

**Special Issue:**

Emerging and Re-emerging Animal Health Challenges in Low and Middle-Income Countries

Evaluation of Oil Vaccines of Avian Influenza H5 Virus in Layer by Enzyme-Linked Immunosorbent Assay

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Abstract | Highly contagious avian influenza illness is caused by high-path and low-path flue viruses. Chicken vaccinations are intramuscular or subcutaneous injections that reduce illness severity and transmission. To stimulate the immune system, large-scale immunization campaigns may utilize live-attenuated vaccines. Aim of study: This research aimed to assessing the effectiveness of oil vaccines against the H5 virus of avian influenza using an enzyme-linked immunosorbent test in layers. After inoculation with the killed oil vaccine against influenza were tested for H5 immunity in Iraqi Layers hens (LOHMAN Brown). Four treatment groups of 192 chicks were immunized at 1 to 210 days. AI titration by enzyme-linked immunosorbent assay was performed in blood serum to determine immunity. First-day of measurements, ELISA titer was similar across research groups. Significant changes were seen 30 days post-intervention, with MEVAC leading (883.17 ± 173.94). At 50 days post-intervention, MEVAC group had the highest level (2767.33 ± 445.10). At 70 days post-intervention, MEVAC group had the highest level (3269.00 ± 244.67). At each time point assessed, the MEVAC group had the highest ELISA values (90 days 5671.00 ± 330.52 ; 210 days 10573.75 ± 775.74). Results indicated that the vaccine consistently outperformed other groups in ELISA testing across all evaluated intervals. This indicates a high and prolonged vaccination immunological response.

Keywords | Oil vaccines, Avian influenza, H5N1, H5N8, Enzyme-linked immunosorbent assay

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INTRODUCTION

The influenza A viruses affect poultry globally (Asha and Kumar, 2019). Avian influenza viruses including Highly Pathogenic Avian Influenza and low Pathogenic Avian Influenza affects chickens. LPAI viruses infect Anseriformes and Charadriiformes (Gonzales *et al.*, 2021). After being found in wild birds in 2010, H5N8 HPAI spread to Chinese domestic birds. The 2014–2015 H5N8 HPAI pandemic in Asia, Europe, and North America killed 50 million birds. Asia's 2016–2017 H5

HPAI pandemic, Europe's biggest bird flu epidemic, killed domestic and wild birds (Blagodatski *et al.*, 2021). The influenza A genome has eight negative-sense RNA regions. HA and NA surface glycoproteins distinguish influenza A subtypes. H5 and H7 HPAI viruses destroy flocks, devastating the poultry sector (Gulyaev *et al.*, 2016). Effectiveness of oil-based layer hen avian flu vaccinations. An oil-based adjuvant suspends or emulsifies avian influenza virus antigen in an oil-based vaccination (Lu *et al.*, 2016). Scientists blend avian influenza viral antigens with oil adjuvant to make the vaccine. Adjuvants

boost and sustain antigen-specific immunity. Scientists suggest layer hen vaccine experiments (Toussi and Massari, 2014). Scheduled layer hen vaccines. One vaccine administration technique is injections. Immune responses in immunized chickens are explored (Birhane and Fesseha, 2020). These studies assess blood antibody levels, cellular immunological responses, and immunity duration (Dan *et al.*, 2020). Researchers may test the vaccine by infecting vaccinated and untreated chickens with H5 avian influenza. Comparing sickness severity and survival rates between groups may determine protection (Swayne and Kapczynski, 2016). Controlling and preventing chicken bird flu requires immunization. Bird vaccines boost antibody production and minimize influenza severity and transmission (Suarez and Pantin-Jackwood, 2017). Avian influenza prevention and healthy chicken populations need vaccination. When vaccinating, species and vaccine details matter most (Gupta *et al.*, 2021). Vaccinations utilize latent or dead avian influenza viruses. Many of these formulations include immunity-boosting adjuvants. Subcutaneous or intramuscular inactivated vaccines are given. This immunizes the immune system best (Mehmood *et al.*, 2023). Transgenic avian viruses are used in live-attenuated flu vaccinations. Less dangerous viruses may multiply in hosts. The immune system reacts forcefully because they imitate sickness. Water, spray, and nasal drops may deliver live attenuated vaccinations. These bulk chicken flock immunization methods are appropriate for mass vaccination (Lopez and Legge, 2020).

