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Vaccine Quality Challenges in Secretly Traded H5 and H9 Avian Influenza Vaccines in Nigeria

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ABSTRACT

Avian Influenza Virus (AIV) especially the highly pathogenic subtype H5Nx causes Avian Influenza (AI) disease and it is a major threat to the poultry industry and public health worldwide. Vaccination is one of the control measures in many countries, though restricted in Nigeria. As such, some poultry farmers secretly employ imported H5 and H9 AI vaccines to vaccinate their chickens. This study investigated the antigenic quality of these imported vaccines. Four imported vaccine brands were tested using Hemagglutination (HA) test and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). All four vaccine samples tested negative in the HA test, indicating a lack of detectable HA antigen titer. Similarly, RT-PCR failed to amplify the targeted region of the viral matrix gene in any of the vaccine samples against reference control. These negative results are of great concern and suggest that the imported H5 and H9 vaccines lack essential antigens that could stimulate antibodies. Thus, potentially rendering them ineffective against circulating AI subtypes. The failure in antigenic quality could be due to myriads of factors including improper storage, transport (often due to illegal import), or limitations in the original vaccine production process. Regardless of the specific cause, these findings highlight the potential risks associated with the use of unregulated vaccines. Poultry farmers who use these imported vaccines may be incurring unnecessary costs while receiving a false sense of security for their flocks. In conclusion, due to the apparent lack of efficacious antigens, we suggest a quality monitoring of imported H5 and H9 vaccines in Nigeria for compliance with local regulations on vaccine use, biosecurity measures and investigation into the root causes of the vaccine failure observed in this study.

Key words: Vaccine quality, handling, antigen, Avian influenza, HA, RT-PCR, Nigeria

INTRODUCTION

Avian influenza (AI) is a viral disease in birds caused by Avian Influenza virus (AIV) (Sendor *et al*., 2024). AIV are classified as type A influenza viruses and belong to the family Orthomyxoviridae (Wolff *and* Veit, 2021; Sendor *et al*., 2024). Type A influenza viruses generally possess a negative-sense, segmented RNA genome. This segmentation strategy allows for genetic reassortment, a process where different AIV strains co-infect a host cell and exchange RNA segments (Gong *et al*., 2021). This reassortment can generate a novel viral progeny with potentially altered host tropism, virulence, and antigenic properties, posing a significant challenge for control efforts (Vijaykrishna *et al*., 2015). Projecting from the envelope of all Type A influenza viruses, including AIV, are viral surface proteins: Hemagglutinin (HA) and Neuraminidase (NA) which classify AIV into serological subtypes (CDC, 2023). AIV exhibit a spectrum of virulence in avian hosts, categorizing them into highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) viruses based on established criteria encompassing viral characteristics and their ability to induce mortality in experimentally infected chickens (WAOH, 2023). Avian influenza A viruses (AIV) primarily infect avian hosts, but a restricted number of subtypes have been documented to cause zoonotic transmission to humans (CDC, 2024). The introduction of avian influenza into Nigeria is attributed to migratory birds traveling from Asia and Europe; Nigeria lies on the route of major bird migrations (East-Africa-Asia flyway, Atlantic-America, and Black Sea/Mediterranean flyway (Ducatez *et al*., 2006: Meseko *et al*., 2018). Highly pathogenic avian influenza (HPAI) subtype H5N1 clade 2.2 was first identified in Nigeria in 2006. To control the outbreak, Nigeria implemented strict biosecurity measures and a modified stamping-out policy. This approach remains the country's primary method for controlling HPAI outbreaks to date. Vaccines or other immunoprophylactic substances were not utilized for control (Fusaro *et al*., 2009; Oladokun *et al*., 2012). In 2015, another Highly Pathogenic strain H5N1 clade 2.3.2.1c was reported in Nigeria. Within a few weeks, it spread within and between countries, affecting nearby West African countries (Niger, Cameroon, Ghana, Ivory Coast, and Burkina Faso) from 2015 to 2016. The virus was still detected in 2017 and 2018 (Monne *et al*., 2015; Tassoni *et al*., 2016; Laleye *et al*., 2018).

