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Protective efficacy of a prime-boost protocol using H5-DNA plasmid as prime and inactivated H5N2 vaccine as the booster against the Egyptian avian influenza challenge virus

H. A. HUSSEIN[1], B. M. AHMED[1], S. M. ALY[2], A. H. EL-DEEB[1], A. A. EL-SANOUSI[1], M. A. ROHAIM[1], A. A. ARAFA[3], M. R. GADALLA[1]

[1]Department of Virology, Faculty of Veterinary Medicine, Cairo University, Giza 12211, Egypt; [2]Department of Immunology, Animal Health Research Institute, Dokki 12618; Egypt; [3]Reference Laboratory for Veterinary Quality Control on Poultry Production (RLQP), Animal Health Research Institute, Dokki 12618, Egypt

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Summary. – In this study, a recombinant DNA plasmid was constructed, encoding for *HA1* of a selected Egyptian H5N1 virus (isolated during the 2012 outbreaks). In the immunization and challenge experiments, SPF chickens received 1 or 2 doses of H5-DNA plasmid prime, and boosted with the inactivated H5N2 vaccine. Haemagglutination inhibition (HI) titers, protection levels, and the magnitude of virus shedding were compared to that of the chickens that received either DNA plasmid or inactivated H5N2 vaccine alone. H5N1 virus A/chicken/Egypt/128s/2012 (H5N1) highly pathogenic avian influenza (HPAI) clade 2.2.1/C was used for the challenge. Chickens immunized with 1 or 2 doses of H5-DNA vaccine failed to overcome the challenge with 0% and 10% protection, respectively. Quantitative real-time reverse transcription-PCR revealed virus shedding of 2.2 x 10^4 PCR copies/ml 3 days post challenge (dpc) in the only surviving bird from the group that received 2 doses of plasmid. However, chickens immunized with 1 or 2 doses of H5-DNA plasmid as prime and inactivated H5N2 vaccine as booster, showed 80% protection after challenge, with a viral shedding of 1.2 x 10^4 PCR copies/ml (1 dose) and 1.6 x 10^4 PCR copies/ml (2 doses) 3 dpc. The surviving birds in both groups did not shed the virus at 5 and 7 dpc. In H5N2-vaccinated chickens, protection levels were 70% with relatively high virus shedding (1.8 x 10^4 PCR copies/ml) 3 dpc. HI titers were protective to the surviving chickens. This study reports the efficacy of H5-DNA plasmid to augment reduction in viral shedding and to provide better protection when applied in a prime-boost program with the inactivated AI vaccine.

Keywords: avian H5N1 influenza; inactivated H5N2 vaccine; DNA plasmid; hemagglutinin; prime-boost

Introduction

Avian influenza virus (AIV) H5N1 (the genus *Influenza virus A*, the family *Orthomyxoviridae*) contains a segmented single-stranded negative sense RNA genome, with an envelope that harbors 2 major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). The viral genome encodes at least 10 proteins (Webster *et al*., 1992). Disease caused by the highly pathogenic avian influenza (HPAI) H5N1 virus has been endemic in Egypt since 2008, after the first cases were reported in 2006 (Aly *et al*., 2008). The disease has caused massive economic losses in the poultry industry, directly by ravaging the poultry population and indirectly by forcing approximately 1.5 million people working in this industry to lose their main source of livelihood (Abdelwhab *et al*., 2010). Moreover, HPAI H5N1 viruses are a public health concern due to the annual human fatalities

Email: husvirol@cu.edu.eg; phone: +201002159364.

Abbreviations: AI(V) = avian influenza (virus); dpc = days post challenge; EID_{50} = egg infective dose (50%); HA1 = hemagglutinin one (the part representing influenza hemagglutinin globular head); HI = hemagglutination inhibition; HPAI = highly pathogenic avian influenza; qRT-PCR = quantitative real-time reverse transcription-polymerase chain reaction; SPF = specific pathogen free

caused by these viruses (Fasina *et al.*, 2010). Thus, there is an urgent need to develop novel, safe, and potent influenza vaccines given the rapid global spread of influenza viruses in poultry and human populations.

