RESEARCH ARTICLE

A Novel DNA Vaccine Coding For H5 and N1 Genes of Highly Pathogenic Avian Influenza H5N1 Subtype

MS Elnagar Eman[1], AN Gamal Maha[2], FF Zaki[2], MA Saad[1], YA Soliman[2]

Abstract

Control of avian influenza infection requires a good vaccine that could induce both humoral and cell-mediated immune response, specifically IFN-ɣ production, to maintain a high level of protection along with the minimal level of viral shedding after the infection to prevent secondary epidemics. In the current study, deoxyribonucleic Acid (DNA) vaccine coding for full-length H5 and N1 genes have been produced and evaluated in SPF-chicken. Humoral immune response estimated by haemagglutination inhibition (HI) assay revealed that the DNA vaccine gave a high titer of antibodies at the day 28-post vaccination and 14 days post-challenge. However, the shedding level was minimal with the DNA vaccine (0.1 Log 10 EID_{50}). The IFN-ɣ transcript was upregulated at a higher level in the DNA vaccinated group. The results revealed that the DNA vaccine could induce a high level of humoral and IFN-ɣ level that maintains a high level of protection (92%) with the advantage of limiting the shedding level and thus, prevent secondary epidemics.

Keywords: Avian influenza, DNA vaccine, Haemagglutinin gene (H5), IFN-ɣ, Neuraminidase gene (N1).

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Introduction

Avian influenza infection is caused by a group of viruses in the family Orthomyxoviridae, genus influenza virus A. Diagnosis of avian influenza based on virus isolation followed by molecular characterization of the haemagglutinin cleavage site to identify its pathogenicity. Infections in birds can give rise to a wide variety of symptoms that may vary depending on the host species, strains of virus, the host's immune response, environmental conditions, and the presence of secondary exacerbating organisms (OIE 2012).

Vaccination programs, along with biosafety and biosecurity measures, would be a powerful tool to support the eradication of the infectious virus from the environment through increase resistance to field challenge, eliminating viral shedding from infected birds and thus reduce viral transmission and secondary epidemics (Capua *et al*., 2004). Although vaccination is the primary method for control of the influenza disease, the lack of proofreading activity by the RNA polymerase leads to the recurrent appearance of several mutations in the virus progeny (Deng *et al*., 2006).

The most widely used completely inactivated influenza virus, vaccine, only confer optimized protection to the circulating strain of the virus (Xu *et al*., 2011). This type of vaccine cannot induce a cellular immune response, and thus viral shedding will not be hindered. Therefore due to the reasons mentioned above and due to logistical, surveillance and monetary constraints, current vaccination approaches are not sufficient. Furthermore, due to the antigenic variant characteristics of the virus, vaccines may need to be updated with frequency to new circulating strains.

DNA vaccines provide an alternative approach to maintain both humoral and cell-mediated immune responses to a higher degree. DNA vaccine, when administrated I/M or S/C, would give both humoral and cellular immune responses presented to the MHC-I and MHC-II via the resident APC such as Langerhans cells, dendritic cells and even by myocytes. The importance of induction cellular immune response may be the fundamental advantage of the use of DNA vaccine in the elimination of infection and eradication of the disease as it will prevent the shedding of the pathogen after infection (Shedlock and Weiner 2000).

In the current study, DNA vaccine based on the expression of H5 and N1 genes from locally circulating field isolate was produced and tested for its protective efficacy in chickens. Also, its ability to reduce viral shedding from dead or diseased birds was under investigation to maintain the environmental load of the virus at the minimal.

[1]Vet. Serum and vaccine research Institute, Abbasia, Cairo Agriculture Research Center (ARC)

[2]Central Laboratory for Evaluation of Veterinary Biologics, Abbasia, Cairo. 131, Elsekka Elbeida , St. Abbasia, Cairo. Egypt.

Corresponding Author: Y.A. Soliman, Central Laboratory for Evalu ation of Veterinary Biologics, Abbasia, Cairo. 131, Elsekka Elbeidast, Abbasia, Cairo. Egypt., e-mail: dryousefadel@gamil.com

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Materials and Methods

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. In order to develop a novel DNA vaccine coding for H5 and N1 genes of highly pathogenic avian influenza H5N1 subtype following steps were undertaken.

- Amplification of the full-length H5 and N1 genes of the H5N1 isolate by RT-PCR amplification
- Sequencing of the full-length H5 and N1 genes
- Cloning and expression vector (DNA vaccine) coding the full H5 and N1 genes
- Preparation of the DNA vaccine coding for H5 and N1 genes
- Vaccination of the chickens with DNA vaccine carrying Full H5 and N1 genes
- Evaluation of the protective efficacy of the DNA vaccine**:** Four parameters viz.antibody titer, protection percentage, shedding test, and measurement of the IFN-ɣ transcript level using qRT-PCR.

