Research Article

Evaluation of Codon Optimized DNA Vaccine Against Avian Influenza A Viruses using Local Egyptian Strain of H9N2

Wesam Hasan Mohamed Mady [1*], Bing Liu[2], Dong Huang[2], A. Arafa[1], M.K. Hassan[1], M.M. Aly[1], Pucheng Chen[2], Yongping Jiang[2*] and Hualan Chen[2]

[1]Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Agriculture Research Center, Egypt; [2]National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, CAAS, Harbin Heilongjiang, 150069.

Abstract | The low pathogenic avian influenza viruses (LPAIV) subtype H9N2 are highly prevalent in the Middle East and are causing economic losses in the commercial poultry production sectors. Due to potential of H9N2 infections in human, LPAI H9N2 poses serious public health risk. Therefore, development of new potent and cost-effective influenza vaccines is urgently needed to safeguard poultry and to contain zoonosis. The primary goal of this study was to develop H9 plasmid-based DNA vaccine against the currently circulating AIV subtype H9N2 targeting HA gene of the virus A/Turkey/Egypt/1341V/2013(H9N2). The full length sequence of HA gene was codon optimized to the chicken biased codons and subcloned into the pCAGGS plasmid vector under the control of the chicken β-actin promoter. The *in vitro* expression of recombinant plasmid DNA was performed by transfection of 293T (HEK) cell line. The HA protein was analyzed using SDS-PAGE followed by Western blot and immunofluorescence assays. The pCAGG-optiH9 vaccine efficacy was evaluated by intramuscular immunization of SPF chickens with different concentrations of plasmid DNA and the sera were collected weekly post vaccination for antibody detection by HI test. All immunized chickens shown high HI antibody titers ($9Log_2$) two weeks post-booster dose. The chickens were then challenged using homogenous AI H9N2 strain. During the course of infections, oropharyngeal and cloacal swabs were collected from all chickens at 3, 5 and 7 days post-challenge (p.c.) for virus titration in eggs. Data indicated that all chickens vaccinated with pCA-Egy-H9 were fully protected without clinical signs and virus shedding. Our results revealed that the pCA-Egy-H9 plasmid DNA vaccine could induce complete protection in chickens against H9N2 virus challenge and may propose an alternative strategy to control virus infections in the poultry industry.

Editor | Muhammad Munir, The Pirbright Institute, UK.
Received | February 04, 2017; **Accepted** | February 28, 2017; **Published** | March 04, 2017
***Correspondence** | Wesam Hasan Mohamed Mady, National Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, P.O. Box 264-Dokki,Giza-12618, Egypt; **Email**: wesammady83@hotmail.com; Yongping Jiang, Harbin Veterinary Research Institute, CAAS, 678 Haping Road, Harbin 150069, P. R. China. **Email:** ypjiang99@163.com
DOI | http://dx.doi.org/10.17582/journal.hv/2017/4.2.21.27
Citation | Mady, W.H.M., B. Liu, D. Huang, A. Arafa, M.K. Hassan, M.M. Aly, P. Chen, Y. Jiang and H. Chen. 2017. Evaluation of codon optimized DNA vaccine against avian influenza a viruses using local Egyptian strain of h9n2. *Hosts and Viruses*, 4(2): 21-27.
Keywords: DNA vaccine, Avian influenza virus, Protein expression, H9N2 avian influenza, Codon optimization

Introduction

The H9N2 subtype of avian influenza virus A (AIV), member of the family *Orthomyxoviridae*, are causing panzootic in the Middle East and Asia over the last decade and have been recorded in different sectors of poultry worldwide (Guo et al., 2000). It is associated with serious disease in poultry and is occasionally accompanied by considerable mortality (Brown et al., 2006). Infection with H9N2 virus-

es often result in decreasing the laying rate of hens, and co-infection with other viruses or bacteria, can cause severe morbidity and high mortality in chickens (Kishida et al., 2004 and Kim et al., 2006).

