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Full Length Research Paper

Approaches toward the development of DNA vaccine for influenza virus

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The main goals of this investigation were to prepare a viral DNA vaccine to help stimulate the immune system of poultry and to increase the efficiency of this vaccine. To accomplish this work, a strain of H5N1 circulating in Egypt was confirmed using rapid diagnostic methods and also, reverse transcriptase polymerase chain reaction (RT-PCR) to hemagglutinin and neuraminidase genes. The virus was propagated in MDCK cell line and the viral genes were extracted and reverse transcribed individually. Individual genes were cloned in gene expression vector (PHW2000) and were used as DNA vaccine. The level of maternal antibodies was determined by ELISA to appoint the right time to give the vaccine. The chicks were divided into eight groups and each group was vaccinated by the couple of DNA NP with one of the other genes. The efficiency of coupled DNA vaccine was determined by neutralization assay and compared with the inactivated vaccine. The results showed that the vaccine that had NP with NS had adequate protection for poultry.

Key words: H5N1, virus, vaccine, poultry, DNA.

INTRODUCTION

Immunity to conserved viral components can provide broad protection against different influenza A strains and subtypes. Research immunity has been long studied in animals (Lamb and Krug, 2001) and there is evidence that it may exist in humans (Fouchier et al., 2005; Horimoto and Kawaoka, 2005). Vaccines based on such cross-protection would not require knowing the identity of the strains that would circulate during the coming season, information which is never certain and could avoid hurried manufacturing in response to outbreaks. Inactivated vaccines given intranasal can induce cross-protection (Johnson and Mueller, 2002; Reid et al., 2004), but DNA prime-viral boost regimens offer the advantage of endogenously expressed antigens that may induce a broader range of immune effectors.

DNA vaccination to conserved influenza nucleoprotien NP or NP and matrix proteins (M), has been studied in animal models and protection against both matched and mismatched challenge viruses has been demonstrated (Gamblin et al., 2004; Horimoto and Kawaoka, 2005), albeit with relatively low dose challenges. H5N1 viruses from the 1997 human outbreak in Hong Kong presented a demanding test because of their virulence and rapid kinetics of infection (Matrosovich et al., 2000). Limited protection was achieved by DNA vaccination with NP + M against some lethal H5N1 challenges (Subbarao et al., 1998). To improve the efficacy of this approach, Epstein et al. (2005) explored the ability of a recombinant adenovirus (rADV) vector to enhance potency of vaccination to NP, an antigen with >90% protein sequence homology among influenza A isolates (Buxton et al., 2000) and containing dominant target epitopes (Xu et al., 1999). The ability of the regimen to protect against high virus challenge doses and against challenge with mismatched highly pathogenic H5N1 strains was tested. The DNA prime-rADV boost regimen is more effective than vaccination with NP construct, conferring protection against doses of challenge virus that were lethal to mice vaccinated only with DNA or rADV. In this study, investigation was carried out to find effective system for protection against lethal doses of H5N1 by preparing DNA

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vaccine from mixture of the NP construct with other viral gene constructs to find the one that is more effective in poultry protection.

An epidemic of disease occurs when there are more cases of that disease than normal, while pandemics are worldwide epidemic of a disease. An influenza pandemic may occur when a new influenza virus appears against which the human population has no immunity. The avian influenza viruses AI have devastating impact on poultry causing nearly 100% mortality within 48 h of infection (Roberts and Lim, 2008).

Inactivated vaccines are the main stream of influenza prophylaxis (Horimoto and Kawaoka, 2006). The first of the inactivated vaccine formulation is the whole virion, the experimental use of which dates back to 1940s. The second is split virion which is derived by disrupting whole virus particles with detergents and finally, the subunit form, which is prepared by enriching for the viral surface glycoproteins HA and neuraminidase (NA) following disruption of viral particles, although, arguably more immunogenic.

This system consists of a plasmid that synthesizes the six gene segments for the internal proteins (PB2, PB1, PA, NP, M and NS) and a second plasmid that synthesizes the HA and NA segments. A total of four plasmids are used for transfection into Vero cells, (Horimoto and Kawaoka, 2006) which results to reassortant viruses. This four-plasmid system might therefore, be valuable in the future generation of pandemic vaccine seed viruses. The HA and NA were derived from a human H5N1 virus and the rest of their genes were derived from a virus (known as backbone virus) that grows well in eggs (Horimoto et al., 2006).

Previous vaccination with seasonal trivalent, inactivated or live attenuated influenza vaccines did not elicit a cross-reactive antibody response to the novel strains of H1N1 and H5N1 viruses. The currently licensed seasonal influenza vaccines are subtype specific, thus, do not induce protective immune responses against newly emerged influenza viruses. Up till now, a protective vaccine for the H5N1 influenza virus is not available due to several problems that include shortening the time from vaccine strain selection to vaccination, increasing the capacity of production, broadening the vaccine-induced immune response, and evaluating the vaccine's effectiveness and safety (Monto and Ohmit, 2009).

DNA vaccines represent a novel and powerful alternative to conventional vaccine approaches. Their novelty and usefulness stems form the fact that, they are noninfectious, nonreplicating, extremely stable and can be produced en masse at low cost. Most importantly, DNA vaccines against emerging pathogens or bioterrorism threats can be quickly constructed based solely upon the pathogen's genetic code (Kim and Jacob, 2009; Donnelly et al., 2005). The aims of this study were to develop eight DNA constructs of influenza A virus in mammalian expression system, immunization of chicken by mixing the NP construct with other viral gene constructs, and evaluation study for the most effective mixture by determination of the immune response.

