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PAMJ One Health

Research

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**Received:** 15 Jan 2025 - **Accepted:** 19 Jun 2025 - **Published:** 01 Aug 2025

**Keywords:** Avian influenza, vaccine quality, immunogenicity, Nigeria, poultry health, biosecurity

**Funding:** This work was supported by the African Union Commission, Ethiopia, through the Pan African University Life and Earth Sciences Institute (Including Health and Agriculture)'s scholarship (Grant number PAU020110MB). The sponsors had no role in the design, execution, interpretation, or writing of the study.



**Cite this article:** Franklyn Ayomide Oluwadare et al. Seroreactivity of clandestinely traded avian influenza vaccines in Nigeria. PAMJ-One Health. 2025;17(13). 10.11604/pamj-oh.2025.17.13.46568

**Available online at:** https://www.one-health.panafrican-med-journal.com/content/article/17/13/full

**Seroreactivity of clandestinely traded *avian influenza* vaccines in Nigeria**

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## Abstract

***Introduction:*** *highly pathogenic avian influenza strains like H5N1, pose a significant threat to poultry production in Nigeria. In spite of Nigeria's 'no vaccination' regulation against Avian influenza (AI), there is a clandestine practice of AI vaccination by some poultry farmers. However, the effectiveness of this practice is uncertain. This study was therefore carried out to determine the effectiveness of this practice.* ***Methods:*** *two commercial vaccines were evaluated for antigen content (antigenicity) and immunogenicity. These vaccines were tested using Hemagglutination (HA) test and Reverse Transcription Polymerase Chain Reaction (RT-PCR). Subsequently, the vaccines were administered to experimental chickens, Hemagglutination inhibition and ELISA were used to assess seroconversion.* ***Results:*** *results indicated low levels of hemagglutinating antigen and M1 transcripts, as evidenced by hemagglutination titers below 4 log2 and qPCR cycle threshold values of 30 and 32 in the two vaccines. Seroconversion studies in vaccinated chickens showed GMTs ranged from 12 to 80. ELISA at day 28 post first vaccination revealed 40% and 80% seropositivity for vaccine 1 and 2 respectively.* ***Conclusion:*** *the results indicate that the vaccines being used covertly may be of poor quality, and could give false confidence, resulting in inadequate bio-security measures in poultry farms thereby increasing the risk of AI outbreak. On the basis of these findings, regulatory authorities are to enforce the ban on importation, distribution and uses of clandestinely traded AI vaccines. Furthermore, poultry farmers must be enlightened on risks of the use of unregulated vaccines and also encouraged to adopt appropriate biosecurity practices.*

## Introduction

*Avian influenza* (AI) is a devastating viral disease in birds caused by *avian influenza* virus (AIV), it is a member of the *Orthomyxoviridae* viral family, and the *influenza A*genus [1,2]. Influenza A viruses are responsible for severe health effects in birds and humans, causing respiratory diseases in both animals and humans, with severe devastation in poultry [3]. These viruses are classified into two pathotypes highly pathogenic *avian influenza* (HPAI) and low pathogenic *avian influenza* (LPAI) viruses based on morbidity and mortality patterns in experimentally infected chickens *(Gallus gallus domesticus)* [4]. Wild water birds particularly mallards are considered as the natural reservoir and reassortment vessels for AIV [5].

Since 2006, Nigeria has faced repeated outbreaks of various *avian influenza* virus (AIV) strains [6,7]. In response to these outbreaks, biosecurity measures, including bio-exclusion and biocontainment, have been the primary strategies for controlling AI in poultry production systems in Nigeria and many other countries [4,8]. However, in developing countries such as Nigeria, strict adherence to biosecurity principles is challenging in certain contexts due to social and economic constraints [9]. Other control strategies for *avian influenza* (AI) often include culling infected and exposed poultry, a practice known as stamping out, to prevent further spread. This approach is based on the belief that vaccination may be ineffective and could lead to virus mutations capable of infecting humans [10]. Providing financial compensation is crucial to encourage early disease reporting and support the implementation of effective disease control measures [4].