H9N2 AIV is prevalent in chickens, ducks and other poultry species, but has demonstrated the ability to infect human beings (Li et al., 2003), as the virus possesses human virus-like receptor specificity, and it can infect humans producing flu-like illness (Wan and perez, 2007).

H9N2 AIV infections in pig farms have also been confirmed in several provinces in China (Xu et al., 2004), suggesting that H9N2 AIV can cross the species barrier to infect other animals in addition to humans. Thus, transmission to other poultry species may cause significant genetic and antigenic changes (Park et al., 2011).

Based on phylogenetic analysis of the HA gene of H9N2 viruses, there are, so far, two major genetic lineages; the North American and Eurasian lineages (Banks et al., 2000). In the latter, several sublineages have been distinguished: The G1-like sublineage was established in the Middle East and on the Indian subcontinent in the 1990s while other sublineages (Y280 and Ck/bei-like) circulate mainly in countries of the Far East (Shahsavandi et al. 2011). Recent detection of avian influenza H9N2 subtype has been recorded in bobwhite quails in Egypt in 2011 with genetic relatedness to the G1-lineage represented by A/quail/Hong Kong/G1/97 (Qa/HK/G1/97) indicates that this lineage is still circulating in the Middle East region (El-Zoghby et al., 2012). Phylogenetically, the Egyptian H9N2 virus was closely related to viruses of the G1-like lineage isolated from neighboring countries, indicating possible epidemiological links (El-Zoghby et al., 2012). Genetic and phylogenetic analysis of the HA gene of Qa/Egypt/11 showed the closest overall relationship to the Israeli strains (Tu/IS/1608/06 and Ck/IS/182/08) in comparison with other H9N2 viruses that were previously circulating in the Middle East region from 2006 to 2010 (Arafa et al., 2012). The topologies of all of the eight phylogenetic trees revealed substantial genetic diversity and frequent re-assortment events for the Egyptian H9N2 virus, which could be schematically identified as intra-subtype exchanges (between different H9 genetic clusters) (Arafa et al., 2012). These studies collectively indicate the potential of the H9N2 infections to prevail and to post threats to public health and commercial poultry.

Therefore, the development of cost-effective vaccine that could protect chickens from H9N2 avian influenza and prevent the spread of the virus is urgently needed. DNA-based immunization offers a promising strategy to prevent viral diseases (Wu et al., 2011). Thus, the primary aim of this study was to develop an H9 DNA vaccine, which can be produced on a large scale.

Materials and Methods

Virus strain

Low pathogenic avian influenza virus strain A/Turkey/Egypt/1341V/2013(H9N2) isolated in Reference Laboratory for Veterinary Quality Control on Poultry Production (RLQP) was used. The virus belong to genotype G1 and is available in the GenBank under accession No. KJ781214.

Construction of pCA-Egy-H9 plasmid DNA

The full length sequence of HA gene was codon optimized to the chicken biased codons using DNASTAR from a codon usage database. The Kozak sequence (GCCGCCACC) was inserted before the ATG start codon, restriction endonuclease site sequence for EcoRI was inserted upstream of the Kozak sequence and the XhoI site was inserted to the 5' end of the downstream primer followed by the codon optimized HA gene was chemically synthesized by GenScript (Nanjing) Co., Ltd., China. The synthetic HA gene with codons optimized for chicken usage (optiHA) was subcloned into the pCAGGS plasmid vector under the control of the chicken β-actin promoter (provided by Harbin Veterinary Research Institute, China) to generate the DNA construct pCA-Egy-H9.

The plasmid pCA-Egy-H9 was transformed in Top10 competent *E. coli* cells, screened by PCR using specific primers designed in Harbin Veterinary Research Institute (HVRI) available upon request and confirmed with digestion analysis using EcoR1 and XhoI restriction enzymes, and extracted by Axygen miniprep plasmid extraction kit.