MATERIALS AND METHODS

Virus

The avian influenza H5N1 (A/chicken/Qalubiya/1/2006(H5N1)), (Egyptian strain) was kindly provided by Prof. Dr. Mohamed Ali, National Research Centre in Cairo. The obtained virus was previously identified as AVI H5N1virus by both RT-PCR and nucleotide sequencing.

The nucleotide sequence of the HA gene was deposited in the GenBank under accession No. FJ472343.

Oligonucleotide primers

For H5, the following forward (F) and reverse (R) primers were designed based on previously published sequence using the Lasergene sequence analysis software (DNASTAR Inc., Madison, USA) to amplify partial sequence of a 311 bp segment: H5-F: 5'-CCTCCA GARTATGCMTAYAAAATTGTC-3'; H5-R 5'-TACCAACCGTCTACCATKCCYTG -3'.

For neuraminidase (N1), the following previously published (Wright et al., 1995) F and R primers were used to amplify the partial sequence of 615 bp segment: N1-F: 5'-TTGCTTGGTCGGCAAGTGC-3'; N1-R: 5'-CCAGTCCACCCATTTGGATCC-3'.

Another set of universal primers (Hoffmann et al., 2001) that was recomended for amplification of eight segments for all the influenza "A" viruses were used. These primers were: PB2-1F tattggtctcagggAGCGAAAGCAGGTC; PB2-2341R atatggtctcgtattAGTAGAAACAAGGTCGTTT; PB1-1 F tattcgtctcagggAGCGAAAGCAGGCA; PB1-2341R atatcgtctcgtattAGTAGAAACAAGGCATTT; PA-1 F tattcgtctcagggAGCGAAAGCAGGTAC; PA-2233R atatcgtctcgtattAGTAGAAACAAGGTACTT; HA-1 F tattcgtctcagggAGCAAAAGCAGGGG; NS-890R atatcgtctcgtattAGTAGAAACAAGGGTGTTTT; NP-1 F tattcgtctcagggAGCAAAAGCAGGGTA; NP-1565R atatcgtctcgtattAGTAGAAACAAGGGTATTTTT; NA-1 F tattggtctcagggAGCAAAAGCAGGAGT; NA-1413R atatggtctcgtattAGTAGAAACAAGGAGTTTTTT; M-1 F tattcgtctcagggAGCGAAAAGCAGGTAG; M-1027R atatcgtctcgtattAGTAGAAACAAGGTAGTTTTT; NS-1T F tattcgtctcagggAGCGAAAAGCAGGGTG; NS-890R atatcgtctcgtattAGTAGAAACAAGGGTGTTTT; Uni 12 primer 5'-AGCAAA AGCAGG-3'

Materials for cDNA cloning in expression vector

Cloning vector

pHW2000 vector was kindly provided by Prof. Dr. Mohamed Ali, of the National Research Centre in Cairo, from Dr. Richard Webby, St.Jude Children's Research Hospital and Memphis, USA through Materials Transfer Agreement (MTA) (Figure 1).

RT-PCR: Virus RNA extraction

Viral RNA extraction was accomplished using the viral RNA extraction reagent kit and the manufacturing instructions as procedures.

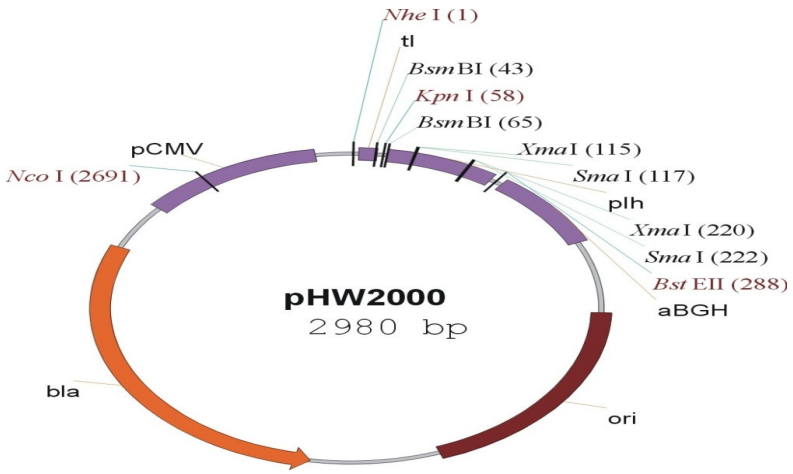

Figure 1. Schematic of pHW2000 plasmid.

Reverse transcription of extracted RNA

Reverse transcription of the extracted RNA was performed for synthesis of cDNA, using the method of Hoffmann et al. (2000). Polymerase chain reaction (PCR) of both H5 and N1 partial sequences and visualization of RT-PCR product was carried out according to the method of Wright et al. (1995).

Production of polyclonal antibodies against the killed H5N1: Egyptian strain

Madin Darby canine kidney cell (MDCK) tissue culture was grown to confluence the complete growth media (DMEM) in 75 cm^2 culture flasks as route protocol stander work.

Propagation of H5N1: Egyptian isolate in MDCK

Avian influenza H5N1 virus (A/chicken/Qalubiya/1/2006(H5N1)) was propagated in MDCK as route protocol stander work.

H5N1 virus titration

Plaque forming unit (PFU) assay was used for H5N1 virus titration, applying the method of Rashad and Ali (2006).

Preparation of killed H5N1: Egyptian isolate

HPAI-H5N1virus was inactivated by 0.1% formaldehyde (1 µl/ml), and then was treated with sodium thiosulfate solution (1 mg/ml) to remove the residual formaldehyde. The killed virus was mixed with four volumes of *Negilla sativa* oil that was previously reported to induce both cellular and humoral immune responses upon being used as an adjuvant (Salem, 2005).