Avian influenza virus: (A/chicken/Qalubia/ch1.12.61/2017 (H5N1))highly pathogenic Egyptian field strain clade2.2 was used in the current study for both DNA vaccine preparation and challenge test. The strain was propagated on 9 days old SPF.ECE and the allantoic fluid was collected and clarified and stored at -20 °C

Amplification of the full-length H5 and N1 genes:

Viral RNA was extracted from the clarified Allantoic fluid according to the method of Sambrook *et al*. (1989***)*** using QIAamp Viral RNA Mini Kit (Qiagen Germany, cat #52904), as per the manufacturer's instructions. The full-length H5 and N1 genes of the H5N1 isolate were amplified by two steps RT-PCR. First, the cDNA was synthesized using M-MuLV First Strand cDNA Synthesis Kit (Biomatik cat # K5147) according to the manufacture instruction. The second step PCR was done using PfuUltra II Hotstart PCR Master Mix (Agilent USA cat # 600850). Primers that target full *orf* of H5 and N1 genes (Table 1) were designed and verified by Lasergene DNAStare software V 15 using the full-length *orf* of H5 and N1 sequences of avian influenza H5N1 Egyptian isolates retrieved from the gene bank database. The amplicons were electrophoresed on a 1% agarose and the size of the amplicons were determine using SynGene tool software V4.01 (SynGen Corporation, Cambridge, England)

Sequencing of the full-length H5 and N1 genes:

For the preparation of the full H5 and N1 genes for sequencing, the PCR products were separated on 1% low melting agarose. The bands were sliced off and purified with the biospin PCR purification kit (Biobasic cat # BSC03S1), as described by the manufacturer. Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using ABI PRISM 3730XL Analyzer BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using the primer used for PCR amplification. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Cloning of full H5 and N1 genes

The amplified full-length H5 and N1 genes were cloned separately in the donor vector using pENTR™ Directional (pENTR/SD/D-TOPO) Cloning Kits (life technologies USA Cat # K2420-20) according to the manufacture instructions, followed by transformation in TOPO 10 E.coli and inoculated on LB-Kanamycin agar and incubated overnight at 37°C. Transformants were subcultured on LB broth-kanamycin (50 ng/mL) at 37°C in a shaker incubator at a speed of 150 rpm for 24 h. (Soliman *et al*., 2016)

The transformants were analyzed by QPCR for the presence of the gene of interest. Briefly, the plasmid DNA

Table 1: Sequences of primers used in the current study

Target gene	*Primer Sequence (5'- 3')*		*Ref*
full-length orf of H5 gene	AI-H5- f	5' – **CACC**ATGGAGAAAATAGTGCTTCTTCTT- 3'	This study
	AI-H5- r	5' –AATGCAAATTCTGCATTGTAACGA- 3'	
full length orf of N1 gene	AI-N1- f	5' – **CACC**ATGAATCCAAATCAGAAGATAAT- 3'	This study
	AI-N1- r	5' – CTTGTCAATGGTGAATGGCAACT - 3'	
QPCR for H5 gene	HA5-f	5' – CAAGACTCTATCAAAACCCAACCAC- 3'	Naksupan *et al*., 2008
	HA5-f	5' – CACCCTCTCACYATCGGRGAATGCC- 3'	
QPCR for N1 gene	NA-f	5' – GTCTTTGACAGTCCCRTTGGAGTGC- 3'	Naksupan *et al*., 2008
	NA-r	5' – ACTACCKGTTCCATCATTGGGGCGT- 3'	
QPCR IFN-ɣ	IFN-ɣ-f	5'–GTGAAGAAGGTGAAAGATATCATGGA- 3'	Kaiser *et al*., 2003
	IFN-ɣ -r	5'–GCTTTGCGCTGGATTCTCA- 3'	
	IFN-ɣ p	Fam- 5'–TGGCCAAGCTCCCGATGAACGA- 3'-Tamra	
QPCR β- actin	β- actin f	5'–CTCCATCATGAAGTGTGACGTT- 3'	Hong *et al*., 2006
	β- actin –r	5'–ATCTCCTTCTGCATCCTGTCAG- 3'	
	β- actin-p	Fam-5'–CAAGGACCTCTATGCCAACACAGTGCT-- 3'Tamra	

was extracted from the overnight culture using Pure Link. HQ Mini Plasmid Purification Kit (Invitrogen Cat. # K2100-01). Ten ng of the DNA plasmid was mixed with 12.5µl of the Brilliant II QPCR Master Mix (Agilent Cat # 600804), 100nM of each of the forward and reverse primers (Table 1) and 200 nM of the dual-labeled probe. The program was adjusted at 95°C for 10 min initial denature then 40 cycles at 95°C for 30 seconds, annealing at 58°C for 30 sec and extension at 72°C for 30 sec.

Transformants that give positive C_t (indicating the presence of H5 or N1 insert) were further sub-cultured on LB-broth-kanamycin, and plasmids were purified and subjected to homologous recombination with the Gateway pcDNA-DEST40 vector (life technologies USA Cat # 12274-015) according to the manufacturer's instructions. Recombinant destination vector carrying either H5 (pDEAST40/H5) or N1 (pDEAST40/N1) genes were transformed in TOPO 10 E.coli, cultured on LB-ampicillin (100IU/ml) overnight at 37°C. Growing colonies were sub-cultured overnight on LB-ampicillin broth in shaker incubator and tested for the presence of H5 or N1 genes using QPCR as before.

Preparation of the DNA vaccine coding for H5 and N1 genes

A single colony of E.coli harboring the destination vector carrying either H5 or N1 genes was picked up in 10 mL of LB broth containing 100 IU/mL ampicillin and incubated overnight at 37°C with agitation (200 rpm) in shaker incubator. The whole culture was added to 1 litter LB broth with antibiotics and incubated as before. The culture was then cooled down on the ice and subjected to plasmid purification using Maxi prep kit (BioBasic cat # BS466). The plasmid preparation (DNA vaccine) was quantified using Qubit® dsDNA BR Assay Kit (Life technologies Cat# Q32850). The concentration was adjusted at 1 µg/µL and stored at -20°C till used for vaccination.