Several vaccine strategies have been considered for the development of potential vaccines against H5N1 influenza viruses. All the approaches adopted so far have made provisions for obtaining vaccine strains that can be used without the risk of transmission to birds or to humans during vaccine manufacturing. These strategies include (1) the use of a related low pathogenic (LP) avian influenza strain (Chen and Bu, 2009), (2) Preparation of purified recombinant hemagglutinin protein and virus-like particles (Treanor *et al.*, 2001; Tao *et al.*, 2009; Gadalla *et al.*, 2014), (3) Reverse genetics-based vaccines (Subbarao and Katz, 2004), (4) DNA vaccines (Rao *et al.*, 2008) and, (5) Recombinant viral-vectored vaccines (Tripp and Tompkins, 2014). Current AIV vaccines rely on a highly time-consuming production process of growing the viruses in eggs or tissue culture. There are ongoing efforts worldwide for finding alternatives to produce influenza vaccines in systems other than eggs or tissue culture cells, and to produce a universal vaccine that doesn't require annual reformulation (Chen *et al.*, 2014).

DNA vaccination is an alternative approach that involves cloning the gene of interest into a bacterial plasmid under the control of a mammalian promoter, which drives intracellular protein expression following intramuscular or intradermal injection (Gurunathan *et al.*, 2000; Rajcani *et al.*, 2005). DNA vaccination offers many potential advantages, including lower costs as plasmid DNA can be generated very inexpensively in a large scale from *Escherichia coli*-based culture. Following purification, plasmid DNA can be stably stored without a cold chain, reducing the cost of vaccine distribution, which is of particular concern in developing countries (Steel *et al.*, 2010). Another advantage is the ability of recombinant DNA vaccines to induce both humoral and cell mediated immune responses via the intracellular expression of viral antigens, in a manner resembling natural infection, without the risk of disease (Khan, 2013).

Hemagglutinin is the key influenza antigen encoded by genome segment 4 and is synthesized as a precursor, HA0, which then undergoes cleavage into subunits HA1 and HA2. These subunits trimerize [3(HA1-HA2)] to give rise to the active form of hemagglutinin (Wiley and Skehel, 1987). In this active form, only HA1 is exposed and loaded with immunogenic epitopes (Chiu *et al.*, 2009). HA gene-based DNA vaccines are an attractive option for several reasons. HA is the major surface immunogen of influenza A virus and antibodies against it are able to neutralize viral infectivity through the interference with viral binding or endosomal fusion (Brown *et al.*, 1992). These vaccines can induce both humoral and cellular immune responses to influenza viruses in different species, including chickens, swine, mice, and ferrets (Robinson *et al.*, 1993; Larsen and Olsen, 2002). Moreover, HA gene-based DNA vaccination does not generate antibodies to nucleoprotein (NP), which are often used for avian influenza serological surveillance, and allows for DIVA (Differentiating Infected from Vaccinated Animals) strategy (Swayne, 2003). These advantages make HA-DNA vaccines potentially a very effective means for rapid control of avian influenza. The present study evaluated the protective efficacy of pCDNA3.1™ plasmid containing HA1 gene sequence of Egyptian H5N1 AIV (pcDNA3.1D/HA1) against a challenge of H5N1 strain A/chicken/Egypt/128s/2012(H5N1) HPAI sub-clade 2.2.1/C. In addition, the ability of pcDNA3.1D/HA1 plasmid to reduce virus shedding was investigated in a prime-boost vaccination protocol with inactivated H5N2 vaccine.

Materials and Methods

Vaccine. Volvac® AI KV Inactivated low pathogenic avian influenza (LPAI) H5N2 oil emulsion Vaccine Mexican strain virus A/chicken/Mexico/232/94 (H5N2) was used to immunize chicken either alone or as booster following DNA plasmid prime.

Viruses and cells. Egyptian HPAI virus used in this study: A/chicken/Egypt/VRLCU/2012 (H5N1) with GenBank Acc. No. KC625532.1, isolated and characterized by Hussein and colleagues (Hussein *et al.*, 2015) was used for HA cloning into the pcDNA3.1 vector, and A/chicken/Egypt/128s/2012 (H5N1) with GenBank Acc. No. JQ858485.1, isolated and characterized by Reference Laboratory for Quality Control on Poultry Production (RLQP), Animal Health Research Institute (AHRI), Egypt (Arafa *et al.*, 2012) was used for infection of chickens (challenge). Vero cell line (ATCC® CCL-81™) was grown and maintained in monolayer cultures in calcium- and magnesium-free Eagle's Minimal essential media (Sigma-Aldrich, USA) supplemented with 10% FCS without antibiotics.