AIM OF STUDY

This research aimed to assessing the effectiveness of oil vaccines against the H5 virus of avian influenza using an enzyme-linked immunosorbent test in layers.

MATERIALS AND METHODS

STUDY DESIGN

Results were conducted from June 2023–February 2024 comprised 192 one-day-old Lohmann Brown-Classic Layer chicks at The University of Baghdad College of Agriculture/Department of Animal Production. Parent flocks were not inoculated against avian flu had chicks without maternal protection. The ELASA was conducted on one day old showed no maternal immunity in the chicks. Spraying and disinfecting the chicken house with Virkon and iodine before delivering the chicks. Clean sawdust was utilized as litter after chlorine cleaned food and drink dishes. Lighting and ventilation were managed according to standards protocols. The layer chicks were separated into four groups: MEVAC (H5N1, H5N8), MSD (H5N2), Zoites (H5N3), and control. G1, G2, and G3: Inactivated oil-killed vaccines for MEVAC, MSD, and Zoites.

COLLECTION OF THE SAMPLES

In order to extract serum, 5 milliliters of blood were drawn from each chick and placed in a gully tube. Every sample was flash-frozen at -20°C before analysis. At 1, 30, 50, 70, 90, 110, 130, 150, 170, 190, and 210 days of age, blood serum was collected every 20 days to determine immunity by monitoring the titration of AI using ELAZA.

ID screen® influenza A nucleoprotein indirect ELISA kit The interactive ELISA kit detects influenza A virus nucleoprotein antibodies. This quantitative test found antibodies in chicken, turkey, duck, and geese serum, plasma, or egg yolk. Microwells coated with purified NP antigen. Wells received test samples and controls. If present, anti-NP antibodies form an antigen-antibody complex. In the wells, an anti-chicken horseradish peroxidase (HRP) conjugate bound to the sample antibodies to form an antigen-antibody-HRP complex after washing. The substrate solution (TMB) was added after washing off conjugation. Unique antibody count determines specimen color. The stop solution yellows blue antibodies. Absence of antibodies prevents coloration. At 450 nm, this microplate was read.

STATISTICAL ANALYSIS

Statistics were done using SPSS 27. It was used frequencies and percentages for categorical variables. Continuous variables were shown as (Means $\pm$ SD). ANOVA compared means of three or more groups. A p-value < 0.05 was deemed significant.

RESULTS AND DISCUSSION

In Table 1 and Figure 1, the mean differences of ELISA test which done at days 1 among study groups including (MEVAC, MSD, Zoites and Control group) were studied, and the results showed that, there were no significant differences of ELISA test among study groups at days 1.

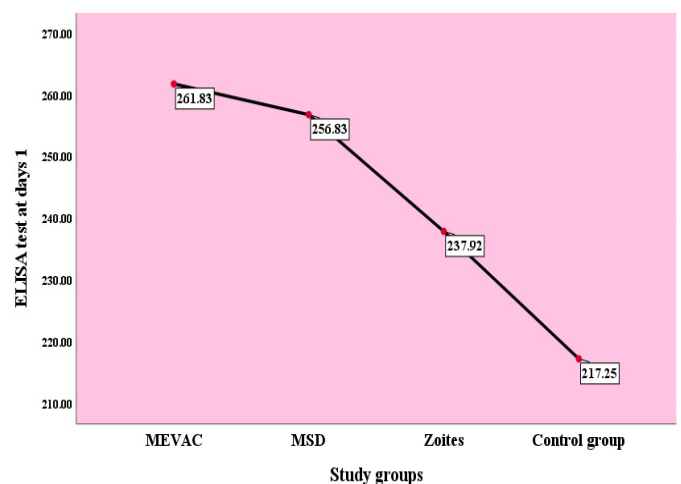


Figure 1: The mean differences of ELISA test at day 1 for study groups (N=48).