Immunization of chicks for production of polyclonal antibodies

Three groups of three days old chicks (each of five) were used for the production of polyclonal antibodies. The first group was immunized with intramuscularly injection with 200 µl solution containing 10^6 PFU killed virus. The second group was immunized with lysate of uninfected MDCK host cells and the third group was a control left unimmunized. Immunized chickens received a booster dose of the killed virus three weeks after the first immunization. Serum samples were collected from the three groups before immunization and then weekly post-immunization.

Preparation of construct DNA vaccine

RT-PCR amplification of each viral gene using full length primers

Each viral gene of H5N1 was amplified by RT-PCR using full length primers as applied by Hoffmann et al. (2000).

Purification of the PCR products

Five volumes of buffer PB was added to one volume of the PCR sample and was mixed by the QIAquick spin column.

Cloning of amplified viral segments for preparation of DNA constructs

Digestion of the amplified genes and pHW2000 was with *BsmB1*endonuclease and the amplified genes and pHW2000 were digested with *BsmB1*endonuclease using the method of Hoffmann et al. (2002).

Ligation of each gene in plasmid terminal with T4 ligase

The ligation reaction was done using the T4 DNA ligase kit (Promega). Briefly the vector was centrifuged and DNA tubes were inserted to collect the contents at the bottom of the tube. The reaction mixture was prepared with 8 µl of the digested vector (BsmBI), 2 µl 10X buffer, 8 µl digested PCR of NA (BsaI) and 2 µl T4 DNA ligase. The mixture was incubated at 4°C overnight.

Transformation of each construct in *E. coli* (Top 10 competent cells)

To transform each construct in *E. coli*, 50 µl of the TOP10 compe-

tent bacterial vial and 5 µl ligation mixtures were added according to the routine stander method protocol.

Selection of the transformed bacterial colony and extracted plasmid by miniprep

This was done according to the manufacturer's instructions (plasmid minipreparation kit, promega).

Confirmation of the constructed DNA

PCR for each constructed DNA was performed according to routine stander method protocol.

Large-scale preparation of plasmid DNA

The method was done according to the manufacturer's instructions (plasmid maxi preparation kit; QIAGEN, Hilden, Germany).

Determination of maternal antibodies against H5N1 virus

Maternal IgG antibodies against H5N1 virus were determined by ELISA according to the method of Bahgat et al. (2006).

Vaccination of chicks

A suspension of the NP construct DNA with each of the seven constructed DNA was prepared individually according to routine stander method protocol.

Detection of immunoglobulin G (IgG) against constructed DNA

Antibodies produced against each DNA vaccine constructs were detected by ELISA according to the method of Bahgat et al. (2006).

Neutralization assay for antibodies titration

Procedure of virus titration

Microtitre plates were seeded with MDCK cells. Monolayer of MDCK cell in the T-75 flask was gently rinsed with 5 ml trypsin EDTA. Trypsin-EDTA (5 ml) was added to cover the cell monolayer. The flask was laid flat and incubated at 37℃ in 5% CO_2 until the monolayer detached (approximately 10 min). MDCK medium (5 ml) was added to each flask and cell was removed and transferred to centrifuge tube.

The cells were washed with PBS (5 min at 12,000 rpm). Cells were resuspended in D-MEM cell growth medium and counted with a hemocytometer. Cells number was adjusted to $1.5x10^5$ cell/ml with D-MEM growth medium. Cells suspensions (100 µl/well) were added into microtiter plate. Plate was incubated overnight at 37℃, at 5% CO_2 for 18 to 22 h. Plate was used when cell just reached confluence. For optimum results, the cells were in the growth phase.

Preparation of virus dilutions

Virus was diluted 1/10 in the virus growth media VGM (working stock dilution of virus) as route protocol stander work.

Virus titration

Virus titration was done according to routine stander method protocol. The TCID50 was calculated by the Read-Meunch method.

Preparation dilutions of test sera

Collected sera were diluted in DMEM media. A volume of 12 µl from each serum was added to 108 µl of DMEM media free FBS and was decontaminated by antibiotic mixture in 0.5 ml tubes. Wells in the microtiter plate were coated with 60 µl DMEM media free FBS and were decontaminated by antibiotic mixture except the first row (uncoated). Wells in the first row received 60 µl of the serum dilution. Equal volumes (60 µl) of DMEM media free FBS and decontaminated by antibiotic mixture were added to each well of the first row. Twofold serial dilution was prepared by transferring 60 µl from row to row (A, B, C to H) and 60 µl was discarded from the last row (row H).

The highest dilution of each serum completely protecting the cell sheet from CPE in at least two wells of four was taken to be the viral antibody titer.

RESULTS

Propagation and titration of the H5N1 Egyptian isolate

The virus isolate was propagated in MDCK cells with daily microscopic examination. Due to virus propagation after 2 days of post infection, CPE appeared on the infected cells as demonstrated by marked morphological changes including rounding, clumping, darkness and destruction in the cell monolayer and this effect increased dramatically by day 4 (Figure 2).

Harvested virus from MDCK-infected cells at day 4 was titrated by plaque infectivity assay. Results showed that the virus titer was approximately 10^6 PFU/ml (Table 1).

Amplification of H5N1 Full length of eight segments

Amplification of the eight viral segments was carried out using RT-PCR and specific primers for each segment. The results clearly demonstrated that amplification of 2341, 2341, 2233, 1565, 1027, 890, 1778 and 1413 bp fragments, respectively, corresponded with the expected molecular weights (band size) of PB2, PB1, PA, NP, M, NS, HA and NA genes (Figure 3).

cDNA cloning of extracted RNA in expression vector

The RT-PCR products of each segment were purified with Qiagen PCR purification reagent and individually cloned in pHW2000 mammalian expression vector. The cloned pHW2000 of each segment was transformed in top 10 competent bacterial cells for propagation. The constructed plasmid was extracted from the propagated

Table 1. Plaque titration of HPAIV H5N1.

