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Comparative Assessment of Seroconversion in Poultry Vaccinated with Two Avian Influenza Vaccines

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Abstract: From August to December 2020, a new outbreak of H5 avian influenza caused another serious animal health emergency in Kazakhstan, leading to the deaths or culling of more than 500 000 chickens. This outbreak renewed interest in developing prevention strategies for this re-emerging infection. In this study, we evaluated poultry seroconversion levels after two H5 vaccines. Regardless of age, productivity, and scheme of vaccination Super Nick layers (a total of 368 heads) received a single dose of an inactivated whole H5 vaccine or a baculovirus-derived H5 vaccine, respectively. We assessed the seroconversion by Hemagglutination Inhibition (HI) and Enzyme-Linked Immunosorbent (ELISA) assays. Cloacal and tracheal swabs were tested for Influenza A Virus (AIV) by real-time PCR to monitor the field AIV circulation. The analysis of 368 serum samples showed that the inactivated vaccine provided a significantly higher humoral immune response when compared to the baculovirus-derived vaccine as evaluated by both ELISA and HI in 30, 60, and 120 Days Post-Vaccination (DPV). Thus, our study demonstrates that under farm conditions classical Inactivated Avian Influenza (AIV) vaccine induces a higher seroconversion level against the H5N1 virus predicting better protection against field infection, than a baculovirus-derived H5 vaccine.

Keywords: Avian Influenza, Inactivated Vaccine, Baculovirus-Derived Vaccine, Seroconversion

Introduction

The problem of Avian Influenza (AIV) has been acute in the Republic of Kazakhstan since 2005. Outbreaks of avian influenza occur in Kazakhstan every 3-4 years, resulting in huge numbers of poultry on private and industrial farms dying. Four subtypes of avian influenza virus (AIV) have been identified in Kazakhstan so far, including H9N2, H5N2, H5N1, and H5N8 (WAHID Report N° 18, 2021). In September 2020, the Ministry of Agriculture of the Republic of Kazakhstan announced an outbreak of AIV in the Akmola region and later in Kazakhstan's Kostanay and southern regions. In total, the outbreak of AIV in 2020 resulted in the death or culling of more than 24,000 heads on private farms and more than 500,000. According to unauthorized resources the mortality rate reached 98 – 100%.

Anti-AIV vaccination was prohibited in Kazakhstan until 2005 (Vet. Rules, 2006). After severe AIV outbreaks in 2005–2008 and 2012 vaccination was authorized on the condition of a special state permit (Vet. Rules, 2015).

Significance of the Study

After AIV outbreaks in 2020, it became clear that AIV routine vaccination should be the main control tool on big commercial poultry farms containing 1,000,000 heads or over. Several types of vaccines have been developed to protect poultry against AIV. In 2008, Research Institute for Biological Problems in Kazakhstan developed and registered an H5 inactivated vaccine frequently used on big farms. However, other imported vaccines are also applied, and two of the currently used vaccines are observed in this study. There are currently two main types of AIV vaccines in use: Inactivated hole AIV virus vaccine and live recombinant vaccine containing such subtypes as H5N1, H5N2, H5N3, H7N1, H7N3 (Suarez and Schultz-Cherry, 2000; Yee *et al*., 2009). The H5 Hemagglutinin (HA) of inactivated whole AI virus

vaccines, subunit vaccines, and live recombinant vaccines are either homologous or heterologous, depending on the choice of the viral strain. The homologous type is produced from epidemic isolates or standard strains possessing the same Hemagglutinin (HA) as the circulating field virus. Inactivated whole virus and subunit vaccines induce a humoral response whereas live vectored vaccines can also induce cellular immunity (Swayne *et al*., 2020). Vaccines that only induce a response to the HA protein (like vector vaccines with an H5 gene insert and subunit vaccines with H5 antigen) enable the Differentiation of Infected animals from Vaccinated Animals (DIVA strategy) when combined with suitable diagnostic tests. The immune response to various vaccines against H5N1 in chickens is relatively well researched (Swayne, 2006a; Swayne *et al*., 2007), but less is known about how well these vaccines perform under farm conditions. Discrepancies between laboratory and field results can occur due to the immunity of vaccinated birds (immunosuppressive factors such as feed, temperature, co-infections) or the technical issues related to vaccination such as storage and administration of the vaccine, dosage, and time of vaccination.