Table 1: The mean differences of ELISA test at days 1 for study groups (N=48).

Study variable	Study groups	Number	Mean $\pm$ SD	P value
ELISA test at days 1	MEVAC	12	261.83 $\pm$ 133.92	0.841
	MSD	12	256.83 $\pm$ 136.44	
	Zoites	12	237.92 $\pm$ 120.37	
	Control group	12	217.25 $\pm$ 141.89	

In Table 2 and Figure 2, the mean differences of ELISA test which done after 30 days among study groups including (MEVAC, MSD, Zoites and Control group). The results showed that, there were significant differences of ELISA test among study groups after 30 days from intervention with highest level in MEVAC group (883.17 $\pm$ 173.94).

Table 2: The mean differences of ELISA test after 30 days for study groups (N=48).

Study variable	Study groups	Number	Mean $\pm$ SD	P value
ELISA test after 30 days	MEVAC	12	883.17 $\pm$ 173.94	<0.001*
	MSD	12	773.33 $\pm$ 161.76	
	Zoites	12	797.50 $\pm$ 175.42	
	Control group	12	114.00 $\pm$ 66.02	

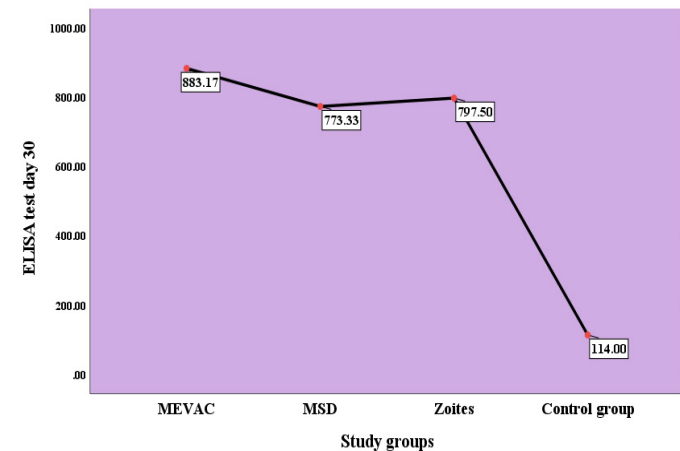


Figure 2: The mean differences of ELISA test after 30 days for study groups (N=48).

In addition, in Table 3 and Figure 3, the mean differences of ELISA test which done after 50 days among study groups including (MEVAC, MSD, Zoites and Control group), and the results showed that, there were significant differences of ELISA test among study groups after 50 days from intervention with highest level in MEVAC group (2767.33 $\pm$ 445.10).

However, In Table 4 and Figure 4, the mean differences of ELISA test which done after 70 days among study groups including (MEVAC, MSD, Zoites and Control group). The results showed that, there were significant differences

of ELISA test among study groups after 70 days from intervention with highest level in MEVAC group (3269.00 $\pm$ 244.67).

Table 3: The mean differences of ELISA test after 50 days for study groups (N=48).

Study variable	Study groups	Number	Mean $\pm$ SD	P value
ELISA test after 50 days	MEVAC	12	2767.33 $\pm$ 445.10	<0.001*
	MSD	12	2274.00 $\pm$ 161.76	
	Zoites	12	2444.33 $\pm$ 409.67	
	Control group	12	77.00 $\pm$ 56.02	

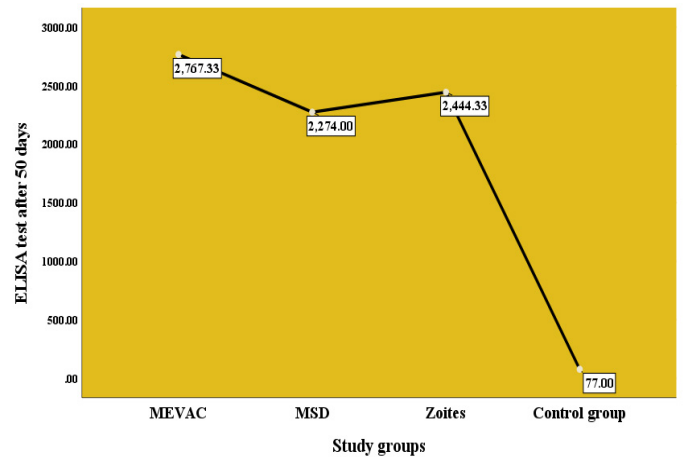


Figure 3: The mean differences of ELISA test after 50 days for study groups (N=48).