Vaccination of the chickens with DNA vaccine carrying Full H5 and N1 genes

Three weeks old, White Leghorn SPF chickens (Nile-SPF-eggs farm, Koom Oshiem, Fayom, Egypt) were housed in closed system Bio-isolators with sterilized food and water supply. The chickens were divided into 3 groups 25 chicken each and were vaccinated as in Table (2). At day 28 of vaccination, chicken challenged with 0.2 mL (virus titer= 6 Log 10 EID_{50}) of the challenging virus strain (A/chicken/Qalubia/ch1.12.61/2017 (H5N1)) via eye drop route.

Blood samples were taken from the jugular vein of all birds before vaccination, 15, 21, and 28 days post-vaccination (before challenge) and after 7 and 14 days post-challenge from live birds. Heparinized blood samples were taken to measure the level of the IFN-ɣ transcript at days 3, 5, 7, 15 and 28 post vaccination (before challenge), and 3,7 and 14 days post-challenge from live birds. Cloacal swaps were taken from life birds 14 days post-challenge and, when possible, from the recently died chickens.

Evaluation of the vaccine potential of the DNA vaccine coding for H5 and N1 gene.

Protection rate

The protection rate was calculated 14 days post challenge using the following equation:

$$\text{Protection percentage} = \frac{\text{(total number of bird in a group} - \text{number of dead birds}}{\text{total number of birds)}} X\ 100$$

Shedding test

Cloacal swaps were swirled in 1 mL of sterile saline and the titer of the viral shedding was measured using EID_{50} following the standard method in routine practice.

IFN-ɣ transcript quantitation using qRT-PCR

- *Purification of Avian influenza protein antigen:*

Viral proteins of the strain (A/chicken/Qalubia/ch1.12.61/2017 (H5N1) was purified using Trizol Reagent (Invitrogen cat No 15596026) according to the manufacturer's instructions. The purified protein was resuspended in CAHPS and the concentration of the protein was measured using Qubit II assay (Invitrogen Cat # Q33211) and adjusted at 5 µg/ml. The protein preparation kept in aliquots in low bind poly-propelling tubes at -80°C till used.

- *Purification of peripheral blood mononuclear cells (PBMCs) from chicken groups:*

PBMCs were separated by density-gradient centrifugation using Ficoll-Paque (biowest cat# L0560-500) following the method of Harrington *et al*. (2007). To induce the IFN-γ transcript, the cells were pulsed with either 10 µl of avian influenza protein (specific stimulant) or with PBS (unstimulated negative control cells). The cells incubated at 37 °C for 20 h in a 5% CO2 incubator. After 20 h, the cells were

Table 2: Vaccination program and doses of the experimental infection study

Group	*Symbol*	*Vaccine type and dose*
Group one (25 birds)	G1	0.5mL of DNA vaccine containing 50µg (pDEAST40/H5) and 50µg (pDEAST40/N1)
Group two (25 birds)	G2	Commercially inactivated oil adjuvanted avian influenza vaccine
Group three (25 birds)	G3	0.5 mL saline as a non-vaccinated group (control)

harvested and the total cytoplasmic RNA was purified using Trizol reagent according to the manufacturer's instructions. All RNA samples were treated with RNase-free DNase I (Qiagen cat# 79254) to remove any traces of genomic DNA contamination.

- *Quantitation of the IFN- γ transcript:*

The level of IFN-γ mRNA from cultured PBMCs either pulsed with avian influenza proteins or the un-pulsed culture was measured using Q-PCR assay (Yeong *et al*., 2014: Pete *et al*., 2003).

Total RNA was reverse transcribed to cDNA with the Omniscript cDNA Synthesis kit (Qiagen Cat # 205111). The real-time PCR reaction was performed from resultant cDNA following the method of Harrington *et al*. (2007) using 200 nM of the probe and 100 nM each of forward and reverse primers (sequence shown in table 1), and 1X of the brilliant II QPCR master mix (Agilent cat # 600804).

β actin genes were used as housekeeping non-regulated reference genes for normalization of target gene expression. The results were analyzed using Livak method. Relative transcript abundance of the IFN-γ gene equals ΔCt values (ΔCt = C_t target – C_t reference). Relative changes in the transcript were expressed as $2^{-\Delta\Delta Ct}$ values (*Schmittgen and Livak 2008*).

Statistical analysis

The statistical analysis was done using SPSS V21. ANOVA test with LSD as post hook analysis was used to calculate the significance of value at 95% confidence interval for both HI titer and IFN-γ assay.

Results and Discussion

Amplification of the full length of H5 and N1 genes of HPAI H5N1 subtype

Avian influenza virus (A/chicken/Qalubia/ch1.12.61/2017 (H5N1)) was used to amplify the full-length orf of both H5 and N1 gens to be used in the subsequent cloning procedures, as seen in Fig. 1, clear visible fragments with a molecular size of ~ 1700 bp and1400bp corresponding to the full-length H5 and N1genes respectively. The specific amplified fragments were sliced off and subjected to purification for cloning

Sequencing analysis of the full H5 and N1 genes:

Nucleotide sequence and the phylogenetic tree for the full-length H5 gene and the full-length N1 gene (Supplementary file), was created by MegAlin suit of DNASTARE software. The phylogenetic tree was built using the maximum likelihood method using the sequence of Egyptian isolates found on the gene bank and it was found that the strain used in this study has a maximum identity 98-99% with most of them. The phylogenetic tree revealed that there was minimum substitution with all test retrieved genes that make the strain suitable for DNA vaccine production

