Original Article

Immunogenicity of Inactivated H5 Avian Influenza Vaccine Used in Commercial Laying Pullet in Tehran Province, Iran

Karami, S[1], Karimi, V[1*], Barin, A[2], Rouigari, MR[3], Bozorgmehri Fard, MH[1]

1. *Department of Poultry Diseases, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran*
2. *Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran*
3. *Department of Poultry Diseases, Iran Veterinary Organization, Tehran, Iran*

How to cite this article: **Karami S, Karimi V, Barin A, Rouigari MR, Bozorgmehri Fard MH. Immunogenicity of Inactivated H5 Avian Influenza Vaccine Used in Commercial Laying Pullet in Tehran Province, Iran. Archives of Razi Institute Journal. 2024;79(3):499-506.** DOI: 10.32592/ARI.2024.79.3.499

ABSTRACT

Highly pathogenic avian influenza (HPAI) is a viral disease caused by some H5 and H7 subtypes of influenza virus type A in most species of birds, especially poultry. HPAI viruses are among the most challenging viruses that threaten both human and animal health. Consequently, various strategies, such as the use of vaccines have been proposed to control the disease. After a catastrophic pandemic and the failure of conventional methods (elimination and extermination) in Iran, multiple vaccines have been used to control the disease. This study investigates the immunogenicity of two recombinant inactivated commercial vaccines of H5N1 and H5N3 subtypes in laying pullet flocks in Tehran Province, Iran. From 32 halls in six breeding units of laying pullets, 3,200 sera, and 800 tracheal and cloacal swabs were collected. After collecting the samples, Serum neutralisation (SN) and hemagglutination inhibition (HI) tests were conducted on sera to determine the serum titers of H5 specific antibody obtained from vaccine inoculation in three steps: before, after the first vaccination, and after the second vaccination (booster). The SN and HI tests were carried out by the alpha and beta methods on the pooled samples by the vaccine type (as antigen for HI and SN), and the results were compared. The PCR was performed on the tracheal and cloacal swab samples to possibly detect the HA (H5) virus in the studied flocks. The HI test results showed that both vaccines had a Serum antibody titre above 5 (log_2) after two vaccination rounds, indicating a desirable immunogenic response. The SN test results also showed a neutralisation index above $10^{4.5}$ for both vaccines, indicating more than 50% reduction in antigenicity of the virus. The PCR results were negative. This study was the first investigation of immunogenicity following two-time vaccination against H5 subtype vaccines in Iranian poultry flocks, indicating suitable antibody titer against the influenza virus in vaccinated flocks.

Keywords: Heamagglutination inhibition, Highly pathogenic avian influenza, PCR, Serum neutralisation, Vaccine

Article Info:
Received: 20 November 2023
Accepted: 4 February 2024
Published: 30 June 2024