In vitro expression of pCA-Egy-H9 plasmid DNA

Monolayer of 80–90% confluent 293T-HEK cells in 6-well plate were transfected with 4 μg of the pCA-

Egy-H9 plasmid using Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. A total of 200 µl OptiMEM was mixed with 10 µl Lipofectamine and incubated 5 minutes at room temperature. In another tube, a total of 200 µl of optiMEM was added in 4 µg plasmid DNA. DNA and Lipofectamine were then mixed (2.5µl of Lipofectamine 2000/µgDNA), incubated at room temperature for 30 min, and added directly to the cells. Six hours later, the DNA-transfection reagent mixture was replaced by DMEM (GIBCO/BRL, Carlsbad, CA) containing 10% fetal calf serum.

Protein analysis and *in vitro* confirmation of protein expression

Immunofluorescence Assay (IFA): Forty-eight hours after transfection, the media was aspirated and the cells was fixed by 4% paraformaldehyde for 30 min at room temperature, and then washed triple times by PBS. 0.1% triton X-100 was added for 10 minutes and then washing again with PBS followed by 1% BSA (Bovine serum albumin) addition for 30-60 minutes at room temperature. Cells were stained using primary antibodies diluted with a ratio of 1:200 in BSA for 1 hour at 37˚C and the cells were washed triple times with 0.5% tween 20 in PBS (PBS-T). Secondary antibodies, diluted 1:500 in BSA, was added for 1 hour at 37˚C and cells washed again with PBS-T, DAPI (100 ng/ml) was added for 10 minutes at room temperature and the cells washed one time with PBS-T then imaged under the fluorescent microscope.

Western Blotting: Forty-eight hours after transfection, the cells were reconstituted in RIPA buffer and 100X PMSF before loading and separation on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membrane. The membrane was blocked in 5% milk at 4ºC overnight and then probed with primary antibodies, which are raised in chicken against H9-AIV (specific antiserum) diluted in 1:200, followed by addition of secondary antibodies (conjugate) diluted in 1:1000.

Evaluation of pCAGGS-optiH9 plasmid DNA vaccine efficacy

Three weeks old SPF chickens were divided into 3 groups in HEPA-filtered isolators, the plasmid DNA was re-suspended in phosphate-buffered saline (PBS, pH 7.2) at a final concentration of 15 µg for the 1st group and 60 µg for the second group, and the 3rd group is positive control (challenged non-vaccinated) group. The chickens were immunized by intramuscular injection of 0.2 ml plasmid DNA in the thigh muscle. Three weeks later, the chickens were given booster vaccinations using the same dose and delivery route. Two weeks later after the booster dose, the chickens were challenged with homologous A/chicken/Egypt/A2-D/2011(H9N2) (10^6EID_{50})intranasally. Chickens were observed daily for disease signs and death for 2 weeks, Oropharyngeal and cloacal swabs were collected from all chickens at 3, 5 and 7 days post-challenge (p.c.) for virus titration in eggs, and sera were collected weekly post-vaccination (p.v.) for detection of hemagglutination inhibition (HI) antibody by using HI test according to recommendation of the OIE, 2009.

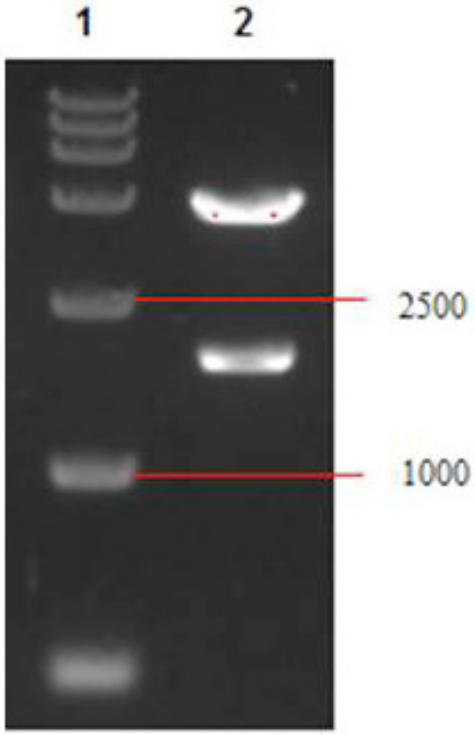

Figure 1: *The restriction enzyme digestion analysis. Lane (1) is the molecular weight marker, and lane (2) is the digested opti-H9 gene with EcoR1 and XhoI restriction enzymes and showing the bands of expected sizes (1.7 Kb for HA and 4.7 Kb for pCAGGS plasmid vector).*