Cloning of the full –length H5 and N1 genes in the donor vector

To produce the DNA vaccine coding for full-length H5 and N1 genes, gateway technology was adopted. First, the full-length H5 and N1 amplicons were cloned in the donor vector (pENTR™/SD/D-TOPO). After the transformation of the competent *E. coli* Topo 10 cells with the recombinant donor vector, many colonies were grown after overnight incubation at 37°C. (Figures 2.A and B)

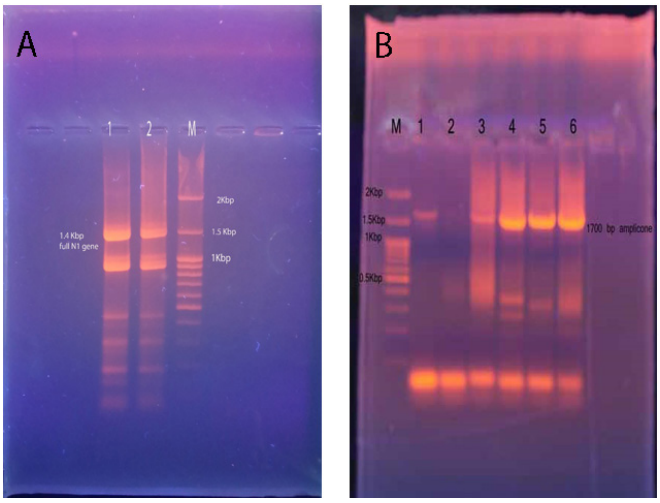

Fig. 1: The PCR amplification of the full-length N1 and H5 genes. (A) Clear visible fragment migration about 1400 bp corresponding to the full-length NA1 gene. (B) Fragment migration of about 1700 bp was seen (B). M is DNA ladder

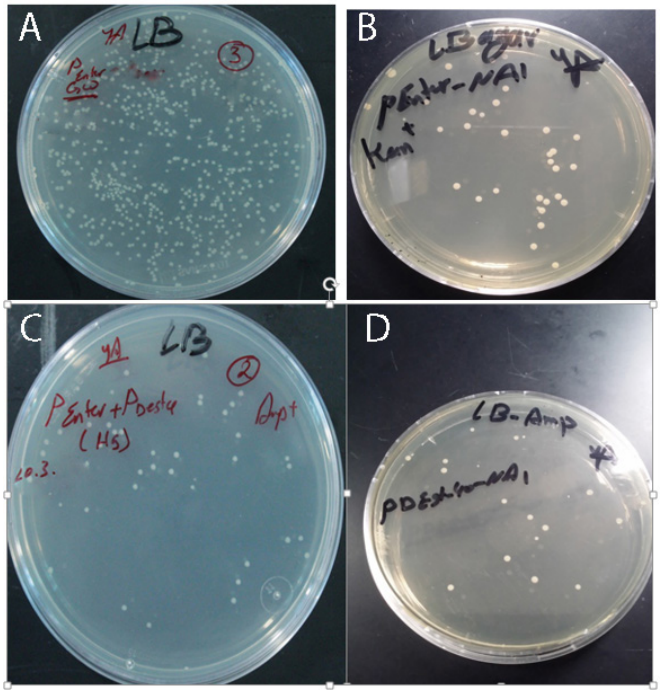

Fig. 2: .E.coliTopo 10 transformed with recombinant pENTR/SD/D-TOPO / H5 (A), pENTR/SD/D-TOPO / N1 (B), pDEST40/ H5 (C) and pDEST40/N1 (D). Note the growth of many colonies with nearly the same size after overnight culture.

Analyzing the transformants

Analyzing the transformants with miniprep

The preparation of the plasmids from the overnight culture was done to verify the size of the recombinant entry vector. The donor vector, pENT R™/SD/D-TOPO, is a 2601bp, so the expected size or the recombinant vector should be ~ 4301bp for the H5 and 4001bp for the N1 recombinant vectors respectively. As seen in Fig. 3 a very clear band migrating about 4.3 and 4.1 Kbp were seen with all the tested 8 colonies. Bands with molecular size longer also observe, which represents plasmid dimmers or trimmers. The supercoiled plasmid also has seen at the much lower size (about 3Kbp).

After analyzing the transformants with miniprep QPCR

To verify the presence of the H5 or N1 inserts in the corresponding donor vector, a real-time PCR assay was performed using miniprep as a templet and primer sets that target either H5 or N1 gens. As seen in Figures 4 A and B, the pENTR™/SD/D-TOPO carrying the full-length H5 gene gave a positive Ct range from 14.18 -15.89, and pENTR™/SD/D-TOPO carry the full-length N1 gene gave a positive Ct range from 22.25-23.14.

Homologous recombination between the entry vector and destination vector

In order to produce a mammalian expression vector, a gateway destination vector pDEST 40 was used for the homologous recombination with the entry vector. The resulted from recombinant destination vector (pDEST 40/H5) and (pDEST 40/N1) were transformed into one shot E.coli Topo 10 competent cells. After overnight culture with selective antibiotics (ampicillin), few colonies have been observed (Fig. 2 C, D).

The maxi prep of the destination plasmid was done using Maxi prep kit (Bio Basic cat # BS466), and the concentration was measured using Qubit II Fluorometer. The concentration was ranging from 80-100ng/µl (in 4mL elution volume) from 4 litter overnight culture (the total concentration thus is 320µg/litter). The DNA vaccines (pDEST 40/H5) and (pDEST 40/N1) preparations were concentrated, combined and adjusted in a concentration of 50 µg of each vaccine per 0.5 ml sterile saline

Evaluation of the protective efficacy of the DNA vaccine

Four parameters viz.antibody titer, protection percentage, shedding test, and measurement the IFN-ɣ transcript level.