Corresponding Author's E-Mail:
vkarimi@ut.ac.ir

1. Introduction

The avian influenza virus belongs to the *Orthomyxoviridea* family and causes avian influenza (AI), a critical respiratory and infectious disease in poultry. This virus spreads rapidly and has been reported in various parts of the world (1). Until now, four types as A, B, C, and D are reported based on the antigenic indices in the nucleoprotein (NP) and matrix protein (MP) (2, 3). Among the various virus types, influenza virus type A is divided based on the heamaglutinin glycoprotein, and H5 and H7 are classified as highly pathogenic avian influenza (HPAI) viruses, which cause widespread disease and mortality in birds (4, 5). There are many reports on causalities and damages caused by the influenza viruses in Iran in the last decade. Laboratory diagnoses are conducted by isolating the virus by injecting it into embryonated eggs. The virus is detected by PCR and the antibody is detected by heamagglutination inhibition (HI). Indirect and direct detection of the virus is done by HI and PCR, respectively. There are many reports on causalities and damages caused by the influenza viruses in Iran in the last decade (6, 7). Laboratory diagnoses are conducted by isolating the virus by injecting it into embryonated eggs and detecting the presence of the virus through HI and RT-PCR tests and viral culture (8, 9). Vaccines are the most effective way to prevent from being infected by the influenza virus. However, prevention by vaccination is facing challenges due to antigenic changes in the influenza viruses (10). Inactivated virus and live attenuated virus (LAV) vaccines are among the conventional influenza vaccines (11). For the first time, a completely inactivated vaccine formulation was tested in 1940, which is still used today (12). Inactivated virus vaccines cannot produce local IgA antibodies or stimulate cellular immune responses. In contrast, LAV vaccines can produce a neutralising antibody and stimulate cellular immune responses (13, 14). However, the LAV type of vaccine has limitations, *e.g.*, two rounds of vaccination are required to obtain favourable immune responses. Antigen changes are sometimes very severe and since the influenza virus A is capable of gene exchange between various bird species and humans, a new virus is produced by combining the genomes of human and avian influenza viruses that is called Antigenic shift. In this case, antigen shift occurs, and the new virus is usually more pathogenic and destructive with higher pathogenicity than the previous two viruses (15, 16). AI is one of the most important diseases in the poultry industry. Despite all protective measures, the current H5N1 pandemic started in 2003 has been unprecedented in terms of geographical extent. In addition to causing heavy economic damage to the poultry industry in most countries (through eradication, prevention costs, etc.), it can be transferred to humans, causing serious deadly diseases (17). Concerns about the occurrence of pandemics caused by the recombination of human virus and HPAI virus genes and the 2009 unexpected outbreak promoted research to use recombinant inactivated vaccines or conserved proteins against the virus (3, 18). This study investigated the immunogenicity of killed H5 avian influenza vaccines in commercial pullet flocks in Tehran Province, Iran.

2. Materials and Methods

2.1. Selection of laying flocks

Six laying pullet farms with 32 halls and 910000 commercial laying pullets in the west (Shahriyar and Malard), east (Pakdasht), south (Varamin), and southwest (Eslamshahr) of Tehran were selected for random sampling. The chickens in the farms were mainly prepared for same laying hen units. In addition to the HPAI (H5) vaccine, the flocks were vaccinated against Newcastle disease, H9 subtype influenza, Gumboro, and infectious bronchitis several times, according to the vaccination program.

2.2. Vaccines and vaccination

Vaccine A: The recombinant and inactivated vaccines included the hemagglutinin (H) subunit of the H5N1 influenza virus (parent strain A/dk/China/E319-2/2003) encoded in a Baculovirus along with the inactivated LaSota strain of the Newcastle virus.

Vaccine B: Inactivated H5N3 vaccine (A/chicken/Vietnam/C58/2004)

The vaccinated chickens were separated from non-vaccinated ones. The tests were conducted in a dark place to reduce the stress in the flock. Then, vaccination was carried out subcutaneously in the back of the neck by individually tying the chickens and injecting 0.5 mL of the vaccine, and the vaccinated chickens were kept in a special place. According to the instructions, all pullet breeding units were vaccinated two times: first in 30-day old and second 4-6 weeks later.

2.3. Sampling

Serum sampling. To serologically study the vaccines' effectiveness, 1 mL blood samples were taken three times from the wing veins of 20 pullets in each breeding hall.

- Blood sampling from chickens before vaccination to investigate the level of antibody against H5 antigen before using the vaccine
- Blood sampling 3 to 5 weeks after the first round of vaccination
- Blood sampling 3 to 5 weeks after the second round of vaccination

The sentinel (control) group consisted of 20 non-vaccinated chickens from each hall. In two rounds of sampling after vaccination, serum samples were also taken from the control birds.

Swab sampling. To investigate the possible infection of studied farms with HPAI, tracheal and cloacal swabs were taken from sentinel groups simultaneously with serum sampling.

- Sampling 20 tracheal swabs and 20 cloacal swabs before vaccination
- Sampling 10 tracheal swabs and 10 cloacal swabs after the first vaccine
- Sampling 10 tracheal swabs and 10 cloacal swabs after the second vaccine

Sera and swab samples were kept at -20°C for serum tests.