Dilution	Replica 1	Replica 2	Replica 3	Average number of plaque	PFU/ml
10-1	Unc	Unc	Unc	Unc	Und
10-2	Unc	Unc	Unc	Unc	
10-3	110	95	105	103	103x10x103=1x106
10-4	15	7	12	25	9x10x104=9x105
10-5	0	0	0	0	0

Unc, Uncountable; Und, undetermined. Titration = $\frac{1\times10^6 + 9\times10^5}{2} = 22\times10^5 = 2\times10^6$ pfu/ml.

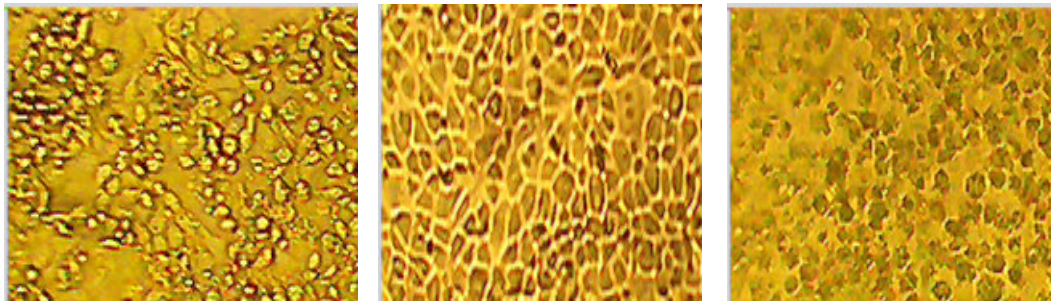

Figure 2. MDCK cells infected with H5N1 HPAIV. (A), Control uninfected cells; (B and C), infected cells with two levels of CPE as rounding and detachment of the cell monolayer.

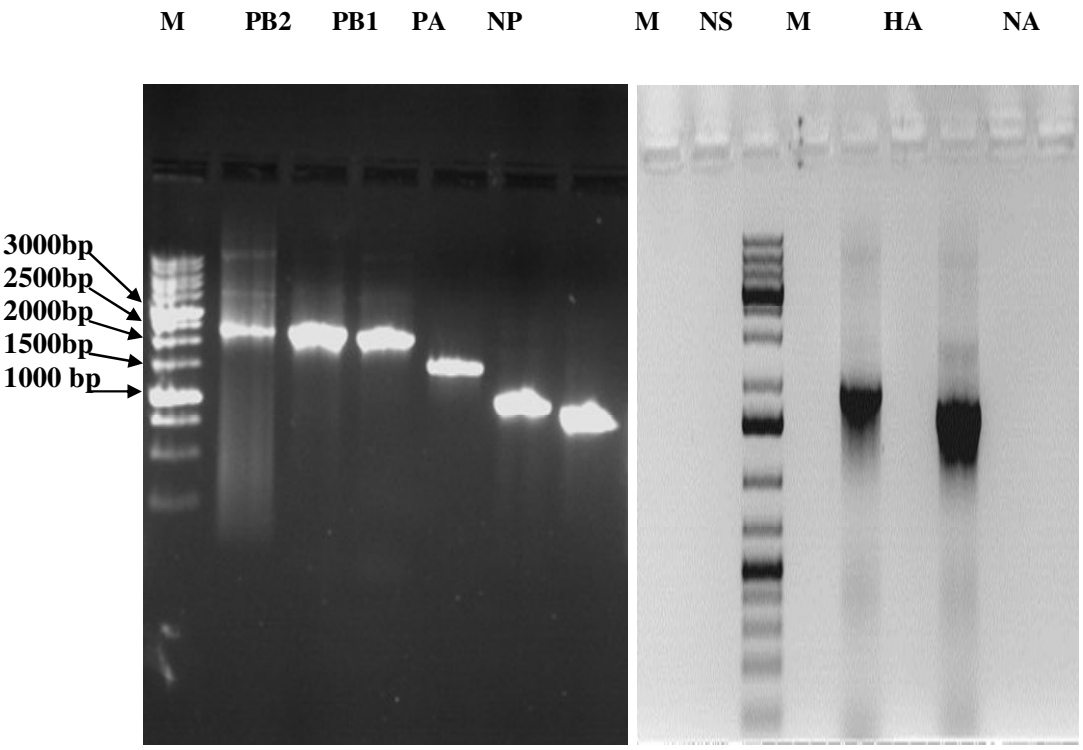

Figure 3. RT-PCR amplification products of the eight virus full length segments. M is 1Kb molecular weight marker.

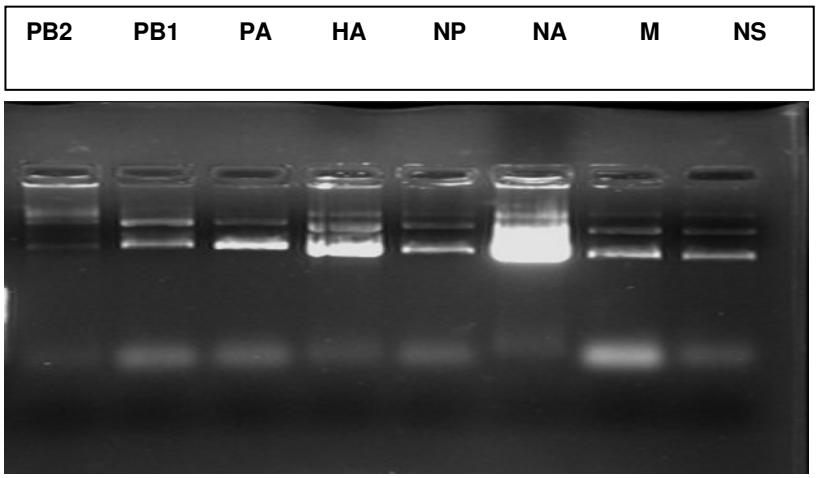

Figure 4. Electrophoretic analysis of the constructed plasmid with individual segment of H5N1.