Oligonucleotide primers and RT-PCR assay. Viral RNA was extracted using TRIZOL reagent (Invitrogen, San Diego, CA) according to the manufacturer's protocol. One-step RT-PCR amplification of the HA1 gene fragment was carried out using VERSO one-step RT-PCR kit (Thermo Scientific, UK) with forward primer H5DF 5'-CACCATGGAGAAAATAGTGCTTCTTC-3' and reverse primer HA1R 5'-TTAAGAGCTATTTCTGAGCCCAG-3', to amplify 975bp fragment of the HA gene, which included the globular HA immunogenic epitopes but excluded the polybasic cleavage site. Briefly, 50 µl reaction was set up in a thin-walled PCR tube containing 5 µl RNA, 2 µl each of 15 pmol oligonucleotide primers, 25 µl 2x master mix, 2 µl enzyme mix, 2.5 µl RT enhancer and 11.5 µl nuclease-free water. The reaction mix was incubated at 50°C for 15 min followed by 3 min at 95°C. The PCR conditions were as follows: 95°C for 30 sec (denaturation); 56°C for 30 sec (annealing); 72°C for 1 min (extension); 72°C for 10 min. (final extension). The PCR was run for 35 amplification cycles. The amplification product was run on 1.2% agarose gel and the correct fragment

visualized and compared with the help of 100 bp DNA marker (Vivantis, Malaysia).

Construction of the recombinant DNA plasmid (pcDNA3.1D/HA1). The amplified HA1 sequence was cloned into pCR™II-TOPO® Vector (Invitrogen, San Diego, CA) and then sub-cloned into pcDNA3.1D/V5-His-TOPO® mammalian expression vector with the aid of Platinum® *Pfx* DNA polymerase. The recombinant expression vector was then transformed in One Shot® TOP10 chemically competent *Escherichia coli* cells (Invitrogen, San Diego, CA). All procedures were conducted according to the manufacturer's instructions. Positive colonies containing the recombinant plasmid were identified by colony PCR. Briefly, 25 µl volume PCR reaction was set up using Go-Taq green 2X master mix (Promega, USA) containing 12.5 µl PCR mix, 1 µl each of 15 pmol H5DF and HA1R primers, 2 µl extracted DNA and 8.5 µl water. PCR cycle conditions were as mentioned above. To check for correct orientation of cloned HA1 gene fragment, PCR was performed using H5DF primer and vector reverse BGH primer 5'-TAGAAGGCACAGTCGAGG-3'. Sequencing of the amplified fragment was carried out using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster city, CA).

Indirect immunofluorescence to detect the expressed protein. Small-scale plasmid preparation (4 µg/µl) was prepared and used to transfect Vero cell line using Lipofectamine 2000™ transfection reagent (Invitrogen, USA) according to the manufacturer's instructions. Forty-eight hr post-transfection, Vero cells were fixed with absolute methanol for 20 min at 4°C. Fixed cells were then incubated with 2% bovine serum albumin for 30 min at 37°C followed by incubation with reference H5N1 AIV chicken antiserum (Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, China) at a dilution of 1/50 in PBS for 1 hour at 37°C. After 3 washes with PBS of 5 min each, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-chicken IgG antibody (Sigma, USA) for 1 hr at 37°C followed by 3 successive washes with PBS of 5 min each. Following washing, the cells were covered by one drop of mounting buffer (50% glycerol in PBS) before being examined under fluorescence microscope (Olympus IX70).