Results and Discussions

Construction of pCAGGS-optiHA9 plasmid DNA

The constructed pCA-Egy-H9 was transformed in Top10 competent *E. coli* cells and the positive clones were screened by PCR, and restriction enzymes digestion analysis for identification of synthetic full-length codon optimized HA gene. The HA gene showed the expected size (1.7 Kb) by PCR and the restriction enzyme digestion analysis of the inserted opti-HA gene

in pCAGGS expression plasmid showed the expected sizes 1700 bp for HA and 4746 bp for pCAGGS plasmid (Figure 1). Further characterization of the construct was done by sequencing for confirmation of the fidelity and direction of the inserted optiHA gene. The alignment showed that the sequence of optiHA was consistent as expected.

Protein analysis and *in vitro* confirmation of protein expression

Expression of H9 HA gene was confirmed by immunofluorescence assay and Western blotting in the transfected 293T-HEK cells with pCA-Egy-H9.

Indirect immunofluorescence assay (IFA): Expression of Opti-H9 gene was confirmed by immunostaining of 293T-HEK cells transfected with pCA-Egy-H9 after incubation with H9 specific chicken antisera. The transfected cells showed bright specific fluorescence indicating successful H9 protein expression (Figure 2).

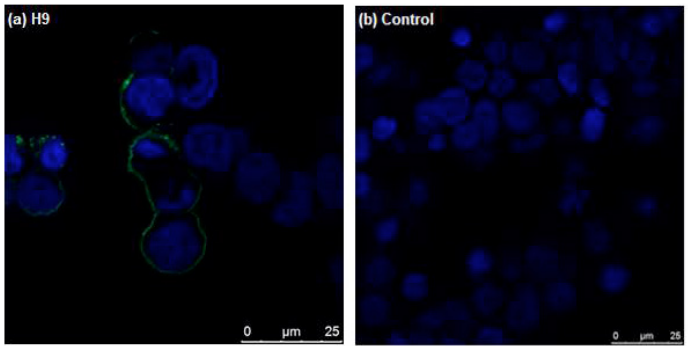

Figure 2: (a) *Immunofluorescence staining of 293T-HEK cells transfected with pCAGG-optiH9 showing the specific fluorescence.* **(b)** *Negative control 293T-HEK cells showing no fluorescence.*

Western blot (WB): The expression of opti-H9 gene in 293T-HEK cells was analyzed by SDS-PAGE and western blotting 48 hours after transfection. The expressed H9 protein was successfully detected giving the specific band (Figure 3).

Antibody response induced by pCAGG-optiH9 plasmid DNA

After immunization of chickens with different concentrations of pCA-Egy-H9 (15 µg and 60 µg), all the vaccinated chickens developed detectable HI antibody titers which increased sharply post booster immunization. The HI antibodies were detectable up to $5Log_2$ two weeks after immunization with the 1st dose of pCAGG-optiH9 plasmid DNA and the antibody titers were increased up to $9Log_2$ two weeks after the booster immunization (Figure 4).

