Highly immunogenic prime-boost DNA vaccination protects chickens against challenge with homologous and heterologous H5N1 virus

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Abstract

Highly Pathogenic Avian Influenza Viruses (HPAIVs) cause huge economic losses in the poultry industry because of high mortality rate in infected flocks and trade restrictions. Protective antibodies, directed mainly against hemagglutinin (HA), are the primary means of protection against influenza outbreaks. A recombinant DNA vaccine based on the sequence of H5 HA from the H5N1/A/swan/Poland/305-135V08/2006 strain of HPAIV was prepared. Sequence manipulation included deletion of the proteolytic cleavage site to improve protein stability, codon usage optimization to improve translation and stability of RNA in host cells, and cloning into a commercially available vector to enable expression in animal cells. Naked plasmid DNA was complexed with a liposomal carrier and the immunization followed the prime-boost strategy. The immunogenic potential of the DNA vaccine was first proved in broilers in near-to-field conditions resembling a commercial farm. Next, the protective activity of the vaccine was confirmed in SPF layer-type chickens. Experimental infections (challenge experiments) indicated that 100% of vaccinated chickens were protected against H5N1 of the same clade and that 70% of them were protected against H5N1 influenza virus of a different clade. Moreover, the DNA vaccine significantly limited (or even eliminated) transmission of the virus to contact control chickens. Two intramuscular doses of DNA vaccine encoding H5 HA induced a strong protective response in immunized chicken. The effective protection lasted for a minimum 8 weeks after the second dose of the vaccine and was not limited to the homologous H5N1 virus. In addition, the vaccine reduced shedding of the virus.

Keywords: avian influenza, H5N1, chicken, poultry immunization, DNA vaccine

1. Introduction

DNA vaccines are new-generation vaccines offering many advantages over conventional ones (Liu, 2011). They are relatively simple, easy and fast to produce, generate low costs in storage and transport, and are more stable than protein formulations. Numerous data show the effectiveness of experimental DNA immunizations against various viral, bacterial, parasitic and cancer diseases. However, only a few veterinary products have been registered to date in the USA and Canada, and despite several clinical trials, no human DNA vaccine is available (Ferraro et al., 2011; Kutzler and Weiner, 2008). Various experimental DNA vaccines have been tested in poultry (Oshop et al., 2002). The high potential of DNA immunization, particularly in cases requiring a rapid response to an influenza pandemic have led to the development of this technology and increase of report on DNA vaccines for chickens against influenza (Chen et al., 2001; Jiang et al., 2007; Kodihalli et al., 2000; Lee et al., 2006; Lim et al., 2012; Rao et al., 2008; Shan et al., 2011; Suarez and Schultz-Cherry, 2000)

The influenza virion has several structural and non-structural antigens, namely hemagglutinin (HA), neuraminidase (NA), capsid protein (M1), ion channel protein (M2), nucleoprotein (NP) and the components of the viral polymerase PA, PB1 and PB2 (Steinhauer and Skehel, 2002). Although detectable antibody responses are observed against many viral proteins, the major determinants for a protective response are antibodies produced against surface glycoprotein HA, the most prominent antigen of the virus (see the review (Skehel and Wiley, 2000) and references therein). HA is synthesized as a precursor polypeptide H0 and is then cleaved into subunits H1 and H2. The HA cleavage site is the main determinant of the pathogenicity of influenza viruses. In low-pathogenic avian influenza viruses (LPAIVs) the cleavage site can be limited to a single arginine residue recognized by extracellular trypsin-like proteases, while in high-pathogenic viruses (HPAIV) the H0 precursor contains a sequence that can be recognized by proteases present in nearly all cell types, which facilitates systemic spread of the virus (Skehel and Wiley, 2000; Steinhauer and Skehel, 2002).

In the EU, permission to vaccinate poultry against H5N1 HPAI can be granted after the fulfillment of strict requirements laid down in the EU Directive for the Control of AI (Council Directive 2005/94/EC). The Directive is concerned with the high risk of a “silent spread” of the virus due to incomplete protection at a flock level, leading to the impossibility of differentiating the infected from the vaccinated individuals in case of usage of inactivated vaccines. Therefore, considering the needs of the DIVA (Differentiating Infected from Vaccinated Animals) strategy, there is a great demand for new-generation vaccines (Capua,

2007; European_Commission, 2006; Savill et al., 2006). It is strongly recommended by the OIE and the EU that preventive and emergency vaccination should be an additional method of controlling and fighting the virus in case of disease outbreak, by protecting valuable flocks and reducing the spread of virus in restriction and buffer zones.

The H5N1 strain of HPAIV which is the object of our studies and caused the Asian epidemic in 2003 was first identified in domestic gees in China in 1996 (Xu et al., 1999). After several years of spreading and genetic diverging in South Eastern Asia, some strains have crossed the Russian border and reached the Middle East and Europe (Cattoli et al., 2009). Several local outbreaks appeared in almost all European countries, both on poultry farms and among wild birds. In March 2006, the first disease outbreak was reported in Poland in mute swans (Minta et al., 2007; Śmietanka et al., 2008). Despite the high standards of food and animal trade in the EU due to the intense human and animal movement the risk of virus re-emergence is high. In this study the immunization experiments were conducted with common broiler type chicken grown in a biologically secure poultry-house. The duration of the immunization experiments was 6 weeks, because such is the length of broilers' life. Two intramuscular doses of DNA vaccine were sufficient to stimulate the anti-HA response in sera of immunized chickens. The second series of experiments involved challenge with HPAI H5N1 viruses and were conducted in a P3 laboratory using SPF chickens of laying type, which allowed the time of the experiments to be extended to 8-13 weeks in order to test for the long-term protection. The challenge experiments indicated a high protective potential of the tested DNA vaccine. The immunized SPF chickens were protected in 100% against H5N1 virus from a homologous clade (clade 2.2) and in 80% against the H5N1 virus from a heterologous clade (clade 1).