2.4. Haemagglutination inhibition (HI) assay

The HI assay was performed as previously described (19). Briefly, 25 µL of phosphate-buffered saline (PBS) was added to all wells of a round-bottomed 96-well plate. Subsequently, 25 µL of the serum was added to the first row and mixed well. Twofold serial dilutions were made of 25 µL H5 virus suspension crossways the plate and incubated for 45 min at room temperature (RT). Finally, 25 µl of 1% chicken's red blood cells in PBS was added to each well, and HA activity was determined after 25 min incubation at RT. HI titres are given as the reciprocal of the highest serum dilution, producing complete inhibition of haemagglutination.

HI (Beta). The HI assay was performed as previously described (20). Briefly, for each tested serum, 25 µL PBS was poured into 12 wells of the HI test microplate. Twenty-five microliters of the as-prepared serums were poured into the first wells of the microplate. Then, ten 25 µL consecutive dilutions of the antigen were prepared. Thereafter, 25 µL of the antigen containing four hemagglutinin units (HAU) was added. After 45 min of incubation at room temperature (the time required for the formation of the antigen-antibody complex), 25 µL of 1% chicken red blood cells (RBC) were added to all wells. The test result was read after 25 min of incubation at room temperature. The last dilution in which RBCs were precipitated completely was selected as the serum antibody titer. The wells 11 and 12 were considered the control without antigen and the control without antiserum, respectively.

HI (Alpha). Unlike the beta method, a constant amount of serum was added to various antigen dilutions in this test.

2.5. Serum neutralisation (SN) assay

To carry out the LD50 test for the virus, -3 to -8 dilutions of the H5 virus were prepared and injected into the eggs in the same way as the conventional method (without mixing with the serum), and the rest of the steps were carried out like serum samples. Before virus dilution, the HA test was conducted to determine its titer. Then, 0.5 mL of virus dilution was added to 18 tubes (six dilutions for each three serum groups). The serum-virus mixture was incubated. Thereafter, 0.2 mL of the mixture dilution was injected into four embryonated eggs (9-11 days) through the allantois. The injected eggs were incubated. After incubation, the eggs were kept at 4°C overnight. After taking samples from the allantoic liquid of eggs, 25 µL of the allantoic liquid was poured into the wells of a 96-well plate. The HA test was conducted on the collected allantoic liquid samples. According to the results of the HA test on the allantoic liquid samples to determine the LD50 level, the relative distance (Proportionate distance; PD_{50}) was determined for each serum group by the Reed & Muench method (21).

2.6. Polymerase Chain Reaction (PCR)

To detect possible viruses in the studied flocks and in the case of disease, 800 tracheal and cloacal swabs were taken from the sentinels birds. When no disease symptoms were observed in the vaccinated birds, especially in non-vaccinated (sentinel) birds, after pooling, the swabs were randomly studied by the conventional and real-time methods based on the World Organization for Animal Health (WOAH) guidelines (22)

3. Results

The HI test results showed that both vaccines had a serum titer above 5 after two rounds of vaccination, indicating their favorable protecting effect. Regarding the mortality reduction performance, vaccine B showed 78% titer above 5 after the first round of vaccination, but both vaccines showed favorable performance after two rounds of vaccination. Regarding preventing the proliferation and release of the virus, none of the vaccines were effective after one round of vaccination. After two rounds of vaccination, vaccine B showed better performance than vaccine A in this regard.

3.1. HI assay

HI (Beta). Vaccine A. The mean serum titer in the HI test on the samples taken from the vaccinated group in the whole flock from the first to third farms was 3.01, 3.10, and 3.23 after the first round of vaccination. It was 5.22, 4.96, and 4.88 after the second round of vaccination (Figure 1).

Vaccine B. The mean serum titer in the HI test on the samples taken from the vaccinated group in the whole flock from the first to third farms was 5.18, 5.42, and 4.42 after the first round of vaccination. It was 5.92, 6.43, and 6.07 after the second round of vaccination (Figure 1).