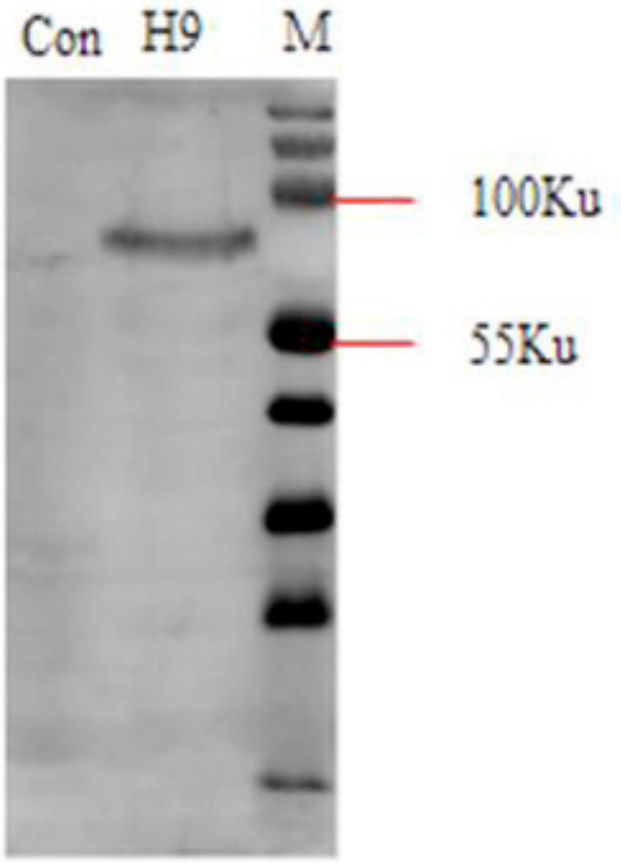

Figure 3: *Western blot analysis of pCAGGS plasmid expressing opti-H9 HA, M is the molecular weight marker, H9 is the expressed HA protein of pCA-Egy-H9 showing the specific band of approximately 70 KDa, and con is the negative control.*

Protective efficacy of pCA-Egy-H9 in chickens against the low pathogenic H9N2 avian influenza virus challenge

The pCA-Egy-H9 induced strong immune response and complete protection up to 100% after challenge with H9N2 low pathogenic avian influenza virus strain. After challenge chickens were observed daily for signs of disease for 2 weeks, all immunized chickens with pCA-Egy-H9 were greatly protected from virus challenge and no disease signs were observed. Oropharyngeal and cloacal swabs were titrated for virus shedding in SPF embryonated chicken eggs (ECE). No virus shedding was observed in all the chickens immunized with pCA-Egy-H9 plasmid DNA **(**Table 1**)**.

Low pathogenic avian influenza (LPAI) H9N2 virus has been recorded in Egypt since 2011 (El Zoghby et al., 2012 and Arafa et al., 2012). The presence of avian influenza virus subtype H9N2 in poultry in Egypt is a major concern since it becomes widely distributed in Egypt and since many AI H5N1 and H9N2 natural co-infections were recorded because of the high pos

Table 1: *The virus titrations from swabs post H9N2 virus challenge*[A]

Vaccines	Doses	Virus isolation from swabs: shedding/total (titer, $log_{10}EID_{50}$/ml)											
		Day 3		Day 5		Day7		Day9		Day11		Day13	
		Oro-pharyngeal	Cloacal	Oro-pharyn-geal	Cloacal	Oro-pharyngeal	Cloacal	Oro-pharyn-geal	Cloacal	Oro-pharyn-geal	Cloacal	Oro-pharyn-geal	Cloacal
pCA-Egy-H9	15µg	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
	60µg	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Control	PBS	10/10(2.3)	6/10(2.2)	5/10(1.2)	6/10(1.8)	2/10(2.4)	1/10(3.5)	2/10(+)	1/10(+)	1/10(+)	2/10(+)	0/10	0/10

[A] *Groups of 3-week-old SPF chickens were inoculated intramuscularly with 15 µg and 60 µg of pCA-Egy-H9 in 100 mL of PBS or with 100 mL of PBS as a control. Animals were challenged intranasally with 10^6EID_{50} of A/chicken/Egypt/A2-D/2011(H9N2) virus. Swabs were suspended in 1 mL of PBS and titrated for virus shedding in eggs at an initial dilution of 1:10, or undiluted if negative at the lowest dilution.*