The emergence of large-scale outbreaks of H5 and H7 *avian influenza* (AI) subtypes along with the economic impacts of stamping out has led to the exploration of vaccinations as preventives strategy for controlling AI outbreaks. Countries such as China, Egypt and Mexico have implemented mass

poultry vaccination using inactivated and live recombinant vaccines to prevent AI outbreaks in their regions [11]. However, despite the advantages of AI vaccination, challenges such as the emergence of vaccine resistant strains in birds and the potential for increased viral circulation highlight the need for rigorous vaccination monitoring to detect vaccine failures and mutations [11].

In Nigeria, AI vaccination is currently prohibited, because poorly managed vaccinations can lead to vaccine breaks or failure which may subsequently result in the virus attaining an endemic nature [9]. Poor vaccination practices may result in the emergence of new AI variants [12]. The perceived limitations of biosecurity measures in AI prevention and control have prompted farmers in Nigeria to seek alternative control strategies, resulting in the use of unauthorized imported vaccines [13]. However, the efficiency of this practice and the antigenic and immunogenic compatibility of the used vaccines with local AI strains has not been characterized, raising questions about their efficacy and potential implications for disease dynamics. The present study was, therefore, designed to fill these critical knowledge gaps by investigating the antigenicity and immunogenicity profiles of two clandestinely traded AI vaccines in Nigeria. Particularly, it seeks to ascertain the capability of these vaccines to induce protective immune responses in experimental chickens.

## Methods

This study is an experimental study conducted at the Regional Laboratory for Transboundary Animal diseases in the National Veterinary Research Institute, Vom, Plateau State Nigeria. This Laboratory is the national reference laboratory for the diagnosis of *Avian influenza* viruses in Nigeria.

**Study design**

For the purpose of this study two commercially available *Avian influenza* vaccines were procured from regular vendors under confidence of non-disclosure due to government restrictions. Vaccine 1 was a water-in-oil emulsion vaccine that protects birds against highly pathogenic (HPAI) H5N1 and H5N8 strains, and low pathogenic (LPAI) H9N2 strain. Vaccine 2 was an oil-in-water emulsion designed to protect birds from the H5N2 (LPAI) strain of AI. Both vaccines were weakened and chemically inactivated vaccines. Vaccine antigen detection was carried out using the hemagglutination (HA) assay and Reverse Transcription Polymerase Chain Reaction (RT-PCR) to amplify the matrix (M1) gene, following procedures outlined by Spackman *et al.* [14]. Afterward these vaccines were administered to experimental chickens to evaluate their immunogenicity using serological tests (HI and ELISA).

**Laboratory analysis**

***Haemagglutination test***

Hemagglutination assay (HA) was performed to determine the potency of the vaccine sample following Food and Agriculture Organization (FAO) guidelines [15]. Twenty-five (25) µL of phosphate-buffered saline (PBS) was dispensed into each well of a V-bottomed microtiter plate, 25 µL of the vaccine sample was added to the first well. Two-fold serial dilutions of the virus were prepared across the plate, ranging from 1: 2 to 1: 4096, using PBS as the diluent. An additional 25 µL of PBS was dispensed into each well to maintain volume consistency.

Following dilution, 25 µL of prepared 1% red blood cell (RBC) suspension was added to each well. The plate was gently tapped to mix the contents and incubated at 20°C for 30 minutes. After incubation, the RBC settling pattern in each well was visually inspected. The presence or absence of "tear-shaped streaming" of RBCs after tilting the

plate, was used to assess agglutination. Wells exhibiting hemagglutination (absence of streaming) indicated the presence of the virus, while negative controls (no virus) displayed a flow rate similar to the free RBC suspension. Titer expressed as hemagglutination units (HAU) was determined as the highest dilution of the virus that caused agglutination of RBCs. Each dilution step represented one HAU. This assay provided a quantitative measure of the virus's ability to agglutinate RBCs, which is indicative of its relative potency within the vaccine.