The antibody titer as measured by HI

Dynamic changes of antibody titers in chickens as measured by HI assays (Table 3) revealed a significant difference ($p < 0.05$) between G1 (DNA vaccine) and G2 (available commercial vaccine) at 28 days post-vaccination and 14 days post-challenge, where there is a great difference ($p<0.05$) when G1 or G2 when compared with G3 (non- vaccinated group) at all time points.

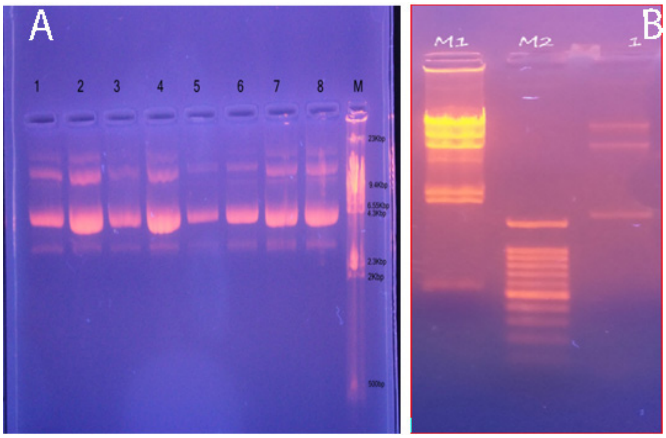

Fig. 3: Electropherogram of the miniprep from E. coli Topo10 colonies transformed with recombinant entry clone pENTR™/SD/D-TOPO carrying the full-length H5 (A) and N1 (B). Note the migration of the 4.3 Kbp recombinant vector carrying the H5 gene and 4.1 Kbp recombinant vector carrying the N1 gene. Other bands migrating at a size over 9Kbp also observed, which represents plasmid dimmers.

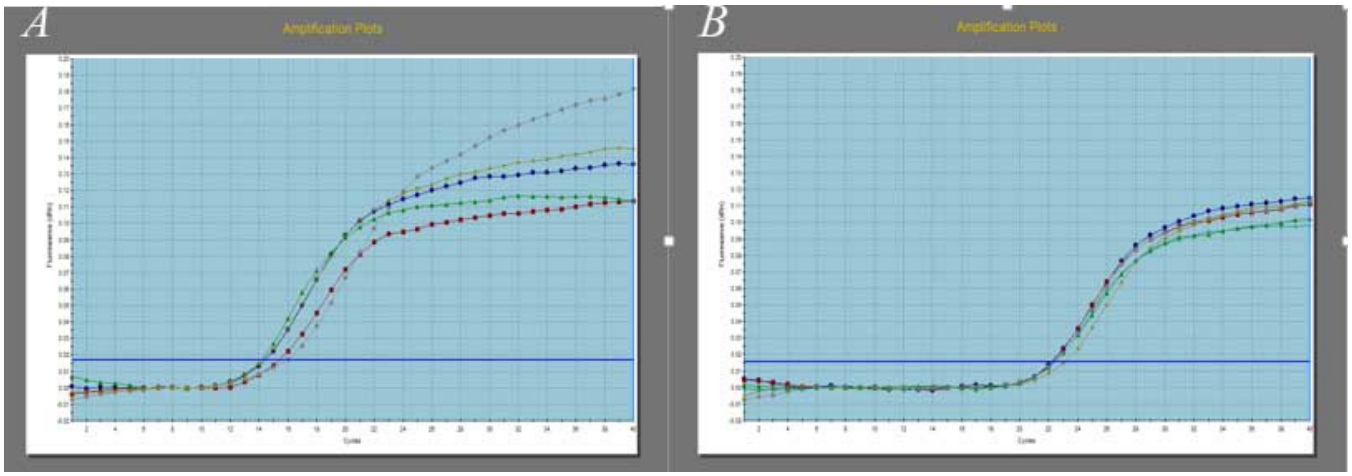

Fig. 4: Real time qPCR amplification of the H5 gene (A) and N1 gene (B) from the pENTER SD/D/H5 miniprep. All the tested miniprep gave positive Ct, indicating successful cloning.

Table 3: Mean antibody responses to pDEAST40/H5 , pDEAST40/N1, and the commercially available inactivated vaccine, in comparison with the non-vaccinated control SPF chickens as measured by HI assay. Results expressed as mean HI titer (Log 2) ± SE

	Mean HI titer (Log2) ± SE					
	Days post-vaccination				*Days post-challenge*	
Groups	*0*	*15*	*21*	*28*	*7*	*15*
Group 1 (G1) pDEAT40/H5 and (pDEST 40/N1) DNA vaccines	0	8.88 ± 0.441	9.5 ± 0.25	11.63 ± 0.42	8.5 ± 0.423	13.63 ±0.42
Group 2 (G2) Commercially available inactivated vaccine	0	5.75 ± 0.412	8.25 ± 0.164	9.5 ± 0.327	7.5 ± 0.327	10.13 ± 0.398
Group 3 (G3) Control non vaccinated	0	0	0	0	No bird survive	

Protection percentage

After the challenge, DNA vaccinated chickens (G1) showed no sign of disease. The birds remained healthy, active with eating and drinking rates normal. The survival rate in this group was 92%, and the dead birds (deaths were at day 10-12) showed very mild post mortem signs not more than slight cyanosed combs (Table 4).