Challenge experiments. Large-scale cultures (4 x 0.5 liter) of *E. coli* containing the recombinant plasmid (pcDNA3.1D/HA1) were prepared and plasmid isolation was performed using Qiagen Maxi-prep extraction kit (Qiagen, GMBH). Immunization and challenge experiments were conducted in biosafety level 3 (BSL-3) chicken isolators at the Reference Laboratory for Veterinary Quality Control on Poultry Production (RLQP), Agriculture Research Center (ARC), Giza, Egypt. Seventy of 3-weeks old SPF chickens were divided into groups (n = 10) that were kept and reared in BSL-3 chicken isolators and were monitored daily for 38 days. Chicken groups were immunized with single or double dose of 100 µg of the purified plasmid DNA (500 µl) by direct intramuscular injection in thigh muscle. In addition, a prime-boost strategy was employed using the purified recombinant DNA plasmid (pcDNA3.1D/HA1) as prime, followed by boosting with inactivated H5N2 AI vaccine 10 days later. Chicken groups were challenged with 10^6 EID_{50}/bird 3 weeks after the 2nd immunization. Detailed experimental protocol is presented in Fig. 1. Protection % of the challenged chicken groups was calculated. Sera from vaccinated birds were tested for anti-HA antibodies with the haemagglutination inhibition test (OIE, 2009). Quantitative real-time reverse transcription-PCR (qRT-PCR) was performed on tracheal swabs to determine virus shedding levels from different chicken groups at 3, 5 and 7 days post-challenge, and the virus titer was calculated as genome equivalents. Animal experimental protocols were in accordance with the Animal Health Research Institute regulations and were approved by its scientific and ethics committee.

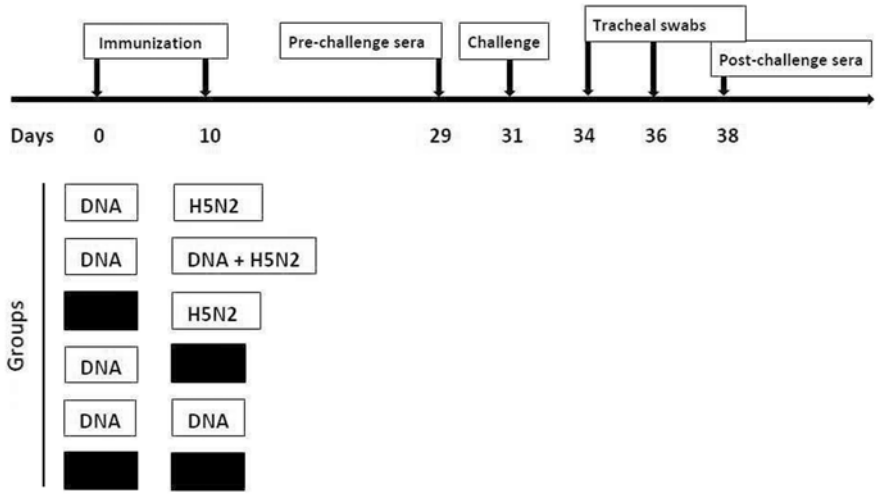

Fig. 1

Time schedule for SPF chicks' immunization and challenge

Six groups (n = 10) were immunized. Black boxes indicate no vaccination. Group 6 was the non-vaccinated challenge (positive control) group. Additional non-vaccinated non-challenged group was also included as negative control. Tracheal swabs were collected 3, 5, and 7 days post-challenge.