2. Materials and methods

2.1. Plasmids and vaccine design

Based on the predicted amino acid sequence of HA from H5N1 A/swan/Poland/305-135V08/2006 strain of HPAIV (EpiFluDatabase [http://platform.gisaid.org]; Accession No. EPI156789), a synthetic gene optimized to the domestic chicken codon bias and containing deletion of the proteolytic cleavage site (from Arg-341 to Arg-346) was designed (GenBank Accession No. KC172926). Two variants of the DNA vaccine were prepared: (i) long, codon-optimized HA (aa 1-568) with the original N-terminal signal peptide of 16 amino acids (aa 1-16) and a deletion of the proteolytic cleavage site RRRKKR (Δ341-346) and (ii) short, codon-optimized HA, containing only aa 17-340 (only H1 subunit, without signal peptide). The non-optimal codons in the native HA gene sequence were replaced by codons optimized

to chicken codon usage and the sequence was also checked for the absence of cryptic splice sites (commercial service by GenScript USA Inc.). The inserts were cloned into the pCI (Promega) between immediate-early enhancer/promoter from Cytomegalovirus (CMV) and a terminator/polyadenylation signal from SV40. Plasmid DNA was purified using NoEndo JETSTAR Plasmid Kit (Genomed, Germany) and suspended in PBS pH 7.4, and the appropriate amount of DNA (62-250 μg) was mixed with the Lipofectin transfection reagent (Life Technologies, USA) as recommended by the manufacturer. In each trial the same ratio of DNA amount (w):Lipofectin (v), 6:1 was used. The volume of one dose of vaccine was 160 μl.

2.2. Influenza viruses and stock preparation

Table 1 lists the used influenza viruses. The HPAIVs were propagated in the allantoic cavities of embryonated chicken eggs (Valo-Biomedia, Germany) in biosafety level 3 conditions of the National Veterinary Research Institute (Pulawy, Poland) and stored in aliquots at -70°C (for challenge purpose) or inactivated with 0.1% formaldehyde (Sigma–Aldrich, MO, USA) for 2 h at 37°C (for hemagglutination inhibition test). The LPAIVs were either purchased or kindly provided by others. The viral stocks stored at -70°C were titrated before use.

2.3. Immunization and challenge experiments

Broilers (Ross 308) were housed in poultry-house in cages, in standard commercial conditions including temperature, photoperiod, litter and fodder. Five independent immunizations of broilers were conducted. Depending on the experiment, animals (7-15 per group) were immunized subcutaneously in the neck or intramuscularly in the breast muscle with the indicated amount of DNA complexed with Lipofectin. Blood was collected from the wing veins, allowed to coagulate, and centrifuged. The collected sera were kept at -20°C.

Specific pathogen free (SPF) White Leghorn chickens, housed in a biosafety level 3 containment of the National Veterinary Research Institute, Pulawy, were immunized intramuscularly twice (using 1-ml syringe with 0.5x 1.6 mm needle) with the DNA vaccine containing 125 μg of plasmid DNA complexed with Lipofectin. Prior to the challenge, the chickens were placed in separate isolators (Montair Andersen B.V., Holland) equipped with HEPA filters. Three challenge experiments were performed. The immunized chickens (10 birds/group in Experiments 1 and 3, and 5 birds in Experiment 2) as well as control (untreated, fully susceptible chickens, 2-5/group) were inoculated occulonasally with 10^6 50% egg infectious dose (EID_{50}) of the respective virus in the volume of 100 μl (50 μl into the nares and 50 μl into the eye per bird). Approximately 24 h after inoculation, 6-week-old

151 contact SPF chickens (1 or 2 per group) were placed in the same isolators as the vaccinated
152 chickens to monitor virus transmission. Other details are shown in Table 2.

153 **2.4. Ethic statements**

154 The experiments were approved by the Second Local Ethical Committee for Animal
155 Experiments at the Medical University of Warsaw, Permit Number 17/2009 (broilers) or the
156 Second Local Ethical Committee for Animal Experiments at the University of Life Sciences
157 in Lublin, Permit Number 26/2012 (SPF chickens). All efforts were made to minimize
158 suffering. The chickens were monitored twice a day (morning and afternoon), including
159 weekends. The immunized chickens were sacrificed (humanely euthanized by decapitation)
160 about 3 weeks after the final immunization (about 6 weeks after hatching).

161 **2.5. ELISA**

162 The 96-well polystyrene plates (Nunc, Denmark) were coated overnight at 4°C with 300 ng of
163 HA antigen (A/swan/Poland/305-135V08/2006 (H5N1)) produced in a baculovirus system
164 (Oxford Expression Technologies, UK). Bound IgY were detected using goat anti-chicken
165 IgY (Fc-specific)-HRP (Pierce/Thermo Scientific, IL, USA) antibodies. Results were
166 analyzed using the STATISTICA program (StatSoft, Poland).