***Viral RNA isolation from vaccine samples***

Total viral RNA was extracted from vaccine samples using the QIAamp Mini Spin Column (QIAGEN, Cat. no. 59604) according to the manufacturer's instructions with minor modifications. Briefly, 560 µL of Buffer AVL containing carrier RNA was added to a 1.5 mL microcentrifuge tube, followed by 140 µL of the vaccine sample. The mixture was incubated at room temperature (25°C) for 10 minutes after a brief 15-second vortexing. The lysate was then centrifuged briefly to remove any drop from the lid. Next, 560 µL of ethanol (96%) was added, and the mixture was pulse-vortexed for 15 seconds, followed by another brief centrifugation. The lysate was loaded onto a QIAamp Mini Spin Column and placed in a 2 mL collection tube without wetting the rim. After closing the cap, the column was centrifuged at 6,000 x g (8,000 rpm) for 1 minute. The flow-through was discarded, and the QIAamp Mini Spin Column was transferred to a clean collection tube.

Two washes were performed with Buffer AW1 (500 µL) each using the same centrifugation conditions (6,000 x g for 1 minute). For complete drying of the silica membrane, the column was centrifuged at full speed (14,000 rpm) for 1 minute in a new 2 mL collection tube. Finally, the purified viral RNA was eluted by placing the QIAamp Mini Spin Column in a clean 1.5 mL microcentrifuge tube and adding 60 µL of Buffer AVE equilibrated to room temperature. The column was centrifuged at 6,000 x g (8,000 rpm) for 1 minute to collect the eluate. The eluted viral RNA was stored at -20°C.

***Reverse Transcription-Polymerase Chain Reaction (RT-PCR)***

One-step RT-PCR was performed using the Qiagen OneStep RT-PCR kit with a 20 µL reaction mixture containing: 0.8 µL enzyme mixture (including reverse transcriptase and hot-start Taq polymerase), 10 pmol of each forward and reverse primer, 0.3 µM probe specific for the target sequence, 400 µM of each deoxynucleoside triphosphate (dNTP), 3.75 mM MgCl2, and 6.5 U of RNase inhibitor.

The thermal cycling conditions included a reverse transcription step at 50°C for 30 minutes followed by enzyme activation at 94°C for 15 minutes. This was followed by 45 cycles of amplification with denaturation at 94°C for 0 seconds and annealing/extension at 60°C for 20 seconds (Spackman *et al.*, 2002). Fluorescence data were collected at the end of each annealing step to monitor amplicon generation. Nuclease-free water was used as a negative control to detect any potential contamination, while lab-grown allantoic fluid containing the target virus served as a positive control to ensure proper assay functionality.

**Experimental chickens and vaccination**

Thirty Specific Antibody Negative (SAN) chickens (Isa Brown and White Leghorn) of about 3 weeks of age were procured from the poultry farm of the National Veterinary Research Institute (NVRI) Vom, Plateau State, Nigeria. The animal house and cages were cleaned, disinfected and fumigated to ensure biosecurity, proper ventilation and temperature control prior to the arrival of the chickens. Habitat enrichment was also provided for the chickens. Hemagglutination inhibition assay was carried out to confirm the specific antibody negative status of the chickens. The chickens were divided into three groups, each consisted of ten chickens. Group 1 was vaccinated

with Vaccine 1, Group 2 received Vaccine 2 while Group 3 served as the unvaccinated control group. Chickens in Groups 1 and 2 were vaccinated at 8 weeks of age according to the manufacturer's instructions (0.5ml per bird subcutaneously at the caudal 3rd of the neck) while PBS was administered to chickens in Group C at the same time and site. Booster shots were administered at 14 days and 28 days post first vaccination (pfv).