In some countries, AI vaccines are employed within comprehensive control approaches to safeguard poultry from highly pathogenic AI strains like H5N1. These vaccinations play a crucial role in diminishing disease prevalence and minimizing viral shedding among infected poultry (Swayne and Kapczynski, 2008). Most of the vaccines used to date target the viral surface hemagglutinin (HA)(Dey *et al*., 2023) .

Studies have shown that vaccination with inactivated whole-virus vaccines (WIV) can protect vaccinated chickens and is an effective way to prevent poultry birds from AIV infection (Dong *et al*., 2022). Many Avian influenza WIVs are very immunogenic because of their crude production methods, especially chemical inactivation methods. As a result, the remaining virus RNA triggers the innate immune signaling

pathways (Furuya, 2011). Chemical inactivation preserves the structural shape and integrity of the HA and NA, the two main targets for neutralizing antibodies (Krammer, 2019). Whole Inactivated vaccines also have internal antigens, which induce cellular immunity (Dey *et al*., 2023). However, vaccination of birds against avian influenza in Nigeria is currently prohibited (Meseko *et al*., 2023).

Nigeria has experienced recurrent HPAI H5N1 outbreaks since 2006, with clade 2.2, 2.3.2.1c, 2.3.4.4b strains identified (Fusaro *et al*., 2009; Monne *et al*., 2015; Meseko *et al*., 2023)). The persistent occurrence and spread of bird flu have increased the need for finding better ways of controlling this viral disease besides improved biosecurity measures. Biosecurity limitations and unrestricted poultry movement also contribute to viral spread (Meseko *et al*., 2023). Furthermore, trade in poultry and poultry products across a large area of different ecological zones of Nigeria has also led to the circulation of H5N1 clade 2.3.2.1c, and the occasional detections of H5N8 clade 2.3.4.4 in farms and live bird markets (LBMs) in 2017 and 2018 (OIE, 2017; FAO, 2018). The repeated outbreaks call for adopting additional immunoprophylactic control measures such as vaccination (Meseko *et al*., 2023). The limitations of biosecurity measures in preventing HPAI outbreaks have driven Nigerian poultry farmers to seek alternative control strategies. This has led to the unauthorized use of imported vaccines, highlighting the need for improved biosecurity protocols and potentially re-evaluating poultry vaccination policies (Meseko *et al*., 2021). However, the efficacy of these practices against local strains remains unknown.

This study was designed to investigate farmers vaccination practices and evaluated the effectiveness of the imported avian influenza vaccines. The evaluation focused on the levels and concentrations of antigens within the vaccines, as crucial factors influencing the immune response (immunogenicity) after vaccination.

MATERIALS AND METHODS

Four commercially available avian influenza vaccines were procured from regular vendors under confidence of non disclosure due to government restrictions.

- Vaccine A is an oil-in-water emulsion designed to protect birds from the H5N2 strain of Avian Influenza. The vaccine uses a weakened and chemically inactivated type A influenza A virus.
- Vaccine B is a water-in-oil emulsion vaccine that protects birds against: the highly pathogenic (HPAI) H5N1 and H5N8 strains, and the low pathogenic (LPAI) H9N2 strain.
- Vaccine C is an oil-in-water emulsion vaccine that protects birds against the H9N2 strain of Avian Influenza. The vaccine uses an inactivated (killed) virus.
- Vaccine D is a combination vaccine that protects birds against four diseases: Newcastle disease, Infectious Bronchitis, Avian Influenza (H9 subtype), and Infectious Bursal Disease (IBD).

Vaccine antigen detection was carried out on the procured vaccines using the Hemagglutination (HA) test and vaccine antigen characterization using Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) to amplify the matrix (M1) gene, following procedures outlined by Spackman *et al.* (2002).