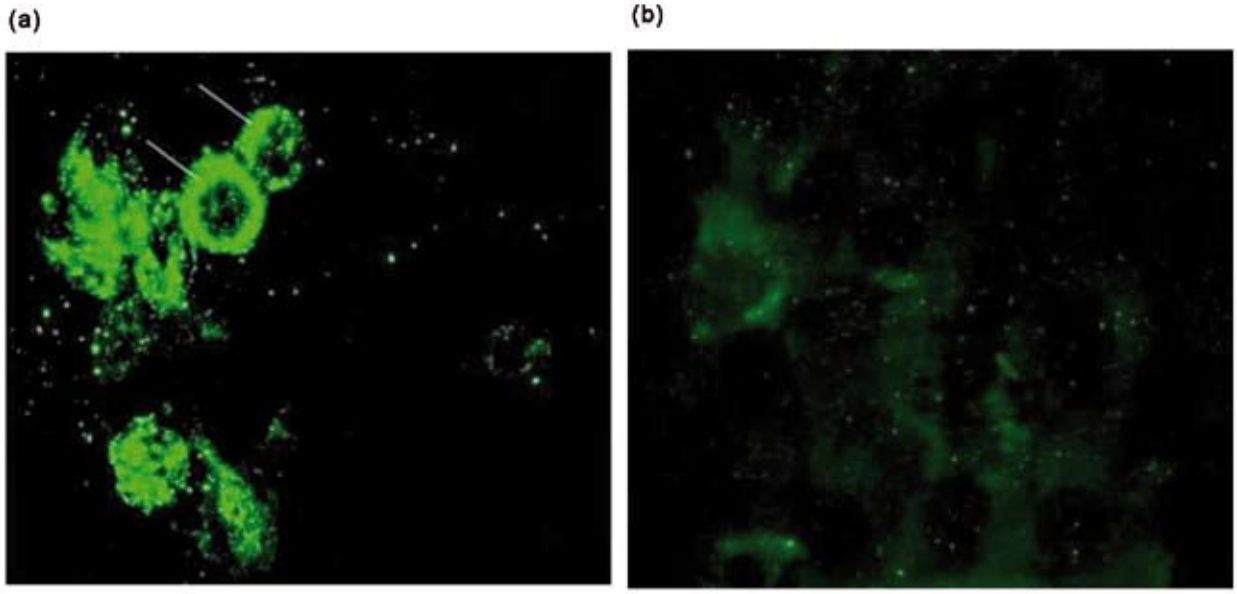

Fig. 2

Indirect immunofluorescence assay of transfected Vero cells

(a) Transfected Vero cells with the recombinant pcDNA3.1D/HA1 showing positive cytoplasmic fluorescence specific for HA1 protein. (b) Cells mock-infected with PBS. Cells were stained using FITC-labeled rabbit anti-chicken antibodies (Sigma-Aldrich) after treatment with anti-H5 antibodies (Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, China) on methanol-fixed pcDNA3.1D/HA1 plasmid-transfected Vero cells.

Results

Construction of the recombinant (pcDNA3.1D/HA1) DNA plasmid and in-vitro expression

PCR amplification of HA1, TOPO cloning, transformation, colony PCR (to check cloning) and orientation PCR (to check insert direction) were conducted and achieved successfully (data not shown).

Partial sequencing of the cloned fragment revealed complete identity to its published sequence (KC625532.1) (data not shown); thus maintained 99% sequence identity to H5 sequence of the challenge virus (JQ858485.1). Both viruses showed about 80% sequence homology to H5 sequence (AY497096.1) in the vaccine H5N2 strain.

Expression of HA1 protein in transfected Vero cells was confirmed using fluorescent microscopy. Characteristic cytoplasmic and sub-membranous fluorescence indicated successful expression of HA1 protein in transfected Vero cells in comparison with control non-transfected cells (Fig. 2a,b).

Challenge test results

Protection levels in the challenged chicken groups. SPF chicken groups that received either 1 or 2 doses (10 days apart) of pcDNA3.1D/HA1 purified plasmid failed to overcome virulent challenge with 0% (all chickens died 2 dpc) (Fig. 3b) and 10% protection (one chick survived) (Fig. 3a,b), respectively. The groups that received inactivated H5N2 AIV vaccine alone showed a protection level of 70% against challenge (7 chickens survived and 2 died 2 dpc and 1 chick died 3 dpc) (Fig. 3a,b), whereas the groups primed with either one or two doses of HA1-DNA plasmid and boosted by inactivated H5N2 vaccine showed higher protection levels of 80% (8 chickens survived). All birds in the control unvaccinated group died (Fig. 3a,b).

HI titers and virus shedding. Determination of serum HI titers corroborated the observed protection %. The chicken groups that were primed by pcDNA3.1D/HA1 purified plasmid and boosted by inactivated H5N2 vaccine showed significantly high HI titers, which further increased post-challenge. Titers in the chicken groups that received inactivated H5N2 vaccine alone were lower. Groups that received either 1 or 2 doses of pcDNA3.1D/HA1 plasmid did not show any HI titers prior to challenge. A single bird from the group that received 2 doses of pcDNA3.1D/HA1 survived the challenge and demonstrated significantly higher post-challenge HI titer (Fig. 4).