167 **2.6. Hemagglutinin Inhibition**

168 The HI test was conducted according to the standard procedure ([O.I.E.] World Organization
169 for Animal Health, 2012). Shortly, the collected sera (25 µl of sera in serial two-fold
170 dilutions) were incubated for 25 min in a titration plate with four HA units of the inactivated
171 antigen. Next, a suspension of 1% hen erythrocytes was added and incubated for 30 min. The
172 HI titer was determined as the reciprocal of the highest dilution in which hemagglutination
173 was inhibited. Sera from Trials 1-5 were tested using the heterologous strain H5N2
174 A/chicken/Belgium/150/1999 (GD Deventer, Netherlands). Sera from the Challenge
175 Experiments 1-3 were tested with a much broader range of antigens (see Table 1).

176 **2.7. Determination of viral titers**

177 The level of viral RNA in samples from challenged chickens was assayed by quantitative real
178 time RT-PCR/M using RNA isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany)
179 as described (Spackman et al., 2002). Oligonucleotides M-25 (5'-AGATGAGTCTTCTAA
180 CCGAGGTCG-3') and M-124 (5'-TGCAAAAACATC TTCAAGTCTCT-3') were used as
181 primers and M-64 (5'-FAM-TCAGGCCCCCTC AAAGCCGA-TAMRA-3') served as a
182 probe. Quantitative standards of RNA extracted from 10-fold dilutions of a titrated virus
183 homologous to the challenge strain were used to convert and express the QRT-PCR Ct values
184 as equivalent EID_{50} ($eqEID_{50}$) per milliliter of swab fluid from oropharyngea and cloaca.

3. Results

3.1. Immune responses of chickens to DNA vaccines

Five independent immunization experiments with slightly different time schedule of priming, boosting and blood collection were conducted in experimental farm conditions (Table 2, Figure 1). The duration of each experiment did not exceed 6 weeks, the life span of broilers. In the initial experiment (Trial 1) we focused on the evaluation of the immunogenic potential of two variants (short and long) of the DNA vaccine. The plasmid containing the long sequence appeared to be better vaccine candidate than the one with the short (H1) sequence (Figure 1A), therefore we concentrated only on the long DNA vaccine in further experiments. The minimal amount of plasmid DNA inducing satisfactory response by subcutaneous route was assessed in the next experiment (Trial 2) as 125 μg (Figure 1B). When two high doses of 250 μg DNA were administered without Lipofectin the anti-HA response was significantly lower than the response to two doses of 250 μg or two doses of 125 μg administered with the carrier.

Subsequent trials (Trials 3-5) (i) allowed us to choose the intramuscular route as superior to the subcutaneous, (ii) confirmed that the intramuscularly injected dose of 125 μg of the long variant of the DNA vaccine is sufficient to induce anti-HA humoral response in 100% of chicken, and (iii) demonstrated that two doses of the vaccine are necessary to induce antibodies capable of hemagglutination inhibition (Figure 1C). The results of all trials consistently indicated that two weeks after priming (just before boosting) anti-HA antibodies could be detected in sera. However, the second dose of the vaccine (boosting) resulted in a strong increase of anti-HA antibody titers in most groups (as detected 3 weeks after the boost) and in increase of hemagglutinin inhibition (HI) activity (Figure 1 and Supplementary Figure 1). Generally, irrespective of the experimental group, no HI activity was detected in sera with low titers of anti-HA antibodies.

3.2. Dynamics of serological response

The previously optimized immunization schedule (intramuscular route, 125-μg dose, prime/boost on days 7/21) was applied to SPF chickens. Then, on day 42 (Experiment 1 and 3) or 77 (Experiment 2) chickens were challenged with either H5N1 HPAIV from clade 2.2 (Experiments 1-2) or H5N1 HPAIV from clade 1 (Experiment 3). The HI response in sera of immunized and challenged chickens is shown in Figure 2 and Supplementary Figure 2. Consistently, the best HI response was against an antigen homologous to the one encoded by the DNA vaccine. At 3 weeks post booster (wpb) (challenge day in Experiment 1 and Experiment 3) all birds were HI-positive with the following values of Geometric Mean Titers

(GMT): 169 (Experiment 1), 97 (Experiment 2) and 111 (Experiment 3). However, only 60-80% of birds tested on the challenge day (3 wpb or 8 wpb) were found positive with antigen from heterologous and antigenically distant clade and the percentage of seropositive chickens never reached 100%. The strong elevation of HI antibody was always observed 2 weeks after challenge.