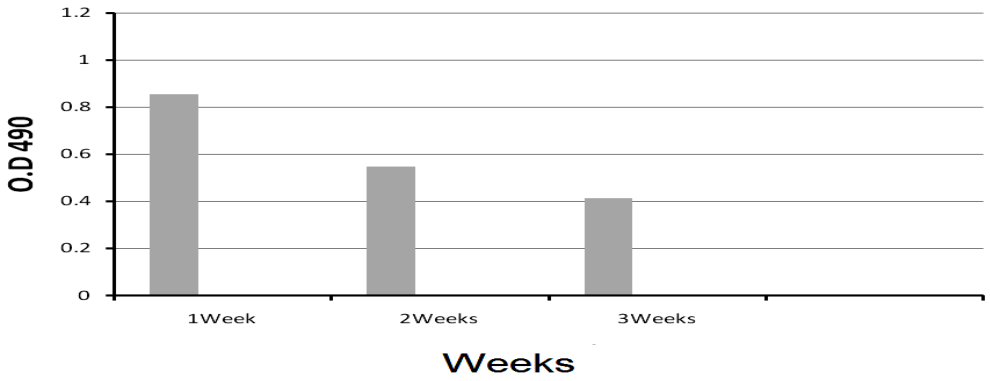

Figure 5. IgG maternal antibody levels of the chicks.

transformed bacteria and run in gel-electrophoresis (Figure 4).

Confirmation of cloning

Confirmation of the constructed DNA was achieved by amplifying each constructed plasmid containing segment using PCR and specific primers for each segment. The results clearly demonstrated that, amplification of 2341, 2341, 2233, 1565, 1027, 890 and 1778 bp, respectively and 1413 fragments, corresponded to the expected molecular weights (band size) of PB2, PB1, PA, NP, M, NS, HA and NA gene.

Determination of IgG maternal antibodies of the chicks groups against H5N1 isolate

The success of vaccination program of poultry depends on the absence of maternal antibodies against avian influenza H5 in the vaccinated chicks. So, the recommended age for the prime dose was one day old. Because all chicks came from vaccinated mothers, the maternal antibodies interfered with vaccine efficiency. Accordingly, the maternal antibodies against H5N1 virus was monitored weekly using ELISA to determine the maximum drop in maternal antibodies. The optical density (O.D) of the IgG maternal antibody against H5N1 in one week old chicks was 0.84. After the second week, the value of O.D. dropped to 0.54. The maximum drop in IgG maternal antibodies was observed at the third week (0.42) (Figure 5).

Detection of IgG antibodies against each couple of construct DNA vaccine

Antibodies produced against each DNA vaccine constructs and inactivated vaccine were detected by ELISA (Figure 6). Although, each serum was diluted 1:1, the results of ELISA according to the OD value indicated

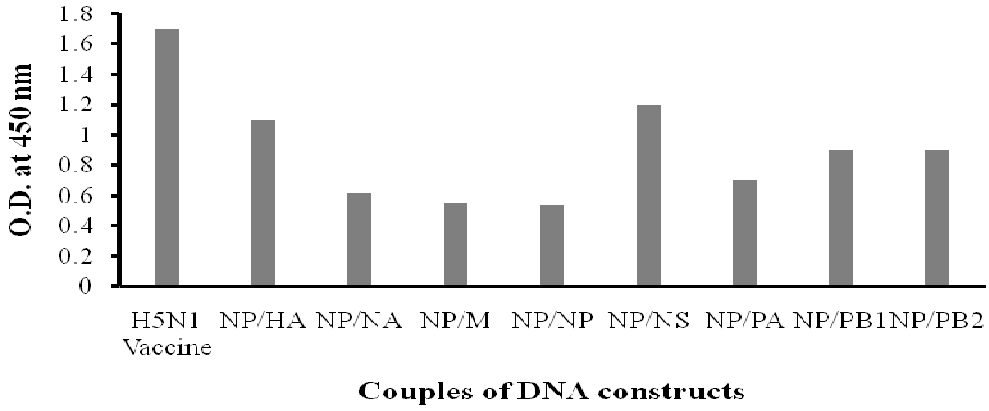

Figure 6. Detection of antibodies against each couples of DNA constructs and inactivated vaccine by ELISA.

Table 2. Detection of IgG antibodies against each couple of construct DNA vaccine and inactivated vaccine.

Type of vaccine	OD
H5N1 vaccine	1.7
NP/HA	1.1
NP/NA	0.62
NP/M	0.55
NP/NP	0.53
NP/NS	1.2
NP/PA	0.7
NP/PB1	0.9
NP/PB2	0.9

high variations in the concentration of antibodies produced against each couple of DNA construct. The highest OD was obtained with inactivated vaccine of H5N1 (1.7). The lowest OD was obtained with NP/NP, where the highest OD was obtained with DNA construct vaccine was with NP/NS (1.2). Table 2 indicates all the OD values obtained through all the couples of DNA construct vaccine.

Titration of IgG antibody against each couples of DNA constructs and inactivated vaccine by neutralization assay

The neutralizing titer of the H5N1 genome segment couples was determined by neutralization assay. The titers were compared with the prepared H5N1 oil emulsion inactivated vaccine. Results clearly demonstrated that, titers of 2 , 8, 8, 2, 16, 2, 2, 2, 64 and 128, respectively corresponded with NP/NP, NP/PB1, NP/PB2, NP/PA, NP/HA, NP/NA, NP/M, NP/NS and H5N1 vaccine. The results revealed that, the maximum neutralizing efficiency was obtained by the NP/NS couple of genes as shown in Table 3 and Figure 7.