Aim of the Study

This study aimed to evaluate the immune response in commercial layers following vaccination with two vaccines widely used in Kazakhstan: A classic inactivated vaccine (Vaccine I) and a baculovirus-derived vaccine (Vaccine II).

Materials and Methods

Birds

The research was carried out on a farm located in the Akmola region, Kazakhstan. Super Nick layers were vaccinated at different ages during the autumn-winter period, 2020. In total, 450,000 birds over 5 weeks old placed in 6 houses and 750,000 birds over 5 weeks old placed in 10 houses were vaccinated with Vaccine I and Vaccine II, respectively. As the experiment was conducted on the operating farm and there was a risk of AIV infection we didn't have the opportunity to take control of the unvaccinated group. It should be noted that all chickens were obtained from AIV non-immune parents, thus, no birds had maternally derived antibodies to AIV. Twenty-three birds from each house (368 birds in total) were sampled and analyzed serologically, forming, thereby, 16 experimental groups. All birds were monitored serologically before vaccination (pre-vaccination). Age or productivity did not vary significantly between the groups. All the birds were kept ensuring proper biosecurity measures. All the experimental procedures with birds were performed following the International Guiding Principles for Biomedical Research Involving Animals and Local Ethical Committee Protocol (#2, approved December 7, 2021).

Vaccines

Two commercially available vaccines against H5N1 were used in this independent non-sponsored study. The first vaccine used was the monovalent H5N1 inactivated vaccine (referred to as Vaccine I), derived from the extraembryonic fluid of chicken embryos infected with AIV (strain A/Chicken/Novosibirsk/64/05(H5N1), clade 2.2., NCBI: txid346232), inactivated with formalin, with the addition of oil adjuvant ISA 70 (SEPPIC). The second vaccine (Vaccine II) used was an oil adjuvanted inactivated vaccine for the prevention of H5 AIV type A and Newcastle Disease (ND) in chickens. According to the manufacturer's instruction, the AIV component in this vaccine was specifically engineered by inserting the Hemagglutinin (HA) viral sequence of the AIV H5N1 subtype clade 2.3.2 into the baculovirus genome (parent strain A/dk/China/E319-2/2003 (H5N1), GenBank accession AY518362.1). The insert was generated using a recombinant vaccine technology to highly resemble the HA protein of currently circulating H5 viruses. During the replication process, the baculovirus expresses the recombinant HA protein of the H5 virus. The recombinant HA protein was then collected and assembled with the conventional inactivated ND (LaSota) virus in oil emulsion. Both vaccines were injected once subcutaneously into the middle third of the neck at a volume of 0.5 mL by the manufacturers' recommendations. Both vaccines were stored and transported according to manufacturers' instructions. These vaccines have been registered and recommended for chicken vaccination in Kazakhstan.

Sampling

Twenty-three birds were selected for random sampling from each birdhouse regardless of flock size for the reproducibility and reliability of the serological analysis results and the true mean flock titer as per commonly known sampling method for effective flock monitoring. In practice, 23 to 30 samples are ideal and will accurately reflect the vaccination or infection rate with a 90-95% confidence level.

PCR

PCR analysis of the tracheal and cloacal swabs was performed using a RealPCR Influenza A Virus RNA Mix kit (IDEXX) according to the manufacturer's instruction. Viral RNA was isolated by the column method using a RealPCR DNA/RNA Spin Column Kit (IDEXX). The amplification was carried out in a Bio-Rad CFX96. Samples with the cycle threshold ≥ 45 were considered negative.