HI (Alpha). The titer obtained from the HI test by the alpha method on the serums collected from all sampled farms before vaccination, after vaccination by vaccine A (second round of vaccination), and after vaccination by

vaccine B (second round of vaccination) was 8, 5, and 3, respectively.

3.2. PCR

To detect possible viruses in the studied flocks and since no disease symptoms were found in vaccinated birds, particularly in non-vaccinated (sentinel) birds, 130 swabs were tested by conventional PCR test. All results were negative. To ensure the accuracy of the results, some samples were tested by the real-time PCR method and showed negative results.

3.3. SN assay

Hemagglutination was obtained based on injection dilutions and according to tested vaccines (Table 1).

3.4. Proportionate distance (PD) and Neutralisation index (NI)

Considering the HA test results for the allantoic liquid samples to determine LD_{50} according to (Table 2) and the formula given, PD_{50} for serums 1 and 2 was $10^{3.5}$ and 10^{5}, respectively. The SN test results showed a neutralization index (NI) above $10^{4.5}$ for both vaccines, indicating more than 50% reduction in the antigenicity of the virus.

$$PD = \frac{\%\ \text{positive above 50\%} - 50\%}{\%\ \text{positive above 50\%} - \text{positive below 50\%}}$$

NI = Log of control virus titer - log of serum and virus

$$\frac{80-50}{80-50} = \frac{30}{30} \qquad 5$$

$PD_2 = \qquad = 1 \rightarrow T_2 = 10^2 \times 10 = 10^3$

$NI_2 = 10^8 - 10^3 = 10^5$

Table 1. Hemagglutination based on injection dilutions and tested vaccines

LD_{50} of virus		Serum 2 (vaccine B)		Serum 1 (vaccine A)	
Dilution	Result	Dilution	Result	Dilution	Result
-3	+1	-1	+3	-1	+4
-4	+2	-2	+2	-2	+3
-5	+4	-3	+1	-3	+1
-6	+4	-4	0	-4	0
-7	+3	-5	0	-5	0
-8	+4	-6	0	-6	0