DISCUSSION

The 1997 outbreak of H5N1 avian influenza in humans in Hong Kong and frequent subsequent outbreaks in China and Eastern Europe resulted to great concern in the world health community (Subbarao et al., 1998). This is because, the outbreaks was caused by highly pathogenic strains of an influenza subtype to which humans lack immunity and hence, poses the potential to cause an influenza pandemic, as seen in 1918. Considering the molecular basis for the virulence of H5N1 viruses (Hatta et al., 2001), the most promising method of controlling a pandemic is the use of antiviral drugs (Laver and Garman, 2001). However, these drugs partially reduce the symptoms and duration of the disease and drug resistance has been found in multiple isolates (Schunemann et al., 2007). Genomic and antigenic analyses of H5N1 viruses isolated since 2004 have

Table 3. The neutralizing titer of the H5N1 genome segment for each couples and prepared H5N1 oil emulsion inactivated vaccine.

Neutralizing titer	NP/NP	NP/PB1	NP/PB2	NP/PA	NP/HA	NP/NA	NP/M	NP/NS	H5N1 vaccine
	*No. infected / total	*No. infected / total	*No. infected / total	*No. infected / total	*No. infected / total	*No. infected / total	*No. infected / total	*No. infected / total	*No. infected / total
1:1	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
1:2	2/4	0/4	0/4	2/4	0/4	2/4	2/4	0/4	0/4
1:4	3/4	0/4	0/4	3/4	1/4	3/4	3/4	0/4	0/4
1:8	4/4	2/4	2/4	4/4	0/4	34	3/4	0/4	0/4
1:16	4/4	3/4	3/4	4/4	2/4	4/4	4/4	1/4	0/4
1:32	4/4	4/4	4/4	4/4	3/4	4/4	4/4	0/4	1/4
1:64	4/4	4/4	4/4	4/4	4/4	4/4	4/4	2/4	0/4
1:128	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	2/4

*= Number of infected wells/total number of wells.

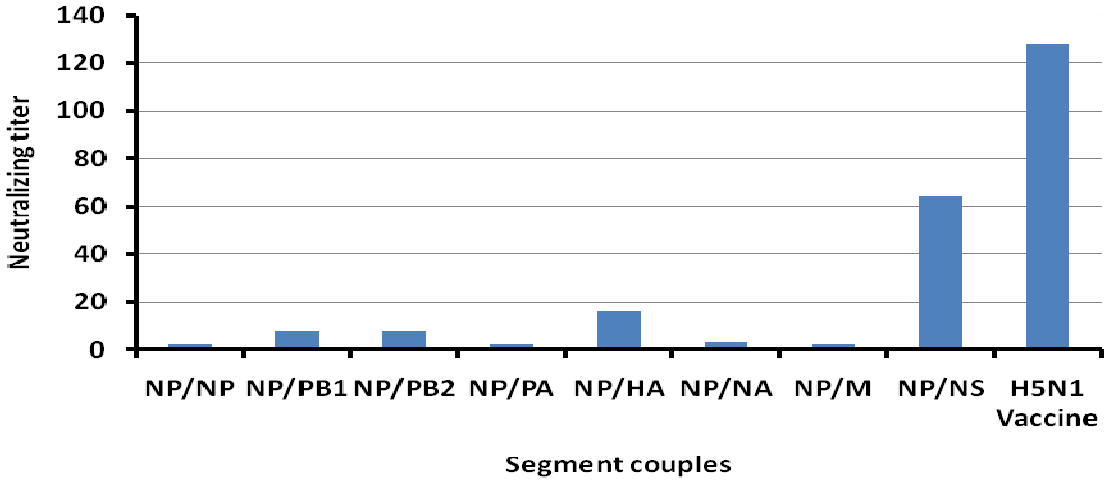

Figure 7. Neutralizing titer as a result of vaccination with couples of DNA constructs.

revealed at least two distinct sub-lineages with different geographic distributions, designated clades 1 and 2 (Webster and Govorkova, 2006). This raises the concern that it is not possible to predict which strain may emerge in a future pandemic; therefore, a vaccine generated from a single selected strain may not be able to protect against a diverse set of viruses. In addition, there are several inherent difficulties associated with H5N1 vaccines (Stephenson et al., 2006; Subbarao and Luke, 2007). H5 viruses are highly pathogenic, yet H5-HA is poorly immunogenic for unknown reasons. Little is known about the antigenic sites on avian HAs and immune correlation of protection from avian influenza infections. In comparison with human-adapted influenza viruses, the yield of candidate vaccines of H5N1 in embryonic chicken eggs was reduced and limited. The manufacturing capacity represents an additional obstacle in the development of H5N1 vaccines. Thus, the major focus in H5N1 vaccine development is on testing vaccine candidates for priming, cross-reactivity and cross-protection against infection with viruses from different clades and subclades.

Studies have explored the possibility of developing DNA vaccines against H5N1 influenza viruses (Epstein et al., 2005; Laddy et al., 2007). As in human influenza viruses, the protective ability of HA-based DNA vaccines of H5N1 virus is limited to homologous strains of virus. Kodihalli and colleagues showed that, a DNA vaccine encoding HA from the index human influenza isolate A/HongKong/156/97, provided immunity against homologous H5N1 infection of mice (Kodihalli et al., 1999).