Birds in G2 (vaccinated with the commercially available vaccine) showed depression with loss of appetite, roughen feather. This vaccine could not provide more than 80% survival rate with extreme PM lesions externally (the cyanosed combs and wattles, hemorrhage on the chunk) or internally (blood spots on the coronary fat, hemorrhagic tracheas)

The shedding as measured by EID50:

Cloacal swaps were taken from all birds survived after the challenge and from dead birds within 24hours of death. The cloacal swaps were kept in sterile saline at -20C until the EID50 test was performed. As shown in Table 4, the DNA vaccine managed to decrease the shedding level to as minimal 10 in comparison to the inactivated commercially available vaccine (3.25 Log 10) . In contrast, the level of shedding in the non-vaccinated group were as high as 5.24 Log 10.

Measurement the IFN-γ transcript level:

After vaccination, G1 showed slight elevation of the transcript level by the 5th day and seen till the 7th days post-vaccination only while G2 and G3 showed no detectable level during the 28 days post-vaccination (Table 5 and Fig. 5)

However, after challenge, G1 group showed an increased level of IFN-γ transcript by the 3rd days post-challenge (912838), which reach its maximum by the 7th-day post-challenge (4E+06) then begin to decline (558037), yet it remains significantly higher than the G2 ($p < 0.05$). G2 showed a moderate increase of the transcript level by the 3rd-day post-challenge that lasts till the 14th days post-challenge. All birds in G3 group were dead by the 3rd day post-challenge.

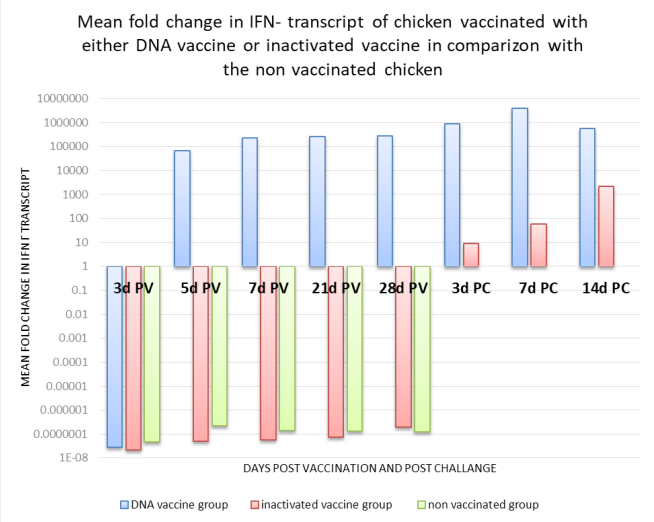

Fig. 5: Mean fold change in IFN- transcript of chicken vaccinated with either DNA vaccine or inactivated vaccine in comparison with the non-vaccinated chicken

Table 4: The protection rate, the lesions and the shedding level recorded with each group after 14 days of challenge (- No lesions:+ Very mild lesions (one sign only externally or internally): ++ Moderate lesions (more than one sign): ++++sever lesions (typical for natural infection)

Group	*Vaccine type*	*#of deaths*	*Survival rate %*	*Presence of signs on dead birds*	*Presence of P/M lesions*	*Mean EID 50 of the shedding*
G1	pDEAST40/H5 and pDEAST40/N1	2/25	92	-	-	0
G2	Commercially available vaccine	5/25	80	++	++	3.25
G3	Control non vaccinated	25/25	0	++++	++++	5.24

Table 5: Transcriptional level of the target IFN-γ gene expressed as fold change post-vaccination and post-challenge.

	Transcriptional level of the target IFN-γ gene expressed as fold change							
Groups	*Days post-vaccination(PV)*					*Days post-challenge(PC)*		
	3d	*5d*	*7d*	*21d*	*28d*	*3d*	*7d*	*14d*
G1	2.83908E-08	64633.7	221969.2	267652.2	269513.8	912838	4E+06	558037
G2	2.15162E-08	4.8414E-08	5.75743E-08	7.49238E-08	1.96359E-07	9.1896	59.302	2241.1
G3	4.70901E-08	2.25558E-07	1.39813E-07	1.27765E-07	1.20873E-07	All birds were died		

Discussion

Vaccination programs against avian influenza would be a powerful tool for viral eradication if used along with biosafety measures. Two main parameters should be established with a good vaccine candidate. First, the vaccine should give a high percentage level; second, the vaccine should decrease and limit viral shedding to prevent secondary epidemics and could eliminate the virus from the environment. In the current study, DNA vaccine coding for full-length H5 and N1 have fulfilled these two main parameters.

The full-length H5 and N1 genes (orf) were amplified from the selected strain (A/chicken/Qalubia/ch2.2.64/2017 (H5N1)). The specific amplicon migrating about 1700 and 1400bp corresponding to H5 and N1 respectively, were seen; many nonspecific amplification fragments have also seen, which may result from the relatively lower annealing temp and long extension time changing these condition resulted in negative results. After cloning in the entry vector, the miniprep revealed the 4 Kbp product corresponding to the recombinant pENTR™/SD/D-TOPO/N1 and 4.3Kbp product corresponding to the recombinant pENTR™/SD/D-TOPO/H5 with other bands at 9Kbp representing polymer forms of the plasmids. This phenomenon always is seen in the high copy number plasmids. qPCR quantitation of the miniprep gave positive Ct values (Ct range 14.18-15.86) for pENTR™/SD/D-TOPO/H5 and Ct values (Ct range 22.25-23.14) for pENTR™/SD/D-TOPO/N1, this difference in the concentration of plasmids may be due to difference in the efficiency of cloning. The mammalian expression vector (pDEST40) has a molecular size of 7.1kb. This vector derived from the pcDNA 3.1/V5-His vector. It is designed to allow high-level, constitutive expression of the gene of interest (e.g. haemagglutinin H5 and neuraminidase N1) in a variety of mammalian hosts. PDEST40 contains the essential elements for controlling the selective and high-level expression of the gene inserts in the mammalian cells, including Human cytomegalovirus immediate-early (CMV) promoter/enhancer for high-level expression (Boshart, *et al.,* 1985 and Southern and Berg 1982).