***Serological tests***

For serology test, two millilitres (2 ml) of blood were aseptically extracted from the chicken's axillary veins using disposable needles and syringes. To separate the serum, the samples were centrifuged at 695.75 g for 10 minutes after they arrived at the lab. Before being used for additional testing, the serum was meticulously removed from each tube and kept in 2-mL cryovial tubes at -20°C. Blood sampling were done before vaccination to ascertain specific antibody negativity. Afterwards, blood samples were also collected at days 14, 21, 28, 35, and 42 post first vaccination. Hemagglutination Inhibition (HI) and Enzyme linked Immunosorbent Assay (ELISA) tests were used to evaluate humoral immune response to the vaccines.

***Hemagglutination inhibition assay***

Phosphate buffered saline (25 µl) was dispensed into all wells of a plastic microtiter plate with V-bottomed wells after which 25 µl of serum was added to the first wells of each row of wells. Two-fold serial dilution of sera were made across the plate. Following this, 25 µl of antigen (OIE H5N1) containing 4 HAU was added to each well. The plate was gently tapped to mix and then incubated at 20°C for 30 minutes. After incubation, 25 µl of a 1% c-RBC suspension was added to each well. The plate was again gently tapped to mix and incubated at 20°C. After another 30 minutes, the plates were read by tilting and observing the presence or absence of tear-shaped streaming at the same rate as the control wells containing only RBCs (25 µl) and PBS (50 µl). The presence of HA inhibition by serum, indicating a positive result, or the absence of HA inhibition, indicating a negative result, was noted. The HI titer was determined as the highest dilution of antiserum causing complete inhibition of 4 HAU of the virus. The results were considered valid if the negative control serum had a titer of less than 3 $\log_2$ for 4 HAU, and the positive control serum had a titer within one dilution of its known titter.

***Enzyme-Linked Immunosorbent Assay (ELISA)***

A competitive ELISA test kit (ID Screen®, manufactured by Innovative Diagnostics, France was used to detect specific antibodies against the H5 antigen of the influenza A virus in sera of the chickens on day 28 post first vaccination. Diluent (25 µl) was pipetted into all the wells of the microtiter plate and 25 µl of the positive control was added into wells A1 and B1 while 25 µl of the negative control was added into wells C1 and D1. Following this, 25 µl of each serum sample was dispensed into one well at a time. The plate was covered and incubated for 1 hour at 37°C. The wells were then washed three times with at least 300 µl of washing solution for each well, ensuring that they did not dry out between washes. Conjugate was prepared by diluting the concentrated conjugate with diluent and 100 µl was dispensed into each well. The plate was covered and incubated for 30 minutes at 25°C after which each well was washed three times as before. Substrate solution (100 µl) was then dispensed into each well and the plate was covered and incubated for 15 minutes while excluding light. Finally, 100 µl of the stop solution was dispensed into each well to end the reaction. Optical densities were measured at 450 nm.

**Ethical approval**

Ethical approval for animal experimentation was obtained from the National Veterinary Research Institute Animal Use and Care Committee, Vom, Plateau State, Nigeria (AEC/02/161/24) before the experiment commenced.

**Data analysis**

The HI assay results recorded as logarithmic titters in vaccinated groups, were analyzed to calculate the arithmetic mean titters using Microsoft Excel 2016 software. The mean titles were then converted to geometric mean titles (GMT) following the method described by Brugh *et al.* [16]. For this study, GMT of <160 was considered negative.

## Results

**Antigenicity of clandestinely traded AI vaccines in Nigeria**

***Haemagglutination assay results:*** haemagglutination test results indicated < 2log 2 HAU for both vaccine samples.

***Reverse Transcription -Polymerase Chain Reaction (RT-PCR) results:*** RT-PCR revealed high cycle threshold values of 35.97 and 31.68 (Cq) in Vaccines 1 and 2, respectively, as indicated in Figure 1.