However, a DNA vaccine encoding the HA from A/Ty/Ir/1/83 (H5N8), which differs from A/HK/156/97 (H5N1) by 12% in HA1, prevented death but not H5N1 infection (Kodihalli et al., 1999). The possibility of protection conferred by NA-based DNA vaccines has also been explored (Sandbulte et al., 2007). Based on the idea that the NA of H5N1 viruses (avN1) and endemic human H1N1 viruses (huN1) are classified in the same

serotype, Sandbulte's group tested whether an immune response to huN1 could mediate cross-protection against H5N1 influenza virus infection (Sandbulte et al., 2007).

A DNA vaccine encoding huN1 from A/PR/8/34 (H1N1) partially protected mice from lethal challenge with H5N1 virus or recombinant PR8-avN1. These findings suggest that, a portion of the human population could have some degree of resistance to H5N1 influenza (Sandbulte et al., 2007). More promising results regarding cross-protection are found in studies where internal protein-based DNA vaccines were applied. Epstein and colleagues showed that, DNA vaccination encoding the PR8 (H1N1)-NP and M1 proteins, reduced replication of A/HongKong/486/97, a non-lethal H5N1 strain in mice and completely protected and minimized morbidity upon lethal challenge with more virulent A/HongKong/156/97 (Epstein et al., 2002). Upon challenge with a highly virulent strain of HK/483, half of the vaccinated mice survived (Epstein et al., 2002). The strategy of combined DNA vaccination against H5N1 virus was also shown to be effective against other viruses, such that H5- and H7-encoding DNA vaccines, protected chickens against lethal infection by both A/Ck/Vic/1/85 (H7N7) and A/Ty/Ir/1/83 (H5N8) (Kodihalli et al., 2000).

In this study, the effect of maternal antibodies which could decrease or increased the efficiency of the construct DNA vaccine or H5N1 vaccine where countered by vaccinating the chick till the dropping of maternal antibodies. At this moment, the chicken will be ready for vaccination with oil emulsion inactivated vaccine. The use of DNA vaccination may resolve this problem but the selective gene segment is needed to try different couples of segments and that was what was performed in this study. The NP protein segment was selected to be the main DNA for vaccination because it is known that this segment is highly conserved. The results revealed that the couples of NP and NS gave the highest neutralizing titer than all other DNA couples against H5N1 (Egyptian isolate).

Conclusions

Avian influenza viruses are still the most infectious agents of the 21st century worldwide. No expected changes or mutations can be predicted for these viruses. The use of DNA vaccines may be the promising tool for virus control and eradication. This is beside the low cost for vaccine preparation and use. The main advantage of this type of vaccines is that, it may lead to a universal vaccine for influenza A infection. In this study, production of antibodies against H5N1 virus by chick immune system could be achieved using construct DNA vaccine. As it was shown in the results, the couple construct DNA vaccine of NP/NS gave the highest antibodies titer (1:64) compared with other couple construct DNA vaccines and it was almost the next titer of H5N1 inactivated vaccine (1:128). The two folds difference would not be an obstacle of using this type of vaccine instead of inactivated vaccine in the comparison of the advantages and disadvantages of the application of these two types of vaccines.

Surveillance for influenza viruses is an urgent need for developing and developed countries. The developing countries must obtain the technology of vaccine preparation to avoid what happened in swine influenza pandemic of 2009. Researches must continue in the field of virus characterization and vaccine evaluation. This study could be considered as pilot study for the production of a DNA vaccine against H5N1 virus. As long as the production of antibodies was enhanced in this study by using DNA vaccine, further studies for vaccine evaluation are required. Trials and investigations concerning the efficacy of this vaccine against the infection with other strains and subtypes of influenza viruses should be done.

REFERENCES

Bahgat M, Sorgho H, Ouédraogo JB, Poda JN, Sawadogo L, Ruppel A (2006). Enzyme-linked immunosorbent assay with worm vomit and cercarial secretions of *Schistosoma mansoni* to detect infections in an endemic focus of Burkina Faso. J. Helminthol. 80(1): 19-23.

Buxton BC, Katz JM, Seto WH, Chan PK, Tsang D, Ho W, Mak KH, Lim W, Tam JS, Clarke M, Williams SG, Mounts AW, Bresee JS, Conn LA, Rowe T, Hu-Primmer J, Abernathy RA, Lu X, Cox NJ, Fukuda K (2000). Risk of influenza A (H5N1) infection among health care workers exposed to patients with influenza A (H5N1), Hong Kong. J. Infect. Dis. 181: 344-348.

Donnelly JJ, Wahren B, Liu MA (2005). DNA vaccines: progress and challenges. J. Immunol. 175: 633-639.

Epstein SL, Kong WP, Misplon JA, Loa CY, Tumpey TM, Xub L, Nabel GJ (2005). Protection against multiple influenza A subtypes by vaccination with highly conserved nucleoprotein. Vaccine, 23: 5404–5410.

Epstein SL, Tumpey TM, Misplon JA, Lo CY, Cooper LA, Subbarao K, Renshaw M, Sambhara S, Katz JM (2002). DNA vaccine expressing conserved influenza virus proteins protective against H5N1 challenge infection in mice. Emerg. Infect. Dis. 8(8): 796–801.

Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, Rimmelzwaan GF, Olsen B, Osterhaus AD (2005). Characterization of a novel influenza a virus hemagglutinin subtype (H16) obtained from black-headed gulls. J. Virol. 79: 2814-2822.

Gamblin SJ, Haire LF, Russell RJ, Stevens DJ, Xiao B, Ha Y, Vasisht N, Steinhauer DA, Daniels RS, Elliot A, Wiley DC, Skehel JJ (2004). The structure and receptor binding properties of the 1918 influenza hemagglutinin. Science, 303: 1838-1842.

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.