3.3. Protection against challenge and viral shedding

The survival ratio after the challenge is shown in Figure 3, while the amount of viral RNA in swabs collected from the immunized/infected and the contact groups is summarized in Table 3. All control chickens (non-immunized, challenged) died by 3 dpi and large amounts of the virus (usually > 5 $\log_{10}$ EID_{50} per ml swab fluid) were found. In Experiment 1 (challenge with the homologous virus 3 weeks after the boosting), no clinical signs or mortality were observed in immunized chickens or contact birds (Figure 3A). The small amount of the virus (up to 3 $\log_{10}$ EID_{50}) was detected in oropharyngeal swabs of single birds tested at 3 dpi and 14 dpi. This seems, however, to be probably below the infecting dose, which is reflected in the survival of the contact birds. In Experiment 2 (challenge with the homologous virus 8 weeks after boosting), the vaccinated and challenged chickens remained clinically healthy, but the contact bird developed clinical symptoms and died 7 days after the vaccinated birds had been challenged (Figure 3B). The virus was found at 3 dpi in the oropharyngeal swabs collected from 2 immunized birds (amount of RNA equivalent to 3.3 $\log_{10}$ EID_{50}) while in dead contact chicken a the viral RNA was present both in respiratory and digestive tracts (quantity corresponding to 6.2 and 4.6 $\log_{10}$ $EID_{50,}$ respectively). Regarding Experiment 3 (challenge with the virus from the heterologous clade 3 weeks after boosting), three immunized and infected birds died on days 5, 10 and 13, but viral RNA was never detected in swabs taken from the immunized chicken found dead at 13 dpi. Both contact chickens were found dead 2 days after they had been placed in the isolator with the challenged birds (Figure 3C). The vaccinated and challenged chickens shed the virus up to 10 dpi with the peak at 3 dpi (seven birds, amount of RNA equivalent to 2.4-5.8 $\log_{10}$ EID_{50}, see Table 3).

4. Discussion

This work focused on testing the DNA vaccine effective in chickens that can be used in case of influenza outbreak. Comparison of two immunization routes resulted in choosing the intramuscular injection as better and more reliable than the subcutaneous one (Figure 1C). This route of injection was also more convenient for the personnel and, in our evaluation,

better tolerated by birds. In other reports this route was also often used for DNA immunizations with HA-encoding plasmids (Jalilian et al., 2010; Jiang et al., 2007; Lim et al., 2012; Oveissi et al., 2010) and gave good results; however, other, more sophisticated methods like gene gun (Kodihalli et al., 2000), electroporation (Shan et al., 2011) or needle free jet injector (Rao et al., 2008) were sometimes found more effective. We have also established the minimal dose inducing the satisfactory response as 125 μg. In other works a very broad range of doses was tested and showed to be effective. Usually around 10-200 μg ensured high or average level of response and protection (Jalilian et al., 2010; Jiang et al., 2007; Kodihalli et al., 2000; Lim et al., 2012; Oveissi et al., 2010; Rao et al., 2008; Shan et al., 2011).

The reported here immunization protocol, initially optimized in broilers in experimental farm conditions, was used in three challenge experiments with H5N1 HPAIVs using SPF White Leghorn chickens kept in a biosafety level 3 laboratory. Two HPAIV strains were used for challenge. The first was A/turkey/Poland/35/07 from clade 2.2 that reached Europe at the end of the year 2005 and circulated for several months in European countries causing small local outbreaks. The second was A/crested eagle/Belgium/01/04 from the distinct clade 1 which was circulating in southern Asia for several years and was once found at the Brussels airport in smuggled birds (Van Borm et al., 2005). The mortality rate of the challenged animals was monitored, virus shedding and transmission were tested, and the dynamics of serological response (HI test) followed. All immunized birds were protected against the homologous virus used in challenge Experiments 1 and 2. Transmission of the virus was absent in the challenge Experiment 1 (infection at 3 wpb), while it was observed in Experiment 2 (infection at 8 wpb). As a result, a control contact chicken died 7 days after inoculation. It should be pointed out that shedding of the virus was mild and infection in the contact chicken developed with a delay of several days. Of the ten chickens vaccinated in Experiment 3 (challenge with heterologous H5N1 HPAIV) two died and had moderate to high levels of viral RNA in oropharyngeal and cloacal swabs, as well as in the lung, brain, kidney and spleen samples. The third chicken died also at the last day of observation. However, it was clinically healthy throughout the experiment and no virus RNA was detected at any time; therefore, we assumed that the reason of its death could be other than viral infection. The two contact chickens also died, proving transmission of the virus from vaccinated chickens. However, in order to get statistically significant results concerning virus transmission from the vaccinated to not-vaccinated birds it would be necessary to use a larger group of contact birds.

The H5N1 virus is highly variable and the existence of multiple antigenic and genetic variants makes the generation of a universal vaccine difficult or even impossible. A partial

solution to the problem may be a “multi-clade” DNA vaccine which should potentially protect against a broader spectrum of viruses (Zhou et al., 2012). Vaccination against avian influenza is one of the tools in the control of the disease and generation of a vaccine that elicits even partial protection can greatly help limit the spread of infections in susceptible populations. The reported DNA vaccine provides 100 and 70% protection against, respectively, a homologous and heterologous virus.and fulfills the criteria established by the World Organization for Animal Health for an efficient AI vaccine (Swayne, 2012). The major advantage of this vaccine is that it can be used as a part of a DIVA strategy since the antibodies are produced exclusively against viral hemagglutinin. Although it still needs to be optimized in order to reduce its cost, optimize the immunization schedule and/or administration route, we believe that the similar DNA vaccines can be used in future as part of prophylactic, preventive or emergency strategies in the protection of valuable flocks against H5N1.