Table 4: The mean differences of ELISA test after 70 days for study groups (N=48).

Study variable	Study groups	Number	Mean $\pm$ SD	P value
ELISA test after 70 days	MEVAC	12	3269.00 $\pm$ 244.67	<0.001*
	MSD	12	2932.00 $\pm$ 264.26	
	Zoites	12	3015.00 $\pm$ 267.37	
	Control group	12	44.25 $\pm$ 26.90	

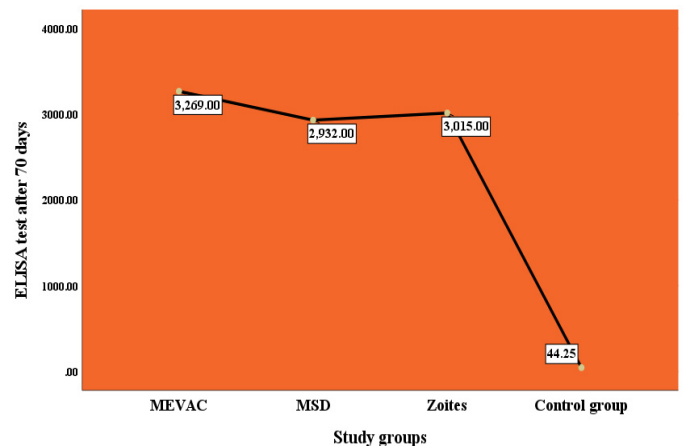


Figure 4: The mean differences of ELISA test after 70 days for study groups (N=48).

In Table 5 and Figure 5, the mean differences of ELISA test which done after 90 days among study groups including (MEVAC, MSD, Zoites and Control group). The results showed that, there were significant differences of ELISA test among study groups after 90 days from intervention with highest level in MEVAC group (5671.00 ± 330.52).

Table 5: The mean differences of ELISA test after 90 days for study groups (N=48).

Study variable	Study groups	Number	Mean $\pm$ SD	P value
ELISA test after 90 days	MEVAC	12	5671.00 ± 330.52	<0.001*
	MSD	12	5154.33 ± 326.77	
	Zoites	12	5293.67 ± 291.96	
	Control group	12	37.00 ± 23.35	

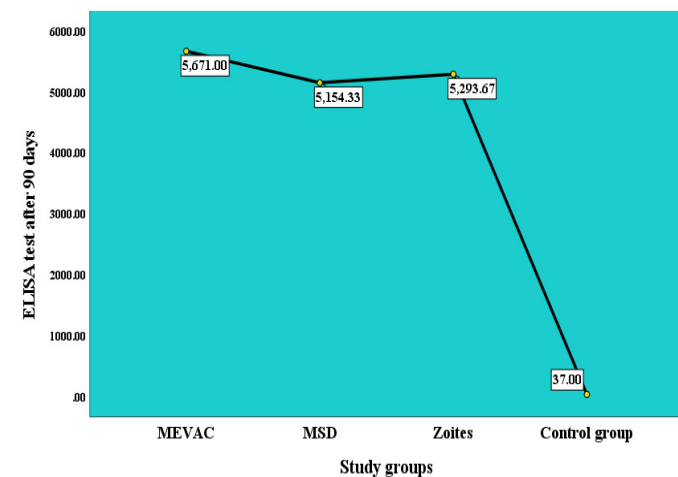


Figure 5: The mean differences of ELISA test after 90 days for study groups (N=48).

In Table 6 and Figure 6, the mean differences of ELISA test which done after 110 days among study groups including (MEVAC, MSD, Zoites and Control group). The results showed that, there were significant differences of ELISA test among study groups after 110 days from intervention with highest level in MEVAC group (6321.33 ± 340.70).