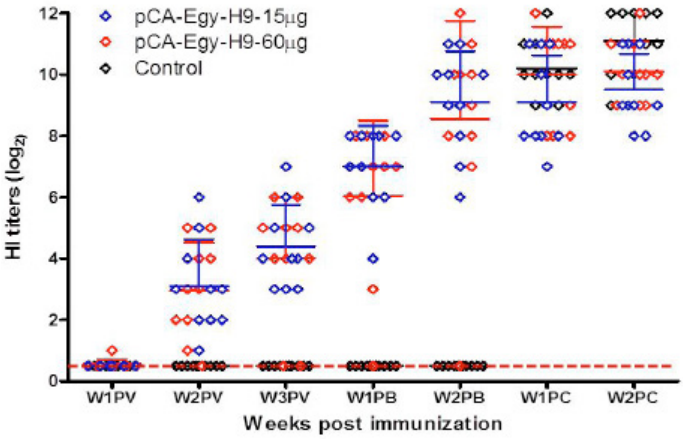

Figure 4: *HI antibody titer (Log2) in the chickens immunized with the pCA-Egy-H9 plasmid DNA vaccine.*

sibilities for reassortant between both HPAI H5N1 and LPAI H9N2 viruses, which may leads to generation of new AI viruses that may have higher public health hazard and causing higher economic losses. The current available inactivated whole virus vaccines for H9N2 AIV require large numbers of specific pathogen free embryonated chicken eggs and about 6 months to propagate the viruses and it give poor protection against the existing strains in Egypt. So the development of AI vaccine from the same strain circulating in the field is of great need for H9N2 AI control. The development of cost-effective avian influenza vaccine is a priority to prevent pandemic flu outbreaks. Because hemagglutinin (HA) protein is a major viral surface antigen for which the most of neutralizing antibodies are elicited, recombinant HA remained a candidate avian influenza DNA vaccine.

In our study, we constructed pCAGGS vector encoding codon optimized HA gene. The full-length sequence of HA gene was codon optimized to the chicken biased codons and the Kozak sequence (GCCGCCACC) was inserted before the ATG start codon. The pCA-Egy-H9 administered by intramuscular injection induced high HI antibody titers for H9 antibodies and this reported that optimization of the HA gene and expression vector and insertion of Kozak sequence resulted in the high seropositive conversion.

According to Jiang Y.P. et al. 2007, the use of chicken optimized codons in the HA gene resulted in great increase in the expression of the gene in chickens. Previous studies reported that the high GC ratio in the optiHA gene may increase its mRNA stability, processing and nucleocytoplasmic transport. Also, the number of CpG motif was increased to 16 in the optiHA, while there are only 2 in the wild-type HA gene, and it is possible that these motifs may also contribute to the increased immunogenicity of the optiHA sequence (Krieg et al., 1998).

Our results also were also in agreement with Songhua shan et al. 2011, who have reported that insertion of Kozak sequence and use of the high efficiency pCAGGS vector synergistically contributed to higher level of HA expression than other plasmid vectors and result in measurable antibody response in chickens. The Kozak consensus sequence plays a major role in the initiation of the translation process, and it is important and required for optimal translational efficiency of expressed mammalian genes (Kozak, 1987 and 1997).

According to Jiang et al. (2006), groups treated with both 100 µg and 10 µg the optiHA5 constructs induced higher levels of antibody responses in chickens than the wild-type HA constructs. This trend was also observed with the vector pCAGGS compared to the pCI constructs (Jiang et al., 2007) and this may be due to the promoter/enhancer elements or other transcriptional elements in pCAGGS. The pCAGGS

vector contains the β-actin/CMV promoter/enhancer and the bovine growth hormone (BGH) poly(A) signal, which previous studies have indicated leads to stronger expression than RSV, SV40, PGK or CMV promoters (Bu et al., 2003; Xu et al., 2001). Therefore, the promoter activities may have led to high gene expression, which could influence the immune responses elicited by the pCAGGS-based constructs (Jiang Y.P., 2007).

To evaluate the protective efficacy of the pCAGG-optiH9 plasmid DNA, the chickens were challenged with H9N2 Low pathogenic avian influenza virus strain. No disease signs were observed in the chickens immunized with pCAGGS-optiH9 plasmid DNA. For evaluation of virus shedding, the collected oropharengeal and cloacal swabs were inoculated in SPF ECE for virus isolation and titration. All chickens immunized with pCA-Egy-H9 were virus free and no virus shedding was observed (Table 1) implying complete protection from low pathogenic avian influenza virus.