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399 **Table 1**. Influenza viruses used in this study

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Experiment	Antigen	Clade	Source
DNA vaccine	H5N1 A/swan/Poland/305-135V08/2006	Clade 2.2	Department of Poultry Diseases, National Veterinary Research Institute, Pulawy, Poland
HI tests in Trials 1-5 (broilers)	H5N2 A/chicken/Belgium/150/1999	-	GD Deventer, Netherlands
Challenge Experiment 1 and 2	H5N1 A/turkey/Poland/35/07	Clade 2.2	Department of Poultry Diseases, National Veterinary Research Institute, Pulawy, Poland
Challenge Experiment 3	H5N1 A/crested eagle/Belgium/01/2004	Clade 1	Dr. T. van den Berg (CODA-CERVA, Brussels, Belgium)
HI tests in Experiments 1-3 (SPF chickens)	H5N1 A/Ck/Scotland/59 Inactivated Antigen (Sc)	Eurasian group	Animal Health and Veterinary Laboratories Agency, Waybridge, UK
	H5N1 A/turkey/Poland/35/07 (Po)	Clade 2.2	Department of Poultry Diseases, National Veterinary Research Institute, Pulawy, Poland
	H5N1 A/crested eagle/Belgium/01/2004 (Be)	Clade 1	Dr. T. van den Berg (CODA-CERVA, Brussels, Belgium)

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403 **Table 2**. Design of immunization and challenge experiments

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Experiment / route		Group size			Respective treatment day after hatching		
		Imm	Cntr	Cnct	**Prime/Boost**	**Blood**	**Challenge**
Immunization (broilers)	Trial 1 / sc	14	10	n/a	10/22	21/42	n/a
	Trial 2 / sc	28	14	n/a	5/19	18/40	n/a
	Trial 3 / sc, im	30	10	n/a	6/21	20/42	n/a
	Trial 4 / im	24	5	n/a	7/21	20/38	n/a
	Trial 5 / im	24	3	n/a	7/21	20/42	n/a
Challenge (SPF)	Exp 1 / im	10	3	2	7/21	21/35/42/56	42
	Exp 2 / im	5	2	1	7/21	21/35/42/63/ 70/77	77
	Exp 3 / im	10	5	2	7/21	21/35/42/56	42

405

406 Routes of immunization (sc - subcutaneous; im - intramuscular). Imm - immunized; Cntr -
407 control (non-immunized); Cnct – contact; Prime - first immunization; Boost - second
408 immunization; Blood - blood collection; Challenge - experimental infection with H5N1 virus;
409 n/a – non-applicable. For all experiments with experimental infection (challenge) SPF
410 (specific pathogen free) chickens were used.
411

Table 3. Viral RNA detection after challenging of vaccinated chickens

		Days post infection (d.p.i.) 3		7		10		14	
		O (+/t)	**C (+/t)**	**O (+/t)**	**C (+/t)**	**O (+/t)**	**C (+/t)**	**O (+/t)**	**C (+/t)**
Exp 1	Immun	1/10 (3log$_{10}$ EID$_{50}$)	0/10	0/10	0/10	0/10	0/10	1/10 (<2 log$_{10}$ EID$_{50}$)	0/10
	Contact	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
Exp 2	Immun	2/5 (<2-3.3log$_{10}$ EID$_{50}$)	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	Contact	0/1	0/1	1/1 (6.2 log$_{10}$ EID$_{50}$)	1/1 (4.6log$_{10}$ EID$_{50}$)	-[a]	-	-	-
Exp 3	Immun	7/10 (2.4-5.8log$_{10}$ EID$_{50}$)	0/10	3/9[b] (3.0-5.3log$_{10}$ EID$_{50}$)	1/9 (3.3log$_{10}$ EID$_{50}$)	0/9	1/9 (<2 log$_{10}$ EID$_{50}$)	0/7[c]	0/7
	Contact	2/2 (5.3-6.9log$_{10}$ EID$_{50}$)	2/2 (6.1-7.0log$_{10}$ EID$_{50}$)	-[d]	-	-	-	-	-

Results are shown for immunized (Immun) and contact chickens. Results for corresponding control groups (non-immunized and infected) are not shown. These chickens always died by 3 dpi and had large amounts of the virus (usually > 5 log$_{10}$ EID$_{50}$ per ml of swab). The number of virus-positive (+) and of all individuals (total, t) in each group is provided. The amount of viral RNA in shedding chickens is shown in brackets. O – oropharyngeal swabs, C – cloacal swabs; [a] bird died on 7 dpi, [b] bird died on 5 dpi, [c] one bird died on 10 dpi and one bird died on 13 dpi, [d] birds died on 3 and 4 dpi.

Experimental infection (challenge) was performed on 42nd (Exp 1 and Exp 3) and on 77th day after hatching (Exp 2) with either virus from the homologous clade (Exp 1 and Exp 2) or virus from a heterologous clade (Exp 3). Results obtained with the H5N1 virus from the clade homologous to the one used for preparing the DNA vaccine are highlighted.

Figure Legends

Figure 1. Chicken immune responses to DNA vaccine. Anti-HA Ab measured by ELISA (A, B and C) and HI of corresponding sera (C) are shown for individual chickens in each group. The number (n) of chicken per group is indicated; sc, subcutaneous; im, intramuscular; buffer - chickens received two doses of buffer; vector – chickens received empty vector; (-)carrier – chickens received DNA without lipid carrier; doses of DNA (250, 125 or 62 μg) are specified; chickens were immunized twice unless indicated otherwise, where 1x denotes the group which received only the first dose. In Trial 1 (A) either the short or long variant of the vaccine was used, while in Trials 2 (B) and Trials 3-5 (C) only the long variant was used.