Serological Test

For serological studies, blood samples were taken from the axillary vein 30 days before the vaccination and 30, 60, and 120 Days Post-Vaccination (DPV). The HI and ELISA tests were used to evaluate humoral immune responses to the vaccines. The HI test was performed using two commercial antigens: H5N1 nonrecombinant antigen (Antigen I, GD Animal Health, inactivated strain A/chicken/Netherlands/SP00153/2014(H5N1)) and H5 recombinant antigen (Antigen II, Boehringer Ingelheim) which is highly homologous to Vaccine II. Both antigens were diluted in phosphate-buffered saline to make a concentration of four HA units. Twofold serial dilutions of each serum sample were made to a final dilution of 1:4096. Fifty microliters of diluted antigens were added per well of a 96-well plate containing serial dilutions of serum samples. Plates were incubated for 30 min at room temperature before 0.5% chicken red blood cell suspension was added. After that plates were shaken for 15 sec and incubated for 45 min at room temperature. Results were interpreted as the reciprocal of the last serum dilution, where complete inhibition of hemagglutination was observed (Swayne, 2006b). ELISA test was performed using the Influenza A Ab Test kit (IDEXX). The kit is designed to measure antibodies to any AIV A type subtype. According to the manufacturer's protocol, antibodies in the sample were determined by calculating the sample-to-positive control (S/P) ratio with a positive cut-off value of S/P > 0,50. The xChekPlus (IDEXX) software was used for analyzing and managing ELISA results. ELISA titers were calculated using the following formula: Titers (log 10) = 1.09 (log10 S/P) +3.36 which is mentioned in the instruction for the test kit. The coefficient of variation (CV%) was considered for the evaluation of the homogeneity of the birds' immunity (Toffan *et al.*, 2010).

To exclude circulation of the low pathogenic influenza H9N2 virus in poultry, serum samples were analyzed by HI test using the commercial H9 antigen (GD Animal Health).

Statistical Analysis

For all groups of studied birds, the Geometric Mean Titer (GMT) and the level of seroconversion were calculated. GMT was calculated as the antilogarithm of the mean of logarithms in each group of birds studied. A titer of <1:16 was considered negative. The seroconversion rate was calculated as the ratio of the total number of birds to the number of birds with a post-vaccination titer level of ≥1:16. The groups were comparing the two-way ANOVA. Significant differences were obtained at p values of <0.05.

Results

PCR Test Results

PCR analysis of 320 tracheal and cloacal swabs for influenza A viral RNA performed before vaccination and on day 60 after vaccination demonstrated negative results suggesting that the AIV did not circulate among the birds during the study (Table 1).

Pre – Vaccination Titers of Antibodies to AIV Type A

All the studied birds were tested for the presence of AIV type A and H5 antibodies before vaccination. None of the tested birds showed pre-vaccination antibodies to AIV type A and H5 (Table 1). As it is well known, low pathogenic AIV H9N2 can circulate without any obvious clinical signs in birds and boost anti-influenza type A antibody titers. To exclude active ongoing H9N2 infection in the studied flocks, we performed the HI test of 368 samples using the H9 antigen. No H9 antibodies were detected in the studied birds (data not shown).

Post-Vaccination HI Titers of Antibodies to H5N1 Highly Pathogenic AIV

The results of the HI assay of 368 serum samples obtained from birds vaccinated by either Vaccine I or Vaccine II are presented in Fig. 1A. Overall, 88±4,9% of the birds that received Vaccine I were seroconverted in 30 DPV with a further increase in the percentage of seroconverted birds in 60 and 120 DPV. As expected, this increase failed to reach statistical significance. On the contrary, only 13±5,8% of birds seroconverted in 30 DPV receiving Vaccine II. And the level of seroconversion fell to 7±3,5% in 120 DPV. Moreover, the same HI test revealed higher antibody titers 30, 60, and 120 DPV in birds receiving Vaccine I (≥8 log2) as compared to Vaccine II (≤5 log2). Groups of birds immunized with Vaccine II showed no H5 AIV antibodies 120 DPV (Fig. 1B).