Results of determination of viral shedding demonstrated that although the level of protection in the group that received 2 doses of DNA plasmid was 10%, the single surviving bird from this group showed viral shedding only at 3 days post-challenge with a shedding titer of (2.2×10^4 PCR copies/ml). All prime-boost groups demonstrated greater reduction

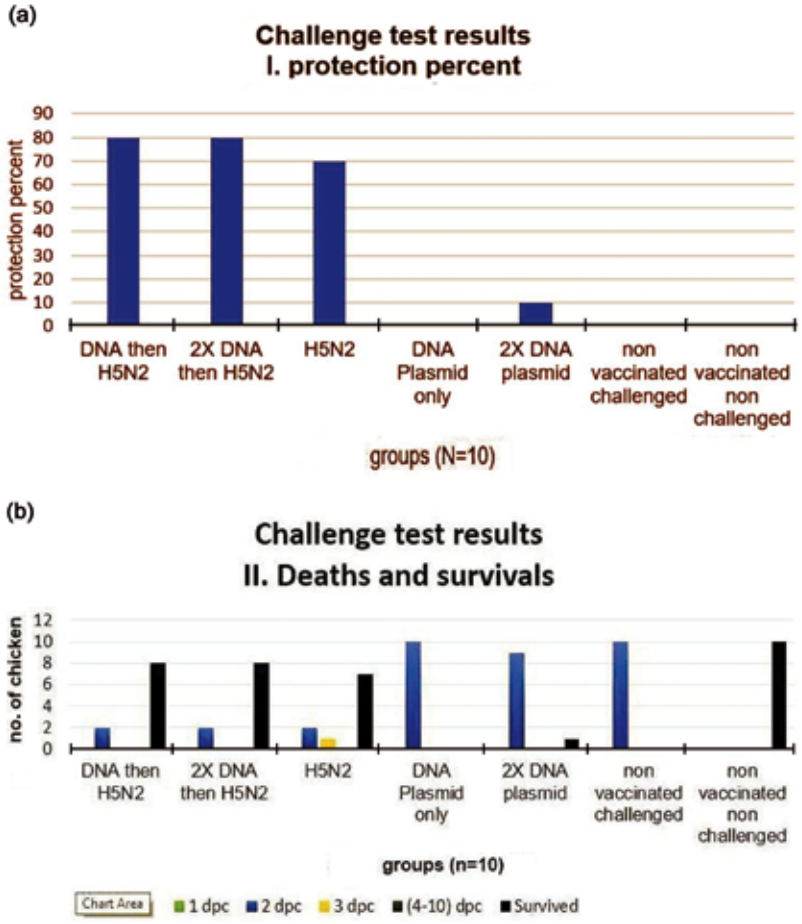

Fig. 3

Challenge test

(a) Results of protection percentage in different chicken groups. Vertical axis represents percent of protection by decimal increment; horizontal axis represents different groups treatment. Bars represent respective protection percent for each group. (b) Results of post-challenge deaths and surviving chickens through the period of observation. Vertical axis represents number of chickens; horizontal axis represents different group treatments.

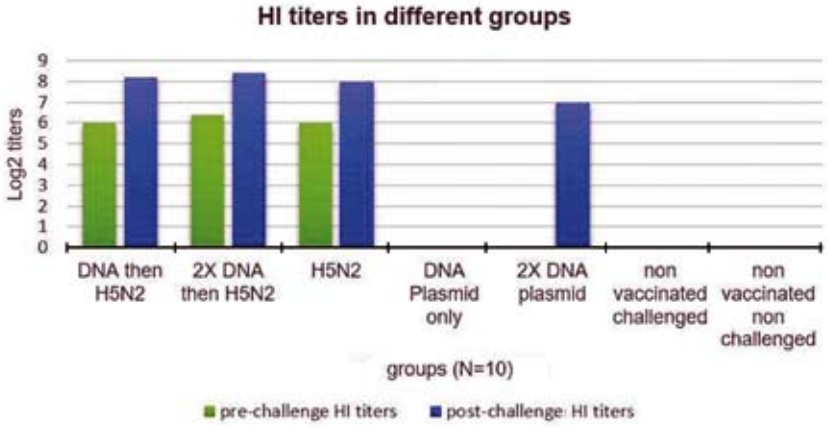

Fig. 4

Hemagglutination inhibition titers in different groups

Vertical axis represents a $\log_2$ HI titers, horizontal axis represents different group labels. Blue represents pre-challenge titers; orange represents post-challenge titers.