Hoffmann E, Krauss S, Perez D, Webby R, Webster RG (2002). Eight-plasmid system for rapid generation of influenza virus vaccines, 19-20 (25-26): 3165-3170.

Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG (2000). A DNA transfection system for generation of influenza A virus from eight plasmids. Proc. Natl. Acad. Sci. U. S. A. 23-97(11): 6108-6113.

Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR (2001). niversal primer set for the full-length amplification of all influenza A viruses. Arch. Virol. 146: 2275–2289.

Horimoto T, Kawaoka Y (2005). Influenza: lessons from past pandemics, warnings from current incidents. Nat. Rev. Microbiol. 3: 591-600.

Horimoto T, Kawaoka Y (2006). Strategies for developing vaccines

against H5N1 influenza A viruses. Division of Virology, Department of Microbiology and Immunology, International Research Center for Infectious Diseases, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo, pp. 108- 8639.
Horimoto T, Takada A, Fujii K, Goto H, Hatta M, Watanabe S, Iwatsuki-Horimoto K, Ito M, Tagawa-Sakai Y, Yamada S, Ito H, Ito T, Imai M, Itamura S, Odagiri T, Tashiro M, Lim W, Guan Y, Peiris M, Kawaoka Y (2006) The development and characterization of H5 influenza virus vaccines derived from a 2003 human isolate. Vaccine, 24: 3669–3676.
Johnson NP, Mueller J (2002). Updating the accounts: global mortality of the 1918–1920 'Spanish' influenza pandemic. Bull. Hist. Med. 76: 105–115.
Kim JH, Jacob J (2009). DNA vaccines against influenza viruses. Curr. Top. Microbiol. Immunol. 333: 197-210.
Kodihalli S, Goto H, Kobasa DL, Krauss S, Kawaoka Y, Webster RG (1999). DNA vaccine encoding hemagglutinin provides protective immunity against H5N1 influenza virus infection in mice. J. Virol. 73: 2094–2098.
Kodihalli S, Kobasa D, Webster RG (2000). Strategies for inducing protection against avian influenza A virus subtypes with DNA vaccines. Vaccine, 18:2592–2599.
Laddy DJ, Yan J, Corbitt N, Kobasa D, Kobinger GP, Weiner DB (2007). Immunogenicity of novel consensus-based DNA vaccines against avian influenza. Vaccine. 25: 2984–2989.
Lamb RA, Krug RM (2001). Orthomyxoviridae: the viruses and their replication. In Fields Virology (4th edn) (Knipe, D.M. et al., eds), pp. 1487–1531, Lippincott-Raven.
Laver G, Garman E (2001). Virology: The origin and control of pandemic influenza. Science. 293: 1776–1777.
Matrosovich M, Tuzikov A, Bovin N, Gambaryan A, Klimov A, Castrucci MR, Donatelli I, Kawaoka Y (2000). Early alterations of the receptor-binding properties of H1, H2 and H3 avian influenza virus hemagglutinins after their introduction into mammals. J. Virol. 74: 8502–8512.
Monto AS, Ohmit SE (2009). Seasonal influenza vaccines: evolutions and future trends. Expert. Rev. Vaccines, 8: 383–389.
Rashad E, Ali MA (2006). Synthesis and antiviral screening of some thieno[2,3-d]- pyrimidine nucleosides. Nucleosides Nucleic Acids. 25: 17-28.
Reid AH, Taubenberger JK, Fanning TG (2004). Evidence of an absence: the genetic origins of the 1918 pandemic influenza virus. Nat. Rev. Microbiol. 2: 909–914.
Roberts H, Lim WS (2008). Viral lung infections and the potential for a human pandemic. Medicine 36(6): 291-294.
Salem ML (2005). Immunomodulatory and therapeutic properties of the Nigella sativa L. seed. Int Immunopharmacol. 5 (13-14):1749-1770.
Sandbulte MR, Jimenez, GS, Boon AC, Smith LR, Treanor JJ, Webby RJ (2007). Cross-reactive neuraminidase antibodies afford partial protection against H5N1 in mice and are present in unexposed humans. PLoS Med. 4:e59.
Schunemann HJ, Hill SR, Kakad M, Bellamy R, Uyeki TM, Hayden FG, Yazdanpanah Y, Beigel J, Chotpitayasunondh T, Del Mar C, Farrar J, Tran TH, Ozbay B, Sugaya N, Fukuda K, Shindo N, Stockman L, Vist GE, Croisier A, Nagjdaliyev A, Roth C, Thomson G, Zucker H, Oxman AD (2007). WHO Rapid Advice Guidelines for pharmacological management of sporadic human infection with avian influenza A (H5N1) virus. Lancet Infect. Dis. 7: 21–31
Stephenson I, Gust I, Pervikov Y, Kieny MP (2006). Development of vaccines against influenza H5. Lancet Infect. Dis. 6: 458-460.
Subbarao K, Luke C (2007). H5N1 viruses and vaccines. PLoS Pathog., 3:e40.
Subbarao K, Klimov A, Katz J, Regnery H, Lim W, Hall H, Perdue M, Swayne D, Bender C, Huang J, Hemphill M, Rowe T, Shaw M, Xu X, Fukuda K, Cox N (1998). Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. Science, 279: 393-396.
Webster RG, Govorkova EA (2006). H5N1 influenza—continuing evolution and spread. N. Engl. J. Med. 355: 2174–2177.
Wright KE, Wilson GAR, Novosad D, Dimock C, Tan D, Weber JM (1995). Typing and Subtyping of Influenza Viruses in Clinical Samples by PCR. J. Clin. Microbiol. 33: 1180–1184.
Xu X, Subbarao K, Nancy J, Yuanji C, Guo Y (1999). Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. Virology, 261: 15-19.