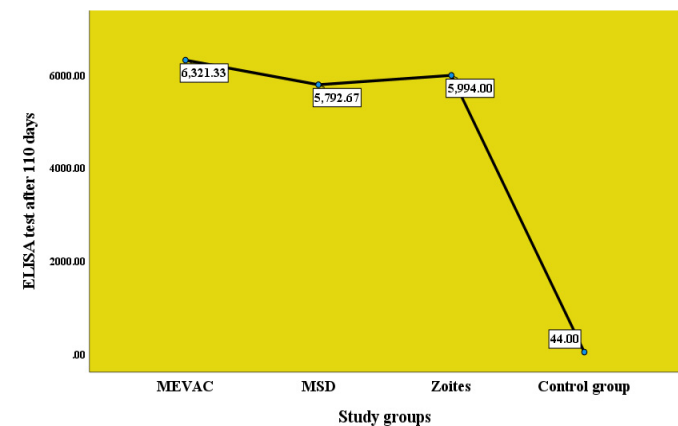


Figure 6: The mean differences of ELISA test after 110 days for study groups (N=48).

Table 6: The mean differences of ELISA test after 110 days for study groups (N=48).

Study variable	Study groups	Number	Mean $\pm$ SD	P value
ELISA test after 110 days	MEVAC	12	6321.33 ± 340.70	<0.001*
	MSD	12	5792.67 ± 355.90	
	Zoites	12	5994.00 ± 423.67	
	Control group	12	44.00 ± 26.04	

In Table 7 and Figure 7, the mean differences of ELISA test which done after 130 days among study groups including (MEVAC, MSD, Zoites and Control group). The results showed that, there were significant differences of ELISA test among study groups after 130 days from intervention with highest level in MEVAC group (7599.17 ± 561.92).

Table 7: The mean differences of ELISA test after 130 days for study groups (N=48).

Study variable	Study groups	Number	Mean $\pm$ SD	P value
ELISA test after 130 days	MEVAC	12	7599.17 ± 561.92	<0.001*
	MSD	12	6737.67 ± 452.39	
	Zoites	12	7027.50 ± 827.61	
	Control group	12	46.00 ± 28.35	

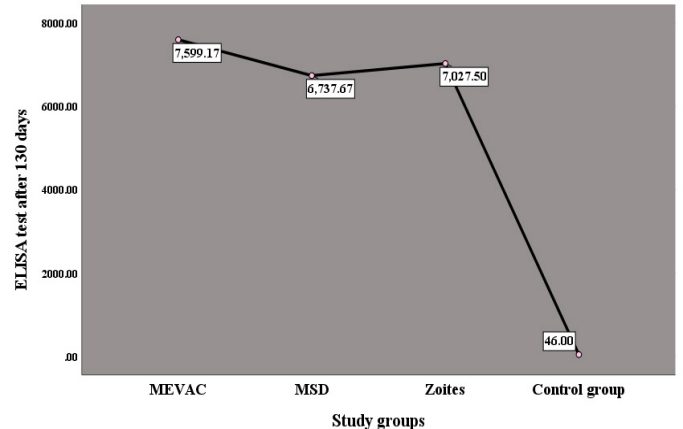


Figure 7: The mean differences of ELISA test after 130 days for study groups (N=48).

In Table 8 and Figure 8, the mean differences of ELISA test which done after 150 days among study groups including (MEVAC, MSD, Zoites and Control group). There were significant differences of ELISA test among study groups after 150 days from intervention with highest level in MEVAC group (8433.08 ± 547.21).

In Table 9 and Figure 9, the mean differences of ELISA test which done after 170 days among study groups including (MEVAC, MSD, Zoites and Control group), the results showed that, there were significant differences of ELISA

test among study groups after 170 days from intervention with highest level in MEVAC group (8712.25 ± 325.48).

Table 8: The mean differences of ELISA test after 150 days for study groups (N=48).