Table 1. Virus shedding titers from immunization and challenge experiment

Groups	Post-challenge virus shedding (PCR copies/ml)		
	Day 3	Day 5	Day 7
DNA then H5N2	$1.2x10^4$ (2 birds)	Neg.	Neg.
2x DNA then H5N2	$1.6x10^4$ (2 birds)	Neg.	Neg.
H5N2	$1.8x10^4$ (3 birds)	Neg.	Neg.
DNA vaccine only	NA	NA	NA
2X DNA vaccine	$2.2x10^4$ (1 bird)	Neg.	Neg.
Non-vaccinated, non-challenged	NA	NA	NA

Sampling and qRT-PCR testing was performed 3, 5, and 7 days post-challenge. All groups stopped shedding after 5 days post-challenge. NA = not applicable; Neg. = negative.

in viral shedding compared with the groups that received either H5N2 vaccine or DNA plasmid alone (Table 1). Moreover, a single dose of DNA plasmid was more effective in the reduction of viral shedding when used in a prime-boost regimen with the inactivated H5N2 vaccine. There were no detectable virus titers in the chicken groups at 5 and 7 days post-challenge, confirming the efficacy of DNA plasmid to reduce viral shedding.

Discussion

The idea of DNA vaccination was first introduced and experimentally validated in 1993 (Fynan *et al.*, 1993a,b). Successful implementation of this methodology has been demonstrated by the introduction of the West Nile-Innovator DNA vaccine (Pfizer) on the market in 2005 (Davis *et al.*, 2001). HA-based DNA immunization confers antigen-specific protective immunity in vaccinated susceptible chickens (Fynan *et al.*, 1993a). *De novo* synthesis of HA is initiated in muscle cells at the injection site, the synthesized HA protein is then further processed, and the antigens are presented in the context of both MHC type I and II molecules, resulting in the activation of both humoral and cell-mediated immune responses. DNA vaccination, therefore, has the advantage of eliciting immune responses similar to those in natural infection without the risk of handling live infectious agents (Khan, 2013). Previous studies have explored the use and efficacy of HA-based DNA vaccination against avian influenza in chicken (Fynan *et al.*, 1993a; Robinson *et al.*, 1993; Jiang *et al.*, 2007). In the present study, HA1 gene of an Egyptian HPAI H5N1 virus was successfully cloned into pcDNA3.1D TOPO mammalian expression vector and expressed *in vitro* to be used in prime-boost strategy with inactivated H5N2 vaccine.

The selection of Mexican inactivated H5N2 vaccine was based on the fact that it was one of the first vaccines used to combat clade 2.2 HPAI in Egypt since its introduction in 2006 (Aly *et al.*, 2008). Following emergence of the variant HPAI 2.2.1.1 H5N1 viruses in Egypt in 2007, failure of Mexican H5N2-based vaccines to confer either clinical protection or to decrease virus shedding due to strain heterogeneity was reported (Abdelwhab *et al.*, 2011; Hassan *et al.*, 2012). Low protection percent of the inactivated H5N2 Mexican strain vaccine (70% in the study) was expected and accepted by the research team to better assess the positive effect of DNA plasmid prime.

Chickens that were immunized with the purified pcDNA3.1D/HA plasmid alone (either once or twice) failed to produce detectable anti-HA antibody response measured as the serum HI titers. Consequently, they failed to overcome HPAI challenge with only 10% protection observed in the group that received 2 doses of the DNA plasmid. The sole surviving chicken from this group was able to produce significantly high HI titer post-challenge, indicating proper induction of B-cell memory. The absence of anti-HA antibodies in such DNA-vaccinated groups was not surprising because previous dose-response studies have demonstrated variability in induction of HI antibodies, which play a major role in protection against avian influenza (Jiang *et al.*, 2007). Moreover, poor humoral response has been previously reported with a homologous DNA-DNA prime-boost vaccination strategy (Suguitan *et al.*, 2011). It is worth noting that in the present study, the absence of HI titers was directly related to the lowering of protective efficacy, in contrast to previous studies that showed protection in chickens despite the lack of any detectable anti-HA antibody titer (Kodihalli *et al.*, 1997; Jiang *et al.*, 2007).