Figure 2. Dynamics of HI titer in immunized and infected groups of chickens. Chickens in Experiments 1 and 2 were challenged with H5N1 from clade 2.2 (homologous to the vaccine), while in Experiment 3 with H5N1 from heterologous clade 1. Challenge was on day 42 in Experiments 1 and 3 and on day 77 in Experiment 2. Chickens from the control (non-immunized) groups had no detectable serological response to any of the antigens tested and they are not shown. Annotations on horizontal axes refer to the type of virus used for HI test (Sc - A/Ck/Scot/59; Po - A/turkey/Poland/35/07; Be - A/crested eagle/Belgium/01/2004) and to the day of blood collection.

Figure 3. Results of challenge experiments. (A) Experiment 1 – infection with homologous HPAIV three weeks post boost (wpb). (B) Experiment 2 – infection with homologous HPAIV eight wpb. (C) Experiment 3 – infection with heterologous HPAIV three wpb. The data are shown as percentage survival in respective groups (Cntr – control chickens, Cnct – contact chickens, Imm – immunized chickens).

Supplementary Figure 1. Statistical analysis of chicken immune responses to DNA vaccine. The values for individual chickens and other explanations are as in Figure 1.

Supplementary Figure 2. Dynamics of HI titer in individual immunized and infected chickens. Chickens in Experiments 1 and 2 were challenged with H5N1 from clade 2.2 (homologous to the vaccine), while in Experiment 3 with H5N1 from heterologous clade 1. Challenge was on day 42 in Experiments 1 and 3 and on day 77 in Experiment 2. Numbers on horizontal axes indicate individual chickens. Only data for chickens from immunized groups are shown. Chickens from the control (non-immunized) groups had no detectable serological response to any of the antigens tested. Asterisks indicate that the test was not

performed. Three birds from the immunized and challenged group in Experiment 3 died on days 5, 10 and 13 (birds No. 24, 23 and 25, respectively).