Study variable	Study groups	Number	Mean $\pm$ SD	P value
ELISA test after 150 days	MEVAC	12	8433.08 ± 547.21	<0.001*
	MSD	12	7640.33 ± 509.17	
	Zoites	12	7947.25 ± 553.38	
	Control group	12	42.08 ± 27.95	

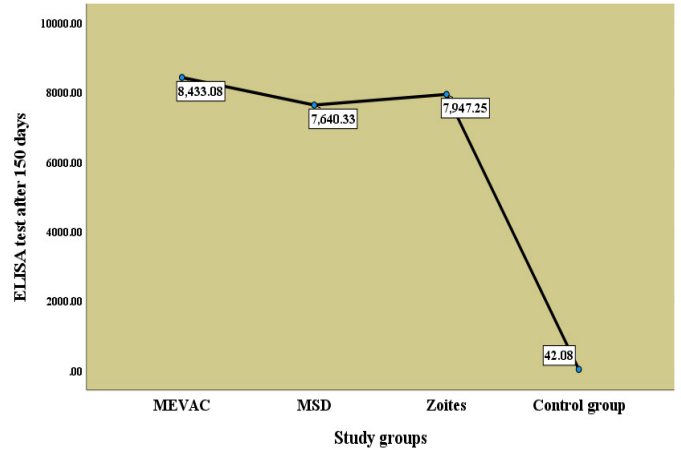


Figure 8: The mean differences of ELISA test after 150 days for study groups (N=48).

Table 9: The mean differences of ELISA test after 170 days for study groups (N=48).

Study variable	Study groups	Number	Mean $\pm$ SD	P value
ELISA test after 170 days	MEVAC	12	8712.25 ± 325.48	<0.001*
	MSD	12	8037.00 ± 107.84	
	Zoites	12	8300.00 ± 263.24	
	Control group	12	43.08 ± 27.22	

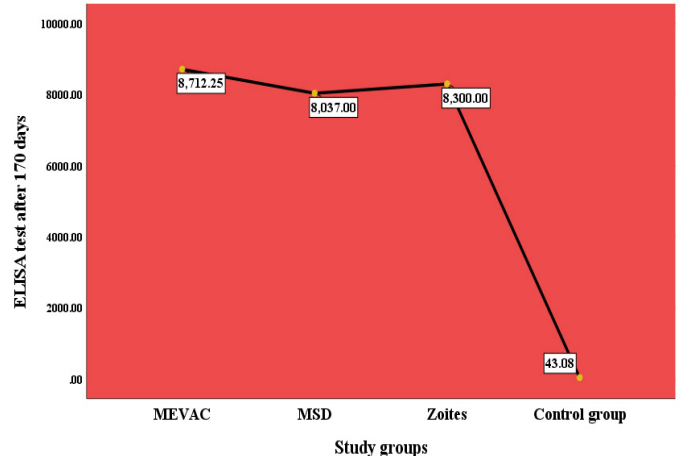


Figure 9: The mean differences of ELISA test after 170 days for study groups (N=48).

In Tables 4-10 and Figures 4-10, the mean differences of ELISA test which done after 190 days among study groups including (MEVAC, MSD, Zoites and Control group). The results showed that, there were significant differences of ELISA test among study groups after 190 days from intervention with highest level in MEVAC group (10332.00 ± 597.66).

Table 10: The mean differences of ELISA test after 190 days for study groups (N=48).

Study variable	Study groups	Number	Mean $\pm$ SD	P value
ELISA test after 190 days	MEVAC	12	10332.00 ± 597.66	<0.001*
	MSD	12	9161.08 ± 851.42	
	Zoites	12	9580.92 ± 722.76	
	Control group	12	38.83 ± 18.07	

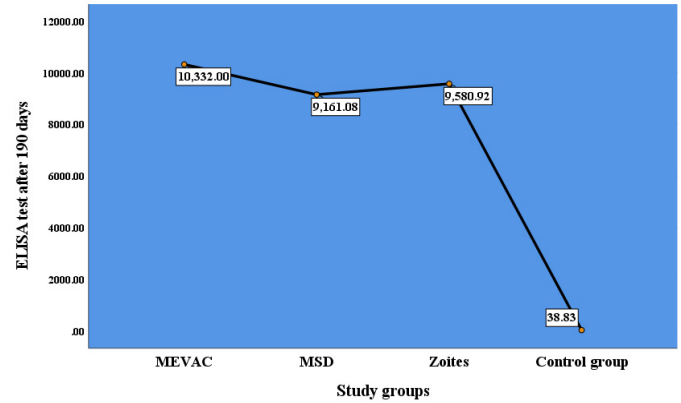


Figure 10: The mean differences of ELISA test after 190 days for study groups (N=48).