Taken together, our results showed that pCA-Egy-H9 could completely protect chickens from H9N2 virus challenge and this was revealed by absence of virus shedding, and clinical signs. Since the AI antibody is the major correlate of protection and is the major determinants for a protective response to avian influenza virus, the high titer of H9 antibodies in this study reflected the high protective efficiency of the the pCAGG-optiHA DNA vaccine.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Contribution

W.H. Mady, A. Arafa and Yongping Jiang conceived the study; W. H. Mady, Bin Liu and Dong Huang performed the research; W.H. Mady, Bin Liu, Dong Huang, A. Arafa, M.K. Hassan, M.M. Aly, Pucheng Chen, Yongping Jiang and Hualan Chen were involved in drafting the work and revising it critically for important intellectual content. W. H. Mady, A. Arafa and Yongping Jiang wrote the manuscript. And W.H. Mady, Bin Liu, Dong Huang, A. Arafa, M.K. Hassan, M.M. Aly, Pucheng Chen, Yongping Jiang, Hualan Chen compiled the final approved version to be published.

References

Arafa, A.S., N. Hagag, A. Erfan, W. Mady, M. El-Husseiny, A. Adel and S. Nasef. 2012. Complete genome characterization of avian influenza virus subtype H9N2 from a commercial quail flock in Egypt. virus Gen. https://doi.org/10.1007/s11262-012-0775-0

Banks, J., E.C. Speidel, P.A. Harris, D.J. Alexander. 2000. Phylogenetic analysis of influenza A viruses of H9 haemagglutinin subtype. Avian Pathol. 29:353–359. https://doi.org/10.1080/03079450050118485

Brown, I.H., J. Banks, R.J. Manvell, S.C. Essen, W. Shell, M. Slomka, B. Londt, and D.J.Alexander. 2006. Recent epidemiology and ecology of influenza A viruses in avian species in Europe and the Middle East. Dev. Biol. (Basel). 124:45–50.

Bu Z., Ling Y., Compans R.W., Yang C., 2003. Enhanced cellular immune response against SIV Gag induced by immunization with DNA vaccines expressing assembly and release-defective SIV Gag proteins. Virology 309: 272–281.

El-Zoghby, E.F., A. Arafa, M.K. Hassan, M.M. Aly, A. Selim, W.H. Kilany, U. Selim, S. Nasef, M.G. Aggor, E.M. Abdelwhab and M.H. Hafez. 2012. Isolation of H9N2 avian influenza virus from bobwhite quail (Colinus virginianus) in Egypt. Arch. Virol. https://doi.org/10.1007/s00705-012-1269-z

Guo, Y.J., S. Krauss, D.A. Senne, I.P. Mo, K.S. Lo, X.P. Xiong, M. Norwood, K.F. Shortridge, R.G. Webster and Y. Guan. 2000. Characterization of the pathogenicity of members of the newly established H9N2 influenza virus lineages in Asia. Virol. 267:279–288. https://doi.org/10.1006/viro.1999.0115

Jiang Y.P., H. Zhang, C. Li, Z. Bu, G. Deng, K. Yu and H. Chen. 2006. Comparison of protective effects among H5 subtype avian influenza DNA vaccines with optimized HA gene and expressive vectors. J. Agric. Biotech. 14(3): 301–306

Jiang Y.P., K. Yu, H. Zhang, P. Zhang, C. Li, J. Tian, Y. Li, X. Wang, J. Ge, Z. Bu and H. Chen. 2007. Enhanced protective efficacy of H5 subtype avian influenza DNA vaccine with codon optimized HA gene in a pCAGGS plasmid vector. Antiviral Res., 75: 234–241. https://doi.org/10.1016/j.antiviral.2007.03.009