Fig.1

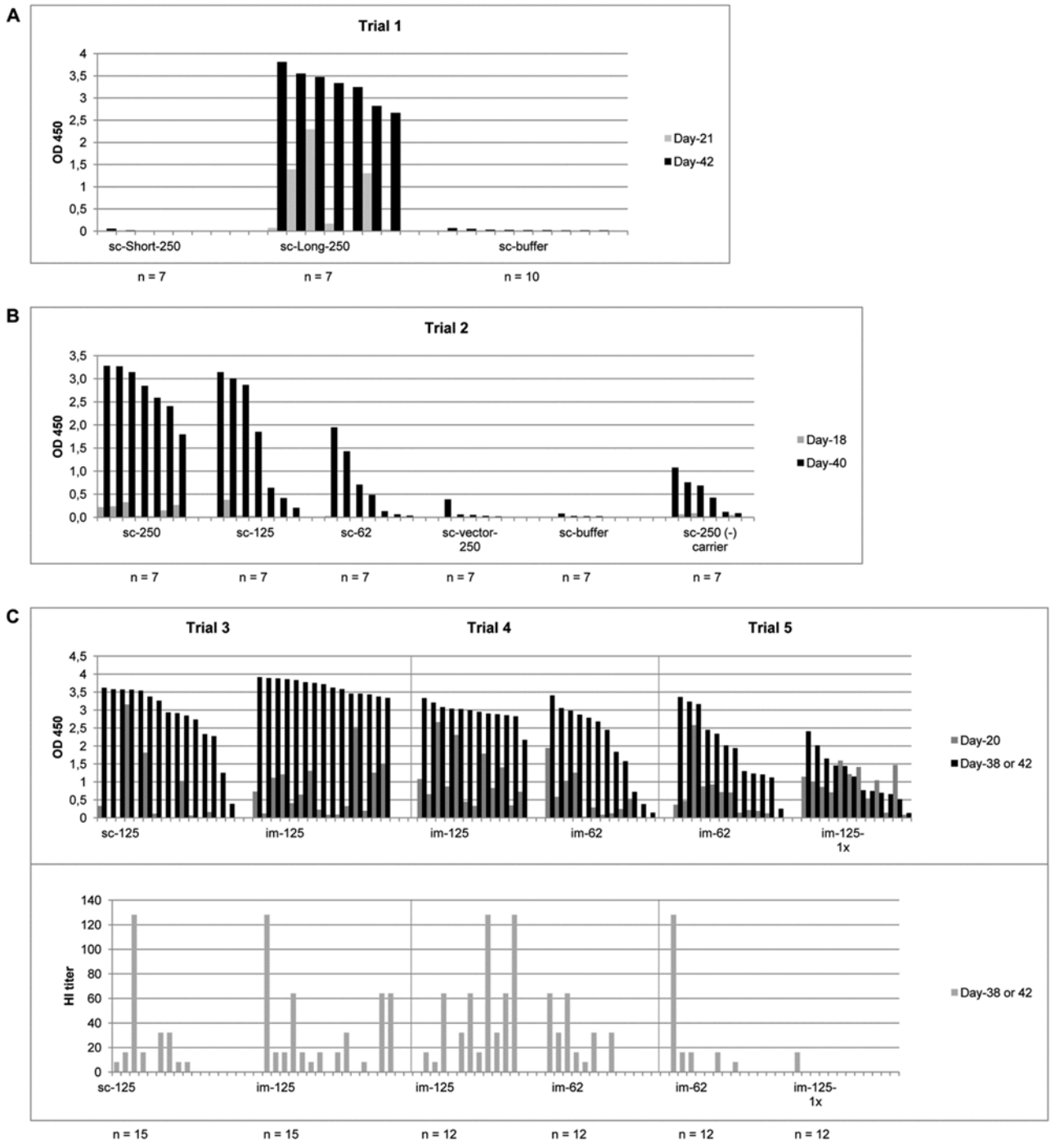


Fig.2

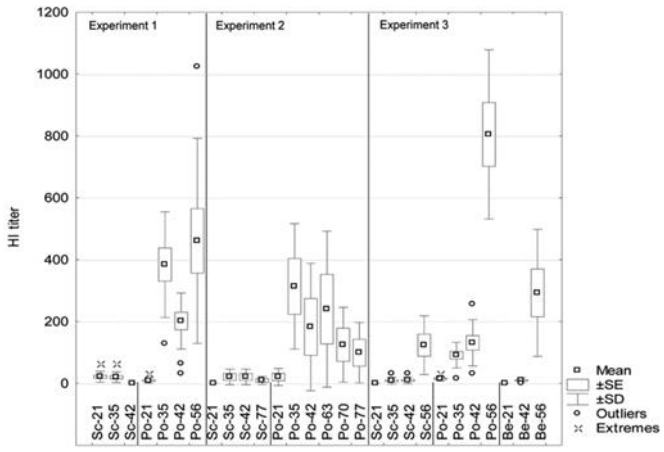
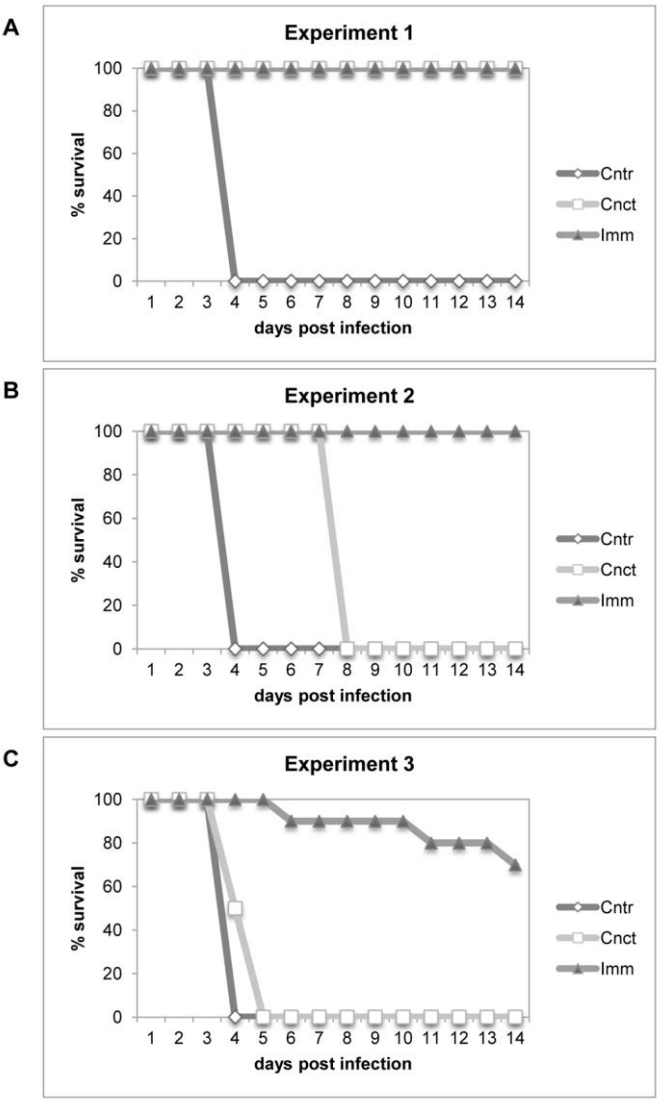
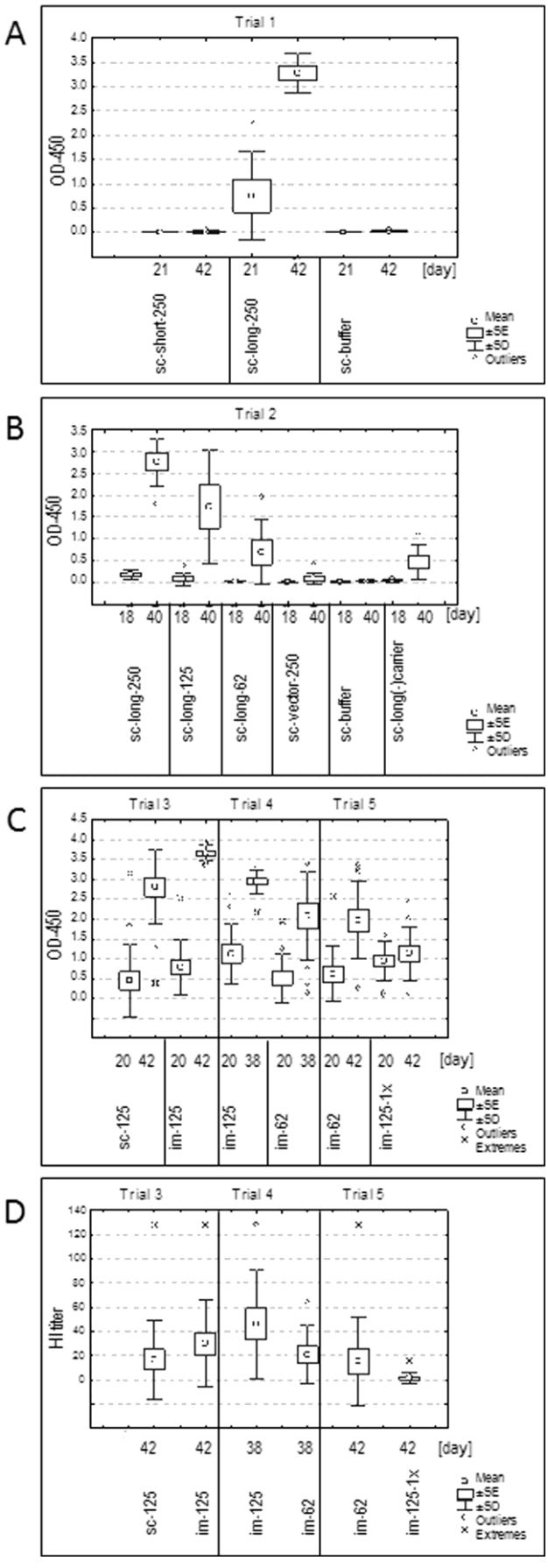