The ELISA results with the use of a quantitative ELISA test kit showed that on day 30 DPV (a single injection of Vaccine I) layers exhibited significantly higher antibody titer (GMT = 5463 ±1356) ($p < 0.05$) compared to the group vaccinated with Vaccine II (GMT = 229±141). Later, 60 and 120 DPV, GMT increased to 6775±1857 in groups, vaccinated with Vaccine I and slightly dropped in groups, vaccinated with Vaccine II. These differences were non-significant when compared with the GMT on day 30 DPV in the same groups (Fig. 2).

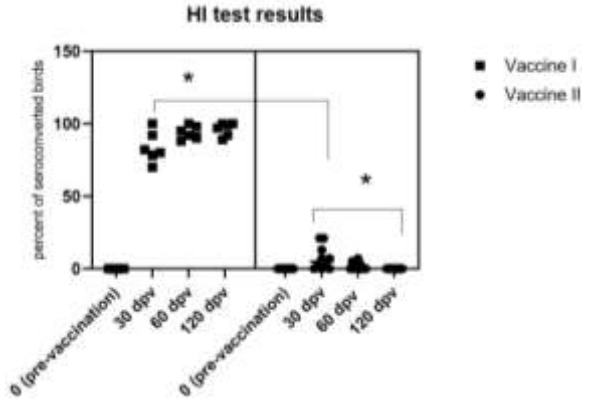

Fig. 1A: Percent of seroconverted birds tested in HI test using AIV H5N1 antigen at days 30, 60, and 120 DPV with Vaccine I and Vaccine II. The seroconversion rate is the ratio of all tested birds to the number of birds in which the titer after vaccination was ≥1:16. * - shows the significant difference ($p < 0.05$) in seroconversion rate after vaccination

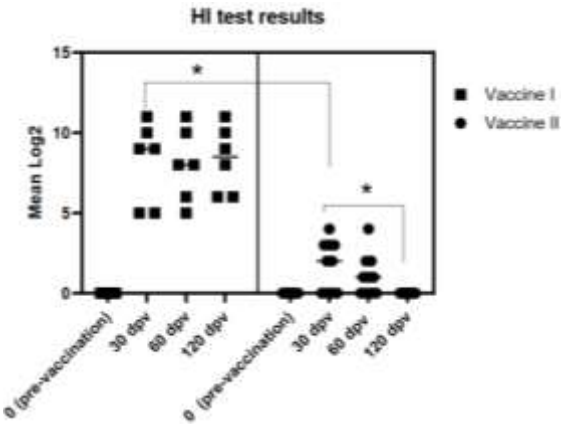

Fig. 1B: Geometric mean titer log2 is for each birdhouse tested in HI test using AIV H5N1 antigen at 30, 60, and 120 DPV with two vaccines. * - shows the significant difference ($p < 0.05$) in H5 antibody titers after vaccination

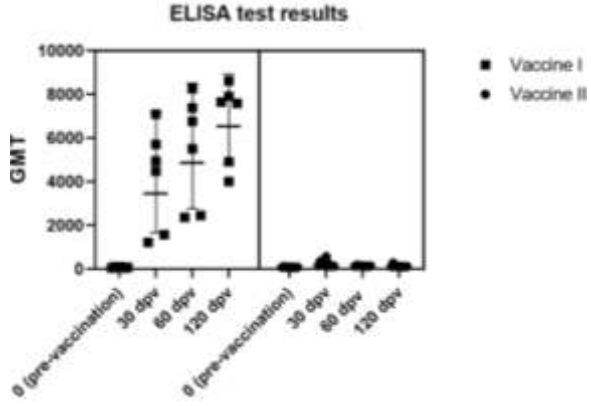

Fig. 2: Geometric mean titer log10 using Influenza A Ab ELISA test kit. Twenty-three samples per vaccinated group (368 samples totally) were collected and tested with 30, 60, and 120 DPV. ELISA titers were calculated by using following formula Titers (log10) = 1.09 (log10 S/P) +3.36. ELISA data are presented in a scatter dot plot diagram with lines as geometric mean and SD. The above and below bars are error bars