**Serological responses to clandestinely traded AI vaccines in experimental chickens in Nigeria:** haemagglutination inhibition screening of experimental chickens prior to vaccination revealed a GMT of < 160 across all experimental chickens.

***Humoral antibody response to vaccination:*** the serological tests revealed variability in the vaccine immunogenicity. Haemagglutination inhibition test results for both Groups 1 and 2 revealed geometric mean titre (GMT) of 12 and 13, respectively, at 14 days pfv. At days 21, 28, 35 and 42 pfv, GMT values were 13, 15, 20 and 12, respectively, for Group 1 and 46, 80, 226 and 80, respectively, for Group 2 (Figure 2). Qualitative ELISA results on day 28 pfv revealed 40% AI antibody positive and 80% positive for group 1 and 2 chickens, respectively, as indicated in Figure 3.

## Discussion

This study assessed the antigenicity and immunogenicity of two inactivated, oil-based, secretly used *avian influenza* vaccines in Nigeria. The poor haemagglutinating activity and subsequent low viral antigen concentration in both vaccines as determined by RT-PCR could be responsible for the low antibody responses recorded in the chickens. A similar study conducted by Brugh *et al.* [16] revealed that the magnitude of the antibody response to these oil-based AI vaccines is largely dependent on the concentration of the vaccine antigens present in the vaccine. This implies that higher antigen concentrations in the vaccine formulations caused a stronger immune response in the chickens. Similarly, it can be inferred from a study by Webster *et al.* [17] that antibody response post-vaccination is directly proportional to antigen concentration in experimental birds.

In this study, seroconversion, in terms of AI antibody production was measured by HI assay used to evaluate vaccine efficacy according to the OIE International Manual [18]. Usually, immunogenicity testing is confirmed by the ability of the vaccine to induce titres of 4-5 log2 (GMT 160-320) [19]. Previous studies on inactivated AI vaccines in experimental birds by Kumar *et al.*, [20] and van der Goot *et al.*, [21] revealed a reduction in viral shedding, transmission and mortality rates in birds with HI titles > 4 $\log_2$ indicating a direct relationship between HI titres and protection. Therefore, it can be inferred that Group 1 chickens (vaccinated with Vaccine 1) might not have been protected against any H5Nx challenge because the HI titres were consistently less than 4 $\log_2$ (GMT160) while Group 2 chickens (vaccinated with Vaccine 2) showed greater seroactivity with protective HI titter of 226 GMT on day 35 pfv which is protective. However, this seroactivity was not sustained as HI title dropped to 80 GMT by day 42 pfv after it peaked on day 35 pfv.

This implies that vaccine 2 may not be an economically prudent option for vaccination because despite the two booster doses on days 14 and 28 pfv, antibody titters still waned quickly. Overall, the results from this study is consistent with the previous report by Meseko *et al.* [13] where poor antibody responses were detected in a commercial poultry farm that was suspected to have clandestinely vaccinated against AI in spite of the no-vaccination policy of the Nigerian government.

This study reveals that there are numerous shortcomings associated with AI vaccines that are being secretly used in Nigeria. Farmers obtain and administer these ineffective vaccines being oblivious of these shortcomings resulting in significant financial losses without improving the health of their birds. In some cases, a vaccinated population gives the farmers false confidence resulting in the relaxation of bio-security measures, which might exacerbate an outbreak. In the absence of a technically guided vaccination policy in Nigeria, institution of biosecurity measures such as regulated farm access, regular disinfection, and bird health monitoring, is the most effective technique for controlling HPAI.