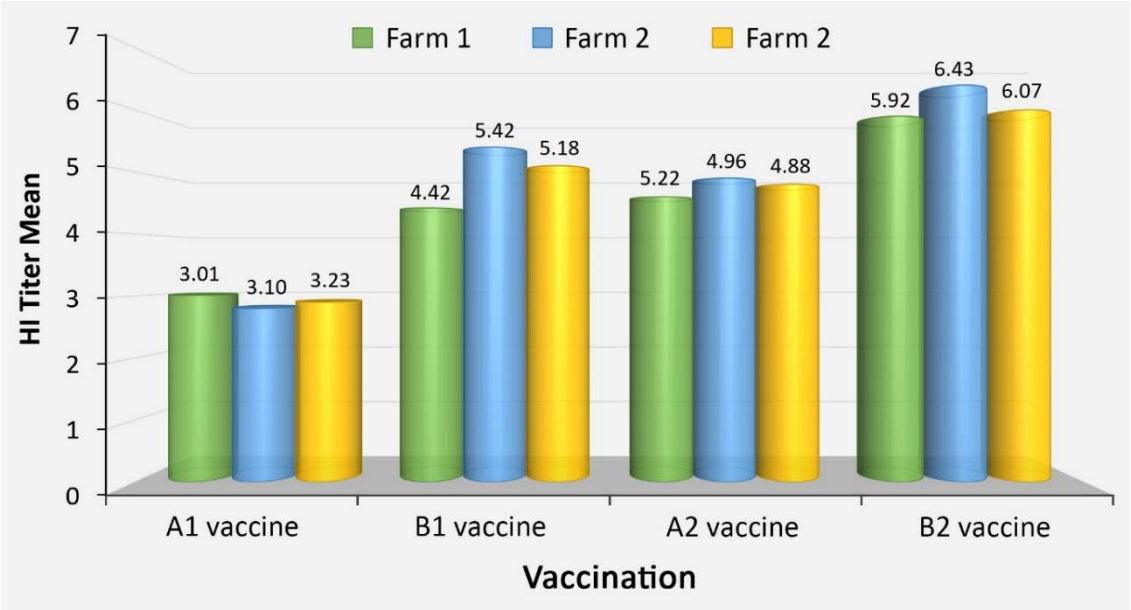

Figure 1. Mean HI titer graph of samples

Table 2. PD for each of the serum groups

Serum	Positive response (%)	Positive response to total	Cumulative amount of negative response +-	Cumulative amount of positive response ++	Negative response -	Positive response +	Dilution
1	100	8.80	0	8	0	4	-1
	80	4.50	1	4	1	3	-2
	20	1.80	4	1	3	1	-3
	0	0.80	8	0	4	0	-4
2	86	6.70	1	6	1	3	-1
	50	3.60	3	3	2	2	-2
	14	1.70	6	1	3	1	-3
	0	0.10	10	0	4	0	-4

* Hemagglutination for allantoic control serum samples was all negative.

The HI titer frequency showed the highest frequency of 34.8% in the first round of vaccination by vaccine A in titer 4. It was 46.4% for vaccine B in titer 6. The highest frequency in the second round of vaccination by vaccine A and vaccine B was 37.7% in titer 5 and 39.7% in titer 7 (Table 3).

Table 3. Frequency of HI titers

	Vaccine (A)			**Vaccine (B)**		
Vaccination	**%**	**Number**	**titer**	**%**	**Number**	**titer**
First	3.5	13	0	0.8	2	0
	9.3	35	1	0.0	0	1
	13.8	52	2	3.1	8	2
	26.6	100	3	3.4	9	3
	34.8	131	4	11.5	30	4
	10.4	39	5	33.3	87	5
	1.6	6	6	46.4	121	6
	0.0	0	7	1.5	4	7
	0.0	0	8	0.0	0	8
	100.0	376		100	261	
Second	0.0	0	0	0.0	0	0
	0.0	0	1	0.0	0	1
	2.4	9	2	0.0	0	2
	8.3	31	3	2.3	6	3
	17.9	67	4	6.1	16	4
	37.7	141	5	13.7	36	5
	20.9	78	6	31.7	83	6
	12.8	48	7	39.7	104	7
	0.0	0	8	6.5	17	8
	100	374		100	262	