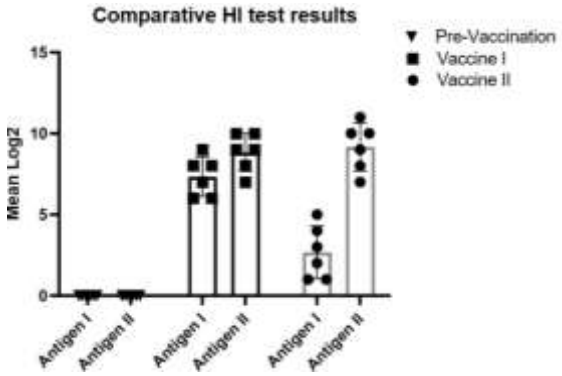

Fig. 3: Comparative HI testing using two antigens. Statistical analysis using two-way ANOVA showed a significant difference (p=0,003) between post-vaccination H5 antibodies after Vaccine II revealed in HI test using recombinant H5N1 Antigen II and homologous H5N1 Antigen I. The scatter dot plot diagram presents mean SD and error bars

Table 1: Response of chickens vaccinated with the AIV inactivated vaccine and baculovirus-derived H5 vaccine 30, 60, and 120 DPV

Vaccine	HI mean log2	RT- PCR	ELISA GMT	SR%
pre- vaccinated	0	Neg. ≥ 45	79±21	0%
Inactivated vaccine				
30 dpv	≥8	N/A	5463±1356	88±4,9%
60 dpv	>8	Neg. ≥ 45	5447±2033	100%
120 dpv	>8	N/A	6775±1857	100%
Baculovirus-derived H5 vaccine				
30 dpv	≤5	N/A	229±141	13±5,8%
60 dpv	<4	Neg. ≥ 45		
120±72	7±3,5%			
120 dpv	0	N/A	0	0%

Geometric mean titers (GMT), seroconversion rate (SR) – the percentage of birds demonstrating a titer above or equaling 16 log2. RT-PCR was conducted in all bird groups before vaccination and 60 DPV to monitor the absence of field AIV infection. NA = not applicable

The seroconversion rate in the ELISA test was higher (100% ± 3,2%) after Vaccine I when compared with the seroconversion rate in HI on day 30 DPV (88 ± 4,9%). However, after Vaccine II, the seroconversion rate in ELISA was low (26% ± 6,5%). But this is anyway higher than in the HI test (13 ± 5,8%). Some authors notice low sensitivity of the HI test as suggested by Rauw *et al*. (2012) because the HI test measures antibodies against only HA, whereas other tests such as ELISA measure antibodies against all viral proteins.

HI Titers using Different Antigens

There is evidence that chickens vaccinated with subunit or recombinant vaccines showed a good humoral immune response in the HI test only with the homologous recombinant antigen (Oliveira *et al*., 2017). However, this is not true for inactivated vaccines. Hence, parallel HI testing was conducted using homologous (H5N1) and recombinant (H5N1) AIV antigens (4HA unit) for the vaccine virus on the collected serum samples. On 30 DPV with Vaccine I, HI geometric mean (GM) AIV antibody titers were 6.3 ± 2.5 and 8 ± 2 log2 (mean ± SD) for Antigen I and Antigen II, respectively, the revealed difference was non-significant. On the other hand, Antigen I and Antigen II HI tests conducted on the samples from birds vaccinated with Vaccine II showed fundamentally different titers (p = 0,003). The HI GMT on 30 DPV (Vaccine II) was much higher when evaluated using recombinant antigen (Antigen II) highly homologous to the Vaccine II (9 ± 2.3 log2). On the contrary, GMT revealed in the HI test using homologous antigen (Antigen I) was 3 ± 2,1 log2, while half of the samples (66/138) showed negative results (Fig. 3). Thus, Vaccine I induced a high antibody response to both homologous and recombinant antigens.