Haemagglutination Test Procedure

A hemagglutination assay (HA) was performed to determine the potency of the vaccine sample. 25 µL of phosphate-buffered saline (PBS) was dispensed into each well of a V-bottomed microtiter plate. A volume of 25 µL of each vaccine sample was added to wells A1 to D1, 25 µL of PBS was added to well E1 to F1 and served as negative controls. Serial two-fold 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 a 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 was used to assess agglutination. Wells exhibiting hemagglutination (absence of streaming) indicated the presence of the virus (vaccine HA antigen), while negative controls (no virus) displayed a flow rate similar to the free RBC suspension.

The HA titer, expressed in hemagglutination units (HAU), was determined as the highest dilution of the virus (vaccine HA antigen) 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 sample.

Viral RNA Isolation from Vaccine Samples

Total viral RNA was extracted from vaccine samples using the QIAamp Mini Spin Column (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. 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 (15-25°C) for 10 minutes after a brief 15-second vortexing. The lysate was then centrifuged briefly to remove any drops 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). An optional step included a third wash with Buffer AW1 to eliminate any residual carryover. For complete

drying of the silica membrane, the column was centrifuged at full speed (20,000 x g or 14,000 rpm) for 1 minute in a new 2 mL collection tube (not provided).

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

To detect the Influenza A matrix gene (M-gene), RNA extracts underwent thermal cycling conditions. Different regions of the M-gene, a conserved gene in all Influenza A viruses, were amplified using this protocol (Spackman et al., 2002). Primers and fluorescence probes were sourced from Macrogen in the Netherlands. A 25ul RT-qPCR reaction mix was prepared, made up of 1.8ul nuclease free water, 12.5ul RT buffer, 1.5ul each of primer at a final concentration of 0.32uM, 2.5UL of probe at a final concentration of 0.08uM, 0.2 UL of enzyme mix and 5ul of RNA template. Gene-specific primers and probes designed for the generic matrix gene (F: 5′-AGATGAGTCTTCTAACCGAGGTCG-3′ R: 5′-TGCAAAGACACTTTCCAGTCTCTG-3′, FAM- 5′- TCAGGCCCCCTCAAAGCCGA-3′) were used. The RT-PCR was conducted using a Rotor-Gene Q thermocycler (Qiagen, Hilden, Germany). 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 controls to ensure proper assay functionality. The reaction included a reverse transcription step at 50°C for 30 minutes, followed by enzyme activation at 94°C for 15 minutes. Then, 45 cycles of denaturation at 94°C for 0 seconds and annealing/extension at 60°C for 20 seconds were performed (Spackman *et al*., 2002). Fluorescence data were collected at the end of each annealing step to monitor amplicon generation.

RESULTS

Table 1 and Figure 1: shows that four samples tested negative in the Hemagglutination tests, presenting with $< 2^1$ HA titer.

Figure 1: Hemagglutination tests plate

Table1: Haemagglutination Test Result

Vaccine	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
A	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ
B	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ
C	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ
D	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ
negative controls	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ

Key: Θ = Negative, + = Positive

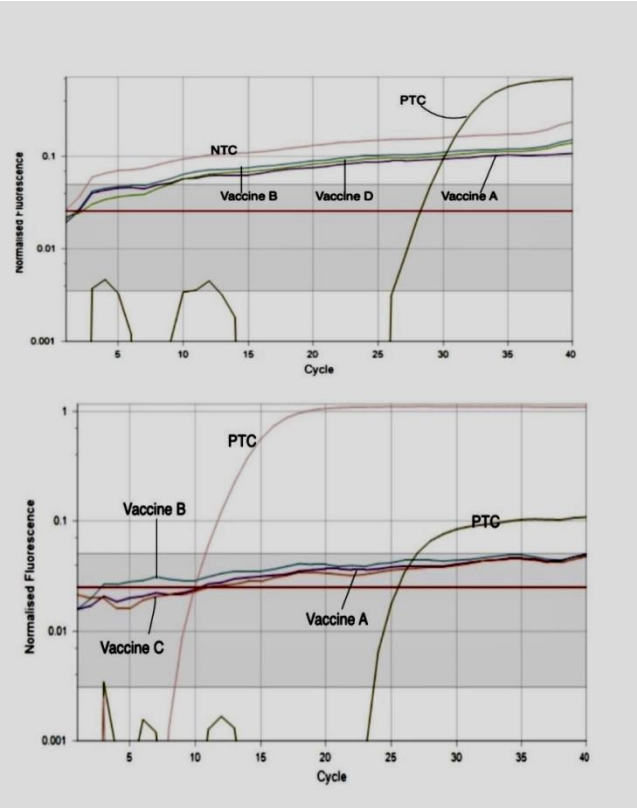

Figure 2: RT- PCR Results

No amplification was detected by RT-PCR in any of the four analyzed samples.

Table 2: Result of RT-PCR of the Four Samples analyzed.

Well	Cq	Efficiency	Efficiency R^2	Results
Vaccine A	-	-	-	**Excluded**
Vaccine B	-	-	-	**Excluded**
Vaccine C	-	-	-	**Excluded**
Vaccine D	-	-	-	**Excluded**
Negative Control	-	-	-	**Excluded**
Positive Control 1	**9.95**	**2.20**	**0.972528**	
Positive Control 2	**25.46**	**1.26**	**0.94233**	

DISCUSSION

In an attempt to protect poultry flocks against Highly Pathogenic Avian Influenza (HPAI), some poultry farmers have resorted to a covert practice: vaccinating their poultry with vaccines not approved for use in the country, as reported by Meseko *et al.* (2021). A visual examination of the vaccine B label, revealed that it was manufactured to protect against AI-stain H5N1 clades 2.2.1.1 and 2.2.1.2, which is different from the known circulating H5N1 clades 2.2, 2.3.2.1c, and 2.3.4.4b strains in Nigeria. As reported by Yamayoshi *and* Kawaoka (2019), antigenic mismatch causes low vaccine efficacy; therefore, a major concern for this vaccine is the lack of antigen specificity to the indigenous known strains in Nigeria, which can result in a possible mismatch as vaccines are highly antigen specific.

Furthermore, as indicated in Table 1, all four vaccines lacked hemagglutination-detectable antigens, which indicates that the vaccine may lack the HA antigen, which is the major antigen in AI vaccines, as reported by Swayne *and* Spackman (2013).

Likewise, the RT-PCR assay suggests the absence of the targeted region of the matrix gene in the extracted RNA, as indicated on Table 2. This may be due to the low viral concentration in the original vaccine samples, resulting in insufficient detection after RNA extraction and cDNA synthesis. The lack of hemaglutinating and PCR-Detectable Influenza antigen infers that all the vaccines studied lacked AI antigen and therefore cannot induce any form of protection in vaccinated subjects. The possible causes of the failure of these vaccines to meet the primary antigenic requirements and quality may include improper storage or transportation conditions, which is often a consequence of bypassing established channels; additionally, vaccines sourced from various global manufacturers may lack essential antigens specific to the prevalent Nigerian strains, further compromising their effectiveness. The lack of regulatory oversight by regulatory agencies due to the illegal import status of these vaccines raises serious concern about their potency. While the intent of farmers to protect

flocks is commendable, this approach carries a significant psychological risk of possible relaxation of biosecurity measures on poultry farms in the false assertion that birds vaccinated with such vaccines are protected against AI; this may potentially exacerbate an outbreak. To address this issue, further investigation is necessary to pinpoint the exact cause of vaccine potency loss; if it is manufacturing error, inadequate cold chain management during transportation, or the lack of essential antigens in the vaccines. By discouraging off-label vaccination and promoting robust biosecurity practices, Nigerian authorities can work with farmers to create a more sustainable and effective approach to HPAI control.

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