**Limits of the study**

The present study assessed only two clandestinely traded *avian influenza* vaccines, which cannot be assumed to represent the totality of vaccines clandestine use within the country. Besides, the assessment was limited to antigenicity and immunogenicity testing, with no considerations of other critical parameters that could further define the vaccines qualities, such as conditions of storage of the vaccine, distribution practices, or even the genetic compatibility of vaccine strains with the circulating viruses. Besides that, due to the discretion concerning the procurement of vaccines, more detailed data from the manufacturers was hardly accessible that would give a deeper look at vaccine composition and production standards.

## Conclusion

The present study gives insight into the quality and efficacy of clandestinely traded *avian influenza* vaccines in Nigeria. Both vaccines tested had low antigenicity, as evidenced by the weak hemagglutinating activities and low viral antigen content, confirmed through RT-PCR. Immunogenicity evaluation showed that both vaccinations would give very inconsistent and poor immune responses among vaccinated chickens, one inducing very minimal seroconversion and the other inducing short-term antibody response. These deficiencies leave the vaccinated poultry populations vulnerable to infections of *avian influenza* and undermine any perceived protection provided by these vaccines. These findings, therefore, calls for immediate action on the attendant risks of such vaccines, as well as on protection of poultry health without further delay. Regulatory authorities are to take up strict measures with a view to preventing importation, distribution, and uses of clandestine traded AI vaccines, in respect of Nigeria's "no vaccination" policy. The farmers must also be enlightened on risks from unregulated vaccines, besides being persuaded to adopt appropriate biosecurity practices that include controlled access to farms, regular disinfection, and close monitoring of the health of flocks to reduce the risk of outbreaks.

***What is known about this topic***

- *Highly pathogenic avian influenza (HPAI) strains such as H5N1 represent a critical threat to poultry health and production globally, with outbreaks causing severe economic and public health impacts;*
- *Nigeria enforces a "no vaccination" policy for avian influenza due to concerns about improper vaccine administration, which can result in the emergence of vaccine-resistant viral strains and endemic virus circulation;*

- *Clandestine vaccination practices with unregulated vaccines are known to occur among Nigerian poultry farmers, but the quality and efficacy of these vaccines remain largely unverified.*

***What this study adds***

- *The clandestinely traded avian influenza (AI) vaccines used in Nigeria exhibit poor antigenicity and inadequate immunogenicity;*
- *The clandestine AI vaccines used in Nigeria produce inconsistent and insufficient immune responses in vaccinated chickens, leaving them unprotected;*
- *The use of clandestine AI vaccines in Nigerian poultry farms increases the risk of avian influenza outbreaks and drives the persistence and spread of the virus.*

## Competing interests

The authors declare no competing interests.

## Authors' contributions

The research concept was developed by Franklyn Ayomide Oluwadare, Bitrus Inuwa, Olayinka Asala, Olusegun Fagbohun, Clement Adebajo Meseko. Data collection was carried out by Franklyn Ayomide Oluwadare, Mkpuma Nicodemus, Ally Omary Killo, Oluyemi Ogunmolawa, Olanrewaju Igah. Laboratory Assays were conducted by Franklyn Ayomide Oluwadare, Mkpuma Nicodemus, Ally Omary Killo and Anthony Darang. Statistical analysis was done by Franklyn Ayomide Oluwadare, and Edmond Onidje. The draft of the manuscript was prepared by Franklyn Ayomide Oluwadare, Omolade Oladele, Edmond Onidje and Clement Adebajo Meseko. All authors read and approved the final version of the manuscript and contributed equally to its content.

## Acknowledgments

The authors wish to express their sincere gratitude to the Pan African University Life and Earth Sciences Institute (PAULESI) and the National Veterinary Research Institute (NVRI) for providing the necessary facilities for this research, which constitutes a significant portion of the first author's MSc thesis.