Experiment 1
Experiment 2
Experiment 3
HI titer
1200
1000
800
600
400
200
0
Sc-21
Sc-35
Sc-42
Po-21
Po-35
Po-42
Po-56
Sc-21
Sc-35
Sc-42
Sc-77
Po-21
Po-35
Po-42
Po-63
Po-70
Po-77
Sc-21
Sc-35
Sc-42
Sc-56
Po-21
Po-35
Po-42
Po-56
Be-21
Be-42
Be-56
Mean
±SE
±SD
Outliers
Extremes

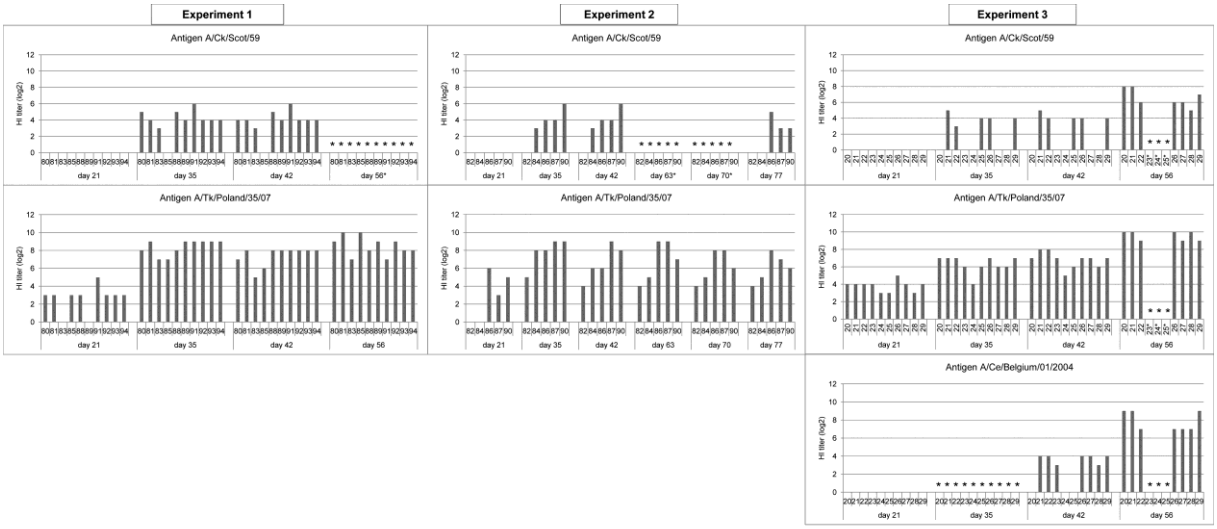
Fig.3


A
Experiment 1
% survival
days post infection
Cntr
Cnct
Imm
B
Experiment 2
% survival
days post infection
Cntr
Cnct
Imm
C
Experiment 3
% survival
days post infection
Cntr
Cnct
Imm

Suppl fig.1


A
Trial 1
OD-450
[day]
sc-short-250
sc-long-250
sc-buffer
Mean
±SE
±SD
Outliers
B
Trial 2
OD-450
[day]
sc-long-250
sc-long-125
sc-long-62
sc-vector-250
sc-buffer
sc-longL-jcarrier
Mean
±SE
±SD
Outliers
C
Trial 3
Trial 4
Trial 5
OD-450
[day]
sc-125
im-125
im-125
im-62
im-62
im-125-1x
Mean
±SE
±SD
Outliers
Extremes
D
Trial 3
Trial 4
Trial 5
HI titer
[day]
sc-125
im-125
im-125
im-62
im-62
im-125-1x
Mean
±SE
±SD
Outliers
Extremes

Suppl. Fig 2


Experiment 1
Experiment 2
Experiment 3
Antigen A/Ck/Scot/59
Antigen A/Tk/Poland/35/07
Antigen A/Ce/Belgium/01/2004
HI titer (log2)
day 21
day 35
day 42
day 56*
day 63*
day 70*
day 77