4. Discussion

HPAI (H5N8 and H5N1) is still considered a potential threat that can cause causalities and financial losses to the human and poultry population (23). Among the commonly used vaccines for dealing with diseases caused by H5 viruses, inactivated recombinant (which contains the H5 gene) and inactivated whole virus vaccines have been extensively studied (24). Our study showed that despite the fact that both vaccines (recombinant and inactivated vaccines) could not cause a minimum mean serum titer of 5 log 2 after the first round of vaccination (which is acceptable for reducing disease symptoms and mortality), the inactivated vaccine outperformed the recombinant vaccine by at least one log. Boltz et al. (2009) showed that after one round of vaccination by rgH5N3, the mean HI titer was 4.5, and the serum level increased by 20%. After two rounds of vaccination, the mean titer increased to 6.04, and the serum level increased by 75% (by the homologous antigen) (25). In our study, after two rounds of vaccination by the heterologous vaccine H5N3, the mean titer was 6.07, and the serum level increased by 100% in the vaccinated birds. Evaluating neutralizing antibodies in the SN test showed that a minimum neutralizing index of $10^{4.5}$ was obtained after two rounds of vaccination by the killed vaccine H5N3, showing a 5 times higher performance than the recombinant vaccine. Betran et al. (2017) found that one round of vaccination by the dual recombinant vaccine (rHVT-H5) and inactivated vaccine (rgH5N1) increased the serum level by 40% with a mean titer of 8. However, the dual recombinant vaccine + RNA vaccine increased the serum level by 30% and showed a mean titer of 4.7. The recombinant vaccine increased the serum level by 80% and showed a mean titer of 3.3 (26). In our study, the recombinant (A) and inactivated (B) vaccines increased the serum level by 30 and 85% and showed a mean titer of 3.01 and 5.1, respectively. In another research, Astemirov et al. (2022) studied a recombinant vaccine and an inactivated vaccine. The use of the inactivated vaccine (H5N1) caused a higher level of HI antibodies (more than 8 log2), and the serum level increased by 88% after one round of vaccination. In contrast, the serum level increased by only 13% and the titer was 5 log2 in birds who received the recombinant Baculovirus vaccine (27). After one round of vaccination by vaccine A (recombinant Baculovirus) and vaccine B (inactivated H5N3) in our study, the serum levels respectively increased by 50 and 95%, and the highest serum titers of 6 log2 and 7 log2 were obtained, which were relatively similar to those found by Astemirov et al. The frequency of the HI titer in this study showed the highest frequency of 34.8% in titer 4 for the first round of vaccination by vaccine A. The highest frequency of 46.4% in titer 6 was obtained after the first round of vaccination by vaccine B. The highest frequency of 37.7% in titer 5 was obtained for the second round of vaccination by vaccine A. The highest frequency of 39.7% in titer 7 was obtained after the second round of vaccination by vaccine B. Comparing the performance of antibody production by the two vaccines shows a significant difference between vaccines B and A in terms of the logarithm of titer in the first and second rounds of vaccination and the frequency of the maximum titer. The serum level (seroconversion) increased by 46.8 and 92.7% after the first round of vaccination by vaccines A and B, respectively. After two rounds of vaccination by vaccines A and B, the serum level increased by 89.3 and 98.03%, respectively. Therefore, vaccine B is better than vaccine A in terms of immunogenicity and increased serum level. The mean titer of serums tested in the first round of vaccination showed that vaccine B had 2 logarithmic units above vaccine A. In the second round of vaccination, vaccine B outperformed vaccine A with a logarithmic difference of 1 unit above vaccine A. The highest titer for vaccines A and B in the first round of vaccination was 6 and 7, respectively. The highest titer for vaccines A and B in the second round of vaccination was 7 and 8, respectively. The results showed the highest titer after two rounds of vaccination by vaccine B. According to WOAH guidelines, a titer above 5 in the HI test can protect birds against mortality (8). In the first round of vaccination, vaccines A and B showed 12% and 81.2% of this capability. In the second round of vaccination, vaccines A and B showed a performance of 71.4 and 91.6% in this regard. When the titer is above 7, in addition to protecting the birds against mortality, it can also reduce proliferation and the release of the virus from the birds. As a result, the titer above 7 for vaccine A was zero after the first round of vaccination, and none of the serums had this titer. The corresponding value was 1.5% after the first round of vaccination by vaccine B. These values reached 12.8 and 46.2% after the second round of vaccination by vaccines A and B, respectively. Regarding protecting against death, vaccine B caused 81.2% of the titer above 5 after one round of vaccination, but both vaccines showed a favorable mortality protection performance after two rounds of vaccination. None of the vaccines effectively prevented the proliferation and release of the virus after one round of vaccination. Vaccine B outperformed vaccine A after two rounds of vaccination. Regarding the neutralizing performance of antibodies produced by the vaccines, according to the results of the serum neutralization test by the SN (invivo) method, there is only a 0.5 logarithmic unit difference between the two vaccines, so vaccines A and B with an NI of $10^{4.5}$ (56%) and 10^{5} (62%) reduced virus antigenicity. The results

showed that the SN test is a more realistic test than the HI test in measuring the protective power of the vaccines. The results of some studies showed that despite a lower HI titer in some cases, the results were desirable in terms of reduced virus release and reduced disease signs in the challenge test. According to our results, regardless of the limitations of the challenge test, the results may indicate the effect of at least two rounds of vaccination by effective vaccines in the immunization of the flock, leading to reduced clinical symptoms, mortality rate, and reduced virus release. Inactivated vaccines (whole virus) can produce HI serum antibodies even after one round of vaccination to reduce disease symptoms and mortality. In contrast, recombinant Baculovirus vaccines show a weak immune response after one round of vaccination. Regardless of problems related to repeated vaccination in laying flocks (lowering the production indices), at least two rounds of vaccination by effective vaccines are recommended to achieve an immune level for reducing virus proliferation and release and protecting the birds against the circulatory virus.