Kim J.A., S.H. Cho, H.S. Kim, S.H. Seo. 2006. H9N2 influenza viruses isolated from poul-

try in Korean live bird markets continuously evolve and cause the severe clinical signs in layers.Vet. Microbiol. 118: 169–176. https://doi.org/10.1016/j.vetmic.2006.07.007

Kozak, M.1987. At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. J. Mol. Biol.196:947-950. https://doi.org/10.1016/0022-2836(87)90418-9

Kozak, M. 1997. Recognition of AUG and alternative initiator codons is augmented by G in position +4 but is not generally affected by the nucleotides in positins +5 and +6. EMBO J. 16: 2482-2492. https://doi.org/10.1093/emboj/16.9.2482

Li K.S., K.M. Xu, J.S.M. Peiris, L.L.M. Poon, K.Z. Yu, K.Y. Yuen, K.F. Shortridge, R.G. Webster and Y. Guan. 2003. Characterization of H9 subtype influenza viruses from the ducks of southern China: a candidate for the next influenza pandemic in humans. J. Virol. 77: 6988–6994. https://doi.org/10.1128/JVI.77.12.6988-6994.2003

Kishida, N., Y. Sakoda, M. Eto, Y. Sunaga and H. Kida. 2004. Co-infection of Staphylococcus aureus or Haemophilus paragallinarum exacerbates H9N2 influenza A virus infection in chickens. Arch. Virol. 149: 2095–2104. https://doi.org/10.1007/s00705-004-0372-1

Krieg A.M., Yi A.- K., Schorr J., Davis H.L. 1998. The role of CpG dinucleotides in DNA vac cines. Trends Microbiol. 6, 23–27.

OIE Terrestrial Manual. 2009. Avian influenza. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Chapter 2. 3. 4.

Park K.J., H.I. Kwon, M.S. Song, P. Noriel, Q. Pascua, Y.H. Baek, J.H. Lee, H.L. Jang, J.Y. Lim, I.P. Mo, H.J. Moon, C.J. Kim and Y.K. Choi. 2011. Rapid evolution of low-pathogenic H9N2 avian influenza viruses following poultry vaccination programmes. J. Gen. Virol. 92: 36–50. https://doi.org/10.1099/vir.0.024992-0

Shahsavandi, S., A.H. Salmanian, S.A. Ghorashi, S. Masoudi, F. Fotouhi and M.M. Ebrahimi. 2011. Development of rHA1-ELISA for specific and sensitive detection of H9 subtype influenza virus. J. Virol. Method. 171: 260–263. https://doi.org/10.1016/j.jviromet.2010.11.012

Shana, S., Y. Jiang, Z. Bu, T. Ellis, X. Zeng, J. Edwards, G. Tian, Y. Li, J. Ge, H. Chen and S. Fenwick. 2011. Strategies for improving the efficacy of a H6 subtype avian influenza DNA vaccine in chickens. J. Virol. Method. 173: 220–226. https://doi.org/10.1016/j.jviromet.2011.02.008

Wan, H. and D.R. Perez. 2007. Amino acid 226 in the hemagglutinin of H9N2 influenza viruses determines cell tropism and replication in human airway epithelial cells. J. Virol. 81(10): 5181–5191. https://doi.org/10.1128/JVI.02827-06

Wu, H., K.S. Gunn, S.R. Singh, N.K. Singh, S.C. Ou, J.J. Giambrone. 2011. A DNA vaccine against an H1N1 avian influenza virus induced humoral and cell-mediated immunity in SPF chickens. J. Biotech. Res. 3:27-36

Xu C., W. Fan, R. Wei, H. Zhao. 2004. Isolation and identification of swine influenza recombinant A/Swine/Shandong/1/2003(H9N2) virus. Microbes Infect. Inst. Pasteur. 6: 919–925. https://doi.org/10.1016/j.micinf.2004.04.015

Xu Z.L., Mizuguchi H., Ishii-Watabe A., Uchida E., Mayumi T., Hayakawa T., 2001. Optimization of transcriptional regulatory elements for constructing plasmid vectors. Gene 272, 149–156.