The HI titer revealed that DNA vaccine in a single dose (which was significant higher than inactivated vaccine) could elicit a powerful humoral immune response that prevent the viral cell entry through binding to the HA protein antigen with subsequent reducing both mortalities and the clinical signs observed with infected birds (Angeletti *et al*., 2017). One of the very important outcomes of the use of the DNA vaccine is the control of the shedding. The shedding seen in the group vaccinated with the pcDEST40/H5, and N1 was zero, which is significantly different ($p < 0.05$) than that of the group received the inactivated vaccine(3.25 Log 10 EID_{50}) and with the control non-vaccinated chicks (5.24 Log 10 EID_{50}). In the current study, the level of IFN-γ transcript was very high even after 14 days post-challenge, which explains the zero shedding level in the group received the DNA vaccine. The fold change in the transcriptional level of IFN-γ was significantly higher ($p < 0.05$) in the DNA vaccinated group when compared with either the group vaccinated with the inactivated commercial vaccine or the negative control group at all time point tested.

Based on the current study, the DNA vaccine coding for H5 and N1 genes would be a suitable alternative to the traditional oil adjuvanted inactivated vaccines mainly due to its inexpensive, rapidly produced, stable, and safe properties (Kutzler, and Weiner 2008) along with its capability to produce both humoral and cellular immune response that gave both high protection level with zero viral shedding after challenge. Other advantages, including the ability to express diverse antigens, rapid production, and inability to revert into virulent forms, easy storage, long shelf life, and possible avoidance of the cold chain, also have much consideration in the commercialization of this type of vaccines.

Conclusion

In conclusion, the pDEST 40/H5 and pDEST 40/N1 gave a very high percentage of protection (92%) and very high HI titer and IFN-γ level, this DNA vaccine also give a zero level of viral shedding after challenge which will prevent secondary epidemics and might be useful in eradication of the virus from the country.

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Appendix

Fig. 1: nucleotide sequence of the full-length H5 gene of HPAI-H5N1 subtype isolate (A/chicken/Qalubia/ch1.12.61/2017 (H5N1))