Acknowledgment
The authors express their gratitude to the staff of the University of Tehran for their valuable contributions to this project.

Authors' Contribution
Study concept and design: V. K.
Acquisition of data: S. K. and A. B.
Analysis and interpretation of data: MR. R. and MH. BF.
Drafting of the manuscript: S. K.
Critical revision of the manuscript: V. K.
Statistical analysis: S. K.

Ethics
It is declared that all ethical considerations were taken into account in the preparation of the submitted manuscript.

Conflict of Interest
The authors declare that there are no conflicts of interest.

Data Availability
The data that support the findings of this study are available on request from the corresponding author.

References

1. Kanaujia R, Bora I, Ratho RK, Thakur V, Mohi GK, Thakur P. Avian influenza revisited: concerns and constraints. Virusdisease. 2022;33(4):456-5. doi: 10.1007/s13337-022-00800-z
2. Wolff T, Veit M. Influenza B, C and D viruses (Orthomyxoviridae). Encyclopedia Virol. 2021;561-74. doi: 10.1016/B978-0-12-809633-8.21505-7
3. Dey P, Ahuja A, Panwar J, Choudhary P, Rani S, Kaur M, et al. Immune control of avian influenza virus infection and its vaccine development. Vaccines (Basel). 2023;11(3):1-8. doi: 10.3390/vaccines11030593
4. Pascha MN, Thijssen V, Egido JE, Linthorst MW, van Lanen JH, van Dongen DAA, et al. Inhibition of H1 and H5 influenza a virus entry by diverse macrocyclic peptides targeting the hemagglutinin stem region. ACS Chem Biol. 2022;17(9):2425-36. doi: 10.1021/acschembio.2c00040
5. Šantak M, Matić Z. The role of nucleoprotein in immunity to human negative-stranded RNA viruses-not just another brick in the viral nucleocapsid. Viruses. 2022;14(3):1-9. doi: 10.3390/v14030521
6. Nili H, Asasi K. Avian influenza (H9N2) outbreak in Iran. Avian Dis. 2003;47(3):828-31. doi: 10.1637/0005-2086-47.s3.828
7. Fallah Mehrabadi MH, Tehrani F, Shoushtari A, Bahonar A, Rabiee MH, Ghalyanchilangeroudi A, et al. Outbreak investigation of officially reported and highly pathogenic avian influenza (H5N8 subtype) in Iran during 2016. Arch Razi Inst. 2021;76(1):17-29. doi: 10.22092/ari.2019.124904.1291
8. World Organisation for Animal Health (WOAH). (2021). Manual of diagnostic tests and vaccines for terrestrial animals, 12ed. Chapter 3.3.4. https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/A_summry.htm
9. Eisfeld AJ, Neumann G, Kawaoka Y. Influenza A virus isolation, culture and identification. Nat Protoc. 2014;9(11):2663-81. doi: 10.1038/nprot.2014.180
10. Nuwarda RF, Alharbi AA, Kayser V. An overview of influenza viruses and vaccines. Vaccines (Basel). 2021;9(9):1-7. doi: 10.3390/vaccines9091032
11. Brett IC, Johansson BE. Immunization against influenza A virus: Comparison of conventional inactivated, live-attenuated and recombinant baculovirus produced purified hemagglutinin and neuraminidase vaccines in a murine model system. Virology. 2005;339(2):273-280. doi:10.1016/j.virol.2005.06.006
12. Kim YH, Hong KJ, Kim H, Nam JH. Influenza vaccines: Past, present, and future. Rev Med Virol. 2022;32(1):1-6. doi: 10.1002/rmv.2243
13. Rajaram S, Boikos C, Gelone DK, Gandhi A. Influenza vaccines: The potential benefits of cell-culture isolation and manufacturing. Ther Adv Vaccines Immunother. 2020;8:1-8. doi: 10.1177/2515135520908121
14. Wiggins KB, Smith MA, Schultz-Cherry S. The Nature of immune responses to influenza vaccination in high-risk