The groups primed with pcDNA3.1D/HA1 purified recombinant plasmid and boosted with inactivated H5N2 vaccine, showed 80% protection post-challenge with significant pre- and post-challenge anti-HA antibody titers. The prime-boost protocol, using pcDNA3.1D/HA1 purified recombinant plasmid, enhanced protection by 10% compared to the protection levels in the group that received inactivated H5N2 vaccine alone. Enhancement could be attributed to the induction of cell-mediated immune response as DNA vaccines are potent mobilizers of cellular immune response (Donnelly *et al.*, 1997; Khan, 2013) via direct transfection of antigen presenting cells and/or cross priming (Iurescia *et al.*, 2014). Earlier studies have shown that priming with HA-DNA is as effective as immunization with live attenuated influenza vaccine (LAIV) or with inactivated adjuvanted influenza vaccine (Suguitan *et al.*, 2011). Results of the present study demonstrate that a prime-boost regimen using pcDNA3.1D/HA1 purified recombinant plasmid with the inactivated H5N2 vaccine is more immunogenic than the use of either pcDNA3.1D/HA1 purified recombinant plasmid or inactivated H5N2 vaccine alone. There is evidence that DNA vaccine is effective in inducing a long lasting B-cell memory (Wang *et al.*, 2008). Others have suggested

that the low-dose antigen delivery is more effective in the induction of better antibody response and B-cell memory (Gonzalez-Fernandez and Milstein, 1998; Nichol, 2003). This enhancement could be further improved by using a larger DNA plasmid dose for priming (≥250 μg/chick) (Ogunremi *et al.*, 2013) or by extending the time interval between vaccine doses as shown in recent studies, where intervals longer than 4 weeks between vaccinations had a positive effect on protection (Ledgerwood *et al.*, 2011).

It should be pointed out that a greater reduction in viral shedding post-challenge was noticed in the chickens that received pcDNA3.1D/HA1 prime-inactivated H5N2 vaccine boost than in the chickens immunized with the H5N2 inactivated vaccine alone. However, in order to get statistically significant results for viral shedding, it would be necessary to use larger groups of chickens as well as non-vaccinated contact groups. The results obtained indicated that a greater reduction in viral shedding was achieved with the administration of a single, rather than a double, dose of pcDNA3.1D/HA1 plasmid in the prime-boost regimen. Notably, previous studies have shown that the administration of antigen at different immunization sites unexpectedly decreased the overall protection, suggesting that a single site injection of antigens could improve T-cell priming and enhance the immune response to the second dose (Kedl *et al.*, 2002; Patel *et al.*, 2012). Single dose DNA prime is beneficial in field application as it reduces production costs and bird handling.

The current study provides evidence for heterologous prime-boost vaccination as an attractive approach to control AI infections. The use of DNA vaccine expressing the HA1 gene of H5 HPAI virus as a prime, followed by boosting with inactivated AI vaccine resulted in an enhanced anti-influenza response, thus inhibited virus replication and contributed to the reduction in virus shedding. Although immunization with the HA-DNA vaccine alone resulted in minimal protection, the use of a single dose of DNA plasmid in a prime-boost regimen with the inactivated H5 vaccines may represent an effective prophylactic approach for vaccination against H5N1 AIV virus in the future.

Conclusion. In an epizootic situation with the absence of homologous H5 vaccine, HA1 cloning in mammalian expression vector and its use in priming followed by boosting with any H5Nx AI vaccine could provide a fast solution. Priming with H5 HA1 DNA plasmid presumably enhances the immunological response, resulting in better protection and a reduced viral load. This strategy could be improved by the use of chicken-based promotors, codon optimization, co-expression of immune modulators (chemokines and interleukins), and co-injection of antigen presenting cells or stimulatory adjuvants.

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