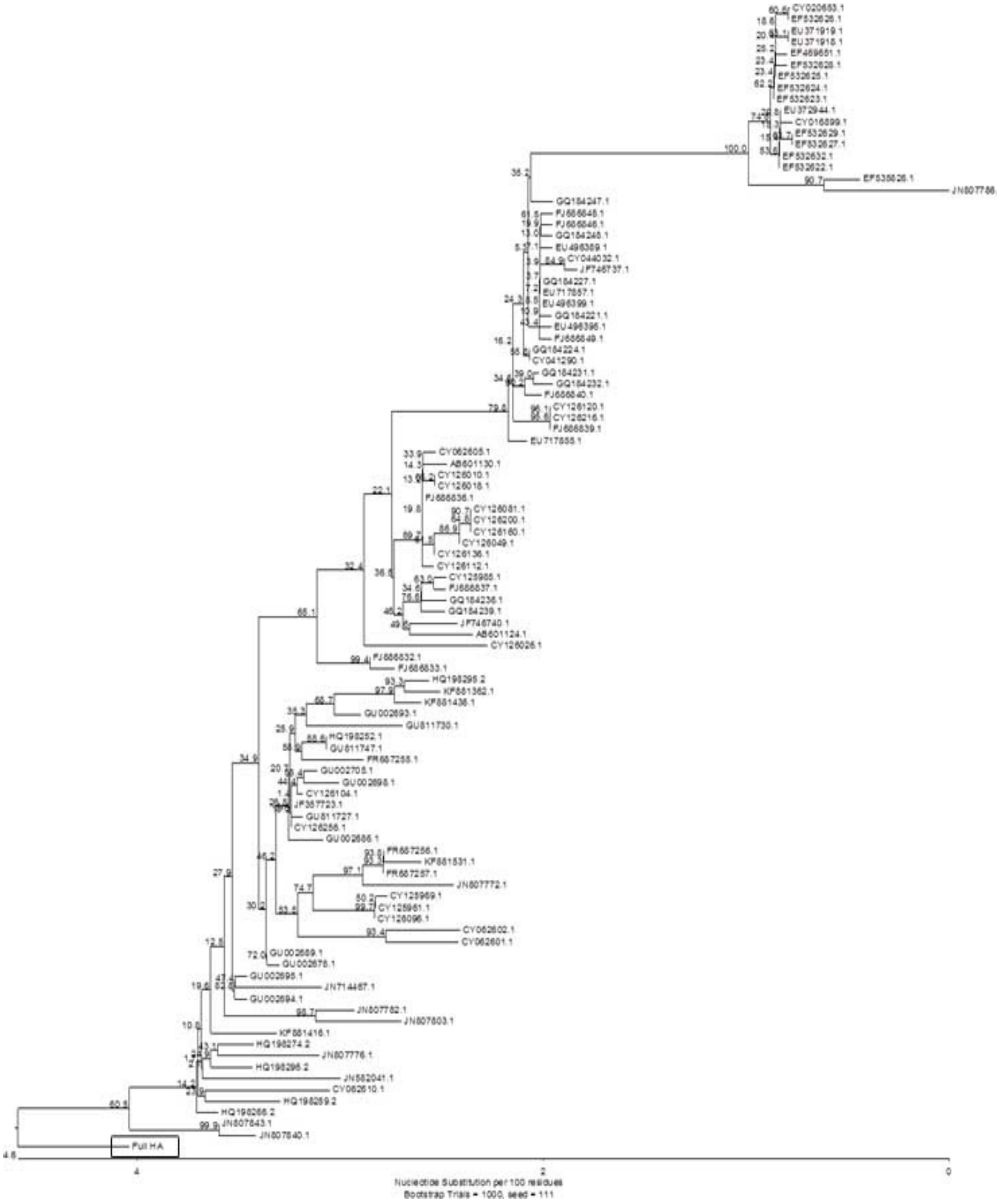

Fig. 2: Phylogenetic trees for the HA gene of the Full length HA of H5N1 used for cloning (A/chicken/Qalubia/ch1.12.61/2017 H5N1) (red arrow). The trees were generated by using DNASTAR V15 software by the maximum likelihood method (Bootstrap test: 1000). The length of each pair of branches represents the distance between the sequence pairs, and the units at the bottom of the tree indicate the number of substitution events.

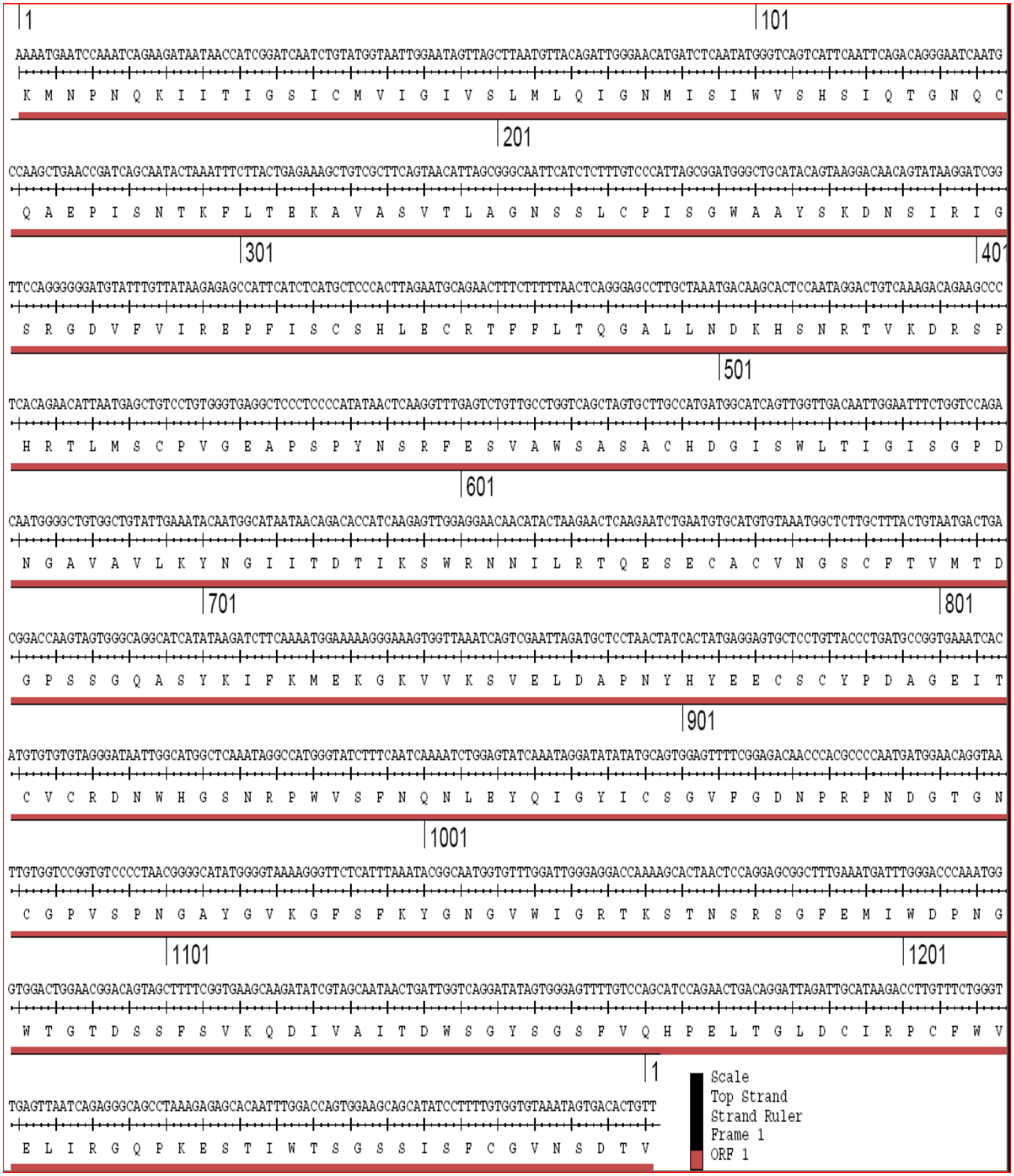

Fig. 3: nucleotide sequence and deduced amino acid sequence of the full length N1 gene of the isolate (A/chicken/Qalubia/ch1.12.61/2017 (H5N1)) used in the cloning procedure in the current study