In Table 11 and Figure 11, the mean differences of ELISA test which done after 210 days among study groups including (MEVAC, MSD, Zoites and Control group), the results showed that, there were significant differences of ELISA test among study groups after 210 days from intervention with highest level in MEVAC group (10573.75 ± 775.74).

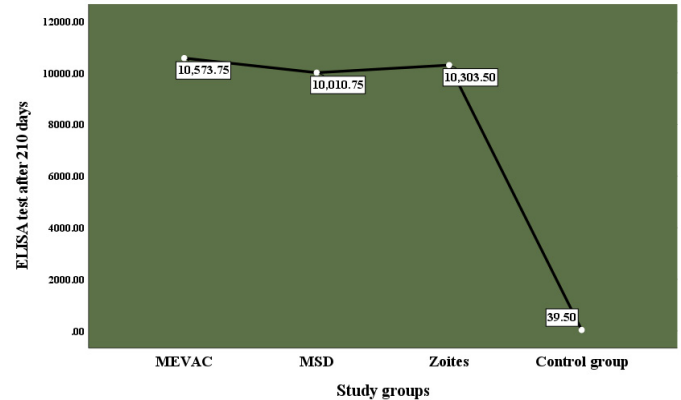


Figure 11: The mean differences of ELISA test after 210 days for study groups (N=48).

Table 11: The mean differences of ELISA test after 210 days for study groups (N=48).

Study variable	Study groups	Number	Mean $\pm$ SD	P value
ELISA test after 210 days	MEVAC	12	10573.75 $\pm$ 775.74	<0.001*
	MSD	12	10010.75 $\pm$ 743.86	
	Zoites	12	10303.50 $\pm$ 635.49	
	Control group	12	39.50 $\pm$ 21.77	

Yitbarek *et al.* (2019) have found no significant differences on day 1, suggesting the hens had similar influenza strain antibodies at the start of the investigation. If they had never been exposed to similar strains or antigens, this result was likely. It was conceivable that antibody baseline levels were similar. The vaccines worked and were formulated identically. Immune response differences may not become obvious until after vaccination. Follmann *et al.* (2023) found that day 1 antibodies defined the initial immunological status. This enabled researchers to ensure that any immune response alterations afterward were due to vaccines and not pre-existing antibody levels. Establishing a baseline helps standardize vaccination group comparisons. It ensures that all groups start from the same spot, making antibody increases easier to link to immunizations. Astill *et al.* (2018) found that on day 1, the chickens had not been immunized, therefore antibody levels were expected to be low or equivalent across all groups. This finding happened because the chickens had not been exposed to the vaccination antigens. On day 1, there were no significant changes, suggesting that all hens had similar immunological states before inoculation. Eladl *et al.* (2019) stressed the importance of preserving this uniformity for studying the effects of immunizations on future immune responses. Between 30 and 70 days following avian influenza immunization, laying hens' ELISA results changed significantly. Disparities may be caused by many factors. By 30–70 days following inoculation, the hens adaptive immune system had enough time to respond to the vaccination. B cells, which produced avian influenza antibodies, may develop and mature during this period. Hu *et al.* (2020) employed ELISA assays to measure these antibodies, therefore significant differences were seen as antibody levels increased. Most antibody production peaks in the first several weeks following inoculation. Memory B cells were the first vaccination-induced immune response. Memory B cells may generate large amounts of antibodies when exposed to the vaccination again. These antibodies reached significant levels between 30 and 70 days after delivery, causing considerable test results differences, according to Stachyra *et al.* (2019). The kind of vaccination (live attenuated or inactivated) significantly affected its immune response. Kongchanagul *et al.* (2019) state that successful vaccines will generate a strong and long-lasting immune response, resulting in higher antibody titers that

