R, Guan Y, Yang Z, Chen J, Wang H, Chen Q, Sui Z, Fang F, Chen Z (2010): A live bivalent influenza vaccine based on a H9N2 virus strain. Vaccine 28, 673–680. http://dx.doi.org/10.1016/j.vaccine.2009.10.102

Zhang W, Tu J, Zhao Z, Chen H, Jin M (2012): The new temperature-sensitive mutation PA-F35S for developing recombinant avian live attenuated H5N1 influenza vaccine. Virol. J. 23, 97. http://dx.doi.org/10.1186/1743-422X-9-97