populations. Viruses. 2021;13(6):1-9. doi: 10.3390/v13061109
15. Ciabattini A, Nardini C, Santoro F, Garagnani P, Franceschi C, Medaglini D. Vaccination in the elderly: The challenge of immune changes with aging. Semin Immunol. 2018;40:83-94. doi: 10.1016/j.smim.2018.10.010
16. Tripp RA, Bakre A, Stambas J. Editorial: Understanding the limitations of current influenza vaccine strategies and future development of more efficacious preventative and therapeutic interventions. Front Immunol. 2019;10:1-6. doi: 10.3389/fimmu.2019.02804
17. Rehman S, Effendi MH, Witaningruma AM, Nnabuikeb UE, Bilal M, Abbas A, et al. Avian influenza (H5N1) virus, epidemiology and its effects on backyard poultry in Indonesia: A review. F1000Res. 2022;11:1-10. doi: 10.12688/f1000research.125878.2
18. Zhang N, Zheng BJ, Lu L, Zhou Y, Jiang S, Du L. Advancements in the development of subunit influenza vaccines. Microbes Infect. 2015;17(2):123-34. doi: 10.1016/j.micinf.2014.12.006
19. Ma N, Xia ZW, Zhang ZG, Nian XX, Li XD, Gong Z, et al. Development of an mRNA vaccine against a panel of heterologous H1N1 seasonal influenza viruses using a consensus hemagglutinin sequence. Emerg Microbes Infect. 2023;12(1):1-8. doi: 10.1080/22221751.2023.2202278.
20. Geeraedts F, Goutagny N, Hornung V, Severa M, de Haan A, Pool J, et al. Superior immunogenicity of inactivated whole virus H5N1 influenza vaccine is primarily controlled by Toll-like receptor signalling. PLoS Pathog. 2008;4(8):1-8. doi: 10.1371/journal.ppat.1000138
21. Ramakrishnan MA. Determination of 50% endpoint titer using a simple formula. World J Virol. 2016;5(2):85-6. doi: 10.5501/wjv.v5.i2.85.
22. World Organisation for Animal Health (WOAH). (2021). Manual on animal influenza diagnosis and surveillance. World Organisation for Animal Health. https://apps.who.int/iris/handle/10665/68026
23. Trombetta CM, Kistner O, Montomoli E, Viviani S, Marchi S. Influenza viruses and vaccines: The role of vaccine effectiveness studies for evaluation of the benefits of Influenza vaccines. Vaccines (Basel). 2022;10(5):1-7. doi: 10.3390/vaccines10050714.
24. Soejoedono RD, Murtini S, Palya V, Felföldi B, Mató T, Gardin Y. Efficacy of a recombinant HVT-H5 vaccine against challenge with two genetically divergent Indonesian HPAI H5N1 strains. Avian Dis. 2012;56(4):923-7. doi: 10.1637/10169-041012-ResNote.1
25. Boltz DA, Douangngeun B, Sinthasak S, Phommachanh P, Midouangchanh P, Walker D, et al. Field assessment of an H5N1 inactivated vaccine in chickens and ducks in Lao PDR. Arch Virol. 2009;154(6):939-44. doi: 10.1007/s00705-009-0385-x
26. Bertran K, Balzli C, Lee DH, Suarez DL, Kapczynski DR, Swayne DE. Protection of white leghorn chickens by U.S. emergency H5 vaccination against clade 2.3.4.4 H5N2 high pathogenicity avian influenza virus. Vaccine. 2017;35(46):6336-44. doi: 10.1016/j.vaccine.2017.05.051
27. Astemirov BA, Mamadaliyev SM, Perfiliyeva, YV, Kopochenya MA. Comparative assessment of seroconversion in poultry vaccinated with two avian influenza vaccines. Am J Anim Vet Sci. 2022;17(3):211-8. doi: 10.3844/ajavsp.2022.211.218