Discussion

Various vaccine technologies have been developed and have shown efficacy in experimental studies to protect against AIV (Swayne, 2004). The most frequently licensed AI vaccine technology has been inactivated whole AIV adjuvanted vaccines followed by chemical inactivation and oil emulsification (Swayne, 2006ab; 2009). Live recombinant fowlpox virus, herpesvirus of turkeys, and Newcastle disease vaccines with AI H5 gene inserts (rFPV-AIV-H5, rHVT-AIV-H5, and rNDV-AIV-H5, respectively) have been licensed, and are used in a few countries (Swayne and Kapczynski, 2016) From 2002–2010, the majority of AIV vaccine used in national routine vaccination programs are inactivated oil-emulsified vaccine which accounts for 95.5% and 4.5% are recombinant vaccines (Swayne and Sims, 2021).

It is widely known that the AIV vaccine must protect the vaccinated animals against clinical signs, prevent mortality, reduce virus shedding and increase the minimum dose of virus required to infect a bird, therefore limiting contact infection and spread of the disease. Protection against AIV is also conferred by the production of antibodies against HA viral protein (Katz *et al*., 2000; Swayne, 2003). There is much evidence of the direct relationship between HI titers and protection after inactivated vaccines. Kumar *et al*. (2007) and van der Goot *et al*. (2005) have shown that viral shedding was reduced and transmission was prevented with HI titers>4 log2; (Kumar *et al*., 2007; Van der Goot *et al*., 2005; Lee *et al*., 2007) have shown that no shedding was observed for HI titers>5 log2 (Lee *et al*., 2007).

Therefore, the level of seroconversion in terms of anti-HA antibodies [measured by HI test] is used to evaluate vaccine efficacy. According to the OIE International Manual, HI titers are considered positive when the inhibition of the hemagglutination occurs for a serum dilution of at least 32 (5 log2) against 4 Haemagglutinin Antigen Units (HAU) antigen (OIE, 2021).

However, this is not true for recombinant vector vaccines (Swayne *et al*., 2006; Swayne, 1997; Swayne *et al*., 2000). Previous studies conducted in Vietnam (Yuk *et al*., 2017) and South Africa (Abdelwhab and Hafez, 2012) showed no titers in conventional ELISA and HI assays but revealed a mean of 8.6 log2 HI titer with the recombinant antigen in chickens vaccinated with recombinant baculovirus-derived H5 vaccine. Previously in 2006 and 2009 (Qiao *et al*., 2006; 2009*)*. showed that an inconsistent or low antibody response after vaccination of chickens with a recombinant avian influenza vaccine may be associated with the antigen used in the HI, while this is not the case for inactivated vaccines (Qiao *et al*., 2006; 2009). Moreover, the efficacy of the recombinant vaccine depends on a carrier vector along with the other factors (Rauw *et al*., 2012; Cui *et al*., 2013). For instance, the recombinant vaccines which contain baculovirus expressed H5 antigen have possible differences in glycosylation of insect versus avian cells and this may have contributed to the low H5 HI antibody titers in vaccinated birds. Thus, in our studies, we decided to test the same samples collected from both vaccinated groups in the HI test using homologous (Antigen I) and recombinant (Antigen II) antigens. In our studies, the HI test results using two different antigens showed very little difference in titers 30 DPV when using the inactivated vaccine (Vaccine I). Conversely, birds vaccinated with the baculovirus-derived vaccine (Vaccine II) and tested 30 DPV in HI applying the two antigens showed significantly different results (p=0,003). Birds vaccinated with the recombinant baculovirus-derived vaccine (Vaccine II) showed a good humoral immune response with the recombinant H5 antigen (Antigen II) and a very poor response with the non-recombinant H5N1 antigen (Antigen I).

On the contrary, there is ample evidence of sufficient protection of inactivated AIV vaccines from clinical signs

and the virus shedding after the AIV challenge (Bertran *et al.*, 2015; Brugh *et al*., 1979; Brugh and Stone, 2003; Swayne *et al*., 2012; Stone, 1987). Swayne *et al.* (2006) undertook a study to determine whether two inactivated commercial vaccines, one based on a European and the other a North American low pathogenicity AI virus strain, could protect chickens against the Asian H5N1 HPAI virus. The North American and European vaccine viruses had 84 and 91% deduced amino acid sequence similarity to the HA1 segment of haemagglutinin protein of Indonesia H5N1 HPAI challenge virus, respectively. Both vaccines provided complete protection from clinical signs and death. The vaccines reduced the number of chickens infected and shedding viruses from the respiratory and intestinal tracts at the peak of virus replication (Swayne *et al*., 2001).