may be measured by ELISA testing. Some vaccinations included a booster dose to improve immunity. An additional booster would stimulate the immune system, increasing antibody levels and ELISA test variances. When re-exposed to the antigen, the immunological memory from the initial immunization causes a robust and quick secondary immune response. ELISA treatments showed a significant increase in antibody titers, according to Crawt *et al.* (2019). Putri *et al.* (2019) found significant differences in H5N1 and H5N8 groups compared to H5N2 and H5N3 groups. Chickens immune responses were assessed on day 30 following influenza vaccines. This was done to study chicken immunity. By the thirty-first day, the chickens immune systems have had time to respond to the vaccine and produce antibodies. The vaccination's effectiveness was assessed during this period, allowing for a more accurate assessment. Day 30 was generally chosen as the vaccination date since it represented the peak antibody levels following immunization. With this information, the maximal immune response from numerous immunizations may be compared (El-Shall *et al.*, 2021). Keitel *et al.* (2019) found that maximal antibody response occurred 30 days following immunization. Researchers were able to determine the injections' peak antibody levels using ELISA. According to Haveri *et al.* (2020), H5N1 and H5N8 vaccines may be more efficient at sustaining antibody levels beyond the first peak. H5N1 and H5N8 strains may be more immunogenic, resulting in a stronger immunological response over time. Thus, antibody levels increase and stay longer, which ELISA may detect on day 130. Day 130 marks a long time since vaccination. Zhou *et al.* (2021) state that this time point allows researchers to assess immunizations long-term effects on antibody levels and immune response lifetime. According to Haveri *et al.* (2020), using the ELISA at this time provides a full view of the immune response across time and allows for the evaluation of vaccine formulations sustained effectiveness. H5N1 and H5N8 vaccinations may have elicited and sustained a longer-lasting immunological response than H5N2 and H5N3 vaccines. Zhang *et al.* (2020) suggest that H5N1 and H5N8 vaccines may sustain higher antibody levels after 130 days. Testing at day 210 provides a full comparison of vaccine formulation effectiveness throughout time. This made it easier to identify immunizations that elicit persistent immunity. Compared to H5N2 and H5N3 vaccinations, H5N1 and H5N8 vaccines may induce a stronger enduring and sustained immune response (Mosa *et al.*, 2022; Ibrahim *et al.*, 2020). Zhang *et al.* (2020) suggest that H5N1 and H5N8 vaccines may maintain higher antibody levels after 210 days. The formulations or adjuvants used in H5N1 and H5N8 vaccines may have fostered a more prolonged immune response. This may explain antibody levels differences.

CONCLUSIONS AND RECOMMENDATIONS

At all times (30, 50, 70, 90, 110, 130, 150, 170, 190, and 210 days), MEVAC had the highest ELISA test values. Over time, MEVAC elicited an immune response better than MSD, Zoites, and Control. Post-intervention immune response improved as ELISA test results varied significantly over time. The MEVAC group saw a considerable increase in ELISA levels (10573.75 ± 775.74) after 210 days, indicating a sustained immune response. MEVAC's long-term effectiveness was crucial for therapeutic evaluation. MSD, Zoites, and Control had different immunological responses throughout the experiment, confirming the claim that MEVAC was more effective. MEVAC's increased immune response implies further research was required to understand its mechanisms and possible applications.

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We thank all the staff of Department of Physiology and chemistry for their support me to complete the study.

NOVELTY STATEMENT

This study highlights the superior and sustained immune response of the MEVAC oil-based vaccine against H5 avian influenza in Iraqi layer hens, providing valuable insights for enhancing poultry vaccination strategies.

AUTHOR'S CONTRIBUTION

Both authors work equally in this research.

ETHICAL APPROVAL

Prior to the commencement of this research, approval was obtained from the local committee overseeing the care and use of animals at the University of Baghdad, College of Agriculture/Department of Animal Production.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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