## Figures

**Figure 1**: RT-PCR for *avian influenza* vaccine for the detection of matrix (M1) gene

**Figure 2**: *avian influenza* antibody heamagglutination inhibition geometric mean titres in vaccinated chickens

**Figure 3**: Enzyme Linked Immunosorbent Assay (ELISA) result at 28 days pvf in chickens vaccinated with AI vaccines

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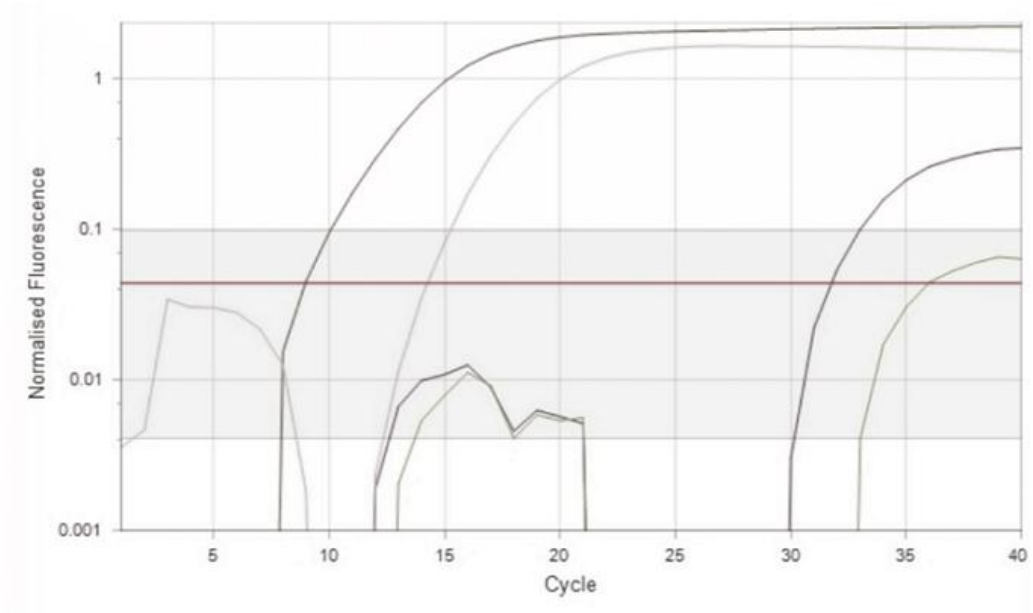


| Well | Colour | Cq | Efficiency | Efficiency $R^2$ | Result |
|---|---|---|---|---|---|
| **NTC** | | | | | $\bar{x}$ = 35.52 σ = 0.00 |
| 7 | | 35.52 | - | - | Excluded from WOL |
| **POS ALF 028** | | | | | $\bar{x}$ = 14.19 σ = 0.00 |
| 6 | | 14.19 | 1.92 | 0.98587 | |
| **POS ALF 029** | | | | | |
| 5 | | - | - | - | Excluded |
| **Vaccine 1** | | | | | $\bar{x}$ = 35.97 σ = 0.00 |
| 2 | | 35.97 | - | - | Excluded from WOL |
| **Vaccine 2** | | | | | $\bar{x}$ = 31.68 σ = 0.00 |
| 1 | | 31.68 | 2.00 | 0.93052 | |