In our study the inactivated vaccine (Vaccine I) induced a high level of HI antibodies (≥ 8 log2 (1:256)) and the level of seroconversion reached 88% in 30 DPV in a one-dose regimen, suggesting high protection against AIV. On the contrary, the level of seroconversion did not reach the protective level in 30, 60, and 120 DPV in layers after a single vaccination with the recombinant baculovirus-derived H5 vaccine (Vaccine II), whereas HI titers were shown after Vaccine II when using recombinant highly homologous Antigen II. Thus, in our trial, the inactivated vaccine (Vaccine I) surpassed the baculovirus-derived one (Vaccine II) in its ability to induce high levels of HI antibodies and a higher level of seroconversion in commercial layers. Moreover, the ELISA method was used along with the HI test. And in agreement with the results obtained in the HI assay, significant differences in post-vaccination titers were observed between the two tested vaccines. According to the ELISA results, the inactivated vaccine (Vaccine I) induced titers were 50 times higher than the baculovirus-derived recombinant vaccine (Vaccine II).

Per many previous studies, our field trial showed consistent humoral immune response 30 DPV using the inactivated vaccine (Vaccine I) in the single-dose regimen. A sufficient humoral immune response is observed both in HI test using homologous (Antigen I) and recombinant (Antigen II) antigens and in conventional ELISA using total influenza A antigen. It is economically important for big commercial layers farms as subcutaneous or intramuscular vaccination brings stress for layers and demands much labor costs.

Thus, our study showed the practical results of seroconversion detection in commercial layers after two different vaccines in single-dose regimens in the field conditions. Based on the above described, the best choice for timely AIV protection of birds at an operating poultry farm is the systematic use of an inactivated vaccine with constant monitoring of humoral immunity in birds and revaccination when antibody titers fall below 4 log2.

Research Limitations

The study has several limitations. Manufacturers of both studied vaccines recommend a two-dose vaccination with 14 and 28-day intervals according to the epizootiology situation in the territory. However, the suggested approach is difficult to maintain when working on big farms or with broiler chicken with a relatively short lifespan. It is widely known that repeated subcutaneous or intramuscular vaccination of layers can also affect production indicators due to stress factors for birds (Beato *et al*., 2007). Therefore, in our studies, we evaluated only a one-dose regimen to maintain high production indicators on the operating farm.

Also, since this study was carried out at an operating commercial layer enterprise, we had no opportunity to evaluate the protective effect of the studied vaccines against challenges with avian influenza virus isolates.

Conclusion

Application of inactivated vaccine in a single-dose regimen provides significantly higher protection based on antibody level both in classical serological tests and in the HI test using a recombinant H5 antigen. The use of a baculovirus-derived H5 vaccine shows a low humoral response in classical serological tests, predicting lower protection. Overall, the inactivated vaccine provided a superior humoral immune response in a single vaccination strategy compared to the baculovirus-derived H5 vaccine suggesting a better choice of a single vaccination strategy aiming at both AIV protection and maintaining good production indices.

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Author's Contributions

Belan A. Astemirov: Contributed to the original ideas of the research, research design, and data collection. Analysis and interpretation and manuscript writing.

Seidigapbar M. Mamadaliyev: Contributed to the original ideas of the research, and coordinate the manuscript preparation.

Yuliya V. Perfiliyeva: Contributed to data analysis and manuscript preparation.

Marina A. Kopochenya: Contributed research ideas, data analysis, and manuscript writing.

Conflict of Interest

The authors declare no potential conflicts of interest to the research, the authorship, and/or publication of this article.

Ethics

This article is original and contains unpublished material before. The authors declare that there are no ethical issues may arise after the publication of this study.

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