**Figure 1**: RT-PCR for avian influenza vaccine for the detection of matrix (M1) gene

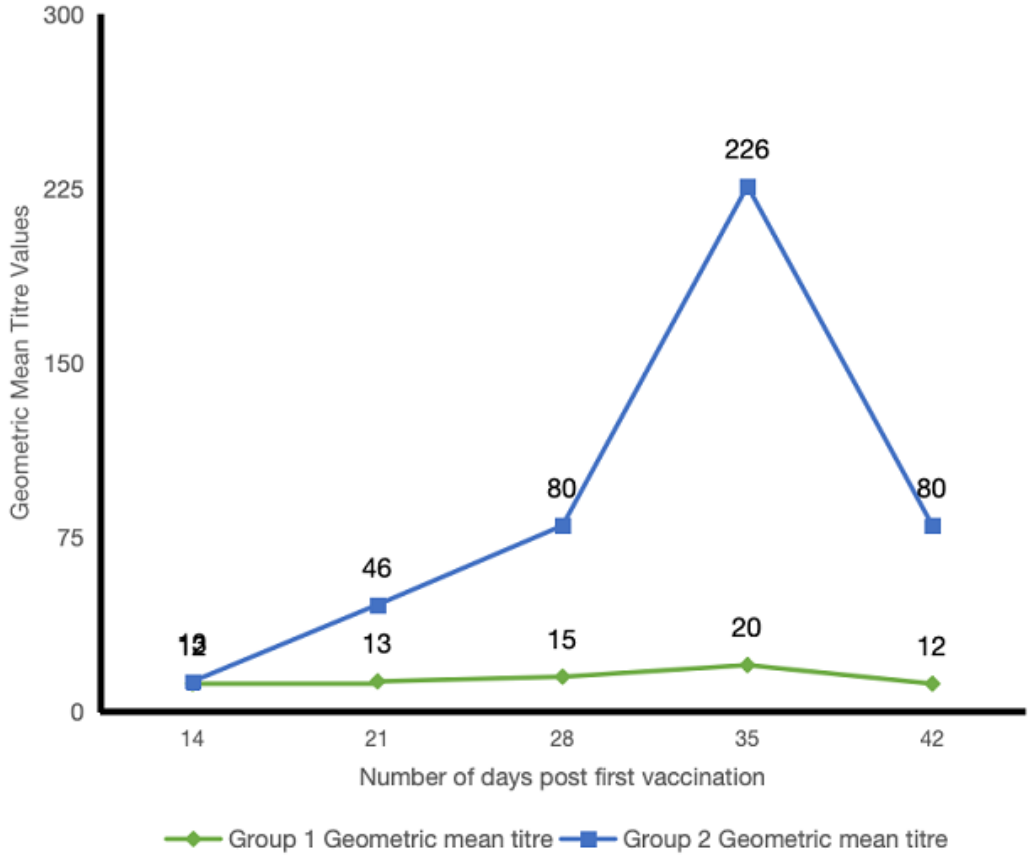


**Figure 2**: avian influenza antibody heamagglutination inhibition geometric mean titres in vaccinated chickens

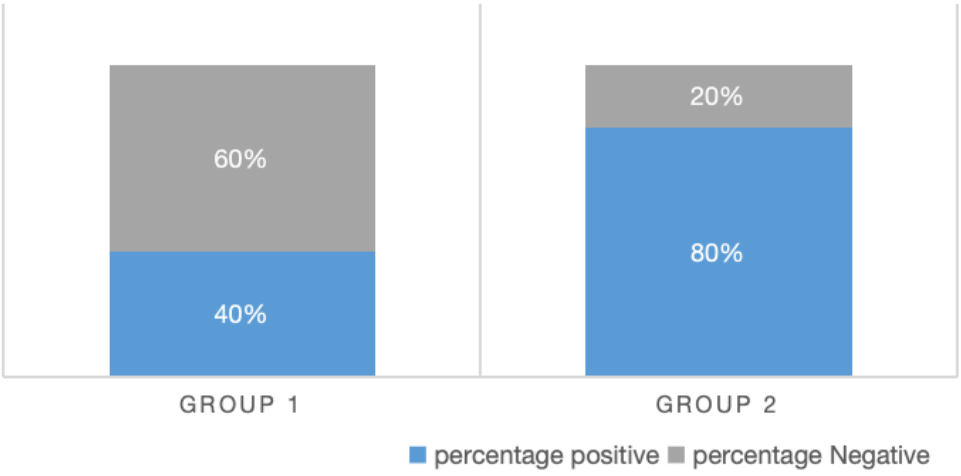


**Figure 3**: Enzyme Linked Immunosorbent Assay (ELISA) result at 28 days pvf in chickens vaccinated with AI vaccines